

benzene, 827-52-1; 1-acetyl-4-(3,4-dichlorobenzyl)benzene, 77529-35-2; *p*-bromoacetophenone, 99-90-1; 2-phenylindole, 948-65-2; (*E*)-4-phenyl-3-buten-2-one, 1896-62-4; 2-undecanone, 112-12-9; 4'-nonylacetophenone, 37593-05-8; (*E*)-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one, 79-77-6; 4'-phenyl-

acetophenone, 92-91-1; 4-acetyl-4'-(benzylthio)[1,1'-biphenyl], 85763-20-8; 4-acetyl-4'-[[2-(4-pyridyl)ethyl]thio][1,1'-biphenyl], 85763-21-9; 4-acetyl-4'-[[[3,4-dihydro-3-hydroxy-2*H*-1,5-benzodioxepin-3-yl)methyl]thio][1,1'-biphenyl], 85763-22-0; 4'-(1,2,3,4-tetrahydronaphth-1-yl)acetophenone, 40845-96-3.

1,3-Dipalmitoylglycerol Ester of Chlorambucil as a Lymphotropic, Orally Administrable Antineoplastic Agent

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A glyceride derivative of chlorambucil (**2**), 1,3-dipalmitoyl-2-[4-[bis(2-chloroethyl)amino]benzenebutanoyl]glycerol (**1**), was synthesized and tested as an orally administrable antineoplastic drug endowed with lymphotropic properties. A significantly higher efficacy (increased life span) and a reduced toxicity of **1**, relative to **2**, were apparent when both compounds given per os were evaluated against P388 leukemia subcutaneously implanted in mice, a situation where the tumor cells disseminate along the lymphatic route. In order to assess the selective absorption of **1** by the intestinal lymphatic system after oral administration, we determined plasma and intestinal lymphatic concentrations and compared them with that obtained with **2**. The results clearly demonstrate that the esterification of **2** to a diacylglycerol moiety brings about considerably higher levels in the lymph and reduces plasma levels. Moreover, pharmacokinetic and biological data suggest that **1** is most probably acting by itself rather than as a prodrug of chlorambucil.

In recent years, there has been increasing interest in the design of lymphotropic delivery systems for anticancer agents.¹ Indeed, a selective lymphatic administration of antineoplastic drugs can be of major significance for cancer chemotherapy in the frequent situation where tumor cells spread along the lymphatic pathways and metastasize in the lymph nodes. Various types of emulsions and liposomes administered via the parenteral route have been used to deliver drugs into lymphatics for the treatment of metastasis.²⁻⁸ Similarly, selective absorption of bleomycin from intestinal loops has been observed in rat by using a bifunctional delivery system that combines the lymphotropic properties of dextran sulfate with the absorption-inducing properties of lipid-surfactant mixed micelles.^{9,10}

As an alternative to these supramolecular vectorized forms of antineoplastic drugs, we are presently interested in the design of synthetic acylglycerols incorporating a chemotherapeutic moiety in the 2-position of glycerol and fatty acids in the 1- and 3-positions, which might be absorbed via enteral route, preferably through the intestinal lymphatic system. The rationale behind this monomolecular approach is based on the well-known essential role played by this system in the physiological process of lipid absorption. Moreover, the use of a glyceride-like structure as a lymphotropic carrier of anticancer agents currently administered per os is expected to reduce the gastrointestinal toxicity of these compounds. As a matter of fact, such an effect has been previously reported in the group of antiinflammatory agents with a dipalmitoyl derivative of acetylsalicylic acid.¹¹

In order to explore this possibility, we synthesized 1,3-dipalmitoyl-2-[4-[bis(2-chloroethyl)amino]benzenebutanoyl]glycerol (**1**), a glyceride derivative of chlorambucil (**2**). Chlorambucil was selected as a model on the basis of the following considerations: (a) it is one of the most effective agents in the treatment of Hodgkin's disease and other lymphomas, (b) it is a standard orally administered

antineoplastic drug, and (c) it can be easily attached to glycerol via an ester linkage, with its alkylating bis(2-chloroethyl)amino head remaining free; consequently, the resulting derivative **1** might be expected to have by itself carcinostatic activity.

In the present paper, we describe the preparation of **1** and the comparative study of the antitumor activity of **1** and **2**, determined against P388 leukemia in mice. This tumor disseminates lymphatically when it is transplanted subcutaneously (sc).¹² Experimental results reveal a highly significant increase in activity of **1** compared with **2** when both compounds are orally administered. Additional evidence of the lymphotropic properties of **1** is presented, resulting from a pharmacokinetic experiment designed to study the intestinal lymphatic uptake of the radiolabeled compound after oral administration.

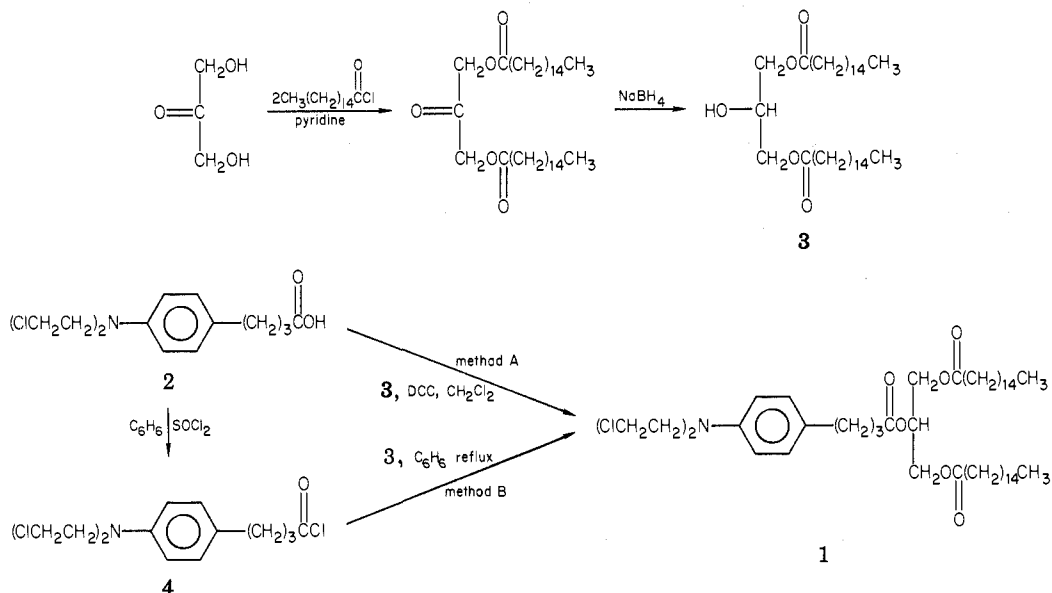
Chemistry. Compound **1** was synthesized as outlined in the Scheme I. Initially, chlorambucil (**2**) was allowed to react with 1,3-dipalmitin (**3**) in solution in CH₂Cl₂ in

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Scheme I



the presence of dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine at ambient temperature. Subsequently, it was found that 4-(dimethylamino)pyridine was detrimental to the stability of 1 and that higher yields could be obtained in the absence of this catalyst (method A). However, the highest yields (85%) were obtained by reacting the acid chloride of chlorambucil (4) with 3 in refluxing benzene (method B). The ^{14}C -labeled 1 was prepared according to method B.

Results and Discussion

Antitumor Activity in Vivo. A comparative study of effectiveness of compound 1 and chlorambucil (2) was carried out by using the ascites cell form of P388 leukemia, inoculated in CDF₁ mice. The in vivo testing was evaluated by recording median survival time (MST) of the treated animals (T) as compared to that of the control animals (C). Body weight change was followed up during the first 4 days after the beginning of the treatment.

In a preliminary experiment, P388 leukemic cells were inoculated intraperitoneally (ip), and compounds 1 and 2, in equimolecular amounts, were administered to animals by the same ip route. As shown in Table I, the antineoplastic activity of 2 is fully retained in its glyceride derivative 1. Indeed, the survival time prolonging effects on tumor-bearing mice produced by 1 at 0.052 and 0.069 mmol/kg were slightly higher, although not significantly, and the weight loss, which is indicative of host toxicity, was roughly similar as compared to chlorambucil (2).

The second set of experiments was designed to assess the lymphotropic properties of 1. The assay was performed according to the same conditions as outlined above, except that the leukemic cells were implanted subcutaneously. As described by Tsuruo et al.,¹² the ascites cell form of P388 leukemia inoculated by this route invades the body through the lymphatics. Equimolecular doses of 1 and 2 were administered either ip or orally (po) to different groups of animals.

Intraperitoneal injections of 1 and 2 did not result in any difference of antitumor activity at 0.033 and 0.050 mmol/kg doses (Table II), while a difference in favor of 1 was noted at 0.066 mmol/kg. In contrast to this, the effectiveness of 1 was overall more important than that of 2 ($p < 0.001$) when both compounds were orally administered. Moreover, while 2 was toxic at 0.11 and 0.16 mmol/kg doses according to the definition of the National

Table I. Antitumor Activity of Intraperitoneally Administered Chlorambucil (2) and Its Analogue (1) against Intraperitoneally Inoculated P388 Leukemia in CDF₁ Mice^a

drug	dose, mmol/kg	equiv dose, mg/kg	BWC, ^b g	MST, ^c days	T/C, ^d %
2	0.033	10	-2.2	16.9 (14-18)	169
	0.050	15	-2.9	19.5 (17-21)	195
	0.066	20	-3.4	23.0 (20-25)	230
1	0.035	30	-1.6	17.0 (14-19)	170
	0.052	45	-2.0	22.0 (19-23)	220
	0.069	60	-2.6	26.5 (21-28)	265
control			+1.90	10.0 (9-12)	

^a Each group of six CDF₁ mice (18-22 g) received ip inoculations of 1×10^6 viable leukemic cells on day 0. Treatment started 24 h after tumor implantation during 4 consecutive days. ^b BWC (body weight change) is the mean weight variation between day 1 and day 5. ^c MST (median survival time) is the range of survival days of mice. ^d T/C is the ratio of the MST of treated animals (T) to the MST of untreated controls (C), expressed in percent.

Cancer Institute (Bethesda, MD),¹⁴ at the same doses the efficacy of 1 was maintained at a high level, as indicated above. These results strongly suggest that the superior life span prolonging effect of 1 specifically observed by oral administration and in a situation where inoculated tumor cells spread by the lymphatic pathway might be due to preferential absorption of 1 via the intestinal lymphatic route rather than via the portal vein system, as compared with the free drug 2.

Intestinal Lymphatic Uptake. The selective absorption of 1 by the intestinal lymphatic system as compared to that of 2 was investigated in male Wistar rats after oral intubation of the same dose of both ^{14}C -labeled compounds. The intestinal lymphatic was cannulated according to the method described by Warshaw.¹³ Lymph samples were collected at 1-h intervals for as long as 7 h and analyzed for radioactivity. The kinetics of appearance of total radioactivity can be seen in Figure 1. Higher concentrations are observed at all times for compound 1,

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Table II. Antitumor Activity of Chlorambucil (2) and Its Analogue (1) against Subcutaneously Inoculated P388 Leukemia in CDF₁ Mice^a

drug	dose, mmol/kg	equiv dose, mg/kg	route of administration	BWC, ^b g	MST, ^{c,e} days	T/C, ^{d,e} %
2	0.033	10	ip	-2.55	19.5 (18-23)	134
	0.050	15	ip	-2.95	21.5 (17-25)	148
	0.066	20	ip	-3.95	toxic	toxic
	0.066	20	po	-4.15	22.5 (19-24)	155
	0.11	35	po		toxic	toxic
	0.16	50	po		toxic	toxic
1	0.035	30	ip	-2.35	20.5 (16-22)	141
	0.052	45	ip	-2.55	21.5 (17-24)	148
	0.069	60	ip	-3.95	23.5 (20-25)	162
	0.11	100	po	-3.5	24.5 (22-28)	168
	0.17	150	po	-3.9	34.0 (31-37)	234
	0.23	200	po		toxic	toxic
control				+0.35	14.5 (13-16)	

^a CDF₁ mice (18-22 g) were administered sc on day 0 with 10⁶ viable leukemic cells. Treatment started 24 h after tumor implantation during 4 consecutive days and on day 10. ^{b-d} See corresponding footnotes in Table I. ^e Toxic refers to the norms published by the National Cancer Institute (page 25 in ref 14).

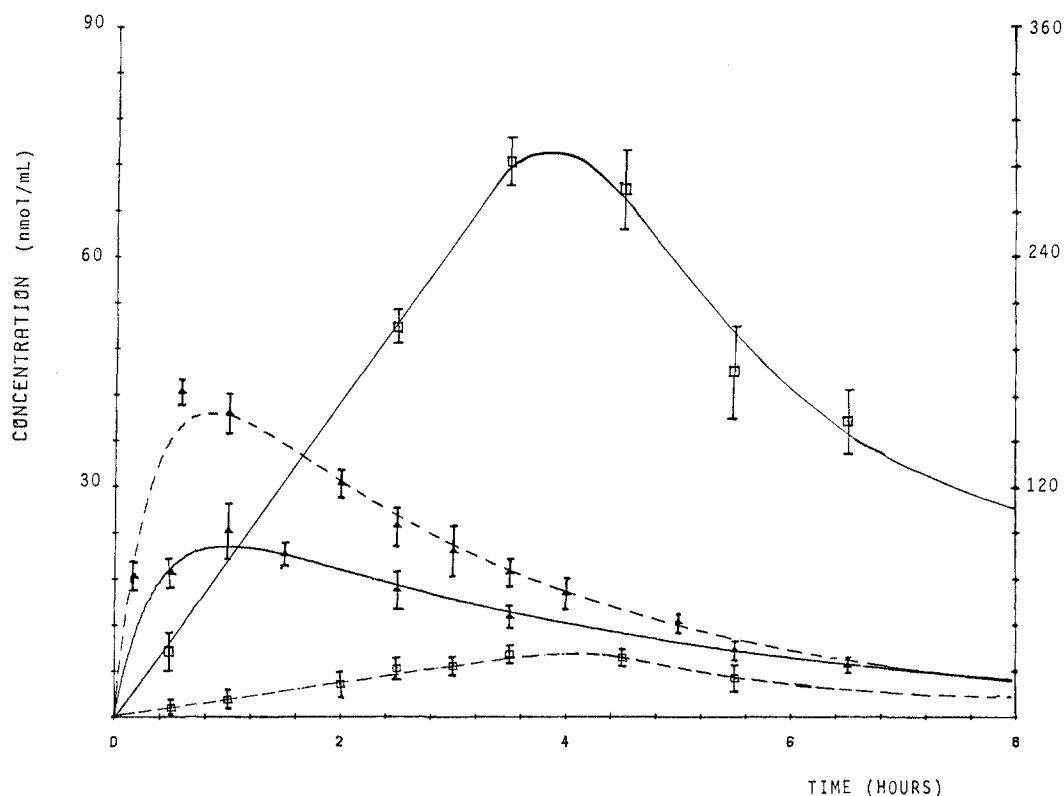


Figure 1. Appearance of 1 (□) and 2 (▲) in intestinal lymphatic system (—) and in plasma (---) after administration of an identical dose of 23 μmol/kg by gastric intubation. The curve of 1 in lymph is referred to the right scale; other curves are referred to the left scale. Values are the means plus or minus standard error of five rats. The resultant curves were fitted by a steepest descent method.

culminating at 3.5 h (22.2 times than that of 2). Seven hours after administration of 1, $26.4 \pm 4.6\%$ of the radioactivity was recovered in the lymph, and only $3.4 \pm 1.5\%$ was recovered when 2 was given. Lymph samples from rats dosed with ¹⁴C-labeled 1 were checked both by TLC and HPLC for radioactivity distribution. The chromatographed radioactivity was found essentially associated with the spot and the peak corresponding to unlabeled 1. No radioactivity was recovered as free chlorambucil. Incidentally, one should consider that the present TLC and HPLC observations do not rule out the possibility of the presence in the lymph of homologues of 1, resulting from the removal of the palmitoyl groups followed by the reesterification by other fatty acids.

These results clearly demonstrate that the attachment of chlorambucil to a diacylglycerol moiety considerably

enhances the extent of its absorption by the intestinal lymphatic system and brings about much higher concentrations for longer periods.

In order to confirm the selectivity of the lymphotropic properties of 1, we measured the time course of 1 and 2 concentration in plasma after oral intubation of equimolar doses of ¹⁴C-labeled compounds under the same experimental conditions as those used for the lymphatic absorption study. Here (Figure 1), in contrast with the results of the latter study, the concentration levels of 1 are extremely low and lower at all times than those of 2. This may explain the reduced toxicity exhibited by 1 after oral administration, as shown in Table II. As already observed with lymph, the peak concentration of 1 is delayed in comparison to 2: 3.5 and 0.5 h, respectively. This effect appears to be related to the preliminary lymphatic passage.

Following enteral administration of 1, about 10% of the radioactivity present in the plasma was recovered as free chlorambucil. This indicates that 1 can be hydrolyzed to some extent in the blood. However, the resulting concentrations of 2 are too low to account for the observed antineoplastic activity. This consideration, as well as the absence of 2 in the lymph and the effectiveness of 1 against sc implanted P388 lymphocytic leukemia, suggests that compound 1 is most probably acting by itself rather than as a prodrug of chlorambucil.

Conclusion

This preliminary report describes the synthesis and the in vivo evaluation of a chlorambucil-dipalmitoylglycerol conjugate (1) and shows its potential applicability as an orally administrable antineoplastic drug with genuine lymphotropic properties. A much higher efficacy (increased life span) and a reduced toxicity of the conjugate relative to the parent drug were apparent when both compounds were evaluated against P388 lymphocytic leukemia subcutaneously implanted in mice and spreading along the lymphatic pathway. These observed effects are fully consistent with the selective uptake of 1 by the intestinal lymphatic system, which is clearly demonstrated by pharmacokinetic data. Further work will be necessary to evaluate the potential advantages of the present approach, particularly with respect to promoting drug absorption through the intestinal lymphatic route. In this regard, 1 can be considered as a prototype.

Experimental Section

Melting points (uncorrected) were determined with a Thomas-Hoover capillary melting point apparatus. Mass spectra were recorded on a LKB 9000 S coupled instrument using the direct way of sample introduction (70 eV). The ^{13}C NMR spectra were obtained with a Bruker WP 80/SY spectrometer. Chemical shifts (δ) were expressed in parts per million downfield from an internal reference of Me_4Si . Elemental analyses were performed by Continental Pharma (Louvain-la-Neuve, Belgium). Where analytical results are indicated only by symbols of the elements, the observed values differed from the calculated values by not more than $\pm 0.4\%$. Radioactive samples (100 μL) were counted for ^{14}C on a Philips liquid scintillation analyzer after the addition of 10 mL of Lumagel (Lumac, Schaesberg, Netherlands). Lymph and blood samples were checked both by TLC [Merck silica gel 60 F₂₅₄, hexane-ether (70:30 v/v)] and HPLC [Waters 6000 A solvent-delivery system equipped with a variable-wavelength UV detector, Model LC3 Pye Unicam, and a radial-pack 10- μm C-18 reversed-phase column, using a mixture of acetone-acetonitrile (64:36, v/v)]. Detection was carried out at 320 nm, and a typical flow rate was 3 mL min^{-1} .

1,3-Dipalmitoyl-2-[4-[bis(2-chloroethyl)amino]benzenebutanoyl]glycerol (1). Method A. Compound 2 (200 mg, 0.65 mmol), DCC (137 mg, 0.66 mmol), and compound 3 (374 mg, 0.65 mmol) were dissolved in 5 mL of CH_2Cl_2 and kept at ambient temperature for 48 h. The precipitate of dicyclohexylurea was filtered, and the filtrate was washed successively with water, 5% aqueous acetic acid, and water. After the organic layer was evaporated in vacuo, the residue was crystallized from hexane-ether (70:30, v/v) to yield 220 mg (40%). The purity was confirmed by TLC (hexane/ether, 70:30, v/v): R_f 0.74; mp 69–71 °C; ^{13}C NMR δ 173.1, 172.4 (CO), 144.7, 130.8, 129.7, 112.7 (aromatics), 75.5 (CH), 69.2–22.6 (CH_2), 14.0 (CH_3); MS, m/e 855 (M + 2), 853 (M), 755, 256, 239. Anal. ($\text{C}_{49}\text{H}_{85}\text{NO}_6\text{Cl}_2$) C, H, N.

Method B. A solution of 20 mg (0.065 mmol) of 2 in 0.15 mL of dry benzene was treated with 0.05 mL of freshly distilled SOCl_2 (0.69 mmol) at 0 °C. The reaction mixture was stored overnight at 4 °C, then treated with 37.5 mg (0.066 mmol) of 3 dissolved in 2 mL of dry benzene, heated to reflux for 72 h, and evaporated in vacuo to yield a residue, which was chromatographed over 0.05–0.2-mm silica gel with *n*-hexane/ether (70:30, v/v) as eluting solvent. The component having R_f 0.74 was collected. The yield was 47 mg (85%), mp 69–71 °C. 1,3-Dipalmitoyl-2-[4-[bis(2-chloroethyl)amino][^{14}C]benzenebutanoyl]glycerol was prepared according to this method. Radiochemical purity was confirmed by TLC, carried out in a mixture of hexane-ether (70:30, v/v).

Antileukemic Evaluation in Mice. Protocol 1.200 of the National Cancer Institute (Bethesda, MD) was followed throughout the experimentation.¹⁴ Tumor and mice: P388 leukemia was maintained by weekly serial transplantation in DBA/2 mice. For experimentation, the ascitic fluid was removed from the peritoneal cavity on the 7th day after tumor implantation, the number of cells was counted on a hemacytometer, and the cells were suspended in chilled saline solution (0.9%) and maintained in melting ice during transplantation. CDF₁ mice received 1×10^6 viable leukemic cells either ip or sc. Drug treatment started 24 h later.

Drugs were suspended in Klucel [0.3% of (hydroxypropyl)-cellulose in saline], and 0.5 mL of suspension was daily administered ip or po to CDF₁ mice. Drug suspensions were prepared daily and injected into animals.

Survival was recorded daily until death or up to day 40, and median survival time (MST) was estimated for both treated and control groups. The relative increase in survival time is expressed by the ratio of the MST of treated animals (T) to the MST of untreated control animals (C), expressed in percent, further referred to as T/C.

A reproducible T/C value of 120% is representative of biologically significant activity. For these experiments, 6 male mice per dose and 24 animals per control group were used.

Intestinal Lymphatic Uptake After Oral Administration. All studies were carried out in overnight-fasted male Wistar rats weighing 200 g. One hour before the experiment, they were given 3 mL of concentrated milk in order to facilitate the visualization of the lymphatics. Following anesthesia with 35 mg/kg of sodium pentobarbital (ip), the animals were positioned on a surgical board. The intestinal lymphatic was cannulated according to the method described by Warshaw.¹³ Oral doses (23 $\mu\text{mol}/\text{kg}$) of ^{14}C -labeled 1 or 2 (98.0 kBq/mmol) were administered by gastric intubation just before the rat woke up. The animals were kept in restraining cages with access to water ad libitum. Lymph samples (0.57 ± 0.15 mL/h) were collected at 1-h intervals for 7 h and digested in Lumagel. Counting efficiency was estimated by external standardization. Concentrations in nanomoles per milliliter were plotted as a function of the midpoint of the collection interval (Figure 1). Results are expressed as the mean plus or minus standard error of five animals.

Plasma Concentrations After Oral Administration. Oral doses (23 $\mu\text{mol}/\text{kg}$) of ^{14}C -labeled 1 or 2 (98.0 kBq/mmol) were administered to male Wistar rats weighing 200 g. Plasma samples were obtained from tail blood vessels at various time intervals for 6 h and analyzed for radioactivity. In Figure 1, results are expressed as the mean plus or minus standard error of five animals.

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