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

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In order to study the role of the steroidal moiety on the expression of anti-leukemic activity, we synthesized six derivatives of chlorambucil (CHL), and tested them on leukemias P388 and L1210 *in vivo* and in normal human lymphocytes *in vitro*. Five of the six tested compounds produced submultiple toxicity, while the measured anti-leukemic potency was significantly increased. The lactamization of the B-steroidal ring rendered the molecules more potent, but the corresponding 7-oxidized derivatives proved better in both leukemias tested. The lactamization of the D-steroidal ring afforded potent compounds, regardless of the configuration of the B-ring. The best among all derivatives contains both chemical modifications and is intended as a promising key molecule that must be further studied. We speculate that in leukemic cells a tumor-specific protein is overexpressed, the steroid has the ability to bind and block this protein from carrying out its normal function, and the drug–protein complex prevents the repair of the adducts. The synthesis, physicochemical and spectroscopic data of these compounds and a modified route for the synthesis of CHL are also reported. *Anti-Cancer Drugs* 17:511–519 © 2006 Lippincott Williams & Wilkins.

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Keywords: anti-leukemic activity, B-steroidal lactams, chlorambucil, D-steroidal lactams, structure–activity relationship, steroidal esters

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Introduction

DNA-alkylating agents have had an important role in cancer chemotherapy since the introduction of nitrogen mustards [*N*,*N*-bis(2-chloroethyl)amines] more than 50 years ago [1]. The alkylation of DNA and the formation of DNA interstrand crosslinks are considered as the main cytotoxic lesions of nitrogen mustards [2–4]. Despite their long history in cancer research, only a few of these compounds, such as chlorambucil (CHL) and melphalan [5,6], remain in clinical use today.

A common disadvantage of all DNA alkylators is their high chemical reactivity, which can result in loss of drug by reaction with other cellular nucleophiles, particularly proteins and low-molecular-weight thiols [7], and render them sensitive to cellular resistance mechanisms, such as increased levels of glutathione [8–10]. Other limitations, mainly for aniline mustards, are the lack of intrinsic DNA-binding affinity of the core *N*,*N*-bis(2-chloroethyl)amine pharmacophore and the requirement for bifunctional crosslinking in order to be fully cytotoxic. These characteristics diminish their potency and the observed high ratio of genotoxic monoadducts to crosslinks (up to 20:1) contributes to their known carcinogenicity [11].

For these reasons, the rational concepts of chemically tethering nitrogen mustards onto carrier structures in order to achieve increased selectivity and effectiveness towards DNA alkylation, as well as reducing systemic activity by increasing the drug concentration in the vicinity of DNA, would mean less chance for active drug loss by reaction with other cell components [12–16].

Steroid 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 [17]. They have been used as effective carriers of nitrogen mustards since they can lead them directly to the nuclei where they naturally act as transcription factors through binding to their receptors [18]. Estramustine and prednimustine are typical representatives of this category, and have been used in clinical practice against several types of cancers [19,20].

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Our ongoing research in this field has shown that esters in which aromatic nitrogen mustard is linked to a modified steroidal moiety show enhanced anti-leukemic activity and reduced toxicity compared to nitrogen mustards themselves [21–24]. Specifically, structure–activity relationship (SAR) studies show that compounds that carry a -NHCO- group in the D-steroidal ring are much more effective *in vitro* and *in vivo* compared with the corresponding esters in which the alkylating agent is linked to a simple steroidal skeleton [25–27]. This evidence has led us to the suggestion that the lactam moiety confers the anti-leukemic activity of the esteric steroidal nitrogen mustards and establishes the indispensability of its presence at the steroidal part of these molecules [21]. Moreover, other studies proved that the insertion of a keto group at the 7 position of D-modified as well as of non-D-modified steroidal skeletons considerably enhanced the anti-leukemic and cytogenetic effectiveness of the final esteric derivatives of CHL and their active metabolites [28,29], leading us to assume that functional changes at the B-steroidal ring are crucial regarding the SAR studies of these complex compounds.

In order to further elucidate the influence of the B-steroidal ring's configuration on the anti-leukemic potency, in an earlier study a -NHCO- endocyclic moiety was introduced to the B-ring of dehydroepiandrosterone (DHEA) and 17-keto-D-homo-aza-DHEA [30]. The anti-leukemic evaluation of their esters with CHL's active metabolite [4-*N*,*N*-bis(2-chloroethyl)amino phenylacetic acid (PHE)] [31,32] provided interesting results. The presence of the -NHCO- group in the B-steroidal ring did not afford the same impressive positive influence on the effectiveness of CHL's active metabolite esters as in the D-ring. However, the lactamic B-ring rendered the final esteric derivatives much more toxic compared to the corresponding esters with a simple six-membered B-ring, indicating that there might be a biological effect induced by the lactamic B-ring that could be expressed in a positive manner if another nitrogen mustard was used instead of PHE [30].

As the next step of our studies concerning the SAR of these compounds, we rationally designed and synthesized a new series of steroidal esteric analogs replacing PHE by its parental nitrogen mustard, CHL [6]. The comparative study presented herein concerns esters of 4-*N*,*N*-bis(2-chloroethyl)amino phenylbutyric acid (CHL) with the two B-lactamic steroids, 3β-hydroxy-7a-aza-B-homo-androst-5-en-7,17-dione (1a) and 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, 3β-hydroxy-androst-5-en-7,17-dione (1b) [28] and 3β-hydroxy-17α-aza-D-homo-androst-5-en-7,17-dione (2b) [29], and their parental non-oxidized steroids, 3β-hydroxy-androst-5-en-17-one (1c) [33] and 3β-hydroxy-17α-aza-D-homo-androst-5-en-17-one (2c) (Fig. 1) [34].

CHL 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*.

The results of this study in comparison with the previous results obtained for the corresponding B-lactamic esters of PHE are also discussed.

Methods

Synthetic procedures

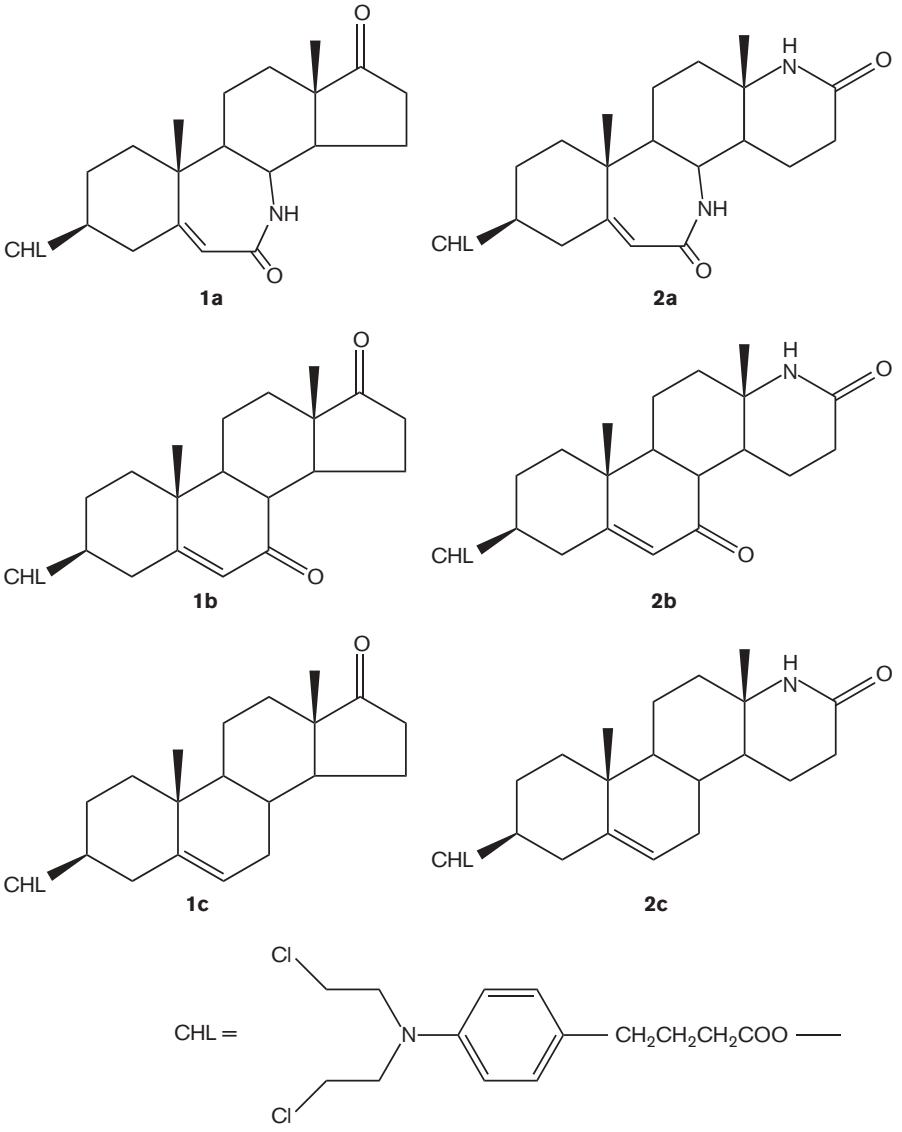
3β-Hydroxy-androst-5-en-17-one was purchased from Steraloids (Newport, Rhode Island, USA). 3β-Hydroxy-17α-aza-D-homo-androst-5-en-17-one was prepared by a method described in the literature [35]. The t-BuOOH/CuI-TBAB biphasic oxidizing method was applied for the allylic oxidation of the Δ^5-steroids [36], while the B-lactamic-DHEA and the B,D-bilactam were prepared according to a new synthetic procedure developed in our laboratory [37]. For the synthesis of CHL, phenylbutyric acid was nitrated according to the method described in the literature [38] followed by the synthetic steps described in Fig. 2. The final steroidal esteric derivatives of CHL were synthesized via the asymmetric anhydrides procedure [28] (Fig. 3).

Table 1 gives 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, Germany). For SCE demonstration, 5 μg/ml 5-bromodeoxyuridine (BrdUrd) and the chemicals were added at the beginning of culture. 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 and stained by the FPG procedure [39]. 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 statistical evaluation of the experimental data, the χ^2-test was performed for cell kinetic comparisons. For SCE frequencies, 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

Fig. 1



Chemical structures of CHL and its steroidal esteric derivatives

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 (HPLC purified) used in this study were prepared immediately before use. They were suspended in corn oil in the desired concentration following initial dissolution in 5% DMSO. This concentration by itself produced no observable toxic effects.

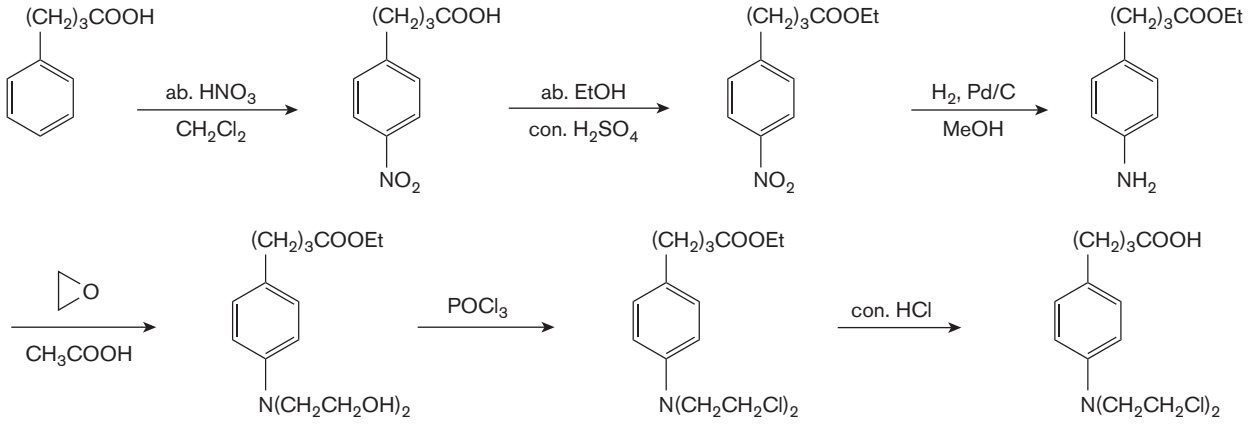
Mice

BALB/c, DBA/2 and BDF1 mice of both sexes, weighing 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

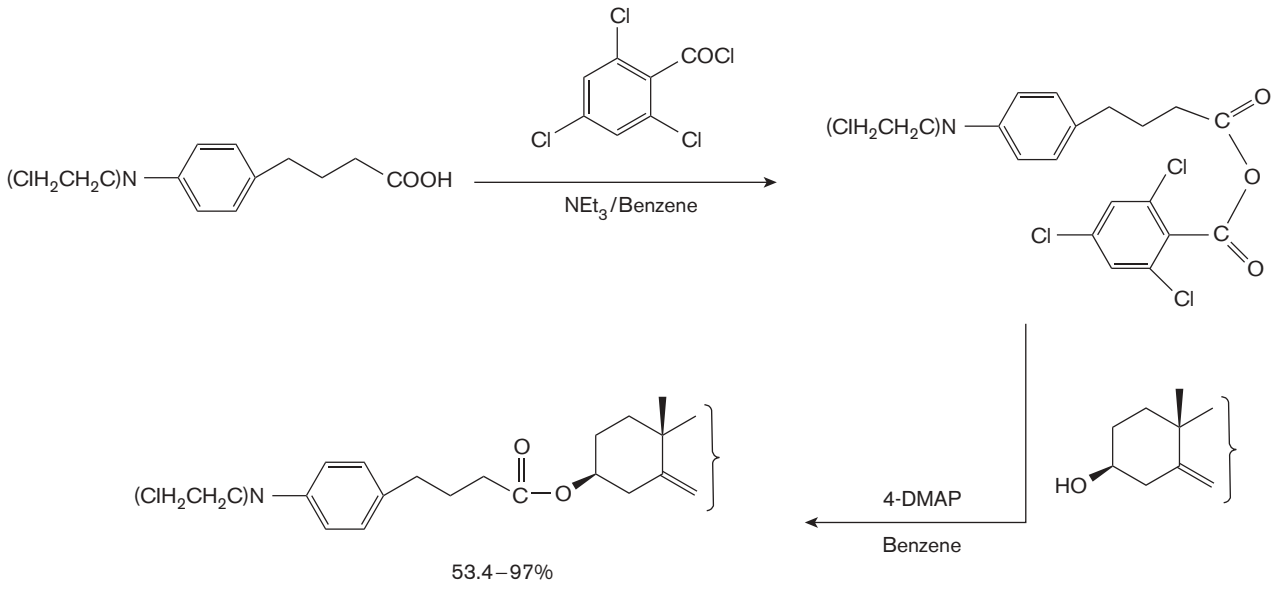
Leukemia P388- and L1210-bearing BDF1 (DBA/2 × C57BL) mice were used to evaluate the cytostatic effect. Lymphocytic P388 and lymphoid L1210 leukemias were maintained in ascitic form by injection of 10^6 and 10^5 cells, respectively, at 7-day intervals into the peritoneal cavity of DBA/2 mice.

Fig. 2



Synthetic procedure for the preparation of CHL.

Fig. 3



General synthetic procedure for the preparation of the final compounds.

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 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, whereas the tumor control group included eight mice; equal numbers of male and female mice were used in each group. Experiments were initiated by implanting mice with tumor cells according to the protocol of the National

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

Compound	Yield (%)	Recrystallization solvent	Melting point (°C)	IR (cm^{-1})	^{1}H-NMR (CDCl3) δ	Elemental analysis					
						Calculated (%)			Found (%)		
						C	H	N	C	H	N
1a	97.0	ethylacetate	97–98	3192, 1738, 1662, 1616, 804, 736	7.08d, 6.56d, 6.12s, 5.86s, 4.75m, 3.69t, 3.54t, 3.49t, 2.58t, 2.3t, 1.98m, 1.3s, 0.89s	65.66	7.35	4.64	65.63	7.36	4.62
1b	64.5	ethylacetate	120–121	1736, 1725, 1681, 806	7.21d, 6.61d, 5.71s, 4.63m, 3.71t, 3.68t, 2.56t, 2.31t, 1.98m, 1.21s, 0.91s	67.34	7.36	2.38	67.33	7.35	2.34
1c	92.0	ethylacetate	119–120	1734, 1725, 810	7.22d, 6.62d, 5.42s, 4.61m, 3.72t, 3.68t, 2.55t, 2.29t, 1.87m, 1.21s, 0.91s	72.85	8.09	2.74	72.83	8.10	2.73
2a	58.0	ethylacetate	180–182	3232, 1728, 1660, 1616, 817	7.09d, 6.65d, 6.91s, 6.42s, 5.85s, 4.72m, 4.71t, 3.62t, 3.20t, 2.61t, 2.31t, 1.93m, 1.27s, 1.17s	64.07	7.33	6.79	64.08	7.31	6.76
2b	53.4	ethylacetate	182–183	3500–3050, 1730, 1672, 1650, 804	7.12d, 6.6d, 6.82s, 5.64s, 4.58m, 3.7t, 3.66t, 2.51t, 2.29t, 1.85m, 1.22s, 1.11s	65.66	7.35	4.64	65.64	7.3	4.76
2c	84.5	ethylacetate	143–144	3170, 3055, 1720, 1655, 1610, 800	7.18d, 6.62d, 6.81s, 5.37s, 4.59m, 3.68t, 3.66t, 2.52t, 2.3t, 1.83m, 3.45s, 1.22s, 1.1s	67.23	8.12	4.73	67.80	8.08	4.62

Cancer Institute [40]. Treatments were given as an intermittent dose ($LD_{10}/2 \times 3$, days 1, 5 and 9). The experiments were terminated on day 90. Statistical evaluation of the experimental data was performed by the Wilcoxon test.

Results and discussion

As shown in Table 2, all the steroidal derivatives of CHL gave LD_{50} values higher than for CHL, indicating that in all cases the conjugation of CHL with the steroid resulted in a reduction of its toxicity.

Table 2 **Toxicity of the CHL and its steroidal esters**

Compound	LD_{50}(mg/kg)[a]	LD_{10}(mg/kg)
CHL	24	15
1a	56	14
1b	110	70
1c	220	135
2a	98	40
2b	62	43
2c	133	80

[a]LD_{50} values were estimated graphically, where the percentage of deaths due to the toxicity of each dose is shown on 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.

Comparing the toxicity of the compounds bearing a simple B-steroidal ring (1c and 2c) with that of the B-modified steroidal esters (1a, 1b and 2a, 2b), it is obvious that the introduction of the 7-ketone as well as the further modification to B-lactams resulted in an enhancement of toxicity. When a 7-keto group was introduced to the simple steroidal skeleton of DHEA (1c) the toxicity was doubled (1b). The -NHCO- moiety rendered the final molecule 1a even more toxic with a LD_{10} value almost equal to that of CHL. Almost a half reduction in the LD_{50} value was observed when 1c was converted to its lactamic analog 2c. Any further modification to 2c on the B-ring transformed the final molecules (2b and 2a) into more toxic compounds. These results confirm once more previous studies that have shown that these steroidal functional groups (7-keto and -NHCO-) render the esteric derivatives of nitrogen mustards more toxic than the derivatives with common steroidal skeletons [29,30,41].

Table 3 summarizes the results of the in-vivo anti-leukemic activity against P388 and L1210 leukemias. Since the treatment schedule $D/2 \times 3$ has proven superior to the single-dose schedule in several previous studies [29,30,41], we chose to test this series of compounds using the $D/2 \times 3$ treatment.

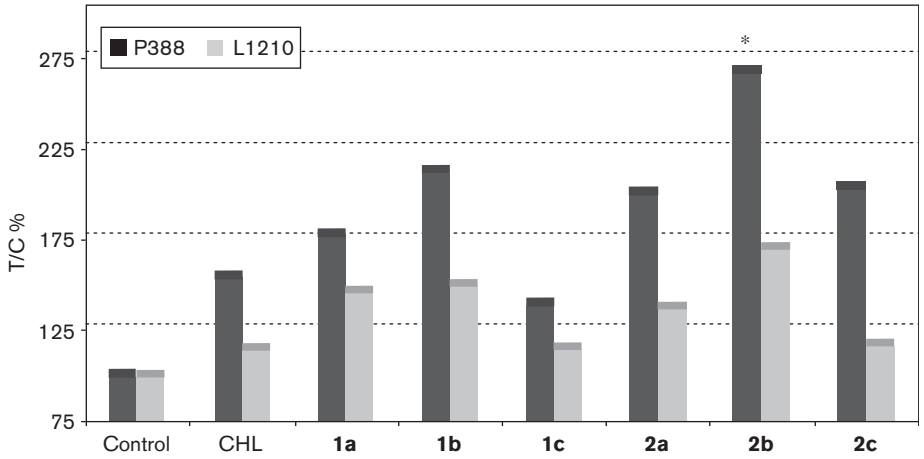
All compounds tested gave better T/C% values than CHL except for 1c, which bears a simple steroidal ring. This supports the observation that the conjugation of a nitrogen mustard to the appropriate steroidal molecule leads to more potent anti-leukemic activity [21–24].

Table 3 Anti-tumor activity of CHL 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	11.3	100	0/6	10.8	100	0/6
CHL	1,5,9	7.5	17.5	155	0/6	12.3	114	0/6
1a	1,5,9	7	20.1	178	0/6	15.6	145	0/6
1b	1,5,9	35	23.8	211	0/6	16.1	149	0/6
1c	1,5,9	67.5	15.6	138	0/6	12.3	114	0/6
2a	1,5,9	20	22.5	199	0/6	14.7	136	0/6
2b	1,5,9	21.5	30.2	267	**1/6**	18.4	170	0/6
2c	1,5,9	40	23.0	204	0/6	12.6	117	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. 4



The effect of the B-lactamic ring on the anti-leukemic potency of CHL steroidal esters. *Cures.

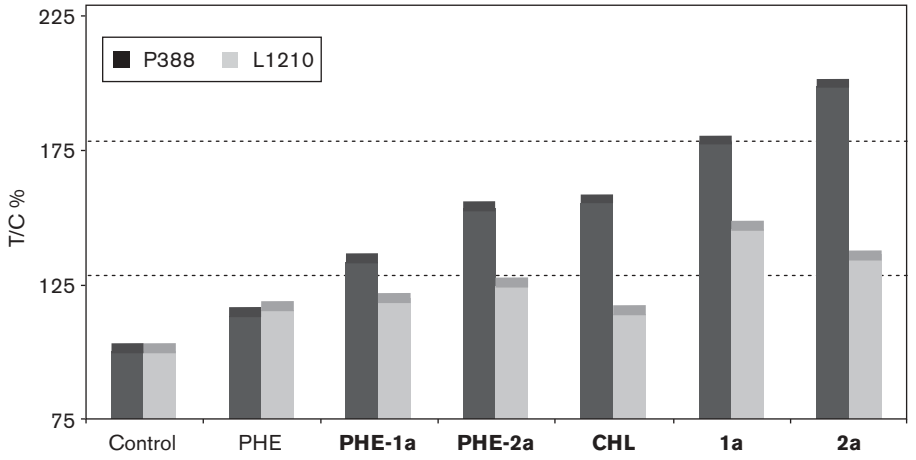
The introduction of a -NHCO- moiety in the B-steroidal ring of DHEA had a positive influence on the anti-leukemic activity of the esteric derivatives of CHL, but not to the same extent as the 7-keto group. In the case of derivative 1a, the modification of the B-ring to lactamic resulted in an enhancement of the T/C% value in both leukemias compared with 1c. However, when compared to the 7-oxidized derivative, which is less toxic, 1a proved almost equipotent with 1b in leukemia L1210 and less potent in leukemia P388.

Comparing the derivatives with a D-lactamic ring, the results were almost the same. By introducing a second -NHCO- moiety to the steroidal skeleton the final ester 2a gave a better T/C% value in leukemia L1210 (which is more aggressive than P388) than 2c and was found to be almost equipotent with 2c in P388. The 7-oxidized derivative 2b proved the most potent of all, although it is not proved to be the most toxic. (See Fig. 4).

As previously mentioned, in an earlier study [30] we examined the anti-leukemic potency of B-lactamic-DHEA and B,D-bilactam conjugated with PHE (the active metabolite of CHL). These esters (named PHE-1a and PHE-2a herein, see Fig. 5) proved impressively inferior compared to the corresponding esters of PHE with a simple or a 7-oxidized B-ring. From these data, we can estimate that the same conclusions do not apply in the case of CHL esters. The conjugation of these B-lactamic steroidal skeletons with CHL instead of PHE improved the potency of the final esteric derivatives, validating in that way our initial notion that they can be used as efficient and proper modules as long as the appropriate nitrogen mustard is used. (See Fig. 5).

Combining the results of the present study with other studies [29,30,41], it is once more confirmed that the steroidal part of these molecules is not only a simple biological carrier, as has been speculated for many years, but also plays a significant role in their mechanism of action. The presence of the -NHCO- moiety on the steroidal skeleton as well as the 7-keto group alters the biological behavior of these compounds, showing that these steroidal groups interfere with the ability of the

Fig. 5



The role of the nitrogen mustard on the anti-leukemic potency of B-lactamic steroidal esters.

nitrogen mustard to interact with different sequences of bases in the minor or major groove of DNA, thus producing an enhancement or a decrease of anti-leukemic specificity depending on the nitrogen mustard used.

SCEs have been frequently used as highly sensitive indicators of DNA damage and/or subsequent repair [42,43]. Non-repaired damage expressed as SCEs in normal cells, caused by certain chemicals, may indicate inability to repair 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* [44] 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 [45]. 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 alterations in cell cycle kinetics [46]. In the present study, a good correlation ($P < 0.02$) between SCE enhancement and PRI suppression was observed.

The in-vitro results are shown in Table 4. Compound 1c was the least effective inducer of SCEs among all of the compounds, which is in agreement with the in-vivo studies where it proved almost inactive, indicating that the conjugation of CHL with a common steroidal skeleton reduces the effectiveness towards SCEs induction. The derivatives with a 7-oxidized steroidal skeleton proved to be better inducers, on a molar basis, than the derivatives with a B-lactamic ring at both concentrations tested (0.2 and 0.6 µmol/l). PRI is used as a criterion for cytostatic activity. The best cell division delays were achieved by treating cells with derivatives 1b and 2b at the concentration of 0.6 µmol/l. Among all compounds tested, 2b gave the lowest PRI value – a result that correlates well with the in-vivo study where 2b proved to be the most potent of all compounds.

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

Compound	Concentration (µmol/l)	SCE/cell ± SE	PRI
Control	–	10.16 ± 0.63	2.52
CHL	0.2	24.9 ± 1.21	2.31
	0.6	31.12 ± 1.77	1.55
1a	0.2	15.89 ± 1.50	2.52
	0.6	30.36 ± 1.24	2.10
1b	0.2	25.37 ± 2.26	2.47
	0.6	33.47 ± 2.73	1.50
1c	0.2	13.4 ± 1.10	2.5
	0.6	14.51 ± 1.22	1.97
2a	0.2	14.62 ± 1.20	2.50
	0.6	24.75 ± 1.6	1.75
2b	0.2	20.8 ± 1.90	2.45
	0.6	32.98 ± 2.66	1.36
2c	0.2	21.12 ± 0.96	2.41
	0.6	30.11 ± 1.14	1.61

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

Comparing the results obtained from the six steroidal esters of CHL with the corresponding esters of PHE [30], we can estimate that when the simple or 7-oxidized steroidal skeletons are esterified with PHE the final molecules produced are more potent than the corresponding derivatives of CHL. On the contrary, the B-lactamic steroidal derivatives of CHL are better than those of PHE. This observation supports the notion that the anti-leukemic specificity of nitrogen mustard's steroidal esters depends on the configuration of the

whole molecule and the appropriate combination of a nitrogen mustard with a steroidal module. In order to substantiate this conclusion, however, further SAR studies have to be performed including other alkylating agents as well as studies on the mechanism of action of these molecules.

Conclusion

The B-ring of these complex molecules seems to play a crucial role in the anti-leukemic activity and specificity of action of these compounds. A minor alteration such as the introduction of a 7-keto group or its modification to a lactamic ring drastically influences both the cytotoxicity and the anti-leukemic activity. This influence is expressed, however, in a positive or negative way, probably depending on how these functional groups interfere with the ability of the alkylator to bind at specific sequences on the DNA. The B-lactamic steroidal derivatives of CHL proved more potent than the corresponding derivatives of PHE, in contrast to 7-oxidized derivatives, indicating that the -NHCO- moiety on the B-ring enhances and/or decreases the inert ability of CHL to interact effectively with the DNA, while the 7-keto group has the same impact on PHE. Consequently, the anti-leukemic potency of these compounds depends on the proper combination of the specific alkylator with a suitable steroidal skeleton in order to increase the specificity of its interaction. In addition, we speculate that in leukemic cells a tumor-specific protein may be overexpressed, and the steroidal part of the molecule, depending on its configuration, has the specific ability to bind and block the adduct repaired by DNA repair enzymes and prevent this protein from carrying out its normal function.

In order to prove this concept, more alkylating agents and further modifications on the steroidal part, as well as different biological functions (e.g. apoptosis, transcription, signaling), must also be examined.

Acknowledgments

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References

1 Kohn K. Beyond DNA cross-linking: history and prospects of DNA-targeted cancer treatment. Fifteenth Bruce F. Cain Memorial Award Lecture. *Cancer Res* 1996; **56**:5533–5546.
2 Ross WE, Ewig RA, Kohn KW. Differences between melphalan and nitrogen mustard in the formation and removal of DNA cross-links. *Cancer Res* 1978; **38**:1502–1506.
3 Zwelling L, Michaels S, Schwartz H, Dobson PP, Kohn KW. DNA crosslinking as an indicator of sensitivity and resistance of L1210 leukemia cells to *cis*-diaminedichloroplatinum(II) and L-phenylalanine mustard. *Cancer Res* 1981; **41**:640–649.
4 Brookes P, Lawley PD. The reaction of mono- and di-fuctional alkylating agents with nucleic acids. *Biochem J* 1961; **80**:496–503.
5 Dusenbery KE, Bellairs EE, Potish RA, Twiggs LB, Boente MP. Twenty-five year outcome of sequential abdominal radiotherapy and melphalan: implications for future management of epithelial carcinoma of the ovary. *Gynecol Oncol* 2005; **96**:307–313.
6 Shamash J, Dancey G, Barlow C, Wilson P, Ansell W, Oliver RT. Chlorambucil and lomustine (CL56) in absolute hormone refractory prostate cancer: re-induction of endocrine sensitivity an unexpected finding. *Br J Cancer* 2005; **92**:36–40.
7 Brendel M, Ruhland A. Relationships between functionality and genetic toxicology of selected DNA-damaging agents. *Mutat Res* 1984; **133**:51–85.
8 Morgan AS, Ciaccio PJ, Tew KD, Kauvar LM. Isozyme specific glutathione S-transferase inhibitors potentiate drug sensitivity in cultured human tumor cell lines. *Cancer Chemother Pharmacol* 1996; **37**:363–370.
9 Green JA, Vistica DT, Young RC, Hamilton TC, Rogan AM, Ozols RF. Potentiation of melphalan cytotoxicity in human ovarian cancer cell lines by glutathione depletion. *Cancer Res* 1984; **44**:5427–5431.
10 Panasci L, Xu Z, Bello V, Aloyz R. The role of DNA repair in nitrogen mustard drug resistance. *Anticancer Drugs* 2002; **13**:211–220.
11 Kaldor JM, Day NE, Hemminki K. Quantifying the carcinogenicity of antineoplasmatic drugs. *Eur J Cancer Clin Oncol* 1988; **24**:703–711.
12 Denny WA. DNA minor groove alkylating agents. *Curr Med Chem* 2001; **8**:533–543.
13 Bielawska A, Bielawski K, Chrzanowski K, Wotczynski S. Prolidase-activated prodrug for cancer chemotherapy, cytotoxic activity of proline analogue of chlorambucil in breast cancer. *Farmaco* 2000; **55**:736–741.
14 Soyez H, Seymour LW, Schacht E. Macromolecular derivatives of *N,N*-di-(2-chloroethyl)-4-phenylene diamine mustard. 2. *In vitro* cytotoxicity and *in vivo* anticancer efficacy. *J Control Release* 1999; **57**:187–196.
15 Kasiotis KM, Magiatis P, Pratsinis H, Skaltsounis AL, Abadji V, Charalambous A, *et al.* Synthesis and biological evaluation of novel daunorubicin–estrogen conjugates. *Steroids* 2001; **66**:785–791.
16 Ferguson LR, Turner PM, Denny WA. The mutagenic spectrum of acridine-linked aniline nitrogen mustards in AS52 cells: implications of DNA targeting with high selectivity for adenine or guanine bases. *Mutat Res* 2000; **469**:115–126.
17 Wasan H, Waxman J. In: Pusztai LL, Lewis CE, Yap E (editors): Cell proliferation cancer. Oxford: Oxford University Press; 1996. pp. 260–281.
18 Lupulescu AP. Hormones, vitamins, and growth factors in cancer treatment and prevention. A critical appraisal. *Cancer* 1996; **78**:2264–2280.
19 Albrecht W, Van Poppel H, Horenlas S, Mickisch G, Horwich A, Secretta V, *et al.* Randomized phase II trial assessing estramustine and vinblastine combination chemotherapy vs estramustine alone in patients with progressive hormone-escaped metastatic prostate cancer. *Br J Cancer* 2004; **90**:100–105.
20 Brandt L, Konyves I, Moller TR. Therapeutic effect of LEO 1031, an alkylating corticosteroid ester, in lymphoproliferative disorders. I. Chronic lymphocytic leukemia. *Acta Med Scand* 1975; **197**:317–322.
21 Nikolaropoulos S, Tsavdaridis D, Arsenou E, Papageorgiou A, Karaberis E, Mourelatos D. Synergistic antineoplastic and cytogenetic effects by the combined action of two homo-aza-steroidal esters of nitrogen mustards on P388 and L1210 leukaemias *in vivo* and *in vitro*. *Anticancer Res* 2000; **20**:2745–2752.
22 Nikolaropoulos SS, Arsenou ES, Papageorgiou A, Mourelatos D. Antitumor and cytogenetic effects of esteric (ASE) and amidic (ASA) steroidal derivatives of p-bis(2-chloroethyl)amino phenylacetic acid (CAPA). A comparative study. *Anticancer Res* 1997; 17:4525–4530.
23 Anastasiou A, Catsoulacos P, Papageorgiou A, Margariti E. On the formation of homo-azasteroidal esters of *N,N*-bis(2-chloroethyl)aminobenzoic acid isomers and their antitumor activity. *J Heterocycl Chem* 1994; **31**:367–373.
24 Athanasiou K, Demopoulos N, Catsoulacos P. Chromosome damage and SCE induced by the cytostatic factor homo-aza-steroidal ester of *p*-bis(2-chloroethyl) aminophenyl acetic acid in CHO cells in culture. *Environ Mutagen* 1983; **5**:279–283.
25 Wall ME, Abernethy Jr GS, Caroll FJ, Taylor DJ. The effect of some steroidal alkylating agents on experimental animal mammary tumor and leukemia systems. *J Med Chem* 1969; **12**:810–818.
26 Catsoulacos P, Politis D, Wampler GL. A new steroidal alkylating agent with improved activity in advanced murine leukemias. *Cancer Chemother Pharmacol.* 1979; **3**:67–70.
27 Catsoulacos P, Wampler GL. Activity of 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-*p*-bis(2-chloroethyl)-aminophenylacetate. *Oncology* 1982; **39**:109–112.
28 Karayianni V, Papageorgiou A, Mioglou E, Iakovidou Z, Mourelatos D, Fousteris M, *et al.* 7-Keto hybrid steroidal esters of nitrogen mustard: cytogenetic and antineoplastic effects. *Anticancer Drugs* 2002; **13**: 637–643.

29 Arsenou E, Fousteris M, Koutsourea A, Papageorgiou A, Karayianni V, Mioglou E, *et al.* The allylic 7-ketone at the steroidal skeleton is crucial for the anti-leukemic potency of chlorambucil's active metabolite steroidal esters. *Anticancer Drugs.* 2004; **15**:983–990.
30 Papageorgiou A, Koutsourea A, Arsenou E, Fousteris E, Mourelatos D, Nikolaropoulos S. Structure–anti-leukemic activity relationship study of B and D-ring modified and non-modified steroidal esters of chlorambucil's active metabolite. *Anticancer Drugs* 2005; **16**:1075–1082.
31 McLean A, Woods RC, Catovsky D, Farmer P. Pharmacokinetics and metabolism of chlorambucil in patients with malignant disease. *Cancer Treat Rev* 1979; **6**(**Suppl**):33–42.
32 Pettersson-Fernholm T, Vilpo J, Kosonen M, Hakala K, Hovinen J. Reactions of 4-bis(2-chloroethyl)aminophenylacetic acid (phenylacetic mustard) in physiological solutions. *J Chem Soc Perkin Trans 2* 1999; 2183–2187.
33 Pairas G, Camoutsis Ch, Catsoulacos P. A new route for the synthesis of steroidal esters of carboxylic derivatives of *N*,*N*-bis(2-chloroethyl)aniline with DCC. *Eur J Med Chem Chim Ther* 1985; **20**:287–288.
34 Catsoulacos P, Boutis L. Cytostatic action of 3β-hydroxy-13α-amino-13,17-seco-5-androsten-17-oic-13,17-lactam-*p*-*N*,*N*-bis(2-chloroethyl)aminophenylacetate. *Eur J Med Chem Chim Ther* 1974; **9**:211–213.
35 Regan BM, Hayes FN. 17 and 17α-aza-D-homo steroids. *J Am Chem Soc* 1956; **78**:639–643.
36 Arsenou EA, Koutsourea AI, Fousteris MA, Nikolaropoulos SS. Optimization of the allylic oxidation in the synthesis of 7-keto-Δ^5-steroidal substrates. *Steroids* 2003; **68**:407–414.
37 Koutsourea A, Arsenou E, Fousteris M, Nikolaropoulos S. Synthetic approaches for the synthesis of a cytostatic steroidal B–D bilactam. *Steroids* 2003; **68**:659–666.
38 Strazzolini P, Giumanini G, Runcio A, Scuccato M. Experiments on the Chaperon effect in the nitration of aromatics. *J Org Chem* 1998; **63**: 952–958.
39 Goto K, Maeda S, Kano Y, Sugiyama T. Factors involved in differential Giemsa-staining of sister chromatids. *Chromosoma* 1978; **66**: 351–359.
40 Goldin A, Sofina Z, Syrkin A. Experimental evaluation of antitumor drugs in the USA and USSR and clinical correlations. *Natl Cancer Inst Monogr* 1980; **55**:25–26.
41 Fousteris MA, Koutsourea AI, Arsenou ES, Papageorgiou A, Mourelatos D, Nikolaropoulos S. Antileukemic and cytogenetic effects of modified and non-modified esteric steroidal derivatives of 4-methyl-3-bis (2-chloroethyl)amino benzoic acid (4-Me-CABA). *Anticancer Res* 2002; **22**: 2293–2300.
42 Kasal A. Epalons: 6-substituted derivatives of 7-norepiallopregnanolone. *Tetrahedron* 2000; **56**:3559–3565.
43 Deen DF, Kendall LA, Marton LJ, Tofilon P. Prediction of human tumour cell chemosensitivity using the SCE assay. *Cancer Res* 1986; **46**: 1599–1602.
44 Mourelatos D, Chromosomes study as predictor of chemoresponse of tumours. *Cancer J* 1996; **9**:136–141.
45 Tolifon P, Basic I, Milas L. Prediction of *in vivo* tumor response to chemotherapeutic agents by the *in vitro* SCE assay. *Cancer Res* 1985; **45**:2025–2030.
46 Mourelatos D, Dozi J, Kotsis A, Gourtsas C. Enhancement of cytogenetic damage and of antineoplastic effect by caffeine in Ehrlich ascites tumour cells treated with cyclophosphamide *in vivo*. *Cancer Res* 1988; **48**: 1129–1131.