Structure–antileukemic activity relationship study of B- and D-ring modified and nonmodified steroidal esters of 4-methyl-3-*N,N*-bis(2-chloroethyl)amino benzoic acid: a comparative study

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This study was designed as a rational continuation of our research regarding the functional requirements essential for the antileukemic activity of compounds comprising an alkylating moiety and a modified steroid. The steroidal esteric derivatives of 4-methyl-3-*N,N*-bis(2-chloroethyl) amino benzoic acid were tested on leukemias P388 and L1210 *in vivo* and in normal human lymphocytes *in vitro*. Among them the B-lactamic steroidal esters proved more potent antileukemic agents than the 7-oxidized and those with a simple B-ring, but not more effective inducers of DNA damage and cell cycle arrest *in vitro*. We speculate that these results indicate a different mechanism of action induced by the lactamized B steroidal ring, in comparison to the 7-keto or the D-lactamic groups, which involves the interaction of the –NHCO– moiety with cellularcomponents essential for tumor growth. 4-Methyl-3-*N,N*-bis(2-chloroethyl)amino benzoic acid proved a more proper module for the B-lactams than chlorambucil and phenyl acetic acid's nitrogen mustard probably because the esteric bond is less cleaved by the esterases, resulting in an increased concentration of the drug in the vinicity of the target site essential for an antineoplasmatic response. *Anti-Cancer Drugs* 18:997–1004 © 2007 Lippincott Williams & Wilkins.

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Introduction

DNA-damaging agents that form cross-links were the first anticancer drugs used in clinical practice and remain until today some of the most effective chemotherapeutics for the treatment of a variety of neoplasms. Owing to their high toxicity, however, and the inherent or developed resistance of some tumors to these compounds, much effort has focused on the development of strategies that can defeat the molecular barriers responsible for clinical failure [1,2]. One of the approaches used to increase the vulnerability of certain malignancies to alkylating drugs is the design of new cross-linking derivatives capable of directing the antineoplasmically relevant activity predominantly to the target tumor [3–6].

In site-directed chemotherapy of malignancies the goal is to utilize drugs with selective toxicity for tumor cells. The discovery of steroid hormone receptors and hormone responsiveness in a series of human tumors [7–10] offers the opportunity for targeting using drug–hormone conjugates [11–15]. The initial approach used for the design of complex molecules, comprising a nitrogen mustard and a steroidal skeleton, was based on the concept that the steroidal part would act as a 'biological platform' enabling the alkylating moiety to approach its site of action by altering its physicochemical properties (e.g. lipophilicity). Although researchers in the past have used this notion [16–18] to explain the antileukemic activity of such compounds, recently new molecules of this category which were designed to possess small but effective differences (e.g. a simple keto group on a specific position of the steroidal skeleton) proved that the observed significant differentiations on the antileukemic potency of these compounds could not be explained only on the basis of the change of the physicochemical parameters [19,20]. Apparently the effectiveness of these molecules involves the existence of other mechanisms of actions as a number of recent studies have revealed. Indications for a relative enrichment of DNA-damaging effects in the tumor tissue and for reduced myelotoxicity have been obtained with specific hormone conjugates [21,22], whereas in a recent study a rationally designed hormone-linked genotoxicant was found to possess the ability to block repairing enzymes by camouflaging the DNA-adducts formed [23].

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Our ongoing structure–antileukemic activity relationship studies in this field have indicated some structural requirements to design effective antileukemic analogs. Specifically it has been found that when nitrogen mustards are tethered to steroidal skeletons that carry a D-lactamic ring, the final molecules are more effective antileukemic agents than derivatives with a common or nonmodified steroidal congener [24–26]. Moreover, recent studies have indicated that minor functional changes in the B steroidal ring (e.g. 7-keto group) had considerable effects on the final molecules' antileukemic, genotoxic and cytotoxic activity, leading us to assume that its modification is fundamental for the design of more effective molecules [19,27].

To further investigate the role of the B steroidal ring and at the same time acquire additional data concerning the biological effects caused by the presence of lactamic moieties on the steroidal congener, in a recent study a –NHCO– group was introduced to the B-ring of a simple steroidal skeleton (B-lactamic-dehydroepiandrosterone, B-lactamic-dehydroepiandrosterone (DHEA)) and to the corresponding D-lactamic one (B,D-bilactamic-DHEA). The two steroids were first tethered to phenylacetic acid mustard (4-*N*,*N*-bis (2-chloroethyl) amino phenylacetic acid (PHE) chlorambucil's active metabolite, [28,29]), and evaluated for their antileukemic, genotoxic and antiproliferative activity [20]. 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. This new modification of the B-ring, however, rendered the final esteric derivatives much more toxic, compared with the corresponding esters of PHE with a simple B-ring, indicating that there might be a biological effect induced by the B-lactamic ring that could be exerted in a positive way if another nitrogen mustard was used instead of PHE. On the basis of this hypothesis a subsequent study concerning the same steroidal derivatives of chlorambucil (CHL) [30] indicated that when B-lactamic-DHEA and B,D-bilactamic-DHEA were tethered to CHL the antileukemic activity was enhanced in comparison to the corresponding derivatives of PHE.

On the basis of these structure–activity relationship studies we decided to further investigate the biological profile of these two B-lactamic steroidal skeletons. Thus, this study concerns the synthesis, the in-vivo antileukemic evaluation and the in-vitro genotoxic and cytotoxic effects caused by the esters of 4-methyl-3-*N*,*N*-bis(2-chloroethyl)amino benzoic acid (4-Me-CABA) with the two B-lactamic steroidal skeletons, i.e. 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). To have comparative results with the previous studies [20,30] the esters of 4-Me-CABA with the corresponding steroids with a simple and a 7-oxidized B steroidal ring were also synthesized and tested, i.e. 3β-hydroxy-androst-5-en-7,17-dione (1b), 3β-hydroxy-17α-aza-D-homo-androst-5-en-7,17-dione (2b) [27] and their parental non-oxidized steroids, that is 3β-hydroxy-androst-5-en-17-one (1c) and 3β-hydroxy-17α-aza-D-homo-androst-5-en-17-one (2c) [31] (Fig. 1).

The nitrogen mustard 4-MeCABA was selected for this study in a continuation of our studies with the specific alkylating agent [27,31,32], and because its esters have been proven more effective and more potent among a series of steroidal esters of *N*,*N*-bis(2-chloroethyl)amino-benzoic acid isomers tested [33].

The nitrogen mustard 4-Me-CABA and its six steroidal esters, were tested against leukemias P388 and L1210 *in vivo*, and for the induction of sister chromatid exchange (SCE) and the reduction of the proliferation rate index (PRI) in normal human lymphocytes *in vitro*.

Methods

Synthetic procedures

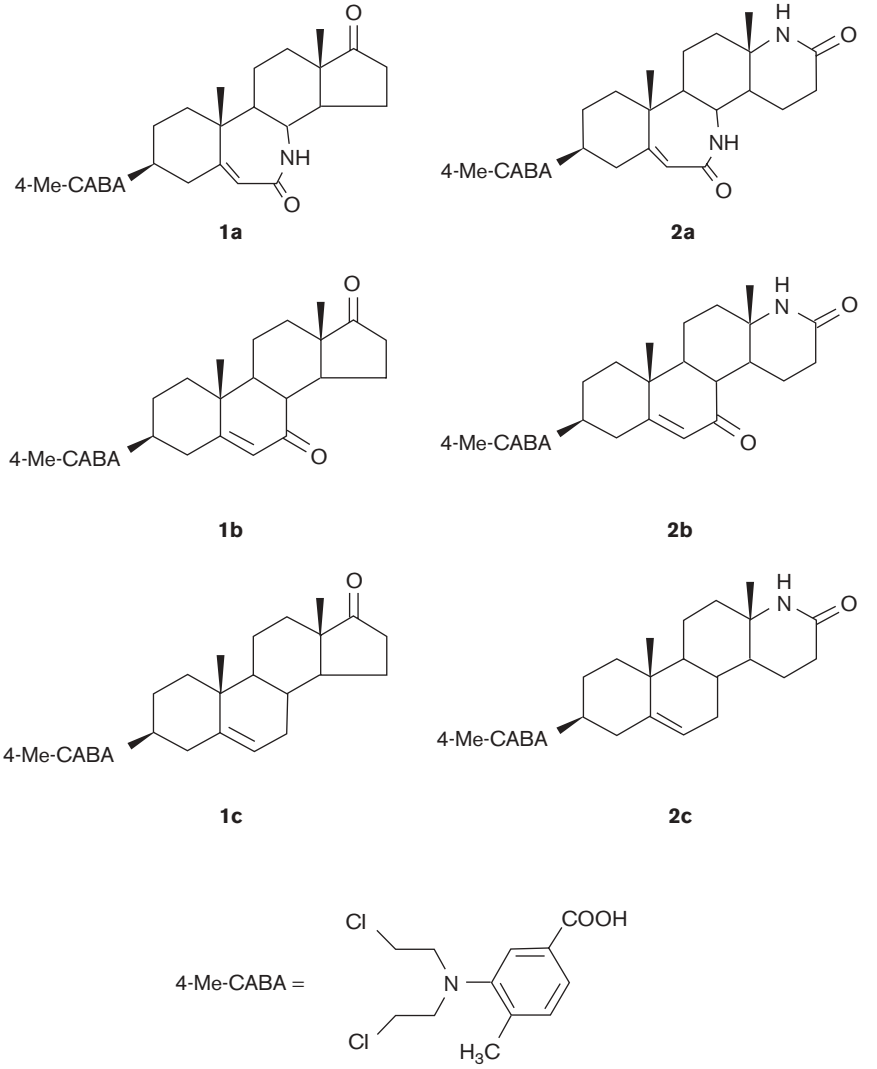
3β-Hydroxy-androst-5-en-17-one was purchased from Steraloids (Newport, Rhode Island, USA). 3β-Hydroxy-17α-aza-D-homo-androst-5-en-17-one was prepared by the method described in the literature [34]. The *t*-BuOOH/CuI-TBAB biphasic oxidizing method was applied for the allylic oxidation of the Δ^5-steroids [35], whereas the B-lactamic-DHEA and the B,D-dilactam were prepared according to a new synthetic procedure developed in our laboratory [36]. The synthesis of 4-Me-CABA was achieved according to methods described in the literature [37,38]. The final steroidal esteric derivatives of 4-Me-CABA were synthesized via the asymmetric anhydrides procedure [32] (Fig. 2).

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

In-vitro sister chromatid exchange and proliferation rate index 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 and the chemicals were added at the beginning of culture life. Throughout, all cultures were maintained in the dark to minimize photolysis of bromodeoxyuridine. 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 [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

Fig. 1



Chemical structures of 4-Me-CABA and its steroidal esteric derivatives. 4-Me-CABA, 4-methyl-3-*N,N*-bis(2-chloroethyl)amino benzoic acid.

both chromatids light staining). Twenty suitably spread second division cells from each culture were blindly scored for SCEs. For PRIs, 100 cells at least 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 intraperitoneal treatment, stock solutions of the compounds used in this study were prepared immediately before use. They were suspended in corn oil in the desired concentration following initial dissolution in 5% dimethylsulfoxide. This concentration by itself produced no observable toxic effects.

Mice

BALB/c, DBA/2 and BDF1 mice of both sexes, weighting 20–23 g, 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-bearing and L1210-bearing BDF1 (DBA/2XC57BL) 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.

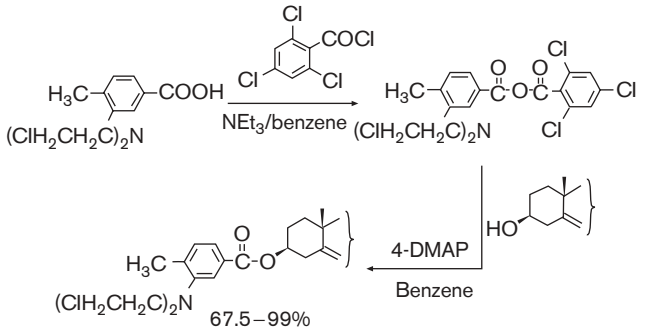
Estimation of acute toxicity

The acute toxicity of the compounds was determined following a single intraperitoneal 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 increase in median life span 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 the 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, whereas 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, USA [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 made by the Wilcoxon test.

Fig. 2



General synthetic procedure for the preparation of the final compounds.

Results and discussion

The toxicity values of the compounds tested are illustrated in Table 2. In all cases the esterification of the alkylating agent with the steroidal skeletons resulted in an increase of the LD_{50} values.

Among the derivatives tested, those with a simple B steroidal ring (1c, 2c) were the least toxic. The insertion of a 7-keto group rendered the final molecules 1b and 2b more toxic, whereas the modification of the B steroidal ring to lactamic further reduced the LD_{50} value in the case of derivative 1a. In the case of the derivatives with a D-lactamic ring the toxicity values, however, remained almost at the same levels when the B-ring was converted to lactamic (2a) from 7-oxidized (2b). These results are in agreement with the previous studies concerning the esters of the same steroidal skeletons with PHE [20] and CHL [30]. The modification of a simple B steroidal ring either to oxidized or to lactamic resulted in a increment of the cytotoxicity in all cases (Fig. 3), showing that the configuration of this ring is important for the activation

Table 1 Physicochemical and analytical data of the final steroidal esteric derivatives of 4-Me-CABA

Compound	Yield (%)	Recrystallization solvent	Melting point (°C)	IR (cm^{-1})	^{1}H-NMR ($CDCl_3$) δ	Elemental analysis					
						Calculated (%)			Found (%)		
						C	H	N	C	H	N
1a	92.0	Ethylacetate	247–248	3300, 1734, 1658, 1616, 1286 761, 736	7.70s, 7.60d, 7.30d, 5.90s, 5.70s, 5.00m, 3.40m, 3.20t, 2.30s, 1.32s, 0.90s	64.69	7.00	4.87	64.70	7.02	4.84
1b	67.5	Ethylacetate	145–146	1735, 1719, 1670, 1257, 763, 728	7.72s, 7.61d, 7.21d, 5.71s, 4.81m, 3.45m, 2.31s, 1.21s, 0.77s	66.42	7.01	2.50	66.31	7.07	2.55
1c	99.0	Ethylacetate	176–177	1729, 1717, 1255, 760, 730	7.82s, 7.74d, 7.33d, 5.39s, 4.82m, 3.44m, 2.38s, 1.18s, 0.89s	68.12	7.56	2.56	68.23	7.39	2.55
2a	80.0	Ethylacetate	168–170	3238, 1716, 1658, 1616, 1257, 761, 736	7.80s, 7.70d, 7.20d, 7.00s, 6.50s, 5.90s, 4.95m, 3.4m, 3.10t, 2.3s, 1.26s, 1.16s	63.04	7.00	7.12	63.06	7.00	7.14
2b	77.7	Ethylacetate	171–173	3182–3070, 1714, 1674, 1656, 1257, 761, 732	7.74s, 7.66d, 7.24d, 6.55s, 5.71s, 4.85m, 3.36m, 2.39s, 1.20s, 1.09s	64.69	7.00	4.87	64.78	6.92	4.95
2c	72.0	Ethylacetate	215–216	3180, 3055, 1710, 1660, 760, 730	7.83s, 7.71d, 7.26d, 5.40s, 4.81m, 3.46m, 2.38s, 1.19s, 1.01s	69.93	7.69	5.62	69.90	7.70	5.62

IR, infrared; 4-Me-CABA, 4-methyl-3-*N,N*-bis(2-chloroethyl)amino benzoic acid, NMR, nuclear magnetic resonance.

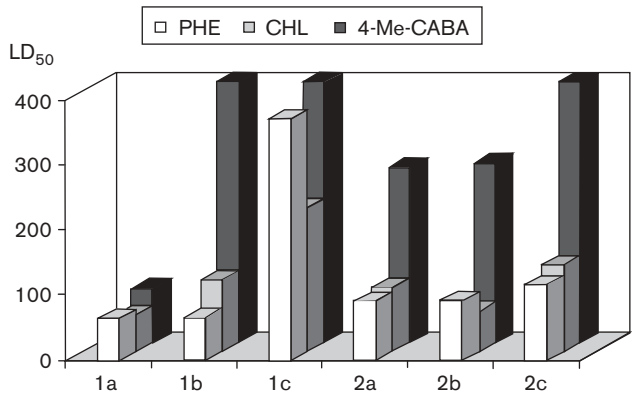
of cellular mechanisms responsible for the biological response of these complex compounds. Comparing the derivatives of 4-Me-CABA with those of PHE and CHL it is clearly shown that they are less toxic. This result can be explained by the fact that the esteric bond of 4-Me-CABA's derivatives is less cleaved by the esterases because of the stereochemical hindrance, resulting in that way in a decrease of the percentage of the free alkylating agent in the cell and subsequently of the toxic effects derived from the nitrogen mustard alone.

Table 2 Toxicity of the 4-Me-CABA and its steroidal esters

Compound	LD_{50}[a] (mg/kg)	LD_{10} (mg/kg)
4-Me-CABA	18	10
1a	82	42
1b	680	450
1c	870	540
2a	270	220
2b	275	170
2c	570	250

4-Me-CABA, 4-methyl-3-*N,N*-bis(2-chloroethyl)amino benzoic acid.
[a]LD_{50} values were estimated graphically, where the percentage of deaths owing to the toxicity of each dose is shown in the ordinate, whereas 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.

Fig. 3



Toxicity of the steroidal esteric derivatives of PHE, CHL and 4-Me-CABA. PHE, 4-N,N-bis (2-chloroethyl) amino phenylacetic acid; CHL, chlorambucil; 4-Me-CABA, 4-methyl-3-*N,N*-bis(2-chloroethyl)amino benzoic acid.

The results from the antileukemic evaluation of the compounds against leukemias P388 and L1210 are shown in Table 3.

All modified steroidal esteric derivatives gave better T/C% values than 4-Me-CABA in both leukemias. Compound 1c, with a simple steroidal congener, proved inactive with a T/C% value even lower than that of the alkylating agent. Comparing compounds 1c with 1b we can see that a small modification on the steroidal skeleton, e.g. the 7-keto group, was adequate to render the former inactive agent in a compound more active even than 4-Me-CABA. The lactamization of the B steroidal ring (1a) further improved the antileukemic potency in a considerable degree.

Comparing compounds 2a, 2b and 2c, an analogous result is observed concerning the modification of the B steroidal ring. The introduction of a 7-ketone gave compound 2b which proved a more effective antileukemic agent against both leukemias than 2c with a simple B-ring. Its lactamization further enhanced the activity against leukemia P388 (also giving one out of six cures), whereas the T/C% value of 2a in L1210 remained at the same levels as 2c.

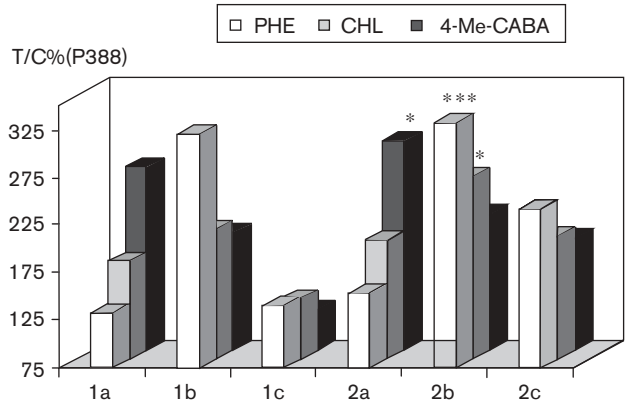
Through the comparison of 1a, 1b and 1c with the corresponding D-lactamic derivatives 2a, 2b and 2c we can see that the lactamization of the D steroidal ring improved the antileukemic potency of the compounds against leukemia P388, whereas in leukemia L1210 the D-lactamic ring had the same positive effect except for compound (2a). This result is in agreement with the previous studies [20,30] showing the importance of the presence of the D-lactamic ring in these complex molecules.

Table 3 Antitumor activity of 4-Me-CABA and its steroidal esters on P388-bearing and L1210-bearing mice leukemia, using doses on the basis of 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	10	100	0/6	7	100	0/6
4-Me-CABA	1,5,9	5	14.4	144	0/6	9.0	129	0/6
1a	1,5,9	21	26.8	268	0/6	13.6	194	0/6
1b	1,5,9	225	19.8	198	0/6	10.8	155	0/6
1c	1,5,9	270	11.7	117	0/6	8.2	117	0/6
2a	1,5,9	110	29.5	295	1/6	11.3	161	0/6
2b	1,5,9	85	21.9	219	0/6	12.5	179	0/6
2c	1,5,9	125	19.3	193	0/6	11.5	165	0/6

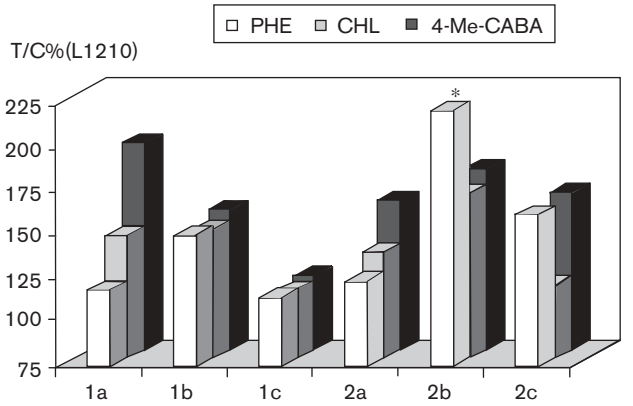
4-Me-CABA, 4-methyl-3-*N,N*-bis(2-chloroethyl)amino benzoic acid.
[a]MST, mean survival time of mice inoculated with lymphocytic leukemia P388 or lymphoid leukemia L1210 cells and treated with compounds.
[b]T/C, increase median life span of the drug-treated animals (T) versus corn-oil-treated animals (C).

Fig. 4



The effect of structural modifications on the antileukemic potency of PHE, CHL and 4-Me-CABA steroidal esters in P388 leukemia. PHE, 4-N,N-bis (2-chloroethyl) amino phenylacetic acid; CHL, chlorambucil; 4-Me-CABA, 4-methyl-3-*N,N*-bis(2-chloroethyl)amino benzoic acid. *Cures.

Fig. 5



The effect of structural modifications on the antileukemic potency of PHE, CHL and 4-Me-CABA steroidal esters in L1210 leukemia. PHE, 4-*N,N*-bis(2-chloroethyl)aminophenylacetic acid; CHL, chlorambucil; 4-Me-CABA, 4-methyl-3-*N,N*-bis(2-chloroethyl)amino benzoic acid. *Cures.

Figures 4 and 5 summarize the results of the in-vivo studies against leukemias P388 and L1210 of the steroidal derivatives of PHE [20], CHL [30] and 4-Me-CABA. Concerning the derivatives with a B-lactamic steroidal ring, we can see that their conjugation with 4-Me-CABA enhanced considerably the antileukemic potency against both leukemias in comparison to the previous studies where they were tethered to PHE and CHL whereas at the same time they were found less toxic as previously mentioned (Fig. 3). These results confirm our initial notion discussed in the two previous studies [20,30], that the lactamization of the B steroidal ring can enhance the antileukemic activity if a different nitrogen mustard is used. A possible explanation for this, as previously suspected [20], may be that the insertion of the –NHCO– moiety in the B steroidal ring enables the interaction of these molecules with different cellular sites that can cause an antileukemic response. The fact that PHE and CHL were not proved suitable as the alkylating congener may be attributed to the high hydrolysis rate of their steroidal esters, in contrast to 4-Me-CABA's esters studied herein, whose esteric bond is less cleaved by the esterases because of the stereochemical hidrance thus resulting in an increased concentration of the drug at the specific binding site and consequently to a more effective antileukemic treatment.

The hypothesis that the B-lactamic steroidal esters act through different mechanistic pathways is further substantiated by the fact that the 7-oxidized steroidal derivatives did not have the same behavior when tethered to 4-Me-CABA. This is clearly stated in leukemia P388, where the replacement of PHE and CHL with 4-Me-CABA had the opposite effect in the antileukemic activity of the 7-keto steroidal derivatives in comparison to the B-lactamic ones. This kind of behavior can only be attributed to the ability of the steroidal part of these molecules to interact with different sites in the cell and consequently have different mechanisms of action. Specifically the configuration of the B steroidal ring seems to alter the biological profile of these molecules and thus may be considered as a key site for the interactions responsible for the antineoplasmatic potency.

Studies concerning the mechanism of action of such compounds have shown that except for the interaction of the alkylating congener with the nucleic acids, there are also other cellular sites implicated in the expression of the antineoplasmatic activity. Estramustine [41–44] is a typical example of this category of compounds that has been found to also act through the disruption of interphase and mitotic microtubule network in cells, thus achieving cell death. Moreover, recent studies have shown that hormone-linked genotoxicants have the ability to interact with tumor-specific proteins and block their action, whereas at the same time the drug–protein complex formed camouflages the DNA adducts from being repaired by excision repair enzymes [21–23]. Maybe the antileukemic potency of the new steroidal alkylating agents studied herein is a result of an analogous mechanism of action.

To elucitate the mechanistic pathways responsible for the biological profile of these molecules we examined their ability to induce SCEs and to reduce the PRI in normal human lymphocytes *in vitro*. SCEs have been frequently used as highly sensitive indicators of DNA damage and/or subsequent repair [45,46]. Nonrepaired 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 antitumor agents in cancer cells *in vitro* and *in vivo* [47] is positively correlated with 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 [48]. 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 [49].

In this study a good correlation ($P < 0.02$) between SCE enhancement and PRI suppression was observed. The results of the in-vitro experiments are illustrated in Table 4.

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

Compound	Concentration (μmol/l)	SCE/cell ± SE	PRI
Control	–	10.16 ± 0.63	2.52
4-Me-CABA	0.2	14.77 ± 0.84	2.41
	0.6	26.85 ± 2.04	1.47
1a	0.2	15.06 ± 0.95	2.49
	0.6	13.82 ± 0.85	2.08
1b	0.2	17.28 ± 1.29	2.73
	0.6	23.43 ± 2.58	1.71
1c	0.2	12.13 ± 0.59	2.51
	0.6	15.93 ± 1.07	2.38
2a	0.2	10.56 ± 0.86	2.35
	0.6	14.16 ± 1.52	2.20
2b	0.2	13.90 ± 0.9	2.54
	0.6	24.46 ± 1.68	1.49
2c	0.2	15.22 ± 1.13	2.45
	0.6	21.55 ± 1.49	2.01

SCEs have been correlated with corresponding PRI values ($r = -0.51$, $t = 2.63$ and $P < 0.02$).
4-Me-CABA, 4-methyl-3-*N,N*-bis(2-chloroethyl)amino benzoic acid; PRI, proliferation rate index; SCE, sister chromatid exchange.

At the concentration of 0.2 μmol/l, the bilactamic derivative 2a proved inactive, whereas all other derivatives induced the SCEs in relation to the control. Additionally, at the concentration of 0.6 μmol/l the B-lactamic compounds (1a, 2a) proved the worst inducers among all compounds whereas the 7-oxidized derivatives were the most potent (1b, 2b). These results do not agree with the in-vivo antileukemic activity where the B-lactamic agents proved more potent. As SCEs indicate the damage induced by chemicals and the subsequent repair of DNA, it is obvious that the introduction of the –NHCO– moiety at the B steroidal ring enhances the antileukemic potency of the final compounds through the interaction with other cellular sites, in contrast to the 7-keto group and the D-lactamic steroidal ring. 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, whereas compounds 1a and 2a did not show a good cytostatic activity for normal human lymphocytes. This indicates that their cytostatic activity against leukemic cells *in vivo* involves the blockage of specific components expressed in tumor cells that are essential for the tumor's survival and growth.

Conclusion

The lactamization of the B-ring rendered the steroidal esteric derivatives of 4-Me-CABA more potent antileukemic agents than the 7-oxidized or the derivatives with a simple B-ring in contrast to previous studies where it was found that the 7-keto group enhanced to a greater extent the antileukemic activity of PHE and CHL hormone conjugates. Nevertheless, the B-lactamic steroidal derivatives were neither effective inducers of SCEs nor good cytostatics against normal human lymphocytes as the 7-oxidized ones. These results suggest a different mechanism of antileukemic action induced by the presence of the –NHCO– moiety in the B steroidal ring, probably through the interaction of this functional group with components in the leukemic cells essential for tumor growth. It is once more confirmed that to obtain the desirable biological effect there are multiple factors that must be examined concerning the whole configuration of these multifunctional compounds as the same functional changes on the steroidal skeleton did not have the same impact on the antileukemic activity of PHE and CHL derivatives. The esterification of the B-lactamic steroidal skeletons with 4-Me-CABA probably resulted in a diminuation of the hydrolysis they suffer from the esterases and thus the concentration essential for producing an antineoplasmatic effect in the vinicity of their binding site was attained. Further studies which will elucitate the mechanism of action of these compounds are in process.

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