Experimental comparison of some methods to assess cytotoxicity

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Abstract

We have been witnessing an intensive growth in the medical and pharmaceutical fields in the recent years. New therapies are being introduced and elements of potential medicines are being developed, both of which require numerous trials and tests before they can be universally used. Prerequisite stage of many laboratory tests is cytotoxicity assessment. For that reason, it is vital to check reliability of such techniques.

In this study, we compared ATPlite and ATPlite 1step tests based on the assessment of ATP released by cells and the measurement of chemiluminescence as well as DELFIA® EuTDA Cytotoxicity assay based on labeling of target cells with Europium and evaluating TRF.

As target cells, the following cell lines were used: Jurkat, HL-60, RPMI 8226, and CCRF 913. In order to determine the linearity, we assessed values of the signals read with increasing numbers of target cells. A reliable test should be quantitative, reproducible and should ensure high sensitivity with a minimal number of cells.Test DELFIA® EuTDA Cytotoxicity has the most advantages and fulfils these requirements.

Key words: cytotoxicity test, cell line, cytotoxicity, ATP, time resolved fluorescence, TRF.

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Introduction

Cytotoxicity assays are widely used in the fields of toxicology, pharmacology, immunology and medicine.

The need for reliable, easy to handle, and fast cytotoxicity tests has led to the development of several assays which are now routinely used to detect cytotoxic effects in *in vitro* cellular systems [1]. There are many tests available on the market to assess cell cytotoxicity. Cytotoxicity assays have been developed by using different parameters associated with the cell death and proliferation. Many of them measure cytotoxicity based on the amount of fluorochrome release [2-6] from labelled target cells.

Second type of assay appraises cytotoxicity by measuring a decrease in a metabolic activity, which is directly proportional to the number of viable cells. Another parameter used is adenosine triphosphate (ATP) [7-9]. It presents metabolical activity of cells and can be determined by measurement of bioluminescence. The assay based on the production of light is produced through the reaction of ATP with added luciferase and D-luciferin. The ATP assay can be used to assess the specific immune response against cancer cells [10] which is an important issue in immunology, cancer immunotherapy and oncological diseases.

Time-resolved fluorometry assay are used to measure NK and LAK cytotoxic activity [11-13]. To choose an appropriate assay, various parameters, such as test compounds, detection mechanism, specificity, and sensitivity need to be considered [1]. In this study, three cytotoxicity assays were compared.

To identify the differences between tests which result from various factors and phenotypic differences, we investigated four cell lines, including Jurkat, HL-60, RPMI 8226 and CCRF 913.

Material and methods

Cell lines

The cell lines used were: Jurkat, HL-60 (human promyelocytic leukemia cells), RPMI 8226 and CCRF 913. Jurkat (acute T cell leukemia) cells are an immortalized line

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of T lymphocyte cells. HL-60 cells are predominantly a neutrophilic promyelocyte (precursor) [14]. RPMI 8226 (plasmacytoma; myeloma), is a B lymphocyte type cell line. CCRF913 (acute lymphoblastic leukemia) is a T lymphoblast cell lines. All cells were cultured in a humidified atmosphere of 5% CO_2, at 37°C in media consisting RPMI 1640 (PAA, Austria) containing 10% FCS (Biochrom AG, Germany), 1% combination of antibiotics (Sigma-Aldrich, Germany). The cell lines were subcultured two or three times a week. In this experiment, cell lines in the log phase growth with a viability of > 98% were used. Before the test, they were washed twice in PBS and counted.

Cultures of 5000-50000 (100 µl volume) cells per well were tested in a 96-well round-bottomed microplate for both tests based on ATP release. DELFIA® EuTDA Cytotoxicity used 500-5000 cells per well.

ATPLite

ATP quantification is a widely accepted method which is used to assess viability of healthy cells that contain closely regulated levels of this biomarker [15].

ATP can be measured using bioluminescence based on luciferin–luciferase reaction [16]. The emitted light is proportional to the ATP concentration and the reaction generates light of the wavelength of 562 nm [17]. This is the reaction scheme in Figure 1.

The test was performed according to the manufacturer's protocol. For 96-well microplates we added 50 µl of mamamlian cell lysis solution to each well containing cells and shook it for five minutes. This lyses the cells and stabilizes the ATP. Then 50 µl substrate solution was added to the wells, shaken for five minutes, and after an appropriate period of time, the luminescence was measured. The result was measured on Victor3 multireader (Perkin Elmer, USA).

ATPLite 1 step

The principle of the method is similar to the previous one, as it is also based on the luciferin-luciferase reaction. However, contrary to the previous one, this method consists of one step. The test was performed according to the manufacturer's protocol.

For microplate we added 100 µl ATPlite 1step reagent, shook it for two minutes and measured luminescence.

DELFIA® EuTDA Cytotoxicity

The procedure is based on loading target cells with fluorescence enhancing ligand (BATDA, bis(acetoxymethyl) 2,2':6',2"-terpyridine-6,6'-dicarboxylate). The hydrophobic ligand penetrates the membrane. Within the cell hydrolysed estarbonds form a hydrophilic ligand which does not pass through membrane. After cytolysis, the ligant is released and introduced to the DELFIA® Europium Solution and creating fluorescence. The measured signal correlates directly with the amount of lysed cells.

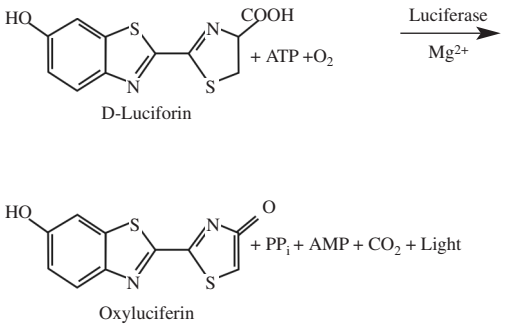

Fig. 1. Luciferin–luciferase reaction scheme

The test was performed according to the manufacturer's protocol. At the beginning cells were washed and incubated with fluorescence enhancing ligand for 20-30 minutes at 37°C. After the incubation, they were washed 3 to 5 times.

Cell suspension were seeded at 500-5000 cells per well in volume of 100 µl in the 96-well microplate. After adding 10 µl of lysis buffer, centrifuge, we transferred 20 µl to the next plate, add 200 µl of the DELFIA® Europium solution, shook for 15 minutes, and measured the fluorescence.

Results

In order to determine the linearity correlation between the cell number and fluorescence, we assessed the values of the signal received with increasing quantity of target cells.

Test DELFI® EuTDA Cytotoxicity showed complete linearity for all cell lines used with the target cells from 500 to 5000. As shown in the Figure 2. However, the ATPlite and ATPlite 1step linearity was evaluated on the number of target cells amounting from 5000 to 50000.

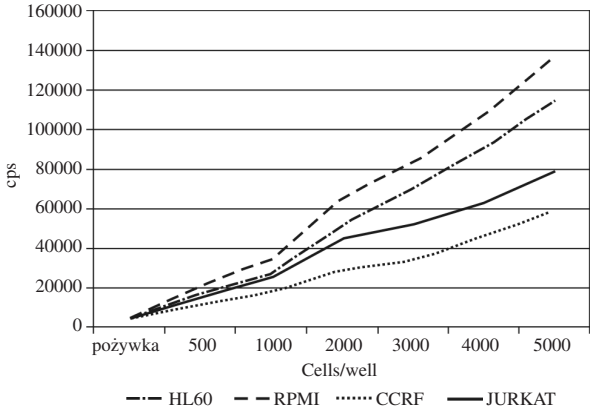

Fig. 2. Corelation between cell number (500-5000) and values of the signal (cps) in DELFIA® EuTDA Cytotoxicity assay. The first section presents the measurement of the medium

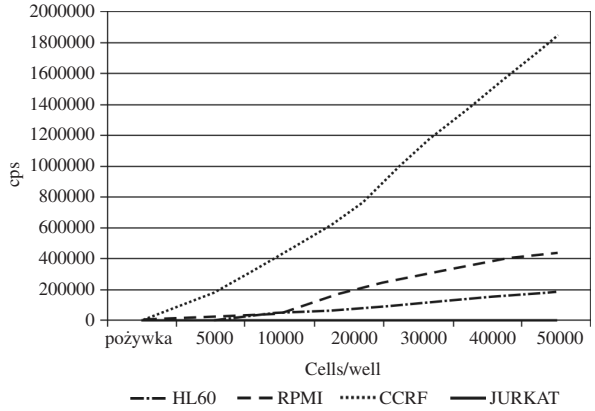

Fig. 3. Luminescence measured as a function of cell viability. Corelation between cell number (5000-50000) and values of the signal (cps). ATPlite 1step assay

The test ATPlite 1step gave good results. Linearity for cell lines was obtained only with the exception of the Jurkat cell (Fig. 3), which gave correct results in Delfi test.

The least expected results came from th ATPlite test. The linearity for the studies failed to respond beyond the RPMI 8226 line. CCRF line showed a small deviation from the curve. Higher fluorescence values and linearity in the over test were observed with RPMI 8226 cell line. While Jurkat cell line did not show linearity in any assays measuring ATP concentration. The Delfia assay showed linearity and good values for Jurkat cell line.

Discussion

When selecting a suitable test for our research, we have to take into consideration such parameters as sensitivity, reproducibility, the type of cells used and the mechanism of cell death [18].

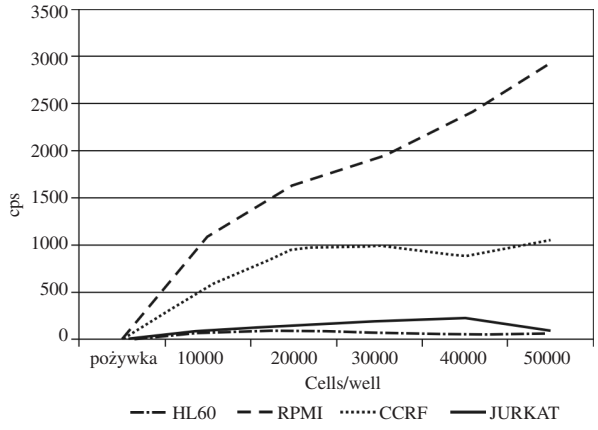

Fig. 4. Corelation between cell number (5000-50000) and values of the signal (cps). ATPLite assay

Comparative experiment is designed to select the method of cytotoxicity evaluation which would meet high expectations and would be a useful immunological technique.

Test DELFIA® EuTDA Cytotoxicity assay demonstrated full linearity results. In addition this test requires only 5000 target cells which makes it the most sensitive test. This test seems to be very useful when large quantities of target cells and effectors are not available, e.g. with biopsy material or the material collected by endoscopy. In the case of larger quantities of biological materials the ATPlite 1step can be used. However, our results suggest that it may not give reliable results for certain types of cells. The less sensitive the test is the less receptive ATP based assay. The disadvantage of this method is the luminescence-readout, influenced by quenching side effects in the samples. Moreover, the luminescence intensity is time dependent and can lead to systemic errors [18].

An important aspect of comparative tests is also time performance. Tests which are based on measuring the ATP concentration are less time-consuming when compared with tests based on release of markers from labelled cells.

K. Saarinen *et al.* (2000) [19] studied the cytotoxicity tests which used neutrophils from human peripheral blood. He based his research on fluorescence signal of labelled cells. Results were stable and reproducible which showed good correlation with our research. In addition, Blomberg *et al.* (1996) [11] used time-resolved fluorimetric assay for the cytotoxicity measurment. He used the K-526 (chronic myelogenous leukemia) cell line. This demonstrated a high linear relationship between the number of lysed cells and the measured signal. However, in our work, DELFIA® EuTDA Cytotoxicity test also gave a hight correlation between fluorescence signal and number of cells lysed.

To characterize the differences between these three cytotoxicity assays, we investigated four cell lines. Andreotti *et al.* (1995) [7] used ATP luminescence assay to measure chemosensitivity of cells. Human cells from AML and lymphoma show sensitivity, linearity and reproducibility for the measurment of cellular ATP. We used acute myelogenous leukemia (AML), which is a cancer of the myeloid line that includes HL-60 cells. Our tests measuring the concentration of ATP did not give such results. ATPlite 1step showed linearity but produced small values. And what is more, ATPlite test did not concur with that test in any of the parameteres. A possible, reason accounting for these differences might that different cells type were used. In our study we used culture cell lines but in the above mentioned experiment, directly isolated cells were used. There are reports, that cells in culture can change. It is likely the reason for the discrepancy in results.

In conclusion, to select cytotoxicity assessment method, many aspects need to be taken into account.

Our study depicts the differences between various tests evaluating cytotoxicity. Although these test, which measured different parameters, were conducted on the same

lines, they gave noticeably different results. Our studies suggest that tests evaluating ATP might not be reliable for certain types of cells. The best of the comparative tests proved to be the one based on the measurement of fluorescence coming from labelled cells. This was the most repetitive and reliable test.

The results which best reflect the actual cell condition after reacting with a toxic substance are the ones obtained from DELFIA® EuTDA Cytotoxicity test.

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References

1. Bopp SK, Lettieri T (2008): Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line. BMC Pharmacol 8: 8.
2. Blomberg K, Ulfstedt AC (1993): Fluorescent europium chelates as target cell markers in the assessment of natural killer cell cytotoxicity. J Immunol Methods 160: 27-34.
3. Lichtenfels R, Biddison WE, Schulz H et al. (1994): CARE-LASS calcein-release-assay , an improved fluorescence-based test system to measure cytotoxic T lymphocyte activity. J Immunol Methods 172: 227-239.
4. Kolber MA, Quinones RR, Gress RE, Henkart PA (1988): Measurement of cytotoxicity by target cell release and retention of the fluorescent dye bis-carboxyethyl-carboxyfluoresce (BCECF). J Immunol Methods 108: 255-264.
5. Blomberg K, Granberg C, Hemmila I, Lövgren T (1986a): Europium-labelled target cells in an assay of natural killer cell activity. I. A novel non-radioactive method based on time-resolved fluorescence. J Immunol Methods 86: 225-229.
6. Blomberg K, Granberg C, Hemmila I, Lövgren T (1986b): Europium-labelled target cells in an assay of natural killer cell activity. II. A novel non-radioactive method based on time-resolved fluorescence. Significance and specificity of the method. J Immunol Methods 92: 117-123.
7. Andreotti EP, Cree IA, Kurbacher CHM, et al. (1995): Chemosensitivity Testing of Human Tumors Using a Microplate Adenosine Triphosphate Luminescence Assay: Clinical Correlation for Cisplatin Resistance of Ovarian Carcinoma. Cancer Res 55: 5276-5282.
8. Maehani Y, Anai H, Masuda H (1986): In vitro chemosensitivity testing evaluated by intracellular level: ATP assay. Gan To Kagaku Ryoho13: 2342-2345.
9. Sevin BU, Peng Z, Perras J, et al. (1988): Application of an ATP bioluminescence assay in human tumor chemosensitivity testing. Gynecol Oncol 31: 191-204.
10. Cree IA, Pazzagli M, Mini E, et al. (1995): Methotrexate chemosensitivity by ATP luminescence in human leukaemia cell lines and in breast cancer primary cultures: comparison of the TCA-100 assay with a colonogenic assay. Anticancer Drugs 6: 398-404.
11. Blomberg K, Hautala R, Lovgren J, et al. (1996): Time-resolved fluorometric assay for natural killer activity using target cells labelled with a fluorescence enhancing ligand. J Immunol Methods 193: 199-206.
12. Blomberg K (1994): Simultaneous measurement of natural killer cell cytotoxicity against each of three different target cell lines. J Immunol Methods 168: 267-273.
13. Lovgren J, Blomberg K (1994): Simultaneous measurement of NK cell cytotoxicityagainst two target cell lines labelled with fluorescent lanthanide chelates. J Immunol Methods 173: 119-125.
14. Gallagher A, Collins S, Trujillo J, et al. (1979): characterization of the continuous, differentiating myeloid cell line from a patient with acute promyelocytic leukemia. Blood 54: 713-733.
15. Lundin A (1986): Estimation of biomass in growing cell lines by ATPassay. Methods Enzymol 133: 27-42.
16. Cree IA, Andreotti PE (1997): Measurement of cytotoxicity by ATP-based luminescence assay in primary cell cultures and cell lines. Toxicol In Vitro 11: 553-556.
17. Bradbury DA, Simmons TD, Slater KJ, Crouch SPM (2000): Measurement of the ADP: ATP ratio in human leukaemic cell lines can be used as an indicator of cell viability, necrosis and apoptosis. J Immunol Methods 240: 79-92.
18. Weyermanna J, Lochmanna D, Zimmerb A (2005): A practical note on the use of cytotoxicity assays. Int J Pharm 288: 369-376.
19. Saarinen K, Kivisto K, Blomberg K, et al. (2000): Time-resolved fluorometric assay for leukocyte adhesion using a fluorescence enhancing ligand. J Immunol Methods 236: 19-26.