Synthesis and Biophysical Characterization of Chlorambucil Anticancer Ether Lipid Prodrugs

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The synthesis and biophysical characterization of four prodrug ether phospholipid conjugates are described. The lipids are prepared from the anticancer drug chlorambucil and have C16 and C18 ether chains with phosphatidylcholine or phosphatidylglycerol headgroups. All four prodrugs have the ability to form unilamellar liposomes (86−125 nm) and are hydrolyzed by phospholipase A_2, resulting in chlorambucil release. Liposomal formulations of prodrug lipids displayed cytotoxicity toward HT-29, MT-3, and ES-2 cancer cell lines in the presence of phospholipase A_2, with IC_{50} values in the 8−36 μM range.

Introduction

Ever since Gregoriadis et al.[1] suggested liposomes as drug carriers in 1974, serious efforts have been put into the development of liposomes as efficient drug delivery systems for the treatment of cancer. The discovery that liposomes accumulate to a high degree in tumor tissue[2] if their surface is covered with poly(ethylene glycol) was a major improvement over earlier formulations and made these nanoparticles applicable as drug carriers of chemotherapeutics to tumor tissue. Such liposomal drug delivery systems based on the enhanced permeation and retention (EPR[a]) effect were utilized in the commercially successful liposomal formulation of doxorubicin. However, it is now apparent that this formulation is not generally useful for the majority of potentially interesting drug candidates because of the lack of a controlled drug release.[3] An optimal drug delivery formulation should be able to retain and stabilize the carried drug during blood circulation and effectively release the drug in the target tissue.[3] This calls for the utilization of site specific release mechanisms, and several have been investigated, e.g., enzymatic release[4] and pH,[5] light,[6] and heat sensitive liposomes.[7]

Liposomal drug delivery has mainly relied on the encapsulation of hydrophilic drugs in the aqueous core[3,8] or on trapping hydrophobic molecules in the lipid bilayer.[9] Although this approach is successful, it does suffer from some limitations such as the potential for leakage and the fact that the release of the active drug is not directly coupled to the mechanism activating the carrier. One strategy that addresses both these issues is the formulation of a lipid−prodrug conjugate that is susceptible to selective degradation by endogenous enzymes in the target tissue, serving to simultaneously degrade the carrier and release the drug.[3] By covalent incorporation of the active chemotherapeutic agent in the delivery system, the problem with premature leakage is effectively circumvented. Herein, we describe the synthesis and characterization of prodrugs (**1** and **2**, Figure 1) that are suitable for liposomal delivery to cancerous tissue and susceptible to secretory phospholipase A_2 activation.

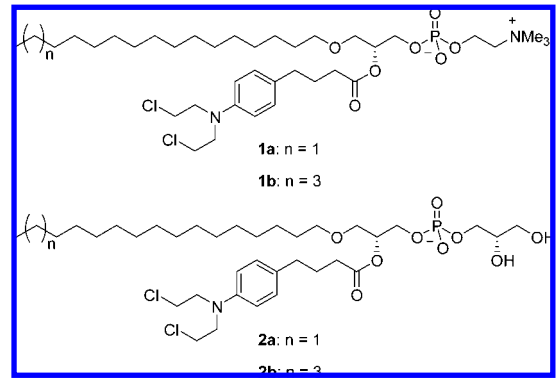

Figure 1. Four target chlorambucil prodrug ether lipids. Prodrugs **1a** and **1b** have a phosphatidylcholine headgroup with a C16 and a C18 ether chain, respectively. Target compounds **2a** and **2b** have the negatively charged phosphatidylglycerol headgroup.

Secretory phospholipase A_2 ($sPLA_2$) is overexpressed in cancer tissue,[10] and has previously been exploited in liposomal drug delivery.[11] Subtype $sPLA_2$ IIA has been identified in several human tumors including breast,[12] stomach,[13] colorectal,[14] pancreatic,[15] prostate,[10a,16] and liver cancer.[17] The prodrugs are based on covalently attaching an anticancer drug in the *sn*-2 position of *sn*-1 ether phospholipids. The principle is illustrated in Figure 2: $sPLA_2$ will hydrolyze the *sn*-2 ester bond, releasing both the anticancer drug bound to the *sn*-2 position and an anticancer ether lipid (AEL). Ether lipids were chosen because of their higher stability and because of the cytotoxicity of the lyso-lipids released upon $sPLA_2$ hydrolysis.[11] Furthermore, the lyso-ether lipids have potential to attenuate the toxicity of chlorambucil by increasing the cellular uptake of the drug,[3] and thus, the two molecules released by $sPLA_2$ hydrolysis will work in unison against cancer cells.

It is crucial for the prodrug strategy that suitable drug candidates are available. Since the incorporated drug will be part of the lipophilic membrane, a hydrophobic nature is an

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[a] Abbrevations: EPR, enhanced permeation and retention; $sPLA_2$, secretory phospholipase A_2; AEL, anticancer ether lipid; PMB, *p*-methoxybenzyl; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; DBU, 1,8-diazabicyclo-[5.4.0]undec-7-ene; DLS, dynamic light scattering; SUV, small unilaminar vesicles.

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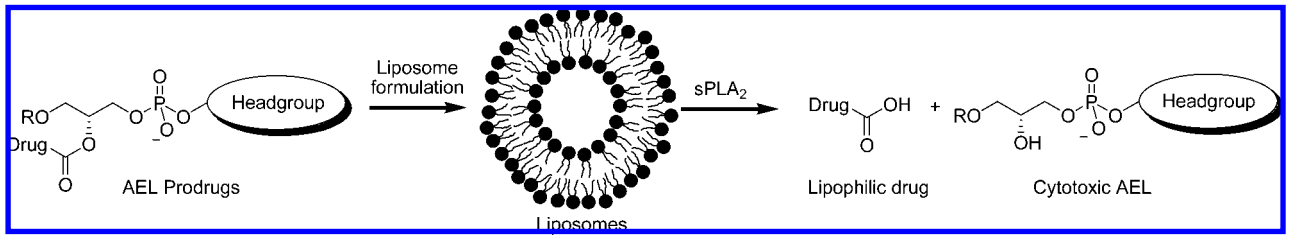
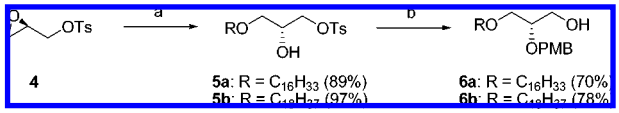


Figure 2. Schematic overview of the drug delivery concept. The AEL prodrugs are formulated as liposomes. Because of the EPR effect, the liposomes will accumulate in cancer tissues and sPLA2, which is up-regulated in cancer tissue, will hydrolyze the AEL prodrug lipids, releasing two anticancer drugs.

Scheme 1. Synthesis of Lipid Precursors **6a** and **6b**[a]



[a] Reagents: (a) $C_{16}H_{33}OH$ or $C_{18}H_{37}OH$, $BF_3 \cdot OEt_2$, CH_2Cl_2; (b) (i) PMBTCA, $La(OTf)_3$, toluene; (ii) CsOAc, DMSO, DMF; (iii) NaOMe, MeOH.

obvious requirement and, furthermore, a carboxylic acid moiety is needed for the attachment of the drug to the AEL backbone. We have identified a number of candidates such as chlorambucil (**3**),[18] all-*trans* retinoic acid,[19] and prostaglandins.[20] In the present study prodrugs made from chlorambucil are investigated. Chlorambucil (**3**) is a chemotherapeutic agent of the mustard gas type,[21] and it was originally synthesized by Everett et al. in 1953.[18a] It is used clinically for the treatment of lymphocytic leukemia[22] typically in combination with other drugs. Chlorambucil is orally administrated but undergoes rapid metabolism, and as a result, the stability in aqueous environments is low and **3** has an elimination half-life of 1.5 h.[23] The prodrug formulation could remedy this, since this system will shield chlorambucil from degradation through the incorporation in the lipophilic part of the liposomal membrane and deliver it directly to the tumor, decreasing metabolism compared to the oral administration route. To investigate the effect of *sn*-1 ether chain length and headgroup charge on enzymatic activity, prodrugs **1** and **2** were prepared with both C16 and C18 ether chains and a choline and a glycerol phosphate headgroup, respectively. Biophysical and biological characterization of the synthesized chlorambucil prodrugs (**1**, **2**) is included, with focus on liposome formulation, particle size determination, and in particular $sPLA_2$ activity. Proof-of-principle of the strategy is demonstrated in three cancer cell lines, providing the first successful example of this prodrug approach to liposomal drug delivery.

Synthesis of Chlorambucil AEL Prodrugs

Anticancer ether lipids have previously been synthesized via different routes, e.g., starting from D-mannitol[24] or glycidols.[25] Commercially available (*R*)-glycidyl tosylate (**4**) served as our starting material, and the aliphatic ether chain was introduced by ring-opening of the epoxide under Lewis acid catalysis,[26] resulting in yields of 89% and 97% for **5a** and **5b**, respectively (Scheme 1). The *p*-methoxybenzyl (PMB) group was chosen for protection of the secondary alcohol[27] and introduced by using *p*-methoxybenzyl trichloroacetimidate with $La(OTf)_3$ catalysis.[28] The resulting tosylate was converted to the acetate with CsOAc in a 9:1 mixture of DMSO and DMF and the ester hydrolyzed with NaOMe in MeOH at 40 °C, yielding the primary alcohols **6a** and **6b** in overall yields of 70% and 78%, respectively, over three steps. It was essential to carry out the hydrolysis at elevated temperature in order to obtain homogeneous reaction mixtures and achieve full conversion in the transformations.

The choline headgroup was attached to the primary alcohols **6a** and **6b** by reaction with phosphorus oxychloride and NEt_3, followed by addition of choline tosylate, pyridine, and finally H_2O (Scheme 2).[11a,29] Excess choline tosylate was removed on an MB-3 ion-exchange column, and after purification by flash column chromatography the PMB-group was removed with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in an 18:1 mixture of CH_2Cl_2 and H_2O,[30] which resulted in full conversion within 3 h with isolated yields of 99% and 79%. The final attachment of chlorambucil to the lipid was achieved via a Steglich esterification with DCC and a catalytic amount of DMAP.[31] When the acylation of **7a** was performed in ethanol-free chloroform at 20 °C or CH_2Cl_2 in the temperature range from 0 °C to reflux, we did not observe any incorporation of chlorambucil, but when the conditions were changed to reflux in 1,2-dichloroethane, the acylation occurred in a 75% yield, albeit only after adding 5 equiv of chlorambucil and DCC in portions over 31 h. The acylation of **7b** led to an isolated yield of 76% in refluxing chloroform, and that was not improved by using 1,2-dichlorethane as the solvent. Changing the coupling reagents to EDCI and DMAP[32] did not improve the conversion of the AELs.

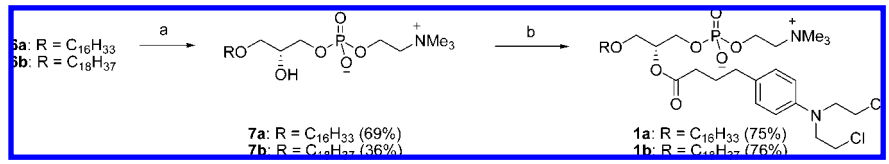
The phosphoramidite **11** needed for the installment of the glycerol headgroup was synthesized in four steps from allyl *p*-methoxybenzoate (**8**) (Scheme 3). The key step was a Sharpless asymmetric dihydroxylation[33] of **8**, which occurred with excellent enantioselectivity (97% ee, chiral HPLC, and Mosher ester analysis; see Supporting Information). TBDMS protection and reduction of the *p*-methoxybenzoate with DIBAL-H at −78 °C afforded the TBDMS-protected glycerol **10**. The coupling between **10** and the commercially available phosphorylating agent $(i\text{-Pr})_2NPClO(CH_2)_2CN$ resulted in the desired phosphoramidite **11** in a very satisfactory yield, isolated as a 1:1 diastereomeric mixture as evident from ^{31}P NMR.

The glycerol headgroup was attached to the lipid backbone (**6a** and **6b**) via reaction with **11** under activation of tetrazole and successive oxidation with ^{t}BuOOH (Scheme 4). Deprotection of the PMB-group was achieved with DDQ in moist CH_2Cl_2. Acylation with chlorambucil in CH_2Cl_2 followed by 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) mediated deprotection of the cyanoethylene group[34] afforded the lipids **12a** and **12b** in good yields over five steps. Finally, removal of the TBDMS protection groups was achieved by treatment with HF in $MeCN/H_2O$, providing **2a** and **2b**.

Biophysical and Biological Data

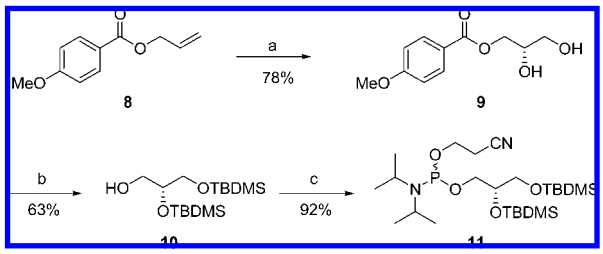
The chlorambucil AEL prodrugs (**1**, **2**) were formulated as liposomes by extrusion in HEPES buffer using the dry lipid film technique.[35] The lipid solutions were analyzed by dynamic

Scheme 2. Synthesis of Chlorambucil AEL Prodrugs **1**[a]



[a] Reagents: (a) (i) $POCl_3$, Et_3N, CH_2Cl_2; (ii) choline tosylate, pyridine; (iii) H_2O; (iv) DDQ, CH_2Cl_2, H_2O; (b) **3**, DCC, DMAP, $CHCl_3$ or 1,2-dichloroethane.

Scheme 3. Synthesis of the Phosphoramidite **11**[a]



[a] Reagents: (a) $K_2OsO_4 \cdot 2H_2O$, $(DHQD)_2PHAL$, $K_3Fe(CN)_6$, K_2CO_3, ^{t}BuOH, H_2O; (b) (i) TBDMSOTf, DIPEA, CH_2Cl_2; (ii) DIBAL-H, CH_2Cl_2; (c) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2.

light scattering (DLS) in order to investigate the particle size, and DLS analysis revealed that **1** and **2** form particles in the liposome size region (Table 1) and with a low polydispersity, indicating formation of small unilaminar vesicles (SUVs). Initial confirmation of enzymatic hydrolysis was obtained by treating the liposome solutions with snake (*Naja mossambica mossambica*) venom $sPLA_2$ for 24 h at 37 °C. Snake venom $sPLA_2$ is a convenient model enzyme, since it is not sensitive to the charge of the interfacial region, unlike human $sPLA_2$, but shows the same substrate specificity.[10d,36] DLS measurements of the resulting solutions confirmed that the liposomes had been degraded, as only particles with a diameter of less than 5 nm were present. Incubation of the liposomes for 24 h without enzyme did not result in a change in particle size as measured by DLS (data not shown).

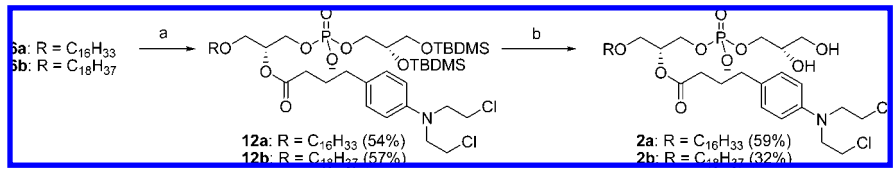
In order to investigate the hydrolysis on a molecular level, we applied MALDI-TOF MS and HPLC. MALDI-TOF MS has recently been exploited as a very fast and sensitive technique for detection of lipids,[37] and we decided to study the enzyme activity with this method in order to verify that the lipids were consumed and the anticancer drugs released. Figure 3 shows the digestion of the chlorambucil AEL prodrugs **1a** and **2a** and the release of AELs catalyzed by snake (*Agkistrodon piscivorus piscivorus*) venom $sPLA_2$. The spectra show the disappearance over time of the prodrugs signals ($M + H^+$ and $M + Na^+$) and the emergence of the expected AEL signals ($M + H^+$ and $M + Na^+$). From the spectra it is also possible to get information about the conversion rate, and whereas **2a** is almost fully consumed after 2 h, **1a** needs more than 24 h for full digestion by $sPLA_2$. The MALDI-TOF MS analysis of **1b** and **2b** (see Supporting Information) revealed that full degradation is obtained in 2−6 h. These results were verified by HPLC (Figure 4 and Supporting Information). Neither HPLC nor MALDI-TOF MS was capable of detecting the released chlorambucil, but that was not surprising given the low stability of free chlorambucil in an aqueous environment.[23] Chatterji et al. report 15 min as the half-life of chlorambucil in a buffer like the HEPES buffer at 37 °C.[23b] MALDI-TOF analysis (see Supporting Information) of liposome solutions of **1b** and **2b** stored for 6 weeks at 20 °C showed that the prodrug lipids were intact. No significant hydrolysis of the chloroethyl groups of chlorambucil was detected, proving that the liposomal formulation enhances chlorambucil stability significantly. These findings were further supported by the 4-nitrobenzylpyridine alkylating assay,[38] which showed that alkylation by chlorambucil occurred when liposomes of **1b** and **2b** were subjected to $sPLA_2$, whereas no alkylation of **1b** and **2b** was detected in the absence of $sPLA_2$ (see Supporting Information).

To demonstrate the $sPLA_2$-dependent cytotoxicity of the chlorambucil AEL prodrugs, we investigated the activity of **1b** against HT-29 and MT-3 cancer cells for 24 h and **2b** against the same two cell lines in addition to ES-2 cells (Table 2). None of these cells secrete $sPLA_2$, which is an advantage in these studies because it enables us to control the presence or absence of the enzyme in each experiment. Before addition of snake (*Agkistrodon piscivorus piscivorus*) venom $sPLA_2$, all liposome formulations have IC_{50} values significantly higher than that of free chlorambucil (entries 1−3), demonstrating that cytotoxicity is associated with the prodrug activation by $sPLA_2$. Upon addition of the enzyme, both prodrugs display IC_{50} values below that of chlorambucil itself for all three tested cell lines (entries 4 and 5), suggesting a cooperative effect of the two cytotoxic compounds released, chlorambucil and AELs. The lyso-ether lipid **7b** has activity in the same range as the prodrugs with added $sPLA_2$ (compare entries 4−5 and entry 6). Earlier studies of *sn*-1 ether lipids with a fatty acid in the *sn*-2 position have shown that AEL alone is more cytotoxic than the liposome formulation,[11a] lending further support to our belief that the two active components work in unison to kill the cancer cells. Phospholipase A_2 alone has no effect on cell viability (entry 7). Taken together, the data in Table 2 clearly show the potential of this prodrug strategy for $sPLA_2$ mediated degradation of liposomes consisting of *sn*-1 ether lipids with an anticancer drug covalently bound in the *sn*-2 position.

Conclusion

In the present study we have synthesized a series of novel prodrugs and shown that they form small unilamellar vesicles that are stable in size over time. It was found that $sPLA_2$ can hydrolyze all the prodrugs of this type, showing how diverse $sPLA_2$ substrates can be, which makes $sPLA_2$ an excellent target for future prodrug strategies. The approach described here is a new application of prodrugs in liposomal formulations, and we believe it has significant advantages over conventional liposomal drug delivery system, where hydrophilic drugs are encapsulated. Problems with leakage during circulation are circumvented because of the covalent attachment of the active compound to the phospholipids. Furthermore, since the drugs are contained in the liposome membrane until activated by the enzyme, our strategy opens up for the use of lipophilic compounds that would otherwise be too toxic if employed systemically because of their affinity for biological membranes. Lastly, the use of prodrug strategies where the drug is protected in the membrane may open new possibilities with respect to drugs with low stability

Scheme 4. Synthesis of Chlorambucil AEL Prodrugs **2**[a]



[a] Reagents: (a) (i) **11**, tetrazole, CH_2Cl_2, MeCN; (ii) ^{t}BuOOH; (iii) DDQ, CH_2Cl_2, H_2O; (iv) **3**, EDCI, DMAP, CH_2Cl_2; (v) DBU, CH_2Cl_2; (b) HF, MeCN, H_2O.

Table 1. DLS Analysis of Chlorambucil AEL Prodrugs[a]

	before $sPLA_2$ addition		after $sPLA_2$ addition, diameter (nm)
prodrug	diameter (nm)	polydispersity	
1a	124	0.12	<5
1b	125	0.22	<5
2a	104	0.08	<5
2b	113	0.05	<5

[a] Determined before and after addition of snake venom (*Naja mossambica mossambica*) $sPLA_2$.

in a biological environment, as evident from the stability of chlorambucil in the liposomal formulation.

Experimental Section

General. Starting materials, reagents, and solvents were purchased from Sigma-Aldrich Chemical Co. and used without further purification. Reactions involving air or moisture sensitive reagents were carried out under N_2, and flasks were dried by flame heating under reduced pressure. DMF, DMSO, MeCN, CH_2Cl_2, $CHCl_3$, 1,2-dichloroethane, and toluene were dried over 4 Å molecular sieves. Pyridine and NEt_3 were dried over KOH. Evaporation of solvents was done under reduced pressure (in vacuo). TLC was performed on Merck aluminum sheets precoated with silica gel 60 F_{254} plates. Compounds were visualized by charring after dipping in a solution of *p*-anisaldehyde (10 mL of H_2SO_4 and 10 mL of *p*-anisaldehyde in 200 mL of 95% EtOH), $KMnO_4$ (1.5 g of $KMnO_4$, 10 g of K_2CO_3, and 2.5 mL of 5% NaOH in 150 mL of H_2O), or Cemol (6.25 g of $(NH_4)_6Mo_7O_{24}$ and 1.5 g of $Ce(SO_4)_2$ in 250 mL of 10% aqueous H_2SO_4). Flash column chromatography was performed using Matrex 60 Å silica gel. Purity of all compounds was found to be equal to or greater than 95% by elemental analysis or HPLC (see below).

NMR spectra were recorded using a Varian Mercury 300 MHz spectrometer or a Varian Unity Inova 500 MHz spectrometer. Chemical shifts were measured in ppm and coupling constants in Hz, and the field is indicated in each case. The solvent peaks from $CDCl_3$ (7.26 ppm in ^{1}H NMR and 77.16 ppm in ^{13}C NMR) or acetone-d_6 (2.05 ppm in ^{1}H NMR) were used as standards.[39] HPLC was performed on a Waters Alliance HPLC equipped with a DAD, using a LiChrospher Si 60 column and eluting with water/isopropanol/heptane mixtures. Elemental analyses were obtained from H. Kolbe, Mikroanalytisches Laboratorium, Mülheim/Ruhr, Germany. IR analysis was carried out on a Perkin-Elmer 1600 series FTIR spectrometer, as KBr pills or neat between AgCl plates. Melting points were measured by a Buch & Holm melting point apparatus and given in degrees Celsius (°C) uncorrected. HRMS was recorded on an Ionspec Ultima Fourier transform mass spectrometer.

Liposome Preparation and Particle Size Determination. The chlorambucil AEL prodrugs were dissolved in $CHCl_3$ in a glass tube, dried under a stream of N_2, and then placed under vacuum for 3−15 h to form a thin film. The film was solubilized by addition of aqueous buffer (0.15 M NaCl, 2.5 mM HEPES, pH 7.4) and vortexed periodically over 1 h at 20 °C. Subsequently, the solutions were extruded through a 100 nm polycarbonate cutoff membrane using a Hamilton syringe extruder (Avanti Polar Lipids, Birmingham, AL), yielding unilamellar vesicles with a diameter ranging from 86 to 125 nm and with a low polydispersity as measured by DLS. The DLS measurements were obtained using a BI-200SM goniometer from Brookhaven Instruments (New York), applying a fixed scattering angle of 90° with a 632.8 nm HeNe laser.

$sPLA_2$ Activity Measurements Monitored by DLS. The chlorambucil AEL prodrugs (2 mL, 0.05 mM), formulated in an aqueous buffer (0.15 M NaCl, 5 mM $CaCl_2$, 2.5 mM HEPES, pH 7.4), were incubated at 37 °C with snake venom $sPLA_2$ from *Naja mossambica mossambica* (1.93 nmol) for 24 h. The $sPLA_2$ from *Naja mossambica mossambica* was purchased from Sigma-Aldrich Chemical Co. The resulting solutions were analyzed by DLS as described above.

$sPLA_2$ Activity Measurements Monitored by HPLC and MALDI-TOF MS. The chlorambucil AEL prodrugs (2 mM) were hydrated in an aqueous buffer (0.15 M KCl, 30 μM $CaCl_2$, 10 μM EDTA, 10 mM HEPES, pH 7.5) for 1 h at 60 °C and then sonicated for 1 h at 60 °C, providing a clear solution. The formulated chlorambucil AEL prodrugs (0.40 mL, 2 mM) were diluted in an aqueous buffer (2.1 mL, 0.15 M KCl, 30 μM $CaCl_2$, 10 μM EDTA, 10 μM HEPES, pH 7.5), and the mixture was stirred at 37 °C in a container protected from light. The catalytic reaction was initiated by addition of snake (*Agkistrodon piscivorus piscivorus*) venom $sPLA_2$ (20 μL, 42 μM). The purified snake venom $sPLA_2$ was donated by Dr. R. L. Biltonen (University of Virginia). Sampling was done after 0, 2, 6, 8, 20, 24, and 90 h by collecting 100 μL of the reaction mixture and rapidly mixing it with a solution of $CHCl_3$/MeOH/H_2O/AcOH 4:8:1:1 (0.5 mL) in order to stop the reaction. The mixture was washed with water (0.5 mL), and the organic phase was isolated by extraction. For HPLC 30−75 μL of the organic phase was injected on a 5 μm diol column and eluted with an isocratic eluent ($CHCl_3$/MeOH/H_2O 730:230:30 for **1a** and **2a**; $CHCl_3$/MeOH/25% aqueous NH_3 800:195:5 for **2a** and **2b**). An evaporative light scattering detector was used for detection. For the MALDI-TOF MS measurements 9 μL of the organic phase was mixed with 3 μL of 2,5-dihydroxybenzoic acid (DHB) matrix (0.5 M DHB, 2 mM CF_3COONa in MeOH), and 0.5 μL of this mixture was used for the MS analysis.

Cytotoxicity. Colon cancer HT-29 and ovarian cancer ES-2 cells were cultured in McCoy's 5A medium in the presence of 10% FCS and 1% Pen-Strep (InVitrogen). Breast cancer MT-3 cells were cultured in RPMI medium. Cells were plated in 96-well plates at a density of 1×10^4 cells per well 24 h prior to addition of the tested compound. Chlorambucil (**3**) was solubilized in DMSO and water (final DMSO concentration max of 0.5%). Liposomes were diluted in PBS, and initial lipid concentration in the liposome solutions was determined by phosphorus analysis.[40] After 24 h of incubation, the substances were removed and the cells were washed and incubated in complete medium for another 48 h. Cytotoxic activity was assessed using a standard 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cell Proliferation Kit I, Roche, Germany).[41] Cell viability is expressed as percentage reduction of incorporated MTT.

1-*O*-Hexadecyl-2-(4-(4-(bis-(2-chloroethyl)amino)phenyl)butanoyl)-*sn*-glycero-3-phosphocholine (1a). Compound **7a** (67 mg, 0.139 mmol) was dissolved in anhydrous 1,2-dichloroethane (3.5 mL), and the mixture was heated to reflux under an atmosphere of N_2. DMAP (10 mg, 0.082 mmol), chlorambucil (**3**) (64 mg, 0.209 mmol), and DCC (1 M in CH_2Cl_2, 0.2 mL, 0.2 mmol) were added, and after 5 and 19 h additional portions of chlorambucil (**3**) (64 mg, 0.209 mmol) and DCC (1 M in CH_2Cl_2, 0.2 mL, 0.2 mmol)

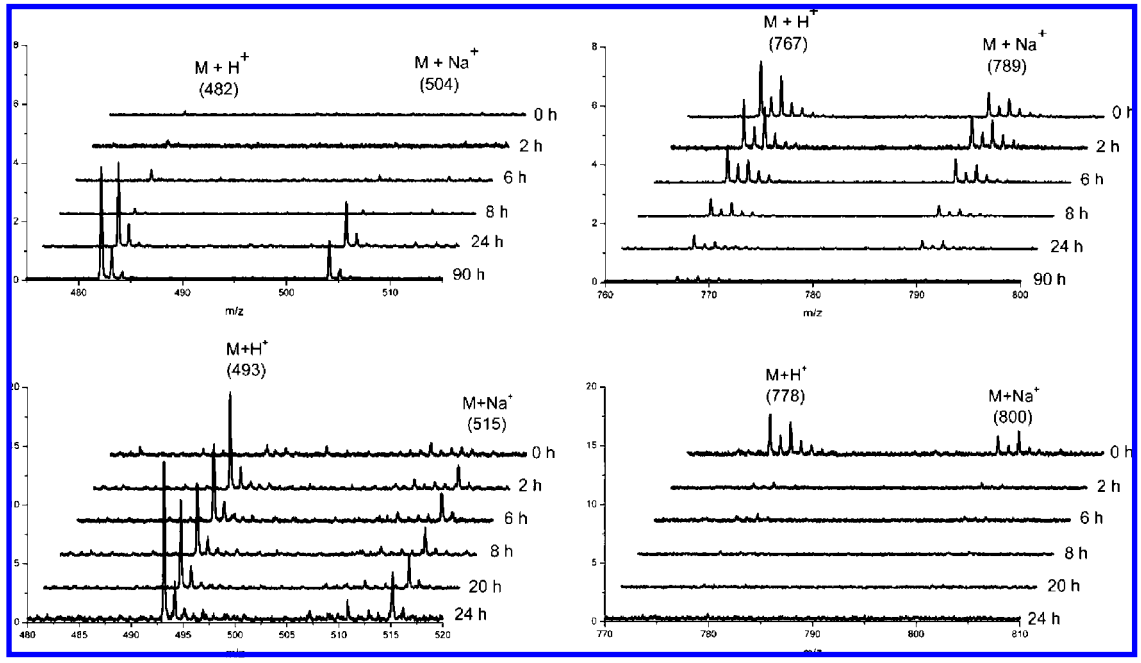


Figure 3. MALDI-TOF MS monitoring of snake (*Agkistrodon piscivorus piscivorus*) venom $sPLA_2$ activity on chlorambucil AEL prodrug **1a** (top) and **2a** (bottom). The spectra demonstrate that the prodrugs (right) are consumed and the AELs (left) are released.

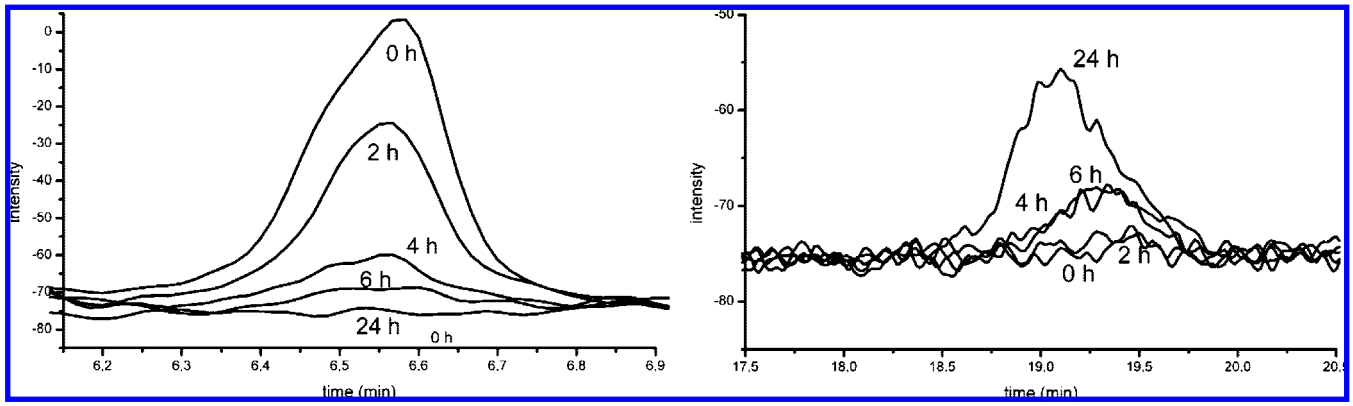


Figure 4. HPLC chromatogram for the snake (*Agkistrodon piscivorus piscivorus*) venom $sPLA_2$ hydrolysis experiment on chlorambucil AEL prodrug **1a** showing the amount of prodrug (left) and AEL (right) before the addition of the enzyme and after 2, 4, 6, and 24 h.

Table 2. IC_{50} Values (μM) of Chlorambucil (**3**), a Lyso-Ether Lipid (**7b**), and the Prodrugs **1b** and **2b** in the Presence and Absence of $sPLA_2$[a]

entry	compd	IC_{50} HT-29	IC_{50} MT-3	IC_{50} ES-2
1	**3**	70 ± 10	95 ± 21	34 ± 3
2	**1b**	>200	>200	nd
3	**2b**	>200	>200	97 ± 2
4	**1b** + $sPLA_2$	32 ± 2	36 ± 4	nd
5	**2b** + $sPLA_2$	10 ± 1	36 ± 4	8 ± 0,5
6	**7b**	18 ± 5	35 ± 1	30 ± 1
7	$sPLA_2$	*b*	*b*	*b*

[a] Cytotoxicity was measured using the MTT assay as cell viability 48 h after incubation with the indicated substances for 24 h and shown by mean ± SD (n = 3); nd = not determined. Snake (*Agkistrodon piscivorus piscivorus*) venom $sPLA_2$ was added to a final concentration of 5 nM. [b] No change in cell viability was observed after 24 h.

were added. After 27 h the mixture was concentrated in vacuo and purified by column chromatography (CH_2Cl_2/MeOH 4:1; then CH_2Cl_2/MeOH/H_2O 30:20:1) to give 80 mg (75%) of **1a** as an oil. R_f = 0.18 (CH_2Cl_2/MeOH/H_2O 30:20:1). ^{1}H NMR (500 MHz, 4:1 $CDCl_3$/CD_3OD) δ 7.08 (d, J = 8.6 Hz, 2H), 6.65 (d, J = 8.6 Hz, 2H), 5.16 (p, J = 5.9 Hz, 1H), 4.22 (s, 2H), 4.03−3.99 (m, 2H), 3.72 (t, J = 7.1 Hz, 4H), 3.64 (t, J = 7.1 Hz, 4H), 3.63−3.59 (m, 2H), 3.56 (s, 2H), 3.49−3.39 (m, 2H), 3.19 (s, 9H), 2.56 (t, J = 7.6 Hz, 2H), 2.36 (t, J = 7.6 Hz, 2H), 1.90 (p, J = 7.6 Hz, 2H), 1.54 (p, J = 6.8 Hz, 2H), 1.32−1.26 (m, 26H), 0.88 (t, J = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, 4:1 $CDCl_3$/CD_3OD) δ 174.1, 144.8, 130.8, 130.0 (2C), 112.6 (2C), 72.2 (d, J = 8.2 Hz), 72.1, 69.6, 66.8, 64.4 (d, J = 5.2 Hz), 59.2 (d, J = 5.2 Hz), 54.5, 54.4, 54.4, 53.9 (2C), 40.9 (2C), 34.2, 34.0, 32.3, 30.1, 30.0, 29.9, 29.9, 29.7, 27.2, 26.4, 23.0, 14.3. IR (neat) 2923, 2852, 2366, 1734, 1091 cm^{-1}. m/z (M + H$^+$) 767.43.

1-*O*-Octadecyl-2-(4-(4-(bis(2-chloroethyl)amino)phenyl)butanoyl)-*sn*-glycero-3-phosphocholine (1b). Compound **7b** (67 mg, 0.131 mmol) was dissolved in anhydrous $CHCl_3$ (4 mL), and the mixture was heated to reflux under an atmosphere of N_2. DMAP (10 mg, 0.082 mmol), chlorambucil (**3**) (40 mg, 0.131 mmol), and DCC (1 M in CH_2Cl_2, 130 μL, 0.130 mmol) were added, and after 3, 6.5, 23.5, 26.5, and 31 h additional portions of chlorambucil (**3**) (40 mg, 0.131 mmol) and DCC (1 M in CH_2Cl_2, 130 μL, 0.130 mmol) were added. After 48 h the mixture was concentrated in vacuo and purified by column chromatography (CH_2Cl_2/MeOH 4:1, then CH_2Cl_2/MeOH/H_2O 30:20:1) to give 79 mg (76%) of **1b** as an oil. R_f = 0.15 (CH_2Cl_2/MeOH/H_2O 30:20:1). ^{1}H NMR (500 MHz, 4:1 $CDCl_3$/CD_3OD) δ 7.07 (d, J = 8.5 Hz, 2H), 6.65 (d, J = 8.5 Hz, 2H), 5.16 (p, J = 5.8 Hz, 1H), 4.22 (s, 2H), 4.06−3.94 (m, 2H), 3.72 (t, J = 6.8 Hz, 4H), 3.64 (t, J = 6.8 Hz, 4H), 3.63−3.59 (m, 2H), 3.56 (s, 2H), 3.48−3.40 (m, 2H), 3.20 (s, 9H), 2.56 (t, J =

7.5 Hz, 2H), 2.36 (t, $J = 7.5$ Hz, 2H), 1.90 (p, $J = 7.5$ Hz, 2H), 1.54 (m, 2H), 1.32−1.26 (m, 30H), 0.88 (t, $J = 6.9$ Hz, 3H). ^{13}C NMR (75 MHz, 4:1 $CDCl_3/CD_3OD$) δ 174.1, 144.9, 130.8, 130.1 (2C), 112.6 (2C), 72.3 (d, $J = 8.6$ Hz), 72.1, 69.6, 66.9, 64.5 (d, $J = 5.2$ Hz), 59.4 (d, $J = 5.2$ Hz), 54.5, 54.4, 54.4, 53.9 (2C), 40.9 (2C), 34.3, 34.0, 32.3, 30.1, 30.0, 29.9, 29.9, 29.8, 27.3, 26.4, 23.1, 14.3. IR (neat) 2923, 2852, 2366, 1734, 1518, 1247, 1088, 750 cm^{-1}. *m/z* (M + Na^+) 817.44.

(*S*)-(2,3-Di-*O*-*tert*-butyldimethylsilyl)glyceryl 2-Cyanoethyl-*N*,*N*-diisopropylphosphoramidite (11). Alcohol **10** (904 mg, 2.82 mmol) and diisopropylethylamine (1.0 mL, 5.92 mmol) were dissolved in anhydrous CH_2Cl_2 (10 mL) under an atmosphere of N_2. 2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (1.0 g, 4.22 mmol) was added dropwise, and the mixture was stirred at 20 °C for 1.5 h, after which EtOAc (20 mL) and saturated $NaHCO_3$ (50 mL) were added and the organic layer was isolated by extraction with EtOAc (2 × 50 mL). The combined organic phases were concentrated in vacuo, and the residue was purified by column chromatography (EtOAc) to give 1352 mg (92%) of **11** (two diastereoisomers, 1:1) as a colorless oil. $R_f = 1.0$ (EtOAc). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.87−3.46 (m, 5H), 2.67−2.61 (m, 2H), 1.20−1.17 (m, 12H), 0.90 (s, 9H), 0.89 (s, 9H), 0.09−0.06 (m, 12H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 117.7, 73.3, 65.0, 58.6, 43.1 (2C), 26.1 (3C), 26.0 (3C), 24.7 (4C), 20.5, 18.5, 18.3, −4.4, −4.5, −5.2, −5.3. ^{31}P NMR (202 MHz, $CDCl_3$): δ 149.0, 148.5. IR (neat): 2958, 2929, 2883, 2857 cm^{-1}. *m/z* (M + Na^+) 543.32.

1-*O*-Hexadecyl-2-(4-(4-(bis-(2-chloroethyl)amino)phenyl)butanoyl)-*sn*-glycero-3-(2-cyanoethylphospho)-(*S*)-2,3-di-*O*-*tert*-butyldimethylsilylglycerol (12a). To a solution of **6a** (0.79 g, 1.8 mmol) and **11** (1.3 g, 2.5 mmol) in CH_2Cl_2 (10 mL) was added molecular sieves (3 Å). After the mixture was stirred for 30 min, 1*H*-tetrazole in acetonitrile (5.5 mL, 0.45 M, 2.5 mmol) was added and the mixture stirred for another 30 min before 5.5 M *tert*-butyl hydroperoxide in decane (0.50 mL, 2.8 mmol) was added and the mixture stirred for 1 h before being concentrated in vacuo. The residue was purified by column chromatography (EtOAc/heptane 1:1) to yield 1.63 g of an oil. ^{31}P NMR showed two signals at −0.66 and −0.82 in the 1:1 ratio set by the amidite. The product was subsequently treated with DDQ (0.45 g, 2.2 mmol) in CH_2Cl_2 (10 mL) and water (0.6 mL) for 2 h before Na_2SO_3 was added and the mixture diluted with CH_2Cl_2. The mixture was filtered and concentrated in vacuo before purification by column chromatography (CH_2Cl_2, then EtOAc/heptane 1:1) afforded 1.16 g. ^{31}P NMR showed two signals at −0.10 and −0.18 ppm. The deprotected compound was dissolved in CH_2Cl_2 (12 mL) together with chlorambucil (0.70 g, 2.3 mmol), and EDCI (0.59 g, 3.1 mmol) and DMAP (0.38 g, 3.1 mmol) were added. After being stirred for 2 h, the mixture was concentrated in vacuo and purified by column chromatography (EtOAc/heptane 1:1) and the resulting 1.36 g product dissolved in CH_2Cl_2 (10 mL) and treated with DBU (0.20 mL, 1.3 mmol) for 30 min. The reaction mixture was concentrated in vacuo and the residue was purified by column chromatography (CH_2Cl_2/MeOH 9:1) to give the phospholipid (0.96 g, 54%). $R_f = 0.73$ (CH_2Cl_2/MeOH/H_2O 65:25:4). ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$ 3:1): δ 6.90 (d, $J = 8.5$ Hz, 2H), 6.47 (d, $J = 8.5$ Hz, 2H), 5.00 (q, $J = 5$ Hz, 1H), 4.08 (m, 1H), 3.83−3.76 (m, 2H), 3.71−3.59 (m, 3H), 3.55−3.53 (m, 4H), 3.47−3.38 (m, 8H), 3.30−3.21(m, 2H), 2.38 (t, $J = 7.5$ Hz, 2H), 2.19 (t, $J = 7.5$ Hz, 2H), 1.73 (q, $J = 7.3$ Hz, 2H), 1.36 (m, 2H), 1.09 (s, 26 H), 0.72 (s, 18H), −0.06 (s, 6H), −0.11 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$ 3:1): δ 173.1, 144.0, 130.0, 129.3, 111.8, 72.4 (d, $J = 9.5$ Hz), 71.5, 71.4 (d, $J = 6.3$ Hz), 68.8, 66.5 (d, $J = 5.6$ Hz), 64.6, 63.5 (d, $J = 5.2$ Hz), 53.2, 40.1, 33.5, 33.3, 31.5, 29.3, 29.3, 29.2, 29.1, 29.0, 26.4, 25.6, 25.5, 25.4, 22.3, 17.9, 17.7, 13.6, −5.1, −5.1, −5.8, −5.8. ^{31}P NMR (202 MHz, $CDCl_3/CD_3OD$ 3:1): δ −1.82. IR (neat): 3450, 2922, 1732, 1616, 1519, 1463, 1360, 1252, 1102 cm^{-1}. *m/z* (M + Na^+) 1006.53.

1-*O*-Octadecyl-2-(4-(4-(bis-(2-chloroethyl)amino)phenyl)butanoyl)-*sn*-glycero-3-(2-cyanoethylphospho)-(*S*)-2,3-di-*O*-*tert*-butyldimethylsilylglycerol (12b). The synthesis was performed as for **12a**, starting from **6b** (650 mg, 1.40 mmol) and **11** (1.0 g, 1.9 mmol), affording 810 mg (57%) of **12b** as a colorless oil. $R_f = 0.73$ (CH_2Cl_2/MeOH/H_2O 65:25:4. ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$ 3:1): δ 6.96 (d, $J = 8.7$ Hz, 2H), 6.53 (d, $J = 8.7$ Hz, 2H), 5.08 (q, $J = 5.4$ Hz, 1H), 3.82−3.75 (m, 2H), 3.70−3.58 (m, 3H), 3.54−3.51 (m, 4H), 3.46−3.36 (m, 8H), 3.29−3.22 (m, 2H), 2.45 (t, $J = 7.7$ Hz, 2H), 2.24 ($J = 7.7$ Hz, 2H), 1.77 (q, $J = 7.4$ Hz, 2H), 1.42 (m, 2H), 1.15 (s, 30H), 0.78 (s, 18H), −0.01 (s, 6H), −0.05 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$ 3:1): δ 173.2, 144.1, 130.2, 129.4, 111.8, 72.4 (d, $J = 9.6$ Hz), 71.5, 71.5 (d, $J = 7.8$ Hz), 68.8, 66.6 (d, $J = 5.5$ Hz), 64.6, 63.6 (d, $J = 5.4$ Hz), 53.3, 40.2, 33.6, 33.3, 31.6, 29.4, 29.4, 29.3, 29.2, 29.1, 26.5, 25.7, 25.6, 25.5, 22.4, 18.0, 17.8, 13.8, −4.9, −5.0, −5.7, −5.7. ^{31}P NMR (202 MHz, $CDCl_3/CD_3OD$ 3:1): δ −2.00. IR (neat): 3448, 2926, 2854, 1735, 1617, 1519, 1464, 1360, 1252, 1108 cm^{-1}. *m/z* (M + Na^+) 1034.57.

1-*O*-Hexadecyl-2-(4-(4-(bis(2-chloroethyl)amino)phenyl)butanoyl)-*sn*-glycero-3-phospho-(*S*)-glycerol (2a). Compound **12a** (0.42 g, 0.43 mmol) was dissolved in MeCN (9 mL) and cooled to 0 °C. Then 40% aqueous HF (1 mL) was added, and the mixture was allowed to reach 20 °C while being stirred vigorously for 2 h. The reaction mixture was then poured into saturated aqueous $NaHCO_3$ (20 mL) and extracted with CH_2Cl_2 (3 × 10 mL) and EtOAc (10 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo and the residue was purified by column chromatography (CH_2Cl_2/MeOH/H_2O 65:25:4) to afford **2a** (0.19 g, 59%). $R_f = 0.56$ (CH_2Cl_2/MeOH/H_2O 65:25:4). ^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD 3:1): δ 6.88 (d, $J = 8.6$ Hz, 2H), 6.45 (d, $J = 8.6$ Hz, 2H), 4.98 (q, $J = 5$ Hz, 1H), 3.84−3.71 (m, 3H), 3.60 (q, $J = 5$ Hz, 1H), 3.54−3.51 (m, 4H), 3.46−3.39 (m, 8H), 3.30−3.20 (m, 2H), 2.37 (t, $J = 7.5$ Hz, 2H), 2.17 (t, $J = 7.6$ Hz, 2H), 1.71 (q, $J = 7.3$ Hz, 2H), 1.35 (t, $J = 6.6$ Hz, 2H), 1.07 (s, 26H), 0.69 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$ 3:1): δ 173.2, 144.0, 129.9, 129.2, 111.7, 71.4 (d, $J = 5.4$ Hz), 71.3, 70.4 (d, $J = 4.8$ Hz), 68.7, 66.1 (d, $J = 4.9$ Hz), 63.8 (d, $J = 4.8$), 61.8, 53.1, 40.1, 33.5, 33.4, 33.2, 33.0, 31.5, 29.3, 29.2, 29.1, 29.1, 28.9, 26.4, 25.6, 22.2, 13.5. ^{31}P NMR (202 MHz, $CDCl_3/CD_3OD$ 3:1): δ −0.99. IR (neat): 3345, 2923, 2853, 1732, 1616, 1519, 1466, 1356, 1248, 1115, 1002 cm^{-1}. *m/z* (M + H^+) 778.36.

1-*O*-Octadecyl-2-(4-(4-(bis(2-chloroethyl)amino)phenyl)butanoyl)-*sn*-glycero-3-phospho-(*S*)-glycerol (2b). The synthesis was performed as for **2a**, starting from **12b** (334 mg, 0.33 mmol) and affording **2b** (83 mg, 32%). $R_f = 0.56$ (CH_2Cl_2/MeOH/H_2O 65:25:4). ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$ 3:1): δ 6.89 (d, $J = 8.5$ Hz, 2H), 6.46 (d, $J = 8.2$ Hz, 2H), 4.99 (q, $J = 5$ Hz, 1H), 3.84−3.72 (m, 3H), 3.60 (q, $J = 5$ Hz, 1H), 3.53 (m, 4H), 3.47−3.41 (m, 8H), 3.30−3.20 (m, 2H), 2.37 (t, $J = 7.5$ Hz, 2H), 2.17 (t, $J = 7.5$ Hz, 2H), 1.71 (q, $J = 7.5$ Hz, 2H), 1.35 (t, $J = 6.5$ Hz, 2H), 1.07 (s, 30H), 0.70 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$ 3:1): δ 173.3, 144.0, 130.0, 129.2, 111.8, 71.5, 71.4 (d, $J = 6.3$ Hz), 70.5 (d, $J = 4.8$ Hz), 68.7, 66.1, 63.8 (d, $J = 5.6$ Hz), 61.9, 53.2, 40.1, 33.5, 33.2, 31.5, 29.3, 29.3, 29.2, 29.1, 29.0, 26.4, 25.6, 22.3, 13.6. ^{31}P NMR (202 MHz, $CDCl_3/CD_3OD$ 3:1): δ −0.31. IR (neat): 3332, 2923, 2853, 1733, 1616, 1519, 1466, 1355, 1236, 1115, 1002 cm^{-1}. *m/z* (M + Na^+) 828.37.

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Supporting Information Available: Analytical and spectral data for all synthesized compounds, experimental procedures for the synthesis of **5a**, **5b**, **6a**, **6b**, **7a**, **7b**, **9**, and **10**, prodrugs stability data, Mosher ester analysis data of **10**, further MALDI-TOF MS and HPLC data for $sPLA_2$ degradation experiments, and alkylating assay data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Gregoriadis, G.; Wills, E. J.; Swain, C. P.; Tavill, A. S. Drug-carrier potential of liposomes in cancer chemotherapy. *Lancet* **1974**, *1*, 1313–1316.

(2) (a) Maeda, H.; Matsumura, Y. Tumoritropic and lymphotropic principles of macromolecular drugs. *Crit. Rev. Ther. Drug Carrier Syst.* **1989**, *6*, 193–210. (b) Seymour, L. W. Passive tumor targeting of soluble macromolecules and drug conjugates. *Crit. Rev. Ther. Drug Carrier Syst.* **1992**, *9*, 135–187. (c) Yuan, F.; Leunig, M.; Huang, S. K.; Berk, D. A.; Papahadjopoulos, D.; Jain, R. K. Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Res.* **1994**, *54*, 3352–3356. (d) Jain, R. K. Delivery of molecular and cellular medicine to solid tumors. *J. Controlled Release* **1998**, *53*, 49–67.

(3) Andresen, T. L.; Jensen, S. S.; Jørgensen, K. Advanced strategies in liposomal cancer therapy: problems and prospects of active and tumor specific drug release. *Prog. Lipid Res.* **2005**, *44*, 68–97.

(4) (a) Pak, C. C.; Ali, S.; Janoff, A. S.; Meers, P. Triggerable liposomal fusion by enzyme cleavage of a novel peptide−lipid conjugate. *Biochim. Biophys. Acta* **1998**, *1372*, 13–27. (b) Meers, P. Enzyme-activated targeting of liposomes. *Adv. Drug Delivery Rev.* **2001**, *53*, 265–272. (c) Davidsen, J.; Jørgensen, K.; Andresen, T. L.; Mouritsen, O. G. Secreted phospholipase A2 as a new enzymatic trigger mechanism for localised liposomal drug release and absorption in diseased tissue. *Biochim. Biophys. Acta* **2003**, *1609*, 95−101.

(5) (a) Connor, J.; Yatvin, M. B.; Huang, L. pH-sensitive liposomes: acid-induced liposome fusion. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 1715–1718. (b) Ellens, H.; Bentz, J.; Szoka, F. C. pH-induced destabilization of phosphatidylethanolamine-containing liposomes: role of bilayer contact. *Biochemistry* **1984**, *23*, 1532–1538. (c) Collins, D.; Litzinger, D. C.; Huang, L. Structural and functional comparisons of pH-sensitive liposomes composed of phosphatidylethanolamine and three different diacylsuccinylglycerols. *Biochim. Biophys. Acta* **1990**, *1025*, 234–242. (d) Shin, J.; Shum, P.; Thompson, D. H. Acid-triggered release via dePEGylation of DOPE liposomes containing acid-labile vinyl ether PEG-lipids. *J. Controlled Release* **2003**, *91*, 187–200.

(6) (a) Miller, C. R.; Bennett, D. E.; Chang, D. Y.; O'Brien, D. F. Effect of liposomal composition on photoactivated liposome fusion. *Biochemistry* **1996**, *35*, 11782–11790. (b) Bondurant, B.; Mueller, A.; O'Brien, D. F. Photoinitiated destabilization of sterically stabilized liposomes. *Biochim. Biophys. Acta* **2001**, *1511*, 113–122. (c) Shum, P.; Kim, J. M.; Thompson, D. H. Phototriggering of liposomal drug delivery systems. *Adv. Drug Delivery Rev.* **2001**, *53*, 273–284.

(7) (a) Yatvin, M. B.; Weinstein, J. N.; Dennis, W. H.; Blumenthal, R. Design of liposomes for enhanced local release of drugs by hyperthermia. *Science* **1978**, *202*, 1290–1293. (b) Gaber, M. H.; Hong, K.; Huang, S. K.; Papahadjopoulos, D. Thermosensitive sterically stabilized liposomes: formulation and in vitro studies on mechanism of doxorubicin release by bovine serum and human plasma. *Pharm. Res.* **1995**, *12*, 1407–1416. (c) Kono, K.; Nakai, R.; Morimoto, K.; Takagishi, T. Temperature-dependent interaction of thermo-sensitive polymer-modified liposomes with CV1 cells. *FEBS Lett.* **1999**, *456*, 306–310. (d) Needham, D.; Anyarambhatla, G.; Kong, G.; Dewhirst, M. W. A new temperature-sensitive liposome for use with mild hyperthermia: characterization and testing in a human tumor xenograft model. *Cancer Res.* **2000**, *60*, 1197–1201.

(8) (a) Gabizon, A.; Catane, R.; Uziely, B.; Kaufman, B.; Safra, T.; Cohen, R.; Martin, F.; Huang, A.; Barenholz, Y. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. *Cancer Res.* **1994**, *54*, 987–992. (b) Gabizon, A.; Shmeeda, H.; Barenholz, Y. Pharmacokinetics of pegylated liposomal doxorubicin. Review of animal and human studies. *Clin. Pharmacokinet.* **2003**, *42*, 419–436.

(9) Graybill, J. R.; Craven, P. C.; Taylor, R. L.; Williams, D. M.; Magee, W. E. Treatment of murine cryptococcosis with liposome-associated amphotericin-B. *J. Infect. Dis.* **1982**, *145*, 748–752.

(10) (a) Abe, T.; Sakamoto, K.; Kamohara, H.; Hirano, Y.; Kuwahara, N.; Ogawa, M. Group II phospholipase A2 is increased in peritoneal and pleural effusions in patients with various types of cancer. *Int. J. Cancer* **1997**, *74*, 245–250. (b) Graff, J. R.; Konicek, B. W.; Deddens, J. A.; Chedid, M.; Hurst, B. M.; Colligan, B.; Neubauer, B. L.; Carter, H. W.; Carter, J. H. Expression of group IIa secretory phospholipase A2 increases with prostate tumor grade. *Clin. Cancer Res.* **2001**, *7*, 3857–3861. (c) Laye, J.; Gill, J. H. Phospholipase A_2 expression in tumours: a target for therapeuticintervention. *Drug Discovery Today* **2003**, *8*, 710–716. (d) Murakami, M.; Kudo, I. Phospholipase A_2. *J. Biochem.* **2002**, *131*, 285–292.

(11) (a) Andresen, T. L.; Jensen, S. S.; Madsen, R.; Jørgensen, K. Synthesis and biological activity of anticancer ether lipids that are specifically released by phospholipase A_2 in tumor tissue. *J. Med. Chem.* **2005**, *48*, 7305–7314. (b) Andresen, T. L.; Davidsen, J.; Begtrup, M.; Mouritsen, O. G.; Jørgensen, K. Enzymatic release of antitumor ether lipids by specific phospholipase A_2 activation of liposome-forming prodrugs. *J. Med. Chem.* **2004**, *47*, 1694–1703.

(12) Yamashita, S.; Yamashita, J.; Sakamoto, K.; Inada, K.; Nakashima, Y.; Murata, K.; Saishoji, T.; Nomura, K.; Ogawa, M. Increased expression of membrane-associated phospholipase-A2 shows malignant potential of human breast-cancer cells. *Cancer* **1993**, *71*, 3058–3064.

(13) (a) Murata, K.; Egami, H.; Kiyohara, H.; Oshima, S.; Kurizaki, T.; Ogawa, M. Expression of group-II phospholipase-A2 in malignant and nonmalignant human gastric-mucosa. *Br. J. Cancer* **1993**, *68*, 103–111. (b) Leung, S. Y.; Chen, X.; Chu, K. M.; Yuen, S. T.; Mathy, J.; Ji, J. F.; Chan, A. S. Y.; Li, R.; Law, S.; Troyanskaya, O. G.; Tu, I. P.; Wong, J.; So, S.; Botstein, D.; Brown, P. O. Phospholipase A2 group IIA expression in gastric adenocarcinoma is associated with prolonged survival and less frequent metastasis. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 16203–16208.

(14) (a) Kennedy, B. P.; Soravia, C.; Moffat, J.; Xia, L.; Hiruki, T.; Collins, S.; Gallinger, S.; Bapat, B. Overexpression of the nonpancreatic secretory group II PLA(2) messenger RNA and protein in colorectal adenomas from familial adenomatous polyposis patients. *Cancer Res.* **1998**, *58*, 500–503. (b) Edhemovic, I.; Snoj, M.; Kljun, A.; Golouh, R. Immunohistochemical localization of group II phospholipase A2 in the tumours and mucosa of the colon and rectum. *Eur. J. Surg. Oncol.* **2001**, *27*, 545–548. (c) Praml, C.; Amler, L. C.; Dihlmann, S.; Finke, L. H.; Schlag, P.; Schwab, M. Secretory type II phospholipase A(2) (PLA2G2A) expression status in colorectal carcinoma derived cell lines and in normal colonic mucosa. *Oncogene* **1998**, *17*, 2009–2012.

(15) Kashiwagi, M.; Friess, H.; Uhl, W.; Berberat, P.; Abou-Shady, M.; Martignoni, M.; Anghelacopoulos, S. E.; Zimmermann, A.; Buchler, M. W. Group II and IV phospholipase A(2) are produced in human pancreatic cancer cells and influence prognosis. *Gut* **1999**, *45*, 605–612.

(16) Jiang, J.; Neubauer, B. L.; Graff, J. R.; Chedid, M.; Thomas, J. E.; Roehm, N. W.; Zhang, S.; Eckert, G. J.; Koch, M. O.; Eble, J. N.; Cheng, L. Expression of group IIA secretory phospholipase A2 is elevated in prostatic intraepithelial neoplasia and adenocarcinoma. *Am. J. Pathol.* **2002**, *160*, 667–671.

(17) Ying, Z.; Tojo, H.; Komatsubara, T.; Nakagawa, M.; Inada, M.; Kawata, S.; Matsuzawa, Y.; Okamoto, M. Enhanced expression of group-II phospholipase A(2) in human hepatocellular-carcinoma. *Biochim. Biophys. Acta* **1994**, *1226*, 201–205.

(18) (a) Everett, J. L.; Roberts, J. J.; Ross, W. C. J. Aryl-2-halogenoalkylamines. XII: Some carboxylic derivatives of *N*,*N*-di-2-chloroethylaniline. *J. Chem. Soc.* **1953**, 2386–2392. (b) Urbaniak, M. D.; Bingham, J. P.; Hartley, J. A.; Woolfson, D. N.; Caddick, S. Design and synthesis of a nitrogen mustard derivative stabilized by apo-neocarzinostatin. *J. Med. Chem.* **2004**, *47*, 4710–4715. (c) Sienkiewicz, P.; Bielawski, K.; Bielawska, A.; Palka, J. Amidine analogue of chlorambucil is a stronger inhibitor of protein and DNA synthesis in breast cancer MCF-7 cells than is the parent drug. *Eur. J. Pharmacol.* **2004**, *494*, 95–101.

(19) Altucci, L.; Gronemeyer, H. The promise of retinoids to fight against cancer. *Nat. Rev. Cancer* **2001**, *1*, 181–193.

(20) (a) Forman, B. M.; Tontonoz, P.; Chen, J.; Brun, R. P.; Spiegelman, B. M.; Evans, R. M. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. *Cell* **1995**, *83*, 803–812. (b) Naitoh, T.; Kitahara, M.; Tsuruzoe, N. The effect of activation of peroxisome proliferator-activated receptor gamma (PPARγ) on human monocyte function: PPARγ ligands do not inhibit tumor necrosis factor-α release in human monocytic cell line THP-1. *Cell Biol. Toxicol.* **2000**, *16*, 131–135.

(21) Kundu, G. C.; Schullek, J. R.; Wilson, I. B. The alkylating properties of chlorambucil. *Pharmacol., Biochem. Behav.* **1993**, *49*, 621−624.

(22) Montserrat, E.; Rozman, C. Chronic lymphocytic leukaemia treatment. *Blood Rev.* **1993**, *7*, 164–175.

(23) (a) Ehrsson, H.; Eksborg, S.; Wallin, I.; Nilsson, S. O. Degradation of chlorambucil in aqueous solution. *J. Pharm. Sci.* **1980**, *69*, 1091–1094. (b) Chatterji, D. C.; Yeager, R. L.; Gallelli, J. F. Kinetics of chlorambucil hydrolysis using high-pressure liquid chromatography. *J. Pharm. Sci.* **1982**, *71*, 50–54. (c) Pettersson-Fernholm, T.; Vilpo, J.; Kosonen, M.; Hakala, K.; Hovinen, J. Reactions of 4-bis(2-chloroethyl)aminophenylacetic acid (phenylacetic acid mustard) in physiological solutions. *J. Chem. Soc., Perkin Trans. 2* **1992**, *2*, 2183–2187. (d) Löf, K.; Hovinen, J.; Reinikainen, P.; Vilpo, L. M.; Seppälä, E.; Vilpo, J. A. Kinetics of chlorambucil in vitro: effects of fluid matrix, human gastric juice, plasma proteins and red cells. *Chem.-Biol. Interact.* **1997**, *103*, 187–198. (e) Balboa, M. A. H.; Arévalo, V. V.; Reyes, V. H. A.; Velázquez, A. M.; Ganem-Quintanar, A.; Quintanar, D.; Camacho, B.; Arzaluz, G. N.; Rosales-Hoz, M.; Leyva, M. A.; Angeles, E. Study of chlorambucil and chlorambucil-trimethyl-*b*-cyclodextrin inclusion complex by CE. *Chromatographia* **2008**, *67*, 193–196.

(24) (a) Peters, U.; Bankova, W.; Welzel, P. Platelet-activating-factor synthetic studies. *Tetrahedron* **1987**, *43*, 3803–3816. (b) Massing, U.; Eibl, H. Synthesis of enantiomerically pure 1-*O*-phosphocholine-2-*O*-acyl-octadecane and 1-*O*-phosphocholine-2-*N*-acyl-octadecane. *Chem. Phys. Lipids* **1994**, *69*, 105–120. (c) Massing, U.; Eibl, H. New optically pure dimethylacetals of glyceraldehydes and their application for lipid and phospholipid synthesis. *Chem. Phys. Lipids* **1995**, *76*, 211–224.

(25) (a) Hirth, G.; Barner, R. Synthesis of glyceryl etherphosphatides. 1. Preparation of 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine (platelet activating factor), of its enantiomer and of some analogous compounds. *Helv. Chim. Acta* **1982**, *65*, 1059–1084. (b) Guivisdalsky, P. N.; Bittman, R. Novel enantioselective synthesis of platelet activating factor and its enantiomer via ring-opening of glycidyl tosylate with 1-hexadecanol. *Tetrahedron Lett.* **1988**, *29*, 4393–4396. (c) Guivisdalsky, P. N.; Bittman, R. An efficient stereocontrolled route to both enantiomers of platelet activating factor and analogues with long chain esters at C2. Saturated and unsaturated ether glycerolipids by opening of glycidyl arenesulfonates. *J. Org. Chem.* **1989**, *54*, 4643–4648. (d) Lindberg, J.; Ekeroth, J.; Konradsson, P. Efficient synthesis of phospholipids from glycidyl phosphates. *J. Org. Chem.* **2002**, *67*, 194–199.

(26) Guivisdalsky, P. N.; Bittman, R. Regiospecific opening of glycidyl derivatives mediated by boron trifluoride. Asymmetric synthesis of ether-linked phospholipids. *J. Org. Chem.* **1989**, *54*, 4637–4642.

(27) Wang, P.; Blank, D. H.; Spencer, T. A. Synthesis of benzophenone-containing analogues of phosphatidylcholine. *J. Org. Chem.* **2004**, *69*, 2693–2703.

(28) (a) Nakajima, N.; Horita, K.; Abe, R.; Yonemitsu, O. MPM (4-methoxybenzyl) protection of hydroxyl functions under mild acidic conditions. *Tetrahedron Lett.* **1988**, *29*, 4139–4142. (b) Rai, A. N.; Basu, A. An efficient method for *para*-methoxybenzyl ether formation with lanthanum triflate. *Tetrahedron Lett.* **2003**, *44*, 2267–2269.

(29) (a) Hirth, G.; Barner, R. Synthesis of glyceryl etherphosphatides. 1. Preparation of 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine (platelet activating factor), of its enantiomer and of some analogous compounds. *Helv. Chim. Acta* **1982**, *65*, 1059–1084. (b) Chupin, V. V.; Ostapenko, O. V.; Klykov, V. N.; Anikin, M. V.; Serebrennikova, G. A. Formation of a structural isomer of platelet-activing-factor during 1-alkyl-*sn*-glycero-3-phosphocholine acetylation. *Bioorg. Khim.* **1993**, *19*, 1111–1121.

(30) Horita, K.; Yoshioka, T.; Tanaka, T.; Oikawa, Y.; Yonemitsu, O. On the selectivity of deprotection of benzyl, MPM (4-methoxybenzyl) and DMPM (3,4-dimethoxybenzyl) protecting groups for hydroxyl functions. *Tetrahedron* **1986**, *42*, 3021–3028.

(31) Neises, B.; Steglich, W. Simple method for the esterification of carboxylic acids. *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 522−524.

(32) Boden, E. P.; Keck, G. E. Proton-transfer steps in steglich esterification: A very practical new method for macrolactonization. *J. Org. Chem.* **1985**, *50*, 2394–2395.

(33) (a) Kolb, H. C.; VanNieuwenhze, M. S.; Sharpless, K. B. Catalytic asymmetric dihydroxylation. *Chem. Rev.* **1994**, *94*, 2483–2547. (b) Corey, E. J.; Guzman-Perez, A.; Noe, M. C. The application of a mechanistic model leads to the extension of the sharpless asymmetric dihydroxylation to allylic 4-methoxybenzoates and conformationally related amine and homoallylic alcohol derivatives. *J. Am. Chem. Soc.* **1995**, *117*, 10805–10816.

(34) Evans, D. A.; Gage, J. R.; Leighton, J. L. Asymmetric synthesis of calyculin A. 3. Assemblage of the calyculin skeleton and the introduction of a new phosphate monoester synthesis. *J. Org. Chem.* **1992**, *57*1964–1966.

(35) (a) Lichtenberg, D.; Barenholz, Y. Liposomes. Preparation, Characterization and Preservation. *Methods Biochem. Anal.* **1988**, *33*, 337–462. (b) Tirrell, D. A.; Takigawa, D. Y.; Seki, K. Interactions of synthetic polymers with cell-membranes and model membrane systems. 7. pH sensitization of phospholipid-vesicles via complexation with synthetic poly(carboxylic acid)s. *Ann. N.Y. Acad. Sci.* **1985**, *446*, 237–248.

(36) (a) Singer, A. G.; Ghomashchi, F.; Le Calvez, C.; Bollinger, J.; Bezzine, S.; Rouault, M.; Sadilek, M.; Nguyen, E.; Lazdunski, M.; Lambeau, G.; Gelb, M. H. Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A(2). *J. Biol. Chem.* **2002**, *277*, 48535–48549. (b) Bahnson, B. J. Structure, function and interfacial allosterism in phospholipase A2: insight from the anion-assisted dimer. *Arch. Biochem. Biophys.* **2005**, *433*, 96–106. (c) Gadd, M. E.; Biltonen, R. L. Characterization of the interaction of phospholipase A(2) with phosphatidylcholine−phosphatidylglycerol mixed lipids. *Biochemistry* **2000**, *39*, 12312–12323. (d) Leidy, C.; Linderoth, L.; Andresen, T. L.; Mouritsen, O. G.; Jørgensen, K.; Peters, G. H. Domain-induced activation of human phospholipase A_2 type IIA: local versus global lipid composition. *Biophys. J.* **2006**, *90*, 3165–3175. (e) Peters, G. H.; Møller, M. S.; Jørgensen, K.; Rönnhölm, P.; Mikkelsen, M.; Andresen, T. L. Secretory phospholipase A(2) hydrolysis of phospholipid analogues is dependent on water accessibility to the active site. *J. Am. Chem. Soc.* **2007**, *129*, 5451–5461.

(37) (a) Harvey, D. J. Matrix-assisted laser desorption/ionization mass spectrometry of phospholipids. *J. Mass Spectrom.* **1995**, *30*, 1333–1346. (b) Schiller, J.; Arnhold, J.; Benard, S.; Müller, M.; Reichl, S.; Arnold, K. Lipid analysis by matrix-assisted laser desorption and ionization mass spectrometry: a methodological approach. *Anal. Biochem.* **1999**, *267*, 46–56. (c) Petković, M.; Muller, J.; Muller, M.; Schiller, J.; Arnold, K.; Arnhold, J. Application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for monitoring the digestion of phosphatidylcholine by pancreatic phospholipase A_2. *Anal. Biochem.* **2002**, *308*, 61–70.

(38) (a) Epstein, J.; Rosenthal, R. W.; Ess, R. J. Use of *p*-(4-nitrobenzyl)pyridine as analytical reagent for ethylenimines and alkylating agents. *Anal. Chem.* **1955**, *27*, 1435–1439. (b) Friedman, O. M.; Boger, E. Chlorimetric estimation of nitrogen mustard in aqueous media. *Anal. Chem.* **1961**, *33*, 906–910. (c) Genka, S.; Deutsch, J.; Shetty, U. H.; Stahle, P. L.; John, V.; Lieberburg, I. M.; Ali-Osmant, F.; Rapoport, S. I.; Greig, N. H. Development of lipophilic anticancer agents for the treatment of brain tumors by the esterification of water-soluble chlorambucil. *Clin. Exp. Metastasis* **1993**, *11*, 131–140.

(39) Gottlieb, H. E.; Kotlyar, V.; Nudelman, A. NMR chemical shifts of common laboratory solvents as trace impurities. *J. Org. Chem.* **1997**, *62*, 7512–7515.

(40) Chen, P. S.; Toribara, T. Y.; Warner, H. Microdetermination of phosphorus. *Anal. Chem.* **1956**, *28*, 1756–1758.

(41) Carmichael, J.; DeGraff, W. G.; Gazdar, A. F.; Minna, J. D.; Mitchell, J. B. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.* **1987**, *47*, 936–942.

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