From: **Johannes Delp** johannes.delp@uni-konstanz.de
Subject: AW: AW: eu-toxrisk: CS4 data on neurons
Date: 25 February 2019 at 21:54
To: fredomatic fredomatic@free.fr
Cc: Marcel Leist marcel.leist@uni-konstanz.de, Wang GAO (UTC Pers) wang.gao@utc.fr

Dear Frederic,

it would be nice if we could collaborate on this qAOP! Rotenone and deguelin are for sure a good starting point.

Here is the list of KE assays that we consider to be most relevant to start with:
KE1 (cI inhibition): Measurements of cI activity in permeabilized cells, so that the mitochondria are directly accessible.
KE2 (mito dysfunction): overall "basal" mitochondrial respiration of intact cells, acutely treated with rot/deg.
KE3 (proteostasis): activity of the proteasome (assessed by proteasomal modification of a fluorescent proteasome substrate).
KE4 (neuronal degeneration): neurite outgrowth assay (decrease in neurite outgrowth assessed in parallel to blunt cytotoxicity).
KE5 (neuro-inflammation): assay still in development.

I've attached you some protocols (for KE1-3 assays) and an accepted DB-ALM SOP for the KE4 assay. However, I think a TC might be more helpful than reading through all these paper works to understand how the data is generated and what it means. If you agree, lets schedule a short TC or lets meet at the SOT, if you're going there.

Concerning the intracellular concentrations: we have the simulations form Simcyp, which I've also attached (Excel file). "Real" measurements confirming/challenging these values are planned, but not performed yet.

Best
Johannes

-----Ursprüngliche Nachricht-----
Von: fredomatic [mailto:fredomatic@free.fr]
Gesendet: Freitag, 22. Februar 2019 11:45
An: Johannes Delp <johannes.delp@uni-konstanz.de>
Cc: Marcel Leist <marcel.leist@uni-konstanz.de>; 'Wang GAO (UTC Pers)' <wang.gao@utc.fr>
Betreff: Re: AW: eu-toxrisk: CS4 data on neurons

Dear Johannes,

We are aiming to quantify the AOP ETR07N (Inhibition of mitochondrial complex I of nigrostriatal neurons leads to Parkinsonian motor deficits), using Bayesian networks within task 10.2 of WP10.

Marcel just sent us some general indications of what was done experimentally (which sounds very extensive). I think we should for now focus only on deguelin and rotenone. Do you agree that this is a good choice? It seems that you have rather complete data on both.

Next is the fact that you have several measurements for most KEs. They should go in the same direction, but they may have slightly different mechanisms. May be we should start by just looking at the "most relevant" measurement data. Can you tell us what the most relevant assay is, in your opinion, for each KE? And for those, can you tell us what the protocols were (at least roughly) or where we can find them? We need to understand how the data were generated to model them correctly.

Also: do you have cellular concentration data or estimates for rotenone and deguelin, or just nominal concentrations in medium?

Thanks for your help.

Frederic

On 22/02/2019 09:23, Marcel Leist wrote:

> Dear Fred,
> thanks for following up on this. It would be great to start a collaboration on this.
>
> I think the BioStudies are not very helpful, if you want to obtain an
> idea on data structure I give a rough overview, so you can judge what
> couldbe interesting and to define more detailed questions to Johannes
>
> We have (pr can retrieve) data on:
>
> MIE (binding data simmulatons by G. Ecker)
>
> KE1: Mito inhibition
> ATP, oxygen consumption rate, concentration dependent for at least 7
> C-I inhibitors
>
> KE2: Mito dysfunction
> (overlaps with KE1. On this level, we have some mechanistic data, like
> glutathione levels (ox stress indicator), gene regulation data (ox

stress and other stress responses) and some metabolomics data on 2 inhibitors (about 300 metabolites)

We also have the mito-inhibition data for cells grown in glucose (mainly using glycolysis) and cells grown in galactose (mainly using mitochondria), to better define the dependence on mitochondrial function at this step

KE3: proteostasis
We have two data sets on proteasome inhibition and on altered glycoprotein synthesis and sorting. We also have some data on mitochondrial transport along axons

KE4: Degeneration
Neuro/specific neurie degeneration data - concentration -dependent for several inhibitors (in glucose and galactose)

KE5: No data yet, but we look into this in a co-culture model.

The presentation attached gives some overview on the AOP...
best
marcel

Marcel Leist
University of Konstanz
Tel: +49-7531885037
marcel.leist@uni-konstanz.de

-----Ursprüngliche Nachricht-----
Von: fredomatic [mailto:fredomatic@free.fr]
Gesendet: Donnerstag, 21. Februar 2019 16:37
An: marcel.leist@uni-konstanz.de
Cc: Wang GAO (UTC Pers) <wang.gao@utc.fr>
Betreff: eu-toxrisk: CS4 data on neurons

Dear Marcel,

We talked briefly in Egmond ann Zee about Wang Gao analyzing your CS4 data on neurons. They seem to be a perfect example of data suitable for AOP quantification.
Probably, the best to start with would be getting an idea of the data collected, the exact protocols and the data structure: on which KEs (of AOP ETR07N "Inhibition of mitochondrial complex I of nigrostriatal neurons leads to Parkinsonian motor deficits", tell me if I'm wrong)? which chemicals, etc. There is probably a summary document, but a data sheet for one chemical would be good. You will probably tell me that they are all on Biostudies, which would be fine, but just in case...

Best regards.

Frederic


CS4_Simpcyp_IV_predic...96.xlsx


KE4_DB-ALMPr...8.docx


KE1_20180913_JD_Indiv.....docx


KE2_20180913_JD_Mit...ol.docx


KE3_20180918_JD_Pro...ol.docx

3.3 Inhibition of mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (ETR07N)

Rotenone is associated with neuronal dysfunction and Parkinson-like disease in farm workers and laboratory animals. In addition, rotenone is a known inhibitor of mitochondrial complex I. Based on these finding, an AOP has been proposed by UKN, namely "Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficit". This AOP assumes that the Parkinsonian motor deficits are directly related to MRC complex 1 inhibition followed by formation of reactive oxygen species leading to impaired proteostasis and thereafter neuronal cell injury (schema below):

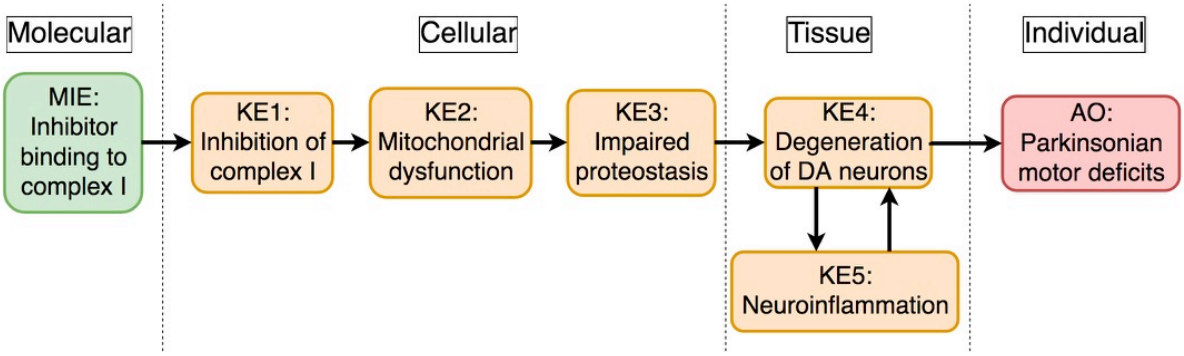

Figure 5: AOP Mitochondrial Inhibition of mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits.

We have seen previously that the inhibition of complex I leads to mitochondrial dysfunction. Then, the correlation between mitochondrial dysfunction and impaired proteostasis is based on the considerations that (*i*) proteostasis is an energy-consuming process requiring ATP from mitochondria, and that (*ii*) components of the proteasomal system are subject to inhibition by ROS, generated from dysfunctional mitochondria (Finley, 2009).
Proteostasis describes a coordinated balance between the synthesis, modification, transport, and degradation of proteins in a cell. Disturbances in proteostasis can lead to a loss of the genuine function of a protein or to the gain of undesired properties. Two major controllers of proteostasis are protein degradation and cellular transport mechanisms. The two major degradation systems in a cell are the ubiquitin proteasomal system (UPS) and autophagy lysosomal pathway (ALP). Disturbed protein degradation or trafficking often leads to inappropriate protein accumulation in cells, ranging from protein aggregates e.g. alpha synuclein (ASYN) to whole mitochondria.

ASYN protofibrils interact with intracellular organelles such as neurotransmitter vesicles or mitochondria, and lead to an uncontrolled release of DA and an impairment of mitochondrial function (Chinta, 2010). DA-modified ASYN not only blocks its own degradation by the chaperone-mediated autophagy (CMA) pathway, but also prevents the degradation of other proteins. Aggregates of wild-type or mutant forms of ASYN disturb controlled axonal transport of mitochondria. In neurons, key steps, such as mitochondrial fission/fusion or mitophagy, are conducted in the cell body. Impaired axonal transport of mitochondria hence leads to limited ATP supply and elevated levels of ROS, generated by dysfunctional mitochondria.

Neurons of the substantia nigra project into the striatum to release dopamine. In the striatum, DA has an excitatory (D1 receptors) and inhibitory (D2 receptors) influence on GABAergic striatal interneurons; DA augments (by both pathways) the thalamic output to the motor cortex. A decline of striatal DA therefore leads to a decreased thamalic input to the cortex from the basal ganglia motor control loop. In idiopathic and genetic forms of Parkinson's disease, or

after exposure to toxicants such as MPTP, a preferential degeneration of nigrostriatal DA neurons is observed. Also, neuroinflammation describes the activation of microglia and astrocytes, manifested by a shape change, induction of pro-inflammatory enzymes and cytokines, and a migration towards the site of damage. In response to pathogens or to damaged neurons, microglia are initially activated and subsequently they promote the reaction of astrocytes. Reactive glial cells represent rich sources of nitric oxide ($^{\bullet}NO$), superoxide ($^{\bullet}O_2^-$), and cytokines, thus possibly contributing to the damage of adjacent neurons. Chronic neurodegenerative diseases such as Parkinson's disease are characterized by a persistent inflammatory activation of glial cells
Analysis of nigrostriatal tissue of patients with parkinson's disease (PD) has suggested an impairment in the activity of the 20/26S proteasome (McNaught, 2001). Similar observations were made in fibroblasts obtained from patients with PD, which exhibited elevated basal levels of ubiquitinated proteins and impaired 20S proteasomal activity (Ambrosi, 2014). The brain-region selective impairment of proteasomal activity correlates with the selective demise of DA neurons in this region. Disturbances in the ubiquitin proteasomal system are also directly associated with prominent examples of mutations (e.g., parkin, ubiquitin C-terminal hydrolase L1) identified in genetic PD cases. Both are sufficient to cause preferential degeneration of nigrostriatal DA neurons.
More information about this AOP can be found on the OECDiLibrary (https://www.oecd-ilibrary.org/environment/adverse-outcome-pathway-on-inhibition-of-the-mitochondrial-complex-i-of-nigro-striatal-neurons-leading-to-parkinsonian-motor-deficits_b46c3c00-en.)

To complement this AOP, integrating approaches to testing and assessment (IATA) was developed to support a KEs-based testing strategy. Compounds that are produced by the agrochemical industry as pesticides, insecticides or fungicides were selected. The hypothesis is that all MRC pesticides have a liability to cause Parkinsonian motor deficits and that this effect is due to the increased sensitivity of neuronal cells to mitochondrial complex (MRC) inhibitors compared to other target organ cells. Rotenone was used as the source compound. One structural analogue that is commercially available was also considered. In addition, a set of complex II inhibitors were selected (some of these inhibitors being structurally similar). Finally, a group of complex III inhibitors were selected; here a large class was structurally similar and belonging to the class of strobilurins; several non- related complex 3 inhibitors were selected (Table 1).

For most compounds in vivo data is available, but no neuronal toxicity is defined. Primarily rotenone has been used as an in vivo model compound to induced Parkinson-like phenotypes in laboratory animals. A risk assessment based on the available in vivo data for rotenone was performed, since all other compounds do not have the appropriate in vivo data. The results of 23 test representing different human organs exposed to Rotenone is depicted in Figure 6A. The toxicity of the different target chemicals will be predicted based on the NAM data that will be generated.

For 14 compounds (table 1) a gene expression analysis on neuroblastoma cell line (SH-SY5Y) and Immortalized Human Dopaminergic Neuronal Precursor Cells (LHUMES) have been performed at different concentrations and 24h using the TempO-seq technology.

Complex I	Complex II	Complex III
Deguelin	Carboxine	Antimycin
Rotenone	Thifluzamide	Pyraclostrobin
Pyrimidifen	Mepronil	Azoxystrobin
Tebufenpyrad		Picoxystrobin
Fenpyroximate		Cyazofamid

Capsaicin		

Table 1: 14 compounds known to inhibit MRC

To perform this experiment, human dopaminergic neurons (LUHMES cells) were differentiated for two days in a UKN4 test, before they were plated at a density of 100,000 cells/cm² (ca. 30,000 cells/well) into 96-well plates, treated one hour after re-plating with the indicated mitochondrial complex I inhibitors and analyzed after 24 h of compound treatment at day 3 of differentiation. To evaluate the degeneration of neurons by these compounds, neurone outgrowth and viability were determined by high content imaging and concentration-response curves are given for three independent experiments (Figure 6B).
Although the outcomes of TempO-Seq have not been analyzed yet, this analysis will indicate the concentration that lead to deregulation of MRC and might suggest alternative key events.
The protease activity of these compounds is also planned to assess how these compounds disturb the balance between the synthesis, modification, transport, and degradation of proteins in a cell.

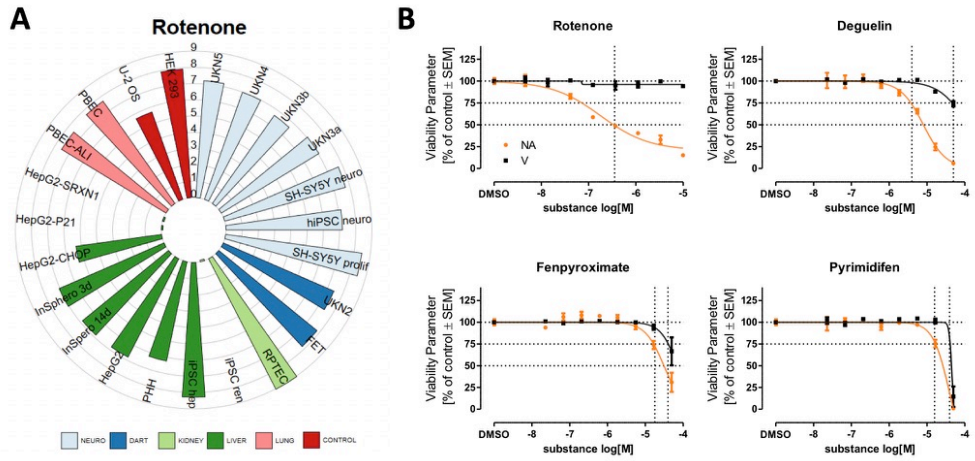

Figure 6: Effect of mitochondrial complex I inhibitors in various tests. **(A)** 23 tests representing different human organs (represented by color code) were treated with the mitochondrial complex I inhibitor rotenone. The concentration of the BMC15 of the most sensitive endpoint of each test is depicted in –log[M]. If the BMC15 was not met within the applied concentration range, the bar was set to -0.1. **(B)** Human dopaminergic neurons (LUHMES cells) were differentiated for two days, before they were plated at a density of 100,000 cells/cm² (ca. 30,000 cells/well) into 96-well plates, treated one hour after re-plating with the indicated mitochondrial complex I inhibitors and analyzed after 24 h of compound treatment at day 3 of differentiation. Neurite area (NA, orange) and viability (V, black) were determined by high content imaging. Concentration-response curves are given for three independent experiments.

3.4 Peripheral neuropathy caused by microtubule interacting drugs (ETR09N)

Peripheral neuropathy is regularly observed as a side-effect in microtubule-targeted chemotherapies. The peripheral nervous system (PNS) connects the central nervous system with peripheral tissues and can be divided into the visceral and the somatic nervous system. The somatic nervous system consists of sensory and motor neurons. While the motor neurons control the contraction of skeletal muscles, the sensory neurons receive information from joints, muscles and skin and send it to the CNS. Motor neuron cell bodies lie in the spinal cord but cell bodies of the sensory neurons are located in the dorsal root ganglia (DRG). In contrast to the

Assessment of individual mitochondrial respiratory chain complex activity with permeabilized LUHMES cells

Introduction

Aim

This protocol describes the analysis of how to assess the activity of the mitochondrial respiratory chain complex I-IV activities by using selectively permeabilized LUHMES cells (human neuronal).

Purpose

Many toxic substances inhibit mitochondrial function. Also many compounds in research for pharmaceutical use inherit mitochondrial off-target effects. This assays aims to identify direct inhibition of the MRC complexes I-IV.

Limitations

Since the analysis of each MRC complex activity is based on the oxygen consumption at cIV, sometimes only indirect conclusions can be drawn. However control experiments can confirm the suggested results. Additionally, adverse effects on cytochrome c or ubiquinone are not assessed.

Method outline

Cells get selectively, i.e. only their plasma membrane, permeabilized before the experiment starts. Then, their MRC complexes are sequentially fed with specific substrates, i.e. first cI, then cII, then cIII, finally cIV. At the same time, inhibitors for upstream complexes are added with the fuel substance for the next downstream complex to enable the analysis. Finally, complex activity is analyzed simultaneously in treated samples relative to solvent control samples, based on the cIV-mediated oxygen consumption using the Seahorse device.

Method description

Chemicals and buffers

Table 1: Chemicals used in experiments

Product	Supplier	Cat. No.	Lot. No.	Cas. No.
ADP	Sigma	A5285	-	72696-48-1
Digitonin	Sigma	D141	0001432565	11024-24-1
D-Mannitol	Sigma	M4125	007K0166	69-65-8
DMSO	Merck	1.09678	K48040378 727	67-68-5
Duroquinol	TCI	T0822	5QCSN	527-18-4
EGTA	Sigma	E0396	SLBP2807V	67-42-5
Fatty-Acid free BSA	Roth	8076.5	197254757	90604-29-8
HEPES	Roth	HN77.4	468101696	-
KH_2PO_4	Ridel-deHaen	30407	51530	7778-77-0
L-Ascorbic Acid	Sigma	A-4544	21H01855	50-81-7
L-Glutamine	Sigma	G3126	046K0009	56-85-9
Malic acid	Sigma	M6413	BCBJ3883V	97-67-6
Malonic acid	Sigma	M1296	BCBV3859	141-82-2
$MgCl_2$-Hexahydrate	Roth	2189.1	387253790	7791-18-6
Potassium Hydroxide	Sigma	30603	SZBE1180V	1310-58-3
Pyruvic acid	Sigma	107360	MKCC8726	127-17-3
Succinic acid	Sigma	S9512	057K01281	110-15-6
Sucrose	Merck	1076511000	K47396351613	57-50-1
TMPD	Sigma	T7394	STBC1186V	100-22-1
XF calibrant solution	Agilent/Seahorse	100840-000	090	-

Table 2: reagent preparation

Reagent	Stock	Final (on cells)	10 x port solution	Solvent
ADP	100 mM	1 mM	10 mM	MAS
Ascorbate	200 mM	2.0 mM	20 mM	MAS
Digitonin	25 mg/ml	25 µg/µl	250 µg/ml	MAS
Duroquinol	500 mM	0.250 mM	2.5 mM	DMSO
Glutamate	200 mM	2 mM	20 mM	MAS
Glutamine	200 mM	2 mM	20 mM	Cell culture in H2O
Hydroquinone	500 mM	5 mM	50 mM	Water
Idebenone	250 mM	0.25 mM	2.5 mM	DMSO
Malate	250 mM	2.5 mM	25 mM	MAS
Malonate	250 mM	5 mM	50 mM	MAS

Pyruvate	500 mM	5 mM	50 mM	MAS
Succinate	250 mM	10 mM	100 mM	MAS
TMPD	500 mM	0.125 mM	1.25 mM	Ethanol

Rotenone & antimycin used a as in mitostress test (0.5 µM each)

Table 3: Formulation of 1x MAS buffer

Reagent	1x MAS	Amount for 1.0 liter of 1x MAS
Sucrose	70 mM	23.96 g
Mannitol	220 mM	40.08 g
KH_2PO_4	10 mM	1.36 g
$MgCl_2$ hexahydrate	5 mM	1.02 g (for hexahydrate; for pure MgCl2: 0.48 g)]
HEPES	2 mM	0.48 g
EGTA	1 mM	0.38 g
Fatty acid free BSA	4 mg/ml	4 g

Reagents were dissolved in MiliQ-water and pH was adjusted to 7.2 by using KOH. After 0.22 µm filter sterilization, MAS buffer was stored at 4°C.

Preparation upfront

- Hydrate seahorse cartridge
- Have cells at 95% confluency in coated (normal PLO/fibronectin LUHMES coating, optionally with additional laminin) Seahorse plates ready, cultured at least 18 h in these plates for equilibration
- 1x MAS buffer
- Substrates and tool compounds

Experimental procedure

1. Cells were grown in T175 cell culture flasks until they reached a confluency of 95%
2. For assays, conducted one day after cell seeding in Seahorse cell culture plates, proliferating cells were seeded at a density of 60.000 cpw in 100 µl proliferation medium (PM). For assays, conducted two days after cell seeding in Seahorse cell culture plates, proliferating cells were seeded at a density of 40.000 cpw in 100 µl PM.
3. After one hour of attachment, 150 µl of PM was added to proliferating cells.
4. The Seahorse cell culture plates were incubated at 37°C in a humidified 5%-CO_2 incubator until the day of assay.
5. At the day of assay, the reagents for the respective assay were prepared. Therefore 1x-MAS buffer was warmed to 37°C in the water bath.
6. The prepared stock solutions of mitochondrial effectors and oxidizable substrates had to be thawed if they were frozen.
7. MAS-buffer is used to prepare the 10x port solutions, which are loaded into the ports of the cartridges. The desired final concentrations for oxidizable substrates and mitochondrial inhibitors are listed in above tables.
8. After preparing the desired concentrated port solutions, they are loaded into the appropriate ports of the cartridge, by adding 56 µl/62 µl/69 µl/75 µl into ports A/B/C/D, respectively.
9. Until the start of the assay, the cartridge was incubated at 37°C without CO2.

10. Create an XF assay template with Mix/Wait/Measure times of 2 min/1 min/2 min, respectively. The equilibration step is excluded from the assay template. Each step is repeated two cycles.
11. After the calibration step of the XF assay template is finished, the cell culture plate is removed from the incubator.
12. The medium was replaced with pre-warmed 1x MAS-buffer (37°C) supplemented with digitonin and ADP. Therefore, the medium was carefully aspirated, and 500 µl of MAS-buffer was added to each well of the cell culture plate.
    a. MAS 12.857 ml + digitonin 13 ul (25 µg/ml on cells) of stock and ADP 130 µl of stock (1 mM on cells)
13. The utility plate was removed from the analyzer and was discarded, while the cartridge remained in the instrument.
14. Finally, the cell culture plate was inserted into the XF analyzer and the assay was started.

Data analysis

- Each experiment has to contain control treated (solvent) samples
- Oxygen levels have to be checked before deeper data analysis (mmO2, not OCR!)
- If oxygen doesn't get depleted, the data can be analyzed.
- First, basal respiration of all wells gets normalized to the last measurement value before compound injection via port A
- Then the oxygen consumption rate of the first measurement cycle after each tool substrate/tool inhibitor mix injection undergoes comparison. Inhibition results in OCR values smaller in treated samples than in control samples, uncoupling vice versa.
- Compound effects are expressed as percent inhibition relative to control
- Over several experiments (4 weeks, 30 compounds, 4 complexes assessed), the SD of the measurement was 12.5%. Thus compound effects of >25% (=2xSD) were considered as "biologically significant"

Mitochondrial stress test using LUHMES cells

Introduction

Aim

This protocol describes the analysis of how to assess the utilized and spare mitochondrial activity of LUHMES cells (human neurønal).

Purpose

Many toxic substances impair mitochondrial function. Also many compounds in research for pharmaceutical use inherit mitochondrial off-target effects. This assays aims to identify direct or indirect impairment of mitochondrial function.

Limitations

This assay asses only the mitochondrial and non-mitochondrial oxygen consumption, but gives not directly an explanation why these parameters might be impaired. E.g. mitochondrial pyruvate uptake inhibitors might result in the same reduction in oxygen consumption as complex I inhibitors. Additionally, redox cyclers might result in the same increased oxygen consumption as mitochondrial uncouplers. Therefore follow-up experiments might be needed.

Method outline

Cells are cultured in Seahorse assay plates (100,000 cells per well in 24 well plates) and allowed to equilibrate to their environment. At the day of the assay, their normal cell culture medium gets replaced by Seahorse assay medium, supplemented with pyruvate (1 mM), glucose (18 mM), glutamine (2 mM), N2 supplement (1x) and tetracycline (2.25 µM) 1 h prior to the assay.

Then the Agilent Seahorse Mitostress test is performed according to the manufacturer's recommendation. Port A is used for the injection of the compound of interest or the solvent control, while ports B-D are used for oligomycin, FCCP and rotenone/antimycin a, respectively.

Finally, mitochondrial oxygen consumption is analyzed simultaneously in treated samples relative to solvent control samples, based on the oxygen consumption using the Seahorse device, as the manufacturer recommends.

Method description

Chemicals and buffers

Agilent Seahorse Mitostress test:

Oligomycin: final concentration on cells is 1 µM

FCCP: final concentration on cells is 1.5 µM

Rotenone/antimycin A: final concentration on cells is 0.5/0.5 µM

Agilent Seahorse basal DMEM

Glucose, pyruvate, glutamine, N2 supplement and tetracycline

Preparation upfront

- Hydrate seahorse cartridge
- Have cells at in coated (normal PLO/fibronectin LUHMES coating, optionally with additional laminin) Seahorse plates ready, cultured at least 18 h in these plates for equilibration
- Substrates and tool compounds

Experimental procedure

1. Change the normal cell culture medium to the assay medium, place the plate in a 37°C non-CO2 incubator for at least 1 h before the start of the experiment
2. Prepare the cartridge with the compounds solutions (10x solution in ports A/B/C/D, 56 µl/62 µl/69 µl/77 µl, respectively), incubate in a 37°C non-CO2 incubator for at least 1 h before the start of the experiment
3. Set up the Seahorse analyzer and the measurement program, use mix/wait/measure times of 3/2/3 minutes, respectively. Enable “calibrate” and “equilibrate”
4. Calibrate the cartridge and when the instrument is ready, the utility plate is removed from the analyzer and, while the cartridge remained in the instrument.
5. Finally, the cell culture plate was inserted into the XF analyzer and the assay was started.

Data analysis

- Each experiment has to contain control treated (solvent) samples
- Oxygen levels have to be checked before deeper data analysis (mmO2, not OCR!)
- If oxygen doesn’t get depleted, the data can be analyzed.
- First, basal respiration of all wells gets normalized to the last measurement value before compound injection via port A
- Then the oxygen consumption rate of the first measurement cycle after each tool inhibitor injection undergoes comparison. Inhibition results in OCR values smaller in treated samples than in control samples, uncoupling vice versa.
- Compound effects are expressed as percent inhibition relative to control
- The manufacture’s Excel sheets were used for data analysis.

Assessment of proteasome activity in live cells

Introduction

Aim

This protocol describes the analysis of how to assess the proteasome activity of live LUHMES cells (human neuronal).

Purpose

Proteasomal activity is an essential process of cellular homeostasis. It can be directly inhibited, e.g. by proteasomal inhibitors, or decreased as a consequence of e.g. energy depletion, since the proteasome is ATP-dependent.

Limitations

The proteasome has three major catalytic activities: the β1 subunit has a caspase-like activity (i.e. cleaves after acidic amino acids), the β2 subunit has a trypsin-like activity (i.e. cleaves after basic amino acids) and the β5 subunit has a chymotrypsin-like activity (i.e. cleaves after hydrophobic amino acids). The used substrate (MeOSuc-Gly-Leu-Phe-AMC) is thus most likely mainly cleaved by the β5 subunit, while the activity of the other subunits might not be assessed to a similar extent. The positive control (e.g. MG-132) is an inhibitor which impairs all catalytic subunits.

Additionally, for proper cellular proteasomal protein degradation, also the ubiquitin system has to work functionally, which is not assessed by this assay.

During the assay (while the proteasomal substrate is incubated), no treatment substances are on the cells, thus treatment substances might be diluted out of the cells during assessment of proteasomal activity.

On top, other intracellular proteases could cleave the substrate. This background/ non-blockable cleavage should be assessed by controls, e.g. broad-spectrum proteasome inhibitors.

Method outline

Cells are cultured normally according to established lab protocols in 96 well plates. Around the time of endpoint assessment, the medium is removed from the wells and the assay mix with the proteasomal substrate is added to replace the medium. The proteasomal substrate is cell-permeable and diffuses into the proteasome, as well as the cleavage products diffuse out of the proteasome.

The proteasome activity dye consists of a fluorophore which is linked to a small peptide chain which quenches the fluorescence activity. Upon proteasomal cleavage, the fluorescence increases. Thus the increase in fluorescence activity between directly after addition of the substrate and 2 h later is correlated with the proteasomal activity.

Method description

Chemicals and buffers

- Proteasome substrate: MeOSuc-Gly-Leu-Phe-AMC from Bachem (I1430).
  Prepare 10 mM Stock in DMSO, store at -20°C.
- HBSS buffer
- Cells in 96 well plates (otherwise adapt the volumes)
- Fluorescence plate reader

Preparation upfront

- Prepare working mix of proteasome substrate: dilute 1:400 in HBSS, final concentration 25 µM

Experimental procedure

- Remove medium from the wells (2 h before the endpoint should be determined)
- Add 100 µl/well working mix (25 µM proteasome substrate in HBSS)
    - Measure fluorescence directly after addition (=baseline measurement; ex. 360 nm, em: 465, fixed gain)
- Incubate the cells in their standard incubation conditions, thereby wait for 1-3 h, depending on the cellular activity
    - Measure fluorescence again (=end measurement; ex. 360 nm, em: 465, fixed gain – same as for baseline)
    - Time course measurements can be performed to find the best waiting time
- Use assay positive controls: cells treated with a sufficient concentration of a proteasome inhibitor, e.g. MG-132 (alternatively: lactacystin, bortezomib, epoxomicin or a combination of them)

Data analysis

- Subtract from each value of the end measurement its baseline value
- Calculate fluorescence intensity relative to control
    - Positive controls can be used to determine the amount of not proteasome inhibitor blockable substrate cleavage

DB-ALM Protocol

Template for Data Content

Last Update: September 2013 (v.9.4)

EURL ECVAM DATABASE SERVICE ON ALTERNATIVE METHODS TO ANIMAL EXPERIMENTATION

http://ecvam-dbalm.jrc.ec.europa.eu

The present template is based on the Content Criteria for Protocols, which are designed for the provision of technical details that enable the documented alternative method to be transferred to other laboratories without the need of additional information.

The Content Criteria, on which the presented template is based, are generic and based on the analysis of common descriptors from hundreds of different non-animal experimental methods and techniques. However, not all parameters or sections indicated are applicable to all protocols. It is the responsibility of the author to provide content where relevant and as appropriate related to a precise protocol. The compilation is to be performed by completing the individual sections with the information related to your method. A review for consistency, completeness in relation to the technique described and compliance with the Content Criteria in place is always performed by the JRC staff. Furthermore, before any protocol is published *via* the DB-ALM, the final draft is reviewed and approved by designated contact person(s) (method's owner and/or experienced user).

A summary description of the main method features bringing it in a context regarding its intended purpose(s) and application(s), as well as the scientific rationale is always to be provided with each protocol. The content criteria for the method summary will be sent in due course.

The DB-ALM is operated by the European Reference Laboratory for Alternatives to Animal Testing of the Joint Research Centre.

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NOTE: All statements shall be supported with references within the text and full details listed under "**Bibliographic References***"* data sector.

Part A. Protocol Introduction

Protocol Name: UKN4 assay to test compound-derived impairment in neurite outgrowth in human mature dopaminergic neurons.

Abstract: This protocol describes the UKN4 assay to assess cell viability and neurite outgrowth after 24h of compound treatment. It includes information about the differentiation of human dopaminergic neurons.

Résumé

This assay is based on high-content automated microscopy and images dopaminergic neurons during differentiation (day 3 of differentiation). It assesses not only cell viability (by co-staining with Hoechst and calcein, the latter one being taken up only by viable cells), but also neurite outgrowth. Neurite outgrowth has been shown to be a more sensitive readout than cell viability, enabling to identify substances that specifically impaired neurite area while not affecting cell viability.

Experimental Description

Biological Endpoint and Endpoint Measurement:

Neurite outgrowth is an essential feature for a developing brain to acquire unimpaired function. Impaired development of dopaminergic neurons can be triggered either genetically or by exposure to toxicants and chemicals.
The current protocol is used as an *in vitro* tool to detect compounds that impair neurite outgrowth of developing and differentiating dopaminergic neurons.
The test captures cell viability and neurite area as neuron-specific functional readout after short term exposure to toxicants (24 h).

Endpoint Value:

Endpoint values are inhibitory concentrations (IC) and benchmark concentrations (BMC) derived from concentration-response curves of both readouts (viability and neurite area) and the ratio of these.

Experimental System:

The human LUnd Human MESencephalic cell line (LUHMES (ATCC® CRL-2927™) is used to generate morphologically and biochemically mature dopamine-like neurons. The differentiating and developing stage of these neurons are then used in the UKN4 assay.

Discussion

In the UKN4 assay developing human dopaminergic neurons are used. No specific ethical approval is required. Duration of the assay is minimum 3 days due to cell differentiation (2 days) plus treatment period.

Frozen cell batches (each thawed cell batch/vial can be passaged 12-15 times) are not varying much (expression of characteristic genes during differentiation are checked after generating new baches: βIII-tubulin, TH, DAT, DRD2, EN1 and NURR1). The results of one experiment should be normalized with the untreated controls within the same experiment.

The performance of the UKN4 assay requires an automated array microscope (e.g. Cellomics ArrayScanVTI, Thermo Fischer) equipped with a 20x lense. The operator should be trained in good cell culture and good laboratory practice and microscope handling. Otherwise no special handling is required. Operators can get trained within 2-4 weeks. Cell

seeding and medium change should be performed as fast as possible to keep cells as short as possible at room temperature. The more practice an operator has, the faster the critical steps can be performed.

To prevent negative edge effects, only the inner 60 wells of a 96-well plate are used and the edge wells should be filled with PBS.

The UKN4 assay is a medium throughput assay. With three technical replicates the results of the assay are reproducible and robust within the lab and trained operators (standard deviation of viability of technical replicates of DMSO controls ~5% of average, standard deviation of neurite area of technical replicates of DMSO controls ~10% of average). Positive hits are meaningful. The 'negative hits' provide little information and require other tests in a developmental toxicity test battery. The assay can be combined with other viability assays like ATP quantification or resazurin reduction.

Possible problems: replating the cells at a too high density can then cause problems with cell density later within the assay. Improper coating can result in problems with cell attachment and cell clumping. Also problems with the microscope can affect the efficient performance of the assay, e.g. when autofocus is not working properly.

Status

In Development:

Method is fully developed and established.

Known Laboratory Use:

Test system (UKN LUHMES cells) has been transferred and established to numerous other labs. The assay itself has been used only at the University of Konstanz by one operator and hasn't been transferred or applied in other labs.
Used in EU-ToxRisk (H2020 EU-funded project no No 681002).

Participation in Evaluation Study:

Used in EU-ToxRisk project, used in NTP 80 compound library screen.

"Assessment of chemical-induced impairment of human neurite outgrowth by multiparametric live cell imaging in high-density cultures."
Stiegler NV, Krug AK, Matt F, Leist M.
Toxicol Sci. 2011 May;121(1):73-87. doi: 10.1093/toxsci/kfr034. PMID: 21342877

"Evaluation of a human neurite growth assay as specific screen for developmental neurotoxicants."
Krug AK, Balmer NV, Matt F, Schönenberger F, Merhof D, Leist M.
Arch Toxicol. 2013 Dec;87(12):2215-31. doi: 10.1007/s00204-013-1072-y. PMID: 23670202

Participation in Validation Study:

No participation in a validation study.

Regulatory Accepted:

Not submitted to regulatory acceptance.

Proprietary and/or Confidentiality Issues

No proprietary or confidentiality issues known.

Health and Safety Issues

General precautions:

No safety and health issues known to be related to the method. Risk and Safety Statements of toxicants, compounds and staining dyes should be considered and followed.

MSDS Information:

The positive control narciclasine may cause genetic defects.
https://www.caymanchem.com/msdss/20361m.pdf
In addition to the safety measures regarding the compounds in use, there are no safety measures needed for the performance of this method.

Abbreviations and Definitions

BMC:	benchmark concentration
DMEM:	Dulbecco's Modified Eagle Medium
LUHMES:	LUnd Human MESencephalic cell line
N-2:	medium supplement by Thermo Fisher Scientific, chemically defined serum-free supplement based on Bottenstein's N-1-formula.
cAMP:	cyclic Adenosine MonoPhosphate
GDNF:	Glial cell-Derived Neurotrophic Factor
FGF:	Fibroblast Growth Factor
IC:	inhibitory concentration
PLO:	poly-L ornithine
PBS:	Phosphate-Buffered Saline
rh:	recombinant human
SOP:	Standard Operating Procedure
T75:	cell culture flask with 75cm^2
T175:	cell culture flask with 175cm^2
FCS:	Fetal Calve Serum
HCS:	High Content Screening
PD:	Parkinson's Disease
DMSO:	Dimethyl sulfoxide
RT:	room temperature
T175:	cell culture T-flask with a cell attachment area of 175cm^2
T75:	cell culture T-flask with a cell attachment area of 75cm^2
FBS:	fetal bovine serum
2D	2-dimensional cell culture
3D	3-dimensional cell culture
MES 2.10:	human mesencephalicderived cell line characterized at and originating from Lund University (Lund, Sweden) (Lotharius et al., 2002).
VCS :	viable cellular structures
VCSA :	virtual cell soma area

Last Update: March 2018

Part B. Technical Description

Procedure Details, Latest Version: March 2018

Protocol Name: UKN4 assay to test compound-derived neurite outgrowth impairment in differentiating human dopaminergic neurons.

Contact person

Prof. Dr. Marcel Leist
In vitro toxicology and biomedicine
Dept inaugurated by the Doerenkamp-Zbinden foundation
University of Konstanz
Box M657
D-78457 Konstanz
marcel.leist@uni-konstanz.de
Tel: +49-7531-88-5037

Materials and Preparations

CELL OR EXPERIMENTAL SYSTEM

LUHMES cells are the ony test system of this protocol. Cells were originated from the ventral mesencephalon of an 8 week old human, female fetus. They exhibit the same characteristics as MESC2.10 cells (they are a subclone of the tetracycline-controlled, v-myc-overexpressing human mesencephalic-derived cell line MESC2.10).

They can be differentiated into morphologically and biochemically mature dopamine-like neurons following exposure to tetracycline, GDNF (glial cell line-derived neurotrophic factor), and db-cAMP for 6 days.

They are usually cultured in a 2D monolayer, but have also been shown to grow into 3D structures (Smirnova et al. 2015).

Here the LUHMES cells are used for the UKN4 assay to assess the effect of compounds on neurite outgrowth after 24 h of exposure.

EQUIPMENT

Fixed Equipment

Benchtop centrifuge
Laminar flow hood for sterile atmosphere (type II classified)
Liquid nitrogen storage
Freezer (-20°C and -80°C)
Fridge (4°C)
Humified incubator (37°C 5% CO_2)
Mr. Frosty™ freezing container
Light microscope for cell counting
Neubauer counting chamber
Pipettes (various volume, 1 µl – 1000 µl)
Pipetboy Pipeteur
Cellomics Array Scan VTI HCS reader
Computers, screens and software for data analysis (e.g. Microsoft Office)
Multichannel pipettes (Integra)
Multistepper pipettes (Eppendorf)

Consumables

15 ml and 50 ml conical plastic tubes (such as Falcon™ REF 352096 and REF352070)
175 cm^2 cell culture flasks with filtered cap (Sarsted REF 83.3912.002)
96-well plates (Sarsted REF 83.3924)
Deepwell plates (such as Greiner Bio-One REF 786261)
5 ml, 10 ml, 25 ml and 50 ml plastic pipettes (such as Sarsted REF 86.1685.001; REF 86.1689.001; REF 86.1253.001 and REF 12.54.001)
Neubauer counting chamber
Cyrovials (such as Nalgene Cat. No. 5000-0020)
Eppendorf tubes (such as various sizes)
Filter tips (such as Biosphere REF 70.760.212; REF 70.1130.210
Gloves (such as nitrile: VWR Cat. No. 112-2373 or latex: MaiMed REF 74175)

MEDIA, REAGENTS, SERA, OTHERS

Coating:
Poly-L-ornithine hydrobromide (Sigma-Aldrich; P3655-100mg)
Fibronectin solution (Sigma-Aldrich; F1141-5mg)

Basic medium:
Advanced DMEM/F12 (Gibco™, Thermo Fisher Scientific, Cat. No.12491015)
N-2 supplement (100x ; Thermo Fisher Scientific, Cat. No.17502048)
Glutamine (Sigma-Aldrich, G7513)

Components of Proliferation medium:
rhFGF-2 (R&D Systems; 4114-TC)

Components of Differentiation medium:
Tetracycline (Sigma-Aldrich, T7660-5g)
cAMP (Sigma-Aldrich sodium salt; D0627-1g)
rhGDNF (R&D Systems; 212-GD)

Staining:
Calcein-AM (Sigma-Aldrich; 17783-1MG)
Hoechst Bisbenzimide 33342 (Sigma; 14533-100MG)

other:
Trypsin 0,05% (ThermoFisher Scientific; 25300-062)
PBS without Ca^{2+}/Mg^{2+} (Biochrom AG L1825)
DMSO (Sigma D2650)
FBS (PAA A15-101)

PREPARATIONS

Media and Endpoint Assay Solutions

Freezing medium:
Advanced DMEM/F12
FBS to a final concentration of 20 %
DMSO to final concentration of 10 %
The freezing medium is always prepared freshly prior to use. No information about stability can be stated. Prepare under sterile conditions.

Aliquots of reagents:
Poly-L ornithine (PLO) (1mg/ml):
dissolve 100 mg in 100 ml of sterile distilled water

filter sterile (using filter pore of 0.22 µm)
aliquots of 5-10 ml, aliquotting under sterile conditions
store at -20°C
Can be stored up to 3 months. After thawing the solution is stored at 4°C till use and not re-frozen.

Fibronectin (1mg/ml):
aliquots of 150 µl, aliquotting under sterile conditions
store at 4°C
Can be stored up to 3 months. Do not freeze.

N-2:
ready to use
store at -20°C
Stable till use-by date of provider. As one vial is enough for one bottle of medium it is used after thawing and never re-frozen again. However re-thawing and re-freezing is possible up to 10 times.

L-Glutamin (200mM):
thaw in water bath, mix thorougly
aliquots of 10 ml, aliquotting under sterile conditions
store at -20°C
After thawing, L-Glutamin can be re-frozen and re-thawn one more time.

Fibroblast Growth Factor rhFGF-2 (160µg/ml):
prepare 0.1% (m/v) BSA in PBS
sterile filter (using filter pore of 0.22 µm)
dissolve 1 mg FGF in 6.25 ml of sterile 0.1% BSA/PBS
aliquots of 100 µl, aliquotting under sterile conditions
store at -20°C

Glial cell-Derived Neurotrophic Factor (rhGDNF) (20µg/ml):
dissolve in 0.1% (m/m) BSA in PBS and sterile filter (using filter pore of 0.22 µm)
stocks 1 ml (store at -80°C)
aliquots of 20 µl, aliquotting under sterile conditions
store aliquots at -20°C
Can be re-frozen and re-thawn. Stable up to 3 months.

Tetracyclin (1mg/ml):
weigh 25 mg and dissolve in 25 ml sterile distilled water
sterile filter (using filter pore of 0.22 µm)
aliquots of 500 µl, aliquotting under sterile conditions
store at -20°C
Can be re-frozen and re-thawn. Stable up to 3 months.

cAMP (100mM):
dissolve 1g in 20,4 ml of sterile distilled water
sterile filter (using filter pore of 0.22 µm)
1 ml aliquots in brown Eppis, as cAMP is light sensitive
store at -20°C
Can be re-frozen and re-thawn. Stable up to 3 months.

0.05% Trypsin:
thaw in water bath
aliquots of 12 ml, aliquotting under sterile conditions
store at -20°C
After thawing trypsin can be stored at 4°C till aliquot is finished. No re-freezing and re-thawing when aliquot is in use. Aliquots at -20°C are stable up to 3 months.

Coating solution:

Components of medium	**Volume required per 1 ml**
Sterile MilliQ H_2O	955 µl
Polyornithin (1 mg/ml in water)	44 µl
Fibronectin (1 mg/ml in water)	1 µl

Prepare freshly and use immediately.

Proliferation medium:

Components of medium	Volume required per 10 ml
Advanced DMEM/F12	9.8 ml
L-Glutamine (200mM)	100 µl
N2 (100x)	100 µl
FGF-2 (160µg/ml)	2.5 µl

Prepare freshly and use immediately.

Differentiation medium:

Components of medium	Volume required per 10 ml
DMEM/F12 Advanced	9.7 ml
L-Glutamine (200mM)	100 µl
N2 (100x)	100 µl
cAMP (100mM)	100 µl
Tetracycline (1 mg/ml)	10 µl
GDNF (20 µg/ml)	1 µl

Prepare freshly and use immediately.

Staining solution

- for 96-well plates 10 µl per well; staining solution: 10 µM calcein-AM and 10 µg/ml Hoechst 33342 dyes in PBS. Calcein-AM stock solution is 4 mM in DMSO (-> dilute calcein stock solution 1:400) and Hoechst stock solution is 1 mg/ml in MilliQ water (-> dilute Hoechst stock solution 1:100).
- Prepare freshly and use immediately. No long-term storage.

Test Compounds

Test compound are stored according to manufacturer's instructions. Stock solutions should be dissolved in sterile water or DMSO, if possible 1000x more concentrated than the working solution. The used DMSO is stored in a lightproof, air-tight bottle at room temperature.

The stock solutions are aliquoted into volumes sufficient for one experiment and frozen at -80°C. The aliquots are only used once and discarded after first thawing. This avoids repeated freezing and thawing and therefore to damage the compounds stability and efficiency. At 80°C the compound aliquots are considered to be stable for several months.

Compound dilutions are usually prepared in steps of 1:3, but the ratio can be increased up to 1:1.5 if needed. The dilutions are prepared in deep well plates using medium containing 1% DMSO (as the addition of 10 µl to 90 µl results in a 1:10 dilution, the final concentration of DMSO on the cells is 0.1%).

Positive Control(s)

Positive control is Narciclasine, which reduced neurite area to 50% of DMSO control, while cell viability was reduced only by 10% (50 nM final concentration). The Narciclasine preparation follows the same indications as for the test compound: stock solution in DMSO (50 µM), aliquots of 10 µl, storage at -80°C, discarding after use, no re-freezing and re-thawing.

Negative Control(s)

Negative control is 0.1% DMSO final concentration.

Method

EXPERIMENTAL SYSTEM PROCUREMENT

Storage of cells

Frozen cell batches are stored in liquid nitrogen.

Thawing of cells

Prepare and pre-warm proliferation medium. Prepare a falcon with 9 ml of Advanced DMEM/F12 medium without supplements. Take a LUHMES cell vial out of liquid nitrogen and thaw at 37°C in the water bath until almost completely unfrozen. In sterile conditions, quickly transfer the cell suspension to the falcon with medium without supplements to dilute the DMSO. Centrifuge (5min at 300g) to get rid of the freezing medium. Aspirate the medium, resuspend the cells in 1 ml of pre-heated proliferation medium and seed them in a pre-coated T75 flask with 14 ml proliferation medium, so that the total volume is 15 ml.

ROUTINE PROCEDURES

Coating

LUHMES cells only grow on coated plastic ware.
The coating solution is added in the following volumes to flasks and plates:
10 ml per T75 flask
20 ml per T175 flask
50 µl/well in 96 well plate

and incubated overnight at 37°C and 5% CO_2 in the incubator. The next day the coating solution is aspirated and washed once with sterile MilliQ water (10 ml per T75 flask, 20 ml per T175 flask, and 100 µl/well in 96 well plate) to remove potential leftovers.

Cell passaging

- For maintenance, grow LUHMES cells in flasks
- At 60-80% of confluency, aspirate medium and wash once gently with pre-warmed PBS (37°C)
- Add 2 ml of pre-warmed (37°C) trypsin (T0.05%) to a T75 flask, 4ml to a T175 flask
- Incubate shortly 2-3 min at 37 °C untill the cells detach. Rigorous tapping to the flask speeds up the detachment (hold flask with one hand, keep it planar, harshly tap against the flask with the other hand).
- Add Advanced DMEM/F12 without supplements to the flask (18 ml for T75 flasks or 36ml for T175 flasks) and resuspend cells by pipetting vigorously up and down several times to obtain a single cell suspension
- Transfer cell suspension into 50 ml Falcon tube
- Centrifuge at 300g at room temperature for 5 min

- Discard supernatant carefully and resuspend pellet in an appropriate volume of medium (estimated cell number should be 5-10 million cells / ml) and count living cells in a Neubauer chamber.
- Plate 2 x10^6 cells per T75 in 20 ml of proliferation medium and let the cells grow for 2 days in an incubator at 37°C, 5% CO_2.

Freezing of cells

For preparation pre-cool Mr. Frosty containers at -20°C. Label cryotubes with ethanol-proof pen. These procedure should be done when the LUHMES cells are at approx. 75% confluency.

Wash with PBS and then detach cells with trypsin as described previously. After resuspension in 18 ml Advanced DMEM / F12 without supplements, determine cell number by counting with a Neubauer chamber. Cell number is determined before centrifugation, as cells cannot last in freezing medium. The amount of cells lost in the centrifugation process is neglectable. Centrifuge the cell suspension to get rid of old medium and trypsin. Resuspend cells in an appropriate volume of cold freezing medium to obtain a suspension of 3-5*10^6 cells/ml and quickly distribute through pre-labelled cryovials. Put cryovials to pre-cooled Mr. Frosties and freeze at -80°C for at least 3h (better overnight). Transfer the vials to liquid nitrogen.

TEST MATERIAL EXPOSURE PROCEDURES

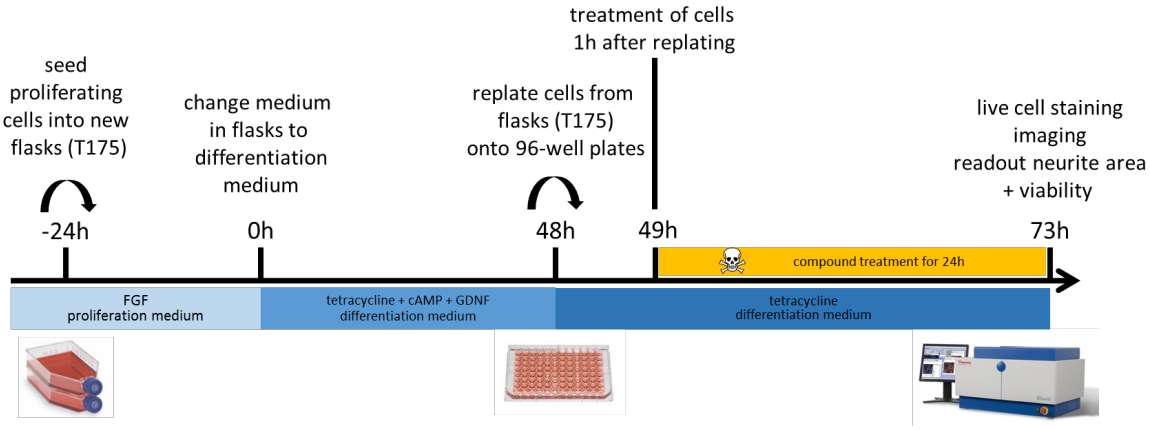

Fig. 1: exposure scheme for UKN4. Medium supplements are indicated in blue underneath arrow. Compound exposure is highlighted in yellow.

The following steps are conducted subsequently:

-24h: pre-differentiation:

To expand cells before differentiation is started, 7*10^6 cells per T175 flask are seeded in 20 ml proliferation medium. One T175 flask with 7*10^6 cells will result in 30*10^6 cells on d2 (48h in Fig. 1) of differentiation, so depending on cell number needed on d2 (48h in Fig. 1), (replating to 96-well plates), several flasks need to be seeded.

0h: start of differentiation:

To start differentiation, the cells are either seeded directly into differentiation medium (20*10^6 cells in T175), or the medium in the flasks seeded on d-1 (-24h in Fig. 1) is changed to differentiation medium without detaching or re-seeding cells.

48h: seeding in 96-well plates on day 2 of differentiation:

The cell amount per T175 flask should be around 30-40*10^6, equivalent to 75-80% confluency. Cells are trypsinized and resuspend in a concentration of 30.000 cells/ml. Distribute 90µl/well.

49h: treatment with toxicant:

On day 2 of differentiation, 1 h after seeding to 96 well plates, the compounds are added (in 10µl of volume). The cells are exposed to the toxicant for exactly 24 h (toxicant treatment and staining) from day 2 to day 3 of differentiation.

Typical 96-well plate layout:

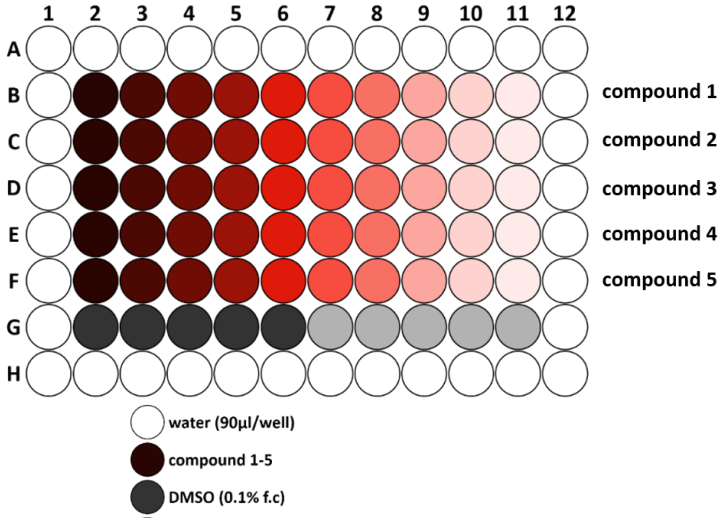

Fig. 2: recommended plate layout of UKN4 assay. Dilution series from left to right, with highest concentration of the left, lowest concentration on the right. Red colour indicates compound, the colour getting lighter and lighter represents the increasing dilution. Dark grey is the negative solvent control (DMSO 0.1% final concentration), light grey is positive control (Narciclasine 50 nM final concentration). Due to evaporation effects the outer wells are not used for the assay and only filled with water.

d6: staining and readout:

23.5 h after exposure (on day 6 of differentiation) cells should incubate with the staining solution at 37°C and 5% CO_2 in the incubator for at least 30min before imaging. Once stained, the staining remains stable up to 2 h. 10 µl of staining solution are added to each well. The readout using the automated microscope is done ideally exactly 24 h after compound treatment.

ENDPOINT MEASUREMENT

There are two endpoints measured in the UKN3b assay (viability and neurite area) both based on the fluorescent readout of the staining solution, using a fluorescent microscope (Cellomics ArrayScan VTI HCS microscope).

To measure viability and neurite area, the cells are stained 0.5 h before imaging. After staining the cells are incubated at 37°C and 5% CO_2 in the incubator until imaging. The cell staining is imaged in a Cellomics Array Scan VTI HCS reader automated microscope. Hoechst H-33342 staining is imaged in channel 1 (UV-Hoechst); calcein staining is imaged in channel 2 (Green-FITC). Exposure times are set manually to enable ideal quality for the image analysis algorithms (depending on incubation time with staining in our hands ~1-4 msec). While images in channel 1 (Hoechst) are exposed within the range (no

overexposure), images acquired in channel 2 (calcein) are on the limit to overexposure. See Fig. 3, left, upper row for example images.

Quantification of cell viability and neurite area are performed using the Cellomics Scan software (Insight version 1.6.2.4 – 1.00 x (Build 6390); Thermo Fisher Scientific).

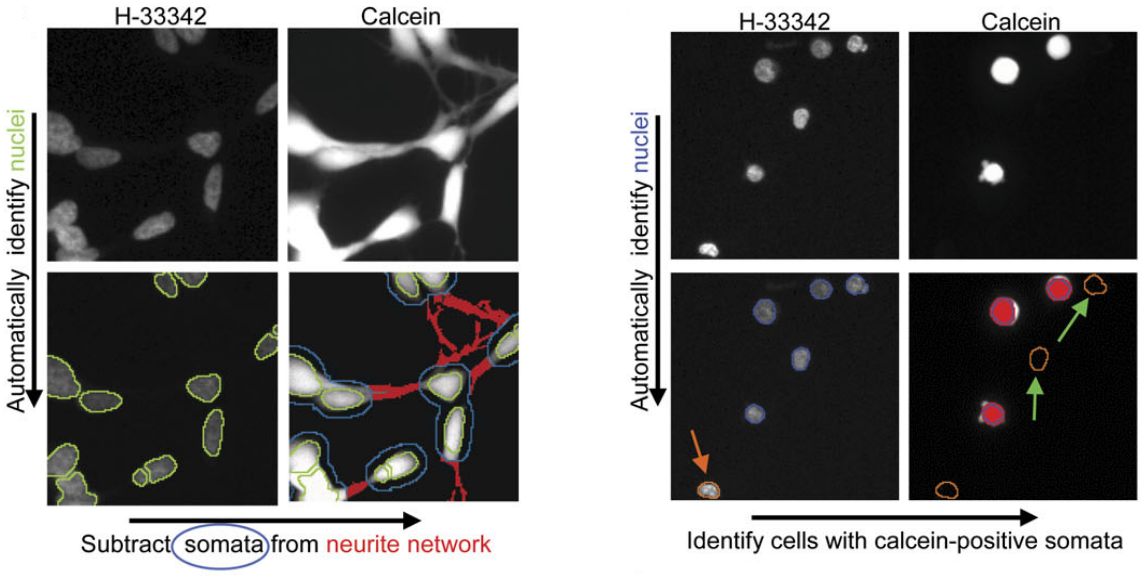

Fig. 3: example images of algorithm quantifying neurite area and viable cells. Left side: quantification of neurite area. Right side: Quantification of viable cells. Details in text below.

Quantification of neurite outgrowth (Fig. 3, left)

- For image acquisition, an **automated microplate reading microscope** (such as Array-ScanII HCS Reader, Cellomics, PA) equipped with a Hamamatsu ORCA-ER camera (resolution 1024 x 1024; run at 2 x 2 binning) should be used.
- Ten fields per well are imaged. Images are recorded in **2 channels** using a 20x objective and excitation/emission wavelengths of 365 ± 50/535 nm ± 45 nm to detect H-33342 in channel 1 and 474 ± 40/535 nm ± 45 nm to detect calcein in channel 2.
- In both channels, a fixed **exposure time** and an intensity histogram-derived threshold are used for object identification.
- Neurite pixels are identified using the following image analysis algorithm: nuclei are identified as objects in channel 1 according to their size, area, shape, and intensity which are predefined on untreated cells using a machine-based learning algorithm, and manual selection of nuclei to be classified as intact. The nuclear outlines are expanded by 3.2 µm in each direction, to define a virtual cell soma area (VCSA). based on the following procedure: The average width of the cytoplasm ring (distance nucleus - cell membrane) of LUHMES cells was experimentally determined to be 2.3 µm. Size irregularities are not always due to growing neurites, as determined by combined F-actin/tubulin beta-III staining. To avoid scoring of false positive neurite areas, the exclusion ring (VCSA) is made bigger than the average cell size. We found 3.2 µm to be optimal both to detect neurite growth over time and to identify reduced neurite growth with high sensitivity.
- All calcein-positive pixels of the field (beyond a given intensity threshold) are defined as viable cellular structures (VCSs). The threshold is dynamically determined for each field after flat field and background correction and intensity normalization to 512 gray values and is set to 12% of the maximal brightness (channel 63 of 512).
- The VCS defines the sum of all somata and neurites without their assignment to individual cells. In an automatic calculation, the VCSAs, defined in the H-33342 channel, are used as filter in the calcein channel and subtracted from the VCS. The remaining pixels (VCS - VCSA) in the calcein channel are defined as neurite area.

Quantification of individual viable cells by imaging (Fig. 3, right)

- For a quantitative assessment of viable cells, the same images used to assess neurite area are analyzed using another image analysis algorithm: nuclei are identified in channel 1 as objects according to their size, area, shape, and intensity. Nuclei of apoptotic cells with increased fluorescence are excluded.
- A virtual cell soma area (VCSA) is defined around each nucleus by expanding it by 0.3 µm into each direction.
- Calcein-AM staining, labeling live cells, is detected in channel 2. The algorithm quantifies the calcein intensity in the VCSA areas. Cells having an average calcein signal intensity in the VCSAs below a predefined threshold are classified by the program as “not viable”. Valid nuclei with a positive calcein signal in their cognate VCSA are counted as viable cells.

ACCEPTANCE CRITERIA

Positive control narciclasine:
Neurite area ≤ 75% of DMSO control
Viability ≥ 90% of DMSO control (or not significantly changed)

Negative control DMSO:
Neurite area ≥ 35.000

Data Analysis

- Array Scan VTI HCS Reader (Cellomics, PA) takes images (optionally bitmap or tiff-format; 512 x 512 pixels, 8bit or 16bit)
- Images are locally analyzed using the Array Scan software, algorithms quantify neurite area and cell count (nuclei)
- data are copy-pasted into an Excel sheet (see Figure 4), further analysis is done with Excel + KNIME + GraphPad Prism
- The data are analyzed and represented with GraphPad Prism.
- For the concentration curve, a nonlinear regression fit is calculated. The fitting method is least squares. If a non-linear curve fit is not possible, a linear curve fit is performed. The curve deriving from the fit is a 4-parameter log function.
- To calculate the EC50 value, this log-function is solved for y=50% of the total scale, not for 50% of the min-max scale.
- Treated concentrations are analyzed for deviation from control. Sometimes it is analyzed whether the deviation of neurite growth is different from the deviation of viability. This is done by two-way ANOVA + Tukey-Kramer post hoc testing.
- Statistics applied are one-way ANOVA (and nonparametric) with Dunnett’s post test.

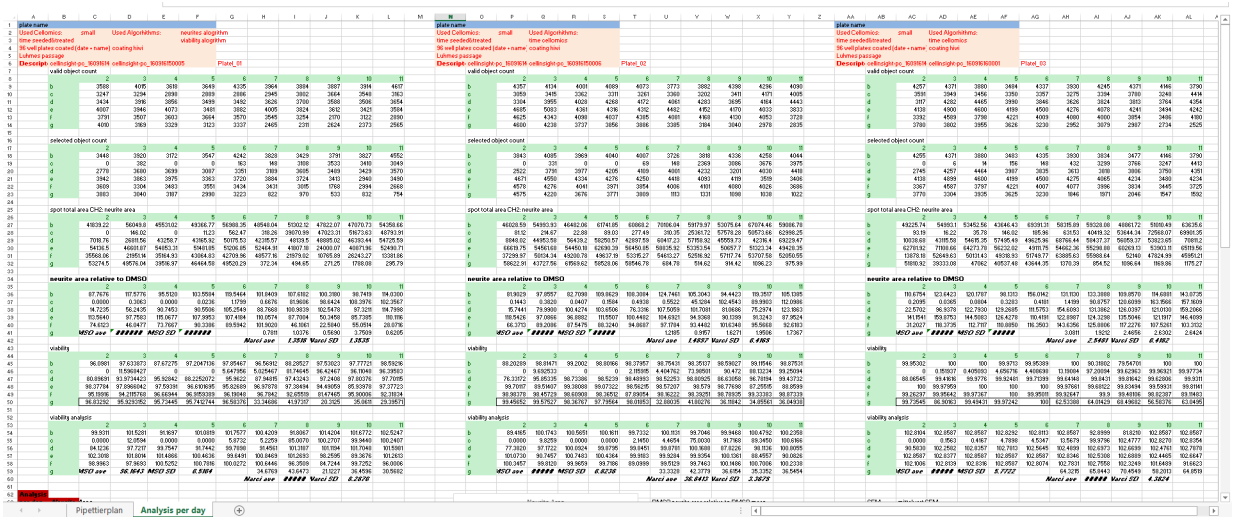

Fig. 4: screenshot of an Excel sheet used for analysis. Each column represents one plate, e.g. one technical replicate. The different readouts (neurite area, cell nuclei, viable cells) are listed underneath each other. Percentage of viable cells and normalization to DMSO control are calculated in this Excel sheet.

Prediction Model

Two different models are used:

1. prediction model for screening:
hit = decrease/increase in neurite area while viability is not changed (compare to narciclasine positive control:
Neurite area ≤ 75% of DMSO control
Viability ≥ 90% of DMSO control

2. prediction model for compound hazard evalaution:
hit confirmation testing;

EC50 Viability (V) / Neurite Area (NA) ≥ 4 → specifically neurotoxic

Annexes

No annexes available.

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