Pergamon

European Journal of Cancer Vol. 31A, No. 10, pp. 1677–1681, 1995

0959-8049/95 $9.50+0.00

0959-8049(95)00245-6

Original Paper

Activity of NK 611, a New Epipodophyllotoxin Derivative, Against Colony Forming Units from Freshly Explanted Human Tumours *In Vitro*

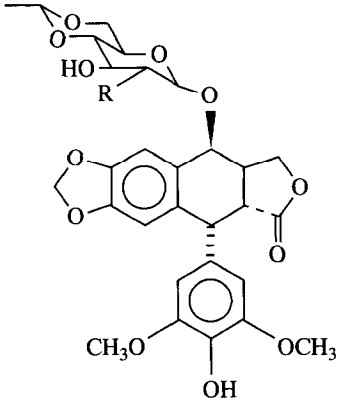
A.-R. Hanauske, K.C. Wüster, A. Lehmer, M. Rotter, P. Schneider, A. Kaeser-Fröhlich, J. Rastetter and H. Depenbrock

NK 611 is a new semisynthetic analogue of etoposide, which presumably also acts through inhibition of topoisomerase II, and has been found to be more potent against several cancer cell lines *in vitro* than etoposide. The objectives of our study were to determine the activity of NK 611 against freshly explanted clonogenic cells from human tumours and compare this agent with etoposide and other clinically useful agents. After exposure for 1 h in 45 evaluable tumour specimens, NK 611 showed clear concentration-dependent antitumour activity. At 51 μM, 49% of specimens were markedly inhibited. Using a long-term (21–28 day) exposure at 6.8 μM, 58% of 50 evaluable specimens were profoundly inhibited. At equimolar concentrations, NK 611 was as active as etoposide. Across all tumour types studied, NK 611 was as active as vinblastine, bleomycin, doxorubicin, 5-fluorouracil, mitomycin-C and cisplatin. Our results showed cross resistance to etoposide in the majority of specimens. Activity of NK 611 was greater with long-term exposure than with short-term exposure indicating schedule dependency. We conclude that NK 611 has a wide spectrum of *in vitro* antitumour activity. Since preliminary clinical information suggests that this drug is well tolerated at high doses, further development of this agent in Phase II trials with multiple dosing schedules is warranted.

Key words: epipodophyllotoxin derivatives, NK 611, etoposide, activity, *in vitro*
Eur J Cancer, Vol. 31A, No. 10, pp. 1677–1681, 1995

INTRODUCTION

ETOPOSIDE IS a semisynthetic epipodophyllotoxin derivative which is clinically active against a wide range of haematological and solid tumours [1]. Its main mechanism of action is inhibition of topoisomerase II [2]. NK 611 is a newly developed epipodophyllotoxin derivative which differs from etoposide in the replacement of a glucose moiety with a 2-*N*,*N*-dimethylglucosamine side chain (Figure 1). Like etoposide, NK 611 inhibits topoisomerase II activity. However, NK 611 is 120-fold more soluble in water than etoposide. In various murine tumour models including L 1210, P 388, Lewis lung cancer, M 5076 fibrosarcoma, B 16 melanoma, and C 26 colon carcinoma, significant antitumour activity of NK 611 has been observed [3].



ETP: R = OH
NK611: R = $N(CH_3)_2 \cdot HCl \cdot 2H_2O$

Figure 1. Structure of NK 611 and etoposide. ETP, etoposide.

These experiments also indicate a greater potency of NK 611 as compared with etoposide presumably due to faster intracellular uptake of NK 611. Animal experiments have also indicated that the oral absorption rate of NK 611 is significantly higher than that of etoposide potentially allowing for a safer and more

Correspondence to A.-R. Hanauske at the Division of Haematology and Oncology, I. Medizinische Klinik und Poliklinik, Klinikum rechts der Isar der Technischen Universität München, Ismaninger Str. 22, D-81675 München, Germany.
A.-R. Hanauske, K.C. Wüster, J. Rastetter and H. Depenbrock are at the Division of Hematology and Oncology; A. Lehmer is at the Department of Urology; M. Rotter is at the Department of Pathology; P. Schneider is at the Department of Surgery, Klinikum rechts der Isar der Technischen Universität München; and A. Kaeser-Fröhlich is at Asta Medica AG, Frankfurt/M., Germany.
Revised 23 Dec. 1994; accepted 6 Jan. 1995.

effective oral treatment [3]. Analytical methods have been developed for the determination of NK 611 and its *N*-demethyl metabolite in plasma samples, and clinical Phase I trials with intravenous and oral preparations are currently being performed in Japan and Europe [4–8]. In this study we investigated the activity of NK 611 against freshly explanted human tumour colony forming units *in vitro*.

MATERIALS AND METHODS

Biochemicals

NK 611 was provided by Asta Medica GmbH (Frankfurt/M., Germany). Stock solutions and final solutions of NK 611 were prepared in 5% glucose, pH 3.0. Based on *in vitro* cloning and clinical pharmacokinetic data for etoposide, NK 611 was studied at final concentrations of 0.35, 3.5, and 35 μg/ml (corresponding to 0.51, 5.1, and 51.0 μM) using a short-term incubation of 1 h followed by removal of the agent. For etoposide, peak plasma concentrations of ≤ 51 μM have been reported for intravenous doses of ≤ 290 mg/m^2 [9, 10]. Clinical data on peak plasma concentrations of NK 611 from an ongoing German phase I trial indicate that peak plasma concentrations of 71–87 μM may be achieved with a bolus injection of 120 mg/m^2.

Human tumour cloning system

Tumour specimens were collected by sterile standard procedures as part of routine clinical measures. Biopsies of solid tumours were stored in McCoy's 5A medium containing 5% fetal calf serum (FCS), 10 mM HEPES, 90 U/ml penicillin, 90 μg/ml streptomycin and 1 mM sodium pyruvate (all Gibco, Paisley, U.K.) for transport to the laboratory. Preservative-free heparin (10 U/ml, Novo Nordisk, Mainz, Germany) was added immediately after collection of fluids to prevent coagulation. Solid specimens were minced and repeatedly passed through metal meshes with mesh widths of 100 and 50 μm (Linker, Kassel, Germany) to obtain a single cell suspension. Effusions were centrifuged at 110 *g* for 5–7 min and passed through 25 g needles to obtain single cell suspensions when necessary.

Soft agar tumour cloning experiments were performed in glass capillary tubes as described earlier [11–13]. Cells were plated at a density of 5 × 10^4/capillary in 100 μl glass capillaries in a mixture of 0.3% agar in CMRL medium 1066 (Gibco) containing 15% horse serum, 2% FCS, 5 mg% vitamin C (Sigma, Deisenhofen, Germany), 90 U/ml penicillin, 90 μg/ml streptomycin, 0.1 mM non-essential amino acids, 2 mM glutamine (all Gibco), 50 U/ml catalase (Serva, Heidelberg, Germany), epidermal growth factor (10^{-10} M; Flow, Meckenheim, Germany) and 4 ng/ml hydrocortisone (Sigma). Immediately prior to plating, HEPES (10 mM final concentration), asparagine (Sigma, 100 μg/ml final concentration), and sodium pyruvate (2 mM final concentration) were added.

Tumour cells were incubated with the drug for 1 h prior to transfer into capillaries. In the same experiment, other clinically important antitumour agents for the specific tumour type were tested. For each compound, the concentration tested was 0.1-fold of clinically achievable peak plasma concentrations after conventional dosing.

For long-term incubation (21–28 days), final concentrations of NK 611 were 4.68, 2.34, and 1.17 μg/ml (6.8, 3.4, and 1.7 μM). Again, the choice of concentrations was based on available data for the parent compound etoposide administered at 50 mg/m^2 daily for 21 days. With this regimen, trough plasma concentrations were in the range of 1 μg/ml (1.7 μM) and peak plasma concentrations were 4–6 μg/ml (6.8–10.0 μM).

Each experiment included a control with ammonium monovanadate (10^{-3} M, Merck, Darmstadt, Germany) to assure the

Table 1. Types of tumours studied and evaluable per tumour type

Tumour	Continuous incubation No. evaluable*/No. attempted	1 h incubation No. evaluable*/No. attempted
Renal cell	15/21	16/21
Breast	3/10	2/10
Stomach	3/9	2/9
Colorectal	6/8	5/8
Sarcoma	2/7	2/7
Head and neck	3/5	2/5
Ovarian	3/4	3/4
Lung	3/4	2/4
Unknown primary site	1/4	1/4
Thymus	2/3	2/3
Non-Hodgkin's	1/3	1/3
Prostate	1/3	1/3
Endocrine system	2/2	1/2
Melanoma	1/2	1/2
Bladder	1/2	1/2
Corpus uteri	1/1	1/1
Liver	1/1	1/1
Pancreas	1/1	1/1
Hodgkin	0/1	0/1
Other urinary	0/1	0/1
Total	50/92 (54%)	45/92 (49%)

In the short-term 1 h drug exposure schedule, 45/92 (49%) tumours had adequate growth in controls. In long-term drug exposure experiments 50/92 (54%) tumour specimens were evaluable.

* ≥ 3 colonies/capillary in controls.

Table 2. Inhibitory activity of NK 611 against tumour colony forming units from freshly explanted human tumours in vitro using a short-term exposure schedule (1 h)

Tumour	NK 611 [μM] 0.51	5.1	51	Etoposide 5.1 [μM]
Renal	1/16*	5/16	6/16	5/16
Colorectal	1/5	1/5	4/5	1/5
Ovarian	0/3	0/3	2/3	1/3
Breast	1/2	1/2	1/2	1/2
Thymus	0/2	0/2	1/2	0/2
Others†	3/17	5/17	8/17	5/17
Total	6/45 (13%)	12/45 (27%)	22/45 (49%)	13/45 (29%)

NK 611 has profound and concentration-dependent antitumour activity. At equimolar concentrations, the agent is at least as active as the parent compound etoposide.

* Number of inhibited specimens (≤ 50% survival of tumour colony forming units) over number of evaluable specimens; †Bladder, corpus uteri, head and neck, liver, lung (non-small cell and small cell), non-Hodgkin's lymphoma, melanoma, endocrine system, pancreas, prostate, sarcoma, stomach, unknown primary site.

presence of a good single-cell suspension (positive control) [14]. Capillaries were incubated at 37°C, 5% CO_2, 100% humidity. After 21–28 days, colonies were counted with an inverted microscope. An experiment was considered evaluable when the water control had ≥ 3 colonies/capillary and the positive control showed ≤ 30% colony formation compared to the solvent control. A decrease in tumour colony formation was considered relevant if survival of colonies was ≤ 0.5-fold compared to the control.

Table 3. Comparison of in vitro activity of NK 611 with seven common clinical antitumour agents

Compound	NK 611 (5.1 μM)* Resistant†	Sensitive‡
Vinblastine (0.05 μg/ml)	$P = 0.077$	
Resistant	8	1
Sensitive	7	13
Bleomycin (0.2 μg/ml)	$P = 0.11$	
Resistant	7	2
Sensitive	8	6
Cisplatin (0.2 μg/ml)	P = N.S.	
Resistant	11	1
Sensitive	1	1
Doxorubicin (0.04 μg/ml)	P = N.S.	
Resistant	5	1
Sensitive	3	3
5-Fluorouracil (6.0 μg/ml)	P = N.S.	
Resistant	10	4
Sensitive	3	1
Mitomycin-C (0.1 μg/ml)	P = N.S.	
Resistant	8	1
Sensitive	2	1
Etoposide (0.3 μg/ml)	P = N.S.	
Resistant	27	5
Sensitive	6	7

* Short-term incubation (1 h); † Number of tumours with colony formation >0.5 × control; ‡ Number of tumours with colony formation ≤0.5 × control; N.S., not significant, McNemar's test.

Statistical analysis

Data were expressed as means and standard deviations of six capillaries per data point. Per cent survival was calculated by expressing the average number of tumour colony forming units from treated cells as a percentage of the average number of tumour colony forming units from untreated controls. Statistical analyses were performed using McNemar's test.

RESULTS

A total of 102 tumours was studied for the antitumour effects of NK 611. Ten specimens had to be excluded from further evaluation (two benign histology, five bacterial/fungal contamination, three technical problems). Fifty-two of the remaining 92 specimens (57%) showed adequate growth in controls. All tumour specimens were tested simultaneously using short-term and long-term drug exposure schedules. Seven specimens showed adequate colony formation only in controls in the 1 h schedule while two specimens only grew in the long-term exposure schedule. Table 1 summarises the tumour types studied and evaluable per tumour type. The major tumour types accrued were renal cell cancer, breast cancer and gastrointestinal cancers.

As shown in Table 2 for the short-term incubation, NK 611 had a profound inhibitory effect on tumour colony formation. The antitumour effect was concentration-dependent. At 0.51 μM, *in vitro* growth of 6/45 (13%) evaluable specimens was inhibited, at 5.1 μM 12/45 (27%) specimens were inhibited, and at 51 μM colony formation of 22/45 (49%) tumours was inhibited. When compared with etoposide (5.1 μM, 0.1-fold peak plasma concentration), *in vitro* response rates were comparable (27% versus 29%). Of interest is that colorectal cancer specimens showed a clear concentration-dependent sensitivity to NK 611 with 4 of the 5 specimens inhibited at 51 μM. However, at 5.1 μM the chemosensitivity patterns of NK 611 and etoposide were identical, indicating that NK 611 and etoposide target identical tumour types.

A total of 15 clinically used anticancer agents were tested in direct comparison with NK 611. Sufficient data for statistical analyses were obtained for bleomycin, cisplatin, doxorubicin,

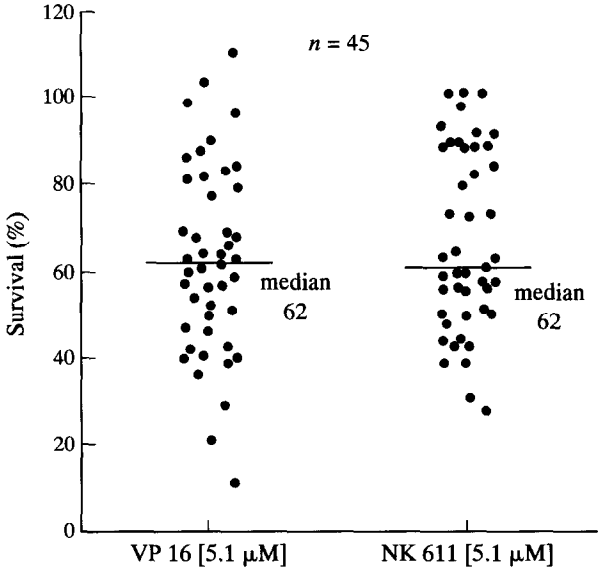


Figure 2. Comparison of colony formation expressed as a percentage of control between NK 611 and etoposide. Tumour cells were incubated with 5.1 μM of each agent for 1 h. The median decrease in colony formation was identical for both agents. VP 16 = etoposide.

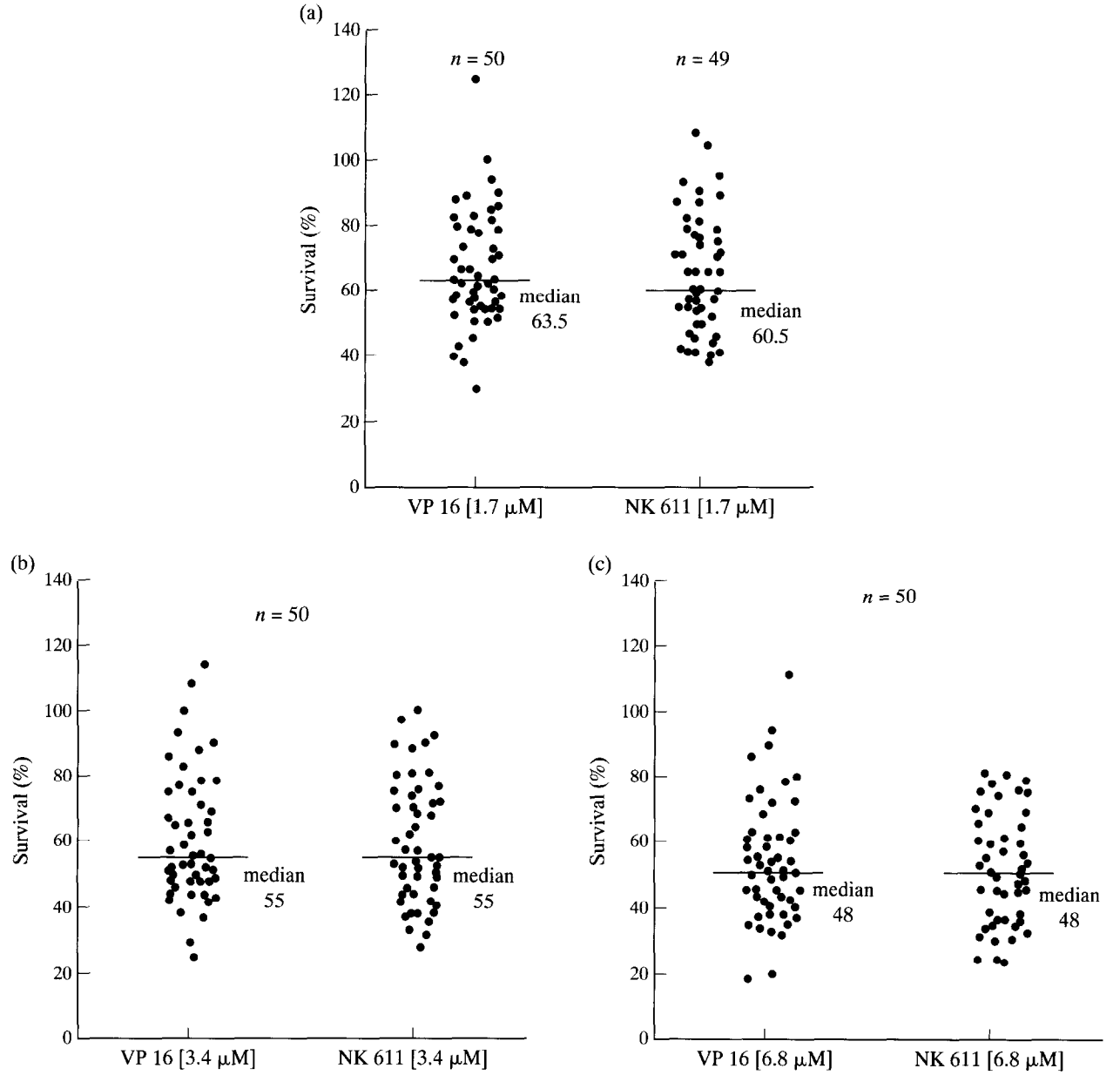


Figure 3. Comparison of colony formation expressed as a percentage of control between NK 611 and etoposide using a long-term exposure schedule. For each concentration evaluated, the median decrease in colony formation was identical for both agents. VP16 = etoposide. (a) 1.7 μM; (b) 3.4 μM; (c) 6.8 μM.

5-fluorouracil, mitomycin-C, vinblastine and etoposide. Across all tumour types, no statistically significant difference between the antitumour activity of NK 611 and any of these agents was noted. For vinblastine and bleomycin a statistical trend for superior activity was noted (Table 3). The comparison of median reductions of colony formation by NK 611 and etoposide also demonstrated that NK 611 is as active as etoposide (Figure 2).

For continuous exposure experiments, concentrations of NK 611 and etoposide ranged from 1.7 to 6.8 μM. Again, NK 611 showed a profound and concentration-dependent antitumour activity. The activity of NK 611 compared well with that of etoposide. No difference in chemosensitivity was noted for the tumour types tested. Similarly, for all concentrations examined the overall effects of NK 611 on tumour colony formation were similar to etoposide (Figure 3a–c). Furthermore, in the majority of individual tumour specimens, NK 611 was as active as etoposide in decreasing colony formation (data not shown). These data argue for a cross resistance between NK 611 and etoposide in the majority of tumour specimens.

In an attempt to define further whether the *in vitro* activity of NK 611 is schedule-dependent, we performed head-to-head comparisons of short-term and long-term drug exposures. The inhibition of tumour colony formation was slightly more pronounced with the long-term exposure schedule. This indicates a potential schedule dependency of the activity of NK 611. It may, therefore, be hypothesised that clinical trials with multiple dosing will give superior results compared with single dose schedules.

DISCUSSION

NK 611 is a chemically modified epipodophyllotoxin derivative with certain advantages compared with etoposide when tested *in vitro* in early screening systems. Although both agents have a similar spectrum of antitumour activity, NK 611 is 3–9-

fold more potent than etoposide *in vitro* and *in vivo* [3]. It is also 120-fold more water soluble than etoposide. Cellular uptake of NK 611 was twice that of etoposide. In dogs, 85% of orally administered NK 611 was absorbed indicating a markedly improved gastrointestinal availability compared with etoposide. These characteristics prompted further interest in the development of NK 611 as a clinical anticancer agent.

We have investigated the antiproliferative effects of NK 611 using freshly explanted human clonogenic tumour cells. We have also performed head-to-head comparisons with etoposide and other, clinically used antitumour agents. Finally, we have compared different schedules of exposure to NK 611. Final concentrations of NK 611 were chosen on the basis of pharmacokinetic data obtained in a presently ongoing clinical phase I study. In this trial, NK 611 is administered intravenously over 30 min every 28 days producing peak plasma concentrations of up to 23.0 μg/ml (33 μM) at 120 mg/m^2 [7]. Since this study is continuing at higher doses, peak plasma levels in the range of 51 μM may be clinically achievable.

Our first finding was that NK 611 had profound, concentration-dependent antitumour activity against a variety of human tumours *in vitro*. This is in agreement with other preclinical information using murine tumour systems and tumour cell lines and raises interest in further development of this agent. However, our results do not confirm earlier reports with human cell lines indicating a significantly higher potency of NK 611 over etoposide. In these studies, Ekimoto and associates have found a 2 to 6-fold increase in activity of NK 611 compared with etoposide in 20 human tumour cell lines *in vitro* [3]. In our experiments, equimolar concentrations of NK 611 and etoposide yielded similar response rates in both exposure schedules. The reasons for this difference may include different sensitivities of established cell lines and freshly explanted specimens as well as differences in drug stability since NK 611 is sensitive to a pH of > 3.0. Significant decomposition of the agent during the long-term exposure appears unlikely since the antiproliferative effects of NK 611 were greater even at lower concentrations than with the short-term incubation. In addition, short-term incubation with NK 611 for 1 h also failed to have a superior effect to etoposide. Our second finding was that long-term incubation resulted in slightly increased inhibition of *in vitro* colony growth compared with short-term incubation. This is in accordance with published reports on other topoisomerase II inhibitors recommending repeated drug administrations to exploit cell-cycle dependent cytotoxicity [15]. This finding argues for the use of multiple dosing schedules for clinical phase II studies with NK 611. Using human tumour xenografts, Ekimoto and associates reported that while antitumour activity of NK 611 was observed with single administration, multiple administrations gave superior results. The differences in antitumour activity appeared to be a function of the total dose administered [16]. Our third finding was that, across all tumours tested, NK 611 was as active as six other clinically used antitumour agents. When compared with etoposide, 11 of the 45 specimens showed lack of cross resistance. This finding is in agreement with the report by Ekimoto and colleagues who found partial cross resistance between NK 611 in cell lines that were resistant to etoposide and expressed the *MDR*1 gene product [3]. Most of the human tumours in our experiments, however, were cross resistant to NK 611 and etoposide.

In summary, NK 611 is a new compound active against a variety of cancer cells. Although some of the cell line data cannot be reproduced in freshly explanted human tumour cells, our results indicate that further clinical development of NK 611 is warranted.

1. Dorr RT, Von Hoff DD. *Cancer Chemotherapy Handbook*, 2nd edn. Norwalk, Appleton & Lange, 1993.
2. Minocha A, Long B. Inhibition of the DNA catenation activity of type II topoisomerase by VP-16-213 and VM-26. *Biochem Biophys Res Commun* 1984, **122**, 165–70.
3. Ekimoto H, Okamoto K, Maruyama S, *et al.* Preclinical characterization of NK611, a novel water-soluble etoposide analog. *Ann Oncol* 1992, **3** (Suppl. 1), 98.
4. Machida M, Tanaka S, Nakamori K. Simultaneous determination of NK611, a novel water-soluble derivative of etoposide, and its metabolite (DeNK611) in dog plasma by column-switching high performance liquid chromatography. *Biomed Chromatogr* 1993, **7**, 82–85.
5. Hüttmann A, Mross K, Hossfeld DK. Determination of the new podophyllotoxin derivative NK 611 in plasma by high-performance liquid chromatography with ultraviolet detection. *J Chromatogr* 1993, **620**, 233–238.
6. Zucchetti M, De Fusco M, Sessa C, Frohlich A, Reichert S, D'Incalci M. High-performance liquid chromatographic assay for the determination of the novel podophyllotoxin derivative dimethylaminoetoposide (NK611) in human plasma. *J Chromatogr B Biomed Appl* 1994, **654**, 97–102.
7. Hanauske A-R, Schilling T, Mross K, *et al.* Phase I clinical and pharmacokinetic trial of NK 611, a new podophyllotoxin derivative. *Proc Am Soc Clin Oncol* 1994, **13**, 145.
8. Kudoh S, Fokuoka M, Furuse K, *et al.* Phase I trial and pharmacokinetic study of the new etoposide analog NK 611 in patients with advanced solid tumors. *Ann Oncol* 1992, **3**, (Suppl. 1), 146.
9. Allen LM, Creaven PJ. Comparison of the human pharmacokinetics of VM-26 and VP-16, two antineoplastic epipodophyllotoxin glucopyranoside derivatives. *Eur J Cancer* 1975, **11**, 697–707.
10. Slevin ML, Clark PI, Joel SP, *et al.* A randomized trial to evaluate the effect of schedule on the activity of etoposide in small-cell lung cancer. *J Clin Oncol* 1989, **7**, 1333–1340.
11. Hanauske A-R, Hanauske U, Von Hoff DD. The human tumor cloning assay in cancer research and therapy. *Curr Probl Cancer* 1985, **9**, 1–50.
12. Maurer HR, Ali-Osman F. Tumor stem cell cloning in agar-containing capillaries. *Naturwiss* 1981, **68**, 381–383.
13. Von Hoff DD, Forseth BJ, Huong M, Buchok JB, Lathan B. Improved plating efficiencies for human tumors cloned in capillary tubes versus Petri dishes. *Cancer Res* 1986, **46**, 4012–4017.
14. Hanauske U, Hanauske A-R, Marshall MH, Muggia VA, Von Hoff DD. Biphasic effects of vanadium salts on *in vitro* tumor colony growth. *Int J Cell Cloning* 1987, **5**, 170–178.
15. Hainsworth JD, Johnson DH, Frazier SR, Greco FA. Chronic daily administration of oral etoposide—a phase I trial. *J Clin Oncol* 1989, **7**, 396–401.
16. Ekimoto H, Okamoto K, Kusama K, *et al.* Treatment schedule dependency of antitumor effect of NK611, a novel water-soluble analog of etoposide. *Proc Am Assoc Cancer Res* 1992, **33**, 436.

Acknowledgement—This work was supported by a grant from Asta Medica, Frankfurt/M., Germany.