Biochemical Pharmacology, Vol. 32, No. 3, pp. 535–539, 1983.
Printed in Great Britain.

0006-2952/83/030535-05 $03.00/0
© 1983 Pergamon Press Ltd.

UPTAKE AND DECOMPOSITION OF CHLORAMBUCIL BY L5178Y LYMPHOBLASTS *IN VITRO**

ASHER BEGLEITER† and GERALD J. GOLDENBERG
The Manitoba Institute of Cell Biology and the Department of Medicine, University of Manitoba, Winnipeg, Manitoba R3E 0V9, Canada

(*Received* 14 *April* 1982; *accepted* 28 *July* 1982)

Abstract—The uptake of [^{14}C]chlorambucil by L5178Y lymphoblasts was studied using thin-layer chromatography to identify the various radioactive components that enter or leave cells. Theoretical calculations predicted that entry of chlorambucil into cells by simple diffusion would be rapid and essentially complete in 45 sec or less. Uptake of intact chlorambucil was rapid, reaching a cell/medium ratio of approximately 1.5 in less than 15 sec at both 37° and 4°, consistent with a simple diffusion mechanism. In cells treated with [^{14}C]chlorambucil for 60 min, the intracellular level of intact drug decreased with time, and this decay was attributed to hydrolysis and alkylation. The level of intact drug in the medium decreased at a similar rate resulting in a nearly constant cell/medium distribution ratio. Intact chlorambucil in the cells was found to be entirely ethanol- and trichloroacetic acid-soluble. Efflux of intact chlorambucil was very rapid and temperature-insensitive. These findings suggest that chlorambucil efflux, as well as influx, is by a simple diffusion mechanism. A derivative of chlorambucil was found in ethanol solutions of the drug. This derivative, which may be the ethyl ester of the drug, is highly concentrated in cells and may interfere with pharmacological investigations of chlorambucil.

Chlorambucil, a nitrogen mustard analog containing the phenylbutyric acid moiety, has been used extensively in the treatment of chronic lymphocytic leukemia [1, 2] and malignant lymphoma, especially well-differentiated lymphocytic lymphoma [3, 4]. The mechanism of antitumor action of this agent is believed to involve alkylation and cross-linking of cellular DNA [5, 6]; however, because of its ability to interact with various cell components including the cell membrane [7, 8] and nuclear proteins [9], other possible cytotoxic effects have been implicated [7, 8, 10, 11].

The uptake of chlorambucil by sensitive and resistant Yoshida ascites sarcoma cells *in vitro* has been studied [12–14]. Evidence was presented that uptake occurs by passive diffusion; the drug was not accumulated against a concentration gradient, and uptake was temperature-insensitive and was unaffected by the presence of metabolic inhibitors. Furthermore, a temperature-sensitive loss of drug from the cells was observed, which was attributed to degradation of the aromatic ring of chlorambucil [12].

In this study we describe the use of thin-layer chromatography (TLC) to separate intact chlorambucil from hydrolysis and alkylation products, providing a more detailed evaluation of chlorambucil uptake and decomposition by L5178Y lymphoblasts.

MATERIALS AND METHODS

[^{14}C]Chlorambucil [*p*-(di-2-chlorethyl)amino-γ-[^{14}C]phenylbutyric acid; sp. act. 6.1 mCi/mmole] was supplied by Drs. A. L. Weinberg and W. G. Duncombe of the Wellcome Research Laboratories, Beckenham, Kent, England. TLC studies to separate [^{14}C]chlorambucil from other radiolabeled components were carried out on silica gel 60 precoated plastic sheets (E. Merck, Darmstadt, West Germany) in ether–heptane (5:1) by procedures described previously [15].

Transport studies were performed on suspension cultures of L5178Y lymphoblasts incubated *in vitro* at cell concentrations of 2–4 × 10^6 cells/ml in Fischer's medium as described previously [16, 17]. The cells were washed and, then, lysed in water at 4° for 15 min, and the cell lysate and transport medium were analyzed by TLC. Uptake of intact drug was expressed as the ratio of radioactivity of intact drug in the cell to that of an equivalent volume of extracellular medium [16, 18]. Cell volume was determined in a Coulter counter, model Z_{B1} (Coulter Electronics, Hialeah, FL) calibrated with paper-mulberry spores (mean cell diameter, 12.5 μm) obtained from Coulter Diagnostics, Inc., Miami Springs, FL. Efflux studies were performed on cells in Fischer's medium as described previously [19].

For ethanol and trichloroacetic acid (TCA) solubility studies, washed cells were treated with ethanol or 10% TCA for 1 hr at 4° and filtered through 0.45 μm cellulose filters (Millipore Corp., Bedford, MA). Cell suspensions were analyzed by TLC before filtering to determine the total concentrations of each of the radiolabeled components and after filtration to determine the ethanol or TCA solubilities of the components.

RESULTS AND DISCUSSION

As discussed previously [20], Lieb and Stein [21, 22] have shown that the permeability of a cell

* This work was supported by the National Cancer Institute of Canada.
† Author to whom all correspondence should be addressed.

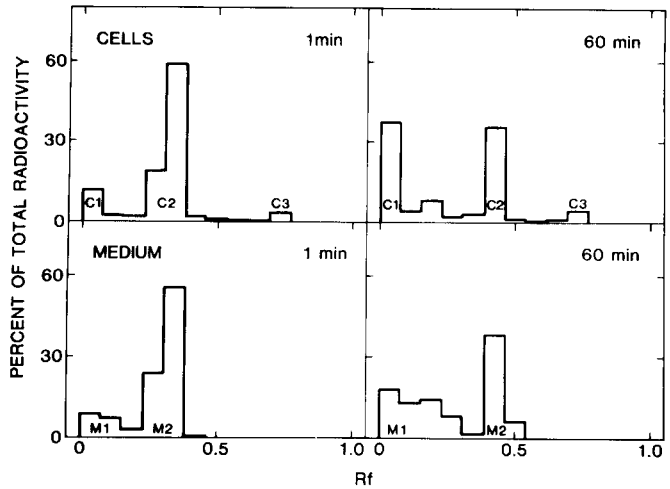

Fig. 1. Results of thin-layer chromatography of [^{14}C]chlorambucil and other radiolabeled constituents on plastic sheets precoated with silica gel 60 (E. Merck, Darmstadt, West Germany). Lysed cell suspensions and supernatant medium were chromatographed after treating 2–4 × 10^6 L5178Y lymphoblasts with 100 μM [^{14}C]chlorambucil for 1 and 60 min at 37°, in Fischer's medium. Separation was achieved using a solvent system consisting of ether–heptane (5 : 1); sequential 1 cm strips of silica gel were cut, and radioactivity was determined by liquid scintillation spectrometry as described previously [24].

membrane to a compound can be predicted from the equation

$$P_s = P_0 M^{-S_m} K^{S_k}$$

where P_0 is a measure of the overall permeability of the membrane; M is the relative molecular weight of the compound compared to methanol, and S_m is the mass selectivity coefficient or the ability of the membrane to discriminate between permeants by virtue of size; K is the partition coefficient for distribution of the permeant between a model solvent and an aqueous phase, and S_k is the accuracy with which the model solvent describes the solvent properties of the membrane under consideration.

The partition coefficient for chlorambucil was determined by measuring the distribution of ^{14}C-labeled drug in equal volumes of octanol and water. The partition coefficient K (mean ± S.E.) was found to be 40.2 ± 6.5, indicating that chlorambucil, which is a weak acid, is only slightly dissociated in water. Using the Lieb and Stein equation, as we have done previously for procarbazine [20] and for hexamethylmelamine and pentamethylmelamine [23], entry of chloroambucil into cells by simple diffusion was predicted to be very rapid, being essentially complete in 45 sec or less.

In order to identify free, intact drug, uptake of radioactivity by L5178Y cells incubated with [^{14}C]chlorambucil was analyzed using TLC; in these studies total recovery of total radioactivity ranged from 80 to 100%. The results of the chromatography of cell contents and medium of cell suspensions treated with freshly prepared 100 μM [^{14}C]chlorambucil are shown in Fig. 1. After 1 min, approximately 80% of the radioactivity in the medium was in a major peak (M2) with an R_f of approximately 0.37, which was identical to that of intact chlorambucil; the remainder of the radioactivity migrated at or near the origin (M1). The radioactivity in the cells appeared in three peaks; one peak (C2), accounting for approximately 80% of the radioactivity in the cells, represented intact chlorambucil; a small peak (C1) at or near the origin, consisting of 15% of the radioactivity in the cells, probably represented hydrolyzed drug and/or drug bound to macromolecules; and a third peak (C3), amounting to less than 5% of the radioactivity in the cells had an R_f value of approximately 0.70. After 60 min the peak due to intact drug had decreased to approximately 40% of the radioactivity in both the cells and the medium, while the origin peaks had increased to approximately 40 and 30% in the cells and medium respectively. The bifid peak at and above the origin probably represents di- and mono-hydrolyzed drug respectively.

The $T_{\frac{1}{2}}$ (mean ± S.E.) for disappearance of intact chlorambucil from the medium was 43.6 ± 1.5 min and that for appearance of radioactivity at and above the origin was 43.9 ± 10.9 min. These values are essentially identical to the $T_{\frac{1}{2}}$ of 42 min reported previously for hydrolysis of chlorambucil in blood [25]; this finding suggests that the disappearance of intact drug from the medium resulted primarily from non-enzymatic hydrolysis and alkylation rather than from cell-mediated metabolism and subsequent efflux.

A time course of uptake of intact chlorambucil by L5178Y cells incubated with 100 μM radiolabeled drug at 37° and 4° is shown in Fig. 2. Uptake of chlorambucil reached a steady-state level within 1 min and the cell/medium drug distribution ratio remained constant at approximately 1.5, for 60 min (Fig. 2, upper panel). Additional influx studies of intact chlorambucil with incubation times of less than 1 min showed that the steady state was reached in less than 15 sec. Furthermore, evidence that uptake was non-saturable was the observation that the cell/medium distribution ratio remained essentially

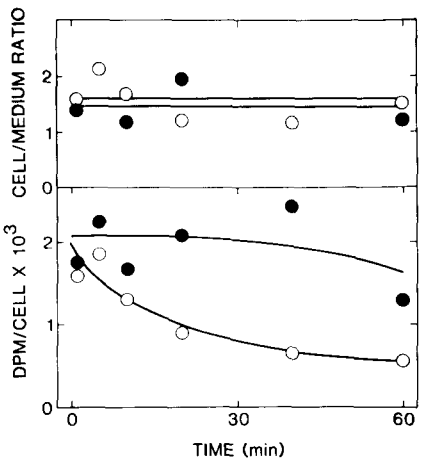

Fig. 2. Time course of uptake of 100 μM [^{14}C]chlorambucil by L5178Y cells *in vitro* at 37° (○) and 4° (●). Upper panel: uptake is expressed as the cell/medium ratio of intact chlorambucil determined by TLC analysis as described in the text. Lower panel: uptake is expressed as the dpm/cell × 10^3 of intact drug as determined by TLC analysis.

constant at extracellular chlorambucil concentrations ranging from 1 to 100 μM.

The level of intact drug within the cell decreased with time and this decay was greater at 37° than at 4° (Fig. 2, lower panel). The level of intact drug in the medium also decreased (Fig. 1), and this apparently occurred at a rate similar to that in the cells, thereby explaining the constant cell/medium ratio observed (Fig. 2, upper panel). A corresponding increase in origin counts was observed in both the cell and supernatant fractions, suggesting that disappearance of intact drug was due to hydrolysis and alkylation.

Thus, uptake of chlorambucil was rapid, reaching a steady-state level in less than 15 sec as predicted by theoretical calculations for a simple diffusion mechanism; the cell/medium ratio in the steady state was approximately 1.5, and uptake was non-saturable and temperature independent. These results

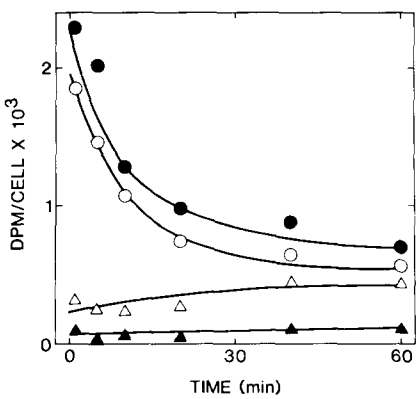

Fig. 3. Time course of radioactivity of intact chlorambucil and of the origin fraction in L5178Y cells incubated with 100 μM [^{14}C]chlorambucil at 37°. The data are presented as dpm/cell × 10^3 for total (○) and ethanol-soluble (●) intact drug, and for total (△) and ethanol-soluble (▲) components of the origin fraction. Ethanol-soluble counts were determined as described in the text.

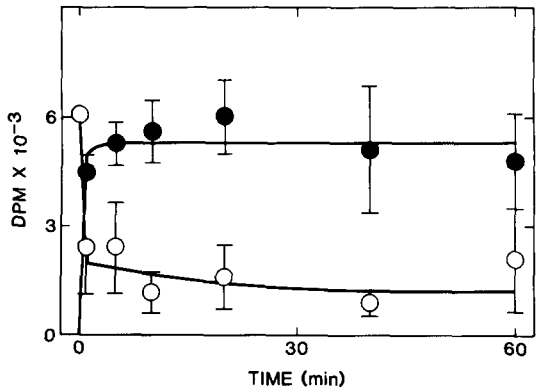

Fig. 4. Time course of efflux of intact chlorambucil from L5178Y lymphoblasts preloaded with 100 μM [^{14}C]chlorambucil for 10 min at 4°, washed through sucrose to remove extracellular radioactivity, and resuspended at approximately 3 × 10^6 cells/ml in Fischer's medium at 37°. Aliquots (1 ml) of cell suspensions were removed, and the radioactivity due to intact chlorambucil (dpm × 10^{-3}) remaining in the cells (○) or appearing in the medium (●) is plotted as a function of efflux time. The data represent the means ± S.E. of three determinations.

provide strong evidence that influx of intact chlorambucil is by a simple diffusion mechanism.

Ethanol extraction of cells incubated with [^{14}C]chlorambucil showed that all of the intact drug in the cell was ethanol-soluble (Fig. 3). Total activity of the origin fraction appeared to increase with time; the ethanol-soluble portion of origin counts appeared to increase only slightly, so that the major increase in the origin fraction was due to ethanol-insoluble components (Fig. 3). The ethanol-soluble component of the origin fraction probably represented hydrolyzed drug, whereas the insoluble fraction may have represented drug bound to cell components. Similar results were obtained in extraction studies with TCA.

Of interest was that the major decrease within the cell of intact drug was associated with only a slight increase of bound drug and little change in hydrolyzed drug. There are at least two possible explanations for this disparity: first, there may have been efflux of intact drug as a consequence of extracellular hydrolysis or, second, there may have been intracellular hydrolysis followed by a relatively rapid efflux of this polar species. Although a clear choice is not possible, the first alternative might be more likely since non-polar compounds generally traverse the cell membrane rapidly by simple diffusion.

Efflux of intact chlorambucil from L5178Y cells was very rapid at 37° (Fig. 4) with greater than 60% of the drug being lost from the cells in 1 min. A correspondingly rapid increase of chlorambucil in the medium was also observed. Thereafter there appeared to be a slow loss of intact drug in the cells probably due to hydrolysis and alkylation. The rapid efflux, which was also observed at 4°, is consistent with a simple diffusion mechanism for drug exit.

The non-polar component (C3) observed in cells treated with chlorambucil was of interest since it was possible that it represented a metabolite of the drug. However, chromatographic analysis of ethanol solutions of chlorambucil showed the presence of a

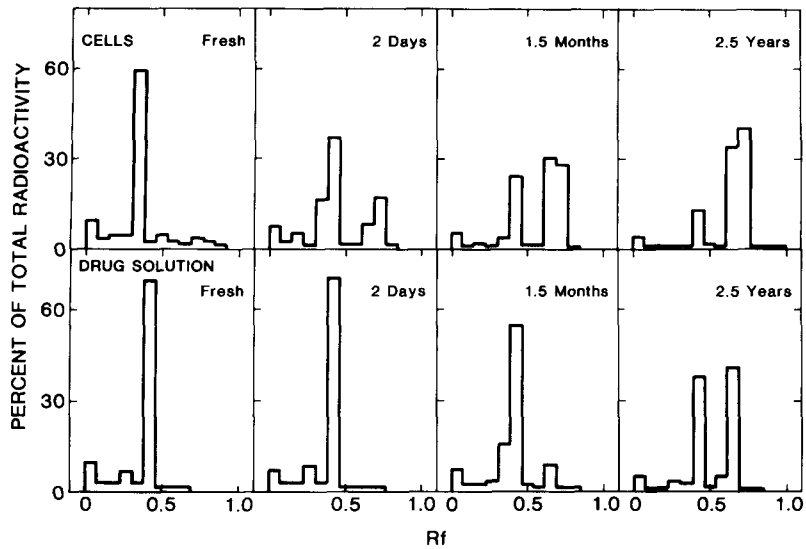

Fig. 5. Thin-layer chromatograms of radioactive components of cells treated with ethanolic solutions of [^{14}C]chlorambucil of different ages (upper panels) and of the same solutions of chlorambucil that had been used to treat these cells (lower panels). Cells were treated with 100 μM [^{14}C]chlorambucil for 1 min at 4° in Fischer's medium in a final concentration of 1:100 of ethanol. The actual concentrations of intact chlorambucil varied from 75 μM in the fresh preparation to 40 μM in the 2.5-year-old preparation.

peak with an R_f value identical of that of C3 (Fig. 5). Furthermore, this peak increased in prominence with increasing age of the drug solution (Fig. 5, lower panels). The non-polar nature of this component and its slow formation in ethanol solution suggests that C3 may have been the ethyl ester of chlorambucil, a compound that has been reported previously [26]. Attempts to isolate and characterize this compound have been confounded by the unavailability of an authentic sample with which to conduct comparative studies.

The chlorambucil derivative appeared to be highly concentrated in cells (Fig. 5, upper panels), reaching apparent cell/medium ratios as high as 150-fold. The apparent concentrative accumulation of the ethyl ester suggests that it might be more potent than the parent compound; however, the ethyl ester of chlorambucil demonstrated less antitumor activity than the parent drug against the Walker carcinoma *in vivo* [26].

The finding of a derivative of chlorambucil in ethanol solutions of the drug poses a potential pitfall in pharmacological investigation of this compound. Even minor contamination of chlorambucil solutions by this derivative may complicate studies since the derivative is rapidly concentrated by cells in suspension. This problem may be avoided by preparing drug solutions immediately prior to use or by using solvents other than ethanol.

The findings in this study that the rate of uptake of intact chlorambucil was of the order of magnitude expected for a simple diffusion system, that uptake was independent of temperature, and that drug was not concentrated against a concentration gradient suggested that chlorambucil uptake was by simple diffusion. The temperature-sensitive loss of drug from L5178Y cells appeared to be due to alkylation and hydrolysis. Finally, rapid efflux of chlorambucil from cells at both 37° and 4° suggested a simple diffusion mechanism for drug exit.

Acknowledgements—We thank Judy Grover for excellent technical assistance and Dorothy Faulkner and Janis Zamick for typing this manuscript.

REFERENCES

1. D. A. G. Galton, E. Wiltshaw, L. Szur and J. V. Dacie, *Br. J. Haemat.* **7**, 73 (1961).
2. E. Z. Ezdinli and J. Stutzman, *J. Am. med. Ass.* **191**, 444 (1965).
3. H. Kersten and H. M. Rauwen, *Nature, Lond.* **190**, 1195 (1961).
4. S. E. Jones, S. A. Rosenberg, H. S. Kaplan, M. E. Kadin and R. F. Dorfman, *Cancer, N.Y.* **30**, 31 (1972).
5. P. Brookes and P. D. Lawley, *Biochem. J.* **80**, 596 (1961).
6. P. D. Lawley, *Prog. nucleic Acid Res.* **5**, 89 (1966).
7. J. H. Linford, A. Froese and L. G. Israels, *Nature, Lond.* **197**, 1068 (1963).
8. J. H. Linford, W. Hryniuk and L. G. Israels, *Biochem. Pharmac.* **18**, 2723 (1969).
9. P. G. Riches and K. R. Harrap, *Cancer Res.* **33**, 389 (1973).
10. P. G. Riches, E. W. Gascoigne, C. L. Leese and K. R. Harrap, *Biochem. Pharmac.* **24**, 951 (1975).
11. K. R. Harrap and M. E. Furnass, *Eur. J. Cancer* **9**, 343 (1973).
12. K. R. Harrap and B. T. Hill, *Biochem. Pharmac.* **19**, 209 (1970).
13. B. T. Hill, M. Jarman and K. R. Harrap, *J. med. Chem.* **14**, 614 (1971).
14. B. T. Hill, *Biochem. Pharmac.* **21**, 495 (1972).
15. A. Begleiter, H-Y. P. Lam and G. J. Goldenberg, *Cancer Res.* **37**, 1022 (1977).
16. G. J. Goldenberg, C. L. Vanstone, L. G. Israels, D. Ilse and I. Bihler, *Cancer Res.* **30**, 2285 (1970).
17. G. J. Goldenberg, C. L. Vanstone and I. Bihler, *Science* **172**, 1148 (1971).

18. G. J. Goldenberg, *Cancer Res.* **34**, 2511 (1974).
19. A. Begleiter, J. Grover and G. J. Goldenberg, *Cancer Res.* **42**, 987 (1982).
20. H-Y. P. Lam, A. Begleiter, W. Stein and G. J. Goldenberg, *Biochem. Pharmac.* **27**, 1883 (1978).
21. W. R. Lieb and W. D. Stein, *Nature, Lond.* **224**, 240 (1969).
22. W. R. Lieb and W. D. Stein, in *Current Topics in Membranes and Transport* (Eds. A. Koeinzeller and F. R. Bronner), Vol. 2, p. 1. Academic Press, New York (1971).
23. A. Begleiter, J. Grover and G. J. Goldenberg, *Cancer Res.* **40**, 4489 (1980).
24. G. J. Goldenberg, M. Lee, H-Y. P. Lam and A. Begleiter, *Cancer Res.* **37**, 755 (1977).
25. W. C. J. Ross, *Biological Alkylating Agents*, p. 98. Butterworths, London (1962).
26. J. L. Everett, J. J. Roberts and W. C. J. Ross, *J. chem. Soc.* 2386 (1953).