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THE SYNTHESIS OF ^3H LABELLED STEROID-NITROGEN MUSTARD DERIVATIVES AND STUDIES ON THEIR ACTION IN RAT AND HUMAN PROSTATE

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SUMMARY

Tissue receptor proteins are thought to be a key factor in selective retention and nuclear translocation of steroid hormones in their target tissues. Steroid-cytotoxic derivatives with high affinity for these receptor proteins may, therefore, be useful as site directed agents in the treatment of hormone dependent cancers. The prostatic retention and subcellular localisation of estramustine and a 17β nitrogen mustard derivative of 5α-dihydrotestosterone have been compared since the latter compound has higher androgen receptor binding affinity than the former. The 5α-dihydrotestosterone derivative was not retained more avidly than estramustine by either human or rat ventral prostatic tissue. Nuclear localisation could not be detected with either compound.

The cytotoxic action of these compounds requires the liberation of the nitrogen mustard moiety. The cleavage rate of both compounds was low but similar in both benign and malignant prostatic tissues.

We conclude that steroids are unlikely to be suitable agents for directing cytotoxic compounds to prostatic tissue particularly if the steroid-cytotoxic derivative requires metabolism for the expression of the cytotoxic activity.

INTRODUCTION

Since steroid hormones can be accumulated by their target tissues with some selectivity [1, 2] they may be used to direct agents specifically to these tissues. This idea has considerable appeal for the development of site directed cytotoxic agents for the treatment of cancers of the breast and prostate.

An initial attempt to develop such a compound was the drug ESTRACYT (A. B. Leo, Helsingborg, Sweden), a nitrogen mustard derivative of oestradiol-17β. This drug had little effect against its putative target, carcinoma of the breast, but has been found to be efficacious in the treatment of some prostatic adenocarcinoma [3, 4]. ESTRACYT a phosphate derivative of estramustine, is administered by mouth. On absorption the phosphate group is rapidly removed [5] and the resulting estramustine (E_2-NM) is the form of the drug that is found in plasma and which we have used in our studies.

The action of steroidal hormones in their target tissues involves interaction with specific cytosolic receptor proteins and subsequent translocation of the steroid receptor complex to the nucleus [6]. Binding to receptor proteins is thought to be a key factor in steroid retention by target cells. It thus may be argued that a steroid-cytotoxic derivative with high receptor binding affinity would be retained more selectively by target tissues than by other tissues. Furthermore, receptor binding may also permit the translocation of these compounds to the nucleus perhaps potentiating their cytotoxic action, particularly in the case of alkylating agents.

In the prostate the major receptor protein has a high affinity for 5α-dihydrotestosterone [7]. This protein has virtually no binding affinity for estramustine. A nitrogen mustard derivative of 5α-dihydrotestosterone (DHT-NM) was reported to be bound with higher affinity than estramustine by the receptor [8]. We were interested to compare the action of these two compounds with respect to prostatic accumulation relative to other tissues and subcellular localisation.

Despite the similarities between rat ventral and human prostate with regard to the mechanism of action of androgens and specifically their respective androgen receptor proteins [9] this animal is not an ideal model for this study. The rat ventral prostate contains very large amounts (some 20% of total protein) of a protein that binds estramustine with high affinity [10]. This protein is in much lower concentration in human prostatic tissue [21]. Furthermore this protein has little affinity for DHT-NM (Forsgren, personal communication). We have, therefore, attempted to develop an *in vitro* method to study the retention of these compounds by human prostate.

Nitrogen mustards are cytotoxic because of their action as alkylating agents. Their chemical bonding to the steroid prevents the alkylating action. Thus the

Trivial names: Estramustine—oestradiol-17β-3*N*-*bis*-(2-chlorethyl) carbamate. DHT-NM—5α-dihydrotestosterone 17β*N*-*bis*-(2-chlorethyl) carbamate.

cytotoxic effect of the steroid-nitrogen mustards is likely only to be expressed if the molecules undergo metabolism to liberate the cytotoxic moiety [11]. We have, therefore, studied the metabolism of estramustine and DHT-NM in both benign and carcinomatous human prostatic tissue.

Our results are discussed in relation to the design of steroid linked cytotoxic agents.

MATERIALS AND METHODS

Materials

The reagents D-[U-^{14}C]-glucose, [6,7-^{3}H]-oestradiol and 5α-dihydro[1α,2α(*n*)-^{3}H]-testosterone were purchased from the Radiochemical Centre, Amersham, Bucks, U.K., unlabelled steroids from Steraloids, Croydon, U.K., *bis*-(2-chloroethyl)-amine hydrochloride from Aldrich Chemical Co., Gillingham, Dorset, U.K., 13181 Silica gel on plastic thin layer plates No. 6060 from Eastman Kodak Co, Rochester, NY 14650, U.S.A., alumina for column chromatography from ICN Pharmaceuticals GmBH and Co., 3440 Eschwege, G.F.R., Soluene-350 from Packard Instrument Co, Caversham, Berks., U.K.

All other chemicals were of the highest available purity and purchased from BDH, Poole, Dorset, U.K.

Tissues

Human benign prostatic tissue was obtained after retropubic prostatectomy. Human prostatic andenocarcinoma tissue was obtained after surgery by the transurethral route.

Tissues were cut using a Stadie Riggs microtome [12] into 0.5 mm slices unless otherwise indicated. Slices from benign tissue were cut from dissected nodules. Only slices cut from the centre of the tissue chips were used in the case of material obtained after transurethral prostatectomy.

Rat ventral prostates were coarsely chopped with scissors. Slices (0.5 mm) were cut from all other rat tissues used.

All tissues were used within 10 min of removal.

Incubation

All incubations were conducted in Krebs–Ringer bicarbonate buffer containing glucose. Incubations were carried out for a total of 1 h at 37°C, the medium was renewed after 0.5 h of incubation. Where appropriate steroids and steroid-nitrogen mustards were dissolved in 1,4 dioxan and added to the medium at a final concentration of 1% 1,4 dioxan.

Synthesis of steroid-nitrogen mustards

(a) *Unlabelled.* E_2-NM was synthesized according to the published method [13], and DHT-NM by the procedure described for androsterone [13].

E_2-NM was purified by column chromatography on neutral alumina (activity 1 on the Brockman scale) using a chloroform eluant. Fractions were screened by silica gel thin layer chromatography, developed in chloroform and stained with dilute sulphuric acid. Fractions containing E_2-NM were then combined and taken to dryness under a stream of N_2. The E_2-NM was purified by recrystallisation from isopropyl ether–hexane (1:1, v/v).

DHT-NM was purified by column chromatography on fully activated neutral alumina using gradient elution (20% chloroform in toluene to 50% chloroform in toluene). Fractions were screened by silica gel thin layer chromatography, developed in chloroform and stained with Zimmerman reagent. Fractions containing DHT-NM were then combined and taken to dryness under a stream of N_2. DHT-NM was purified by recrystallisation from a mixture of 1,4 dioxan and water.

Both recrystallised E_2-NM and DHT-NM gave single spots after thin layer chromatography. The structures of E_2-NM and DHT-NM were confirmed by n.m.r. spectroscopy. The m.p. of E_2-NM corresponded to that published (101–103°C). The m.p. of DHT-NM was determined (253-255°C).

(b) *Radioactively labelled.*

[^{3}H]-E_2-NM.

5 mCi (SA 50 Ci/mmol) of [6,7-^{3}H]-oestradiol-17β were taken to dryness on a rotary evaporator. Dry pyridine (2 ml) was added and taken to dryness, this step was repeated. [^{3}H]-oestradiol-17β was finally taken up in dry pyridine (1 ml). *Bis*-(2-chlorethyl)-amine (2.3 mmol) freshly liberated from its hydrochloride was extracted into toluene (2 × 10 ml) and dried over $MgSO_4$ for 2 h. The amine (0.3 mmol) was added to 1.8 mmol of phosgene in dry toluene under dry conditions. The mixture was allowed to stand at room temperature with occasional swirling for 3 h. The resulting mixture, containing a white precipitate, was rapidly filtered and reduced to ½ volume by rotary evaporation to remove excess phosgene. Dry pyridine was added, and the mixture reduced by rotary evaporation to the original volume. This step was repeated until no smell of phosgene remained. This mixture (0.5 ml, approximately 0.1 mmol of material) was added to the [^{3}H]-oestradiol-17β, and the mixture allowed to stand at room temperature for a minimum of 72 h. After this time the reactants and products were extracted into ethyl acetate (3 × 10 ml). The ethyl acetate extracts were combined and washed with 1 N HCl (1 × 10 ml) and water to neutrality. The ethyl acetate was removed by rotary evaporation. [^{3}H]-E_2-NM was purified by descending paper chromatography in a modified Bush B3 system (O/N equilibration, 1.5 h development). [^{3}H]-E_2-NM was located approximately, by comparison with authentic E_2-NM run on an adjacent line stained with molybdophosphoric acid, and accurately by strip scanning using a Packard Radiochromatogram scanner. [^{3}H]-E_2-NM was recovered from the paper chromatogram by Soxhlet extraction using chloroform. The resulting [^{3}H]-E_2-NM gave a single radioactive spot coinci-

dent with authentic E_2-NM after thin layer chromatography. The procedure gave a 50% yield.

[^3H]-DHT-NM.

2 mCi of 5α-dihydro[1α,2α(*n*)-^{3}H]-testosterone (SA 50 Ci/mmol) were taken up in 1 ml dry toluene (see procedure for E_2-NM). Excess phosgene (1.5 mmol) in dry toluene was added and the reactants allowed to stand at room temperature, with the exclusion of moisture, for a minimum of 72 h. 5 mmol of *bis*-(2-chlorethyl)-amine (see preparation [^{3}H]-E_2-NM) in dry toluene (10 ml) was added to the DHT-phosgene reaction mixture and the reactants allowed to stand at room temperature for a further 72 h. The reaction mixture was diluted with chloroform (20 ml) and exhaustively washed with water. The remaining organic phase was taken to dryness on a rotary evaporator. The residue was applied to a paper chromatogram which was developed in a descending direction in a Bush A system (O/N equilibration, 3 h development). The area corresponding to [^{3}H]-DHT-NM was located approximately by staining an adjacent lane loaded with authentic DHT-NM with Zimmerman reagent, and accurately by strip scanning. The area corresponding to [^{3}H]DHT-NM was eluted with chloroform in a Soxhlet apparatus. The resulting [^{3}H]-DHT-NM gave a single radioactive spot after thin layer chromatography coincident with authentic DHT-NM. The procedure gave a 100% yield.

Uptake and exchange rates

Tissue specimens (~2.0 g) were incubated in a total of 50 ml of medium containing ^{3}H labelled test compound (12.5 μCi, specific activity 50 Ci/mmol). At the end of the incubation period tissues were washed with normal saline at 37°C.

Approximately 1.5 g of tissue was transferred to a superfusion apparatus [14]. Medium containing unlabelled test compound at the same concentration (10^{-8} M) as that used in the incubation was pumped over the tissue at a rate of 40 ml/h unless otherwise indicated. Fractions were collected every 20 min over a 3 h period. Aliquots of the fractions (0.5 ml) were counted directly in 10 ml aqueous scintillation fluid. The total amount of radioactive material appearing in the superfusate was determined for each fraction. From the knowledge of the radioactive concentration in the tissue (see below) at the start of the superfusion the total amount of radioactivity in the tissue during the time course was calculated. A graph was drawn of log d.p.m. remaining in the tissue against time. The results approximated to a straight line and first order kinetics were assumed. The half life ($t\frac{1}{2}$) of the compound under these exchange conditions was calculated.

The remaining tissue after incubation (~0.5 g) was immediately frozen in liquid nitrogen. The tissue was pulverised. Approximately 0.25 g samples were taken to glass scintillation vials and 1 ml Soluene-350 added. After incubation at 40°C for 48 h 10 ml of non-aqueous scintillation fluid was added to the now solubilized tissue. After counting the radioactive concentration (d.p.m./g) in the tissue was calculated and taken as a measure of the uptake of the test compound by the tissue.

Tissue viability

Glucose utilization was measured as a test of tissue viability.

Samples of tissue (0.25 g) were incubated at 37°C for various times up to 4 h in Krebs–Ringer buffer (1 ml) containing 0.2 μCi D-[U-^{14}C]-glucose. Tissue incubations were carried out in rubber sealed vessels containing above the level of the medium a small plastic cup. At the end of the incubation period the reaction was stopped by addition of 2 M H_2SO_4 (1.0 ml). The plastic cup was filled with hyamine (0.5 ml) and the still sealed vessel allowed to stand for 16 h to allow adsorption of liberated CO_2. Both sulphuric acid and hyamine were introduced using a hypodermic syringe through the rubber seal. The hyamine was removed and added to 10 ml non-aqueous scintillation fluid and taken to radioactive counting.

Nuclear localisation

Following incubation in a total of 50 ml of medium containing 12.5 μCi of ^{3}H labelled test compound (E_2-NM or DHT-NM) diluted with unlabelled test compound to a final concentration of either 10^{-8} M or 10^{-5} M, tissues (1.5–2.0 g) were washed with ice cold saline (3 ×). Nuclei were purified from the tissue. Human benign prostatic tissue was frozen in liquid nitrogen, stored and processed to yield purified nuclei by the method of Symes *et al.*[16]. Rat prostatic tissue was processed immediately and nuclei purified according to the method of Mainwaring[15].

Isolated nuclei were resuspended in water (1 ml) and extracted with ethyl acetate (2 × 5 ml). The combined ethyl acetate extracts were taken to dryness under a stream of N_2. Appropriate unlabelled steroids were added. In the case of [^{3}H]-E_2-NM incubations 10 μg each of oestrone, oestradiol-17β and E_2-NM, in the case of [^{3}H]-DHT-NM incubations 10 μg each of 5α-dihydrotestosterone and DHT-NM were added. An aliquot (1/10) was taken to liquid scintillation counting for measurement of total radioactivity. The remainder was analysed by thin layer chromatography, developed in chloroform, for identification of the radioactive metabolites. By comparison with standard lanes areas corresponding to oestrone, oestradiol-17β and E_2-NM or 5α-dihydrotestosterone and DHT-NM were identified. The thin layer chromatograms were divided into 1 cm sections and counted directly in 10 ml non-aqueous scintillation fluid after overnight incubation at room temperature. There was a total recovery of counts by this method.

Metabolic studies

Tissue specimens (1.0–2.0 g) were incubated in a total 50 ml medium containing 12.5 μCi of ^{3}H labelled test compound (E_2-NM or DHT-NM) adjusted to a final concentration of 10^{-8} M or 10^{-5} M with unlabelled test compound. At the end of the incubation (1 h at 37°C) media were extracted with ethyl acetate (2 × 100 ml.) The combined ethyl acetate extracts were taken to dryness on a rotary evaporator. To aliquots (1/10) were added appropriate unlabelled steroids and steroid–nitrogen mustards and analysis of radioactive products was performed as above. Further aliquots (1/10) were taken for determination of total radioactivity. From a knowledge of the total ^{3}H applied to the thin layer plate and the ^{3}H associated with the free steroid regions (either 5α-dihydrotestosterone with DHT-NM incubation or oestrone plus oestradiol-17β-after E_2-NM incubations) the cleavage rate of the test compound was calculated.

Radioactive counting

Counting was performed in a Packard 3375 liquid scintillation counter. D.p.m. were calculated using the automatic external standard.

Non-aqueous scintillation fluid contained 10 g PPO and 250 mg POPOP in 2.5 l toluene. Aqueous scintillation fluid contained 9 g PPO, 60 mg POPOP, 600 ml Triton X-100 in 1800 ml toluene.

RESULTS

Androgen receptor binding affinity

The receptor binding affinity of the two compounds were kindly performed for us by Dr A. Wakeling of ICI Pharmaceuticals. The relative binding affinities of these compounds as compared to 5α-dihydrotestosterone were 100:0.1:0.005 (5α-DHT:DHT-NM:E_2-NM).

Distribution in rat tissues in vivo

Following the intra-peritoneal injection of [^{3}H]-E_2-NM or [^{3}H]-DHT-NM (25 μCi, specific activity 50 Ci/mmol) to 24 h castrate rats the concentration of ^{3}H in a number of tissues was determined over a 4 h time course. The results are depicted in Fig. 1. There were small differences in the relative distribution of [^{3}H]-E_2-NM and [^{3}H]-DHT-NM between different tissues. During the first 2 h less ^{3}H could be detected in the liver after injection of [^{3}H]-DHT-NM than after injection of [^{3}H]-E_2-NM. Slightly more ^{3}H could be detected in prostate relative to all other tissues after the injection of [^{3}H]-DHT-NM than after the injection of [^{3}H]-E_2-NM. At 4 h these trends were reversed.

The % of the total dose recovered in the tissues studied was however very different being some 5–10 times greater after injection of [^{3}H]-E_2-NM than after injection of [^{3}H]-DHT-NM.

Uptake and exchange rates in vitro

We reasoned that compounds that might be selectively accumulated by the prostate *in vivo* would show high uptake and/or slow exchange rates in this tissue *in vitro*. Using human benign prostatic tissues we have measured the uptake and exchange rates of a number of steroids, estramustine and DHT-NM. As can be seen (Table 1) the uptake of these steroids and estramustine were similar but significantly greater than the uptake of DHT-NM.

Exchange rates were determined from the rate of loss of ^{3}H during superfusion of tissue that had been preincubated in ^{3}H labelled test compound. The experimental design necessitated the use of tissue *in vitro* for 4 h. Tissue viability, as judged by glucose utilisation, over this time course was established for both human benign and malignant prostates. In each case no change in glucose utilisation was detected.

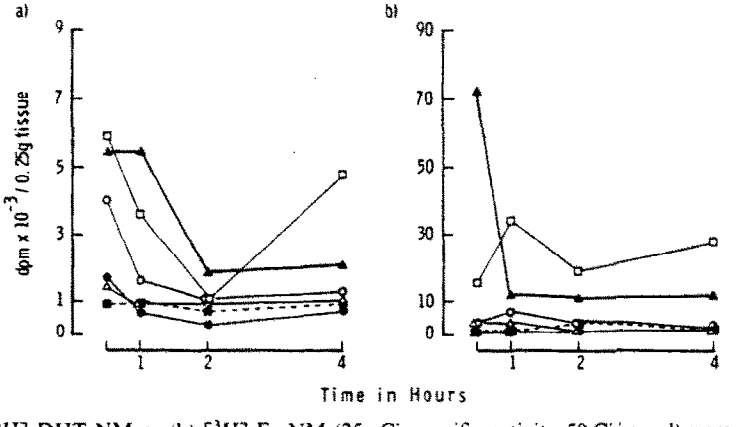

Fig. 1. (a) [^{3}H]-DHT-NM or (b) [^{3}H]-E_2-NM (25 μCi, specific activity 50 Ci/mmol) were dissolved in 1,4 dioxan and made 1% with respect to normal saline and injected i.p. to 24 h castrate rats. ^{3}H concentration in various tissues was determined (by total tissue combustion in a Packard carbon/tritium oxidiser and subsequent liquid scintillation counting) at periods up to 4 h post injection. The mean results of two experiments are plotted. □——□ liver, ○——○ kidney, ▲——▲ prostate, △——△ heart, ■-----■ blood, ●——● brain.

Table 1. Uptake of steroids and steroid-nitrogen mustards by human benign prostatic tissue *in vitro*

Test compound	Uptake (pmol/g tissue)
Testosterone	22.5 ± 9.4 (11)
5α-dihydrotestosterone	34.0 ± 14.6 (4)
Oestradiol-17β	31.0 ± 10.7 (9)
E_2-NM	16.8 ± 6.4 (6)
DHT-NM	3.4 ± 0.6 (4)

Slices of tissue were incubated at 37°C for 1 h in medium containing 12.5 μCi ^{3}H labelled test compound at 10^{-8} M. Uptake is expressed as pmol/g tissue. Number of experiments performed quoted in brackets. Mean ± SD quoted.

The method of measuring exchange rates has been described. Variables of this procedure were considered, namely the thickness of the tissue slices, the flow rate of the superfusion medium, the concentration of test compound at which exchange took place. The data are summarised in Table 2. As can be seen variations in any of these parameters produced little change in the rate of exchange.

The exchange rates of testosterone, 5α-dihydrotestosterone, oestradiol-17β, E_2-NM and DHT-NM were determined using benign human prostatic tissue (Table 3). There was considerable variation in the estimate of exchange rates of the steroid-nitrogen mustards between tissues. Furthermore the half lives of the steroid-nitrogen mustards measured in human benign prostatic tissue were greater than those measured for testosterone. The $t_{\frac{1}{2}}$ of the compounds followed the order: E_2-NM > DHT-NM ⩾ oestradiol-17β > 5α-dihydrotestosterone ⩾ testosterone. Exchange rates, determined in one carcinomatous prostate, of the two steroid-nitrogen mustards were similar.

In order to assess whether the *in vitro* procedure reflected the *in vivo* situation we turned to the rat model. We had established (unpublished observations) that the uptake and retention of testosterone, 5α-dihydrotestosterone and oestradiol-17β by rat ventral prostrate followed the order: testosterone > 5α-dihydrotestosterone ⩾ oestradiol17β. Exchange rates of these compounds were measured using rat ventral prostates from both normal and 24 h castrate animals. The measured $t_{\frac{1}{2}}$ *in vitro* appeared to reverse the trend seen *in vivo*, that of oestradiol being greater than that of 5α-dihydrotestosterone which was either equal or greater than that of testosterone.

Table 2. Exchange rates ($t_{\frac{1}{2}}$) of testosterone, estramustine and DHT-NM under different experimental conditions

Experimental conditions			Half Life (h)		
Concentration (M)	Slice thickness (mm)	Flow rate (ml/h)	Testosterone	E_2-NM	DHT-NM
10^{-8}	0.5	20	2.1	7.6	4.3
10^{-8}	0.5	40	2.4	7.9	5.1
10^{-8}	0.25	20	3.1	—	—
10^{-8}	0.25	40	—	7.9	—
10^{-5}	0.5	40	2.0	—	—

Slices of tissue from the same specimen were incubated in either [^{3}H]-testosterone, [^{3}H]-E_2-NM or [^{3}H]-DHT-NM for 1 h at 37°C. The concentrations of test compounds and subsequent conditions of superfusion were varied. The half life time (h) of the compound in the tissue under exchange conditions is quoted.

Table 3. Exchange rates ($t_{\frac{1}{2}}$) of steroids and steroid-nitrogen mustards in human and rat prostatic tissue *in vitro*

Tissue	Experiment	Half Life (h)				
		Testosterone	5α-dihydrotestosterone	estradiol	E_2-NM	DHT-NM
Human Benign	1	4.5	4.2	8.2		
	2				7.8	
	3					4.7
	4	2.0		4.6		
	5	4.2			24.0	21.2
Human Carcinoma	1				4.0	5.5
Normal rat ventral	1	2.5	2.2	7.2		
24 h castrate rat ventral	1	16.5	2.4	24.0		

The half life (h) of the compounds in various human and rat prostatic tissues under exchange conditions are quoted. All experiments were performed at 10^{-8} M with 0.5 mm slices and 40 ml/h superfusion flow rate.

Table 4. Cleavage rate of estramustine and DHT-NM by human benign and carcinomatous prostatic tissue

Tissue	Concentration	Cleavage rate (μg/g tissue/h)	
		E_2-NM	DHT-NM
Benign	10^{-5} M	39.7 ± 30.9	62.9 ± 44.9
		13.2 – 110.3	17.9 – 157.2
		$n = 11$	$n = 6$
Benign	10^{-8} M	0.035 ± 0.027	0.018 ± 0.009
		0.022 – 0.053	0.009 – 0.031
		$n = 6$	$n = 4$
Carcinoma	10^{-5} M	3.3 ± 2.2	3.0 ± 2.0
		1.0 – 5.0	1.0 – 5.0
		$n = 5$	$n = 5$

Slices of human benign or carcinomatous prostatic tissue were incubated at 37°C for 1 h in medium containing ^{3}H labelled test compound. Cleavage rate was calculated from liberated free steroid. Rate is quoted in μg/g tissue/h.
Mean ± SD, range and number of experiments are quoted.

Subcellular localisation

Nuclei were isolated from human benign and rat ventral prostatic tissue following incubation in medium containing [^{3}H]-E_2-NM or [^{3}H]-DHT-NM at concentrations of 10^{-8} M or 10^{-5} M. The total radioactivity extracted from nuclei was very low in every case and could not be resolved after thin layer chromatography into discrete areas.

Metabolic studies

The cleavage rates of E_2NM and DHT-NM were determined by measuring the free steroid liberated after incubation in media containing ^{3}H labelled test compound. In the case of E_2-NM this was measured as a sum of [^{3}H]-oestrone and [^{3}H]-oestradiol-17β since it has been shown that E_2-NM at least in rat prostatic tissue, is first oxidised to oestrone-nitrogen mustard [18]. In the case of DHT-NM the cleavage rate was measured from liberated [^{3}H]-5α-dihydrotestosterone. Although 5α-dihydrotestosterone is metabolised to androstanediols in prostatic tissue [22] these compounds were not detected. The thin layer system used would have resolved these compounds.

The cleavage rates of the two steroid-nitrogen mustards were similar (Table 4) but considerably greater during incubation at 10^{-5} M than at 10^{-8} M. The cleavage rates were low only representing on average some 10–15% of the available material. There were, however, considerable differences in cleavage rates between individual tissues. It was of interest to examine whether a similar variation occurred between carcinomatous tissue. To date five specimens have been examined; all were moderately differentiated adenocarcinomata. The cleavage rates were similarly low in each case.

The tissue specificity of the steroid-cytotoxic compounds with respect to cytotoxicity might be affected by the relative cleavage rates of these compounds in different tissues. It is difficult to examine this question in human so the rat model was used. Various rat tissues were tested for their ability to cleave DHT-NM. As can be seen in Table 5 the cleavage rate was similar in all tissues studied with the exception of brain which had a very low cleavage rate.

DISCUSSION

[^{3}H]-DHT-NM and [^{3}H]-estramustine were readily synthesized in good yield following published procedures [13] from 5α-dihydro[1α-2α(*n*)-^{3}H]-testosterone and [6,7-^{3}H]-oestradiol respectively.

Injection of these compounds into the 24 h castrate rat revealed that a much higher proportion of the total dose was recovered in the ventral prostate after injection of [^{3}H]-estramustine than after [^{3}H]-DHT-NM. The proportion of the injected dose measured in other tissues was also higher after [^{3}H]-estramustine administration. That is the relative distribution of these two compounds between the various tissues studied was similar. These data are surprising since rat ventral prostate contains much larger amounts of

Table 5. Cleavage rate of DHT-NM by rat tissues

Tissue	Cleavage rate (μg/g tissue/h)
Blood	20 ± 2
Prostate	22 ± 6
Brain	9 ± 4
Kidney	15 ± 2
Spleen	32 ± 27
Lung	18 ± 4
Liver	22 ± 6

Slices of rat tissue were incubated in medium containing [^{3}H]-DHT-NM at 10^{-5} M for 1 h at 37°C. Cleavage rate, measured from the liberated free steroid, is quoted in μg/g tissue/h.
The mean and standard deviation of 3 experiments are quoted.

a high affinity estramustine binding protein [10, 21] than other tissues and since DHT-NM has little affinity for this protein. The published data on tissue distribution is limited and conflicting. Appelgren *et al.*[19] showed a higher concentration of estramustine in liver compared to ventral prostate while Forsgren *et al.*[20] report a higher or equal concentration of estramustine in ventral prostate compared to liver. Both measurements were made 2 h after initial injection of [^{3}H]-estramustine, Appelgren *et al.* injected five times the dose of estramustine that was used by either Forsgren *et al.* or us.

Our attempts to compare the retention of [^{3}H]-estramustine and [^{3}H]-DHT-NM by human prostate tissue, as judged by their respective exchange rates, did not appear to be reliable. The half lives of both steroid-nitrogen mustards differed markedly between individual tissues. Also, studies using the rat as model indicated that the *in vitro* procedure did not seem to follow *in vivo* observations.

We were unable to demonstrate nuclear localisation of either DHT-NM or estramustine in either rat or human prostatic tissue. Although the androgen receptor binding affinity of DHT-NM was higher than that of estramustine it was weak by comparison with 5α-dihydrotestosterone. It is known [16] that the 17β hydroxyl group of 5α-dihydrotestosterone is important in receptor protein binding site recognition. A more suitable derivative with higher receptor binding affinity and more likelihood of nuclear translocation might be prepared by attaching the nitrogen mustard through a less critical position in the steroid molecule.

The potential free and active alkylating moiety in tissues was estimated from the rate of cleavage of the steroid-nitrogen mustards as measured by the yield of free steroid after incubation. The rate of cleavage was concentration dependent. Considerable variation was detected between individual human benign prostatic tissue specimens. It was of interest in view of the reported success of estramustine treatment in only some cases of carcinoma of the prostate [3, 4] to establish if a similar variation exists between different prostatic carcinoma. To date five tumour specimens had been examined and in each case a low cleavage rate was obtained.

The rate of cleavage of the DHT-NM was no higher in ventral prostatic tissue than other tissues of the rat. This finding parallels the reported data on estramustine metabolism in different rat tissues [18]. These data suggest that no particular advantage will be accrued by the prostate with respect to metabolic activation of the compounds.

In conclusion no advantage with respect to selective tissue retention, metabolic activation or nuclear translocation has been achieved by using a 5α-dihydrotestosterone linked nitrogen mustard with higher androgen receptor protein binding affinity than an oestradiol derivative, estramustine.

From our results we conclude that there are real difficulties in the design of steroid linked cytotoxics for treatment of prostatic cancer. The factors that determine the degree of selective retention, of steroids, by the prostate, include the nature of the steroid and the dose administered. Selective prostatic retention of testosterone is significant at physiological but not at pharmacological doses [23]. It is unlikely that steroid-cytotoxics, even if they displayed the same tissue specificity as testosterone, will be useful at physiological doses unless the cytotoxic moiety is very potent. Furthermore, compounds which require metabolic activation to become cytotoxic will be less useful than those that do not, since the rate of metabolic activation will be low at low doses.

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