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Development of a propidium iodide fluorescence assay for proliferation and cytotoxicity assays

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A propidium iodide fluorescence assay (PIA) was developed to characterize the *in vitro* growth of human tumor cell lines as well as to test the cytotoxic activity of standard compounds. Propidium iodide (PI) was used as a dye which penetrates only damaged cellular membranes. Intercalation complexes are formed by PI with double-stranded DNA which effect an amplification of the fluorescence. Incubation of the total cell population with PI and subsequent fluorescence detection allowed assessment of the number of non-vital cells (first measurement). After freezing the cells at −20°C for 24 h PI had access to total DNA leading to total cell population counts (second measurement). The number of viable cells was calculated by the difference between these two measurements. In the proliferation and cytotoxicity assays 5×10^3 cells per well were plated in 96 multiwells and finally stained with 50 μg/ml PI in 25 μl for 10 min. A correlation between the log of cell number and the log of flurorescence units could be demonstrated over a 2.5–3 log range ($r = 0.97$). The lower limit of cell detection was 150–500 cells/wells. In cytotoxicity assays eight clinically used cytostatics were tested which effected a clear dose–response relationship ($r = 0.93$–0.98) and high reproducibility ($r = 0.92$). In conclusion, this assay is a simple and rapid test system, the main advantages are the absence of any washing steps and the small number of tumor cells necessary for drug testing. The PIA can easily be used for cell number determinations in biological and pharmacological studies.

Key words: Chemosensitivity testing, cytotoxicity assay, fluorescence assay, proliferation assay, propidium iodide.

Introduction

The synthesis and development of novel antineoplastic compounds is one of the main subjects of current oncological research. Preclinical evaluation of potential anticancer drugs in highly efficient *in vitro* test systems is of crucial importance.

Assay systems used in drug development may be divided into the following categories:[1] (i) measurement of cell viability (dye exclusion assays, chromium release assays), (ii) inhibition of cellular metabolism [MTT, sulforhodamine B (SRB)], (iii) inhibition of radioactive precursor incorporation ([^{3}H]thymidine incorporation) and (iv) determination of cellular reproductive capacity *in vitro* after drug exposure [human tumor stem cell assay (HTSCA)]. These test assays[2–6] are widely used in drug testing, but each of them has some restrictions.

Application of the clonogenic assay for large-scale drug screening is limited by different factors. Some tumors show an insufficient cloning efficiency and not all tumor types grow at high frequency.[1] In addition, the test procedure is labor intensive and colony formation takes 7–20 days depending on tumor type and growth rate. However, the clonogenic assay[7,8] demonstrated an excellent correlation to the response behavior in patients[9] as well as in nude mice.[10,11] Radioactive methods (determination of [^{3}H]thymidine or [^{125}I]iododexuridine incorporation) allow us to test a large number of samples, but difficulties in handling the radioactive samples promoted the search for other techniques. Besides these methods, the measurement of metabolic activity can be used to determine drug efficacy. The MTT assay described by Mosmann[12] is widely used for this purpose, but its application in the National Cancer Institute (NCI) drug screening program revealed disadvantages in laboratory handling, e.g. several washing steps. Furthermore, measurements must be performed after a given period which limits its practicability in a broad testing program.[5] As part of ongoing efforts to optimize laboratory efficiency, an alternative assay endpoint based on the use of a protein-binding dye, SRB, was introduced in the NCI system. Although used in drug screening, the staining protocol uncovers time-consuming and time-sensitive steps,[5] which should be avoided in novel assays.

For a broad primary screening a rapid and quantitative assay is required, capable of testing large numbers of samples. The assay system should be as simple as possible, and suitable for proliferation assay and drug sensitivity testing. To avoid methodical pitfalls the experimental setup should ex-

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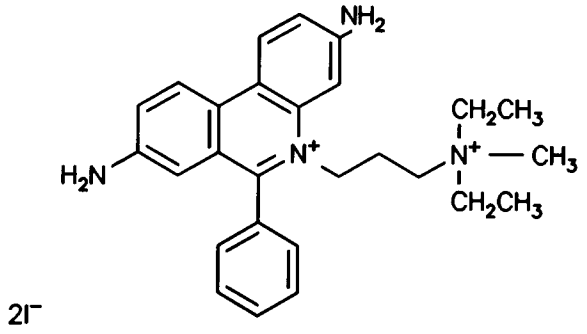

Figure 1. Chemical structure of PI.

clude washing steps or time-sensitive reactions. These requirements are fullfilled with propidium iodide [(PI) Fig. 1] which is used for nucleic acid staining of dead cells in flow cytometry.[13] It intercalates with double-stranded DNA but not with other macromolecules. These complexes produce an intense fluorescence signal correlating with the DNA content.[13] Since PI is a polar dye and highly soluble in water, cell membranes and nuclear envelopes have to be lysed before PI can enter the nuclus and stain the double-stranded DNA of viable cells.[14] This property, that an intact membrane of live cells excludes dye penetration into the cell, allows us to differentiate between the DNA of dead cells and the total DNA of a given cell population. Therefore, PI is an attractive candidate for a rapid screening system which allows differential staining. In a first measurement, the fluorescence signal of dead cells is detected. After permeabilization of the cell membrane and the nuclear envelope, PI has access to total cellular DNA which can be quantified in a second measurement. Determination of these two values allows for assessment of the DNA of viable cells. In this way the total cell number as well as the rate of viable and dead cells can be estimated by two measurements with one dye.

In this study the development and standardization of a novel PI assay (PIA) is described. Different parameters concerning the staining protocol with PI were analyzed. Finally, the applicability of the PIA was investigated to test the cytotoxicity of standard compounds as well as to study the growth kinetics of tumor cells.

Material and methods

Cell lines and cell culture

Twenty one cell lines of different solid tumor types were used in the study. They were established from human tumor xenografts growing in serial passage in nude mice (Table 1). Stock cultures were grown in 75 cm^2 flasks (Costar, Cambridge, MA) using Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% heat-inactivated fetal calf serum (Pan Systems, Aidenbach/Passau, Germany), 2 mM glutamine and streptomycin (100 μg/ml)/penicillin (100 U/ml). Cultures were incubated at 37°C and 7% CO_2 in a humidified atmosphere, with medium change or subcultivation once or twice a week. The cells were dissociated with trypsin/EDTA (0.05%/0.02% w/v). All lines were grown as monolayers except the small cell lung cancer LXFS 650L which grew in suspension culture. Cell viability of the cultures was above 98% as determined by the Trypan blue exclusion test. All cell lines were free of *Mycoplasma*.

Dye and staining procedure

In all experiments, PI (Aldrich-Chemie, Steinheim, Germany) was used as the staining dye. PI was solubilized in water to a final concentration of 50 μg/ml and stored in aliquots at −20°C for a maximum of 3 months. Staining was performed by adding 25 μl PI to each well without removing the culture medium. After an incubation period of 10 min in the dark at room temperature, fluorescence intensity was determined using an automatic fluorescence scanner (Millipore Cytofluor™ 2350, Bedford, MA). The excitation wavelength of the reader was 530 nm (bandwidth: 25 nm) with an emission filter of 620 nm (bandwidth: 40 nm). Since PI penetrates only leaky or lysed membranes, DNA of dead cells can be stained and measured, while living cells will not be stained. In order to estimate the living part of the cell population, cells were permeabilized by freezing the plates at −20°C for 24 h and then thawed at room temperature. Thereby, cellular membranes of live cells were lysed and PI could form intercalation complexes with nucleic acids. A second measurement followed, in which the total DNA-related fluorescence was assessed. Calculation of the fluorescence of live cells resulted from the difference between these two measurements.

Cell membrane permeabilization methods

Since a permeable cellular membrane and nuclear envelope are essential to allow PI access to the DNA of viable cells, several methods to disrupt the mem-

Table 1. Tumor and patient characteristics of established human tumor cell lines selected for the development of the PIA

Tumor cell lines		Tumor origin		Patient data		
Designation[a]	PDT[b] (days)	Type	Histology	Sex	Age (years)	Survival time (days)
CXF 94L	4	colorectal	adeno	m	70	245
CXF 164L	4	colorectal	adeno	m	50	171
GXF 251L	6	gastric	undiff	m	60	107
LXFA 289L	4	lung	adeno	m	63	17
LXFA 526L	5	lung	adeno	m	58	42
LXFA 629L	8	lung	adeno	m	59	101
LXFL 529L	3	lung	large	f	34	115
LXFL 1072L	5	lung	large	f	41	88
LXFL 1121L	6	lung	large	f	57	>810
LXFS 650L	6	lung	small	m	64	53
MEXF 276L	5	melanoma	melanotic	m	23	321
MEXF 514L	6	melanoma	melanotic	m	40	>1420
MEXF 989L	5	melanoma	amelanotic	m	32	88
OVXF 899L	7	ovarian	pap.serious	f	77	18
PXF 1118L	5	pleural mesothelioma		f	73	101
RXF 486L	4	renal		f	51	186
RXF 631L	6	renal		m	55	42
RXF 944L	4	renal		f	56	312
RXF 1218L	6	renal pelvis		f	74	
SCLS 1442[c]	3	soft tissue		m	32	330
UXF 1138L	8	uterus	carcinosarcoma	f	66	780

[a]Developed from: CXF, colorectal xenograft; GXF, gastric; LXF, lung; A, adeno; L, large cell; E, epidermoid cell; S, small cell; MEXF, melanoma xenograft; OVXF, ovarian cancer xenograft; PXF, mesothelioma; RXF, renal; SCLS, soft tissue; UXF, uterus.
[b]Population doubling time *in vitro*.
[c]Directly established from patient tumor material.

brane were studied. A total of 100 000 cells suspended in 50 μl medium from three different tumor types (CXF 164L, MEXF 514L and RXF 944L) were seeded per well in 96 multiwells. Membrane lysis was performed thermically by freeze-thawing or chemically by addition of 50 μl detergents (0.1% Triton X-100, 0.04% saponin) or absolute alcohols (methanol, ethanol). In parallel, a vitality test with Trypan blue exclusion was performed to demonstrate successful complete cell membrane permeabilization. Subsequently, cells were stained with PI and measured.

Proliferation assay

To study growth kinetics of tumor cells the development and increase of the DNA-associated fluorescence signal was observed over a period of 10–16 days depending on the time necessary to reach the plateau phase of cell growth. For this 5×10^3 trypsinized cells were seeded in triplicate for each measuring point in 96 multiwells (Costar, no. 3595). Each plate contained blank controls to detect background fluorescence and a positive control with 1000 μg/ml 5-fluorouracil (5-FU). The first measurement was performed on day 0, representing the fluorescence of the initial cell number. Cultures were incubated at 37°C and 7% CO_2 in a humidified atmosphere and monitored daily using an inverted microscope. Daily measurements were performed to determine the lag phase, the phase of exponential growth and the shift to the plateau phase (beginning at day 7–14 dependant on doubling time). At each measuring point cells were stained with the standardized protocol as described earlier.

Cytotoxicity assay

After dissociation with trypsin, 5000 cells suspended in 50 μl medium corresponding to 1×10^5

cells/ml were seeded per well into 96-well flat-bottomed microtiter plates (Costar, no. 3595) in the same medium as used for stock cultures. Plate design contained 12 blank wells to correct for background fluorescence, nine control wells, one positive control with 1000 μg/ml 5-FU (three wells) to document a potential drug effect and treatment groups plated in triplicate. In addition, a negative control was plated to measure the initial fluorescence on day 0. For chemosensitivity testing, cytostatic drugs were applied in 50 μl medium 1 day after plating, the control group received medium only. Anticancer agents bleomycin, cisplatin, dacarbazine, doxorubicin, etoposide (VP16-213), mitomycin-C, vincristine and vindesine were studied for cytotoxic efficacy at six dose levels ranging from 0.0001 to 10 μg/ml (log steps). Each compound was solubilized in either water or saline. Stock solutions were stored in aliquots at −20°C for a maximum of 3 months. By continuous drug exposure, cultures were incubated at 37°C and 7% CO_2 in a humidified atmosphere and visually controlled by microscopy. At the beginning of cell confluence (endpoint: maximum fluorescence signal intensity in the control) experiments were stained as described above.

Evaluation

The mean measured fluorescence (FU) of a control or treated group was corrected for background considering mean blank values:

$$FU_{Corrected} = FU_{Measured} - FU_{Background} \quad (1)$$

The fluorescence of viable cells (equation 4) was calculated from the first (equation 2) and second measurement (equation 3) according to the following formula:

$$FU_{Dead\ Cells} = FU_{First\ Measurement} - FU_{Background\ First\ Measurement} \quad (2)$$

$$FU_{Total\ Cells} = FU_{Second\ Measurement} - FU_{Background\ Second\ Measurement} \quad (3)$$

$$FU_{Live\ Cells} = FU_{Total\ Cells} - FU_{Dead\ Cells} \quad (4)$$

Correlation of FU with cell number was performed by a double logarithmic regression line.

Experiments were fully evaluable if mean fluorescence of the control group reached a minimum of 500 fluorescence units correlating with sufficient cell growth until the end of the experiment (monolayer formation). Initial fluorescence on day 0 should be ≤30% of the final control group fluorescence. The positive reference compound 5-FU (at the toxic dose of 1000 μg/ml) had to effect a reduction of fluorescence to ≤30% of the control at the end of the experiment.

In cytotoxicity assays, drug effects were expressed in terms of survival, obtained by comparison of the mean number of fluorescence units (FU) of live cells in the treated groups with the untreated controls (test versus control value [T/C]):

$$T/C[\%] = \frac{FU_{Treated\ Live\ Cells} \times 100}{FU_{Control\ Live\ Cells}} = \frac{(FU_{Treated\ Total\ Cells} - FU_{Treated\ Dead\ Cells}) \times 100}{FU_{Control\ Total\ Cells} - FU_{Control\ Dead\ Cells}}$$

A drug was considered active if it reduced FU of viable cells in the treatment group to less than 30% of the control group value (T/C ≤ 30%). Data evaluation was automated using software specifically developed in our laboratory.

Results

Factors involved in the staining procedure

Concentration of PI. The optimum concentration of PI was determined in four different cell lines (CXF 164L, LXFL 529L, MEXF 514L and RXF 486L). A total of 100 000 cells per well were plated and after membrane permeabilization PI was immediately added in 12 concentrations ranging from 0.1 to 100 μg/ml (Figure 2). Incubation time was varied from 10 to 90 min, which gave similar results. The concentration of 50 μg/ml effected a plateau in DNA staining (Figure 2) which was therefore used in all subsequent studies.

Determination of minimum incubation time of PI staining. The effect of incubation time on fluorescence intensity was evaluated in four different cell lines (CXF 164L, LXFL 529L, MEXF 514L and RXF 486L) plated at seeding densities of 1000 to 100 000 cells per well. The staining of freeze-thawed cultures with 50 μg/ml PI was required for a few minutes only at room temperature (25°C) and a plateau level of fluorescence was reached within 10 min irrespective of cell number (Figure 3). A prolonged incubation time had no additional effects on the

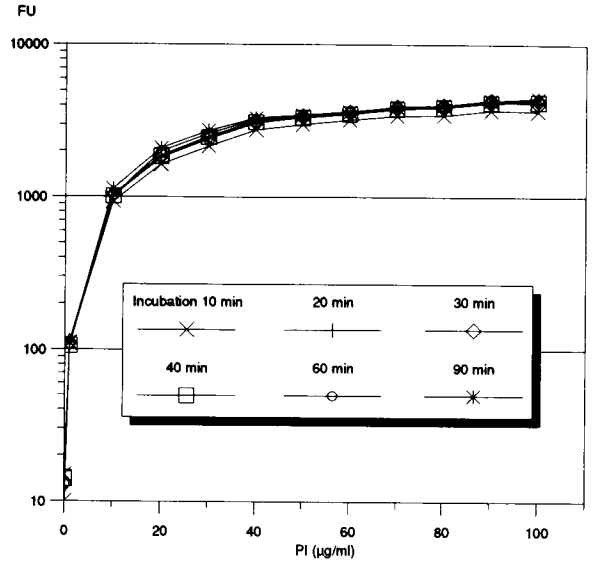

Figure 2. Determination of the optimum PI concentration. FU in relation to PI concentration after different incubation times in cell line MEXF 514L (50 µg/ml was considered optimal and used in further experiments).

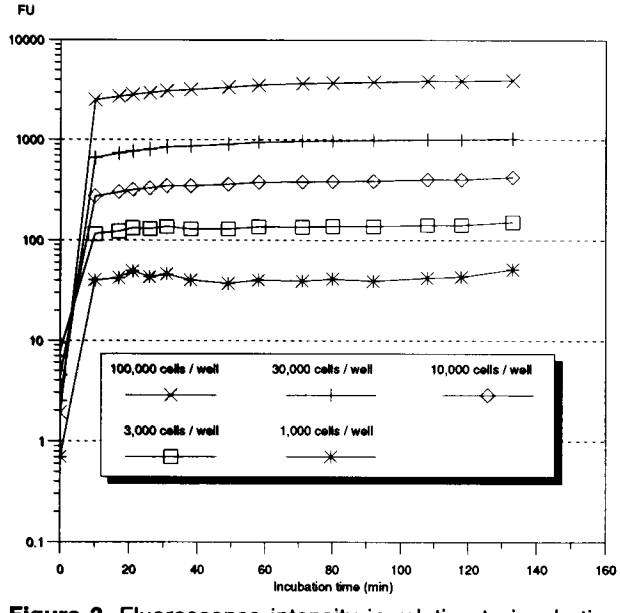

Figure 3. Fluorescence intensity in relation to incubation time of PI and cell number in RXF 486L. PI concentration was 50 µg/ml.

fluorescence level. Therefore, a 10 min incubation period was chosen for the further investigations.

Cell membrane permeabilization methods. As shown in Figure 4, the detergents Triton X-100

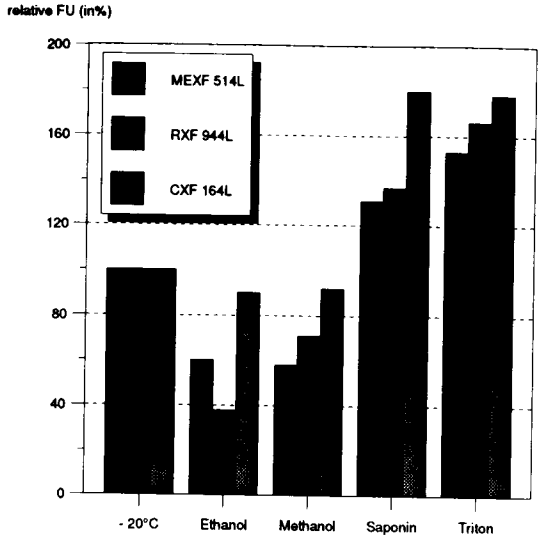

Figure 4. Cell membrane permeabilization methods tested in the cell lines CXF 164L, MEXF 514L and RXF 944L. The fluorescence units are expressed relative to the freeze-thaw method (100%).

(0.1%) or saponin (0.04%) produced an enhanced fluorescence intensity. In contrast, cellular fluorescence was quenched by the use of ethanol or methanol. The freeze-thawing is arranged between both methods inducing chemical membrane leakage and it was preferred since no additional steps are necessary and artefacts produced by drug interaction with chemicals are excluded. A single freezing to −20°C and thawing to room temperature was sufficient to produce maximal permability to exogeneous PI. These advantages resulted in use of the freeze-thawing procedure for the subsequent experiments.

Linearity of cell numbers and FU

Linearity of PI fluorescence units with cell number was evaluated by plating cells of eight different tumor cell lines at densities ranging from 150 to 150 000 cells per well. Cutlures were stained with PI as described earlier. In all eight cell lines studied (Table 2 and Figure 5) the DNA related fluorescence signal was proportional to the cell number at the range 150–150 000 cells per well. The lower limit of cell detection for eight cell lines studied ranged from 150 to 1500 cells per well with a median of 500 cells. A clear correlation between the log of cell number and the log FU could be demonstrated over a 2.5–3 log range with a median correlation coefficient of $r = 0.98$ (Table 2).

Table 2. Linearity of cell number with measured fluorescence and limit of cell detection in the PIA

Cell line	Fluorescence intensity[a] depending on cell numbers per well							Minimal cell no. detected/well	Coefficient of correlation
	150	500	1500	5000	15000	50000	150000		
LXFL 529L	0	13 ± 0.13	16 ± 0.16	68 ± 0.68	248 ± 2	280 ± 3	1372 ± 178	500	0.98
LXFL 629L	0	0	39 ± 0.39	116 ± 3	407 ± 8	1176 ± 59	2710 ± 136	1500	0.92
LXFL 1121L	0	4 ± 0.08	19 ± 0.19	46 ± 0.46	133 ± 1	382 ± 11	NE[b]	500	0.95
LXFS 650L	28 ± 0.28	44 ± 0.88	52 ± 0.52	105 ± 1	286 ± 3	778 ± 23	1856 ± 111	150	0.96
RXF 944L	6 ± 0.36	24 ± 0.24	43 ± 0.42	201 ± 8	584 ± 18	1440 ± 72	1496 ± 135	150	0.99
RXF 1218L	0	10 ± 0.1	91 ± 2	306 ± 9	969 ± 39	2294 ± 115	4246 ± 425	500	0.98
UXF 1138L	0	0	12 ± 0.12	66 ± 0.66	177 ± 7	575 ± 11.5	1302 ± 104	1500	0.99
SCLS 1442	0	14 ± 0.14	36 ± 0.36	99 ± 0.99	307 ± 6	694 ± 49	1341 ± 134	500	1.00

[a]Mean fluoresence units ± SD, $n = 3$.
[b]Not evaluable.

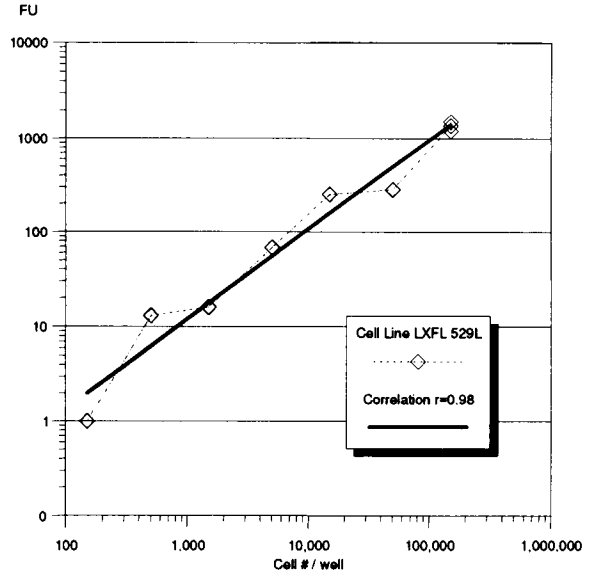

Figure 5. Linearity of cell number versus fluorescence intensity in the cell line LXFL 529L.

Proliferation assays

Cell lines established in our laboratory were characterized with respect to their growth behavior. Cells (10^5/ml) were seeded and growth was observed until the end of the plateau phase. Table 3 summarizes the time course of growth, logarithmic growth and beginning and end of the plateau phase. The logarithmic growth phase lasted 7–14 days. The cell lines LXFA 629L and RXF 944L demonstrated a marked lag phase of 3 days suggesting that these cell lines are influenced by the assay setup procedure (dissociation by trypsin, plating) or growth factor dependence. The optical monitoring revealed that confluent monolayer formation coincided with the maximum measured fluorescence as well as with the plateau phase of cell growth.

Cytotoxicity assays

The assay provides a simple and efficient method of evaluating *in vitro* chemosensitivity of human tumor cell lines in 96-multiwell microtiter plates. In this way we have characterized 17 cell lines established in our laboratory. A panel of eight standard antineoplastic agents was tested in six dose levels ranging from 0.0001 to 10 μg/ml (log steps). A clear dose–response relationship was found for all drugs tested (r = 0.93–0.98). A representative dose–response curve of human lung carcinoma LXFL 529L is shown in Figure 6. Drug response of the various cell lines is summarized in Tables 4 and 5 listing the calculated concentrations resulting in 70% growth inhibition (IC_{70}) on the basis of the response curve for each cell line tested. The data clearly reveal the differential sensitivity pattern of each cell line. The most potent compounds in descending order were doxorubicin (IC_{70} 0.05 μg/ml), followed by vincristine (0.06 μg/ml), vindesine (0.1 μg/ml), etoposide (0.12 μg/ml), mitomycin (0.16 μg/ml), bleomycin (0.79 μg/ml), cisplatin (1.1 μg/ml) and darcarbazine (5.44 μg/ml).

Reproducibility. Reproducibility of the *in vitro* data has been studied by repeated testing of eight standard antineoplastic agents (see Table 6, remarks) in the same tumors in cell culture. In the cell lines LXFL 529L, LXFL 1072L, LXFS 650L, RXF 486L and RXF 944L the results fo the first experiment have been compared with the second experiment (Table 5). Statistical analysis of the distribution of T/C values

Table 3. Growth characterization of human tumor cell lines with the PIA

Cell line	Day 0	Lag Phase		Logarithmic growth	Plateau phase	
	$FU^{a}_{at\ beginning}$	from day	$FU_{at\ minimum}$	day	day	$FU^{b}_{at\ endpoint}$
GXF 251L	61	—	—	0–9	9[c]	1689
LXFA 629L	365	0–3	171	3–7	7–10	1256
LXFL 430L	6	—	—	0–9	9–13	1090
LXFL 529L	131	—	—	0–10	10–14	2148
LXFL 1121	90	—	—	0–7	7–10	1876
MEXF 514L	158	—	—	0–14	14[c]	735
PXF 1118L	85	—	—	0–12	12–15	2879
RXF 944L	328	0–2	306	2–10	10–13	3206
RXF 1218L	430	–	–	0–7	7–9	3179
UXF 1138L	98	—	—	0–7	7–8	1708

[a]Fluorescence units.
[b]Of the end of the logarithmic growth.
[c]No plateau observed within the daily measurement.
—, immediately increase of FU indicating no lag phase by daily measurement.

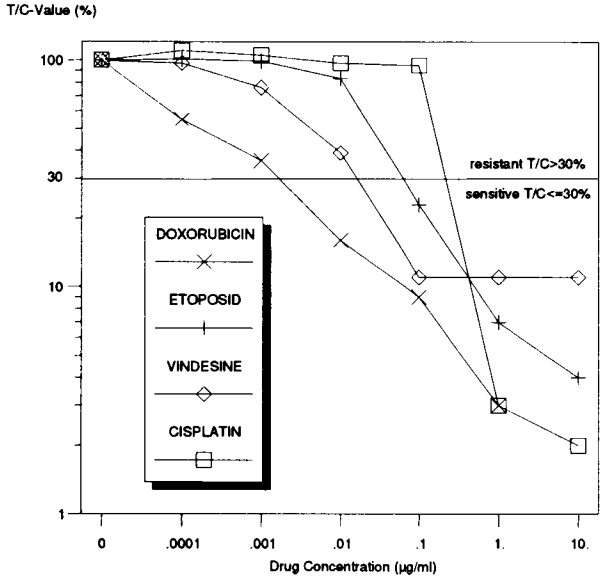

Figure 6. *In vitro* effect of cisplatin, doxorubicin, etoposide and vindesine in the cell line LXFL 529L.

obtained in this way yielded a median coefficient of correlation $r = 0.92$ with a range of 0.79–0.98.

Relationship between sensitivity, incubation time and growth characteristics of different cell lines. With regard to the diversify of tumor cell growth behavior (initial cell number, doubling time) and the fact that an increase in drug concentration and incubation time can lead to an increased response rate of tumor cells, the optimal time of measurement (end point) was determined with constant initial cell numbers in eight experiments with different tumor cell lines and various drugs with distinct mechanism of action. The responsiveness to the agent used was evaluated at days 1, 3, 5, 7, 9, 11, 15 and 17. Figures 6 and 7 demonstrate in parallel the DNA related fluorescence emission and the responsiveness (T/C values) under the influence of doxorubicin in dependence of drug concentration and evaluation day in the cell line LXFL 529L. The beginning of confluence of cultured adherent cell lines (day 7–9) gave the maximal difference between test and control groups. The point marking the change from logarithmic growth to the plateau phase gave the best sensitivity for drug testing. This observation was found in all cell lines tested. Therefore, the optimal endpoint of chemosensitivity testing was determined to be the end of the logarithmic growth phase. Since this endpoint can vary by up to 3 days in the same cell lines, close optimal monitoring is necessary to recognize confluent monolayer formation and to define the end of an experiment.

Discussion

In the present study we describe a novel proliferation and cytotoxicity assay based on PI, a water-soluble DNA fluorescence dye, structurally derived from phenylanthren and widely used in flow cytometry.[13] Two polar groups in the molecule prohibit

Table 4. Dose–response relationship for standard drugs in 17 cell lines in the PIA

Drug	Percentage of tumors sensitive at drug concentration (μg/ml)						Coefficient of Correlation
	0.001	0.01	0.1	1	10	100	
Vincristine	38	50	69	69	75	94	0.97
Vindesine	27	50	50	63	73	73	0.97
Etoposide	19	19	41	65	82	100	0.98
Doxorubicin	13	18	53	100	100	100	0.93
Mitomycin C	13	33	60	60	80	100	0.98
Cisplatin	6	6	18	47	82	100	0.96
Bleomycin	6	13	25	56	75	94	0.98
Darcarbazine	6	6	13	31	50	75	0.95

Dose levels around 30% are underlined.

Table 5. *In vitro* responsiveness of human tumor cell lines in the PIA

Cell line	Evaluation day	Inhibitory concentration 70% (μg/ml) of drug							
		BLEO[a]	DOX	DTIC	MITO	PLAT	VCR	VIND	VP16
CXF 94L	12	24.04	0.06	3.480	48.77	6.48	0.03	0.24	0.78
CXF 164L	12	2.56	0.07	7.44	43.94	3.34	0.007	<0.001	2.92
GXF 251L	8	3.45	0.05	0.50	0.005	0.45	<0.001	0.001	0.01
LXFA 289L	6	0.84	0.05	14.92	46.81	4.51	10.00	0.16	0.07
LXFL 529L	7	0.77	0.001	86.60	0.05	0.51	0.03	0.02	0.08
LXFA 629L	12	0.05	0.20	0.66	0.02	0.13	0.003	0.001	0.23E-5
LXFL 1121L	12	0.23	0.05	0.07	0.003	0.21	<0.001	ND[2]	0.03
LXFS 650L	12	0.21	<0.001	0.07	0.004	0.04	<0.001	<0.001	0.02
MEXF 276L	8	ND	4.28	ND	ND	5.18	ND	>10.00	<10.00
MEXF 514L	10	0.001	0.14	>100.00	0.08	2.37	25.12	>100.00	2.63
PXF 1118L	9	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	<0.001
RXF 486L	5	>100.00	1.00	112.53	2.74	50.43	10.00	1.89	39.24
RXF 631L	9	35.34	0.57	70.17	4.56	75.43	41.46	>100.00	11.16
RXF 944L	11	43.80	0.18	104.62	3.06	55.37	59.12	>100.00	1.70
RXF 1218L	7	10.00	0.01	28.610	0.001	0.756	<0.001	<0.001	<0.001
SCLS 1332	5	0.83	0.38	171.91	ND	1.14	0.49	10.00	10.00
UXF 1138L	8	0.02	0.002	0.39	0.03	0.08	<0.001	<0.001	0.006
Mean IC_{70}		0.79	0.05	5.44	0.16	1.10	0.06	0.10	0.12

[a]BLEO, bleomycin; DOX, doxorubicin; DTIC, darcarbazine; MITO; mitomycin; PLAT, cisplatin; VCR, vincristine; VIND, vindesine; VP16, etoposide.
[b]Not done.

penetration through an intact cell membrane and, therefore, only membrane damaged cells are stained by intercalation of PI into double-stranded DNA. In fact PI is usually used in double-staining fluorescence protocols to determine the dead cell number. Cell membrane permeabilization by freezing allows for assessment of total cell numbers,[15,16] the viable cell number can be determined as the difference between total and dead cell counts. The minimum number of viable cells which can be detected in this assay system was 500 cells per well in a total of eight experiments (Table 2). In the logarith-

Table 6. Comparison of first and second experiments

Cell line	No. of drugs tested	No. of doses applied	Total no. of comparisons	Coefficient of correlation
LXFL 529L	8	6	48	0.98
LXFL 1072L	4	6	24	0.98
LXFS 650L	8	6	48	0.79
RXF 486L	8	6	48	0.92
RXF 944L	8	6	48	0.89
Total	36	6	216	0.92

Drugs tested: 5-FU, bleomycin, cisplatin, darcarbazine, doxorubicin, etoposide (VP16-213), vincristine, vindesine and vinblastine.

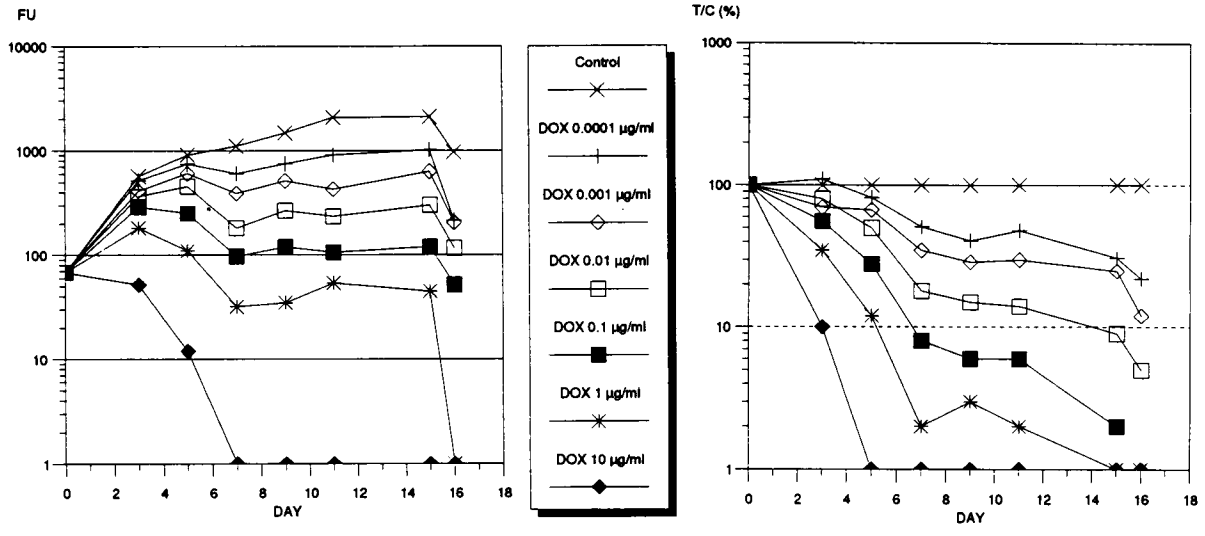

Figure 7. Relationship between fluorescence intensity, incubation time and doxorubicin sensitivity in the human lung cancer cell line LXFL 529L: (a) absolute fluorescence units and (b) test/control values.

mic scale a linear relation between cell count and fluorescence could be demonstrated over a cell density range of at least 3 orders of magnitude (Figure 5 and Table 2). The main advantage consists in the rapidity and simplicity of the methodology in contrast to other similar assays (e.g. MTT or SRB).

The PIA is useful for proliferation assays as well as for cytotoxicity tests. In this way cell lines were characterized with regard to growth phases. In anticancer testing a clear dose–response relationship could be demonstrated with correlation coefficients ranging between $r = 0.93$ and 0.98. The optimum sensitivity to cytostatics was determined with the beginning of monolayer formation (range: day 7–14 dependent on tumor doubling time) in contrast to short time assays, such as MTT or SRB, evaluated at day 4.[2] Theoretically, the PIA can be used as a short time incubation assay; however, this leads to a reduced drug sensitivity.

The PI technique measures the total and, indirectly, the viable cell number of a given cell population. The intercalation itself is independent of metabolic side effects. In contrast, the MTT assay, which uses the reaction of tetrazolium salts to formazan,[5,17] can be influenced by glucose, pH, NADH and NADPH.[18,19] Although many laboratories worldwide have adopted this assay procedure, some disadvantages (washing steps or time-sensitive staining procedures) are limiting large-scale screening application. To optimize laboratory efficiency, the NCI has changed their screening system from the MTT to the SRB assay. The latter technique allows only the determination of total protein content, a differentiation between live and dead cells is not possible. Although the SRB assay has advantages, the comparison of the major features of MTT and SRB assay protocols reveals both time-sensitive and time-consuming steps (like plate washing or

cell fixation).[5,14] The PIA circumvents these problems. The assay can be used for cell lines of solid tumors and also for hematologic lines growing as suspension cultures.

Like the above mentioned monolayer assays, clongenic assays have been widely used for *in vitro* investigations of anticancer drug cytotoxicity.[20] However, clonogenic assays are labor-intensive and plated cells take 10–20 days to form colonies of sufficient size to be counted. Despite these potential problems, the clonogenic assays are the golden standard in radiobiology and chemosensitivity testing.[21] One reason for their popularity may be that they have proven relevance with respect to clinical response to chemotherapy.[9,22] The main difference between the clonogenic assay and the PIA exists in the cell populations affected by drugs. While in the clonogenic system only the stem cell population is growing, the PIA provides growth of all proliferating cells. Therefore, the PIA might be a useful supplement to our *in vitro/in vivo* test procedure for anticancer drug development.[23,24] At first the PIA should identify and select new compounds for anticancer activity; in a secondary step, drug efficacy is confirmed *in vitro* in a clonogenic assay and *in vivo* in human tumor xenografts growing s.c. in nude mice, which have proven to be reliable tumor models.[25,26]

In chemosensitivity assays drugs can show quite different inhibitory effects depending on various parameters, e.g. inoculum density, culture conditions, duration of drug exposure, duration of recovery period before drug exposure and the determination of the endpoint.[27,28] Critical in all assays is the time of measurement. A fixed chosen endpoint does not take into account that rapidly proliferating tumors with short cell cycle intervals are more vulnerable to cytostatics than slowly growing ones.[25] Based on this background we specified in the PIA the end point individually for each cell line depending on its growth potential, which can easily be measured by growth kinetics in the PIA. The time-dependent cytocidal effect of cisplatin published by Bernhardt[25] illustrates the importance of extending the assay incubation time to account for delayed drug actions and/or loss of membrane function. In addition, different types of cells may require different periods of time following lethal injury. As a consequence, an individually chosen assay endpoint seems to be the optimum for drug sensitivity testing in the PIA, but further experiments will be necessary to validate this concept. Furthermore, a critical factor could be the recovery period between cell plating and drug application. In the experiments performed, cells were preincubated 1 day before drugs were applied. However, studies of tumor growth behavior showed that two out of 10 tumor lines demonstrated a lag phase of up to 3 days (Table 3), suggesting that a 1 day recovery period might be not sufficient. Cells damaged by the plating procedure could be more sensitive than cells not exposed to exogeneous stress factors. Additional studies have to be carried out to clarify the relevance of drug administration in relation to the recovery period of an individual cell line in order to prevent false positive test results.

Some potential limitations of the PIA should be considered. These may include artifacts due to possible drug fluorescence as observed with doxorubicin at higher concentrations (> 1 μg/ml, data not shown). This disadvantage may be overcome by initial estimation of drug-related fluorescence for every new compound by determination of viable cells only. In this case additional drug fluorescence is a constant factor which disappears by subtraction of the two fluorescence measurements under the condition that no loss of substance activity by metabolism or drug dissociation will occur.

Conclusion

The PIA offers several methodical advantages and practical benefits over standard assays like the MTT, SRB or clonogenic assays. It is less sensitive to environmental fluctuations produced by staining procedures just as it is less influenced by intermediary metabolism. The performance is excellent with respect to other traditional assay systems. This microculture test provides sensitive and reproducible indices of cell growth as well as cytotoxicity, and it appears well suited for several applications, including the first stage *in vitro* screening of compounds with potential antitumor activity. Originally designed for use with established cell lines in culture, the PIA can also be adapted for the study of fresh human biopsy material for predictive drug testing in the patient.

References

1. Carney DN, Winkler CF. *In vitro* assays of chemotherapeutic sensitivity. In: DeVita TV, Samuel H, Rosenberg SA, eds. *Important advances in oncology.* Philadelphia: Lippincott 1985: 78–103.
2. Skehan P, Storeng R, Scudiero D, *et al.* New colorimetric cytotoxicity assay for anticancer drug screening. *J Natl Cancer Inst* 1990; **82**: 1107–12.
3. Nygren P, Kritensen J, Jonsson B, *et al.* Feasibility of the fluorometric microculture cytoxicity assay (FMCA) for cytotoxic drug sensitivity testing of tumor cells from

patients with acute lymphoblastic leukemia. *Leukemia* 1992; **6**: 1121–8.
4. Friedman HM, Glaubiger DL. Assessment of *in vitro* drug sensitivity of human tumor cells using [^{3}H]thymidine incorporation in a modified human tumor stem cell assay. *Cancer Res* 1982; **42**: 4683–9.
5. Rubenstein LV, Shoemaker RH, Paull KD, *et al.* Comparison of *in vitro* anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel human tumor cell lines. *J Natl Cancer Inst* 1990; **82**, 1113–8.
6. Marshall ES, Finlay GJ, Matthews JHL, *et al.* Microculture-based chemosensitivity testing: a feasible study comparing freshly explanted human melanoma cells with human melanoma cell lines. *J Natl Cancer Inst* 1992, **84**: 340–5.
7. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. *Science* 1977; **197**: 461–3.
8. Courtenay VD, Mills J. Assay for tumors grown in immune-supressed mice and tested *in vivo* with cytotoxic agents. *Br J Cancer* 1978; **37**: 264–8.
9. Von Hoff DD. He's not going to talk about *in vitro* predictive assay again, he is? *J Natl Cancer Inst* 1990; **82**: 96–101.
10. Fiebig HH. Comparison of tumor response in nude mice and in the patients. In: Winograd B, Peckham MJ, Pinedo HM, eds. *Human tumor xenografts in anticancer drug development.* Berlin: Springer 1988: 25–30.
11. Berger DP, Henß BR, Winterhalter BR, *et al.* The clonogenic assay with human tumor xenografts: evaluation, predictive value and application for drug screening. *Ann Oncol* 1990; **1**: 333–1.
12. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity. *J Immunol Methods* 1983; **65**: 55–63.
13. Krishan A. Rapid DNA content analysis by the propidium iodide–hypothonic citrate method. *Methods Cell biol: Flow Cytometry* 1990; **33**: 121–5.
14. Eddin M. A rapid, quantitative fluorescence assay for cell damage by cytotoxic antibodies. *J Immunol* 1970; **104**: 1303–6.
15. Lambalgan R, Lelieveld P. The PIT method: an automated *in vitro* technique for drug toxicity testing. *Invest New Drugs* 1987; **5**: 161–5.
16. Skehan P, Bokesch H, Williamson K. A new lymphoma assay. In: Kaspers GJ, Pieters R, Twentyman PR, *et al.* eds. *Drug resistance in leukemia and lymphoma.* Harwood: New York 1993: 409–13.
17. Carmichael J, DeGraff WG, Gazdar AF, *et al.* Evaluation of a tetrazolium based semiautoamted colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987; **47**: 936–42.
18. Jabbar SAB, Twentyman PR, Watson JV. The MTT assay underestimates the growth inhibitory effects of interferons. *Br J Cancer* 1989; **60**: 523–8.
19. Vistica DT, Skehan P, Scuderio D, *et al.* Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production. *Cancer Res* 1991; **51**: 2515–20.
20. Hoffman RM. *In vitro* sensitivity assays in cancer: a review, analysis and prognosis. *J Clin Lab Anal* 1991; **5**: 133–43.
21. Weisenthal LM, Lippman ME. Clonogenic and nonclonogenic *in vitro* chemosensitivity assays. *Cancer Treat Rep* 1985; **69**: 615–32.
22. Perez RP, Godwin AK, Handel LM, *et al.* A comparison of clonogenic, microtetrazolium and sulforhodamine B assay for determination of cisplatin cytotoxicity in human ovarian carcinoma cell lines. *Eur J Cancer* 1993; **29A**: 395–9.
23. Fiebig HH, Schmid JR, Bieser W, *et al.* Colony assay with human tumor xenografts, murine tumors and human bone marrow. Potential for anticancer drug development. *Eur J Cancer Clin Oncol* 1987; **23**: 937–48.
24. Fiebig HH, Berger DP, Dengler WA, *et al.* Combined *in vitro/in vivo* test procedure with human tumor xenografts for new drug development. *Contrib Oncol* 1992; **42**: 321–51.
25. Hajdu SI, Lemos LB, Kozkewich H, *et al.* Growth pattern and differentiation of human soft tissue sarcoma in nude mice. *Cancer* 1981; **47**: 90–8.
26. Bender K, Steiert A, Berger DP, *et al.* Characterization of 53 human tumor xenografts by polymorphic enzyme analysis. *Contrib Oncol* 1992; **42**: 87–97.
27. Bernhardt G, Reile H, Birnböck H, *et al.* Standardized kinetic microassay to quantify differential chemosensitivity on the basis of proliferative activity. *J Cancer Res Clin Oncol* 1992; **118**: 35–43.
28. Freshney RI, Paul J, Kane IM. Assay of anti-cancer drugs in tissue culture; conditions affecting their ability to incorporate ^{3}H-leucine after drug treatment. *Br J Cancer* 1975; **31**, 89–99.

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