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Note

A practical note on the use of cytotoxicity assays

Jörg Weyermann[a], Dirk Lochmann[a], Andreas Zimmer[b,*]

[a] *Johann Wolfgang Goethe-University, Institute for Pharmaceutical Technology, Marie-Curie-Str. 9, D-60439 Frankfurt am Main, Germany*
[b] *Institute of Pharmaceutical Sciences, Pharmaceutical Technology, Karl-Franzens-University, Schubertstraße 6, A-8010 Graz, Austria*

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Abstract

In this study, four cytotoxicity detection assays and four cytotoxic mechanisms were compared in one cellular system. Cellular responses and their effects were characterized. The assays used are based on different modes of detection like LDH release, MTT metabolism, neutral red uptake and the ATP content of treated cells. As cytotoxic mechanisms were used the model agents triton X-100, chloroquine and sodium azide (which are common in cell culture) as well as an ion channel (NMDA) mediated excitotoxicity cell death (which is specific for the cell line used). We found major differences in the calculated EC_{50}-values for the cytotoxic effect of choroquine (0.1 up to 200 mM) and for sodium azide (4 up to 1300 mM) depending on the assay used. Therefore, it is important to choose a suitable cytotoxicity assay depending on the supposed cell death mechanism. As this study compares the strengths and weaknesses of the most common assays, it can help to find the appropriate one.

Keywords: LDH; MTT; Neutral red; Cytotoxicity assay

1. Introduction

In past years, a number of methods have been developed to study cell viability and proliferation in cell culture (Cook and Mitchell, 1989). The most convenient, modern assays have been optimized for the use of microtiterplates (96-well format). This miniaturization allows many samples to be analyzed rapidly and simultaneously. Colorimetric and luminescence based assays allow samples to be measured directly in the plate by using a microtiterplate reader or ELISA plate reader. Cytototoxicity assays have been developed which use different parameters associated with cell death and proliferation. We chose four of these common assays to have a closer look on their comparability.

One parameter for cell death is the integrity of the cell membrane, which can be measured by the cytoplasmic enzyme activity released by damaged cells. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell cul-

* Corresponding author. Tel.: +43 316 380 8881; fax: +43 316 380 9100.
E-mail address: Andreas.Zimmer@uni-graz.at (A. Zimmer).

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ture supernatant upon damage of the plasma membrane (Korzeniewski and Callewaert, 1983). The LDH activity is determined in an enzymatic test. The first step is the reduction of NAD^+ to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. In a second step, the catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), which is reduced to a red formazan (Decker and Lohmann-Matthes, 1988; Lappalainen et al., 1994; Nachlas et al., 1960).

Another parameter used as the basis for colorimetric assays is the metabolic activity of viable cells. Tetrazolium salts (Smith, 1951) are reduced only by metabolically active cells. Thus, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) can be reduced to a blue colored formazan (Mosmann, 1983).

Neutral red (3-amino-*m*-dimethylamino-2-methylphenazine hydrochloride) has been used previously for the identification of vital cells in cultures (DeRenzis and Schechtman, 1973). This assay quantifies the number of viable, uninjured cells after their exposure to toxicants; it is based on the uptake and subsequent lysosomal accumulation of the supravital dye, neutral red. Quantification of the dye extracted from the cells has been shown to be linear with cell numbers, both by direct cell counts and by protein determinations of cell populations (Borenfreund and Puerner, 1985, 1986).

Adenosine triphosphate (ATP) that is present in all metabolically active cells can be determined in a bioluminescent measurement. The bioluminescent method utilizes an enzyme, luciferase, which catalyses the formation of light from ATP and luciferin. The emitted light intensity is linearly related to the ATP concentration (Crouch, 2000; Crouch and Slater, 2000; Crouch et al., 1993; Slater, 2001).

To characterize the differences between these four cytotoxicity assays, we have investigated four cytotoxicity models caused by four different mechanisms.

Our first model used was t-octylphenoxypolyethoxyethanol (triton X-100) a non-ionic surfactant, which is widely common for the solubilisation of membranes under non-denaturing conditions. The permeabilisation of the membrane leads to a fast cell death by necrosis (Borenfreund and Puerner, 1985; Jones, 1999).

As a second model we used chloroquine, which is known to facilitate some type of cellular exocytosis (e.g. lysosomal enzyme exocytosis) (Claus et al., 1998; Isobe et al., 1999). It is also described as causing a significant decrease in the activities of mitochondrial inner membrane enzymes such as NADH dehydrogenase, which implies a decreased ATP synthesis (Deepalakshmi et al., 1994).

A third model was introduced with sodium azide (NaN_3) as an inhibitor of the mitochondrial respiratory chain, and induces cell death by apoptosis (Chen et al., 1998; Inomata and Tanaka, 2003). Therefore, it is commonly used for a chemical sterilisation.

In our study we used a cell line, which stably expressed the functional recombinant *N*-methyl-D-aspartate (NMDA) receptors (NR's) which are ligand gated ion channels.

The overexpression and stimulation with glutamate of these ion channels leads to an influx of Ca^{2+}-ions resulting in a cellular toxic effect (excitoxicity). This cellular system represents our fourth cytotoxicity model. For this purpose, the mouse fibroblast cell line L(tk-) was co-transfected stably with cDNA's encoding the NR subunits NR1-1a and NR2A. The expression of NR's could be induced by incubation with 4 μM dexamethasone. This system was introduced and well characterized by Steinmetz et al. (2002). Stimulation of NR with L-glutamate and glycine resulted in a necrosis of the cells. This kind of cell damage and the resulting response of the different cytotoxicity assays could be interesting for researchers who are working e.g. with ion channels. Thus, we investigated the protective effects of an open channel blocker, ketamine.

The aim of this study was to compare four commonly used cytotoxicity assays and the influence of four different mechanism of cell death on the outcome of these assays.

2. Material and methods

2.1. Fibroblast culture

From our earlier studies, we used mouse L(tk-) cells (ATCC CCl1.3), which were stably cotransfected with NR1-1a and NR2A. For the transfection, the dexamethasone-inducible eukaryotic expression

vectors pMSG NR1-1a and pMSG NR2A were used (Bourguignon et al., 1997; Le Bourdelles et al., 1994).

These cells were a generous gift from Ralf D. Steinmetz from the Institute for Pharmaceutical Chemistry of the University of Frankfurt.

The NR expression and functionality was verified by RT-PCR, Western blotting, immunocytochemistry and fluo-4 calcium imaging. Stimulation of NR-ion channels with L-glutamate and glycine resulted in necrosis of cells within 1 h (Steinmetz et al., 2002). The cells were grown in minimal essential medium eagle (MEM, PAA, Cölbe, Germany) containing 10% fetal calf serum (FCS, Biochrom, Berlin, Germany). All other reagents and substances were obtained form Sigma-Aldrich (Deisenhofen, Germany).

2.2. Experimental design

Individual wells of a 96-well plate (Nunc, Wiesbaden, Germany) were inoculated with 200 μl medium containing 15×10^3 cells. Transparent plates were prepared for the LDH, MTT and neutral red assay. White plates were prepared in parallel for the ViaLight assay. All plates were incubated for 24 h to achieve 60–70% confluency.

Only for the excitotoxicity model, which is mediated by the NR stimulation we induced the expression of NR by incubating the cells with 4 μM dexamethasone for 16 h under the protective effect of 100 mM ketamine, which was carefully washed off with medium containing 1% bovine serum albumin. For a negative control we used non-induced cells.

Thereafter, the medium was replaced by 100 μl fresh unmodified medium (control) or with medium containing various concentrations of the test agents.

2.3. Cytotoxicity assays

2.3.1. LDH release

The LDH release from necrotic cells into the extracellular fluid was determined after 4 h of treatment with the test agents by using the commercially available Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Mannheim, Germany). The plates were centrifuged at 400 g and 4 °C for 4 min and an aliquot of 50 μl was taken to quantify the LDH. The test was performed according to the manufacturers instructions including a positive control with LDH-standard solution form rabbit muscle.

2.3.2. MTT metabolism and neutral red uptake

The cells were treated for 4 h with each test agent. Initially, fresh medium was applied to the treated cells. After 2 h, 50 μl of a 0.25% (w/v) solution of MTT in PBS buffer (NaCl 136.9 mM, KCl 2.68 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.47 mM, pH 7.4) was added to the media. Similarly, 100 μl of 30 mg/l neutral red in PBS was added. Two hours later, the cells were centrifuged at 400 g and 4 °C (Eppendorf Centrifuge 5804 R, Eppendorf, Hamburg, Germany) for 4 min, washed twice with PBS, and dissolved in a mixture of dimethyl-sulfoxide (Sigma-Aldrich, Deisenhofen, Germany), 5% (w/v) sodium dodecyl sulphate (SDS) and 1% (v/v) 1N hydrochloric acid. After an additional brief agitation on a microtiter plate shaker we measured the absorption at 550 nm with a plate reader (FluoStar, BMG Labtechnology, Offenburg, Germany). Mean values from eight wells were determined ($n = 8$).

2.3.3. ATP content

The ATP content of the cells was determined after 4 h of treatment with the test agents by using the ViaLight HS cytotoxicity assay (Cambrex, Belviers, Belgium) based upon the bioluminescent measurement of ATP after lysis of the cells. The assay was performed according to the manufacturers instructions.

2.4. UV adsorption measurements

UV absorption as a time scan of 1000 s ($\lambda = 485$ nm) were measured using a Hitachi U-3000 spectrophotometer (Scientific Instruments, Schwäbisch Gmünd, Germany). The sample interval was 0.5 s and the slit width was set to 1 nm. The derived data were analyzed using the Hitachi software UV-solutions, Version 1.2.

2.5. Data analysis

The viability was calculated with regard to the untreated cell control [y_0], which was set to 100% viability. A lysis control [y_{100}] where the cells were treated with 0.5% triton X-100 was set to 0% viability, which was found to be sufficient to induce 100% cell death.

Each data point is the mean value of four independent experiments ($n = 4$). The resulting curves were fitted by SigmaPlot 2001 (SPSS Science Inc., Chicago, IL) to a hill equation with four parameters (Eq. (1)). From these data, it was possible to calculate EC_{50}-values for the concentration[c] at which the viability of the cells reaches 50%. The Hill coefficient is given as [H].

$$y = y_0 + \frac{(y_{100} - y_0)[c]^H}{[EC_{50}]^H + [c]^H} \quad (1)$$

3. Results and discussion

3.1. Triton X-100

The first set of studies to characterize the differences between the four assays was done with triton X-100, a non-ionic surfactant (Fig. 1). The resulting viability curves adjoin in a close manner and showed a parallel progression. Low concentrations of triton X-100 seemed to induce the mitochondrial activity (MTT) and the uptake of the neutral red dye. We found a good correlation of the calculated triton X-100 EC_{50}-values (31–80 μM) in all models applied (Table 1). This good comparability of the assays could be explained by the mechanism of action, which leads to a rapid cell death by necrosis caused by the permeabilisation of the cell membranes.

3.2. Chloroquine

Surprisingly, we found deviating results (EC_{50} 0.1–200 mM) for our second test agent, chloroquine. We found in the LDH and MTT assay higher EC_{50}-values (200 and 10 mM) in comparison to the neutral red uptake and ATP content (both 0.1 mM), which correlated well (Table 1). Another interesting effect is that the LDH and MTT assays showed an apparent increase in viability at higher doses (Fig. 2A). Therefore, the data were fitted only up to the minimum viability (1e-2 mol). Trypan blue staining confirmed these results after microscopic examination. For the LDH assay, we could explain this with a reduced turnover of the substrate by inhibition of the involved enzymes, which leads to a false-positive result. We were able to reproduce this inhibition in a reaction tube measurement with substituted LDH extracted form rabbit muscle (Fig. 2B). It is described in the literature that chloroquine inhibits the activities of different enzymes (Deepalakshmi et al., 1994). However, the increased MTT formazan values cannot be explained in detail from our study, but this phenomenon seems to be an artificial side reaction.

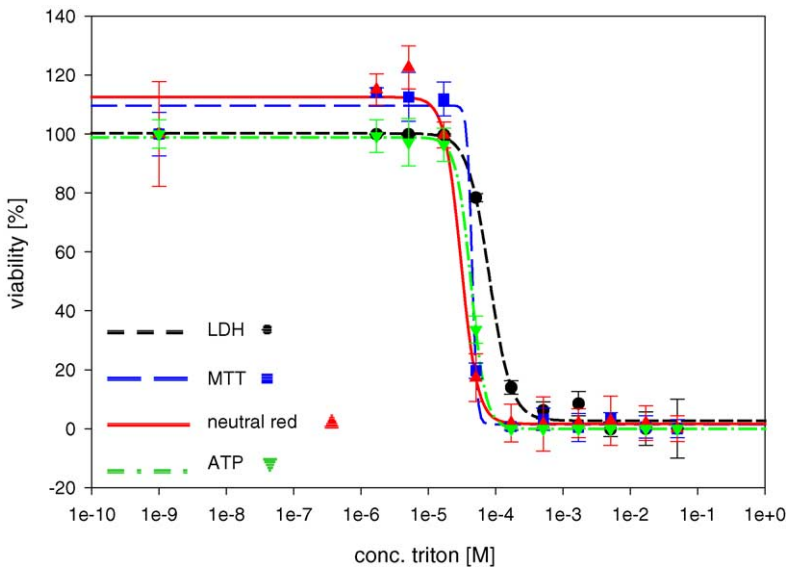

Fig. 1. Concentration–viability curve of triton X-100 (data points as scatter plot and Hill fit as line).

Table 1
EC_{50}-values calculated on basis of a hill fit with four parameters Eq. (1) of the effect curves

	Triton X-100	Chloroquine	Sodium azide	Inhibition by ketamine
LDH	80 μM (2, 7)	200 mM (1, 1)	1300 mM (0, 9)	13 μM (2, 0)
MTT	44 μM (11, 2)	10 mM (0, 6)	300 mM (2, 6)	6 μM (1, 2)
Neutral red	31 μM (3, 5)	0.1 mM (1, 6)	3.7 mM (1, 0)	200 μM (0, 3)
ATP	43 μM (4, 0)	0.1 mM (4, 5)	8.2 mM (1, 0)	28 μM (0, 7)

The Hill coefficient (H) is given in round brackets.

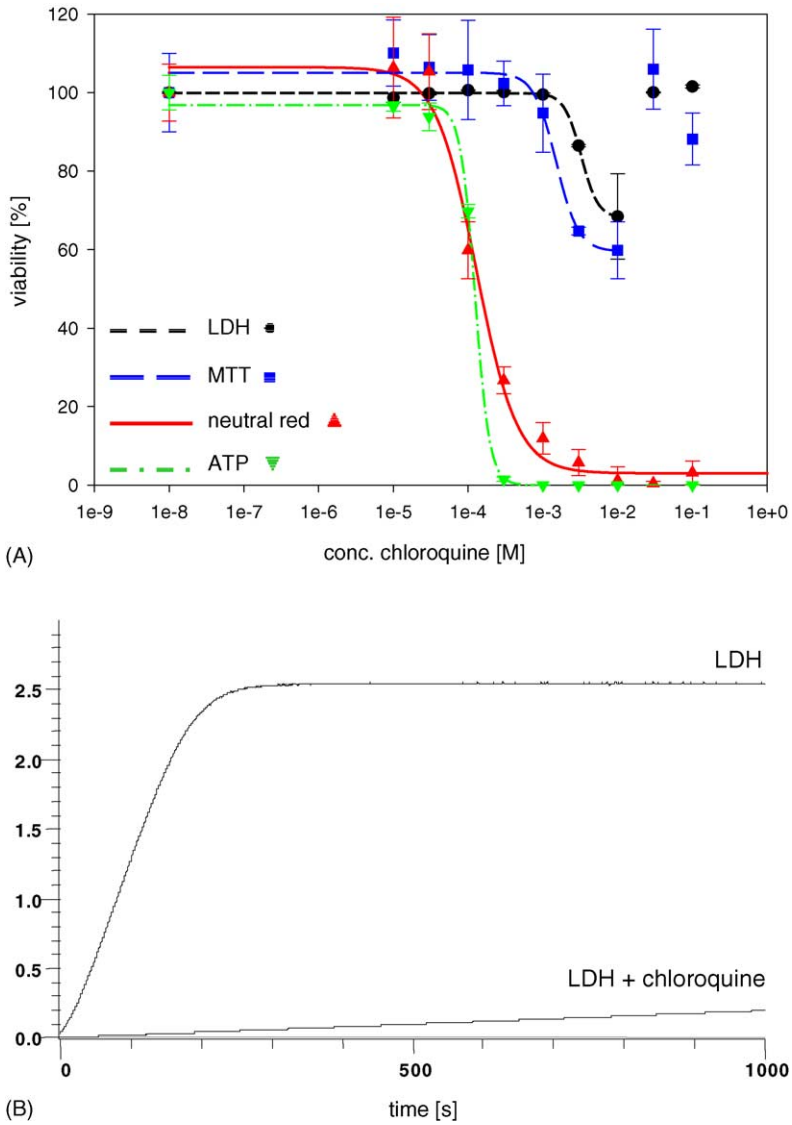

Fig. 2. (A) Concentration–viability curve of chloroquine (data points as scatter plot and Hill fit as line). (B) Time scan of the absorption (λ = 485 nm) of LDH standard with and without 200 mM chloroquine in assay media.

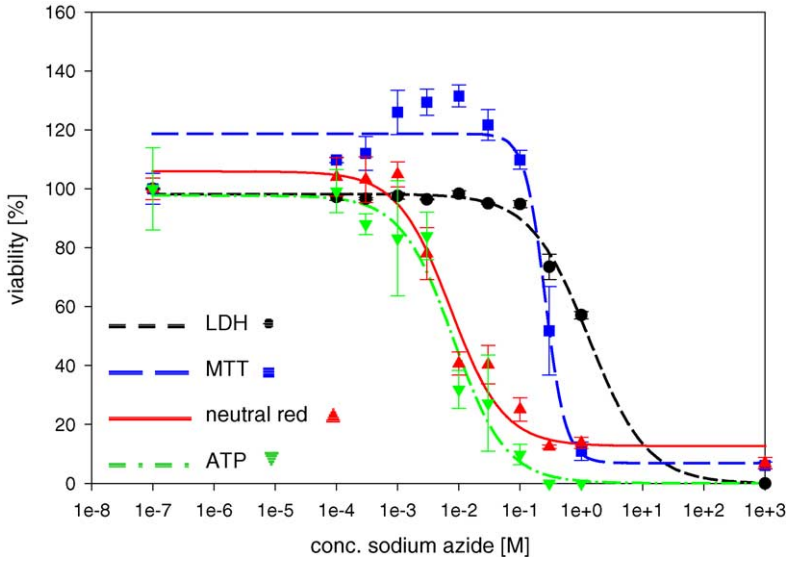

Fig. 3. Concentration–viability curve of sodium azide (data points as scatter plot and Hill fit as line).

3.3. Sodium azide

The initial induction of the MTT was observed at low concentrations of sodium azide (0.1–100 mM), which resulted in viability values of more than 100% (Fig. 3). A similar effect was described previously in literature (Isobe et al., 1999). Sodium azide showed only an effect in LDH release and MTT metabolism at concentrations of about 1 M respectively 300 mM which are hypertonic solutions. These results are in contrast

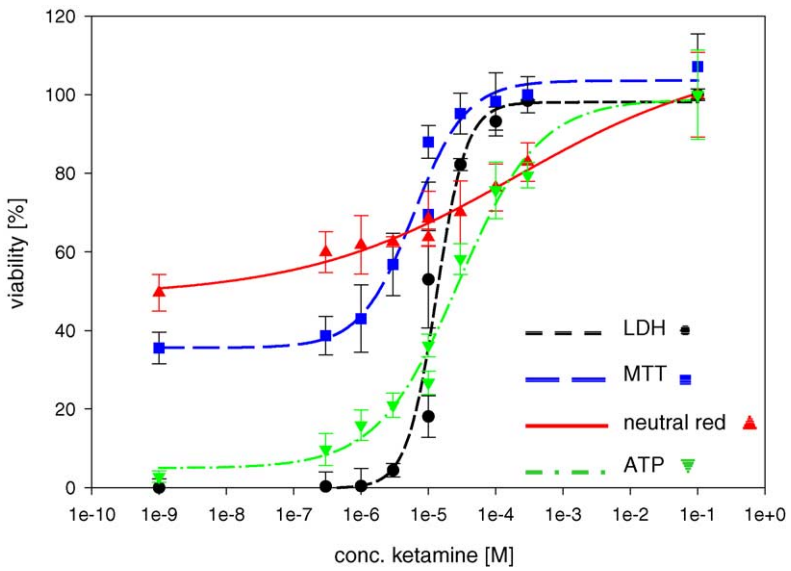

Fig. 4. Concentration–viability curve of ketamine (data points as scatter plot and Hill fit as line).

to the neutral red uptake and ATP level of the cells, where we found EC_{50}-values of about four respectively 8 mM.

3.4. NR mediated cell death (inhibition by ketamine)

Our last set of trials was dedicated to the excitotoxic effect of the expression and stimulation of NMDA receptors. The rapid and massive influx of Ca^{2+} should lead to cell death within 1 h by necrosis (Steinmetz et al., 2002). This mechanism could be prevented by the use of the open channel blocker ketamine. We investigated the dose dependency of ketamine on the viability determined by the four cytotoxicity assays. The EC_{50}-values in LDH, MTT and ATP sensitive assay varied in a small range of about 6 up to 28 μM (Table 1). The neutral red assay seems to be not suitable for this application because of the gentle course of the concentration–viability curve and the maximal reduction of about 50%, which is not satisfactory (Fig. 4).

3.5. Comparison of the different assays

The sensitivity of the cytotoxicity assay used differs depending on the different mechanisms, which lead to cell death. Some of these differences might be minimized by longer incubation with the agents, but in every case it is important to have a close look on the expected mechanism to get the right decision for or against an assay.

The LDH assay give satisfactory responses by using cell membrane damaging agents like triton X-100, but can be misleading if the toxic agent only influences intracellular activities e.g. sodium azide which inhibits the respiratory chain.

The MTT assay is simple to use. Using this assay, the metabolic activity of the mitochondria can be determined. Unfortunately, this assay shows more or less the same disadvantages as the LDH assay.

Neutral red is an inexpensive possibility to measure cell death. In some cases, this assay is less sensitive (excitoxic model) and we cannot recommend it for the use in ion channel studies.

The most sensitive assay in our study was the ATP based assay. The disadvantage of this method is the luminescence-readout, which could be influenced by quenching side effects in the samples. Furthermore, the luminescence intensity is time dependent and can lead to systemic errors.

4. Conclusion

Concluding our results, we found differences in the viability of the treated cells depending on the test agent and the cytotoxicity assay used. For this reason, it is important to consider what effect is expected, respective of what cell death mechanism is predicted. Assays like LDH and MTT, which are dependant on enzymatic reactions, might be influenced by enzyme inhibitors like chloroquine. Sometimes an inexpensive assay like the neutral red uptake assay is sufficient when a more expensive test kit fails. One has to be careful with the suitability of the assay used to prevent false-positive or false-negative results.

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