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m-N,N-bis(2-chloroethyl)aminocinnamic acid and four new homo-aza-steroidal esters induce chromosomal abnormalities and affect protein synthesis in human lymphocytes *in vitro*

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The alkylating agent *m-N,N*-bis(2-chloroethyl)aminocinnamic acid (*m*-ACA) and four new homo-aza-steroidal esters were studied for their ability to induce chromosomal abnormalities and to affect protein synthesis in human lymphocytes *in vitro*. A mitotic index reduction and an increase in the total number of aberrations were observed. Analysis of chromosomal abnormalities has shown that these are mainly chromatid breaks. A decrease in protein synthesis was also observed that seems to fit with the order of activity of the above compounds reflected in the induction of chromosomal aberrations. The observation that protein synthesis and the induction of chromosomal aberrations are affected by these chemicals may reflect interactions between these molecules and DNA that result in structural chromosome changes and decreased protein synthesis.

Key words: Anticancer drugs, chromosomal aberrations, homo-aza-steroidal esters, protein synthesis.

Introduction

Alkylating agents are known for their use in cancer chemotherapy,[1] and most of them are also known for their mutagenic and carcinogenic properties.[2] These chemical substances, being widespread in the environment, have been shown to be useful tools in studying mutagenesis because of their ability to react directly with DNA.[3] On the other hand, modified steroids, such as lactams, have been used as biological vehicles for the transport of alkylating molecules to tumors under attack, after the observation that steroid hormones reduce systemic toxicity and improve the specificity of cancer therapy.[4] In this study *m-N,N*-bis(2-chloroethyl)aminocinnamic acid (*m*-ACA) and four different homo-aza-steroidal esters are tested for their ability to induce chromosomal aberrations.

The esters are 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-3,17-lactam-*m-N,N*-bis(2-chloroethyl)aminocinnamate (*m* - ACAE1), 17β - hydroxy - 3 - aza - A - homo - 4α - androsten - 4 - one - *m* - *N*, *N* - bis(2 - chloroethyl)aminocinnamate (*m*-ACAE2), 3β-hydroxy-13α - amino - 13,17 - seco - 5 - androsten - 17 - oic - 13,17 - lactam-*m-N,N*-bis(2-chloroethyl)aminocinnamate (*m*-ACAE3), 3α-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-*m*-*N*-*N*-bis(2-chloroethyl) aminocinnamate (*m*-ACAE4). These compounds possess a modified steroidal moiety as a carrier that is linked to the alkylating agent in different ways. It is also known that similar chemical properties of antineoplastic drugs usually result in similar biological responses, while at the same time small differences in chemical properties may result in large differences in biological responses.[5] The chemical compounds studied have been shown to express slight antitumor activity in different types of experimental tumors including L1210 leukemia, P388 leukemia Ehrlich ascites tumor (EAT) and B16 melanoma.[6] In addition to DNA, proteins are known to be another group of biomolecules influenced by alkylating agents.[7] Thus, it would be of interest to study any possible influence of the above molecules on cellular proteins. As a first step the effects of these homo-aza-steroidal esters on protein synthesis in human lymphocyte cultures *in vitro* was studied.

Materials and methods

Test compounds

Four steroidal lactams of the A- and D-ring have been used for the esterification in the C-3 or C-17 position of their nuclei with (*m*-ACA).

The condensation reaction of the hydroxy group of the steroidal lactams with the mustard was effected in dichloromethane in the presence of *p*-

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dimethylaminopyridine as catalyst 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 (C,H,N), and IR and UV spectra.[6] The chemical formulae are given in Figure 1.

The compounds were dissolved in DMSO. Different quantities of the compounds were added to the cultures to obtain different concentrations. Cultures containing only DMSO, in the appropriate concentration, were used as negative controls.

Cell cultures and chromosome preparations

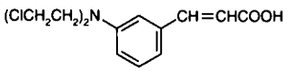
Chromosome preparations were made from human lymphocyte cultures. Whole blood (0.2 ml) derived from two healthy adult men was added to 6.5 ml Ham's F-10 (Gibco/BRL, European Division), 1.5 ml fetal calf serum (Gibco/BRL), 0.3 ml phytohemagglutinin (Gibco/BRL). The cultures were incubated at 37°C for 72 h. Cells were exposed for the last 24 h of the incubation period to the corresponding chemical solution. Two hours before fixation 20 μg/ml of colcemid (Gibco/BRL) was added. The cells were then collected by centrifugation, exposed to 0.075 M KCl hypotonic solution for 20 min and fixed three times in methanol:acetic acid (3:1). Drops of a concentrated suspension of cells were placed on microslides and allowed to air dry. The slides were then stained in 10% buffered Giemsa (Ferak, Berlin, Germany) solution for 10 min. Microscopic analysis was performed in a 'blind' fashion. In each case two replicate samples were handled identically and 100 metaphases were examined. All the aberration data were recorded according to Savage's description.[8] The determination of mitotic index (MI) was based on the scoring of 2000 cells for each point and was calculated according to:

$$\text{MI} = \frac{\text{no. of cells in division}}{\text{total no. of cells}} \times 100$$

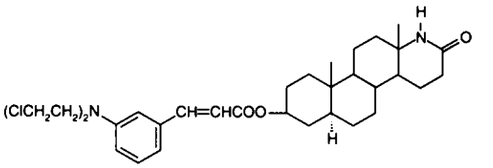
Regression analysis was used for the dose–response relationships.[9]

Labeling of proteins in human lymphocytes

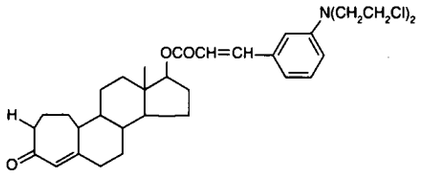
Whole blood samples were derived from the same healthy individuals. Samples (3 ml), collected in



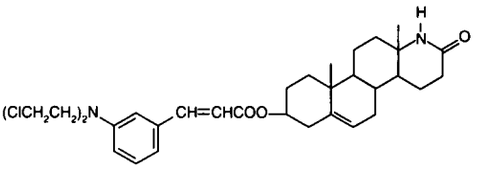
m-ACA : *m-N,N*-bis(2-chloroethyl)aminocinnamic acid.



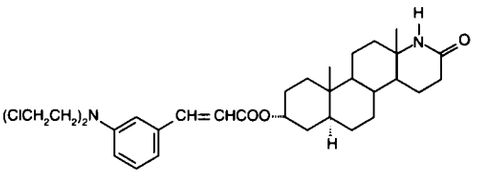
m-ACAE1 : 3β-Hydroxy-13a-amino-13,17-seco-5a-androstan-17-oic-13, 17-lactam-*m-N,N*-bis(2-chloroethyl)aminocinnamate.



m-ACAE2 : 17β-Hydroxy-3-aza-A-homo-4a-androsten-4-one-*m-N,N*-bis (2-chloroethyl)aminocinnamate.



m-ACAE3 : 3β-Hydroxy-13a-amino-13,17-seco-5-androsten-17-oic-13, 17-lactam-*m-N,N*-bis(2-chloroethyl)aminocinnamate.



m-ACAE4 : 3a-Hydroxy-13a-amino-13,17-seco-5a-androstan-17-oic-13, 17-lactam-*m-N,N*-bis(2-chloroethyl)aminocinnamate.

Figure 1. Chemical formulae of *m-N,N*-bis(2-chloroethyl)aminocinnamic acid and its homo-aza-steroidal esters.

heparinized tubes, were centrifuged at 3000 r.p.m. for 10 min, and blood plasma was removed and substituted by the same volume of MEM-meth (Gibco). Then 40 μg/ml of the compounds under study was added and the samples were incubated at 37°C for 2 h. At the end of that period [^{35}S]methionine (Amersham; specific activity 1361 Ci/mmol) was added at a final concentration of 17 μCi/ml and incubation was continued for a further 2 h. Labeling was terminated by adding an equal volume of ice-cold HBSS and the samples were layered on an equal volume of histopaque-1077 (Sigma) at room temperature. Samples were centrifuged at 2000 r.p.m. for exactly 30 min. The interface containing lymphocytes was transferred into a clean conical centrifuge tube and the lymphocytes

were washed twice with HBSS. Cells were lysed in a buffer containing 0.0625 M Tris–HCl, pH 6.8 and 1% β-mercaptoethanol and sonicated using a Soniprep 150, MSE sonicator. Cell lysates were centrifuged in an Eppendorf Microcentrifuge for 2 min and supernatant was collected and stored at −70°C until used.

Protein synthesis

Protein synthesis was measured by precipitation in 5 μl aliquots of homogenized cells with 10% trichloroacetic acid on Whatman 3MM filters as described by Mans and Novelli.[10] The radioactivity was counted in a LKB 1215 RACBETA liquid Scintillation counter.

Results

Human lymphocyte cultures have been used in order to study chromosomal abnormalities induction as well as protein synthesis. For the study of chromosomal aberrations the follow concentrations of the test compounds were used: 1, 2, 10 and 40 μg/ml for *m*-ACA, and 1, 10, 40 and 80 μg/ml for its homo-aza-steroidal esters (*m*-ACAE1, *m*-ACAE2, *m*-ACAE3, *m*-ACAE4). The results are shown in Table 1. It can be shown that MI is not affected by the presence of *m*-ACA at concentrations of 1 and 2 μg/ml, but it is decreased to 2.22 at 10 μg/ml and 0.74 at 40 μg/ml. The percentage of abnormal metaphases is increased to 23 at the lowest concentration (1 μg/ml) up to 100 at the highest (40 μg/ml). The frequency of total number of aberrations is 0.40 at 1 μg/ml and 5.15 at 40 μg/ml. Chromosome gaps do not seem to be induced but the frequency of chromosome breaks is increased up to 0.56. On the other hand, chromatid-type aberrations have shown an increase in every class examined. Regression analysis, in terms of t_{reg} (Table 1), shows that there is a dose–response relationship for every cytogenetic end-point except for chromosome gaps. The effect of *m*-ACAE1 ester on MI and chromosomal aberrations is also presented in Table 1. MI does not seem to be affected at the lowest concentration (1 μg/ml), but there is a clear reduction at concentration of 10, 40 and 80 μg/ml to 3.41, 1.44 and 1.12, respectively. The percentage of abnormal metaphases is increased to 30 as the concentration of the chemical increases while the frequency of the total number of aberrations is increased. Chromosome-type aberrations are

Table 1. The cytogenetic effects of *m*-ACA and its homo-aza-steroidal esters in human lymphocyte cultures *in vitro*

Compound concentration (μg/ml)	MI	Abnormal metaphases (%)	Total number of aberrations (frequency)	Chromosome-type aberrations		Chromatid-type aberrations		
				Gaps (frequency)	Breaks (frequency)	Gaps (frequency)	Breaks (frequency)	Exchanges (frequency)
0	3.95	11	21 (0.21)	—	5 (0.05)	—	16 (0.16)	—
m-ACA								
1	3.92	23	40 (0.40	2 (0.02)	5 (0.05)	11 (0.11)	21 (0.21)	1 (0.01)
2	3.81	28	49 (0.49)	3 (0.03)	10 (0.10)	7 (0.07)	28 (0.28)	1 (0.01)
10	2.22	54	132 (1.32)	6 (0.06)	22 (0.22)	28 (0.28)	69 (0.69)	7 (0.07)
40	0.74	100[b]	412 (5.15)	6 (0.08)	45 (0.56)	117 (1.46)	232 (2.90)	12 (0.15)
t_{reg}[a]	−5.55*	7.23*	50.36*	2.72	17.09*	18.89*	30.71*	8.06*
m-ACAE1								
1	3.94	12	18 (0.18)	—	6 (0.06)	1 (0.01)	11 (0.11)	— —
10	3.41	22	41 (0.41)	1 (0.01)	3 (0.03)	4 (0.04)	33 (0.33)	—
40	1.44	23	41 (0.41)	1 (0.01)	8 (0.08)	5 (0.05)	27 (0.27)	— —
80	1.12	30	42 (0.42)	4 (0.04)	2 (0.02)	9 (0.09)	27 (0.27)	— —
t_{reg}[a]	−4.79*	3.54*	1.72	0.58	−0.62	5.52*	1.12	—

Table 1. (*Continued*)

Compound concentration (μg/ml)	MI	Abnormal metaphases (%)	Total number of aberrations (frequency)	Chromosome-type aberrations		Chromatid-type aberrations		
				Gaps (frequency)	Breaks (frequency)	Gaps (frequency)	Breaks (frequency)	Exchanges (frequency)
m-ACAE2								
1	3.94	13	26	—	4	3	19	—
			(0.26)		(0.04)	(0.03)	(0.19)	—
10	3.51	26	41	1	5	10	25	—
			(0.41)	(0.09)	(0.05)	(0.10)	(0.25)	—
40	1.38	22	50	—	6	5	39	—
			(0.50)	—	(0.06)	(0.05)	(0.39)	—
80	1.05	27	57	4	6	5	42	—
			(0.57)	(0.04)	(0.06)	(0.05)	(0.42)	—
t_{reg}[a]	−4.81*	1.75	3.57*	—	1.89	0.34	4.42*	—
m-ACAE3								
1	3.87	13	24	—	5	2	17	—
			(0.24)	—	(0.05)	(0.02)	(0.17)	—
10	3.07	19	35	—	3	2	30	—
			(0.35)	—	(0.03)	(0.02)	(0.30)	—
40	1.18	22	46	2	7	7	30	—
			(0.46)	(0.02)	(0.07)	(0.07)	(0.30)	—
80	0.91	39	89	3	4	11	70	1
			(0.89)	(0.03)	(0.04)	(0.11)	(0.70)	(0.01)
t_{reg}[a]	−4.37*	7.52*	9.37*	—	−0.64	9.79*	5.16*	—
m-ACAE4								
1	3.91	10	20	1	1	3	15	—
			(0.20)	(0.01)	(0.01)	(0.03)	(0.15)	—
10	3.93	18	33	1	5	5	22	—
			(0.33)	(0.01)	(0.05)	(0.05)	(0.22)	—
40	1.34	35	69	5	11	9	44	—
			(0.69)	(0.05)	(0.11)	(0.09)	(0.44)	—
80	0.53	61	146	1	12	32	101	—
			(1.46)	(0.01)	(0.12)	(0.32)	(1.01)	—
t_{reg}[a]	−6.01*	40.41*	15.29*	0.51	3.43*	6.20*	10.23*	—
DMSO (0.56%)	3.94	12	18	—	3	7	8	—
			(0.18)	—	(0.03)	(0.07)	(0.08)	—

[a] $t_{(0.05)} = 3.182$, d.f. = 3.
[b] Only 80 metaphases have been scored.
* $p < 0.05$.

not induced. Chromatid gaps seem to be increased in a dose–response manner while chromatid breaks show a slight increase. Regression analysis (Table 1) for MI, abnormal metaphases and chromatid gaps, shows that there is a dose–response relationship. In Table 1, it is shown that treatment of lymphocytes with *m*-ACAE2 causes a MI decrease to 1.05 at 80 μg/ml in relation to control. The percentage of abnormal metaphases and the frequency of the total number of abnormalities at the same concentration are 27 and 0.56 respectively. Chromatid breaks is the only class of chromosomal aberrations that has been induced. MI, total aberrations and chromatid breaks are the classes that regression analysis shows dose–response relationships. MI is reduced by *m*-ACAE3 to 0.91 at 80 μg/ml. At the same concentration the percentage of abnormal metaphases is 39 and the frequency of the total number of aberrations increases as the chemical concentration increases. Chromatid gaps and chromatid breaks have also been induced by the same ester. A dose–response relationship is observed for MI, abnormal metaphases, total aberrations and chromatid-type aberrations. The effect of the last ester, *m*-ACAE4, is also presented in Table 1. There is also a MI reduction to 0.53 at 80 μg/ml and an increase of abnormal metaphases. The same is also true for the total number of aberrations including chromosome

breaks, chromatid gaps and chromatid breaks. Regression analysis shows that a dose–response relationship exists for every class exept chromosome gaps.

In order to study the effect of *m*-ACA and its esters on human lymphocyte protein synthesis the incorporation of [^{35}S]methionine into the total proteins of human lymphocytes *in vitro* at 40 μg/ml was measured. At this concentration an increase of the total number of abberations was observed for every chemical examined and for this reason it has been selected to be tested. DMSO at 0.56% was also tested for its ability to affect protein synthesis. This was done because the compounds are dissolved in DMSO. The results are presented in Table 2. As shown there is a clear reduction of protein synthesis caused by *m*-ACA to 56% and by *m*-ACAE4 to 61%. The esters *m*-ACAE1 and *m*-ACAE3 decreased protein synthesis to 76 and 74%, and *m*-ACAE2 only to 92% in relation to control preparations. DMSO treatment seems to slightly reduce protein synthesis (90%). Additionally a comparison between samples treated with compounds and samples treated only with DMSO was made. There was also a profound decrease with four of the five chemicals. The higher decrease was observed in cultures treated with *m*-ACA and the ester *m*-ACAE4, i.e. 63 and 67%, respectively. Protein synthesis was reduced to 85 and 82% for the esters *m*-ACAE1 and *m*-ACAE3, while there was no reduction for the ester *m*-ACAE2, in relation to DMSO treatment.

Discussion

Chemotherapy is one of the main approaches used in the treatment of cancer. Many anticancer drugs can also act as carcinogenic and/or mutagenic agents.[11] Nitrogen mustards, referred to as alkylating agents, belong to this category and it is known that they express their antitumor as well as carcinogenic and mutagenic activity by their interactions with DNA.[12] Different derivatives of nitrogen mustard and *N,N*-bis(chloroethyl)-aniline have been found to express selective cytotoxicity against certain types of tumors.[13,14] The *ortho*, *meta* and *para* isomers of *N,N*-bis(2-chloroethyl)aminocinnamic acid have also been found to be active against experimental tumors in animals.[15] Lactams, being modified steroids, have been used as biological vehicles of different alkylating agents to transport them to the tumor site.[16] The four esters of *m*-ACA tested in this study belong to this category. Treatment of human lymphocyte cultures *in vitro* by these chemicals shows a differential response in different cytogenetic endpoints (Table 1). As to the MI, *m*-ACA is the most effective compound while the four homo-aza-steroidal esters seem to be less cytotoxic. Concerning the percentage of abnormal metaphases, *m*-ACA is also more effective. From the four esters, *m*-ACAE4 induces the higher numbers of abnormal metaphases. The induction of total number of aberrations is greater in the case of *m*-ACA, whereas *m*-ACAE4 is the most active between the four esters. Chromatid breaks is the only class of aberrations that is induced by all these chemical compounds, *m*-ACA being the stronger inducer followed by *m*-ACAE4. Chromosome gaps do not seem to be induced by any of these compounds, chromosome breaks are induced by *m*-ACA and slightly by *m*-ACAE4, and chromatid gaps by *m*-ACA, *m*-ACAE1, *m*-ACAE3 and *m*-ACAE4 (Table 1). Our findings are in accordance with previous results[6] that indicate the ability of *m*-ACA and its homo-aza-steroidal esters to induce sister

Table 2. Incorporation of [^{35}S]methionine into total proteins of human lymphocyte cultures *in vitro* after treatment with *m*-ACA and its homo-aza-steroidal esters

Chemical compound	Concentration	Mean c.p.m.[a]	Incorporation in relation to no treatment (%)	Incorporation in relation to DMSO treatment (%)
m-ACA	40 μg/ml	4868	56	63
m-ACAE1	40 μg/ml	6571	76	85
m-ACAE2	40 μg/ml	7934	92	102
m-ACAE3	40 μg/ml	6339	74	82
m-ACAE4	40 μg/ml	5217	61	67
None	—	8216	—	—
DMSO	0.56%	7752	90	—

[a]Mean numbers have been extracted from at least three different experiments

chromatid exchanges and cause cell cycle delay. In this case, also, *m*-ACA is the most drastic molecule, while its esters are able to cause the same cytogenetic phenomena in a lesser way. Voutsinas *et al.*[17,18] have found that the above molecules induce base-pair substitutions in strains TA 100 and TA 1535 in the *Salmonella*/microsome mutagenicity assay. Moreover three homo-aza-steroidal esters of *p*[*N*,*N*-bis(2-chloroethyl)amino]phenylacetate which present antitumor activity have been found to induce sister chromatid exchange in human lymphocytes *in vitro*.[19]

Most of the chemicals which exert mutagenic, carcinogenic or cytotoxic properties affect DNA synthesis, disturb the cell cycle and influence protein synthesis in individual cells.[20] In this study the effects of *m*-ACA and its four esters on protein synthesis in human lymphocyte cultures *in vitro* were also tested. Our results show that there is a clear inhibition of [^{35}S]methionine into proteins in the presence of *m*-ACA and *m*-ACAE4 ester. This is also true for the esters *m*-ACAE3 and *m*-ACAE2 but to a lesser extent, while the *m*-ACAE2 ester decreases the incorporation only to about the same level as in DMSO. These results are in accordance with other findings in which *m*-ACA acid and its homo-aza-steroidal esters inhibit [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine incorporation in BHK cells and in experimental tumors,[6] and enhances our previous data in which the antitumor alkylating agents NSC 294859 and ASE reduce protein synthesis in the ovaries of *Drosophila melanogaster*.[21]

In the present study the order of inhibition of protein synthesis seems to fit with the order of activity of the above compounds reflected in the induction of chromosomal aberrations, in which it was found that the *m*-ACA and *m*-ACAE4 molecules are the most effective. The observation that protein synthesis and the induction of chromosomal aberrations are affected by these chemicals may reflect interactions between these molecules and DNA that result in structural chromosome changes and decreased protein synthesis.

The use of modified steroids as biological vehicles for the transport of different alkylating molecules to the tumor site results to a higher antineoplastic action and restricted cytotoxicity against normal tissue.[16] In our experiments with human lymphocytes from healthy donors, linkage of the most potent molecule *m*-ACA with the modified steroid resulted in a reduced cytogenetic and cytotoxic activity as well as protein synthesis. This decrease is not of the same level for the different esters as it depends on the chemical stuctures of these isomers.

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