Eur. J. Med. Chem. 34 (1999) 645–649

Short communication

On the formation of steroidal amidoesters of 4-[N,N-bis(2-chloroethyl)amino]benzoic acid and their cytotoxic activity#

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(Received 18 November 1998; accepted 27 January 1999)

Abstract – The 4-[N,N-bis(2-chloroethyl)amino]benzoate of 17β-acetamido-5α-androstan-3β-ol, 17β-acetamido-5-androsten-3β-ol, 3β-acetamido-5α-androstan-17β-ol and 3α-acetamido-5β-androstan-17β-ol have been prepared and their antineoplastic effect evaluated against MIA Pa-Ca-2 pancreatic carcinoma, T47D breast carcinoma and A431 squamus cell carcinoma. Among the compounds tested, the compound 17β-acetamido-3β-hydroxy-5-androsten-4-[N, N-bis(2-chloroethyl)amino]benzoate appeared to possess a significant cytotoxic effect against A431 cells. © 1999 Éditions scientifiques et médicales Elsevier SAS

steroidal 4-[N,N-bis(2-chloroethyl)amino]benzoate amidoesters / MTT assay / cytotoxicity / MIA Pa-Ca-2, T47D, A431 cells

1. Introduction

Recent studies in our laboratory have been concerned with the synthesis and anticancer evaluation of compounds synthesized from modified steroids, namely steroidal lactams, esterified with carboxylic derivatives of N,N-bis(2-chloroethyl)aniline, in either the A or D ring. By preparing these compounds, which contain a modified steroid as a carrier molecule for transporting the alkylating agents to the tumour site, it is possible to diminish toxicity and improve selectivity.

The biological action of lactams, characterized by the -NHCO- group, may be structurally specific and therefore of more prolonged activity, because of the multiple interactions of this group with similar groups that exist in proteins and nucleic acids. Therefore, the -NHCO- group of the steroidal lactam molecule may be essential for antitumour activity. Whereas unmodified steroidal esters have generally been reported to be inactive in the treatment of L1210 leukaemia [1], most homo-aza-steroidal esters gave satisfactory results in early and advanced P388, L1210 leukaemias [2–4] and solid tumours [5–7], with substitution in either the A or D ring by an easily cleaved ester bond.

These results prompted us to continue our research on the synthesis of four new steroidal amido-esters of 4-[N, N-bis(2-chloroethy)amino]-benzoic acid (p-ABA) [8] containing the -NHCO- group outside the steroidal nucleus and study their antineoplastic effect on epithelial tumours and adenocarcinomas in vitro.

2. Chemistry

As biological platforms of the 4-[N,N-bis(2-chloroethyl)amino]benzoic acid, we used A and D ring steroidal amides namely, 17β-acetamido-5α-androstan-3β-ol **Ia** [9], 17β-acetamido-5-androsten-3β-ol **Ib** [10], 3β-acetamido-5α-androstan-17β-ol **IVa** [11] and 3α-acetamido-5α-androstan-17β-ol **IVb** [11].

The usual procedures for the esterification of C-3 and C-17 hydroxylic groups of the amido-steroids were effected by the symmetrical acid anhydride method, as outlined in *scheme 1*. Reaction of the appropriate amido-steroids **Ia–b** and **Iva–b**, with the symmetrical acid anhydride **II** in refluxing benzene afforded the corresponding steroidal amidoesters **IIIa–b** and **Va–b**.

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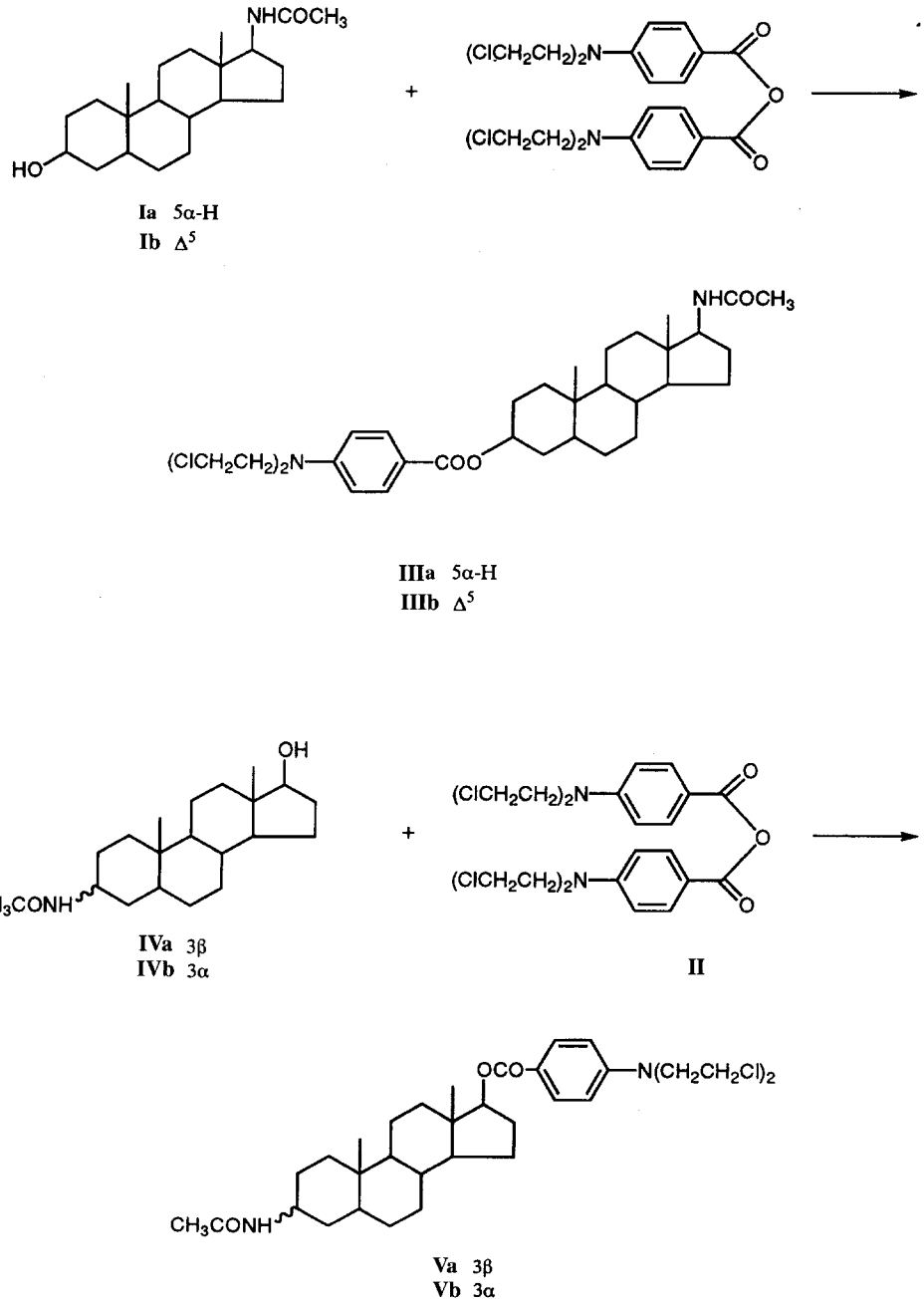
# Dedicated to the memory of Professor Panayotis Catsoulacos



Scheme 1.

3. Results and discussion

As previously shown, several homo-aza- steroidal esters of benzoic acid mustard tested for their antitumour activity were found to possess significant activity against cancer cells [12]. In order to diminish toxicity and to increase the antineoplastic effects, we esterified 4-[N, N-bis(2-chloroethyl)amino]benzoic acid (p-ABA) with amidosteroids having the -CONH- group outside the steroidal nucleus.

We tested these new compounds **IIIa, IIIb, Va, Vb** and the p-ABA for antineoplastic activity against three tumour cell lines (A431, T47D, MIA Pa-Ca-2) in vitro. The

Table I. Cytostatic effect of the compounds tested (p-ABA, **IIIb, IIIa, Va, Vb**), as indicated by percentage of growth (PG), on A431, TD47 and MIA Pa-Ca2 cell lines in different concentrations.

Compounds-tested	Cell lines	Log10 Drug Concentration (M)					
		0.0	−8.0	−7.0	−6.0	−5.0	−4.0
		Percentage growth (%)					
p-ABA	A431	100	105	99	79	61	49
	MIA	100	112	103	98	87	78
	T47D	100	107	101	93	75	63
IIIb	A431	100	99	72	50	23	-4
	MIA	100	108	101	99	95	89
	T47D	100	112	90	79	67	49
IIIa	A431	100	114	108	99	81	79
	MIA	100	99	100	93	80	82
	T47D	100	103	97	87	79	74
Va	A431	100	107	104	101	97	90
	MIA	100	103	105	103	95	89
	T47D	100	110	99	101	92	83
Vb	A431	100	112	104	95	86	71
	MIA	100	98	90	88	81	79
	T47D	100	95	90	88	76	63

results are presented in *table I* and *figure 1*. As it is shown, none of the compounds proved significantly effective in growth inhibition of T47D and MIA Pa-Ca-2 tumour cells *(table I)*. On the other hand, compound **IIIb** appeared to have significant cytostatic effects (G150: 10^{-6} M) against A431 cells *(figure 1)*. Moreover, unpublished results indicate that none of the compounds tested caused toxicity in mice treated with high doses up to 800 mg/kg i.p.

These results may partially lie under the low cytostatic activity of the acid (p-ABA). Furthermore, although the

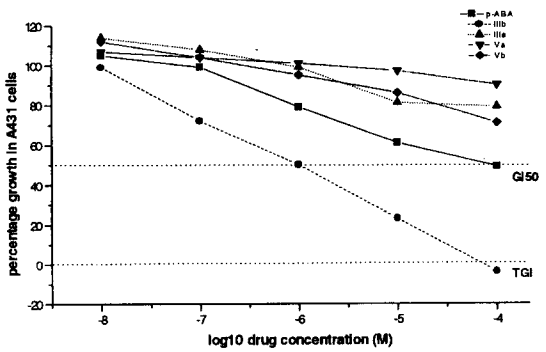


Figure 1. Growth inhibition (GI) activity of the compounds tested (p-ABA, **IIIb, IIIa, Va, Vb**), against the A431 cell line in different concentrations.

three treated tumour cell lines are sensitive in different degrees to steroid hormones (especially T47D cells which carry a high concentration of receptors for a variety of steroids) [13–15], their growth was not affected by the tested steroidal esters.

In conclusion, the hybrid compound 17β-acetamido-3β-hydroxy-5-androsten-4-[N,N-bis(2-chloroethyl)amino] benzoate (**IIIb**) showed a significant (and possibly specific for EGF-dependant tumours) cytostatic effect versus A431 cells, as well as, non toxic effects on mice. Our results are in accordance with previously published data concerning the antitumour effectiveness of a hybrid of 3β-hydroxy-13α-amino-13,17-seco-5-androsten-17-oic-13, 17-lactam-4-[N,N-bis(2-chloroethyl)amino]benzoate [12]. Both studies suggest that the double bond in the steroidal B-ring seems to be of great importance in antineoplastic activity which probably lies in its effect on the stereochemistry.

4. Experimental protocols

4.1. 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 ($DMSO\text{-}d_6$) and are reported in δ units relative to tetramethylsilane (TMS) as an internal standard. Column chromatography was performed utilizing 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.

4.1.1. General procedure for the synthesis of steroidal amidoesters of p-[N,N-bis(2-chloroethyl)amino]benzoic acid

A stirred mixture of 10 mmol of the 4-[N,N-bis(2-chloroethyl)-amino]benzoic acid in 200 mL of dry benzene, 7.55 mmol of dicyclo-hexylcarbodiimide in 20 mL of dry benzene and 7.55 mmol of 4-dimethylamino-pyridine in 20 mL of dry benzene was allowed to stay at room temperature for 6 h. The precipitate of N, N-dicyclohexylurea was filtered off. To the solution, 5.33 mmol of steroid was added and the mixture was refluxed for 72 h. After the reaction time, the solution was washed several times with 3% aqueous hydrochloric acid solution, with aqueous $NaHCO_3$ solution and after, with water. The solvent, after drying over anhydrous magne-

sium sulfate, was removed by evaporation under reduced pressure. The remaining residue was dissolved in 30 mL of chloroform and chromatographed on a column (5.0 × 2.5 cm) of silica gel (60 × 230 mesh, 120 g), that was eluted successively with chloroform.

4.1.1.1. 17β-Acetamido-3β-hydroxy-5α-androstan-4-[N, N-bis(2-chloroethyl)amino]benzoate (**IIIa**)

Yield 48%. M.p. 207–208 °C. I.R. (cm^{-1}): 1 700 (C=O), 1 655 (NHCO), 830, 770, 725, 695 (phenyl). Anal. $C_{32}H_{46}Cl_2N_2O_3$ (C, H, N). ^{1}H-NMR: 0.70 (CH_3-18), 1.07 (CH_3-19), 1.86 (NHCOCH_3), 3.82 [$N(CH_2CH_2)_2$], 4.68 (C_3-H), 7.51 (*NH*$COCH_3$), 6.85–7.84 (arom. prot.).

4.1.1.2. 17β-Acetamido-3β-hydroxy-5α-androsten-4-[N, N-bis(2-chloroethyl)amino]benzoate (**IIIb**)

Yield 45%. M.p. 264–265 °C. I.R. (cm^{-1}): 1 710 (C=O), 1 630 (NHCO), 830, 760, 695, 660 (phenyl). Anal. $C_{32}H_{44}Cl_2N_2O_3$ (C, H, N). ^{1}H-NMR: 0.70 (CH_3-18), 1.00 (CH_3-19), 2.07 (NHCOCH_3), 3.82 [$N(CH_2CH_2)_2$], 4.70 (C_3-H), 5.32 (C_6-H), 7.75 (*NH*-$COCH_3$), 6.89–7.89 (arom. prot.).

4.1.1.3. 3β-Acetamido-17β-hydroxy-5α-androstan-4-[N, N-bis(2-chloroethyl)amino]benzoate (**Va**)

Yield 47%. M.p. 214–215 °C. I.R. (cm^{-1}): 1 710 (C=O), 1 635 (NHCO), 830, 770, 700 (phenyl). Anal. $C_{32}H_{46}Cl_2N_2O_3$ (C, H, N). ^{1}H-NMR: 0.82 (CH_3-18), 0.90 (CH_3-19), 1.80 (NHCOCH_3), 3.82 [$N(CH_2CH_2)_2$], 4.72 (C_{17}-H), 7.71 (*NH*$COCH_3$), 6.86–7.83 (arom. prot.).

4.1.1.4. 3α-Acetamido-17β-hydroxy-5α-androstan-4-[N, N-bis(2-chloroethyl)amino]benzoate (**Vb**)

Yield 40%: M.p. 140–142 °C. I.R. (cm^{-1}): 1 700 (C=O), 830, 765, 695, 660 (phenyl). Anal. $C_{32}H_{46}Cl_2N_2O_3$ (C, H, N). ^{1}H-NMR: 0.82 (CH_3-18), 0.90 (CH_3-19), 1.80 (NHCOCH_3), 3.82 [$N(CH_2CH_2)_2$], 4.70 (C_{17}-H), 7.65 (*NH*$COCH_3$), 6.86–7.83 (arom. prot.).

4.2. Biology

4.2.1. Materials and methods

In order to determine the cytostatic and/or cytotoxic effects of the tested compounds, we used three established human cancer cell lines: MIA Pa-Ca-2 pancreatic carcinoma, T47D breast carcinoma, and A431 scams cell carcinoma. MIA Pa-Ca-2 and A431 cells were cultured in DMEM medium, supplemented with 10% FCS, glutamine and antibiotics. T47D cells were cultured in complete RPMI 1640 medium. Cells were plated in 96-well microtitre plates at a cell density of 10^3 cells/mL and cultured in a CO_2 incubator. The cells were treated with the five test compounds for 48 h in 5 dose concentrations ranged between 10^{-8}–10^{-4} M. All the test compounds were dissolved in DMSO in appropriate dilutions.

The antineoplastic effects of the test compounds were evaluated using the MTT tetrazolium salt assay [16]. 50 μL of MTT stock solution (5 mg/mL) were added to each well and incubated for 3 h at 37 °C to allow metabolization. The formazan crystals were solubilized by adding 100 μL acidic isopropanol (0.04 N HCl in absolute isopropanol) per well. Absorbance of converted dye was measured at a wavelength of 540 nm on an ELISA reader. The MTT assay gives a quantitative estimation of the mitochondrial and cytoplasmic reductase system activity of the viable cells [13].

The in vitro screening methodology and the measurement of the effect of the compounds on the cell lines were carried out in accordance with the National Cancer Institute (NCI) standard procedures [17, 18]. The measured effect of the compounds on each cell line was calculated according to one or the other of the following two expressions:

i) If (mean OD_{test} – mean OD_{tzero}) ≥ O, then PG (percentage growth) = 100 × (mean OD_{test} – mean OD_{tzero})/(mean OD_{ctrl} – mean OD_{tzern}).

ii) If (mean OD_{test} – mean OD_{tzero}) < O, then PG = 100 × (mean OD_{test} – mean OD_{tzero})/mean OD_{tzero}.

where: mean OD_{tzero} = the average of optical density measurements of MTT-derived colour just before exposure of cells to the test compound, mean OD_{test} = the average of optical density measurements of MTT-derived colour after 48 h exposure of cells to the test compound and mean OD_{ctrl} = the average of optical density measurements of MTT-derived colour after 48 h with no exposure of cells to the test compounds.

References

[1] Wall M.E., Abernethy J.R.S., Carroll E.I., Taylor D.J., J. Med. Chem. 12 (1969) 810–818.

[2] Wampler G.L., Catsoulacos P., Cancer Treat. Rep. 61 (1977) 37–41.

[3] Catsoulacos P., Politis D., Wampler G.L., Cancer Chemother. Pharmacol. 10 (1983) 129–132.

[4] Catsoulacos P., Politis D., Wampler G.L., Cancer Chemother. Pharmacol. 3 (1979) 67–70.

[5] Catsoulacos P., Boutis L., Cancer Chemother. Rep. 57 (1973) 365–367.

[6] Catsoulacos P., Wampler G.L., Oncology 39 (1982) 109–112.

[7] Catsoulacos P., Cancer Lett. 22 (1984) 199–202.

[8] Everett S.L., Ross W.C.J., J. Chem. Soc. (1949) 1972–1983.

[9] Tanabe S., Onda M., Yakugaku Zasshi 72 (1952) 944–945.

[10] Schmidt-Thome J., Ber. 88 (1955) 895–899.

[11] Janot M.M., Khuong Huu Q., Gutarell R., Memoires Presentes a la Societe Chimique (1966) 1640–1643.

[12] Anastasiou A., Catsoulacos P., Papageorgiou A., Margariti E., J. Heterocycl. Chem. 31 (1994) 367–374.

[13] Berridge M.Y., Tan A.S., Arch. Biochem. Biophys. 303 (1993) 474–482.

[14] Benz C., Hollander C., Miller B., Cancer Res. 46 (1986) 2276–2281.

[15] Blomgoist C.H., Leung B.S., Zhang R., Zhu Y., Chang P.M., J. Cell. Biochem. 59 (1995) 409–417.

[16] Mosmann T., J. Immunol. Meth. 65 (1983) 55–63.

[17] Carmichael J., Mitchell J.B., De Graff W.G., Gamson J., Gazdar A.F., Johnson B.E., Glatstein E., Minna J.D., Br. J. Cancer 57 (1988) 540–547.

[18] Chabner B.A., J. Nat. Cancer Inst. 82 (1990) 1083–1085.