CASY System, Multi-Parameter Cell Counter and Analyzer

Focus Application
Oncology Studies

Featured Study:
Using the CASY Cell Counter and Analyzer for Proliferation and Viability Measurement in Oncological Studies

- Use the CASY System for label-free multi-parameter quality control of cell cultures.
- Exclude necrotic cell death using the Roche LDH assay when testing antileukemia/antitumor compounds.
- Obtain quantitative information about apoptosis early after treatment (<24 hours) using the CASY System.
- Confirm apoptosis using the Roche Annexin-PJ and DNA-fragmentation assays.

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Introduction

In recent decades, great efforts have been made to improve the outcome of individuals with acute leukemia. Today, most children suffering from acute leukemia can be cured using standardized, combined chemotherapeutics. Nevertheless, the development of drug resistances limits the efficiency of therapy and poses a challenge to find new substances that can overcome multiple drug resistance in leukemia and tumor cells.

The focus of our research group is the examination of new antileukemia and antitumor compounds that induce apoptosis in a wide range of human tumor cell lines and can overcome the multiple drug resistance in leukemia and tumor cells. Our group established leukemia and tumor cell lines which are resistant to common cytostatics clinically used for the treatment of children with acute lymphoblastic leukemia (ALL). Different chemosensitivity assays are used and described below, which show the ability of newly synthesized compounds to overcome drug resistance *in vitro* using drug resistant leukemia cells, as well as *ex vivo* with drug resistant primary lymphoblasts from children with ALL.

Here we identify specific anticancer properties of substance A in Burkitt-like lymphoma cells (BJAB cells). Using the CASY Cell Counter and Analyzer, BJAB cells were diluted and cultured at a cell concentration of 1 x 10^5/mL for the following experiments.

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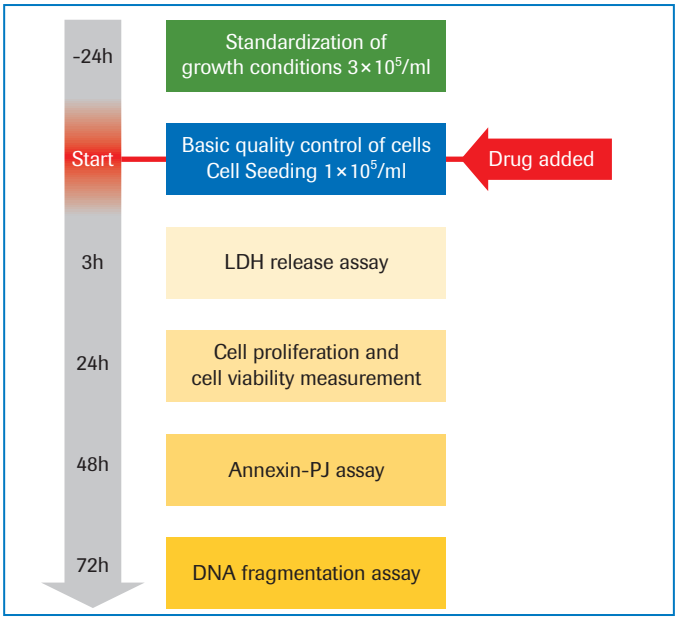

Figure 1: Typical workflow.

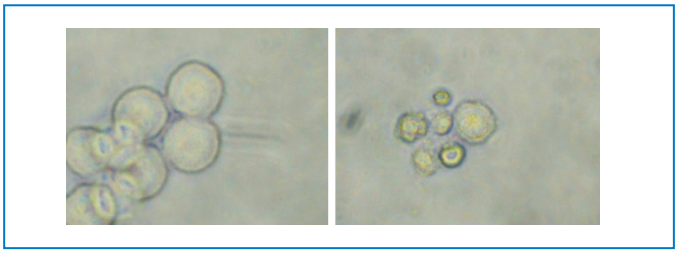

Figure 2: Untreated lymphoma cells (BJAB cells, left). Shrinking and blebbing are typical signs of apoptosis induction in lymphoma cells (BJAB cells, right).

The LDH release assay in BJAB cells demonstrates that necrotic damage is negligible after 3 hour incubation with substance A. Treatment with substance A for 24 hours was shown to inhibit proliferation and decrease cell viability, as measured using CASY Cell Counter and Analyzer. Using the Annexin-PJ-Assay, a precise distinction between necrotic, early and late apoptotic cells was found after 48 hour incubation. Specific apoptotic cell death was detected using the DNA fragmentation assay after treatment for 72 hours.

Materials and Methods

Cell Culture Conditions

The CASY Cell Counter and Analyzer was used for basic quality control, including measurements of cell number, cell viability, cell volume, cell aggregation, and cell debris. BJAB (Burkitt-like lymphoma) cells were subcultured every 3–4 days by dilution to a concentration of 1 x 10^5/mL.

All experiments were performed in DMEM or RPMI 1640 (GIBCO, Invitrogen), supplemented with 10% heat inactivated fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.56 g/L L-glutamine. Twenty-four hours before the assay was setup, cells were cultured at a concentration of 3 x 10^5 /mL to attain standardized growth conditions. For apoptosis assays, cells were diluted to a concentration of 1 x 10^5/mL immediately before addition of the different drugs.

LDH-Release Assay

Nonspecific necrotic cell death is characterized by the early release of lactate dehydrogenase (LDH), whereas apoptotic cells initially retain their membrane integrity and do not exhibit rapid release of large intracellular proteins such as LDH. Cytotoxicity of substance A was measured by the release of LDH as described previously [1,6,7,8]. After treatment with different concentrations of the test agent for 3 hours, LDH release in BJAB cells was measured in cell culture supernatants using the Cytotoxicity Detection Kit from Roche Diagnostics (Mannheim, Germany). Supernatants were centrifuged at 1500 rpm for 5 min; 20 µL of cell-free supernatants were diluted with 80 µL phosphate-buffered saline (PBS), and then 100 µL reaction mixture containing 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium chloride (INT), sodium lactate, NAD+ and diaphorase was added. Time-dependent formation of the reaction product was quantified photometrically at 490 nm. The maximum amount of LDH activity released by the cells was determined after cell lysis using 0.1 % Triton X-100 in culture medium. This value was used to represent 100% cell death.

Determination of Cell Concentration and Cell Viability

Cell viability was determined using the CASY Cell Counter and Analyzer (Roche Diagnostics, Mannheim, Germany) [9,10]. With the CASY System, cell concentration can be analyzed simultaneously in three different size ranges. Cell debris, dead cells, and viable cells are determined in a single measurement. A cell-specific setup was used to define dedicated range settings for the differentiation of dead and viable cells. This was done by measuring a mixture of viable cells (>90% viability) with a standardized sample of dead cells, prepared using CASY blue (Roche Diagnostics, Mannheim, Germany).

Cells were seeded at a density of 1 x 10^5 cells/mL and treated with different concentrations of the test substance; non-treated cells served as controls. After a 24 hour treatment, cells were resuspended completely and 100 µL from each well was diluted in 10 mL CASY ton ready-to-use isotonic saline solution (Roche Diagnostics, Mannheim,

Germany) for automated multi-parameter cell counting. The CASY System can not be used to differentiate between necrotic and apoptotic cell death. Necrotic cell death can be excluded using the LDH Release assay. The CASY System provided significant early information about apoptosis induction.

Annexin-V-Propidium Iodide Assay

Apoptotic cells can be detected by membrane alterations, such as phosphatidylserine translocation, and differentiated from necrotic cells by flow cytometry using the Annexin-V-FLUOS Kit (Roche Diagnostics, Mannheim, Germany). This method provides simultaneous staining of cell surface phosphatidylserine with Annexin-V-FLUOS and necrotic cell labeling with propidium iodide (PI). During apoptosis, the phospholipid phosphatidylserine is exposed to the outer leaflet of the plasma membrane [2, 3]. Annexin-V–FLUOS then binds to phosphatidylserine leading to an increase in fluorescence. In contrast, PI is excluded from non-necrotic cells with intact membranes. A positive PI result is therefore a sign of cell necrosis, whereas cells which are annexin-V–FLUOS positive, but PI negative, are generally defined as early apoptotic [4]. For the Annexin-V/PI assay, 1 x 10^5 cells were measured using the CASY Cell Counter and Analyzer, and prepared according to the manufacturer´s instructions. Analyses were performed on a FACScan using the CellQuest analysis software.

Measurement of DNA Fragmentation

Apoptosis, in contrast to nonspecific necrosis, is a controlled and regulated program leading to cell death. DNA fragmentation (hypoploidy) is a typical characteristic of apoptotic cell death. DNA fragmentation was measured as previously described [5]. Apoptosis triggered by the applied agent was quantified using flow cytometric measurements of DNA fragments after incubating BJAB cells for 72 hours with the agent to be tested. The modified cell cycle FACS analysis detects both nuclear DNA fragmentation at a single cell level and hypodiploid DNA.

Cells were seeded at a density of 1 x 10^5 cells/mL, and treated with different concentrations of the test substance. After a 72 hour treatment at 37°C, cells were collected by centrifugation at 1500 rpm for 5 min, washed with PBS at 4°C and fixed in PBS / 2% (v/v) formaldehyde on ice for 30 min. After fixation, cells were pelleted, incubated with ethanol/PBS (2:1, v/v) for 15 min, pelleted and resuspended in PBS containing 40 µg/mL RNase. RNA was digested for 30 min at 37°C, after which the cells were pelleted again and resuspended in PBS containing 50 µg/mL propidium iodide. Data were collected and analyzed using a FACScan equipped with CELL Quest software. Data are given in percent hypoploidy (subG1), reflecting the number of apoptotic cells.

Results

Necrotic cell death which is accompanied by the early release of lactate dehydrogenase (LDH) could be ruled out using the LDH release assay. Figure 2 demonstrates that LDH release from BJAB cells after 3 hour treatment is not correlated with increasing concentrations of the applied test agent. This result clearly indicates that the test agent induces negligible nonspecific cell damage, indicating that is acting as a proper anticancer agent by inducing apoptosis.

The inhibition of cell proliferation and the decrease in cell viability induced by substance A, was evaluated *in vitro* in BJAB cells. After treatment of 24 hours, cell viability and cell number were measured using the CASY Cell Counter and Analyzer. Figure 3 shows the concentration dependent decrease of cell proliferation induced by the test compound. Figure 4 shows the change of cell size distribution used

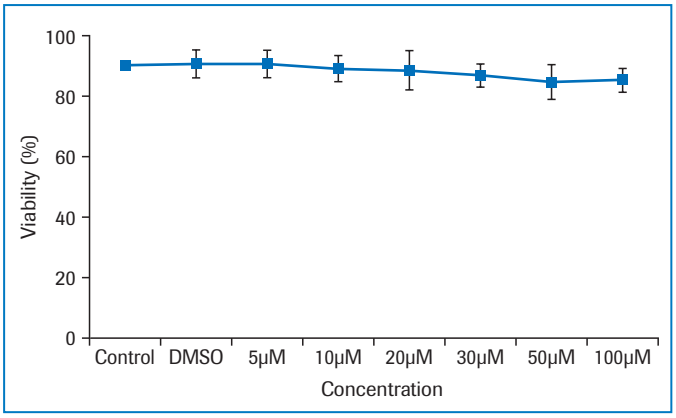

Figure 3: Significant necrosis in the presence of substance A was not observed in BJAB cells. Cell viability was determined using the LDH release assay after an incubation period of 3 hours.

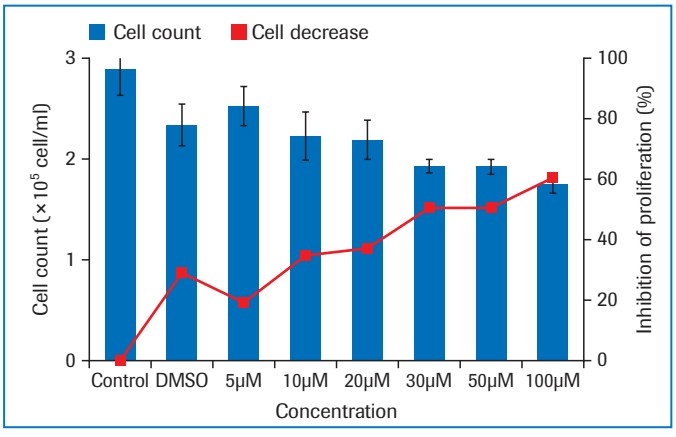

Figure 4: Substance A inhibits cell proliferation in a dose-dependent manner. BJAB cells were seeded at a density of 1 x 10^5 cells/mL, and cell proliferation was measured after 24 hours using the CASY Cell Counter and Analyzer.

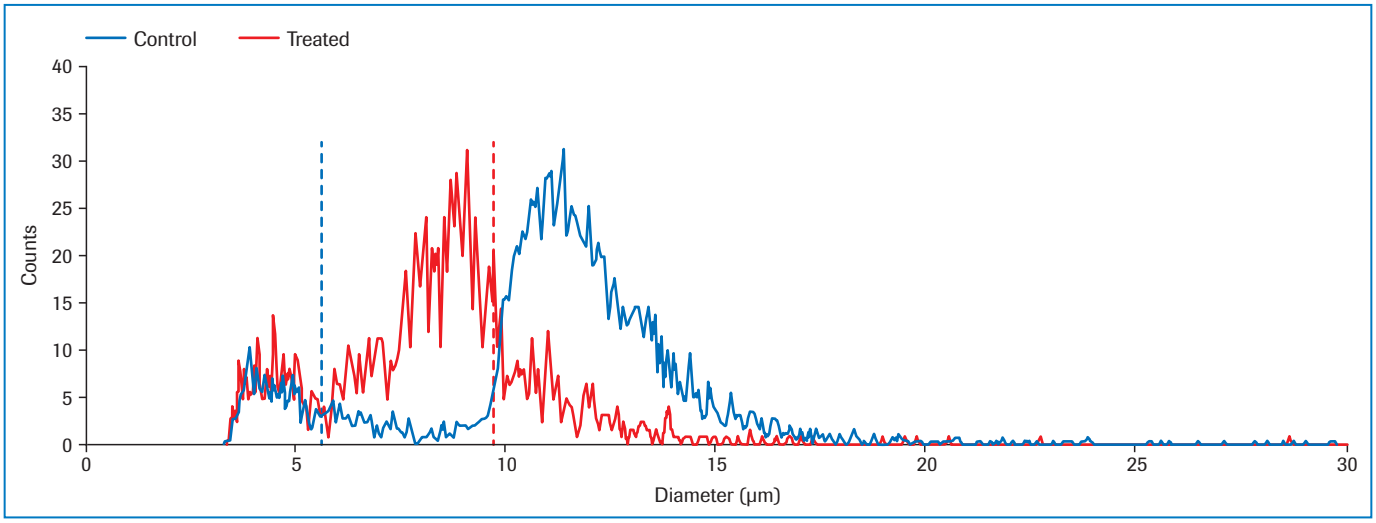

Figure 5: Decrease in cell viability based on cell size distribution. Substance A causes a dose-dependent decrease in cell viability. The cell size distributions of BJAB cells incubated with 50 µM of the compound for 24 hours were recorded using the CASY Cell Counter and Analyzer and compared with the cell size distribution of the control. Cell viability decreased from 92% to 39%.

to calculate cell viability caused by the substance A. As necrotic cell death was shown to be negligible, these findings clearly indicate apoptotic induction as early as 24 hours after treatment.

To differentiate between necrotic and apoptotic cells, we used annexin V-FLUOR and propidium iodide staining after 48 hours of treatment. As shown in Figure 5, the number of early apoptotic cells increases in a concentration-dependent manner, whereas the number of necrotic cells is comparatively low, confirming the observation obtained by LDH release assay, that necrosis does not have a significant role in the potency of substance A to induce cell death.

DNA fragmentation (hypodiploidy) is a characteristic effect specific for apoptotic cell death. The induction of DNA fragmentation during apoptosis was quantified using flow cytometric measurements of DNA fragments after incubating BJAB cells for 72 hours with substance A. As shown in Figure 6, high level DNA fragmentation is observed in treated BJAB cells.

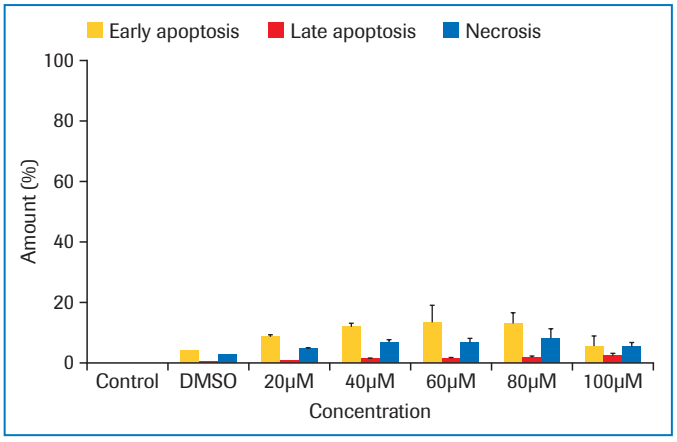

Figure 6: Apoptosis induction by substance A. Early and late apoptosis in BJAB cells measured after 48 hours of incubation with cytarabine and Annexin/PI staining. The amount of phosphatidylserine was measured using flow cytometry. Values of phosphatidylserine exposure are given as percentages of Annexin-V-positive/PI-negative cells ± SD (n=3). Concentration (DMSO): 100 µM.

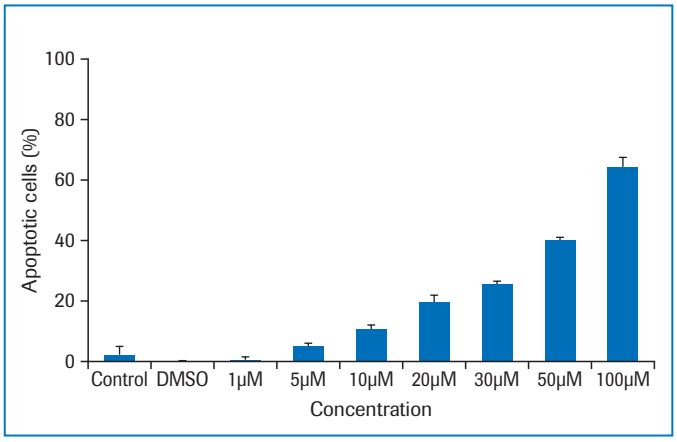

Figure 7: BJAB cells were treated for 72 hours with different concentrations of the antitumor agent. Nuclear DNA fragmentation was quantified by flow cytometric determination of hypodiploid DNA. Data are given in % hypoploidy (subG1), which reflects the number of apoptotic cells ± SD (n=3). Apoptotic cell death was identified as the major cause of cell death.

Conclusions

The CASY Cell Counter and Analyzer is an important improvement in our workflow to investigate the anticancer properties of substance A in lymphoma cells. In addition to using the CASY System for multi-parameter quality control of the cell cultures, including cell concentration, cell viability, cell volume, cell aggregation and cell debris, the system can be used to examine apoptosis induction as early as 24 hours after treatment. This means a saving of 24 and 48 hours, respectively, compared to using the Annexin-V and DNA-Fragmentation assays. The CASY System accurately measures cell viability, but cannot differentiate between necrotic and apoptotic cells. Combining the CASY measurements with an early exclusion of nonspecific necrosis using the LDH release assay, clearly showed the correlation between decreasing cell proliferation, decreasing cell viability, and the induction of apoptosis.

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Cytotoxicity Detection Kit^{PLUS} (LDH)	2000 tests	04 744 934 001
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In Situ Cell Death Detection Kit, Fluorescein	50 tests	11 684 795 910

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Key Words:
CASY System, cell proliferation, cell viability, oncology, chemotherapeutics, anti tumor compounds, acute leukemia, multiple drug resistance, lymphoma cells (BJAB), cell death, necrosis, apoptosis, LDH release, Annexin-PJ-assay, DNA fragmentation.

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