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A label-free, impedance-based real time assay to identify drug-induced toxicities and differentiate cytostatic from cytotoxic effects

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ABSTRACT

Cell-based assays are key tools in drug safety assessment. However, they usually provide only limited information about time-kinetics of a toxic effect and implementing multiple measurements is often complex. To overcome these issues we established an impedance-based approach which is able to differentiate cytostatic from cytotoxic drugs by recording time-kinetics of compound-effects on cells. NIH 3T3 fibroblasts were seeded on xCELLigence® E-plates and impedance was continuously measured over 5 days. The obtained results reflected cytotoxicity and cell proliferation, as confirmed by neutral red uptake in vitro. Based on known toxicants, we established an algorithm able to discriminate cytostatic, cytotoxic and non-toxic compounds based on the shape of the impedance curves. Analyzing impedance curve patterns of additional 37 compounds allowed the identification and differentiation of these distinct effects as results correlated well with previous in vivo findings. We show that impedance-based real-time cell analysis is a convenient tool to characterize and discriminate effects of compounds on cells in a time-dependent and label-free manner. The presented impedance assay could be used to further characterize toxicities observed in vivo or in vitro. Due to the ease of performance it may also be a suitable screening tool.

1. Introduction

During the drug development process, cell-based assays are a key tool to support the selection of safe drug candidates before entering animal testing and are crucial to fulfill the concept of 3R, i.e. reducing, refining and replacing animal testing (Russell, 1995). Today, a plethora of commercial as well as non-commercial cellular assays exist to identify potential toxic liabilities of compounds based on in vitro experiments (Kepp et al., 2011; O'Brien and Haskins, 2007). The parameters analyzed to assess cytotoxicity differ considerably among the published methods. For example, some assays measure cell death by analyzing membrane disruption while others measure the uptake of a specific dye as an indicator for cell viability (O'Brien and Haskins, 2007; O'Brien et al., 2006). Recently, more specific cell death assays have become available which are able to differentiate multiple mechanisms such as apoptosis or necrosis by targeting specific enzymes involved in these processes. These assays have in common that the information provided is usually limited to the time points investigated, disregarding the kinetics of the effects. They typically measure only one or at most two time points and thus differentiation of late from early toxic events is usually not possible (Xia et al., 2008). Real time cell analysis based on impedance could overcome this issue. This novel technology enables living cells to be constantly and non-invasively monitored in terms of cell growth, cell shape and cell damage over an extended period of time. Impedance describes an electrical parameter that measures opposition to an alternating current and relates to resistance in direct current. In the current setting, gold electrodes placed at the bottom of each cell culture well are submitted to an alternate current and act as electronic sensors that measure changes in impedance caused by adherent cells. When cells are seeded into the dish the signal increases with cells attaching to the bottom of the culture vessel because they impede the current between the electrodes (Irelan et al., 2011; Ke et al., 2011; Xi et al., 2008). Using dividing cells, an increase in impedance over time reflects cell proliferation whereas a decrease can be considered as a sign of cytotoxicity as cells detach when they die (Atienza et al., 2006). Impedance based microelectrode sensor arrays have been used earlier by Giaever and colleagues

Abbreviations: NRU, neutral red uptake assay; RTCA, real time cell analysis; CI, cell index; LD, low density cultures; HD, high density cultures.

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to monitor cell proliferation (Giaever and Keese, 1986) and demonstrated that impedance correlated well with cell activity. Later on it was shown that impedance measurements could reflect drug-induced changes in cell attachment and cell coverage of 3T3 fibroblasts (Huang et al., 2003). Similarly, changes in shape due to pharmacological intervention with specific receptors or the cytoskeleton as well as beating frequency (e.g. cardiomyocytes) will cause a measurable effect on the impedance (Denelavas et al., 2011; Guo et al., 2011). These changes in the impedance readout will also be reflected by an altered shape of the impedance curve over time (Ke et al., 2011; Xi et al., 2008). Thus, impedance technology offers a label-free, multi-parametric alternative to analyze compound induced effects over time in contrast to established endpoint-based biochemical analysis.

The goal of the present study was to establish a simple, robust and easy to use cytotoxicity assay able to differentiate cytotoxic from cytostatic compounds in vitro by applying impedance-based real-time cell analysis. Knowledge on both the direct cytotoxic potential of a drug candidate as well as its ability to interfere with e.g. the cell-cycle machinery leading to growth arrest is an important aspect to identify compound liabilities which may lead to adverse drug reactions in vivo (Rich, 2003; Sundman-Engberg et al., 1998). A cytostatic effect is generally considered one where a treatment leads to inhibition or suppression of cellular growth and multiplication which is the goal of many anti-cancer therapies aiming to stop uncontrolled growth of tumor cells (Valeriote and van Putten, 1975). These drugs typically interfere with cellular growth pathways, either at the level of membrane receptors or at the level of intracellular signaling molecules such as kinases involved in control of cell proliferation (Shawver et al., 2002). A cytostatic effect therefore is characterized by prior cessation of growth and cell division before induction of cell death (Hoffman, 1991). Specialized assays using e.g. Propidium Iodide then can be applied to identify the stage of cell cycle where cells are arrested (Bergamo et al., 1999). On the other hand, a cytotoxic effect encompasses a wide range of potential detrimental effects of treatments directly leading to necrosis, apoptosis and/or cell lysis (Melino, 2005; Xia et al., 2008). A set of 40 compounds that had been previously analyzed in vivo and in vitro for toxic effects was analyzed. We could demonstrate that label-free impedance analysis is a valid tool to asses and discriminate compound mediated effects in a simple in vitro cytotoxicity assay.

2. Materials and methods

2.1. Cell culture

NIH 3T3 Fibroblasts (3T3 Balb/C A31; ATCC No CCL 163) were cultured in Dulbecco's Modified Eagles Medium (DMEM; Invitrogen) containing 10% FCS in a humidified atmosphere at 37 °C and 5% CO_2. Medium was changed every second day and subcultivation of the cells was performed before the cells reached full confluence. 3T3 Fibroblasts used for presented experiments were between passages 95 and 120.

2.2. Neutral red assay

To differentiate cytostatic from cytotoxic effects the neutral red uptake assay (NRU assay) was performed with two different cell seeding densities: 1.250 (low density; LD) or 20.000 (high density; HD) cells per well were seeded into a 96 well plate (reflecting 3,900 and 62,500 cells/cm^2, respectively). On the next day, cells were treated with the test compounds. For this, the medium was replaced with medium supplemented with indicated concentrations of test compound, with a final solvent concentration of 0.1% DMSO in each well. After 24 h the high density cultures were assayed for neutral red uptake whereas the low density cultures were incubated for further 48 h before performing the measurement.

The NRU assay was performed as published elsewhere (Borenfreund and Puerner, 1985; Repetto et al., 2008). Briefly, cells were incubated for 3 h with neutral red solution (0.2 mg neutral red/ml in DMEM with 10%FCS) in the incubator. After washing, 150 μL Ethanol/Acetic Acid solution (50% Ethanol, 1% Acetic Acid in water) was added and the plate was incubated on a rocking platform for 15 min. Finally, neutral red concentration in each well was measured using a multi plate reader at 550 nm (Spectramax, Molecular Devices). Cell viability was calculated as follows: $OD550_{treated}/OD550_{control} \times 100$. Data presented in graphs are average values of 8 biological replicates; error bars represent standard deviation.

2.3. Monitoring of cell growth using real time cell analysis (RTCA)

Cell growth was continuously monitored for at least 90 h using the xCELLigence RTCA MP instrument (Roche). Background impedance signal was measured with 50 μL cell culture medium per well. The final volume in a single well was adjusted to 100 μL cell culture medium by adding additional 50 μL medium containing 2.500 3T3 Fibroblasts (12,500 cells/cm^2). After plating, impedance was routinely recorded in 15 min intervals. One day after seeding, test compounds were added to the culture. All incubations were performed in 150 μL volume and with a final solvent concentration of 0.1% DMSO. For each compound and concentration four replicates on an E-Plate 96 were performed and each compound was analyzed at 8 different concentrations (0.03, 0.1, 0.3, 1, 3, 10, 30, 100 μM; except for Sprycel and RC28: 0.001, 0.003, 0.01, 0.03, 0.1, 1, 10, 100 μM). After compound administration, impedance was measured every 5 min for the following 12.5 h and afterwards every 15 min until the end of the experiment. The impedance signal was analyzed by normalizing data of each singe well to the first measurement after starting the treatment: $CI_{(normalized)} = CI_{time\ x}/CI_{norm\ time}$ (termed here "normalized cell index"). This normalized cell index was used for graphical result representation and exported for further processing using Microsoft Excel. Finally, each E-Plate was subject to a NRU assay at the end of the impedance analysis which was performed as described above.

2.4. FACS-based cell cycle analysis

3T3 fibroblasts were seeded at 40,000 cells per T25 culture flasks and in parallel treated for 24 h with compounds in the corresponding concentrations. For FACS-based cell cycle analysis cells were fixed with Ethanol after detachment. After centrifugation cells were resuspended in Propidium Iodide labeling solution (PBS, 50 μg/ml Propidium Iodide and 100 μg/ml RNAse added) and incubated for 75 min at 37 °C in the dark. Finally cells were analyzed by FACS (FACS-calibur, Becton–Dickinson). Results were displayed as average of 3 biological replicates calculated as percentage of cell present in the corresponding cell cycle; error bars represent standard deviation.

2.5. Impedance-data processing for toxicity assessment

Out of 4 normalized cell index values per time point the median curve was calculated and the area below and above "1" was calculated as shown in Fig. 2 A. Based on the calculated areas, the prediction of the compound's mode of action (cytotoxic, cytostatic or negative) was performed by a decision tree in Microsoft Excel using the "IF" function (for more details please refer to the Results section and see Fig 3). The graphs of xCELLigence data depicted in this manuscript were prepared in Microsoft Excel using a subset of