A Simple Non-destructive Test of Cellular Activity (NTCA) for *In Vitro* Assessment of Cancer Cell Chemosensitivity/Resistance

IRENA NETIKOVA[1], EVA BURSIKOVA[2,3,4], PAVEL VESELY[3,4], MARKETA PRCHALOVA[2,4], ZDENEK KLEIBL[2] and EVA MATOUSKOVA[2,3,4]

[1]*Department of Cytostatics Preparation, General Faculty Hospital Prague, Prague;*
[2]*Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine, Charles University Prague, Prague;*
[3]*Department of Cell Biology, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague;*
[4]*Prague Burn Centre, Third Faculty of Medicine, Charles University Prague, Prague, Czech Republic*

Abstract. *Determination of chemosensitivity/chemoresistance is becoming increasingly important for individualization of breast cancer chemotherapy. We developed a simple non-destructive test of cellular activity (NTCA) for assessment of the cytopathic effect of antitumour drugs in vitro. Contrary to routinely used methods (e.g. MTT), besides the comparative evaluation of metabolic activity using pH (given by the medium colour), the NTCA enables the simultaneous assessment of proliferation and morphology of cultured cells (phase-contrast microscopy) at any time during the incubation with cytostatics. Moreover, the regenerative potential of the cells can be examined by cell recovery and growth after drug removal. We provide evidence for the relevance of NTCA in chemosensitivity testing of primary breast cancer cells and breast cancer cell lines for cisplatin, gemcitabine and tamoxifen. NTCA represents a simple addition to the chemosensitivity assessment and could also serve for rapid screening of new antitumour strategies.*

Individualization of breast cancer chemotherapy by determining the chemosensitivity of tumour cells is becoming increasingly important at a time of chemotherapy tailoring. Various *in vitro* assays have been developed for screening drug efficacy and toxicity, as well as for individual chemosensitivity/chemoresistance testing (1). These assays are usually based on evaluation of drug effects on the metabolic activity of the cells. In most cases, the endpoints of the tests are destructive for the cells used, as in *e.g.* the dye exclusion (2), radioactive incorporation (3), MTT (4, 5) or ATP bioluminescence assays (6). It means that cells used in the assay are destroyed when the result is determined, usually immediately after the antitumour drug exposure. Other types of tests are based on the selective growth of transformed cells in agar culture systems (7-9), however, many types of human tumour cells do not grow in semi-solid media.

Seldom have dynamic tests based on a running evaluation of living cell responses been used. This is surprising as non-destructive tests have some advantages, mainly the possibility to observe and measure several types of cell responses, such as metabolic activity, cell growth and cytopathic effect (CPE), repeatedly and in parallel in the course of the test. Moreover, regeneration of cells can be followed for a prolonged period of time as needed. Therefore readjustment of the test according to the early results is possible.

Biochemical activity tests can be represented by the MTT assay, which is based on the ability of living cells to reduce a soluble yellow tetrazolium salt (MTT) into an insoluble blue formazan precipitate (4). Our laboratory has long-standing experience with this assay (10). It is a simple and rapid method, suited to routine testing. However, it is impossible to differentiate the cytotoxic from the cytostatic effect because of the destructive endpoint after a 3-day incubation.

Another common obstacle to the wider use of chemosensitivity/chemoresistance testing of individual tumours is insufficient cellular yield from biopsy specimens, which are smaller with earlier diagnosis. We have developed a culture method to achieve sufficiently large yields of epithelial cells from small biopsy specimens of breast tumours to be used for the prediction of drug resistance using the MTT assay (10).

Correspondence to: Eva Matouskova, Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine, Charles University, U Nemocnice 5, 12108 Prague 2, Czech Republic. e-mail:matous@beta.img.cas.cz

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The aim of the current work was to develop and verify a non-destructive chemosensitivity assay on cells cultured from primary breast tumours that could complement or substitute the MTT assay. We aimed at a running test with which we could simply and repeatedly evaluate the cytopathic/cytostatic effect, metabolic activity, proliferation, cell changes, or regenerative ability of tumour cells and/or their alteration after the exposure to cytotoxic agent(s). Such an assay should be useful for individual chemosensitivity/chemoresistance assessment as well as for the screening of potentially new antitumour drugs.

Materials and Methods

Cell cultures. A new human mammary cell line, EM-G3, established in our laboratory (11) and four primary cell cultures isolated from biopsies of human breast carcinomas (L179, L180, L181, L182) were used in this study. The medium used for all mammary epithelial cells was H-MEM supplemented with all non-essential amino acids, 0.12 g/l sodium pyruvate, 1 g/l $NaHCO_3$, 10% bovine serum, 2% foetal bovine serum, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin (Novo Nordisk, Coppenhagen, Denmark), 10^{-10} M choleratoxin, 5 ng/ml EGF (Sigma, St. Louis, USA), 200 U/ml penicillin and 100 μg/ml streptomycin. Primary cells were cultured on a feeder layer of lethally irradiated 3T3 cells (10). The cells for propagation were cultured in tightly closed tissue culture flasks. Culture dishes and microplates for cytotoxicity tests were maintained in a 3.5% CO_2 humidified atmosphere at 37˚C. For NTCA tests the medium contained phenol red for pH indication. In the MTT test the medium was used without phenol red.

Cytostatics. The antitumour drugs tested were the nucleoside analogue gemcitabine (gem; Gemzar, Eli Lilly, Canada) and the alkylating agent cisplatin (cDDP; Platidiam, Pliva-Lachema a.s., Brno, Czech Republic). Their tested concentrations are given in multiples of the peak plasma concentration (ppc), which is the concentration of the drug in the patient's serum achievable with therapeutic dose regimens (gem ppc 1x=30 μg/ml; cDDP ppc 1x=5 μg/ml).

Tamoxifen (Tx; Sigma, St. Louis, USA), which is an antagonist of the oestrogen receptor, was used in combination with cisplatin. Tx at a concentration of 10 μM was applied for 48 h; medium with tamoxifen was then removed and changed with the medium containing cisplatin in concentrations 1x, 2x and 3x ppc.

MTT assay. The epithelial cells were plated into 96-well microplates (10,000 cells per well in 80 μl media without phenol red). After 24 h, 20 μl of tested drugs were added for 24 h, 72 h or 3 x 24 h. A volume of 10 μl of 5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) solution in PBS were added. Six hours later, 100 μl of 10% SDS solution (lauryl sulfate; Sigma), pH 5.3, were added and the plates incubated overnight. The optical density of wells (intensity of blue colour depending on mitochondrial activity) was measured at the end of the incubation using an ELISA plate reader (570 nm test wavelength and 630 nm background wavelength).

Table I. *Review of evaluation criteria.*

Growth (cell density)		Evaluation
25% of culture area covered by cells		+
50% of culture area covered by cells		++
75% of culture area covered by cells		+++
100% of culture area covered by cells		++++
CPE (damage)	**Description of damage**	
25%	First changes in morphology, enlarged cells, vacuoles	*
50%	Visible cell destruction, decrease in cell number	**
75%	Majority of cells irreversibly disintegrated	***
100%	All cells destroyed	****

Metabolic activity criteria (pH): colour	pH range	Metabolic activity	
Yellow	6.6-6.8	high	++++
Yellow-orange	6.9-7.1	medium	+++
Orange	7.2-7.3	medium/low	++
Orange-red	7.4-7.6	low	+
Red	7.7-8.0	none	-

In NTCA cell growth was evaluated using cell density (phase-contrast microscopy), CPE was evaluated by cell damage (phase-contrast microscopy or stained cells), metabolic cellular activity was examined using pH as given by the medium colour. The pH of the medium should be evaluated within 1-5 min after removing cell cultures from the CO_2 incubator. The medium colour is visually compared with the colour of phosphate buffers containing phenol red set to a range of pH 6.6 to 8.0 and kept in sealed glass ampoules.

Development of the NTCA assay. The design of the test had to enable simultaneous readings using phase-contrast microscopy of conditions of a set of attached cell cultures exposed to various concentrations of the drug(s) *versus* controls. Changes of cell spreading, morphology, proliferation, CPE and cell death could thus be evaluated. For running readings of the metabolic activity, estimation of pH given by the colour of the medium (based on H-MEM) containing phenol red was used. The duration of the assay can be modified if the regenerative potential of the cells after drug removal is of interest. At the end, fixed and stained cultures demonstrate the final effect of the drug(s) and can be kept as evidence.

NTCA layout: 24-well multi-dish plates (16 mm well diameter, 200 mm^2 area, 4 rows, 6 columns) were selected for an optimal compromise between optical properties for phase-contrast imaging and area related to the necessary number of cells that was set at 250 cells/mm^2, *i.e.* 50,000 cells/well in 1.5 ml culture medium. In the basic layout, four cell types were seeded in rows for five concentrations of the tested cytostatic against the control in columns. One to two days after cell seeding the cytostatics were added at pre-calculated concentrations for 24, 48 or 72 h incubation.

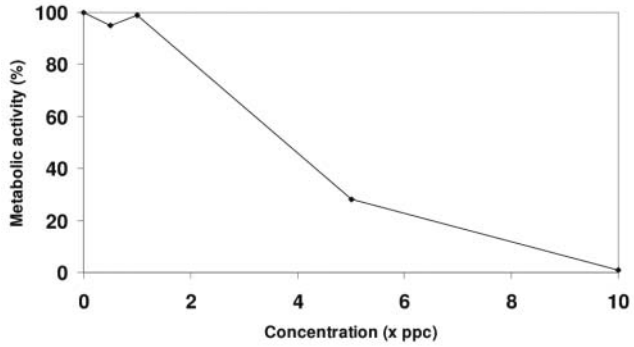

Figure 1. *Effect of cisplatin on EM-G3 cells determined using the MTT assay. Cisplatin at concentrations 0.5-10x ppc was applied for 3 days, in fresh medium daily. Note that at 0.5x and 1x ppc, the metabolic activity was practically the same as in the control (0x ppc).*

NTCA running daily evaluation (Table I): pH (medium colour) was examined as representative of cellular metabolic activity. Cell density and morphology revealing the emerging cytopathic effect were assessed microscopically. At the end of the test, the microplate is fixed and stained using May-Grünwald and Giemsa-Romanowski solutions to show final cell density and morphology of damaged cells.

Results

Validation of the NTCA assay (comparison of NTCA with MTT). Comparison of NTCA with MTT is illustrated by the effect of cisplatin on EM-G3 cells. Results measured using MTT are shown in Figure 1, results determined using NTCA are shown in Figure 2. The cisplatin was added to subconfluent cells approximately 48 h after seeding and applied for a total of 72 h (three applications of fresh solution at 24 h intervals) at concentrations 0.5x, 1x, 5x and 10x ppc. While the MTT test was abruptly ended after cisplatin exposure, the NTCA continued for another 15 days after cisplatin removal.

MTT results: Figure 1 shows results of the MTT test measured after 72 h of cisplatin exposure. The graph indicated that after application of low concentrations of cisplatin (0.5x and 1x ppc) the metabolic activity was similar to the control, nearly 100%. At a concentration 5x ppc of cisplatin the cells were partially damaged (27% of metabolic activity compared to the control) and at a concentration 10x ppc the cells died.

NTCA results (compared to MTT): Figure 2A shows images of the metabolic activity six days after cytostatic removal as expressed by the medium colour (pH) with its corresponding graphic evaluation. Figure 2B shows cell growth and CPE under phase-contrast microscopy of living cells and their corresponding graphic evaluation. Figure 2C shows cell growth and CPE on the final day of the experiment (15 days after cytostatic removal) when the cells were fixed and stained.

Metabolic activity as evaluated using the pH/colour of the media (Figure 2A) in NTCA corresponded well with MTT results. Application of cisplatin at concentrations 0.5x and 1x ppc did not influence metabolic activity of the cells, which was similar to that of the control (90%). Application of cisplatin at 5x ppc resulted in a decrease of metabolic activity to ~25%. The metabolic activity after 10x ppc cisplatin exposure was 0%.

Morphology reflecting cell growth and CPE could only be evaluated in the NTCA test. The morphology of living cells during the NTCA assay (Figure 2B, phase-contrast) and final morphology (Figure 2C, stained cells) after 0.5x and 1x ppc cisplatin exposure indicated a decrease in cell proliferation and changes in morphology (fewer cells of larger size, Figure 2B and C). These changes were not determinable using MTT. After exposure to cisplatin at 5x ppc most cells were damaged (growth +, CPE***), and at a concentration 10x ppc the cells died (CPE****) (Figure 2B, 2C). The gradual changes in metabolic activity, cell growth and CPE can easily be evaluated for several days, or even weeks, as the NTCA test is not destructive for the tested cells until the decision on experimental termination is taken. The possibility to observe cell regeneration for a prolonged period of time is important.

By comparison of NTCA and MTT, the higher sensitivity of NTCA is apparent, particularly when the criteria of living cell morphology using phase-contrast microscopy (running examination) and stained cells (final cell density and morphology) were considered. By repeated microscopic observations, the course of subsequent cell changes could be seen.

Sensitivity of EM-G3 cells and primary breast cancer cells to gemcitabine. The effect of gemcitabine was tested on the EM-G3 cell line and on four primary cell populations derived from human breast carcinomas. Five concentrations of gemcitabine (1x, 3x, 10x, 30x, 50x ppc), were applied for 24 h (Figures 3, 4). The results were evaluated with the MTT test immediately after drug exposure (Figure 3) and NTCA (24 h gemcitabine exposure, change on fresh medium, 3 weeks for regeneration, staining) (Figure 4).

While the results of MTT did not show any differences between chemosensitivity of the tested cell samples early after drug exposure (Figure 3), in a parallel performed NTCA test obvious differences were revealed (Figure 4). Differences were observed during the entire test on living cells (pH, morphology under phase-contrast microscopy) and emphasized by final staining. The highest concentration at which cells survived is indicative of their resistance. From the staining pattern in the NTCA assay,

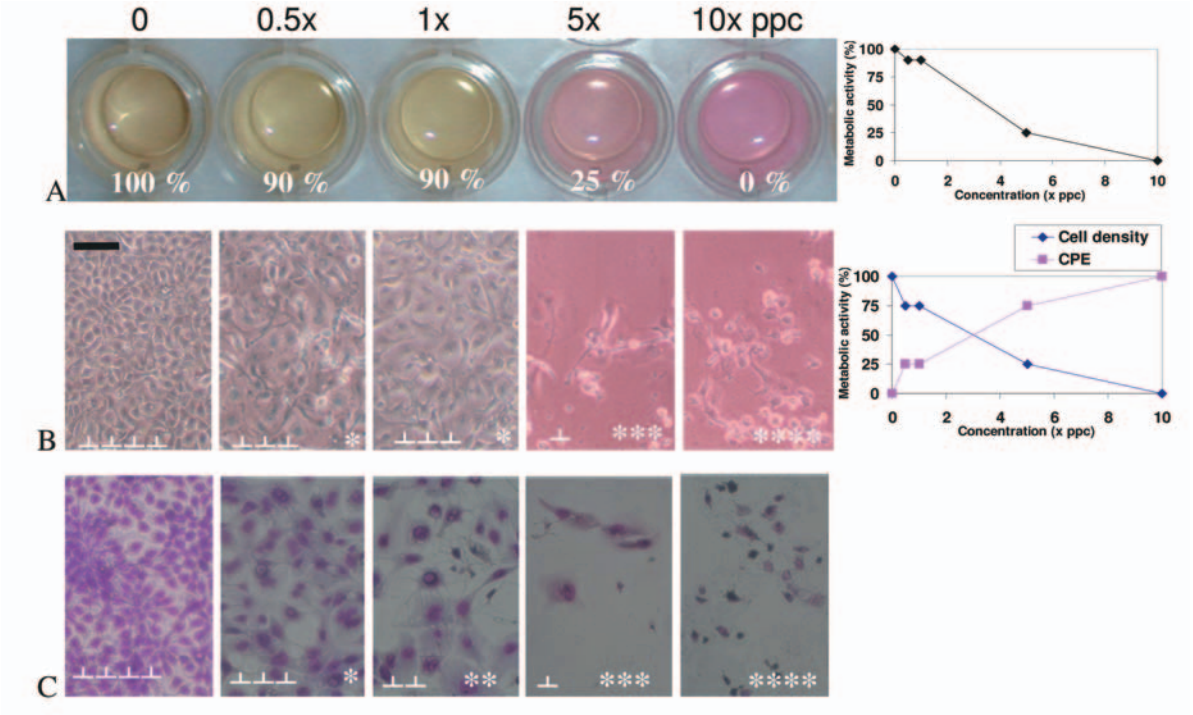

Figure 2. *Effect of cisplatin on EM-G3 as determined using the NTCA assay. A) Metabolic activity shown by the pH/colour of the media and its graphic presentation. B) Cell growth/CPE (+/*) shown by the morphology of cells under phase-contrast microscopy 6 days after cisplatin removal and its graphic presentation (cell density: blue curve, CPE: red curve). C) Growth/CPE shown by the morphology of stained cells 15 days after cisplatin removal. Note in B and C changes in morphology and cell density at 0.5x and 1x ppc compared to control and survival of some cells at 5x ppc. Cell growth: + to ++++, CPE: * to ****, bar 25 mm.*

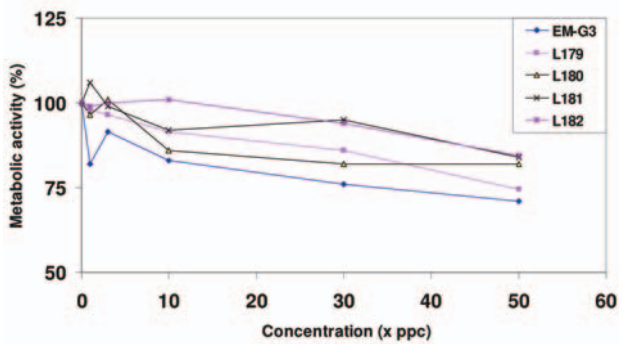

Figure 3. *Sensitivity of the EM-G3 cell line and four primary cell populations derived from breast tumours to gemcitabine as measured using the MTT assay. Gemcitabine at different concentrations was applied for 24 h and metabolic activity assessed.*

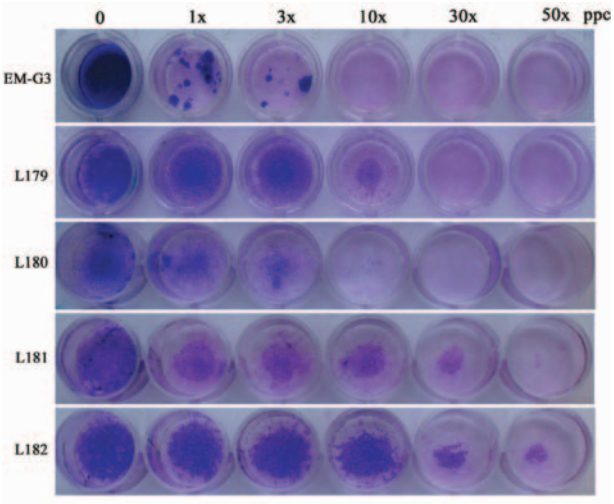

Figure 4. *Sensitivity of the EM-G3 cell line and four primary cell populations derived from breast tumours to gemcitabine as shown by NTCA-final staining. Gemcitabine at different concentrations was applied for 24 h to the EM-G3 cell line and to primary cells L179, L180, L181 and L182. After three weeks the cells were fixed and stained. Note that the different sensitivity to gemcitabine of individual cell types is clearly visible.*

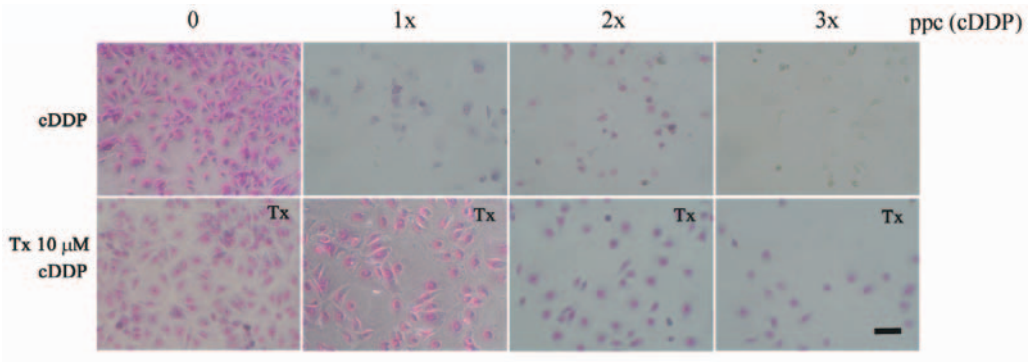

Figure 5. *Effect of tamoxifen/cisplatin on MCF7 cells. The upper row shows the effect of cisplatin alone, cisplatin was applied to subconfluent MCF7 cells in a single application on the first day of the experiment. The lower row shows the effect of cisplatin on cells pre-treated with 10 μM tamoxifen; tamoxifen was applied for 48 h to subconfluent MCF7 cells on the first day of the experiment; cisplatin at concentrations 1x ppc, 2x ppc or 3x ppc was then added by a single application for 72 h exposure. After this period all cells were stained (bar 20 μm).*

the chemosensitivity/chemoresistance of individual cell types is simply observable, as described further: EM-G3 cell line: regeneration of cells (growth of cell colonies) was observed at gemcitabine concentrations 1x ppc and 3x ppc; at gemcitabine 10x-50x ppc no cells survived (Figure 4, 1st row); L179: a fraction of cells survived at a concentration of 10x ppc (Figure 4, 2nd row); L180: a fraction of cells survived at a concentration of 3x ppc, small colonies were also observed at 10x ppc (Figure 4, 3rd row); L181: resistant cells, a fraction of cells survived at a concentration of 30x ppc, small colonies survived even at 50x ppc (Figure 4, 4th row); L182: resistant cells, substantial cell fractions survived at concentrations 30x ppc and 50x ppc (Figure 4, 5th row).

In conclusion, the EM-G3 cell line and L180 primary cells are the most sensitive cells to gemcitabine, L181 and L182 primary cells are the most resistant.

Effect of cisplatin on ER-positive MCF7 cells pre-treated with tamoxifen. An important possibility is provided with the NTCA assay, *e.g.* for monitoring the effects of several drugs applied sequentially to the cells. Our aim was to determine whether the pre-treatment of cells with anti-oestrogen tamoxifen would increase the effect of cisplatin in ER-positive MCF7 cells. Tamoxifen at a concentration of 10 μM was applied for 48 h to subconfluent MCF7 cells. Cisplatin at concentrations of 1x ppc, 2x ppc and 3x ppc was then added by a single application for 72 h. The cells influenced by cisplatin alone served as a control. Figure 5 indicates that tamoxifen did not increase the effect of cisplatin, but in fact slightly protected the cells. At a cisplatin concentration 1x ppc the cells were less damaged than when using cisplatin alone. By using MTT these differences were not recognizable.

Discussion

A simple non-destructive test of cellular activity (NTCA) for assessment of cancer cell chemosensitivity/resistance *in vitro* was developed. The test is based on a simple running comparative evaluation of metabolic activity and morphology of attached cells cultured from primary tumours and treated with cytostatics *versus* appropriate controls.

Despite new treatment strategies, disease progression resulting from intrinsic and acquired drug resistance still presents a major therapeutic challenge. The attempts to individualize chemotherapy by determining the sensitivity and resistance of tumour cells *in vitro* started shortly after the introduction of chemotherapy into the fight against cancer. A number of chemosensitivity assays have been introduced and significant predictive correlations between *in vitro* drug response assays and cancer patients' responses have been demonstrated. By eliminating ineffective agents, the patient is spared toxic treatment without benefit, while selection of agents active *in vitro* may increase the probability of a response *in vivo* (1). In different chemosensitivity tests alternative endpoints were developed, including measurements of mitochondrial function (*e.g.* MTT and ATP assays), membrane integrity assay, or differential staining cytotoxicity assay (1, 4, 6, 8).

The low cellular yield of a breast cancer sample represents a limiting factor for *in vitro* chemosensitivity testing. The diagnosis of tumours is shifting to their earlier developmental stages and therefore smaller amounts of tissue are available for diagnostic purposes. Cells multiplied *in vitro* from original tumours are then the only source for chemosensitivity testing (12-15). Although it is known that only selected cell subpopulations grow *in vitro* (16, 17), the assays using them proved to be capable of predicting

resistance of breast cancer patients to cytostatics (12, 18).

Using a combination of the feeder-layer technique for cultivation of mammary epithelial cells with the MTT test, we developed a method permitting extensive drug chemosensitivity/chemoresistance testing of cells isolated from thin-needle biopsy breast cancer samples (10, 19). The relatively low information capability of the MTT assay due to terminal destruction of the tested cells led us to develop an alternative/complementary test, NTCA, based on running evaluation of changes in living cells. In contrast to biochemical tests, it is possible to evaluate the developing topical effect of the cytostatics comparatively among several cell types. The cytopathic effect is signalled by the slower change of medium colour/pH compared to control wells (reflecting the degree of metabolic activity) and by cell morphology as examined under phase-contrast microscopy (*e.g.* changes in cell number, cell size, cell surface activity, degree of cell damage) even several times during the assay. The regenerative potential can be examined by cell growth after drug removal. An advantage is that NTCA can be terminated at a chosen appropriate time-point. By fixation and staining of cells, a durable image/evidence of affected cells is then obtained.

The applicability of NTCA was demonstrated on several examples. The effect of cisplatin on EM-G3 cells revealed that the degree of metabolic activity measured by MTT corresponded well with that measured by NTCA. However, dynamic microscopy showed the higher sensitivity of NTCA capable of registering even small subsequent changes in morphology and cell density in the course of the test. Cytostatic and cytopathic effects of gemcitabine were evaluated on EM-G3 and four primary cell cultures derived from solid breast tumours using NTCA and MTT. After 24 h of gemcitabine treatment there were no substantial differences between sensitivity/resistance of cell populations determined using the MTT assay. However, differences in chemosensitivity were revealed by NTCA 1-3 weeks post gemcitabine treatment. This event is understandable knowing the mechanism of gemcitabine action (antimetabolite with consecutive induction of apoptosis), which is manifested with a delay of several days. For less well-known effects of drugs, the long observation period available with NTCA should therefore be of advantage. By comparison of different types of finally stained cells in NTCA, the individual sensitivity and regenerative potential was clearly observable.

The effect of tamoxifen in combination with cisplatin was assessed on the oestrogen receptor-positive cell line MCF7. Surprisingly, in contrast to our expectations, pretreatment of cells with tamoxifen slightly decreased the effect of cisplatin. Using MTT, differences in the metabolic activity of tamoxifen-treated or untreated cells were not recognizable. We therefore suppose that NTCA can also be preferentially used for the prediction of two or more drug effects on individual cell types, be it in simultaneous or sequential applications. Moreover, besides individualized drug chemosensitivity/chemoresistance assessment, NTCA can be useful in screening of new potentially antitumour agents.

Conclusion

The main contribution of NTCA lies in its simplicity of operation, evident comparative evaluation and flexibility of the test management. Compared to the *e.g.,* MTT test, it is possible to reliably recognise a cell's condition before the actual end of the test.

Acknowledgements

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