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New Estradiol-linked Nitrosoureas: Can the Pharmacokinetic Properties Help to Explain the Pharmacodynamic Activities?

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Abstract—*The pharmacokinetics of 1-(2-chloroethyl)-1-nitrosocarbamoyl-L-alanine-estradiol-17-ester (CNC-alanine-estradiol-17-ester) a new estradiol-linked anticancer drug and the unlinked DNA-crosslinking agent 1-(2-chloroethyl)-1-nitrosocarbamoyl-L-alanine (CNC-alanine) have been studied in methylnitrosourea-induced female Sprague–Dawley rats after equimolar intravenous and oral administration.*

In comparison with the unlinked single agent, the CNC-alanine-estradiol-17-ester showed a 3-fold longer halflife in plasma and a three times larger volume of distribution. The distribution after intravenous administration was nearly three times faster. The absorption after peroral administration was likewise two times faster. The bioavailability of the estradiol-linked drug was determined to be 52%. After application of CNC-alanine-estradiol-17-ester the cytostatic metabolite CNC-alanine was found, indicating the cleavage of the ester bond. CNC-alanine generated from CNC-alanine-estradiol-17-ester showed a 50% longer halflife than when applied directly.

The results indicate that linking 2-chloroethyl-nitrosoureas to estradiol can result in new anticancer agents with modified properties in comparison to the unlinked single agent. The higher antineoplastic activity of the hormone-linked drug can mainly be attributed to differences in the pharmacokinetic behaviour.

1. INTRODUCTION

2-CHLOROETHYLNITROSOUREAS are known to be highly active antineoplastic agents in a wide variety of experimental tumour models [1]. However, the clinical use of these compounds is still limited due to their unselective toxicity towards all rapidly proliferating tissues. One main toxicity is delayed bone marrow suppression [2]. There is continued interest in the development of new analogue compounds with higher antineoplastic activity and lower toxicity [3–5].

The work of our group has been directed to the development of steroid hormone-linked anticancer agents. Linking cytotoxic drugs to steroid hormones can deliver new antineoplastic agents with a higher therapeutic ratio. The steroid hormone has the function of a carrier molecule [6] and should increase the affinity of the drug towards hormone receptor positive tumours [7, 8].

Two representatives of steroid hormone-linked compounds that are currently used in oncological clinics are prednimustine (a prednisoloneester of chlorambucil) and estramustine phosphate (an estradiol-3-*N*-lost-carbamate-17-phosphate). The main advantage of these drugs is their reduced toxicity in comparison with the unlinked single agents [9–11]. It has, however, been shown that prednimustine is very rapidly cleaved *in vivo* thus not allowing to detect intact compound following p.o. application [12], whereas estramustine phosphate has no estradiol receptor affinity and is not an alkylating agent [13].

Several preclinical therapeutic studies with steroid hormone-linked nitrosoureas in rats with chemically induced mammary tumours have demonstrated that CNC-alanine linked to estradiol in position 17 (CNC-alanine-estradiol-17-ester) provides a drug with a higher antineoplastic activity and lower toxicity in comparison to the unlinked

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single agents CNC-alanine and estradiol [14–17]. Systematic comparative pharmacokinetic studies with the two corresponding compounds (CNC-alanine and CNC-alanine-estradiol-17-ester) described in this article were to investigate whether the differences in the therapeutic ratio are due to differences in the pharmacokinetic behaviour.

MATERIALS AND METHODS

Drugs

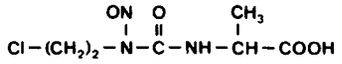
The test compounds (Fig. 1), 1-(2-chloroethyl)-1-nitrosocarbamoyl-L-alanine (CNC-alanine) and CNC-alanine-estradiol-17-ester were synthesized according to known methods [17].

The chemical purity of the two compounds (yellow, crystalline powders) was determined by thin-layer chromatography and NMR spectrometry. The drugs were dissolved immediately before use. CNC-alanine-estradiol-17-ester was injected in dimethylsulphoxide (DMSO) and CNC-alanine was given in physiological saline. The concentrations of the solutions were 132 mg/ml (CNC-alanine-estradiol-17-ester) and 60 mg/ml (CNC-alanine). The compounds were given on an equimolar basis at 137 μmol/kg which corresponded to 66 and 30 mg/kg, respectively.

Animals and tumours

Female Sprague–Dawley rats with an average body weight of 250 ± 50 g were used for all experiments. All animals were maintained on a standard diet (Altromin® 1320) and water *ad libidum*. After a period of adaption (8–10 days) mammary carcinomas were induced with methylnitrosourea (MNU) as described elsewhere [18]. Before administration of drugs two venous catheters that were especially developed for pharmacokinetic studies in rats [19] were implanted under ether anesthaesia. The first catheter positioned in the vena jugularis served for drug application and the second in the vena cava was used for blood sampling. The animals were fasted 24 h before the experiments.

CNC-L-alanine



CNC-L-alanine-estradiol-17-ester

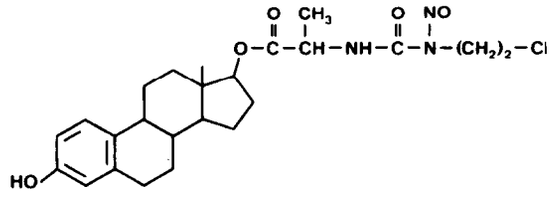


Fig. 1. Chemical structure of the tested drugs.

Experimental design

For pharmacokinetic studies following intravenous application the drugs were given in a quick bolus injection via the vena jugularis. Immediately before, and 1, 3, 5, 10, 15, 30, 60, 120, 240, and 360 min after application, blood samples were taken from the vena cava. For pharmacokinetic studies after oral application the drugs were given by gastric intubation. Blood samples were taken 15, 30, 60, 120, 240 and 360 min after administration via the vena cava catheter. The volume of the samples varied from 0.5 to 2 ml depending on the time point they were taken: 0.5 ml (1–15 min after application) 1 ml (15–120 min after application) and 2 ml (240–360 min after application). Since the total volume sampled did not exceed 2 ml in one animal, rats were replaced after 10, 30, 120 and 240 min. The blood was collected in heparinized tubes that contained 100 μl of an aqueous solution of 1-(2-chloroethyl)-1-nitroso-3-(2-hydroxyethyl)-urea (HECNU) as internal standard. Plasma was obtained by centrifugation (800 ***g***, 4°C) for 10 min. The samples were stored at −30°C until analysis.

Sample extraction and derivatization

The drugs were extracted from plasma with a vacuum extraction system (Baker, Groß Gerau, F.R.G.). Columns packed with C18 silica gel were conditioned with 1.0 ml methanol followed by 1.0 ml distilled water. After absorption of the blood sample the column was washed with 2 ml distilled water prior to elution with 2 ml methanol. This method gave recoveries of 75 ± 5% for the CNC-alanine-estradiol-ester and 70 ± 5% for the CNC-alanine.

Methanol extracts were derivatized with ethereal diazomethane (see Fig. 2) for 30 min prior to concentrating to dryness under a stream of nitrogen.

The residue was taken up in methanol (1 ml). During the complete procedure the samples were maintained at −4°C using an ice/salt bath for cooling.

HPLC method

Sample aliquots (15 μl) were analysed by means of high pressure liquid chromatography (HPLC) as detailed in Table 1.

The quantities of the compounds were determined with calibration curves that were linear in a range of 0.1–500 μg/ml. Concentrations are corrected for internal standard recovery and are normalized to nmol/ml. The limit of detection was 1 nmol/ml. In samples showing several unknown peaks in the region of interest u.v. spectra were recorded at the specified retention times (Fig. 3).

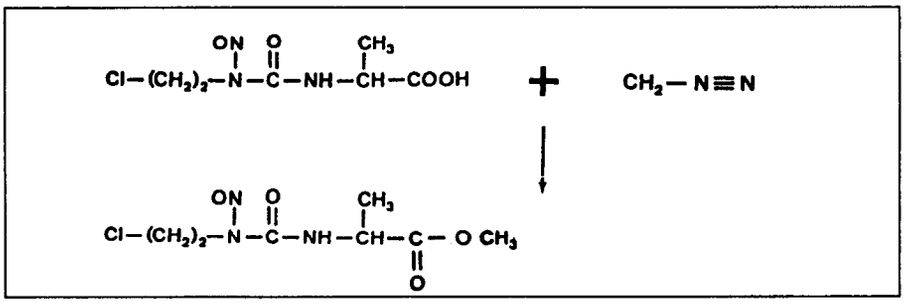
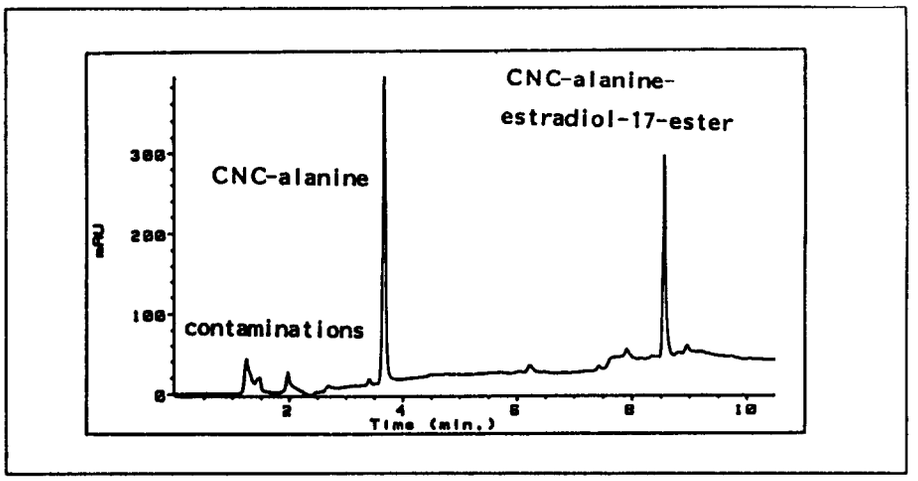


Fig. 2. Methylation of CNC-alanine.

I: typical chromatogram measured at 230 nm



II: typical chromatogram measured from 230 nm – 300 nm

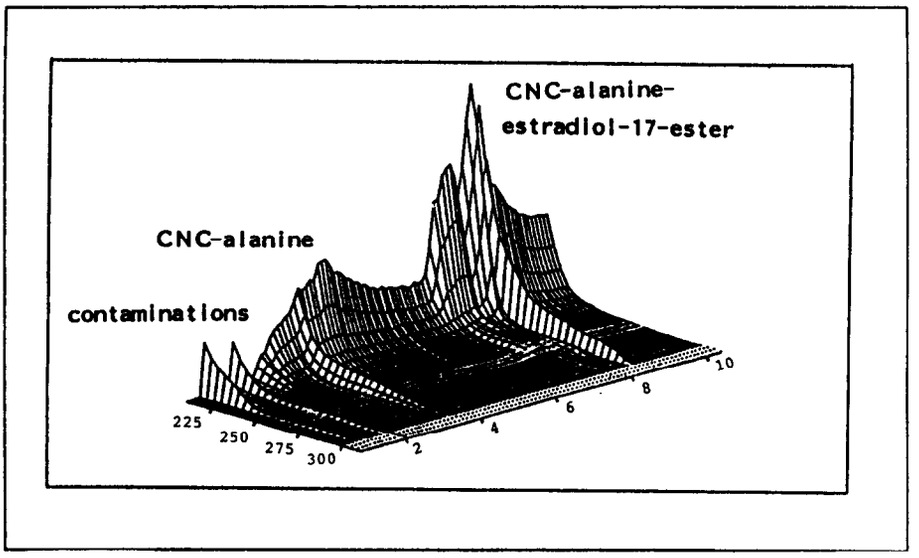


Fig. 3. Identification of the compounds by the retention time (I = fixed detector) and by the retention time plus shape of u.v. spectrum (II = diode array detector).

Calculation of pharmacokinetic parameters

Arithmetic means and standard deviations (S.E.) were calculated. The pharmacokinetic calculations were performed according to standard methods [20–22]. The pharmacokinetic parameters (see Table 2) were calculated by means of a personal computer (IBM XT, Model 286, U.S.A.) and a special software for pharmacokinetics (MK model, Elsevier Biosoft, Cambridge, U.K.).

RESULTS

Pharmacokinetics of CNC-alanine

The plasma levels of CNC-alanine after intravenous administration of 30 mg/kg followed a bi-

Table 1. Chromatographic conditions

Equipment	
HPLC:	HP 1090 (Hewlett Packard)
Data processing:	HP 79994 (Hewlett Packard)
Separation	
Column:	Spherisorb C_{18} 250 × 4 mm 25 µ
Mobile phase:	Methanol/acetonitrile/water
Gradient	Begin: 10/20/70
Flow rate:	End: 80/0/20
	1 ml/min
Detection	
Technique:	u.v. absorption
Wavelength:	200–400 nm

phasic decay that could be described best with a two compartment model (see Fig. 4).

The distribution phase showed a halflife of 3.5 ± 0.96 min, whereas the elimination phase had a halflife of 50.1 ± 7.5 min. The volume of distribution for CNC-alanine was 0.81 ± 0.34 l/kg, its clearance amounted to 211.6 ± 70.3 ml/h (see Table 3). The area under the curve from start of application till infinity (AUC) was measured as 171.3 ± 55.5 nmol × h/ml.

After oral application of 30.0 mg/kg CNC-alanine, the plasma levels showed a fast absorption phase, which after peaking was followed by the elimination phase (see Fig. 5). As a suitable model for the curve, a one compartment model after oral application was used.

CNC-alanine was absorbed after oral application with a halflife of 50 ± 38.3 min. The concentration in plasma reached a peak of 53.0 ± 17.3 nmol/ml at 90.0 ± 60.0 min after administration. The following elimination phase had a halflife of 60.0 ± 15.6 min (see Table 4). The AUC showed values of 122.0 ± 10.9 nmol × h/ml. The ratio of the AUCs for the two routes (i.v. and p.o.) gave a mean bioavailability of 71%.

Pharmacokinetics of CNC-alanine-estradiol-17-ester

After intravenous application of 66 mg/kg CNC-alanine-estradiol-17-ester the plasma level followed a biphasic decay. The curve was best described with a two compartment model (see Fig. 6).

The halflifes of the distribution and elimination phases were 1.02 ± 1.57 min and 167.4 ± 23.2 min, respectively. Total body clearance was measured as 174.3 ± 12.6 ml/h (see Table 5). For the AUC 205.9 ± 14.7 nmol × h/ml was calculated.

After an oral dose of 66 mg/kg CNC-alanine-estradiol-17-ester the concentration–time curve was comparable to that of CNC-alanine. Again, the curve could be best described by a one compartment model after oral application (see Fig. 7).

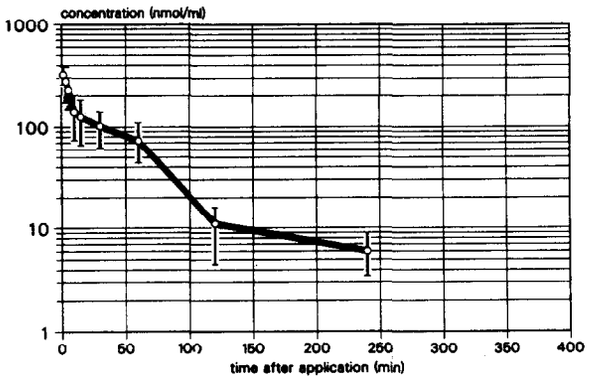


Fig. 4. Concentration–time curve of CNC-alanine in plasma after intravenous administration of 30 mg/kg *in female Sprague–Dawley rats (points are mean ± S.E.;* n = 3).

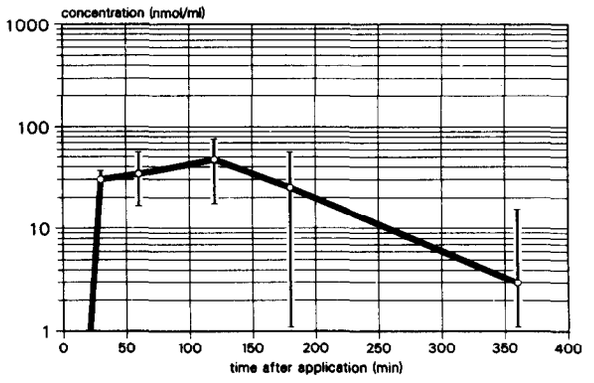


Fig. 5. Concentration time curve of CNC-alanine in plasma following oral administration of 30 mg/kg *in female Sprague–Dawley rats (points are mean ± S.E.;* n = 3).

Table 2. Pharmacokinetic parameter abbreviations and dimensions

Parameter	Abbreviation	Dimension
Halflife of the distribution phase	$T_{1/2\ alpha}$	min
Halflife of the elimination phase	$T_{1/2\ beta}$	min
Halflife of the absorption phase	$T_{1/2\ abs}$	min
Total body clearance	Cl	ml/h
Volume of distribution	V_{diss}	l/kg
Bioavailability	Bioavail	Percentage of given dose
Elimination constant of the distribution phase	K_{alpha}	min^{-1}
Elimination constant of the elimination phase	K_{beta}	min^{-1}
Velocity constant of the absorption phase	K_{abs}	min^{-1}

Table 3. Pharmacokinetic parameters of CNC-alanine in plasma after intravenous administration of 30 mg/kg *in female Sprague–Dawley rats (values are mean ± S.E.;* n = 3)

Parameter	Mean	± S.E.	Dimension
K_{alpha}	0.2	0.07	min^{-1}
K_{beta}	0.014	0.0002	min^{-1}
$T_{1/2\ alpha}$	3.5	0.96	min
$T_{1/2\ beta}$	50.1	7.5	min
Cl	211.6	70.3	ml × h^{-1}
V_{diss}	0.81	0.34	l × kg^{-1}

Table 4. Pharmacokinetic parameters of CNC-alanine in plasma after oral administration of 30 mg/kg *in female Sprague–Dawley rats (values are mean ± S.E.;* n = 3)

Parameter	Mean	± S.E.	Dimension
K_{abs}	0.03	0.02	min^{-1}
K_{beta}	0.0115	0.0014	min^{-1}
$T_{1/2\ abs}$	50.0	38.3	min
$T_{1/2\ beta}$	60.0	15.6	min
C_{max}	53.0	17.3	nmol × ml^{-1}
T_{max}	90.0	60.0	min

Table 5. Pharmacokinetic parameters of CNC-alanine-estradiol-17-ester in plasma after intravenous administration of 66 mg/kg *in female Sprague–Dawley rats (values are mean ± S.E.;* n = 3)

Parameter	Mean	± S.E.	Dimension
K_{alpha}	2.6	2.9	min^{-1}
K_{beta}	0.0042	0.0007	min^{-1}
$T_{1/2\ alpha}$	1.02	1.57	min
$T_{1/2\ beta}$	167.4	23.2	min
Cl	174.3	12.6	ml × h^{-1}
V_{diss}	2.36	0.73	l × kg^{-1}

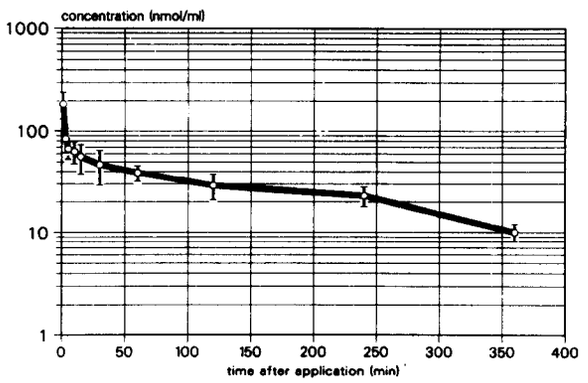


Fig. 6. Concentration time curve of CNC-alanine-estradiol-17-ester in plasma following intravenous administration of 66 mg/kg *in female Sprague–Dawley rats (points are mean ± S.E.;* n = 3).

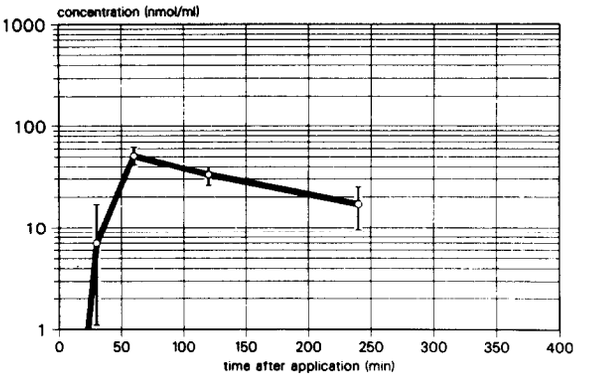


Fig. 7. Concentration–time curve of CNC-alanine-estradiol-17-ester in plasma following oral administration of 66 mg/kg *in female Sprague–Dawley rats (points are mean ± S.E.;* n = 3).

The absorption halflife of CNC-alanine-estradiol-17-ester after oral application was 21.0 ± 0.8 min. The absorption maximum reached 51.6 ± 10.3 nmol/ml after 60.0 ± 0 minutes. The terminal halflife after the absorption maximum amounted to 146.0 ± 27.6 min (see Table 6). For the area under the curve 106.3 ± 10.4 nmol × h/ml was calculated. The ratio of the areas under the curve after i.v. and p.o. application indicated a mean bioavailability of 52%.

Following intravenous application of CNC-alanine-estradiol-17-ester, its metabolite CNC-alanine could be detected concomitantly, indicating cleavage of the ester bond of the linked agent (see Fig. 8).

The halflife of CNC-alanine released from the ester thus was longer compared to its halflife after direct application (75.5 ± 11.6 min in contrast to 50.1 ± 7.5 min).

DISCUSSION

The observed differences in pharmacokinetic parameters between the hormone-linked agent and CNC-alanine can mainly be attributed to the markedly differing physiocochemical properties of the two molecules. CNC-alanine is a relatively strong acid and therefore dissociates to a large extent at physiological pH. In contrast to CNC-alanine, being preferentially anionic, the steroid conjugate

Table 6. Pharmacokinetic parameters of CNC-alanine-estradiol-17-ester in plasma after oral administration of 66 mg/kg *in female Sprague–Dawley rats (values are mean ± S.E.;* n = 3)

Parameter	Mean	± S.E.	Dimension
K_{abs}	0.03	0.0	min^{-1}
K_{beta}	0.0048	0.0004	min^{-1}
$T_{1/2abs}$	21.0	0.8	min
$T_{1/2beta}$	146.0	27.6	min
C_{max}	51.6	10.3	nmol × ml^{-1}
T_{max}	60.0	0.0	min

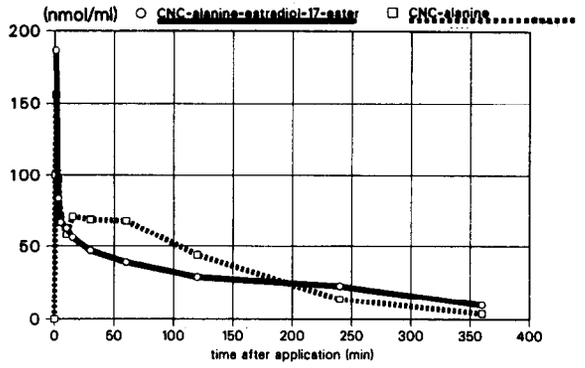


Fig. 8. Concentration–time curve of CNC-alanine-estradiol-17-ester in plasma and its metabolite CNC-alanine following intravenous administration of 66 mg/kg *in female Sprague–Dawley rats (points are mean;* n = 3).

is neutral and highly lipophilic. This correlates well with the observed shorter halflife of the distribution phase following i.v. administration, the quicker absorption after p.o. application and the three times larger volume of distribution of CNC-alanine-estradiol-17-ester compared to CNC-alanine.

The 3-fold longer terminal plasma halflife of the lipophilic steroid ester is not surprising, since high lipophilicity usually is related to a high extent of protein binding which contributes to delay metabolism. Moreover it has been found that steroid-linked CNC-amino acid esters are inhibitors of serum esterases [23].

The observed differences explain in part the antineoplastic superiority of the estradiol linked agent as compared to CNC-alanine [14–17]: this applies to the longer halflife of the former compound that ensures a longer exposure of tumour cells to drug concentrations, which exert cytotoxic effects *in vitro* [24]. In addition, the latter agent which is generated from CNC-alanine-estradiol-17-ester by cleavage of the ester bond displayed a 50% extended halflife. This phenomenon may be interpreted as a continuous release of an active metabolite. Although CNC-alanine has not been found to be of remarkable anticancer activity following bolus injection [17], its continuous release from the ester might well contribute to higher antineoplastic efficacy. A prolonged halflife alone, however, does not suffice to explain increased anticancer action, which can be exemplified by comparing with the effects of BCNU: this similarly lipophilic agent has a halflife in rats approaching that of the steroid ester [25], but is poorly active in chemically induced mammary carcinoma [26]. Thus, the target tissue also is of major importance. On the other hand, whereas BCNU is very effective in rodent leukaemias L1210 and L5222 [27, 28], CNC-alanine as well as its steroid ester are similarly less active in these models (Fiebig *et al.*, in prep.; Zeller *et al.*, in prep.).

Evidence has been accumulated from animal experiments [17] that the estradiol receptor affinity of CNC-alanine-estradiol-17-ester plays a role in its anticancer activity against tumours containing estradiol receptors. This is supported to some extent by the volume of distribution of the steroid-linked agent, which exceeds more than two times the body volume. Investigations on the tissue distribution of CNC-alanine-estradiol-17-ester in both estradiol receptor-positive and -negative organs will clarify a possibly receptor-mediated accumulation.

In summary, the pharmacokinetic properties of this new class of anticancer agents are highly important to explain pharmacodynamic effects; however, obviously tumour tissue characteristics also play a major role.

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