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Report

7-Keto hybrid steroidal esters of nitrogen mustard: cytogenetic and antineoplastic effects

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Four newly synthesized antitumor steroidal compounds were compared, on a molar basis, regarding their ability to induce sister chromatid exchanges (SCEs) and cell division delays. The concept of designing and developing these compounds (1–4) is to enhance the anticancer activity of esteric steroidal derivatives of nitrogen mustard by introduction of a keto group at the 7-position of the D^5 steroidal skeleton, and a double bond between positions 6 and 7 of the B ring of the steroidal nucleus. In our study, the cytogenetic and antileukemic effects of these newly synthesized compounds are reported. The four substances induced statistically significant enhancement of SCEs and of cell division delays, and in both schedules used, therapeutic effects. However, compounds 1 and 3 showed increased genotoxicity towards human lymphocytes ($p < 0.001$) and antileukemic activity towards P388 leukemias ($p < 0.001$), compared to compounds 2 and 4. It seems that the introduction of a keto group at the 7-position of the steroidal skeleton enhances the antitumor effect of these substances in comparison with our previous studies with the corresponding compounds characterized by the absence of the 7-keto group. Therefore, the *in vivo* antitumor effect of the four compounds appears to correlate well with the *in vitro* cytogenetic effect caused by these chemicals. [© 2002 Lippincott Williams & Wilkins.]

Key words: 7-Keto-steroidal derivatives, antitumor effects, cell kinetics, chlorambucil, genotoxicity, sister chromatid exchange, steroidal lactam.

Introduction

Nitrogen mustards have been used as anticancer agents since the end of World War II.[1,2] Later, it was discovered that if nitrogen mustards were linked with a steroid, the newly synthesized compound has an improved chemotherapeutic effect. This happens because the steroid acts as a biological platform that carries the alkylating agent and helps it to interact with the DNA of tumor cells. Moreover, it is believed that the steroidal esters with alkylating agents decrease the systemic side effects caused by the alkylating congener.[3] Since then, multiple modifications have been made to the steroidal nucleus in order to create more potent antitumor drugs. This revealed the chemotherapeutic potency of the homo-aza-steroidal esters of nitrogen mustard, in both *in vivo* and *in vitro* studies.[4]

The compounds (**1**–**4**) studied are shown in Figure 1. All compounds have a keto group at the 7 position of the steroidal nucleus. Compound **1** has a keto group at position 17 of the steroidal nucleus and is a phenylacetate derivative. Compound **2** has also a keto group at the 17 position, but it is a phenylbutyrate derivative (Chlorambucil). Compound **3** has a lactamic moiety at the D ring of the steroidal nucleus and is also a phenylbutyrate derivative. Finally, compound **4** has the same lactamic configuration at the D ring of the steroidal nucleus, but it is a benzoate derivative having a methyl group at the *para* position and the *N*,*N*-bis(2-chloroethyl)amino agent at the *meta* position, while all other compounds have the latter agent at the *para* position.

Sister chromatid exchanges (SCEs) have been proposed as a very sensitive method for detecting genotoxicity, and lately as one of the methods for evaluating chemotherapeutic efficiency *in vitro* and *in vivo*.[5,6] In addition, studies have shown that the determination of proliferation rates in lymphocyte

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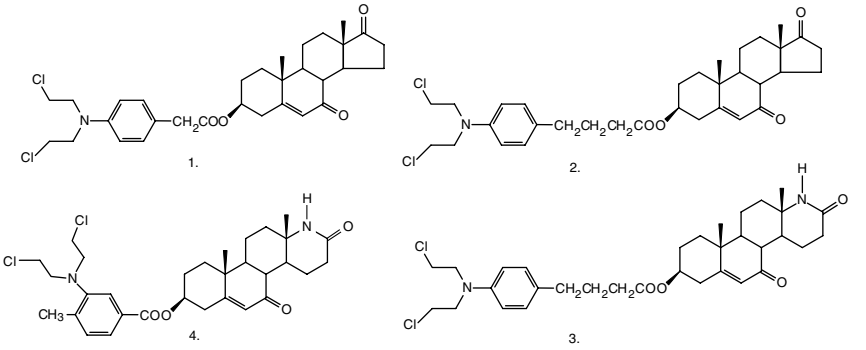

Figure 1. Chemical structure of compounds **1–4**. (**1**) 3β-hydroxy–5-androsten-7,17-dione-*p*-*N*,*N*-bis(2-chloroethyl)amino phenyl acetate, m.p.=154–156°C. (**2**) 3β-hydroxy-5-androsten-7,17-dione-*p*-*N*,*N*-bis(2-chloroethyl)amino phenyl butyrate, m.p.=120–121°C. (**3**) 3β-hydroxy-17α-aza-D-homo-5-androsten-7,17-dione-*p*-*N*,*N*-bis(2-chloroethyl)amino phenyl butyrate, m.p.=182–183°C. (**4**) 3β-hydroxy-17α-aza-D-homo-5-androsten-7,17-dione-*p*-methyl-*m*-*N*,*N*-bis(2-chloroethyl)amino benzoate, m.p.=171–173°C.

cultures should be a useful and sensitive indicator of the cellular toxicity of chemotherapeutic agents.[5,6] In the present work a comparative study of these compounds, on a molar basis, was undertaken regarding their ability to induce SCEs and cell division delays; the results were compared with the antitumor activity of these agents established at equitoxic doses on leukemia P388-bearing mice.

Materials and methods

General procedure for the synthesis of steroidal esters of nitrogen mustard

For the synthesis of the four steroidal esters, which have been reported previously, the general method of esterification by using the asymmetric anhydride of the carboxylic acid of the nitrogen mustards with the 2,4,6-trichlorobenzoyl chloride was used (Figure 2). According to this method, in a round-bottom flask the desired amount (1 mmol) of the carboxylic acid of each nitrogen mustard (M-COOH) is placed and diluted in dry benzene. 2,4,6-Trichlorobenzoyl choride (1 mmol) and triethylamine (1 mmol) is added, and the mixture is refluxed under Ar and a tube of $CaCl_2$ for 1 h. A solution of the steroidal alcohol in dry benzene and a catalytic amount of 4-(dimethyl)aminopyridine (4-DMAP) are then added in the mixture mentioned above. The reflux is continued for 2–3 h. The benzene is totally removed by evaporation in vacuum and the remaining oil is diluted with CH_2Cl_2. The resulting mixture is extracted with a 5% HCl aq. solution and the organic layer is then washed with a 7% $NaHCO_3$ aq. solution, and finally with water. After being dried over Na_2SO_4, the solvent is removed under reduced

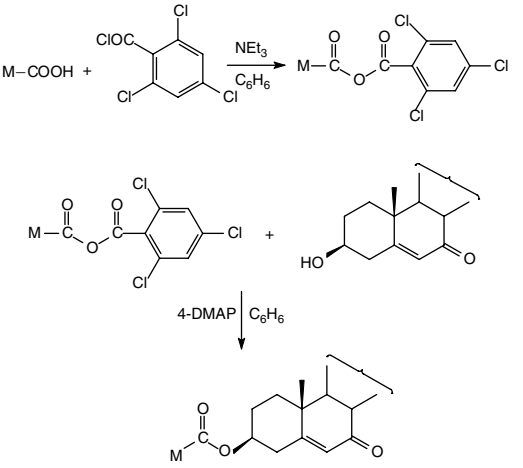

Figure 2. General method of esterification for the synthesis of steroidal esters.

pressure. The residue is chromatographed on a silica gel column and the desired esters are isolated after elution with CH_2Cl_2 (for the **1** and **4** products) and with CH_2Cl_2/MeOH 2% (for the **2** and **3** products). The yields of the synthetic procedure for the esteric derivatives varies from 65 to 87%.

All four compounds were recrystallized from ethyl acetate and their structure was identified with IR, ^{1}H-NMR and ^{13}C-NMR spectroscopy, and by elemental analysis.

In vitro SCE assay

Lymphocyte cultures were set up by adding 11 drops of heparinized whole blood from three normal subjects to 5 ml of chromosome medium 1A (RPMI 1640; Biochrom, Berlin, Germany). For SCE

demonstration, 5 μg/ml 5-bromodeoxyuridine (BrdUrd) and the four compounds were added at the beginning of culture life. During the experiment all cultures were maintained in the dark to minimize photolysis of BrdUrd. The cultures were incubated for 72 h at 37°C. Metaphases were collected during the last 2 h with colchicine at 0.3 μg/ml. Air-dried preparations were stained by the FPG procedure.[7] The preparations were scored for cells in their first mitosis (both chromatids dark staining), second mitosis (one chromatid of each chromosome dark staining), and third and subsequent divisions (a portion of chromosomes with both chromatids light staining). Twenty suitably spread second division cells from each culture were blindly scored for SCEs. For proliferation rate indices (PRIs), at least 100 cells were scored. The PRI was calculated according to the formula $PRI=(M_1+2M_2+3M_3)/N$, where M_1, M_2 and M_3 indicate those metaphases corresponding to first, second and third or subsequent divisions, and N is the total number of metaphases scored. For the statistical evaluation of the experimental data, the χ^2-test was performed for the cell kinetic comparisons. For the SCE frequencies, Student's *t*-test was used.

In vivo experiments

Compounds for i.p. treatment. Stock solutions of the compounds used in this study were prepared immediately before use. They were suspended in corn oil in the decided concentration following initial dissolution in 10% DMSO. This concentration by itself produces no observable toxic effects.

Mice. DBA/2 mice of both sexes and BDF1 male mice, weighing 20–23 g, 6–8 weeks old, were used for toxicity and antitumor testing, respectively. They were obtained from the Experimental Laboratory of Theagenion Anticancer Hospital, and were kept under conditions of constant temperature and humidity in sterile cages with water and food *ad libitum*.

Tumors. Lymphocytic P388 leukemia was maintained in ascitic form in DBA/2 mice by injection of 10^6 cells at 7-day intervals into the peritoneal cavity.

Acute toxicity experiments. DBA/2 mice in groups of 10 were used to determine the LD_{10} and LD_{50}, which are the lethal doses for 10 and 50% of the mice used, respectively.[8] They were injected with a single i.p. injection per dose at three different dosages ranging from 50 to 200 mg/kg. The mice were observed for 30 days, and the LD_{50} and LD_{10} values were estimated graphically, where the percentage of deaths due to toxicity for each dose is shown in the ordinate, while the administered doses are indicated on the abscissa on semilogarithmic paper.

Antitumor experiments. These were initiated on day 0 by i.p. implantation of 10^6 ascites cells of P388 leukemia into male BDF1 mice, according to the protocol of the National Cancer Institute.[9] Drug administration started 24 h later, either as a single injection at the LD_{10} on day 1 or three injections with $LD_{10}/2$ on days 1, 5 and 9. Antitumor activity was assessed from the percent increase in median lifespan of treated animals versus the untreated controls. Eight BDF1 male mice were used as the control group and six male mice for each drug concentration. For the statistical evaluation of the experimental data the *t*-test was performed.

Results

The cytogenetic results of compounds **1**–**4** are shown in Table 1. The parameters used for estimating the

Table 1. The effect of compounds **1**–**4** on SCE rates and PRI in normal human lymphocyte culture

Compound	Concentration (μM)	SCE/cell ± SEM	PRI
Control	–	10.16 ± 0.63	2.52
1	0.2	32.0 ± 3.22**	1.76***
	0.4	66.86 ± 6.79	1.97***
	0.6	–[a]	–[a]
	1.2	69.58 ± 4.39	–[b]
2	0.2	25.37 ± 2.26	1.82***
	0.4	30.46 ± 2.24	2.47
	0.6	33.47 ± 2.73	1.50***
	1.2	–[a]	–[a]
3	0.2	20.80 ± 1.90	1.36***[c]
	0.4	–[a]	–[a]
	0.6	32.98 ± 2.66	–[b]
	1.2	–[a]	–[a]
4	0.2	13.90 ± 0.90	1.49***
	0.4	16.45 ± 1.04*	2.31
	0.6	24.46 ± 1.68	1.49***
	1.2	–[a]	–[b]

[a]No results were obtained.
[b]Insufficient numbers of cells were evaluated for PRI calculations.
[c]Significant decrease ($p < 0.001$, χ^2-test) over the same concentration of compounds **1**, **2** and **4**.
*Significant increase ($p < 0.01$, *t*-test) over the control.
Significant increase ($p < 0.001$, *t*-test) over the same concentration of compounds **2, **3** and **4**.
***Significant decrease ($p < 0.001$, χ^2-test) over the control.

Table 2. Acute toxicity of compounds **1**–**4** in DBA/2 mice

Compound	LD_{50} (mg/kg body weight)	LD_{10} (mg/kg body weight)
1	65	40
2	110	70
3	50	34
4	275	170

genotoxic and cytostatic effects of the compounds were the SCEs and the PRIs respectively. According to the results, compound **1** was by far the most effective of all compounds with SCE values that reach an average of even 69.58 at 1.2 μM versus the control with 10.16. Compound **2** caused an average SCE increase to 33.47 at 0.6 μM. A similar result was obtained for compound **3** with an average SCE value of 32.98 at 0.6 μM. Compound **4** was the less effective SCE inducer with an average value of 24.46 at 0.6 μM. Compound **1** induces statistically ($p<0.001$) higher SCE levels compared with the rest of the compounds at all concentrations tested (Table 1). The most cytostatic compound, however, seemed to be compound **3** with a PRI value of 1.36 at 0.2 μM. This value is statistically lower compared to the PRI values of compounds **1**, **2** and **4** at the same concentration ($p<0.001$, χ^2-test). Compound **4** followed with a PRI of 1.49 at 0.2 μM, while compounds **1** and **2** caused a PRI depression of 1.76 and 1.82 at the same concentration. However, the PRI depression caused by compound **2** was similar to compound **4** with a PRI value of 1.50 at 0.6 μM. Compound **3** seemed to be very toxic as there were not enough cells for the determination of the PRIs at the concentrations of 0.4 and 0.6 μM (Table 1). At 0.4 μM, compound **1** appeared superior in PRI depression compared to compounds **2** and **4** (Table 1).

Table 2 shows the acute toxicity of compounds **1**–**4** in DBA/2 mice. The parameters for estimating acute toxicity were the LD_{10} and LD_{50}. The most toxic compound was **3**, with an LD_{50} of 50 mg/kg and an LD_{10} of 34 mg/kg, followed by compound **1** with an LD_{50} of 65 mg/kg and an LD_{10} of 40 mg/kg. Next in order of toxicity was compound **2** with an LD_{50} of 110 mg/kg and an LD_{10} of 70 mg/kg, followed by compound **4** with an LD_{50} of 275 mg/kg and an LD_{10} of 170 mg/kg.

The antileukemic effect of the compounds, given at equitoxic doses, is shown in Table 3. Treatment with the compounds at a dose of $LD_{10}/2$ using the days 1, 5 and 9 schedule was more effective than the single administration at a dose of the LD_{10} on day 1 for all compounds. Compound **1** given at 20 mg/kg on days 1, 5 and 9 increased significantly the lifespan of leukemia P388-bearing mice, achieving 321% of the control. Compound **3** increased the lifespan to T/C=257% at 17 mg/kg (days 1, 5 and 9). Treatment with compounds **1** and **3** (days 1, 5 and 9) resulted in a significantly increase in lifespan, compared to treatment with compounds **2** and **4** in the same schedule. Compound **2** effected a T/C=198%, while compound **4** caused T/C=219%.

Discussion

All compounds showed effective genotoxicity with compound **1** being the most effective SCE inducer.

Table 3. Antitumor activity of compounds **1**–**4** on P388 leukemias in BDF1 mice, given at equitoxic doses

Compound	Treatment schedule	Dosage (mg/kg/day)	Medial survival time of each mice group ± SEM	T/C% [a]
Control		corn oil	7.7 ± 0.21	100
1	day 1	40[b]	17.3 ± 0.33*	225**
	days 1, 5 and 9	20[c]	22.5 ± 1.23*	321**[d]
2	day 1	70	10.5 ± 0.34*	136
	days 1, 5 and 9	35	13.8 ± 0.30*	198
3	day 1	34	16.5 ± 0.22*	214**
	days 1, 5 and 9	17	19 ± 0.22*	257**
4	day 1	170	12.5 ± 1.06*	161
	days 1, 5 and 9	85	15.3 ± 0.55*	219

[a]Percent increase in median lifespan of each group of treated mice over the controls.[8]
[b]Dose corresponds to the LD_{10} (Table 2).
[c]Dose corresponds to $LD_{10}/2$.
[d]Significant increase ($p<0.001$, *t*-test) over the same treatment schedule of compounds **2**, **3** and **4**.
*Significant increase ($p<0.001$, *t*-test) over the control.
Significant increase ($p<0.001$, *t*-test) over the same treatment schedule of compounds **2 and **4**.

According to the cytogenetic results (Table 1), a very remarkable increase of SCE rates was caused by compound **1** compared to the control and to the rest of the compounds ($p<0.001$, *t*-test). However, compounds **2**–**4** induced significant increase of SCEs ($p<0.01$, *t*-test) over the control.

All tested compounds were proved to be cytostatic since they induced a significant decrease of PRI ($p<0.001$, χ^2-test) compared to the control. However, in some concentrations there was insufficient number of cells for evaluating PRI. This could be attributed to the fact that the drug (e.g. compound **2** at 1.2 μM) was so toxic that the cells of certain donors that were not destroyed by it were not numerous enough for calculating the PRI.

Compound **2** showed a slightly higher SCE induction than compound **3**. However, compound **3** induced a PRI of 1.36 at 0.2 μM, while compound **2** decreased the PRI at 1.82 at the same concentration. It is obvious that the values in the SCE assay obtained for compounds **2** and **3** (both with the chlorambucil moiety) are very similar, and compound **1** [with the active metabolite 4-*N*,*N*-bis(2-chloroethyl)amino phenyl acetic acid] was a better SCE inducer compared to them and to compound **4**. At 0.4 μM compound **1** also appeared superior in PRI depression compared to compounds **2** and **4**. The most cytostatic compound, however, seemed to be compound **3** compared to the rest of the compounds at a concentration of 0.2 μM (Table 1). In previous studies the alkylating agent component chlorambucil (part of compounds **2** and **3**) and 4-*N*,*N*-bis(2-chloroethyl)amino phenyl acetic acid (part of compound 1) were shown to be quite less effective inducers of cytogenetic effects than compounds **2**, **3** and **1**, respectively.[10,11]

At the *in vivo* antileukemic experiments, all compounds showed therapeutic effects, in both treatment schedules used. However, the results obtained after administration of the drugs on days 1, 5 and 9 at a dose of $LD_{10}/2$ were better than those obtained by single administration on day 1 at a dose of the LD_{10}.

All compounds showed a significant increase ($p<0.001$, *t*-test) of the mean lifespan of each group of mice compared to the control. Among them, compounds **1** and **3** gave significantly higher ($p<0.001$, *t*-test) antileukemic values compared to those of compounds **2** and **4** at the same treatment schedule, while compound 1 was proved to be the most effective (T/C%=321).

Both the cytogenetic and antileukemic experiments showed that **1** and **3** were more potent chemotherapeutics than **2** and **4**. The difference between compounds **2** and **3** is focused on the configuration of the D steroidal ring, which on the latter compound is a six-membered lactam, while the alkylating agent remains the same. Since compound **3** was considered to be more potent than **2**, the suggestion that the lactamic modification on the steroidal D ring enhances the antineoplastic activity seems to be confirmed.[12]

Compound **1** was proved to be superior in SCE induction and PRI depression compared to the corresponding analog, where the 7-keto group is not present.[11] The same analog proved completely inactive in *in vivo* experiments against P388 and L1210 leukemias.[13] Similar conclusions are derived by the comparison of compound **3** to a similar molecule, which also lacks the 7-keto group.[14,15] Additionally, comparing the biological activity of compound **4** with that of the corresponding non-7-oxidized molecule, we found that compound **4** gave also higher SCE values and depressed the PRI of normal human lymphocyte cultures more,[16] while its T/C% value at *in vivo* studies was higher at the LD/2×3 treatment schedule.[17]

These findings indicate that the introduction of a ketone at the 7-position of the steroidal ligand of these molecules probably confers their increased activity and are in agreement with several studies reporting that the $\triangle^5$-7-keto steroids are more toxic towards cancerous than non-cancerous cells,[18] while they are able to inhibit cell replication.[19]

The keto group found at position 7 of the steroidal skeleton of all newly synthesized compounds might interact in a favorable manner with similar species of DNA and proteins, and enhance the chromosome damage and antitumor effect induced by these agents.

Additionally, the guanine N^7 adducts, which the nitrogen mustards primarily form at 5′-GNC sequences (where N is any nucleotide) of the DNA strands as their major cytotoxic effect, are among the most easily repaired.[20,21] The effective SCE induction obtained herein may be also a result of other DNA's alkylating lesions, as it has been reported that chlorambucil (the alkylating moiety in compounds **2** and **3**) and its active metabolite,[22–24] 4-*N*,*N*-bis(2-chloroethyl)amino phenylacetic acid (the alkylating moiety in compound **1**), can alkylate adenines at the minor groove N3 position apart from N^7 of guanines, presumably because of the weak DNA-targeting ability of the aromatic ring.[25]

There are findings indicating that the effectiveness in SCE induction and in PRI depression of potential antitumor agents in cancer cells *in vitro*[5,8] and *in vivo*[26,27] are positively correlated with the *in vivo*

tumor response to these agents. This suggests that the SCE assay could be used to predict both the sensitivity of human tumor cells to chemotherapeutics and the heterogeneity of drug sensitivity of individual tumors.[26–29] Furthermore, the SCE assay has been proposed as having predictive value as a clinical assay for drugs for which a strong correlation between cell killing and induction of SCEs has been established.[6,8,26–29] Studies for establishing a correlation between SCE induction and other expressions of genotoxicity have shown a positive relationship between SCEs, reduced cell survival and alteration in cell cycle kinetics.[5,6,8,26] In the present study all tested compounds were proved to induce a significant decrease of the PRI ($p < 0.001$, χ^2-test). SCE is a reliable method for estimating unrepaired DNA damage.[5,6,8,26] It is assumed that cancer cells have similar repair mechanisms to normal cells. As a result, the DNA damage caused by potential chemotherapeutics and measured in normal human cells with SCEs could reflect the same unrepaired damage caused by the same chemotherapeutics in rodent cancer cells.[5,6,8,26] Human cells, like some rodent cells, are proficient in DNA repair. Therefore, it is expected that the DNA repair mechanisms in both cell types would be disturbed in a similar manner by the same potential chemotherapeutics.[6]

In this study a good correlation was observed between the *in vitro* genotoxic and the *in vivo* antitumor growth effect of the newly synthesized compounds.

Conclusion

The chemical conjugation of nitrogen mustards with modified steroidal molecules, via an esteric bond, produces derivatives with reduced toxicity and significantly increased antitumor activity.[12] This indicates that steroidal molecules are not just carriers for efficient transportation of the alkylating agent to the target sites of DNA, but also confer its activity. Such carriers are the modified steroids bearing the NH–CO moiety in the D ring of their molecule and, moreover, the 7-keto-Δ^5-steroids.

The introduction of the allylic 7-keto group in the skeleton of the steroidal carrier induces the antitumor activity of the final esteric derivatives; rather, it confers activity as it alters the inactive compounds to strongly active (i.e. dehydroepiandrosterone's derivative) and improves the effectiveness of the already active compounds (3β-hydroxy-17α-aza-D-homo-5-androsten-7,17-dione derivatives).

Additionally, it is further indicated that SCEs and PRIs are sensitive indicators for determining cytogenetic damage *in vivo* and *in vitro*, and predicting the chemotherapeutic effect of new compounds *in vivo*.[5,8,14,27–29]

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