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ORIGINAL ARTICLE

Preclinical studies of steroid-linked nitrosoureas in murine pancreatic adenocarcinoma PANO2

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Summary

Purpose: *In earlier studies, this laboratory carried out research on the synthesis and anticancer evaluation of hybrid compounds, which combine two molecules in one such as homo-aza-steroidal esters (HASE) of carboxylic derivatives of N, N-bis (2-chloroethyl) aniline. In this combination, steroidal hormones are employed as carriers for transporting the alkylating agents to specific targeted tissues. Aiming to continue our research, we used alkylating agents, as nitrosoureas, instead of nitrogen mustards. In this work the N-[N-(2-chloroethyl)-N-nitroso-carbomoyl]-L-alanine (CNC-ala) has been used and was bound to 7 newly synthesized modified steroidal esters (carrier molecule) of nitrosourea and the hybrid molecules were tested for antitumor activity against PANO2 murine pancreatic adenocarcinoma.*

Materials and methods: *PANO2 adenocarcinoma was used in this study. C57Bl mice were used for chemotherapy evaluation. The activity was assessed from the inhibition of tumor growth and the oncostatic parameter T/C %.*

Results: *The antitumor activity displayed by 7 hybrid steroidal esters of nitrosourea was quite interesting. It was able to discern 4 of 7 compounds that exhibited considerable antitumor activity, increasing the lifespan of the tumor-bearing mice by inhibiting the tumor growth.*

Conclusion: *The comparative study of 7 newly synthesized hybrid steroidal esters of nitrosourea shows that the antitumor effects of compound 7, which has an enlarged (7 carbon atoms) A-lactamic ring and nitrosourea esterified at the position 17, which seems to be the most appropriate for the connection of a DNA cross-linking amino acid derivative is superior.*

Key words: experimental chemotherapy, nitrosoureas, pancreatic adenocarcinoma

Introduction

The discovery of steroid hormones and hormone responsiveness in a series of human tumors, such as breast cancer [1,2], prostate cancer, ovarian and endometrial cancers [3,4], gastrointestinal cancers [5,6], and small cell lung carcinoma [7], has led to the concept of a receptor-mediated chemotherapy. Several investigators have focused on the synthesis of steroid hormone-linked cytotoxic agents in order to direct the cytotoxic moiety specifically to the hormone receptor containing tumor tissue and by doing so to achieve higher antitumor efficacy and lower toxicity.

These studies have indicated that the chemical link between cytotoxic moiety and hormone should allow for release of the cytotoxic moiety by hydrolysis or by enzymatic cleavage. Consequently, an ester-type link has appeared to be appropriate [8,9]. At present only prednimustine (prednisolone ester of chlorambucil) and estramustine are hormone-linked antitumor compounds that carry clinical promise [10].

Nitrosoureas such as N, N-bis (chloroethyl)-N-

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nitrosourea (BCNU), N-(2-chloroethyl)-N-cyclohexyl-N-nitrosourea (CCNU) and N-(2-chloroethyl)-N-(4-methylcyclohexyl)-N-nitrosourea (MeCCNU) are important antitumor agents in the treatment of human cancer. In addition, a number of new nitrosoureas with better therapeutic properties have been synthesized and tested for cytostatic activity. Among those with good therapeutic activity against various tumor models were nitrosourea in which the nitrosocarbomoyl residue was linked to amino acid.

In this work the N-[N-(2-chloroethyl)-N-nitrosocarbomoyl]-L-alanine has been used and 7 steroidal esters of nitrosourea have been synthesized, namely 17β-[N- [N-(2-chloroethyl)-N-nitrosocarbamoyl]-L-alanyl]-androst-4 en-3-one (1), 17β-[N- [N-(2-chloroethyl)-N-nitrosocarbamoyl]-L-alanyl]-5a-androstan-3-one (2), 3β-[N-[N-(2-chloroethyl)-N-nitrosocarbamoyl]-L-alanyl]-androst-5-en-17-one (3), 3β-[N-[N- (2-chloroethyl)-N-nitroso-carbamoyl]-L-alanyl]-5a-androstan-17-one (4), 17β-[N-[N-(2-chloroethyl)-N-nitroso-carbamoyl]-L-alanyl]-17-methyl-androst-4-en-3-one (5), 3β-[N-[N-(2-chloroethyl)-N-nitrosocarbamoyl]-L-alanyl]-androst-5-en-17β-acetamid (6), and 17β-[N-(2-chloroethyl)-N-nitroso-carbamoyl]-L-alanyl]-3-aza-A-homo-4a-androsten-5-one (7) in order to study structure and biological activity relationship (Figure 1).

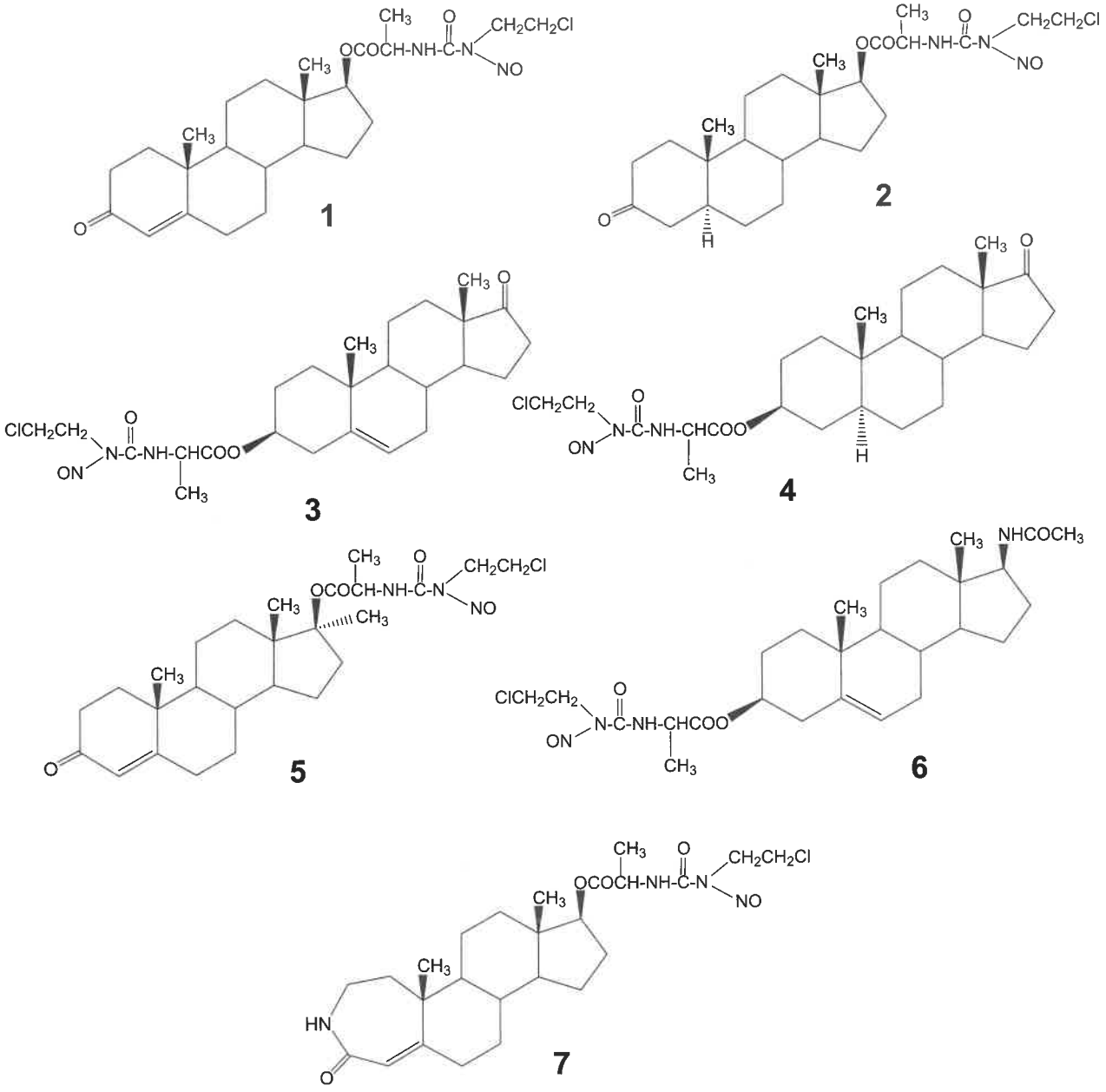


Figure 1. Chemical structure of steroid-linked nitrosoureas.

Testosterone, epiandrosterone, 17a-methyl testosterone, 3β´hydroxy-androst-5-en-17-acetamide and 17β-hydroxy-3-aza-A-homo-4a-androsten-5-one were used as carrier hormones and were linked to cytotoxic N-[N-(2-chloroethyl)-N-nitrosocarbamoyl]-L- alanyl by an esteric bond. However, the usual procedures for the esterification of C-3 and C-17 hydroxylic group of the above steroids were affected in anhydrous dichloromethane in the presence of p-dimethylaminopyridimide as catalyst and of dicyclohexylcarbodimide as dehydrating agent. Elemental analysis (C, H, N) and both infrared (IR) and nuclear magnetic resonance (NMR) spectra supported the structure of the prepared compounds 1-7.

Materials and methods

Chemicals

The compounds 1-7 were freshly formulated by dissolution in a small amount of diethyl sulfoxide (DMSO, 10% of final volume) and dilution in corn oil, to produce a fine suspension at the desired concentration.

Animals

Male and female C57 Bl mice, 4-6 weeks of age and weighing 12-28 g, were used for antitumor evaluation. The mice were provided by the Experimental Animal Production Laboratory, Theagenion Cancer Hospital, and were under conditions of constant temperature (22° C) and humidity (55±10%), with sterile bedding, water and food.

Tumor

The pancreatic ductal adenocarcinoma PAN02 was used in this study. The tumor was purchased from the DCTD, NCI, Frederick, MD, USA. C57 Bl mice of both sexes were used for chemotherapeutic evaluation. The tumor was implanted in the axillary region with puncture in the inguinal region. The sacrificed donor animal was pinned to a dissecting board, dorsal surface up. The tumor was transferred to a sterile Petri dish placed over ice and cleared of any necrotic material. The tumor was cut into cubes, usually about 2×2×2 mm. With forceps, the tumor fragments were placed into the bevel of a 13-gauge trocar. The area of the implant was swabbed with 70% ethanol and the tumor fragment was inoculated subcutaneously. The animals were allowed to rest for 24 h and subsequently divided into the indicated number of groups, which received intraperitoneal treatment.

Antitumor evaluation

Standard propagation methods were used for testing the cytostatic effect of the compounds. The antitumor activity against PAN02 was assessed from the inhibition of tumor growth by volume in cubic centimetres (cm^3), according to the protocol of the experimental evaluation of antitumor drugs of the NCI. The tumor size was measured on days 22, 25, 28 with a microvernier and calculated from 3-dimensional measurements using the formula $a \times b \times c/2$ cm^3, where a = length, b = width, c = depth at the site of transplantation (average tumor volume, ATV). The oncostatic parameter T/C%, the median survival time of drug-treated animals (T) vs. saline-treated controls (C), was also used.

Treatment

Experimental groups of 6 mice in each drug-treated group and 8 mice in the control group were used. The experiments were initiated by implanting the mice with the tumor cells. Treatments were given as a single dose (LD10) on day 1 after transplantation; intermittent doses (LD10/2×3 days) were given on days 1, 4, 7 or on days 1-9 (LD10/4×9 days).

Results

The results of the antitumor activity of compounds 1-7 are presented in Table 1. Based on single, intermitted or consecutive treatment schedules, we were able to discern 4 of 7 compounds that displayed considerable antitumor activity against pancreatic adenocarcinoma PANO2. These chemicals were determined to increase strongly the lifespan of the tumor-bearing mice by inhibiting the tumor growth.

Compound 7

This compound was superior, causing the maximum cytostatic effect. Treatment with 500 mg/kg (single dose), 250 mg/kg (intermitted treatment schedule) or 125 mg/kg (consecutive treatment), produced T/C values of 187, 203 and 191%, whereas the inhibition of tumor growth was 72, 89 and 75%, respectively. Evidently, higher antitumor effect was obtained when the intermitted treatment on days 1, 4 and 7-post implantation of the tumor was used.

Compounds 2 and 6

Administration of compounds 2 and 6 on a inter-

Table 1. Antitumor activity of compounds 1-7 on pancreatic adenocarcinoma PANO2

Compound	*Treatment schedule*	*Dosage (mg/kg)*	*TI% (on day 28)*	*T/C%*	*Cures*
1	day 1	500	7	114	0/6
	days 1, 4, 7	250	34	137	0/6
	days 1-9	125	10	125	0/6
2	»	500	43	166	0/6
	»	250	**74**	**189**	0/6
	»	125	51	171	0/6
3	»	500	9	115	0/6
	»	250	38	138	0/6
	»	125	17	124	0/6
4	»	400	13	121	0/6
	»	200	41	139	0/6
	»	100	23	127	0/6
5	»	400	37	152	0/6
	»	200	**70**	**177**	0/6
	»	100	42	164	0/6
6	»	500	37	151	0/6
	»	250	**73**	**182**	0/6
	»	125	40	159	0/6
7	»	500	72	187	0/6
	»	250	**89**	**203**	0/6
	»	125	75	191	0/6

TI: tumor inhibition, T/C: tumor bearing mice/controls
Numbers in bold indicate significant T/C values

mitted treatment schedule yielded almost equal antitumor effect, increasing the lifespan of PANO2-bearing mice by 103 and 89% and inhibiting the tumor growth by 81 and 74%.

Compound 5

The effect obtained with the compound 5 was slightly lower compared with that of compounds 7, 2 and 6.

Compounds 1, 3 and 4

These compounds were the less active and we accept that their effect was marginal.

Discussion

Nitrosoureas are important bifunctional antitumor agents that have demonstrated activity against a wide spectrum of human malignancies. A large number of high lipophilicity nitrosoureas derivatives have been reported as highly active in experimental tumor systems. BCNU has been used since 1971 as an antineoplastic agent in the treatment of Hodgkin's lymphoma, multiple myeloma and primary or metastatic brain tumors. Although the mechanism of action is not yet fully established, it is generally assumed that the derivatives of nitrosoureas, which include compounds such as BCNU, CCNU and methyl-CCNU exert their cytotoxicity through the liberation of alkylating and carbomoylating moieties [11,12].

The antineoplastic nitrosoureas have the capacity to undergo spontaneous non- enzymatic degradation with the formation of variety of products such as the methyl carbonium ion (BCNU compounds) and the 2-chloroethyl carbonium ion (CCNU compounds), strongly electrophilic and alkylating, a variety of substances (guanine, cytosine and adenine), a number of which are derived from the attachment of halo ethyl group to nucleophilic sites on DNA bases. Displacement of halogen atom can then lead to inter- or intrastrand cross-linking of DNA. The formation of cross-links after the initial alkylation is a relatively slow process and can be interrupted by a DNA repair enzyme. It is generally accepted that interstrand cross-linking is associated with the cytotoxicity of nitrosoureas, as with the nitrogen mustards [11,12].

In addition to the generation of carbonium ions, the spontaneous degradation of nitrosoureas liberates organic isocyanates that are capable of carbomoylat-

ing lysine residues of proteins. The nitrosourea-type moiety has been attached to various carrier molecules with alterations in crucial properties such as tissue specificity, distribution and toxicity. On the other hand the concept of a receptor-mediated chemotherapy has been based on the discovery of steroid hormone receptors and hormone responsiveness in a series of human tumors such as breast, prostate, ovarian, endothelial and gastrointestinal cancers [6]. In order to specifically direct a cytotoxic group to a hormone receptor-containing tumor and so to achieve higher antitumor activity and lower toxicity, steroid hormone-linked cytotoxic agents have been synthesized. The chemical link between the cytotoxic group and a hormone should be hydrolyzed or enzymatically degradated, allowing for the release of the cytotoxic moiety. An ester-type link has appeared to be appropriate.

In the present comparative study of 7 newly synthesized steroidal esters of nitrosourea, the antitumor effect of compound 7 was superior. It is remarkable that only this compound has an enlarged (7 carbon atoms) A-ring, modified with a lactam group (-NHCO-) and the nitrosourea moiety esterified at the position 17 (Figure 1). It seems that the position 17 of the steroid is the most appropriate for connection of a DNA-cross-linking amino acid derivative. We suppose that the lactam group interacts with similar groups existing in DNA, RNA and proteins and, also, has the possibility to be transformed by a metabolic process or at least by an enzymatically catalyzed reaction to the active species: $-NHCO^+$, $-N-H^-$ and $-C=O^+$. These species could attack the constituents of rodent and human cancer cells, acting synergistically with the alkylating congener.

In the present study we have indicated that the structure alteration of the most potent in inducing antineoplastic effect compound 7 confirm our previous findings that the biological activity of molecules is directly correlated with their chemical structure. Finally, we conclude that further preclinical investigation appears warranted.

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