OMNI Life Science

Application Note

How Chemicals influence Cell Proliferation

Straightforward Cell Culture Monitoring using CASY TT

Annegret Becker, Almke Bader, Anne Klett & Inken Hertrampf

Institut für Biophysik, AG Zellphysiologie & Zelluläre Mechanik Prof. Dr. Ngezahayo, Leibniz Universität Hannover, Germany

Introduction

Monitoring mammalian cell cultures is an important aspect in quality control of experiments. When working with drugs and agents to activate or inhibit different signaling pathways, it is important to be able to determine the cytotoxic and cell growth-affecting properties of these substances. This information is critical in order to obtain reproducible results allowing a reliable comparison between differently treated cell populations, especially when experiments are performed after a long-lasting exposure to the drugs.

A simple and convenient method to analyze cell proliferation is achieved by counting the number of living cells after different incubation periods with the drug of interest. This can easily be achieved using the CASY system. Besides counting, CASY also determines the respective amount of dead and viable cells as well as cell debris - important parameters for the quality control of a cell culture.

In this study we compared the cell proliferation of two different cell lines, GM-7373 and Caco-2 subjected to two different chemicals, the antithrombotic drug dipyridamole and dextran sodium sulfate (DSS), which is used to mimic colitis *in vitro*.

Methods

GM-7373 bovine aortic endothelial cells (Grinspan et al. 1983, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and Caco-2 human epithelial colorectal adenocarcinoma cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) were cultured in DMEM/Ham's F-12 (1:1) supplemented with 10 % fetal calf serum (Biochrom GmbH) and penicillin/streptomycin (100 U/ml and 0.1 mg/ml, respectively, Biochrom GmbH).

Cells were cultivated in a humidified incubator at 37 °C and 5 % CO_2. For cell proliferation assays, 1 · 10^5 GM-7373 cells and 3 · 10^5 Caco-2 cells per well were seeded into 12 multiwell plates (TPP) and were allowed to adhere and grow for 24 h. Then, GM-7373 cell culture medium was exchanged to either untreated medium or medium with 0.1 % (v/v) dimethyl sulfoxide (DMSO, Carl Roth) as vehicle control or 25 µm dipyridamole (dip., Boehringer Ingelheim International GmbH, dissolved 1:1,000 from stock solution in DMSO). Culture medium of Caco-2 cells was supplemented with 0.5 or 2 % DSS (Dextran sodium sulfate) or without DSS supplement. For cell number determination, the cells were washed once with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) + EDTA (3.4 mM) and incubated with 250 µl trypsin (0.25 % (w/v), Biochrom GmbH, dissolved in PBS + EDTA) per well for 5 min. Trypsinization was stopped by addition of 500 µl cell culture medium. Cells were carefully rinsed from the culture surface and the cell suspension was pipetted up and down a few times before transferring it into 1.5 ml reaction tubes.

50 µl of each cell suspension was diluted in 10 ml CASYton (dilution factor 201) and the cell number counted using the appropriate setup for each cell line in the CASY. The GM-7373 cell culture medium including chemicals was renewed every 24 h. For Caco-2 cells, the DSS-medium was changed every 48 h. Cells were counted immediately after adding the chemicals or DSS (0 h) and every 24 h afterwards for 4 to 6 days, respectively.

The average cell numbers and SEM (Standard Error of the Mean) of three individual biological replicates for each treatment were calculated. Cell morphology was additionally monitored and documented with a transmission light microscope (Nikon Eclipse TS100 with NIS Elements, Nikon).

Results

GM-7373 and dipyridamole

The drug dipyridamole is used for its antithrombotic effect on platelets and administered to prevent secondary strokes (Eisert 2006). Furthermore, dipyridamole is known for its anti-proliferative effects in endothelial and smooth muscle cells (Liem et al. 2001, Zhuplatov et al. 2006).

Attenuation of cell proliferation

We analyzed the anti-proliferative effect of dipyridamole on the bovine aortic endothelial cell line GM-7373, which was shown to respond to dipyridamole application with an increased intercellular coupling via gap junctions within 6 h (Begandt et al. 2010, 2013). An incubation period of 24 h with 25 µM dipyridamole did not change the amount of viable cells compared to untreated or vehicle-treated cells (Fig. 1). However, after 48 h a decrease in the number of viable dipyridamole-treated GM-7373 cells was observed which corresponded to about 90 % of the respective cell number in DMSO-treated samples. Further increasing the cultivation time, cell proliferation was strongly attenuated with dipyridamole. 72 and 96 h after dipyridamole treatment, viable cells made up for about 50 % of the number of DMSO-treated cells (Fig. 1). Growth inhibition by dipyridamole could also be observed by comparing cell doubling times of untreated, vehicle- and dipyridamole-treated cells (Tab. 1). It has to be noted that the vehicle for dipyridamole, DMSO, which was used as a control, did not alter cell proliferation compared to untreated cells up to an incubation time of 72 h. However,it possibly showed a slight growth inhibitory effect after an incubation for 96 h (Fig. 1).

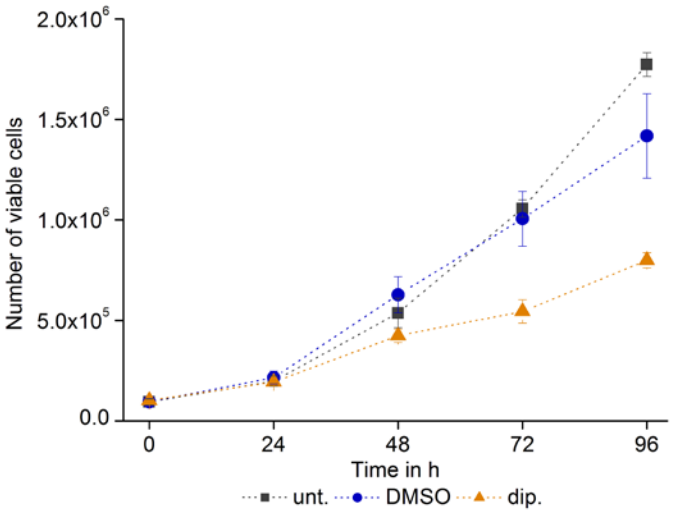


Fig. 1: **Increasing cultivation time with dipyridamole leads to a strong attenuation of cell proliferation.** Development of the cell number of GM-7373 cells after cultivation with untreated (unt.) medium, 0.1 % DMSO (DMSO) or 25 µM dipyridamole (dip.) for 96 h. It can clearly be seen that cultivation with dipyridamole for 48 h or more leads to a strongly decreased number of viable cells. Viable cell numbers are shown as average ±SEM from three replicates.

Growth inhibition

The antiproliferative effect of dipyridamole seems to be a result of growth inhibition rather than dying of the cells as cell viability was not affected by addition of the drug. Furthermore, the size distribution of particles counted by the CASY system did not differ between control cells and dipyridamole-treated cells (Fig. 2). Additionally, cell numbers still increased in the presence of dipyridamole but the doubling time clearly increased with prolonged dipyridamole incubation periods (Tab. 1).

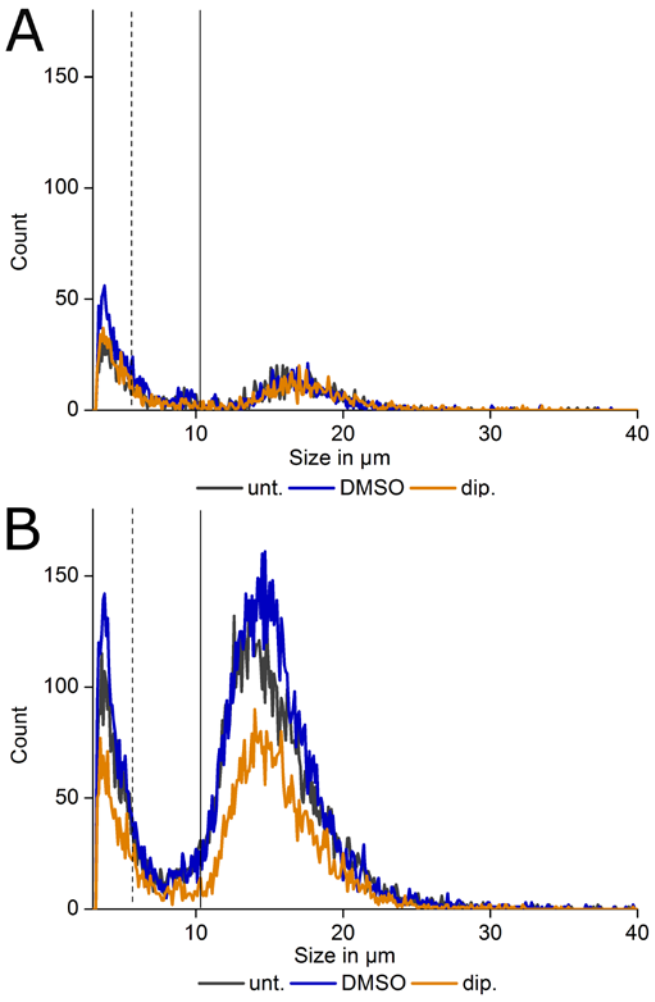


Fig. 2: **Antiproliferative effect of dipyridamole is a result of growth inhibition rather than dying of the cells.** Exemplary graphs showing cell counts and size distributions after cultivation of GM-7373 cells with untreated (unt.) medium or medium supplemented with 0.1 % DMSO (DMSO) or 25 µM dipyridamole (dip.) for 0 h (A) and 72 h (B). Vertical lines represent the cut-off sizes for cell debris (dotted) and dead cells (solid line). Even after dipyridamole treatment for 72 h (B), cell viability was not affected as there is no peak of dead cells.

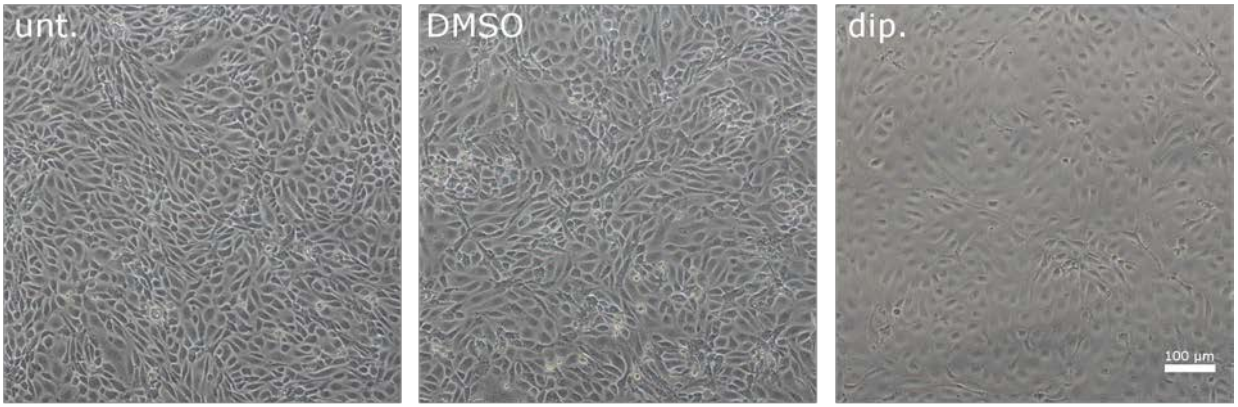


Fig. 3: **Dipyridamole-treated cells appear bigger and less squeezed than untreated or DMSO-treated cells but exhibit a normal morphology.** Exemplary micrographs showing GM-7373 bovine endothelial cells after cultivation in untreated (unt.) medium or medium with 0.1 % DMSO (DMSO) or 25 µM dipyridamole (dip.) for 72 h.

Results, *continued*

Caco-2 and DSS

DSS is widely used to induce colitis in mice with symptoms similar to ulcerative colitis, an inflammatory bowel disease (Laroui et al. 2012). DSS was shown to disrupt tight junctions of mouse intestine cells as well as of human epithelial colorectal adenocarcinoma cells (cell line Caco-2) (Samak et al. 2015).

DSS clearly decreased cell proliferation

We analyzed the effect of 0.5 % and 2 % DSS on cell proliferation using Caco-2 as a model for intestinal epithelium. Cells were treated with DSS 24 h after seeding. Viable cell numbers were recorded daily using the CASY TT for 6 days. Cells treated with 0.5 % DSS showed a clearly decreased cell proliferation with 40 % of viable cells from day 4 compared to untreated cells. However, an administration of 2 % DSS decreased the viable cell number by even 80 % during the entire incubation period compared to untreated cells (Fig. 4). Hence, DSS seems to inhibit cell proliferation in a dose-depended manner. Cell numbers still increased during treatment with 0.5 % DSS whereas 2 % DSS resulted in nearly unchanged cell numbers at any time.

Cell viability remains unaffected

Based on size distributions, DSS did not affect cell viability since the number of dead cells did not increase compared to untreated cells (Fig. 5). Monitoring of cell morphology by light microscopy showed altered cell morphology and a leaky cell monolayer caused by 2 % DSS compared to 0.5 % DSS-treated cells and untreated cells (Fig. 6).

Table 1: Average doubling times ± SEM of GM-7373 cells in untreated medium or medium with 0.1 % DMSO or 25 µM dipyridamole compared to the respective previous cultivation period.

	untreated	DMSO	dipyridamole
24 h	22 ±1.0	20 ±2.1	26 ±3.1
48 h	17 ±1.6	16 ±0.6	21 ±0.8
72 h	25 ±3.5	35 ±2.5	74 ±15.2
96 h	32 ±0.5	49 ±2.6	47 ±10.7

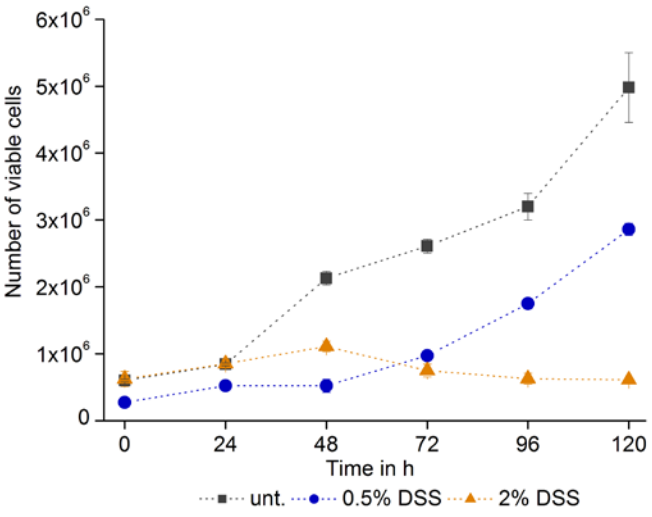


Fig. 4: **DSS inhibits cell proliferation in a dose-dependent manner**. Caco-2 cells were untreated (unt.) or treated with 0.5 or 2 % DSS, respectively, for 6 days (120 h). DSS treatment and cell proliferation were measured in 24 h steps after cell seeding. Results are shown as averages ±SEM from three replicates.

Fig. 5: **DSS did not affect cell viability.** Exemplary graph showing cell counts and size distributions of Caco-2 cells incubated with 0.5 and 2 % DSS, respectively, and without treatment (unt.) for 6 days. Size distributions were determined by CASY. Depending on DSS concentration, proliferation of viable Caco-2 cells was inhibited, indicated by a reduced peak height (solid line), while viability maintained, indicated by the absence of dead cells (dotted line).

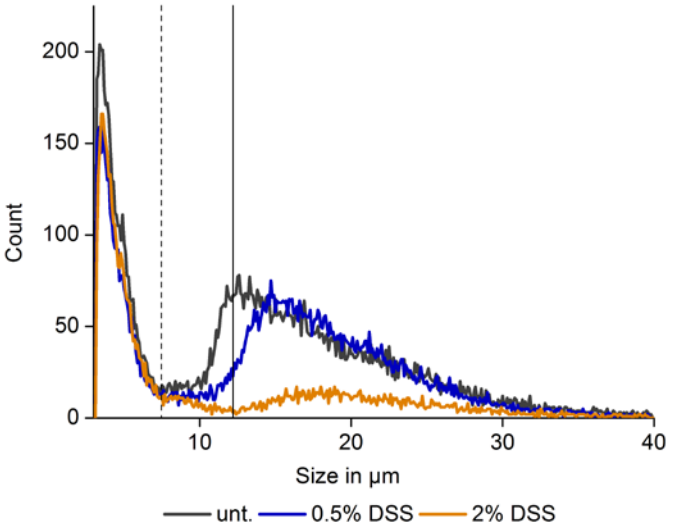
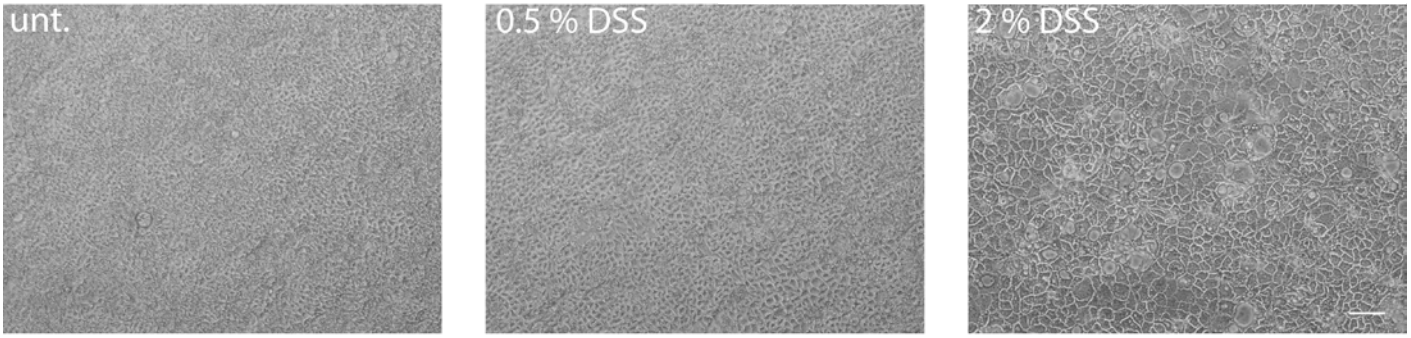


Fig 6: **Bigger cells, leaky monolayer.** Micrographs of Caco-2 cell morphology alteration after treatment with 0.5 and 2 % DSS for 5 days compared to untreated cells (unt.). Cells treated with 2 % DSS appear bigger than untreated cells and cells treated with 0.5 % DSS. Moreover, cells treated with 2 % DSS seem to have a leaky cell monolayer compared to untreated cells. 0.5 % DSS barely effected cell morphology.



Summary

Cell counting and viability analysis

Both, reliable cell counting together with CASY's capabilities for cell viability analysis, enabled us to set up a study monitoring possible cytotoxic and growth-affecting properties of drugs and chemicals to be used in subsequent experiments.

Parameters for quality control of cell cultures

CASY has clearly demonstrated its usability in QC related assays such as proliferation control, viability assays or cell debris monitoring by its reliable, label-free and straightforward measurements and comprehensive data evaluation tools.

Contact

Almke Bader
a.bader@biophysik.uni-hannover.de

Annegret Becker
a.becker@biophysik.uni-hannover.de

Institut für Biophysik, AG Zellphysiologie & Zelluläre Mechanik Prof. Dr. A. Ngezahayo, Leibniz Universität Hannover, Herrenhäuser Straße 2, 30419 Hannover, Germany.

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