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EFFECTS OF HOMO-AZA-STEROIDAL ESTER OF *p-BIS*(2-CHLOROETHYL)AMINO PHENYL ACETIC ACID ON LYMPHOCYTES STIMULATED WITH PHYTOHEMAGGLUTININ *IN VITRO*

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SUMMARY

The effect of 2-*bis*(2-chloroethyl)amino phenyl acetic acid(**I**) of lactam(**II**) and of lactam ester(**III**) were tested in a 48 h culture of human lymphocytes from normal subjects. The experiments yielded evidence that (a) for compound **I** there is a concentration-dependent increase progressively in the cell damage from the lowest (0.128×10^{-8} M/ml) the highest concentration used (0.384×10^{-8} M/ml).

On the whole, it was indicated that compound **I** was rendering the cells almost unable to divide and to incorporate [^{3}H]-thymidine proportionally to the concentration used; (b) for compound **II** no cell damage was observed and its role remains obscure; (c) for compound **III** there was evidence that in a concentration of 1.42×10^{-9} M/ml (which is actually half the corresponding concentration of compound **I**) it exhibits inhibition of DNA synthesis and low number of mitosis as well as chromosome damage. A fact worth mentioning is that mitotic index and [^{3}H]-thymidine incorporation showed the same pattern of response in the lymphocytes examined.

INTRODUCTION

Alkylating agents have been used for some years as cytotoxic agents mainly in the treatment of lymphocytic leukemias and lymphomata. Chlorambucil (4[*p-bis*(2-chloroethyl)]amino phenyl butyric acid) is said to alkylate DNA *in vitro* and to cause chromosome-type damage in bacteria, plants and insects [1]. However, when human lymphocytes were used in *in vitro* experiments there was suggestive evidence that exposure of the cells to chlorambucil over the whole period of culture demonstrated damage predominantly of chromatid-type but including more complex changes and, in extreme cases chromosome pulverization [2, 3].

Furthermore, Stevenson and Patel (1973) have found that chlorambucil could stimulate a high proportion of lymphocytes from normal subjects to DNA synthesis and that only a few cells proceeded to the cycle of prophase or even metaphase. Similar findings have been reported by Hill [4] using lymphocytes from patients with chronic leukemia.

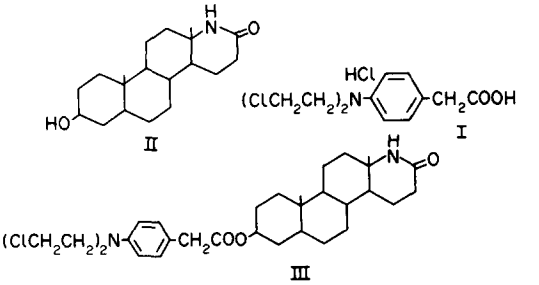
Heterocyclic steroidal ester of *p-bis*(2-chloroethyl)-amino phenyl acetic acid gives >50% increase life span (ILS) over controls in the treatment of L1210 leukemia. Similarly it gives >150% ILS in the treatment of P388 leukemia [5] in contrast to steroidal esters of *p-bis*(2-chloroethyl)amino phenyl acetic acid in which Wall and co-workers [6] have shown that in L1210 leukemia were inactive according to the criteria of CCNSC.

Since there are no reports on *in vitro* experiments of modified steroidal esters of *p-bis*(2-chloroethyl)-amino phenyl acetic acid which are known for their cytostatic action [7–9] and particularly in the treatment of animal leukemias [5] we thought it to be of interest to investigate the effect of the above mentioned compound on cultures of human lymphocytes.

MATERIALS AND METHODS

All experiments described were performed on human peripheral-blood lymphocytes that were obtained from heparinized blood. The culture techniques employed are based on those described by Moorhead *et al.*[10] and Hungerford[11].

Phytohemagglutinin (Reagent grade, Wellcome, U.K. reconstituted) was used at a concentration, of 0.1 ml/10 ml of culture medium and was added 10 min after the compounds. In all the experiments reported below the compounds (**I, II** and **III**) were dissolved in ethanol and appropriate dilutions were made in Eagle's medium and added at the beginning of the culture. The same amount of alcohol 0.16 μl/ml of culture medium was added to the control cultures.



I. *p-bis*(2-chloroethyl)amino phenyl acetic acid.
II. 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17 lactam.
III. 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17 lactam-*p-bis*(2-chloroethyl)amino phenyl acetate.

Cultures were maintained at 37°C for 48 h. At 41 h half of the culture suspension was removed and 1 μCi [methyl-^{3}H]-thymidine per ml of culture medium was added (S.A. 5 Ci mmol^{-1}) and after 7 h of labeling cells were harvested. In the remaining half suspension colchicine (0.3 μg/ml of culture medium) was added 3 h before harvesting.

Both the cell suspensions were treated according to Moorhead *et al.*[10] and Hungerford[11] techniques (fixing with a mixture of methanol–acetic acid (3:1, v/v) and dry smears of the lymphocytes were prepared. Authoradiographs from the dry smears of labeled lymphocytes were prepared using Kodak nuclear track emulsion NTB2 and exposed it for 2 days. The percentage of labeled interphase nuclei were calculated from 800–1000 analyzed cells per sample. For autoradiography the cultures were harvested without prior addition of colchicine. Cells having up to 15 grains were considered as unlabeled cells.

RESULTS AND DISCUSSION

Cultures of human lymphocyte from 10 normal subjects (30–40 years old) were exposed to single doses of the three compounds, **I**, **II** and **III** outlined above.

The same donors offered blood for the three sets of *in vitro* experiments which were undertaken.

Lymphocytes from each subject were exposed to three different concentrations of the compound **I**, 0.128×10^{-8} M, 0.256×10^{-8} M and 0.384×10^{-8} M/ml of the culture medium and cultured in one batch. This experiment yielded evidence that exposure of human lymphocytes in culture over a 48 h period showed cell damage. This damage increased sharply from concentration of 0.128×10^{-8} M/ml to 0.384×10^{-8} M/ml. The results showed that, using a concentration of 0.128×10^{-8} M/ml, no different effect could be detected as compared to non compound-treated cultures (control). The percentage of labeled lymphocytes as well as the number of lymphocytes in the stage of mitosis is of the same order in both, although examination of the slides suggests that killing of transformed cells is sufficient to partly invalidate this numerical equality.

Using a concentration of 0.256×10^{-8} M/ml it was found that compound **I** was causing serious damage to the cells resulting in lowering the mitotic index as well as the number of labeled lymphocytes by a factor greater than 2 as compared to the untreated cultures (see Table 1). The relatively low mitotic index suggests a blocking effect in late S or G_2. In support of this, evidence from cytological appearance, indicates that in this concentration fewer cells reached metaphase as compared to the control and a smaller proportion of cells were transforming. However, there were many early transformed cells which were clearly unhealthy—or even dead as indicated by staining with Trypan blue—despite the fact that they had been treated by phytohemagglutinin in the presence of which in culture is considered that partially protects them an enables a considerable proportion of the cells to reach and pass through metaphase [2]. Subsequently the concentration of 0.384×10^{-8} M/ml was tested. In this case it was observed that almost all of the cells were damaged and no cell reached metaphase.

It is clear from these results that there is a concentration-dependent increase in the cell damage. On the whole, this series of experiments seemed to strongly indicate that under these experimental conditions compound **I** was rendering the cells unable to incor-

Table 1. Experiments of human lymphocytes from ten normal donors stimulated with PHA and cultured for 48 h 0.256×10^{-8} M/ml 0.260×10^{-8} M/ml and 1.42×10^{-9} M/ml of culture medium of compounds **I**, **II** and **III** respectively, 1000 cells analysed

	Control cultures (the amount of ethanol added in the culture medium) (0.16 μl/ml)		Compound **I**		Compound **II**		Compound **III**	
Exp	Number of lymphocytes in mitosis	Number of labeled lymphocytes	Number of lymphocytes in mitosis	Number of labeled lymphocytes	Number of lymphocytes in mitosis	Number of labeled lymphocytes	Number of lymphocytes in mitosis	Number of labeled lymphocytes
1	34	598	15	230	37	634	22	204
2	33	580	16	223	38	600	21	180
3	33	572	18	192	36	654	19	210
4	34	575	19	200	37	595	21	213
5	31	547	17	184	40	585	25	200
6	32	545	16	233	39	650	21	184
7	30	542	15	240	38	606	20	210
8	37	586	19	220	40	620	23	204
9	35	570	18	243	40	630	25	195
10	36	560	17	215	39	605	22	203
	Control (without ethanol)							
	34	544						
	28	583						

Table 2. Human lymphocytes treated at indicated concentration of compound I (48 h cultures, 1000 cells analyzed)

Concentration of compound I (M/ml)	Number of lymphocytes in mitosis	Number of labeled lymphocytes
0.00	42	631
0.128×10^{-8}	28	520
0.256×10^{-8}	15	204
0.384×10^{-8}	None	95

porate [^{3}H]-thymidine and to divide proportionally to the concentration used (see Table 2).

In a second series of experiments the effect of the compound **II** was tested. It was added at a concentration of 0.260×10^{-8} M/ml which was found to be a suitable dose for assessing relative sensitivities of lymphocytes to the compound. The findings are set out in Table 1 and it is strongly suggested that on the whole, no cell damage was observed from exposure to the compound **II**. The [^{3}H]-thymidine incorporation and the number of lymphocytes in mitosis resembled those of the control.

In an attempt to clarify whether the combined form **III** (1 part of **I**:1 part of **II**) was inducing more detectable damage than compound **I** alone, further experiments were undertaken. In this case lymphocytes were exposed to the combined form **III** at a concentration of 1.42×10^{-9} M/ml and the data are shown in Table 1. As is seen the damage is a little lower than with compound **I**. Although the numerical difference is small, it has to be taken into account that compound **III** is composed of almost equal amounts of compounds **I** and **II**. This results in lowering the concentration of compound **I** roughly to 0.128×10^{-8} M/ml. In this concentration as it has been shown (Table 1) compound **I** alone is ineffective while in the combined form it produced a high level of cell damage.

A possible explanation could be that many of the unlabeled cells could have entered S but inhibition of DNA synthesis and [^{3}H]-thymidine incorporation was so great at the 1.42×10^{-9} M/ml concentration of **III** that they were unable to incorporate enough isotope to be registered as labeled under the experimental conditions employed. Such cells, although

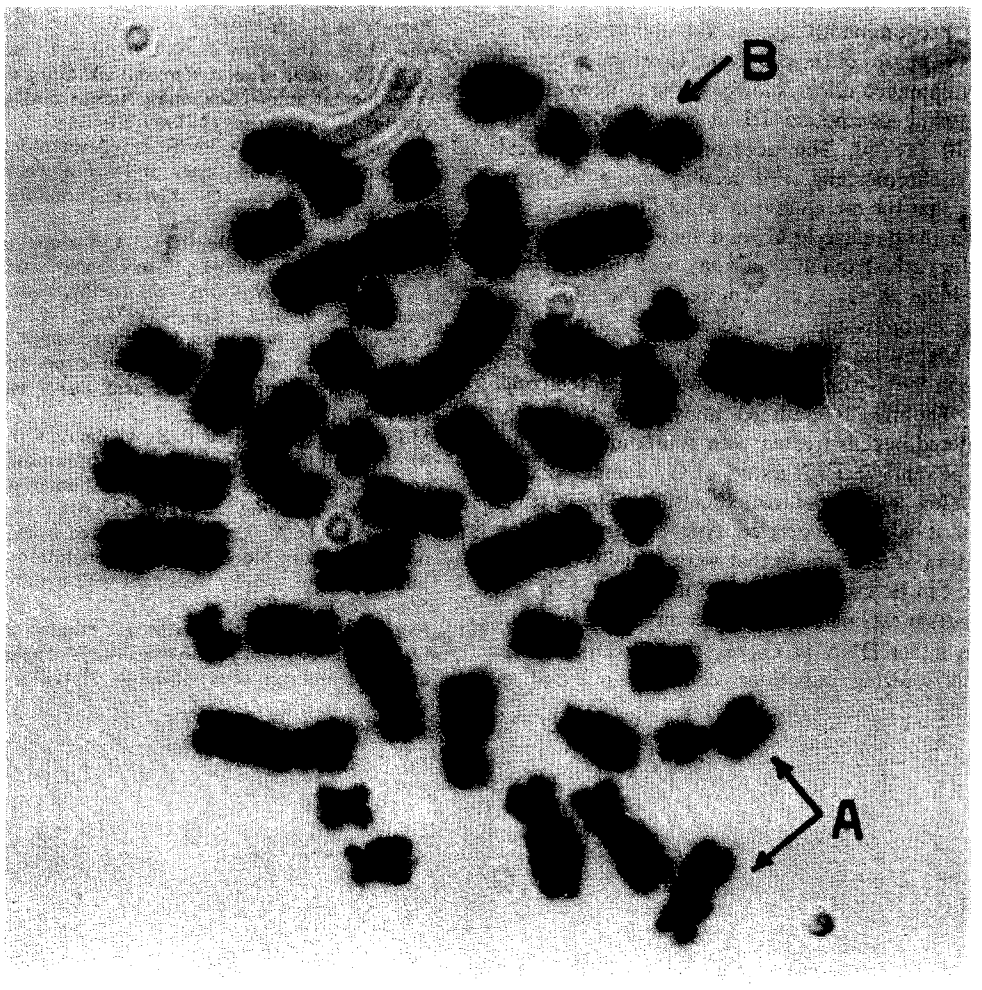


Fig. 1. A well spread metaphase lymphocyte cell from a 48 h culture where 1.42×10^{-9} M/ml of compound **III** was presented. A. Dicentric chromosome and acentric fragment. B. Chromatid break.

capable of dividing, may have been affected by the compound **III** and hence they have been unable to initiate DNA synthesis.

Alternatively compound **III** could have completely inhibited initiation of DNA-synthesis in some cells which already existed in G_1 at the beginning of the treatment. Our experiments cannot distinguish between these two possibilities. In connection with these findings we considered it of crucial importance to mention the corresponding therapeutic dose of this steroidal ester which is 10 mg/kg [7].

From the results obtained it could be suggested that although the effect of compound **II** alone appears not to produce any cell damage it cannot be ruled out that compound **II** is acting as a biological carrier capable of transporting the alkylating agent into the cell. The results from the combined form **III** give strong evidence to sustain this hypothesis.

A fact worth mentioning derived from all experiments outlined above is that both criteria used, i.e. the mitotic index and the [^{3}H]-thymidine incorporation showed a linearly proportional pattern in the 48 h culture.

As is suggested from the experimental evidence several workers have presented some drugs that were causing predominantly chromatid aberrations in S and/or G_2 phases of the cell cycle. However, a typical severely damaged cell is shown in Fig. 1 from a 48 h culture using compound **III** at a concentration of 1.42×10^{-9} M/ml. The occurrence of one typical dicentric chromosome with accompanying fragment suggests that the metaphase in which it is seen is the first after the damage in G_1 and such an interchange figure does arise from discontinuity on a unduplicated chromosome in G_1. Furthermore, in the same cell another chromosome with chromatid damage is found, suggesting that it is most likely that the damage in the two chromosomes was due to the compound. The results outlined above are frequently encountered in cells treated with compound **III.** This finding is somewhat suggestive of G_1 damage occurring at a low level in the treated cultures. The possibility that it is of the derived type resulting from a duplication of aberrations that were initially chromatid-type can be excluded for a 48 h culture [12].

A possible explanation is that compound **III** might damage intact DNA in G_0/G_1 and this, unlike the effects of ionizing radiations, could be reflected in chromatid damage as detected in the subsequent metaphase.

In view of the evidence presented, further experiments are in progress in order to define the stage in which the compounds used, are inducing chromosomal damage.

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