Genetic effects caused by potent antileukemic steroidal esters of chlorambucil's active metabolite

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Three steroidal esters with a common alkylating agent (chlorambucil's active metabolite, PHE) and PHE were studied with regard to their genetic activity in human lymphocyte cultures treated *in vitro.* The cytokinesis block micronucleus assay was used in combination with fluorescence *in situ* hybridization and the cytosine arabinoside method (ARA-C). The aim of this study was (i) to examine if the modified analogs (EA-72 and SOT-19) of the parent compound (ASE) exerted the same genetic activity with ASE and to correlate the genetic activity with the chemical structure, (ii) to investigate whether these steroidal esters are able to induce excision repairable lesions, through the alkylation of DNA, and (iii) to collect data in order to evaluate the exact role of the steroidal skeleton on the expression of the antileukemic activity. We found that PHE and its steroidal esters are cytotoxic for human lymphocyte cultures, as indicated by the reduction of Cytokinesis Blocked Proliferation Index, PHE being the most cytotoxic molecule. All studied compounds are capable of inducing both chromosome breakage and chromosome delay as indicated by the increased $C^{-}MN$ and $C^{+}MN$ frequencies. The steroidal derivatives gave reduced genetic activity. The conjugate ketone at the B ring of the steroidal skeleton resulted in decreased genetic activity mainly due to decreased chromosome delay. All studied compounds are capable of inducing DNA excision repair. *Anti-Cancer Drugs* 16:67–75 © 2005 Lippincott Williams & Wilkins.

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Introduction

Nitrogen mustards are the earliest and most extensively studied bifunctional alkylating agents, and their cytotoxic and therapeutic effects derive primarily from their ability to cross-link DNA [1]. However, only a few members of these anticancer compounds, such as melphalan and chlorambucil, are used in clinical cancer chemotherapy today [2,3]. Due to their high inert chemical activity, they can bind covalently to the nucleophilic sites of most of biomolecules. Subsequently, their relatively low affinity and only slight selectivity for longer DNA sequences, and the rapid hydrolysis they suffer before reaching the DNA target, diminish effective alkylation [4].

The chemical conjugation of nitrogen mustards to molecular ligands is one of the strategies which researchers apply in order to reduce the toxicity of these alkylating agents, and increases the selectivity and the effectiveness towards alkylation of DNA [5–7]. Estramustine and prednimustine, nitrogen mustard carbamates of steroids with clinical use [8], are typical examples of conjugation of an alkylating agent to steroids. Estrogens appear to show efficacy through a combination mechanism of systemic slow release and targeting to hormone-sensitive tumors [9]. From our team's previous work with a series of aromatic aniline mustards linked to simple or D-ring modified steroidal molecules, strong evidence was obtained for a significant enhancement of antileukemic activity and reduced toxicity of these compounds compared with the nitrogen mustards themselves [10–13].

In the present study we selected three specific steroidal esters, i.e. ASE [3β-hydroxy-13α-aza-D-homo-5α-androstan-17-one-*p*-*N*,*N*-bis(2-chloroethyl)aminophenyl acetate], EA-72 [3β-hydroxy-androst-5-en-7,17-dione-*p*-*N*,*N*-bis-(2-chloroethyl)aminophenyl acetate] and SOT-19 [3β-hydroxy-13α-aza-D-homo-androst-5-en-7,17-dione-*p*-*N*,*N*-bis-(2-chloroethyl)aminophenyl acetate], which contain the very same alkylating agent, i.e. PHE [*p*-*N*,*N*-bis(2-chloroethyl)aminophenyl acetic acid], chlorambucil's active metabolite [14]. The chemical structures are shown in Fig. 1.

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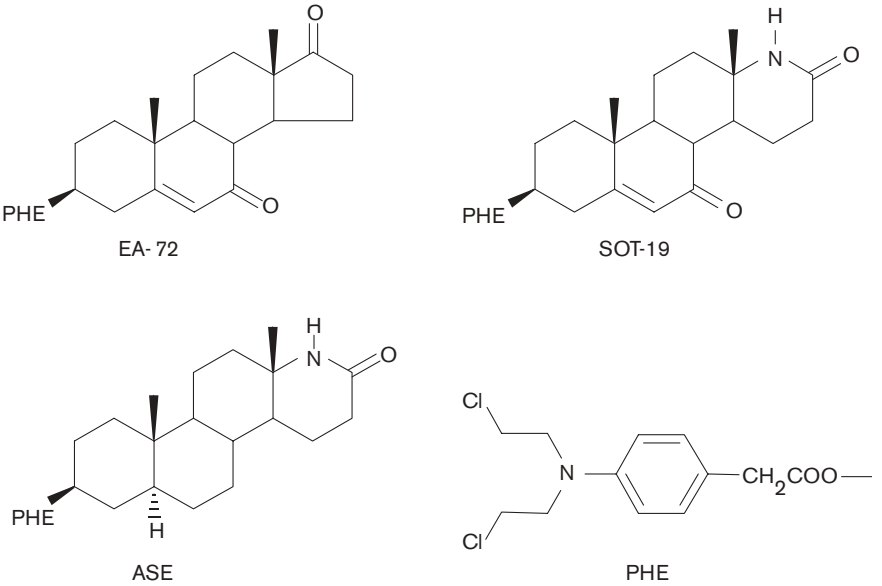
Fig. 1



Chemical structures of the compounds prepared and studied.

ASE is known to display good antineoplastic activity in experimental tumors [15–18]. Moreover, ASE has been shown to exert mutagenic potential [19] and clastogenic properties [17,20,21]. Recent findings by our group have shown that ASE is capable of inducing both chromosome breakage and chromosome delay in human lymphocyte cultures treated *in vitro* [22]. In addition, EA-72 and SOT-19 possess remarkable antileukemic properties in L1210 leukemia in mice and enhance sister chromatid exchange frequencies in human lymphocyte cultures *in vitro* ([23] and recent and yet unpublished results). In both EA-72 and SOT-19 molecules there is a conjugate ketone at the 7 position of the B-ring, which is absent on ASE, while in EA-72 the D-steroidal ring is the common five member and not a lactamic one as in ASE and SOT-19. The choice of these oxidized steroidal molecules as vehicles of the nitrogen mustard was also based on the observation that some of these oxidized Δ^5-steroids are more toxic toward cancerous cells than non-cancerous cells [24]. Additionally, although the biological profile of these compounds has not been clarified as yet, various information about their activity [25] supports this suggestion.

Micronucleus analysis has been widely used as a biomarker to evaluate the genotoxic effects of various environmental agents on humans. Since first described [26], the cytokinesis block micronucleus assay (CBMN) in lymphocytes cultured from peripheral blood has been one of the main methods used for the identification of genetic damage in genetic toxicology studies in humans [27,28]. The main advantage of this method is the identification of cells that have divided once after treatment and this is achieved by inhibiting cytokinesis using cytochalasin B. Micronuclei (MN) are scored in cells that have completed their first nuclear division in culture and cytoplasm division has been blocked by cytochalasin B, so they are recognized as binucleated cells. Chromosome breakage and chromosome delay during anaphase of meiosis or mitosis are the two main mechanisms leading to MN formation, and thus MN contain either acentric chromosome fragments or/and whole chromosome or chromatids. In combination with the application of the anti-kinetochore antibody and immunofluorescence technique (CREST) or fluorescence *in situ* hybridization (FISH) with centromeric probes, the content of MN can be identified [22,29].

A limitation of the method is that, due to the origin of MN in lymphocytes, it is relatively insensitive when detecting exposure in G_0 or G_1 phase by agents that mainly induce excision repairable DNA lesions, such as UV light, pyrimidine dimers or abasic sites. However, this limitation forced the development of the so-called ARA/CBMN assay [30,31]. This approach has made it possible to convert excision repairable DNA lesions induced in human lymphocytes to MN by using cytosine arabinoside (ARA-C). ARA-C exerts its effect by inhibiting the DNA polymerization step (gap filling) that normally occurs after excision of damaged bases during nucleotide excision repair (long-patch repair) or base excision repair (short-patch repair) [32]. As a consequence, excision

repair sites are converted to single-strand breaks (SSBs), which in turn are converted to double-strand breaks (DSBs) after replicative DNA synthesis. These chromatid breaks are lost at anaphase and appear in the cytoplasm of divided cells (binucleated cells) as MN [31]. Thus, we can conclude that the increase in the MN frequency observed with this method would be a direct consequence of the repair response of cells to genotoxins.

The aim of this study was (i) to examine if the modified analogs (EA-72 and SOT-19) of the parent compound (ASE) exert the same genetic activity as ASE as well as to correlate the genetic activity with the chemical structure, (ii) to investigate whether these steroidal esters are able to induce excision repairable lesions through the alkylation of DNA and (iii) to collect data in order to evaluate the exact role of the steroidal skeleton on the expression of the antileukemic behavior. The alkylating agent (PHE) was also studied. For this purpose, the CBMN assay was used in human lymphocyte cultures in combination with FISH and ARA-C methods.

Materials and methods

Donors

Four healthy, non-smoking individuals, two males and two females, aged 24–28 years old, who in the last 6 months had received no treatment, had not been exposed to X-rays and had no evidence or history of infection were used as blood donors to establish whole-blood lymphocyte cultures.

Chemical compounds

3β-Hydroxy-androst-5-en-17-one was purchased from Steraloids (Newport, RI). The parent steroidal alcohols and t-PHE were prepared by methods described in the literature [33–35]. The t-BuOOH/CuI-TBAB biphasic oxidizing method was applied for the allylic oxidation of the Δ^5-steroids [23], while the final steroidal esteric derivatives of PHE were synthesized accordingly to the asymmetric anhydrides procedure [10]. All the compounds under study were purified by HPLC.

Combined CBMN/FISH experiments

Cultures, cell harvest and slide preparation

Whole blood (0.5 ml) was added into culture medium consisting of 6.5 ml Ham F-10 (Gibco, Grand Island, NY), 1.5 ml fetal calf serum (Gibco) enriched with antibiotics (Penstrep; Gibco) and Glutamine (Sigma, St Louis, MO). Lymphocytes were stimulated to divide with 0.3 ml phytohemagglutinin (PHA; Gibco) at the onset of the cultures. Separate cultures were established from each of the four donors corresponding to the different experimental points, control and treated. Chemical compounds (ASE, EA-72, SOT-19 and PHE) were added into the cultures 41 h after culture initiation to give final concentrations, 1×10^{-5} M, at which ASE provoked both chromosomal breaks and chromosome delay [22]. Cytochalasin-B (Sigma), 6 μg/ml, was added into the culture medium 44 h after culture initiation. The cultures were incubated at 37°C for 72 h in a 5% CO_2 atmosphere with 95% humidity. Cells were harvested by centrifugation at 400 *g* for 10 min. A mild hypotonic treatment with a solution of Ham F10:ddH_2O = 1:1 was given for 3 min at room temperature and was followed by a 10-min fixation with a fresh solution of methanol:acetic acid = 3:1 (Merck, Darmstadt, Germany). Cell drops were layered onto clean slides from a very low distance.

FISH procedure

FISH was performed using an α-satellite probe for all human centromeres (Oncor, Gaithersburg, MD; P 5095:DG 5). Slides were pretreated in pepsin (Sigma) solution in 0.01 M. HCl, pH 3, for 5 min, then washed in distilled sterile water and PBS, 2 min each time, and then submerged in 1% formaldehyde (Merck) in PBS at 4°C for 5 min and washed with PBS and distilled sterile water, 2 min each time, followed by dehydration with increasing series of ethanol (Merck). Nuclear DNA denaturation was achieved with 70% formamide (Merck) in 2 × SSC at 70°C for 2 min chilled in 70% ethanol and dehydrated in increasing series of ethanol. Probe denaturation was performed at 70°C for 5 min and chilled on ice. Slides were then incubated with the probe to hybridize (15 μl for each slide) overnight at 37°C in a humidified atmosphere. At the end of the hybridization time, slides were washed twice for 10 min with 50% formamide in 2 × SSC, followed by two washes in 2 × SSC, 4 min each and one, 5-min, wash in 4 × SSC/0.05% Tween 20 (Sigma) buffer. Slides were then incubated with 5% skimmed milk as blocking reagent at 37°C for 15 min. After a short wash in 4 × SSC/Tween 20 buffer, monoclonal anti-digoxigenin antibody (Sigma; D-8156) dialyzed in 0.5% skimmed milk (1:200) was laid onto the slides and allowed to bind to the probe by incubation at 37°C for 50 min. Three washes with 4 × SSC/Tween 20 buffer followed and slides were then incubated with sheep anti-mouse Ig digoxigenin conjugated secondary antibody (Chemicon, Temecula, CA; AQ300D) dialyzed in 0.5% skimmed milk (1:50) at 37°C for 50 min followed by a subsequent incubation with anti-digoxigenin–fluorescein, fab fragments (Roche, Mannheim, Germany; 1207741), also dialyzed in 0.5% skimmed milk (1:10) at 37°C for 50 min. Every incubation was followed by extensive washes in 4 × SSC/Tween 20 buffer. Counterstain was performed using a mixture of DAPI-PI (Sigma) in 4 × SSC/Tween 20 buffer at room temperature for 5 min, washed in tap water, air-dried and mounted in Vectashield Mounting Medium (Vector, Burlingame, CA). Slides were kept in the dark at 4°C and analyzed in a Zeiss Axioskop fluorescence microscope. The pass band filters used were of 546, 490 and 360 nm for green, blue and UV light, respectively.

Combined CBMN/ARA-C experiments

Two separate sets of cultures were established from two of the four donors, which were included in the combined CBMN/FISH experiments: one set of cultures received ARA-C (Sigma) at a final concentration of 1 μg/ml and the other one received only the solvent of ARA-C (sterile saline). The appropriate chemical compounds solutions (ASE, EA-72, SOT-19 and PHE), diluted in DMSO to give final concentrations of 1×10^{-6} M, were added at the moment of cultures initiation (G_0 phase), for a total exposure period 16 h. Methylnitrosourea (MNU) (Sigma) at a final concentration 30 μg/ml was used as positive control. One hour later, lymphocytes were stimulated to divide by adding 0.3 ml PHA. Ten minutes later, the inhibitor, ARA-C, was added to the inhibited cultures. Treatments were interrupted 16 h later by centrifuging the cells at 1200 r.p.m. After two rinses in phosphate-buffered saline (PBS), 6.5 ml Ham F-10 (Gibco) and 1.5 ml fetal calf serum (Gibco) without PHA and supplemented with 10 μg/ml of deoxycytidine were added to the remaining cell pellet. Deoxycytidine (Sigma) was added to compete with residual ARA-C bound to the DNA polymerase and to allow DNA replication to run normally. Cells were harvested 72 h after culture initiation according to the above protocol. Cells were stained with 7% Giemsa (Ferak, Berlin, Germany) for 10 min.

Microscopy

For the estimation of MN frequency, at least 1000 binucleated cells were scored for each donor and for each treatment. Standard criteria [36] were used for the scoring of MN for both analyses. To determine possible cytotoxic effects, 2000 cells were counted for the calculation of the Cytokinesis Blocked Proliferation Index (CBPI), which is given by $CBPI = M_1 + 2M_2 + 3(M_3 + M_4)/N$, where M_1, M_2, M_3 and M_4 correspond to the numbers of cells with one, two, three and four nuclei, and N is the total number of cells. In combined CBMN/FISH experiments, at least 30 MN were analyzed for the presence of centromere-positive signals for each experimental point and for each donor. To identify a micronucleus as containing a centromere-positive signal, this should be of the same intensity as those of the main nuclei.

Statistical analysis

Statistical analysis of MN data comparing, in individual donors, each treatment with the control was achieved by the G-test [37] for independence on 2×2 tables. This test is based on the general assumption of the χ^2 analysis, but offers theoretical and computational advantages. The ANOVA test was performed for estimating the cytotoxicity and cytogenetic activity between chemical compounds.

Results

Combined CBMN/FISH experiments

Whole-blood lymphocyte cultures were set up from four different donors. In a previous paper we have shown that ASE in human lymphocyte cultures is capable of increasing MN frequencies in a linear dose-dependent manner. In addition, ASE at the concentration of 10^{-5} M induced both chromosome breakage and chromosome delay, while at lower concentrations it induced only chromosome breakage [22]. To compare the genetic activity between the studied compounds, these were tested at the concentration at which ASE induced both genetic phenomena. The outcome of the treatment of human lymphocytes with the four compounds in relation to micronucleated binucleated (BNMN) cell frequency, MN frequency and CBPI is presented in Table 1.

All four compounds induce cell cycle delay by decreasing CBPI in every donor studied. One-way ANOVA analysis has shown that PHE causes the highest decrease in CBPI, while the reduction is less extensive in the case of EA-72 and SOT-19, and even less in the case of ASE. This means that PHE causes the greatest delay in cell cycle, while ASE has a smaller cytotoxic effect among the compounds studied. As expected [22], ASE statistically significantly increased the BNMN and MN frequencies compared to control in all four donors ($p = 0.0001$, G-test). In addition, the two ASE analogs EA-72 and SOT-19 provoked BNMN and MN frequencies in the same donors ($p = 0.0001$, G-test) and this was also true for the common moiety PHE ($p = 0.0001$, G-test). Comparing mean MN frequencies among different treatments (one-way ANOVA), it seems that PHE is the most potent MN inducer followed by ASE, which is able to increase the MN frequency to a statistically significant higher level than EA-72 and SOT-19.

The mechanism by which MN are formed, i.e. chromosome breakage (clastogenic effect) or chromosome delay (aneuploidogenic effect), was also studied. The content of MN was identified by FISH using an α-satellite pancentromeric probe. This probe consists of a selection of alphoid sequences and hybridizes to the centromeres of all human chromosomes, and thus any chromosome included in a micronucleus can be identified by probe imunodetection with a series of antibodies, the last of which is fluorescently labeled. The results are presented in Table 2.

C^-MN and C^+MN frequencies were calculated according to the type: $‰C^-MN = ‰MN \times \%C^-MN/100$ or $C^+MN = ‰MN \times \%C^+MN/100$, where ‰MN is the respective MN frequency from Table 1, $\%C^-MN$ is the percentage of MN with no hybridization signal and $\%C^+MN$ is the percentage of MN presenting hybridization signal from Table 2. The FISH analysis has shown

Table 1 Spontaneous and induced frequencies of BNMN and MN in lymphocyte cultures of four donors treated *in vitro* with 10^{-5} M of the steroidal esters ASE, EA-72, SOT-19 and PHE

Compound	Donor	BN cells analyzed	MN scored	MN (‰)	BNMN scored	BNMN (‰)	CBPI
Untreated	1	5962	37	6.2	35	5.9	2.02
	2	6025	44	7.3	40	6.6	2.00
	3	5484	39	7.1	37	6.8	2.02
	4	4120	38	9.2	37	9.0	1.88
total/mean ± SE		21591	158	7.5 ± 0.6	149	7.1 ± 0.7	1.98 ± 0.03
ASE	1	1657	76	45.9[a]	71	42.9[a]	1.82
	2	2210	81	36.7[a]	78	35.3[a]	1.81
	3	2342	112	47.8[a]	109	46.5[a]	1.84
	4	2061	60	29.1[a]	60	29.1[a]	1.73
total/mean ± SE		8270	329	39.9[b] ± 4.3	318	38.5[b] ± 3.9	1.80[b] ± 0.0
EA-72	1	1658	51	30.8[a]	46	27.7[a]	1.76
	2	2180	54	24.8[a]	50	22.9[a]	1.76
	3	4093	109	26.6[a]	104	25.4[a]	1.78
	4	2171	58	26.7[a]	57	26.3[a]	1.61
total/mean ± SE		10102	272	27.2[b,c] ± 1.3	257	25.6[b,c] ± 1.0	1.73[b] ± 0.04
SOT-19	1	1711	51	29.8[a]	50	29.2[a]	1.71
	2	5608	131	23.4[a]	126	22.5[a]	1.74
	3	3850	119	30.9[a]	104	27.0[a]	1.76
	4	3397	95	28.0[a]	91	26.8[a]	1.67
total/mean ± SE		14566	396	28.2[b,c] ± 1.7	371	26.4[b,c] ± 1.4	1.72[b,c] ± 0.02
PHE	1	1050	59	56.2[a]	54	51.4[a]	1.63
	2	1492	75	50.3[a]	69	46.3[a]	1.65
	3	1782	101	56.7[a]	93	52.2[a]	1.64
	4	2316	126	54.4[a]	124	53.5[a]	1.69
total/mean ± SE		6640	361	54.4[b–e] ± 1.5	340	50.9[b–e] ± 1.6	1.65[b,c,e] ± 0.01

[a] $p = 0.0001$ (*G*-test).
[b] $p < 0.05$ in comparison with untreated cultures (one-way ANOVA).
[c] $p < 0.05$ in comparison with ASE-treated cultures (one-way ANOVA).
[d] $p < 0.05$ in comparison with EA-72-treated cultures (one-way ANOVA).
[e] $p < 0.05$ in comparison with SOT-19-treated cultures (one-way ANOVA).

Table 2 Spontaneous and induced frequencies of MN with (C^+MN) and without (C^-MN) a hybridization signal in lymphocyte cultures of four donors treated *in vitro* with 10^{-5} M of the steroidal esters ASE, EA-72, SOT-19 and PHE, as evaluated after FISH analysis using α-satellite probe for all human centromeres

Compound	Donor	MN analyzed	C^-MN	C^+MN	C^-MN ‰	C^+MN ‰
Untreated	1	34	20	14	3.7	2.6
	2	36	23	13	4.7	2.6
	3	32	22	10	4.9	2.2
	4	34	22	12	6.0	3.3
total/mean ± SE		136	87	49	4.8 ± 0.5	2.7 ± 0.2
ASE	1	61	44	17	33.1[a]	12.8[a]
	2	55	38	17	25.3[a]	11.3[a]
	3	103	74	29	34.4[a]	13.5[a]
	4	53	35	18	19.2[a]	9.9[a]
total/mean ± SE		272	191	81	28.0[b] ± 3.6	11.9[b] ± 0.8
EA-72	1	47	35	12	22.9[a]	7.9[a]
	2	51	34	17	16.5[a]	8.3[a]
	3	103	72	31	18.6[a]	8.0[a]
	4	56	44	12	21.0[a]	5.7
total/mean ± SE		257	185	72	19.8[b] ± 1.4	7.5[b,c] ± 0.6
SOT-19	1	51	40	11	23.4[a]	6.4[a]
	2	102	75	27	17.2[a]	6.2[a]
	3	119	84	35	21.8[a]	9.1[a]
	4	92	72	20	21.9[a]	6.1
total/mean ± SE		364	271	93	21.1[b] ± 1.3	7.0[b,c] ± 0.7
PHE	1	50	39	11	43.8[a]	12.4[a]
	2	35	25	10	35.9[a]	14.4[a]
	3	101	74	27	41.5[a]	15.2[a]
	4	126	98	28	42.3[a]	12.1[a]
total/mean ± SE		312	236	76	40.9[b–e] ± 1.7	13.5[b,d,e] ± 0.8

[a] $0.0001 \leq p < 0.01$ (*G*-test).
[b] $p < 0.05$ in comparison with untreated cultures (one-way ANOVA).
[c] $p < 0.05$ in comparison with ASE-treated cultures (one-way ANOVA).
[d] $p < 0.05$ in comparison with EA-72-treated cultures (one-way ANOVA).
[e] $p < 0.05$ in comparison with SOT-19-treated cultures (one-way ANOVA).

that the steroidal esters ASE, EA-72 and SOT-19 as well as PHE increased the C^-MN frequencies ($p = 0.0001$, *G*-test) in every donor examined, indicating a clastogenic activity. This activity seems to be higher for PHE, and lower for the three esters ASE, EA-72 and SOT-19 (one-way ANOVA analysis). An increase in C^+MN frequencies

Table 3 Frequencies of BNMN and MN induced in human whole blood lymphocyte cultures treated *in vitro* with MNU, ASE, EA-72, SOT-19 and PHE (10^{-6} M) in the presence or absence of 1 μg/ml of the inhibitor of the gap-filling step of excision repair, ARA-C

Compound	Donor	In the absence of ARA-C			In the presence of ARA-C		
		BN	MN (‰)	BNMN (‰)	BN	MN (‰)	BNMN (‰)
Untreated	1	1206	8 (6.6)	8 (6.6)	1137	25 (22.0)[a]	23 (20.2)[a]
	4	1393	13 (9.3)	12 (8.6)	1290	36 (27.9)[a]	34 (26.4)[a]
total (mean ± SE)		2599	21 (8.0 ± 1.4)	20 (7.6 ± 1.0)	2427	61 (25.0 ± 3.0)[b]	57 (23.3 ± 3.1)[b]
MNU	1	1155	10 (8.7)	10 (8.7)	1219	38 (31.2)[a]	31 (25.4)[a]
	4	1092	16 (14.7)	16 (14.7)	1157	43 (37.2)[a]	42 (36.3)[a]
total (mean ± SE)		2247	26 (11.7 ± 3.0)	26 (11.7 ± 3.0)	2376	81 (34.2 ± 3.0)[b]	73 (30.9 ± 5.5)[b]
ASE	1	1000	31 (31.0)	31 (31.0)	1020	100 (98.0)[a]	89 (87.3)[a]
	4	1054	34 (32.3)	33 (31.3)	1048	86 (82.1)[a]	77 (73.1)[a]
total (mean ± SE)		2054	65 (31.7 ± 0.7)	64 (31.2 ± 0.2)	2068	186 (90.1 ± 8.0)[b]	166 (80.2 ± 7.1)[b]
EA-72	1	1000	18 (18.0)	17 (17.0)	1000	140 (140.0)[a]	125 (125.0)[a]
	4	1132	39 (28.3)	38 (33.6)	1264	95 (75.2)[a]	83 (65.7)[a]
total (mean ± SE)		2132	57 (23.2 ± 5.2)	55 (25.3 ± 8.3)	2264	235 (107.6 ± 32.4)[b]	208 (95.4 ± 29.7)[b]
SOT-19	1	1027	19 (18.5)	18 (17.5)	1001	96 (95.9)[a]	85 (84.9)[a]
	4	1950	72 (36.9)	70 (35.9)	1484	97 (65.4)[a]	95 (64.0)[a]
total (mean ± SE)		2977	91 (27.7 ± 9.2)	88 (26.7 ± 9.2)	2485	193 (80.7 ± 15.3)[b]	180 (74.5 ± 10.5)[b]
PHE	1	1040	36 (34.6)	34 (32.7)	1031	108 (104.8)[a]	89 (86.3)[a]
	4	1522	46 (30.2)	46 (30.2)	1553	102 (65.7)[a]	97 (62.5)[a]
total (mean ± SE)		2562	81 (32.4 ± 2.2)	79 (31.5 ± 1.3)	2584	210 (85.3 ± 19.6)[b]	186 (74.4 ± 11.9)[b]

[a] $p < 0.005$ in comparison with respective cultures in the absence of ARA-C (*G*-test).
[b] $p = 0.0001$ in comparison with respective cultures in the absence of ARA-C (*G*-test). BN: binucleated cells.

was also induced for ASE and PHE in all donors studied ($p < 0.01$, *G*-test), also indicating aneuploidogenic activity. With regard to EA-72 and SOT-19, elevated C^+MN frequencies were observed in three of the four donors. Comparing mean C^+MN frequencies, it seems that PHE and ASE are stronger inducers of MN exhibiting a hybridization signal, while EA-72 and SOT-19 show a statistical significant weaker effect (one-way ANOVA analysis) in relation to ASE and PHE.

Combined CBMN/ARA-C experiments

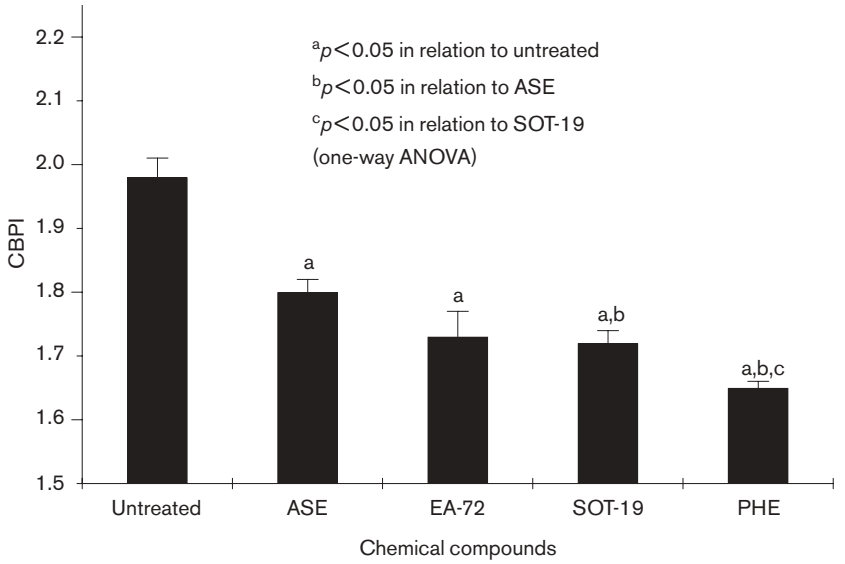
In order to investigate whether the steroidal esters ASE, EA-72 and SOT-19 are able to induce excision repairable lesions, two series of experiments were carried out in the presence and absence of inhibitor of the DNA polymerization step, ARA-C. PHE was also included in these experiments. Cultures treated with MNU (30 μg/ml) were used as positive control. Lymphocyte cultures were established from the two of the four donors enrolled in the former experiments. The final concentration of the four compounds studied was 1×10^{-6} and not 1×10^{-5} M as in the combined CBMN/FISH experiments. This was done because, according to this methodology, the lymphocytes are treated during G_0 and G_1 phase, where cells are more sensitive to the compound's activity [22]. The results are presented in Table 3.

ARA-C caused a statistically significant increase in MN frequencies in both donors and in all cultures, treated with the compounds or untreated, compared to the respective cultures to which no ARA-C was added. In the untreated cultures, the mean MN frequency was increased after ARA-C addition from 8.0 to 25.0‰, while for the cultures treated with ASE, EA-72, SOT-19 and PHE, an increase from 31.7 to 90.1, 23.2 to 107.6, 27.7 to 80.7 and 32.4 to 85.3‰ was found, respectively. The same observation holds true for MNU-treated cultures in which ARA-C addition increased the MN frequency from 11.7 to 34.2‰. Thus, it seems that the chemical compounds under study, i.e. ASE, EA-72, SOT-19 and PHE, are able to induce excision repairable DNA lesions.

Discussion

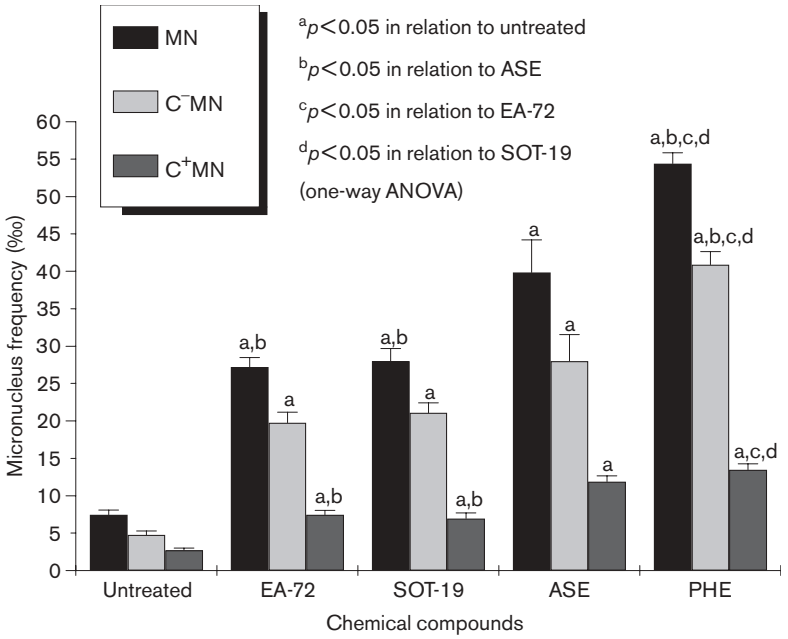
Although nitrogen mustards are used in the chemotherapy of neoplastic diseases, their importance for cancer chemotherapy is limited due to their chemical activity on non-cancerous cells. Modified steroids used as biological vehicles transport the alkylating molecule to the tumor site in a rather specific manner [38] and homo-aza-steroidal esters of nitrogen mustard produce satisfactory activity [35]. In this paper, three selected steroidal esters of nitrogen mustard, i.e. ASE, EA-72 and SOT-19, and their common moiety PHE (nitrogen mustard) were studied with regard to their genetic activity on human lymphocyte cultures *in vitro*. Human lymphocytes cultured *in vitro* are widely used as an appropriate system to identify the genetic activity of various chemical compounds. Lymphocytes obtained from normal volunteers are used to test environmental agents for their clastogenic and/or aneugenic activity. The CBMN assay in human lymphocytes is extensively used in genetic toxicology studies. Our results show that the compounds studied decreased CBPI and enhanced MN frequencies. With respect to CBPI, PHE is the more cytotoxic compound since it caused a higher decrease in proliferation index, while the esters induce a lower cytotoxic effect (Fig. 2). Thus, it seems that chemical conjugation of the steroid to the nitrogen mustard results in molecules which exert

Fig. 2



Comparison of the effect of the studied compounds on CBPI in whole-blood lymphocyte cultures treated *in vitro*.

Fig. 3



Comparison of MN, C^-MN (MN not exhibiting hybridization signal) and C^+MN (MN exhibiting hybridization signal) frequencies induced in whole-blood lymphocyte cultures treated *in vitro* by the compounds studied.

less cytotoxicity and this is in accordance with previous studies [17]. With regard to MN frequencies, PHE seems to be the most potent molecule followed by ASE, while EA-72 and SOT-19 enhance MN frequencies at the same level, which is lower than that of ASE. FISH experiments were carried out in order to identify the mechanism by which MN are formed. In a previous paper it was shown [22] that ASE provoked both chromosome breakage as well as chromosome delay. Our results confirmed these previous experiments with ASE and showed that the two

analogs, EA-72 and SOT-19, also induced chromosome breakage and chromosome delay since they increased MN containing both chromosome fragments (C^-MN), which do not show any hybridization signal, and whole chromosomes (C^+MN), which show hybridization signal. The same observation holds true for their congener PHE. Comparing the cytogenetic effects of the molecules under study (Fig. 3), it seems that the steroidal esters exert clastogenic activity at the same level, PHE being the most potent molecule, whereas they differ significantly in relation to their aneuploidogenic effect. PHE and ASE are stronger inducers of chromosome delay, while EA-72 and SOT-19 exhibit a lower effect. We assume that the lower effectiveness of EA-72 and SOT-19 in inducing overall MN frequency is due to their weaker aneuploidogenic effect. Since progenitor or stem cells are the likely targets in the development of leukemia and other bone marrow disorders, it would also be of interest to study the genetic effects of these antileukemic steroidal analogs in bone marrow progenitor cells.

The observation that agents that primarily act as clastogens can additionally induce aneuploidogenic effects has also been mentioned by other researchers. For example, butadiene and its major metabolites, epoxybutene and diepoxybutane, are known to express both clastogenic and aneuploidogenic activity [39–42]. With regard to the mechanism by which clastogens induce aneuploidy, it is believed that the interaction of some mutagens with the centromeric region may cause aneuploidogenic effects [43]. It has been found that when primary human cells are irradiated *in vitro* by X-rays at G_1 and G_2 phase, chromosome loss and non-disjunction were determined [44]. Since higher frequencies of chromosome loss were observed in the G_1 phase it is assumed that aneuploidy during the G_1 phase might be preferentially attained by interactions with DNA targets. In addition, aneuploidogenic activity was observed when the effect of X-rays and low-energy protons was studied in human primary fibroblasts assuming that the protons induced chromosome loss by kinetochore detachment or by breakage in the centromeric region, whereas X-rays induced aneuploidy through a non-DNA damage mechanism [45].

The combined CBMN/ARA-C experiments have shown that all the studied chemical compounds ASE, EA-72, SOT-19 and PHE as well as the positive control MNU induced higher MN frequencies after ARA-C addition, indicating that these compounds are able to convert excision-repairable lesions into MN in a single cell cycle. The degree of synergism (DS) between ARA-C and the studied compounds was calculated according to the equation $DS = (MN_{t+i} - MN_c)/[(MN_t - MN_c) + (MN_i - MN_c)]$, where MNc is the MN frequency of the untreated cultures, MN_{t+i} is the MN frequency treated with both the chemical compound and the ARA-C, MN_t is the MN frequency of the cultures treated only with the chemicals and MN_i is the MN frequency of the cultures treated only with the inhibitor ARA-C [32]. The results are presented in Table 4, which shows that DS is between 2.0 and 3.4, indicating a synergism between the inhibitor and all studied compounds, and confirming their ability to induce excision repairable DNA lesions. Taking into consideration that one of the mechanisms that tumor cells use to overcome the effects of alkylating agents is enhanced excision repair mechanisms, further experiments investigating the effects of these novel agents in a tumor cell model would support the results of this study.

Table 4 DS between the inhibitor of the gap-filling step of excision repair, ARA-C, and MNU, ASE, EA-72, SOT-19 and PHE

Donor	DS				
	MNU	ASE	EA-72	SOT-19	PHE
1	1.4	2.3	5.0	3.3	2.3
4	1.2	1.7	1.8	1.3	1.5
Mean ± SE	1.3 ± 0.1	2.0 ± 0.3	3.4 ± 1.6	2.3 ± 1.0	1.9 ± 0.4

In conclusion, the results of the present study indicate that:

- The steroidal esters studied are cytotoxic for human lymphocyte cultures, as indicated by the reduction of CBPI, but chlorambucil's active metabolite PHE proved the most cytotoxic molecule.
- All compounds studied are capable of inducing both chromosome breakage (clastogenic effect) and chromosome delay (aneuploidogenic effect) as indicated by the increased C^-MN and C^+MN frequencies.
- The conjugation of the alkylating agent PHE with the steroidal molecule resulted in molecules (ASE, EA -72 and SOT-19) with clearly reduced genetic activity.
- The insertion of a conjugate ketone at the B-ring of the steroidal skeleton (EA-72 and SOT-19) resulted in decreased genetic activity mainly due to decreased chromosome delay, while these compounds possess potent antileukemic activity.
- All studied compounds are capable of inducing DNA excision repair.

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