Original Paper

In Vitro Cell Death Discrimination and Screening Method by Simple and Cost-Effective Viability Analysis

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Key Words

Viability analysis • Apoptosis • Necrosis • Cell death • Cytotoxicity

Abstract

Background/Aims: For *in vitro* cytotoxicity testing, discrimination of apoptosis and necrosis represents valuable information. Viability analysis performed at two different time points post treatment could serve such a purpose because the dynamics of metabolic activity of apoptotic and necrotic cells is different, i.e. a more rapid decline of cellular metabolism during necrosis whereas cellular metabolism is maintained during the entire execution phase of apoptosis. This study describes a straightforward approach to distinguish apoptosis and necrosis. **Methods:** A431 human epidermoid carcinoma cells were treated with different concentrations / doses of actinomycin D (Act-D), 4,5,6,7-tetrabromo-2-azabenzimidazole (TBB), Ro 31-8220, H₂O₂ and photodynamic treatment (PDT). The resazurin viability signal was recorded at 2 and 24 hrs post treatment. Apoptosis and necrosis were verified by measuring caspase 3/7 and membrane integrity. **Results:** Calculation of the difference curve between the 2 and 24 hrs resazurin signals yields the following information: a positive difference signal indicates apoptosis (i.e. high metabolic activity at early time points and low signal at 24 hrs post treatment) while an early reduction of the viability signal indicates necrosis. For all treatments, this dose-dependent sequence of cellular responses could be confirmed by independent assays. **Conclusion:** Simple and cost-effective viability analysis provides reliable information about the dose ranges of a cytotoxic agent where apoptosis or necrosis occurs. This may serve as a starting point for further in-depth characterisation of cytotoxic treatments.

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Introduction

Cell death has been characterised to occur in several different forms depending on the cell type and stimulus studied [1-3]. However, in many circumstances and regarding morphology, the most important types of cell demise are apoptosis (programmed / active cell death) and necrosis (passive cell death).

Apoptosis is a strictly regulated physiological energy-consuming process essential for normal development, elimination of degenerated cells, metabolism, cell turnover, as well as functioning and development of the immune system [4, 5]. It is characterised by cell shrinkage, pyknosis, karyorrhexis and eventual phagocytosis of the dying cell [1, 3, 4, 6]. Contrary to this, necrosis leads to cytoplasmatic swelling, a dehiscence of the plasma membrane and subsequent release of intracellular material into the extracellular environment, causing inflammatory responses in the surrounding tissue [4, 6]. Both cell death mechanisms can occur simultaneously in different subpopulations of cells and – in most cases – are dependent on the applied concentration or dose of a cytotoxic agent as well as the cell type [4].

Quantification of cell death and identification of the cell death modes is an important information for general physiological questions and drug development including *in vitro* studies on cytotoxicity in different cell types as well as the dose- and time- dependency of the cytotoxic action [7]. Most assays that are currently used for detection and discrimination of apoptosis and necrosis are based on measurement of cellular characteristics: mitochondrial membrane potential decrease and cytochrome c release [8], caspase activation [9-11], morphological changes (cell swelling or shrinkage, membrane blebbing) [12-14], nuclear fragmentation [15, 16], DNA cleavage i.e. 'DNA laddering' [17], membrane integrity [11], release of intracellular compounds [18] and phosphatidylserine (PS) exposure [19]. Complementing these approaches, several types of cell viability assays exist for estimation of overall viability or viable cell mass that measure different surrogate parameters such as reducing potential and metabolic activity (e.g. resazurin, AlamarBlue®, CellTiter-Blue® [7, 11, 20, 21]), enzyme activity (conversion of tetrazolium salts such as MTT, MTS, WST [22, 23]) and adenosine triphosphate (ATP) levels [11, 24, 25]. The resazurin assay is based on the reduction of the oxidised, non-fluorescent, blue state (resazurin) to the fluorescent, pink state (resorufin) by components of the mitochondrial electron transport chain and mitochondrial and cytoplasmatic enzymes of viable and metabolically active cells (see [26-28] for details). A decreased rate of reduction (resazurin conversion) therefore indicates impairment of cellular metabolism [27].

Based on the fact that apoptosis is an energy-requiring, controlled and active process which takes several hours (depending on the cell type and probably the apoptosis inducer) to be completed, we have previously used an *in vitro* assay system based on viability analysis to simultaneously detect and discriminate apoptosis, necrosis and survival responses after photodynamic treatment (PDT) [25, 29, 30]. This approach is based on the observation that during PDT-induced apoptosis, cells maintain constantly high ATP levels until late in the apoptotic process to ensure sufficient energy supply for the energy-dependent steps in the apoptotic cascade [29-31]. As this requires active cellular metabolism, these metabolic dynamics during apoptosis – contrasting necrosis with a fast or immediate ATP and plasma membrane integrity breakdown – can be employed in viability assays if performed at two time points following treatment: at an early time point (e.g. 2 hrs), necrotic cells have already lost their metabolic activity whereas apoptotic cells are still metabolically active. At a later time point (e.g. 24 hrs), these cells have completed the apoptotic process and have undergone secondary necrosis (*in vitro*, in the absence of phagocytising cells) [32]. The difference curve calculated from early and late measurements can be interpreted to distinguish all three conventional cellular responses (survival at low, apoptosis at intermediate and necrosis at higher concentrations / doses), as discussed in the context of PDT-induced cytotoxicity in previous reports [25, 29-31].

To provide a definitive validation of this methodological approach, we tested the viability-based detection and discrimination using the resazurin assay for several different

apoptosis inducers (4,5,6,7-tetrabromo-2-azabenzimidazole (TBB), Ro 31-8220, actinomycin-D (Act-D), H₂O₂, photodynamic treatment) in an *in vitro* model system (A431 human epidermoid carcinoma cells). Confirmed by independent assays for apoptosis and necrosis, the results obtained for dose-dependent analyses demonstrate that time-dependent viability analysis can reliably detect and discriminate cell survival / proliferation, apoptosis and necrosis. Provided that the test is performed in 96-well or even 384-well microplates to ensure comparability of all samples and includes a sufficient number of different drug doses or concentrations (dose-dependent cell response characteristics), the approach described in the present report represents a cost-efficient and simple method to gain first information on the dose / concentration-dependent type of cytotoxicity within an *in vitro* treatment.

Materials and Methods

Cell culture and treatment with apoptosis inducers

Human epidermoid carcinoma cells (A431, ATCC-Nr. CRL-1555) were cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 4.5 g/l glucose, 3.7 g/l NaHCO₃, stable glutamine (Biochrom, Berlin, Germany) and supplemented with 5% foetal bovine serum (FBS) (FBS Superior, Biochrom), 1 mM Na-Pyruvat (Sigma-Aldrich, Vienna, Austria), antibiotic antimycotic solution (100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 µg/ml amphotericin-B; Sigma-Aldrich), in a humidified atmosphere at 37°C and 5% CO₂. For measurement of cell viability, caspase 3/7 activity and membrane integrity, 10,000 cells per well were seeded into transparent 96-well microplates (Sarstedt, Nümbrecht, Germany). Prior to treatment (24 hrs after seeding the cells), cells were washed once with 100 µl 0% FBS DMEM (further referred to as 'DMEM'). As established apoptosis inducers, the following drugs or treatments were used: RO 31-8220 (bisindolylmaleimidine IX, pan-PKC inhibitor, Selleckchem, Munich, Germany [33]), TBB (casein kinase II inhibitor, Sigma-Aldrich [34]), actinomycin D (DNA-dependent inhibitor of RNA synthesis, Abcam Biochemicals, Cambridge, United Kingdom [35]), H₂O₂ (Sigma-Aldrich [36]) and a PDT treatment (Foscan®, BioLitec Pharma, Dublin, Ireland [37]). Cells were incubated with eleven different concentrations (serial 1+1 dilution) of RO 31-8220 (starting at 10 µM), TBB (40 µM), Act-D (20 µM), or H₂O₂ (40 mM) in DMEM. Based on previous experience [25, 29, 30], the samples were incubated at 37°C for 2 or 24 hrs for the resazurin viability analysis, 5 hrs for caspase 3/7-activity [38] and 2 hrs for membrane integrity measurements, respectively. For PDT treatment, cells were incubated with 0.3 µM of Foscan® in DMEM for 20 hrs, washed once with 100 µl DMEM per well and illuminated with a 660 nm LED array [39] with energies ranging from 0.07 to 1.05 J/cm² (0.5-7.5 min illumination) and processed as described above. The final volume per well after treatment is 100 µl for the cell viability assays, whereas in all other assays it is 50 µl per well.

Time-dependent cell viability assay

The procedure for time-dependent viability analysis as well as the theoretical basis for interpretation is summarised in Fig. 1. After incubation for 2 or 24 hrs, cell viability was measured in each half of the 96-well microplate using the resazurin assay (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt; Sigma-Aldrich). The supernatant was replaced by 100 µl DMEM containing 0.5 mM resazurin (stock solution 2.5 mM in phosphate-buffered saline, PBS). After 1 hr of incubation at 37°C, 90 µl of the supernatant were transferred to a new 96-well microplate and stored at -20°C until measurement. The fluorescence of the product (resorufin) was detected at λ_{ex}=535 nm and λ_{em}=595 nm (intensity-top-measurement) using a Zenyth microplate reader (Anthos, Salzburg, Austria). Mean viability values were corrected for blank values (without cells) and related to untreated controls (UTC, without apoptosis inducer). All treatments were measured in quadruplicate wells, repeated three times and final results represent mean percentages of untreated controls ± SEM.

Apoptosis assay – caspase 3/7

Five hours post-treatment, caspase 3/7 activity was measured by means of the Caspase-Glo® 3/7 Assay based on the luminogenic peptide substrate DEVD (Promega, Mannheim, Germany) following the manufacturer's instructions. Cell treatments were performed using 50 µl supernatant in single wells and after addition of the substrate (50 µl/well) and 20 min of incubation at 37°C, 90 µl of the supernatant were transferred to a white 96-well microplate (Greiner, Kremsmünster, Austria). The luminescence was measured with the Zenyth microplate reader (integration time: 1 s). Results represent mean values ± SEM of at least three independent experiments.

Necrosis assays

Early loss of membrane integrity accompanied by release of intracellular material, i.e. necrosis, was verified using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega), which is based on the amount of active lactate dehydrogenase (LDH) released into the supernatant by necrotic cells. According to the manufacturer's instructions, after 100 min, the plate was transferred to room temperature (22°C) and after additional 20 min (i.e. at 2 hrs post-treatment), 50 µl of the CytoTox-ONE™ reagent were added to all wells. After incubation for 10 min at room temperature, 25 µl of stop solution were added to all wells and the fluorescence signal was measured with an Infinite M200 microplate reader (Tecan, Grödig, Austria). All samples were related to maximum LDH release values (positive control) and were measured in single wells. Results represent mean values ± SEM of four independent experiments. Positive control wells (maximum LDH release values) were generated by addition of Triton X100 (final concentration 1% v/v; Scharlau, Scharlab, Spain) 10 min prior to addition of the CytoTox-ONE™ reagent and subsequently processed as described above.

As the CytoTox-ONE™ Assay did not give reliable LDH-release signal after PDT treatment in preliminary experiments, for PDT samples, necrosis was measured using the CytoTox-Glo™ Cytotoxicity Assay (Promega). This assay uses a luminogenic peptide substrate (AAF-Glo™ Substrate) to measure specific protease activity, which is only detectable after membrane integrity loss. For this purpose, 2 hrs post treatment, 25 µl of CytoTox-Glo™ reagent were added to each well, accompanied by positive control wells (Triton X100, as described above). After 15 min of incubation at room temperature, the luminescence signal was measured using the protocol described above for the CytoTox-ONE™ Assay.

Results

Time-dependent viability analysis

Figure 2A shows the results from the resazurin-based viability analysis obtained at the two time points (2 and 24 hrs) post treatment: for Ro 31-8220, H₂O₂ and PDT (row a, d, e), a concentration-dependent continuous decrease of the 2 hrs viability signal can be noted (e.g. down to <10% of untreated controls at H₂O₂ concentrations >10 mM) while for TBB and Act-D (row b and c), the early reading (2 hrs) indicates no or only small decreases of viability even at the highest drug concentrations. In contrast, viability measured at 24 hrs post treatment drops to nearly 0% for all treatments – either at the highest concentration (Ro 31-8220, TBB) or already at lower concentrations (Act-D, H₂O₂, PDT).

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Fig. 1. Plate layout and experimental workflow of the time-dependent viability analysis. (A) Plate layout for 2 measurement time points, 11 different drug doses, blanks and untreated control wells. (B) Raw data for early and late viability measurement. (C) Calculation of the early versus late reading difference curve. (D) Interpretation relating to the drug / treatment dose ranges. Abbreviations: UTC, untreated control; Viab, viability. Modified from [25].

Fig. 2. Establishment and validation of viability-based cell death discrimination. For several cytotoxic agents including Ro 31-8220, TBB, Act-D, H_2O_2 and photodynamic treatment (PDT), the proposed viability-assay and specific tests for apoptosis and necrosis were performed for a range of concentrations / doses. (A) Cell viability values measured with resazurin assays at 2 (filled squares) and 24 hrs (filled circles) post treatment and related to the signal of untreated controls (UTC, 100%). Filled triangles represent the difference curve calculated by subtraction of the late from the early viability reading. (B) Caspase Glo® 3/7 activity at 5 hrs post treatment. (C) Necrosis measured by LDH release (CytoTox-ONE™) indicating loss of membrane integrity for Ro 31-8220, TBB, Act-D and H_2O_2 . For PDT treatments, necrosis was measured based on the detection of dead cell protease-release with AAF-Glo™ Substrate (CytoTox-Glo™).

Interpretation and identification of cell death modes

As outlined in Fig. 1, the peak of the curve calculated by subtraction of the late from the early viability reading should indicate the range of concentrations (treatment doses) where cells undergo apoptosis. The 'delta' curves in Fig. 2A show distinct peaks for H_2O_2 and PDT treatment, while for Ro 31-8220, TBB and Act-D the difference curve continuously increases with increasing drug concentrations. In case of H_2O_2 and PDT, higher concentrations or treatment doses beyond the peak of the difference curve result in its decrease back down to ~0%. This is due to the small (absent) viability difference between early and late viability readings at these (highest) concentrations indicating treatment doses where all cells lose their viability early after treatment. As described in Fig. 1, this dynamics can be interpreted as necrotic cell death characterised by early loss of membrane integrity and metabolic activity.

Verification of apoptosis

Figure 2B shows the results for apoptosis induction (caspase 3/7 activity) at 5 hrs post treatment (based on previous studies [29, 30]) using the identical concentrations and treatment doses as for the viability analysis: the peak of caspase activity corresponds well with the peak of the viability difference curve ('delta') in case of Ro 31-8220, H_2O_2 and PDT treatment. For TBB and Act-D, only the left (raising) part of the caspase activation peak is observable – well reflecting the continuous increase of the viability difference curve up to the highest concentrations. Beyond the maximum of caspase activity, higher drug / treatment

doses (in case of Ro 31-8220, Act-D, H_2O_2 and PDT) cause a decrease of the caspase signal within the concentration ranges that are presumably causing necrotic cell death.

Verification of necrosis

In figure 2C, results from the necrosis induction measurements are shown. Necrosis induction was measured by LDH activity or necrosis-specific release of cell proteases into the supernatant from leaky, i.e. necrotic cells. While for the 'biochemical' apoptosis inducers (Ro 31-8220, TBB and Act-D) no increase of necrosis can be observed at any concentration employed, high concentrations (>5 mM) of H_2O_2 and PDT doses (>0.2 - 0.4 J/cm²) cause a continuous increase of the LDH signal. This increase again corresponds well to the 'necrosis range' indicated by the viability difference analysis (Fig. 2A) which identifies the same treatment ranges as necrosis-inducing – based on the absence of a difference between early and late viability readings.

Discussion

Today, several distinct cell death mechanisms are described including anoikis, autophagic cell death, cornification, mitotic catastrophe, excitotoxicity, entosis, pyroptosis, netosis, oncosis and parthanatos [2, 3] which question the 'formal' classification of cell death including only three variants: i) apoptosis (type I), ii) autophagy (type II, extensive cytoplasmic vacuolization), and iii) necrosis (type III, showing neither apoptotic nor autophagic characteristics) [40]. However, the morphologically most distinct and experimentally most often observed death types are apoptosis and necrosis. The discrimination of cell survival, apoptosis and necrosis represents an important basic information for determining appropriate drug concentrations for many *in vitro* experiments and hence importantly also for drug development including characterization of a drug's cytotoxic, dose- and time-dependent mode of action [7]. While qualitative and quantitative identification of apoptosis and necrosis is based on morphological and biochemical characteristics ascribed to these types of cell demise (see introduction), notably, these 'hallmarks' do not represent definitive and stand-alone criteria for discrimination of the cell death mode [40]. Examples include that mitochondrial cytochrome-c release was observed in cells undergoing necrosis [41], caspase inhibition could delay cell death after plasma membrane disruption [42], caspase-3 activation occurs under physiological conditions not related to cell death [43], apoptosis may occur without DNA cleavage while oncosis may involve such changes [44], PS exposure is involved in numerous (patho-)physiological processes (e.g. platelet activation and swelling-induced erythrocyte haemolysis [45-47]), and, canonical apoptotic stimuli may induce initial cell swelling [48, 49].

The aim of this study was to validate a rapid and cost-effective *in vitro* method that can serve as an easy-to-use tool to distinguish between apoptosis and necrosis – based on simple viability analyses. Viability assays represent a cost-effective method to distinguish living from dead cells based on metabolic activity (mostly as enzymatic activity / reducing potential). As the temporal kinetics of this activity is different for apoptotic and necrotic cells [25, 29-31], performing viability assays at an early and a late time-point after death induction gives information about when and at which doses cells undergo apoptosis and secondary necrosis. The late time point (e.g. at 24 hrs post treatment) measures the viability present after completion of apoptosis and – in cell culture, i.e. in the absence of phagocytising cells – secondary necrosis [31]. In contrast, an early viability signal can be attributed to cells either having survived the treatment or being in the process of apoptosis which requires active metabolism and energy supply until late steps in the apoptotic process [29, 30]. Positive values (peak) of the calculated difference between early and late measurements stand for the proportion of cells which have lost their viability compared to the early time point of measurement and, therefore, indicate the dose range where apoptosis has been triggered. Table 1 summarises viability assays based on metabolic activity which are suitable for the proposed time-dependent assay approach.

After cell death induction with TBB and Act-D, the difference curve is increasing continuously indicating a homogeneous apoptotic population at the highest drug dose. Contrary to that, the difference curve after H_2O_2 -treatment has a narrow peak: this well reflects the apoptotic population of cells as verified by the caspase assay and is followed by increasing loss of membrane integrity indicating necrotic cells. Reflecting the nature of PDT as a cytotoxic treatment which is based on (unspecific) generation of reactive oxygen species (ROS), the viability difference curve shows a rather broad peak – i.e.

a broad range of apoptosis induction in a subpopulation of cells and a dose-dependent continuous increase of a necrotic subfraction of cells.

While the interpretation of the viability reads in our experiments is confirmed by independent and specific assays for apoptotic and necrotic cell death, application of this approach for other cell types or treatment conditions may require establishment of particular assay conditions. Most importantly, the time points of the viability assay must be chosen to allow for correct interpretation: if a cell type requires more time to complete the apoptotic programme (e.g. >24 hrs), the time point for 'late' measurement has to be adjusted accordingly. Furthermore, the results obtained by the proposed viability analyses should be confirmed by tests more specific for apoptosis / necrosis for each new cell type to ensure validity of the results.

Conclusion

Provided that appropriate measurement time points are known for a certain cell type and cytotoxic agent, simple and cost-effective cell death discrimination is possible by viability analysis and may serve as screening method for evaluation of new drugs or cytotoxic treatments.

Disclosure Statement

The authors declare no conflict of interest.

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Table 1. Suitable microplate-based (viability) assays (see [21] for an overview). ¹ Numerous vendors offer commercial assay kits (trade names for resazurin-based assays, e.g. CellTiter-Blue® Cell Viability Assay or AlamarBlue® Cell Viability Reagent). Abbreviations: ATP, adenosine-5'-triphosphate; MTS, 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl)tetrazolium, inner salt; MTT, methylthiazolyldiphenyl-tetrazolium bromide; WST-1, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium; XTT, sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate

Assay ¹	cell lysis required	Readout		
		absorbance λ (nm)	fluorescence $\lambda_{ex} / \lambda_{em}$ (nm)	luminescence λ_{max} (nm)
Resazurin	no	570-600 nm	530-560 / 590	-
MTT	yes	550-600 nm	-	-
XTT	no	450-500 nm	-	-
MTS	no	490-500 nm	-	-
WST-1	no	420-480 nm	-	-
ATP	yes	-	-	550-570 nm

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