Structure–anti-leukemic activity relationship study of B- and D-ring modified and non-modified steroidal esters of chlorambucil's active metabolite

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We have studied the effect of modification of the B-steroidal ring to lactamic on the anti-leukemic potency of D-modified and D-non-modified steroidal esters of chlorambucil's active metabolite. The compounds synthesized were studied against leukemias P388 and L1210 after the subsequent estimation of their toxicity *in vivo*, and for their ability to induce sister chromatid exchanges (SCEs) and to inhibit cell proliferation in normal human lymphocytes *in vitro*. The *in vitro* results correlated well, on a molar basis, with the results obtained from the study of the anti-leukemic potency. In a comparative study, the B-lactamic steroidal derivatives proved less active than the 7-oxidized ones against both leukemias. The presence of the -NHCO- group in the B-steroidal ring did not have the same positive effect on the biological action of chlorambucil's active metabolite esters as in the D-lactamic ring. However, this new modification of the B-ring rendered the final esteric derivatives much more toxic, compared with to the corresponding esters with a simple B-ring. This loss of the anti-leukemic specificity, which occurs from the modification of the B-ring, is additional evidence for the role of the steroidal part on the mechanism of action of these promising compounds. This provides support for the notion that the steroidal part of these molecules is not just a simple biological carrier, as has been speculated for many years. *Anti-Cancer Drugs* 16:1075–1082 © 2005 Lippincott Williams & Wilkins.

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Introduction

Nitrogen mustards are among the oldest and most extensively studied agents in human cancer chemotherapy [1]. These cytotoxic drugs exert their biological activity by inducing interstrand cross-links in the major groove of DNA [2], which represents the most toxic of all alkylation events [3,4]. Despite that, only a small fraction forms effective cross-links due to their high inert chemical activity, which results in their covalent binding to the nucleophilic sites of other biomolecules [5]. Additionally, simple mustards undergo rapid hydrolysis before reaching the DNA target; then have relatively low affinity and only slight selectivity for longer DNA sequences, and the guanine N^7 adducts which they primarily form are among the most easily repaired [6].

Although numerous mustard structural modifications have been made during the last 30 years [7–9], in order to increase their target affinity and reduce their toxic effects only a few members of these compounds, such as chlorambucil and melphalan, are used in clinical cancer chemotherapy today [10–12]. That is why recent research has focused on the exploitation of the cytotoxic activity of already known nitrogen mustards in new therapeutic strategies [13–16] (new therapeutic schemes, combination with other anti-neoplasmatic drugs, pro-drug strategies, etc.), rather than on the synthesis of new simple alkylating analogs.

Chemical linkage with a steroidal moiety has been proven one of the most effective strategies in order to reduce the high toxicity and ameliorate the selectivity of alkylating agents [17–19]. Steroidal hormones have been reported to influence the growth of many cancers and the presence of tumor-associated receptors for these hormones offers the opportunity for targeting using drug–hormone conjugates [20]. Estramustine and prednimustine are typical examples of conjugation of alkylating agents to steroids in clinical use [21–23].

From our laboratory's previous work with a series of steroidal esters of chlorambucil and its analogs, there is

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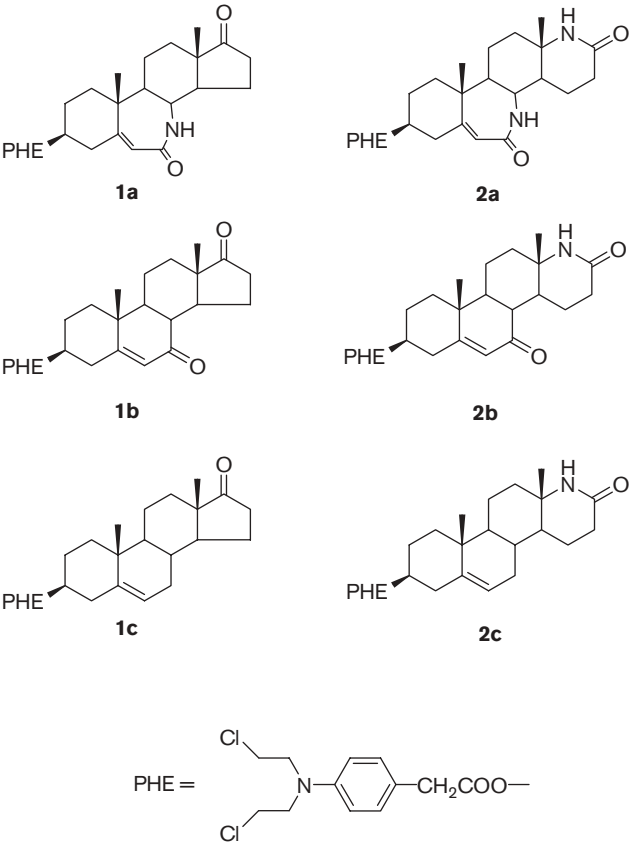
strong evidence for a significant enhancement of anti-leukemic activity and reduced toxicity of these compounds compared with nitrogen mustards themselves [24–27]. Among them, simple steroidal skeletons, such as dehydroepiandrosterone (DHEA), although they significantly reduce the toxicity of the alkylating agents, have no effect on the leukemias tested [28], while D-homo-aza-steroidal esters of the same alkylating agents have been proven more effective *in vitro* and *in vivo* [27,29,30]. These results have led us to the suggestion that the lactamic moiety confers anti-leukemic activity to the esteric steroidal nitrogen mustards, which additionally have been proven less toxic than the alkylating agents themselves [24]. Moreover, recent studies have shown that the insertion of a 7-keto group in DHEA's skeleton as well as its D-lactamic analog had considerable effects on the cytotoxic and anti-leukemic activity of the final esteric derivatives [31,32].

The fact that this slight modification in the B-steroidal ring had such an impressive influence on the anti-leukemic potency of a series of compounds tested [32], combined with previous studies which established the importance of the presence of a -NHCO- moiety at the D-ring of the steroidal skeleton [27,29,30], prompted us to further modify the B-ring by introducing an endocyclic -NHCO- group in the simple DHEA and a second lactamic moiety in the already modified D-lactamic-DHEA. Another fact that contributed to this decision was the observation that the introduction of an acetamide group at the 17-position on an A-lactamic steroid resulted in a derivative with inherent cytostatic activity [33].

In order to investigate the significance of the B-steroidal ring configuration and its effect on the anti-leukemic activity, the final B-lactamic esteric analogs were compared with the corresponding nitrogen mustard esters of DHEA and 7-keto-DHEA, as well as D-homo-aza-DHEA and 7-keto-D-homo-aza-DHEA. Thus, the present study concerns esters of 4-*N*,*N*-bis(2-chloroethyl) amino phenylacetic acid (PHE) with two B-lactamic steroids, i.e. 3β-hydroxy-7a-aza-B-homo-androst-5-en-7,17-dione (**1a**), 3β-hydroxy-7a,17a-diaza-B,D-dihomo-androst-5-en-7,17-dione (**2a**) as well as with their analogous 7-keto-Δ^5-steroids, i.e. 3β-hydroxy-androst-5-en-7,17-dione (**1b**) [31] and 3β-hydroxy-17α-aza-D-homo-androst-5-en-7,17-dione (**2b**) [32], and their parental non-oxidized steroids, i.e. 3β-hydroxy-androst-5-en-17-one (**1c**) [34] and 3β-hydroxy-17α-aza-D-homo-androst-5-en-17-one (**2c**) [35] (Fig. 1).

The nitrogen mustard PHE is chlorambucil's active metabolite [36,37] and was selected for this study in a continuation of our studies with the specific alkylating agent [32], and because it has been proven more effective and more potent than chlorambucil.

Fig. 1



Chemical structures of PHE and its steroidal esteric derivatives.

The nitrogen mustard PHE and its six steroidal esters were tested against leukemias P388 and L1210 *in vivo*, and for the induction of sister chromatid exchange (SCE) and reduction of the proliferation rate index (PRI) in normal human lymphocytes *in vitro*.

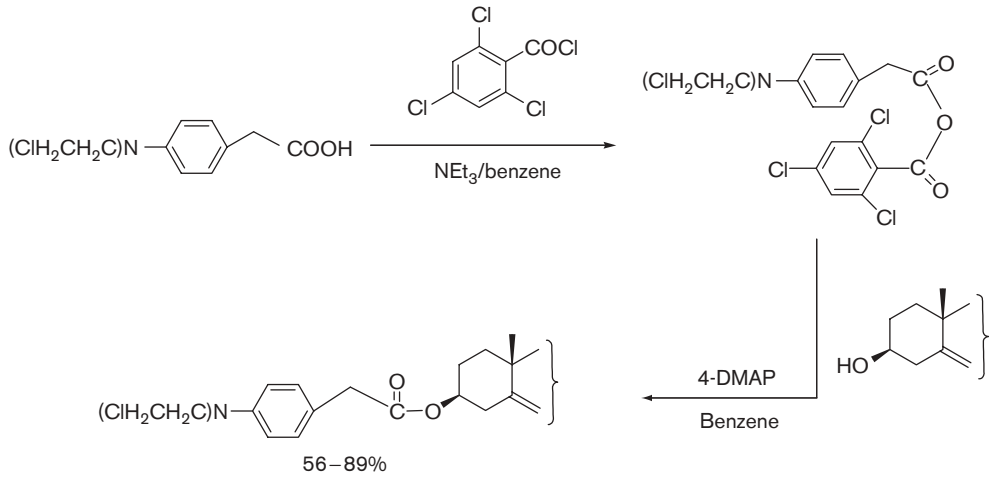
Methods

Synthetic procedures

3β-Hydroxy-androst-5-en-17-one was purchased from Steraloids (Newport, Rhode island, USA). 3β-hydroxy-17α-aza-D-homo-androst-5-en-17-one and *p*-*N*,*N*-bis(2-chloroethyl) amino phenylacetic acid were prepared by methods described in the literature [38,39]. The t-BuOOH/CuI-TBAB biphasic oxidizing method was applied for the allylic oxidation of the Δ^5-steroids [40], while the B-lactamic-DHEA and the B,D-dilactam were prepared according to a new synthetic procedure [41]. The final steroidal esteric derivatives of PHE were synthesized via the asymmetric anhydrides procedure [31] (Fig. 2).

Table 1 illustrates the physicochemical and spectroscopic measurements of the final compounds.

Fig. 2



General synthetic procedure for the preparation of the final compounds.

In vitro SCE and PRI assay

Lymphocyte cultures were set up by adding 11 drops of heparinized whole blood from three normal subjects to 5 ml of chromosome medium 1A (RPMI 1640; Biochrom, Berlin, Germany). For SCE demonstration, 5 μg/ml 5-bromodeoxyuridine (BrdUrd) and the chemicals were added at the beginning of culture. Throughout, all cultures were maintained in the dark to minimize photolysis of BrdUrd. The cultures were incubated for 72 h at 37°C. Metaphases were collected during the last 2 h with colchicines at 0.3 μg/ml. Air-dried preparations were stained by the FPG procedure [42]. The preparations were scored for cells in their first mitosis (both chromatids dark staining), second mitosis (one chromatid of each chromosome dark staining), and third and subsequent divisions (a portion of chromosomes with both chromatids light staining). Twenty suitably spread second division cells from each culture were blindly scored for SCEs. For PRIs, at least 100 cells were scored. For the statistical evaluation of the experimental data, the χ^2-test was performed for the cell kinetic comparisons. For the SCE frequencies, the Student's *t*-test was used. We also calculated the correlation between SCEs and PRI values. The formula for the Pearson product moment correlation coefficient *r* was applied. Then a criterion for testing whether *r* differed significantly from zero was applied, whose sampling distribution is Student's *t*-test with $n-2$ d.f.

In vivo experiments

Compounds

For i.p. treatment, stock solutions of the compounds used in this study were prepared immediately before use. They were suspended in corn oil at the desired concentration following initial dissolution in 5% DMSO. This concentration by itself produced no observable toxic effects.

Mice

BALB/c, DBA/2 and BDF1 mice of both sexes, weighting 20–23 g and 6–8 weeks old, were used for toxicity studies and anti-tumor evaluation. Mice obtained from the experimental section of the Research Center of Theagenion Anticancer Hospital, Thessaloniki, Greece, were kept under conditions of constant temperature and humidity, in sterile cages, with water and food.

Tumors

Leukemia P388- and L1210-bearing BDF1 (DBA/2 × C57BL) mice were used to evaluate the cytostatic effect. Lymphocytic P388 and lymphoid L1210 leukemias were maintained in ascitic form by injection of 10^6 and 10^5 cells, respectively, at 7-day intervals, into the peritoneal cavity of DBA/2 mice.

Estimation of acute toxicity

The acute toxicity of the compounds was determined following a single i.p. injection into BALB/c in groups of 10 mice per dose at three different dosages. The mice were observed for 30 days and the therapeutic dose of the compounds was determined after graphical estimation of the LD_{50} (30-day curves). The highest dose used for a single treatment was equal to the LD_{10} value.

Anti-leukemic evaluation

For the survival experiments, the anti-leukemic activity of the tested compounds against the above-mentioned murine tumors was assessed from the oncostatic parameter T/C%, i.e. the mean of the median survival time of the drug-treated animals (T) excluding long-term

Table 1 Physicochemical and analytical data of the final steroidal esteric derivatives of PHE

Compound	Yield (%)	Recrystallization solvent	Melting point (°C)	IR (cm^{-1})	1H-NMR ($CDCl_3$) δ	Elemental analysis					
						Calculated (%)			Found (%)		
						C	H	N	C	H	N
1a	75.0	ethylacetate	241–242	3194, 1738, 1662, 1616, 806	7.16d, 6.65d, 5.94s, 5.79s, 5.06m, 3.74t, 3.65t, 3.44t, 3.51s, 1.32s, 0.90s	64.69	7.00	4.87	*64.72*	*7.00*	*4.85*
1b	81.4	ethylacetate	154–156	1736, 1670, 806	7.21d, 6.62d, 5.73s, 4.62m, 3.72t, 3.63t, 3.42t, 1.22s, 0.91s	66.42	7.01	2.50	*66.43*	*7.01*	*2.48*
1c	89.0	ethylacetate	124–125	1734, 1655, 810	7.22d, 6.62d, 5.41s, 4.59m, 3.72t, 3.64t, 3.42s, 1.20s, 0.95s	68.12	7.56	2.56	*67.95*	*7.49*	*2.55*
2a	56.0	ethylacetate	148–150	3192, 1732, 1660, 1616, 806	7.17d, 6.66d, 6.9s, 6.48s, 5.83s, 4.72m, 3.73t, 3.64t, 3.18t, 3.51s, 1.26s, 1.16s	63.04	7.00	7.12	*63.05*	*7.02*	*7.16*
2b	71.3	ethylacetate	183–185	3196, 1729, 1668, 1650, 801	7.13d, 6.92s, 6.62d, 5.63s, 4.59m, 3.65t, 3.676t, 3.48s, 1.21s, 1.07s	64.69	7.00	4.87	*64.56*	*7.12*	*4.89*
2c	85.6	ethylacetate	149–150	3182, 1725, 1660, 1648, 804	7.17d, 7.03s, 6.64d, 5.38s, 4.61m, 3.66t, 3.68t, 3.45s, 1.22s, 1.09s	66.30	7.54	4.99	*66.22*	*7.55*	*5.06*

survivors versus corn oil-treated controls (C) expressed as a percentage. The other index of the anti-leukemic activity used was the number of long-term survivors defined as mice alive for 90 days after tumor inoculation. Each drug-treated group consisted of six mice, while the tumor control group included eight mice; in each group, equal numbers of male and female mice were used. Experiments were initiated by implanting mice with tumor cells according to the protocol of the National Cancer Institute [43]. Treatments were given either as an intermittent dose ($LD_{10}/2 \times 3$, days 1, 5 and 9) or as a single dose ($LD_{10} \times 1$, day 1). The experiments were terminated on day 90. Statistical evaluation of the experimental data was performed by the Wilcoxon test.

Results and discussion

The toxicities of the compounds tested are reported in Table 2. The LD_{50} values of the steroidal esteric derivatives are higher than those of nitrogen mustard, showing that this conjugation reduces the cytotoxicity of the alkylating agent.

The further modification of the B-steroidal ring to lactamic rendered the final molecules (**1a** and **2a**) more toxic than the derivatives **1c** and **2c,** which bear a simple B-ring. Even in the case of the **1c** derivative, which is almost non-toxic ($LD_{50} = 370$), the alteration of the configuration of its B-ring to lactamic had a considerable effect on the toxicity of the final ester **1a** ($LD_{50} = 64$), which also gave the same LD_{10} value with the alkylating agent itself. Despite that, the enhancement of the cytotoxicity induced by the B-lactamic ring remained at the same levels with the corresponding enhancement observed with the introduction of the allylic keto group in the 7-position of the steroidal skeleton (**1b** and **2b**). These results are in agreement with previous studies showing that the esters of nitrogen mustards with the common and non-modified steroidal skeletons are less toxic than those carrying a -NHCO- moiety [32,44].

From the results shown in Table 3 we can estimate that all esteric derivatives tested gave better T/C% values than the nitrogen mustard itself in the treatment schedule $D/2 \times 3$, which subsequently proved more effective in all cases (except for PHE in P388) than the single-dose schedule. Although the introduction of the allylic 7-keto group in the B-steroidal ring had an impressive influence on the anti-leukemic potency of the compounds tested (T/C > 300% for all the oxidized compounds in P388 leukemia) [32], similar results were not observed in the case of the B-lactamic steroidal derivatives.

In the case of derivative **1a**, the introduction of the -NHCO- moiety in the B-steroidal ring resulted in a reduction of the T/C% values in both leukemias compared with the 7-oxidized derivative **1b**. The anti-leukemic potency achieved in both treatment schedules was equivalent to that obtained by the parental non-oxidized ester **1c**. However, such a relationship was not observed concerning their toxicity, since **1a** proved almost 6-fold more toxic than **1c** and equitoxic with **1b**. It is remarkable that two derivatives with the same toxicity (**1a** and **1b**) differed markedly in anti-leukemic

activity (especially in leukemia P388, see Fig. 3), in contrast to the general observation that the more active a compound is, the more toxic it proves to be.

The results obtained with derivative **2a** in which the B- and D-steroidal rings are lactamic were almost similar. Comparing derivative **2a** with the corresponding D-lactamic, 7-oxidized **2b,** although they have the same toxicity, the former proved marginally active in P388 in the D/2 × 3 treatment schedule and inactive in L1210 leukemia in both treatment schedules, whereas **2b** was the most potent compound of all, and also gave three out of six cures in P388-bearing mice and one out of six cures for the treated L1210-bearing mice (Table 3) [32]. Consequently, although the insertion of a 7-keto group in the steroidal skeleton improved the anti-leukemic potency of the non-oxidized D-lactamic derivative **2b** to a marked degree, further modification of the B-steroidal ring to lactamic had the opposite result, since it turned an active molecule (**2b**) into an almost inactive molecule (**2a**). See Fig. 3.

Table 2 Toxicity of PHE and its steroidal esters

Compound	LD_{50}(mg/kg)[a]	LD_{10}(mg/kg)
PHE	20	10
1a	64	10
1b	65	37
1c	370	155
2a	90	58
2b	90	56
2c	115	65

[a]LD_{50} values were estimated graphically, where the percentage of deaths due to the toxicity of each dose is shown in the ordinate, while the administered doses are indicated on the abscissae on semilogarithmic paper. For chemotherapy testing, the highest dose used for a single treatment was LD_{10}. Therefore, the drugs in the following experiments were compared at equitoxic doses.

From the above-mentioned results, it seems that the insertion of a -NHCO- moiety in the B-steroidal ring does not have a positive impact on the anti-leukemic potency of chlorambucil's active metabolite steroidal esters. However, it seems that it has a remarkable effect on the toxicity of these compounds since both derivatives (**1a** and **2a**) were found to be more toxic than the corresponding derivatives bearing a simple B-steroidal skeleton (**1b** and **2b**).

The presence of the -NHCO- moiety in the steroidal skeleton appears to alter the biological behavior of the molecule. In previous studies [24,44] it has been claimed that these properties may result from the multiple interactions of this group with similar groups present in proteins and nucleic acids; after its metabolic transformation *in vivo* to active species they might destabilize DNA structure, facilitating DNA–protein or DNA–DNA interstrand cross-links and detectable strand breaks. Still, very little is known about the sequence specificity of DNA interstrand cross-link formation. It seems that the preferential cross-links produced by nitrogen mustards occur in a 5′-GNC sequence [45]. The existence of the -NHCO- group probably enhances the ability of the nitrogen mustard to interact with different bases on specific major and minor grooves of DNA sequences.

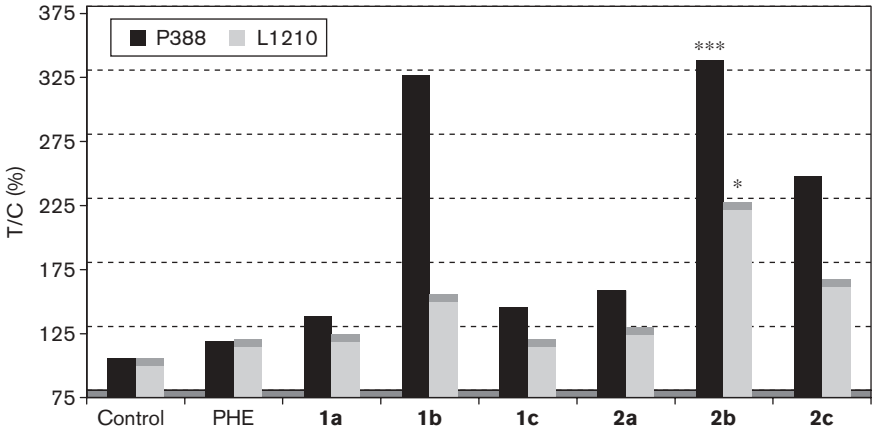
As previous studies have shown [27,29,30], the presence of the D-lactamic group improved the activity of the final derivatives. This conclusion can also be derived from the present study. Comparing **1a–c** with **2a–c**, correspondingly, we can see that in all cases the conversion of a simple D-ring to lactamic enhances the anti-leukemic activity in both leukemias. Since an analogous result is not observed in the case where the -NHCO- group was introduced in the B-steroidal ring, one can conclude that

Table 3 Anti-tumor activity of PHE and its steroidal esters on P388- and L1210-bearing mice leukemia, using doses based on toxicity studies

Compound	Treatment schedule (day)	Dosage (mg/kg/day)	P388			L1210		
			MST(days)[a]	T/C(%)[b]	Cures	MST (days)	T/C (%)	Cures
Control	–	corn oil	8.5	100	0/6	9.3	100	0/6
PHE	1	10	10.2	120	0/6	10.1	108	0/6
	1,5,9	5	9.6	113	0/6	10.7	115	0/6
1a	1	10	9.8	115	0/6	10.5	113	0/6
	1,5,9	5	11.3	133	0/6	11.0	119	0/6
1b	1	37	19.1	225	0/6	13.5	145	0/6
	1,5,9	18.5	27.3	321	0/6	14	150	0/6
1c	1	155	10.8	127	0/6	10.5	113	0/6
	1,5,9	77.5	11.9	140	0/6	10.7	115	0/6
2a	1	58	12	141	0/6	10.8	116	0/6
	1,5,9	29	13	153	0/6	11.5	124	0/6
2b	1	56	27.7	326	0/6	15.3	165	0/6
	1,5,9	28	28.3	333	**3/6**	20.6	222	**1/6**
2c	1	65	19.2	225	0/6	12.3	132	0/6
	1,5,9	32.5	20.6	242	0/6	13.8	162	0/6

[a]MST = mean survival time of mice inoculated with lymphocytic leukemia P388 or lymphoid leukemia L1210 cells and treated with compounds.
[b]T/C = mean median survival of the drug-treated animals (T) versus corn-oil-treated animals (C).

Fig. 3



The effect of the B-lactamic ring on anti-leukemic potency.

it is not only the presence, but also the specific position of this group that contributes to the potency of the molecules tested. Likewise, another important conclusion that can be derived from this study is from the comparison of derivatives **1a** with **2b**, where there is an inversion of the functional groups between the B- and D-ring. This structural modification rendered the most potent derivative of all (**2b**) to the least potent derivative of all (**1a**), confirming once more that the position of the functional groups (-NHCO- and keto groups) is very important and, consequently, the configuration of the whole steroidal part of the molecules plays a crucial role for the anti-leukemic activity of chlorambucil's active metabolite steroidal esters. However, we cannot yet exclude these steroids as potential carriers of other nitrogen mustards as well, since the toxicity values indicated that there might be a biological effect induced by the B-lactamic ring that could be exerted in a positive way (concerning the anti-leukemic activity) if less-toxic alkylating agents were used.

SCEs have been frequently used as highly sensitive indicators of DNA damage and/or subsequent repair [46,47]. Non-repaired damage expressed as SCEs in normal cells, caused by certain chemicals may indicate inability for repair of the damage induced by the same chemicals in cancer cells. There are findings indicating that the effectiveness in SCE induction by potential anti-tumor agents in cancer cells *in vitro* and *in vivo* [48] is positively correlated with the *in vivo* tumor response to these agents. This suggests that the SCE assay could be used to predict both the sensitivity of human tumor cells to chemotherapeutics and the heterogeneity of drug sensitivity of individual tumors [49]. Other studies investigating a relationship between SCE induction and other expressions of genotoxicity have also shown a positive relationship between SCE and reduced cell survival and alteration in cell cycle kinetics [50]. In the present study, a good correlation ($P < 0.02$) between SCE enhancement and PRI suppression was observed. The results from the *in vitro* experiments (Table 4) indeed showed relevance with these from the *in vivo* studies.

Table 4 Induction of SCEs and cell division delays by PHE and its steroidal esters in human lymphocytes

Compound	Concentration (μmol/l)	SCE/cell ± SE	PRI
Control	–	10.16 ± 0.63	2.52
PHE	0.2	15.45 ± 0.83	2.40
	0.6	32.38 ± 1.01	1.67
1a	0.2	17.99 ± 1.21	2.02
	0.6	39.93 ± 1.54	1.97
1b	0.2	31.12 ± 3.22	2.52
	0.6	69.58 ± 4.39	1.72
1c	0.2	14.21 ± 1.12	2.48
	0.6	15.81 ± 1.37	2.13
2a	0.2	14.26 ± 1.34	2.48
	0.6	20.97 ± 1.54	2.01
2b	0.2	30.83 ± 2.47	1.95
	0.6	38.03 ± 2.71	1.80
2c	0.2	23.15 ± 1.12	2.40
	0.6	30.44 ± 1.71	1.63

SCEs have been correlated with corresponding PRI values ($r = -0.51$, $t = 2.63$ and $P < 0.02$).

All the compounds studied induced a statistically significant increase in SCE rates at both concentrations tested (0.2 and 0.6 μmol/l). The increases were directly related to the concentrations used. The 7-keto-Δ^5-steroid esters (**1b** and **2b**) were the most effective inducers, on a molecular basis, of SCEs, while the B-lactamic derivatives (**1a** and **2a**) proved less effective. Among them, compound **1a** showed the second highest value for SCE induction for the 0.6 μmol/l concentration, while **2a** was even less effective than the nitrogen mustard itself.

Compound **1c** was the less effective, indicating that the conjugation of PHE with the simple steroidal skeleton of 3β-hydroxy-androst-5-en-17-one reduces its effectiveness towards SCE induction. PRI is used as a criterion of cytostatic activity. There is a good correlation between SCE induction and PRI depression (Table 4). The best cell division delays were achieved by treating cells with **1b** and **2b**, which gave the minor PRI values, while the B-lactamic derivatives proved once more less effective than the 7-oxidized derivatives.

Conclusion

The biological action of chlorambucil's active metabolite esters bearing the -NHCO- moiety may be structurally specific and therefore more prolonged, as long as this group is inserted to the proper position in the steroidal skeleton. Its presence in the B-steroidal ring produced derivatives marginally active, but at the same time more toxic, indicating that they interact by a different way or by different cellular moieties. This kind of behavior offers additional support for the concept that these molecules produce their anti-leukemic activity by interacting and/or binding at specific sites and that the steroidal moiety is not only a simple carrier for the nitrogen mustard. We need more structural modifications on the steroidal skeleton to prove this concept, but obviously slight changes at the steroidal moiety dramatically influence the anti-leukemic activity, while the corresponding steroid itself is completely inactive whether or not it contains any major or minor chemical alterations. Finally, the conjugation of the same B-lactamic steroidal skeletons with less-toxic alkylating agents must also be examined before excluding these steroidal skeletons as efficient and proper modules.

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