and antithrombin III deficiency in adult lymphoblastic leukemia during L-asparaginase therapy. *Acta Haematol* 1983, **69**, 188–191.
21. Homans AC, Rybak ME, Baglini RL, Tiarks C, Steiner ME, Forman EN. Effect of L-asparaginase administration on coagulation and platelet function in children with leukemia. *J Clin Oncol* 1987, 5, 811–817.
22. Hansen JF, Carpenter RH. Fatal acute systemic anaphylaxis and hemorrhagic pancreatitis following asparaginase treatment in a dog. *J Am Anim Hosp Assoc* 1983, **19**, 977–980.
23. Swanson JF, Morgan S, Green RA, Woody BJ. Cerebral thrombosis and hemorrhage in association with L-asparaginase administration. *J Am Anim Hosp Assoc* 1986, 22, 749–755.
24. Liu UP, Chabner BA. Enzyme therapy: L-asparaginase. In Chabner B, ed. *Pharmacologic Principles of Cancer Chemotherapy*. Philadelphia, WB Saunders, 1982, 435–443.

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An Oestradiol-linked Nitrosourea and Site-directed Chemotherapy in Mammary Carcinoma

B. Betsch, M.R. Berger, B. Spiegelhalder, D. Schmähl and G. Eisenbrand

The half-life, peak concentration, peak accumulation and tissue availability of the DNA-crosslinking nitrosourea 1-(2-chloroethyl)-1-nitrosocarbamoyl-L-alanine (CNC-alanine) and its oestradiol-linked derivate (CNC-alanine-oestradiol-17-ester) were studied in liver, lung, spleen, uterus and mammary carcinomas in female Sprague-Dawley rats with chemically induced mammary carcinomas. Compared with CNC-alanine, the ester had a longer half-life, higher peak concentration, increased peak accumulation and enhanced tissue availability in all tissues. In oestradiol receptor positive mammary carcinomas, the oestradiol-linked drug showed a 2 times higher peak concentration, a 5 times longer half-life, a 10 times increased peak accumulation and a 20 times greater tissue availability compared with CNC-alanine. Oestradiol-linked nitrosoureas may offer new perspectives for site-directed chemotherapy of oestradiol receptor positive breast cancer.
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INTRODUCTION

OESTRADIOL-LINKED nitrosoureas have modified pharmacokinetic [1–3] and antitumour properties compared with the corresponding single agents. Increased half-life ($T_{1/2}$) and volume of distribution of the conjugate cannot fully explain the pharmacodynamic superiority of 1-(2-chloroethyl)-1-nitrosocarbamoyl-L-alanine-oestradiol-17-ester (CNC-alanine-oestradiol-17-ester). For the improved antineoplastic activity and decreased toxicity that have been observed in preclinical studies [4–6], a specific pattern of drug distribution can be supposed [3, 7]. Although preferential drug distribution into oestradiol receptor positive tumours is the rationale for the use of oestradiol-linked anticancer agents, there is hardly any knowledge about the disposition of such drugs [8].

For site-directed chemotherapy of receptor positive breast cancer the hormone should function as a carrier [9] that leads to receptor-mediated drug accumulation in receptor positive mammary carcinoma cells [10, 11]. In this way, at least some sparing of non-target tissues should be achievable, allowing more selective, less toxic chemotherapy.

We have studied the disposition of CNC-alanine and its oestradiol conjugate in receptor positive tissues (uterus and mammary carcinomas), the liver and in receptor negative tissues (lung and spleen) of rats with chemically induced mammary carcinoma.

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MATERIALS AND METHODS

Drugs

CNC-alanine and CNC-alanine-oestradiol-17-ester were synthesized [7] and checked for purity by thin-layer chromatography and nuclear magnetic resonance. CNC-alanine and CNC-alanine-oestradiol-17-ester were dissolved in physiological saline and dimethylsulphoxide, respectively. Equimolar doses (137 μmol/kg) were administered via the tail vein, corresponding to 30 mg/kg CNC-alanine and 66 mg/kg CNC-alanine-oestradiol-17-ester.

The relative binding affinity (RBA) to an oestradiol receptor preparation from calf uterus cytosol is known for both compounds [7]. For CNC-alanine no oestradiol receptor affinity was detectable (RBA = 0%), while for CNC-alanine-oestradiol-17-ester an RBA of 1% was measured compared with that for oestradiol (100%).

*Table 1. Concentration–time courses of CNC-alanine after intravenous administration of 30 mg/kg**

Time (min)	Tissue concentration (nmol/g) Liver	Lung	Spleen	Uterus	Mammary carcinoma
1	20.0 (5.3)	7.3 (2.1)	13.0 (3.5)	ND	2.0 (1.7)
15	13.3 (3.5)	2.3 (1.9)	11.3 (4.9)	ND	1.0 (1.7)
30	8.3 (5.7)	0.4 (0.6)	2.3 (4.0)	ND	ND
60	4.3 (0.6)	ND	ND	ND	ND
120	ND	ND	ND	ND	ND

*Mean (S.D.), n = 3. ND = not detectable.

Animals and tumours

Female Sprague-Dawley rats (Institut für Versuchtierkunde, Hannover) with an average body weight of 250 (S.D. 50) g were used. They had free access to 'Altromin 1320', and water. After an adaption period of approximately one week mammary carcinomas were induced by intravenous injection of methylnitrosourea (MNU) [7, 14]. Animals were fasted for 24 h before the experiments.

Experimental design

Drugs were given in a quick bolus injection via the tail vein. Immediately before and at 1, 15, 30, 60, 120 and 240 min after injection, for each time 3 animals were killed by cervical dislocation under ether anaesthesia. Samples of liver, lung, spleen, uterus and mammary carcinomas were immediately frozen in liquid nitrogen and stored at −60°C. An aqueous solution of elmustine was added as an internal standard (100 μl; concentration 1 mg/ml).

Assay

Drugs were extracted from tissues by homogenizing three times ('Ultraturax IKA') in methanol at a tissue/methanol ratio of 1/2. After centrifugation (800 *g*, 4°C, 30 min) supernatants were concentrated to a defined volume (1 ml) under nitrogen. Samples were maintained at −4°C in an ice/salt bath [3, 8].

Analysis was done by high-pressure liquid chromatography. Aliquots of 15 μl of clear supernatant were injected. Separation was done on a 'Spherisorb' C_{18} column (250 × 4 mm, 25 μm) with a solvent gradient of methanol/acetonitrile/water (1 ml/min). Substances were detected with a diode array detector between 200 and 400 nm. Calibration curves were linear between 0.1 and 500 nmol/g of tissue. Concentrations were expressed after correction for internal standard recovery. The limit of detection was 0.1 nmol/g. In samples with several unknown peaks in the region of interest ultraviolet spectra were recorded at the specified regions of interest [3, 8].

Table 2. Pharmacokinetic indices of CNC-alanine

Tissue	Index*	Mean (±S.D.)
Liver	$T_{1/2}$	31.9 (14.2)
	C_{max}	20.0 (4.0)
	C_{accum}	10.4 (2.3)
	Tissue availability	5.5 (0.3)
Lung	$T_{1/2}$	12.0 (3.2)
	C_{max}	7.3 (2.3)
	C_{accum}	2.2 (0.6)
	Tissue availability	0.7 (0.06)
Spleen	$T_{1/2}$	17.3 (12.6)
	C_{max}	14.6 (3.6)
	C_{accum}	4.1 (1.1)
	Tissue availability	1.5 (0.16)
Mammary carcinoma	$T_{1/2}$	17.3 (12.6)
	C_{max}	2.0 (0.5)
	C_{accum}	0.9 (0.4)
	Tissue availability	0.2 (0.05)

*$T_{1/2}$ in min, C_{max} in nmol/g, C_{accum} as % of plasma level and tissue availability as % of dose.

Table 3. Concentration–time courses of CNC-alanine-oestradiol-17-ester after intravenous administration of 66 mg/kg

Time (min)	Tissue concentration (nmol/g) Liver	Lung	Spleen	Uterus	Mammary carcinoma
1	12.6 (0.6)	16.0 (6.2) 12.0	7.3 (1.5)	6.6 (0.6)	3.0 (1.4)
15	7.0 (5.1)	(1.4)	5.6 (0.6)	5.0 (1.0)	4.0 (2.0)
30	6.0 (1.7)	8.5 (0.7)	4.6 (5.0)	4.6 (1.1)	3.0 (0.2)
60	5.0 (4.4)	6.0 (1.4)	2.6 (1.5)	4.0 (1.4)	3.5 (0.7)
120	4.6 (0.6)	2.0 (3.4)	1.0 (0.1)	3.1 (1.4)	1.0 (0.1)
240	3.0 (1.0)	ND	ND	1.0 (1.5)	0.8 (0.1)
480	1.3 (1.1)	ND	ND	ND	ND

Pharmacokinetics

Pharmacokinetic values were calculated according to standard methods [15–17] and were given as arithmetic means. Peak accumulation (C_{accum}) was obtained by the ratio of the concentration in tissue to that in plasma. Tissue availability was determined by the ratio of the area under the curves (AUC) of $AUC_{tissue}/AUC_{plasma}$. For specified time points the plasma concentrations and the AUC_{plasma} were taken from earlier studies [8].

RESULTS

CNC-alanine

In all tissues, the concentration–time curve after intravenous administration of 30 mg/kg CNC-alanine followed a one-compartment model, reaching peak concentration (C_{max}) 1 min after injection. 2 h after injection all tissue drug concentrations were below the detection limit (Table 1). The highest values for $T_{1/2}$, C_{max}, C_{accum} and tissue availability were recorded in the liver (Table 2).

CNC-alanine-oestradiol-17-ester

After intravenous application of 66 mg/kg CNC-alanine-oestradiol-17-ester the concentration-time courses in all tissues followed a one-compartment model. C_{max} was reached 1 min after injection. 4 h after injection intact drug was still detected in liver, uterus and mammary carcinoma. 8 h after administration drug concentrations in uterus and mammary carcinoma were below the detection limit whereas in the liver intact drug was still present (Table 3). Again, the longest $T_{1/2}$ and highest tissue availability were found in the liver (Table 4).

Table 4. Pharmacokinetic indices of CNC-alanine-oestradiol-17-ester

Tissue	Index	Mean (±S.D.)
Liver	$T_{1/2}$	250.6 (72.6)
	C_{max}	12.6 (0.6)
	C_{accum}	18.0 (1.7)
	Tissue availability	16.6 (7.9)
Lung	$T_{1/2}$	84.3 (76.6)
	C_{max}	16.6 (5.6)
	C_{accum}	21.0 (2.8)
	Tissue availability	4.8 (1.9)
Spleen	$T_{1/2}$	41.1 (4.8)
	C_{max}	7.6 (2.3)
	C_{accum}	9.6 (0.6)
	Tissue availability	3.2 (1.0)
Mammary carcinoma	$T_{1/2}$	105.5 (2.5)
	C_{max}	4.6 (1.3)
	C_{accum}	8.8 (1.6)
	Tissue availability	4.2 (0.5)
Uterus	$T_{1/2}$	114.8 (102.0)
	C_{max}	6.6 (0.6)
	C_{accum}	10.6 (4.2)
	Tissue availability	4.1 (3.0)

DISCUSSION

CNC-alanine-estradiol-17-ester and the corresponding unlinked CNC-alanine had different tissue distribution. In comparison with CNC-alanine the ester had a longer $T_{1/2}$ and higher C_{max}, C_{accum} and tissue availability in all tissues studied. In mammary carcinomas, the oestradiol-linked drug had a 2 times higher C_{max}, a 5 times longer $T_{1/2}$, a 10 times higher C_{accum} and, above all, a 20 fold higher tissue availability. These differences can partly be explained by the highly increased lipophilicity of the hormone-linked drug which facilitates diffusion through membranes.

Binding to the oestradiol receptor is obviously a prerequisite for preferential drug uptake in receptor-containing tissues. In oestradiol receptor positive tissues, such as uterus and mammary carcinomas, a significantly longer $T_{1/2}$ was observed for CNC-alanine-oestradiol-17-ester (RBA = 1%) than in the receptor negative tissues (lung and spleen). For CNC-alanine (RBA = 0%), no significant differences in $T_{1/2}$ between receptor positive and receptor negative tissues were found. In receptor positive mammary carcinomas, CNC-alanine-oestradiol-17-ester had strongly enhanced tissue availability compared with CNC-alanine. A strong and long-lasting depletion of cytosolic oestradiol receptor contents in MNU-induced mammary carcinomas has been observed with CNC-alanine-oestradiol-17-ester. This effect was not seen with CNC-alanine [7, 13]. As a consequence of the linking to oestradiol, CNC-alanine has higher antineoplastic activity and lower toxicity in the therapy of oestradiol receptor positive mammary carcinomas. This appears to result from affinity for oestradiol receptor positive tumour cells which obviously leads to sparing of oestradiol receptor negative tissues, concomitantly with an accumulation of the drug in oestradiol receptor positive mammary carcinomas [4–6]. In oestradiol receptor negative tumours the CNC-alanine-oestradiol-17-ester lost its therapeutic advantage and displayed in terms of antineoplastic activity and toxic side-effects no differences from unlinked CNC-alanine [7].

Future investigations with steroid-linked anticancer agents should be focused on the development of drugs with further improved antineoplastic activity based on enhanced steroid hormone receptor affinity. This could be achieved by coupling highly antineoplastic drugs (e.g. cyclophosphamide, nitrosoureas) to hormone derivatives with strong receptor affinity. For the connection between hormone and cytotoxic moiety a more solid linkage should be used than the ester linkage [8]. This could preserve the drug integrity required for a receptor mediated activity. Our results indicated that oestradiol-linked nitrosourea agents offer new perspectives for site-directed chemotherapy of oestradiol receptor positive mammary carcinomas.

1. Betsch B, Berger MR, Spiegelhader B, Eisenbrand G, Schmähl D. Estradiol-linked nitrosoureas: new anticancer agents with clearly modified pharmacokinetic properties. *Eur J Clin Pharmacol* 1989, **36** (Suppl.), 308.
2. Betsch B, Berger MR, Spiegelhalder B, Eisenbrand G, Schmähl D. Pharmacokinetics and bioavailability of N-(2-chloroethyl)-N-nitrosocarbamoyl-L-alanine-estradiol-17-ester (CNC-ala-E_2-E) in mammary tumor bearing Sprague-Dawley rats. *J Cancer Res Clin Oncol* 1989, **115**, 62.
3. Betsch B, Berger MR, Spiegelhalder B, Eisenbrand G, Schmähl D. New estradiol-linked nitrosoureas: Can the pharmacokinetic properties help to explain the pharmacodynamic activities? *Eur J Cancer Clin Oncol* 1985, **25**, 105–111.
4. Berger MR, Floride J, Schreiber J, Schmähl D, Eisenbrand G. Evaluation of new estrogen-linked-2-chloroethylnitrosoureas. I. Short term anticancer efficacy in methylnitrosourea-induced rat mammary carcinoma. *J Cancer Res Clin Oncol* 1984, **108**, 148–153.
5. Berger MR, Henne T, Schmähl D. Differential affinity of two new estradiol-linked nitrosoureas in comparison with their respective single agents to tumour and bone marrow of methylnitrosourea-induced SD rats. *Recent Adv Chemother* 1985, 483–484.
6. Zeller WJ, Schreiber J, Eisenbrand G. Cytostatic activity of steroid-linked nitrosoureas on the MXT mammary carcinoma. *Proc AACR* 1985, **26**, 250.
7. Berger MR, Floride J, Schmähl D, Schreiber J, Eisenbrand G. Estrogen-linked 2-chloroethylnitrosoureas: anticancer efficacy in MNU-induced rat mammary carcinoma, uterine activity in mice and receptor interactions. *Eur J Cancer Clin Oncol* 1986, **22**, 1179–1191.
8. Betsch B. Pharmacokinetische Studien mit östradiolgekoppelten Nitrosoharnstoffderivaten an Ratten mit chemisch induziertem Mammakarzinom. Nachweis der erhöhten Affinität eines östradiolgekoppelten Zytostatikums zu Östradiolrezeptor positven Geweben. 1988, M.D. Thesis, Faculty of Medicine, University of Heidelberg, F.R.G.
9. Muggia FM, Lippman M, Heuson J-C (eds). Reports from the workshop on the use of steroid as carriers in cytotoxic in breast cancer. *Cancer Treat Rep* 1978, **62**, 1239–1268.
10. Druckrey H, Raabe S. Organspezifische Chemotherapie des Krebses. *Klin Wsch* 1952, **30**, 882–884.
11. Eisenbrand G, Müller N, Schreiber J, *et al.* Drug design: nitrosoureas. In: Schmähl D, Kaldor IM (eds). *Carcinogenicity of Alkylating Cytostatic Drugs*. IARC Sci. Publ. No. 78. 1986, 281–293.
12. Eisenbrand G, Berger M, Fischer J, Scheider MR, Zeller J, Tang W. N'-(2-chloroethyl)-N'-nitrosocarbamoyl amino acid derivatives of steroid hormones. *Cancer Treatment Rev* 1987, **14**, 285–290.
13. Fischer J. Synthese von Steroid-verknüpften N-[N'-(2-Chloroethyl)-N'-nitrosocarbomyl]-aminosäurederivaten und Untersuchung ihrer biologischen Wirkung. PhD Thesis, University of Kaiserslautern, 1988.
14. Berger MR, Habs M, Schmähl D. Noncarcinogenic chemotherapy with a combination of vincristine, methotrexate and 5-fluorouracil (VMF) in rats. *Int J Cancer* 1983, **32**, 231–236.
15. Dost FH. *Grundlagen der Pharmakokinetik*. Stuttgart, Georg Thieme 1969.

16. Wagner JC. *Fundamentals of Clinical Pharmacokinetics*. Hamilton Illinois, Drug Intelligence Publications, 1976.
17. Ritschel WA. *Handbook of Basic Pharmacokinetics*. Hamilton, Illinois, Drug Intelligence Publications, 1976.
18. Corr R, Berger MR, Betsch B, Brix HP, Floride J, Schmähl D. Modulation of cytosolic sexual steroid receptors in autochthonous methylnitrosourea-induced rat mammary carcinoma following application of 2-chloroethylnitrosocarbamoyl-L-alanine linked to oestradiol or dihydrotestosterone. *Br J Cancer* 1990, **62**, 42–47.

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Activity of Cytostatic Drugs in Two Heterotransplanted Human Testicular Cancer Cell Lines with Different Sensitivity to Standard Agents

Andreas Harstrick, Hans-Joachim Schmoll, Jochen Casper, Hansjochen Wilke and Hubert Poliwoda

Two established human testicular cancer cell lines were used in a mouse xenograft model to assess the antitumour activity of 15 anticancer agents. Line H 12.1 was highly sensitive to cisplatin, bleomycin and vinblastine, resembling non-pretreated testicular tumours, whereas line H 23.1 showed resistance to cisplatin and vinblastine, comparable to tumours with acquired or intrinsic drug resistance. In line H 12.1 several drugs were highly active, including cyclophosphamide, ifosfamide, nimustine and vincristine; carmustine, vindesine, doxorubicin, epidoxorubicin, pirarubicin, mitoxantrone, carboplatin and iproplatin had only moderate activity. In line H 23.1 only cyclophosphamide, ifosfamide, nimustine, vincristine and bleomycin had antitumour activity. These two cell lines represent a useful model for preclinical evaluation of new agents with presumed activity in testis cancer. *Eur J Cancer*, Vol. 26, No. 8, pp. 898–901, 1990.

INTRODUCTION

THE RATE of complete remissions achieved with cisplatin-based combination chemotherapy in non-seminomatous testicular cancer ranges between 85 and 95%, and most patients can expect to be long-term survivors [1–3]. But patients with advanced disease or patients relapsing after first-line chemotherapy have a poor prognosis [4, 5], and all chemotherapy protocols currently in use have serious side-effects [6, 7].

Clinical investigation of new agents in testicular cancer is hampered by the numbers of eligible patients being too small, which makes preclinical evaluation of new drugs important. Human tumour tissue, transplanted into congenitally athymic nude mice, is a valid and reliable test system for *in vivo* evaluation of chemotherapeutic drugs. The heterotransplanted tumours preserve their histological and biological characteristics. Furthermore, the response of xenografted tumours is correlated with clinical response [8–11].

We have used two established human testicular cancer cell lines with differences in response to standard drugs to assess the activity of a panel of new agents.

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MATERIALS AND METHODS

Cell lines

Cell lines H 12.1 and H 23.1 were both established by J.C. in our laboratory. The origin and histology after heterotransplantation of both lines are shown in Table 1. Neither of the two patients had received chemotherapy before orchiectomy. The cell lines were grown as continuous monolayer cultures in RPMI 1640 supplemented with 15% fetal calf serum (Biochrom), penicillin 2 IU/ml, streptomycin 2 μg/ml and L-glutamine

Table 1. Cell line characteristics

	Cell line	
	H 12.1	H 23.1
Origin, date of establishment	Testis, 21/5/81	Testis, 27/4/84
Primary histology	S,EC,T,CC	EC
Histology after heterotransplantation	EC,T,STGC	EC,YS
Doubling-time after heterotransplantation (days)	13.5	13

S = seminoma, T = teratoma, EC = embryonal carcinoma, CC = chorioncarcinoma, STGC = syncytiothrophoblastic giant cells and YS = yolk sac.