Research Article

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Comparative analysis of XTT assay and xCELLigence system by measuring cytotoxicity of resveratrol in human cancer cell lines

İnsan kanser hücre hatlarında resveratrol sitotoksisitesinin XTT testi ve xCELLigence sistemi ile karşılaştırmalı analizi

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Abstract

Objective: In vitro preliminary oncological and translational studies are mainly based on evaluating the cytotoxic effects of a specific compound on cultured cells. Resveratrol is a commercially available compound which is originally isolated from the roots of white hellebore and later from *Polygonum cuspidatum*. The objective of the study was to compare cytotoxicity data of Resveratrol from XTT end point assay with a real-time cell based xCELLigence system in terms of accuracy, sensitivity, speed and reproducibility in a panel of human cancer cell lines.
Methods: XTT end point assay and real-time cell based xCELLigence system were used to evaluate cytotoxicity. Cytotoxicity results were verified by monitoring cells under phase-contrast microscope which were treated with IC_{50} values of resveratrol.
Results: Resveratrol decreased cell viability in a time- and concentration-dependent manner in all cancer cell lines when tested by both the XTT assay and xCELLigence system. Standard deviations of the xCELLigence data were found to be lower than the data from XTT assay.
Conclusion: The data from this study strongly imply that xCELLigence system has higher precision, more enlightening and more reproducible than XTT end point assay.

Keywords: Resveratrol; Cytotoxicity; XTT; xCELLigence; Human cancer cell lines.

Zusammenfassung

Amaç: In vitro preliminer onkolojik ve translasyonel çalışmalar, spesifik bir ilacın sitotoksik etkilerinin hücre kültürleri üzerinde aydınlatılmasına dayanır. Resveratrol ticari olarak satışta olan beyaz zambak bitkisinin köklerinden ve *Polygonum cuspidatum* bitkisinden izole edilmiş bir bileşiktir. Bu çalışmada resveratrolün sitotoksik etkilerinin, hedeflenen zaman noktalarında ölçüm yapan XTT testi ve sürekli ölçüm yapan xCELLigence sistemi kullanılarak tutarlılık, hassasiyet, hız ve tekrarlanabilirlik yönünden karşılaştırılması amaçlanmıştır.
Yöntem: Sitotoksisitenin belirlenmesinde, hedeflenen zaman noktalarında ölçüm yapmayı sağlayan XTT testi ve sürekli veri toplayarak ölçüm yapan xCELLigence sistemi kullanıldı. Resveratrolün IC_{50} konsantrasyonu ile muamele edilen hücreler faz kontrast mikroskobunda görüntülenerek sitotoksisite verileri doğrulandı.
Bulgular: XTT yöntemi ve xCELLigence sistemi ile test edilen Resveratrolün, tüm kanser hücre hatlarında canlılığı konsantrasyona ve zamana bağlı olarak azalttığı belirlendi. xCELLigence sisteminden elde edilen verilere ait standart sapma değerlerinin XTT yöntemine göre elde edilenlerden daha düşük olduğu belirlendi.

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Sonuç: Bu çalışmada, XTT testi ile karşılaştırıldığında, xCELLigence sisteminin daha yüksek duyarlılığa sahip olduğunu ve tekrarlanabilir veriler elde etmede daha verimli olduğunu belirten veriler elde edilmiştir. Hücre kültürlerinin sürekli izlenmesinin, hücre canlılığının erken dönemde oluşabilecek değişikliklerin belirlenmesine olanak sağlayacağını ve hücre ölümünün başladığı zamanı doğru tahmin etmede yararlı olacağını belirten veriler elde edilmiştir.

Anahtar Kelimeler: Resveratrol; Sitotoksisite; XTT; xCELLigence; İnsan kanser hücre hatları

Introduction

In vitro translational research studies are mainly based on evaluating the possible cytotoxic effect of a specific compound in cultured cells. Several methods are in use to investigate the cytotoxic effects of anticancer compounds. Current standard approaches include the assays measuring distinct cell viability and metabolism related endpoints such as the activity of intracellular enzymes, integrity of cellular membranes, DNA synthesis and ATP content [1].

Since their initial development, tetrazolium salts such as 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT), 2,3-bis (2-Methoxy-4-nitro-5-sulfophenyl)- 2H-tetrazolium-5-carboxanilide (XTT) and 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) have been widely used as colorimetric cell viability tests in eukaryotic cells for more than 50 years. Tetrazolium salts act as an electron acceptor that is colorless in its native form but forms colored formazan derivatives when reduced by mitochondrial dehydrogenase enzymes in viable cells. The amount of the formazan product is measured by using a spectrophotometer to estimate relative cell viability. One of the most common forms of tetrazolium salts is XTT [2].

Recently, the Real-Time Cell Analyzer (RTCA) system under the name of xCELLigence™ has been launched by Roche Applied Science and ACEA Biosciences. The xCELLigence system allows for label-free and real-time monitoring of cell proliferation in adherent cell cultures. It utilizes an electronic readout called impedance to quantify cellular status non-invasively and in real-time. In other words, the system monitors continuously the cellular status and collects real-time information about cell growth, morphological changes and cell death during the experiment [3]. Currently used viability assays are designed to provide information about changes in cell viability and proliferation induced by tested compounds. However, the nature of compounds, varying endpoints used by particular assays and the type of cell model may produce inconsistent results in cell culture studies. Therefore, cytotoxicity data of a certain compound should be compared in either different cell lines or by different viability methods in terms of accuracy, sensitivity and reproducibility parameters [1].

Resveratrol (RV) (3,5,4′-trihydroxy-trans-stilbene) is a commercially available compound, originally isolated from the roots of white hellebore and later from *Polygonum cuspidatum*. As a potential anti-cancer agent, RV has been shown to inhibit the growth of various cancer cells in vitro and in vivo [4–8].

The main objective of this study was to compare RV cytotoxicity data from XTT end point assay and a real-time cell-based xCELLigence system in a panel of human cancer cell lines including prostate (PC-3 and DU-145), breast (MCF-7 and MDA-MB-231) and ovarian (OVCAR-3 and MDAH-2774). Moreover, second aim was to compare two different commercially available in vitro assays in terms of accuracy, sensitivity, cost and reproducibility.

Materials and methods

Cell culture and RV treatment

Human breast cancer cells (MCF-7 and MDA-MB-231) were obtained from HPA (Health Protection Agency, UK) and the other human cancer cells (PC-3, DU-145, OVCAR-3 and MDAH-2774) were obtained from ICLC (Genova, Italy). The cells were grown as adherent cell lines and were routinely cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin in 75 cm^2 polystyrene flasks (Corning Life Sciences, UK) and maintained at 37°C in a humidified atmosphere with 5% CO_2. RV was obtained from Sigma Chemical Co. (St Louis, MO, USA) and stock solutions (43.8 mM) were prepared in ethanol. Cell culture supplies were obtained from Biological Industries (Kibbutz Beit Haemek, Israel).

XTT cell viability assay

After verifying cell viability using trypan blue dye exclusion test by Cellometer automatic cell counter (Nexcelom Inc., USA), cells were seeded at approximately 1×10^4/well in a final volume of 100 µL in 96-well flat-bottom microtiter plates. After an overnight incubation, cells were treated with different concentrations of RV (30-500 µM). Plates

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were incubated at 37°C in a 5% CO_2 incubator for the indicated time periods. At the end of incubation, 100 µL of XTT (Roche Applied Science, Mannheim, Germany) was added to each well, and plates were incubated at 37°C for additional 4 h. Absorbance was measured at 450 nm against a reference wavelength at 650 nm using a microplate reader (Beckman Coulter, DTX 880 Multimode Reader). The mean of triplicate experiments for each dose was used to calculate the half maximal inhibitory concentration (IC_{50}) by CalcuSyn 2.1 (Cambridge, UK) software. Viability data are calculated using following formula: (A450–A650) of test cells × 100/(A450–A650) of control cells.

Real time monitoring of cell proliferation by xCELLigence system

The xCELLigence system (Roche Applied Science and ACEA Biosciences, Mannheim, Germany) uses a special 96-well micro plate that differs from standard 96 well plates with its incorporated gold microelectrode cell sensors in the bottom of the wells. These sensors allow real time monitoring of cells in each well [9]. Application of a low voltage (20 mV) produces a small electric field between the electrodes. This field can be impeded by the presence of adherent cells leading to large changes in the measured impedance. The extent of change is proportional to the number of cells and their morphological and adhesive characteristics. The more cells that are growing on the electrodes, the more the value of electrode impedance increases [10]. Cell index (CI) is derived to represent cell status based on the measured relative change in electrical impedance that occurs in the presence and absence of cells in the wells, which is calculated based on the following formula: $CI = (Z_i - Z_0)/15$, where Z_i is the impedance at an individual point of time during the experiment and Z_0 is the impedance at the start of the experiment [9].

The xCELLigence system was used to determine the time point that different RV concentrations have their most potent effects. To obtain background readings, 50 µL serum free medium was added to E-plate 96. Then, human cancer cells were seeded in 75 µL (10^4 cells/well) growth medium and plates were incubated for 30 m at room temperature. E-Plate 96 was then moved to incubator. Cell attachment and proliferation were monitored every 1 h by using the RTCA SP instrument. After seeding, the cells were incubated for 24 h and exposed to 25 µL serum free medium containing the following agent RV (30–500 µM). Controls received either medium-only, ethanol-only or medium + ethanol. Cells were monitored every 2 m for 1 h after RV addition for short term responses. For the long term responses, they were monitored every 30 m from 1 h to 72 h.

The integrated RTCA software (Version 1.2) calculates the CI and IC_{50} values measured in the 96 well plate from the time of seeding the cells up to end of the experiment 72 h. The RTCA software performs a curve-fitting of selected "sigmoidal dose-response equation" to the experimental data points and calculates CI values at specific time points.

Visualizing RV treated cells under microscope

In order to confirm the viability data obtained from cytotoxicity assays, we visualized RV treated cells under microscope to visualize the morphological changes during the experiment. Cells in 75 cm^2 flasks were detached using a trypsin-EDTA solution followed by the addition of culture medium to neutralize trypsin, and then the cells were pelleted by centrifugation at 1000 rpm for 5 min at 4°C. Supernatants were removed, and cell pellets were re-suspended in assay medium and counted using a Cellometer automatic cell counter. Cell densities were adjusted to 2×10^4 cells/1000 µL in 6-well plates. Plates were placed in an incubator overnight to allow for attachment and recovery. Then, cells were treated with IC_{50} values of RV for 56 and 72 h. After incubation, cells were monitored using inverted microscope (Euromex Microscope BV, Arnhem, The Netherlands).

The LIVE/DEAD viability/cytotoxicity assay

In order to verify our results derived from xCELLigence system, we performed LIVE/DEAD Viability/Cytotoxicity Assay (Molecular Probes, Invitrogen) at both 56 and 72 h. Then, we have compared the viability of cells exposed to RV at indicated time points. Calcein-AM stains live cells via intracellular esterase activity and ethidium homodimer (EthD-1) stains dead cells that have lost plasma membrane integrity [10]. For each sample, all treatments were removed and mixture of 2 mM calcein-AM and 5 mM EthD-1 solution was added into each well. Plates were incubated for 30 min at room temperature [11]. Living cells were labeled in green and dead cells were labeled in red. Stained cells were examined with a fluorescence microscope (Leica Microsystems, Heerbrugg, Switzerland) and images were obtained.

Assessment of reproducibility of each method

Coefficient of variation (CV) is used for assessment of reproducibility of each method since lower CV value is interpreted as higher reproducibility of the method. CV

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values were derived from the IC_{50} values calculated for each of three individual experiments.

Statistical analysis

All experiments were conducted in triplicate and the results expressed as the mean ± SD. The mean of triplicate experiments for each dose was used to calculate the IC_{50} values using CalcuSyn 2.1 software (Biosoft CalcuSyn, Ferguson, MO, USA) and graphs were done by Graphpad Prism 5.0 software (CA, USA). Experimental data for RV dose range effect were analyzed by ANOVA test. Student's t-test was used to compare the results between two groups ($p < 0.05$ was considered significant).

Results and discussion

Two independent assays, XTT cell viability assay and xCELLigence system were performed to assess cytotoxic effect of RV on human cancer cell lines. Cells were exposed to the increasing concentrations of RV (30–500 μM) in both assays. XTT assay was performed at 24, 48 and 72 h end points; while the data was collected for 96 h in xCELLigence system, continuously.

RV decreased cell viability in a time- and concentration-dependent manner in all cancer cell lines when tested by XTT assay. The cytotoxicity data calculated from XTT assay for investigated cell lines was illustrated in Figure 1A–F. Results from the XTT assay showed that cytotoxicity of RV appeared at 100 μM for the first time and

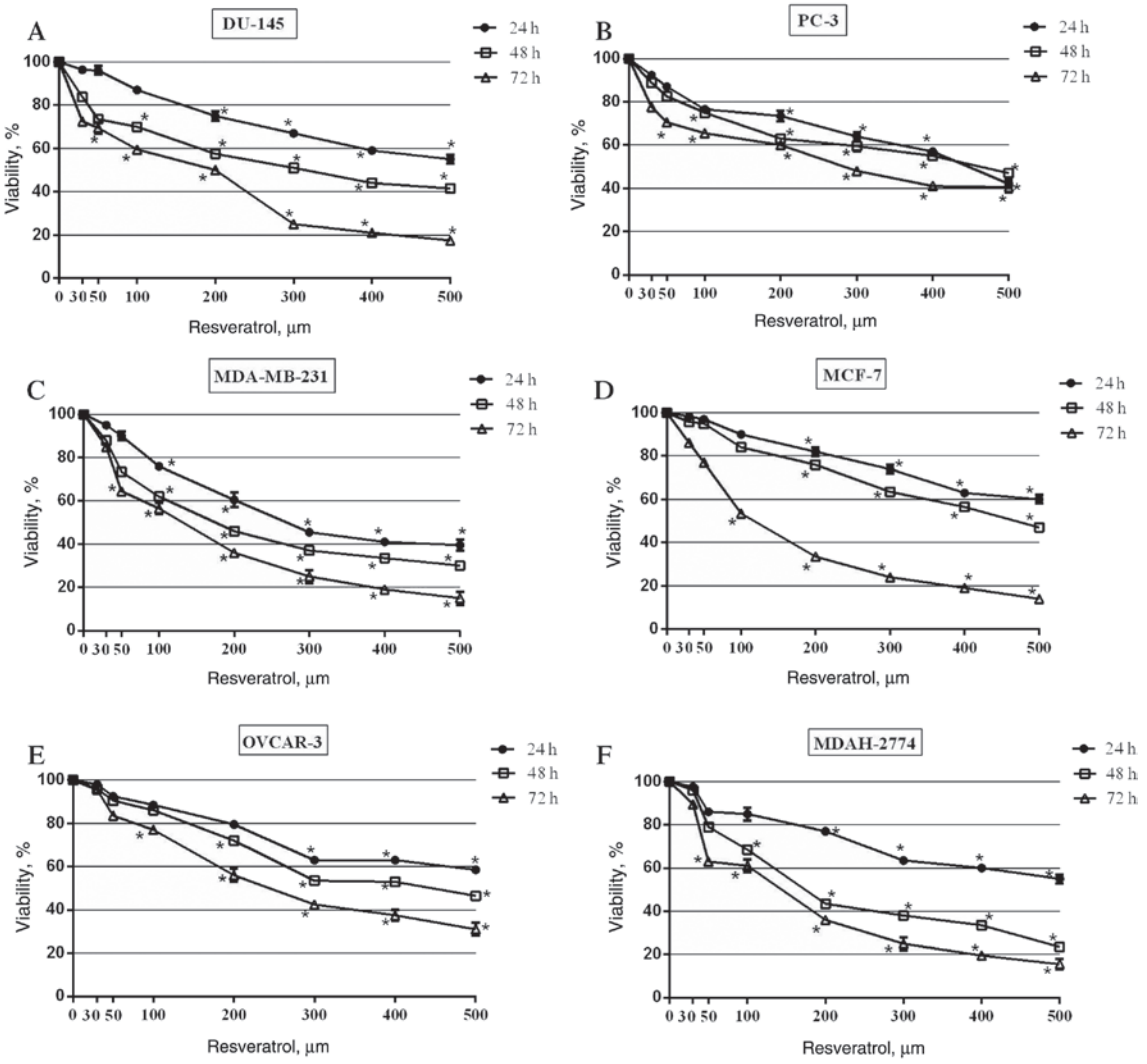


Figure 1: Concentration-dependent effects of RV on the viability of DU-145 (A), PC-3 (B), MDA-MB-231 (C), MCF-7 (D), OVCAR-3 (E) and MDAH-2774 (F) cells at 24, 48 and 72 h.
The results are expressed as the mean of three independent experiments (*$p < 0.05$).

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increased by the above concentrations in all investigated cell lines. IC_{50} values for each cell line were calculated by using Calcusyn 2.1 software and demonstrated in Table 1. Based on the methodological principles, the reagent used in XTT assay is cytotoxic and destroys the cells under investigation, thus it allows only a single evaluation at a single time point which is the most important limitation of this widely used method.

Table 1: IC_{50} values of investigated human cancer cell lines treated with Resveratrol for 72 h from both XTT assay and xCELLigence system.

Cell Lines	Resveratrol, μM			
	XTT assay		xCELLigence system	
	(IC_{50}) (±SD)	CV, %	(IC_{50}) (±SD)	CV, %
PC-3	115.6±7.7	6.6	103±0.1	0.09
DU-145	84.5±5.5	6.5	38±0.07	0.25
MCF-7	131±6.2	9.8	92±0.07	0.52
MDA-MB-231	82.5±8.4	4.7	27±0.2	0.07
OVCAR-3	62.6±4.9	6.9	44.6±0.3	0.67
MDAH-2774	50.7±3.5	7.8	25±0.07	0.28

Coefficient of variation (CV, calculated as a%) data also shown for the effectiveness of the two methods. The higher CV indicates greater level of dispersion in the data.

CI versus time curves from the xCELLigence system of six cancer cell lines tested with the increasing concentrations of RV are shown in Figure 2A–F. Each cell line responded differently to RV exposure by means of time and concentration. For example, in MDAH-2774 ovarian cancer cells, CI was decreased by all of the tested concentrations at 24 h indicating that MDAH-2774 ovarian cancer cells were more sensitive than OVCAR-3 cells to RV.

To compare the viability data from xCELLigence and XTT, we transformed CI values which are obtained from xCELLigence system into percentage of viability. As shown in Figure 3, viability data showed differences by increasing concentrations of RV at 72 h. Comparison of IC_{50} values from both assays were also different from each other and IC_{50} values detected in the xCELLigence system were significantly lower than those in the XTT assay (Table 1). As mentioned above, RV sensitivity was quite different in the tested cell lines. For example, ovarian MDAH-2774 and MDA-MB-231 breast cancer cell lines were the most sensitive cells to RV with IC_{50} values of 25 and 27 μM, respectively; whereas PC-3 prostate and MCF-7 breast cancer cells

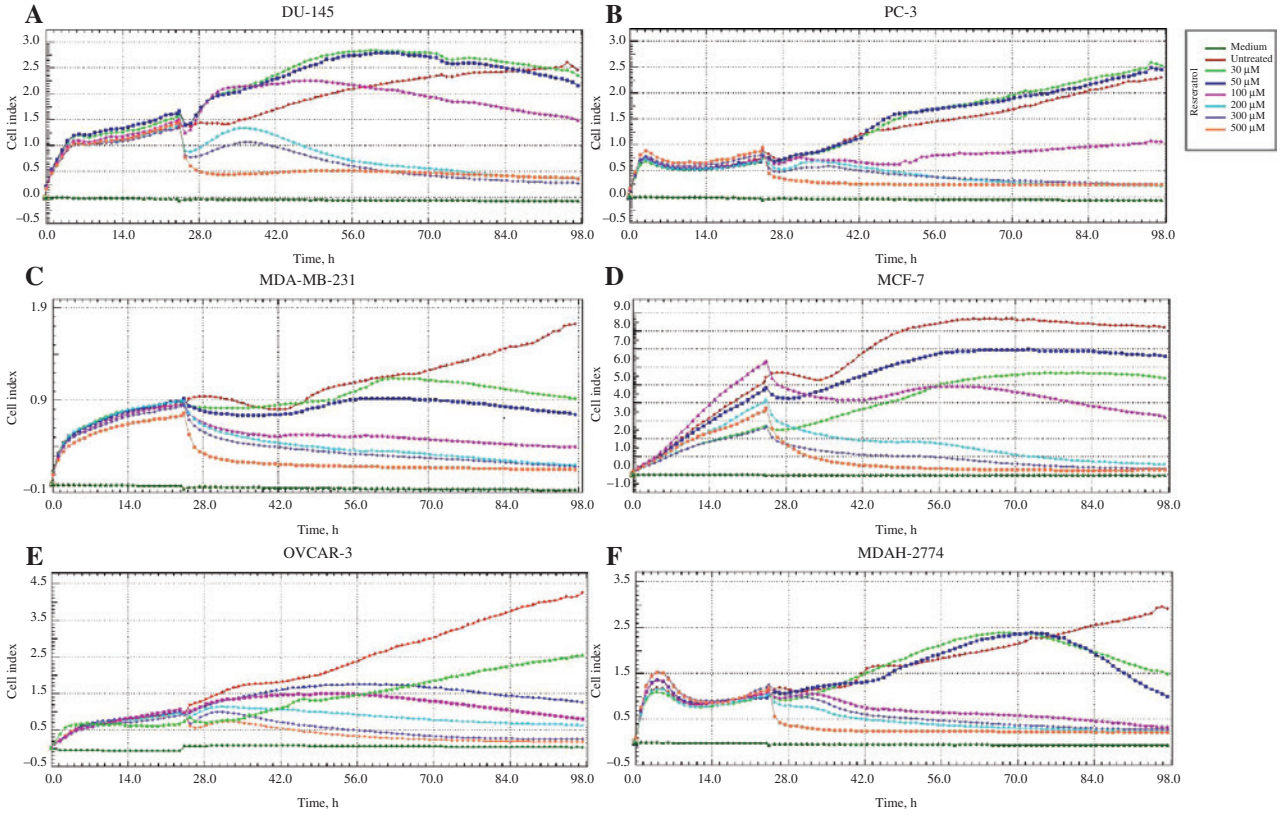


Figure 2: Dose-response curves of the cell index (CI) of human cancer cell lines which were treated by different concentrations of RV. After seeding 24 h, cells were exposed to 25 μL serum free medium containing the following concentrations of RV (30-500 μM).

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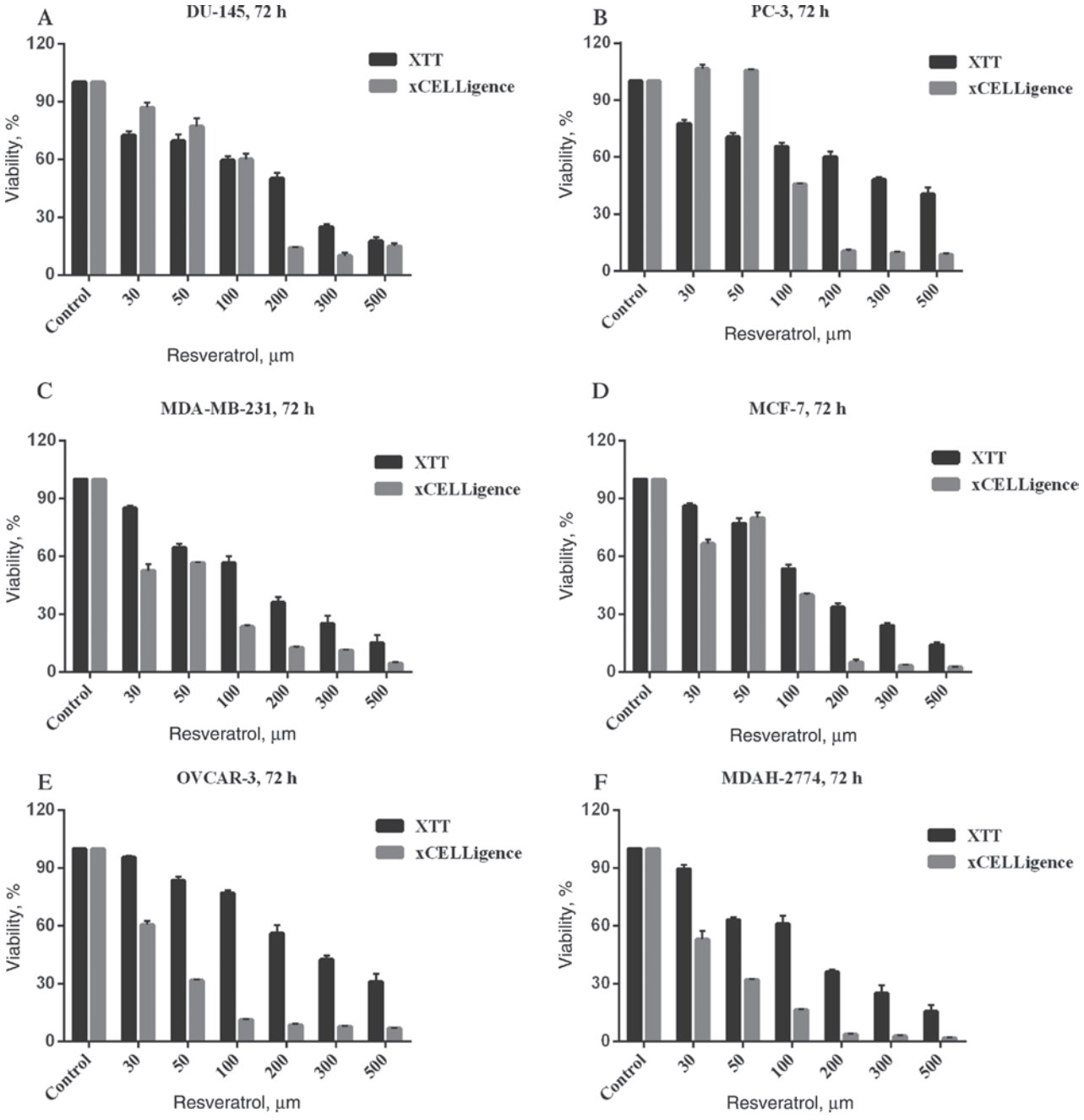


Figure 3: Comparison of viability data of DU-145 (A), PC-3 (B), MDA-MB-231 (C), MCF-7 (D), OVCAR-3 (E) and MDAH-2774 (F) cells from xCELLigence system and XTT assay at 72 h.

were the most resistant cell lines with the IC_{50} values of 103 and 92 µM at 72 h, respectively. The highest cytotoxicity was achieved at 72 h by XTT end point assay for all investigated cell lines. However, in the xCELLigence system, maximum effect of RV was observed at ~56 h due to continuous and real-time data collection. To confirm the data obtained from xCELLigence system, cell samples were collected from both 56 and 72 h time points and visualized under inverted microscope (Figure 4). As seen in Figure 4, number of viable cells was lower at 72 h as compared to 56 h treated cells. Moreover, the live/dead viability/cytotoxicity assay was used to demonstrate live and dead cells in both 56 and 72 h. Similar results were also obtained by this method (Figure 5).

Two different cytotoxicity methods were compared in terms of precision by using triplicate samples within each experiment. Standard deviations of the XTT data were higher than the xCELLigence data, indicating that XTT

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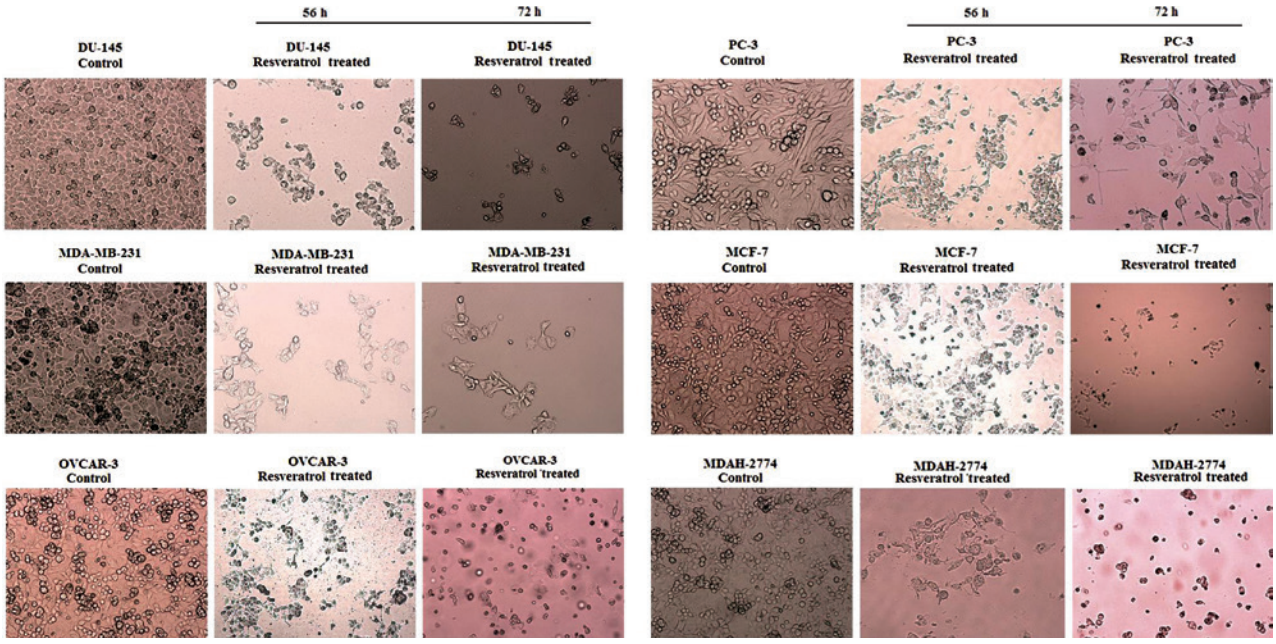


Figure 4: Micrographs (40× magnification) using phase contrast microscopy of single point screen after the addition of IC_{50} values of RV at both 56 and 72 h.

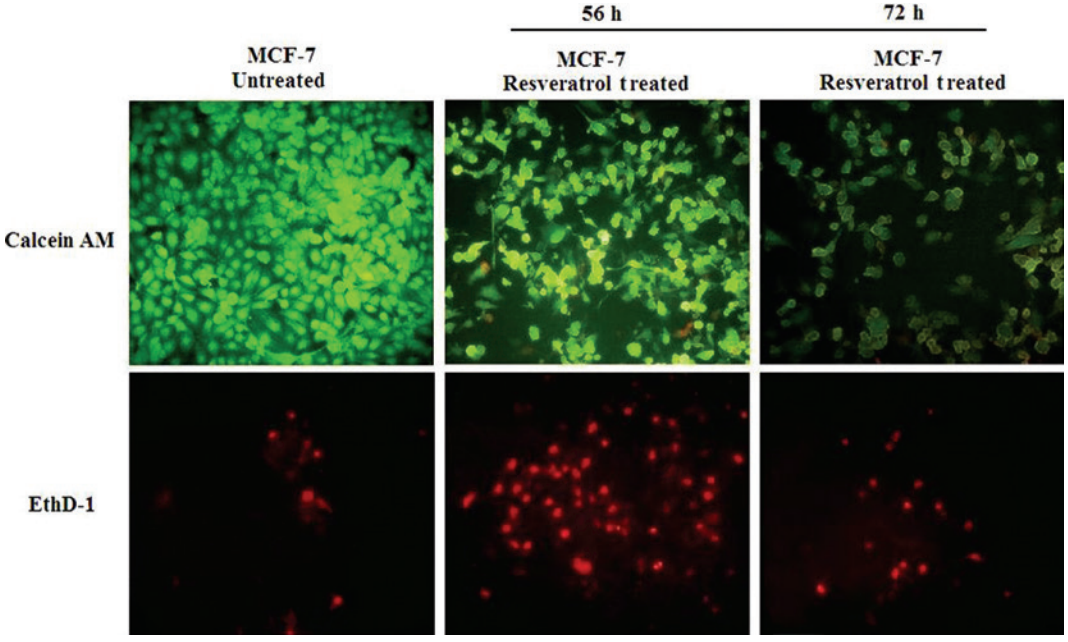


Figure 5: Immunofluorescence images of MCF-7 cells using live/dead cell reagent. After incubating the cells with 100 µM of RV for 56 and 72 h, cells were treated with 1 µL calcein AM/1 mL of D-PBS.
The red and green fluorescence are the markers of dead and live cells, respectively. The Live/Dead Viability/Cytotoxicity Assay Kit (Invitrogen) allows determination of live and dead cells with two probes using calcein AM and ethidium homodimer (EthD-1). Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells.

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assay has lower precision. Since the xCELLigence system lets the investigator to have a continuous data flow, it has more enlightening and more realistic data than those in XTT end point assay.

Reproducibility of two methods was also evaluated comparing inter-assay variability based on CV values of independent experiments (Table 1). CV values from XTT assay were high at lower concentrations of RV, but low in its higher doses, whereas the xCELLigence system showed consistent low CV values at all doses.

Several methods have been proposed for the rapid and continuous detection of cell viability in cultured cells. XTT is a widely used tetrazolium salt in mammalian cell cultures and allows single end point quantitative measurement of cell viability. Relative cytotoxicity is calculated indirectly by using the viability data. Typically several single time points are used to assess the cytotoxic effect of compounds in one experimental design. XTT comprises a number of manipulation steps that potentially lead to perturbational changes of the end point measurements. It requires substantial reagent optimization. Absorbance data from optic-based detection are vulnerable to distortions such as pH, polyphenols, pyruvate analogs and nanomaterials [12]. However, XTT is inadequate in continuously monitoring cell viability in experimental design. Therefore, it is an important requirement for the quantitative and continuously monitoring cellular biological parameters in cell culture model systems [3].

In regards to xCELLigence, it is a novel, quantitative, real-time and label-free cell based system using an electronic cell sensor technology that addresses solutions for some of the limitations of XTT assay. It allows continuous monitoring of the cell status, proliferation and also cell death in a real- time based system. Thus, the importance of xCELLigence system is enabling to detect and monitor early changes related to cell viability dynamics in cell population. Moreover, it allows for the calculation of time dependent IC_{50} values, which can be more informative than single IC_{50} end points of classical toxicity testing [2]. The other advantage of this system is to give some clues at which time point the cell death exactly begins.

When comparing both systems, the cost of each assay was also calculated. The price per well of the xCELLigence system is 1.07 euro/well, whereas 0.3 euro/well for XTT assay. The xCELLigence system is obviously more expensive than XTT; however, the higher price of xCELLigence is, surely, compensated for obtaining more reproducible and reliable results.

By this study, we have found out that the cytotoxicity data of RV was not similar in two assays. This can be explained in several terms. Firstly, each assay has completely different detection principle. XTT assay is mainly based on the enzymatic conversion of XTT in the mitochondria, whereas, xCELLigence system has completely different mechanism based on measurement of impedance. Since xCELLigence system does not require any stain or label, component interference during measurement is not in question. Secondly, it was interpreted that differential sensitivity to RV may be due to perturbations in drug uptake dynamics and/or imply genomic differences between the human cancer cell lines.

In the literature, IC_{50} values of RV in human cancer cell lines are between 20 and 100 μM at 72 h [12–15]. Differences in IC_{50} values from the literature may be due to dissolving conditions of RV, different medium content, different incubation times, perturbations in drug uptake dynamics and the difference of methods that have been used (i.e. MTT, XTT). However, to the best of our knowledge, there is not any data regarding the cytotoxic effect of RV investigated by xCELLigence system. Thus, our study is the first one to investigate this new and reliable cell cytotoxicity method for RV. The data from this study strongly imply that reliable and reproducible assessment of drug cytotoxicity requires complementary tests to confirm the results. Secondly, real-time based continuously monitoring treated and untreated cell cultures gives more insight into early changes in cell viability dynamics. To determine the correct time point of the beginning of cell death and early changes in cell population allows us to describe correct hypothesis in order to enlighten the cellular targets of tested compound which will allow for new aspects for drug research studies in cancer.

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