Microplate spectrophotometry for high-throughput screening of cytotoxic molecules

C. Tomelleri*, C. Dalla Pellegrina† and R. Chignola†‡

*Department of Clinical and Experimental Medicine, University of Verona, Verona, Italy, †Department of Biotechnology, University of Verona, Verona, Italy, and ‡National Institute for Nuclear Physics, Trieste Section, Trieste, Italy

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Abstract

Objectives: High-throughput chemical and biochemical technologies are now exploited by modern pharmacology and toxicology to synthesize a multitude of new molecules with bioactive potential, or to isolate them from living matter. Testing molecules in cell systems on a large scale, however, is a rate-limiting step in drug discovery or in toxicity assessment. In this study, we developed a low-cost high-throughput method for first-level screening of cytotoxic molecules.

Materials and methods: We used microplate spectrophotometry to measure growth kinetics of human tumour cells that grow in suspension (Molt3) or adherent to the plastic surface of culture wells (HeLa) in standard RPMI medium. Cells were treated with colchicin, idarubicin or paclitaxel under various treatment schedules. The effects were quantified and compared with those measured by optical microscopy using the trypan blue dye exclusion method to reveal dead cells.

Results: Proliferation kinetics of tumour cells can be quantified by measuring variations in optical densities of cell samples at 410 and 560 nm wavelengths. For cells that grow in suspension, one single reading at 730 nm may be sufficient to reconstruct growth curves that parallel those obtained by direct cell counting. Effects of the cytotoxic treatments could also be quantified and results compared very favourably with those obtained using standard techniques.

Conclusions: Microplate spectrophotometry is a robust and sensitive method to monitor growth of animal cell populations both in the absence and in

the presence of cytotoxic drugs. This method implements existing technologies and can be fully automated.

Introduction

Chemistry and biology have undoubtedly faced a molecular revolution over the last decades that has driven development of technologies for characterization, isolation or synthesis of new molecules. Thousands of potentially bioactive molecules are now isolated from living matter or synthesized *de novo* at an incredibly high rate, an effort which is justified by the never-ending need to search for new drugs and consequently to overcome evolving resistance of biological systems to therapeutics. Drug research, however, cannot avoid biological testing (1), despite the fact that integration of chemistry and biochemistry with computer sciences is driving towards high-throughput screening (HTS) of those molecules that have, amongst many others, potential effects on specific molecular targets (2). Biological testing with cell-based systems is nonetheless, required when cytotoxicity of natural compounds, or that of synthetic molecules, must be assessed.

Cytotoxicity assays measure cell-killing potential of molecules (for example, anti-tumour drugs and also natural toxicants) *in vitro*. To this end, animal cells isolated from different tissues are used to collect appropriate information with predictive power, as no individual cell system can reproduce the complexity of the whole organism. In addition, cytotoxicity of a generic compound is always a function of concentration and exposure time on cells, thus no single end-point of its effects can be considered in toxicity assessment. Cytotoxicity assays therefore require planning experiments accurately with various cell types and with different concentrations of the compound that must also be administered to cells for different exposure time periods.

Basically, cytotoxicity tests measure how growth of cell populations is modified by presence of the toxic

Correspondence: R. Chignola, Dipartimento di biotecnologie, Università di Verona, Strada Le Grazie, 15 - CV1, I-37134 Verona, Italy. Tel.: +39 045 8027953; Fax: +39 045 8027929; E-mail: roberto.chignola@univr.it

compounds. A number of different strategies can be followed for this purpose, which may include (3): determination of probes incorporated into biological molecules, such as a labelled amino acid into proteins or a labelled base into DNA; quantification of probes retained and/or metabolized by live cells (for example, crystal violet, tetrazolium salts); quantification of chemical or fluorescent dyes that selectively enter dead cells (for example, trypan blue) or of enzymes released by dead cells (for example, lactate dehydrogenase); quantification of energy store provided by ATP. These and other experimental approaches have been critically reviewed under the light of their possible application for HTS of cytotoxic compounds, and here we wish to summarize the main criteria for developing appropriate assays that are relevant also to the present study (4). First, reagents (for example, probes dissolved in appropriate buffers) that must be added to cell cultures to carry out measurements of cell viability must not be toxic or to react with growth media components, and they must allow unequivocal discrimination between dead and live cells. This might not be the case for certain fluorescent probes that permeate membranes of dead cells, as cells that have been mortally wounded by a drug may retain membrane integrity for a long time (4,5). Secondly, HTS of cytotoxic compounds should be performed in such a way to avoid filtration, separation and washing steps that are time-consuming and difficult to automate. Thus, these assays need to be performed in microplates where incubation and analysis can be achieved in the same plate (4). Thirdly, HTS assays ideally should be capable of detecting a small number of cells that would allow for miniaturization of the tests. Finally, assays for HTS of cytotoxic compounds should be able to measure the absolute number of surviving cells rather than the percentage of dead cells (as determined by flow cytometry) to take into account cell proliferation during drug treatment (4–6).

A few experimental procedures satisfy the above points and these include bioluminescence detection of ATP (4,7,8), spectrofluorimetric detection of fluorescent dyes such as alamar blue (9) and the use of chemical, biochemical or optical sensors to monitor oxygen in cell cultures (10–12).

We have recently studied growth variability of tumour cell clones *in vitro*, using a human T-lymphoblastoid cell line (13). To quantify growth kinetics of cell populations, generated by hundreds of individual clones, has inspired well known procedures used in microbiology. We have exploited microplate spectrophotometry to measure turbidity of cell samples and have investigated under which limitations this technique could be used for animal cells. We found the method to be robust for this purpose and it has major advantages

over other methods, the most important being that measurements did not perturb cell population growth. Thus, the technique could be used to monitor proliferation of small populations of animal cells seeded in individual culture wells for long time periods. However, although turbidity measurements can successfully measure growth of cell suspensions, they can not do this for adherent cells.

In this article, we discuss how to exploit microplate spectrophotometry for quantitative HTS of cytotoxic molecules in cells that grow either in suspension or adherent to the plastic surface of their culture plates.

Materials and methods

Cells and reagents

Cells from the human T-lymphoblastoid line Molt3 (ATCC number CRL-1552) and from the human cervix carcinoma line HeLa (ATCC number CCL-2) were cultured in RPMI 1640 medium (Biochrom AG, Berlin, Germany; cat. no. F1215) supplemented with 2 mM glutamine (Sigma, St. Louis, MO, USA), 20 mg/l gentamycin (Biochrom AG), and 10% heat-inactivated foetal bovine serum (Biochrom AG, cat. no. S0115). Cells were grown at 37 °C in a humidified 5% CO₂ atmosphere in T75 culture flasks (Greiner Bio-One, Frickenhausen, Germany) and were periodically diluted with fresh medium to avoid starvation.

Therapeutic drugs such as colchicine, idarubicin and paclitaxel were from Sigma, and were stored lyophilized at –20 °C and dissolved at appropriate concentrations in complete medium immediately before use.

Population growth assays

Cells from exponentially growing cultures were seeded at a density of 1000 cells/well into the wells of 96-well culture plates (Greiner Bio-One) in 150 µl complete RPMI medium. We used both flat-bottomed (cat. no. 650 180) and round-bottomed (cat. no. 655 180) 96-well plates. Cell population growth was monitored using a microplate spectrophotometer (Powerwave; Bio-Tek Instruments, Winooski, VT, USA) and raw data were analysed using dedicated KC4TM software. For each well, absorption spectra in the wavelength range between 380 and 750 nm were collected and were compared with those measured for culture wells containing medium only. Cell population growth was also measured in parallel assay by direct cell counting, using light phase-contrast microscopy (Olympus IX51; Olympus, Tokyo, Japan), a Burkert chamber and vital dye trypan blue (dilution 1:2) to exclude dead cells.

Cytotoxicity assays

Colchicin, idarubicin and paclitaxel were dissolved at appropriate concentrations (please see Results section) and used to treat cells as described elsewhere in this article. In all protocols, we used 96-well microplates to seed the cells. Questions concerning geometry of the wells will be addressed below.

Protocol 1: Cells were seeded at initial density of 1000 cells/well in 150 µl growth medium. After 24 h, they were treated with different drug concentrations (please see Results section) and incubated for further 24 h. Then, drugs were washed away by centrifuging plates at 300 g for 15 min and cells were allowed to grow in fresh medium without further washing.

Protocol 2: Cells were initially treated as described in Protocol 1, but in this case, the drugs were not removed from culture media and were allowed to react for the whole assay.

Protocol 3: Here, a fixed drug concentration was used to treat cells seeded at initial cell density of 1000 or 5000 cells/well.

In all experiments, cell population growth was monitored each day as described above. Each drug concentration was assayed in six independent wells and effects were compared to those of untreated cells, also in six replicates.

Quantification of the cytotoxic effect

Raw data (please see above) from six replicates were averaged and SD calculated. To quantify cytotoxic effects of the drugs, we first calculated the area beneath the growth curves as follows:

$$A = \sum_i x(t_i) \cdot \Delta t \quad (1)$$

where, A is the area beneath growth curves, $x(t_i)$ is the average experimental measure carried out at time i -th and Δt is the time-interval between two successive measures. We then considered the following parameter:

$$R_A = \frac{A - A_{\min}}{A_{\max} - A_{\min}} \quad (2)$$

where, $A_{\max}$ is the area beneath the growth curve calculated for control untreated cells and $A_{\min}$ is the area calculated for the background (that is, for cells that did not grow as a consequence of the full cytotoxic effect of the drug). Parameter R_A , therefore, varies from 0 (that is, complete cytotoxic effect) to 1 (no effect) and measures the normalized average effect for each treatment. The error around R_A values was calculated from SD of raw data, using common equations from error propagation theory.

Results and discussion

Measuring population growth of tumour cells by microplate spectrophotometry

Left panels of Fig. 1 show average absorbance spectra sampled at different time-points for both HeLa (top) and Molt3 (bottom) cells grown in wells of round-bottomed 96-well culture plates. Major differences in spectra measured for adherent cells and cells grown in suspension were observed, and these were dependent upon well-known properties of cells that grow in suspension, but not of adherent cells, to cluster by gravity at the bottom of the wells. Only a slight variation in absorption spectra was measured for wells containing growth medium only [not shown, see however (13)]. Two peaks at 410 and 560 nm were visible in both cases and these corresponded to absorbance properties of pH-sensitive dye phenol-red dissolved in RPMI growth medium. Absorbance intensities of the two peaks varied in time as a consequence of cell population growth and hence of acidification of the medium (because of proton secretion by cells). The ratio between optical density (OD) measured at 410 and 560 nm is a parameter proportional to population size. Top-right panel of Fig. 1, compares time-dependent variations of this parameter – measured for six independent HeLa cell cultures – with actual cell density data. Cell density was measured by direct cell counting by light microscopy, of live cells using trypan blue dye to exclude dead cells. This experiment revealed that OD ratio 410/560 nm can be used to quantify cell population increase in a well. Figure 1 (bottom-right panel), also shows that the method could be further simplified for cells that grow in suspension, by measuring absorbance at 730 nm only. At this wavelength, OD is not influenced by phenol-red absorption and level of sample turbidity is, nonetheless, proportional to population size (13).

Figure 2, however, shows that turbidity measurements could be carried out successfully for cell suspensions grown in round-bottomed, but not in flat-bottomed, 96-well culture plates, due to the reason that in the first case, cells can aggregate by gravity at the centre of the well, which lies on the axis of the optical system. On the contrary, OD ratio is a measure of cell population growth that is independent of well geometry (Fig. 2). Turbidity measurements here did not allow for quantification of cell population growth of adherent HeLa cells, grown either in round-bottomed microplates or in flat-bottomed microplates (data not shown). Thus, of the two proposed spectrophotometric methods, OD ratio should be considered to be the most reliable one to measure growth of different cell types, cultured in microplates with different well geometry.

Figure 1. Measuring tumour cell growth in individual wells by microplate spectrophotometry. *Left panels:* Traces in both panels represent mean absorbance spectra measured for six wells, where HeLa (top) or Molt3 (bottom) cells were cultured. Standard deviation is not reported for the sake of clarity; however, experimental error is shown in the right panels. *Right panels:* growth kinetics of HeLa (top) or Molt3 (bottom) cells measured for six cell populations grown in independent wells by two different assays: microplate spectrophotometry (HeLa cells: OD ratio 410/560 nm; Molt3 cells: OD at 730 nm) and direct cell counting of live cells, by light microscopy, using the trypan blue exclusion technique to reveal dead cells.

Microplate spectrophotometry does not allow for quantification of spontaneous cell death that occurs at the end of assays due to cell starvation, as dead cells are not removed from the wells (turbidity measurements), nor can they buffer environmental acidity (410/560 OD ratio). This is the reason why growth curves determined by spectrophotometry in Figs 1 and 2 deviate from cell density measurements at later times [please see Fig. 1 and also (13)]. Growth curves measured by microplate spectrophotometry, therefore, have a logistic shape (13) and can be fitted and analysed automatically using available algorithms developed for high-throughput analysis of logistic dose-response curves (14).

Treatment of tumour cells with cytotoxic drugs

Microplate spectrophotometry provides measures that are proportional to cell biomass. In addition, the method does not perturb cell population growth, nor requires additional reagents beside those that are already present in common growth media. As a consequence, one should be able to measure easily whole growth kinetics of cell populations subjected to cytotoxic treatments administered at different concentrations, and avoiding sampling and washing steps that are required by most conventional methods. Information on both dose and time of activity of a cytotoxic compound could be, in principle, obtained in the same

Figure 2. Turbidity measurements are affected by geometry of the wells in which cells are grown. Molt3 cells were grown in round-bottomed (U) or flat-bottomed (F) 96-well culture plates. At indicated time points, cell populations were measured using microplate spectrophotometry, and OD ratio 410/560 nm was calculated or OD at 730 nm was sampled. Data are mean $\pm$ SD values from six independent cell populations.

experiment. This is important, as no single end-point can be considered to define cytotoxic potential of a drug.

To demonstrate this, both HeLa and Molt3 cells were treated with three cytotoxic drugs, colchicin, paclitaxel and idarubicin, which are currently used in clinics, and which act with different molecular mechanisms (15–17). Here, cells were treated according to the three different protocols described in the Materials and methods section and results are shown in Figs 3–5. Each curve in Figs 3–5 has been obtained by averaging data measured in six independent wells where cells were allowed to grow for the whole assay. Thus, each well represents an individual experimental setting.

Figures 3–5 show that both spectrophotometric methods can be exploited to assess cytotoxic effects of drugs as a function of administered dose (panels a and d, 24 h treatment), of both dose and exposure time (panels b and e) and of initial cell density (panels c and f). Growth of tumour cell populations was inhibited by all used drugs at a given combination of concentration, exposure time and cell density. However, the data also show that our method is capable of highlighting transient cytotoxic effects, which are unlikely to be observed in conventional end-point assays. Clear examples can be observed in Fig. 5, panels b (HeLa cells continuously treated with 10 ng/ml paclitaxel) and D (Molt3 cells treated for 24 h with 1.25 ng/ml paclitaxel), where the

cytotoxic effects induced growth delay after which cells started to re-proliferate at a rate similar to that of control cells.

Cytotoxic effects of colchicin, idarubicin and paclitaxel on both HeLa and Molt3 cells were then quantified. Data were analysed as described in the Materials and methods section and were compared with those obtained measuring cell population size by direct counting of live cells under the microscope, using the well-established trypan-blue exclusion method to label dead cells. Figure 6 shows the two spectrophotometric methods that provide data which are in quantitative agreement with those obtained by direct cell counting; these results further demonstrate that microplate spectrophotometry can be used to measure cell population size in a well and its reduction upon therapy with cytotoxic drugs.

Although it was not the main focus of the present study to investigate cytotoxic properties of colchicin, idarubicin and paclitaxel on HeLa and Molt3 cells, it is worth noting that concentrations inhibiting 50% cell growth (IC₅₀) – as estimated by data in Fig. 6 – were in good agreement with previously published values, measured for different human tumour cells, following conventional experimental procedures [Colchicin, IC₅₀ $\approx$ 7 ng/ml (18), Idarubicin, IC₅₀ $\approx$ 1.9–20 ng/ml (19,20), Paclitaxel, IC₅₀ $\approx$ 3–6 ng/ml (18,19)].

Spectrophotometric measurements vary as a function of sample volume, that is, of path length of the optical ray. During the course of our studies with cytotoxic drugs, we have observed mild evaporation of culture medium in wells, along borders of plates and this can affect measurements. To overcome this problem, these wells were filled with distilled water. Thus, overall, 60 wells/plate were actually available for cytotoxicity assays.

Inter-experimental variability

Data in Figs 3–6 show that within the same assay, experimental variability was rather limited in spite of calculations performed with raw data. It should be taken into consideration that experimental error propagates after each mathematical operation on mean $\pm$ SD values (for example, division between OD values measured at 410 and 560 nm; calculation of parameter R_A with eqn (2); (21).

To assess inter-experimental variability, we compared six growth curves measured for both HeLa and Molt3 cells, in independent assays carried out over 4 months; results are shown in Fig. 7. Growth curves appear to overlap for data measured using the OD ratio method, whereas turbidity measurements provided growth curves that were somewhat shifted along the time axis, but seem, nonetheless, to have the same

Figure 3. Measurement of growth of colchicine-treated tumour cells by microplate spectrophotometry. Results obtained with HeLa (panels a–c) and Molt3 (panels d–f) cells treated with the chemotherapeutic drug colchicine according to the three protocols described in the Materials and methods section. Panels *a* and *d*: Protocol 1. Panels *b* and *e*: Protocol 2. Panels *c* and *f*: Protocol 3; treatment was carried out with colchicine at 5 ng/ml. In all panels, CTRL refers to cells treated with PBS only. Data are mean $\pm$ SD of the measurements taken on six independent wells.

shape. To quantify inter-experimental variability, we fitted the data in Fig. 7 with a 4-parameter logistic function (13) and estimated both growth rate and saturation level of each growth curve, parameters that collectively characterize any logistic growth process. Growth curves measured for HeLa cells (Fig. 7, panel a) showed mean $\pm$ SD growth rate of 1.30 ± 0.23 /day and saturation level of 1.47 ± 0.01 (r^2 ranging between 0.97 and 0.99); growth curves measured for Molt3 cells (Fig. 7, panel b) showed mean $\pm$ SD growth rate of 1.24 ± 0.37 /day and saturation level of 0.84 ± 0.06 (r^2 ranging between 0.98 and 0.99). The coefficient of variation (CV = SD/mean) of growth rates measured by the OD ratio method was 0.18, whereas that of growth rates measured by the turbidity method was 0.30. Both methods provided good estimations of saturation levels among different experiments (CV = 0.007 and 0.07 for the OD ratio and the turbidity method respectively). Thus, inter-experimental variability was acceptable.

Conclusions

We have investigated microplate spectrophotometry as a rapid and cost-effective technique for high-throughput screening of cytotoxic compounds. As any other method, microplate spectrophotometry has advantages and disadvantages and here we wish to summarize both aspects following the lines indicated by Slater (4) and as provided here, in the Introduction section. The disadvantages are: (i) Required initial cell sample is quite large (1000 cells/well) compared to other methods [10 cells/well using online bioluminescence determination of ATP; See (4) and references cited therein]. We attempted to reduce the initial cell density and in a previous paper, we started from 1 cell/well (13). However, this resulted in long delays before measured values could overcome the background levels. Taking into account the estimated growth rate of the cells and assuming exponential growth, it could be calculated that the measurements provided values above

Figure 4. Measurement of growth of idarubicine-treated tumour cells by microplate spectrophotometry. Panels are as in Fig. 3. Panels c and f show data obtained for cells treated with idarubicine at 5 ng/ml.

background when cells reached density of approximately 1000 cells/well. As in 96-well culture plates, cell density reaches maximal values typically in the order of 100 000–200 000 cells/well, the dynamic range of the method is two orders of magnitude and hence limited when compared to other methods, such as ATP determination or alamar blue detection by spectrofluorimetry (4); (ii) Our method does not discriminate between different death mechanisms (apoptosis versus necrosis). This is the reason why we judge that our method can be useful for first-level screening of cytotoxic molecules. Detailed mechanisms of action of compounds selected for their inhibitory effects on target cells should therefore be assessed using the present method along with more specific experimental approaches such as flow cytometry, which is currently not suited to the demands of HTS of cytotoxic compounds [See (4) and references cited therein]; and (iii) Our method may not be suitable for compounds that absorb at the specified wavelengths. It should, nonetheless, be taken into consideration that cytotoxicity potential of compounds is assayed at very low molecular concentrations and therefore absorbance properties of the molecule under

study are unlikely to interfere with spectrophotometric readings. Indeed, in this study, we have demonstrated that the methods can be exploited to measure idarubicin cytotoxicity, a molecule which, in the visible region of the spectrum, absorbs between 400 and 550 nm with a peak at 485 nm.

However, the advantages of our method are: (i) The technique does not require the use of further reagents besides those contained in common growth media. This is a novel aspect with respect to other HTS methods. This also implies that no filtration, separation or washing steps are needed to carry out measurements. Thus, assays can be performed in microplates where incubation and analysis can be achieved in the same plate; (ii) The method provides measures which are related to cell biomass, and transient cytotoxic effects can be detected. Both aspects are important to determine actual cytotoxic potential of a novel drug; and (iii) Measuring absorbance in each well of a microplate requires just a few minutes and all intermediate procedures can be fully automated using existing technologies {for example, by using automatic dispensers for cells and molecules, robotic arms for plates handling,

Figure 5. Measuring the growth of paclitaxel-treated tumour cells by microplate spectrophotometry. Panels are as in Fig. 3. Panels c and f show the data obtained for cells treated with paclitaxel at 10 ng/ml.

Figure 6. Quantification of effects of cytotoxic drugs by microplate spectrophotometry. HeLa (circles) and Molt3 (squares) cells were exposed to colchicin, idarubicin and paclitaxel at the indicated final concentrations for 24 h. Growth was measured by microplate spectrophotometry (closed symbols) and then analysed using eqns (1) and (2) to calculate normalized area beneath growth curves (R_A). For comparison, cytotoxic effects of the drugs were also measured in independent tests by direct cell counting (open symbols) using the same experimental and analytical procedures.

Figure 7. Assaying inter-experimental variability of the method. Six independent growth experiments with HeLa (panel a) or Molt3 (panel b) cells are shown. Different symbols refer to different assays. Data are mean $\pm$ SD values from six cell populations.

and others [please see (1,2)]. Overall, we conclude that microplate spectrophotometry is suitable as a first-level HTS method for compounds with unknown cytotoxic potential to animal cells.

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