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Colony Assay with Human Tumor Xenografts, Murine Tumors and Human Bone Marrow. Potential for Anticancer Drug Development*†

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Abstract—*The colony formation of human tumor xenografts from nude mice, of murine tumors, and of human bone marrow (CFU-C) has been investigated* in vitro *using a modification of the double-layer agar assay described by Hamburger and Salmon. Systematic modification of growth conditions and careful selection of viable tumor tissue enhanced the growth rate (at least* 30 *colonies per dish) of human tumor xenografts to* 86% (98/114). *The median plating efficiency was* 0.07% *which is comparable to the results observed by others using fresh human tumors. The growth of human bone marrow was stimulated with a placenta-conditioned medium, which allowed growth of granulocytic stem cell colonies (CFU-C). The median plating efficiency of the bone marrow was* 0.08%. *The murine tumors P*388, *L*1210, *B*16 *melanoma, Lewis lung carcinoma and colon carcinoma* 38 *grew very well* in vitro. *Excluding the Lewis lung carcinoma, the plating efficiency of these tumors was markedly higher than that of the human tumor xenografts and human bone marrow.*

The colony assay may have potential as a secondary screening system for identifying new active structures and also for indicating which tumor types are most responsive to a new antitumor agent. We test new structures in 20 *well-selected human tumor xenografts and in the P*388 *mouse leukemia in dose–response relationships. The two most responsive xenograft tumors are subsequently studied* in vivo *in nude mice in order to determine if a new compound presents antitumor activity in an* in vivo *organism at a dose around the* LD_{10} *level. If a remission or at least no change is observed in the subcutaneously growing tumor, the new compound undergoes large disease-oriented testing usually in* 60 *xenografts.*

The in vivo *studies are necessary in determining whether a compound has a more specific effect on tumor cells than on the dose-limiting normal tissue. The comparison of* in vitro/in vivo *activity allows an assessment of the relevant* in vitro *dose based on* in vivo *pharmacological behavior of a drug. It seems justifiable to apply the conclusions of this approach to the clinical setting because mouse toxicity data, e.g. the* LD_{10}, *correspond well to the maximal tolerable doses in man. Moreover, for compounds whose dose-limiting toxicity is bone marrow suppression, the comparison of drug dosages effective* in vitro *on human bone marrow and tumor xenografts may prove helpful.*

The proposed testing strategy has been applied to TGU and Tiazofurin. At the relevant dosages TGU exhibited very limited activity in 67 *human tumor xenografts studied. Moreover, Tiazofurin was active only in* 2% *of* 52 *xenografts investigated. These findings correlate well with the ineffectiveness observed in clinical phase I and II studies of both compounds, suggesting that the proposed testing procedure may offer reliable predictions for the clinical potential of new compounds.*

INTRODUCTION

OVER the past 30 years, new compounds have mainly been screened for anticancer activity in murine tumors *in vivo*. In 1978 the National Cancer Institute (NCI) introduced a tumor panel which was composed of murine solid tumors as well as three human solid tumors growing in nude mice [1, 2]. Nevertheless, all new compounds were first prescreened in the P388 leukemia. Only compounds active in the prescreen were tested in the mouse and human solid tumors. Unfortunately, this new NCI

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screen has not been successful in developing new agents with activity against solid tumors in man [3, 4]. This may be due to the fact that only three single human tumor xenografts, a mammary cancer (MX1), a lung cancer (LX1) and a colon cancer (CX1), were represented in the screen. The heterogeneous response to chemotherapy seen in most tumor types in the clinic suggests that a panel of human tumors for each tumor category should be represented at least for secondary screening.

Since screening in human tumor xenografts *in vivo* is very expensive, attempts have been made to study the responsiveness of fresh human tumors *in vitro*. The NCI has sponsored a program utilizing the human tumor clonogenic assay as a screen for new anticancer drugs [5]. Initial reports have shown that the colony assay can detect drugs as active that were negative in the P388 leukemia prescreening system [6]. However, these active compounds have not yet been studied clinically in human solid tumors.

The colony assay presents several drawbacks when fresh human tumor specimens are used. For many tumor types only limited growth rates have been observed. In a collaborative study from four laboratories, 24% of 2752 experiments showed evaluable testing results using internal quality measures [5]. Another major obstacle is that experiments are not reproducible. In order to avoid these limitations we have used human tumors growing in serial passages in nude mice as tumor material for the colony assay.

The rate of tumors producing enough colonies for drug testing could be markedly increased [7]. Consequently, new compounds can be tested in several dosages, since tumor tissue is available in large quantities.

For any *in vitro* system, a critical question is the relevant dose level for *in vivo* application in man. Since mouse toxicity data, e.g. the LD_{10}, agree well with the maximal tolerable doses in man [8], *in vitro* responsive tumors were tested *in vivo* in nude mice, in order to determine if activity observed at a given dose *in vitro* could also be found *in vivo*. Furthermore, the effect on human bone marrow (CFU-C) *in vitro* was also assessed to determine the relevant *in vitro* dose independent of *in vivo* pharmacological data. In the present paper we report on the growth rates of human tumor xenografts, murine tumors and human bone marrow in the colony assay, as well as our testing strategy for the evaluation of new drugs. The *in vitro* and *in vivo* activity of TGU and Tiazofurin, two compounds which are active in several murine tumors *in vivo* [9, 10], will be presented.

MATERIALS AND METHODS

Tumors

Human tumors established in serial passage in nude mice (NMRI genetic background) in our laboratory were used as tumor material [12–14]. The animals were housed in macrolon cages set in laminar flow rackets and kept under conditions as described by Fortmeyer [15]. The histological appearance of the mouse-grown tumors was strikingly similar to the original human tumor in most cases, only 20% of the xenograft tumors showed some variation in their degree of differentiation. The human origin of the tumors was demonstrated by isoenzyme techniques [12–14]. Only untreated tumors were used for drug testing. 88% of the tumors were studied *in vitro* after 1–10 passages in nude mice, 12% after later passages. The murine leukemias, P388 and L1210, as well as the solid tumors, B16 melanoma, Lewis lung carcinoma and colon carcinoma 38, were obtained from G. Atassi, Brussels. They were subpassaged and maintained in animals as recommended by the NCI.

Human bone marrow

Human bone marrow cells were aspirated from the iliac crest of consenting healthy volunteers into preservative-free heparinized syringes. Mononuclear cells with a density of less than 1.007 g/ml were separated by density centrifugation in Ficoll Paque, washed and plated as described below.

Preparation of single cell suspension

A single cell suspension of the solid tumors was obtained by mechanical disaggregation with scissors and subsquent incubation with enzymes (collagenase 0.04%, DNase 0.07% and hyaluronidase 0.1%) at 37°C for 30 min. Afterwards the cells were washed and passed through stainless steel sieves with 200 and 50 μm mesh size to remove any remaining clumps. Cell counts were performed in a hemocytometer, and the percentage of viable cells was determined by trypan blue exclusion. Tumor murine leukemias P388 and L1210 were subpassaged by i.p. injection yielding leukemic ascites.

Culture methods

A modification of the two-layer soft agar culture system introduced by Hamburger and Salmon was used [16]. The base layer consisted of 1 ml Iscove's modified Dulbeccos medium with L-glutamine containing 10% fetal calf serum and 0.5% agar which were plated in 35 mm Petri dishes. $2–5 \times 10^5$ viable human tumor cells were added in a volume of 1 ml (0.3% agar, 30% fetal calf serum, and medium) over the base layer. Drugs were given in 1 ml medium containing 30% fetal calf serum, whereas the control group received the vehicle only (Fig. 1). Cultures were incubated at 37° C in a humidified atmosphere containing 7% CO_2. Control plates were monitored for growth every other day using an inverted microscope. At the time of maximum colony formation (7–21 days in culture)

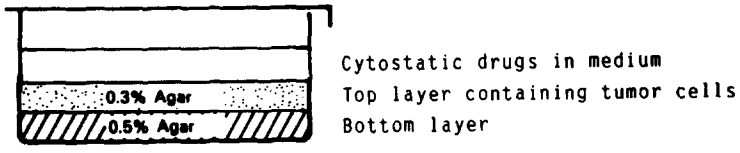

Fig. 1. Schematic illustration of a culture dish in the colongenic assay. Cytostatic drug, applied in 3-fold concentration, reaches the top and bottom layer by diffusion.

final colony counts were obtained with an automatic image analysis system (Bausch & Lomb OMNICON, FAS III). 24 hr prior to final evaluation all dishes were incubated with 1 ml tetrazolium chloride (1 mg/ml), which stained vital colonies and cells only [17]. Objects presenting a circular profile in two dimensions with a minimum diameter of 60 μm—for initially reaggregating adenocarcinomas 80 μm—were scored as colonies. For quality control purposes plates were stained with tetrazolium chloride on day 0 and on day 2 and frozen at −20°C 1 day later after addition of 1 ml glycerin. These plates served as negative controls.

The murine leukemias and human bone marrow required special growth factors to form colonies. P388 and L1210 were incubated additionally with 2-mercaptoethanol (5×10^{-5} mol/l medium), human bone marrow with 0.1 ml of a placenta-conditioned medium which stimulates the growth of human granulopoietic progenitor cells (CFU-C) [18].

Criteria for assays evaluable for drug testing

The methodological pitfalls of the colony assay were previously analyzed by Shoemaker *et al.* [5, 6] who proposed four quality criteria necessary in conducting an evaluable assay. We applied these criteria in 106 *in vitro/in vivo* correlations in testing Adriamycin, Cisplatin, Mitomycin, Vindesin and VP-16 in the colony assay and in nude mice (Fiebig *et al.*, in preparation). The best correlations have been found using the following minor modifications. The mean number of colonies in the control group must be at least 100 for a minimal colony diameter of 60 μm, 50 for an 80 μm dia. Initial plate counts on day 0 or 2 must be smaller than 20% of the final colony count in the control group. A coefficient of variation in the control group of less than 40% is required. The positive reference compound 5-fluorouracil (100 μg/ml, continuous exposure) must effect a colony survival of less than 20% of the controls. Dose–response effects of a drug must be seen. Only experiments fulfilling these criteria (74%) were included in the evaluation of drug testing.

Drugs

TGU (1,2,4-triglycidylurazol, NSC 332488) was obtained from Henkel, Düsseldorf, via Peter Lelieveld, Secretary of the Screening and Pharmacology Group of the EORTC. Tiazofurin (2β-*d*-ribofuranosylthiazol-4-carboxamid, NSC 286193) was obtained from John D. Venditti, National Cancer Institute, U.S.A. Stock solutions of TGU were frozen at −80°C, Tiazofurin at −20°C. Both drugs were dissolved in physiological saline.

Drug treatment in vitro *and* in vivo

In the colony assay TGU and Tiazofurin were applied by continuous exposure until the end of the experiment. Usually three dose levels are studied in triplicate. In each experiment six vehicle-treated cultures were plated for determination of control growth. 5-Fluorouracil (100 μg/ml, continuous exposure) was used as a positive reference compound. A compound was considered active if it reduced colony formation to 30% or less of the control value.

In the treatment of the subcutaneously growing tumors in nude mice TGU was administered at a dose level of 12 mg/kg/day, given days 1–4 and 15–18 i.p. The mortality of tumor-bearing nude mice was 6% (3/50) after 2 weeks and 22% (11/50) after 4 weeks. Tiazofurin was injected i.v. at a dose level of 1200 mg/kg/day days 1–4 and 15–18, resulting in a mortality of 4% (1/28) after 2 weeks and 28% (7/28) after 4 weeks. The LD_{50} in mice after single injection was 55 mg/kg i.p., for TGU and 3.400 mg/kg i.v., for Tiazofurin [9, 10].

Treatment was started after 3–6 weeks when the mean tumor diameter was 8 mm. The product of the tumor diameters was taken as a measure of tumor size. Relative tumor size values were calculated for each single tumor by dividing the tumor size day × by the tumor size day 0 at the time of randomization. The specific growth delay was calculated according to Steel *et al.* [11].

RESULTS

Growth of human tumor xenografts in the clonogenic assay

Of 114 established human tumor xenografts plated, 98 (86%) effected colony growth of at least 30 colonies (Table 1). Most of the tumors were studied in several experiments giving similar results. This high growth rate was observed in all tumor types except soft tissue sarcoma (44%). In some soft tissue sarcomas presenting a pronounced intercellular matrix, it is difficult to obtain a viable single cell

Table 1. Growth of human tumor xenografts in the colony assay

Tumor type	Growth total	
Lung	29/31	94%
Colon	13/14	93%
Stomach	10/13	78%
Melanoma	13/14	93%
Sarcoma	4/9	44%
Kidney	5/6	
Breast	6/6	
Testicle	4/4	
Pleuramesothelioma	3/3	
Ovary	1/2	
Thymoma	2/2	
Head and neck	1/2	
Glioblastoma	1/2	
Thyroid	1/1	
Pancreas	1/1	
Bladder	1/1	
Unknown primary	1/1	
Total	8/114	86%

Growth, minimum of 30 colonies in untreated control plates.

suspension. Approximately 25% of the tumors, particularly adenocarcinomas of the large bowel, stomach and the breast, have a tendency to reaggregate; however, the aggregates are usually smaller than 80 μm. For these tumors we require a minimal colony diameter of 80 μm to render evaluable test assays. Figure 2 presents a typical colony of a small cell cancer of the lung. As observed with fresh tumors from patients the plating efficiency (colony No./seeded cell No.) was low; the median was 0.07% within the range of 0.01–0.40%.

The colony formation vs. time was studied in rapidly and in slowly growing xenografts. For example, the rapidly growing thyroid cancer XF 117 presented some aggregates within the first 2 days with a subsequent rapid colony formation reaching a maximum after 7 days. Afterwards the colonies showed signs of degeneration and disappeared upon exhaustion of the culture medium, accompanied by a colour change of the medium. The slowly growing melanoma MEXF 274 displayed no initial cell aggregates. Maximum colony number was obtained after 21 days with subsequent decrease. These two examples emphasize that growth development must be scored every 2–3 days. Experiments concerning rapidly growing tumors have to be evaluated after 6–8 days, slowly growing tumors usually within 21 days. Further colony formation after 3 weeks was not observed.

Growth of murine tumors and human bone marrow

The five murine tumors used in the NCI screen also formed colonies *in vitro*. The plating efficiency of the two leukemias, the B16 melanoma and the colon carcinoma 38 was markedly higher than that of the human tumor xenografts (Table 2). The Lewis lung carcinoma had a comparable plating efficiency to the human tumors. These differences in plating efficiency reflect the higher proliferation capacity of the experimental murine tumors in comparison to the human tumor xenografts.

Human bone marrow could be cultivated under the same growth conditions except that 0.1 ml of a placenta-conditioned medium had to be added, and the concentration of the agar was increased to 0.4% in the top layer. Under these growth conditions colonies of the granulocytic stem cells (CFUC) can thrive [15]. In 12 experiments the median seeded cell number was 300.000/dish yielding a median colony number of 240. The plating efficiency (0.08%) was similar to that observed in the growth of human tumor xenografts.

Testing procedure for evaluating new compounds

New structures are tested *in vitro* at three dose levels in 20 well-selected and characterized human

Table 2. Growth of murine and human tumors and human bone marrow in the colony assay

Origin	Type	Seeded cells x 1000	Median colony No.	Plating efficiency* (%) Median	Range
Mouse	L1210	2	640	32	(12–56)
	P388	2	240	12	(10–15)
	B16	50	650	1.3	(1.1–1.4)
	Co38	80	624	0.78	(0.11–0.68)
	Lewis lung	500	300	0.06	(0.04–0.08)
Human	Diff. tumors†	200–500	100–800	0.07	(0.01–0.40)
Human	Bone marrow (CFU-C)	300	240	0.08	(0.04–0.12)

*Plating efficiency, colony No./seeded cells.
†From nude mice.

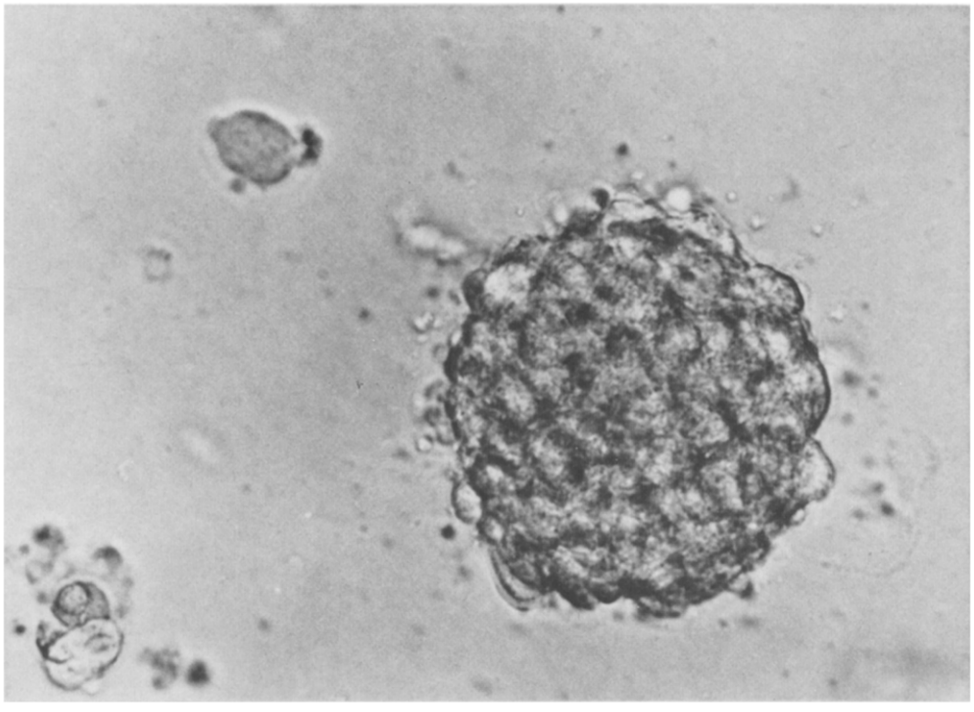

Fig. 2. Morphologic appearance of a small cell lung cancer colony after 12 days in culture.

Table 3. In vitro *effect of TGU in murine tumors, human bone marrow and human tumor xenografts*

Test system	TGU* (μg/ml) 0.1	0.3	1.0	3.0
Murine tumors				
P388, L1210, LL, B16, Co38	1/5	5/5	5/5	3/3
Human bone-marrow (CFU-C)	0/3	2/3	3/3	
Human tumor xenografts				
Lung—non small cell	2/16	5/18	8/18	4/5
—small cell	0/3	0/4	2/3	1/2
Large bowel	0/13	1/13	4/12	1/1
Melanoma	1/7	2/7	2/7	
Pleuramesothelioma	1/2	2/2	2/2	
Stomach	0/7	1/7	1/3	2/2
Breast	0/5	0/5	2/5	
Kidney	0/2	1/2	1/2	
Ovary	0/2	1/2	1/2	
Testes	0/2	1/2	1/2	
Miscellaneous	0/5	0/5	3/5	
Human tumor xenografts	4/64	14/67	27/61	8/10
Sensitive/total	6%	21%	44%	80%

*Continuous drug exposure. Tumors and bone marrow are considered to be responsive if the colony count is ≤ 30% of the control.

tumors growing as xenografts in nude mice as well as in the P388 mouse leukemia. A factor of 10 is chosen for initial dose intervals with reduction to approx. 3 after the first six tumors. Furthermore, the effect on human bone marrow is studied *in vitro*. A new structure is subsequently investigated in the two most responsive tumors in nude mice. If a remission or at least no change is observed the compound undergoes large disease-orientated testing *in vitro* usually in 60 tumor xenografts. For further *in vitro* testing a total of 180 different human tumors have been established and are available in our laboratory.

The described testing procedure has been applied to several drugs. Here we describe the evaluation of TGU and Tiazofurin which showed activity in several murine tumor models *in vivo* and which recently have been examined in clinical studies as well.

Effect of TGU and Tiazofurin

TGU was studied in 67 human tumor xenografts *in vitro* in dosages ranging from 0.1 to 3.0 μg/ml given by continuous exposure (Table 3). The rate of responsive human tumors increased from 6 to 80%. At a dose level of 0.3 μg/ml, 21% of the human tumors were sensitive. At this dose level 2/3 of the human bone marrows were sensitive as well as all five murine tumors (Fig. 3a). *In vivo* only 4/5 of the murine tumors were responsive. The most responsive human tumors were non-small cell cancers of the lung with a response rate of 27% (5/18) and pleuramesotheliomas (2/2). A detailed comparison of the *in vitro* and *in vivo* activity including eight human tumors is shown in Table 4. The activity in the colon cancer xenograft, CXF 280, was additionally observed *in vivo*, where TGU effected a specific growth delay of 7.8 doubling times of the control. Maximal regression was found after 35 days with a relative tumor size of 49% of the initial value. The striking effect in human mesotheliomas *in vitro* was reproducible. However, in subsequent *in vivo* testing the mesothelioma XF 349 grew progressively. This finding suggests that the relevant *in vitro dose* for *in vivo* use will be lower than 0.3 μg/ml, yielding a very low activity.

Tiazofurin was studied in 52 human tumor xenografts over a dose range from 0.1 to 10.0 μg/ml (Table 5). At the likely relevant *in vitro* dose of 1.0 μg/ml, Tiazofurin was effective in 2/52 human tumor xenografts only. At this dose level 4/5 of the human bone marrows were sensitive and 3/5 of the murine tumors responded (Fig. 3b). A detailed comparison of *in vitro* and *in vivo* activity is shown in Table 6. According to our *in vitro* and *in vivo* testing in human tumor xenografts, Tiazofurin will probably have no meaningful therapeutic activity in the clinical setting.

DISCUSSION

Colony assay using human tumor xenografts

Over the past years we have been able to increase the number of tumors producing at least 30 colonies

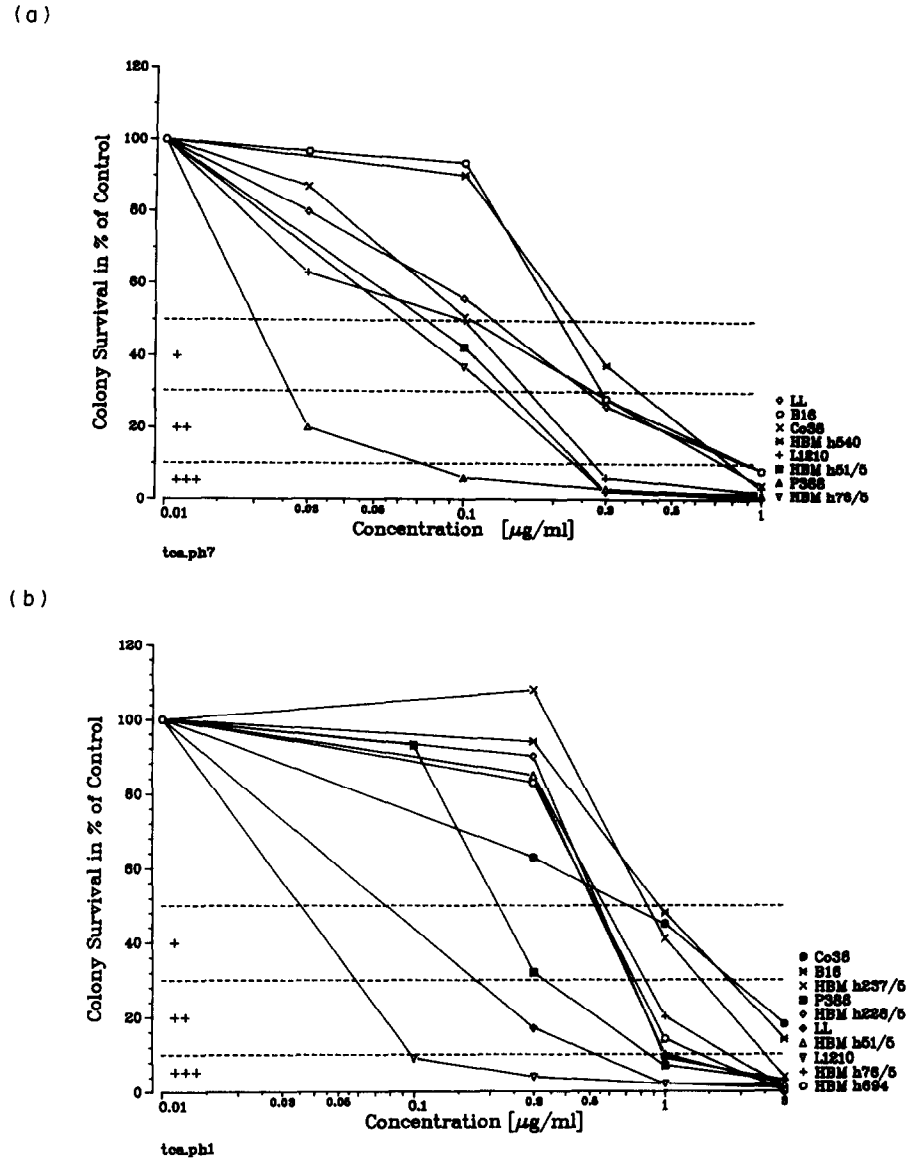

Fig. 3a,b. Colony inhibition after continuous incubation with (a) TGU and (b) Tiazofurin in murine tumors and human bone marrow.

from initially less than 20% to 86%. The same tumor xenografts have been used to study various modifications of the assay. The colony formation has been greater using 30% fetal calf serum (FCS) than 10% from a well-selected batch of FCS. The combined mechanical and enzymatic disaggregation of the solid tumors has yielded a greater amount of viable cells than mechanical preparation only. The most important factor may be that we can carefully choose viable tumor tissue only from the tumor margin. When high numbers of tumor cells are needed, tumors from several nude mice are used for one therapeutic experiment.

Another factor accounting for the remarkable growth rate of xenografts *in vitro* may be that a selection of tumor cells with a high proliferation capacity occurred during the passages in nude mice. Such a process could also influence the response to chemotherapy *in vitro*. However, the high correlation of tumor response in nude mice to that in man [12] suggests that the stem cell population has not changed.

In our preliminary experiments the *in vitro* growth rate of tumors taken directly from patients was about 40%, which is comparable with results from the literature [19].

Problem of the relevant in vitro *dose*

The drug concentrations used *in vitro* were usually derived from concentrations achievable in patients

Table 4. Comparison of in vitro *with* in vivo *activity of TGU in murine tumors and human tumor xenografts*

Tumor system	No. of experiments	Colony assay Control colony No.*	Test/control 0.1 (%)	0.3 μg/ml (%)	Animal experiment	
Murine tumors						
P388	3	820 ± 79	6	3	Active, cures (9)	
L1210	4	635 ± 47	50	7	Active, cures (9)	
B16	4	214 ± 39	94	28	Active, T/C 300% (9)	
Lewis-lung	3	427 ± 57	57	26	Ineffective (9)	
Colon 38	3	491 ± 56	51	29	Active, tumor weight inhibition 82% (9)	
Active/total			3/5	5/5	4/5	
					Relative tumor size day 21	Specific growth delay
Human tumor xenografts†						
CXF 280	3	112 ± 30	51	17	59‡	7.91
PXF 349	2	683 ± 186	65	9	851	−0.09
CXF 158	2	195 ± 22	91	61	277	0.07
GXF 209	1	244 ± 21	97	39	177	−0.03
GXF 218	1	324 ± 62	79	65	264	0.01
GXF 97	1	297 ± 39	79	53	272	0.69
XF 117	1	448 ± 50	84	49	324	0.13
XF 550	1	572 ± 63	86	57	283	0.15
Active/total			0/8	2/8	1/8	

*Data of 1 representative experiment are shown.
†*In vivo* dose was 12 mg/kg, day 1–4, 15–18 i.p.
‡Maximal regression was on day 35, relative tumor size was 49%.
CXF, colon cancer xenograft; PXF, pleuramesothelioma xenograft; GXF, gastric cancer xenograft; XF 117, thyroid cancer xenograft; XF 550, ovarian cancer xenograft.
For *in vitro* activity T/C ≤30% is required.

Table 5. In vitro *effect of Tiazofurin in murine tumors, human bone marrow and human tumor xenografts*

Test system	Tiazofurin* (μg/ml) 0.1	0.3	1.0	3.0	10.0
Murine tumors					
P388, L1210, B16, LL, Co38		2/5	3/5	5/5	
Human bone marrow		0/5	4/5	5/5	
Human tumor xenografts					
Lung—non small cell	0/6	0/14	0/15	4/9	4/5
—small cell	0/1	0/4	0/4	3/4	1/1
Large bowel	0/3	0/8	0/8	3/8	1/1
Melanoma		0/6	1/6	2/6	
Stomach		0/5	0/5	0/1	1/2
Breast	0/3	0/5	0/5	1/4	
Ovary	0/1	0/2	1/2	1/1	
Testes		0/2	0/2	1/2	
Miscellaneous	0/1	0/5	0/5	1/4	
Human tumor xenografts	0/15	0/51	2/52	16/39	7/9
Sensitive/total	0%	0%	4%	41%	78%

*Continuous drug exposure. Tumors and bone-marrow are considered to be responsive if the colony count is ≤ 30% of the control.

Table 6. Comparison of in vitro *with* in vivo *activity of Tiazofurin in murine tumors and human tumor xenografts*

Tumor system	No. of experiments	Colony assay Control Colony No.*	Test control 0.3 (%)	1.0 (%)	3.0 (μg/ml) (%)	Animal experiment	
Murine tumors							
P388	2	820 ± 79	31	7	3	Active, T/C 245% (10)	
L1210	4	635 ± 47	5	2	2	Active, T/C 227% (10)	
B16	3	238 ± 34	94	48	14	Ineffective (10)	
Lewis lung	3	427 ± 57	17	2	2	Active, cures (10)	
Colon 38	2	698 ± 71	63	45	18	Ineffective (10)	
Active/total			2/5	3/5	5/5	3/5	
						Relative tumor size day 21	Specific growth delay
Human tumor xenografts†							
GXF 209	1	244 ± 21	105	99	58	218	0.16
GXF 218	1	324 ± 62	77	65	26	455	0.10
CXF 158	1	197 ± 87	102	64	28	274	0.32
MAXF 449	2	479 ± 82	111	89	60	393	-0.11
XF 117	1	448 ± 50	79	87	56	1154	0.15
Active/total			0/5	0/5	2/5	0/5	

*Data of one representative experiment are shown.
†*In vivo* dose was 1.200 mg/kg, day 1–4, 15–18 i.v.
GXF, gastric cancer xenograft; CXF, colon cancer xenograft; MAXF, mammary cancer xenograft; XF 117, thyroid cancer xenograft.
For *in vitro* activity T/C ≤ 30% is required.

[20, 21]. In general, 10% of the maximal achievable peak plasma level given as 1-hr incubation was administered or 10% of the plasma–concentration–time product. However, the 1 hr incubation of a drug seems to be inadequate for anticancer drugs that are specific for a particular cell cycle [22]. Several drugs such as Vinca-alkaloids, Bleomycin, Hydroxyurea and Ara-C were much more effective when the drugs were incubated continuously in comparison to 1 hr exposure. Since the mode of action for new compounds is usually not known assays for these compounds are performed by continuous exposure.

For all anticancer drugs studied we observed clear dose–response relationships in the colony assay. A critical question is which dose level *in vitro* is relevant for *in vivo* usage. For compounds whose dose-limiting toxicity is bone marrow suppression, the effect on human bone marrow *in vitro* in comparison to the dose active in human tumors allows an assessment of the tumor specific effect of a drug independent of *in vivo* pharmacologic behavior [23]. However, if other tissues *in vivo* are dose-limiting, misleading conclusions can be drawn.

Mouse toxicity data, e.g. the LD_{10}, correspond well with the maximal tolerable doses in man [8]. Therefore, comparison of *in vitro* sensitive tumors with the *in vivo* effects in the same human tumor growing in nude mice seems to be a reliable way to determine the relevant dose *in vitro*. If the *in vivo* effect of a new compound on experimental murine tumors is known, these tumors can also be used. Accordingly, *in vivo* pharmacological behavior of a drug including distribution factors, metabolic activation and inactivation as well as excretion mechanism are included in the final assessment of the relevant *in vitro* dose. Using these criteria, we could demonstrate that TGU has very limited potential in human tumor xenografts. Tiazofurin was inactive.

Potential of the colony assay for drug development

The colony assay may have potential as a secondary screening system for identifying the cytotoxicity of new structures and analogs of known drugs and also for indicating which tumor types are most responsive to a new agent. Therefore, the clinical phase I and II studies should be disease-oriented and focused on the most responsive tumor types. Since human solid tumors with different growth characteristics are included in the early stage of the evaluation of new structures, we hope to identify drugs which could be active particularly in slowly growing human solid tumors. Especially in these tumor types, e.g. cancers of the large bowel, kidney, pancreas, non-small cell of the lung and melanomas,

no substantial progress has been made during the last 10 years [3, 4].

In vitro testing in the previous prescreen, P388, is included in our testing strategy to detect compounds having a higher *in vitro* activity on the more slowly growing human tumor xenografts. Compounds rejected earlier because of *in vivo* inactivity in the P388 with activity in human tumor xenografts should be investigated with special interest.

We use well-characterized human tumor xenografts maintained in nude mice as tumor models. Since early passages are frozen in liquid nitrogen, we revert to the initial frozen tumor at least after eight passages in nude mice. In this way we ensure that no major change in the xenograft tumor has occurred over long time periods. Our experience has shown that the xenograft tumors do not change markedly in their histological appearance, immunohistochemical demonstration of CEA and β-HCG, isoenzyme analysis (esterase-D and LDH) or response to chemotherapy [12–14]. The well known responsiveness to clinically active drugs allows an indication of collateral sensitivity or resistance to a new compound.

In our test procedure approximately 90% of the tests are performed *in vitro* and 10% *in vivo*. The *in vitro* experiments allow the determination of the most sensitive tumors. However, this sensitivity has to be confirmed *in vivo* at a dose tolerable for mice (LD_{10}).

Effect of TGU and Tiazofurin

The detailed *in vitro* and *in vivo* testing of TGU and Tiazofurin yielded the conclusion that TGU had only very limited activity in the 67 human tumor xenografts studied. Tiazofurin was ineffective in 52 xenografts. The relevant dose of TGU is considered to be between 0.1 and 0.3 μg/ml resulting in a response rate of 6% and 21%, respectively. The most responsive tumor types were non-small cell cancer of the lung with a response rate of 13% at 0.1 μg/ml and 28% (5/18) at 0.3 μg/ml. However, the clinical phase II study of TGU (800 mg/m^2 i.v., 4 weeks) showed no partial remission in 16 patients studied [24]. Furthermore, remission rates of 7 and 6% were observed in the phase II study in breast and colorectal cancer [25]. TGU was considered ineffective in phase II studies conducted by the Early Clinical Trial Group of the EORTC in non-small cell cancer of the lung, large bowel, breast cancer and melanomas [26].

Tiazofurin was studied in several phase I and II studies comprising more than 200 patients. In all these studies no antitumor activity was observed. Therefore, our *in vitro* evaluation of TGU and Tiazofurin corresponded well to the negative effects observed in clinical trials. These findings suggest that the proposed testing procedure is able to offer reliable predictions regarding the clinical potential of new compounds.

The apparent disparity between the *in vivo* and *in vitro* activity in murine tumors and the lack of activity in human tumors led to the conclusion that also for TGU and Tiazofurin the murine tumor panel was not able to select drugs with subsequent efficacy in human tumors. The marked differences in growth kinetics are probably responsible for the discrepancy in predictive capabilities of mouse and human tumors.

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