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A bioassay for the simultaneous measurement of metabolic activity, membrane integrity, and lysosomal activity in cell cultures

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ABSTRACT

The aim of this study was the development of an in vitro bioassay that combines several endpoints of general cytotoxicity for the screening of compounds or mixtures of compounds with potential bioactivity. The Alamar Blue assay was employed to assess metabolic activity, the Neutral Red assay was used for the assessment of membrane function and lysosomal activity, and the lactate dehydrogenase leakage assay was employed for the assessment of membrane integrity. Each assay was performed separately and in combination using a human fibroblast cell line (MRC-5). Three fungal secondary metabolites of different chemistry that affect different cellular targets were tested as model compounds: deoxynivalenol, enniatin B1, and 2-amino-14,16-dimethyloctadecan-3-ol. The obtained inhibitive compound concentrations for the assays performed separately and in combination were not significantly different ($P < 0.05$, $n = 9$). The combination of several cytotoxicity endpoints in a single assay increases the chance that potential bioactive/cytotoxic compounds are discovered during the screening of mixtures of natural compounds (e.g., extracts from fungal cultures or plants) when one endpoint fails and, at the same time, might give some basic information on the cellular target.

During the past decade, methods of in vitro toxicology (e.g., based on cell cultures) have become a valuable research tool for the elucidation of the mechanism of toxicity as well as for the screening of chemicals or mixtures of chemicals for possible harmful (or beneficial) effects on animals and humans. Commonly, these methods are inexpensive, are easy to set up, and yield quick results [1]. In vitro toxicity tests have even been applied sporadically for the purpose of risk assessment of food additives [2].

Many different cell types, both continuous and primary, as well as a range of different endpoints have been used for the study of natural toxins (e.g., toxins of fungal origin) [1]. When it comes to the screening of chemical mixtures of unknown composition for bioactivity/toxicity, the first choice is commonly simple, unspecific viability assays, which respond to most compounds that affect the cells in some way. Integrity of the cell membrane is one of the most used parameters for cytotoxicity, and different methodologies have been described for the assessment of cell membrane integrity [2]. The release of stable intracellular enzymes such as lactate dehydrogenase (LDH)[1] has been used in many studies, as has the release of a label such as ^{51}Cr or calcein [3–7]. Assays that measure the metabolic activity of cells are other examples for the evaluation of general cytotoxicity. For example, different tetrazolium compounds have been used for this purpose [1,8]. The Alamar Blue (AB) assay evaluates the metabolic activity of cells based on the reduction of resazurin (blue and nonfluorescent) to resorufin (pink and highly fluorescent) in the presence of metabolically active cells [9]. Finally, the Neutral Red (NR) assay has, in many instances, been used for the quantification of cytotoxicity based on the ability of viable cells to incorporate and accumulate the weakly cationic supravital dye within lysosomes [10,11]. All three assays provide information about basal cytotoxicity and reflect the intrinsic ability of test compounds to cause cell death as a consequence of damage of basic cellular functions. However, it has also been shown that different assays of general cytotoxicity may respond differently to some test compounds [12,13]. As a result, molecules of potential bioactivity/toxicity may be overseen during the screening of potential targets. The combination of several endpoints in a single assay, therefore, would increase the validity and reliability of the assay and decrease the risk that compounds of interest are missed.

The main objective of this study was to combine three commercial cytotoxicity assays such that all three may be performed simultaneously in a single-exposure experiment. Three model compounds of fungal origin were chosen to test whether identical inhibitive concentrations (IC_{50} values) were obtained from the dose–response plots when the assays were performed separately or in combination: a tetracyclic sesquiterpene that inhibits protein synthesis by binding to a ribosomal subunit, deoxynivalenol

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[1] *Abbreviations used:* LDH, lactate dehydrogenase; AB, Alamar Blue; NR, Neutral Red; DON, deoxynivalenol; AOD, 2-amino-14,16-dimethyloctadecan-3-ol; ENN B1, enniatin B1; EMEM, Earl's minimum essential medium; BSS, Earl's balanced salt solution.

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(DON); a sphingosine analogue toxin, 2-amino-14,16-dimethyloctadecan-3-ol (AOD); and a cyclic depsipeptide possibly acting primarily as an ionophore, enniatin B1 (ENN B1) [14–16].

Materials and methods

Reagents

Methanol (Romil, Cambridge, UK) was of HPLC quality. Water was purified and deionized using a Purity Analyst HP water purifier (Oxon, UK). DON (99.4% purity) was purchased from Biopure (Tulln, Austria). AOD and ENN B1 were isolated and purified from rice cultures of the fungus *Fusarium avenaceum* as reported elsewhere [15,17].

Cell culture

The human fibroblast-like fetal lung cell line MRC-5 was obtained from the European Collection of Cell Cultures and grown in Earl's minimum essential medium (EMEM, Cambrex, Verviers, Belgium) containing l-glutamine and Earl's balanced salt solution (BSS). The medium was supplemented with 10% fetal bovine serum (Cambrex). The cells were reseeded after trypsination once or twice per week in a 1:5 split ratio. MRC-5 cells were grown as monolayers in 75-cm^2 cell culture flasks with filter screw caps (Techno Plastic Products, Trasadingen, Switzerland) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Alamar Blue assay

The innate metabolic activity of MRC-5 cells was measured using AB [9]. Experiments to determine the optimal dilution of AB, incubation time, and cell density to obtain linear relationships between fluorescence and cell densities were carried out according to the manufacturer's instructions. Cells were diluted to 2×10^5 cells/ml, and 100 μl of this suspension was transferred to the 60 inner wells of 96-well plates (2×10^4 cells/well, Techno Plastic Products). The outer wells of the plates were filled with 200 μl of medium. The cells were grown overnight to allow the cells to attach and proliferate. On the next day, 100 μl of fresh medium containing 4% of the prepared dilutions of the test compounds in methanol was added to the cell cultures in triplicates. Blank (medium without cells), negative control (medium with cells), and solvent control (2% methanol in medium) were run for each experiment. The plates were incubated for 24 h. Then 8 μl of AB solution was added to each well, and the plates were further incubated for 3 h. The product of the metabolic reduction of AB, resorufin, was quantified using fluorometry on a Victor^2 Multilabel Counter (PerkinElmer, Boston, MA, USA) at an excitation wavelength of 530 nm and an emission wavelength of 580 nm.

Lactate dehydrogenase leakage assay

The release of LDH from the cytoplasm into the surrounding culture medium was monitored using the CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega, Madison, WI, USA). The assay is based on the diaphorase-mediated conversion of resazurin into the fluorescent resorufin product after LDH-catalyzed enzymatic conversion of lactate to pyruvate (Promega technical bulletin). Briefly, 100 μl of cell suspension (2×10^5 cells/ml) was transferred to the 60 inner wells of a 96-well plate (2×10^4 cells/well, Techno Plastic Products). The outer wells were filled with 200 μl of medium. The plate was incubated overnight at 37 °C to allow the cells to attach and proliferate. On the next day, 100 μl of fresh medium containing 4% of the test compounds was added to each well, and the plates were incubated at 37 °C. After 24 h, the plates were removed from the incubator and equilibrated to 22 °C (30 min), and then 100 μl of medium from each well was carefully transferred to a new plate. After that, 100 μl of CytoTox-ONE reagent was added to each well, and the plates were incubated for 10 min at room temperature. The enzymatic reaction was stopped by adding 50 μl of the assay's "stop solution." Resorufin fluorescence was measured using the Victor^2 multilabel counter with an excitation wavelength of 530 nm and an emission wavelength of 580 nm. Each experiment was performed with growth control (medium with cells), lysis control, and solvent control (2% methanol in medium).

Neutral Red assay

The membrane function and lysosomal activity of MRC-5 cells were measured with the NR cytotoxicity assay (Xenometrix, Allschwil, Switzerland). Cells were seeded in 96-well culture plates (Techno Plastic Products) at 2×10^4 cells/well and incubated overnight at 37 °C to allow the cells to attach and proliferate. On the next day, 100 μl of fresh medium containing 4% of the test compounds was added to each well, and the plates were incubated at 37 °C. After 24 h of incubation in the presence of the test compounds, the medium was replaced by 200 μl of medium containing 0.85% NR. The NR labeling solution was diluted with prewarmed media, with the optimal dilution (with regard to the formation of dye crystals) having been determined prior to the study. The cells were incubated for an additional 3 h at 37 °C in a humidified atmosphere containing 5% CO_2. The NR-containing medium was then discarded, and the cells were subsequently treated with 200 μl of fixing and solubilization solutions that are included in the commercial kit. Finally, the absorbance of the dissolved NR was measured at 540 nm using the Victor^2 multilabel counter with 690 nm as the reference wavelength.

Combination of AB, LDH leakage, and NR assays

A cell suspension (100 μl, 2×10^5 cells/ml) was transferred to the 60 inner wells of a 96-well plate (2×10^4 cells/well, Techno Plastic Products). The outer wells were filled with 200 μl of medium. The plate was incubated overnight at 37 °C to allow the cells to attach and proliferate. On the next day, 100 μl of fresh EMEM containing 4% of the test compounds was added to each well, and the plates were incubated at 37 °C. After 24 h, the plates were removed from the incubator and equilibrated to 22 °C (30 min). Then 100 μl of medium from each well was carefully transferred to a new plate, and LDH activity was measured as described above. After that, 100 μl of EMEM containing 1.7% of NR together with 8 μl of AB solution was added to the wells of the original test plate. The original plate was incubated at 37 °C for 3 h. After this incubation period, the metabolic product of AB (resorufin) was measured directly by fluorometry as described above. Finally, the medium was discarded, the cells subsequently treated with 200 μl of fixing and solubilization solutions that are included in the NR kit, and the absorbance of NR was measured at 540 nm using the Victor^2 multilabel counter.

Statistics and data analysis

The IC_{50} values of all test compounds were determined from sigmoidal dose–response plots using GraphPad Prism 4.0 (GraphPad, San Diego, CA, USA). JMP software (version 5.0a, SAS Institute, Cary, NC, USA) was used for the statistical analyses of the collected data. Means were considered as statistically different for $P < 0.05$.