The allylic 7-ketone at the steroidal skeleton is crucial for the antileukemic potency of chlorambucil's active metabolite steroidal esters

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We have investigated the role of the allylic 7-ketone in oxidized Δ^5-steroids on antileukemic activity. We synthesized and studied a series of oxidized and non-oxidized steroidal esters of *p-N,N*-bis(2-chloroethyl) aminophenylacetic acid (PHE), chlorambucil's active metabolite. In a comparative study of these 7-keto derivatives, on a molecular basis, regarding their ability to induce sister chromatid exchanges (SCEs) and to inhibit cell proliferation in normal human lymphocytes *in vitro*, the results with these 7-keto derivatives, on a molecular basis, correlated well with their antileukemic potency against leukemia P388- and L1210-bearing mice, which proved to be significantly increased compared to that of the non-oxidized derivatives. Our results indicate that the role of the steroidal skeleton it is not only for the transportation of the alkylating agent into the cell, but also contributes directly to the mechanism of antileukemic action, by an as-yet unknown way. The main conclusion from this study is that the existence of the allylic 7-keto group in the skeleton of the Δ^5-steroidal esters impressively enhances their antileukemic activity, while the toxicity remains at clinically acceptable levels, suggesting that this structural modification should be further investigated. *Anti-Cancer Drugs* 15:983–990 © 2004 Lippincott Williams & Wilkins.

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Introduction

Several oxidized products of Δ^5-steroids have been found in animal tissues and nutritional additives [1], and reported as inhibitors of mammalian sterol biosynthesis [2,3] and cell replication [4]. Some of these oxidized Δ^5-steroids are more toxic toward cancerous than non-cancerous cells [5], which makes them suitable for application in the field of cancer chemotherapy.

More specifically, it has been reported that 7-keto-dehydroepiandrosterone (7-keto-DHEA) may be responsible for a series of dehydroepiandrosterone's actions including the suppression of spontaneous and carcinogen-induced tumors [6]. DHEA was found to be converted to 7αOH-, 7β-OH- and 7-keto-DHEA in tissues [7–9]. Of great interest is the inhibitory action of 7-keto-Δ^5-androstenes towards the enzyme aromatase [10], where the 7-keto allylic group is reported to be essential for the irreversible inhibition of aromatase [11]. As such, it may have action against hormone-dependent tumors [12]. On the other hand, 7-keto-cholesterol is reported to induce apoptosis in several cell lines such as smooth muscle cells [13] and vascular smooth muscle cells [14] by an as-yet unknown mechanism. In addition, the inhibition of sterol biosynthesis, through the inhibition of the 3-hydroxy-3-methylglutaryl co-enzyme A reductase, is reported to give oxysterols an antiproliferative activity [4], both in *in vivo* and in *in vitro* studies [15–17], even including leukemic blood cells [18].

Nitrogen mustards were the earliest and most extensively studied DNA interstrand cross-linking agents [19], but only a few members of these compounds, such as melphalan and chlorambucil, are used in clinical cancer chemotherapy today [20–23]. Due to their high inert chemical activity, they can bind covalently to the nucleophilic sites of biomolecules. Their relatively low affinity and only slight selectivity for longer DNA sequences, and their rapid hydrolysis before reaching the DNA target, however, diminish their effective alkylation [24].

The chemical conjugation of nitrogen mustards to molecular ligands is one of the strategies which researchers apply in order to reduce their toxicity, and increase

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selectivity and effectiveness towards alkylation of DNA [25–29]. Estramustine and prednimustine, nitrogen mustard carbamates of steroids in clinical use [30,31], are typical examples of conjugation of alkylating agents to steroids. It has been reported that a number of estrogens show efficacy through a combined mechanism of systemic slow release and targeting to hormone-sensitive tumors [32]. From our laboratory's previous work with a series of aromatic aniline mustards linked to simple, or D-ring-modified, steroidal molecules, there is strong evidence for a significant enhancement of antileukemic activity and reduced toxicity of these compounds compared to the nitrogen mustards themselves [33–36]. Moreover, in an early study with four 7-keto steroidal esters of several nitrogen mustards [37], the introduction of a 7-keto group in the steroidal skeleton of the final esteric derivatives produced encouraging results with regard to cytogenetic and antileukemic effectiveness.

Based on the above-mentioned results and in order to further accurately investigate the contribution of the 7-keto group to the antileukemic activity of the steroidal esters of nitrogen mustards, we designed the study presented in this paper. It concerns esters of 4-*N*,*N*-bis(2-chloroethyl) amino phenylacetic acid (PHE) with three 7-keto-Δ^5-steroids, i.e. 3β-hydroxy-androst-5-en-7,17-dione (**1a**) [37], 3β-hydroxy-17β-acetamido-androst-5-en-7-one (**2a**) and 3β-hydroxy-17α-aza-D-homo-androst-5-en-7,17-dione (**3a**), as well as with their parental non-oxidized steroids, i.e. 3β-hydroxy-androst-5-en-17-one (**1b**) [38], 3β-hydroxy-17β-acetamido-androst-5-ene (**2b**) [39] and 3β-hydroxy-17α-aza-D-homo-androst-5-en-17-one (**3b**) [40]. See Figure 1.

This specific nitrogen mustard is the active metabolite of chlorambucil [41,42], which was selected for this study because it has been proved more effective and more potent than chlorambucil.

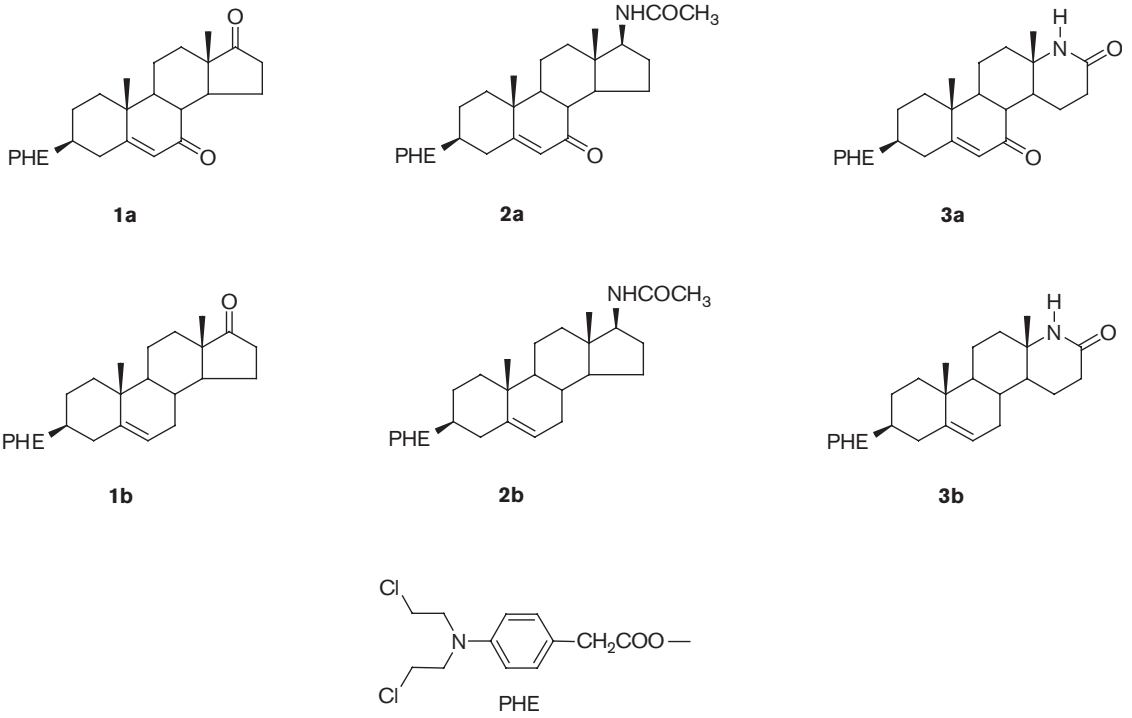
The nitrogen mustard PHE and its six steroidal esters were tested against leukemias P388 and L1210 *in vivo* and for the induction of sister chromatid exchange (SCE) and reduction of the Proliferation Rate Index (PRI) in normal human lymphocytes *in vitro*.

Methods

Synthetic procedures

3β-Hydroxy-androst-5-en-17-one was purchased from Steraloids (Newport, RI). The other two parent steroidal alcohols and the *p*-*N*,*N*-bis(2-chloroethyl) amino phenylacetic acid were prepared by methods described in the literature [43–45]. The t-BuOOH/CuI-TBAB biphasic oxidizing method was applied for the allylic oxidation of the Δ^5-steroids [46], while the final steroidal esteric

Fig. 1



Chemical structures of the compounds prepared and tested.

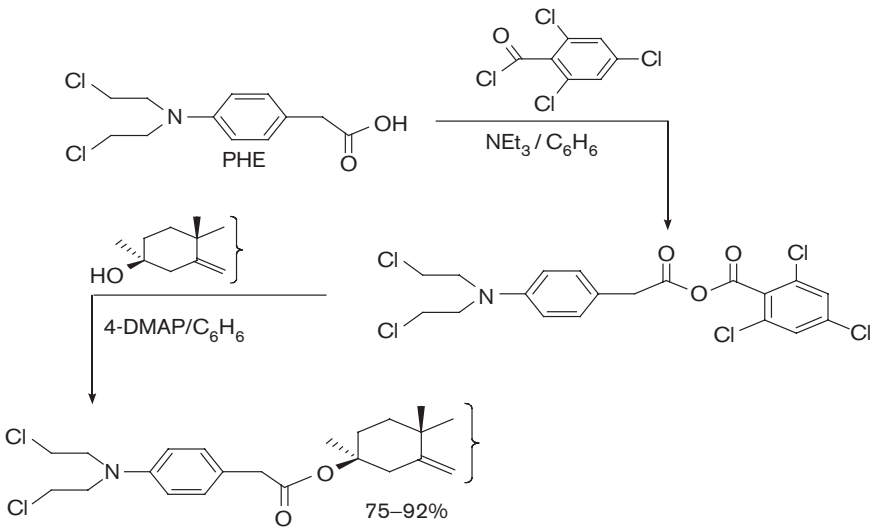
derivatives of PHE were synthesized accordingly to the asymmetric anhydrides procedure [37] (Fig. 2).

Table 1 illustrates the physicochemical and spectroscopic measurements 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, Berlin). 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 made stained by the FPG procedure [47]. The preparations were scored for cells in their first mitosis (both chromatids dark staining), second mitosis (one

Fig. 2



General synthetic procedure for the preparation of the final compounds.

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

Compound	Yield (%)	Recrystallization solvent	m.p. (°C)	IR (cm^{-1})	^{1}H-NMR ($CDCl_3$) δ[a]	Elemental analysis					
						Calculated (%)			Found (%)		
						C	H	N	C	H	N
1a	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[1]	66.42	7.01	2.50	*66.43*	*7.01*	*2.48*
1b	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	64.1	ethylacet/hexane	143–145	3150, 1734, 1672, 1635, 809	7.14d, 7.03s, 6.63d, 5.69s, 4.65m, 3.91m, 3.71t, 3.69t, 3.47s, 2.02s, 1.17s, 0.72s	65.66	7.35	4.64	*65.49*	*7.33*	*4.67*
2b	83.8	ethylacetate	194–196	3139, 1731, 1661, 1628, 803	7.16d, 7.07s, 6.65d, 5.35s, 4.66m, 3.92m, 3.72t, 3.69t, 3.45s, 2.01s, 1.19s, 0.75s	67.22	7.86	4.75	*67.12*	*7.88*	*4.88*
3a	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*
3b	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*

[a]s: single; d: doublet; t: triplet; m: multiple.

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 cell kinetic comparisons. For the SCE frequencies, Student's *t*-test was used. We also calculated the correlation between SCE 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 were prepared immediately before use. They were suspended in corn oil in the desired concentration following initial dissolution in 5% dimethylsulfoxide (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 antitumor 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 degree of 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.

Antileukemic evaluation

For the survival experiments, the antileukemic 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 survivors versus corn-oil-treated controls (C) was expressed as a percentage. The other index of antileukemic 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 [48]. 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 made by the Wilcoxon test.

Results and discussion

The toxicity values of the studied compounds are reported in Table 2. The LD_{50} values showed that in every case the conjugation of the nitrogen mustard PHE with the steroidal molecules resulted in a reduction of its cytotoxicity.

Comparing the toxicity of the 7-keto steroidal esters (**1a**, **2a** and **3a**) with that of the non-oxidized ones (**1b**, **2b** and **3b**), it is obvious that the introduction of the allylic keto group in the 7 position of the steroidal skeleton resulted in an enhancement of cytotoxicity. Even the **1b** molecule, which has been proved practically inactive and almost non-toxic ($LD_{50} = 370$), transformed into a more toxic compound ($LD_{50} = 65$) via the insertion of the 7-keto group. Subsequently, previous studies showed that the esters of nitrogen mustards with the common and non-modified steroidal skeletons always proved less toxic than those modified at the D-ring [49].

The results shown in Table 3 support evidence from previous studies [33–36] that the conjugation of a nitrogen mustard to the appropriate steroidal molecule leads to a more potent antileukemic activity, since all of the esters tested gave better T/C% values than the nitrogen mustard itself. The introduction of the allylic 7-keto group in the steroidal skeleton had an impressive influence on the antileukemic potency of the compounds

Table 2 Toxicity of the PHE and its steroidal esters

Compound	LD_{50}[a] (mg/kg)	LD_{10} (mg/kg)
PHE	20	10
1a	65	37
1b	370	155
2a	75	25
2b	95	58
3a	90	56
3b	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 abscissa 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.

Table 3 Antitumor 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[a] (days)	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	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
1b	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	25	17.30	161	0/6	16.5	178	0/6
	1,5,9	12.5	30.8	314	0/6	22.6	243	0/6
2b	1	50	15.2	179	0/6	12.5	135	0/6
	1,5,9	25	14.8	174	0/6	11.9	128	0/6
3a	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
3b	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).

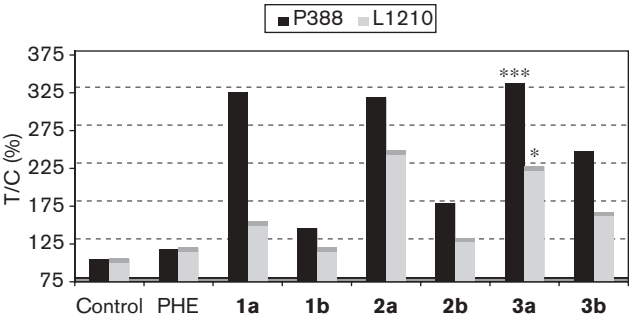
tested (T/C > 300% for all of the oxidized compounds in P388 leukemia).

The results with the **1a** derivative compared with the corresponding non-oxidized **1b** are characteristic for such an influence (Fig. 3). Essentially similar results were also obtained when other pairs of esters (**2a–2b** and **3a–3b**) were compared. A significant enhancement of the antileukemic activity was achieved in all cases of treatment schedule and type of treated leukemia (except for the D1 treatment schedule for **2a–2b**, where these compounds proved equivalent).

The results obtained using **2a** and **3a** against L1210 leukemia were also remarkable; compound **2a** was found to be slightly superior to **3a** against this type of leukemia. In parallel, the total estimation for the antileukemic potency shows that compound **3a** seems to be the most potent derivative while at the same time it was not the most toxic. Moreover, three out of six cures were also recorded for the treated P388-bearing mice and one out of six for the treated L1210-bearing mice.

Thus, the allylic 7-keto group confers antileukemic activity to molecules in which it is introduced. In the case of compounds with already good antitumor activity, such as **3a** where the NH-CO lactamic moiety of the D-ring confers activity, it was obvious from the present results, but also known from previous studies [33–36], that the allylic 7-keto group further enhances the antileukemic potency of the compound. This observation seems to be supplementary evidence that the role of the steroidal skeleton is not only for the transportation of the alkylating agent into the cell, but also contributes directly on the mechanism of antileukemic action by an as-yet unknown way. As has been noted before [50], some of the disadvantages concerning the activity of nitrogen mustards are their slight selectivity for DNA, the rapid hydrolysis they suffer before reaching to the DNA target, and the ability of binding to nucleophilic sites of several biomolecules in cells which results in their deactivation and in some cases development of cellular resistance. The allylic 7-oxidized steroidal carriers, being the corresponding non-oxidized ones, can increase the lipophilicity of the final molecule which contains the nitrogen mustard, in order to permeate more easily and quantitatively the cellular and nuclear membranes [51]. Subsequently, it is already known that the oxygen atom at the steroidal B-ring makes the substrates more soluble in body fluids and that this is potentially useful for an effective treatment as the molecule may reach its target more frequently [52]. However, it is rather difficult to produce such an impressive increment of the antileukemic potency. The most effective treatment schedule in all cases was the D/2 × 3 (except for the **2b** and PHE

Fig. 3



The role of the allylic 7-ketone: The enhancement of the antileukemic potency is clear and sound comparing the pairs **1a–1b**, **2a–2b** and **3a–3b** in both leukemias tested. *Cures.

compounds in P388), as the single-dose schedule D1 produced minor T/C% values and no cures.

SCEs have been frequently used as highly sensitive indicators of DNA damage and subsequent repair [53,54]. Non-repaired damage expressed as SCEs in normal cells, caused by certain chemicals, may indicate inability to repair the damage induced by the same chemicals in cancer cells. There are findings indicating that the effectiveness of SCE induction by potential antitumor agents in cancer cells *in vitro* [55] and *in vivo* [44] is positively correlated with *in vivo* tumor response to these agents. Thus 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 [56]. 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 [57]. 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.

All the compounds studied induced a statistically significant increase in SCE rates at all concentrations tested (0.2, 0.4 and 0.6 μM). The increases are directly related to the concentrations used. The 7-keto-Δ^5-steroids' esters (**1a**, **2a** and **3a**) were the most effective inducers, on a molecular basis, of SCEs. Among them, compound **1a** showed the highest values for SCE induction, and especially for the 0.4 and 0.6 μM concentrations these values were 2-fold higher than those of the other two oxidized esters. Compounds **1b** and **2b** were less effective, with values of SCE induction indicating that the conjugation of PHE with the simple steroidal skeleton of 3β-hydroxy-androst-5-en-17-one or the non-oxidized 17 amidic androgen reduces its effectiveness towards SCE induction. It is obvious, as the *in vivo* study also showed, that the **1a, 2a** and **3a** compounds are effective antitumor agents as well. This is probably due to the above-mentioned characteristics of the molecules tested. Additionally, the major cytotoxic effect which nitrogen mustards produce at the N^7 of the guanine of the DNA strands is among the most easily repaired [58,59]. The effective SCE induction obtained herein may be also a result of other DNA alkylating lesions, as it has been reported that chlorambucil (and, respectively, 4-*N*,*N*-bis(2-chloroethyl)amino phenylacetic acid) can alkylate adenines at the minor groove N^3 position apart from N^7 of guanines, presumably because of the weak DNA-targeting ability of the aromatic ring [60].

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

Compound	Concentration (μM)	SCE/cell ± SE	PRI
Control	–	10.16 ± 0.63	2.52
PHE	0.2	15.45 ± 0.83	2.40
	0.4	22.27 ± 1.14	2.22
	0.6	32.38 ± 1.01	1.67
1a	0.2	31.12 ± 3.22	2.52
	0.4	66.00 ± 6.74	1.97
	0.6	69.58 ± 4.39	1.72
1b	0.2	14.21 ± 1.12	2.48
	0.4	15.42 ± 1.42	2.24
	0.6	15.81 ± 1.37	2.13
2a	0.2	11.81 ± 0.67	2.23
	0.4	31.43 ± 1.55	1.94
	0.6	55.09 ± 1.88	1.55
2b	0.2	15.67 ± 0.82	1.94
	0.4	15.12 ± 0.81	2.32
	0.6	14.53 ± 0.59	1.53
3a	0.2	30.83 ± 2.47	1.95
	0.4	33.43 ± 2.52	1.94
	0.6	38.03 ± 2.71	1.80
3b	0.2	23.15 ± 1.12	2.40
	0.4	25.13 ± 1.41	1.99
	0.6	30.44 ± 1.71	1.63

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

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 **1a**, **2a** and **3a**, which gave the minor PRI values.

Conclusion

The insertion of the allylic 7-ketone at the steroidal skeleton of these molecules produced derivatives (**1a**, **2a** and **3a**) which possess significant antileukemic activity. At the same time, the toxicity remained at clinically acceptable levels and was significantly lower than for the alkylating agent itself.

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