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Estrogen-Linked 2-Chloroethylnitrosoureas: Anticancer Efficacy in MNU-Induced Rat Mammary Carcinoma, Uterine Activity in Mice and Receptor Interactions*

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Abstract—*A series of estradiol-linked* N-(2-*chloroethyl*)-N-*nitrosocarbamoyl*(CNC)-L-*alanines attached in various positions (positions* 3, 6α, 17, 3+17 *of estradiol) have been synthesized and tested in hormone-dependent MNU-induced rat mammary carcinoma. Compounds were given i.p. on day* 1, 8, 22 *and* 29 *after randomization in equimolar dosage. Equimolar mixtures of unlinked agents were tested in comparison. The results show that the* 17-*linked derivative was significantly superior to the other congeners and to the unlinked equimolar mixture. The* 6α-*linked analogue unexpectedly was highly toxic and ineffective. Binding affinities to cytosolic estrogen receptors cannot fully explain the findings of the chemotherapy experiments. Especially the* 3-*linked derivative we found to exhibit much higher values for relative binding affinity than the* 17-*analogue, which was a distinctly more effective antineoplastic agent. Estradiol liberation by facile cleavage of the phenolic* 3-*ester bond might be responsible.*

*Estradiol receptor contents in tumours were diminished or disappeared completely during treatment with individual analogues, progestin receptor contents behaved differently. After a single dose of CNC-*L-*alanine-estradiol-*17-*ester, a long-lasting disappearance of estradiol-receptors, measured for up to* 192 hr, *and a strong induction of progesterone receptors was observed with a maximal value reached at* 16 hr *and return back to normal at* 192 hr. *In the Dorfman uterine weight test in mice, all compounds exhibited distinct uterotrophic activity with no clearcut differences. The relevance of estradiol receptor contents in tumours for antineoplastic activity of the most effective analogue, the* 17-*ester, became evident from the observed reduced responsiveness of MNU-induced rat tumours after ovariectomy. In these hormone-independent tumours the linked compound revealed only the same antitumour efficacy as CNC-*L-*alanine alone.*

INTRODUCTION

2-CHLOROETHYLNITROSOUEAS are highly effective anticancer agents in a wide spectrum of animal tumours [1, 2]. Their clinical application has been limited, however, by their strong, delayed and cumulative toxicity to the bone marrow [3]. Recent developments comprise the synthesis and testing of water-soluble derivatives such as ACNU (1-(2-chloroethyl)-1-nitroso-3-(2-methyl-6-amino-pyrimidyl-5)-urea) and HECNU (1-(2-chloro-ethyl)-1-nitroso-3-(2-hydroxyethyl)-urea) which are in clinical phase II/III studies [4]. The results of experimental and clinical studies suggest that little improvement of therapeutic ratios can be expected without modifying pharmocokinetics and tissue specificity of the CNU group, for example by linking it to an appropriate carrier molecule. Our work concentrates on oligopeptides and steroid hormones as carriers [5–7]. Hormone-linked

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Reprint requests to: Dr. Martin R. Berger.
Abbreviations: MNU, *N*-methyl-*N*-nitrosourea; CNC, *N*-(2-chloroethyl)-*N*-nitrosocarbamoyl; DMSO, dimethylsulfoxid; RBA, relative binding affinity.

cytostatic agents have been synthesized and tested sporadically since Druckrey and his group first developed this principle by synthesizing diethylstilbestrol diphosphate [8]. Recent accomplishments include estramustine and prednimustine (chlorambucil-prednisolone-21-ester), compounds that at present undergo detailed clinical evaluation [9, 10]. Some hormone-linked nitrosoureas have been synthesized and tested [11, 12], but systematic structure-activity studies, with respect to chemical and pharmakological properties in hormone-dependent experimental tumors have not been carried out yet. The present paper describes the results of such a study with newly developed estradiol-linked nitrosoureas and their evaluation with respect to therapeutic ratio in MNU-induced rat mammary carcinoma, influence on receptor contents, receptor binding affinities and uterotrophic activity in mice.

MATERIALS AND METHODS

Chemicals

Crystalline *N*-methyl-*N*-nitrosourea (MNU) was synthesized by Dr. M. Wiessler (Institute of Toxicology and Chemotherapy, German Cancer Research Center, Heidelberg). MNU was dissolved at 1% in Sörensen buffer, pH 6, and distilled water (20/80, v/v).

Chemistry

Preparation of drugs: *N*-(2-chloroethyl)-*N*-nitrosocarbamoyl-L-alanine (CNC-alanine) was prepared by reaction of *N*-(2-chloroethyl)-*N*-nitrosocarbamoylazide [13] with L-alanine by a procedure previously described [14], with some modifications.

N-(2-chloroethyl)-*N*-nitrosocarbamoyl-L-alanyl-L-alanine (CNC-L-alanyl-L-alanine): CNC L-alanine was activated by dicyclohexyl-carbodiimide-mediated condensation with *N*-hydroxy-succinimide to the corresponding CNC-L-alanine-*N*-hydroxysuccinimide-ester. The latter was reacted with L-alanine to give the desired drug.

N-(2-chloroethyl)-*N*-nitrosocarbamoyl-L-alanine-estradiol-3-ester (CNC-L-alanine-estradiol-3-ester) and *N*-(2-chloroethyl)-*N*-nitrosocarbamoyl-L-alanyl-L-alanine-estradiol-3-ester (CNC-L-alanyl-L-alanine-estradiol-3-ester): CNC-L-alanine and CNC-L-alanyl-L-alanine were activated with *N*,*N*-carbonyldiimidazole [15] and converted with estradiol to the corresponding estradiol-3-esters.

N-(2-chloroethyl-*N*-nitrosocarbamoyl-L-alanine-estradiol-3,17-diester (CNC-L-alanine-estradiol-3,17-diester): with an excess of activated CNC-L-alanine, both hydroxy-groups of estradiol were esterified.

N-(2-chloroethyl)-*N*-nitrosocarbamoyl-L-alanine-estradiol-17-ester (CNC-L-alanine-17-ester): The preparation of the ester in 17-position requires protection of the more reactive phenolic hydroxy-group. This was achieved by synthesis of *N*-(2-chloroethyl)-*N*-nitrosocarbamoyl-L-alanine-estradiol-17-ester-3-tetrahydropyranylether, followed by acid-catalyzed cleavage of the tetrahydropyranylether-group.

N-(2-chloroethyl)-*N*-nitrosocarbamoyl-L-alanine-6α-hydroxy-estradiol-6α-ester (CNC-L-alanine-6α-hydroxy-estradiol-6α-ester): Estradiol-diacetate was oxidized by CrO_3 and hydrolized to 6-keto-estradiol [16]. This was reacted with dihydropyran to the 3,17-bis-tetrahydropyranylether-derivative, which was reduced with $LiAlH_4$. The resulting 6α-hydroxy-product was converted to the corresponding ester by activated CNC-L-alanine. The protective groups were removed by acid-catalyzed cleavage of the tetrahydropyranylether linkage to give the endproduct. Identity and purity of the above substances were established by infrared-, ^1H- and ^{13}C-nuclear magnetic resonance spectroscopy and elemental analysis. Detailed information will be published elsewhere (Schreiber *et al.*, in preparation).

Animals and tumor induction

Virgin female Sprague–Dawley rats (Institut für Versuchstierkunde, Hannover, F.R.G.) supplied at an age of 40 ± 1 days were kept under conventional conditions (three animals per size III Makrolon cage during the induction time of tumors and subsequently one animal per size II Makrolon cage during therapy of rats; temperature: 22 ± 2° C; relative humidity: 55 ± 10%). Altromin pellets and tap water were given *ad libitum*.

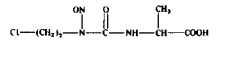
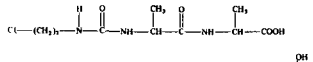
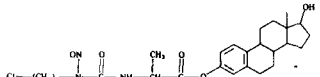
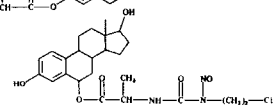
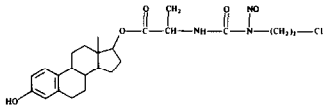
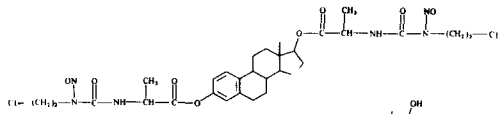
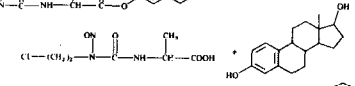
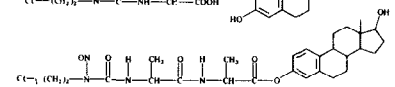
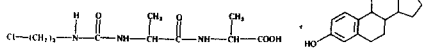
Mammary carcinomas were induced with a slight modification of the original model [17] by three i.v. injections of MNU into the tail vein on days 50, 71 and 92 of life, respectively. Beginning 4 weeks after the first injection of MNU, rats were weighed and palpated twice weekly over the whole experimental period to record tumor manifestation. The investigation comprised three consecutive induction experiments.

Individual tumor volumes were estimated as a product of two vertical axes $(a \times b^2)/2$ as measured by vernier calipers ($a \geq b$). Rats with a total tumor volume of more than 0.8 cm^3 were randomly allocated to experimental groups; therapy started immediately thereafter.

Dorfman test

The Dorfman uterine weight test was used to determine estrogenic activity. Immature, 18-day-old female Swiss mice (Wiga/Sulzfeld, F.R.G.) were randomly distributed into groups of eight animals and injected i.p. daily for 3 days with a DMSO-solution of estradiol or the test compound (for

Table 1. Treatment of MNU-induced rat mammary carcinoma with N-(2-chloroethyl)-N-nitrosocarbamoyl (CNC)-L-alanine- and CNC-L-alanyl-L-alanine-linked to estradiol in comparison with unlinked single agents and ovariectomy

Structures	Compounds and scheme of treatment: Treatment group	Group No.[a]		Single dose[b]	Median total dose (range)[c] (μmol/kg)
	CNC-L-alanine	I	a*	45	180 (180 – 180)
			b*	67	268 (67 – 268)
			c*	101	202 (101 – 404)
	CNC-L-alanyl-L-alanine	II	a***	45	180 (180 – 180)
			b***	67	268 (134 – 268)
			c***	101	404 (202 – 404)
	CNC-L-alanine-estradiol-3-ester	III	a*	75	300 (150 – 300)
			b*	105	420 (105 – 420)
			c*	147	441 (147 – 588)
	CNC-L-alanine-6-α-hydroxy-estradiol-6α-ester	IV	a***	38	152 (152 – 152)
			b***	75	300 (150 – 300)
	CNC-L-alanine-estradiol-17-ester	V	a**	38	152 (152 – 152)
			b**	54	212 (212 – 212)
			c*	75	300 (300 – 300)
			d*	105	420 (420 – 420)
			e*	147	588 (441 – 558)
			f*	206	824 (412 – 824)
	CNC-L-alanine-estradiol-3,17-diester	VI	a**	54	212 (212 – 212)
			b**	75	300 (300 – 300)
			c**	105	420 (315 – 420)
	CNC-L-alanine + estradiol	VII	a**	54 each	212 (212 – 212)
			b*	75 each	300 (225 – 300)
	CNC-L-alanyl-L-alanine-estradiol-3-ester	VIII	a*	75	300 (75 – 300)
			b*	105	420 (210 – 420)
			c*	147	588 (294 – 588)
	CNC-L-alanyl-L-alanine + estradiol	IX	*	75 each	300 (150 – 300)
	Ovariectomy	X	*	–	–
	Untreated controls	XI	a*	–	–
			b**	–	–
			c***	–	–

[a] Numbers of animals in groups I – X: 10 per subgroup
in group XI: 20 per subgroup

[b] Drugs were given at days 1, 8, 22 and 29 after randomization

[c] Range indicates that some animals died prior to completion of dosing.

* = experiment 1; ** = experiment 2; *** = experiment 3

doses see Table 7) in comparison to untreated and vehicle-treated controls. The animals were killed 24 hr after the last injection, body wts were determined, and the uteri carefully removed, weighed and fixed with Bouin's solution for 12 hr [15 parts of a saturated aqueous solution of picric acid, five parts of formaldehyde (40%), and one part of acetic acid].

The uteri were washed with a saturated alcoholic solution of LiCl and dried at 100° C for 24 hr. Thereafter the total weights of uteri per group were determined again. Efficacy was calculated as the quotient: [dried uteri weight (mg)]/[animal weight (g)] × 100 [18]. From this quotient estrogenic activity was expressed in per cent relative to estradiol. The 100% dose of estradiol (0.06 μmol/kg) was determined from a dose–response study with four logarithmically spaced doses (0.0024–0.3 μmol/kg). Estradiol derivatives were given in five dosages using the same escalation factor and a starting dose five times that of estradiol (Table 7).

Therapy and dosages

Substances were applied intraperitoneally, dissolved in DMSO. All solutions were prepared immediately before use. Logarithmically spaced dosages were selected by referring to earlier studies in tumor bearing rats with CNC-L-alanine, which had shown dose-limiting toxicity at single doses exceeding 185 μmol/kg [19]. Therefore in the present study single doses were chosen at levels of 45, 67 and 101 μmol/kg with the median single dose representing 33% of the aforementioned toxic single dose (group I, Table 1). The doses for CNC-L-alanyl-L-alanine were chosen accordingly (group II, Table 1). Estradiol linked analogues were administered in a dose range comprising a median dose equimolar to the maximum dose of CNC-L-alanine and two further doses, selected by multiplying or dividing the median dose by 1.4 (groups III and VIII, Table 1). The starting dose had to be diminished, however, in groups IV and VI, because these derivatives were more toxic (Table 1).

Since in experiment 1 CNC-L-alanine-estradiol-17-ester already showed optimum therapeutic efficacy at the starting dose (75 μmol/kg), the dose range was extended down to 38 μmol/kg and up to 206 μmol/kg, to include also a highly toxic dose level. Mixtures of unlinked anticancer agents and estradiol were applied in dosages equimolar to linked compounds (54 and 75 μmol/kg for group VII, 75 μmol/kg for group IX, Table 1). To compare with an effective standard treatment of this hormone dependent tumour, ovariectomy was also included (group X, Table 1) and performed in rats with established lesions, concomitantly with the start of chemotherapy.

In a separate additional experiment (Table 4), ovariectomy was performed one day after the third MNU-application, before manifestation of mammary carcinomas, in order to induce non-hormone-dependent tumours [20, 21].

Treatment of these tumours was performed with CNC-L-alanine-estradiol-17-ester, the analogue, which in therapy of non-ovariectomized rats had shown the best results.

Evaluation methods

Manifestation and growth of tumours were monitored by recording median tumour number and tumour volume per animal. Total tumour volume per animal was calculated as the sum of all individual tumours. Therapeutic efficacy was measured on the basis of median total tumour volumes of treated groups vs. controls ($T/C \times 100$).

Ranking criteria were chosen according to NCI-recommendations: (active: $T/C \times 100 \leq 42$; highly active: $T/C \times 100 \leq 10$). Statistical significance was based on 95% confidence limits ($P \leq 0.05$) of the median values per group. Additionally equimolar dosages of linked compounds and respective unlinked mixtures of single agents were compared by groups using a non-parametric multivariate test [22]. Significance limit was $P \leq 0.01$.

Receptor assays

Estrogen receptor content: mammary tumours were removed from individual animals under light ether anesthesia immediately before and 4 days after end of therapy, respectively. In a more detailed study with CNC-L-alanine-estradiol-17-ester, tumours were excised immediately before and at given intervals after a single dose.

Tumours were stored in liquid nitrogen until receptor analysis. Frozen tissue was disintegrated in a microdismembrator (Braun, Melsungen) two times, for 45 and 30 sec, respectively, with intermittent cooling in liquid nitrogen for 5 min. The powder was transferred into a glass Potter homogenizer (Schott & Gen., Mainz) and made up with Tris buffer (pH 7.4) to give a 12% mixture (w/v), which was homogenized (five strokes) at 0° C. The homogenate was centrifuged (10 min at 800 ***g***; 60 min at 105,000 ***g***) and the supernatant taken for analysis. Protein content, determined according to Lowry [23], lay between 3 and 6 mg/ml. For determination of specific binding, 100 μl cytosol, 100 μl Tris–EDTA–buffer (pH 7.4) and 100 μl of a solution containing ^{3}H-labelled estradiol (sp. act. 112 Ci/mmol, NEN chemicals) were mixed to give six different final concentrations ranging from 10^{-8}M to 3×10^{-10}M. After 18 hr incubation at 0–4° C, 500 μl of dextran-coated charcoal (0.8%

of activated charcoal Norit A and 0.008% of dextran, MW 60–90.000 in Tris/EDTA buffer pH 7.4) were added and the mixture shaken for 15 min. The charcoal was removed by centrifugation (700***g***/10 min) and 0.2 ml of the supernatant were mixed with 3 ml of scintillation cocktail (Aqualuma, Baker). Radioactivity was measured in a scintillation counter (Mark III, Zinsser Analytik GmbH, Frankfurt); counting efficiency was 38% (10 min). Unspecific binding was determined accordingly with 100 µl of a solution of diethylstilbestrol to yield a final concentration of 5×10^{-6}M.

Progesterone receptor content: progesterone receptor contents were determined in the same way, using 100 µl of a solution of ^{3}H-labelled 16-ethyl-21-hydroxy-19-norpregn-4-ene-3,20-dione (Org 2058, sp. act. 47 Ci/mmol. Amersham Buchler, Braunschweig) to give final concentrations of 2×10^{-8} to 6×10^{-10}M. For unspecific progesterone receptor binding, parallel assays were carried out with the addition of 100 µl of a solution of unlabelled Org 2058, to give a final concentration of 5×10^{-6}M. Receptor contents were determined from Scatchard plots [24].

Relative receptor affinity measurements

Calf uterus cytosol was used with an estradiol receptor content of about 1000 fmol/mg. The cytosol was prepared by the procedure described for tumour cytosol with the exception, that an ultra turrax was used instead of the microdismembrator. One hundred-microlitre aliquots of calf uterus cytosol (5–10 mg ptotein/ml cytosol) were incubated with 100 µl of ^{3}H-estradiol (sp. act. 112 Ci-mmol, NEN chemicals, 5 nmol/l) and 100 µl of test substances (10 values in a range of 10,000 nmol/l to 20 nmol/l) for 45 min at 0° C. The proportion of steroid, not bound to the receptor was removed by dextran-coated charcoal and radioactivity was determined in the same way as described above. Estradiol served as reference. The concentration of the test substance achieving 50% inhibiton of radioactivity was correlated to that of estradiol (100%).

RESULTS

The overall experimental design, including structures of cytostatic agents, dosages and treatment schedule is given in Table 1. The induction of mammary carcinomas as described resulted in multiple tumours occuring at median manifestation times of 59, 57 and 60 days (experiments 1, 2, 3). The mean tumour volume doubling times were 11 days for groups XIa and XIb and 10 days for group XIc. The mean estrogen and progesterone receptor contents apparently decreased during the time interval from randomisation to 33 days thereafter, however this decrease was not significant (group XI, Table 5).

Antitumour effects and toxicity

As can be seen from Table 2, treatment with CNC-L-alanine and CNC-L-alanyl-L-alanine resulted in a distinct tumour inhibition at week 4 (groups Ib,c and IIc) as evidenced by % *T/C* values. The optimum dose exhibiting highest anticancer activity together with lowest toxicity for CNC-L-alanine was 67 µmol/kg and for CNC-L-alanyl-L-alanine 101 µmol/kg. Although the tumour inhibitory effect of the latter compound obviously was relatively short-lived (Table 2), the dipeptide-derivative showed a remarkably smaller toxicity at equimolar doses in terms of % mortality, weight development and median survival times (Table 3).

Therapeutic efficacy appears to be increased for both compounds in the presence of equimolar doses of estradiol (groups VII and IX, compared to groups I and II, Table 2) after 7 weeks, however mortality at this time point seemed to be higher when estradiol was given concomitantly (groups VII and IX compared to groups I and II, Table 3).

After linking CNC-L-alanine to estradiol, the resulting derivatives show remarkably different biological properties. The linkage to position 17 of estradiol yields a compound superior in all respects to derivatives having the linkage in position 3, 6 or 3 and 17 (groups III, IV, VI, Table 2). Compared to the unlinked mixture (groups VIIa, b, Table 2), equimolar doses of the 17-ester exhibit significantly higher tumour inhibitory efficacy (groups V6, c, Table 2). The multivariate nonparametric test according to Koziol and Donna [22] reveals *P*-values of 0.01 for both dosages tested comparatively. A dose-dependent increase in tumouricidal activity ($T/C \times 100$) is evident at week 7 between dosages of 38 and 75 µmol/kg (groups Va-c, Table 2). An even more distinct dose-related effect is seen for median survival times and, correspondingly, for values of % increase in life span (% ILS), the optimum being reached at 75 µmol/kg.

Beyond this plateau of optimum dose, higher doses were antineoplastically less active and more toxic, as indicated by weight loss, rising mortality and decrease in median survival times (groups Vd, e, f, Table 3). Attachment of the cytocidal group in position 3 of estradiol resulted in a compound with inferior antitumour effectiveness in terms of *T/C* values and median tumour volumes compared to the 17-linked analogue ($P < 0.003$). With respect to median tumour number, no significant difference was found between the two compounds (equimolar dosages: IIIa-c and Vc-e). On the other

Table 2. Treatment of MNU-induced rat mammary carcinoma with N-(2-*chloroethyl*)-N-*nitrosocarbamoyl* (*CNC*)-L-*alanine and CNC*-L-*ananyl*-L-*alanine-linked to estradiol in comparison with unlinked single agents and ovariectomy*

Treatment group	Group No.	*Efficacy of treatment* Median tumor number per animal (95% confidence limits) week 1	week 4	week 7	Median tumor volume per animal (cm^3) (95% confidence limits) week 1	week 4	week 7	$T/C \times 100$[a] week 4	week 7
CNC-L-alanine	I a	3.5(1–5)	8(7–10)	8(2–11)	1.2(0.8–2.8)	12.8(3.9–22.8)	18.1(2.7–29.2)	62	63
	b	2.5(1–6)	5(1–11)	6(3–12)	1.4(0.9–4.5)	5.8(1.7–18.1)	11.4(1.1–19.3)	38	39
	c	3.5(3–6)	8(0–8)	6(3–8)	1.0(0.9–1.7)	8.5(2.0–10.5)	13.5(3.6–23.5)	32	50
CNC-L-alanyl-L-alanine	II a	4(2–6)	7(6–9)	8(6–11)	0.9(0.7–7.1)	24.9(11.3–42.8)	21.8(20.4–33.7)	90	71
	b	5(2–6)	10(7–12)	9.5(5–12)	1.0(0.8–1.8)	12.7(8.1–23.6)	17.9(10.9–32.2)	62	58
	c	4(3–5)	6(3–7)	5(1–10)	0.9(0.7–1.5)	7.3(0.3–15.0)	20.1(0.5–43.1)	25	60
CNC-L-alanine-estradiol-3-ester	III a	4.5(3–6)	6(4–8)	5(2–9)	1.1(0.7–4.4)	8.2(4.4–16.6)	13.8(5.1–15.6)	48	44
	b	4(1–4)	3(1–6)	5(1–7)	1.2(0.8–1.5)	2.1(0.5–10.3)	4.1(0.4–27.2)	21	35
	c	3(1–5)	3(1–4)	2(1–3)	1.0(0.7–1.6)	2.9(0.2–5.3)	6.5(0.5–11.5)	15	23
CNC-L-alanine-6-α-hydroxy-estradiol-6α-ester	IV a	3(2–5)	8(7–9)	10(9–11)	0.8(0.7–1.8)	19.6(14.7–24.6)	36.6(11.8–59.5)	72	107
	b	3(2–6)	8.5(4–11)	8.5(8–9)	0.9(0.8–1.3)	23.2(5.8–70.7)	31.7(26.5–36.8)	96	94
CNC-L-alanine-estradiol-17-ester	V a	3(1–5)	3.5(3–5)	4(2–8)	1.0(0.7–1.1)	3.9(1.7–6.7)	15.0(6.7–33.2)	19	51
	b	2.5(2–5)	2.5(2–4)	2(1–4)	0.9(0.7–1.2)	1.7(0.2–3.0)	3.3(1.0–8.9)	9	12(b)
	c	2.5(2–4)	4(2–6)	3(1–7)	1.2(0.9–2.5)	1.8(0.8–8.2)	2.2(0.4–5.7)	18	10(b,c)
	d	5.5(1–8)	5.5(3–8)	6(2–6)	1.3(1.0–2.6)	3.9(1.0–6.7)	6.3(2.0–12.6)	19	26
	e	5(3–6)	5(3–8)	5(3–9)	1.5(0.7–2.4)	3.7(1.5–11.6)	7.7(3.4–21.9)	25	34
	f	4.5(1–6)	4.5(1–10)	3(2–4)	1.1(0.8–1.7)	1.6(0.7–5.8)	5.2(2.4–7.9)	10	19
CNC-L-alanine-estradiol-3,17-diester	VI a	4(3–5)	4(1–5)	5.5(3–12)	1.0(0.8–1.5)	8.2(2.6–15.2)	23.0(7.6–57.5)	38	97
	b	2(1–5)	5(3–7)	6(3–8)	1.0(0.8–2.1)	5.1(0.9–13.4)	15.3(0.4–23.1)	28	48
	c	2.5(1–5)	2(2–5)	3(1–7)	1.2(0.8–2.9)	3.6(0.8–6.9)	10.8(0.3–16.8)	16	29
CNC-L-alanine + estradiol	VII a	3(2–4)	4(3–7)	4(3–7)	1.0(0.7–1.1)	4.1(2.6–9.2)	8.5(6.0–10.9)	25	32
	b	4(3–5)	6(4–6)	6(5–8)	1.2(0.7–2.1)	5.2(1.7–17.2)	4.5(3.9–10.7)	42	24
CNC-L-alanyl-L-alanine-estradiol-3-ester	VIII a	4(3–5)	4(3–7)	4(2–6)	1.1(0.8–1.9)	5.1(2.3–12.9)	6.3(0.7–11.8)	35	25
	b	3(1–6)	4(2–6)	3(2–4)	1.1(0.8–1.7)	3.1(1.7–5.8)	6.1(3.1–9)	18	23
	c	3(2–5)	4(2–5)	4(3–5)	1.3(1.1–1.7)	1.4(0.3–8.3)	3.2(1.8–5.2)	18	13
CNC-L-alanyl-L-alanine + estradiol	IX a	4.5(1–7)	5(3–7)	3(3–8)	1.3(0.8–1.6)	3.6(1.1–10.1)	3.4(0.8–8.8)	32	16
Ovariectomy	X a	4(3–5)	3(2–5)	4(3–6)	1.3(1.0–1.5)	2.0(0.5–4.9)	12.4(4.0–33.3)	12	69
Untreated controls	XI a	4.5(2–6)	7.5(6–9)	7(6–8)	1.0(0.8–1.7)	19.5(13.6–25.0)	22.8(17.6–29.2)	100	100
	b	3(2–4)	7(6–9)	7(6–9)	1.0(0.8–1.5)	23.0(15.0–30.6)	27.9(19.7–39.1)	100	100
	c	4(2–5)	9(7–11)	10(7–12)	0.9(0.7–1.1)	23.1(19.6–36.5)	37.2(20.6–43.3)	100	100

[a] Mean tumor volume of treated rats in % of untreated control.

[b] $P = 0.01$, according to Koziol and Donna, compared to the equimolar mixture (group VIIa, b).

[c] $P < 0.003$, according to Koziol and Donna, compared to the 3-ester (group IIIa).

Table 3. Treatment of MNU-induced rat mammary carcinoma with N-(2-*chloroethyl*)-N-*nitrosocarbamoyl* (*CNC*)-L-*alanine- and CNC-*L-*alanyl-*L-*alanine-linked to estradiol in comparison with unlinked single agents and ovariectomy*

Treatment group	Group No.	% Mortality week 4	week 7	week 10	% Weight difference (week 7–week 1)	Median survival time (days) (95% confidence limits)	% ILS[a]
CNC-L-alanine	I a	0	20	50	+ 8	64.5(43–93)	− 5
	b	20	30	70	− 5	50.5(15–98)	−26
	c	70	70	90	+ 6	13.0(7–46)	−81
CNC-L-alanyl-L-alanine	II a	0	10	30	+ 1	82.0(45–97)	+39
	b	10	20	40	+ 3	69.0(41–81)	+17
	c	20	20	70	+ 2	60.0(23–88)	+ 2
CNC-L-alanine-estradiol-3-ester	III a	10	50	90	−16	49.0(28–62)	−28
	b	30	50	90	−15	38.0(10–63)	−44
	c	40	70	100	−29	24.5(10–50)	−64
CNC-L-alanine-6-α-hydroxy-estradiol-6α-ester	IV a	0	60	90	− 8	40.0(33–79)	−32
	b	30	80	90	− 7	33.0(21.48)	−44
CNC-L-alanine-estradiol-17-ester	V a	0	30	70	+ 3	53.0(44–86)	−30
	b	0	20	30	− 7	65.0(41–94)	−14
	c	0	30	30	− 4	80.0(40–88)	+18
	d	0	10	70	−21	50.0(43–65)	−26
	e	0	50	90	−11	43.0(36–57)	−37
	f	20	80	90	− 6	34.5(16–56)	−49
CNC-L-alanine-estradiol-3,17-diester	VI a	0	20	90	− 2	50.0(39–63)	−34
	b	0	20	80	−10	54.0(39–68)	−29
	c	0	50	100	−19	45.0(32–54)	−41
CNC-L-alanine + estradiol	VII a	0	10	20	− 4	99.0(59–114)	+30.3
	b	0	70	90	− 3	35.0(28–64)	−49
CNC-L-alanyl-L-alanine-estradiol-3-ester	VIII a	10	40	60	− 6	52.0(25–109)	−24
	b	10	80	100	− 9	33.5(26–53)	−51
	c	10	70	90	− 3	35.5(25–57)	−48
CNC-L-alanyl-L-alanine + estradiol	IX	10	30	70	−14	59.0(26–73)	−13
Ovariectomy	X	0	10	30	+29	104(72–127)	+53
Untreated controls	XI a	0	10	50	+15	68.0(51–90)	–
	b	0	20	55	+15	76.0(52–90)	–
	c	5	21	58	+ 7	59.0(44–86)	–

[a] % increase in median life span.

hand, % mortality at all time points clearly was more pronounced after treatment with the 3-ester than with the 17-ester.

The 3,17-diester at 105 μmol/kg (optimal dose) showed an antitumour effectiveness comparable to the equimolar dosage of the 17-monoester but definitely was more toxic, as indicated by increased % mortality (week 10) and reduced life expectancy (% ILS). Moreover, the diester was distinctly less active at the two lower dosages (54, 75 μmol/kg), which represented the optimum dose range for the 17-monoester (group VIa-c, Table 2, 3).

The 6α-ester of 6α-hydroxyestradiol showed no antitumour efficacy at all (groups IVa, b, Table 2). At the same time, it was much more toxic than the 17-ester, even at the lowest dose (7 weeks, 38 μmol/kg, group IVa, b, Table 3). The 3-dipeptide-ester appeared to be a more effective antitumour agent with respect to % *T/C* values than the 3-monopeptide ester (groups VIIIa, c vs. IIIa, c; Table 2), toxicity of the two analogues however was comparable. The antitumour effect of ovariectomy which was performed in rats with established lesions (12% *T/C*, group X, Table 2) was remarkably high at week 4, but was transient and almost completely lost at week 7 (69% *T/C*).

The superiority of CNC-L-alanine-estradiol-17-ester disappeared when ovariectomized animals were treated (group II, Table 4). It is well known that ovariectomy in this tumour model strongly decreases estradiol receptor contents [25, 26]. The data (Table 4) show that therapeutic efficacy is reduced and at the same time toxicity is increased. In fact, activity of CNC-L-alanine-estradiol-17-ester in ovariectomized rats is comparable to that of CNC-L-alanine in non-ovariectomized (group Ib, Table 2) and ovariectomized rats (group III, Table 4).

Table 4. Chemotherapeutic efficacy of CNC-L-alanine-estradiol-17-ester on MNU-induced mammary carcinoma of SD-rats, that have been ovarectomized before manifestion of tumors

Treatment group	Group No.	Median tumor number per animal (95% confidence limits)	Median tumor vol. per animal (cm^3) (95% confidence limits)	$T/C \times 100$	Median weight of animals (g) (95% confidence limits)	Mortality n (%)
Untreated control	I[a]	2(1–2)[d]	1.2(0.9– 3.4)[d]	–	293(260–300)[d]	1 (0)[e]
		3(2–4)[e]	13.9(3.9–28.2)[e]	100[e]	305(290–335)[e]	4 (29)[f]
		3(2–5)[f]	20.5(6.0–52.3)[f]	100[f]	310(290–335)[f]	7 (50)[g]
CNC-L-alanine-estradiol-17-ester	II[b]	1(1–2)[d]	0.8(0.7– 1.2)[d]	–	275(235–285)[d]	2 (15)[e]
		3(2–4)[e]	3.3(2.5–11.5)[e]	24[e]	245(190–280)[e]	7 (54)[f]
		3(1–5)[f]	6.5(1.8–59.2)[f]	32[f]	218(195–270)[f]	11 (85)[g]
CNC-L-alanine	III[c]	2(1–2)[d]	0.8(0.7– 1.0)[d]	–	265(235–310)[d]	5 (38)[e]
		2(1–4)[e]	3.3(1.5–41.4)[e]	24[e]	230(200–285)[e]	7 (54)[f]
		2(1–5)[f]	5.9(2.9–10.5)[f]	29[f]	238(205–295)[f]	7 (54)[g]

[a] Untreated control, $n = 14$ animals.
[b] Treatment with 75 µmol/kg CNC-L-alanine-estradiol-17-ester on days 1, 8, 22 and 29 after randomization, $n = 13$ animals.
[c] Treatment with 75 µmol/kg CNC-L-alanine on days 1, 8, 22 and 29 after randomization, $n = 13$ animals.
[d] Week 1. [e] Week 4. [f] Week 7. [g] Week 10.

Influence of therapy on cytosolic estrogen and progesterone receptor contents

Tumours of untreated controls excised at time points of randomization and 33 days thereafter, showed a loss of about 50% for estradiol receptors and about 75% for progesterone receptors (group XI, Table 5). Treatment with CNC-L-alanine (group I, Table 5) had no overt additional influence on estradiol receptor contents, whereas progesterone receptor levels remained fairly unchanged. An additional influence on receptor levels was observed, however, after treatment by CNC-L-alanyl-L-alanine (group II) or CNC-L-alanine-estradiol-3-ester (group III, Table 5). Ovariectomy caused a massive reduction of progesterone receptors, whereas estradiol receptors were much less affected. Treatment with CNC-L-alanine-estradiol-17-ester (group V) and the 3,17-diester (group VI) resulted in a disappearance of estradiol-receptors, concomitant with an unchanged (group V) or elevated (group VI) level of progesterone receptors. CNC-L-alanyl-L-alanine-estradiol-3-ester likewise induced a complete disappearance of estradiol-receptors and an increased level of progesterone receptors (VIII).

The time course of CNC-L-alanine-estradiol-17-ester-induced variations in cytosolic estrogen and progesterone receptor contents of tumours is given in Table 6. Cytosolic estrogen receptors decreased to barely detectable levels already at 8 hr after therapy, the first time point of analysis. This effect lasted for at least further 88 hr. After 192 hr, first signs of recovery could be observed. During the same interval, progesterone receptor levels strongly increased, attaining a peak level at 16 hr after therapy and returning back to normal at 192 hr.

Uterotrophic activity

Untreated controls showed a mean uterine weight of 58% (groups IX, Table 7), solvent controls of 78% (group VIII, Table 7), respectively, of the mean uterine weight found after 0.06 µmol/kg estradiol. This estradiol dose was defined as optimal dose, achieving 100% estradiol efficacy (group VI, Table 7). As can be seen, the uterotrophic activity of 6α-hydroxyestradiol (VIIa-d, Table 7) was much less than that of estradiol (VIa-c, Table 7) at equimolar doses. This differential effect of the free hormones can be recognized also with the respective derivatives: estradiol-linked derivatives clearly exhibit a marked activity on the uterine tissue, causing increases in mean uterine weight of up to 170% (groups Ie, IIId, IVe, Vc, Table 7), whereas CNC-L-alanine-6α-hydroxyestradiol-6α-ester, albeit being more uterotrophic than 6α-hydroxyestradiol, is by far not as active as the estradiol-linked compounds.

Table 5. Analysis of cytosolic estrogen- and progestin receptor contents in MNU-induced mammary carcinomas before and after a series of four applications with N-(2-*chloroethyl*)-N-*nitrosocarbamoyl* (*CNC*)-L-*alanine- and CNC-*L-*alanyl-*L-*alanine linked to estradiol in comparison with unlinked single agents and ovariectomy*

Treatment group[a]	Group No[b]	Receptor content before randomisation[c] Estrogen (fmol bound/mg cytosolic protein)	Progestin	Receptor content 33 days after randomisation[c] Estrogen (fmol bound/mg cytosolic protein)	Progestin
CNC-L-alanine	I	97 ± 37	68 ± 69	38 ± 26	47 ± 62
CNC-L-alanyl-L-alanine	II	132 ± 96	33 ± 28	11 ± 9	1 ± 2
CNC-L-alanine-estradiol-3-ester	III	87 ± 23	150 ± 205	6 ± 12	16 ± 21
CNC-L-alanine-6-α-hydroestradiol-6α-ester	IV	NT[d]	NT	NT	NT
CNC-L-alanine-estradiol-17-ester	V	44 ± 9	286	0 ± 0	243
CNC-L-alanine-estradiol-3,17-diester	VI	39	78	0	313
CNC-L-alanine + estradiol	VII	NT	NT	NT	NT
CNC-L-alanyl-L-alanine-estradiol-3-ester	VIII	45 ± 25	158 ± 13	0 ± 0	286 ± 346
CNC-L-alanyl-L-alanine + estradiol	IX	NT	NT	NT	NT
Ovariectomy	X	52 ± 49	482 ± 139	38 ± 45	5 ± 7
Untreated controls	XI	105 ± 106	161 ± 129	47 ± 25	29 ± 51

[a] Administration of four doses of 67 μmol/kg (I, II) or 105 μmol/kg (III, V, VI, VIII), respectively.
[b] Five tumors per group were excised before and 4 days after the last therapy, respectively.
[c] Mean ± S.D.
[d] NT = not tested.

Table 6. Time course of cytosolic estrogen- and progesterone receptor contents in methylnitrosourea induced rat mammary carcinomas following a single dose of 75 μmol/kg *CNC-*L-*alanine estradiol-*17-*ester*

Time[a]	Receptor contents[b] Estrogen[c]	Progesterone[c]
0	111 ± 87	223 ± 221
8	1 ± 2	430 ± 376
16	6 ± 8	1077 ± 701
24	2 ± 3	638 ± 703
48	0.5 ± 1	363 ± 323
96	3 ± 4	690 ± 568
192	14 ± 17	188 ± 196

[a] Hours after treatment.
[b] fmol bound/mg protein.
[c] Mean values of six tumors per time point ± S.D.

Estradiol receptor affinity

As can be seen from Table 8, highest affinity of the estradiol-derived compounds is displayed by CNC-L-alanyl-L-alanine (about 10% RBA, Table 8). The corresponding CNC-L-alanine-ester shows about half of this affinity (about 5% RBA) whereas the CNC-L-alanine-estradiol-17-ester reveals distinctly less affinity (about 1% RBA). 6α-hydroxy-estradiol owns only 6.3% affinity relative to estradiol, all other compounds exhibit only negligible RBA values.

DISCUSSION

The present study with estrogen-linked nitrosoureas is part of a comprehensive structure-activity investigation designed to find the optimal sites and types of chemical bonding of a strongly cytocidal group to a hormone carrier molecule. A first contribution dealing with a smaller spectrum of hormone analogues and demonstrating the relevance of MNU-induced mammary carcinoma as an informative model even for short term evaluations, has been published recently [7]. Basically the MNU-model offers distinct advantages in the search for new hormone-linked compounds, since the majority of these tumours are known to be hormone-dependent [20, 21] and display measurable concentration of estradiol- and progesterone receptors [25, 26], an observation which could be found in this study, also. With regard to growth parameters these primary tumours resemble more closely the human situation than transplanted tumours [27] and, concomitantly, are less sensitive to clinically used drugs; this latter finding was established in case of tamoxifen [28] or the combination of cyclophosphamide, methotrexate and 5-fluorouracil [17]. In this study the efficacy of a group with known anticancer activity [7] was evaluated towards MNU-induced tumours after linking it to estradiol in various positions and giving special consideration to long term evaluation

Table 7. Uterine activity of N-(2-*chloroethyl*)-N-*nitrosocarbamoyl* (*CNC*)-L-*alanine and CNC-*L-*alanyl-*L-*alanine linked to estradiol*

Group No.[a]	Compound	Single (total) dose[b] (μmol/kg)	Efficacy[c] $\left(\frac{\text{dry uterus weight (mg)}}{\text{animal weight (g)}} \times 100\right)$	% estradiol[c] efficacy
I a	CNC-L-alanine-estradiol-3-ester	0.012(0.036)	72.7 ± 12.0	110.3 ± 18.2
I b	"	0.06(0.18)	69.3 ± 7.5	105.2 ± 11.4
I c	"	0.3(0.9)	83.4 ± 7.3	126.6 ± 11.1
I d	"	1.5(4.5)	92.6 ± 8.2	140.5 ± 12.4
I e	"	7.5(22.5)	109.9 ± 9.0	166.8 ± 13.7
II a	CNC-L-alanine-6α-hydroxy-estradiol-6α-ester	0.012(0.036)	54.5 ± 11.8	82.7 ± 17.9
II b	"	0.06(0.18)	46.3 ± 6.3	70.3 ± 9.6
II c	"	0.3(0.9)	58.8 ± 8.7	89.2 ± 13.2
II d	"	1.5(4.5)	52.6 ± 16.1	79.8 ± 24.4
II e	"	7.5(22.5)	63.0 ± 11.2	95.6 ± 17.0
III a	CNC-L-alanine-estradiol-17-ester	0.012(0.036)	67.5 ± 22.2	102.4 ± 3.7
III b	"	0.06(0.18)	88.2 ± 17.1	133.8 ± 25.9
III c	"	0.3(0.9)	97.5 ± 12.9	148.0 ± 19.6
III d	"	1.5(4.5)	100.3 ± 18.7	152.2 ± 28.4
III e	"	7.5(22.5)	91.0 ± 18.4	138.1 ± 27.9
IV a	CNC-L-alanyl-L-alanine-estradiol-3-ester	0.012(0.036)	56.9 ± 14.0	86.3 ± 21.2
IV b	"	0.06(0.18)	78.6 ± 8.8	119.3 ± 13.4
IV c	"	0.3(0.9)	82.8 ± 12.0	125.6 ± 18.2
IV d	"	1.5(4.5)	100.7 ± 19.1	152.8 ± 29.0
IV e	"	7.5(22.5)	104.5 ± 9.5	158.6 ± 14.4
V a	CNC-L-alanine-estradiol-3,17-diester	0.012(0.036)	76.5 ± 15.0	116.1 ± 22.8
V b	"	0.06(0.06)	83.3 ± 25.5	126.4 ± 38.7
V c	"	0.3(0.9)	110.4 ± 16.8	167.5 ± 25.5
V d	"	1.5(4.5)	96.7 ± 13.7	146.7 ± 20.8
V e	"	7.5(22.5)	97.4 ± 11.9	147.8 ± 18.1
VI a	Estradiol	0.0024(0.0072)	43.6 ± 6.6	66.2 ± 10.0
VI b	"	0.012(0.036)	48.7 ± 10.3	73.9 ± 15.6
VI c	"	0.06(0.18)	65.9 ± 14.0	100.0 ± 21.2
VI d	"	0.3(0.9)	65.6 ± 8.5	99.5 ± 12.9
VII a	6-Hydroxy-estradiol	0.0024(0.0072)	33.0 ± 5.7	50.1 ± 8.6
VII b	"	0.012(0.036)	39.0 ± 5.8	59.2 ± 8.8
VII c	"	0.06(0.18)	36.9 ± 9.7	56.0 ± 14.7
VII d	"	0.3(0.9)	45.5 ± 5.6	69.0 ± 8.5
VIII	Solvent control	– –	51.3 ± 15.2	77.8 ± 23.1
IX	Untreated control	– –	38.1 ± 14.9	57.8 ± 22.6

[a] Number of animals in groups I–VII: eight per subgroup in groups VIII–IX: 16 each.
[b] Drugs were given at days 19, 20, 21 of life.
[c] Mean ± S.D.

(4 and 7 weeks after start of treatment). Previous studies have suggested that the chemical link should preferably be such that the cytocidal group can be released hydrolytically or by enzymatic cleavage [29]. We therefore choose for this study an ester type of link.

Clearly, remarkable differences in biological activity became apparent with regard to the site of binding to the carrier. The 17-position of estradiol is superior to the other positions tested for linking CNC-L-alanine. Esterification in position 17 not only brings about highest antitumour effectiveness, but also reduced toxicity. Already at 54 μmol/kg the optimal effect of CNC-L-alanine-estradiol-17-ester is reached. This tumour inhibitory effect meets NCI criteria for high activity. This therapeutic effect which extends over two dose levels (54 and 75 μmol/kg) is neither reached by unlinked nitrosoureas [30] nor by the unlinked equimolar mixture of CNC-L-alanine and estradiol ($P = 0.01$ according to Koziol and Donna). At the 54 μmol/kg dose level, the unlinked mixture and the 17-linked derivative display comparably low toxicities. At the following dose, however, the linked compound retains its low toxicity and high therapeutic efficacy, whereas the unlinked mixture becomes highly toxic and barely active. The significant difference between the 17-linked derivative

Table 8. Affinity to estrogen receptor obtained from calf uterus cytosol in relation to estradiol binding affinity

Compound	% RBA[a]	(95% confidence limits)
CNC-L-alanine-estradiol-3-ester	4.7	(3.5– 6.7)
CNC-L-alanyl-L-alanine-estradiol-3-ester	10.2	(8.9–11.9)
CNC-L-alanine-estradiol-17-ester	0.8	(0.6– 1.0)
CNC-L-alanine-estradiol-3,17-diester	< 0.05	
6α-hydroxy-estradiol	6.3	(3.2–13.9)
CNC-L-alanine-6α-hydroxy-estradiol-6α-ester	0.28	(0.23–0.35)

[a] RBA = relative binding affinity, defined as quotient of molar concentrations of estradiol and test compound × 100;
RBA of estradiol = 100.

and the unlinked mixture becomes even more impressive in view of the fact, that estradiol itself obviously displays antineoplastic activity at these pharmacologic dose levels. This becomes clear when comparing groups I vs. VII and II vs. IX from Table 2. The antitumour activity of estradiol might probably be mediated by down regulation of the estradiol receptors [31]. This hypothesis is consistent with the observed strong decrease of estradiol receptor content under therapy with hormone-linked derivatives as shown in Tables 5 and 6. In accordance with the concept that the 3-OH group is important for estradiol receptor binding [32] it is not surprising that the 3-derivative is antineoplastically less active than its 17-congener at the dose of 75 μmol/kg ($P < 0.003$ according to Koziol and Donna). This also holds true for the corresponding 3-dipeptide derivative, which is rather similar in biological activity to CNC-L-alanine-estradiol-3-ester. The 3-derivatives are distinctly more toxic than their 17-congener. This can perhaps be attributed, at least in part, to the blocking of the 3-OH group, which is not available for detoxifying conjugation reactions.

The relevance of position 3 for toxicity is, however, not supported by the results obtained with the derivative of 6α-OH-estradiol. This analogue has been designed and prepared to obtain a derivative having free OH groups in positions 3 and 17, which generally are supposed to be relevant for high receptor binding affinity. A similar rationale has recently been followed by other authors [33], who synthesized an N-Lost adduct in position 6α of estradiol. Unfortunately these authors did not give any data on receptor binding affinity or antineoplastic efficacy of this compound. We found quite unexpectedly that the CNC-L-alanine-6α-hydroxyestradiol-6α-ester not only was highly toxic but also completely inactive. Moreover, this compound has very low binding affinity to the cytosolic estrogen receptor (0.28% RBA). The 3,17-diester also is more toxic than the 17-monoester and displays inferior antineoplastic activity, comparable to the 3-analogue.

With regard to ILS criteria no real benefit was obtained except some marginal prolongation of the median life span in groups IIa and b, Vc, VIIa and X. This is indicative of a considerable toxicity inherent to the CNC-moiety that could not be extinguished fully by linking it to the estradiol carrier. Nevertheless, an interesting improvement was obtained.

Highest estradiol receptor affinity of all compounds is own to the dipeptide derivative. It is reduced to half, when the dipeptide in position 3 of estradiol is replaced by CNC-L-alanine. It is surprising at first glance, that this 3-derivative has higher receptor binding affinity than the corresponding 17-derivative. However, the apparent discrepancy between binding affinity and therapeutic efficacy of these analogues might possibly be result of partial cleavage of the phenolic ester bond resulting in release of estradiol. Liberated estradiol could then be responsible for artificially high RBA values.

The low therapeutic quality of CNC-L-alanine-6αhydroxy-estradiol-6α-ester could be explained in part by its low RBA value; the reasons for its high toxicity however, remain unclear. All CNC-L-alanine-derivatives display uterotrophic effects equal to or higher than those of the corresponding parent hormone. This is not only true for estradiol derivatives, but even for the 6α-hydroxy-estradiol derivative. The disappearance of cytosolic estrogen-receptors and the remarkable increase in cytosolic progestine receptor content observed under therapy with these analogues indicate that they retain estrogen-like effects. We are currently investigating the possibility to exploit these variations in receptor contents, especially the enhancement of progestin receptor contents, for sequential therapy, first with estradiol- and then with progesterone derivatives.

In summary, the relevance of receptor contents for chemotherapy of this mammary tumour model with hormone-linked agents is evidenced by the present study. Further support for the crucial role of receptor contents comes from results obtained in the MXT mammary mouse tumour [34]. In accordance with the results obtained with MNU-induced rat mammary tumours, the mammary MXT mouse tumour is sensitive to therapy only as long as it contains estradiol receptors, with the 17-derivative again being the most active drug. Loss of sensitivity, however, is observed when therapy is carried out at later passage, characterized by loss of receptor contents.

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