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Short communication

On the formation of 4-[*N,N*-bis(2-chloroethyl)amino]phenyl acetic acid esters of hecogenin and aza-homo-hecogenin and their antileukemic activity

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Abstract

The *p*-[*N,N*-bis(2-chloroethyl)amino]phenylacetic acid esters of hecogenin and aza-homo-hecogenin have been prepared and their antineoplastic activity was evaluated against two basic drug screening systems in rodents, P388 lymphocytic and L1210 lymphoid murine leukemias. Among the compounds tested, the *p*-[*N,N*-bis(2-chloroethyl)amino]phenylacetic acid ester of aza-homo-hecogenin was appeared to possess a significant higher antileukemic effect. These results support that the alkylating esters of hecogenin produce important antitumor activity as well as, indicate that the aza-homo-hecogenin ester exhibits significantly higher activity due to lactam group (–NHCO–) modification.

Keywords: Aza-homo-hecogenin; Hecogenin; L1210 lymphoid leukemia; P388 lymphocytic leukemia; *p*-[*N,N*-bis(2-chloroethyl)amino]phenylacetic acid

1. Introduction

In view of the importance of steroid containing alkylating agents, we have used modified steroids, such as aza-homo-steroids, characterized by the amide group in the steroidal nucleus, as a biological carrier that transports the alkylating agent to the tumor site. The idea to design aza-homo-steroids arose from the observation that most steroidal esters of *p*-[*N,N*-bis(2-chloroethyl)amino]phenylacetic acid were proved to be inactive in L1210 leukemia [1], while most aza-homo-steroidal esters of the parent agent, bearing a substitution of an easily cleaved ester bond in A- or D-ring, gave satisfactory results in early and advanced P388 and L1210 leukemia systems [2–5].

The presence of the amide group is important in order to lower systemic toxicity and improve specificity in cancer treatment. As a consequence of this important observation the 3β-hydroxy-12α-aza-C-homo-5α,22α-spirostan-12-one (aza-homo-hecogenin) [6], containing the –NHCO– group inside the C steroid ring was used as carrier of the *p*-[*N,N*-bis(2-chloroethyl)amino]phenylacetic acid, in comparison with the unmodified steroid 3β-hydroxy-5α, 22α-spirostan-12-one (hecogenin).

The high anticancer activity of hecogenin and aza-homo-hecogenin esters with the *p*-[*N,N*-bis(2-chloroethyl)amino]-phenylacetic acid against Lewis Lung carcinoma [7] prompted us to study these compounds against P388 lymphocytic and L1210 lymphoid leukemias in vivo.

2. Chemistry

The alcoholysis of an acid chloride is a general method, which has been widely used for the esterification of steroidal and aza-homo-steroidal alcohols with various carboxylic derivatives of *N,N*-bis(2-chloroethyl)aniline.

Previously the esterification of the C-3 hydrooxylic group of the 3β-hydroxy-5α,22α-spirostan-12-one and 3β-hydroxy-12α-aza-C-homo-5α,22α-spirostan-12-one was effected by

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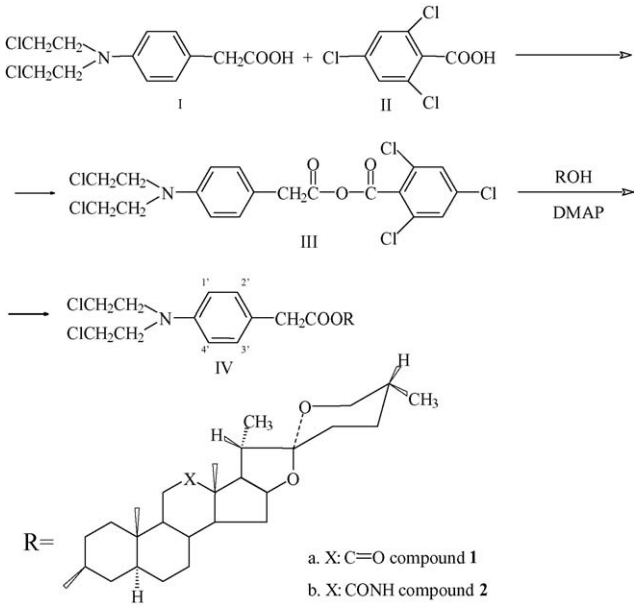

Scheme 1.

condensation of the above steroids with *p*-[*N*,*N*-bis(2-chloroethyl)amino]phenylacetic acid in anhydrous benzene in the presence of *p*-dimethylaminopyridine as catalyst and of dicyclohexylcarbodiimide as dehydrating agent [7].

In the present work the esterification was effected by the mixed carboxylic aniline mustard anhydride, which was prepared using 2,4,6-trichlorobenzoyl chloride as the condensing agent and triethylamine as an auxiliary base. The alcoholysis step was carried out in benzene and in the presence of *p*-dimethylaminopyridine as outlined in Scheme 1. The esters were isolated in good yields, whereas the corresponding 2,4,6-trichlorobenzoic acid esters were isolated as by-products. The formation and the chemical structures of the compounds under investigation are showed in Scheme 1.

3. Antitumor activity

The antitumor activity of compounds **1** and **2** was evaluated against two fundamental anticancer drug screening murine leukemia systems: P388 lymphocytic leukemia and L1210 lymphoid leukemia.

The antitumor activity was assessed from the percent increase in median lifespan (MST: mean survival time) of treated animals over the untreated (control) [(T/C)%]. The minimum criterion for activity is considered a value of T/C > 125% [8]. The results are shown in Table 1.

The in vivo experiments adhered to the "Principles of Laboratory Animal Care" and to the United Kingdom Coordinating Committee on Cancer Research guidelines [9,10].

4. Results and discussion

Both tested compounds induced significant in vivo antileukemic activity against P388 and L1210 leukemia systems. Compound **1** (hecogenin ester) produced %T/C:182 against P388 leukemia ($P < 0.01$) and %T/C:133 against L1210 leukemia ($P < 0.01$) when was administrated on days 1, 5, 9 i.p. at a dose of 125 mg kg^{-1} day^{-1}. Respectively, compound **2** (aza-homo-hecogenin ester) produced %T/C:225 against P388 leukemia ($P < 0.01$) and %T/C:166 against L1210 leukemia ($P < 0.01$) when was administrated on days 1, 5, 9 i.p. at a dose of 75 mg kg^{-1} day^{-1} (Table 1). The compound **2** generated significant higher antileukemic efficacy against both treated leukemia systems, in comparison to compound **1** ($P < 0.001$). Moreover, compound **2** exhibited higher toxicity (LD10: 150 mg kg^{-1}) than compound **1** (LD10: 250 mg kg^{-1}).

The presence of the characteristic group –NHCO– of the homo-aza-steroid molecule was proved important, in order to lower toxicity and improve antitumor activity [12,13]. It was suggested that the –NHCO– lactam group is transformed by a metabolic process or at least by an enzymatically catalyzed reaction to active species which strongly interact with similar groups existing in the DNA and proteins (–NH–CO– → $–NH^{\ominus} + –C = O^{\oplus}$). Modifications of the –NHCO– lactam group by NH methylation (–NCH_3CO–) or by –CO– reduction led to derivatives with lower anticancer activity than the parent compounds [14–16].

In early studies, it was presumed that aza-steroids act due to a fundamental mode on sterol metabolism and interfere

Table 1
The antileukemic effect that was generated by the tested alkylators of hecogenin (1,2) against L1210 and P388 murine leukemias demonstrated by the prolongation of mean survival time (MST) and the T/C (%) survival

Compound	Treatment schedule (days)	Dosage mg kg^{-1} day^{-1} i.p.	L1210 Leukemia		P388 Leukemia	
			MST (days)	T/C (%)	MST (days)	T/C (%)
1	1	250	13.1	149[a]	11.0	122[a,b]
	1,5,9	125	16.0	182[a,b]	12.0	133[a,b]
	1–9	63	14.2	161[a]	10.7	119
2	1	150	14.0	159[a]	12.8	142[a,b]
	1,5,9	75	19.8	225[a,b]	14.9	166[a,b]
	1–9	37.5	13.8	157[a]	13.9	155[a,b]
Control	–	Corn oil	8.8	100	9.0	100

The statistical significance was evaluated with Willcoxon test.
[a] Significant increases of T/C in comparison to the control were produced ($P < 0.01$).
[b] Significant differences between the T/C values that were achieved by respective treatment schedules ($P < 0.01$).

with mitochondrial respiration and/or oxidative phosphorylation [17]. Moreover, the lactam ring of the aza-steroids can react as antagonist or agonist by its binding to certain cellular enzymes as protein kinase C (PKC) with a relative specificity [18–20].

The alkylating component of these esters, effects via the same biochemical pathway of other bifunctional alkylating mustards [21]. Steroid esters can generate high intracellular concentrations due to the lipophilic nature of the steroid carrier. A rate-limiting hydrolysis of the ester bond liberates the two active moieties (one steroid and one alkylating) into the cellular microenvironment [22]. Comparative studies of aza-homo-steroid esters with alkylating agents used in current chemotherapy on antineoplastic activity against experimental systems, including P388 and L1210 leukemias, showed that homo-aza-steroid esters hold a superior or leastwise equal anticancer activity [3,23,24]. Furthermore, meta-analysis of the antileukemic activity of compound **2** in experimental systems, in comparison with the conventional alkylators: mephalan, chlorambucil and cyclophosphamide, showed that compound **2** is more active than chlorambucil, at least equally active with cyclophosphamide, and rather less active than mephalan (data not shown). The stereoisomeric form and chemical structure of the steroidal vectors and alkylating components determine the antitumor effect of these compounds [25,26].

Conclusively, the alkylating esters of hecogenin produce significant antineoplastic effect against two main screening leukemia systems in rodents. The corresponding ester of aza-homo-hecogenin exhibits significantly higher activity indicating the importance of lactam group (–NHCO–) in improvement of antitumor effect. Preclinical research supports that aza-steroids and lactam steroids in esteric forms with nitrogen mustards demonstrate lower acute and sub-acute toxicity as well as superior antitumor activity, particularly against lymphoid leukemia and lymphoma. The important antileukemic efficacy of these compounds adds advantage for further clinical development.

5. Experimental procedures

5.1. Chemistry

Melting points were determined on a Haak melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin–Elmer 298 spectrophotometer in solid phase KBr, with polystyrene as a reference peak. NMR spectra were determined with an AC 300 MHz spectrometer using deuterated dimethylsulphoxide (DMSO-D_6) and are reported in δ units relative to tetramethylsilane (TMS) as an internal standard. Column chromatography was performed using Aldrich silica gel, 70–230 mesh. The C, H, N, analyses were within ± 0.4% of theoretical values and were performed by the Laboratory of Analytical Chemistry, University of Ioannina, Greece.

5.1.1. General procedure for the synthesis of steroidal esters of p-[N,N-bis(2-chloroethyl)amino] phenylacetic acid

A solution of 1 mmole of *p*-[*N,N*-bis(2-chloroethyl)-amino]phenylacetic acid was added in 15 ml of dry benzene, with 1 mmole of 2,4,6-trichlorobenzoyl chloride and 1 mmole of triethylamine and refluxed for 2 h Triethylamine hydrochloride was filtered off and the solvent was removed under reduced pressure. The residue was then dissolved in toluene and the solvent was removed under reduced pressure. The remaining mixed carboxylic anhydride was dissolved in 40 ml of dry benzene and 1 mmole of the corresponding steroidal alcohol and 1 mmole of 4-dimethylaminopyridine were added. The reaction mixture was refluxed for 48 h The solvent was evaporated, the residue was dissolved in chloroform, washed successively with 3% aqueous hydrochloric acid, water, aqueous sodium bicarbonate and water, and dried over sodium sulfate. The solvent was removed under reduced pressure and the residue was chromatographed on silica gel. Elution with chloroform and Chloroform:CH_3OH 98:2, gave the desired compounds.

*5.1.1.1. p-[N,N-bis(2-Chloroethyl)amino]phenylacetic acid ester of 3β-hydroxy-5α,22α-spirostan-12-one **(1)**.* Yield. 72% M.p. 98–99° I.R. (cm^{-4}): 1732 (COO), 1707 (CO), 866, 804, 752, 661 (phenyl). Anal. $C_{39}H_{55}Cl_2NO_5$ (C,H,N). H^1-NMR. 7.24 (d, 2H, 2′-H and 3′-H of ArH, J = 7.92 Hz), 6.85 (d, 2H, 1′-H and 4′-H of ArH, J = 7.92 Hz) 4.73 (m, 1H, 3α –H), 4.35 (m, 1H, 16α-H), 3.87 (s, 8H [$(ClCH_2CH_2)_2N$]), 3.32–3.62 (2xm, 2H, 26-H), 2.52 (m, 1H, 17α-H), 1.14 (s, 3H, 18-H), 1.11 (d, 3H, 21-H, J = 6.72 Hz), 1.04 (s, 3H, 19-H), 0.90 (d, 3H, 27-H, J = 6.72 Hz).

5.1.1.2. p-*[N,N-bis(2-Chloroethyl)amino]phenylacetic acid ester of 3β-hydroxy-12α-aza-C-homo-5α,22α-spirostan-12-one **(2)**.* Yield. 57% M.p. 155–156° I.R. (cm^{-4}): 3232 (HN), 1732 (COO), 1655 (HNCO), 885, 860, 780 (phenyl). Anal. $C_{39}H_{56}Cl_2N_2O_5$ (C,H,N). H^1-NMR. 7.58 (s, 1-H, N–H), 7.31 (d, 2H, 2′-H and 3′-H of ArH, J = 7.95 Hz), 6.92 (d, 2H, 1′-H and 4′-H of ArH, J = 7.32 Hz), 4.81 (m, 1H, 3α-H), 4.45 (m, 1H, 16α-H), 3.95 (s, 8H [$(ClCH_2CH_2)_2N$]), 3.43–3.70 (2xm, 2H, 26-H), 2.74 (m, 1H, 17α-H), 1.45 (s, 3H, 18-H), 1.19 (d, 6H, 21-H and 27-H, J = 9.45 Hz), 0.97 (s, 3H, 19-H).

5.2. In vivo studies

BDF_1 male mice, weighing 20–23 g, 6–8 weeks old were used for evaluation of antitumor activity. 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. Experiments were initiated on day 0 by implanting i.p. of 10^6 ascites cells of lymphocytic P388 leukemia and 10^5 of lymphoid L1210 leukemia according to the protocol of National Cancer Institute, USA [11]. For i.p. treatment stock solutions of compounds were prepared immedi-

ately before use. They were suspended in corn oil at the desired concentration following initial dissolution in a small amount of 10% DMSO. The antileukemic activity of the tested compounds was estimated in separate experiments. Each drug-treated experimental group consisted of six mice and control groups of eight mice. For chemotherapy testing the highest dose used was LD10 (LD10: lethal dose for 10% of the treated animals within 30 days). The antitumor activity was assessed from the percent increase in median lifespan (MST: mean survival time) of treated animals over the untreated (control) [% (T/C)].

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