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Introducing oxygen transfer rate measurements as a novel method for time-resolved cytotoxicity assessment in shake flasks

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Abstract

Background: Determining the cytotoxicity of test substances is essential for the safety assessment of chemicals. To quantify the cytotoxicity, dose–response curves are determined and the half-maximum inhibitory concentration (IC50) is subsequently calculated. Results often rely on a single endpoint evaluation which typically requires manual sampling and subsequent sample analysis to determine the IC50. Hence, no information on culture behavior are available during treatment. Here, measurement of the oxygen transfer rate (OTR) was applied as a method for time-resolved assessment of the cytotoxicity to (a) provide information on culture behavior during treatment and (b) quantitatively assess the cytotoxic effect of a test substance.

Results: To investigate the applicability of the method, different types and concentrations of test substances were added and the OTR was measured for two different CHO suspension cell lines in shake flasks: (a) For CHO cell line one, culture behavior was not affected when 0.1 mM $ZnCl_2$ was added. However, for both cell lines, adding up to 0.2 mM $CoCl_2$ led to a decrease in the OTR that was concentration dependent. For CHO cell line two, adding up to 5% DMSO led to a concentration-dependent decrease in the OTR and in the slope of the OTR; (b) From the measured OTR, dose–response curves were established and used to calculate the IC50. For cell line one, the IC50 for $CoCl_2$ was 0.03 mM while the IC50 from a comparative measurement based on trypan blue exclusion was 0.06 mM. Cell line two was less susceptible to $CoCl_2$ as the IC50 was not reached at the concentrations tested. For DMSO, single OTR values and determined slopes were used for evaluation. In both cases, the IC50 was calculated to 2.3%.

Conclusions: Our approach provides a method for analyzing the cytotoxicity of a test substance based on OTR measurements. This method provides (i) insights on culture behavior, (ii) information on the progression of cytotoxicity, (iii) dose–response curves, and (iv) a first indication of IC50 values. Quantitative assessment of cytotoxicity is possible non-invasively and in real-time during treatment. Compared to traditional endpoint evaluation, our method simplifies experimentations and enhances the comparison of assay results.

Keywords: CHO suspension cells, Oxygen transfer rate, Cytotoxicity determination, Time-resolved monitoring, IC50, Dose–response

Background

A cytotoxicity assay aims to quantitatively describe the effect of a test substance on a cell culture. Hence, cytotoxicity tests play an important role for the safety assessment of chemicals. Different cell lines, including mammalian

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CHO cell lines, may be used in cytotoxicity assays. To quantitatively evaluate the cytotoxicity, dose–response curves are determined. The test substance concentration is plotted (either in linear or logarithmic form) against the measured cytotoxicity. The relationship between dose and effect varies for different substances, resulting in various shapes for dose–response curves [49, 55]. If the test substance concentration is plotted in logarithmic form, a sigmoid curve is usually obtained [40]. However, biphasic relationships between dose and response have been reported as well. In hormesis, a stimulation at low dose and an inhibition at high dose results in a U- or J-shaped dose–response curve [8, 12]. A characteristic value for evaluating and comparing the cytotoxicity of different test substances is the IC50 value. IC50 is the concentration at which the half-maximal inhibition is observed, i.e., the test substance concentration at which the cytotoxicity reaches a value of 50%. This value is obtained from the fitting of the dose–response curve. For curves with a sigmoidal shape, 4-parameter logistic regression (4PL) is usually applied [56].

Despite a recent trend toward a more quantitative risk assessment, most of the currently established assays, including those recommended by the OECD guidelines for the testing of chemicals, rely on endpoint determinations [25]. Established assays can be grouped into different categories. In dye exclusion tests, cell viability is evaluated directly after treatment with the test substance by counting cells that took up the dye (and become colored) and cells that excluded the dye (and remain uncolored) [3]. Examples are the trypan blue exclusion assay or the erythrosine B assay. Among the colorimetric assays, the MTT (3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) assay is most commonly used. Other assays may be based on fluorometric or luminometric evaluation [3]. It needs to be noted that differences were found in the calculated cytotoxicity based on the method used to determine it [18].

Problems of assays relying on endpoint determination

The utilization of endpoint measurement is disadvantageous because cytotoxicity is usually dose and time dependent, and the progression of cytotoxicity varies among test substances [17]. Moreover, the determination of cytotoxicity usually requires several steps after exposure of the cells to the test substance (e.g., staining and cell counting or additional incubation steps). Thus, analysis is often time consuming and requires additional materials. Further, cytotoxicity might be underestimated, depending on the method used. For trypan blue exclusion, it was demonstrated that cytotoxicity is vastly underestimated if performed directly after treatment. The reason is that only cells that are dead at the time that the assay is performed are evaluated [18]. Cells that die after the assay has been conducted, however, are not considered. Moreover, no time-resolved information is provided, as processing and analysis often take considerable time, and only the endpoint is used for evaluation. In particular, no quantitative information of culture behavior is available during treatment with the test substance. However, the treatment time with the test substance and the sampling time for analysis were crucial steps during the determination of cytotoxicity [32, 67]. Differences in these parameters were most likely responsible for different genotoxicity results between CHO and Chinese Hamster Lung (CHL) cultivations [58]. Time-resolved monitoring also offers the advantage to determine changes in cellular response (e.g., changes in growth rate, progression of viability). This way, differences in the mode of action of different test substances and different cell lines might be detected. In addition, the consistency of the assay is monitored, as abnormalities in culture behavior (e.g., insufficient growth of the control culture) become visible in real time.

Real-time measurement of cytotoxicity

Despite the advantages that real-time monitoring of culture progression during treatment offers, real-time monitoring and evaluation of cytotoxicity are yet to be used routinely. Real-time assessment of cytotoxicity was demonstrated by impedance measurement either in 96-well plates [10] and in the commercialized xCELLigence device (ACEA Biosciences/Agilent) [7, 15, 26, 42, 45, 59] with the last publication providing a very comprehensive overview of applications and comparison to endpoint-based assessment In addition, a real-time assay for the determination of cytotoxicity based on the fluorescent luciferase assay has been reported [17]. Furthermore, the Seahorse technology, offered by Agilent, enables measurement of mitochondrial toxicity in microtiter plates based on measurement of the oxygen consumption rate [54].

Online monitoring of the oxygen uptake rate (OUR)

The oxygen uptake rate (OUR) provides a wide variety of information during mammalian cell culture cultivation [23, 28, 29, 41]. As oxygen consumption is directly linked to cell metabolism, the OUR provides information on culture viability. At a constant cell-specific oxygen uptake rate (qO_2), the OUR is directly proportional to the viable cell concentration (VCC). Additionally, the OUR is very close to the oxygen transfer rate (OTR), if the change in the oxygen concentration in the liquid over time is negligibly small. In slow mammalian cell cultures this is always the case, and the OUR may be determined from measurement of the OTR. Measurement of the OTR of CHO

suspension cells has been realized in shake flasks using a prototype device of the Respiration Activity Monitoring System (RAMOS) [28, 29] as well as a commercially available Transfer-Rate Online Measurement (TOM) device [29]. Both systems utilize electrochemical sensors to determine the OTR. For mammalian cells, the overall breathing activities are low and the culture dynamics are slow compared to the measurement times [28]. Additionally, the change of the oxygen concentration in the liquid phase is much smaller than OTR and OUR, it can be assumed that the OUR equals the OTR [28, 44]. For measurement using RAMOS and TOM, a measurement and a gas flow phase were alternately applied. During the gas flow phase, the shake flask is aerated with gas. During the measurement phase, the inlet and outlet valves are closed. Thus, cell respiration results in a linear decrease in the headspace's oxygen partial pressure. The slope of the partial pressure decrease over time is used to calculate the OTR [1, 2]. Further details on the measurement principle are given in Additional file 1: Additional data (Sect. 1.1). Measurement is non-invasive with no manual intervention as the electrochemical sensor is placed outside of the sterile area of the flask [1, 2]. Additionally, measurements are usually carried out in up to eight shake flasks in parallel. However, parallelization is only limited by the number of flasks that can be operated in the incubator simultaneously. Even at the comparably low breathing activity of CHO cells, up to one OTR value per hour can be obtained [28, 29]. For CHO suspension cells, information on the VCC and differences in culture behavior at different initial cell concentrations were determined using RAMOS [29].

The RAMOS technology has already been successfully applied to bacterial systems to determine toxicity [34, 43]. Furthermore, in a miniaturized format in microtiter plates, it successfully has been applied to determine mutagenicity via an improved version of the bacterial Ames fluctuation test [30, 31].

This study aims to evaluate, if time-resolved and non-invasive measurement of the OTR generally allows to evaluate the cytotoxicity of test substances on CHO suspension cells during treatment in real time. Furthermore, it is investigated how the gathered OTR data may be utilized to quantitatively draw conclusions about the cytotoxicity of a test substance. By measuring the OTR, time-resolved information on culture behavior during treatment with the test substance is obtained. For this initial assessment and to determine a possible workflow for interpreting the OTR data, experiments are conducted in shake flasks. To investigate, if the method may be applied in a more general way, different test substances, CHO cell lines and cultivation conditions, including culture media and initial seeding densities, are investigated (Table 1). It should be noted that it is not within the scope of this study to provide a statistically reliable analysis of the cytotoxicity of a certain test substance, but rather to investigate if monitoring of the OTR may in general be suited to gather such data. The advanced and required statistical assessment is left to those more experienced in the field.

Methods

Cell lines and culture media

Two different CHO suspension cell lines were used for cultivation. Cell line one is an industrial CHO suspension cell line producing an IgG1 antibody developed by Rentschler Biopharma SE (see [28, 51]). Cell line two is a CHO suspension cell line obtained from Cell Lines Service (CLS) GmbH, Germany. It is not modified to produce an antibody. Two different cell lines were chosen, to demonstrate that the obtained results are of general nature and not due to a unique characteristic of a single cell line.

In general, the CHO cell lines used in this study were expected to behave differently, because the variability in genotypes and phenotypes is quite substantial among CHO cells [37, 52, 65, 66]. In addition, as mentioned above, CHO cell line one was genetically modified to express a gene of interest. Native and mAb producing CHO cell lines were reported to differ in growth and substrate consumption [39]. This difference might also

Table 1 Overview of the combination of CHO cell lines, cultivation conditions, and test substances investigated within this study

	CHO cell line #1		**CHO cell line #2**	
Culture medium	PowerCHO 2 medium		sciNX medium	
Initial viable cell concentration	$0.2 \cdot 10^6$ cells mL^{-1}		$0.5 \cdot 10^6$ cells mL^{-1}	
IgG1 production?	Yes		No	
Test substance	$ZnCl_2$	$CoCl_2$	$CoCl_2$	DMSO
Test substance concentrations	0.1 mM	0.01–0.3 mM	0.05–0.3 mM	0.1–5% (v/v)
Figure	1	Figures 1, 2, 3, Additional file 1: Fig. S1–3	Figure 4, Additional file 1: Fig. S4	Figures 5 + 6, Additional file 1: Fig. S5–7

influence the sensitivity of the cells toward certain test substances.

When it comes to growth behavior, utilization of suspension cultures has been recommended in the past [33]. Advantages of using suspension cell lines in comparison to adherent CHO cells include shorter cultivation times and no need for trypsinization for detachment of cells [38, 46]. Both cell lines were cultured in a serum-free, chemically defined medium. Different culture media were used for cultivation to explore, if the method is robust and, thus, may be applied to different cell lines cultured in different media. Media were stored at 4 °C until use. Stock solutions for media supplements were stored at − 20 °C in aliquots and thawed as needed. Before use, media and supplements were pre-heated to the cultivation temperature for about 30 min in a water bath (VWB2 12, VWR, USA). Cell line one was cultured in PowerCHO™ 2 serum-free chemically defined culture medium, containing HEPES buffer and Pluronic® F68 (Lonza AG, Switzerland). Here, it should be noted that the cells may uptake Pluronic® [22], which might influence cytotoxicity results. However, as the primary focus of the study was not to provide improved culture media for cytotoxicity assessment, no change to the culture medium was made. This way a comparison to previously published studies [28, 29] is still possible. Directly before use, the medium was supplemented with 6 mM L-glutamine (Gibco Life Sciences, Thermo Fisher Scientific, USA) and 1% (v/v) PenStrep (stock with 10,000 Units mL^{-1} penicillin and 10 g L^{-1} streptomycin) (Sigma-Aldrich, USA). Cell line two was cultured in sciNX medium (CLS GmbH, Germany) and supplemented with 5 mM L-glutamine and 1% (v/v) PenStrep.

Cryopreservation, thawing, and cell passaging

Cryopreservation was performed as described in detail by Ihling et al. [28] (cell line one) and (cell line two) [29] and according to the basic principles described, for example, by Freshney [20]. After thawing, one washing step was included to remove DMSO.

All passages were carried out in 250 mL Corning® polycarbonate flasks closed with a vent cap (Sigma-Aldrich, USA) at a temperature of 36.5 ± 0.25 °C. A filling volume of 20 mL with a shaking frequency of 140 rpm and a shaking diameter of 50 mm was used for cultivation. An orbital shaking incubator with CO_2 and humidity control (ISF1-X, Kuhner AG, Switzerland) set to 5% and 70%, respectively, was used for passaging. Cells were passaged every 3–4 days. The seed cell concentration for subsequent cultures was set to $0.2{\cdot}10^6$ mL^{-1} (cell line one) or $0.25{\cdot}10^6$ mL^{-1} (cell line two), respectively.

Main culture cultivation conditions

After an appropriate number of passages (see Additional file 1: Table S1), cytotoxicity experiments were started. Cells were cultured at a temperature of 36.5 °C (cell line one) or 37 °C (cell line two). A shaking diameter of 50 mm, a filling volume of 20 mL (cell line one) or 50 mL (cell line two), and a shaking frequency of 140 rpm were used in all cases. Cultivation was carried out in 250 mL glass flasks. Flasks were modified in the upper part to allow active aeration but remained unmodified in the lower part that was in contact with the liquid (see Additional file 1: Additional Data Section 1.1 for details). Active aeration of flasks in the TOM device was carried out using incubator air (70% relative humidity and 5% CO_2) (ISF1-X, Kuhner AG, Switzerland). Cells cultured in the RAMOS device were incubated in a temperature-controlled incubator (ISF1-X, Kuhner AG, Switzerland) and aerated using gas (5% CO_2 in synthetic air) supplied by a gas bottle. The initial viable cell concentration (VCC) was set to $0.2{\cdot}10^6$ mL^{-1} (cell line one) and $0.5{\cdot}10^6$ mL^{-1} (cell line two), respectively. The initial VCCs were different, because cultivation protocols were previously independently established for each of the cell lines in the respective culture media.

Trypan blue exclusion method

To determine the viable cell concentration (VCC), a sample was taken from each RAMOS flask after 161 h (experiment 1; Additional file 1: Table S1) and 164 h (experiment 2; Additional file 1: Table S1), respectively. If required, the sample was appropriately diluted with pre-warmed culture medium. The sample was then mixed with trypan blue (1:1) and 10 µL of the mixture was added to a hemocytometer (Counting chamber C-Chip Neubauer improved, Carl Roth, Germany). The number of viable (unstained) cells was manually counted in four quadrants, averaged, and corrected by the dilution factor given by the manufacturer to determine the viable cell concentration.

Addition of test substances

The addition of test substances was either performed directly at the beginning of the experiment or after 24 h (see Additional file 1: Table S1). A 20 mM stock solution of $CoCl_2{\cdot}6H_2O$ (purity ≥ 99%, Carl Roth, Germany) and a 10 mM stock solution of $ZnCl_2$ (Sigma-Aldrich, USA) were each prepared in the culture medium used for CHO cell line 1(experiments 1 + 2). For experiments 3–5, performed with CHO cell line 2, a stock solution of 50 mM $CoCl_2{\cdot}6H_2O$ was prepared using cell culture grade water (HyClone HyPure Cell culture grade water, GE Healthcare, Germany). In experiments 6 + 7, dimethyl sulfoxide (DMSO, ≥ 99.7%, Merck KgA Germany) was added

without dilution to reach the final concentration in the culture medium. DMSO concentrations are given as (v/v).

Logistic fit of the dose–response curve

Obtained dose–response curves were fitted using OriginPro® 2020 (OriginLab Corporation, USA). A 4-parameter logistic fit function (Eq. 1) was chosen to determine the IC50. In the equation, $A1$ is the minimum cytotoxicity if the concentration of the test substance is 0 and $A2$ is the maximum cytotoxicity reached. The value x_0 in the equation represents the IC50. P represents the slope at the steepest part of the curve.

$$\text{Cytotoxicity}[\%] = \frac{A_1 - A_2}{1 + (\frac{x}{x_0})^p} + A_2. \quad (1)$$

Monitoring of the oxygen transfer rate

The oxygen transfer rate (OTR) was monitored in shake flasks using electrochemical sensors. Two different devices, an in-house built Respiration Activity Monitoring System (RAMOS) and a commercially available Transfer-Rate Online Monitoring (TOM) system (Kuhner AG, Birsfelden, Switzerland), were used to monitor the OTR. Single measurement values that deviated more than 30% from the next measurement value were considered outliers and excluded from further evaluation. Further details may be taken from Additional file 1: Additional Data Section 1.1.

Results and discussion

Qualitative determination of cytotoxicity with and without medium exchange

In a first step, monitoring of the OTR was carried out for CHO suspension cell line one in culture medium one (Fig. 1A). Two well-known test substances, $ZnCl_2$ and $CoCl_2$, were used to evaluate culture behavior during treatment. Reduced growth of CHO cells upon exposure to metal salts was previously demonstrated to correlate with strand breaks in the DNA [53]. Exposure with 0.1 mM $ZnCl_2$ for 4 h did not cause DNA strand breaks in CHO cells, and consequently, growth was not affected [53]. On the other hand, an IC50 of 86 µM was determined for CHO cells after 16 h of incubation in the presence of $ZnCl_2$ [60]. Following this literature information, a ZnCl2 concentration of 0.1 mM was investigated (Fig. 1A, yellow triangles).

A strong influence of $CoCl_2$ on cell growth was described for adherently grown CHO cells with the IC50 determined to be about 140 µM [60]. $CoCl_2$ has also been shown to induce apoptosis by excessive generation of reactive oxygen species (ROS) in PC12 cells [68].

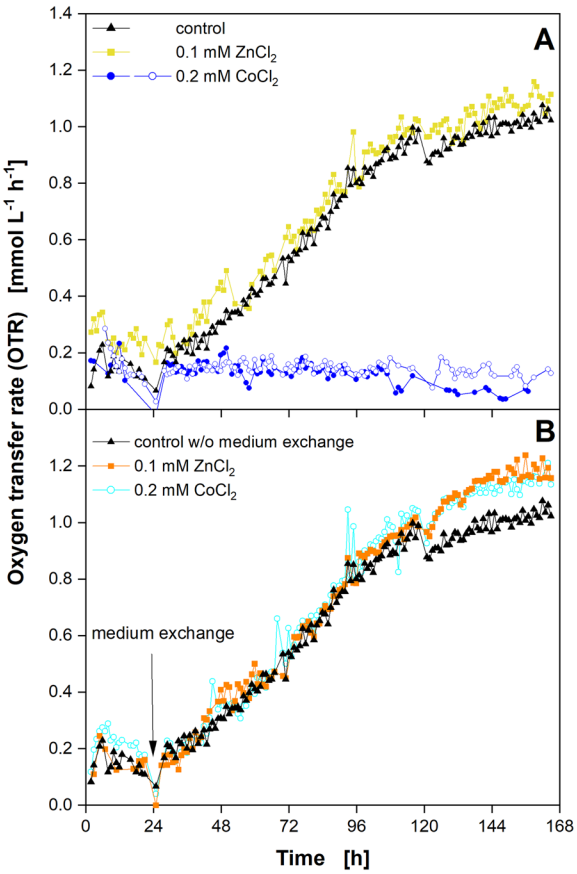

Fig. 1 Oxygen transfer rate (OTR) of CHO cell line one upon addition of $ZnCl_2$ or $CoCl_2$ with and without medium exchange. **A** Cells were cultured without addition of test substance (closed black triangles), with addition of 0.1 mM $ZnCl_2$ (closed yellow squares) or with addition of 0.2 mM $CoCl_2$ (blue circles). Test substances were added at the beginning of the cultivation ($t = 0$ h). **B** Cells were cultured without addition of test substance (closed black triangles), with addition of 0.1 mM $ZnCl_2$ (closed orange squares) or with addition of 0.2 mM $CoCl_2$ (open cyan circles). After 24 h (black arrow), medium was exchanged by fresh culture medium without a test substance added. Data from experiment 1 (see Additional file 1: Table S1)

Exposure to ROS and increasing levels of apoptosis can lead to a decrease in cell growth and viable cell number [35]. As a result, a $CoCl_2$ concentration of 0.2 mM (Fig. 1A, blue circles) was chosen in our study.

A control culture without the addition of test substances (Fig. 1A, closed black triangles) was run for comparison. Culture behavior, represented by the course of the OTR over the cultivation time, was not affected when 0.1 mM $ZnCl_2$ was added to the culture medium (Fig. 1A, closed yellow squares). When 0.2 mM $CoCl_2$ was added (Fig. 1A, blue circles), the OTR did not increase but stayed relatively constant at a value of about 0.15 mmol L^{-1} h^{-1} until the end of the

experiment. Here, it can be concluded that $ZnCl_2$ in the chosen concentration did not harm cell growth and is not cytotoxic under the conditions applied in this study. $CoCl_2$, on the other hand, completely suppressed cell growth at the investigated concentration and for the cell line and cultivation conditions investigated. The influence of $CoCl_2$ on culture behavior will be discussed in detail in Section "Quantitative determination of $CoCl_2$ cytotoxicity."

This first CHO cell line investigated in our study clearly exhibited a lesser sensitivity for $ZnCl_2$ than the CHO cell line used in the study by Tan et al. [60], as cell growth was not affected in the presence of 0.1 mM $ZnCl_2$ in our study, but an IC50 of 86 µM was reported by Tan et al. [60]. In general, $ZnCl_2$, more particularly the availability of Zn^{2+}, has to be considered with caution as various elements influencing the availability, and consequently also the cytotoxicity of Zn^{2+} have been proposed [47]. This includes the presence of fetal calf serum (FCS) in the culture medium, as will be discussed further below. Also, the protein metallothionein may play an important role in intracellular zinc metabolism. Its metal-unsaturated form may act as a chelator for intracellular Zn^{2+} [50], which might help reduce the cytotoxicity of Zn^{2+}.

A more general reason for the observed differences in $ZnCl_2$ sensitivity might be that adherent CHO-K1 cells, which were more sensitive in a cell-mediated cytotoxicity assay [16], were formerly used by Tan et al. [60] instead of the currently tested suspension cells. Growth adaptation from adherent to suspension may in general affect cellular responses [57]. A fundamental difference between adherent and suspension cultures is that suspension cells may interact with a test substance on their complete surface, while the surface of adherently grown cells is not completely accessible. On the other hand, no difference in assay outcome has been observed between cells growing in suspension cultures or adherently [61]. Another reason for the different sensitivity toward $ZnCl_2$ might be the different culture media used. Even though the presence of FCS might reduce the cytotoxicity compared to media with less or no FCS [5, 27], this effect was not observed for all test substances [5]. Further, FCS provides essential Zn^{2+} in a culture medium [47]. The addition of FCS may, thus, result in an unintended and undefined amount of Zn^{2+} being additionally added to the culture medium. Further, and as stated above, the use of Pluronic® F68 might have also affected culture behavior.

To evaluate if the cytotoxic effect was persistent, even when the test substance was removed from the medium, and to simulate a change in cultivation conditions during the performance of a cytotoxicity test, cultivation with an exchange of the medium was carried out (Fig. 1B). Cells were cultured in the presence of $ZnCl_2$ (Fig. 1B, closed orange squares) or $CoCl_2$ (Fig. 1B, open cyan circles) for 24 h. After 24 h (Fig. 1B, arrow), the medium was replaced with freshly prepared culture medium where neither $ZnCl_2$ nor $CoCl_2$ was added. After medium exchange, both cultures grew quite similar compared to the control culture (Fig. 1B, closed black triangles) that was performed in the original culture medium from the beginning on. For $ZnCl_2$, no cytotoxic effect was observed, indeed. For $CoCl_2$, cells could again proliferate once $CoCl_2$ was removed from the medium. Consequently, growth seems to have been suppressed in the presence of $CoCl_2$ but resumed after $CoCl_2$ was removed from the medium.

As demonstrated by this experiment, monitoring of the OTR provided insights on culture behavior during treatment and enabled a direct qualitative assessment of the cytotoxic potential of a test substance. Monitoring of the OTR is also suited to evaluate if cells can recover after test substance exposure and subsequent cultivation in the absence of the test substance. It may further be applied also if changes to the culture conditions are made during the assay.

Quantitative determination of $CoCl_2$ cytotoxicity

As cell growth was strongly affected during treatment with 0.2 mM $CoCl_2$, different concentrations of $CoCl_2$ were tested in a subsequent experiment (Fig. 2A). Besides the IC50 of 140 µM reported in the literature for adherently grown CHO cells [60], addition of 0.3 mM CoCl2 was found to quickly result in cell death [48]. Hence, the CoCl2 concentration was varied between 0.05 and 0.3 mM.

As the data density over time was comparably high, but the overall signal was rather noisy (Additional file 1: Fig. S1), data were smoothed by a moving average of 3 data points.

Culture behavior was reproducible within one experiment, as shown by the duplicate measurements (Fig. 2A). Initial cell growth was comparable for all cultures except when 0.3 mM $CoCl_2$ was added to the medium (Fig. 2A, pink diamonds). However, in all cultures treated with $CoCl_2$, the OTR decreased earlier compared to the control culture. This early decrease indicates an earlier reduction of the VCC and consequently a decreasing viability of the cultures. The results indicate that the cell line might become more sensitive toward $CoCl_2$, the longer the incubation lasts. As mentioned above, this agrees with data from the literature [35]. In addition, the achieved maximum OTR decreased with increasing $CoCl_2$ concentrations, increasingly affecting cell growth as well. Concentrations of $CoCl_2$ of 0.05 mM (Fig. 2A, orange squares) already impacted culture behavior

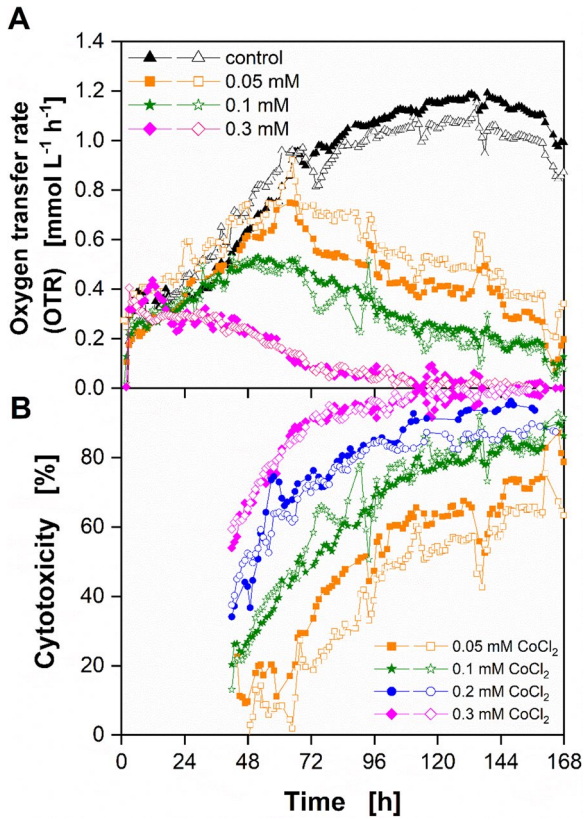

Fig. 2 Oxygen transfer rate (OTR) and cytotoxicity for different $CoCl_2$ concentrations using CHO cell line one. The OTR over time is depicted in duplicate for CHO cells with addition of 0 mM $CoCl_2$ (= control) (black triangles), 0.05 mM $CoCl_2$ (orange squares), 0.1 mM $CoCl_2$ (green stars), or 0.3 mM $CoCl_2$ (pink diamonds). $CoCl_2$ was added at the beginning of the cultivation ($t = 0$ h). Data were smoothed by a moving average of 3 points. Original OTR data are shown in Additional file 1: Fig. S1. **B** Cytotoxicity over time calculated from OTR for different $CoCl_2$ concentrations. Cytotoxicity was calculated by dividing the OTR of the treated culture by the OTR of the control culture at the respective point in time. Data for 0.05 mM $CoCl_2$ (orange squares), 0.1 mM $CoCl_2$ (green stars), and 0.3 mM $CoCl_2$ (pink diamonds) and corresponding control from experiment 2 (Additional file 1: Table S1). OTR data are depicted in **A**. Data for 0.2 mM $CoCl_2$ (blue circles) and corresponding control from experiment 1 (Additional file 1: Table S1). OTR data are depicted in Fig. 1A

visibly. For an IgG producing CHO suspension cell line, inoculated with an initial viable cell density of $3 \cdot 10^6$ cells mL^{-1} and cultured for 72 h, addition of 0.05 mM $CoCl_2$ did not affect cell growth, but 0.3 mM $CoCl_2$ resulted in complete cell death [48]. In our study, cell growth was slightly affected for 0.05 mM $CoCl_2$ (Fig. 2A, orange squares), whereas the OTR of the culture treated with 0.3 mM $CoCl_2$ (Fig. 2A, pink diamonds) had largely decreased after 72 h. As differences in the behavior of even similar CHO cell lines are expected (see "Methods" section), the general trend for $CoCl_2$ compares quite well with data from the literature.

For a quantitative assessment of the cytotoxic potential, it was investigated, if the cytotoxicity may be calculated from a comparison of the OTR values between the treated culture and the control culture. For this, Eq. 2 was used. The obtained absolute value of the OTR at a given $CoCl_2$ concentration and at a given point in time ($OTR_{treated,t=x}$) was divided by the averaged OTR of the two control cultures (Fig. 1A, black triangles) at this point ($OTR_{control,t=x}$) (Eq. 2):

$$\text{Cytotoxicity}_{t=x}[\%] = \left(1 - \frac{OTR_{treated,t=x}}{OTR_{control,t=x}}\right) \cdot 100\%. \quad (2)$$

The presented approach enabled a time-resolved calculation of the cytotoxicity in real time and already during treatment instead of a single endpoint determination. The time-resolved cytotoxicity for different $CoCl_2$ concentrations calculated from the OTR (Fig. 2A) is depicted in Fig. 2B. The calculated cytotoxicity for 0.2 mM $CoCl_2$ (Fig. 2B, blue circles) from experiment 1 (see Additional file 1: Table S1) was also included. As the OTR was initially relatively low, a clear trend for cytotoxicity was only observed after about 40 h. Consequently, the calculated cytotoxicity is depicted from this time onwards.

The cytotoxic effect of $CoCl_2$ increased with increasing cultivation time (Fig. 2B) which agrees with literature results reported for CHO suspension cells [48]. In addition, the time required to reach the maximum cytotoxicity decreased with increasing $CoCl_2$ concentration which points at a dose-dependent effect that also agrees with the literature (see above). For 0.3 mM $CoCl_2$ (Fig. 2B, pink diamonds), the maximum cytotoxicity was approached after around 72 h, while for 0.1 mM $CoCl_2$ (Fig. 2B, green stars), maximum values were reached after around 120 h.

Traditionally, a dose–response curve is typically determined at the end of treatment or even after treatment has ended. As discussed above, dose–response curves are used to determine the IC50 as a characterizing parameter for a test substance. Here, we investigated, if cytotoxicity assessment based on OTR measurements could even be taken a step further by determining not only the course of the cytotoxicity from the OTR data but also a dose–response curve and the IC50 value. Thus, the maximum cytotoxicity of $CoCl_2$ was determined by averaging the cytotoxicity values between 140 and 160 h of cultivation. Here, the cytotoxicity was relatively constant for all concentrations tested (Fig. 2B) and had reached its maximum. As $CoCl_2$ was added from the beginning ($t = 0$ h), this timespan also corresponds to the treatment time.

The determined cytotoxicity was plotted against the added $CoCl_2$ concentration in a semi-logarithmic form to obtain the corresponding dose–response curve (Fig. 3A).

All concentrations tested led to comparably high cytotoxicity above 60% (Fig. 3A). Consequently, values needed to be extrapolated for the calculation of IC50. The values for A1 (minimum cytotoxicity) and A2 (maximum cytotoxicity) (Eq. 1) were set to 0% and 100%, respectively. Logistic fitting (Eq. 1) resulted in an IC50 of 30 µM. As mentioned above, Tan et al. [60] reported an IC50 of 140 µM for adherent CHO-K1 cells in a serum-containing medium. The higher cytotoxicity determined in our study likely resulted from the significantly longer incubation time after which the cytotoxicity was determined (Table 2). As shown in Fig. 2B, the cytotoxicity increased with time, decreasing the IC50 value. Thus, it appears very reasonable that differences in IC50 were observed at different incubation times. In addition, CHO cell lines are known to be genetically and physiologically diverse, which makes comparison of data between different cell lines difficult [64]. In addition, transcriptomic changes were observed for CHO cells upon adaption from adherent to suspension growth [57].

Time-resolved determination of the cytotoxicity enabled the calculation of dose–response curves with high resolution of one dose–response curve per hour. The values at each point in time in Fig. 2B can be used to obtain a dose–response curve at this point leading to coverage of the whole range of cytotoxicity. Exemplarily and for better comparison with the data from Tan et al. [60], a dose–response curve was determined from the OTR after 58 h (Fig. 2B) and used to calculate the IC50 (Fig. 3B). In this case, an IC50 of 111 µM is obtained. This value is closer to the value reported by Tan et al. [60] (140 µM) and appears reasonable, as treatment times are more comparable in this case (Table 2). In addition, lower IC50 levels in real-time assays compared to conventional endpoint determination were also determined for several anticancer drugs [26]. Differences in $CoCl_2$ sensitivity might have also been caused by different culture media utilized (Table 2). Here, partly the same reasons as discussed for $ZnCl_2$ may have affected culture behavior. Particularly, using media with or without FCS as well as differences between adherently grown and suspension grown cells may have affected the culture behavior.

Again, it may be important to note that it was not the aim of this study to provide a statistically reliable analysis of the IC50 values, but rather to investigate if it is in general possible to obtain a dose–response curve using OTR data. Nevertheless, we compared the dose–response curve for CoCl2 that was obtained from OTR measurement (Fig. 3A, Additional file 1: Fig. S2A) to a measurement using trypan blue exclusion (Additional file 1: Fig. S2B). The IC50 determined from trypan blue exclusion measurement is around 60 µM. Even though this is about twofold higher than determined from OTR measurement, the values are in the same order of magnitude. Furthermore, the higher IC50 determined from trypan blue exclusion agrees well with the observation that trypan blue exclusion tends to underestimate the cytotoxicity of a test substance [18]. An underestimation of the cytotoxicity corresponds to a higher IC50. In

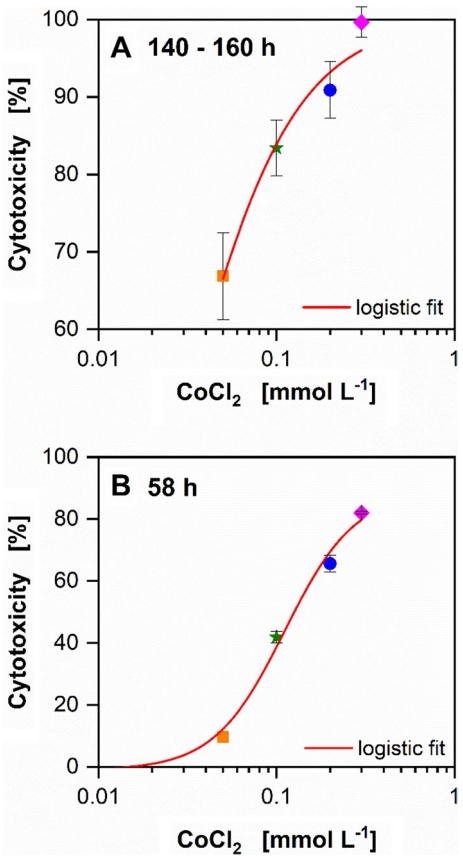

Fig. 3 Dose–response curves for $CoCl_2$ at different points in time using CHO cell line one. **A** Dose–response curve for $CoCl_2$from cultivation data between 140 and 160 h. The following workflow was applied: OTR values were measured time resolved for different $CoCl_2$ concentrations (cf. Fig. 2A). From each measured OTR value, cytotoxicity was calculated using Eq. 2 (cf. Fig. 2B). Calculated cytotoxicity values were averaged in the time interval between 140 and 160 h (cf. Fig. 2B). Obtained cytotoxicity values were plotted against the respective $CoCl_2$ concentration to obtain the depicted dose–response curve. Logistic curve fitting according to Eq. 1 was performed to determine the IC50. IC50 = 30 µM. **B** Dose–response curve for $CoCl_2$ after 58 h of cultivation. The same workflow as in **A** was applied, but single OTR values measured after 58 h were used. IC50 = 111 µM. Data from experiments 1 and 2 (Additional file 1: Table S1)

Table 2 Comparison of cultivation conditions for CHO cell line one from this study and the study by Tan et al. [60]

Parameter	This study	Study by Tan et al. [60]
Growth type	Suspension	Adherent
Medium	PowerCHO 2™ without fetal calf serum **(protein free)**	Ham's F12 medium with 5% (v/v) fetal calf serum **(protein containing)**
Cultivation time after which $CoCl_2$ was added and subsequent treatment time with $CoCl_2$ [h]	0/up to 168 (Figs. 1, 2, 3) 24/ up to 144 (Additional file 1: Fig. S2)	24/16
Evaluation method	Determination of respiration activity	Determination of cellular clonal growth
Cytotoxicity (IC50) [mM] (cultivation time after addition of test substance [h] and subsequent treatment time [h] given in brackets)	0.111 (0/58) 0.030 (0/140–160) 0.036 (24/116–136)	0.140 (24/16)

addition, the IC50 from trypan blue exclusion was determined manually, from a single endpoint measurement and not averaged from multiple measurement values. To investigate, if the cell concentration at the time, when $CoCl_2$ is added, affects the IC50, $CoCl_2$ was added after 24 h in a subsequent experiment (Additional file 1: Fig. S3A). Time-resolved cytotoxicity was calculated from the OTR data (Additional file 1: Fig. S3B). The concentrations tested included CoCl2 concentrations as low as 0.01 mM, as the data obtained (Fig. 3 and Additional file 1: Fig. S2) indicated an IC50 for CoCl2 in the range of 30–60 µM. Cytotoxicity was again similar between 140 and 160 h of cultivation (Additional file 1: Fig. S3B). Thus, a dose–response curve was determined for the 20-h range between 140 and 160 h by dividing the averaged OTR values between treated and control cultures (Eq. 2) (Additional file 1: Fig. S3C). The IC50 was determined from 4-parameter logistic regression (Eq. 1) to 36 µM (Additional file 1: Fig. S3C). No extrapolation of the data was necessary. The results indicate that the cell concentration at the start of the treatment did not impact the maximum cytotoxicity and that IC50 values between consecutive experiments were comparable.

In summary, the IC50 concentration of $CoCl_2$ determined from non-invasive, and time-resolved OTR measurement was in the same order of magnitude compared to conventional endpoint determination by trypan blue exclusion and to data previously reported in the literature. Considering different incubation times, cell lines, initial viable cell concentrations, and media used, as well as differences caused by the methods used to evaluate the cytotoxicity, the IC50s calculated from the OTR seem very reasonable. However, for a statistical reliable analysis of the IC50 values, further tests need to be performed, including a larger number of replicates and further comparison with established cytotoxicity assays. However, in contrast to single endpoint determination, these analyses may be based on a larger data basis.

In comparison to traditional endpoint determinations, where cells may only be cultured for 24–48 h, here evaluation was carried out after up to 160 h. Nevertheless, evaluation may also be performed after about 50 h (see Fig. 3B). For an even earlier evaluation, the noise of the OTR data would need to be reduced. This may, for example, be achieved by increasing the measurement time (see Additional file 1: Additional Data for details), which will result in a decreased temporal resolution. As currently one data point per hour is obtained, this may be acceptable. Further, the IC50 can be determined right after the respective OTR values have been measured with no time for sample analysis required.

Transfer of methodology to a different cell line

The effect of $CoCl_2$ was investigated for a second CHO cell line cultured in another medium to further investigate the applicability of using OTR data to determine the cytotoxicity of a test substance. Furthermore, the negative influence of $CoCl_2$ was exclusively determined for an IgG producing cell line [48] so that comparison with a non-producing cell line appeared interesting. General culture behavior in the utilized medium was described previously [29]. The overall course of the OTR of the control culture (Fig. 4, black squares) was different in shape and OTR values compared to cell line one (Fig. 1, black triangles). The differences can be attributed to different media with different glucose concentrations being used for cultivation. Consequently, the y-axis is seized differently compared to the previous experiments. For CHO cell line two, concentrations up to 0.2 mM $CoCl_2$ were tested (Fig. 4).

The effect of $CoCl_2$ on the growth of the second CHO suspension cell line was much less detrimental compared to the first cell line. Even in the presence of 0.2 mM $CoCl_2$, which entirely suppressed the growth of cell line one (Fig. 1B, blue circles), cells were initially able to grow (Fig. 4, open circles). With increasing cultivation time and $CoCl_2$ concentration, lower overall OTR

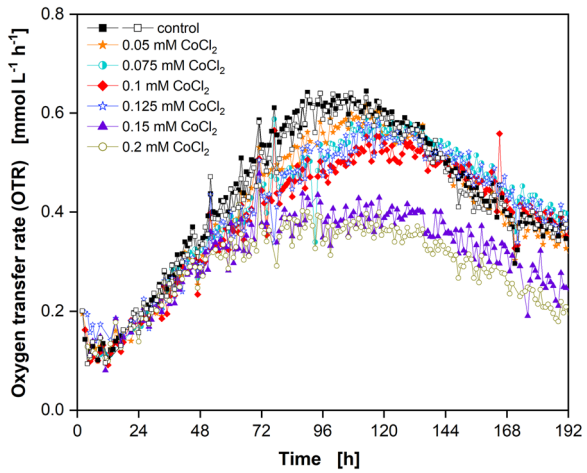

Fig. 4 Influence of $CoCl_2$ on the oxygen transfer rate (OTR) of CHO cell line two. **A** The OTR over time is depicted in presence of 0 mM $CoCl_2$ (= control) (black squares), 0.05 mM $CoCl_2$ (closed orange stars), 0.075 mM $CoCl_2$ (half-filled blue circles), 0.1 mM $CoCl_2$ (closed red diamonds), 0.125 mM $CoCl_2$ (open blue stars), 0.15 mM $CoCl_2$ (closed purple triangles), or 0.2 mM $CoCl_2$ (open yellow circles). $CoCl_2$ was added at the beginning of the cultivation ($t = 0$ h). Data from experiment 4 (Additional file 1: Table S1)

values compared to the control were reached. Maximum deviations between the cultures were reached after around 100 h (Fig. 4) when the OTR of the control had reached its maximum. After the OTR maximum, the VCC decreases and cell behavior is expected to change, as glucose is depleted from the culture medium and lactate is consumed [29]. Consequently, the maximum cytotoxicity was calculated by dividing the OTR values for a $CoCl_2$ concentration of 0.2 mM after 100 h by the OTR of the control culture at the same time (Eq. 2). The calculation resulted in a cytotoxicity of about 40%. The culture behavior of the control and for two $CoCl_2$ concentrations was tested in a subsequent experiment (Additional file 1: Table S1). It was found to be well comparable to the first experiment (Additional file 1: Fig. S4), even though a commercial TOM system was used for experiment 4 (Fig. 4) and an in-house built RAMOS system was used for experiment 5 (Additional file 1: Fig. S3). However, no differences in the OTR data were expected when using two different devices.

Differences in observed IC50 values for $CoCl_2$ between the two different CHO suspension cell lines were expected for the reasons detailed in the "Methods" section and might be attributed to differences in the cell lines, culture media, or seeding densities. For cancer drug screening experiments, the cell line itself was shown to have a more considerable, significant impact on variations in viability compared to medium or seeding density, which had no significant effect [36]. Again, it was not the aim of this study to compare the response of different cell lines to the same test substance, but rather to explore if despite significant differences seen in the course of the OTR for different cell lines, the cytotoxicity may still be determined from the OTR using the presented, generalized workflow.

Influence of DMSO on CHO suspension cell line two

To further investigate the applicability of the method, DMSO was used as a third test substance. DMSO is usually used for the cryopreservation of cells. DMSO is also used to dissolve test substances that are not readily soluble in water, even though replacement of DMSO is recommended [21]. In general, the cytotoxic concentration of DMSO strongly varies among cell types [45]. A DMSO concentration of 10% did not alter membrane permeability in Caco2/TC7 cells but has been used as a positive control for in vitro biocompatibility tests using L929 mouse fibroblasts [4, 13]. Additionally, a DMSO concentration range of 0.1–1.5% (v/v) was previously stated as a common concentration range when used as a vehicle for cellular treatments [62]. Thus, DMSO concentrations between 0.1 and 5% (v/v) were investigated here.

Figure 5 depicts the course of the OTR of CHO cell line two over time for different DMSO concentrations. The control culture reached a maximum OTR of about 0.6 mmol L^{-1} h^{-1} after around 100 h of cultivation (Fig. 5, black squares).

Except for 1% DMSO (Fig. 5, open blue diamonds), the cultures treated with different concentrations of DMSO showed a slower increase of the OTR and reached lower maximum OTR values than the control. In contrast to $CoCl_2$, where initial cell growth was comparable (Fig. 4), culture behavior already deviated from the beginning. With increasing DMSO concentrations, the slope of the OTR over time was lower, and consequently, the OTR ascent was delayed. The observation of an inhibited cell proliferation caused by DMSO was also observed in human cancer cells [45]. Here, the proliferation curve, measured in real time by evaluating the change in impedance, also showed different slopes after DMSO addition [45]. The results underline that attention needs to be paid if cultures are exposed to higher DMSO concentrations under culture conditions. This is consistent with the typical recommendation to quickly remove DMSO after cell thawing.

The point where the maximum OTR of the control was reached (after 100 h) was used to calculate the cytotoxicity of DMSO using Eq. 2 (Fig. 6A). Cytotoxicity was calculated by the ratio of the OTR value of the treated culture and the control culture. The resulting dose–response curve (Fig. 6A) was fitted by 4-parameter logistic fitting (Eq. 1), which resulted in an IC50 of 2.3% (Table 3).

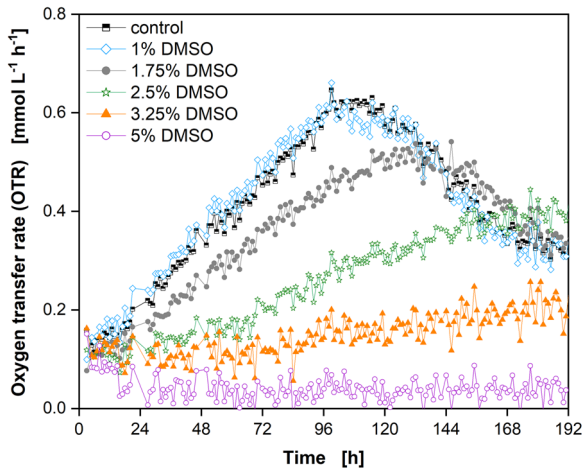

Fig. 5 Influence of DMSO on the oxygen transfer rate (OTR) of CHO cell line two. The OTR over time is depicted in the presence of 0% DMSO (= control) (black squares), 1% DMSO (open blue diamonds), 1.75% DMSO (closed gray circles), 2.5% DMSO (open green stars), 3.25% DMSO (closed orange triangles), or 5% DMSO (open purple circles). DMSO was added at the beginning of the cultivation ($t = 0$ h). Data from experiment 6 (Additional file 1: Table S1)

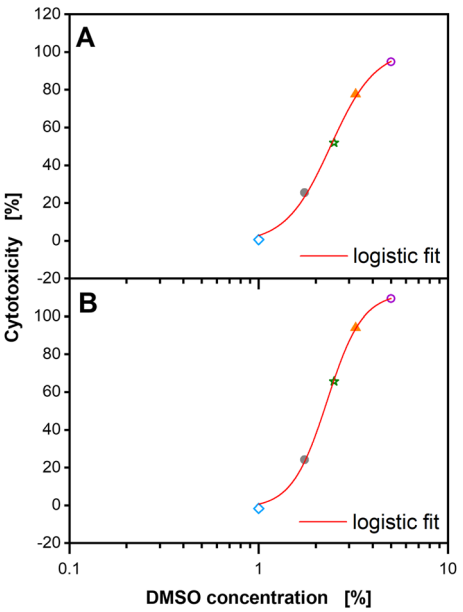

Fig. 6 Dose–response curves of DMSO for CHO cell line two. **A** Dose–response curve for DMSO obtained by curve fitting using Eq. 1 with cytotoxicity values calculated with Eq. 2 by using measured OTR values of treated and control culture after 100 h. **B** Dose–response curve for DMSO obtained by curve fitting using Eq. 1 with cytotoxicity values calculated with Eq. 2 using ratio of determined slopes of treated and control culture. Each slope was calculated by linear fitting of the measured OTR values between 0 and 100 h (Additional file 1: Fig. S6). Data from experiment 6 (Additional file 1: Table S1). OTR data used for calculation are depicted in Fig. 5

As shown in Fig. 5, the increase of the OTR was linear. This linear increase indicated that the cytotoxic effect was not strongly affected by the exposure time. This is also reflected when plotting the time-resolved cytotoxicity calculated from the ratio of the OTR between treated and control culture at each point (Additional file 1: Fig. S5). Consequently, the advantage of the high data density and time-resolved measurement was exploited by calculating the cytotoxicity from the slope of the OTR over time instead of using single OTR values. Accordingly, the slope of the OTR until 100 h was determined by linear fitting (Additional file 1: Fig. S6). For cultures with 5% DMSO addition (Fig. 5, open purple circles), the slope was negative (Additional file 1: Fig. S6, open purple circles). For 1% DMSO, the slope was higher compared to the control culture (Additional file 1: Fig. S5, open blue diamonds).

In the next step, the ratio of the slopes obtained from linear fitting of the OTR was used to calculate the cytotoxicity. This was performed to investigate, if the OTR data could also be exploited in another way. For this purpose, the respective slope values (Additional file 1: Fig. S6) were inserted into Eq. 2. The resulting dose–response curve is depicted in Fig. 6B. For 5% DMSO, a cytotoxicity above 100% was calculated, which could be attributed to the evaluation method applied. A negative slope is calculated, as viable cells are initially present, but the viable cell concentration is decreasing. This negative slope results in a calculated cytotoxicity of more than 100%. For 1% DMSO (Fig. 5, open blue diamonds), a negative cytotoxicity was calculated, because the slope for the culture treated with 1% DMSO was higher than the slope of the control culture. A negative response value might point toward a hormetic dose–response relation characterized by a stimulating effect of the test substance at low concentrations and toxicity at higher concentrations [9]. This stimulating effect is well reflected by the higher slope.

Plateau values (A1, A2) varied, if single OTR values or slopes were used for calculation, because of the method used for evaluation (Table 3). Nevertheless, an IC50 of ~ 2.3% was determined for DMSO by logistic fitting independent of the evaluation method (Table 3). However, calculation using the ratio of the OTR gave a more reasonable value for the maximum cytotoxicity, expressed as A2, reached (Table 3). In addition, utilization of single OTR values enables time-resolved calculation of the cytotoxicity. The time-resolved calculation is not possible if the slopes are used for evaluation. Nevertheless, a comparison of the slopes might also be of interest, if for example, different lag phases between treated and control cultures are observed. Further, using growth rates instead of cell counts in an endpoint determination has been proposed some years ago [24]. Again, the progression

Table 3 Parameters obtained by logistic fitting for evaluation of cytotoxicity of DMSO on CHO cell line two (Fig. 6)

	Figure 6A: Ratio of OTR values between treated and control culture @ 100 h	Figure 6B: Ratio of slopes (0–100 h) calculated from OTR between treated and control culture
A1 [%] (minimum cytotoxicity)	− 1.24 ± 2.48	− 1.70 ± 1.71
A2 [%] (maximum cytotoxicity)	101.55 ± 5.27	112.36 ± 2.79
IC50 [% v/v]	2.39 ± 0.10	2.30 ± 0.05

of the OTR over time for different DMSO concentrations was comparable between consecutive experiments (Additional file 1: Fig. S7) and when two different devices (TOM system for experiment 6, in-house built RAMOS system for experiment 7) were used (see Additional file 1: Table S1).

The determined IC50 of ~ 2.3% is in good agreement with the literature. The IC50 was previously determined for adherent CHO cells in an application note using fluorescence evaluation [11]. A value of 1.8% was reported. Lower IC50 values for the adherent cell line might appear reasonable. Another reason for the good agreement for different CHO cell lines might be that DMSO addition largely increases the osmolality of the culture medium [63]. Consequently, the cytotoxic effect might not result from the compound itself but the effect it has on the culture medium. Thus, it seems reasonable that different CHO cell lines react comparably to an increase in media osmolality and, thus, DMSO concentrations. In addition, our experiments indicate that DMSO addition did not have a large effect on the calculated cytotoxicity between 24 and 96 h of treatment. Consequently, different treatment times may affect the IC50 less and therefore result in better comparability and similar IC50 values between different assay protocols. For a statistical sound analysis, more experiments need to be conducted.

Comparison of cytotoxicity assessment by OTR measurement with other assay methods

In this study, the first assessment of using OTR values to determine cytotoxicity for CHO suspension cells has been conducted in shake flasks. Compared to conventional MTP-based assays, which may only require a volume in the µL range, culture volumes in shake flasks are several orders of magnitude higher. Even though the number of shake flasks to be monitored in parallel may easily be numbered up, the media consumption and footprint are much larger compared to microtiter plate-based assays. Thus, it is well acknowledged, that a transfer to microtiter plate scale is very desirable to increase experimental throughput and to reduce the overall costs of the experiments. For measurement in MTPs, the µRAMOS, which enables monitoring of the OTR in 48- or 96-well microtiter plates [14, 19], may be used. However, it has to the authors knowledge neither been applied for monitoring of mammalian cells in general nor CHO cells specifically, yet.

Assuming that OTR measurement of CHO cells in microtiter plates may be achieved in the future, using measurement of the OTR for cytotoxicity assessment provides an important addition to already established measurement systems. As discussed above, measurement based on impedance (see Additional file 1: Additional Data Sect. 1.2 for a more detailed description of the technology) has been demonstrated (see, for example, [7, 10, 15, 26, 42, 45, 59]). Real-time cell analysis (RTCA) based on impedance measurement is preferably performed for adherent cells [59], while OTR-based evaluation is demonstrated for suspension cells in this study. Additionally, it was hypothesized that additives of drugs, including lactate, could interfere with impedance measurements [7]. As lactate is a common byproduct of CHO cell metabolism, impedance measurement might in general be less suited to be used with CHO cells. Here, measurement of the OTR could provide an alternative measurement option.

Another example, also mentioned above, is the Seahorse technology. It is based on evaluating mitochondrial respiration (see Additional file 1: Additional Data Sect. 1.3 for details) and is intended to be used with adherent cells [6]. This technology may be used for cytotoxicity assessment, but care needs to be taken when evaluating the data [54]. A reason for this is that data normalization to cell numbers may mask a cytotoxic effect [54]. Additionally, the technology is designed to evaluate the effect of a test substance within min to h [54]. Hence, it is not intended for monitoring culture behavior during several days of cultivation. However, as discussed above, the exposure time to a test substance is a critical factor in cytotoxicity assessment and will affect the results. Here, monitoring the OTR, applicable for long cultivation periods lasting several days could again provide a complementary measurement option.

Conclusions

This study investigated the suitability of online monitoring of the oxygen transfer rate (OTR) to assess the cytotoxic effect of different test substances on Chinese hamster ovary (CHO) cells in shake flasks. Two different CHO suspension cell lines cultured in different media were used. Qualitative evaluation of the cytotoxicity was directly possible from the OTR signal. The addition of 0.1 mM $ZnCl_2$ did not affect growth of CHO cell line one. On the other hand, addition of 0.2 mM $CoCl_2$ led to a decrease in the OTR. If test substances were removed from the media after 24 h of incubation, cell cultures subsequently recovered, and growth resumed with a course comparable to the control culture.

Quantitative evaluation of the cytotoxicity was possible by dividing the OTR of the treated and the control culture at each point. The cytotoxicity of $CoCl_2$ increased with exposure time and maximum cytotoxicity was reached earlier with increasing $CoCl_2$ concentrations. This time- and dose-dependent effect is in accordance with literature data.

Dose–response curves obtained from OTR resulted in a sigmoidal correlation between test substance concentration and cytotoxicity that is also observed in conventional assays. For CHO cell line one, 4-parameter logistic fitting of the dose–response curve resulted in an IC50 of 30 µM toward the end of treatment (140–160 h of cultivation). Evaluation of the dose–response curve after 58 h of treatment resulted in an IC50 of 111 µM. For cell line two, no IC50 could be determined, because the calculated cytotoxicity was only at 40% at a $CoCl_2$ concentration of 200 µM after 100 h. The differences observed may be attributed to cell line one producing an IgG1, and/or different culture media used, but further experiments are required to confirm this.

For CHO cell line two, DMSO was investigated in addition to $CoCl_2$. For DMSO, the slope of the OTR decreased with increasing DMSO concentrations at concentrations above 1%. Consequently, evaluation of the cytotoxicity was carried out based on (i) the ratio of the OTR values at each point and (ii) the ratio of the slopes obtained from linear fitting of the OTR. In both cases, an IC50 of 2.3% DMSO was determined. For a DMSO concentration of 1% the growth rate of the culture was higher than the control, pointing at a stimulation at low dose.

In conclusion, a generalized workflow to use measured OTR data to determine the IC50 of a test substance was demonstrated. Future experiments should focus on using the presented method for a statistically sound analysis of individual test substances. Additionally, transfer to the microtiter plate scale is highly desirable. For the demonstrated method, no sampling, no subsequent offline analysis, and no interruption of the cultivation process were required to calculate the IC50 and provide insights on culture behavior. In addition, assessment was possible time resolved with a resolution of one measurement point per hour.

By the presented method, a better comparison of assays performed at different conditions, especially at different treatment times or cell concentrations, is possible. In addition, the time required to reach maximum cytotoxicity can be determined and used to standardize different test protocols. In the future, the approach presented might be implemented into standardized guidelines to assess chemical cytotoxicity in vitro.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-022-00673-5.

Additional file 1. Additional Data, Additional Table S1, Additional Figures S1–S7.

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Author contributions

NI contributed to conceptualization (lead), funding acquisition (equal), data curation (lead), formal analysis (equal), investigation (equal), methodology (lead), visualization (lead), and writing—original draft (Lead); LPM and RP were involved in data curation (supporting), investigation (equal), visualization (supporting), and writing—review and editing (supporting); DL was involved in conceptualization (supporting), resources (supporting), supervision (supporting), and writing—review and editing (equal); JB contributed to conceptualization (supporting), funding acquisition (lead), project administration (lead), supervision (lead), and writing—review and editing (equal). All the authors read and approved the final manuscript.

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Availability of data and materials

The dataset used and/or analyzed during the current study is available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

DL is employed by Rentschler Biopharma SE. The remaining authors declare that they have no competing interests.

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