Cytotoxicity Determination

A Method for the Evaluation of New Active Pharmaceutical Ingredients

With an increasing number of new active pharmaceutical ingredients, it becomes more and more important to find efficient and fast screening approaches for industrial application. The objective of this project was to develop a cytotoxicity assay with high throughput by monitoring the cellular respiration in cell culture.

When cells are treated with cytotoxic compounds, they tend to become apoptotic and do not consume anymore oxygen [1]. If the amount of oxygen diffusion into the medium is greater than the oxygen consumption of the remaining cells that survived, the overall dissolved oxygen content in the medium increases. In contrast to this, when treating cells with cytostatic compounds, the oxygen consumption should stay relatively constant as a result of stopping the proliferating process.

Material and Methods

Cells were cultured in 24-well plates with integrated oxygen sensors, so called OxoDishes by PreSens. The optical sensors located at the bottom of each well are read out online and non-invasively with the SDR SensorDish Reader [2]. For cellular respiration experiments, the Chinese hamster ovary cell line CHO-K1 was used. These cells were maintained in Ham's F-12 medium supplemented with 10 % FCS and 1 % penicillin-streptomycin and incubated at 37 °C in a 5 % CO_2 atmosphere. The optimal cell density was found to be 300.000 cells per well. This optimal density has to be determined for each cell batch, as cell growth changes with the number of cell passages.

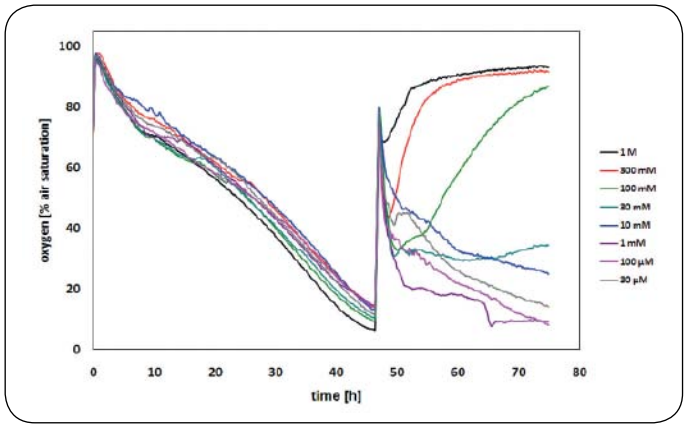

Fig. 1: Online monitoring of oxygen content in medium caused by cellular respiration of CHO-K1 cells before and during treatment with lethal and sub-lethal concentrations of malonic acid. Shown are the average values based on measurements collected from 3 equal wells (n=3) over time.

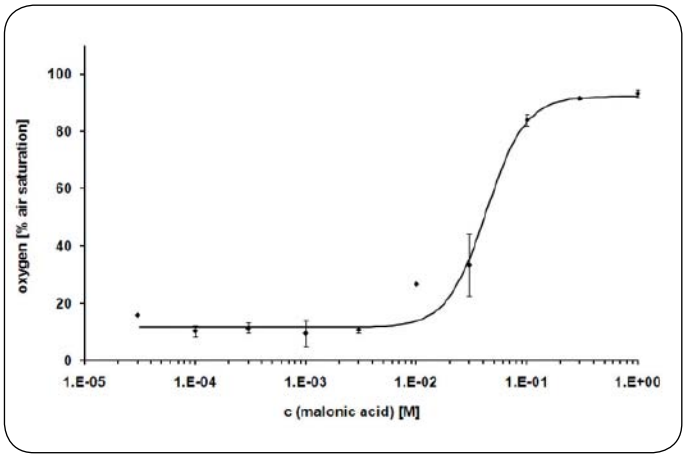

Fig. 2: Dose response curve for malonic acid. Each data point is an average of the response from 3 sensors (n=3). The curve represents a sigmoidal fit of average obtained.

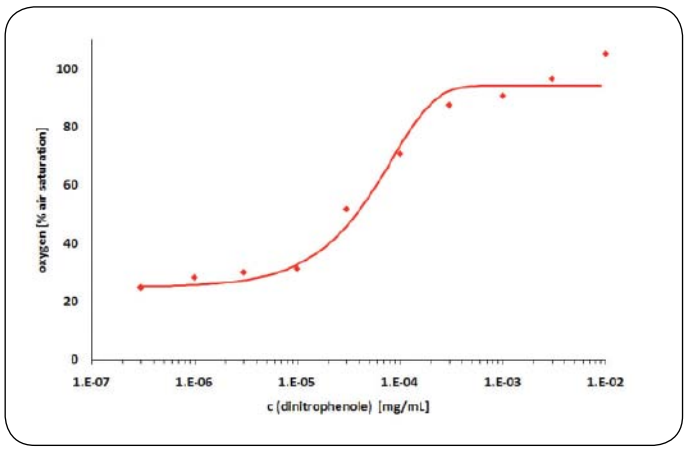

Fig.3: Dose response curve for 2,4-Dinitrophenol. Each data point is an average of the response from 3 sensors (n=3). The curve represents a sigmoidal fit of average obtained.

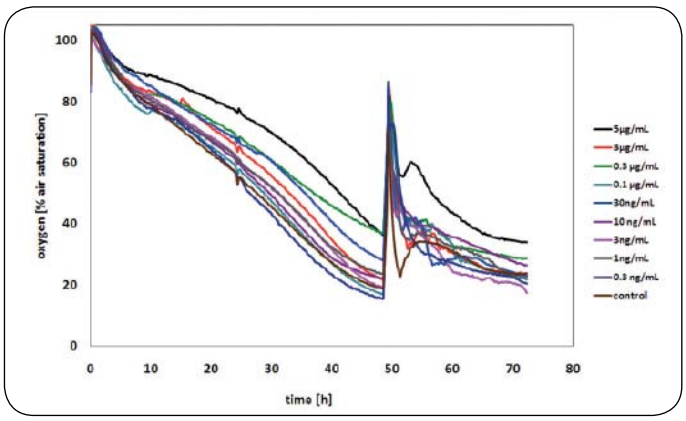

Fig. 4: Online monitoring of oxygen content in medium caused by cellular respiration of CHO-K1 cells before and during treatment with different concentrations of colcemid. Shown are the average values based on measurements collected from 3 equal wells (n=3) over time.

Cells were grown in 1 ml of medium for 24 hours. 1 ml of fresh medium was added containing the cytotoxic or cytostatic substances in different concentrations. The dissolved oxygen content was monitored every 10 minutes for 24 hours. After the respiration measurements, cell viability was directly assessed using the standard MTT test.

All stock solutions of the test compounds were dissolved in Ham´s F-12 medium, if necessary 1 % final concentration of DMSO was used, and then diluted appropriately with medium to yield the desired concentrations. In order to determine the median lethal concentration LC50 of a cytotoxic compound, the dissolved oxygen content after 24 hours of treatment was plotted over the appropriate compound concentration. The resulting curve was fitted with a four-parameter sigmoidal concentration-response curve using OriginPro version 8.0.

Effect of Cytotoxic Compounds on Cellular Respiration

The first cytotoxicity tests were performed with malonic acid. Sublethal concentrations of malonate did not affect the oxygen consumption of the CHO-K1 cells, which led to decreased dissolved oxygen content in the medium. As a result of high malonate concentrations, cell death was induced and thus, no more oxygen is consumed. Due to oxygen diffusion from ambient air, the dissolved oxygen increased up to 100 % air saturation (see figure 1).

The dissolved oxygen content after 24 hours of treatment was plotted over the corresponding concentration of malonic acid to determine the LC50 that was found to be 34 mM (see figure 2).

This value is in very good accordance to the LC50 value determined by the MTT test (31 mM). The literature value (4 mM) was a little lower, which is due to use of a different cell line [3].

In another cellular respiration assay, 2,4-Dinitrophenol (DNP) was used as a cellular metabolic poison.

Adding up to 0.5 % of the solvent DMSO to the stock solution did not impair cell growth. In the cytotoxicity assay, low concentrations of DNP did not seem to impair the cells as they kept consuming oxygen. In contrast, high concentrations quickly led to cell death.

The LC50 from these respiration data was found to be 59 mg/ml (320 mM), whereas for the MTT test, the LC50 value was determined to be 28 mg/ml (152 mM) (see figure 3). Both LC50 values are within the same concentration range indicating that the results of the SDR are a good indication for the LC50 determined by the MTT assay. The published literature LC50 value for DNP was specified to be 3 mg/ml (16 mM) as determined by MTT using BALB/c 3T3 fibroblasts and differs by factor of 10 most likely due to same reasons mentioned above for the values for malonic acid.

Effect of Cytostatic Compounds on Cellular Respiration

The same experimental set-up had been used for the assays with colcemid (demecolcin). Colcemid can depolymerize microtubules by limiting microtubule formation through inhibition of the spindle fiber formation and thus, arresting cells in the metaphase [4,5]. The results gained from these assays proved that the cells treated with colcemid did not die. The oxygen content in the medium was relatively constant indicating a constant cellular respiration. The dissolved oxygen content before and after the treatment is about the same and thus, the cells constantly consume the same amount of oxygen per time period as the cell cycle is inhibited and no cell division is possible (see figure 4). Furthermore, no significant difference in oxygen consumption after 24 hours of treatment with different colcemid concentrations could be found.

The variation of the dissolved oxygen content in the medium can be explained by the slightly different cell numbers in the consecutive wells. Thus, it could be shown that colcemid is not toxic to CHO-K1 cells, but rather a cytostatic agent. This result is supported by the MTT assay that confirmed the cell viability after 24 hours of treatment as none of the samples were found to be non-viable and the different absorbance values might be explained by different cell numbers.

Oxygen Consumption in Synchronized Cells

In order to determine whether the respiration activity of cells in various stages of the cell cycle differs from cells that are synchronized, a serum starvation experiment was performed. With the intention of arresting all cells in the G1-phase, the FCS content during culturing was gradually reduced from 10 % over 5 % and 1 % down to FCS-free medium. After 48 hours incubation in media without growth factors, the medium was replaced with fresh medium containing 10 % FCS again in order to release the cells from their blockage and to achieve synchronization. Cellular respiration was monitored with the SDR and cell viability determined by the MTT assay. It was found that the cells grow normally in medium containing 10 %, 5 % and 1 % FCS (see figure 5). This is facilitated by the fact that only a very small cell density was platted in 1 ml of medium. Even a FCS content of 1 % was found to be sufficient for this small cell number to grow. In contrast to that, the cells did not divide when maintained in FCS-free medium as growth factors were missing. The cells were still viable and consumed oxygen, but due to cell cycle arrest they did not decrease the oxygen content in the medium, which is comparable to the results seen when cells are treated with cytostatic compounds (see colcemid in figure 4). Serum starvation has a reversible cytostatic effect as can be seen in figure 5 as renewed addition of 10 % FCS caused cells growth and a decrease of oxygen

content in the medium. It was found that the course of oxygen consumption was not vastly different before and after the synchronization of the cells, but as a result of the cell growth during the serum starvation, the absolute dissolved oxygen values differ. It can be concluded that no significant variation between synchronized and unsynchronized cells concerning the cellular respiration arise.

Summary

The results show that an explicit distinction between cytotoxic and cytostatic compounds can be made by online monitoring of cellular respiration. While high concentrations of DNP led to an increased dissolved oxygen value, colcemid treatment results in a relatively constant dissolved oxygen concentration. For DNP, the LC50 determined from the dissolved oxygen values was slightly higher than the LC50 from the MTT values, but were found to be within the same range. Overall the cellular respiration can be validated with the commonly used MTT assay. The results obtained with the SDR correlate with the MTT assay and therefore are a good indicator for cytotoxicity. Literature values for cytotoxic compounds are about 10 times lower than the LC50 determined in the experiments shown here. Due to the vast differences between cell lines and primary cells, it is only possible to draw general comparison.

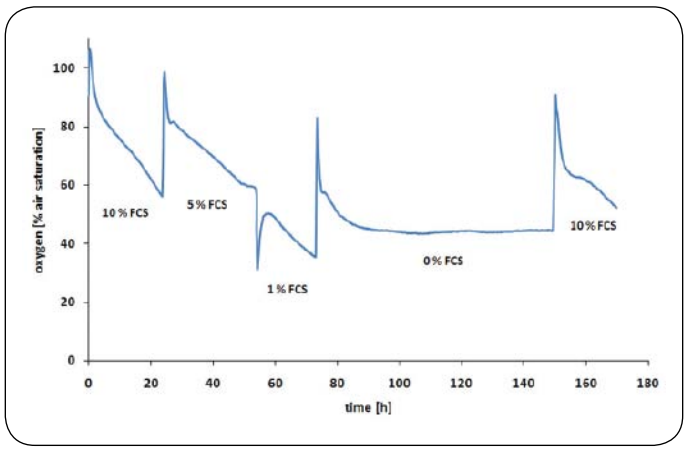

Fig. 5: SDR OxoDishes monitoring of dissolved oxygen change during serum starvation of CHO-K1 cells.

In addition, it could be proven that synchronized cells obtained by serum starvation do not have a different oxygen consumption profile than unsynchronized cells. The removal of FCS inhibits the cell cycle, which was evident in constant oxygen consumption. In summary, the experiments proved that the SDR reader system provides a fast and easy-to-use online method to determine cytotoxicity of new pharmaceutical compounds.

References

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[5] Fujikawa-Yamamoto K.: Apoptosis by Demecolcine in V79 Cells, Cell Structure and Function, 19, 391–396 (1994)

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