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STEROID-NUCLEOSIDE INTERACTIONS WITH RECEPTORS

DAVID T. ZAVA, WILLIAM L. MCGUIRE and JOHAN E. VAN LIER[1]
Department of Medicine, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284, U.S.A., and
[1]Département de Médecine Nucléaire et de Radiobiologie, Centre Hospitalier Universitaire de Sherbrooke, Sherbrooke, Québec, Canada J1H 5N4

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SUMMARY

Steroidal pyrimidines (steroid-nucleosides) have been suggested as potential agents for cancer chemotherapy. In order to evaluate the notion that the steroid moiety of such agents provides organ specificity, a series of steroid-nucleosides have been evaluated for their steroid binding properties. The 21-substituted progesterone derivatives showed affinity for androgen receptor whereas some of the 21-substituted corticoids exhibited affinity for glucocorticoid receptor. None of the 3- or 21-substituted steroids showed significant binding to estrogen or progesterone receptor.

INTRODUCTION

Steroid-nucleosides (SN) have recently been advanced as potential organ-selective agents for cancer chemotherapy [1]. The products consist of steroids coupled via a C–N linkage with naturally occurring pyrimidines. As with conventional steroidal combination drugs [2, 3], the steroid moiety should provide the desired organ specificity. However, steroid-nucleosides differ from such drugs in that neither component is cytotoxic. Instead, the coupled products may behave as nucleoside analogs, with antitumor activity resulting from interference at the DNA duplication level.

Preliminary testing of a series of steroidal pyrimidines linked at C_3 and C_{21} suggested that some of the steroid–base combinations possess antitumor activity against the hormone-sensitive 13762 mammary adenocarcinoma in Fisher 344/CRBL rats [1]. Detailed screening of the drugs against the L-1210 lymphoid leukemia in female mice (NIH screening program, Bethesda, Maryland) showed presumptive activity only for compound 4, NCS 281832 [4]. Further screening of this product is planned. In order to evaluate the potential target specificity of these products, we have now studied the interaction of several 3- and 21-substituted steroids with estrogen, androgen, progesterone and glucocorticoid receptors under cell-free conditions.

EXPERIMENTAL

(1) *Steroid-nucleosides.* Coupling between steroids and pyrimidines was accomplished by modifying established methods of nucleoside synthesis [5, 6]. Briefly, monohydroxysteroids were converted to the bromo- or mesyl-derivative and reacted with persilylated pyrimidines in the presence of an appropriate catalyst. C–N linked products were separated by extensive chromatography. Configurations were assigned to the purified isomeric products on the basis of n.m.r. analysis (manuscript in preparation).

(2) *Interaction of steroid-nucleosides with steroid hormone receptors.* The affinity of steroid-nucleosides for steroid hormone receptors was determined by competition studies using a modified protamine sulfate assay [7]. Cytosols were prepared in phosphate buffer (5 mM sodium phosphate, pH 7.4 at 4°C, 10 mM thioglycerol, and 10% glycerol) from MCF-7 human breast cancer cells (estrogen receptor), uteri of mature castrate Sprague–Dawley rats injected with 1 μg of estradiol for 2 days (progesterone receptor, androgen receptor) and livers of mature castrate male Sprague–Dawley rats (glucocorticoid receptor). These systems have been well characterized by others and in our own laboratory and were chosen as a matter of convenience.

Cytosols were diluted (203 mg protein/ml phosphate buffer) and then 250 μl was added to 12 × 75 mm glass tubes (prewashed with 0.1% bovine serum albumin) containing 250 μl of [^{3}H]-17β-estradiol or [^{3}H]-5α-dihydrotestosterone (final concentration, 5 nM) or [^{3}H]-R5020 or [^{3}H]-dexamethasone (final concentration, 10 nM). Various concentrations of nonradioactive steroids or a 100- and 1000-fold excess of steroid-nucleosides were coincubated in parallel with the radioactive ligands.

After 4 h at 2°C, receptor was precipitated with 250 μl protamine sulfate (1:10 phosphate buffer dilution of USP injection, Eli Lilly Co.). The receptor ligand precipitate was sedimented by centrifugation at 600 *g* for 10 min, then twice washed with 1 ml of phosphate buffer. Radioactivity in the protamine pellets was extracted twice with 2.5 ml toluene based scintillation fluid (4.0 g PPO, 0.05 g POPOP, 1 l.

Table 1. Affinity of steroid-nucleosides for steroid receptors

No.	STEROID-NUCLEOSIDE (SN)	NSC number	% COMPETITION at 100- and 1000-fold excess of SN							
			ER		DHTR		PgR		DexR	
1	3α-(1H-thymin-1-yl)-pregn-5-en-20-one	281829	0	0	0	0	0	9	11	22
2	3β-(1H-thymin-1-yl)-pregn-5-en-20-one	281830	0	0	0	6	17	21	7	27
3	3α-(1H-uracil-1-yl)-pregn-5-en-20-one	281831	0	0	0	0	7	12	20	40
4	3α-(3H-uracil-3-yl)-pregn-5-en-20-one	281832	0	0	0	0	6	16	16	26
5	3α-(1H-5-fluorouracil-1-yl)-pregn-5-en-20-one	281833	4	5	0	3	3	11	0	18
6	21-(1H-thymin-1-yl)-pregn-4-ene-3,20-dione	281834	0	0	21	64	7	24	6	64
7	21-(1H-uracil-1-yl)-pregn-4-ene-3,20-dione	281835	0	2	12	62	4	14	0	60
8	21-(1H-5-fluorouracil-1-yl)-pregn-4-ene-3,20-dione	281836	0	0	9	57	6	25	0	60
9	21-(1H-thymin-1-yl)-pregn-4-en-17α-ol-3,11,20-trione	281837	0	0	0	0	9	0	0	52
10	21-(1H-uracil-1-yl)-pregn-4-en-17α-ol-3,11,20-trione	281838	0	1	0	42	4	9	32	70
11	21-(3H-uracil-1-yl)-pregn-4-en-17α-ol-3,11,20-trione	281839	0	0	0	0	0	2	0	45
12	21-(1H-5-fluorouracil-1-yl)-pregn-4-en-17α-ol-3,11,20-trione	281840	0	2	26	34	0	16	38	64
13	21-(3H-5-fluorouracil-3-yl)-pregn-4-en-17α-ol-3,11,20-trione	281841	0	0	2	34	10	14	17	67
14	21-(3H-thymin-3-yl)-pregn-4-en-17α-ol-3,20-dione	281842	1	7	0	9	1	3	–	28
15	21-(3H-uracil-3-yl)-pregn-4-en-17α-ol-3,20-dione	281843	6	9	0	2	0	4	0	26
16	21-(3H-5-fluorouracil-3-yl)-pregn-4-en-17α-ol-3,20-dione	281844	0	1	0	0	0	0	0	22

The concentration of the [^{3}H]-steroid in each assay was 10^{-9} M for the estrogen (ER) and androgen receptors (DHTR), and 10^{-8} M for the progesterone (PgR) and glucocorticoid receptors (DexR). The competition is expressed as the percentage of [^{3}H]-steroid displaced from the receptor at a 100- and 1000-fold excess of SN.

toluene) and counted in a Beckman LS 233 counter with a counting efficiency of 50%.

The [^{3}H]-steroid bound to receptors in the protamine pellet was measured and the percentage reduction in binding by the competing non-radioactive ligands was calculated.

RESULTS

Table 1 shows the percentage reduction in binding of four [^{3}H]-steroids by a 100- and 1000-fold excess of steroid-nucleoside (SN). Products which competed at both concentrations were considered as having a

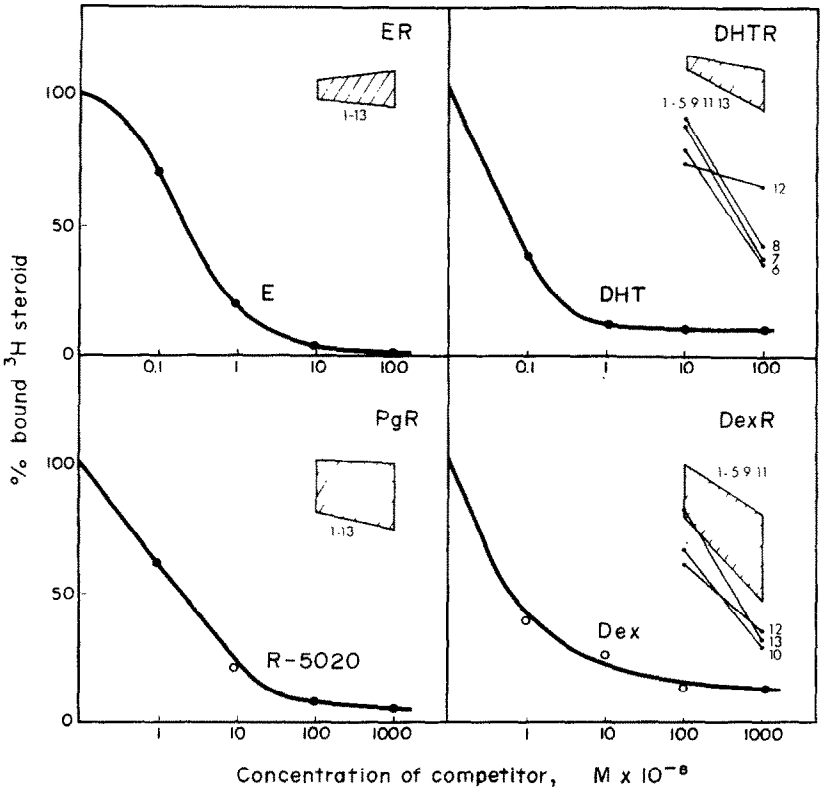

Fig. 1. Competition between steroid-nucleosides 1–16 or unlabeled steroids and [^{3}H]-steroids for cytoplasmic receptors. ER, estrogen receptor; DHTR, androgen receptor; PgR, progesterone receptor; DexR, glucocorticoid receptor; E, estradiol; DHT, dihydrotestosterone; R-5020, 17α,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; Dex, dexamethasone. The percentage of bound [^{3}H]-steroid at each concentration of the competitor is compared to bound [^{3}H]-steroid in the absence of competitor.

significant affinity for the receptor. Figure 1 shows the competition of the active products together with the displacement curve of [^{3}H]-steroid by the corresponding nonlabeled steroids.

Affinity of SN for androgen receptor

Significant displacement of [^{3}H]-dihydrotestosterone from the androgen receptor was observed for all three 21-substituted progesterone derivatives (5, 6, 7) and to a lesser extent for the N_1, 21-linked 5-fluorouracil derivative of 21-desoxycortisone (12). None of the pregnenolone or 17-hydroxyprogesterone analogs showed affinity for androgen receptor.

Affinity of SN for glucocorticoid receptor

Both the N_1 and N_3 5-fluorouracil coupling products with 21-desoxycortisone (12, 13) showed affinity for glucocorticoid receptor. The 21-uracil N_1 analog (10) exhibited a similar activity whereas its N_3-isomer (11) showed much less affinity. The 21-pyrimidine derivatives of progesterone and 17-hydroxyprogesterone showed little competition for binding, while in the pregnenolone series, only the 3α, N_1-uracil derivative showed some affinity.

Affinity of SN for estrogen receptor

None of the steroid-nucleosides tested exhibited competition for binding in this assay, even at a 1000-fold excess over the [^{3}H]-estradiol.

Affinity of SN for progesterone receptor

Little interaction of steroid-nucleosides with progesterone receptor was observed. At a 1000-fold excess over [^{3}H]-R-5020, the 21-substituted progesterone derivatives (5, 6, 7) displaced only up to 25% of the labeled steroid, and at a 100-fold excess, the competition for binding was insignificant.

DISCUSSION

The basic requirements for steroidal combination drugs to function as organ-selective antitumor agents are 2-fold: the product should retain an affinity for steroid receptors, and it should inhibit cell division. Furthermore, a relatively stable linkage between the two moieties of the drug is essential. Liberation of free steroid could result in saturation of the receptors, resulting in a consequent loss of affinity of the target tissue for the therapeutic agent. Most of the earlier reported combination drugs were obtained via ester linkage of the steroid and cytotoxic moiety [2, 3]. Although *in vivo* testing was promising [8], *in vitro* studies suggested that such complexes hydrolyse and that their effect may be attributed to the action of the individual chemical entities [9]. Steroidal alkylating agents linked via more stable C–N bonds have recently been prepared and their potential therapeutic activity has been suggested [10, 11].

In the case of our steroid-nucleosides, *in vivo* stability is of particular importance since their separate components are normal cell constituents devoid of antitumor properties. With the exception of the 5-fluorouracil derivatives, only the coupled products are expected to possess antitumor properties as a result of their resemblance to nucleosides. On the basis of such considerations, we selected the inert C–N bond as a linkage between the steroid and the pyrimidine base and the chemically accessible 3-position of pregnenolone and the 21-position of progesterone and corticoids for base attachment.

Of the SN evaluated in the present study, the 3-substituted pregnenolone analogs show little affinity for any of the four receptors. In contrast, the 21-substituted steroids showed significant affinity: the progesterone derivatives towards androgen receptor with activities comparable to those of antiandrogens, and the corticoid derivatives towards glucocorticoid receptor. Although the affinities are at least 1000 times less than those of the competing ^{3}H-steroids, irreversible binding could have therapeutic value due to progressive exhaustion of cytoplasmic receptor [10]. Accordingly, it will be of interest to evaluate such compounds by *in vitro* studies with tumor cell lines which contain such receptors. Since 21-acetoxyprogesterone is known to possess some affinity for progesterone receptor [12], it is surprising that our 21-pyrimidyl derivatives 5, 6 and 7 lack any such binding properties. It may be that intramolecular hydrogen bonding between the pyrimidine and the acetyl side-chain of the steroid limits the rotational freedom of the latter, preventing steroid–receptor interaction. Although differences in receptor affinity between N_1 and N_3 base attachment, or type of base, can be noted, it is obvious that the affinity properties of the steroid-nucleosides are mainly determined by the coupling site on the steroid molecule and by the nature of the steroid.

The possibility that the observed affinities result from impurities or decomposition products of our SN can be ruled out by the results observed for progesterone derivatives. If samples 6, 7 or 8 decomposed to yield even 0.1% of free progesterone, they would show marked competition for progesterone receptors and less affinity for androgen and corticoid receptors. The results of Table 1 are incompatible with this possibility.

Studies of the relationship between structure and activity of substituted glucocorticoids and estrogens indicate that carbon atoms remote from the functional group are the most suitable candidates for alterations aimed at retaining or augmenting steroid receptor affinity [13, 14]. Accordingly, we are presently pursuing the synthesis and evaluation of 6- and 16-substituted estrogens and corticoids. Further development of our synthetic approach will be guided by *in vitro* evaluation of steroid receptor affinity and by studies of effects on selected tumor cell lines.

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