Cancer Chemother Pharmacol (1992) 29: 297–304

Cancer Chemotherapy and Pharmacology

Cellular kinetics of prednimustine versus chlorambucil plus prednisolone in vitro*

Eugen Musch[1], Mouhamad Malek[2], Jasna Peter-Katalinic[3], Heinz Egge[3], Hermann Rink[4], Bernd Lathan[5], and Eberhard Riedel[6]

[1] Department of Internal Medicine, Mary's Hospital Bottrop, FRG; [2] Department of Internal Medicine, University of Bonn, FRG; [3] Institute for Physiological Chemistry, University of Bonn, FRG; [4] Institute for Radiobiology, University of Bonn, FRG; [5] Department of Internal Medicine, University of Cologne, FRG; [6] Institute for Biochemistry, University of Berlin, FRG

Received 26 November 1990/Accepted 19 August 1991

Summary. Intracellular concentrations of prednimustine (PM), chlorambucil (CLB), phenylacetic acid mustard (PAAM) and prednisolone (P) were measured in different experimental tumor cell lines that had been incubated with either PM or CLB + P. For intracellular analytical determination, we modified a high-pressure liquid chromatographic method for the detection of these substances in plasma. Intact PM could be detected in the intracellular compartment of the incubated tumor cells. PM-incubated cells from PM-injected rats exhibited a higher intracellular concentration-time integral (PAAM) and longer concentration-time profiles for drugs with alkylating capacity than did cells exposed to the CLB + P mixture or to CLB. PAAM was not detectable after incubation of cells with PM, whereas in CLB-incubated cells the AUC of PAAM exceeded that of the parent drug CLB. Our in vitro results therefore favour the concept of a facilitated intracellular uptake and an increased antiproliferative effect for PM versus CLB and CLB + P.

Introduction

Prednimustine is the C-21 prednisolone ester of chlorambucil. The underlying rationale for the design of this drug was that its prednisolone moiety would preferentially bind to glucocorticoid receptors and facilitate the intracellular uptake of the alkylating part of the molecule [13]. In fact, both animal experiments and clinical experience with prednimustine as single-agent therapy in patients presenting with breast cancer as well as in those exhibiting chronic lymphocytic leukemia have demonstrated that prednimustine exerts higher antiproliferative activity and produces less toxicity as compared with its single components [4, 5, 7, 11, 27].

However, prednimustine, the intact steroid ester after oral administration, has thus far been undetectable by chemical analysis in animal as well as in human pharmacology [4, 5, 15, 16, 18, 34]. From the results reported by Ehrsson et al. [8] it has been confirmed that the rate of elimination of chlorambucil following its administration in a formulation of prednimustine is prolonged, with more continuous chlorambucil concentration occurring in blood. The increased anticancer activity of prednimustine versus the single components has thus been attributed to prednimustine's producing a slow-release form of chlorambucil [16, 17].

Recent experiments on the competitive intracellular uptake of radiolabeled cortisol have demonstrated that the transmembrane transport of glucocorticoids to the intracellular compartment represents a very rapid process. Furthermore, they have provided evidence of a specific glucocorticoid carrier located in the cell membrane of glucocorticoid-sensitive rat hepatocytes [1, 2; for reviews see 37, 40]. It has also been hypothesised that the "non-detectability" of the intact ester prednimustine (apart from rapid presystemic hydrolysis) might therefore be attributable to rapid inracellular transport, which would be congruent with the rationale of a more side-specific antitumor effect for prednimustine along with a modulated, more selective anticancer effect on steroid hormone-dependent tumors.

A direct chemical analysis of intracellular prednimustine has never been undertaken. We therefore performed studies on the kinetics of cellular uptake and the intracellular concentration-time profile of prednimustine as compared with chlorambucil and prednisolone to determine whether the rationale of a target-oriented cytostatic approach could be realised using this alkylating steroid conjugate. In the present study we used an in vitro model involving the incubation of experimental tumor cell lines with either prednimustine or chlorambucil plus prednisolone.

* Dedicated to Prof. Dr. H. J. Dengler on the occasion of his 65th birthday. This study was supported by the Ministry of Science and Research of Nordrhein-Westfalen

Offprint requests to: Eugen Musch, Department of Internal Medicine Sigmund-Freud-Str. 25, D-5300 Bonn 1, Federal Republic of Germany

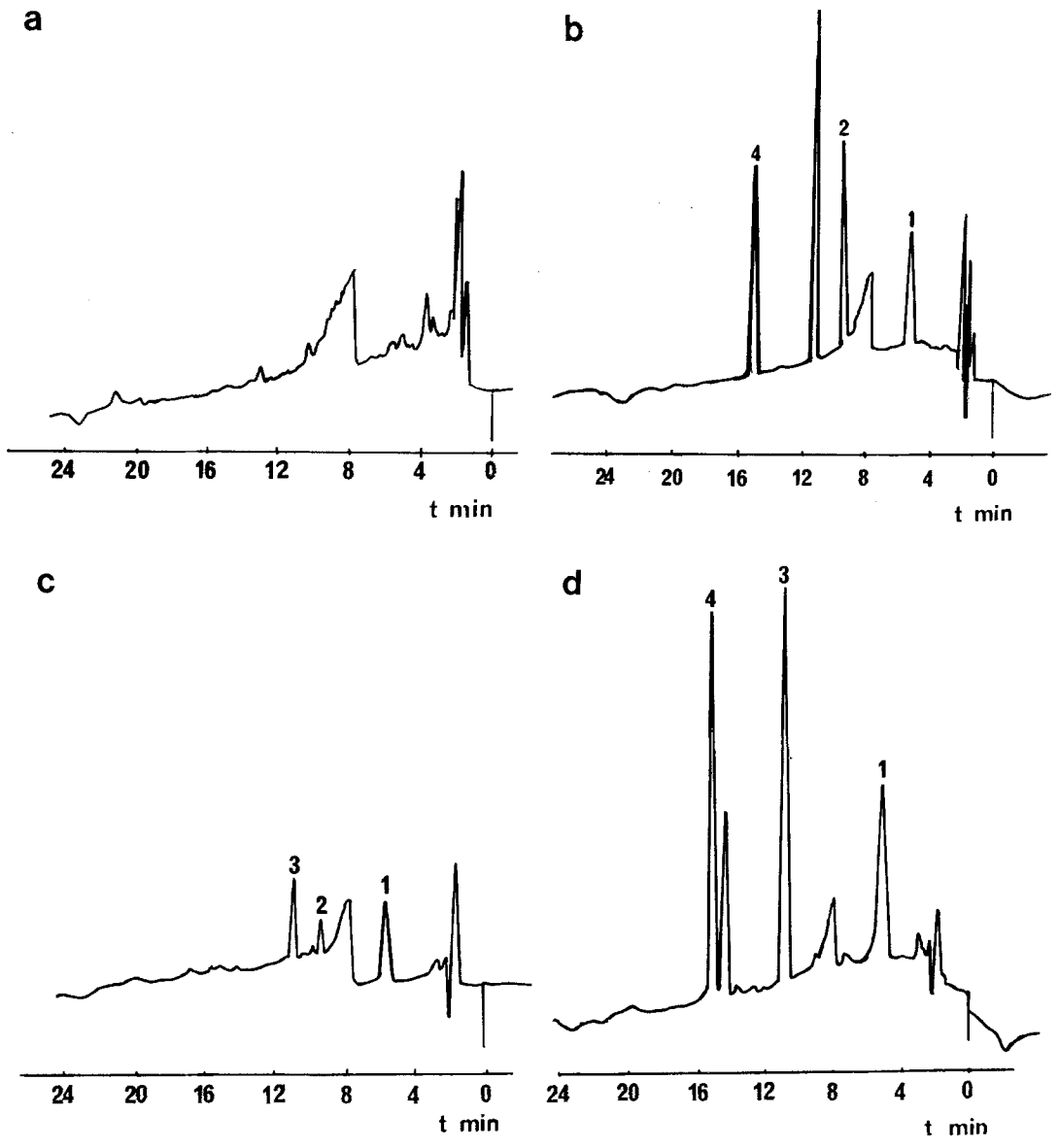

Fig. 1 a–d. Chromatograms of cellular extracts of MCF-7 mammary carcinoma cells. **a** Blank cellular extract. **b** Cellular extract spiked with 500 ng each of prednimustine, chlorambucil, prednisolone and phenylacetic acid mustard. **c** Following incubation with chlorambucil + prednisolone. **d** Following incubation with prednimustine. *Peak 1, Prednisolone; peak 2*, phenylacetic acid mustard; *peak 3*, chlorambucil; *peak 4*, prednimustine

Materials and methods

Chemicals

Pure chlorambucil was contributed by Deutsche Wellcome GmbH (Burgwedel, FRG). A sample of prednimustine was provided by Pharmacia Leo Therapeutics AB (Helsingborg, Sweden). Dr. D. Newell donated the phenylacetic acid mustard, which was synthesised at the Royal Cancer Research Centre (Sutton, UK). Deionized water was used for all experiments. Acetate buffer (pH 3.9) was prepared from acetic acid (0.2 *N*) and a solution of sodium acetate (0.2 *M*; 440 ml acetic acid and 60 ml sodium acetate diluted with deionised water to a final volume of 1 l). This buffer and high-pressure liquid chromatography (HPLC)-grade acetonitrile (Baker Chemicals, Deventer, The Netherlands) were used for chromatography.

Apparatus

The HPLC equipment consisted of an SCL 6A Shimadzu system controller equipped with two 6A LC pumps and an SPD 6A UV spectrophotometric detector (Shimadzu, Darmstadt, FRG) fitted with a Rheodyne injection valve. The UV detector was adjusted to a wavelength of 254 nm. Peaks were quantified using a C-R3A Shimadzu integrator. The analytical column (25 cm × 4.6 mm inside diameter) was filled with Spherisorb C_{18} S5 ODS-1. It was protected by a cartridge-system guard column (3 cm × 4 mm inside diameter) containing the same material. For homogenisation of cells, an ultrasound homogenising disintegrator (Sonifier W-250 model 132 134, Schwäbisch-Gemünd, FRG) was used.

Fast atom bombardment (FAB) mass spectra of the original samples of prednimustine, chlorambucil, 2-phenylacetic acid mustard and of the HPLC-separated material were recorded on a VG Analytical double-focusing mass spectrometer of reversed geometry (Manchester, UK) operating at 7 kV accelerating voltage within the mass range of 15–1000 amu in the downscan mode. Only positive ions were recorded. The atom gun was operated at 9 kV using xenon as the bombarding gas. Thioglycerin (1-mercapto-2,3-dihydroxypropan; EGA, Steinheim, FRG)

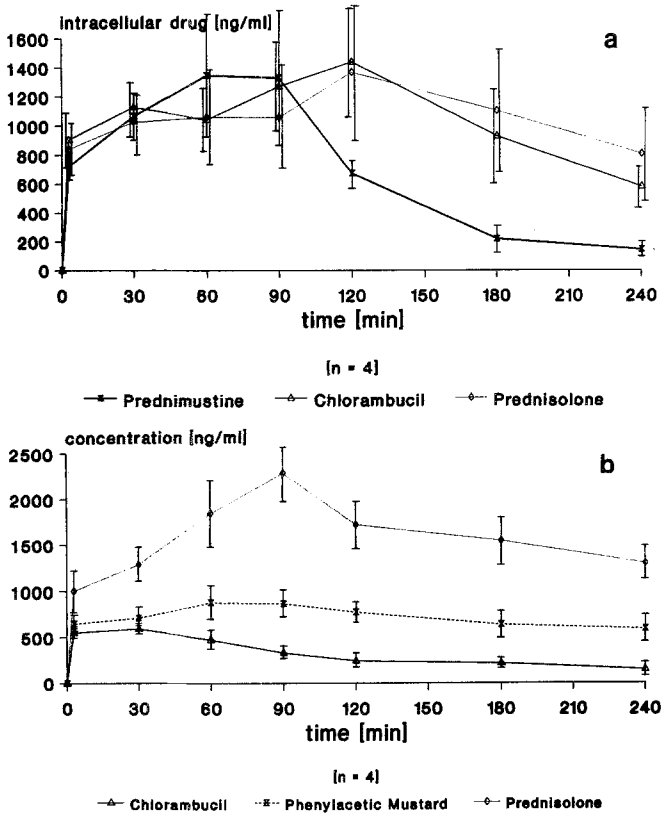

Fig. 2 a,b. Intracellular concentration-time plot of MCF-7-mammary carcinoma cell-line incubated with **a** prednimustine and **b** chlorambucil + prednisolone. Data represent the mean values for 4 incubations

was used as a liquid matrix. Samples dissolved in 1:1 (v/v) chloroform/methanol were applied by syringe to the target coated with the matrix. About 10 µg material was used for reference spectra. The amount of analyte in HPLC fractions was roughly estimated to be 1 µg.

In vitro studies

Four experimental tumor cell lines were cultivated using different media: MCF-7 mammary carcinoma, in MEM (H) (Gibco Ltd., FRG; catalogue number 0421581); MEWO melanoma, in MEM; CEM acute lymphoblastic leukemia, in RPMI 1640 (Boehringer Mannheim, Mannheim, FRG; catalogue number 209945); and HT-29 colon carcinoma, in RPMI 1640.

At 5 days before the experiment, the cell suspensions were distributed into 25-ml glass tubes. Prior to incubation, the cell suspensions were adapted to a constant tumor cell count of 1×10^6 cells/ml. Cells of each line were incubated at 37° C in 10-ml vials. Cell-free aliquots of MEM to which prednimustine or a combination of chlorambucil and prednisolone had been added were simultaneously incubated to serve as controls. Samples were taken at 0, 3, 20, 30, 60, 90, 120, 180 and 240 min after the beginning of the incubation. The samples were centrifuged at 90 *g* for 10 min and cells and supernatant were kept separated at -20° C.

Preanalytical preparations. Following in vitro incubation, cells were withdrawn from the suspension and centrifuged for 5 min at 5000 rpm. The supernatant and the pellet were separated. Cell pellets were washed three times by resuspension of each pellet in 10 ml phosphate-buffered saline (PBS) and centrifugation for 5 min at 5000 rpm. The supernatant was discarded. The cell pellet was resuspended in 1 ml culture medium (RPMI 1640) and lysed using an ultrasound homogeniser (Branson ultrasound disintegrator Sonifier W-250 model 132 134) for 3×15 s in 3 ml ethyl acetate (E. Merck, Darmstadt, FRG; catalogue number 9623), and 3 ml of a mixture of *n*-hexane (E-Merck, catalogue number 4357) and isoamyl alcohol (E. Merck, catalogue number 979; 93:7 v/v) was added. The sample was centrifuged at 10,000 *g* for 10 min; 4 ml of the supernatant was drawn off and evaporated under nitrogen. The residue was redissolved in 250 µl acetonitrile.

Chromatographic assay. For the quantitation of chlorambucil, phenylacetic acid mustard, prednisolone and prednimustine in cell culture media and in the intracellular compartment of cultured cells, we modified our HPLC technique that was previously described in this journal [35]. A mobile phase comprising acetonitrile/acetate buffer (35:65, v/v) was used at a flow rate of 1.5 ml/min. First, 40 µl of the sample was injected and the gradient program was started. After 4.1 min, the portion of acetonitrile was increased to 60%; thereafter, it was increased linearly up to 80% at the 18th min. From the 18th to the 23rd min, the system was returned to its initial condition at a continuous rate.

The column was rinsed with the initial eluent for 3 min prior to the injection of the next sample. Examples of chromatograms obtained for extracts of MCF-7 cells following incubation with chlorambucil plus prednisolone or prednimustine are shown in Fig. 1 c,d. The retention times were 5, 9, 10 and 15 min for prednisolone, phenylacetic acid mustard, chlorambucil and prednimustine, respectively (Fig. 1 a,b). The calibration curves for intracellular concentrations were linear over the range of 25–8,000 ng/ml. The correlation coefficient of the calibration curves was 0.99 for all of these compounds.

Day-to-day precision ranged from 5% for chlorambucil to 14% for prednimustine. The mean recovery rates from cell homogenates in culture medium were 93% for prednimustine and prednisolone, 85% for chlorambucil and 64% for phenylacetic acid mustard. The lower limit of detection was 25 ng/ml for all substances. The main HPLC fractions 4 and 5 were further analysed by fast atom bombardment-mass spectrometry (FAB-MS).

Results

MCF-7 mammary carcinoma, MEWO melanoma, CEM acute lymphoblastic leukemia and HT-29 colon carcinoma cells were incubated with prednimustine and/or the components of this ester of chlorambucil and prednisolone. We succeeded in chromatographic detection of intracellular prednimustine as the intact steroid ester.

The concentration-time plot of intracellular drug in MCF-7 cells (Fig. 2) demonstrates that there was a rapid intracellular uptake of prednimustine (Fig. 2a) that paralleled the increase in intracellular chlorambucil + prednisolone. All three of these substances showed prolonged intracellular concentration-time profiles. In cells that had been incubated with prednimustine, we did not detect phenylacetic acid mustard, the β-oxidation product of chlorambucil. However, the levels of phenylacetic acid mustard exceeded those of the parent compound chlorambucil in cells that had been incubated with chlorambucil + prednisolone (Fig. 2b).

The alkylating potential (i.e. the integrated amount of all drugs exhibiting alkylating potency) in the prednimustine-incubated MCF-7 cells is represented by prednimustine + chlorambucil = 400 µg ml^{-1} min. In cells that had been incubated with chlorambucil + prednisolone, the alkylating protential is represented by chlorambucil, but the major portion corresponds to the metabolite phenylacetic acid mustard (250 µg m^{-1} min; see Table 1). Intracellular prednimustine has also been detected in vivo in CEM and HT-29 tumor cells [1, 2, 4]. Striking differences

Table 1. Cellular concentrations of prednimustine, chlorambucil, phenylacetic acid mustard and prednisolone following in vitro incubation of various tumor cell lines with prednimustine or chlorambucil plus prednisolone

Cell line	Drug	*n*	Substance	Concentration (ng) normalised ot 1×10^7 cells at time							AUC ($\mu g\ ml^{-1}$ min)
				0	3 min	30 min	60 min	90 min	120 min	180 min	240 min	
MCF-7	PM 4 µg/ml	4	PM	0	721±100	1065±181	1346±415	1328±468	674±94	219±93	144±63	151±45
			CLB	0	907±166	1128±206	1042±198	1270±300	1437±366	929±318	583±153	249±63
			P	0	839±194	1026±227	1059±349	1058±357	1368±467	1105±405	810±313	258±85
	CLB 2 µg/ml	4	CLB	0	550±80	594±86	470±68	330±52	240±43	214±43	146±34	77±12
			PAAM	0	650±95	710±106	876±124	864±117	772±99	636±86	592±78	173±24
	P 2 µg/ml		P	0	1000±191	1288±201	1838±278	2276±269	1712±237	1538±216	1296±183	383±24
MEWO	PM 4 µg/ml	2	PM	0	2518	2642	4742	2684	2134	1812	911	568
			CLB	0	452	351	503	435	278	122	31	66
			P	0	293	214	696	641	518	436	402	112
HT-29	PM 4 µg/ml	1	PM	0	2	10	26	100	84	26	14	10
			CLB	0	42	24	26	86	64	18	10	9
			P	0	58	34	22	56	60	42	28	10
CEM 4 µg/ml	PM	1	PM	0	196	873	829	740	686	240	0	120
			CLB	0	220	427	306	284	226	154	0	57
			P	0	141	181	100	87	84	0	0	17

PM, Prednimustine; CLB, chlorambucil; PAAM, phenylacetic acid mustard; P, prednisolone

Table 2. Supernatant concentrations of prednimustine, chlorambucil, phenylacetic acid mustard and prednisolone following in vitro incubation of various tumor cell lines with prednimustine or chlorambucil plus prednisolone

Cell line	Drug	*n*	Substance	Concentration (ng/ml) at time							AUC ($\mu g\ ml^{-1}$ min)
				0	3 min	30 min	60 min	90 min	120 min	180 min	240 min	
MCF-7	PM 4 µg/ml	4	PM	0	3067±288	2265±278	2016±296	1867±324	702±605	299±462	138±434	281±99
			CLB	0	147± 32	559± 88	783±132	1073±170	1327±213	1362±201	1246±181	263±39
			P	0	251± 55	928±128	1461±183	2026±259	2607±318	3085±356	2857±372	523±65
	CLB 2 µg/ml	4	CLB	0	955±307	840±289	713±263	678±207	659±180	427±122	352±103	146±46
			PAAM	0	–	–	–	–	–	–	–	–
	P 2 µg/ml		P	0	1079±266	1181±236	1202±230	1276±206	1319±241	1271±212	1158±191	295±53
MEWO	PM 4 µg/ml	2	PM	0	3246	2518	2096	1928	1531	959	161	372
			CLB	0	–	264	388	390	376	398	292	80
			P	0	236	780	1328	1441	1230	1052	1740	280
HT-29	PM 4 µg/ml	1	PM	0	1949	1438	1320	1074	969	827	743	258
			CLB	0	8	75	224	242	286	180	154	45
			P	0	143	185	332	349	479	500	689	100
CEM	PM 4 µg/ml	1	PM	0	2847	1704	885	275	239	128	109	148
			CLB	0	–	214	224	366	356	228	219	60
			P	0	–	246	341	470	576	646	811	120

PM, Prednimustine; CLB, chlorambucil; PAAM, phenylacetic acid mustard; P, prednisolone

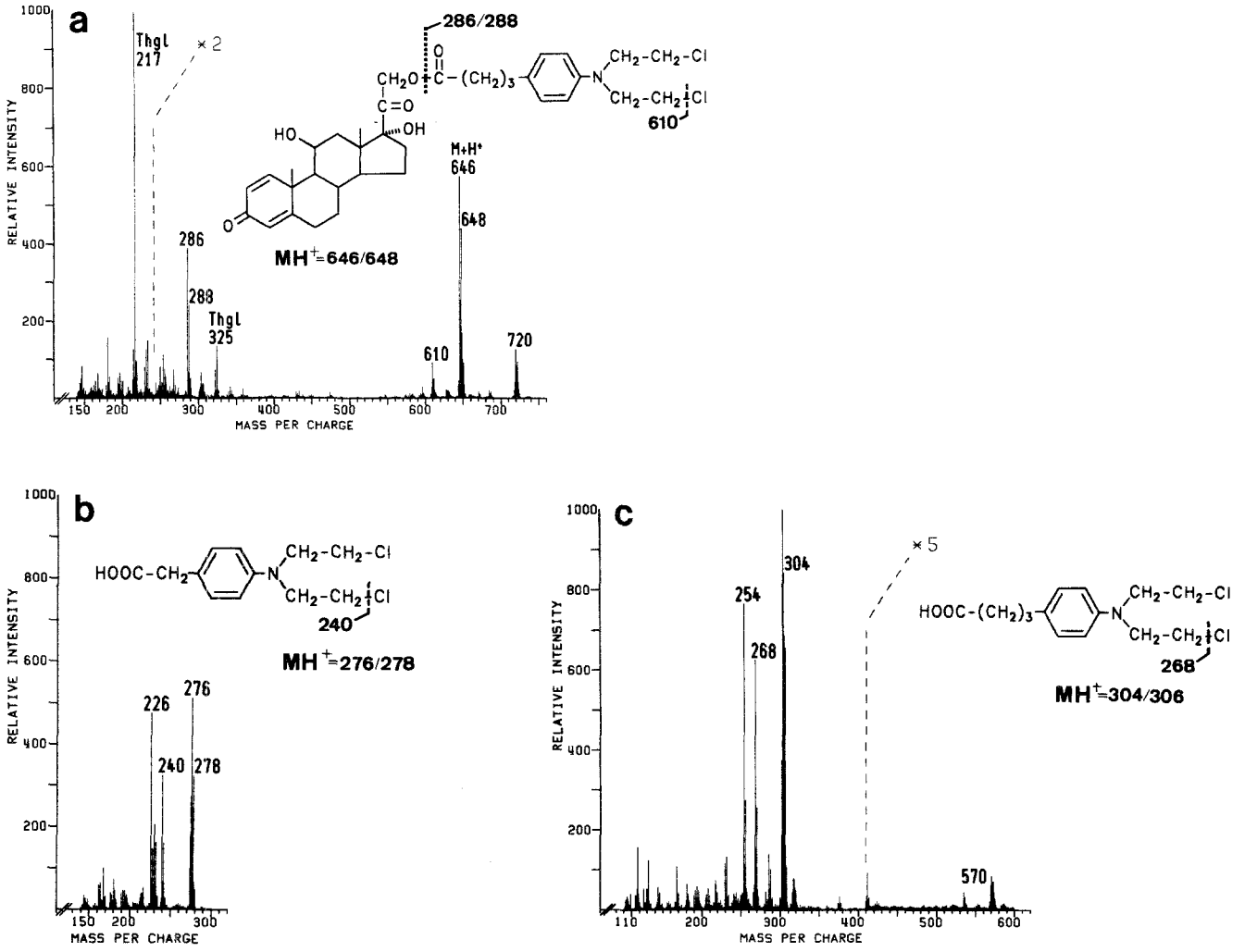

Fig. 3 a–c. Positive-ion FAB-MS of original samples of **a** prednimustine, **b** 2-phenylacetic acid mustard and **c** chlorambucil. Thioglycerin was used as a liquid matrix

in the intracellular concentrations of prednimustine and chlorambucil were found between the different tumor cells tested (Table 1); they were much higher in MEWO cells than in MCF-7, CEM or HT-29 tumor cells.

In ten Sprague-Dawley rats (6 weeks old), we observed tumors measuring 1 cm^3 at 6 weeks after the inoculation of a suspension of HH-9-clone 14 cells. Four rats were given injections of either 2 mg chlorambucil or 1 mg prednimustine into the caudal vein. The difference in the dose of the two formulations (with only one-fourth of the chlorambucil equivalent occuring in the prednimustine-injected animals) was due to the lower solubility of prednimustine in polyethylene glycol, whereby a lower volume of the solute had to be used because it was poorly tolerated by the rats.

In the FAB-mass spectra recorded from original samples of prednimustine, chlorambucil and 2-phenylacetic acid mustard, the molecular MH^+ ions were found at m/z = 646/648, 304/306 and 276/278, respectively (Fig. 3). The presence of two chlorine atoms (natural isotopes 35 and 37) in the molecule is responsible for the distribution of the ions in the molecular ion area. The fragment ions at m/z = 610 (Fig. 3a), m/z = 240 (Fig. 3b) and m/z = 268 (Fig. 3c) arise from the loss of one chlorine atom from the parent molecule. The ion at m/z = 286/288 in the spectrum found for an original sample of prednimustine is the result of fragmentation of the chlorambucil residue in the gas phase (Fig. 3a).

The identity of the substance present in the main HPLC fraction 4 was revealed by FAB-MS to be prednimustine, with the molecular MH^+ ions occurring at m/z = 646/648; the MNa^+ ions, at m/z = 668/670; and the chlorine atom elimination ion, at m/z = 610 (Fig. 4 a). In the FAB-MS of the unknown metabolic product (HPLC fraction 5), molecular ions were found at m/z = 642/644 (MH^+) and m/z = 664/666 (MNa^+), 4 amu lower than those of prednimustine (Fig. 4 b). The ion distribution in the molecular ion area as well as the presence of the characteristic fragment ion arising from the chlorine loss at m/z = 606 (Fig. 4 b) indicate that apparently no metabolic cleavage of the ester bond between the prednisolone moiety and the chlorambucil moiety in the prednimustine molecule took place, as this would have resulted in molecules of lower molecular weight. Moreover, the presence of this metabolically emerging molecule exhibiting 4 amu less than predni-

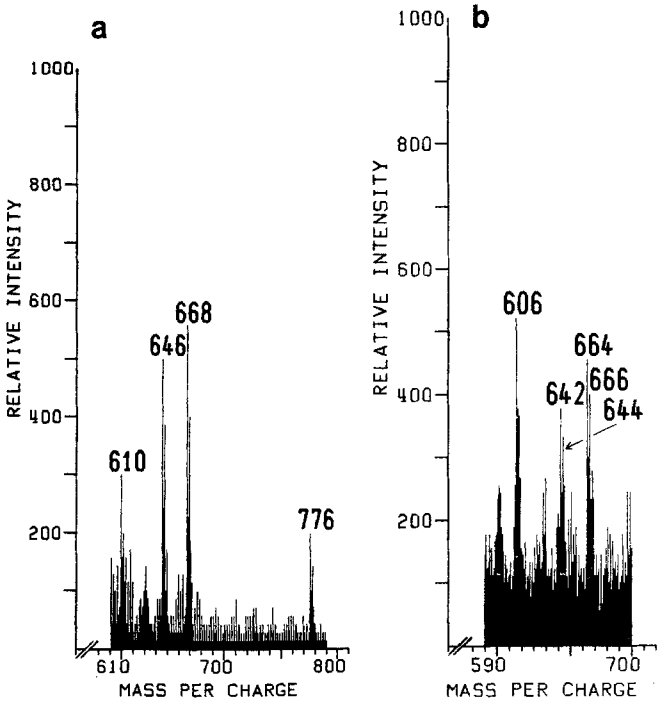

Fig. 4 a,b. Positive-ion FAB-MS of **a** HPLC fraction 4 and **b** the unknown metabolic product (HPLC fraction 5) showing M H^+ = 642/644; M Na^+ = 664/666; M Cl^- = 606. The experimental conditions were identical to those used for the experiment illustrated in Fig. 3

mustine would lead to the conclusion that an oxidation reaction in the steroid moiety probably took place without changing the main molecular skeleton.

Discussion

Experimental antitumor data obtained in vitro have established that prednimustine differs in both its activity and its toxicity from its components chlorambucil and prednisolone [9, 10, 15–17]. Interestingly, we observed that prednimustine was not only highly effective against normal sensitive Yoshida ascites sarcoma tumor but was also effective against a line exhibiting a 50-fold acquired resistance to chlorambucil [16]. In human breast-cancer trials, prednimustine has produced a superior response rate and less toxicity as compared with the combination of chlorambucil and prednisolone [6, 38]. Gandara et al. [11] have confirmed the effectiveness of prednimustine in non-Hodgkin's lymphoma (NHL) that is refractory to standard treatment. In addition, prednimustine has proved to be less toxic to hematopoietic stem cells than chlorambucil or various other cytotoxic drugs [9]. This indicates that prednimustine may represent more than a simple substitute for its two components prednisolone and chlorambucil.

To test the hypothesis of a specific/selective transmembrane transport of prednimustine, we initiated a study of the intracellular uptake of the drug. We performed measurements of the intracellular concentration of prednimustine (PM), chlorambucil (CLB), phenylacetic acid mustard (PAAM) and prednisolone (P) using the experimental in vitro tumour cell lines MCF-7, CEM, MEWO and HT-29 colon carcinoma, which were incubated with either PM or the mixture of CLB + P. To study the cellular uptake and the intracellular kinetics of these substances, we modified the preanalytical preparation and HPLC assay previously described for the determination of CLB, PAAM and PM in plasma [24] (see Fig. 1 for chromatograms). The identity of the main components was established by mass spectrometry.

In MCF-7, MEWO, CEM and HT-29 cells that had been incubated with either PM or the mixture of CLB + P, the intact steroid ester PM was detectable. In the PM-incubated cells, PM was partially hydrolysed to CLB and P. The intracellular area under the curve (AUC) for CLB resulting from the hydrolysis of PM exceeded the intracellular concentration-time integral (AUC) for PM in MCF-7 mammary carcinoma cells and in HT-29 colon carcinoma cells. However, the corresponding integral for PM exceeded that for CLB in CEM lymphoma cells (see Table 1). The CLB metabolite PAAM was not detected in the PM-incubated cells. However, in CLB-incubated cells, this metabolite resulting from β-oxidation of the butyryl side chain of CLB was formed to a high degree. Although PAAM contains the two 2-chloroethyl moieties and therefore exhibits alkylating potential similar to that of CLB, the intracellular concentration-time integral for alkylating molecules (alkylating capacity) was higher in PM-incubated cells (Σ PM + CLB) than in the CLB + P-incubated MCF-7 cells (Σ CLB + PAAM). Although it has been shown that PAAM exerts similar inhibitory effects on the turnover of colony-forming units [13], this metabolite has been ascribed a more general toxic effect [24, 28].

In our previously published pharmacokinetic study [35], patients who had been given the mixture of CLB + P presented appreciably higher plasma concentrations of PAAM than did those who had been treated with PM. In the present in vitro experiments, we confirmed this constellation, with relevant intracellular levels of PAAM occurring in tumor cells that had been incubated with CLB + P but nearly negligible concentrations being observed in PM-incubated tumor cells. The lower toxicity and the higher therapeutic index that has been attributed to PM [4, 5, 15] may therefore result from a reduced β-oxidation of the CLB to PAAM when CLB is given in the form of its P ester.

Identification of PM as the intact steroid ester within the incubated tumour cells and the long-lasting intracellular concentration-time profile of PM are consistent with the postulate of facilitated intracellular uptake of PM and with the production of a slow-release formulation of CLB by PM, as suggested previously [17]. Our in vitro results demonstrate a prolonged, more continuous exposure of cells to CLB following their incubation with PM as compared with the combination of CLB + P. This result is consistent with the hypothesis that the increased anticancer activity of PM demonstrated in a variety of experimental [9, 15] and human tumors [5, 27], which is different from that of CLB and the unlinked components CLB + P, is attributable to a more continuous exposure to and availability of CLB.

However, no intact PM has thus far been detected in serum from animals or patients [8, 21, 24]. It has therefore been postulated that rapid hydrolytic pre- or postabsorptive decomposition of the ester by esterases may occur. However, we could not detect any PAAM, which would arise from β-oxidation, but a previously unknown substance exhibiting molecular ions lower than those of PM (MH^+ = 642/644) were found. It could be formed after an oxidation step in the steroid moiety of the PM molecule. Apart from a pharmacokinetic advantage of PM as a slow-release form of CLB the rationale of a more "side"-specific anticancer effect for PM based on a selective/facilitated intratumoral transport such as that demonstrated for tumor cells in vitro has not been elucidated in vivo.

Thus, in summary, our results confirm the rationale for the design of the drug. One of the main topics of our further investigation will therefore be the intracellular analysis of PM and CLB in the cellular compartment of target cells during therapy with PM (e.g. lymphocytes from patients with chronic lymphocytic leukemia and mammary carcinoma). Fast atom bombardment and related desorption techniques in mass spectrometry can be successfully applied to clinical problems such as the identification of metabolic products or of steroid conjugates [39].

Acknowledgements. The excellent technical assistance of Ms K. Springmann and Ms. R. Lange is gratefully acknowledged. We thank Dr. B. Hartley-Asp and Dr. O. Gunnarsson, Pharmacia Leo, Helsingborg, Sweden, for scientific advice and for reading the manuscript.

References

1. Reference deleted
2. Reference deleted
3. Barber M, Bardoli R, Sedgwick RD, Tyler AN (1981) J Chem Soc Chem Commun 7: 325
4. Berger MR, Habs M, Schmähl D (1986) Long-term toxicology effects of prednimustine in comparison with chlorambucil, prednisolone and chlorambucil plus prednisolone in Sprague-Dawley rats. Sem Oncol 13 [Suppl 1]: 8–13
5. Brandt L, Könyves I, Möller TR (1975) Therapeutic effect of LEO1031, an alkylating corticosteroid ester in lympho-proliferative disorders: I. Chronic lymphocytic leukemia. Acta Med Scand 197: 317–322
6. Cavalli F (1985) Sterecyt beim fortgeschrittenen Mammacarcinom. In: Nagel GA, Hincke A (eds) Hormongebundene Zytostatia (Aktuelle Onkologie, vol 25) W. Zuckschwerdt, München, pp 54–61
7. Catane R, Kaufmann JH, Madajewicz S, et al (1978) Prednimustine therapy of advanced prostatic cancer. Br J Urol 50: 29–32
8. Ehrsson H, Wallin I, Nilsson SO, Johannson B (1983) Pharmacokinetics of chlorambucil in man after administration of the free drug and its prednisolone ester (Prednimustine, Leo 1031). Eur J Pharmacol 24: 251–253
9. Evenaar AH, Wins EHR, Putten LM van (1973) Cell killing effectiveness of an alkylating steroid (LEO 1031). Eur J Cancer 9: 773–774
10. Fredholm B, Gunnarsson K, Jensen G, Muntzing J (1978) Mammary tumor inhibition and subacute toxicity in rats of prednimustine and its molecular components chlorambucil and prednisolone. Acta Pharmacol Toxicol (Copenh) 42: 159–163
11. Gandara DR, Wold G, Redmond J, Kohler M, Reynolds R, Wong P, Forsythe J, Fisher K, Lewis B (1986) Prednimustine in refractory non-Hodgkin's lymphoma. A phase II study of the Northern California Oncology Group. Semin Oncol 13 [Suppl 1]: 14–18
12. Gaver RC, Deeb G, Pittman KA, Issell BF, Mittelman A, Smyth RD (1983) Disposition of orally administered C^{14}-prednimustine in cancer patients. Cancer Chemother Pharmacol 11: 139–143
13. Goodman GE, McLean A, Alberts DS, Chang SY (1980) Inhibition of human tumour clonogenicity by chlorambucil and its metabolites. Br J Cancer 25: 45–52
14. Gunnarson PO, Johansson SA, Svensson L (1984) Cholesterol ester formation by transesterification of chlorambucil: a novel pathway in drug metabolism. Xenobiotica 14: 569–574
15. Harrap KR, Riches PG, Gilby ED, Sellwood SM, Wilkinson R, Könyves I (1977) Studies on the toxicity and antitumor activity of prednimustine, a prednisolone ester of chlorambucil. Eur J Cancer 13: 873–881
16. Hartley-Asp B (1986) The antitumor effect of prednimustine in vitro and in vivo. Semin Oncol 13 [Suppl 1]: 3–7
17. Hartley-Asp B, Gunnarson PO, Liljequist J (1986) Cytotoxicity and metabolism of prednimustine, chlorambucil and prednisolone in a chinese hamster cell line. Cancer Chemother Pharmacol 16: 85–91
18. Ideström K, Kimby E, Bjoerkholm M, Mellstedt H, Engstedt L, Gahrton G, Johansson B, Killander D, Roberts KH, Stalfelt AM, Uden AM, Wadman B, Waehlby S (1982) Treatment of chronic lymphocytic leukemia and well-differentiated lymphocytic lymphoma with continuous low- or intermittent high-dose prednimutine versus chlorambucil/prednisolone. Eur J Cancer 18: 1117–1123
19. Kaufmann JH, Hanjura GL, Mittelman A (1976) Study of LEO1031 (NCS-134087) in lymphocytic lymphoma and chronic lymphocytic leukemia. Cancer Treat Rep 60: 277–279
20. Kirdani RY, Murphy GP, Sandberg AA (1978) Some metabolic aspects of a nitrogen mustard of prednisolone. Oncology 35: 47–53
21. Knospe WH, Loeb V, Huguley CM (1974) Bi-weekly chlorambucil treatment for chronic lymphocytic leukemia. Cancer 33: 555–562
22. Koenyves I, Liljequist J (1976) The steroid group as a carrier of cytotoxic groups. In: Davis W (ed) Biological characterisation of human tumours. Elsevier, Amsterdam, p 98
23. Koenyves I, Nordenskjoeld B, Plym Forshell G, Schryver A de, Westerberg-Larson W (1975) Preliminary clinical and absorption studies with prednimustine in patients with mammary carcinoma. Eur J Cancer 11: 841–844
24. Lee FYF, Coe P, Workman P (1986) Pharmacokinetic basis for the comparative toxicity of chlorambucil, phenylacetic acid mustard and β,β-difluorochlorambucil (CB 7103) in mice. Cancer Chemother Pharmacol 17: 21–29
25. Lele SB, Piver MS, Barlow JJ, et al (1978) LEO 1031 (NSC-134087) in gynecological malignancies. Oncology 35: 10–102
26. Link KH (1986) Zytostatika-Sensitivitätstestung. Fortschr Med 104 [Suppl 5]: 13–14
27. Loeber J, Mouridsen HT, Christiansen IE, Dobernowsky P, Mattson W, Roerth HT (1983) A phase III trial comparing prednimustine (Leo 1031) to chlorambucil plus prednisolone in advanced breast cancer. Cancer 52: 1570–1576
28. Mitoma C, Onodera T, Tagegoshi T, Thomas DW (1977) Metabolic disposition of chlorambucil in rats. Xenobiotica 7: 205–220
29. Mouridsen HT, Kristensen D, Halskov-Nielsen et al (1980) Phase II trial of prednimustine, Leo 1031 (NSC-134087), in advanced breast cancer. Cancer 46: 253–255
30. Musch E, Loos U, Bojar H, Hügl E, Wellens W (1987) Pharmacokinetics of prednimustine and cellular metabolism of prednimustine versus chlorambucil/prednisolone. In: Seeber S, Kaufmann M (eds) Sterecyt/prednimustine. Proceedings of a satellite symposium. AB Leo, Helsingborg, Sweden, pp 19–27
31. Newell DR, Hart LI, Harrap KR (1979) Estimation of chlorambucil,, phenyl acetic mustard and prednimustine in human plasma by high-performance liquid chormatography. J Chromatogr 164: 114–119
32. Newell DR, Shepherd CR, Harrap KR (1981) The pharmacokinetics of prednimustine and chlorambucil in the rat. Cancer Chemother Pharmacol 6: 85–91
33. Newell DR, Calvert AH, Harrap KR, McElwain TJ (1983) Studies on the pharmacokinetics of chlorambucil and prednimustine in man. Br J Clin Pharmacol 15: 253–258

34. Newell DR, Calvert AH, Harrap KR, et al (1983) The clinical pharmacology of chlorambucil and prednimustine. Br J Clin Pharmacol 16: 762–763
35. Oppitz M, Musch E, Malek M, Rüb HP, Unruh G von, Loos U, Mühlenbruch B (1989) Studies on the pharmacokinetics of chlorambucil and prednimustine in patients using a new high-performance liquid chromatographic assay. Cancer Chemother Pharmacol 23: 208–212
36. Petersen-Bjergaard J, Mork-Hansen M, Geisler CH, et al (1980) Clinical trial of prednimustine, LEO 1031 (NSC-134087): Patients with non-Hodgkin's lymphomata and chronic lymphocytic leukemia previously treated with steroids and alkylating agents. Acta Med Scand 207: 215–220
37. Rao GS (1981) Mode of entry of steroid and thyroid hormones into cells. Mol Cell Endocrinol 21: 97–108
38. Sayed A, Howe W van, Vermeulen A (1981) Prednisolone plasma levels after oral administration of prednimustine. Oncology 38: 351–355
39. Shackleton CHL, Chai W (1985) Clinical applications of steroid conjugate analysis by liquid SIMS. In: Burlinggame AL, Castagnoli N Jr (eds) Mass spectrometry in the health and life sciences. Elsevier, Amsterdam, pp 491–505
40. Szego CM, Pietras RJ (1981) Membrane recognition and effector sites in steroid hormone action. In: Litwack G (ed) Biochemical actions of hormones, vol 8. Academic Press, New York, pp 307–463