Anti-Cancer Drugs 1998, 9, pp. 531–538

Research paper

Microphysiometry: new technology for evaluation of anticancer drug activity in human tumor cells *in vitro*

Sara Ekelund, Peter Nygren[1] and Rolf Larsson
Division of Clinical Pharmacology and [1]Department of Oncology, Akademiska Hospital, Uppsala University, 751 85 Uppsala, Sweden. Tel: (+46) 18-665959; Fax: (+46) 18-519237.

Microphysiometry is a non-invasive, physiological method where measurement of metabolic activity can be made on living human tumor cells. Indirect measurement of the extracellular acidification is measured over a pH-sensitive silicon membrane. In this study microphysiometry was employed for the study of cytotoxic agents used in therapy of cancer. Standard cytotoxic drugs with different postulated mechanisms of action were investigated using cell lines as well as primary cultures of patient tumor cells. Each investigated cytotoxic drug induced a characteristic pattern of metabolic activity. From these patterns, key features, like stimulation and inhibition of acidification, the time point when the response curves of the drugs fall below the control curve, and the maximum inhibition of acidification at 20 h, could be quantified. Most of the investigated drugs showed some initial stimulation of acidification rate during the experiments. For drugs producing a reduced metabolic rate at 20 h a concentration–response relationship was observed. The drug effects measured at 20 h were irreversible and correlated reasonably well with parallel measurements of membrane integrity using a standard cytotoxicity test. The results demonstrate the feasibility of 'on-line' measurements of metabolic activity using this approach and also revealed an unexpected variety of drug response profiles. [© 1998 Lippincott-Raven Publishers.]

Key words: Cell metabolism, cytotoxic drugs, cytosensor, microphysiometry.

Introduction

There are a number of different non-clonogenic short-term *in vitro* assays for evaluation of drug-induced cytotoxicity and/or inhibition of proliferation. Most of these assays depend on surrogate endpoints for cell kill or inhibition of cell proliferation, such as measurements of macromolecular synthesis (DNA/protein synthesis), membrane integrity (dye exclusion/inclusion tests) or metabolic activity (redox probes) (reviewed elsewhere[1,2]). A common feature of these methods is that the measurements are performed at a fixed, single time point, mostly after 48–96 h of drug incubation.

These *in vitro* assays have been widely used in the area of anticancer drug discovery and preclinical development using a broad range of cellular systems. The model systems include both established cell lines as well as primary cultures of tumor cells from patients.[3–5] Recent studies have indicated that pharmacological response patterns obtained in cell lines using these assays provide rich information on putative cellular targets for cytotoxic drugs and the information derived from such studies may be of great value in preclinical evaluation of these drugs.[6,7] Whereas the effects of cytotoxic drug interaction with, for example, DNA and pivotal cellular enzymes are not immediately detectable, possible effects on cellular metabolism may be direct and their measurement could provide new knowledge on the action of cytotoxic drugs.

Microphysiometry is a relatively new method for measurements of drug-induced cellular responses using a commercially available instrument, the Cytosensor® (Molecular Devices, Sunnyvale, CA). The endpoint is cellular metabolism measured as the rate of extracellular acidification over a pH-sensitive silicon chip.[8,9] A light-addressable potentiometric sensor is used to determine the very small changes in pH due to excretion of catabolic products from metabolic processes in human cells.[8,10,11] In the field of pharmacology, the main application of the Cytosensor technology has so far been the study of drug-receptor-ligand interactions, since extracellular acid-

This study was supported by grants from the Swedish Cancer Society and the Lions Cancer Research Foundation.

Correspondence to S Ekelund

ification can be detected with the Cytosensor when a wide variety of cellular receptors are activated.[12]

In the present study we tested the applicability of the Cytosensor technology for the study of cytotoxic anticancer agents.

Materials and methods

Cells and experimental drugs

Eight standard cytotoxic drugs with different postulated mechanisms of action were investigated: vincristine, paclitaxel, cisplatin, melphalan, doxorubicin, etoposide, cytarabin and topotecan (Table 1). In addition to the indicated standard concentrations two additional concentrations were tested in separate experiments to evaluate any concentration-effect relationship. The compounds were obtained from commercial sources and were diluted as prescribed.

The cytotoxic drugs were tested on the generally drug-sensitive lymphoma U-937-GTB cell line[13] at concentrations known to be highly cytotoxic (cell survival values less than 10%) after 72 h of continuous drug exposure.

The cell line was grown in cell-culture flasks kept in an atmosphere containing 5% CO_2 and at a temperature of 37°C. Cell culture medium RPMI 1640 (HyClone, Cramlington, UK) was supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone), 2 mM glutamine, 50 μg/ml streptomycin and 60 μg/ml penicillin (HyClone). Growth and morphology of the cell line were monitored two or three times a week.

Patient samples

Additional experiments were made using cryopreserved tumor cells from patients with ovarian carcinoma.

Cell preparation of solid tumors has been described in detail previously.[14] Briefly, tumor tissue from solid tumors was minced with scissors and then enzymatically digested, followed by filtering through a nylon mesh, centrifugation and a Percoll (Pharmacia-Up John Uppsala, Sweden) purification step. Cell viability was determined by the Trypan blue exclusion test.

The Cytosensor method

Acidification rate was investigated in the Cytosensor Microphysiometer using 1.5×10^5 cells/cup. The human histiocytic lymphoma cell line U-937 GTB[13] was used for most of the experiments. The U-937 GTB cells are non-adherent and accordingly need to be immobilized in the cell capsule during the experiments. This was attained by using an agarose mixture (Molecular Devices) that fixed the position of the cells during the 20 h perfusions. The capsule cups were installed into the sensor chambers of the Cytosensor and perfused by medium (National Veterinary Institute, Uppsala, Sweden) at a flow rate of 100 μl/min. The experimental medium was a low buffering-capacity medium with no bicarbonate but with 6 ml/l 4 M NaCl to preserve osmotic balance, and also contained 10 ml/l 200 mM L-glutamine, and 10 ml/l penicillin and streptomycin. A repeated (120 s) pump cycle was used throughout the experiments where the rate of acidification was

Table 1. A comparison of the eight drugs investigated in the Cytosensor experiments

Drug	Concentration (μg/ml)	Mechanism of action	Acidification peak	Maximum stimulation at peak (% of control)	Metabolic activity at 10 h (% of control)	Metabolic activity at 20 h (% of control)
Vincristin	0.5	tubulin active agent	yes	128.0 (2.2)	61.5 (2.7)	19.3 (2.2)
Paclitaxel	0.5	tubulin active agent	no	–	105.9 (6.2)	113.8 (13.8)
Cisplatin	2.5	alkylator	no	–	98.3 (2.3)	94.0 (6.3)
Melphalan	2.5	alkylator	no	–	50.4 (7.6)	8.4 (3.1)
Doxorubicin	2.5	topoisomerase II inhibitor	no	–	73.0 (7.0)	37.9 (8.9)
Etoposide	5.0	topoisomerase II inhibitor	yes	137.2 (12.0)	68.2 (8.8)	20.8 (8.4)
Cytarabin	0.5	antimetabolite	yes	105.2 (3.5)	74.7 (2.8)	56.3 (5.5)
Topotecan	2.5	topoisomerase I inhibitor	yes	103.1 (2.8)	34.4 (3.0)	3.4 (0.4)

The suggested mechanism of action and the concentrations used in the experiments are indicated. These concentrations are known to reduce cell survival to below 10% after 72 h exposure. Values are expressed as per cent of untreated control and SEM are indicated within the parentheses. The results are from three to six experiments for each drug.

measured during a period of 30 s followed by 90 s perfusion. Drugs and solvents were diluted in medium and perfused through a second channel after 1 h of baseline establishment. The rate of acidification of the medium during the recording time was calculated by the Cytosoft program as $-\mu V/s$ and was presented as per cent change from the initial value at start of the experiment when metabolic activity was set to 100%.[11,12] The results were generally presented as acidification rate in per cent of untreated control.

Fluorometric microculture cytotoxicity assay (FMCA) reagents and procedure

Experimental microtiter plates for the FMCA were prepared in advance using a pipetting robot (Propette) filling each well with 20 μl of drug solution of 10 times the final concentration. The plates were stored frozen at -70°C without any change of cytotoxic activity.[15] Fluorescein diacetate (FDA; Sigma, St Louis, MO) was dissolved in DMSO and kept frozen (-20°C) as a stock solution (10 mg/ml) protected from light.

Viability of cells was determined by measuring fluorescence from cells with intact plasma membranes generated from hydrolysis of FDA to fluorescein. The principal steps of the FMCA have been described in detail previously.[15] Cell suspension was added (180 μl/well) to the thawed experimental plates giving 20 000 cells/well. Six blank wells received only culture medium and six wells with cells but without drugs served as control. The plates were incubated for 24 or 72 h at 37°C in humidified atmosphere containing 5% CO_2. After incubation the plates were centrifuged at 200 *g* during 5 min, washed with 100 μl PBS per well, FDA was added and then further incubated for 40 min. Fluorescence was read in a scanning fluorometer (Fluoroscan II, Labsystems Oy, Finland).

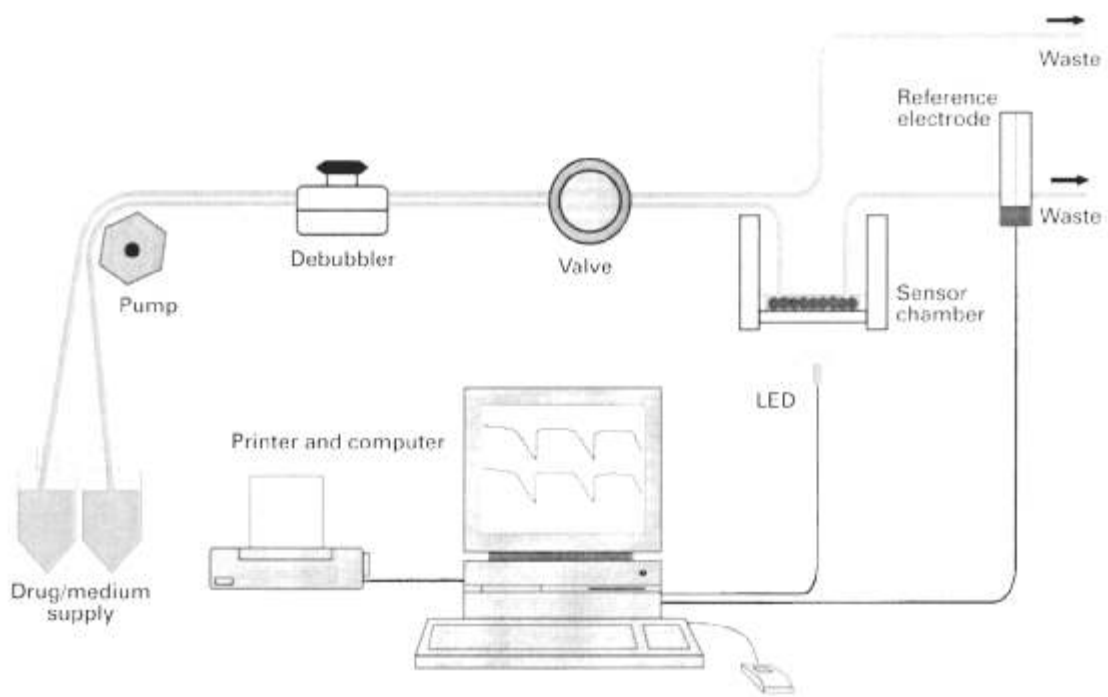

Figure 1. A Cytosensor system contains up to eight cell chambers where parallel measurement of metabolic activity can be made. Every chamber has two separate containers with a channel each for fluid supply, one leading the low buffering medium and the other leading drug diluted in medium. Peristaltic pumps, one for each sensor chamber, pump the fluid to a debubbler, a station with elevated temperature and with a porous, hydrophobic membrane that eliminates air bubbles and reduces the level of dissolved gases in the fluid. A valve then determines which channel that is routed into the sensor chamber and which is directed to the waste. In the sensor chamber the temperature is fixed to 37°C. The silicon chip functions as a light-addressable potentiometric sensor (LAPS) that allows the Cytosensor to detect small changes in pH. An electrical contact and the silicon chip in the censor chamber in combination with the reference electrode completes a circuit. The pH changes detected are visualized on a computer screen as a curve of raw data for each chamber, with the unit $-\mu V/s$ corresponding to 1 mpH/min.[11]

Cell survival is presented as survival index (SI), defined as the fluorescence in experimental wells as a per cent of that in control wells, with blank values substracted.

Results

Figure 1 schematically illustrates the experimental set-up. In Figure 2 the mean of five consecutive microphysiometry recordings from continuous exposure to vincristine is shown along with a mean control curve. Mean control coefficient of variation at 20 h was 15.7% (n=12).

Each investigated drug induced a reproducible and characteristic response pattern, as exemplified in Figure 3. From these patterns, key features, like stimulation and inhibition of acidification, the time-point when the response curves of the drugs fall below the control curve, and the maximum inhibition of acidification at 20 h, could be quantified. The key data are displayed in Table 1. The greatest stimulation at peak, expressed as per cent of control, was seen for etoposide followed by vincristine, and the lowest metabolic activity at 20 h was seen for topotecan and melphalan.

A distinct stimulation was seen for the mitotic inhibitor vincristine, which induced a sharp peak of early stimulation, followed by a steep decline of acidification to an almost complete inhibition of metabolism (Figure 3). A similar curve was seen for cytarabin but the maximum inhibition after 20 h was considerably less. Two of the drugs, topotecan and doxorubicin, showed a tendency to inhibit acidification before the stimulation and then finally turned over to the inhibition phase. Topotecan had a short stimulation while doxorubicin followed the control during 6 h before the acidification rate declined. Topotecan was the drug showing the steepest decline of metabolic activity. After 10 h the acidification rate in response to topotecan was only about 34% of baseline and at the end of the experiments there was essentially no detectable response. The alkylator cisplatin followed the control curve during the whole experiment though the concentration used in the experiments is known to induce complete cell kill after 48–72 h of drug exposure. Melphalan also tended to follow the control the first 5 h before the metabolic activity finally declined. Paclitaxel was the only drug for which the response curve was continuously above the control. For the clinical formulation of etoposide, a topoisomerase II inhibitor, there were indications of effects of the solvent, Tween 80, *per se*. The active substance alone did not give as rapid stimulation compared to the clinical preparation and the solvent alone (not shown).

All of the investigated drugs, except cisplatin, showed some stimulation of acidification rate during the experiments. For all drugs producing a reduced metabolic rate at 20 h a concentration-response relationship was observed, the steepest was seen for vincristine (Figure 4).

At 72 h the SI values were below 8% for all eight drugs, indicating an almost complete cell death, measured as loss of membrane integrity (Figure 5B). To mimic the time frame of the Cytosensor experiments, cells in FMCA experiments were also exposed to drugs during 24 h (Figure 5A) indicating that the time when pronounced cytotoxicity is observed differs between the investigated drugs as well as with assay type (Figures 5 and 6).

The drug effects measured at 20 h with the Cytosensor were irreversible and correlated reasonably well with parallel measurements of membrane integrity in the FMCA, for all drugs except for melphalan and vincristine for which cell survival index using FMCA was higher compared to the inhibition observed in the Cytosensor (Figure 6).

Cytosensor experiments were also made measuring the cytotoxic drug activity in patient tumor cells. In ovarian carcinoma cells the metabolic pattern of doxorubicin resembled those of the cell lines, but for cisplatin an early stimulation was observed (Figure 7).

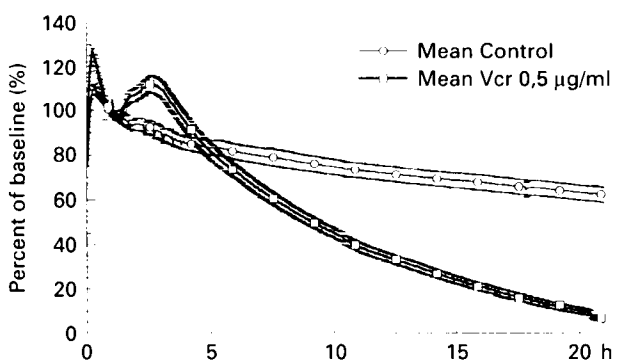

Figure 2. Reproducibility is indicated by a mean curve (n=5) from perfusion with vincristine, 0.5 µg/ml and a mean control curve (n=12) made with the histiocytic lymphoma cell line U-937 GTB. SEM are shown for both curves.

Discussion

Changes in extracellular acidification have been detected with the Cytosensor after drug-induced activation or inhibition of many cellular receptors.

These include muscarinic, α and β adrenergic, dopaminergic, growth factor, and cytokine receptor systems.[9,12,16-18] The kinetic profiles of the receptor responses differ greatly but it is commonly transient

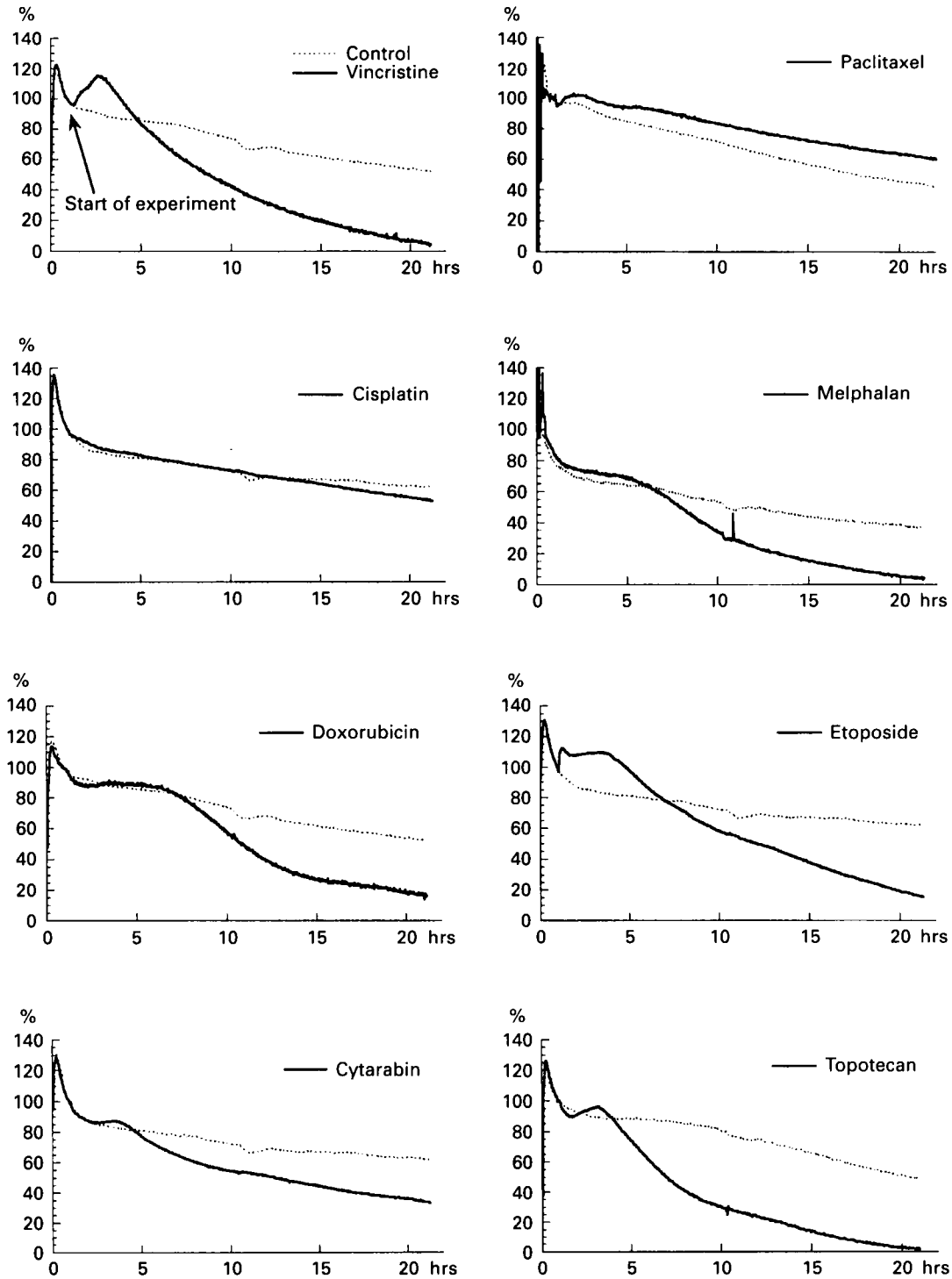

Figure 3. The effect of eight cytotoxic drugs, with the mechanisms of action detailed in Table 1, on the metabolic activity of U-937 GTB cells. Each drug produced a characteristic metabolic pattern. One typical experiment out of three for each drug is shown. In each experiment the first hour of perfusion was with medium alone to establish a baseline as indicated in the vincristine panel. The *y*-axis indicates the per cent of change of cellular metabolism as compared to the baseline value, while the *x*-axis indicates time from start of the experiment.

with an overshoot that settles down to a new steady-state characteristic of the new physiological state in the presence of the ligand. These responses have been found to provide detailed information on the physiological consequences of receptor activation or inhibition, and appear to be relatively independent of the ligand-receptor transduction mechanism and cell type.[8,9]

In the present study Cytosensor measurements of metabolic activity revealed an unexpected variety of characteristic response patterns for the different cytotoxic drugs tested. The metabolic response patterns were reproducible and could also be quantitatively analyzed. Early classification of new agents according to pharmacological similarity or dissimilarity to standard drugs with known mechanism of action is desirable in new drug discovery and development, and the drug-specific patterns generated by the Cytosensor may provide unique and important additional information in this respect. To what extent the response patterns obtained are independent of cell type may be an important question for some applications. For at

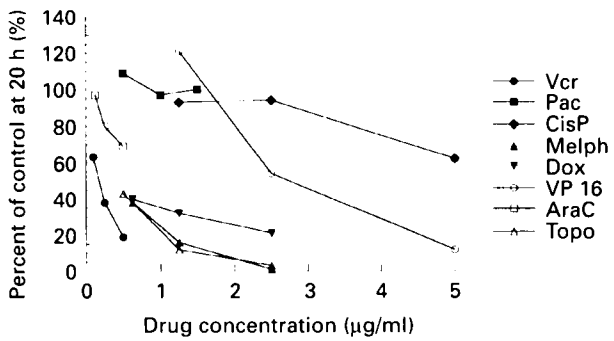

Figure 4. Concentration–response relationships for the eight cytotoxic drugs using U-937 GTB calculated from Cytosensor data at 20 h.

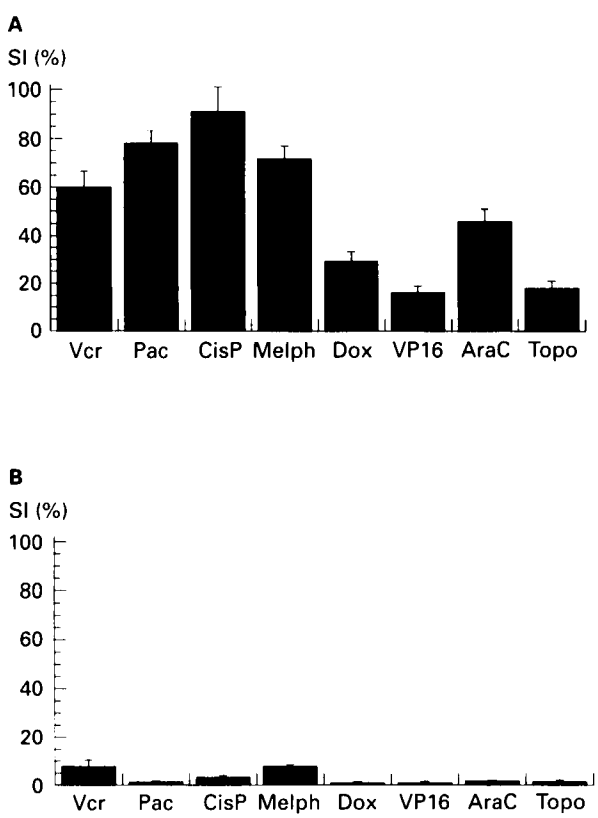

Figure 5. Cell survival (SI) after 24 (A) and 72 (B) h of continuous drug exposure as detected by the FMCA. Drug abbreviations: vincristine (Vcr), paclitaxel (Pac), cisplatin (CisP), melphalan (Melph), doxorubicin (Dox), etoposide (Etopo), cytarabin (AraC) and topotecan (Topo).

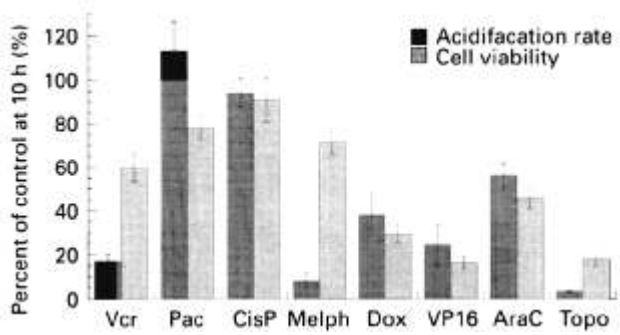

Figure 6. Comparison of the metabolic activity after 20 h of drug exposure and measurements of survival index (SI) as detected by the FMCA.

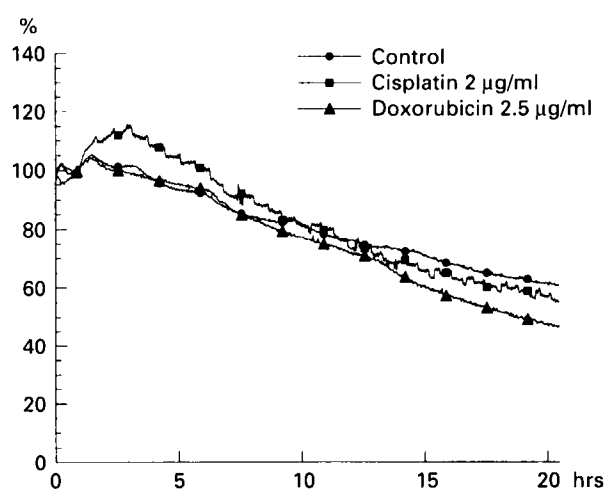

Figure 7. Cytosensor readings of tumor cells from patient samples with ovarian carcinoma.

least two other drug- sensitive cell lines, the qualitative nature of the response patterns induced by doxorubicin and vincristine were found to be similar (not shown). However, this issue remains to be fully clarified in future studies.

The opportunity to continuously follow the metabolic state of the drug-exposed cells appears to be one of the major advantages of this method. This 'on line' capacity facilitates the determination of timepoints of critical events and also when viability of cells declines to an irreversible state, by allowing for recovery under drug-free conditions. This feature makes microphysiometry unique compared to most other *in vitro* assays.

Mechanisms of drug action could also be further elucidated by studying the dependency of the metabolic response on specific signal pathways or target molecules by pharmacological modulation and application of specific cell systems. In the former approach, a variety of pharmacological tools for manipulation of various signal transduction pathways could be used.[19] The latter approach could be based on the parallel testing of cell systems differing in the expression of specific gene products.[20] Other potentially valuable applications for anticancer drug evaluation and characterization could be the detailing of schedule dependency and drug interactions. The feasibility of easy change of exposure protocols in eight parallel channels makes the Cytosensor especially well suited for such applications.

For all drugs producing a fall in acidification rate at 20 h, a concentration-response relationship was observed. The drug effects measured at 20 h correlated reasonably well with parallel measurements of cell viability using the FMCA, for all drugs except for melphalan and vincristine for which cell survival was higher as compared to the inhibition observed in the Cytosensor. This discrepancy may be related to differences in the overall conditions of tests. In the Cytotsensor experiments drug-containing medium is stored at room temperature before being heated and perfused through the cell chamber. The medium surrounding the cells is thus continuously replenished, and metabolites and waste products are removed, which is not the case in the microtiter plate-based assay. The microtiterplates are incubated in 37°C during 72 h and there is no change of medium. In fact, both melphalan and vincristine were shown to decompose more rapidly in 37°C compared to room temperature (data not shown). These features of the Cytosensor has to be kept in mind when comparing results obtained with other cytotoxicity assays.

In the present study we also demonstrated the feasibility of measurement of the cytotoxic drug activity profile in primary cultures of patient tumor cells using the Cytosensor. The response patterns of the drugs tested were qualitatively similar to those obtained in the U-937 cell line, except for cisplatin, which showed initial stimulation of acidification rate in this cisplatin-sensitive tumor type. Whether this reflects the presence of cell type-specific response elements mediating apoptosis remains to be clarified. The mapping of signal transduction pathways that mediate the response to biological agents such as the retinoids, interferon's, interleukins, growth factors and other cytokines in individual fresh patient tumor cell samples is another potentially interesting future application of this technology. Such knowledge could be used to predict clinical outcome for individual patients.

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(Received 16 April 1998; accepted 28 April 1998)