Structure–Anticancer and Structure–Genetic Activity Relationships of Homo-*aza*-steroidal Esters of *N,N*-Bis(2-chloroethyl)aminocinnamic Acid Isomers

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Abstract □ Four steroidal lactams of the A- and D-rings were used for the esterification in the C-3 or C-17 positions, respectively, of their nuclei with the *N,N*-bis(2-chloroethyl)aminocinnamic acid isomers. The condensation reaction of the hydroxylic group of the steroidal lactams with each mustard was effected in dichloromethane in the presence of the catalyst *p*-dimethylaminopyridine and dicyclohexylcarbodiimide as dehydrating agent. The esters were obtained in pure form after column chromatography, and their structures were verified and confirmed by analytical methods (IR and UV spectra). The 12 esters were tested in vivo against P388, L1210 leukemias, Ehrlich ascites tumor, and melanoma B16. The esters 3α-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-*o,m,p*-*N,N*-bis(2-chloroethyl)aminocinnamates, in which the alkylating agents are linked to the modified steroid in the axial position, are inactive in the above experimental animal tumor systems. The effect of the homo-*aza*-steroidal esters of *N,N*-bis(2-chloroethyl)aminocinnamic acid isomers on the incorporation of radioactive precursors into DNA, RNA, and proteins of L1210, P388 leukemias, Ehrlich ascites tumor, and baby hamster kidney cells was investigated. Higher inhibitory effects on the incorporation of the radioactive precursors was obtained with the *ortho*-derivatives, yielding >40% inhibition of thymidine incorporation in all tumor lines tested. The effect of four esters in which the *m*-*N,N*-bis(2-chloroethyl)aminocinnamic acid is linked to the modified steroids on sister chromatid exchanges in human lymphocyte culture was investigated.

Steroidal derivatives have been used as carriers of cytotoxic agents because they reduce systemic toxicity and improve specificity of cancer therapy. Extensive reviews of the synthesis and anticancer activity are available.[1,2] The discovery of new compounds with anticancer activity, originating from compounds with known structure–activity and toxicity relationships, has been accomplished with homo-*aza*-steroids for biological platforms, which could reduce the toxicity of the oncolytic congeners and transport them to the target site.

The idea to design *aza*-steroids in the A- and D-rings of the tetrahydrocyclopentanoperhydrophenanthren nucleus as biological platforms stems from two observations: (a) the biological action of lactams characterized by a –NHCO– group may be structurally specific and therefore more prolonged as the result of the multiple interactions of such a group with similar groups that exist in proteins and nucleic acids; and (b) the –NHCO– group of the lactam molecule may be essential for antitumor activity and could open enzymaticaly, forming a species which could attack the contents of cancer cells.[3]

The satisfactory results of a promising compound [3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-*p*-*N,N*-bis(2-chloroethyl)aminophenylacetate, *aza*-steroidal-ester (ASE), NSC 290205], which has good activity against L1210 and P388 leukemias in B6D2F mice,[4] T8 Guerin tumor in Wistar rats, Theagenion–Bahner angiosarcoma in C3HB mice,[5] B16 melanocarcinoma in the C57BL/6 mouse, colon 26 tumor in the CDF mouse, CDF_1 mammary tumor in the DB12 mouse,[6] and CX-1 colon xenograft, LX-1 xenograft MXT-hormone-dependent transplantable mammary adenocarcinoma, and colon 38 tumor[7] prompted us to continue the synthesis of new homo-*aza*-steroidal esters, and to study their antineoplastic and genetic activity.

Electron effects in compounds containing an aromatic ring are especially pronounced because the inductive effects are readily transmitted through such conjugated systems. An interesting example is found in a series of oncolytic nitrogen mustards, in which there is a decreased percentage of hydrolysis as a result of the inductive effect of halogen, with a consequent reduction in cancer activity of the resulting compounds.[8]

The homo-*aza*-steroidal esters of cinnamic acid mustard isomers have been elected because of the double bond that is introduced into the acyclic side chain of the *N,N*-bis(2-chloroethyl)aminocinnamates and the enhanced pressor effect of these compounds in *ortho* and *para* position caused by the formation of charges by resonance through delocalization of the π-electron cloud. Under these circumstances hydrolysis of the chlorine atoms in the molecules should be more difficult in comparison with the *meta* derivative, and consequently the *ortho* and *para* derivatives should be less active.

Experimental Section

Chemistry—As biological platforms of the cinnamic acid mustard isomers, we used A- and D-ring steroidal lactams; namely, 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam,[9] 3α-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam,[10] 3β-hydroxy-13α-amino-13,17-seco-5-androsten-17-oic-13,17-lactam,[11] and 17β-hydroxy-3-*aza*-A-homo-4α-androsten-4-one.[12] The oncolytic agents *o*-, *m*-, and *p*-*N,N*-bis(2-chloroethyl)aminocinnamic acids were synthesized according to a previously reported method.[13] However, the usual procedures for the esterification of C-3 and C-17 hydroxylic group of the homo-*aza*-steroids were effected in anhydrous dichloromethane in the presence of *p*-dimethylaminopyridine as catalyst and of dicyclohexylcarbodiimide (DCC) as dehydrating agent.[14] Both UV and IR spectra supported the structure of the compounds presented below. Compounds 1–3 have been reported in a previous communication.[15]

Procedures—Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. The IR spectra were recorded on a Perkin-Elmer 298 in solid-phase KBr, with polystyrene as a reference peak. The UV spectra are measured in chloroform on a Perkin-Elmer 515 S instrument. Elemental analyses (C, H, N) were performed by the Laboratory of Organic Chemistry, University of Salonica, Greece.

General Procedure for the Synthesis of the Esters of Steroidal Lactams with *ortho*-, *meta*-, and *para*-[*N,N*-Bis(2-chloroethyl)amino]cinnamic Acid—A stirred mixture of 10 mmol of the steroidal lactam in 200 mL of dry CH_2Cl_2, 13 mmol of DCC in 20 mL of dry CH_2Cl_2, 11.05 mmol of *N,N*-bis(2-chloroethyl)aminocinnamic acid in

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Table I—Physical Properties of Homo-*aza*-steroidal Esters of *N,N*-Bis(2-chloroethyl)aminocinnamic Acid Isomers

Compound[a]	Yield, %	mp, °C
1	70	230–231[b]
2	80	221–222[c]
3	62	215–217[b]
4	20	183–184[b]
5	37	212–213[b]
6	22	147–149[d]
7	31	227–229[d]
8	72	216–217[c]
9	52	209–210[d]
10	36	135–136[d]
11	50	198–199[c]
12	46	191–193[d]

[a] Formulas for **1–6** and **7–12** are $C_{32}H_{44}N_2O_3Cl_2$ and $C_{32}H_{42}N_2O_3Cl_2$, respectively; C, H, N analyses in agreement with calculated values (±0.3%). [b] Solvent, $CH_3COOC_2H_5$. [c] Solvent, $CH_3COOC_2H_5$–$CHCl_3$. [d] Solvent, $CH_3COOC_2H_5$-ηC_6H_{14}.

40 mL of dry CH_2Cl_2, and 13 mmol of 4-dimethylaminopyridine in 20 mL of dry CH_2Cl_2 was allowed to stay at room temperature for 5–6 days. Then, a few drops of acetic acid were added to the reaction mixture to destroy the excess DCC, and the precipitate of *N,N*-dicyclohexylurea was filtered off. The solvent was removed by evaporation under reduced pressure (water aspirator at bath temperature of 50–60 °C). The remaining residue was dissolved in 20 mL of chloroform and chromatographed on a column (40 × 2, 5 cm) of silica gel (60–200 mesh, 100 g) that was eluted successively with chloroform. The physical properties of the compounds prepared are listed in Table I, and the IR and UV data are listed in Tables II and III, respectively.

In Vivo Study—All compounds were dissolved in dimethyl sulfoxide (DMSO), suspended in corn oil, and briefly sonicated prior to use. In all in vivo studies, the drugs were administered by multiple intraperitoneal (ip) injection on days 1, 4, and 7 after tumor inoculation. The treatment schedule experiments were finished when no mice remained alive; that is, on day 62 for Ehrlich ascites tumor (EAT), on day 39 for B16 melanocarcinoma, on day 16 for L1210 leukemia, and on day 19 for P388 leukemia. The toxicity of the homo-*aza*-steroidal esters and the mustards of cinnamic acid isomers are reported in Table IV.

L1210 leukemia was maintained in the laboratory by weekly ip passage of 10^5 L1210 cells in DBA mice. P388 leukemia was maintained by weekly ip passage of 10^6 P388 cells into BALB/C mice.[16] Melanoma B16 was propagated as a solid tumor in C57BL mice, whereas EAT was grown in BALB/C mice by ip injection of 7.10^6 7-day tumor cells. Female mice of 8–12 weeks of age were used. For all experiments, mice (uniform as to sex and age, average weight of 23 g) were kept in groups of six. Group of eight were used for control.

In Vitro Study—The L1210 leukemia cells were grown in RPM1-1640 medium containing 10% calf serum, streptomycin, and penicillin with 42 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES). The P388 leukemia cells were grown in Dulbecco's medium, the EAT cells were grown in medium 199, and the baby hamster kidney (BHK) cells were grown in minimal essential medium (MEM). All of the media were supplemented with 10% calf serum, antibiotics, and 12 mM HEPES.

The incubations were carried out in cell suspensions of 1×10^6 cells/mL at 37 °C. The compounds were dissolved in DMSO. The DMSO concentration in the medium never exceeded 10%. The incubation time was 30 min at a concentration of 25 μg/mL.

The DNA, RNA, and protein syntheses were determined after a 30-min incubation of 10^6 cells and 1 μCi of radioactive precursors ([^{3}H]thymidine, [^{3}H]uridine, [^{3}H]leucine) in a final volume of 1 mL of the various media mentioned above. At the end of the labeling period, cells were washed twice in phosphate-buffered solution (PBS) and resuspended in 50 μL of the same solution. The cell suspension was placed on Whatman filters (no. 41) and, in [^{3}H]leucine incorporation studies, the filters were divided into two identical groups. One group was left in 5% trichloroacetic (TCA) at room temperature and the other group was extracted in 5% TCA at 90 °C for 20 min. All the filters of the three precursors were further washed twice in 5% TCA, twice with commercial alcohol, once with ether:ethanol (1:1), and once with ether. After drying, the filters were placed in scintillation fluid, and the radioactivity was determined with a Packard scintillation counter. In the case of protein determination, the true radioactivity was obtained as the difference of counts in the total cold TCA and the radioactivity remaining after hot TCA extraction.

Cytogenetic Methods—Homo-*aza*-steroidal esters are able to induce cytogenetic endpoints as well as affect protein synthesis.[17,18] In this study, we tested four esters for their ability to induce sister chromatid exchange (SCE); namely, 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-*m*-*N*,*N*-bis(2-chloroethyl)-aminocinnamate,[2] 17β-hydroxy-3-*aza*-*A*-homo-4α-androsten-4-one-*m*-*N*,*N*-bis(2-chloroethyl)aminocinnamate,[11] 3α-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-*m*-*N*,*N*-bis(2-chloroethyl)aminocinnamate,[5] 3β-hydroxy-13α-amino-13,17-seco-5-androsten-17-oic-13,17-lactam-*m*-*N*,*N*-bis(2-chloroethyl)-aminocinnamate,[8] and the alkylating moiety *m*-*N*,*N*-bis(2-chloroethyl)aminocinnamic acid (*m*-ACA). Different concentrations were tested to identify the induction of SCE as well as the effects of these substances on the Mitotic Index (MI) and Replication Index (RI) in human lymphocyte cultures.

Whole blood (0.2 mL) from two healthy adult men was added to 6.5 mL of Ham-F10 (Gibco), 1.5 mL of fetal calf serum (Gibco), 0.3 mL of phytohaemaglutinin (PHA, Gibco). For SCE analysis, the medium contained bromodeoxyuridine (BrdUrd) at 7.5 μg/mL. The cultures were incubated at 37 ± 1 °C in complete darkness to minimize photolysis of BrdUrd. Two hours before fixation, colcemid (20 μg/mL) was added. The cells were then collected by centrifugation, exposed to 0.075 M KCl hypotonic solution for 10 min to spread the chromosomes and hemolyze the red blood cells, and fixed three times in methanol:acetic acid (3:1). Drops of a concentrated suspension of cells were placed on microslides that were allowed to air dry. For SCE analysis, the slides were stained by a modification of the fluorescent plus Giemsa technique[19] to obtain harlequin chromosomes. The slides were stained for 15 min in a solution of 0.5 μg of Hoechst 33258 per milliliter of Sorensen's buffer (pH 6.8). The slides were then washed, dried, mounted with the buffer under a coverslip, and exposed to

Table II—IR Spectra of Homo-*aza*-steroidal Esters of *N,N*-Bis(2-chloroethyl)aminocinnamic Acid Isomers

Compound	NH Stretching Vibrations, cm^{-1}	CH = CH Stretching Vibrations, cm^{-1}	CO–NH Stretching Vibrations, cm^{-1}	COO Stretching Vibrations, cm^{-1}
1	3190, 3030	1635	1660, 1650	1695
2	3190, 3060	1635	1675, 1660	1700
3	3195, 3065	1630	1660, 1640	1705
4	3170, 3060	1630	1680, 1650	1695
5	3220, 3060	1630	1680, 1660	1700
6	3190, 3070	1630	1660, 1650	1705
7	3205, 3040	1635	1660, 1650	1700
8	3180, 3060	1635	1680, 1660	1700
9	3205, 3060	1635	1660	1710
10	3160, 3045	1630	1660, 1650	1700
11	3220	1640	1660	1700
12	3160, 3060	1625	1660	1700

Table III—UV Spectra of Homo-*aza*-steroidal Esters of *N,N*-Bis(2-chloroethyl)aminocinnamic Acid Isomers

Compound	$CHCl_3$, λ_{max}	ε
1	340.1, 312.1, 238	8448, 3372, 3904
2	290.5, 263.5, 233	3423, 8461, 4865
3	278.6, 248.8, 233	8333, 9621, 8318
4	340, 311, 238	18070, 10719, 9938
5	295.3, 267.9, 236	3147, 9105, 6295
6	277.5, 248, 230.5	6545, 8940, 9000
7	340.1, 313, 239	13598, 7347, 6711
8	292.2, 265.4, 233.9	3016, 7481, 4374
9	280.6, 250.1, 234.1	12509, 16487, 16380
10	339, 312, 238.5	16028, 9201, 10944
11	290.1, 264.1, 235.6	4439, 11552, 7848
12	280.2, 250, 238.2	10890, 15710, 15047

Table IV—Toxicity of Homo-*aza*-steroidal Esters and Their Alkylating Congeners *o*-, *m*-, and *p*-ACA

Compound	LD_{50}[a]	LD_{10}[b]
9	500	350
8	700	500
7	650	500
12	280	170
11	620	300
10	350	200
3	450	300
2	580	370
1	900	600
o-ACA	160	50
m-ACA	280	100
p-ACA	140	100
6	600	400
5	530	300
4	700	400

[a] Fifty percent lethal dose. [b] Ten percent lethal dose.

daylight for 24 h. The slides were then stained for 10 min in a 3% Giemsa solution made in the same Sorensen's buffer. Cells dividing for the first (M1), second (M2), and third or more (M3+) times in culture can be determined in such preparation. The M1 cells contain chromosomes with both sister chromatids stained uniformly dark, the M2 cells contain only harlequin chromosomes, with one chromatid darkly stained and its sister chromatid lightly stained, and the M3+ cells contain some harlequin chromosomes and other chromosomes with both sister chromatids stained uniformly lightly. The SCEs were scored in 25 M2 metaphases, having 46 chromosomes completely differentially stained for each point. The data were expressed as the mean number of SCEs per cell ± the standard error (SE). Two hundred cells were scored to determine the percentage of M1, M2, and M3+ cells. Two thousand cells were scored to determine the MI for each case. The determination of RI is based on the frequencies of the first, second, and third or more metaphases and was calculated as follows:

$$RI = \frac{1M1 + 2M2 + 3M3}{100} \quad (1)$$

Results

For the survival experiments, antitumor activity was assessed according to the protocol for experimental evaluation of antitumor drugs in the National Cancer Institute (NCI).[16] The effect of esters and the alkylating agents towards EAT, L1210 and P388 leukemias, and melanoma B16 is shown in Table V. The EATs sensitive to the ester **9** produced a treated/control (T/C) value of 305% and those sensitive to ester **3** produced a 3, T/C value of 240%. In P388 leukemia, **9** and **3** gave 203 and 170% T/C values, respectively, and in L1210 leukemia, **9** and **3** gave 176 and 138% T/C values, respectively. All the compounds tested in melanoma B16 were almost inactive. The stereoisomers of **1–3** [namely, 3α-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-*N*,*N*-bis(2-chloroethyl)aminocinnamates (**4–6**)] were inactive in the above tumor lines.

In vitro incorporation studies demonstrated higher inhibitory effect on the incorporation of the radioactive precursors for the *ortho* derivatives, yielding >40% inhibition of thymidine incorporation in all tumor lines tested (Tables VI, VII, and VIII). The effect of **9** was superior, yielding 81, 70, 84, and 70% inhibition of thymidine incorporation into EAT, L1210 leukemia, P388 leukemia, and BHK cells, respectively, whereas **8** and **7** were less effective on the above tumor lines.

The ability of *m*-ACA to induce SCE, to affect the MI, as well as to influence the cell cycle was studied in human lymphocyte cultures. For this purpose, several concentrations

Table V—Antitumor Activity of Homo-*aza*-steroidal Esters and Their Alkylating Congeners *o*-, *m*-, and *p*-ACA

Compound	Mice Weight, g	Dose, mg/kg/day	T/C, %			
			EAT	L1210	P388	B16
Control	20	Corn oil	100	100	100	100
9	24	175	305	176	203	127
8	23	250	266	147	171	123
7	24	250	235	134	164	118
12	20	85	159	152	187	124
11	25	150	136	124	141	121
10	21	100	110	104	121	103
3	20	150	240	138	170	127
2	22	185	189	128	152	119
1	24	300	175	129	145	120
o-ACA	25	25	132	127	125	107
m-ACA	23	50	141	116	133	109
p-ACA	24	50	224	126	148	98
6	24	200	133	110	109	102
5	23	150	124	114	109	98
4	23	200	120	102	104	100

were tested. In reference to the MI, there is a clear cut reduction (Table IX). The highest concentration (10 μg/mL) reduces the MI to 45.60%. *m*-ACA is also able to induce SCEs in a linear dose response. Therefore, the highest concentration induces 44.08 ± 1.78 SCEs per metaphase, whereas at the control level, the mean number of SCEs per metaphase is 7.96 ± 0.70. Concerning the RI, an obvious reduction is caused. This reduction reaches the level of 1.435 at the concentration 10 μg/mL, whereas the RI for the control is 2.445. This is a clear indication that *m*-ACA can cause cell cycle delay.

We also studied the effect of different esters of *m*-ACA (**2**, **11**, **8**, and **5**) in relation to the above mentioned cytogenetic endpoints (MI, SCE, RI; Table IX). The ester **2** is less toxic than *m*-ACA, but it also reduces the MI and, at the highest concentration tested (100 μg/mL), the MI value is 63.10%. As to the SCE induction, an increase is observed, but it is not correlated with increases in the dose. Concerning cell cycle delay, ester **2** reduces the RI at concentrations 80 and 100 μg/mL, whereas the RI remains near the control level until the concentration of 40 μg/mL is attained.

Esters **11**, **8**, and **5** are more cytotoxic than ester **2**, but less effective than *m*-ACA. So, at the concentration of 100 μg/mL, the MI is 22.69% for ester **11**, 36.52% for ester **8**, and 24.81% for ester **5**. Ester **11** induces 15.60 ± 0.94 SCEs per metaphase at the highest concentration of 100 μg/mL; this is about the same

Table VI—Inhibition of [^{3}H]Thymidine Incorporation by Homo-*aza*-steroidal Esters and Their Alkylating Congeners *o*- and *m*-ACA after Exposure to 25 μg/mL

Compound	% Inhibition of [^{3}H]Thymidine Incorporation			
	EAT	L1210	P388	BHK[a]
9	81	70	84	70
8	61	54	52	53
7	31	47	5	44
12	73	55	79	58
11	60	40	72	51
10	57	17	64	33
3	64	45	46	38
2	39	35	39	25
1	25	11	13	21
o-ACA	57	71	56	48
m-ACA	21	47	30	29
6	61	50	48	54
5	57	30	33	49
4	37	14	29	38

[a] Normal cells.

Table VII—Inhibition of [^{3}H]Uridine Incorporation by Homo-*aza*-steroidal Esters and Their Alkylating Congeners *o*- and *m*-ACA after Exposure to 25 μg/mL

Compound	% Inhibition of [^{3}H]Uridine Incorporation			
	EAT	L1210	P388	BHK[a]
9	60	60	80	60
8	23	21	41	56
7	14	16	13	13
12	51	36	51	65
11	44	29	34	58
10	28	11	30	29
3	34	32	42	55
2	31	15	37	44
1	12	10	15	11
o-ACA	60	45	45	53
m-ACA	37	18	19	41
6	62	39	39	41
5	41	32	27	35
4	34	27	14	29

[a] Normal cells.

Table VIII—Inhibition of [^{3}H]Leucine Incorporation by Homo-*aza*-steroidal Esters and Their Alkylating Congeners *o*- and *m*-ACA after Exposure to 25 μg/mL

Compound	% Inhibition of [^{3}H]Leucine Incorporation			
	EAT	L1210	P388	BHK[a]
9	70	59	46	56
8	36	27	22	27
7	33	14	11	12
12	31	50	29	42
11	29	43	19	12
10	21	24	16	10
3	44	44	22	37
2	16	16	18	9
1	12	12	12	5
o-ACA	64	53	25	31
m-ACA	36	20	9	19
6	41	34	42	23
5	24	25	35	8
4	14	18	23	4

[a] Normal cells.

level of SCE induction as seen with ester 2, but lower than that caused by *m*-ACA. Esters 8 and 5 cause greater mean numbers of exchanges per metaphase at the higher concentration tested than esters 2 and 11. However, these esters are less effective than *m*-ACA. As to the cell cycle duration, esters 11, 8, and 5 cause a clear decrease. This decrease is greater in comparison with that caused by ester 2, but lower than that caused by *m*-ACA. So, at 100 μg/mL, the RI is 1.430 for ester 11, 1.325 for ester 8, and 1.295 for ester 5.

Discussion

Most steroidal alkylating agents have been inactive in L1210 leukemia,[1] but most homo-*aza*-steroidal esters of carboxylic derivatives of *N,N*-bis(2-chloroethyl)aniline have been active with substitution in either the D- or the A-ring.[3,20,21] It is suggested that in addition to an easily cleaved ester, which is a basic requirement for antitumor activity, the lactam moiety is required for activity in leukemia. The evidence that ASE inhibits DNA synthesis[22] directed us to determine if the lactam molecule is synergistic with the oncolytic agent. An indirect chemical approach based on the relationship between structure and mode of action of the new compounds in which the –NHCO– group has been reduced to $-NHCH_2-$ would give some information. In a study

Table IX—Induction of SCEs by Four Different Esters of *m*-ACA in Human Lymphocytes

	Concentration, μg/mL	MI, %	SCE/Metaphase		RI
			Range	Mean ± SE	
m-ACA	0	100	4–18	7.96 ± 0.70	2.445
	0.25	100	7–22	13.6 ± 0.91	2.325
	0.50	100	8–25	16.0 ± 0.87	2.320
	1.00	84.39	7–29	18.44 ± 1.02	1.955
	1.5	82.35	12–32	23.32 ± 1.11	2.23
	2	85.06	16–39	26.16 ± 1.30	2.115
	5	51.46	19–46	30.24 ± 1.50	1.780
	10	45.60	19–59	44.08 ± 1.78	1.435
2	0	100	4–18	7.96 ± 0.70	2.105
	1	100	3–17	10.40 ± 0.81	2.265
	10	100	8–29	15.96 ± 1.14	2.180
	20	100	7–32	16.16 ± 1.08	2.035
	40	84.39	6–23	14.76 ± 0.94	2.000
	80	64.89	8–25	14.60 ± 0.92	1.470
	100	63.10	6–25	14.64 ± 1.00	1.445
11	0	100	4–18	7.96 ± 0.70	2.105
	1	100	6–19	10.72 ± 0.65	1.920
	10	100	6–19	13.04 ± 0.79	2.115
	20	87.75	9–22	15.56 ± 0.65	1.785
	40	44.9	5–26	15.96 ± 1.08	1.470
	80	28.72	6–25	16.36 ± 1.07	1.435
	100	22.69	3–25	15.60 ± 0.94	1.430
8	0	100	4–18	7.96 ± 0.70	2.105
	1	100	4–17	10.32 ± 0.65	2.055
	10	98.40	7–31	14.40 ± 1.18	1.780
	20	76.43	8–27	17.88 ± 0.94	1.650
	40	53.50	11–25	18.48 ± 0.64	1.530
	80	46.10	5–28	17.40 ± 1.18	1.410
	100	36.52	8–31	21.16 ± 1.19	1.325
5	0	100	4–18	7.96 ± 0.70	2.105
	1	75.15	5–17	11.04 ± 0.72	1.930
	10	58.91	9–30	18.68 ± 0.96	1.790
	20	45.22	11–36	23.16 ± 1.07	1.720
	40	25.47	22–41	28.32 ± 1.20	1.470
	80	24.81	9–36	21.60 ± 1.29	1.295

in which ASE and the corresponding reduced compound (–NHCO– → $-NHCH_2-$) carried the same oncolytic agent, *p-N,N*-bis(2-chloroethyl)aminophenylacetic acid, ASE produced T/C values of >200% in P388 leukemia, >180% in L1210 leukemia, >145% in LLC, and >217% in EAT. The reduced compound gave the best activity; that is, T/C values of 152% in P388 leukemia, 143% in L1210 leukemia, 117% in LLC, and 157% in EAT. The results were obtained on days 1, 4, and 7 of treatment, at a dose of 26 mg/kg. The reduced compound was less active than ASE.[3] Given these results, it is possible that the lactam nucleus is transformed by a metabolic process or at least by an enzymatically catalyzed reaction to the active species.

This study of the homo-*aza*-steroidal esters of *N,N*-bis(2-chloroethyl)aminocinnamic acid isomers, in the presence of an extended conjugation system, is suitable for the study of the steric, electronic effects on anticancer activity. In a previous communication, the antineoplastic activity against lymphoid leukemia P388 of the isomeric esters, 1, 2, and 3 were studied.[15] Of the three isomers tested, 3 was active. A good correlation exists between antitumor activity and chemical reactivity assessed by determining the percent hydrolysis of the nitrogen mustard moiety. The higher chemical reactivity of the *ortho*-substituted nitrogen mustards compared with their *meta* and *para* isomers is not uncommon and has been attributed to steric hindrance of mesomerism. Steric repulsion excerted by the *ortho* substituent twists the nitrogen out of cojugation with the π-electrons of the ring and increases its basicity.

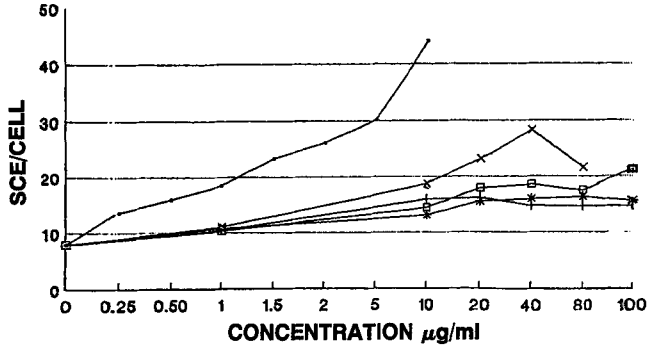

Figure 1—Induction of SCEs by *m*-ACA and its congeners. Key: (●) *m*-ACA; (1) **2**; (*) **11**; (□) **8**; (X) **5**.

In the 12 homo-*aza*-steroidal esters, higher antineoplastic activity was observed in the *ortho*-substituted nitrogen mustard, whereas none was active in the B16 melanoma. Particularly good activity in vivo and in vitro was noted in the homo-*aza*-steroidal ester with the double bond in the C_5 position and with *ortho*-substituted nitrogen mustard. Compounds **1–3** are stereoisomers of **4–6**. The latter were almost inactive in all tumor lines in vivo. This is probably due to the steric arrangement of the alkylating agent (axial). The alkylating congeners, *ortho*-, *meta*- and *para*-ACA (*p*-ACA) are much more toxic than the corresponding esters, whereas in the study for antitumor activity, only *p*-ACA showed activity in EAT and P388 leukemia.

Satisfactory inhibition of RNA, DNA, and protein syntheses was observed in *o*-*N*,*N*-bis(2-chloroethyl)aminoccinamic acid (*o*-ACA). From the cytogenetic point of view the investigation of *m*-ACA and its *meta*-substituted nitrogen mustards has shown that on human lymphocytes, the most effective molecule is the *m*-ACA, whereas its esters are able to cause the same cytogenetic endpoints to a lesser extent.

The exposure of human lymphocytes to different concentrations of *m*-ACA resulted in a linear increase of SCEs (Table IX). This fact indicates the ability of *m*-ACA to interact with DNA molecules during the S-phase. On the other hand, a decrease of the MI to 45.6 is observed when *m*-ACA (10 μg/mL) is present for the last 24 h of the culture period. This decrease shows that this agent is also cytotoxic in human lymphocytes. The determination of the RI for every concentration tested shows a dose-dependent decrease. This observation indicates that *m*-ACA is also able to cause cell cycle delay. Investigation of the genetic activity of *m*-ACA esters has shown that the induction of SCEs is lower for every ester compared with *m*-ACA. The order of the cytogenetic activity is *m*-ACA > **5** > **8** > **11** > **2** (Figure 1). The same order is also true for the effect of these compounds on the MI, whereas all esters are able to cause cell-cycle delay as indicated by their effects on the RI.

The lactam moiety appears to confer activity, and steroid and alkylating congeners combined should diminish the toxicity of nitrogen mustard (see Table IV). The steric arrangement of the alkylating moiety greatly affects toxicity and activity of the drugs. The steric arrangement of the hydrogen atom at position 5 influences these parameters. Further studies of these compounds and newer congeners (particularly in benzoic acid nitrogen mustards) are warranted to refine structure–activity relationships.

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