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Studies on the pharmacokinetics of chlorambucil and prednimustine in patients using a new high-performance liquid chromatographic assay*

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Summary. Following the oral administration of either chlorambucil/prednisolone or prednimustine to patients, the plasma levels of free chlorambucil and phenylacetic acid mustard, the β-oxidation product of chlorambucil, were measured using a new high-performance liquid chromatographic (HPLC) assay. This assay permitted the simultaneous detection of the analyzed compounds with a lower limit of detection of 30 ng/ml. The pharmacokinetics of chlorambucil and phenylacetic acid mustard were found to be entirely different when prednimustine was administered as opposed to its components chlorambucil and prednisolone together. After the ingestion of the conjugate, the plasma concentration-time curves of chlorambucil and phenylacetic acid mustard showed a "delayed" pattern compared with those obtained after the administration of the components. The mean area under the concentration-time curves (AUCs) of prednimustine-derived chlorambucil and phenylacetic acid mustard were 25% and 40%, respectively, of the areas obtained after a stoichiometrically equivalent dose of chlorambucil. Free plasma prednimustine could not be detected at any time. This different pharmacokinetic behavior might offer an explanation for the superior therapeutic effects of prednimustine demonstrated by clinical studies.

Introduction

For more than 30 years, chlorambucil (Fig. 1, *A*) has been widely used in the therapy of chronic lymphocytic leukemia and malignant lymphomas [8]. To improve its clinical antitumor efficacy, chlorambucil was often given together with prednisolone. Subsequently, the prednisolone ester of chlorambucil, prednimustine (Fig. 1, *D*), was introduced into therapy with the expectation that the steroid group in prednimustine would facilitate the uptake of the alkylating compound into tumor cells by way of cellular glucocorticoid receptors [6, 9].

When tested clinically, prednimustine exhibited distinct advantages over the equivalent dose of its constituents [10]; a higher rate of cell kill was observed in vitro with this drug, and in vivo it proved to be less toxic [4, 15]. However, after its administration, no intact prednimustine was found in plasma [1, 2, 14, 15], whereas its constituents chlorambucil and prednisolone could be detected [1, 14, 16]. In addition, phenylacetic acid mustard (Fig. 1, *B*), the β-oxidation product of chlorambucil, was found after the ingestion of prednimustine [14].

The pharmacokinetic parameters and availability of prednimustine-derived chlorambucil were different from those obtained after the administration of chlorambucil itself [1, 14]. It was assumed that prednimustine underwent hydrolysis during absorption and in plasma [7]. In the present study, we compared the bioavailability of chlorambucil and its metabolite in patients treated with chlorambucil/prednisolone and those treated with prednimustine. Additionally, we investigated as to whether any measurable quantity of prednimustine could be found in plasma. For this purpose, a sensitive and specific assay for phenylacetic acid mustard, chlorambucil, and prednimustine was developed and evaluated.

Materials and methods

Chemicals. Pure chlorambucil was generously contributed by Deutsche Wellcome GmbH (Burgwedel, FRG). A sample of prednimustine was provided by AB Leo (Helsingborg, Sweden). Dr. David Newell donated the phenylacetic acid mustard, which was synthesized at the Royal Cancer Research Centre (Sutton, UK). The internal standard, *N,N*-bis-(2-chloroethyl)-para-amino-methoxy-aniline, was synthesized at the Pharmaceutical Institute of the University of Bonn.

Deionized water was used for all experiments. Acetate buffer (pH 3.8) was prepared from acetic acid (0.2 *N*) and a solution of sodium acetate (55 g/l). A total of 440 ml acetic acid and 60 ml sodium acetate solution was poured into a 1-l flask and deionized water was added up to the marker. This buffer and HPLC-grade acetonitrile (Baker Chemicals, Deventer, Holland) were used for high-performance liquid chromatography (HPLC); they were filtered and degassed under vacuum suction prior to their use. For calibration curves, acetonitrile stock solutions of chlorambucil, phenylacetic acid mustard, prednimustine, and internal standard were added to pool plasma obtained from blood donors.

Apparatus. The HPLC equipment consisted of a Spectra-Physics 8700 pump (Darmstadt, FRG) fitted with a programmable dynamic mixer and a Waters 3K injection

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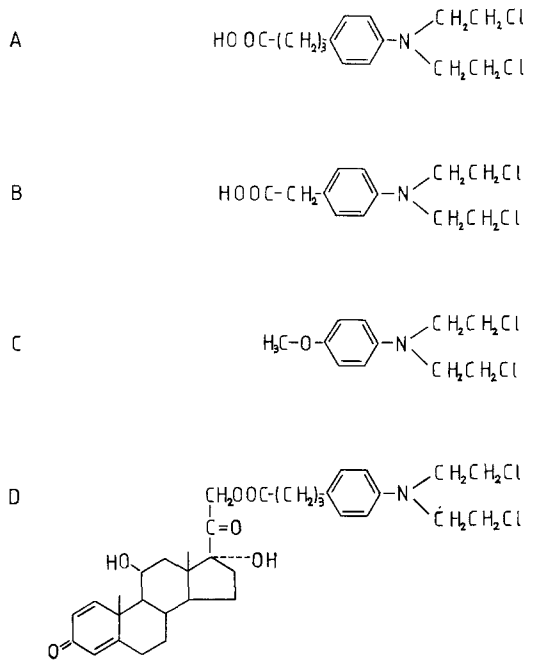

Fig. 1. Structural formulae of (**A**) chlorambucil (*N,N*-bis-(2-chloro ethyl)-amino-phenyl-butyric acid), (**B**) phenylacetic acid mustard (*N,N*-bis-(2-chloroethyl)-amino-phenyl-acetic acid), (**C**) the internal standard (*N,N*-bis-(2-chloroethyl)-para-methoxy-aniline), and (**D**) prednimustine (11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione-21,4-*N,N*-bis-(2-chloroethyl)-amino-phenyl-butyrate)

valve (Eschborn, FRG) with a 100-µl sample loop. The analytical column (25 cm × 4.6 mm inside diameter) was filled with Spherisorb 5 ODS-1; it was protected by a cartridge-system guard column containing the same material. Samples were injected with a 100-µl glass syringe (Hamilton, Bonaduz, Switzerland). The absorption of the eluents was determined by a Pye Unicam LC 871 UV-VIS spectroflow meter (Cambridge, UK) adjusted to a wavelength of 254 nm. Peaks eluted were monitored by UV absorption and quantified using a Chromatopak C3 integrator (Shimadzu, Duisburg, FRG).

Extraction procedure. Frozen plasma standards and samples from patients were thawed at room temperature. A total of 1 ml 0.067 *M* phosphate buffer (pH 7.0) (E. Merck, Darmstadt, FRG) and 1 ml 0.9% sodium chloride solution was added to 1 ml plasma. After being mixed with a mechanical shaker, each sample was transferred onto an extraction column (E. Merck, Extrelut 3, no. 15732). After 15 min the analytes were eluted from the column using 10 ml ethyl acetate followed by 10 ml 85% n-hexane/15% isoamyl alcohol. The solvents were pooled in 20-ml glass vials with pointed tips and evaporated to dryness under nitrogen at 45° C. The residues were redissolved in 250 µl acetonitrile. The concentrates were stored in capped Teflon vials at −20° C prior to analysis.

Chromatographic assay. A mobile-phase acetonitrile/acetate buffer (35%/65% v/v) was used at a flow rate of 1.5 ml/min. In all runs 40 µl sample was injected and the gradient program started. After 4.1 min, the portion of acetonitrile was increased to 60%; after that, 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 before the injection of the next sample.

Clinical pharmacokinetics. Ten patients treated with the combination chlorambucil (Leukeran)/prednisolone (Decortin H) and ten treated with prednimustine (Sterecyt) participated in the study. Both patient groups were comparable with respect to sex, age, and body weight. The patients were given either 30–50 mg chlorambucil (approx. 0.4 mg/kg body weight) and 50 mg prednisolone (approx. 0.67 mg/kg) or 200 mg prednimustine (approx. 2.9 mg/kg) orally. They took the tablets with cocoa after an overnight fast. Blood samples were drawn at 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, and 24 h after administration into heparinized tubes and centrifuged for 15 min. Plasma was isolated and frozen at −20° C until analysis. From the data obtained, the areas under the plasma concentration-time curve (AUCs) were calculated using the trapezoidal rule. All concentration-time plots could be fitted to a one-compartment model with an exponential elimination rate. The elimination half-life ($t_{½}$), the oral plasma clearance (CL), the peak plasma concentration (C_{max}), the time of peak concentration (t_{max}), and the residual area to infinity were calculated. For comparison, the dose-dependent pharmacokinetic parameters were normalized to a reference dose of 30 mg chlorambucil.

Results

We developed an HPLC assay for the quantitation of chlorambucil, phenylacetic acid mustard, and prednimustine in plasma and used it for monitoring the compounds during therapy. Figure 2 (*A*) shows a chromatogram of a plasma spiked with the compounds and Fig. 2 (*B*), the chromatogram of a blank plasma (retention times, 9.1, 12.2, 13.5, and 18.4 min for phenylacetic acid mustard, chlorambucil, internal standard, and prednimustine, respectively). Examples for patient's chromatograms are shown in Fig. 2 (*C*) (4 h after the ingestion of 200 mg prednimustine) and Fig. 2 (*D*) (2 h after the ingestion of 30 mg chlorambucil). The calibration curves were linear over the whole therapeutic range of 30–1500 ng/ml. The coefficients of correlation of the calibration curves were 0.995 for chlorambucil, prednimustine, and the internal standard and 0.992 for phenylacetic acid mustard.

The precision was evaluated by measuring ten extracts from a blank plasma to which chlorambucil, phenylacetic acid mustard, prednimustine, and the internal standard had been added (500 ng/ml each). The coefficients of variation for this series were below 3% for all compounds. Day-to-day precision for the substances was evaluated by analyzing a standard sample (500 ng/ml) on 5 days (Table 1). The day-to-day coefficients proved to be satisfactory (range, from 5% for chlorambucil to 14% for prednimustine).

For recovery evaluation, three blank plasma samples were processed as above. Stock solutions of the compounds were added to the extracts prior to chromatography; the mean values of the three samples were used as the 100% recovery value. Ten blank plasma samples were spiked with the substances and processed as described above. The mean recovery rates were 95% for chlorambucil, 80% for phenylacetic acid mustard, 69% for predni-

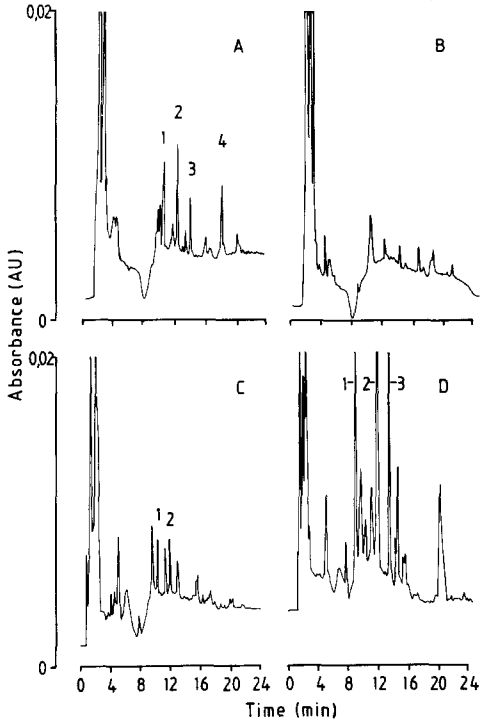

Fig. 2. HPLC chromatograms of pre-column extracts of (**A**) a plasma sample spiked with the reference compounds chlorambucil, phenylacetic acid mustard, the internal standard, and prednimustine (100 ng/ml each); (**B**) plasma of a patient before treatment; (**C**) plasma of a patient, obtained 4 h after the ingestion of 200 mg prednimustine; and (**D**) plasma of a patient, obtained 2 h after the ingestion of 30 mg chlorambucil. *Peak 1,* phenylacetic acid mustard; *peak 2,* chlorambucil; *peak 3,* internal standard; *peak 4,* prednimustine

mustine, and 85% for the internal standard. These percentages remained constant over the whole range of analysis. The lower limit of detection for chlorambucil and prednimustine was 30 ng/ml.

Tables 2 and 3 summarize the pharmacokinetic variables, which revealed striking differences between the patient groups treated with chlorambucil/prednisolone and those given prednimustine. With chlorambucil, high peak plasma concentrations (C_{max}) averaging 510 ng/ml were obtained at 0.8 h after the ingestion of the drug (see Table 2 and Fig. 3). A rapid elimination phase followed, during

Table 1. Day-to-day precision of all compounds (500 ng/ml each), calculated using their peak height

Date	CLB	PRD	PAAM	IS
11/26	14.5	9.3	11.9	8.4
11/28	14.2	9.0	11.2	10.1
12/01	14.0	8.4	11.2	7.8
12/03	14.4	8.8	11.8	6.8
12/05	13.1	8.0	10.3	7.8
x =	14.0	8.7	11.2	8.2
SD =	±0.65	±1.21	±0.76	±0.5
SV =	4.0%	14.2%	6.7%	5.7%

CLB, chlorambucil; PRD, prednimustine; PAAM, phenylacetic acid mustard; IS, internal standard; x, mean peak height (cm); SD, standard deviation; CV, coefficient of variation (%)

Table 2. Pharmacokinetic data of chlorambucil (CLB) and its metabolite phenylacetic acid mustard (PAAM) after the ingestion of chlorambucil. Mean values ± SD are given. The dose-dependent data were normalized to a chlorambucil dose of 30 mg

	t_{max} h	C_{max} ng/ml	$t_{1/2}$ h	CL/kg l/h per kg	AUC ng/ml * h
CLB	0.8 ±0.3	508 ±205	1.2 ±0.5	0.43 ±0.18	1210 ±447
PAAM	1.8 ±0.4	369 ±139	2.0 ±1.1		1434 ±697

Table 3. Pharmacokinetic data of chlorambucil (CLB) and its metabolite phenylacetic acid mustard (PAAM) after the ingestion of prednimustine. Mean values ± SD are given. The data are normalized to an equivalent chlorambucil dose of 30 mg (= 67 mg prednimustine)

	t_{max} h	C_{max} ng/ml	$t_{1/2}$ h	CL/kg l/h per kg	AUC ng/ml * h
CLB	2.7 ±1.1	68 ±47	2.4 ±1.3	2.08 ±1.17	304 ±167
PAAM	4.0 ±1.1	78 ±42	3.4 ±1.7		580 ±319

which the metabolite phenylacetic acid mustard was formed with a mean peak concentration of 369 ng/ml. The maximal concentration of phenylacetic acid mustard was found at 2 h after the administration of chlorambucil/prednisolone. With prednimustine, the peak plasma concentrations of chlorambucil and phenylacetic acid mustard were only 13% and 21%, respectively, of the values obtained after an equivalent dose of its components (see Table 3 and Fig. 4). Chlorambucil and phenylacetic acid mustard reached their maximum 2 h later when prednimustine was given instead of the constituents.

The AUCs of chlorambucil and phenylacetic acid mustard were 1210 and 1434 mg/ml*min, respectively, after the administration of the chlorambucil/prednisolone combination. The corresponding values were 304 and 580 ng/ml*min, respectively, when prednimustine was given. The mean AUC ratio between patients treated with prednimustine and those treated with chlorambucil/prednisolone was 0.25 for chlorambucil and 0.40 for phenylacetic acid mustard, clearly indicating the low amount of chlorambucil in plasma following the use of prednimustine.

We also found differences between prednimustine and the combination therapy with regard to the elimination phase of chlorambucil and phenylacetic acid mustard. Figures 3 and 4 show the plasma concentration-time curves of both compounds for the two treatment groups. After the administration of chlorambucil/prednisolone, the mean half-lives were 1.2 h for chlorambucil and 2 h for phenylacetic acid mustard (Table 2). After prednimustine, these values rose to 2.4 h and 3.7 h (Table 3), suggesting a slower elimination of prednimustine-derived chlorambucil.

Due to the lower availability of chlorambucil after prednimustine, the oral plasma clearance CL of the conjugate-derived chlorambucil was calculated to be 5 times higher than the value obtained after the administration of chlorambucil itself. As shown in Tables 2 and 3, there was

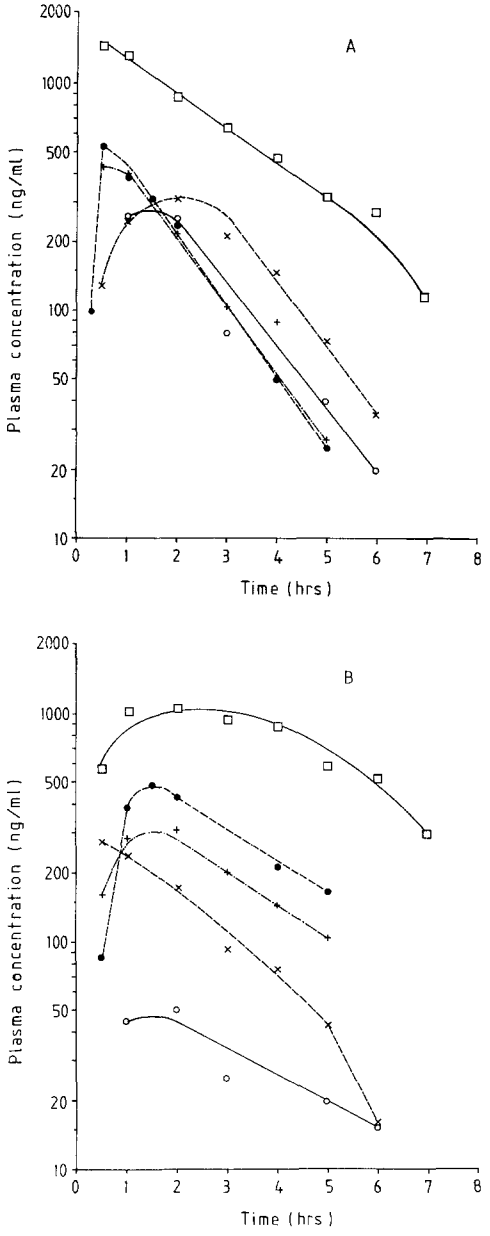

Fig. 3. Plasma concentration-time curves of (**A**) chlorambucil and (**B**) phenylacetic acid mustard in five patients after the administration of chlorambucil (symbols in both **A** and **B** represent the same patients)

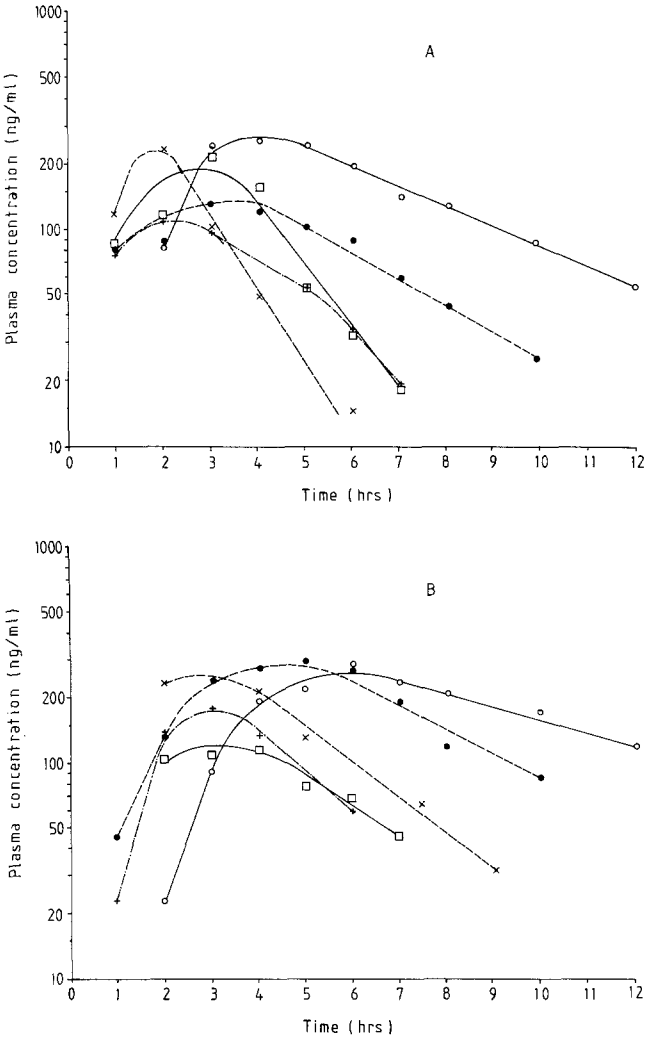

Fig. 4. Plasma concentration-time curves of (**A**) chlorambucil and (**B**) phenylacetic acid mustard in five patients after the administration of prednimustine (symbols in both *A* and *B* represent the same patients)

considerable interindividual variability in the AUCs within each group. The coefficient of variation for the AUC of chlorambucil was approximately 50% in both treatment groups, without distinct differences. Free prednimustine could not be determined in plasma at any time.

Discussion

The validation of the assay showed a sufficient day-to-day precision of 4%, 7%, and 14% for the detection of chlorambucil, phenylacetic acid mustard, and prednimustine, respectively. The calibration curves were linear from the lower limit of detection (30 ng/ml) to the maximal concentration (1500 ng/ml), covering the concentration range for pharmacokinetic analyses. The mean recovery rates of the sample preparation were 95% for chlorambucil, 80% for phenylacetic acid mustard, 69% for prednimustine, and 85% for the internal standard.

The chromatographic peaks of chlorambucil, phenylacetic acid mustard, and the internal standard were sufficiently separated. Endogenous interferences corresponded to <30 ng/ml and determined the lower limit of detection. This method was superior to the previously published HPLC-procedure [13], as (a) chlorambucil and phenylacetic acid mustard could be detected simultaneously, (b) the baseline drift of the gradient was lower, and (c) the extraction efficiency for phenylacetic acid mustard was higher.

Intact plasma prednimustine could not be detected at any time after the ingestion of the drug. Undetectable quantities lower than 30 ng/ml in the blood compartment would have accounted for $<0.1\%$ of the whole drug dose (30 ng/ml * 5000 ml = 150,000 ng, $<0.1\%$ of the 200-mg dose given). This result confirms those of previous studies [1, 2, 14, 15], where free plasma prednimustine could not be detected with different analytical techniques after oral administration.

The variable and low systemic availability of chlorambucil and prednisolone implies [1, 11, 16] that prednimus-

tine may be partially hydrolyzed to its components during absorption and/or in plasma. However, if intact prednimustine reached the plasma compartment, the drug would not be eliminated from the plasma solely by hydrolysis because this process is considerably slow in human plasma [5, 7]. To compare prednimustine and chlorambucil kinetics, we evaluated the concentration-time curves of chlorambucil and its metabolite phenylacetic acid mustard.

The availability of chlorambucil and phenylacetic acid mustard varied by about 50% after chlorambucil as well as prednimustine administration, in agreement with the results of Ehrsson et al. [1]. Newell et al. [15] found a day-to-day variation of approximately 15% for the AUC of chlorambucil in each of two patients treated with this drug. In our study, the availability of chlorambucil was 4 times lower after prednimustine administration than after the ingestion of chlorambucil itself. However, plasma chlorambucil exhibited a lowered and delayed maximum and a decreased elimination rate when prednimustine was given instead of chlorambucil. The latter difference offers an explanation for the therapeutic superiority of prednimustine over its components chlorambucil and prednisolone.

Hartley-Asp et al. [5] have proposed that the persistence of prednimustine-derived chlorambucil could be caused by a slow release of chlorambucil from protein-bound prednimustine. This has not yet been confirmed. The transesterification of chlorambucil from prednimustine to a chlorambucil-cholesterol ester discovered in animals [3] could be another mechanism that would explain the delayed metabolism of chlorambucil derived from prednimustine. Another cause might be a facilitated transport of the conjugate into cells by specific cell membrane carriers. Musch et al. [12] succeeded in detecting intact prednimustine in human lymphocytes isolated from the blood of patients with chronic lymphocytic leukemia who were undergoing prednimustine therapy. This would indicate that after absorption, a portion of the intact prednimustine is rapidly taken up by the cells that are the target of the chemotherapeutic agent. Further studies confirming this mechanism should be conducted. Investigations of the cellular uptake of prednimustine and chlorambucil are currently under way.

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