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Clonogenic assay with established human tumour xenografts: correlation of *in vitro* to *in vivo* activity as a basis for anticancer drug discovery

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Abstract

Pluripotent cells can be grown in clonogenic assays. The tumour stem-cell fraction, which accounts for $<0.4\%$ of the total cells, and which is considered the most relevant cell type in the development of metastases and recurrences, is able to divide and to form colonies in a semisolid matrix (agar or methylcellulose). Major applications of the tumour clonogenic assay (TCA) are chemosensitivity testing of tumours and xenografts, and for assessments within drug discovery programmes. Of critical relevance for the usefulness of the TCA is whether it can predict sensitivity or resistance towards clinically used agents. When we compared the response of human tumours established as xenografts in nude mice in the TCA *in vitro* to that of the clinical response, 62% of the comparisons for drug sensitivity, and 92% of the comparisons for drug resistance were correct. The same percentage of true/false observations was found when tumours were tested after serial passage in nude mice in the TCA *in vitro* and their response compared to *in vivo* activity in corresponding xenografts (60% and 90%, respectively). The highest correct predictive values were, however, found when the clinical response of tumours was compared to their explants established in the nude mouse and treated *in vivo*. Of 80 comparisons performed, we observed a correct prediction for tumour resistance in 97% and for tumour sensitivity in 90%. In our opinion, the TCA with established human tumour xenografts has an important role in current drug discovery strategies. We therefore included the TCA as secondary assay in our approach to anticancer drug discovery and found that a number of novel agents were active; these are now in advanced preclinical development or clinical trials. Thus, the tumour clonogenic assay has proven predictive value in the chemosensitivity testing of standard and experimental anticancer drugs.

Keywords: Clonogenic assay; Chemosensitivity; Xenograft; Drug discovery

1. Introduction

Many normal cells show the phenomenon of adherence, i.e. they grow and divide only if attached to a solid inert support, as is provided for example by the glass or plastic surfaces of tissue-culture dishes. The clonogenic assay is a classical way of evaluating colony formation of pluripotent cells with the potential for anchorage-independent growth in semisolid media, e.g. transformed cells or haematopoietic stem cells. Semisolid media reduce cell movement and allow individual cells to develop into clones that are identified as single colonies. The assay is widespread in oncological research where it is used to test the proliferative capacity of cancer cells after radiation and/or treatment with anticancer agents [1–3].

1.1. Resources for the clonogenic assay

Patients' tumours can be studied directly in the clonogenic assay, or after being established as a permanent xenograft in serial passages in nude mice. The xenograft should be characterised for chemosensitivity and for molecular markers relevant to the pathogenesis of a tumour. Clonogenicity is a hallmark of transformed and malignant cell types; thus, permanent human tumour

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cell lines can also be used, but many of them have changed during long-term serial passaging, with the selection of subclones [4–6]. In addition, murine tumours such as the leukaemias P388 and L1210, as well as the solid models B16, Lewis-Lung, Colon 36, Colon 28, and others, grow very well in the clonogenic assay [7].

Haematopoietic stem cells (the normal tissue being clinically dose limiting for about half of all compounds) are obtained from bone marrow, peripheral blood or umbilical cord blood. The effect of novel compounds can be tested against human tumours and human haematopoietic stem cells, allowing evaluation, based on *in vitro* studies only, of whether a new agent is tumour specific and will have a therapeutic index. As a result, large and expensive up-scaling of compound synthesis or refermentation can be avoided at an early stage.

1.2. Clonogenic assay formats

Most investigators use a three-layer technique with a base layer consisting of 0.5–0.8% agar, a second layer containing cells with 0.4% agar and a third layer containing medium or test drugs [2,3,8]. Human haematopoietic stem cells can be grown to form colonies in semisolid media after the addition of placenta-conditioned medium [7,9], or in methylcellulose media supplemented with defined growth factors (e.g. granulocyte-macrophage-colony-stimulating factor, interleukin 3, erythropoietin) [10–12]. Up to 1990, most studies were done in Petri dishes of 35 mm dia. Since the 1990s the use of 24-well cell-culture microplates of 16 mm dia. has been made possible, allowing for miniaturisation and easier handling [13]. Another aspect of miniaturisation was accomplished by using capillaries of 1–1.5 mm dia. into which agar containing stem cells was introduced [14,15]. The capillaries are 1.5 cm long and the number of colonies is usually small, ranging between 3–10 per capillary however with great variability. In our experience, the 24-well microplate is clearly the most reliable format [13].

1.3. Applications of the clonogenic assay

1.3.1. Sensitivity testing in patients

To individualise chemotherapy regimens by preclinically assessing the chemosensitivity of tumours to registered anticancer agents *in vitro* has been a goal of oncological research for many years. The tumour clonogenic assay (TCA), as described by Hamburger and Salmon [1,16], is one of the most intensively studied *in vitro* methods for chemosensitivity testing. Its role in patient sensitivity testing in addition to *in vitro* methods such as the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay [17,18], the histoculture drug-response assay [19–21], the collagen gel droplet-embedded culture drug-sensitivity test [22,23], or the ATP-based tumour chemosensitivity assay [24–26] is well documented [2,27–30]. However, there are no phase III studies demonstrating a significant increase in survival compared to empirically determined standard chemotherapy. Therefore, the TCA has not found a practical established role in the individualisation of patient therapy.

1.3.2. New drug discovery and the development of experimental agents

In another major application, clonogenic assays have been widely used for assessing the efficacy of novel compounds in anticancer drug discovery programmes, such as that of the Institute for Experimental Oncology in Freiburg [7]. Since the assay is labour intensive and automation not as easy to achieve as in experimental set-ups using adherent or suspended cells, the TCA is not useful as a primary screening method but has its credentials as a secondary screen, e.g. for prioritised compounds after cell-based assays with tumour cell lines [31–34]. We test novel lead compounds from primary screenings in the TCA in 24 models. The IC_{70} and IC_{50} in such a tumour panel are then compared with the sensitivity of human haematopoietic stem cells obtained from cord blood or peripheral blood to define a 'therapeutic window'. In addition, the *in vitro* profile is compared to the fingerprint of standard agents in these tumour models and to 35 known, validated molecular targets in our database. The latter comparison will help to define novelty or similarity to known drugs. Once *in vivo* activity is observed, TCA testing is extended to 48 tumours and the resulting *in vitro* IC_{70} profile can be correlated with our cDNA-expression database (based on the Affymetrix HU133A gene chip; 22000 genes/tumour) in order to identify gene clusters that might be essential for drug activity. With this approach, genes important for the activity of novel compounds with novel mechanisms might be discovered. Large studies demonstrating high correlations between the results of the *in vitro* TCA and the patient's response or resistance to established agents have been published [8,35–38]. Secondary screening of experimental agents for anticancer efficacy has also been described as feasible [7,39].

Established tumour xenografts provide a rich source of regrowable human tumour tissue, which can be broadly characterised. In target-directed drug development, we first determine the expression of a target at the RNA and protein level by using our cDNA gene-expression database and tissue microarrays. Between 12 and 24 tumour models that over-express or are deficient for a particular target are then selected, and potential inhibitors tested in the TCA. This procedure allows us to determine rationally the most sensitive tumours, which can subsequently be evaluated for *in vivo* activity.

1.4. Limitations of the clonogenic assay with patients' tumours or cell lines

The application of the TCA in large-scale anticancer drug development has been hampered by the following factors:

1. Tumours resected for diagnostic or therapeutic purposes provide highly relevant material, but tumour specimens originating from patients have growth rates that range between 40–60% only. Tests are not reproducible and further characterisation of the tumours is mostly impossible [40–42].
2. Cell lines are frequently used as a tumour source for drug screening, but such lines show considerable alterations in biological properties and chemosensitivity pattern as compared to the original tumours [4–6].
3. Interpretation of data is sometimes difficult because of a lack of standardisation of experiments and inadequate quality-control measures [41–44].

By introducing quality-control criteria for the minimum colony number per well, positive controls, background control plates and a coefficient of variation in the control groups of $<50\%$, a substantial increase in assay reliability with a very good reproducibility has been achieved [42].

1.5. Study objectives

In this paper, we report our experience with the growth and predictivity of the TCA by performing the following *in vitro*/*in vivo* correlations comparing the response to standard agents in the same tumour, relating these findings to our earlier work and to published material:

1. Patients' tumours established subcutaneously in nude mice studied in the TCA *in vitro* compared with the same tumour treated in the patient.
2. Patients' tumours grown in nude mice studied in the TCA compared with those treated *in vivo* in the nude mouse.
3. A summary of our earlier experiences in comparing the drug response of a tumour treated *in vivo* in the nude mouse with that in the patient.
4. A literature survey of work in which patients' tumours were studied directly in the TCA and compared with the patients' responses.

We also describe here our concept of integrating the TCA into a combined *in vitro*/*in vivo* drug discovery programme and the advanced preclinical development of experimental anticancer drugs.

2. Materials and methods

2.1. Tumours

For direct testing on patients, living tumour tissue from primary tumours or metastatic lesions, resected for diagnostic or therapeutic purposes, was placed in a sterile tube with RPMI 1640 medium supplemented with 20% fetal bovine serum and 0.05% gentamicin. The tissue was processed within 0.5–2 h of resection. For xenograft testing, fresh human tumour specimens were first cut into slices ($5\times5\times0.5$–1 mm dia.) and implanted subcutaneously into nude mice of NMRI genetic background. The animals were maintained under conditions described previously [45,46]. Tumours were either processed after the first passage (6–16 weeks) or after subsequent passages, at which time they were removed under sterile conditions/laminar flow.

2.2. Preparation of single-cell suspensions for clonogenic assay

Xenografted tumours or fresh human tumour specimens were mechanically minced with scissors and scalpels and subsequently incubated with an enzyme cocktail consisting of 41 U/ml collagenase, 125 U/ml DNase, and 100 U/ml hyaluronidase at 37 °C for approximately 45 min. The cells were passed through stainless-steel sieves of 200 μm and 50 μm dia. mesh size and then washed. The percentage of viable cells was determined by trypan blue exclusion using a haemocytometer.

2.3. Culture method for 24-well microplates

A modification of the clonogenic assay as described by Hamburger and Salmon was used [1]. The bottom layer consisted of 0.2 ml/well, Iscove's modified Dulbecco medium supplemented with L-glutamine (Life Technologies), 20% fetal calf serum and 0.75% agar; $1.5\cdot10^4$–$5\cdot10^4$ cells were added to 0.2 ml of the same culture medium containing 0.4% (w/v) agar and plated in 24-multiwell dishes on top of the bottom layer. Test substances were added (drug overlay) in 0.2 ml culture medium under continuous exposure. Every dish included six untreated control wells and drug-treated groups in triplicate. Cultures were incubated at 37 °C under 7.5% CO_2 in a humidified atmosphere for up to 25 days and monitored closely for colony growth using an inverted microscope. Within this period, *in vitro* tumour growth led to the formation of colonies of >50 μm dia. The culture method for experiments in 35 mm Petri dishes has been reported elsewhere [7].

2.4. Assay quantification

At the time of maximum colony formation, vital colonies were stained with a sterile aqueous solution of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (1 mg/ml, 100 μl/well) for 24 h [47]. Colony counts were then done with an automatic image-analysis system (OMNICON 3600; Biosys GmbH).

The following quality-control measures were implemented:

1. For 35 mm Petri dishes the mean number of colonies in the control group >100 with a minimum 60 μm dia.; for 24-well microplates the mean number of colonies in the control group >20 with a minimum 50 μm dia [3,7].
2. Initial counts on day $1<30\%$ of the final colony count (to exclude initial aggregates as false-positive colony signals from evaluation).
3. Coefficient of variation in the control group $<50\%$.
4. Activity of a reference compound (5-fluoruracil) at the toxic dose of 1000 μg/ml must effect a colony survival of $<30\%$ of the controls (positive control).
5. The dose–response effects of the tested drugs must be observed (except complete resistance).

2.5. Human haematopoietic stem cells

Bone-marrow cells were aspirated from the iliac crest of consenting healthy volunteers into preservative-free heparinised syringes. Alternatively, samples of human umbilical cord blood were diluted 2- to 3-fold with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Peripheral blood mononuclear cells were enriched from the respective samples by Ficoll Paque® (Amersham Biosciences) density-gradient centrifugation and washed twice with PBS containing 0.1% BSA. The resulting cell suspension was stored in aliquots in freezing medium (90% fetal bovine serum, 10% dimethyl sulphoxide) in liquid nitrogen and aliquots were thawed and used for testing. The colony-forming test was performed using 24-well cell-culture microplates and MethoCult GF (Stem Cell Technologies) as culture medium; 42,000 cells/ml of the above-mentioned preparation were seeded in a final volume of 300 μl per well. Solutions of the test substances were added directly to the medium. Every dish included six untreated control wells and drug-treated groups in triplicate. Three wells of the test plate were filled with 1 ml of sterile water to ensure that maximum humidity was attained during the subsequent incubation period. Cultures were incubated at 37 °C under 7.5% CO_2 in a humidified atmosphere for 11 days. Colony growth was evaluated by eye using an inverted microscope.

2.6. Anticancer agents

Drugs for chemosensitivity testing were obtained either as a clinical formulation from the pharmacy, or as pure compounds from Sigma. PKI166 was obtained from Novartis (Basel), an aqueous mistletoe extract (AME) was obtained from Madaus AG, Cologne. Chemosensitivity testing was performed against 12 cytotoxic drugs each in two to three concentrations in triplicate. The relevant dose was determined by comparing the sensitivity of drugs *in vivo* (both in nude mice and in patients) and *in vitro* in sensitive tumour types. In addition, approximately three times higher drug concentrations were tested to ascertain the behaviour of the tumour cells at a non-physiological high dose mimicking the high-dose chemotherapy in the clinic. For drugs with steep dose-effect response curves the relevant dosage and usually twice this dose were tested. Drugs and regimens used are shown for an exemplary patient in Table 8(a), which lists the 12 cytotoxic drugs and their relevant doses employed for chemosensitivity testing. When ample tumour material was available, the tumour cells were also examined for radiosensitivity by exposure at between 1.5 Gy and 10 Gy. A drug was considered effective when colony formation in the therapy plates was less than 30% of the control plates ($T/C \leqslant 30\%$).

2.7. Correlations between clonogenic assays and patients' responses

A tumour was defined as sensitive *in vitro* to a cytotoxic agent if colony formation was reduced to less than 30% of the control value. The *in vivo* reaction of a patient's tumour to chemotherapy was evaluated by the attending oncologist without knowledge of the *in vitro* testing. A complete response was defined as the disappearance of all tumour manifestations for at least one month. A partial response required at least a 50% decrease in measurable tumour area ($a \times b$), and no change, a less than 50% decrease or stabilisation under therapy. Progression was defined as a more than 25% increase in measurable tumour area.

For the correlation of *in vitro*/*in vivo* results, the *in vivo* response in the nude mouse or in the patient was reduced to dichotomy. Only complete and partial remissions were scored as *in vivo* sensitivity. No change and progression were considered to be *in vivo* resistance.

Clinical correlations were possible if the patient received chemotherapeutic agents that were also tested *in vitro*. Since most patients received combination chemotherapies for the treatment of their solid tumours, further clarification is required to explain how *in vivo*/*in vitro* correlations were deduced. Patients achieving clinical responses when treated with two or more drugs that were active *in vitro* were considered to have responded

only to the most active agent in the clonogenic assay. Thus, only one true-positive correlation was recorded in such instances. Conversely, for patients showing clinical tumour resistance while treated with multiple agents, true-negative correlations were established for all corresponding drugs that were inactive *in vitro*. Patients whose tumours progressed clinically while receiving combination chemotherapy, but in whom one or more drugs were active *in vitro*, were considered to have true-negative correlations with the inactive drugs but a false-positive correlation with the drugs that had *in vitro* activity. This method complies with that used by Bertelsen [16] for the analysis of 258 *in vitro*/*in vivo* correlations.

For comparison, the results were analysed with only one correlation for clinically resistant tumours. In this case, only the results for the least active compound were considered; further compounds tested showing lower T/C values were not evaluated.

2.8. Correlation of tumour response in the nude mouse and the patient

Tumour slices averaging 5×5×0.5−1 mm dia from the patient, or 3×3×0.5−1 mm dia in serial passage were implanted subcutaneously into both flanks of the animals. Testing was done in serial passages when tumour growth became regular. For comparing the drug response in the nude mouse and in the patient, testing was done between passages 2 and 10. Treatment was started after 3–6 weeks when the mean tumour diameters were about 5–6 mm, equal to 100 mm^3 or 100 mg. Before the start of treatment, tumour-bearing animals were stratified into treatment and vehicle-control groups according to tumour volume. Each group consisted of 5–6 mice bearing 6–10 evaluable tumours. Drugs to be tested were administered intraperitoneally or intravenously at the maximum tolerated dose as defined by the LD_{10} (14 days after start of treatment with one cycle) or LD_{20} (28 days after start of treatment with two cycles). The treatment regimen corresponded to clinical schedules for single-agent or combination therapy, with the exception that therapy in mice was usually repeated after 2 weeks and two cycles were given (e.g. cisplatin, cyclophosphamide, dacabazine, doxorubicin, etoposide, ifosfamide, mitomycin C), and compounds such as vincristine, vindesine and 5-fluorouracil were administered weekly for 3 weeks [13,48–51]. Remissions were observed in the nude mouse only with two therapy cycles or weekly therapy for 3 weeks. The median relative tumour volume (tumour volume on day X (V_x) divided by the tumour volume on day 0 (V_0) multiplied by 100%) of the respective group was used for drawing growth curves and for treatment evaluation [42].

In combination chemotherapy, drugs were administered at 15-min intervals by different routes to avoid interactions. In two-drug combinations only 70–80% of the dose of the single-agent therapy was given and accordingly only 50–60% in three-drug combinations.

For comparing tumour response in nude mice and in the patient the product of the two diameters was taken as a measure of tumour size. Tumors in nude mice were evaluated after maximum tumour regression or after 3–4 weeks in non-regressing tumours. The effect of treatment was classified in the xenograft system and in the patient as remission (the product of two diameters; $<50\%$ of initial value), minimal regression (51–75%), no change (76–124%), and progression ($>125\%$) of initial value. All patients had measurable lesions; evaluation was usually performed after two treatment cycles or after maximal tumour regression. Different physicians made the evaluation of tumour response in nude mice and in patients.

3. Results

3.1. Biological properties of human tumours grown in the TCA

The properties of both normal haematopoietic and neoplastic cell populations are consistent with a model in which cells with proliferative potential can carry out a limited number of potential divisions or have the capacity to renew the entire cell population, including themselves. These self-renewing and population-renewing cells, which may constitute only a small proportion of the total population, are known as stem cells. Tumour stem cells are the relevant cell population responsible for the development of metastases and late recurrences, and are therefore the primary target for any cytotoxic cancer therapy. The validity of the stem-cell model for human cancer was reported about 20 years ago [52]. Tumours generally used in the TCA are grown as a solid, established tumour xenograft model in immune-deficient mice or are derived directly from patients' cancers. The heterogeneity of solid donor tumours growing subcutaneously in nude mice is very well maintained [51]. Only tumour stem cells, which ensure the self-renewal of normal and malignant tissues, can divide in the agar matrix and form colonies (Table 1). Stromal cells, lymphocytes and differentiated tumour cells are not able to grow. The formation of colonies/spheroids occurs in several layers, and therefore drugs must be able to penetrate over a distance to reach the partly hypoxic centre and achieve complete cessation of growth [7,53,54].

The cell growth occurs in a logarithmic manner (Table 2). One stem cell divides and forms colonies normally containing between 64 and 256 cells as a results of six to eight population doublings. For a presumed doubling time of 24 h, six doublings are reached after 6 days and eight doublings after 8 days. For a

Table 1
Biological properties of human tumours growing in the clonogenic assay

- Only tumour stem cells grow and form colonies
- Differentiated tumour cells, stroma cells and lymphocytes do not grow
- Growth occurs within in the colony's multilayers
- Anchorage independent, three-dimensional growth in a semisolid matrix
- Colonies reach diameters of 50–300 μm
- Initial stem cells divide about 6–8-fold, colonies of 50 μm (>300 μm) diameter contain approx. 64 cells (256 cells)
- Colony growth evaluated after 7–21 days (median 12 days)
- Drugs can be added for 1 h or continously (present over 6–8 cell doublings)
- Colonies are counted by an image analysis system or by eye (very cumbersome)

presumed doubling time of 48 h, six population doublings of a colony are reached after 12 days and eight after 16 days. The latter represents the average time span at which we evaluate and count experiments. Colonies of fast-growing tumours are normally counted after 6–8 days and from slowly growing tumours after 14 up to 21 days. The median incubation time in our hands is 12 days.

3.2. Growth and plating efficiency

The growth of human tumours in the TCA originating directly from patients or from serial passage in nude mice is different (Table 3). The growth rate of primary tumour specimens from the patient was 40% (14 of 35) in our series conducted from 1988 to 1989 and increased to 79% during the years 2001–2003. With today's growth rates of patients' tumours of 70–80% (Table 3), standard agents can be evaluated with reliability and in a period of time that allows the patient to be treated with the most active single agent or combination, normally in second- or third-line therapy.

Tumour specimens can be established as xenografts in immune-deficient mice in serial passage in approximately 25–65% of all cases and for most tumour types except prostate and mammary cancers [13,50,51]. The growth rate of human tumour xenografts in the TCA was 86% (211/251) between 1988 and 1989 in our facility, and was in the same range in the period from 1996 to 2003 (87%) (Table 3). The growth rates of different tumour types in the TCA, together with the median plating efficiency (number of colonies counted related to the number of vital cells plated day 0), are shown in Tables 4 and 5a.

The median plating efficiency for testing different xenograft-derived tumours was 0.37% for the series carried out from 1996 to 2003, and 0.07% in the period from 1988 to 1989, also reflecting the progress in tissue handling and culture conditions (Tables 4, 5a). These data still show that the stem-cell fraction is very small but this is the relevant cell population for recurrences and metastases. Eradicating the tumour stem cells will have the highest positive impact on prognosis. The plating efficiency in human tumour models was similar to that of haematopoeitic stem cells (0.07% versus 0.08% in 1988–1990; 0.37 versus 0.6 in 2002–2003). In contrast the plating efficiency of the transplantable murine leukaemias L1210 and P388 was markedly higher (32% and 12%), and also the solid murine models B16 and Colon 38 showed a more than 10-fold higher plating efficiency than human tumours and human bone marrow in the direct comparison carried out between 1988 and 1990 (Table 4). These differences in plating efficiency clearly demonstrate that the murine

Table 2
Growth simulation in the clonogenic assay for cell doubling times of 24 and 48 h

Doubling time 24 h	day	0	1	2	3	4	5	6	**7**	8	9
Doubling time 48 h	day	0	2	4	6	8	10	12	**14**	16	18
Cell doublings	fold	0	1	2	3	4	5	6	7	8	9
Cell number per colony		1	2	4	8	16	32	64	128	256	512
Colony diameter	μm	~10–15 μm*					~50 μm**		~300 μm***		

*Day 0 single cells with diameters ~10–15 μm are seeded. **Day 6 small colonies of at least 50 μm diameter. ***Large colonies up to 300 μm diameter. Optimal day for colony counting marked in bold.

Table 3
Growth of tumours in the clonogenic assay *in vitro* dependent on tumour origin

Tumour origin	Growth/total no.		%	Cytotoxicity testing (%)
Patient tumours 1989–1989*	14	35	40	31
Patient tumours 2001–2003**	30	38	79	71
Serial passage in nude mice 1988–1989	211	251	86	69
Serial passage in nude mice 1996–2003	148	171	87	92

*>100 Colonies in 35 mm culture dish. **>20 Colonies/well in 24 well cell culture microplates.

Table 4
Growth of human and murine tumours as well as bone marrow in the clonogenic assay *in vitro*

Origin	Designation	Cells seeded ($\times 10^3$)/ml	Median colony number*	Median PE** [%]
Human	Different tumours*** 1996–2003	80–600	112–423	0.37
	Different tumours*** 1988–1990	200–500	140–350	0.07
Human	haematopoietic stem cells 2002–2003	42	77	0.6
	haematopoietic stem cells 1988–1990	300	240	0.08
Mouse (1988–90)	L1210 leukaemia	2	640	32.0
	P388 leukaemia	2	240	12.0
	B16 melanoma	50	650	1.3
	Co38 colon carcinoma	80	624	0.78
	Lewis-lung carcinoma	500	300	0.06

*Range per year; 1988–1990 cultivated in 35 mm dishes, 1996–2003 cultivated in 16 mm dishes (24 well cell culture microplates). **Plating efficiency, number of colonies/number of vital cells plated * 100 (%). ***Derived from xenografts cultivated on nude mice; more than 250 tumours included.

Table 5a
Growth of human tumours derived from nude mouse xenografts in the clonogenic assay according to tumour type, data from 1988–1989 and 1996–2003

Tumour type	Period 1988–1989			1996–2003		
	Growth*/total		Median PE***	Growth**/total		Median PE
	u	rate (%)	(%)	*n*	rate (%)	(%)
Breast	6/7	86	0.06	13/16	81	0.26
Colorectal	35/39	90	0.09	18/19	95	0.26
Kidney	10/10	100	0.09	11/11	100	0.54
Lung	78/84	93	0.09	27/29	93	0.65
Melanoma	23/28	82	0.05	13/17	76	0.44
Miscellaneous	30/37	81	0.09	33/40	83	0.39
Ovary	4/4	100	0.09	9/9	100	0.28
Pancreas	–	–	–	4/4	100	0.26
Prostate	–	–	–	7/8	88	0.49
Sarcoma	9/16	56	0.02	8/11	73	0.32
Stomach	10/13	78	0.04	3/3	100	0.47
Testis	6/7	86	0.05	2/4	50	0.075
Total	211/245	86	0.07	148/171	87	0.37

*(**) > 100 (20) colonies per 35 mm (16 mm) culture dish for a 60 μm (50 μm) colony diameter. ***Plating efficiency, number of colonies/number of vital cells plated*100 (%).

solid tumours and leukaemias have much higher growth kinetics than the human solid tumours or haematopoietic stem cells. Growth rates of between 78% and 100% were observed in the period from 1988 to 1989 for all solid tumours except sarcomas (56%), which in many cases are very difficult to dissociate in order to obtain adequate single-cell suspensions (Table 5a). For the series done between 1996 and 2003, the growth rate ranged mainly between 73% and 100%, with the exception of tumour models of testis, where only four different models were available for testing and two could be successfully tested (Table 5a). Between 2001 and 2003, 37 samples of tumour tissue directly derived from the patient, representing nine different tumour types, were processed for testing in the TCA, and in 27 experiments growth occurred (Table 5b). Thus testing could be successfully carried out in 73% of the cases (Table 5b).

Table 5b
Direct cytotoxicity testing of patient tumours in the clonogenic assay, period 2001–2003, according to tumour type

Tumour type	Cytotoxicity testing*/total		Median PE**
	n	rate (%)	(%)
Breast	3/3	100	0.54
Colorectal	4/4	100	0.59
Kidney	1/2	50	0.18
Lung	0/2	0	
Melanoma	3/3	100	0.14
Miscellaneous	4/7	57	0.39
Ovary	2/4	50	0.20
Pleuramesothelioma	7/7	100	0.27
Sarcoma	3/5	60	0.22
Total	27/37	73	0.29

* > 20 colonies/well for a 50 μm colony diameter in 24 well cell culture microplates. **Plating efficiency, number of colonies/number of vital cells plated* 100 (%).

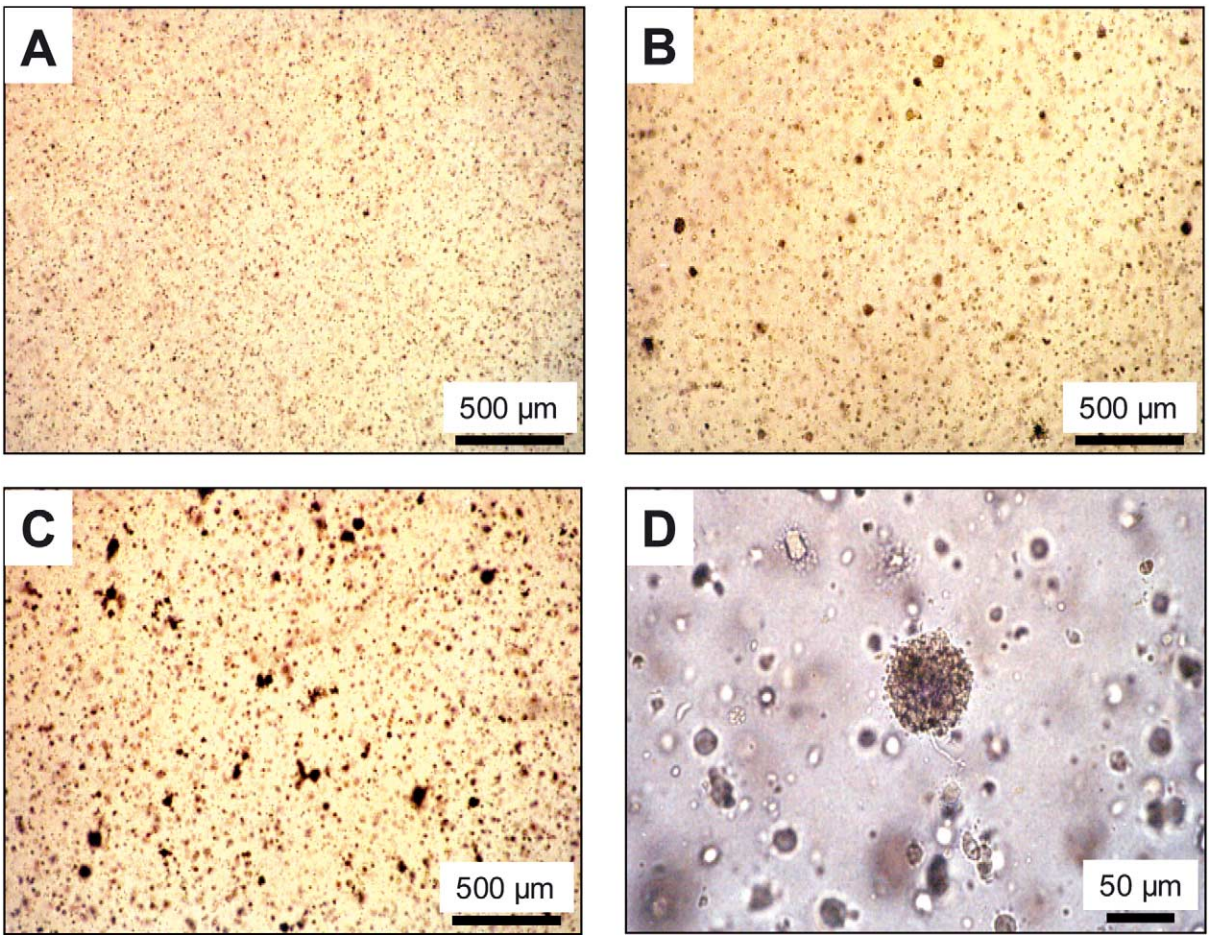

Fig. 1. Growth of the human melanoma model MEXF 384 in the clonogenic assay. Untreated control on day 1 (A), day 10 (B), day 15 (C) (8× magnification), on day 15 (D) (50× magnification).

The development of colonies from a single-cell suspension is shown in Fig. 1. The melanoma MEXF 384 was seeded as a homogeneous single-cell suspension on day 0. After 10 days, colony growth was observed and this increased until day 15, resulting into round colonies (Fig. 1c,d). The time course of colony formation was measured for representative tumour models. Fig. 2 shows as an example the melanoma MEXF 276. Colony growth began after 5 days, and increased logarithmically until day 14; after day 19 no further colony formation was observed. The optimal time to evaluate drug effects in this case was between days 12 and 14. After day 20 the growth medium was exhausted, the colonies became apoptotic, and vital colonies therefore decreased in number.

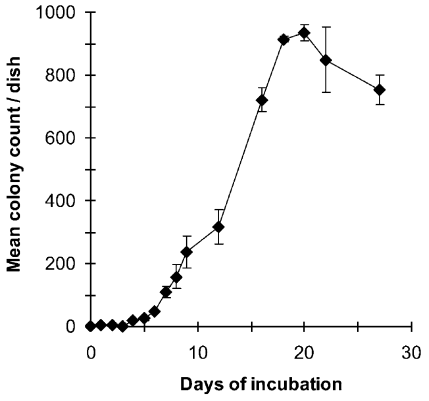

Fig. 2. Time course of colony formation in melanoma MEXF 276.

3.3. Drug testing

We have established a standard set of 12 drugs, which are studied at two to three dose levels in order to identify compounds that can be recommended for second- or third-line therapy in the clinic. The standard agents were initially studied in the TCA to determine which dose level corresponded to an activity of 15–35% (Tables 6, 7). For Adriamycin and gemcitabine the concentration of 0.01 µg/ml was active in 15% and 23% of the tumours, for cisplatin and etoposide 0.1 µg/ml yielded an activity in 16% and 17%, respectively. In Table 7 the activity is divided into tumour types responsive in the clinic and tumour types resistant against the individual agent. Overall, clinically responsive

Table 6
Antitumour efficacy of standard agents in human tumour xenografts and patient primary tumours in the clonogenic assay

	Drug concentration* (μg/ml)				
Adriamycin (ADR)	0.003 4/113** 4%	0.01 40/261 15%	0.03 113/261 43%	0.1 84/152 55%	1.0 72/76 95%
Cisplatin (CDDP)	0.01 3/44 7%	0.03 4/140 3%	0.1 40/245 16%	0.3 90/209 43%	1.0 66/107 62%
Gemcitabine (GEM)	0.001 0/87 0%	0.01 21/91 23%	0.1 34/93 37%	1.0 34/94 37%	10.0 42/83 51%
Etoposide (VP-16)	0.03 6/96 6%	0.1 33/190 17%	0.3 88/224 39%	1.0 66/121 55%	3.0 68/98 69%
Vindesine (VDS)	0.001 16/72 22%	0.003 51/153 33%	0.01 111/246 45%	0.03 128/190 67%	0.1 80/104 77%

*Continuous exposure. **Sensitive tumours (Test/Control $<30\%$)/total.

tumours were sensitive in the TCA in 41% (99 out of 240 different testings). In contrast, clinically resistant tumour types were sensitive only in 11% (19 out of 176). This clearly demonstrates that the TCA is able to differentiate between sensitive and resistant tumour types. The dose levels were selected in order to not to miss an active compound at the expense of having more false-positives.

Representative samples of chemo- and radiosensitivity testing are shown in Tables 8 (a,b). The colon cancer CXF 886 was investigated as a xenograft from the first nude mouse passage. Twelve compounds were investigated in the standard dose and a 3-fold increment, together with radiotherapy ranging from 1.5 to 10 Gy. This colon cancer responded in a typical way in the sense that all 12 compounds were completely inactive at the standard dose and even at the 3-fold higher dose. In addition, the cancer was resistant to radiotherapy (Table 8a). Such a resistance pattern was observed in 12 of 15 colorectal cancers studied, reflecting well the clinical situation (data not shown). However, the newer compounds such as irinotecan and oxaliplatin were not included in the present series.

Table 7
Antitumour efficacy of standard drugs in human tumour xenografts in the clonogenic assay *in vitro*

Drug	Dose*	Responsive tumour types[a]		Resistant tumour types[b]	
	(μg/ml)	active**/total		active**/total	
Adriamycin (ADR)	0.01	21/63	33%	4/45	9%
Cisplatin	0.1	27/74	36%	3/26	12%
Etoposide	0.1	8/10	80%	5/44	11%
Mitomycin	0.005	16/36	44%	5/36	14%
Vindesine	0.01	27/57	47%	2/25	8%
Total		99/240	41%	19/176	11%

*Continuous exposure. **test/control $<30\%$.
[a] Responsive tumour types in clinical studies, e.g. for ADR breast, lung (SCLC and NSCLC), ovary, sarcoma, stomach, testicular.
[b] Resistant tumour types in clinical studies, e.g. for ADR central nervous system, colon, head and neck, kidney, melanoma, oesophagus, pancreas.

Table 8a
Chemo- and radiosensitivity testing in the colon carcinoma CXF 886/1

Drug (abbreviation)	Standard dose [μg/ml]	Activity at standard dose*	Activity at 3-fold standard dose*
Adriamycin (ADR)	0.01	–	–
Bleomycin (BLM)	0.06	–	–
Cisplatin (CDDP)	0.1	–	–
Cyclophosphamide (CTX), active metabolite	0.3	–	–
Dacarbazine (DTIC)	30.0	–	–
5-Fluorouracil (5-FU)	0.2	–	–
HECNU	6.0	–	–
Ifosfamide (IFO), active metabolite	0.3	–	–
Mitomycin (MMC)	0.005	–	–
VP-16 (etoposide)	0.1	–	–
Vinblastine (VLB)	0.003	–	–
Vindesine (VDS)	0.01	–	–
+ +, + + +/total		0/12	0/12
Radiotherapy:	1.5 + 2.5 GY	–	–
	4 + 10 GY	–	–

*Colony count of test groups (T/C), – T/C > 50%.

Table 8b
Chemo- and radiosensitivity testing in the sensitive lung carcinoma LXFE 883/2

Drug	Standard dose [μg/ml]	Activity at standard dose*	Activity at 3-fold standard dose
Adriamycin	0.01	+ +	+ + +
Bleomycin	0.06	+	+
Cisplatin	0.1	+ +	+ + +
Cyclophosphamide, active metabolite	0.3	+ +	+ + +
Dacarbazine	30.0	+	+ +
5-Fluorouracil	0.2	+ +	+ + +
HECNU	6.0	+ +	+ +
Ifosfamide, active metabolite	0.3	+ +	+ +
Mitomycin	0.005	+	+ +
VP-16 (etoposide)	0.1	–	–
Vinblastine	0.003	–	+ + +
Vindesine	0.01	–	+ + +
+ +, + + +/total		6/12	10/12
Radiotherapy:	2 + 5 GY	+	+ +

*For standard doses see Table 8a. –, T/C > 50%; +, 30% < T/C < 50%; + +, 10% < T/C < 30%; + + +, T/C < 10%.

On the other hand, the epidermoid lung cancer LXFE 883 studied as a xenograft derived from the second nude mouse passage responded very well to six out of 12 standard agents at the standard dose, and to 10 out of 12 compounds at 3× standard dose. Moreover, radiotherapy was active at 2 Gy and very active at 5 Gy (Table 8b). Among 22 non-small cell lung cancers (NSCLC) investigated, 10 responded to four to 10 standard drugs studied, six were completely resistant to all agents examined, whereas six were sensitive to one to three agents (data not shown). This demonstrates that NSCLC are more sensitive than for example colon or kidney cancers or melanomas.

3.4. Clonogenic assay using human haematopoietic stem cells

Comparisons of the *in vitro* activity of compounds against human tumour stem cells with that against human haematopoietic stem cells is very helpful in determining tumour-selective activity. We have already reported our first experiences [7]. As an example, the effect of Adriamycin on different tumours and haematopoietic stem cells from five donors as determined in the clonogenic assay is shown in Table 9. At 0.01 μg/ml, Adriamycin was active (T/C < 30%) in 40/261 (15%) tumour preparations tested. At the same concentration, no effect was observed against the haematopoietic stem cells. At 0.1 μg/ml, inhibition of colony formation was observed in preparations of both haematopoietic stem cells and tumour cells in 60% and 55% of the cases, respectively (Table 9). The mean IC_{70} for all tumours was about 0.03 μg/ml. The mean IC_{70} for bone marrow was 0.2 μg/ml, whereas 20% of the very sensitive tumours had mean IC_{70} smaller than 0.02 μg/ml. The results confirmed the known effect of Adriamycin on haematopoiesis.

Another example is decitabine, which today is registered for the treatment of myelodysplastic syndrome (MDS). This compound showed selective activity

Table 9
Effect of doxorubicin on colony formation of hematopoietic stem cells compared to human tumours

Haematopoietic stem cells	Colony no. control	Test/Control (%) at Doxorubicin concentration [μg/ml]				
		0.001	0.01	0.1	1.0	10.0
BM1	50	66 –	34 +	10 + +		
BM2	63	101 –	67 –	26 + +		
BM3	40	76 –	60 –	0 + + +	0 + + +	0 + + +
BM4	152	102 –	70 –	0 + + +	0 + + +	0 + + +
Cord blood	50	77 –	80 –	1 + + +	0 + + +	0 + + +
Active*/total		0/3 0%	0/5 0%	3/5 60%	5/5 100%	3/3 100%
Different human tumours**			40/261 15%	84/152 55%	72/76 95%	

– (T/C > 50%), + (30% < T/C < 50%), + + (10% < T/C < 30%), + + + (T/C < 10%). *T/C < 30%. **Grown as xenografts on nude mice.

against haematopoietic stem cells derived from bone marrow of three healthy donors. The mean IC_{70} of bone marrow was 10-fold lower than for tumours tested in the clonogenic assay; thus human bone marrow was 10 times more sensitive than the most sensitive tumours, suggesting that leukaemias also would be sensitive. This finding was later confirmed in clinical studies. The compound was inactive in eight solid tumours, but activity was seen in acute myelocytic leukaemia and mainly in MDS. Therefore, the stem-cell toxicity approach is very useful in depicting a tumour-specific effect in *in vitro* studies at least for compounds for which haematotoxicity is the dose limiting side-effect.

3.5. *Correlation of* in vitro *drug responses in the clonogenic assay with* in vivo *behaviour in the patient or nude mouse*

Each assay system requires validation for drug testing. The comparison of drug response in the respective test system with the response of the same tumour in the patient is essential. One of the most relevant TCA vs. patient comparisons in the literature compiles data from six series in a total of 2300 cases [26,55]. It reports that of 738 tumours that were sensitive in the clonogenic assay, 512 showed clinical remission with the same treatment. Therefore the positive predicted value was 69%. In contrast, of 1562 tumours predicted as resistant in the clonogenic assay, 1427 were found to be resistant in the clinic. The positive predictive value for tumour resistance in this study was therefore 91% (Table 10). Doses were selected to accept false-positive rather than false-negative results.

Over the past two decades we have also carried out comparisons of drug responses in a systematic way and from several different perspectives in our laboratory. A comparison of response in the TCA from tumours established in nude mice with the patients' responses in the clinic was made in 66 cases. The TCA predicted sensitivity in 29 cases, and the same tumours responded to the same treatment in the patient in 18 cases. Therefore, the correct prediction for tumour sensitivity was 62%. Resistance was observed in the clonogenic assay in 37 cases. The respective finding was obtained in the patient in 34 cases. The correct prediction for tumour resistance was 92% (Table 11). It appears that the initial establishment of the patient tumour as a xenograft in the nude mouse did not influence drug sensitivity when compared with direct testing of the patient's tissues. A relation between the percentage decrease in colony number and the degree of *in vivo* response could be demonstrated (Table 12). Patients who went into complete remission showed the highest average inhibition of colony formation in the clonogenic assay (T/C 10%). The degree of inhibition of colony formation paralleled the clinical behaviour of the tumours *in vivo*. Tumours of patients showing progressive disease gave the lowest average T/C (54%). Details of this study have been published elsewhere [3].

An evaluation of tumour response in the TCA *in vitro* versus response *in vivo* in the nude mouse xenograft was carried out for a total of 108 comparisons. Sensitivity was seen in the clonogenic assay in 40 cases, whilst the same result was observed in the corresponding nude mouse xenograft in 24 cases, equalling a correct positive-predictive value of 60%. Resistance was found in

Table 10
Summary of correlation data from the literature (n = 2300): Clonogenic assay (*in vitro*) versus patient (*in vivo*) [36,55]

Patient	Clonogenic assay	Number	%	
Remission	sensitive	512	22	TP
No remission	sensitive	226	10	FP
No remission	resistant	1427	62	TN
Remission	resistant	135	4	FN
Correct prediction of the clonogenic assay for				
Tumour sensitivity (TP/(TP + FP))		512/738	69%	
Tumour resistance (TN/(TN + FN))		1427/1562	91%	

TP, true positive (patients who are sensitive in vitro and respond to therapy), TN, true negative (patients who are resistant in vitro and do not respond to therapy), FP, false positive (patients who are sensitive in vitro but resistant clinically), FN, false negative (patients who are resistant in vitro but respond clinically).

Table 11
Clonogenic assay (*in vitro*) versus patient (*in vivo*) comparison (*n* = 66) [3]

Patient	Clonogenic assay	Number	%	
Remission	sensitive	18	27	TP
No remission	sensitive	11	17	FP
No remission	resistant	34	52	TN
Remission	resistant	3	4	FN
Correct prediction of the clonogenic assay for				
Tumour sensitivity (TP/(TP + FP))		18/29	62%	
Tumour resistance (TN/(TN + FN))		34/37	92%	

TP, true positive; TN, true negative; FN, false negative; FP, false positive. Data taken from [3].

Table 12
Antitumour efficacy in the clinic (patient) versus inhibition of colony formation (clonogenic assay)

In vivo response in the patient		Inhibition of colony formation *in vitro*	
	n	mean T/C*	T/C range
Complete remission	4	10%	0–34%
Partial remission	14	19%	1–84%
No change	4	31%	5–55%
Progression	37	54%	3–100%

*T/C, colony count of most effective treatment/control group.

Table 13
Clonogenic assay (*in vitro*) versus nude mouse xenograft (*in vivo*) correlations ($n = 108$)

Nude mouse xenograft	Clonogenic assay	Number	%	
Remission	sensitive	24	22	TP
No remission	sensitive	16	15	FP
No remission	resistant	61	56	TN
Remission	resistant	7	7	FN
Correct predicition of the clonogenic assay for				
Tumour sensitivity (TP/(TP + FP))		24/40	60%	
Tumour resistance (TN/(TN + FN))		61/68	90%	

TP, true positive; TN, true negative; FN, false negative; FP, false positive. Data taken from [42]

the TCA in 68 cases, and identical results were seen *in vivo* in the same tumour in 61 cases. Hence, the correct prediction for tumour resistance was 90% (Table 13). Details of this study have been published earlier [42].

Finally, the comparison of the tumour response found *in vivo* in nude mouse xenografts with the patient's response in the clinic was most relevant. We performed 80 comparisons in 55 different tumours; latter number indicates that one-third of the tumours were evaluated for first- as well as second-line therapy, mainly in breast and small-cell lung cancer. 45 of the comparisons were done with combination chemotherapy, mainly in breast, lung, ovarian, and testicular cancer, as compared to single-agent therapy in 35 cases, mainly in colorectal cancers. A remission was obtained in 21 cases in the patient, whereas the same result was observed in the nude mouse system in 19 cases. Therefore, the correct predictivity of the test system for tumour sensitivity was 90%. In contrast, a progression or initial no change was found in 59 patients and the same result in the nude mouse occurred in 57 cases. Here, the correct prediction for tumour resistance was 97% (Table 14). In particular, the high correct predictivity for tumour sensitivity in this study is noteworthy and validates *in vivo* testing as the most reliable and predictive model of response to conventional standard agents. Details of the study have been reported elsewhere [13,48,51]. Additional experiences in other laboratories gave a similar correct prediction. Data are summarised in [56].

Table 14
Comparison ($n = 80$) of nude mouse xenograft (*in vivo*) versus patient (*in vivo*) data

Nude mouse xenograft	Patient	Total	%	
Remission	remission	19	24	TP
No remission	remission	2	3	FP
No remission	no remission	57	71	TN
Remission	no remission	2	3	FN
Correct predicition of the nude mouse assay for				
Tumour sensitivity (TP/(TP + FP))		19/21	90%	
Tumour resistance (TN/(TN + FN))		57/59	97%	

TP, true positive; TN, true negative; FN, false negative; FP, false positive. Data taken form [48].

A summary of the above-described comparisons is shown in Fig. 3. Clearly, the best correct predictivity was seen when the same tumour was treated *in vivo* grown as a nude mouse xenograft and compared to the patient response. Data from the literature as well as our own *in vitro*/*in vivo* comparisons show a correct positive predictive value for resistance of between 90 and 92%, whilst that for tumour sensitivity ranged from 60 to 69% (Fig. 3). A caveat must be added here, as the analysed comparisons rely on the use of standard anticancer therapies that either target DNA and tubulin directly or act as inhibitors of topoisomerase I and II, or as antimetabolites. Whether the same holds true for the new generation of molecularly targeted therapies remains to be seen.

3.6. The clonogenic assay for molecular target-oriented drug discovery

In our group, the major application of the clonogenic assay is in the contemporary anticancer drug discovery programme. When we compared chemosensitivity test results for primary tissue in the TCA derived directly from the patient with those for the corresponding tumour xenograft after up to four *in vivo* passages on nude mice, 22 of 25 comparisons resulted in an identical outcome (Table 15). This finding shows that the chemosensitivity characteristics of tumour xenografts adequately resemble those of the original tumour in the patient, which also holds true for the histological appearance of the xenografts. Although stromal elements and the blood supply are delivered from the murine host, the architecture and morphology of the xenografts closely resemble those of the original

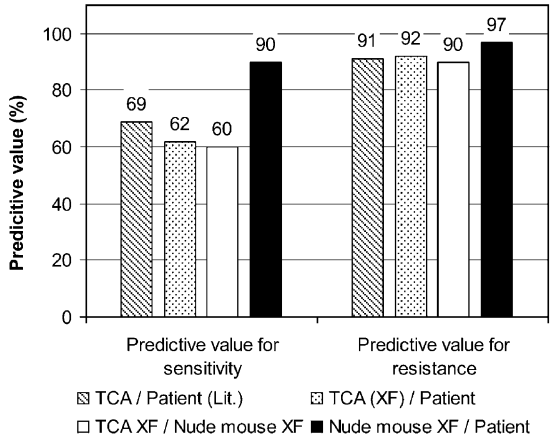

Fig. 3. Correlations between drug response in predictive assays and patients.

Table 15
Comparison of chemosensitivity of patient and xenografted tumours

Tumour*	ADR** 0.01	BLM 0.03	CDDP 0.1	DTIC 30.0	CTX 0.3	IFO 0.3	MMC 005	VP-16 0.1	VCR 0.01	VDS 0.01	5-FU 0.2
OVXF 889/0	–	–	++	++	–	–	+	–	–	–	+++
OVXF 889/2	–	–	–	+++	–	–	–	–	–	–	+++
LXFS 650/0			+++				++			+++	
LXFS 650/4	(+++)		+++				++			+++	(+)
SXF 678/0	–										
SXF 678/1	–										
MEXF 895/0	–	–	–	–		–	–	–	–	–	–
MEXF 895/2	–	–	–	–	(+)	+	–	–	–	–	–

*Tumour No./nude mouse passage: /0 = direct test of patient tumour; OVXF ovary, LXFS small cell lung cancer. SXF sarcoma, MEXF melanoma.
**Cytostatic drug dosages in μg/ml, abbreviations see Table 8a.

specimen [51]. The possibility of conserving vital tumour tissue, and of using that tissue as a renewable and inexhaustible source for antitumour testing *in vitro* in the TCA or *in vivo* as a nude mouse xenograft, provides a valuable tool within anticancer drug discovery.

We follow a dual-testing strategy in order to identify novel anticancer agents (Fig. 4). On the one hand, compound development is target driven in the sense that a target of interest is defined in the tumour models, and tumours showing either up- or downregulation of a specific molecular target are selected for testing [57–59]. On the other hand, a more empirical approach is being exploited. In the rational, target-orientated approach, the selection of the appropriate models is based on RNA and protein, the latter determined by Western blots or immunohistochemistry of arrayed xenograft tissues [60–62]. On average, such a xenograft tumour microarray comprises duplicates of more than 150 different tumours and five normal tissues that can be analysed simultaneously with specific antibodies [59,63]. In another approach, target selection is made possible by using our xenograft gene-expression profile database. The database was generated by determining the transcriptome of 60 xenografts at various passages with the HU 133A-Chip from Affymetrix. Tumour models selected by either method are normally tested in the TCA against 12–24 different tumours that over-express or lack a target of interest. These *in vitro* studies are essential for identifying the most differentially active compounds, for selecting the most sensitive tumours as candidates for subsequent *in vivo* studies in nude mice bearing the respective tumour as xenograft, and for excluding resistant tumour models from testing. This process much reduces the costs of random *in vivo* testing as well as the use of animals from an ethical point of view.

In the more empirical approach to drug discovery, well-defined or combinatorial compound libraries are being screened (Fig. 4) [33,64,65]. We thereby focus

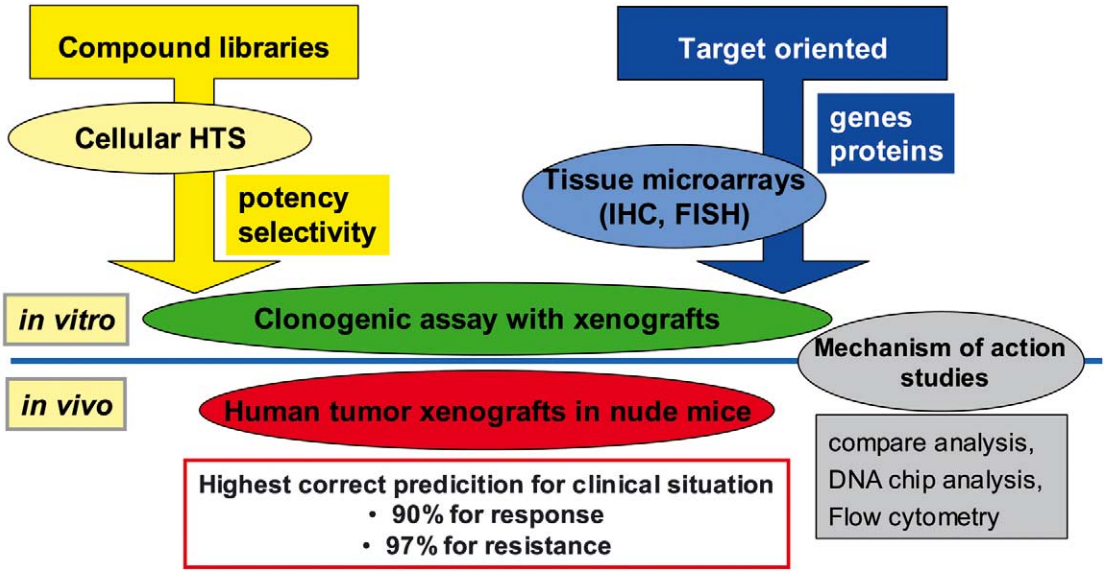

Fig. 4. Drug discovery procedures developed and used at Oncotest, Institute for Experimental Oncology.

mainly on natural products isolated from plants and microorganisms. Our present collection contains more than 8000 pure compounds. In this setting, the TCA is used as a secondary screening and the primary screening is conducted in a high-throughput setting using 8–12 permanent human tumour cell lines, mainly derived from our own xenograft collection [33,64]. Compounds are selected on their antitumour potency and tumour selectivity. The 'hit rate' in the pool of 8000 natural products was about 1–3% depending on the origin of the products.

Promising 'hits' are subsequently tested in the clonogenic assay using human tumour xenograft models *in vitro*. Usually, 24 tumours are studied, e.g. two to three different tumours from eight to 10 histological tumour types, and compounds are tested at six dose levels under continuous exposure. In addition, the effect on haematopoietic stem cells is also evaluated. The most differentially active compounds are selected and tested against two to four of the most sensitive tumour types *in vivo* in nude mice with subcutaneously growing xenografts of the respective tumour type. In order to identify the tumour histological types that should be selected for clinical phase II studies, testing in the clonogenic assay is extended to 40–100 tumours, reflecting four to eight different tumours per tumour type [71,72,66,67]. The tumour models are well selected and representative for a particular tumour entity with respect to chemosensitivity, histology and the expression of oncogenes or tumour markers. With this strategy we are able to identify the most sensitive tumour types.

Examples for the evaluation of a target-directed compound in the clonogenic assay are shown in Fig. 5(a) for PKI166, inhibiting the epidermal growth factor (EGF) receptor-mediated signal transduction. PKI166 inhibited tumour colony formation in a dose-dependent manner, with a mean IC_{70} of 5.18 μg/ml ($n = 29$ tumour models). Inhibitory concentrations of 50% (IC_{50}, T/C 50%) and 70% (IC_{70}, T/C 30%) were calculated and are depicted in a mean graph presentation (Fig. 5a). In the mean graph analysis, the distribution of IC_{70} obtained for a test compound in the individual tumour is given in relation to the mean IC_{70} obtained for all tumours tested. The individual IC_{70} are expressed as bars in a logarithmically scaled axis. Bars to the left demonstrate IC_{70} lower than the mean value (indicating more sensitive tumour models), bars to the right demonstrate higher values (indicating rather resistant tumour models). The mean graph analysis therefore represents a fingerprint of the antiproliferative profile of a compound and sensitive candidate tumour models for further *in vivo* analysis can easily be identified. Antitumour selectivity was pronounced for PKI166, and responsive tumour models for subsequent *in vivo* testing showing individual IC_{70} at least 3-fold below the mean IC_{70} over all 29 tumour models could readily be identified (Fig. 5a). PKI166 was then tested against LXFA 629 the lung adenocarcinoma grown as a xenograft in nude mice (Fig. 5b). This tumour model has been shown to express highly the EGF receptor, as determined by immunohistochemistry (Fig. 5b, inlay). PKI166 given for two cycles of 5 days at 50 and 100 mg/kg per day orally led to dose-dependent growth inhibition with a T/C for relative median tumour volumes of 29.4% (50 mg/kg per day) and 22.2% (100 mg/kg per day), respectively.

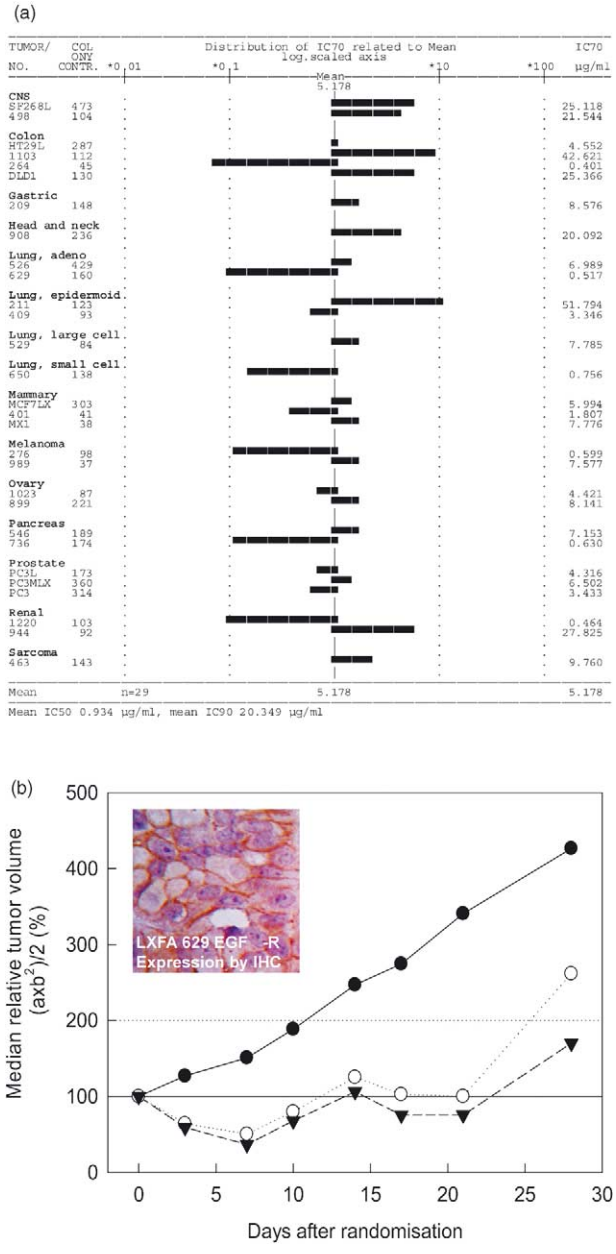

Fig. 5. (a) In vitro efficacy of the epidermal growth factor receptor inhibitor PKI166 in 29 human tumour models in the clonogenic assay. (b) *In vivo* antitumour efficacy of the epidermal growth factor receptor (EGFR) inhibitor inhibitor PKI166 in the lung cancer xenograft LXFA 629. Therapy given orally on days 0–4 and 14–18: ● control 10 ml/kg per day; ○ PKI166 50 mg/kg per day; ▼ PKI166 100 mg/kg per day. Inlay: EGFR expression in the lung cancer LXFA 629 determined by immunohistochemistry.

Median tumour doubling time in the 50 mg/kg per day group was 25 days compared to 11 days for the control (Fig. 5b). Tumour doubling was not reached during the experiment in the 100 mg/kg per day group.

The standard agent vinorelbine (Navelbine®) is shown as an example of broad tumour-panel testing in an empirical approach to the identification of putative phase II-responsive tumour types in the clonogenic assay (Fig. 6). The antitumour effect of vinorelbine was tested against 80 tumours. The compound induced dose-dependent inhibition of colony formation with a mean IC_{70} of 0.48 μg/ml. The mean graph analysis identified mammary cancers (8/8), prostate (4/5), and cervical (1/1) and uterine (2/2) carcinomas as more sensitive than the mean IC_{70}. Vinorelbine has been used clinically in breast cancer [68] and cervical cancer [69,70]. Tumour types such as NSCLC (14/19), melanoma (6/8), ovary cancer (3/3), or bladder cancer (4/6) were rather resistant (Fig. 6).

Similar broad studies using the clonogenic assay have been performed with other standard agents that are commonly used in the clinic, as well as with several compounds in development, e.g. R-roscovitin, in 103 tumour models [71], or with an AME in 47 tumour models [72] (Fig. 7), derivatives of geldanamycin [66,67] and recombinant mistletoe lectin [73].

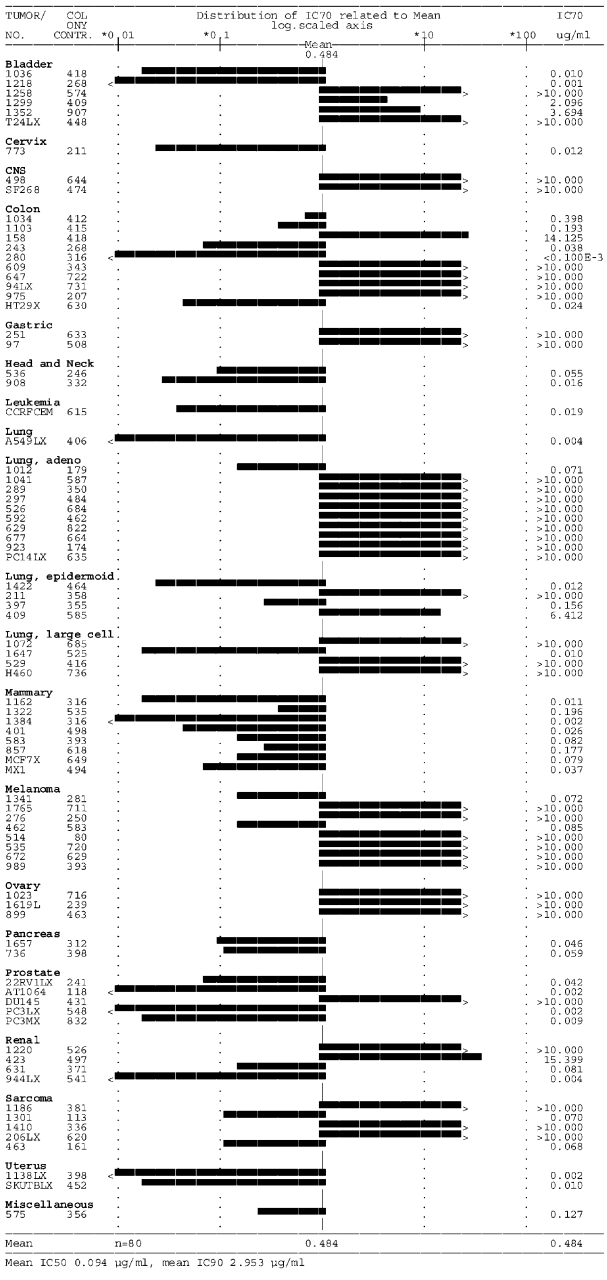

Fig. 6. *In vitro* antitumour efficacy of vinorelbine in 80 human tumour models in the clonogenic assay.

4. Discussion

4.1. Sensitivity testing on patients—future perspectives

Although there is evidence that clinical response rates may be superior for *in vitro* assay-directed chemotherapy rather than chemotherapy selected by an oncologist [37,74,75], there has been no prospective randomised controlled trial comparing survival between patients given an *in vitro*-tested drug, patients treated by surgery alone, and patients treated by standard chemo-

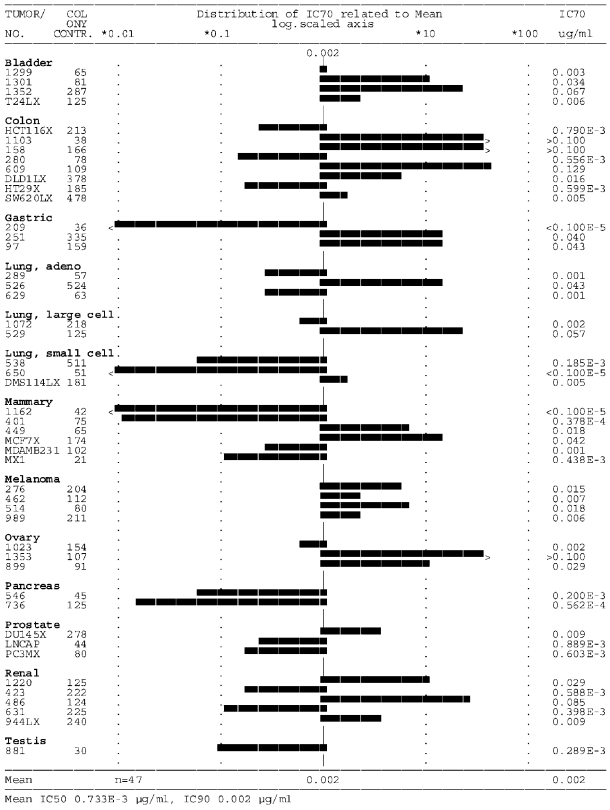

Fig. 7. *In vitro* efficacy of an aqueous mistletoe extract (AME) in 47 human tumour models in the clonogenic assay.

therapy. Many different laboratories have demonstrated the value of the TCA's correct predictivity. In all published studies, its correct prediction for drug resistance ranged from 90% to 97%, and for tumour sensitivity between 60% and 70%. In recent years, the growth rates of primary tumour tissues in the TCA has been significantly improved, in our experience even up to 70–80% depending on tumour type and depending on the time elapsed between tumour removal from the circulation to the test, the careful selection of viable tumour tissue, and the use of appropriate culture media. Thus, the TCA now has the potential to play a practical part in chemosensitivity testing in an individualised treatment protocol.

In general, a number of problems are inherent in predictive *in vitro* assays. Among these are the choice of clinically relevant drug concentrations to be tested *in vitro*, the heterogeneity of patient tumours [76–79], interference by the experimental conditions with the physiological environment for tumour cells that exists in the patient, and selection pressure on tumour cells by the experimental system used. The relation between inhibition of tumour growth *in vitro* and a patient's response to chemotherapy or survival is therefore complex. In fact, in our studies, most had already received a standard first- and sometimes second-line anticancer therapy, and therefore the probability of identifying novel active compounds was not high. However, in this context the value of the correct prediction of resistance by the TCA should be stressed. Whilst the prediction of response and thus the selection of a potential novel treatment option guided by TCA data is most desirable, the prevention of toxic side-effects caused by agents that are unlikely to be effective clinically should be considered an equally important benefit for the cancer patient. In addition, the picture might change when novel compounds with target-directed mechanisms are also included in the TCA testing after having been validated. Relevant studies are now in progress in our institute.

4.2. Role of the clonogenic assay for drug discovery as a secondary screen

The practical application of the TCA must be seen within the concept of a combined *in vitro*/*in vivo* testing procedure. After having selected target-defined tumour models or after a primary prescreening in a high-throughput assay, the TCA has a central role in the profiling of novel compounds. Since, for example, natural products are often available only in very small quantities, a well-balanced evaluation based on the *in vitro* activity of freshly prepared xenografts in the TCA is made possible before compounds are profiled *in vivo* in an animal model that might be not responsive to the class of compounds tested. This process, as outlined above, reduces the costs of random *in vivo* testing and the use of animals.

By using a 'bioinformatics' approach it is also possible to determine if a novel compound has a similar *in vitro* profile (IC_{50}, IC_{70} mean graph analysis) to those of standard or experimental drugs that have been previously tested in the TCA, or if there is a correlation between *in vitro* activity and the expression of specific genes in the respective xenograft. By utilising this testing strategy, our laboratory was able to identify several novel lead compounds with *in vitro* and *in vivo* activity. A number of them are now in development or in clinical phase II trial or I [66,80–84].

4.3. Comparison of the tumour clonogenic assay with monolayer assays

The biological behaviour of tumours growing in the clonogenic assay as compared to monolayer assays is different, and the test results are influenced by this fact. When primary tumour material from patients is used for testing, the solid tissue has to be disaggregated, and the resulting suspension contains not only tumour cells but also other host cells, e.g. mesothelial cells or fibroblasts. In the monolayer assay, fibroblasts in general can overgrow the tumour cells of the suspension (up to 2–3 passages), and the growth of the tumour cells is sometimes delayed. Depending on the experimental set-up of monolayer assays, fibroblast contamination can greatly influence the final results (e.g. DNA content, protein content, metabolic activity of cells). In soft-agar cultures, in contrast, fibroblasts and other normal host cells cease growth and thus present no problem.

In our opinion, the TCA with established human tumour xenografts is important in current drug discovery strategies. We have therefore included the TCA as a secondary assay in our approach to anticancer drug discovery and have found a number of novel active agents that are now in advanced preclinical development or clinical trials. Thus the TCA has recognized predictive value in the chemosensitivity testing of standard and experimental anticancer drugs.

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