Eur J Cancer Clin Oncol, Vol. 24, No. 6, pp. 1027–1032, 1988.
Printed in Great Britain

0277-5379/88$3.00+0.00

In Vitro Evaluation of an Estradiol-linked Nitrosourea in Mammary Carcinomas of Mouse, Rat and Man

E. PETRU,* M.R. BERGER,* W.J. ZELLER* and M. KAUFMANN†

**Institute of Toxicology and Chemotherapy, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, F.R.G. and †Department of Obstetrics and Gynecology of the University, Vosstraße 9, D-6900 Heidelberg, F.R.G.*

Abstract—In vitro *activity of* 1-(2-*chloroethyl*)-1-*nitrosocarbamoyl*-L-*alanine-estradiol*-17-*ester* (*CNC-ala*-17-E_2) *at three concentrations in transplanted MXT mammary carcinoma in* $B_6D_2F_1$ *mice and autochthonous methylnitrosourea* (*MNU*)*-induced mammary carcinoma in Sprague–Dawley rats, as well as in* 30 *human primary breast carcinomas using the bilayer soft agar assay is described. Eighty-five per cent of MXT tumors showed a more than* 70% *inhibition of colony formation following CNC-ala*-17-E_2. *In the MNU-induced model this high degree of inhibition was not observed: only* 5% *of individual tumors showed an inhibition up to* 70%, *but a superiority of the hormone-linked agent over the unlinked single agents was nevertheless discernible. In contrast, in human breast carcinomas a response at this sensitivity level could not be assessed. Thus, in the MXT mammary carcinoma the* in vitro *results paralleled previous findings* in vivo, *whereas in the MNU-induced autochthonous tumor model this close* in vivo–in vitro *correlation was not observed. The discrepancy between* in vivo *and* in vitro *results found in the autochthonous rat model indicates that hormone-linked nitrosoureas should not necessarily be abandoned for the treatment of human breast carcinoma on the basis of negative* in vitro *results alone.*

INTRODUCTION

CHLOROETHYLNITROSOUREAS have been shown to be highly active in a variety of tumor models [1]. The limiting factor for these compounds in the treatment of human carcinomas is their profound, delayed and cumulative bone marrow suppression. Efforts have been directed towards the synthesis of derivatives retaining chemotherapeutic activity but with lower systemic toxicity. Recently, our group has linked the chloroethylnitrosocarbamoyl (CNC) group to a series of carrier molecules such as hormones, oligopeptides or amino acids [2–5]. Of several estrogen-linked nitrosoureas, CNC-ala-17-E_2 exerted superior antitumor activity: *in vivo* this derivative revealed a higher therapeutic index as compared to the respective unlinked single agents in two hormone-dependent rodent models, the transplanted MXT mammary carcinoma in mice and the autochthonous MNU-induced mammary carcinoma in rats [3, 6]. CNC-ala-17-E_2 showed a relative estrogen-receptor affinity of 1% and a distinct estradiol-like activity in the uterine mouse assay [3]. Its half-life in plasma is >40 min. Further pharmacokinetic data will be published elsewhere.

The present report describes investigations on the *in vitro* activity of CNC-ala-17-E_2 compared to the respective unlinked single agent CNC-ala and an equimolar mixture of CNC-ala plus estradiol (Fig. 1). The bilayer soft agar assay [7, 8] was considered an appropriate model to compare the activity of these compounds in both rodent tumors as well as in primary human breast carcinomas.

Accepted 18 January 1988.
Correspondence: Dr. M.R. Berger, Institute of Toxicology and Chemotherapy, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, F.R.G.
Abbreviations used: MNU, *N*-methylnitrosourea; CNC-ala-17-E_2, 1-(2-chloroethyl)-1-nitrosocarbamoyl-L-alanine-estradiol-17-ester; CNC-ala, 1-(2-chloroethyl)-1-nitrosocarbamoyl-L-alanine.

MATERIALS AND METHODS

MXT mammary carcinomas

Fifty milligrams of minced tumor tissue were transplanted subcutaneously into 20 female $B_6D_2F_1$ mice (weight range 18–20 g, source: Charles River Wiga GmbH, Sulzfeld, F.R.G.). Animals were kept under conventional conditions. At the time of inves-

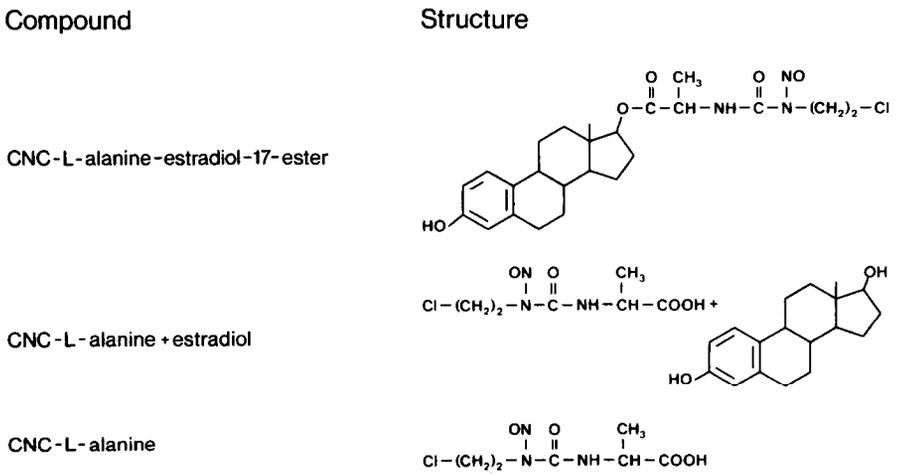

Fig. 1. Chemical structure of 1-(2-*chloroethyl*)-1-*nitrosocarbamoyl*-L-*alanine-estradiol*-17-*ester* (*CNC*-L-*alanaine-estradiol*-17-*ester*) *and the respective unlinked single agents.*

tigation individual tumor volumes were about 1 cm^3.

MNU-induced mammary carcinomas

MNU was synthesized by Prof. M. Wiessler (Institute of Toxicology and Chemotherapy, German Cancer Research Center, Heidelberg). It was dissolved at 1% (pH 6) and injected at 50 mg/kg to 40 female Sprague–Dawley rats (Institut für Versuchstierkunde, Hannover, F.R.G.) on days 50, 71 and 92 of life, respectively. Tumors appeared 60 ± 10 (mean ± S.D.) days following the first injection. Tumors greater than 1.5 cm^3 were used in the present experiment.

Human primary breast carcinoma

Thirty tumor specimens were obtained from breast cancer patients undergoing diagnostic or therapeutic procedures at the Department of Obstetrics and Gynecology, University of Heidelberg. Their mean age was 54 years (range 28–79). Histologic evaluation revealed ductal invasive carcinomas in 25 and lobular invasive carcinomas in five specimens. Receptor assays in all specimens were performed at the Department of Obstetrics and Gynecology, University of Heidelberg according to EORTC criteria [9]. Tumors with an estrogen (progesterone) receptor content of more than 20 fmol/mg cytosol protein were considered estrogen (progesterone) positive. Sixty-one per cent of carcinomas investigated were estrogen- and 50% progesterone-receptor positive.

Clonogenic assay

All tumors used in these experiments were freshly excised prior to plating. The tumor material was placed in plastic Petri dishes and cut into small pieces with scissors under sterile conditions. Tissue pieces were suspended in McCoy's 5A medium (Serva, Heidelberg) containing 10% heat inactivated fetal calf serum (FCS, Gibco, Karlsruhe), penicillin (100U/ml) and streptomycin (100 μg/ml, Serva, Heidelberg), pipetted several times by means of a sharp-edged glass pipette and the resulting cell suspension carefully filtered without pressure through gauze (pore size 200 μm). Cells were collected in culture tubes, centrifuged for 5 min at 150 ***g*** and washed twice with McCoy's 5A medium. Cell viability was checked using the trypan blue exclusion test. Final concentration of viable cells was 3×10^6/ml.

The bilayer soft agar system was used as described in [7], except that no mercaptoethanol, calcium chloride or conditioned medium were added, while CMRL was replaced by basal medium eagle (BME, Gibco, Karlsruhe). In brief, the underlayer consisted of McCoy's 5A medium supplemented with 10% FCS, 5% horse serum (Gibco, Karlsruhe), sodium pyruvate (0.22 mg/ml), L-serine (42 μg/ml), L-glutamine (2.9 μg/ml), sodium bicarbonate (4.5 mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), asparagine (100 μg/ml) (all from Serva, Heidelberg), DEAE–dextran (375 μg/ml, Pharmacia, Freiburg), tryptic soy broth (7.5 mg/ml, Difco, Detroit), and agar at a final concentration of 0.5% (Bacto agar, Difco). One milliliter of the suspension was dispensed immediately into a 35 mm Petri dish.

2-Chloroethylnitrosourea derivatives were synthesized by Prof. G. Eisenbrand, Institute of Food Chemistry and Environmental Toxicology, University of Kaiserslautern, F.R.G. CNC-ala-17-E_2, CNC-ala and CNC-ala plus estradiol were dissolved in a mixture of DMSO (Merck, Darmstadt, F.R.G.) plus McCoy's 5A medium. Final drug concentrations in the incubation tubes were: 100, 10 and 1 μM. The dose levels were chosen on the basis of optimal *in vivo* dosages [3, 6] and pharmacokinetic data which are to be published separately. In detail, 0.5 ml cell suspensions (3×10^6 cells/ml) were

transfered to tubes containing 0.85 ml McCoy's 5A medium with 10% FCS; drugs were added in a constant volume of 0.15 ml. Control tubes contained the respective solvent concentrations. Cells were incubated for 1 h at 37°C, then centrifuged at 150 ***g*** for 10 min, washed twice with McCoy's 5A medium containing 10% FCS and prepared for culture as follows. The upper layer was prepared by resuspension of the cell pellets in 2.7 ml enriched BME plus 0.3 ml agar (final concentration 0.3%). BME was supplemented with 15% horse serum, porcine insulin (2 U/ml, Hoechst, Frankfurt), ascorbic acid (53 μg/ml, Serva, Heidelberg), penicillin (100 U/ml), streptomycin (100 μg/ml), DEAE–dextran (250 μg/ml), and asparagine (66 μg/ml). Plating was performed by aspirating 1 ml of the resultant upper layer mixture containing the incubated cells plus agar and dispensing this volume on top of the lower layer. For each experiment three drug and the respective solvent concentrations were tested in triplicate (5×10^5 cells/plate). Cultures were incubated at 37°C in a humidified 7.5% CO_2 atmosphere.

All samples plated were checked under an inverted-phase microscope (Zeiss, Frankfurt) on day 1 to assure single cell suspension. The average number of stem cell derived colonies (aggregates >30 cells) following drug exposure as a percentage of the respective controls on day 7 after plating was the parameter used to assess tumor growth inhibition. Day 7 was chosen for comparative evaluation of colony numbers because previous studies reported a maximum colony formation on this day in MNU-induced rat mammary carcinoma [10] and human breast neoplasms [11]. In order to detect relevant variations in the number of colonies of human carcinomas at different time points following plating, the colony number was counted additionally on days 14 and 21.

Statistical evaluation

Differences in the mean number of colonies as a percentage of controls were assessed by the distribution-free Kruskal–Wallis test [12].

RESULTS

Table 1 gives mean colony numbers and the respective plating efficiency for the three tumor systems. Evaluation was confined to day 7 following plating, since repeated colony counts of human tumors which had the lowest proliferation revealed no substantial differences compared to day 7 (days 14 and 21, +2.1% and −3.9%, respectively). Plating efficiency was highest in the transplanted mouse model and lowest in the human breast carcinoma.

As given in Table 2, all three drugs showed a well-defined dose–response relationship in the MXT model. CNC-ala-17-E_2 revealed a significantly increased inhibition of colony growth as compared with the respective unlinked single agents. In MNU-induced mammary carcinoma, however, a dose–response relationship was found for the hormone-linked agent, only. Thus, a superiority of CNC-ala-17-E_2 was observed in both hormone receptor-positive rodent models.

Table 3 shows the respective results obtained from 30 human breast carcinomas, which revealed no clear-cut dose–response relationship in all three treatment regimens. Altogether, the estrogen-linked agent exhibited marginally better results. Table 4 presents an analysis of the degree of inhibition of colony formation in individual tumors following *in vitro* exposure to 2-chloroethylnitrosourea derivatives. Eighty-five per cent of MXT tumors showed a more than 70% inhibition of colony formation following CNC-ala-17-E_2; the same degree of inhibition was found in only 20% of tumors following exposure to the respective unlinked single agents.

In contrast, in the MNU-induced autochthonous model and in human breast carcinoma this high degree of inhibition was not observed following CNC-ala-17-E_2. Nevertheless, in the chemically induced model, up to a 50% inhibition of colony formation was found in 48% of individual tumors following CNC-ala-17-E_2 exposure. The respective single agents caused a comparable inhibition in 13 and 15%, only. The same degree of sensitivity to CNC-ala-17-E_2 (inhibition up to 50% of controls) was found in 20% of human carcinomas, whilst CNC-ala plus estradiol and CNC-ala alone resulted in 5 and 16%, respectively. A separate analysis of results in estrogen receptor-positive human tumors revealed only marginal differences compared to findings in all carcinomas irrespective of the estrogen receptor status (Tables 3 and 4).

DISCUSSION

The development of new anticancer agents is directed towards their potential clinical use in cancer treatment. The strategy of infering the human situation from the activity of drugs in panels of transplanted tumors has raised problems, especially with regard to slowly growing tumors [13–16]. The human tumor stem cell assay was developed to establish models closer to the human situation [7, 8].

Since previous *in vivo* experiments revealed superior antineoplastic activity of the new experimental drug CNC-ala-17-E_2 in a series of hormone-linked nitrosoureas [3, 6], we examined its *in vitro* activity in human breast cancer using the bilayer soft agar system. As *in vivo* activity in the MXT and MNU-induced tumors was known, additional *in vitro* activity in both experimental tumor systems was also determined. Consideration of the results in these five systems (Fig. 2) was supposed to clarify

Table 1. Colonies and plating efficiency in control dishes of MXT mammary carcinoma, MNU-induced mammary carcinoma and human breast cancer (evaluation on day 7 after plating)

Species	Tumor origin	No. of tumors	Mean No. of colonies ± S.E.*	95% confidence limits	Plating efficiency (%) (95% confidence limits)
Mouse	Transplanted	20	194.2 ± 6.3	183.5–204.9	0.039 (0.037–0.041)
Rat	Chemically induced, autochthonous	40	117.5 ± 5.3	114.6–120.4	0.024 (0.023–0.024)
Man	Autochthonous	30	35.2 ± 1.4	22.7 47.8	0.007 (0.005–0.009)

*Standard error of the mean.

Table 2. Effect of 100, 10 and 1 μM of treatment on the number of colonies of MXT mammary carcinoma and MNU-induced mammary carcinoma grown in soft agar (percentage of controls, evaluation on day 7 after plating)

Compound	Percentage control plating efficiency ± S.E.*) 100 μM	10 μM	1 μM
MXT mammary carcinoma†			
CNC-ala-17-E_2	27.3 ± 2.4‡§	42.1 ± 4.2‡‖	78.8 ± 6.1§¶
CNC-ala + estradiol	49.7 ± 3.9	78.2 ± 4.1	105.9 ± 2.7
CNC-ala	51.1 ± 5.3	75.0 ± 5.4	96.3 ± 7.9
*MNU-induced mammary carcinoma***			
CNC-ala-17-E_2	54.2 ± 2.3‖††	69.9 ± 1.7	88.5 ± 1.6
CNC-ala+estradiol	75.3 ± 2.0	77.1 ± 2.9	72.6 ± 2.7‡‡
CNC-ala	71.5 ± 2.0	69.3 ± 1.8	78.2 ± 3.1§§

*Standard error of the mean.
†No. of tumors tested: 20.
‡Significantly different from CNC-ala + estradiol ($P < 0.001$).
§Significantly different from CNC-ala ($P < 0.001$).
‖Significantly different from CNC-ala ($P<0.0001$).
¶Significantly different from CNC-ala + estradiol ($P < 0.05$).
**No. of tumors tested: 40
††Significantly different from CNC-ala + estradiol ($P < 0.0001$).
‡‡Significantly different from CNC-ala-17-E_2 ($P < 0.01$).
§§Significantly different from CNC-ala-17-E_2 ($P < 0.0001$).

Table 3. Effect of 100, 10 and 1 μM of treatment on the number of colonies of human breast carcinoma grown in soft agar (percentage of controls, evaluation on day 7 after plating)

Compound	Percentage control plating efficiency ± S.E.* 100 μM	10 μM	1 μM
CNC-ala-17-E_2†	72.3 ± 3.5‡ (71.8 ± 3.1)§‖	81.0 ± 4.6 (77.9 ± 3.7)	85.6 ± 4.6 (81.9 ± 4.9)
CNC-ala + estradiol¶	86.2 ± 2.5 (81.1 ± 3.3)	80.6 ± 3.5 (83.3 ± 4.4)	87.2 ± 3.3 (87.1 ± 4.3)
CNC-ala**	77.1 ± 3.1 (77.0 ± 4.6)	81.2 ± 3.3 (79.4 ± 4.2)	92.3 ± 3.5 (91.8 ± 4.9)

*Standard error of the mean.
†Total No. of tumors tested versus ER+tumors: 30/19.
‡Significantly superior to CNC-ala + estradiol ($P \leq 0.01$).
§Significantly superior to CNC-ala + estradiol ($P \leq 0.05$).
‖Mean values of estrogen-receptor positive (ER+) tumors.
¶Total No. of tumors tested versus ER+ tumors: 22/15.
**Total No. of tumors tested versus ER+ tumors: 26/17.

Table 4. Degree of inhibition in colony formation (percentage of controls) following treatment (1, 10 and 100 μM) of transplanted MXT mammary carcinoma, autochthonous chemically induced mammary carcinoma and human breast cancer

Colony formation, percentage of controls	Number of individual tumors (%)								
	Transplanted MXT			Autochthonous MNU-induced			Human		
	CNC-ala -17-E_2	CNC-ala + estradiol	CNC-ala	CNC-ala -17-E_2	CNC-ala + estradiol	CNC-ala	CNC-ala -17-E_2	CNC-ala + estradiol	CNC-ala
0–30	17 (85)	4 (20)	4 (20)	1 (3)	1 (3)	0 (0)	0 (0) [0 (0)]*	0 (0) [0 (0)]	1 (4) [1 (6)]
31–50	3 (15)	6 (30)	10 (50)	18 (45)	4 (10)	6 (15)	6 (20) [3 (16)]	1 (5) [1 (7)]	3 (12) [2 (12)]
51–70	0 (0)	10 (50)	5 (25)	18 (45)	28 (70)	28 (70)	11 (37) [7 (37)]	7 (32) [3 (20)]	9 (35) [5 (29)]
⩾71	0 (0)	0 (0)	1 (5)	3 (8)	7 (18)	6 (15)	13 (43) [9 (47)]	14 (64) [11 (73)]	13 (50) [9 (53)]

*Results of estrogen receptor positive tumors.

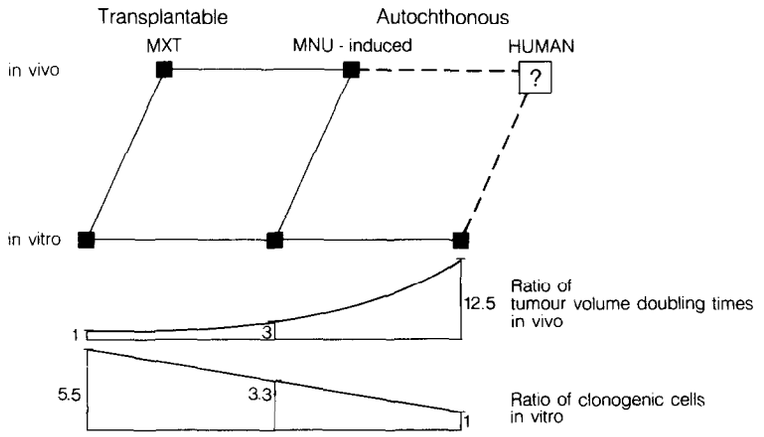

Fig. 2. Experimental systems used to evaluate anticancer activity of 1-(2-chloroethyl)-1-nitrosocarbamoyl-L-alanine-estradiol-17-ester. In addition, the ratio of clonogenic cells of the three systems is opposed to the ratio of tumor volume doubling times.

the relevance of clonogenic assays in the evaluation of this type of compounds.

For investigation of receptor-affinic agents the question arises, to what extent stem cells can be used which *per definitionem* are primitive cells and therefore are expected to possess a lower or no receptor content. Furthermore, differences in the proportion of clonogenic cells in the three tumors have to be taken into account (Table 1, Fig. 2). Whilst *in vitro* results in the MXT mammary carcinoma paralleled previous findings *in vivo* (optimal T/C = 28%; [6]), this close *in vivo–in vitro* correlation was not observed in the MNU-induced rat mammary carcinoma. *In vivo*, CNC-ala-17-E_2 showed highly significant antitumor activity (optimal T/C = 10%; [3]), whereas *in vitro* studies revealed a dose-dependent but less convincing inhibition of colony formation. Only 5% of MNU-induced tumors could be ranked as sensitive, if NCI criteria for *in vitro* drug sensitivity were applied (reduction to 30% or less of the control value; [17]). The different *in vitro* sensitivities of both rodent models cannot be explained on the basis of differences in the receptor contents [3, 18].

In human breast cancer a comparably low degree of sensitivity of individual tumors to CNC-ala-17-E_2 was observed according to this ranking category. If the present *in vitro* data with human mammary carcinomas were extrapolated to the clinical situation, no significant antineoplastic activity would be expected in the treatment of this disease. The discrepancy between *in vivo* and *in vitro* results found in MNU-induced autochthonous rat mammary carcinoma, however, underlines that the predictive value of *in vitro* results obtained with such a receptor-affinic agent might be limited. Future studies with human mammary xenografts grown in nude mice might help in the decision whether this drug should

be a candidate for clinical trials. In conclusion, hormone-linked nitrosoureas for the treatment of human breast carcinoma should not necessarily be abandoned on the basis of negative *in vitro* results alone.

Acknowledgements—The authors are grateful to Prof. G. Eisenbrand for providing the chloroethylnitrosourea derivatives used in this study. We are obliged to Mrs. M. Haarmann, Mrs. S. Heil and Mrs. B. Kaiser for excellent technical assistance. This work was part of the program of the EORTC Screening and Pharmacology Group.

REFERENCES

1. Prestayko AW, Baker LH, Crooke St *et al.* (eds). *Nitrosoureas—Current Status and New Developments*. New York, Academic Press, 1981.
2. Berger MR, Floride J, Schreiber J *et al.* Evaluation of new estrogen-linked 2-chloroethylnitrosoureas. *J Cancer Res Clin Oncol* 1984, **108**, 148–153.
3. Berger MR, Floride J, Schmähl D *et al.* 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.
4. Zeller WJ, Schreiber J, Ho A *et al.* Cytostatic activity of steroid linked nitrosoureas. *J Cancer Res Clin Oncol* 1984, **108**, 164–168.
5. Zeller WJ. New derivatives of CNC-amino acids and -oligopeptides: experimental antitumor activity. *J Cancer Res Clin Oncol* 1986, **111**, 154–156.
6. Zeller WJ, Schreiber J, Eisenbrand G. Cytostatic activity of steroid-linked nitrosoureas on the MXT mammary carcinoma. *Proc AACR* 1985, **26**, 250.
7. Hamburger A, Salmon S. Primary bioassay of human tumor stem cells. *Science* 1977, **197**, 461–463.
8. Salmon S, von Hoff D. *In vitro* evaluation of anticancer drugs with the human tumor stem cell assay. *Semin Oncol* 1981, **8**, 377–385.
9. EORTC Breast Cancer Cooperative Group. Standards for the assessment of estrogen receptors in human breast cancer. *Eur J Cancer* 1973, **9**, 379–381.
10. Manni A, Wright C. Effect of tamoxifen and difluoromethylornithine on clones of nitrosomethylurea-induced rat mammary tumor cells grown in soft agar culture. *Cancer Res* 1983, **43**, 1084–1086.
11. Manni A, Wright C, Davis G *et al.* Promotion by prolactin of the growth of human breast neoplasms cultured *in vitro* in the soft agar clonogenic assay. *Cancer Res* 1986, **46**, 1669–1672.
12. Dunn OJ. Multiple comparison using rank sums. *Technometrics* 1964, **6**, 241–252.
13. Berger MR, Zeller WJ. Wege zur rationalen präklinischen Testung antineoplastischer Chemotherapeutika. *Beitr Onkol* 1984, **18**, 274–286.
14. Atassi G. Do we need new chemosensitive experimental models? *Eur J Cancer Clin Oncol* 1984, **20**, 1217–1220.
15. Martin D, Balis M, Fisher B *et al.* Role of murine tumor models in cancer treatment research. *Cancer Res* 1986, **46**, 2189–2192.
16. Carter SK, Sakuray Y, Umezawa H (eds). *New Drugs in Cancer Chemotherapy*. Recent Results in Cancer Research, 76. Berlin, Springer, 1981.
17. Shoemaker R, Wolpert-de-Fillipes M, Kern D *et al.* Application of a human tumor colony-forming assay to new drug screening. *Cancer Res* 1985, **45**, 2145–2153.
18. Schreiber J. Thesis, Faculty of Natural Sciences, University of Heidelberg, 1985.