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Effects of steroidal carriers of alkylating agents on the phase transition in DPPC membrane bilayers

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Abstract

Several steroidal esters of alkylating agents have been synthesized and tested in vitro and in vivo in various experimental cancer types. 3β-Hydroxy-17α-aza-D-homo-5-androsten-7,17-dione-*N*,*N*-bis(2-chloroethyl) aminophenylacetate (**I**) is a highly active compound. DSC scans show differences between the alkylating agent alone and in conjugation with the steroidal part in the broadening and lowering of the phase transition of DPPC bilayers. These differences may in part explain the better pharmacokinetic profile and lower toxicity of conjugated congener **I** versus the alkylating agent alone.

Keywords: Steroids; Nitrogen mustards; Antitumour steroidal esters; Differential scanning calorimetry; Dipalmitoylphosphatidylcholine bilayers

1. Introduction

3β-Hydroxy-17α-aza-D-homo-5-androsten-7,17-dione-*p*-*N*,*N*-bis(2-chloroethyl)amino phenylacetate (SOT-19, **I**) (Fig. 1), which constitutes a successful combination of the alkylating agent chlorambucil's active metabolite (*p*-*N*,*N*-bis(2-chloroethyl)aminophenylacetic acid) [1] and the modified steroid 3β-hydroxy-17α-aza-D-homo-5-androsten-7,17-dione, possesses high antileukemic activity and moderate toxicity in vitro and in vivo [2]. It has been demonstrated that the chemical conjugation of nitrogen mustards to carrier-molecules reduces the toxicity of these alkylating agents and increases selectivity and effectiveness towards alkylation of DNA [3,4]. The steroidal part of **I** (3β-hydroxy-17α-aza-D-homo-5-androsten-7,17-dione) is a modified androstan derivative that comprises a lactamic D-ring. The thermotropic effects of several androstans on membrane bilayers have been extensively studied by DSC and other techniques, and it is evident that in all cases the structural similarity of these compounds to cholesterol dictates an analogous mode of interaction with membrane bilayers [5–11].

Some anticancer drugs like paclitaxel [12] and tamoxifen [13] have been studied in relation to their thermotropic effects on model membrane bilayers, but to our knowledge no such study has been conducted until now on any alkylating mustard. Our work provides for the first time information on drug-model-membrane interactions of a modified steroid, an alkylating agent and a chemical combination of the two.

The changes in the thermally induced phase transition caused by the steroidal part, the alkylating part, a mixture of the two and the combined molecule when incorporated in lipid membrane bilayers were compared. Different effects were observed among the four samples, which may in part explain the lower toxicity and different pharmacokinetic properties of the conjugated molecule versus the alkylating agent alone.

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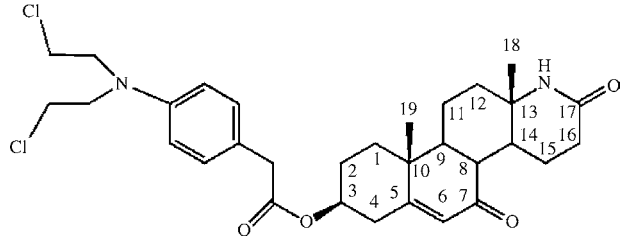

Fig. 1. 3β-Hydroxy-17α-aza-D-homo-5-androsten-7,17-dione-*p*-*N*,*N*-bis (2-chloroethyl)aminophenylacetate (**I**) comprises an alkylating agent and a modified steroid with an expanded lactamic D-ring.

2. Experimental

Thermograms were obtained with a Perkin-Elmer DSC-7 calorimeter (Norwalk, Connecticut, USA). Dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar Lipid Inc. $CDCl_3$ of high purity (>99%) was purchased from Sigma (St. Louis, MO, USA). The samples contained combinations of DPPC bilayers and 3β-hydroxy-17α-aza-D-homo-5-androsten-7,17-dione-*N*,*N*-bis(2-chloroethyl) aminophenylacetate (**I**), 3β-acetoxy-17α-aza-D-homo-5-androsten-7,17-dione (**II**), *N*,*N*-bis(2-chloroethyl)amino-phenylacetic acid (**III**) and a mixture of **II** and **III** (**IV**). The steroidal part and its ester were synthesized in our laboratory, their purity was checked by HPLC (waters, Millipore®) and their structure identity was confirmed by NMR spectroscopy.

After mixing the compound with DPPC, the solvent was evaporated by rotavapore under vacuum (0.1 mmHg) at a temperature above the transition temperature of the phospholipid. For measurements, this dry residue was dispersed in appropriate amounts of bi-distilled water by vortexing. The samples (ca. 5 mg) were sealed into stainless steel capsules of 7.54 mm diameter and 2.79 mm height obtained from Perkin-Elmer. The sample concentrations (calculated in molar ratio of the drug versus the mixture of the phospholipid and the drug) were 0.05, 0.10 and 0.20 (symbolized as $x = 0.05$, 0.10 and 0.20 correspondingly). All samples were scanned at least twice until identical thermograms were obtained with a scanning rate of 2.5 °C/min. The temperature scale of the calorimeter was calibrated with indium ($T_m = 156.6$ °C).

3. Results

Fully hydrated DPPC bilayers show a characteristic thermogram consisting of a broad low enthalpy transition at 35.3 °C and a sharp enthalpy main transition at 41.2 °C. The DPPC bilayer exists in the gel phase ($L_{\beta'}$) at temperatures lower than 33 °C, and in the liquid crystalline phase at temperatures higher than 42 °C ($L_{\alpha'}$). Between 33 and 42 °C the phospholipid bilayer exists in the $P_{\beta'}$ or ripple phase. The DSC scan of fully hydrated DPPC multibilayers shows a pretransition centered at 35 °C and a peak maximum at 41.2 °C. The main phase transition is accompanied by several structural changes in the lipid molecules as well as systematic alterations in the bilayer geometry, but the most prominent feature is the *trans–gauche* isomerization taking place in the acyl chain conformation. The average number of *gauche* conformers indicates the effective fluidity, which depends not only on the temperature, but also on perturbations due to the presence of a drug molecule intercalating between the lipids.

The acquired thermograms for each sample concentration are shown in Fig. 2, while the experimental data are reported in Table 1. The thermal changes caused by **II** are typical of a steroid as already reported in the literature with other steroids [9,10]. Thus, at the 95:5 phospholipid:steroid molar ratio ($x = 0.05$) the presence of the steroid causes broadening of the phase transition and lowers the phase transition temperature. At the 90:10 phospholipid:steroid molar ratio ($x = 0.10$) the broadening is accompanied by a shoulder at the high temperature side, which is abolished at the higher concentration of $x = 0.20$. The pretransition is much reduced at all concentrations of steroid or alkylating agent used. The incorporation of the alkylating agent in the DPPC bilayers causes more significant broadening of the phase transition temperature and lowering of the phase transition temperature. At $x = 0.10$ the broadening is significantly enhanced and clearly two components are visible. At the higher concentration of 80:20 phospholipid:steroid molar ratio ($x = 0.20$) a further lowering and broadening is observed.

At $x = 0.20$, the broadening of the phase transition by the alkylating agent is increased and a shoulder at the high temperature side of the main transition is observed. This thermal profile was also observed by other authors with losartan in DMPC bilayers [14] and is interpreted as a reversible transition from a vesicular suspension to an extended bilayer network. Schneider et al. published an article in which they combine calorimetry, viscosity and electron microscopy methods to explain this thermal profile [15]. They state that these structural transitions arise from two effects: (i) enhanced membrane elasticity accompanying the lipid state fluctuations on chain melting and (ii) solvent-associated interactions

Table 1
T_{onset} is the main transition onset temperature, ΔH the enthalpy change (the average of three measurements ± standard deviation), T_m the main transition maximum temperature, and $T_{m1/2}$ the half-width of the main transition curve

Samples	T_{onset} (°C)	ΔH (J/g)	$T_{m1/2}$ (°C)	T_m (°C)
I ($x = 0.05$)	39.16	44.87 ± 0.34	1.7	40.94
I ($x = 0.10$)	38.76	45.53 ± 0.07	1.6	40.13
I ($x = 0.20$)	38.14	45.06 ± 0.06	1.9	39.64
II ($x = 0.05$)	39.95	43.20 ± 0.27	1.5	41.36
II ($x = 0.10$)	40.27	42.40 ± 0.14	1.3	41.20
II ($x = 0.20$)	40.46	44.32 ± 0.27	1.6	42.01
III ($x = 0.05$)	38.78	44.00 ± 0.38	1.7	40.56
III ($x = 0.10$)	36.98	43.35 ± 0.20	2.7	39.73
III ($x = 0.20$)	30.83	44.46 ± 0.01	4.8	32.64
II + **III** ($x_{tot} = 0.05$)	39.37	43.09 ± 0.11	1.7	41.32
II + **III** ($x_{tot} = 0.10$)	37.87	43.61 ± 0.16	2.5	40.60
II + **III** ($x_{tot} = 0.20$)	34.50	42.74 ± 0.25	4.0	37.59

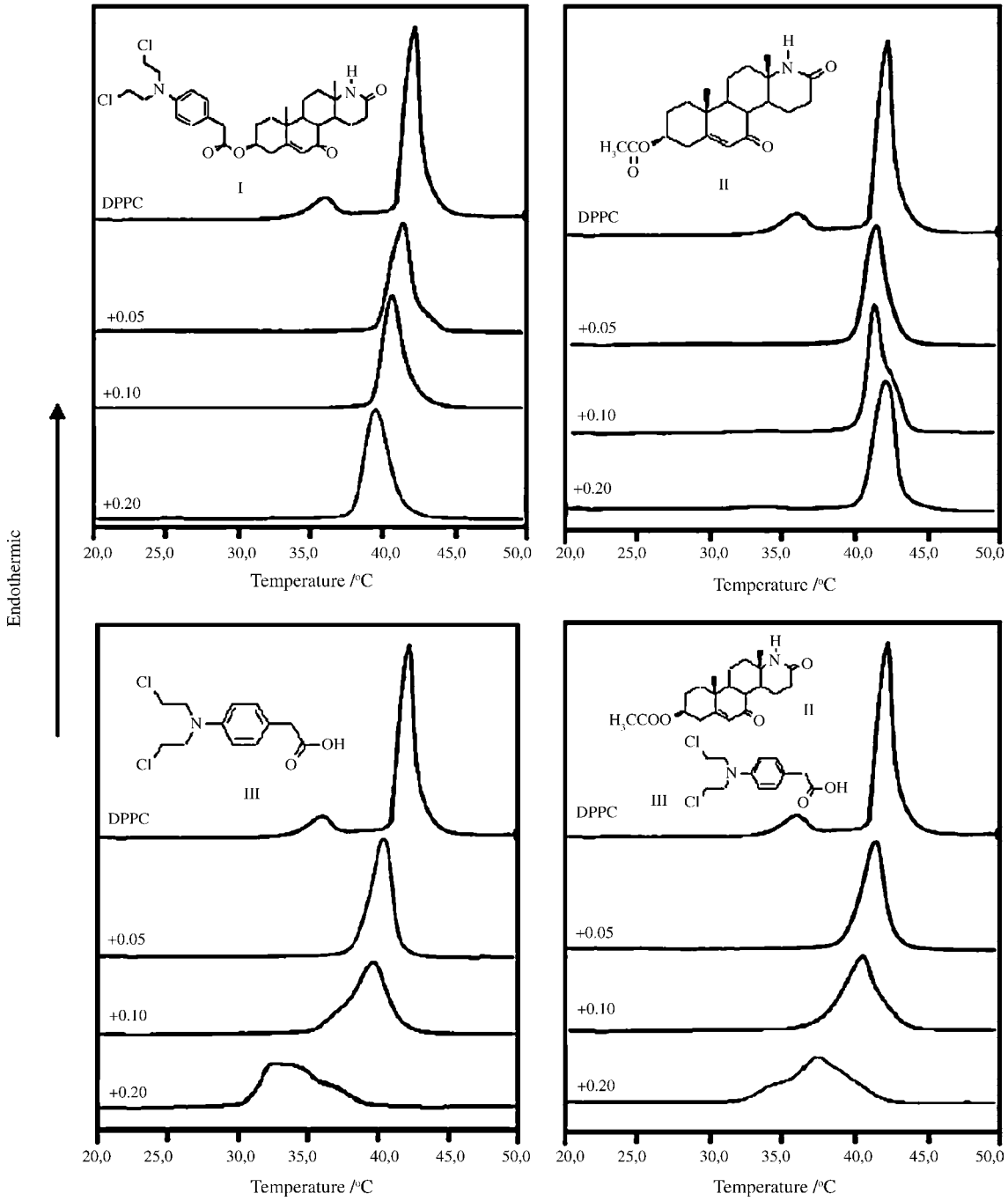

Fig. 2. Thermograms show the perturbations observed on DPPC bilayers by the incorporation of 3β-hydroxy-17α-aza-D-homo-5-androsten-7,17-dione-*N*,*N*-bis(2-chloroethyl)aminophenylacetate (**I**), 3β-acetoxy-17α-aza-D-homo-5-androsten-7,17-dione (**II**), *N*,*N*-bis(2-chloroethyl)aminophenylacetic acid (**III**) and a mixture of **I** and **II** (**IV**), at three different molar ratios with regard to DPPC.

(including electrostatics) that favor a change in membrane curvature.

DPPC bilayers containing a mixture of equimolar concentrations of **II** and **III** showed similar thermal behavior as DPPC bilayers containing only **III**. It is apparent from these thermal scans that the effect of **III** in the mixture is predominant. It appears that the pronounced effect of the alkylating agent masks the smaller effect of the steroid.

When **III** was conjugated with **II** to produce **I**, the thermal scans resembled those of DPPC:**II**. The conjugated compound, **I**, was slightly more effective in lowering the pretransition temperature and broadening of the phase transition of DPPC bilayers compared to the steroid, **II**. However, **I** appeared to be far less perturbing than the mixture of **III**:**II**. It appears that the effects of **III** are masked when **III** is esterified with **II**.

4. Discussion

The differences in the effects on the DPPC phase transition caused by the alkylating agent and its conjugated congener can be attributed to their different interactions with phospholipid bilayers. The alkylating agent possesses an acidic carboxyl group, which probably interacts with the head-group of phospholipid bilayers electrostatically. The anchoring on the head-group may be due to hydrogen bonding between the acidic group and phosphate or carbonyl groups, or the water solvent. The aromatic ring with the two *N*-chloroethyl moieties is oriented toward the upper part of the hydrophobic region of the membrane bilayers. Such topography was observed with the antihypertensive drug losartan which contains similar molecular features. Losartan contains an acidic moiety (tetrazole) and a basic nitrogen atom (imidazole ring). In addition, both molecules contain aromatic rings and chlorine atoms [16]. The conjugated analog, **I**, fits nicely between adjacent phospholipids and perturbs the bilayer less since the acidic moiety is prevented from electrostatic interactions. Both molecules, **I** and **III**, are characterized by amphoteric interactions. However, the conjugated molecule has extended length and can have more productive lipophilic interactions.

Steroid **II** shows similar thermal effects with **I** when incorporated in membrane bilayers, while the incorporation of the steroid and the alkylating agent as a mixture produces a thermogram analogous to that of the alkylating agent alone.

In conclusion, it is evident that the steroidal part modulates the thermal effects of the alkylating agent only if it is conjugated with it, by keeping the latter from interacting with the polar moiety of phospholipid bilayers. This observation may be of biological significance. Alkylating agents are characterized by high toxicity, which may be due to their effects on membrane bilayers or to low permeability. The presence of the steroid may ameliorate the interaction of alkylating agents with biological membranes and exert a beneficial role in reducing their toxicity.

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