Pergamon

European Journal of Cancer, Vol. 33, No. 14, pp. 2404–2410, 1997
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Printed in Great Britain
0959-8049/97 $17.00+0.00

PII: S0959-8049(97)00277-3

Original Paper

Preclinical Activity of *Trans*-indazolium [Tetrachlorobisindazoleruthenate(III)] (NSC 666158; IndCR; KP 1019) Against Tumour Colony-forming Units and Haematopoietic Progenitor Cells

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***Trans*-indazolium[tetrachlorobisindazoleruthenate(III)] (KP 1019) is a new heavy metal complex with promising activity against tumour cell lines and in animal models. We studied the antineoplastic effects of KP 1019 (final concentrations: 1, 10, 100 μg/ml) on *in vitro* proliferation of clonogenic cells from freshly explanted human tumours in a capillary soft agar cloning system, and compared the activity of KP 1019 with conventional antineoplastic agents. 53 of 75 specimens (71%) showed adequate growth in controls. KP 1019 inhibited tumour colony formation in a concentration-dependent manner in both short- (1 h) and long-term (21 d) exposure experiments. KP 1019 at 100 μg/ml with 1 h exposure was as active as bleomycin, cisplatin, doxorubicin, etoposide, 5-fluorouracil, methotrexate, mitomycin-C and vinblastine, with only paclitaxel more active than KP 1019 ($P = 0.002$). The antitumour activity of KP 1019 was more pronounced after long-term exposure, indicating the potential schedule dependency of KP 1019. Activity was observed against non-small cell lung, breast and renal cancer. We conclude that if appropriate plasma levels can be achieved in patients, KP 1019 may have significant clinical activity against a variety of different tumour types. © 1997 Elsevier Science Ltd.**

Key words: ruthenium complexes, clonogenic growth, human tumours
Eur J Cancer, Vol. 33, No. 14, pp. 2404–2410, 1997

INTRODUCTION

SINCE THE discovery of the powerful antitumour activity of *cis*-[$PtCl_2(NH_3)_2$] (cisplatin), several thousand metal complexes have been synthesised and evaluated in preclinical systems. However, the synthesis of new platinum complexes based on structure–antitumoral activity relationships has been rather disappointing. In order to obtain better results, new metal complexes are under current investigation. In particular, titanium and ruthenium complexes appear to be promising candidate heavy metals. Out of a series of analogues, *trans*-indazolium[tetrachlorobisindazoleruthenate(III)] (KP 1019) had the most favourable therapeutic index showing good therapeutic activity and lower toxicity than its congeners [1]. It has substantial antitumour activity *in vitro* and in several experimental murine and rat models [1–6]. KP 1019 is active in various transplantable tumour models, such as the P 388 leukaemia (1.6-fold higher survival time in treated versus controls), the Stockholm Ascitic tumour (2.5-fold), the Sarcoma 180 Ascitic tumour (1.85-fold), the Ehrlich Ascitic tumour (> 3-fold) and the MAC 15A colon tumour (> 3-fold) [6]. Furthermore, KP 1019 produced complete remissions in 30% of chemically induced autochthonous colorectal carcinoma in rats [1].

The precise mechanism by which KP 1019 exerts its cytotoxic effects on tumour cells remains to be determined, but

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Received 29 Nov. 1996; revised 28 Apr. 1997; accepted 6 May 1997.

due to their similarity to iron(III), it has been proposed that antitumour ruthenium(III) complexes could have a high affinity for the plasma protein transferrin [7]. Since neoplastic cells have a high iron requirement and commonly carry large numbers of receptors for the iron-transport protein transferrin, the accumulation of ruthenium(III) complexes in tumours might therefore be mediated by this plasma protein. It has been proposed that cell surface transferrin receptors bind ruthenium-loaded transferrin with high affinity, and that the resultant transferrin–receptor complexes are subsequently endocytosed and transferred to acidic non-lysosomal compartments where ruthenium is released [8]. The transferrin–receptor complexes are subsequently translocated back to the cell surface, and transferrin is released from the receptors. At the molecular level, DNA protein cross-links and DNA interstrand cross-links have been demonstrated [9]. Of importance is also the fact that freshly prepared solutions of the compound fail to inhibit DNA polymerisation *in vitro*, whereas aged solutions containing aquo complexes of KP 1019 inhibit DNA polymerisation to a large extent [6]. This indicates that the pure compound acts as a prodrug, and that intracellular hydrolysis is required for activation. Reduction of ruthenium(III) to ruthenium(II) under hypoxic conditions may be an additional mechanism of cellular activation of ruthenium drugs [6].

KP 1019 is currently being formulated for clinical phase II investigations by the Cancer Research Campaign (U.K.) in cooperation with the Task Force for Anticancer Drug Development (AWO, Germany). In the present study, we determined the activity of KP 1019 against *in vitro* colony formation of freshly explanted human tumours and compared KP 1019 with other clinically relevant antitumour agents.

MATERIALS AND METHODS

Compounds

Trans-indazolium[tetrachlorobisindazoleruthenate(III)] (KP 1019) was synthesised and kindly supplied by Professor B.K. Keppler, Institute of Inorganic Chemistry, University of Heidelberg, Germany (Figure 1). The purity was determined by elemental analysis, IR and ^{1}H nuclear magnetic resonance spectroscopy. Stock solutions and final solutions of KP 1019 were prepared in 0.9% NaCl.

Based on *in vitro* cell line data, KP 1019 was studied at final concentrations of 1, 10 and 100 μg/ml (corresponding to 1.67, 16.7 and 167 μM). For short-term exposure experiments, tumour cells were incubated with the compound for 1 h followed by removal of the agent. In the same experiment, other clinically important antitumour agents for the specific tumour type were tested (Table 1). For each compound, the concentration tested was 0.1-fold the clinical peak plasma concentration observed [10]. For long-term incubations, tumour cells were exposed to KP 1019 for 21–28 days at the concentrations specified.

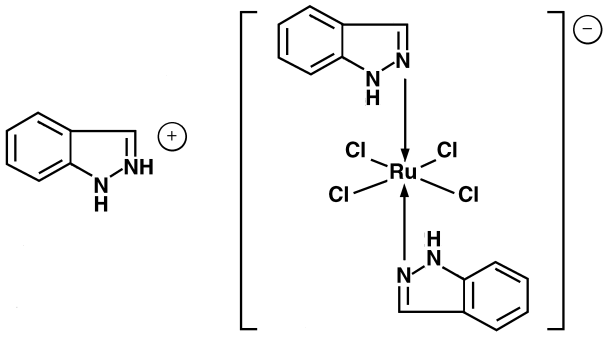

Figure 1. Structure of *trans*-indazolium[tetrachlorobisindazoleruthenate(III)] (KP 1019), MW 598.31.

Human tumour cloning system

Tumour specimens from previously untreated patients and from patients who had received a variety of chemotherapy regimens were obtained 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 hydroxyethylpiperazine ethanesulphonic acid (HEPES), 1 mM sodium pyruvate, 90 U/ml penicillin, and 90 μg/ml streptomycin (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 112 *g* for 5–7 min and passed

Table 1. *Selection of antitumour agents evaluated by tumour type*

Renal cell	Testis
Interferon A_2	Cisplatin
Vinblastine	Etoposide
Bleomycin	Bleomycin
	Vinblastine
Oesophagus	Doxorubicin
Cisplatin	
5-FU	Colorectal
Etoposide	5-FU
Paclitaxel	Interferon A_2
	Mitomycin C
Ovarian	
Cisplatin	Non-small cell lung
Doxorubicin	Cisplatin
5-FU	Vinblastine
Vinblastine	Mitomycin C
Bleomycin	Doxorubicin
Paclitaxel	Etoposide
	Paclitaxel
Unknown primary site	
Doxorubicin	Stomach
Cisplatin	Cisplatin
5-FU	Doxorubicin
Vinblastine	Etoposide
Paclitaxel	5-FU
	Mitomycin C
Corpus uteri	Paclitaxel
Cisplatin	
Doxorubicin	Breast
Bleomycin	Doxorubicin
5-FU	5-FU
Vinblastine	Methotrexate
	Vinblastine
Sarcoma	Mitomycin C
Cisplatin	Paclitaxel
Doxorubicin	
Actinomycin D	Mesothelioma
DTIC	Doxorubicin
Methotrexate	Cisplatin
Paclitaxel	

through 25 gauge needles to obtain single cell suspensions when necessary. Cells were cryopreserved in culture medium containing 10% dimethyl sulphoxide (DMSO, Serva, Heidelberg, Germany) by freezing with a rate of −2°C/min down to −175°C and stored in liquid nitrogen. Both fresh and cryopreserved samples were used in this study.

The capillary soft agar cloning system was used as described earlier [11–13]. Briefly, cells from tumour specimens were seeded at a median density of 5.0×10^4 cells/capillary (range 0.8×10^4–6.5×10^4 cells/capillary) in 100 µl glass capillaries (Brand, Wertheim, Germany) in a mixture of 0.3% agar (Sigma, Deisenhofen, Germany) in double-enriched Connaught Medical Research Laboratories' Medium 1066 (Gibco) containing 15% horse serum (Gibco), 2% fetal calf serum, 0.3 mM vitamin C (Sigma), 90 U/ml penicillin, 90 µg/ml streptomycin, 10 mM HEPES, 2 mM sodium pyruvate, 0.1 mM non-essential amino acids (Gibco), 4 mM glutamine (Gibco), 100 µg/ml asparagine (Gibco), 4 ng/ml hydrocortisone (Sigma), 50 U/ml catalase (Serva) and 0.1 nM epidermal growth factor (Flow, Meckenheim, Germany). For each data point, six capillary tubes were used. Each experiment contained one set of controls with 0.9% NaCl as solvent and a second set with 1 mM ammonium monovanadate (Merck, Darmstadt, Germany) to ensure the presence of a good single cell suspension [14]. Colony formation was evaluated with an inverted microscope after an incubation period of 21–28 days at 37°C, 5% CO_2 and 100% humidity. Colony formation was considered adequate when the NaCl control had a mean of at least 18 colonies per six capillaries and the vanadate control showed $\leq 30\%$ colony formation compared to the NaCl control.

Table 2. Types of tumours studied and evaluability per tumour type. In both the short-term (1 h) and the long-term drug exposure schedule, 51/75 (68%) tumour specimens were evaluable

Tumour type	Long-term exposure No. evaluable*/ No. attempted	1 h exposure No. evaluable*/ No. attempted
Breast	10/12	10/12
Renal cell	8/12	8/12
Non-small cell lung	5/10	6/10
Sarcoma	3/5	3/5
Colorectal	2/5	2/5
Ovarian	4/4	3/4
Testis	4/4	4/4
Mesothelioma	3/3	3/3
Other†	5/11	4/11
Unknown primary site	7/9	8/9
Total	51/75 (68%)	51/75 (68%)

*≥ 18 colonies/6 capillaries in controls. †Stomach, gallbladder, pancreas, corpus uteri, oesophagus, non-Hodgkin's lymphoma, paraganglioma.

Table 3. Inhibitory activity of KP 1019 against tumour colony forming units from freshly explanted human tumours in vitro *using a short-term exposure schedule (1 h) indicating concentration-dependent antitumour activity of KP 1019*

Tumour type	No. specimens inhibited*/No. specimens evaluable KP 1019 (µg/ml)		
	1.0	10.0	100.0
Breast	0/10	0/10	4/10
Renal cell	1/8	1/8	4/8
Non-small cell lung	0/6	0/6	2/6
Testis	0/4	0/4	1/4
Sarcoma	0/3	0/3	3/3
Ovarian	0/3	0/3	0/3
Mesothelioma	0/3	0/3	0/3
Colorectal	0/2	0/2	0/2
Other†	0/4	0/4	0/4
Unknown primary site	0/8	2/8	7/8
Total	1/51 (2%)	3/51 (6%)	21/51 (41%)

*≤ 50% survival of tumour colony-forming units. †Oesophagus, stomach, corpus uteri.

Effects on clonogenic haematopoietic stem cells

Cells from frozen peripheral stem cell harvests were thawed and seeded at a density of 10^5 cells/plate in Petri dishes (Nunc, Naperville, Illinois, U.S.A.) in MethoCult H4431 medium (StemCell Technologies Inc., Vancouver, B.C., Canada) containing 10% fetal calf serum and 1% glutamine. Colony forming units were evaluated with an inverted microscope after an incubation period of 10–14 days at 37°C, 5% CO_2 and 100% humidity and were classified as CFU-GEMM, CFU-GM and clusters.

Table 4. Comparison of in vitro *activity of KP 1019 (short-term incubation) with nine common clinical antitumour agents. Only paclitaxel showed activity against KP 1019-resistant cells*

Compound	KP 1019 (100 µg/ml)* Resistant†	Sensitive‡
Bleomycin (0.2 µg/ml)	P = N.S.	
Resistant	4	2
Sensitive	4	4
Cisplatin (0.2 µg/ml)	P = N.S.	
Resistant	14	7
Sensitive	4	3
Doxorubicin (0.04 µg/ml)	P = N.S.	
Resistant	17	9
Sensitive	5	7
5-Fluorouracil (6.0 µg/ml)	P = N.S.	
Resistant	9	6
Sensitive	8	5
Methotrexate (0.3 µg/ml)	P = N.S.	
Resistant	5	3
Sensitive	2	3
Mitomycin-C (0.1 µg/ml)	P = N.S.	
Resistant	8	5
Sensitive	7	1
Paclitaxel (0.342 µg/ml)	P = 0.002	
Resistant	3	2
Sensitive	16	11
Vinblastine (0.05 µg/ml)	P = N.S.	
Resistant	16	3
Sensitive	6	11
Etoposide (3.0 µg/ml)	P = N.S.	
Resistant	9	2
Sensitive	2	2

*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.

Table 5. *Inhibitory activity of KP* 1019 *against tumour colony forming units from freshly explanted human tumours* in vitro *using a long-term exposure (*21–28 *days). KP* 1019 *has profound and concentration-dependent antitumour activity*

Tumour type	No. specimens inhibited*/No. specimens evaluable* KP 1019 (μg/ml)		
	1.0	10.0	100.0
Breast	1/10	3/10	7/10
Renal cell	1/8	2/8	5/8
Non-small cell lung	2/5	3/5	5/5
Testis	0/4	0/4	4/4
Sarcoma	0/3	0/3	3/3
Ovarian	0/4	1/4	4/4
Mesothelioma	0/3	0/3	2/3
Colorectal	0/2	0/2	0/2
Oesophagus	0/2	1/2	2/2
Stomach	0/1	0/1	0/1
Gallbladder	1/1	1/1	1/1
Corpus uteri	0/1	0/1	1/1
Unknown primary site	1/7	3/7	7/7
Total	6/51 (12%)	14/51 (28%)	41/51 (80%)

*≤ 50% survival of tumour colony-forming units.

Table 6. *Comparison of* in vitro *activity of KP* 1019 *(long-term incubation) with nine common clinical antitumour agents. KP* 1019 *was active against cells resistant to cisplatin, doxorubicin, 5-fluorouracil, vinblastine and etoposide*

Compound	KP 1019 (100 μg/ml)* Resistant†	Sensitive‡
Bleomycin (0.2 μg/ml)	$P = 0.074$	
Resistant	1	5
Sensitive	0	8
Cisplatin (0.2 μg/ml)	$P < 0.001$	
Resistant	2	19
Sensitive	0	5
Doxorubicin (0.04 μg/ml)	$P < 0.001$	
Resistant	5	21
Sensitive	0	10
5-Fluorouracil (6.0 μg/ml)	$P = 0.027$	
Resistant	6	9
Sensitive	1	10
Methotrexate (0.3 μg/ml)	$P = 0.131$	
Resistant	2	6
Sensitive	1	4
Mitomycin-C (0.1 μg/ml)	$P = 0.114$	
Resistant	4	8
Sensitive	2	5
Paclitaxel (0.342 μg/ml)	$P =$ N.S.	
Resistant	2	3
Sensitive	4	21
Vinblastine (0.05 μg/ml)	$P = 0.004$	
Resistant	4	15
Sensitive	2	13
Etoposide (3.0 μg/ml)	$P = 0.016$	
Resistant	0	10
Sensitive	1	4

*Long-term incubation (21–28 d). †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 calculated as means and standard deviations of at least three evaluable determinations per data point. Colony survival was calculated by expressing the average number of colony-forming units from cells exposed to each antitumour agent relative to the average number of colony-forming units from untreated controls. Results were evaluated statistically by the Wilcoxon signed rank test and McNemars's test. P values ≤ 0.05 were interpreted as indicating significant differences.

RESULTS

A total of 80 tumours was studied for the antitumour effects of KP 1019. Five specimens had to be excluded from further evaluation (4 bacterial/fungal contamination, 1 benign histology). 53 of the remaining 75 specimens (71%) showed adequate growth in controls. All tumour specimens were tested simultaneously using short- and long-term drug exposure schedules. Two 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 2 summarises the tumour types studied and the evaluability per tumour type. The major tumour types accrued were breast cancer, renal cell cancer and non-small cell lung cancer.

As shown in Table 3 for the short-term incubation, KP 1019 had a concentration-dependent inhibitory effect on tumour colony formation. At 1.0 μg/ml, *in vitro* growth of 1/51 (2%) evaluable specimens was inhibited, at 10.0 μg/ml 3/51 (6%) specimens were inhibited, and at 100.0 μg/ml colony formation of 21/51 (41%) tumours was inhibited.

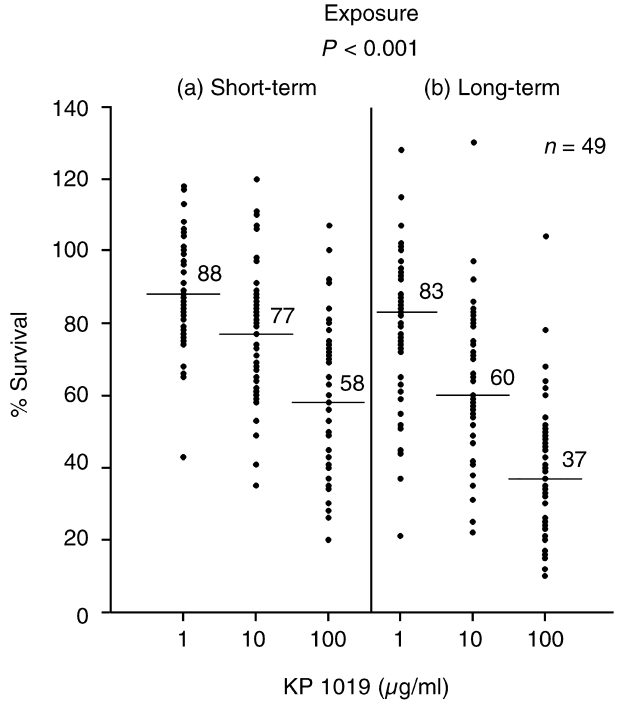

Figure 2. Comparison of colony formation expressed as percentage of control between short- and long-term exposure schedules of KP 1019. The difference in the median values (shown as horizontal lines with median value above) of the two groups is statistically significant ($P < 0.001$). P values were calculated using the Wilcoxon signed rank test.

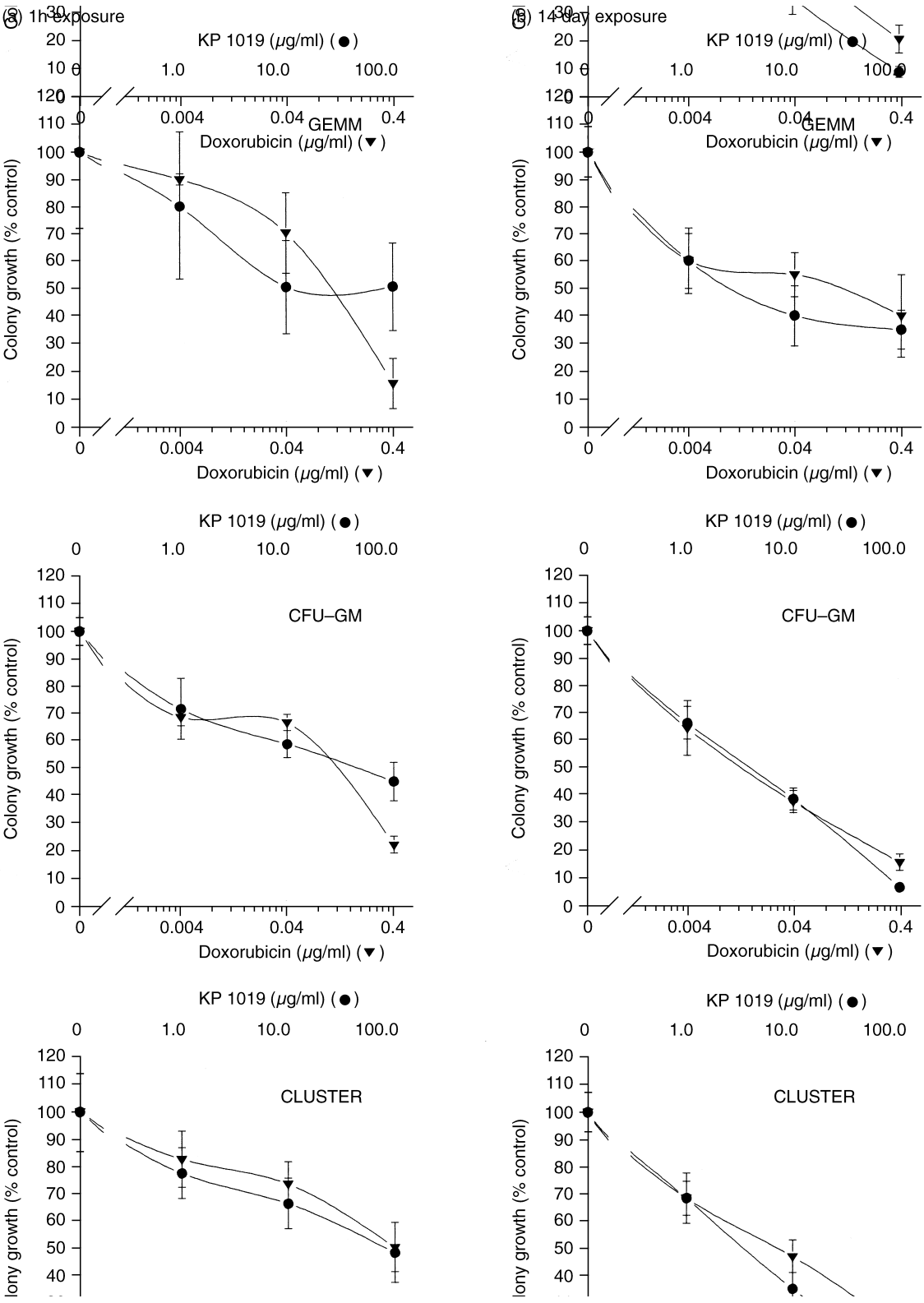

Figure 3. ***In vitro*** **evaluation of toxicity of KP 1019 on haematopoietic stem cells. Concentrations in the range of 1–10μg/ml for KP 1019 showed comparable toxicity to doxorubicin at 1/10 of the peak plasma concentration (0.04 μg/ml) in humans: (a) 1 h exposure; (b) 14 d exposure.**

A total of 12 clinically used anticancer agents were studied simultaneously with KP 1019. Sufficient data for statistical analyses were obtained for bleomycin, cisplatin, doxorubicin, 5-fluorouracil, methotrexate, mitomycin-C, paclitaxel, vinblastine and etoposide (Table 4). Only paclitaxel showed activity against KP 1019-resistant cells.

For continuous exposure experiments, concentrations of KP 1019 ranged from 1.0 to 100 μg/ml. Again, KP 1019 showed concentration-dependent antitumour activity (Table 5). At 1.0 μg/ml, the *in vitro* growth of 6/51 (12%) evaluable specimens were inhibited, at 10.0 μg/ml 14/51 (28%) specimens were inhibited, and at 100 μg/ml 41/51 (80%) specimens were inhibited. At 100 μg/ml, activity was seen against different types of cancer including non-small cell lung, breast and renal cancer. Comparison of the *in vitro* activity of KP 1019 with the long-term exposure with common clinical antitumour agents is shown in Table 6. At 100 μg/ml, KP 1019 was active against cells resistant to cisplatin, doxorubicin, 5-fluorouracil, vinblastine and etoposide. As shown in Figure 2, the inhibition of tumour colony formation was statistically significantly more pronounced with long-term exposure ($P < 0.001$).

The *in vitro* evaluation of the toxicity of KP 1019 on haematopoietic stem cells is summarised in Figure 3. The toxicity of KP 1019 on the GEMM, CFU-GM and clusters was more pronounced with long-term exposure. For GEMMs, the IC_{50} of KP 1019 was 2 μg/ml for long-term incubation and 10 μg/ml for short-term exposure. For CFU-GMs, the IC_{50} was 4 and 50 μg/ml, respectively, and for clusters, the IC_{50} was 4 and 90 μg/ml, respectively. Concentrations in the range of 1–10 μg/ml for KP 1019 showed a comparable toxicity to doxorubicin.

DISCUSSION

Several previous publications have addressed the preclinical activity of KP 1019 against established cancer cell lines *in vitro* and in xenograft models [1–6], but our report focused on tissue freshly obtained from cancer patients and haematopoietic stem cells. It therefore expands the current knowledge about the activity and toxicity of KP 1019 to potentially more clinically relevant models and may provide a guide for subsequent clinical studies. Final concentrations of KP 1019 were chosen on the basis of *in vitro* data obtained in studies on the SW 707 human colon cancer cell line. In this system, the IC_{50} concentration as determined using the MTT assay was 110 μg/ml [3]. Using concentrations ranging from 1 to 100 μg/ml, we found that KP 1019 has concentration-dependent antitumour activity when administered for 1 h. Although, with 1 h incubation KP 1019 had no better activity compared with that of other antitumour agents, the spectrum of activity included tumours with known clinical resistance to conventional chemotherapy. With long-term exposure, KP 1019 again showed concentration-dependent activity, which was much greater in comparison with short-term exposure. This argues for a potential schedule dependency of the activity of KP 1019 *in vitro*. It may thus be postulated that clinical trials with multiple dosing may give superior results compared with single-dose schedules. However, a prerequisite for clinical activity will be that active plasma concentrations can be achieved with tolerable toxicity.

To date, pharmacokinetic data for KP 1019 in humans have not been reported. Toxicological studies with KP 1019 in NMRI mice indicate a high dependence of mortality on the infusion concentration at each dose level. The LD_{50} increased from 50 to 100 mg/kg when dividing the infusion concentration by four and using a 4-fold infusion volume of physiological saline [6]. KP 1019 was also been administered to mice at 50 mg/kg daily for five consecutive days with 4 ml of 0.9% NaCl per 100 g of body weight, resulting in no mortality. KP 1019 is well tolerated by animals even at higher doses, particularly using chronic application schedules. Main target organs for toxicity are kidneys and bone marrow. Nephrotoxicity can be considerably reduced by applying higher volumes of liquid. The major haematological toxicity in mice is erythropaenia [15].

In an attempt to predict the haematotoxicity of KP 1019 in humans, we performed colony-forming assays using human haematopoietic progenitor cells. Concentrations of 1–10 μg/ml of KP 1019 were approximately equitoxic to doxorubicin at a concentration that corresponds to the 1/10 peak plasma concentration in humans. This indicates that, clinically, antitumour activity (observed at concentrations ≥ 10 μg/ml) might be accompanied by marked haematotoxicity. This may be counterbalanced by selective enrichment of KP 1019 in tumour cells due to specific transferrin-mediated uptake. In addition, preclinical evidence support the use of KP 1019 as part of combination strategies. Kreuser and associates have reported that pre-incubation with IFN-α, -β and -γ enhances the cytotoxicity of KP 1019 in human colon cancer cell lines *in vitro* [16]. The combination of KP 1019 and interferon may also be of potential importance for the treatment of renal cell cancer.

With long-term exposure, KP 1019 retained activity against tumours resistant to cisplatin, doxorubicin, vinblastine, etoposide and 5-fluorouracil. It is tempting to speculate that this may be due to a mechanism of action of KP 1019 which overcomes P170-mediated multidrug resistance mechanisms. However, this would not explain the activity of KP 1019 against tumours resistant to cisplatin or 5-fluorouracil. Further studies are needed to understand better the mechanisms of resistance to KP 1019.

In summary, KP 1019 has antiproliferative activity against clonogenic cells from human tumours *in vitro*. If appropriate plasma levels can be achieved in patients, KP 1019 could have significant clinical activity in patients with tumour types sensitive *in vitro*. Haematotoxicity may be clinically relevant but a specific uptake mechanism into tumour cells may help avoid toxic plasma concentrations. Further clinical development of this agent thus seems warranted.

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Acknowledgement—This study was performed in conjunction with the Arbeitsgruppe Wirkstoffentwicklung in der Onkologie der AIO, Germany.