Application Note

Topic:
cardiotox, cardio-oncology
Cells:
H9C2
Tools:
AtlaZ

Chronic cardiotoxicity study on H9C2 cells using *HESI stem cell working group* reference compounds

Nanion Technologies GmbH, Munich.
innoVitro GmbH, Jülich.

Summary

Long-term exposure to cancer-related therapeutics has been linked to alterations of cardiac function in patients. The Stem Cell Working Group as part of the Health and Environmental Science Institute (HESI) currently endeavores to gain further insight into chronic cardiotoxicity. The objective of the HESI study is to optimize non-clinical safety assessment strategies of chronic cardiotoxicity by testing prolonged exposure of compounds on different cell-based assay systems using human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), by investigating e.g. contractility or contractile force.

Here, we used a subset of the HESI chronic cardiotoxicity study reference compounds known to act via different mechanisms of actions (MoAs) in cardiomyocytes, affecting cardiac energetics (doxorubicin, erlotinib, sunitinib), contractility (BMS-986094, Nilotinib), electrophysiology (pentamidine), and myofilament organization (endothelin-1 (ET-1), vincristine) (Figure 1, Table).

Using the AtlaZ platform, we investigated the toxicity of those compounds on H9C2 cell line, which has been differentiated towards a cardiac phenotype. Immortalized cardiomyocyte lines, which are generated from primary cells, are feasible alternatives of primary cultures [1], and amongst those are the rat cardiomyoblast cell line H9C2 originally described in 1976 [2], but also the mouse atrial myocyte derived HL-1 line [3] and the human AC16 line [4]. All these cell lines show quick propagation in culture and homogeneous response to stimuli that allows their use in high throughput drug screening experiments or even in modeling various cardiovascular diseases such as cardiomyopathies or metabolic alternations [1].

The impedance of planar gold-film electrodes that are used here as growth substrate for adherent cells reveals changes in electrode coverage or cell behavior (Figure 1, lower graph). Real-time impedance data provide insights in various cell phenotypes, such as cell morphology, proliferation, lateral migration or cytotoxicity even over prolonged periods of time. A crucial advantage over standard assays, e.g. filter based methods using labels and either an optical or radiometric detection technique, is the continuity of cell monitoring. Endpoint assays using dyes or labels quantify the potency of

Compound	Side effect on cardiac function and MOA	Tested concentration
Pentamidine	hERG trafficking blocker. Induces QT prolongation and arrhythmias.	100nM, 300nM,1µM, 3µ
Vincristine	Antimitotic agent by interacting with tubulin proteins. Causes myocardial ischemia and infarction.	300pM, 3nM, 30nM, 300nM
BMS-986094	Induces contractility impairment by a decrease in calcium transient.	100nM, 300nM, 1µM, 3µM
ET-1	Causes fibrosis of the vascular cells and stimulates production of reactive oxygen species.	300pM, 1nM, 3nM, 100nM
Erlotinib	No cardiotoxic effects are expected.	230nM, 770nM, 2µM, 8µM
Nilotinib	Disruption of CM. Induces QT prolongation & myocardial ischemia.	10nM, 100nM, 300nM, 1µM
Doxorubicin	Mitochondrial toxicity, oxidative stress leading to apoptosis. Induces arrhythmias and heart failure.	100nM, 300nM,1µM, 3µ
Sunitinib	Disruption of cardiomyocyte. Disruption of energy homeostasis and mitochondrial fusion-fission system via inhibition of AMPK signaling. Induces long QT and myocardial infarction.	10nM, 100nM, 300nM, 1µM

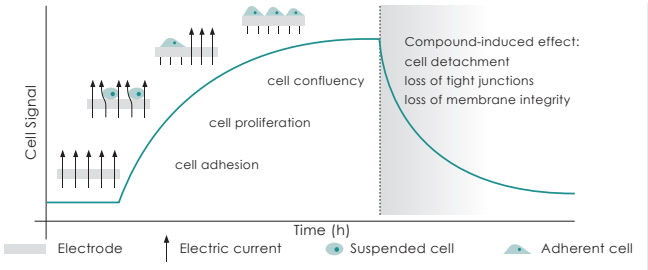


Figure 1: Top *Reference compounds, their different mechanisms of action and tested concentrations.* **Bottom** *The Cell Signal value offers information on cell adherence, proliferation or cell death, e.g. upon treatment with compounds.*

toxins to impact target cells by either measuring target cell viabiliy (i.e. MTT or flow cytometry) or membrane integrity (i.e. LDH). Those assays are labor-intensive and lack kinetic information of the recorded effects.

Unique culture plates with integrated electrodes as used in the AtlaZ system enable long-term measurements over several weeks. Thus real-time data on cell adhesion, proliferation and compound effects can be acquired.

Results

In our assay, we used rat ventricular H9C2 cells (rat cardio-myoblast cell line) which are developed towards a cardiac phenotype. This model cell type represents a homogeneous population of cardiac cells, offering the possibility to simulate cardiac pathologies. The cells lack the capability to contract, furthermore they do not express cardiac ion channels like hERG.

Cells were exposed to compounds for three days to identify chronic toxicity effects. Figure 1 shows H9C2 cells in control conditions as well as in the presence of 100 nM, 300 nM, 1 µM and 3 µM Pentamidine or 230 nM, 770 nM, 2.3 µM and 7.7 µM Erlotinib. Raw traces of Cell Signal are shown on the left, the normalized Cell Signal (normalized to the timepoint of compound addition) is shown on the right. After compound addition, cells continue to proliferate until a 100 % confluency is reached which is represented by the plateau phase.

The H9C2 cell seeding density was 8 k cells/well. The cells then started to proliferate and at t = 100 h the compounds were added. The impedance signal of the cell monolayer was recorded every 15 minutes, and is plotted using the unitless parameter Cell Signal (Figure 2). The data revealed that Pentamidine at concentrations between 100 nM and 3000 nM or Erlotinib at concentrations ranging between 230 nM and 7.7 µM did not prevent the H9C2 cells from proliferating, as cells remained viable and adherent to the surface. As expected, no toxicity was observed upon the application of the hERG trafficking inhibitors Pentamidine and Erlotinib, which are considered as a cardio safe compounds.

Next, we applied Vincristine, BMS-986094 and Doxorubicin. We observed a time- and ratio-dependent decrease of the Cell Signal, representing a structural cell toxicity (Figure 3). Vincristine is an antimitotic agent and is interacting with tubulin proteins, causing myocardial ischemia and infarction [5]. Here, the two highest concentrations induced a decrease of viability, reaching the maximum effect after approx. 30 h. BMS-986094 is known to have an impact on contractility of cardiomyocytes by decreasing the calcium transient. Here, we observed a linear decay of the Cell Signal in a dose- and time-dependent manner. This means BMS-986094 not only has a functional toxicity on cardiomyocytes [6] but also induces cell death. Doxorubicin induces oxidative stress via mitochondria, leading to apoptosis, arrhythmias and heart failure [7]. As expected, we observed a concentration-dependent and time-dependent decay of the Cell Signal, representing loss of cell integrity. ET-1 is known to cause hypertrophy via certain beta adrenergic pathways. We could not observe ET-1 effects in H9C2 cells though (data not shown). Nilotinib and Erlotinib are functionally impacting cardiomyocytes (cells stop contracting), but here in the non-contractile H9C2 cells we could not observe any impact on cell integrity (data not shown).

These results demonstrate the capability of AtlaZ to analyze cardiotoxic compounds when using H9C2 cardiac-like cells.

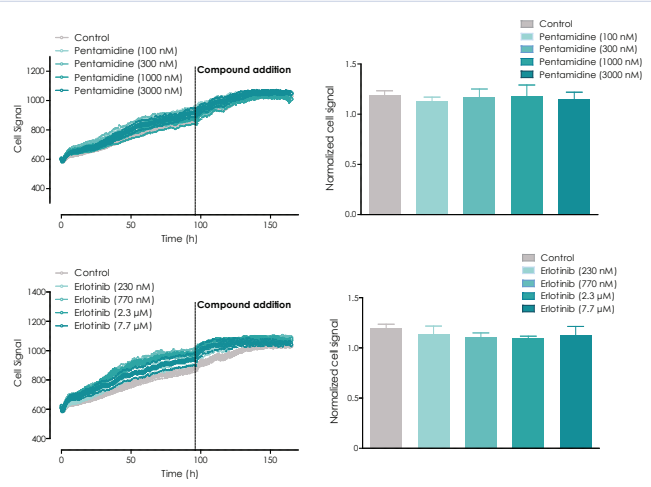
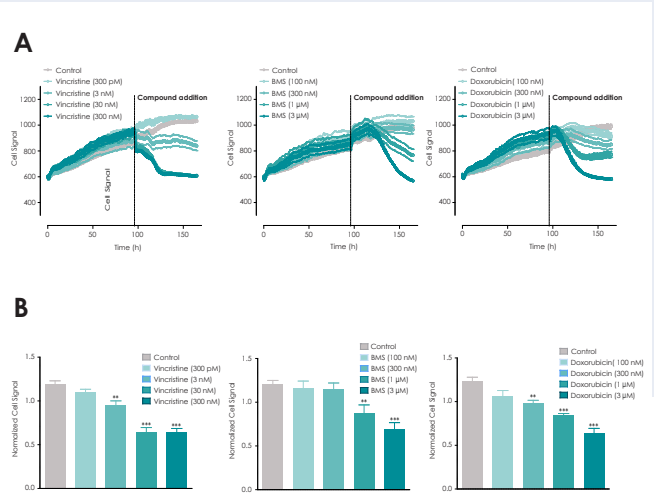


Figure 2: *Pentamidine and Erlotinib did not show toxic effects on H9C2 cells at concentrations up to 3 µM or 7.7 µM, respectively. Data are shown +/- SEM.*

Figure 3: *Chronic exposure to vincristine, BMS-986094 and Doxorubicin showed toxic effects on H9C2 cells in a concentration and time-dependent manner. Data are shown +/- SEM.* **A** *raw impedance and* **B** *data normalized to the timepoint of compound addition.*



In summary, AtlaZ allows for label-free and real-time cellular research on cell adhesion, proliferation and cytolysis. Measurements can be performed in up to 6 x 96-well plates simultaneously or independently. The AtlaZ system provides a versatile tool for in vitro cell monitoring addressing the demands for versatility, physiological relevance and throughput.

Methods

AtlaZ platform

The AtlaZ platform (Nanion Technologies) provides quantitative live-cell analytics by measuring the impedance (Ohm, Ω), displayed as *Cell Signal*, of adherent cells as grown on 96-well plates with embedded planar gold-film electrodes (Nanion Technologies).

Real-time impedance raw data provide insights in various cell phenotypes, such as cell morphology, proliferation, lateral migration or cytotoxicity even over prolonged periods of time. A crucial benefit over standard assays is the continuity of cell monitoring. Continuous measurements reveal the kinetics of cell behavior and allow an in-depth mechanistic understanding without the need for time- and labor-intensive endpoint assays. For example, one of the advantages of data derived from the AtlaZ platform is the possibility to analyse dose-responses at any time during the experiment.

Advanced information content is obtained by using multi-frequency impedance readouts, which is possible with the AtlaZ Control software. The methodology, Electrical Impedance Spectroscopy (EIS), allows to gain a unique richness of information from your cells: depending on the data acquisition frequency, the system detects predominantly the resistive part of cell-cell contacts at lower frequencies and predominantly capacitive currents across the cell membranes at higher frequencies. The signal is dominated by the paracellular cell layer resistance between approx. 1 – 10 kHz and is increasingly influenced by capacitive currents across the cell membranes between 10 – 100 kHz. This renders impedance readouts at different frequencies (here a spectrum of 0.1 kHz – 100 kHz) capable to further dissect physiological responses, zooming into changes in membrane topography, G-protein-coupled receptor (GPCRs) signaling and cell-cell or cell-matrix junctions.

Cell Monitoring assay

Here we used the H9C2 cell line which has been differentiated towards a cardiac phenotype. The readout displayed as the Cell Signal represents raw impedance values. For these specific recordings we have chosen the "Cell Monitoring" mode (10 kHz recording frequency), one of the 3 recording modes available, which is best suited for these type of toxicity investigations. When certain optional corrections are needed, i.e. a correction for background values such as medium control normalization, Cell "indexed" value can be calculated and displayed.

Key findings

1. Our results demonstrate the capability of AtlaZ to analyze cardiotoxic compounds when using H9C2 cardiac-like cells.
2. AtlaZ allows for cellular research on cell adhesion, proliferation and cytotoxicity, label-free and in real-time.
3. Recordings can be performed in up to six 96-well plates simultaneously or independently.

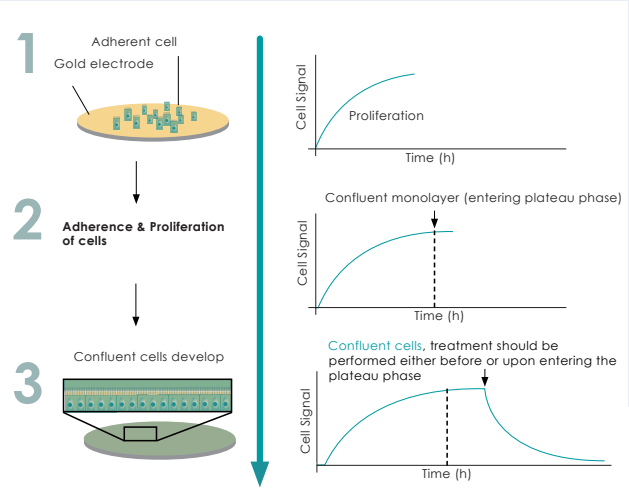


Figure 4: *Workflow of a cell monitoring assay analyzing toxicity of applied treatments (here: potential cardiotoxic compounds). Target cells are seeded in AtlaZ sensor plates, adhere and proliferate (1), upon reaching a plateau phase (2) the treatment can be executed (3). The integrity of target cells is measured and displayed as Cell Signal over time.*

References

1. Onódi Z et al., . Systematic transcriptomic and phenotypic characterization of human and murine cardiac myocyte cell lines and primary cardiomyocytes reveals serious limitations and low resemblances to adult cardiac phenotype. Journal of Molecular and Cellular Cardiology 165 (2022) 19–30, https://doi.org/10.1016/j.yjmcc.2021.12.007.
2. B.W. Kimes, B.L. Brandt, Properties of a clonal muscle cell line from rat heart, Exp. Cell Res. 98 (2) (1976) 367–381, https://doi.org/10.1016/0014-4827(76)90447-x.
3. W.C. Claycomb, et al., HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte, Proc. Natl. Acad. Sci. U. S. A. 95 (6) (1998) 2979–2984, https://doi.org/10.1073/pnas.95.6.2979.
4. M.M. Davidson, et al., Novel cell lines derived from adult human ventricular cardiomyocytes, J. Mol. Cell. Cardiol. 39 (1) (2005) 133–147, https://doi.org/ 10.1016/j.yjmcc.2005.03.003.
5. Di Gregorio F, Favaro G, Fiori MG. Functional evaluation of acute vincristine toxicity in rat skeletal muscle. Muscle Nerve. 1989 Dec;12(12):1017-23. doi: 10.1002/mus.880121210. PMID: 2560143.
6. Yanagida S, Satsuka A, Hayashi S, Ono A, Kanda Y. Chronic cardiotoxicity assessment of BMS-986094, a guanosine nucleotide analogue, using human iPS cell-derived cardiomyocytes. J Toxicol Sci. 2021;46(8):359-369. doi: 10.2131/jts.46.359. PMID: 34334557
7. Wu BB, Leung KT, Poon EN. Mitochondrial-Targeted Therapy for Doxorubicin-Induced Cardiotoxicity. Int J Mol Sci. 2022 Feb 9;23(3):1912. doi: 10.3390/ijms23031912. PMID: 35163838; PMCID: PMC8837080. Wu BB, Leung KT, Poon EN. Mitochondrial-Targeted Therapy for Doxorubicin-Induced Cardiotoxicity. Int J Mol Sci. 2022 Feb 9;23(3):1912. doi: 10.3390/ijms23031912. PMID: 35163838; PMCID: PMC8837080.

Nanion Technologies GmbH I Ganghoferstraße 70a, 80339 Munich, Germany
+49 89 2190 95-0 I info@nanion.de I www.nanion.de