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ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

ANNEX I: DETAILED ASSAY DESCRIPTIONS WITHIN CASE STUDY ON THE USE OF AN INTEGRATEDAPPROACH TO TESTING AND ASSESSMENT FOR MITOCHONDRIAL COMPLEX-III-MEDIATED NEUROTOXICITY OF AZOXYSTROBIN- READ-ACROSS TO OTHER STROBILURINS

Series on Testing and Assessment

The corresponding monograph to this annex is available under the following cotes:
ENV/JM/MONO(2020)23

JT03465714

Methods

Seahorse

- Complexes
- Whole cells

Mitochondrial Membrane Potential

Mitochondrial viability

- lactate
- resazurin

Neurite outgrowth/degeneration

- glucose
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Bioavailability

- model
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Receptor docking

- 3D-modeling
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Some methods are still in the process of being described, see the next table.

Type	sub	Lab	Document	Method	Partner	Type
Seahorse	complex	UKN	Annex 1.1 Assessment of individual mitochondrial respiratory chain complex activity with permeabilised LUHMES cells	seahorse_complex_UKN	Johannes Delp (UKN)	SOP
		Cyprotex	Method will be soon available		Richard Maclennan (Cyprotex)	
	Whole	UKN	Annex 1.2 Mitochondrial stress test using LUHMES cells	seahorse_mitostresstest_UKN	Johannes Delp (UKN)	SOP
		VU	Annex 1.3 CS4 read across HepG2 Seahorse method description	seahorse_mitostresstest_VU	Giada Carta (VU)	description
MMP	JC1	VU	Annex 1.4 CS4 read across RPTEC/TERT1 methods description	Lactate_resazurin_JC1_VU	Giada Carta (VU)	description
	RHO	LU	Annex 1.5 DB-ALM Protocol HepG2 based Rhodamine123 assay	MMP_LU	Wanda van der Stel (LU)	DB-ALM type
	RHO	Swetox	Annex 1.6 Measurements mitochondrial membrane potential in differentiated human neuroblastoma SH-SY5Y cells after exposure to chemicals	MMP_Swetox	Anna Forsby (Swetox)	SOP
Mitochondrial viability	lactate	all partners	Annex 1.7 Lactate Assay (provided by Paul Jennings)	Lactate assay	Giada Carta (VU)	SOP
		VU	Annex 1.4 CS4 read across RPTEC/TERT1 methods description	Lactate_resazurin_JC1_VU	Giada Carta (VU)	description
		Swetox	Annex 1.8 Lactate measurement in supernatant after acute exposure (24h) in differentiated SH-SY5Y cells	Lactate assay_swetox	Anna Forsby (Swetox)	SOP
	resazurin	all partners	Annex 1.9 Resazurin Assay (provided by Paul Jennings)	Resazurin assay	Giada Carta (VU)	SOP
		VU	Annex 1.4 CS4 read across RPTEC/TERT1 methods description	Lactate_resazurin_JC1_VU	Giada Carta (VU)	description
		Swetox	Annex 1.10 Cell viability measurements with Resazurin after acute exposure (24h) in differentiated SH-SY5Y cells	Resazurin assay_Swetox	Anna Forsby (Swetox)	SOP
Neuron outgrowth	glucose	UKN	Method will be soon available		Johannes Delp (UKN)	
		Swetox	Annex 1.11 Neurite degeneration measurements in differentiated SH-Sy5Y cells after acute exposure (24 hours) to chemicals	neurite_degeneration_Swetox	Anna Forsby (Swetox)	SOP
	galactose	UKN	Annex 1.12 NeuriTox/UKN4 assay in galactose conditions	neuron_outgrowth_UKN	Johannes Delp (UKN)	SOP
Bioavailibity	model	Cyprotex	Method will be soon available		Iain Gardner (Certara)	
	collection samples	Cyprotex	Method will be soon available		Richard Maclennan (Cyprotex)	
	mass spec	Cyprotex	Method will be soon available		Richard Maclennan (Cyprotex)	
PBPK	Model	Certara	Annex 1.13 Biokinetic Modelling Description	Biokinetic Modelling	Iain Gardner (Certara)	description
			Annex 1.131. PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELLING TO SUPPORT THE STROBIN READ ACROSS CASE STUDY			
Repeat dosing	RDT	Swetox	Annex 1.14 Multiplex measurement of neurite degeneration, PI staining, ATP content.	Repeat dosing	Anna Forsby (Swetox)	SOP

	UKN	Method will be soon available		Johannes Delp (UKN)	
	LU	Method will be soon available		Wanda van der Stel (LU)	
	VU	Method will be soon available		Giada Carta (VU)	
Docking	UNIVIE	Method will be soon available		Florentina/Barbara	
Similarity	Barcelona	Annex 1.15 Strobilurins SMARTS: Common substructures and structural similarity	Chemical similarity	Jose Carlos	description
		Annex 1.16 Assessment of structural similarity	Chemical similarity	Suzanne Hougaard	description
Summary file	Swetox	Annex 1.17 CS4 read across SH-SY5Y method description (Swetox)	Summary	Anna Forsby (Swetox)	description
	VU	Annex 1.4 CS4 read across RPTEC/TERT1 methods description	Summary	Giada Carta (VU)	description
	LU	Annex 1.18 CS4 read across HepG2 method description (LU)	Summary	Wanda van der Stel (LU)	description
	UKN	Method will be soon available	Summary	Johannes Delp (UKN)	

Annex 1.1. Assessment of individual mitochondrial respiratory chain complex activity with permeabilised LUHMES cells

Method	LUHMES: Individual mitochondrial complex activity assessment
Contact	Johannes Delp (UKN) -> johannes.delp@uni-konstanz.de

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 permeabilised 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, permeabilised 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 analysed 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	2.5 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	MAS
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

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
$MgCl_2$	5 mM	1.02 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 sterilisation, 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 oxidisable 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 oxidisable 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/77 µl into ports A/B/C/D, respectively.
9. Until the start of the assay, the cartridge was incubated at 37°C without CO_2.

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). Therefore, the medium was carefully aspirated, and 500 µl of MAS-buffer was added to each well of the cell culture plate.
13. The utility plate was removed from the analyser and was discarded, while the cartridge remained in the instrument.
14. Finally, the cell culture plate was inserted into the XF analyser 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 (mmO_2, not OCR!)
- If oxygen doesn't get depleted, the data can be analysed.
- First, basal respiration of all wells gets normalised 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"

Annex 1.2. Mitochondrial stress test using LUHMES cells

Method	LUHMES: Mitochondrial stress test
Contact	Johannes Delp (UKN) -> johannes.delp@uni-konstanz.de

Introduction

Aim

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

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 analysed 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, N_2 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-CO_2 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-CO_2 incubator for at least 1 h before the start of the experiment
3. Set up the Seahorse analyser 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 analyser and, while the cartridge remained in the instrument.
5. Finally, the cell culture plate was inserted into the XF analyser 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 (mmO_2, not OCR!)
- If oxygen doesn't get depleted, the data can be analysed.
- First, basal respiration of all wells gets normalised 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.

Annex 1.3. CS4 read across HepG2 Seahorse method description

Method	HepG2: Resazurin Assay
Contact	Giada Carta (VU) -> g.carta@vu.nl

Cell Model:

Human Hepatocellular Carcinoma cells. It is an immortal non-tumorigenic cell line with high proliferation rates and an epithelial-like morphology (Donato, Tolosa, & Gómez-Lechón, 2015). HepG2 don´t need differentiation and use glycolysis as primary source of energy.

Cell splitting and routine culture:

For routine purposes cells are cultured on Sarstedt 10 cm dishes, seeded at the density approximately 1.5 x 10^5 cells/mL and fed every 2nd / 3rd day with 10 mL of cell culture medium Lonza DMEM (25 mM glucose) containing: Pen/Strep (100 U/ml and 100 µg/ml) and 10 % FBS.

Cell splitting for experiments:

For CS4 experiments, cells are seeded as described above in a 96 well plastic seahorse cell culture microplate pre-coated with 5 µg/cm^2 collagen IV, at density of approximately 3 x 10^5 cells/mL one day prior to experiment.

Chemical stock maintenance and use:

Stock solution of chemicals are dissolved in 100% DMSO at the concentration of 50 mM and stored at -20 °C until use. Exposure dilutions are prepared fresh for each experiment in cell culture medium at DMSO concentration of 0.1%. Controls cells are treated with cell culture medium at DMSO concentration of 0.1%

Mitostress assay with Seahorse analyser

The Seahorse mitostress test measures key parameters of mitochondrial function by directly measuring the oxygen consumption rates (OCR) of cells. OCR measured after sequential injections of modulators of respiration that target components of the electron transport chain permits derivation of ATP production, maximal respiration, proton leak, spare respiratory capacity and non-mitochondrial respiration (Agilent Seahorse, 2017).

For this system, cells are injected with test compounds prior to Mitostress modulators.

Cells plate preparation:

Cells are plated on seahorse cell culture microplate one day before experiment. On the day of experiment medium is exchanged one hour prior to chemical exposure with the Seahorse XF Base Medium (DMEM based with no glucose, bicarbonate and phenol red)

supplemented with 1 mM pyruvate, 2 mM glutamine, 10 mM glucose and 5 mM HEPES, pH 7.4.

Assay workflow:

- Baseline OCR of cells is measured for 20 minutes
- Test compound is injected, and the OCR is measured for 30 minutes
- After compound injection mitostress modulators are injected in the following order and OCR is measured for three times after each injection; oligomycin 2 µM (complex V inhibitor, allows ATP production calculations), FCCP 2 µM (uncoupler, allows maximal respiration capacity calculations), mixture of Antimycin A and Rotenone 0.5 µM each (complex III and complex I inhibitors, allows calculation of non-mitochondrial respiration).

CS4 read across reported data:

Reported data consist of a dose response curve given by OCR measurements in intact cells after test compound injection for 30 minutes and expressed as percentage of their own OCR baseline measurements prior to chemical injection.

Re-normalisation of datasets for BMR calculations:

Values of at least two no effect concentrations were forced to upper asymptote exactly at 100 %, data sets were re-normalised as percentage of no effect concentrations. Curve fitting and BMRs values are generated using the *in vitro* toxicology on-line tool provided by AG. Leist, University of Konstanz (http://invitrotox.uni-konstanz.de:3838/BMC/).

Agilent Seahorse. (2017). Mito Stress Test. *Agilent.*

Donato, M. T., Tolosa, L., & Gómez-Lechón, M. J. (2015). Culture and Functional Characterization of Human Hepatoma HepG2 Cells (pp. 77–93). Humana Press, New York, NY. https://doi.org/10.1007/978-1-4939-2074-7_5

Annex 1.4. CS4 read across RPTEC/TERT1 methods description

Method	RPTEC/TERT1: Lactate Assay, Resazurin Assay, MMP assay (JC-1)
Contact	Giada Carta (VU) -> g.carta@vu.nl

Cell Model:

Human Renal Proximal Tubular Cells. Telomerase immortalised, non-cancerous RPTEC/TERT1 (Wieser *et al.*, 2008). Prior to differentiation, the cells are bundle-shaped and highly glycolytic. Upon contact inhibition, RPTEC/TERT1 cells stop proliferating and start differentiating in polarised epithelial cells. Differentiated cells slow down glycolytic rates to increase oxidative phosphorylation as source of energy, furthermore acquire the characteristics of proximal tubule cells, including the expression of numerous transporters. The transport of water and solutes across the monolayer causes the formation of the characteristic "dome" structures on plastic. Microscopic assessment of domes is used as differentiation criteria.

Cell splitting and routine culture:

For routine purposes, cells are cultured on Sarstedt 10 cm dishes, seeded at the density approximately 1.5 x 10^5 cells/mL and fed every 2nd / 3rd day with 10 mL of hormonally defined cell culture medium DMEM/F12 (5 mM glucose) containing: Pen/Strep (100 U/ml and 100 µg/ml), Glutamax (2 mM), ITS (~5 µg/mL, 5 ng/mL), EGF (10 ng/mL), Hydrocortisone (36 ng/mL).

Cell splitting and differentiation for experiments:

For CS4 experiments, cells are seeded as described above in a plastic 96 well plate format at density of approximately 1.5 x 10^5 cells/mL and fed every 2nd / 3rd day with 100 µL cell culture medium. After confluence is reached, 7 days of spontaneous differentiation are counted, time in which formation of domes is assessed.

Experimental exposure to mitotoxins:

Differentiated cells on 96 well plates are fed 24 h prior to chemical exposure. Stock solution of chemicals are dissolved in 100% DMSO at the concentration of 50 mM and stored at -20 °C until use. Exposure dilutions are prepared fresh for each experiment in cell culture medium at DMSO concentration of 0.1%. Controls cells are treated with cell culture medium at DMSO concentration of 0.1%. For this system, cells are treated for 24 h before cell viability and cellular stress assays are performed.

Endpoints:

1. Supernatant lactate quantification:

Most cells use glucose as a primary energy source. The RPTEC/TERT1 medium contains 5 mM glucose that can be used by the cells in glycolysis (leading to the final product lactate) and/or oxidative phosphorylation for the production of ATP. When lactate is produced, it

is quickly extruded from the cells and can be measured in cell culture supernatants. We have previously shown that exposure of RPTEC/TERT1 and other cell lines to chemicals cause an increase in supernatant lactate, likely due to injury or dysfunction of mitochondria leading to or resulting from various types of cellular stress (Limonciel *et al.*, 2011).

24 h after chemical exposure supernatant medium is collected. Produced lactate is quantified using a colorimetric assay. The lactate assay is based on the conversion of lactate to pyruvate by the enzyme lactate dehydrogenase (LDH), reducing the co-factor NAD to NADH (Babson & Phillips, 1965). In the assay, NADH reduces PMS to PMSH which reduces INT to INTH. INTH is the reagent measured by colorimetry. Optical density is read at 490 nm. Unknown lactate values in mM are extrapolated from lactate standard curve using a spline fit function. Values are expressed as percentage of 0.1% DMSO controls.

2. Resazurin reduction quantification:

This method assesses cell viability based on the irreversible reduction of the blue dye resazurin to the pink product resorufin, which is highly fluorescent in red. Although the exact enzyme(s) involved in the reaction have not been identified, the assay is considered to be based on the global REDOX capacity of the cells in culture.

24 h after chemical exposure supernatant medium is collected for lactate quantification, cells are washed with PBS and a fresh cell culture medium containing 44 µM resazurin is applied to the cells for 1.5 h. Fluorescence of the redox product resofurin is read at excitation 540 nm / emission 590 nm. Values are expressed as percentage of 0.1% DMSO controls.

3. Mitochondrial membrane potential assessment with JC-1:

JC-1(tetraethylbenzimidazolylcarbocyanine iodide) is a cationic dye that accumulates in energised mitochondria. At low concentrations (due to low mitochondrial membrane potential) JC-1 is predominantly a monomer that yields green fluorescence with emission of 530±15 nm. At high concentrations (due to high mitochondrial membrane potential), the dye aggregates yielding a red to orange coloured emission (590±17.5 nm). The emission spectra of JC-1 shifts from green to red with increasing concentration (i.e., aggregation) in the mitochondria, thus allowing for a dual-colour (green/red) and radiometric semiquantitative assessment of mitochondrial polarisation states (Perry, Norman, Barbieri, Brown, & Harris, 2011).

24 h after chemical exposure, cells are treated with cell culture medium containing 2 µL/mL JC-1 2 mg/mL and 10 µL/mL Pluronics. After approximately 1.5 h the JC-1 solution was replaced with fresh medium. In a plate reader, cytoplasmatic JC-1 (green monomers) is read at 492 nm excitation, 535 nm emission, and mitochondrial JC-1 (red dimers) at 492 nm excitation and 590 nm emission. The polarisation of the membrane is calculated as ratio of mitochondrial over cytoplasmatic JC-1, the higher the ratio the higher the polarisation of the membrane. Values are expressed as percentage of 0.1% DMSO controls.

Re-normalisation of datasets for BMR calculations:

1. Supernatant lactate:

Values of at least two no effect concentrations were forced to upper asymptote exactly at 100 %, data sets were re-normalised as percentage of no effect concentrations. Curve fitting and ECs values couldn´t be generated using the *in vitro* toxicology on-line tool provided

by AG. Leist, University of Konstanz (http://invitrotox.uni-konstanz.de:3838/BMC/) as the algorithm is suitable only for inhibition curves. Lactate production increase with increase of the insult leading to activation curve, therefore curve fitting and ECs values are generated using point-to-point extrapolation in GraphPad Prism.

2. Resazurin reduction and MMP with JC-1:

Values of at least two no effect concentrations were forced to upper asymptote exactly at 100 %, data sets were re-normalised as percentage of no effect concentrations. Curve fitting and BMRs values are generated using the *in vitro* toxicology on-line tool provided by AG. Leist, University of Konstanz (http://invitrotox.uni-konstanz.de:3838/BMC/).

Babson, A. L., & Phillips, G. E. (1965). A rapid colorimetric assay for serum lactic dehydrogenase. *Clinica Chimica Acta*, 12, 210–215.

Limonciel, A., Aschauer, L., Wilmes, A., Prajczer, S., Leonard, M. O., Pfaller, W., & Jennings, P. (2011). Lactate is an ideal non-invasive marker for evaluating temporal alterations in cell stress and toxicity in repeat dose testing regimes. *Toxicology in vitro*, 25(8), 1855–1862. https://doi.org/10.1016/j.tiv.2011.05.018

Perry, S. W., Norman, J. P., Barbieri, J., Brown, E. B., & Harris, A. (2011). Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. *Biotechniques*, 50(2), 98–115. https://doi.org/10.2144/000113610.Mitochondrial

Wieser, M., Stadler, G., Jennings, P., Streubel, B., Pfaller, W., Ambros, P., … Grillari-Voglauer, R. (2008). hTERT alone immortalizes epithelial cells of renal proximal tubules without changing their functional characteristics. *Am J Physiol Renal Physiol*, 295, 1365–1375. https://doi.org/10.1152/ajprenal.90405.2008.-Telomere-dependent

Annex 1.5. DB-ALM Protocol HepG2 based Rhodamine123 assay

Last Update: Sept 2018 (v.9.4)

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.

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: LU HepG2 Rhodamine123 assay

Abstract:

This protocol describes how to culture HepG2 cells, how to perform compound exposures, how to perform imaging of the reporters, how to perform image analysis and how to perform addition functional assays.

Résumé

With the Rhodamine123 mitochondrial membrane potential sensitive dye the presence of the membrane potential can be monitored over time using confocal imaging systems. This approach provides the option to determine dye intensity and localisation. Furthermore, the dye is not invasive meaning that the cells can subsequently be used assays including cell death assays using propodium iodide, galactose capacity determinations by measuring lactate levels in the supernatant and viability assays by using the resazuring reduction read-out. See references.

Experimental Description

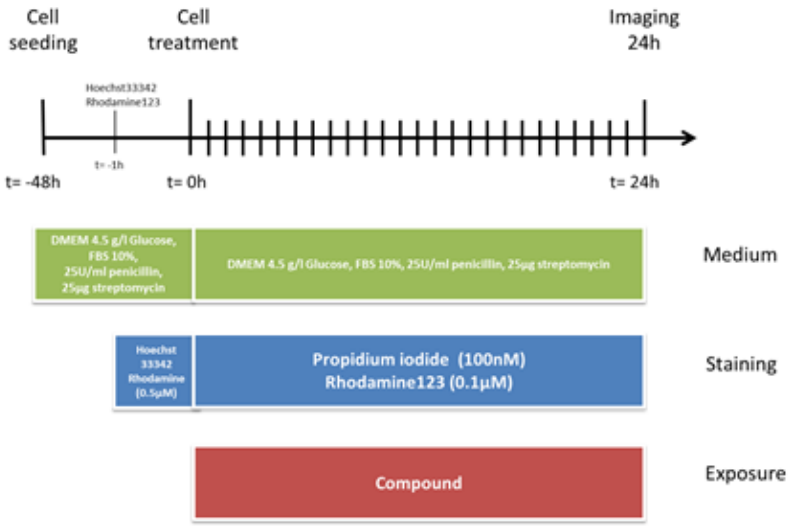
Biological Endpoint and Endpoint Measurement:

The endpoints of our toxicity test system include:

1. Mitochondrial integrity based on membrane potential sensitive dye (rhodamine123)
2. Cytotoxicity based on Propidium Iodide (PI)
3. Glycolytic capacity based on lactate concentrations
4. Viability based on resazurin reduction

The acquisition of microscopic pictures is done at specific timepoints, i.e. 24h/48h/72h or live (every 1h).

Figure 1. Exposure scheme read out every hour live



Endpoint Value:

1. The number of cells is quantified using the Hoechst 3342 staining.
2. The mitochondrial membrane potential is determined based on the EGFP signal intensity of the Rhodamine123 in the cytoplasmic compartment. The intensity is quantified based on the integrated intensity of all pixels in the cytoplasm (circle of 10 pixels around identified nuclei) .
3. The cytotoxicity measurement is based on the percentage of PI positive nuclei.
4. The glycolytic potential of the cell based on lactate concentration
5. The viability measurement is based on the reduction of resazurin to resorufin.
6. Dose response modelling, four-parametric log logistic curve fitting or point of departure calculation might be used to compare responses triggered by different compounds.

Experimental System:

The assay is performed in HepG2 (ATCC® HB8065™) using Rhodamine123, and PI plus lactate concentration measurements and resazuring reduction measurements.

Basic procedure

The HepG2 reporter cell lines are seeded in multi-well imaging plates, stained with Hoechst 33342, propidium iodide, and treated with different concentration of testing compounds. The cells are then imaged live on a confocal laser scanning microscope. The imaging procedure is followed by the collection of supernatant for lactate concentrations. The cells are submerged in the resazurin staining for 2 hours after with the intensity of the reduction product (resaforin) is measured.

Discussion

This bioassay is based live monitoring of the various dyes; therefore, an image acquisition a confocal laser scanning microscope including a cell culture incubator is required. Depending on the test compounds, further safety measures might be necessary.

The person conducting the experiment needs to be properly trained for culturing HepG2 cells, confocal microscopy and high content data analysis; therefore, a Master of Science level education is recommended.

Depending on the desired time point, the experiment itself can be conducted in 3 to 5 days. The amount of time of the data analysis is depending on the computing capacity.

The critical point of this experiment is the seeding of the HepG2 cells as a single cell suspension. HepG2 cells tend to form clumps which negatively affect the image acquisition with a confocal laser scanning microscope and the single nuclei segmentation during the high content image analysis.

Status

In Development:

Data analysis pipeline: Point of departure, EC values

Known Laboratory Use:

The usage of the Rhodamine123 dye for MMP measurements is widely accepted. However, the use of the usage in a live confocal setting is limited to the group of Bob van de Water at the University of Leiden.

The usage of the lactate and resazurin assay in the bellowed described setting are based on the SOP provided by the group of Paul Jennigs at the Vrije University of Amsterdam.

Participation in Evaluation Study:

The SOP was developed within the frame of the EU-ToxRisk project:

http://www.eu-toxrisk.eu/

Grant agreement No 681002

Participation in Validation Study:

The test method did not undergo any validation as defined in OECD Guidance Document No. 34 so far.

Regulatory Accepted:

There are no relevant guidelines available.

Proprietary and/or Confidentiality Issues

The use of rhodamine123 technology is not restricted by patents. The protocols for the lactate concentration assay and the resazurin assay are published (see references). The protocol regarding the experimental and microscopy procedures is open. However, the protocol regarding the automated data-analysis is owned by the Van de Water lab and should not be shared among others.

Health and Safety Issues

General precautions:

Follow general safety precautions of a mammalian cell culture lab. Wear lab coat, gloves and safety googles. For the laser scanning fluorescence microscope, safe work with the involved lasers has to be guaranteed. The involved cell lines are safe to work with in a biosafety level 1 according to council directive 90/679/EEC.

MSDS Information:

For safety data sheets, please contact the compounds' manufacturer.

Abbreviations and Definitions

ATCC…American Type Culture Collection

BAC…Bacterial artificial chromosome

BEVC …best-fit ellipse of Voronoi cell

DMEM… Dulbecco modified eagle medium

DMSO… Dimethyl sulfoxide

EDTA… Sodium ethylenediaminetetraacetate

EGFP…Enhanced green fluorescent protein

FBS…foetal bovine serum

MMP…Mitochondrial membrane potential

PoD…Point of Departure

PI…propidium iodide

P/S…Penicillin/Streptomycin solution

RT…room temperature

T25/T75/T175…Tissue flask 25 cm^2 /75cm^2/175cm^2

WMC…watershed masked clustering

Part B. Technical Description

Procedure Details, Latest Version: 1.

Protocol Name: LU HepG2 Rhodamine123 assay

Contact person

Bob van de Water

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Materials and Preparations

Cell OR Experimental system

The original HepG2 wildtype cells were obtained from ATCC (ATCC® HB-8065™). The obtained cell line has been biobanked and stored at -80°C for short term storage and -150°C for long term storage.

Equipment

Fixed Equipment

Material/Equipment
Centrifuge
TC20™ Automated Cell Counter
Fridge (4°C)
Freezer (-20°C, -80°C, -150°C)
Humidified incubator (37°C, 5% CO_2)
Ice machine
Laminar flow hood for sterile atmosphere
Laminar flow hood for sterile atmosphere
Water Bath
Stereoscopic microscope
Nikon Eclipse Ti or Nikon Eclipse Ti2, equipped with: four lasers (408, 488, 561 and 644 nm lasers), a A1, C1 or C2 box, live cell incubator, automated stage, perfect focus system or equivalent.
Milli-Q water dispenser
FluoStar plate reader

Material/Equipment	Supplier	Catalogue No
Micropipettes Pipet-Lite XLS LTS		
Pipette L-1000XLS+	Rainin	17014382
Pipette L-200XLS+	Rainin	17014391
Pipette L-20XLS+	Rainin	17014392
Pipette L-2XLS+	Rainin	17014393
Multichannel pipets		
Eppendorf Research® plus, 8-channel, variable, incl. epT.I.P.S.® Box, 0.5 – 10 µL, medium gray	Eppendorf	3125000010
Eppendorf Research® plus, 8-channel, variable, incl. epT.I.P.S.® Box, 10 – 100 µL, yellow	Eppendorf	3125000036
Eppendorf Research® plus, 8-channel, variable, incl. epT.I.P.S.® Box, 30 – 300 µL, orange	Eppendorf	3125000052
Software		
NIS viewer	Nikon	
CellProfiler® (version 2.1.1)	Carpenter Lab at the Broad Institute of Harvard and MIT.	
R Studio (version R version 3.3.2 or newer)	RStudio,	
Plastics		
Sterile 15 plastic tubes	Sarstedt	62.554.502
Sterile 50 plastic tubes	Greiner Bio-One	210270
Sterile cell culture flasks T25	Corning	430639
Sterile cell culture flasks T75	Corning	430641U
Sterile cell culture flasks T175	Corning	431080
Sterile pipettes 2 ml	Greiner Bio-One	710180
Sterile pipettes 5 ml	Greiner Bio-One	606180
Sterile pipettes 10 ml	Greiner Bio-One	607180
Sterile pipettes 25 ml	Greiner Bio-One	760180
Cryovials		
Cryo.s U-starand-2ml	Greiner Bio-One	122263
Cryo.s U-starand-2ml	Greiner Bio-One	122277
Cryo.s U-starand-2ml	Greiner Bio-One	122278
Cryo.s U-starand-2ml	Greiner Bio-One	122279
Cryo.s U-starand-2ml	Greiner Bio-One	122280
Micro tube 1.5ml	Sarstedt	72.690.001
Micro tube 2ml	Sarstedt	76.695.500
Sterile Pipette tips		
LTS tips 20ul	Mettler Toledo	17005091
LTS tips 200ul	Mettler Toledo	17005093
LTS tips 1000ul	Mettler Toledo	17005089
Biopur 2-200 ul refills	VWR	613-3569
Biopur 20-300 ul refills	VWR	613-3570
Gloves EcoSHIELD wit econitril PF 250	Boom BV	38055162; 38055163; 38055164
Imaging plates		
96 µclear wells plate	Greiner Bio-One	655090
96 ScreenStar plate	Greiner Bio-One	655 866
384 µclear wells plate	Greiner Bio-One	781 091
Cell Counting Kit, 30 dual-chambered slides, 60 counts, with trypan blue	BIO RAD	1450003
50µm non-sterile CellTrics® filters	SysmexNederland	04-0042-2317

Testing compounds

Compound	abbreviation	Sigma Aldrich Cat. No.	Labelling accord. Reg. (EC) 1272/2008
Rotenone	ROT	R8875	
Deguelin	DEG	D0817	
Azoxystrobin	AZO	31697	
Kresoxim-methyl	KRE	37899	
pyraclostrobin	PYR	33696	
Picoxystrobin	PIC	33658	
trifloxystrobin	TRI	46477	

Media, Reagents, sera, others

Material	Supplier	Catalogue Number
DMEM, high glucose, pyruvate	Fisher Scientific	11504496
Foetal Bovine Serum	South American, Fisher Scientific	S181L-500
Penicillin Streptomycin solution	Fisher Scientific	15070-063
DMSO	Biosolve	04470501/1
Trypan blue	Biorad	1450021
DULBECCO'S PHOSPHATE BUFFERED SALINE MODIFIED	Sigma Aldrich	D8537
Trypsin EDTA	In house	NA
Hoechst 33342, Trihydrochloride, Trihydrate	Life technologies	H1399
Propidium iodide =94.0% (HPLC)	Sigma Aldrich	P4170
Rhodamine123	Sigma Aldrich	R8004-5MG
Distilled Water	Gibco/Thermo Fisher Scientific	15230147

Lactate concentration assay

Material	Supplier	Catalogue Number
Triethanoloamine HCl	Sigma Aldrich	T9534
EDTA.Na_2	Sigma Aldrich	E4884
$MgCl_2$ anhydrous	Sigma Aldrich	M8266
PMS (N-Methylphenazonium methyl sulphate)	Sigma Aldrich	P9625
INT (p-iodonitrotetrazolium violet)	Sigma Aldrich	I8377
Triton X-100	Sigma Aldrich	X100
?NAD	Sigma Aldrich	N7004
Lactate dehydrogenase (LDH)	Sigma Aldrich	L2500-25KU
L-lactic acid sodium salt (standard)	Fluka Chemika	71718

Resazurin assay

Material	Supplier	Catalogue Number
Resazurin	Sigma Aldrich	R7017

PREPARATIONS

Media and Endpoint Assay Solutions

Medium/Solution	Preparation
Foetal Bovine Serum aliquots	Thawn, aliquoted into Sterile 50 plastic tubes, frozen again and stored at -20°C.
PenicillinStreptomycin aliquots	Thaw out Penicillin Streptomycin bottle, prepare aliquots of 2 ml in sterile 15 plastic tubes, frozen again and stored at -20°C.
Complete DMEM	Mix 450 ml of DMEM, 50 ml of FBS and 2 ml PenicillinStreptomycin solution (sterile). Store at 4°C. Medium does not need pre-warming before use.
Trypsin EDTA aliquots	Dissolved in a total volume of 1l in Milli-Q® Type 1 Ultrapure Water : 0.05 % (m/V) trypsine; 8 gr NaCl; 0.25 gr KCl; 1.43 gr Na_2HPO_4*2 H_20; 0.25 gr KHP_2PO_4. pH set to 7.2 with 1M HCl Filtered with a 0.2 µm filter (sterile)
Hoechst 33342 aliquots	1mg/ml Hoechst 33342 in sterile distilled water, Filtered with a 0.2 µm filter (sterile)
Rhodamine123 aliquots	2500uM in DMSO
Propidium iodide aliquots	Stock solution 100M in sterile distilled water, sterile filtered
Hoechst + rhodamine123 staining solution	Create 200ng/ml Hoechst 33342 (1:5000) plus 0.5uM rhodamine (1:5000) in complete DMEM
Propidium iodide + rhodamine123 staining solution	Create 200 nM Propidium iodide aliquot (1:500) plus 0.1uM rhodamine (1:25.000) in complete DMEM
Freezing medium	20% DMSO in FBS
	Test compound stock solution
DMSO stock	DMSO stocks are created by dissolving the provided test compound in in DMSO by vortexing or resuspending it with a pipette. The stock concentration has to be at least 1000 x higher than the highest testing concentration (='0.1%' v/v of DMSO). (The weight provided by JRC was used for concentration calculations)
	Lactate and resazurin assay solutions
Assay solutions	The solutions for the lactate and resazurin assay were created based in the SOPs provided by Paul Jennings lab at the Vrije Unversiteit Amsterdam.

Compound stock solutions

Test compound stock solutions were prepared as described in the following table. All stock compounds solutions were stored in brown glass bottles at -80°C under light exclusion. Furthermore, stocks for usage were stored at -20°C under light exclusion. Freeze thaw cycles were kept minimal. For replicates different aliquots of the same stock solution were used. The stock solutions were diluted out with full DMEM. All treatments, excluding DMEM vehicle, contained 0.1% (V/V).

Compound	Solvent stock solution	Concentration stock solution [mM]	Concentration stock solution [g/l]	Highest testing concentration [uM]
Rotenone	DMSO	10	394,4	10
Deguelin	DMSO	10	394,4	10
Azoxystrobin	DMSO	10	403,39	10
Kresoxim-methyl	DMSO	10	313,4	10
Pyraclostrobin	DMSO	10	387,8	10
Picoxystrobin	DMSO	10	367,3	10
Trifloxystrobin	DMSO	10	408,4	10

Compound	Testing concentration [µM]							
	A	B	C	D	E	F	G	H
ROTENONE	10	2	0.4	0.08	0.016	0.0032	0.00064	0.000128
DEGUELIN	10	2	0.4	0.08	0.016	0.0032	0.00064	0.000128
AZOXYSTROBIN	10	2	0.4	0.08	0.016	0.0032	0.00064	0.000128
KRESOXIM-METHYL	10	2	0.4	0.08	0.016	0.0032	0.00064	0.000128
PYRACLOSTROBIN	10	2	0.4	0.08	0.016	0.0032	0.00064	0.000128
PICOXYSTROBIN	10	2	0.4	0.08	0.016	0.0032	0.00064	0.000128
TRIFLOXYSTROBIN	10	2	0.4	0.08	0.016	0.0032	0.00064	0.000128

Positive Control(s)

Positive controls decrease the membrane potential. Therefore, besides Rotenone we also included FCCP.

Negative Control(s)

Complete DMEM medium, with and without 0.1% DMSO.

Method

Experimental System Procurement

Routine culture procedures

	Cell culture maintenance vessels				Seeding densities *1000	
Cultureware	**Growth Area (cm^2)**	**Media Volume (ml)**	**Trypsin-EDTA Volume (mL)**	**PBS Volume (mL)**	**2 nights**	**3 nights**
T25	25	5	1	5	2000	1300
T75	75	15	2	10	6000	4000
T175	175	30	4	20	12000	8000

	Imaging plates		
Plate format	**Seeded cells per well for 24h of exposure**	**Seeded cells per well for 48h of exposure**	**Seeded cells per well for 72h of exposure**
96 well plate	20000 in 100µl	15000 in 100µl	12000 in 100µl
384 well plate	8000 in 30µl	6000 in 30µl	5000 in 30µl

Thawing of Cryopreserved HepG2 cell lines

1. HepG2 reporter cell lines are stored at -80°C for short term storage and long storage at -150°C.
2. Cells are thawed in 37°C water bath and poured into 4 ml of complete DMEM.
3. After centrifugation (1000 rpm, 5 min, 4°C), the supernatant is replaced by fresh medium (total volume 5 ml).
4. Then the cells were carefully resuspended and transferred into a T25 tissue flask and moved in an incubator (37 °C, 5% CO2 and 95% humidity).
5. After one further passaging step, the cells can be used in an assay.

Splitting of HepG2 cell lines

1. After removing the medium, cells are washed with PBS.
2. Then the cells are incubated with trypsin-EDTA for 5 minutes in an incubator (37 °C, 5% CO_2 and 95% humidity).
3. Before neutralizing the trypsin- EDTA with complete DMEM, the cell suspension is forcefully resuspended 20 times with a 10 ml pipette (smallest opening) in order to achieve a single cell suspension.
4. Passage according to demands in according to table 'Cell culture maintenance vessels'.
5. HepG2 BAC-GFP reporter should not be passaged more than once per 48h.

Cell counting with TC20™ Automated Cell Counter

1. Under sterile condition, remove 10µl of the cell suspension with a microliter pipette (resuspend well before taking the sample).
2. Pipet the 10µl into a dual-chambered slide, avoid air bubbles.
3. Insert dual-chambered slide into TC20™ Automated Cell Counter.
4. Inspect bright field image and histogram of counted particles in order to assess the single cell suspension. Clumps can be removed with Celltrix® 50µm filter.

Cryopreservation HepG2 cell lines

1. Trypsinise cells as described earlier.
2. Add 800 µl cell suspension containing 2*10^6 Cells into cryovials containing precooled (4°C) 800 µl freezing medium.
3. Transfer cells into precooled freezing rack.
4. Freeze at -80°C overnight.
5. Transfer for long term storage to -150°C or maintain on -80°C.

Test Material Exposure Procedures

Seeding HepG2 cell lines

1. Remove medium.
2. Wash with PBS (RT).
3. Add Trypsin EDTA and incubate at 37°C for 5 min.
4. Reduce cell clumps to single cell suspension by pipetting up and down, 20 times, while pressing the pipet's tip to the tissue flasks wall.
5. Determine cell number with TC20™ Automated Cell Counter (see above)
6. If necessary, remove clumps with Celltrix® 50µm filter.
7. Dilute and seed out cells with multichannel pipet in multi-well imaging plates.

Consult table 'Imaging plates' for plating densities.

Added volumes	96 well plate	384 well plate
Hoechst+rho staining solution	100µl	30µl
PI+rho staining solution	50µl	25µl
Compound solution	50µl	25µl

1. Add Hoechst + Rhodamine123 staining solution with multi-channel pipette.
2. Incubate 45 minutes
3. In the meantime: Prepare compound solutions (as mentioned under "test compounds).
4. Aspirate medium containing Hoechst staining solution (3 to 5 rows at a time).
5. Add PI + Rhodamine123 staining solution to each well with multi-channel pipette.
6. Add compounds in the desired concentration with multi-channel pipette.
7. If live imaging: Proceed to the microscope (incubator preheated)
8. If time point imaging: Place imaging plate in incubator until imaging time point arrives.

FLUORESCENT Endpoint Measurement

For the laser and filter setup, consult table 1 Fluorescence Microscope Channels. For live imaging, it is recommended to pre-expose one well with positive control in order to setup the EGFP imaging.

- Preheating incubator of the microscope to 37°C under saturated humidity in time before imaging.
- Hoechst 33342
  - Check in the Hoechst 33342 channel whether the whole plate's cells are in focus.
  - Set intensity to a low enough level to still identify all nuclei, but as low as reasonably possible in order reduce oversaturation as far as possible.
- eGFP
  - Orientate setting of GFP reporter readout at the negative control. Since this measurement ought to be quantified, do not oversaturate.
- Propidium iodide
  - Use a compound concentration which induces cytotoxicity and one negative control to set up microscopic settings to differentiate between background and signal. For PI is an object identification foreseen to classify a cell as positive, therefore a clear visualisation of PI staining of cells is required.

Fluorescence microscopy:

Table 1. Fluorescence Microscope Channels

Fluorophore	Laser	Emission filter [nm]	Filter Range [nm]
Hoechst 33342	408 nm	450/50	425 - 475
eGFP	488 nm	525/50	500 -550
Propidium iodide	561 nm	595/50	570 -620
Annexin V Alexa 633	633 nm	700/75	663 - 738

ACCEPTANCE CRITERIA

The quality of the test is based on the cell death marker (PI) and the GFP intensity

A test is discarded when:

1. More than 15% of the cells are PI-positive in the solvent control (DMSO)
2. To determine if the control provides the expected result, we determine the robust Z score per plate.
3. To be able to do so you need at least 3 wells of your positive control (σ_p and μ_p) and of your negative control (σ_n and μ_n). (σ = standard deviation and μ = mean) The estimated Z-factor should be above 0.25.
4. At least 4 negative controls (complete DMEM), solvent control (complete DMEM + 0.1% (V/V) DMSO), and 4 positive controls should be taken along.

$$\text{Estimated Z-factor} = 1 - \frac{3(\hat{\sigma}_p + \hat{\sigma}_n)}{|\hat{\mu}_p - \hat{\mu}_n|}$$

LACTATE AND RESAZURIN ENDPOINT MEASUREMENT

The lactate and resazurin assay were performed following the lactate and resazurin assay protocol provided by Paul Jennings lab at the Vrije Unversiteit Amsterdam.

Lactate assay

The supernatant was collected after live confocal imaging and stored upon ice until further usages. The provide protocol was followed:

1. 10uL culture medium was combined with 90uL working lactate assay reagent mix
2. The mixture was stored for 7min in the dark
3. Subsequently the absorbance was measured at 490nm using the FluoStar plate reader.

Resazurin assay

The residual medium was removed from the imaging plates. The plates were not washed with PBS (because of the cell loss it would induce).

1. 30uL of pre-warmed 20x diluted resazurin mixture was added to all wells.
2. After 2h of incubation at 37degrees the resorufin fluorescence was measured: excitation at 540nm, emission at 590nm.

Data Analysis

Disclaimer

The nuclei segmentation in cellprofiler relies on the watershed masked clustering developed by Kuan Yan (see Di *et al* 2012). The WMC might not be available to the public. It can be done alternatively via segmentation in imageJ. The segmented pictures can then be read into cellprofiler.

The R-scripts for the data readout out of the HDF5 file is not available publicly.

Image processing

The image format retrieved from the microscopy session is a ND2 file. This .ND2 file contains the images (well /channel/timepoint). Furthermore, it includes the technical information about the microscope composition and settings (laser settings, excitation/emission, detector settings, plate position, etc…). Since the ND2 files can reach a significant size, a fast data connection is recommended. At the Division of Drug Discovery and Safety, the data is retrieved by USB 3 compatible hard drives and an in-house server.

A program freely provided by Nikon (NIS-Elements Viewer) was used to export the .ND2 file to single .tiff images per well /channel/timepoint (xy_c_t).

The exported tiff files were then sorted into image location specific folders (i.e. B02_1, meaning well B02 first imaging location). These were used while reading in the images in cell profiler to associate the images location_ID.

Image analysis

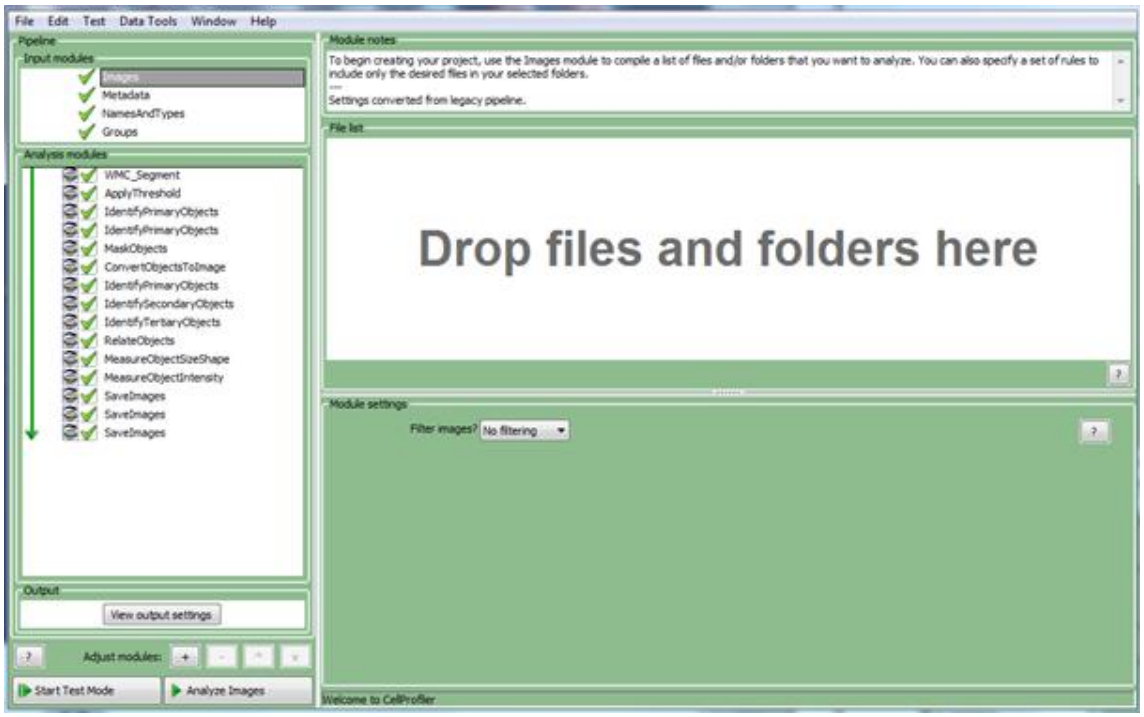
The structured folders with .tiff files are subsequently used for analysis in the free and open-source software program CellProfiler® (version 2.1.1). To be able to study the intensity and shape of objects the following pipeline was used for object identification:

1. Identification of the nucleus based on the Hoechst image
2. After segmentation of the nuclei, a circle of 10 pixels around the nuclei is created.
3. Identification of PI object based on the PI image

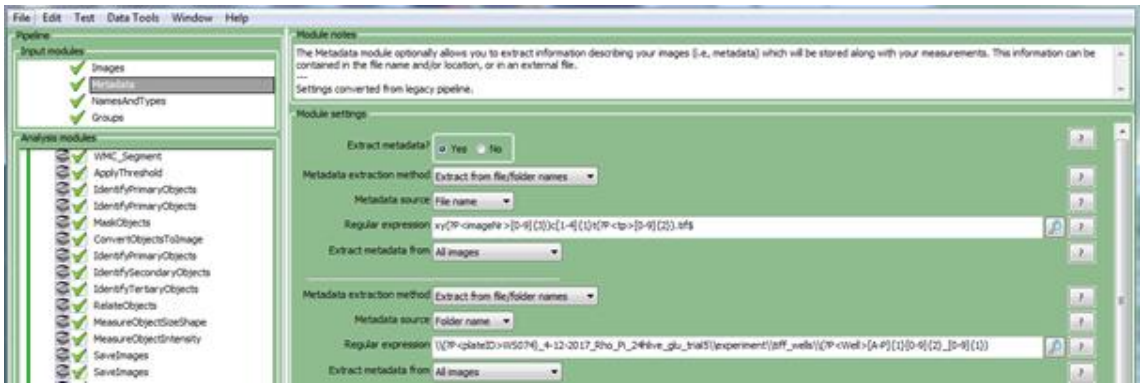
   If there is an overlap between the nucleus and the PI, the cell is considered PI positive.

1. Cell Profiler analysis > set Input modules

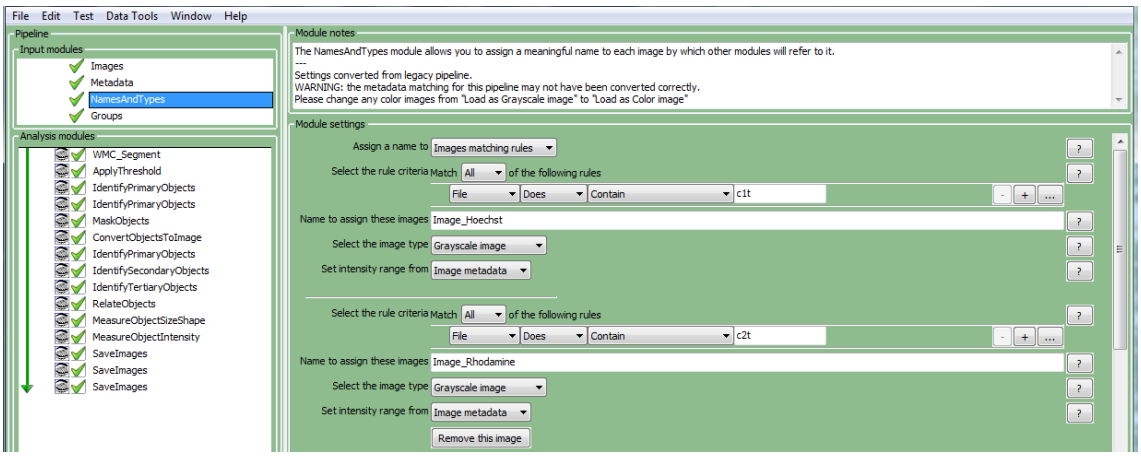
- Open CellProfiler (see what features need to be analysed in the figure above)
- Drag and drop images in CellProfiler:



– Set regular expressions in “metadata”. Add four variables: 1. plateID 2. imageNr 3. Timepoint 4. Well location. Click on update



– Set the different channels (Hoechst, Rho, and PI) in the “names and types” module. Click on update



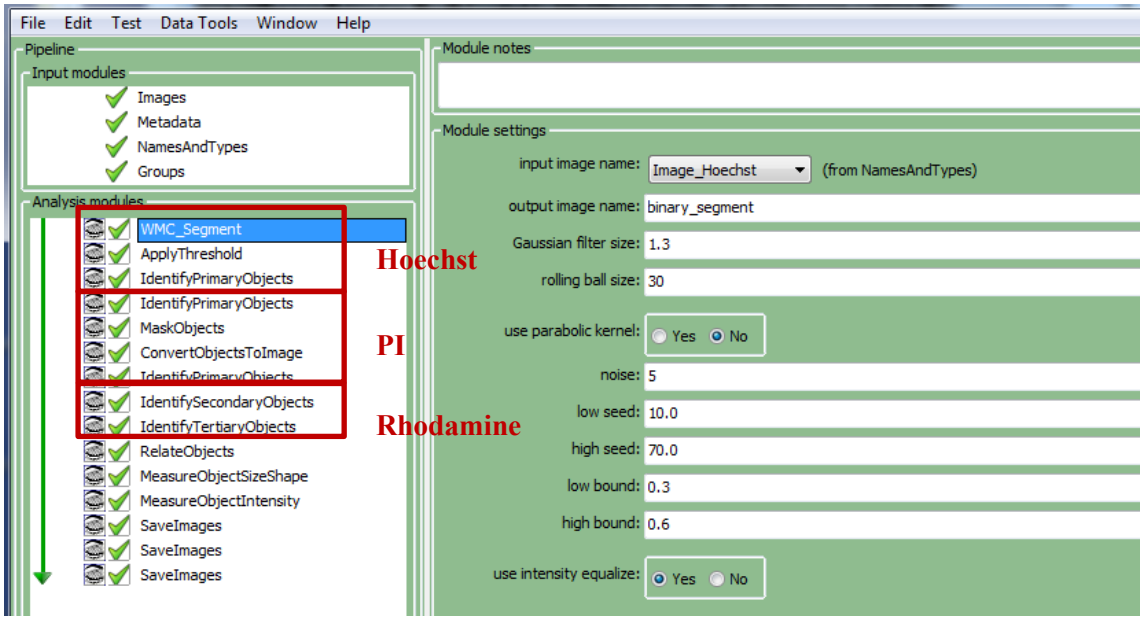
- OPTIONAL: set groups

2. Cell Profiler analysis > set Analysis modules

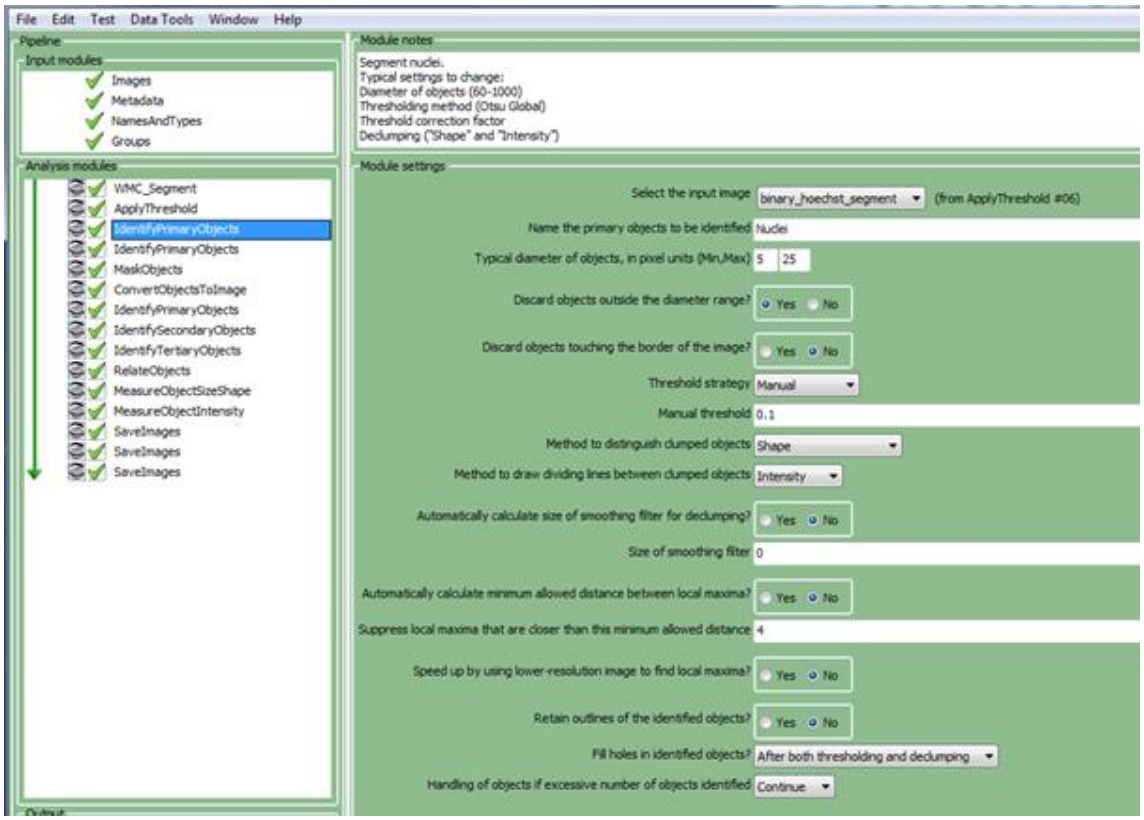
- Start test mode (bottom left corner)

Nucleus identification

- Input = Hoechst Channel
- Check the separation between foreground and background pixel in the Hoechst channel using the watershed masked clustering (WMC) ImageJ plugin.
- The WMC ImageJ plugin was developed by Kuan Yan (see Di *et al* 2012).

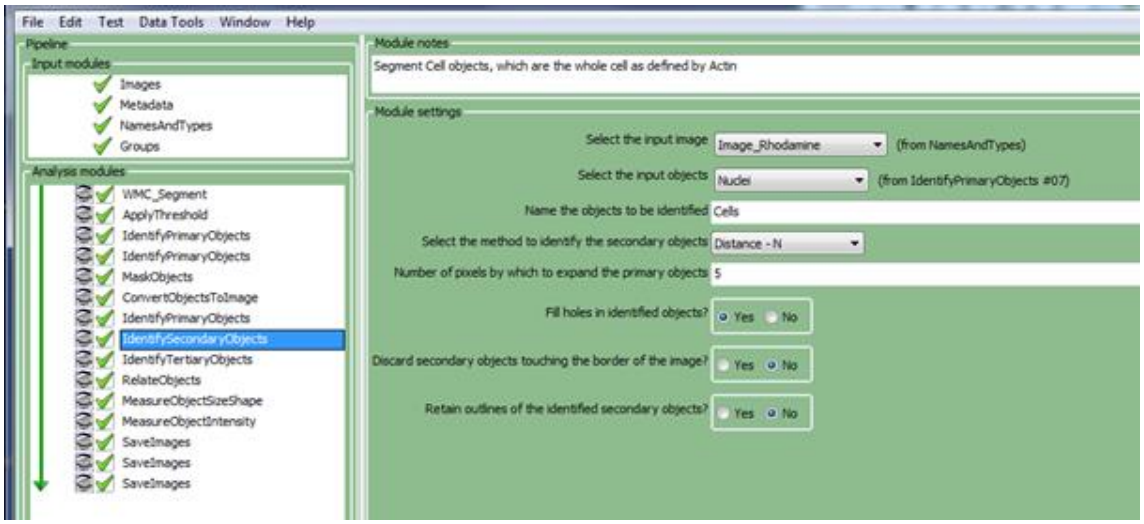


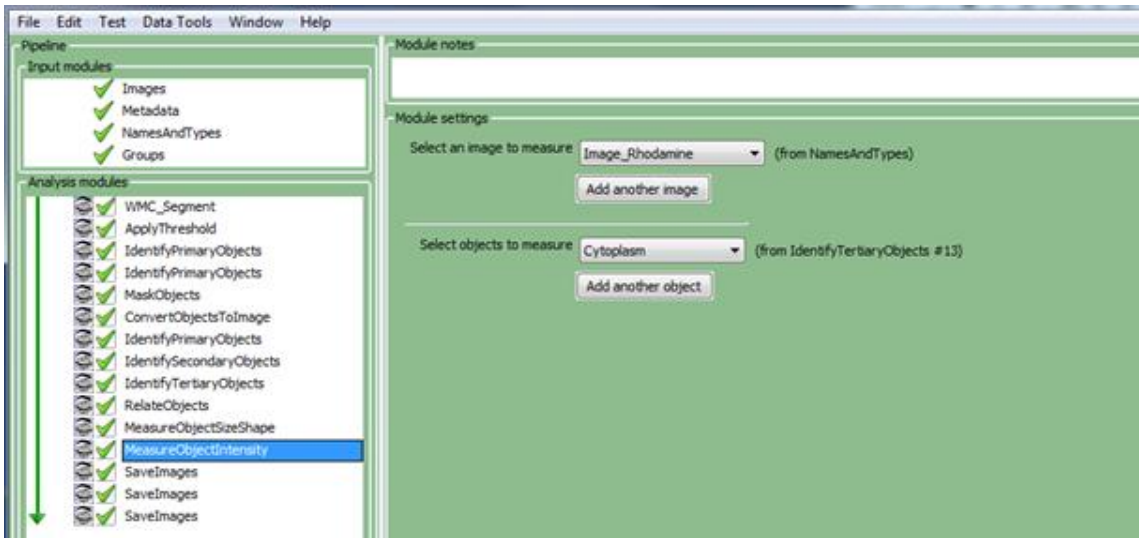
- Check the identification of the different objects in “Identify Primary Objects”



Rhodamine123 signal identification > in the cytoplasm

- Input = eGFP channel
- When identification of the cytoplasm is desired, check the identification of the cytoplasm using the “Identify Secondary Objects” module
- The “Identify Tertiary Objects” is an objected created by subtracting the “Identify Primary Objects” from the “Identify Secondary Objects”.
- MeasureObjectIntensity => Measure GFP intensity in the identified Tertiary Object (Cytoplasm)



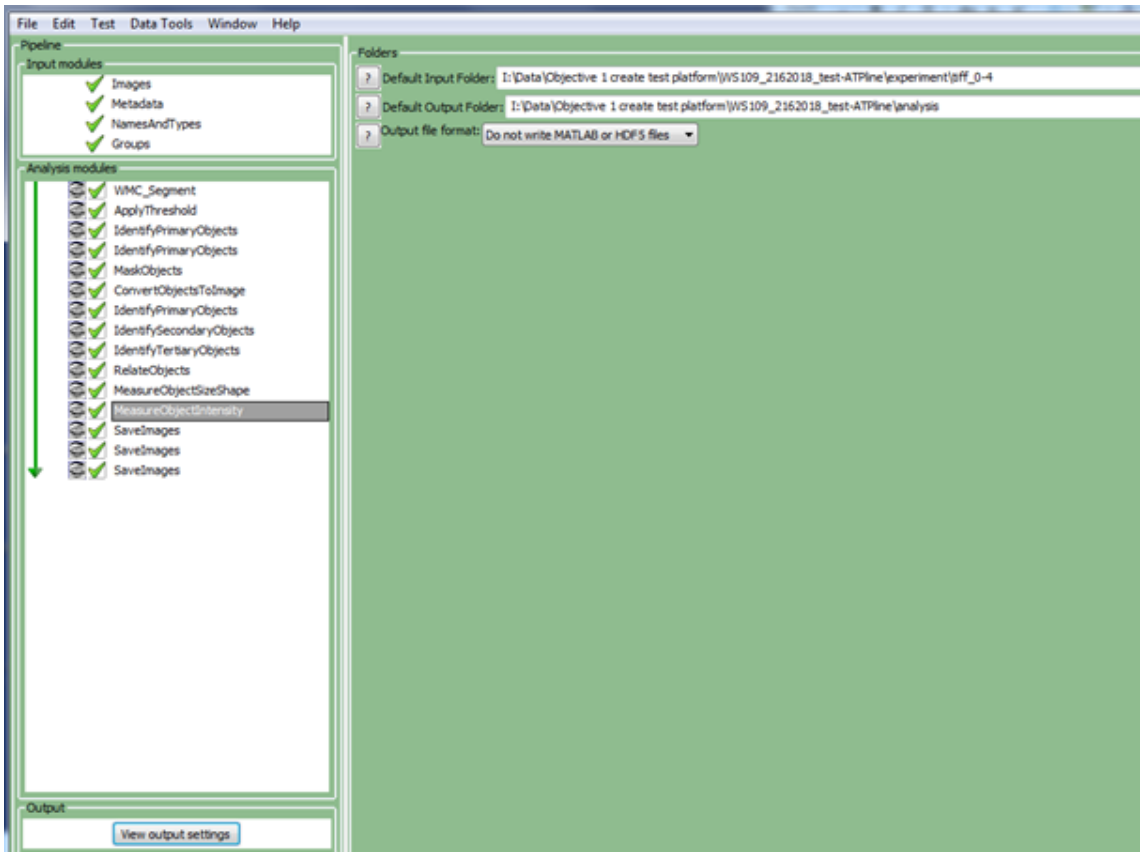


PI staining

- Input = PI/AnV image
- Identify the PI staining in the primary picture (IdentifyPrimaryObject)
- Overlay these PI objects with the nuclei objects and save the overlapping areas (MaskObjects)
- Create new image (ConvertObjectsToImage)
- Identify the PI objects that are still present after the overlay (IdentifyPrimaryObject)

3. Cell Profiler analysis > set Output

- Update “view output settings” by updating the input and output folders



- Start Cellprofiler analysis.
  The output of the CellProfiler run is a HDf5file with all measured parameters and when asked for folders with segmentation images per created .tiff.

Reading out data from an HDF5 can be challenging. The summary data per image can be read out as a .csv file as well. This won't allow single cell data read outs.

Data collection

The output files of the CellProfiler® analysis are the segmentation images (.png) to make a manual take of the segmentation possible. Furthermore the measured objects (nuclei, pi and AnV objects), and intensities are saved in an .HDF5 file.

An R-script assembled in house is used to extract the features of interest for the researcher. Based on the packages: rhdf5, stringr, plyr, data.table, doParallel, ggplot2, reshape2, grid, shiny and ggvis.

Within a graphical user interface, the desired readouts for further analysis were picked:

- Nuclei number (imageCountparentObj)
- Integrated GFP intensity for reporter with GFP signal in the cytoplasm. (Cytoplasm_Intensity_IntegratedIntensity_image_GFP)
- Fraction GFP positive cells (countGFPpos, amount of cells with a GFP intensity more than two times DMSO control).

- Fraction PI positive cells (count_PI_masked_primaryID_AreaShape_Area.DIV.Nuclei_AreaShape_Area_larger_0.1_)

DOSE RESPONSE MODELING

The dose response modelling and calculation of the EC/IC values was based on a Konstanz developed R script.

Step 1 = An in-house script was used to determine the maximal value of the sigmoidal curve. This maximal point was set to hundred per cent to be able to compare all inter lab data.

Step 2 = The created dose responses curves were used to perform the calculation of the EC/IC values using the Konstanz R script.

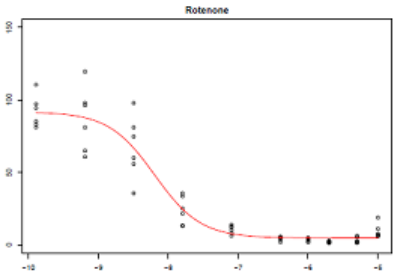
Figure 2. Example of dose response modelling

A) Curve used for normalisation to 100%

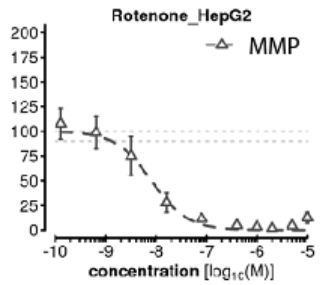
B) Normalised curve used for BMC modelling in Konstanz tool

C) The results from the IC/EC value calculations

A



B



C

BMR	BMC	BMCL	BMCU
BMR5	-9.15	-9.92	-8.89
BMR10	-8.88	-9.33	-8.67
BMR15	-8.72	-9.05	-8.53
BMR20	-8.59	-8.86	-8.43
BMR25	-8.49	-8.71	-8.34
BMR30	-8.4	-8.6	-8.26
BMR50	-8.09	-8.26	-7.98

Prediction Model

Rationale of your test method (= scientific purpose)

The combination of the MMP dye, glycolytic, viability and cell death measurements enables us to quantify and monitor mitochondrial functioning over time in the HepG2 cell line. The usage of automated confocal imaging creates a high throughput manner to study time and dose response relationships. This screening platform provides the opportunity to correlate the various mitochondrial functional read outs, to be able to establish the point of departure in dose and time of mitochondrial perturbation.

Prediction model

This cellular system can be used to predict to what extent, in which timeframe and at which concentration a specific compound induces mitochondrial perturbation and or will be cytotoxic.

Mitochondrial perturbation

The mitochondrial membrane potential can be used as a measure for the integrity of the mitochondria. Furthermore the glycolytic capacity demonstrates the switch in ATP source which is correlated with malfunctioning of mitochondria. The processes can be described with the following read outs.

The dye intensity per cell demonstrates the status of the potential compared to a non-treated condition. The glycolytic capacity of the cell can be based on the lactate concentration which is a by-product of the glycolysis.

Cytotoxicity

The threshold of cytotoxicity is determined by dose response modelling of the PI and resazurin readout.

Prediction model setup

The options for the use of this set of assays as a prediction model are not jet investigation.

Dose response modelling can be done via BMDExpress.

IVIVE In vitro – in vivo extrapolation

The specific measurements are not jet used for *in vivo* extrapolation.

Applicability domain

The applicability of this testing system will be a first tier high throughput testing screen for the identification for the test compounds ability to induce mitochondrial perturbation.

Incorporation in test battery

The mitochondrial functions assays in HepG2 can be used in combination with the other assays for comprehensive testing of a broad range of mitochondrial stress responses.

Strengths	Weaknesses
1. High-content and –throughput	1. Correct handling is crucial for HepG2 cells
2. Live time dynamics	2. Lack of metabolic capacity in HepG2 cell system
3. Identification of AOP while cell death measures can be observed	3. The need of a powerful confocal microscopy system including heater and CO_2 for live cell imaging
4. Stand-alone test system	
5. Single cell measurements	
6. Live monitoring of mitochondrial functioning	

Annexes

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Annex 1.6. Measurements mitochondrial membrane potential in differentiated human neuroblastoma SH-SY5Y cells after exposure to chemicals

Method	SH-SY5Y: MMP using Rho123
Contact	Anna Forsby (Swetox) -> anna.forsby@swetox.se

Standard Operating Procedure (SOP)

1. PURPOSE

The SOP describes how mitochondrial membrane potential in SH-SY5Y cells can be determined with Rhodamine 123 staining.

2. SCOPE

Mitochondrial membrane potential (MMP) is correlated with the cells' ability to produce ATP through oxidative phosphorylation. Acute changes in MMP are monitored by measuring the changes in fluorescence intensity after loading the cells with Rhodamine 123 and exposing them to a selection of chemicals for 24 hours.

3. INTRODUCTION

The mitochondria in all cells produce ATP by means of the proton electrochemical gradient potential (ΔP) which takes place at the inner mitochondrial membrane. The two key factors generating the force need for the movement of electrons across the mitochondrial membrane are the mitochondrial membrane potential (MMP) and to a lesser degree the mitochondrial pH gradient. A variety of dyes designed for measurement of MMP have been design over the years, these dyes have the ability to equilibrate across the mitochondrial membrane displaying a relationship between the accumulation of dye and the MMP of the cells. Thus, factors affecting the polarisation state of the mitochondrial membrane can be assessed by measuring the fluorescence intensity of the mitochondria after incubation with the dye.

4. RESPONSIBILITY

All personnel performing this assay should follow this SOP.

5. PROCEDURES

METHOD OUTLINE

Rhodamine 123 is for staining mitochondria, Hoechst for nuclear staining and Propidium Iodide (PI) for viability measurements.

For experiments, SH-SY5Y cells are plated in 96-well plates (10 000 cells/well) in EMEM with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids, 100g streptomycin/mL and 100 U penicillin/mL, which is exchanged to differentiation medium

with 1 µM RA and without serum (DMEM:F12 [1:1], N2 supplements [diluted 1:100], 1 mM glutamine, 100g streptomycin/mL and 100 U penicillin/mL) after 24 h.

Six (6) days-differentiated SH-SY5Y cells are stained with 1 µM Rhodamine123 for 45 minutes before the exposure with test chemicals. The effect of the test chemicals on the mitochondrial membrane potential is reflected as an increased (hyperpolarisation) or decreased (depolarisation) Rhodamine 123 fluorescence intensity.

DEFINITIONS/ABBREVIATIONS

The following abbreviations are used:

ΔP	Proton electrochemical gradient potential
DMEM/F12	Dulbecco's modified medium and Ham's F12 medium (1:1)
DMSO	Dimethyl sulfoxide
EMEM	Minimum essential medium with Earle's salts
RA	All-trans retinoic acid
MMP	Mitochondrial membrane potential
FBS	Foetal bovine serum
PEST	Penicillin Streptomycin
NEAA	Non-essential amino acids
PI	Propidium Iodide

MATERIALS

A. Cell Culture Type

Human neuroblastoma SH-SY5Y cell line is cultured and differentiated as described in SOP_SH-SY5Y_16_v01.

B. Controls

a Positive controls: FCCP 2µM and 400 nM, Rotenone 10 µM.

b Negative controls: DMSO 0,1%.

C. Technical Equipment

1. Cell cultures

   a Laminar Air Flow cabinet, class 2, for protection of laboratory personnel and material

   b Cell culture incubator with humidified CO_2 atmosphere (5% in air), 37ºC

   c Multichannel pipette (100-200 µl)

2. Mitochondrial membrane potential measurements

   a. Fume hood for work with test chemicals

b. Multichannel pipette (50, 100 µl)

c. Incubator, 37ºC, 5% CO_2

d. ImageXpress® Micro (Molecular Devices, UK)

e. MetaXpress® Software (Molecular Devices, UK)

D. Reagents, Chemicals, Media, Sera, Plastic ware

1. Reagents and chemicals

a Rhodamine 123 (ThermoFisher, cat no R302).

b Hoechst 10 mL (ThermoFisher, cat no H3570), 10mg/mL.

c Propidium iodide solution (Sigma, cat no P4170).

d DMSO (Sigma, cat no D4540).

e Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (Sigma, cat no C2920).

f *All*-trans RA (Sigma, cat no R2625), 10 mM in 99% ethanol.

2. Media, sera and other cell culture reagents

Cell culture medium is prepared as described in SOP_SH-SY5Y_16_v01. Briefly;

a. Cell culture medium, denoted Complete EMEM: EMEM with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids, 100 µg streptomycin/mL and 100 U penicillin/mL.

b. Differentiation medium, denoted **N2-RA medium**: DMEM:F12 [1:1], N2 supplements [diluted 1:100], 1 mM glutamine, 100 µg streptomycin/mL and 100 U penicillin/mL. On the day of start of differentiation, RA [1mM] (dissolved in ethanol) is added to a final concentration of 1 µM (0.1% ethanol).

c. N2 supplements must be aliquoted in 1 mL volumes for 100 mL complete N2-RA medium. The aliquots must be stored at -20ºC.

All-trans retinoic acid

[Note: RA must be handled and stored in dark to avoid degradation.]

1. Prepare a 10 mM stock solution of RA in 99% ethanol. Store the RA stock solution at -20ºC. Moderate heating may be needed to dissolve all RA.
2. Prepare a working solution of 1 mM in 99% ethanol. Store the solution at -20ºC.
3. When applied to cells, dilute the working solution [1mM] 1:1000 in cell (N2) medium. The final concentration of RA will be 1 µM (in 0.1% ethanol).

3. Plastic ware and other materials

a. Black cell culture microplate, 96-well plate with microclear bottom (Greiner, No 655090)

b. 1.5 mL eppendorf tubes, polypropylene (VWR, cat no 700-5239)

c. 2 mL eppendorf tubes, polypropylene (VWR, cat no 211-2120)

d. 50 mL Reagent reservoir, polystyrene (Sigma, cat no CLS4870-200EA)

e. 15 mm syringe filter with regenerated cellulose membrane, pore size 0.2 µM (Sigma, cat no CLS431215-50EA)

METHODS

A. Preparations of Media and Solutions

Cell culture media

Cell culture media are prepared as described in SOP_SH-SY5Y_16_v01. Briefly;

a. Cell culture medium: EMEM with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids, 100 µg streptomycin/mL and 100 U penicillin/mL. To prepare one bottle add:

- 500 mL EMEM
- 50 mL FBS
- 5 mL PEST
- 5 mL L-Glutamine
- 5 mL NEAA

b. Differentiation medium: DMEM:F12 [1:1], N2 supplements [diluted 1:100], 1 mM glutamine, 100 µg streptomycin/mL and 100 U penicillin/mL. On the day of start of differentiation, RA [1mM] (dissolved in ethanol) is added to a final concentration of 1 µM (0.1% ethanol).

B. Preparation of Cells for Assays

[Note: work with cell cultures is performed under sterile conditions.]

1. Detach confluent SH-SY5Y cell cultures by trypsination.
2. Prepare single cell suspension: Count the cells, and dilute the cell suspension to 100 000 cells/mL in EMEM with supplements.
3. Add 200 µl cell culture medium (EMEM without supplements) to the wells in columns 1 and 12 and rows A and H.
4. Plate 100 µl of the cell suspension in each well of rows B-G in columns 2-11 to obtain a final cell density of 10 000 cells per well. Use a multi-channel pipette

with 100 µl aliquot dispenser function. Flush at the lowest possible speed in the centre of each well to get an even cell distribution over the surface.

5. Wrap the plates in plastic foil and place them immediately in the incubator.

Figure 1. Plate setup when seeding. Grey box- 200 µL EMEM and blue box- 100 µL cell suspension

	1	2	3	4	5	6	7	8	9	10	11	12
A	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL
B	200 µL											200 µL
C	200 µL			**100 µL/well 100 000 cell/mL in EMEM**								200 µL
D	200 µL											200 µL
E	200 µL											200 µL
F	200 µL											200 µL
G	200 µL											200 µL
H	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL

C. Differentiation of cells

[Note: RA must be handled and stored in dark to avoid degradation.]

1. On the day after plating, the cells are differentiated by changing the EMEM + supplements medium to N2-RA medium.
2. Prepare the estimated volume of N2-RA medium in a 50 mL reagent reservoir (Corning Costar 4870).

Table 1. Differentiation medium (N2-RA) preparation

Number of plates	N2 medium (mL)	RA [1mM] µL
1	7	7
2	14	14
3	20	20
4	26	26

3. Remove the EMEM + supplements row by row with a 12 channel pipette and immediately replace with 100 µL N2 –RA added gently to avoid flushing away the cells.
4. Wrap the plates in plastic foil and place them immediately in the incubator.
5. After 72 hours remove 50 µL of the N2RA medium inside the wells and replace with 50 µL of freshly prepared N2RA medium. The exposure with test chemicals will be performed after the cells have been differentiated for 6 days.

D. Exposure with chemicals

Preparation of Test Chemicals

General

a. All work with test chemicals must be performed in a fume hood.

b. Test chemicals are stored as recommended by the suppliers.

c. Before use, risk evaluation must be performed for each test chemical. The risk assessment must be approved by the SHE group.

d. Solubility properties are checked in Material Technical Sheets provided by suppliers or at the Santa Cruz website and indicated in the cross systems chemicals table.

e. The test chemicals are dissolved by vortexing or heating. No precipitates must be present in the stock solutions. Careful notes are taken about the solubility of the test chemicals.

Test chemicals

a. Weigh an amount that is close to the indicated amount of the test chemical in a 2 mL Eppendorf tube.

b. Dissolve the test chemical in 100% DMSO yielding a concentration of 10 mM or to the solubility limit.

c. Aliquot the stock solutions and store them at -20 ˚C.

pH of test chemical solutions

1. Check for any pH changes in the medium for the highest concentration by visual analysis of the colour of phenol red.

[Note: The work has to be performed under sterile conditions.]

General

1. Prepare 1-5 mL of a 20 µM solution of the test chemicals dissolved in DMSO in exposure medium, N2-RA.
2. Sterile filter the 20 µM solution into a sterile 1.5 mL eppendorf tube.
3. For each experiment, 5 dilutions of the test chemical are prepared. All dilution steps must contain the same concentration of vehicle, this is usually 0.2% for DMSO. This will be diluted to 0.1% DMSO in the cell plate.
4. The dilutions series are prepared in sterile polypropylene 1.5 mL eppendorf tubes. See Table 3.

Concentrations for main experiment

All compounds are tested at fixed concentrations of 10 µM, 2µM, 400 nM, 80 nM and 16 nM. The cells in the controls are exposed to DMSO 0.2% which will be diluted to DMSO 0.1% when added to the control wells:

Table 2. Preparation of DMSO 0.2% for exposure of controls

Number of plates	N2-RA medium (mL)	DMSO 100% (µL)
1	1	2
2	2	4
3	3	6
4	4	8

Test chemical dilutions

The dilution series are prepared in a stepwise manner to the final well concentrations (table 3). The highest concentration (D1) is prepared by diluting the stock solution 500x in N2RA medium, yielding 0.2% DMSO. Thereafter, the dilution series are prepared in N2-RA medium + 0.2% DMSO. Every dilution step needs to be vortexed before the next dilution step.

Table 3. A typical pipetting scheme for preparation of the dilution series for compounds dissolved in DMSO

Sample name	Conc. In ep-tube (M)	Test chemical (µL)	N2-RA Medium (µL)	Conc. In wells (M)
D1	2,00E-05	1000	0	1,00E-05
D2	4,00E-06	100 of D1	400	2,00E-06
D3	8,00E-07	100 of D2	400	4,00E-07
D4	1,6E-07	100 of D3	400	8,00E-08
D5	3,2E-08	100 of D4	400	1,60E-08

The dilution series and the assay controls are transferred to rows B-G (50 µl per well) according to the cell plate configuration Figure 2.

Figure 2. Plate configuration for MMP measurements.

Four compounds are measured in the same plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM
B	EMEM	DMSO	DMSO	DMSO	DMSO	DMSO	Co3 D1	Co4 D1	Co4 D1	FCCP 400 nM	FCCP 400 nM	EMEM
C	EMEM	Co1 D1	Co1 D1	Co2 D1	Co2 D1	Co3 D1	Co3 D2	Co4 D2	Co4 D2	FCCP 2 µM	FCCP 2 µM	EMEM
D	EMEM	Co1 D2	Co1 D2	Co2 D2	Co2 D2	Co3 D2	Co3 D3	Co4 D3	Co4 D3	FCCP 2 µM	FCCP 2 µM	EMEM
E	EMEM	Co1 D3	Co1 D3	Co2 D3	Co2 D3	Co3 D3	Co3 D4	Co4 D4	Co4 D4	Rotenone 10 µM	Rotenone 10 µM	EMEM
F	EMEM	Co1 D4	Co1 D4	Co2 D4	Co2 D4	Co3 D4	Co3 D5	Co4 D5	Co4 D5	Rotenone 10 µM	Rotenone 10 µM	EMEM
G	EMEM	Co1 D5	Co1 D5	Co2 D5	Co2 D5	Co3 D5	DMSO	DMSO	DMSO	DMSO	DMSO	EMEM
H	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM	EMEM

Preparation of positive control and staining stocks

Rhodamine 123:

Prepare a 1 mM stock solution of Rhodamine 123 as follows:

- Weigh in 25 mg Rhodamine 123 (MW 380.83) and dilute them in 2525 µL 100% DMSO under sterile conditions. The final concentration of the Rhodamine 123 solution will be 26 mM.
- Dilute 39 µL of the previously made 26 mM Rhodamine 123 solution 961 µL 100% DMSO. The final concentration of the Rhodamine 123 solution will be 1 mM.
- Store the solutions at -20˚C.

FCCP:

- Prepare a 50mM stock solution by dissolving 10 mg FCCP in 787 µL 100% DMSO.
- Prepare a 5mM FCCP working solution by diluting 100 µL of the 50mM FCCP stock solution in 900 µL 100% DMSO.
- Store at -20˚C.

Propidium iodide (PI)

- Prepare a 5 mg/mL (7.48 mM) stock solution by diluting 10 mg PI in 2 mL PBS. Store at 4˚C protected from light.
- Prepare a 20 µM PI working solution by diluting 27 µL of the 5 mg/mL PI stock solution in 9973 µL PBS. Store at 4˚C protected from light until further use.

E. Rhodamine staining and exposure to chemicals

1. Approximately 72 hours after start of differentiation remove 50 µL cell medium from the 60 innermost wells and replace with 50 µL fresh N2-RA containing 2µM RA.
2. The exposure starts about 6 days after start of differentiation in N2-RA medium.
3. Prepare a staining solution containing 2µM Rhodamine 123. For two plates, dilute 140 µL of 1 mM Rhodamine 123 in 6860 µL N2-RA medium.
4. Remove 50 µL N2-RA medium from all wells one row at the time with a 12 channel pipette and immediately replace with 50 µL of staining solution containing 2 µM Rhodamine 123 (the final concentration of Rhodamine in the wells is 1 µM).
5. Incubate for 45 minutes at 37˚C with 5% CO_2 in darkness.
6. Meanwhile prepare the dilutions of the test compounds and controls (10 µM rotenone, 2 µM FCCP and 0.4 µM FCCP).
    a. **FCCP:** FCCP in two concentrations is used as a positive control. Dilute 4 µL of the 5mM FCCP stock in 996 µL N2-RA to prepare a 20 µM FCCP working solution. Dilute 100µL of the 20 µM FCCP working solution in 400 µL N2RA to obtain the 4µM FCCP positive control. Repeat the same procedure but this time diluting 100 µL of the 4µM FCCP in 400 µL N2RA to obtain a 0.8 µM FCCP positive control. These solutions will be diluted to 2 and 0.4 µM respectively once added to the cell plate.
    b. **Rotenone:** Rotenone 10µM is used as an internal standard, a decrease in the MMP of about 60% is expected after 24 hours of exposure. To prepare the working solution dilute 2µL of the stock (10mM) in 998 µL N2-RA medium, the final concentration will be 20 µM which will be diluted to 10 µM in the cell plate.

7. After incubation with Rhodamine 123, remove completely the N2-RA + staining solution one row at the time with a 12 channel pipette and wash once with 200 µL pre-warmed DMEM:F12 medium without additives.
8. Add 50 µl N2-RA medium to all wells and 50 µL of each concentration according to the plate layout (Figure 2). Start by adding N2-RA + DMSO 0.2% to the controls.
9. Make a note on the plate lid stating which chemicals were added to the cells, date and time.
10. Place the plate immediately in the incubator (37˚C, 5% CO2) for 24 hours.
11. On the day of measurements prepare a staining solution containing PI and Hoechst as follows:

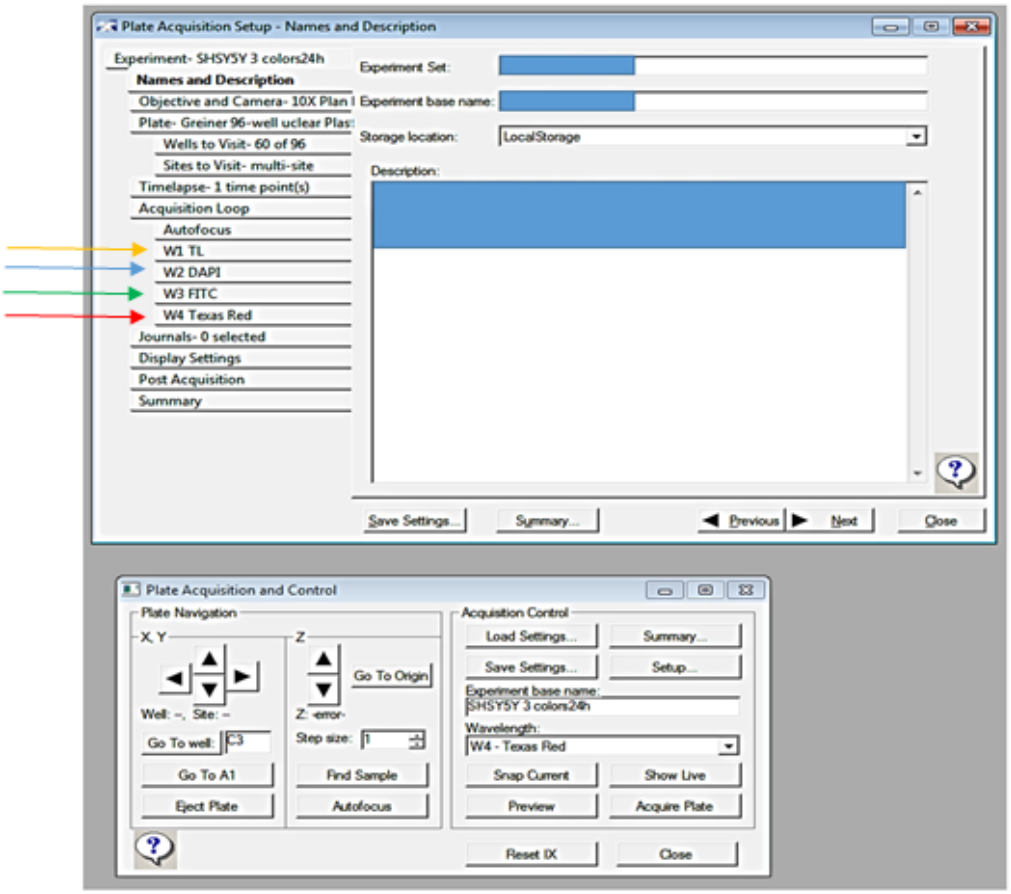
    PI + Hoechst: Sonicate the Hoechst stock for 15 minutes before use. Add 500µL of 20 µM PI and 2.5 µL of 10 mg/mL Hoechst to 1500 µL N2-RA medium. The final concentration of PI in the staining solution will be 5 µM which is diluted to 833 nM in the cell plate. The final concentration of Hoechst in the staining solution will be 20 µM, which is diluted to 3.3 µM in the cell plate.
12. Add 20 µL of the staining solution containing PI + Hoechst to all wells 30 minutes before measuring (after 23 hours 30 minutes incubation with compounds). Place the plate inside the incubator until it is time to start the measurements.

F. Measurement of MMP

ImageXpress® Micro (Molecular Devices, UK) is used to obtain images of the exposed cells. Nine images per well are taken with 4 four channels: DAPI (blue arrow), TL (yellow arrow), FITC (green arrow) and Texas red (red arrow).

1. Turn on the machine 30 minutes before starting the measurements.
2. Open MetaXpress®
3. On the top menu of the MetaXpress® software, select screening → plate acquisition set up.
4. On the top menu of the MetaXpress® software, select screening → plate acquisition and control.
5. Once the plate acquisition set up and plate acquisition and control windows are opened, start by writing the name of the experiment and a description under the "Names and description" tab (see below, Figure 3).

Figure 3. "Names and description" tab.



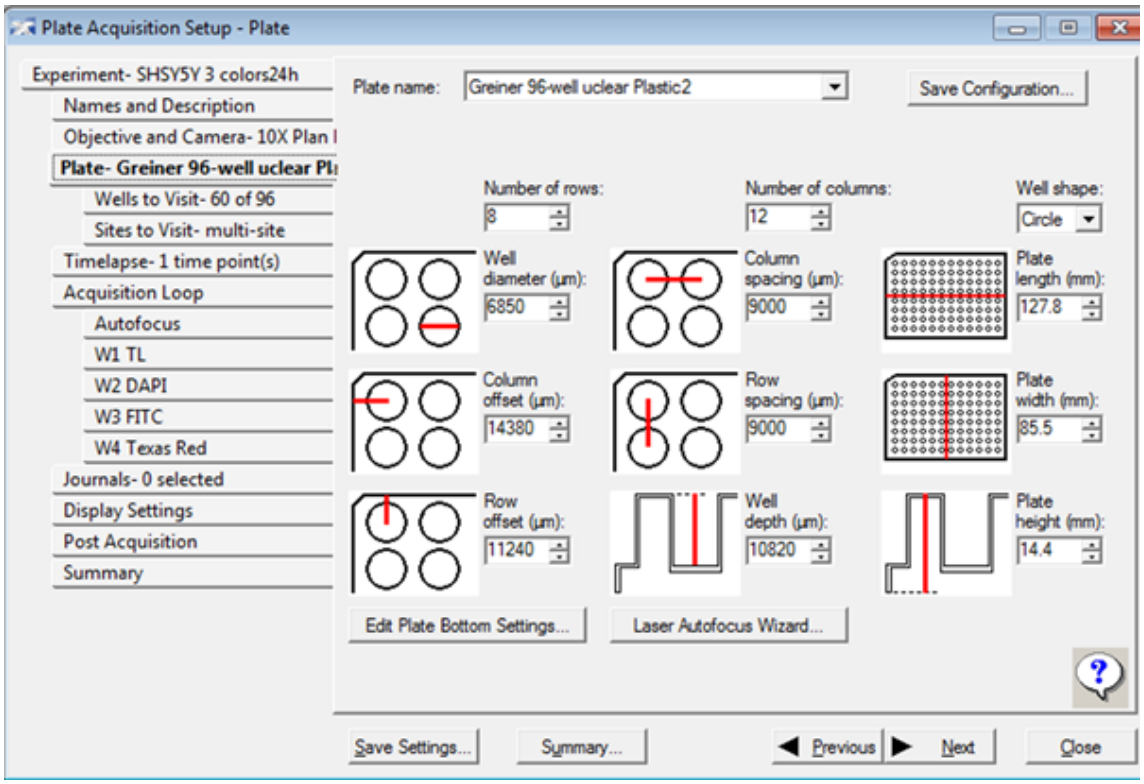
6. Under the "objective and camera" tab, select a 10X magnification, camera binning 1 and gain 1. These settings should only be modified by the instrument's responsible person or technician.

7. Under the "Plate" tab use the settings optimised for 96 well black microclear bottom cell culture plates (Greiner, No 655090). Figure 4 contains a description of the settings used for 96 well plates in this instrument, these settings should only be modified by the instrument's responsible person or technician.

   [Note: different batches of plates can have differences in the well depth within the same plate, this will affect the sharpness of the image taken during the testing of the settings but it will not affect the final images]

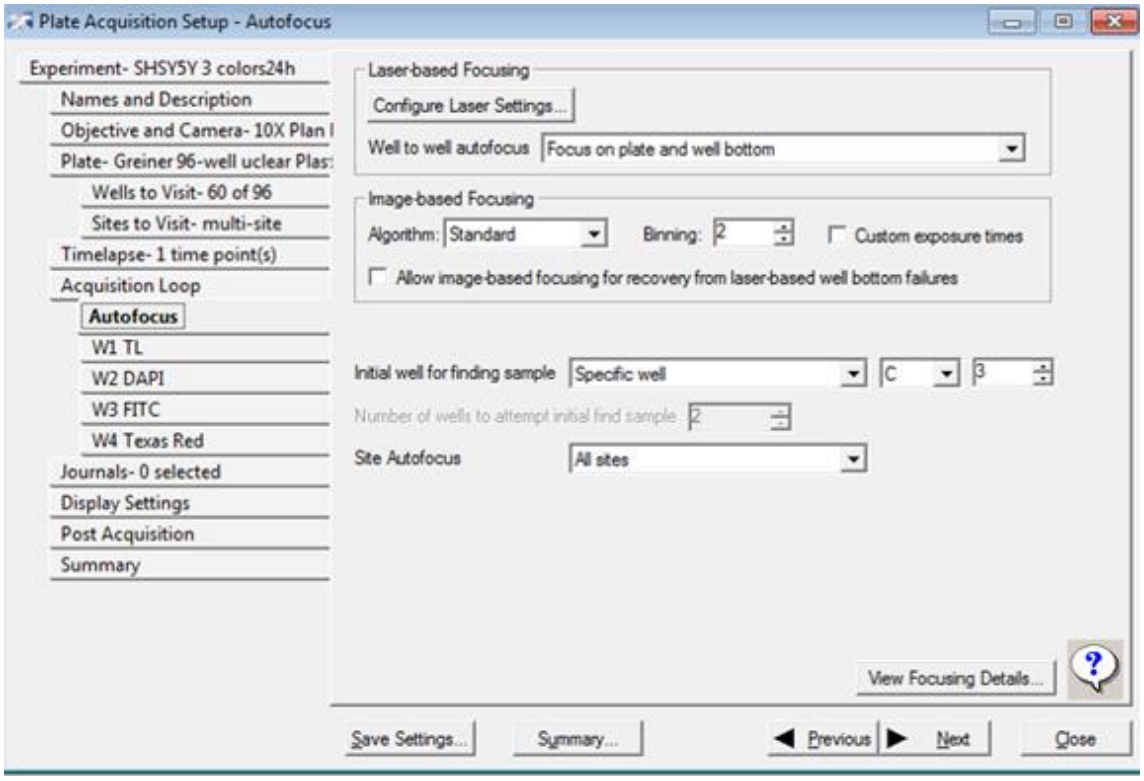
Figure 4. "Plate" tab including the setting that have been standardised for measurements in 96 well plates.

These settings should only be modified by the person responsible for the instrument.



8. Under the "wells to visit" tab, select the 60 innermost wells for analysis. Make sure that the selected wells are showed in a green colour.
9. Under the "sites to visit" tab, select 3 columns and 3 rows to obtain 9 images/well.
10. Under the "timelapse" tab select number of endpoints = 1.
11. Under the "autofocus" tab (see Figure 5) select:
    a. Well to well autofocus: focus on plate and well bottom.
    b. Imaged based focusing:
        i. Algorithm: standard
        ii. Binning: 2
    c. Initial well for finding sample: specific well
    d. Site autofocus: all sites.

Figure 5. "Autofocus" tab with the settings used for measurements in 96 well plates.



12. Under the tabs W1 to W4 the setting for image acquisition in these four wavelengths is specified:

Table 4. Settings used for the acquisition of images with MetaXpress®.

The specific exposure time, target maximum intensity and Z- offset set for each of the four wavelengths used to measure MMP in SH-SY5Y cells are described.

Illumination setting	TL	DAPI	FITC	Texas Red
Exposure time (ms)	5	150	400	400
Target max intensity	16960	34464	33000	33000
Z-offset	8.94	4.48 µm	11	11.96
Calculate Offset (use Z stack and Custom range)	Range: 50 um Step: 1.5 um	Range: 50 um Step: 1.5 um	Range: 50 um Step: 1.5 um	Range: 50 um Step: 1.5 um

13. Insert the plate in the imager
14. With the mouse, right click button select one of the 9 sites and one control well, select W1 TL or W2 DAPI to evaluate cell distribution and morphology within the well. To be able to see the cells click on "find sample" in the plate acquisition and control window.
15. With the mouse right click button select one of the 9 sites and one control well, select W3 FITC to evaluate Rhodamine 123 staining.
16. With the mouse, right click button select one of the 9 sites and one FCCP exposed well. Select W4 Texas Red to evaluate PI staining.
17. Click under the "summary" tab → acquire plate.

18. All obtained images are automatically stored in MetaXpress® in the D disk of the computer. The SOP "procedure for backing up old experiments from the imager" describes the steps to follow in order to create back up files.

Data analysis

MetaXpress® Software (Molecular Devices, UK) is used for data acquisition, the fluorescence intensity of the different dyes is measured using the following settings:

Table 5. Settings used for analysis of the images obtained with MetaXpress® after MMP measurements in SH-SY5Y cells.

Dye	Rhodamine123	PI	Hoechst
Filter	FITC	Texas red	DAPI
Approximate min width	1 µm = 2 pixels	5 µm = 8 pixels	5 µm
Approximate max width	20 µm = 31 pixels	8 µm = 12 pixels	8 µm
Intensity above local background	100 graylevels	50 graylevels	150 graylevels
Measurement	Mean fluorescence intensity per well	Number of cells stained	Number of cells stained

To analyse the images:

1. Open MetaXpress® software.
2. On the top menu, select screening → Review plate data.
3. Select the plate for analysis.
4. Select all the wells to be analysed.
5. On the "Run analysis" tab select:
   a. Analysis: <transfluor HT>
   b. Settings: FITC, Texas red or DAPI. Add the settings described in Table 5.

To export the values to Microsoft Excel:

1. Open MetaXpress® software.
2. On the top menu, select screening → Review plate data.
3. Select the plate for analysis.
4. On the "Measurements" tab select:
   a. Analysis: Hoechst/Rhodamine or PI
   b. Measurement: nuclei (transfluor HT) or Integrated granule intensity (transfluor HT).
   c. Configure log:
      i. Excel file
      ii. Open log
      iii. Log data

In Microsoft Excel, the mean Rhodamine123 fluorescence intensity is normalised against the number of cells stained with Hoechst in the same well. The results are expressed as the

% of change in the Rhodamine123 fluorescence intensity in comparison with the control (DMSO 0.1%).

$$MMP\ (\%) = \frac{\text{Rhodamine123 mean FI exposed cells}}{\text{Rhodamine123 mean FI controls}} * 100\%$$

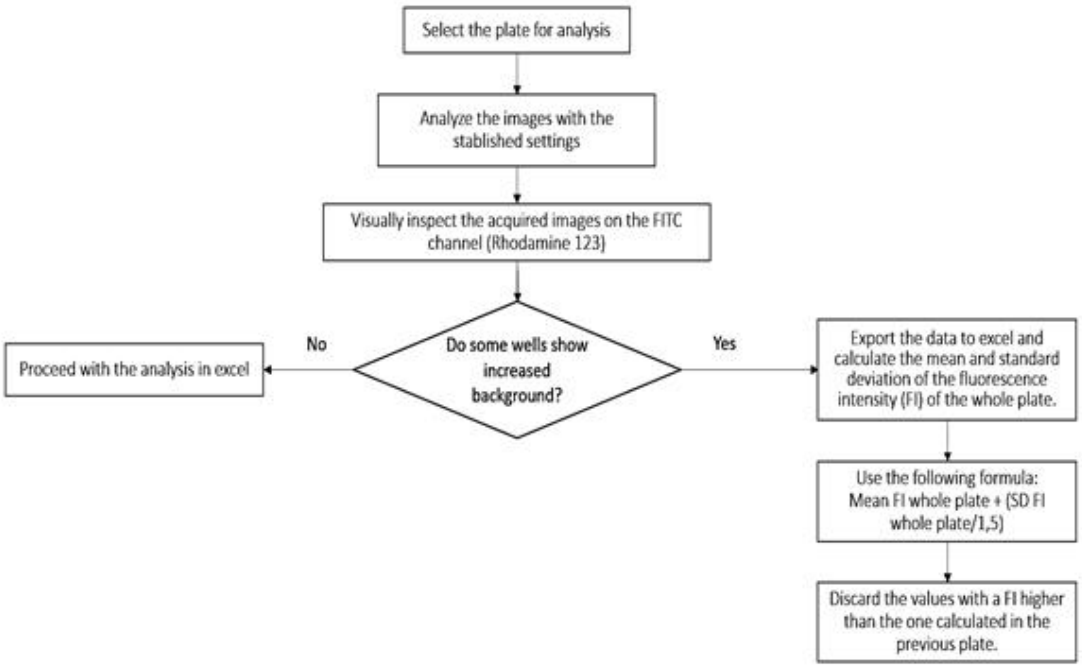
Cell viability is calculated by subtracting the number of PI stained cells from the Hoechst stained cells and normalizing against the number of Hoechst stained cells in the same well. The results are expressed as the % of viable cells in comparison with the control (DMSO 0.1%).

$$Viable\ cells\ per\ well\ (\%) = \frac{(\text{Hoechst stained cells} - \text{PI stained cells})}{\text{Hoechst stained cells}} * 100\%$$

$$Cell\ viability\ (\%) = \frac{\text{Mean viable cells exposed}}{\text{Mean viable cells control}} * 100\%$$

Outliers:

Uneven background can be produced due to difficulties during the washing steps after the first incubation with Rhodamine 123. Wells with higher background can incorrectly report increased fluorescence intensity, to avoid this problem follow these steps:



6. HEALTH SAFETY AND ENVIRONMENT

Cell culture procedures take place in a laminar air flow cabinet, class 2. All work with test chemicals is performed in a fume hood. Experiments are planned in order to reduce the exposure time of the laboratory personnel to the test chemical. Wastes of environmentally hazardous compounds are handled according to local regulations.

7. REFERENCES

ftp://ftp.meta.moleculardevices.com/support/mx/MX51CDR3/Manuals/MetaXpress_Analysis_Guide.pdf

Annex 1.7. Lactate Assay (provided by Paul Jennings)

Method	Lactate Assay
Contact	Giada Carta (VU) -> g.carta@vu.nl

Aim:

This SOP describes the enzymatic analysis of supernatant lactate, a marker of glycolysis, using a colorimetric assay.

Purpose:

Since cells increase glycolysis rates when in stress conditions (for many reasons, including mitochondrial injury) and also in tissue repair due to increased cell proliferation, lactate is a useful indicator of sub-lethal injury (Limonciel et. al.). This is especially so in renal epithelial cells, which have low levels of glycolysis once differentiated and quiescent.

Limitations:

Supernatant lactate generally increases when cells are sub-lethally injured (Limonciel et. al. and Jennings et. al.). However, when there is a significant loss of viable cells supernatant lactate will decrease.

Method Outline:

The lactate assay is based on the conversion of lactate to pyruvate by the enzyme lactate dehydrogenase (LDH), reducing the co-factor NAD to NADH (Babson *et al*). In the assay, NADH reduces PMS to PMSH which reduces INT to INTH. INTH is the reagent measured by colorimetry.

Required

TRAM buffer:

- Triethanoloamine HCl – Sigma T9534, FW=185.65
- EDTA.Na_2 – Sigma E4884, FW=372.24
- $MgCl_2$ anhydrous - Sigma M8266, FW=95.21

Colour reagent:

- PMS (N-Methylphenazonium methyl sulphate) – Sigma P9625, FW=306.3
- INT (p-iodonitrotetrazolium violet) – Sigma I8377) FW=505.7
- 100% Ethanol
- Triton X-100 – Sigma X100

β-NAD - Sigma N7004 / N0632 - FW=663.4 (- 20°C)

Lactate dehydrogenase (LDH) - Sigma L2500-25KU, approx. 12KU/mL

L-lactic acid sodium salt (standard) –Fluka Chemika 71718, FW=112.06

1. Stock TRAM solution

Concentrations: 108 mM Triethanolamine HCl, 10.7 mM EDTA.Na_2, 42 mM $MgCl_2$

Weigh out:

- 20.0 g Triethanolamine HCl
- 4.0 g EDTA.Na_2
- g $MgCl_2$ anhydrous

Bring to 1 L with ddH_20

pH to 7.5

Store at 4°C for up to 12 months

2. Stock Colour reagent

Concentrations: 1.63 mM PMS, 3.95 mM INT, 35% ethanol, 2% Triton-X-100

This solution is light sensitive. Do not leave in direct light.

- weigh out 0.050 g PMS and 0.200 g INT
- dissolve in 35 mL 100% ethanol
- add 63 mL ddH_20
- add 2 mL Triton X-100 for stabilisation (2%)
- sonicate for 5 to 10 min (until powders are fully dissolved)
- store at 4°C for up to 1 year in an amber glass vial
- discard if light yellow colour darkens towards red

3. Lactate standard

Dissolve 56 mg lactate powder into 20 mL ddH_2O to obtain a 25 mM stock.

Store at 4°C for several months.

4. Lactate assay reagent

– prepare immediately before incubation

Sample/mix ratio

For 10 µL supernatant sample, use 90 µL reagent mix (Limonciel *et al*, 2011)

Reagent mix

Concentrations: 326 µM PMS, 790 µM INT, 3.3 mM β-NAD, 4 U/mL LDH

The volume of mix depends on the number of samples to be measured. Where few biological replicates are available, it is recommended to measure each sample with 2 technical replicates.

Typically, the volume of mix in µL is: (X*90) + (16*90) + 1000

where X is the number of samples where lactate levels must be determined, "16" corresponds to the wells for standard curves, "1000" corresponds to an extra volume of 1 mL.

Calculate the volume needed of each mix component for the volume needed for your experiment, relative to the composition below.

For 10 mL reagent:

- 8 mL TRAM
- 1.98 mL colour reagent
- 22.4 mg β-NAD
- 3.3 µL LDH

Mix TRAM and colour reagent, dissolve β-NAD in, add LDH just before starting the incubation.

Mix by inversion and protect from direct light (aluminium)

Note: do not vortex the mix, as 1) it damages the enzyme, 2) it produces a lot of foam and considerably reduces the final volume.

Assay

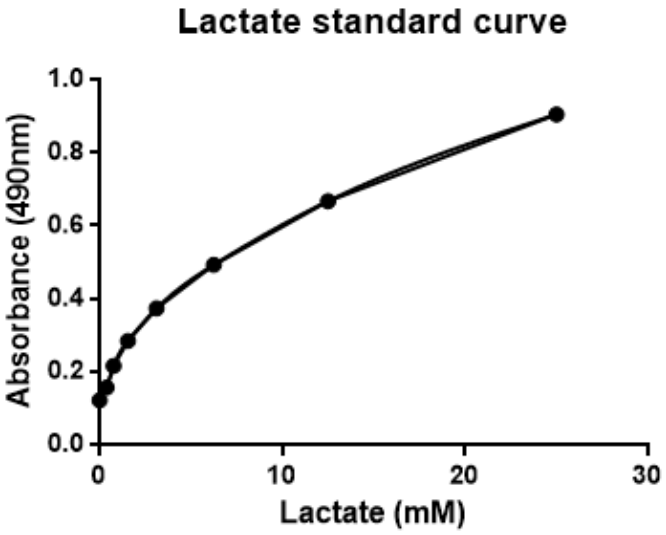
- prepare the lactate assay reagent mix fresh as described
- generate 2 **standard curves** as follows:

  Add 40 µL of 25 mM lactate standard to well A1. Add 20 µL of ddH_20 (or medium) to wells B1 through H1. Take 20 µL of standard from A1 to B1 and then C1, D1, E1, F1 and G1 (not H1). Discard volume left from G1. Using a fresh pipette tip transfer 10 µL H1 to H2. Using the same tip transfer G1 to G2 working upwards and finish with A1 to A2.
- add 10 µL of cell culture supernatant in the blank wells (starting with A3)
- assay all samples in duplicate
- once all samples and standards are added to the 96 well plate, add 90 µL of working lactate assay reagent mix using a multichannel pipette
- leave in dark for about 7 min at room temperature
- the mix goes from yellow to orange, to red. Measure before the samples develop to dark brown.
- if necessary (e.g. there is no close access to the plate reader), the assay can be stopped with 50 µL 1N HCl. Stopped assay should be light protected and read within 1 h

- read optical density (absorbance) at 490 nm (do not use a reference wavelength
- using a **spline fit function**, e.g. Prism, extrapolate unknowns from standard curve values. Do not use a linear fit as there is a slight saturation over 8 mM lactate.

Typical standard curve:

Curve was generated as described using a Tecan Rainbow plate reader with a spline fit / LOWESS (cubic spline) in GraphPad Prism.



References

Babson, A.L. and G.E. Phillips, *A rapid colorimetric assay for serum lactic dehydrogenase.* Clin Chim Acta, 1965. **12**(2): p. 210-5.

Limonciel A, Aschauer L, Wilmes A, Prajczer S, Leonard MO, Pfaller W, Jennings P. Lactate is an ideal non-invasive marker for evaluating temporal alterations in cell stress and toxicity in repeat dose testing regimes. Toxicol *In vitro*. 2011 May 24. [Epub ahead of print] PubMed PMID: 21635945.

Annex 1.8. Lactate measurement in supernatant after acute exposure (24h) in differentiated SH-SY5Y cells

Method	SH-SY5Y: Lactate Assay
Contact	Anna Forsby (Swetox) -> anna.forsby@swetox.se

Standard Operating Procedure (SOP)

1. PURPOSE

The SOP describes how cell viability can be analysed by fluorescence in differentiated SH-SY5Y cells.

2. SCOPE

The assay is a modified version of the protocol provided by Paul Jennings, adapted for differentiated SH-SY5Y cells.

This SOP describes the enzymatic analysis of supernatant lactate, a marker of glycolysis, using a colorimetric assay. Since cells increase glycolysis rates when in stress conditions (for many reasons, including mitochondrial injury) and also in tissue repair due to increased cell proliferation, lactate is a useful indicator of sub-lethal injury (Limonciel *et al.*). This is especially so in renal epithelial cells, which have low levels of glycolysis once differentiated and quiescent.

3. INTRODUCTION

The lactate assay is based on the conversion of lactate to pyruvate by the enzyme lactate dehydrogenase (LDH), reducing the co-factor NAD to NADH (Babson *et al*). In the assay, NADH reduces PMS to PMSH which reduces INT to INTH. INTH is the reagent measured by colorimetry.

4. RESPONSIBILITY

All personnel performing this assay should follow this SOP.

5. PROCEDURES

LIMITATIONS

a) Supernatant lactate generally increases when cells are sub-lethally injured (Limonciel et. al. and Jennings et. al.). However, when there is a significant loss of viable cells supernatant lactate will decrease.

METHOD OUTLINE

For experiments, supernatant collected before the resazurin viability measurements in 24 h exposed, 6 days differentiated SH-SY5Y cells was used for lactate analysis.

DEFINITIONS/ABBREVIATIONS

The following abbreviations are used:

DMEM/F12	Dulbecco's modified medium and Ham's F12 medium (1:1)
DMSO	Dimethyl sulfoxide
EMEM	Minimum essential medium with Earle's salts
RA	All-trans retinoic acid
FBS	Foetal bovine serum
PEST	Penicillin Streptomycin
NEAA	Non-essential amino acids
PMS	N-methylphenazonium methyl sulphate
INT	p-iodonitrotetrazolium violet
LDH	Lactate dehydrogenase

IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

A. Test Chemicals

The cells were pre-exposed to test chemicals before the viability measurements, simulating acute exposure.

B. Controls

a **Negative control:** Cells exposed to N2-RA medium with 0.1% DMSO.

b **Positive control:** Lactate standard curve (25 to 0.78 mM).

MATERIALS

A. Cell Culture Type

Human neuroblastoma SH-SY5Y cell line is cultured and differentiated as described in SOP plating and differentiation.

B. Technical Equipment

a. 96-well standard plate with flat bottom, solvent resistant polypropylene (Corning, cat no 3364); used as compound plate for storage of lactate samples.

b. 96-well plate with clear bottom (Corning, cat no CLS3595-50EA).

c. Triethanoloamine HCl (Sigma, cat no T9534)

d. EDTA.Na_2 (Sigma, cat no E4884)

e. $MgCl_2$ anhydrous (Sigma, cat no M8266)

f. PMS (N-methylphenazonium methyl sulphate) (Sigma, cat no P9625)

g. INT (p-iodonitrotetrazolium violet) (Sigma, cat no I8377)

h. Ethanol 99.8% (VWR chemicals, cat no 20821.296)

i. Triton X-100 (Sigma, cat no X100)
j. B-NAD (Sigma, cat no N7004)
k. Lactate dehydrogenase (LDH) (Sigma, cat no L2500)
l. L-lactic acid sodium salt (standard) (Fluka Chemika, cat no 71718)
m. Tecan® Infinite M200 Pro
n. Tecan® i-control 3.7.3.0 software

METHOD:

A. Preparation of stock solutions

Stock TRAM solution

1. Weigh out:
   - 20 g Triethanolamine HCl
   - 4 g EDTA.Na_2
   - 3,97 g $MgCl_2$
2. Bring to 1 L with ddH_2O. The final concentrations are: 108 mM Triethanolamine HCl, 10.7 mM EDTA.Na_2 and 42 mM $MgCl_2$
3. Adjust pH to 7,5
4. Store at 4 °C for up to 12 months

Stock colour reagent

This solution in light sensitive, do not leave in direct light.

1. Weigh out 0.050 g PMS and 0.2 g INT
2. Dissolve in 35 mL 100% ethanol
3. Add 63 mL ddH_2O
4. Add 2 mL Triton X-100 for stabilisation
5. Sonicate for 5 to 10 min (until powders are fully dissolved)
6. Store at 4 °C for up to 1 year in an amber glass vial

The stock colour reagent should have a light yellow colour. Discard if the colour darkens towards red. The final concentrations of all reagents are: 1.63 mM PMS, 3.95 mM INT, 35% ethanol, 2%Triton X-100

Lactate standard

Dissolve 56 mg lactate powder into 20 mL ddH_2O to obtain a 25 mM stock. Store at 4 °C for several months.

Lactate assay reagent

Prepare **immediately before** incubation

Sample/mix ratio: For 10 µL supernatant sample, use 90 µL reagent mix.

Reagent mix

The final concentrations of all reagents are: 326 µM PMS, 790 µM INT, 3.3 mM β-NAD, 4 U/mL LDH.

The volume of mix depends on the number of samples to be measured.

1. Calculate the volume of mix needed:

   Needed volume of mix in µL = (X*90) + (16*90) + 1000

   Where X is the number of samples were lactate levels must be determined, "16" corresponds to the wells for standard curves, and "1000" corresponds to an extra volume of 1 mL. For the experiment, 12 mL of reagent mix are needed to measure one plate.

2. Calculate the volume needed of each mix component for the volume needed for the experiment, relative to the composition below:

Mix component	To prepare 10 mL reagent	To prepare 12 mL reagent (one plate)
TRAM	8 mL	9,6 mL
Colour reagent	1,98 mL	2,38 mL
B-NAD	22,4 mg	26,88 mg
LDH	3,3 µL	3,96 µL

3. Mix TRAM and colour reagent
4. Dissolve β-NAD in
5. Add LDH just before starting the incubation, mix by inversion (vortexing the mix damages the enzyme and produces foam).
6. Protect from direct light (aluminium)

B. Exposure

SH-SY5Y cells were plated at a density of 10 000 cells per well and exposed for 24 hours to a selection of chemicals for subsequent measurements of cell viability using the resazurin assay. The day the cell viability was performed 150 µL of supernatant were collected and stored at -20 °C for lactate analysis.

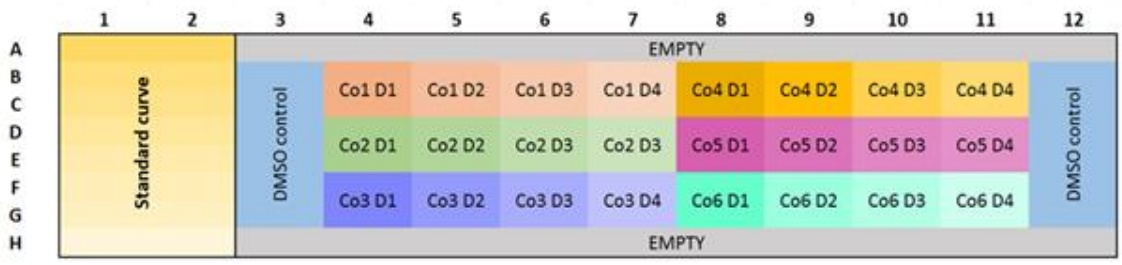
C. Lactate assay

Once all the chemicals have been tested, the collected supernatant from all the plates is analysed.

1. Bring all the plates with supernatant to room temperature
2. Prepare the reagent mix fresh as described in the previous section
3. In a clear 96 well plate generate 2 standard curves as follows:
   a. Add 40 µL of 25 mM lactate standard to well A1. Add 20 µL of ddH_20 (or medium) to wells B1 through H1. Take 20 µL of standard from A1 to B1 and then C1, D1, E1, F1 and G1 (not H1). Discard volume left from G1. Using a fresh pipette tip transfer 10 µL H1 to H2. Using the same tip transfer G1 to G2 working upwards and finish with A1 to A2.

4. Add 10 µL of cell culture supernatant in the blank wells (See plate configuration, Figure 1)
5. Once all samples and standards are added to the plate, add 90 µL of reagent mix using a multichannel pipette
6. Cover the plate with aluminium foil
7. Shake the plate for 2 minutes
8. Incubate at room temperature for 5 minutes (the mix goes from yellow to orange, to red. Measure before the samples develop to dark brown)
9. If needed, the assay can be stopped with 50 µL 1M HCl. The stopped assay should be light protected and read within 1 hour
10. Open the Tecan Infinite M200 Pro reader
11. Read the plate:
    a. Mode: absorbance
    b. Wavelength: 490 nm (Do not use reference wavelength)
    c. Bandwidth: 9 nm
    d. Number of flashes: 25
    e. Settle time: 0 ms
    f. Temperature: off
12. Store the data in the S-disk: S:\Projects\PHC33_EUToxRisk\Case study 4_Mitotox\Lactate.
13. Microsoft Excel is used to analyse the data.
14. The average absorbance of each concentration (duplicates) and standard deviation (SD) are calculated. The average absorbance value of each concentration is normalised against the mean absorbance value of control wells (12 wells) and subsequently multiplied by 100 in order to obtain the % of lactate production in comparison to the negative controls.

Figure 1. Plate configuration used for the lactate assay



IX. HEALTH SAFETY AND ENVIRONMENT

Cell culture procedures take place in a laminar air flow cabinet, class 2. All work with test chemicals is performed in a fume hood. Experiments are planned in order to reduce the

exposure time of the laboratory personnel to the test chemical. Wastes of environmentally hazardous compounds are handled according to local regulations.

X. REFERENCES

1. Babson, A.L. and G.E. Phillips, *A rapid colorimetric assay for serum lactic dehydrogenase.* Clin Chim Acta, 1965. **12**(2): p. 210-5.
2. Limonciel A, Aschauer L, Wilmes A, Prajczer S, Leonard MO, Pfaller W, Jennings P. Lactate is an ideal non-invasive marker for evaluating temporal alterations in cell stress and toxicity in repeat dose testing regimes. Toxicol *In vitro*. 2011 May 24. [Epub ahead of print] PubMed PMID: 21635945.

Annex 1.9. Resazurin Assay (provided by Paul Jennings)

Method	Resazurin Assay
Contact	Giada Carta (VU) -> g.carta@vu.nl

This method assesses cell viability based on the irreversible reduction of the blue dye resazurin to the pink product resorufin, which is highly fluorescent in red. Although the exact enzyme(s) involved in the reaction have not been identified, the assay is considered to be based on the global REDOX capacity of the cells in culture.

Resazurin stock

Resazurin [Sigma R7017], formula weight = 251.2 g/mol.

A 20X resazurin stock (880 µM) can be prepared and stored at 4°C for several months if kept sterile.

To prepare 20X stock:

- weigh out 0.011g resazurin
- add 1mL 0.1 N NaOH
- stir gently to dissolve
- bring to just under 50 mL with PBS
- mix
- adjust pH from about 11 to 7.8
- bring to exactly 50 mL with PBS
- filter sterilise (0.2 µm) under laminar flow
- store cold (4°C) and protected from light (aluminium) for up to 3 years

Assay

- warm up the 20X resazurin stock to 37°C to dissolve resazurin crystals
- **dilute 1 in 20** into warm complete culture medium (final conc: 44 µM)
- remove cell culture supernatant
- wash with sterile PBS (room temperature)
- add xx µL of diluted resazurin to the cells for 1 to 3 h

  Volume depends on the format and should be the same as that used for cell seeding and feeding, in order to cover the whole cell population in the well/dish.

  Duration depends on the cells and their REDOX capacities. For HK-2 and RPTEC/TERT1 cells, 2 h is sufficient. For HepaRG, 1h is enough. Incubation was too long if all the wells have turned from blue to pink.
- include a **blank with no cells** to determine background resazurin fluorescence. This should be incubated in similar conditions as the cells.

- measure resorufin fluorescence: **excitation at 540 nm, emission at 590 nm**. Either measure in the plate from the top or collect supernatant to a 96-well plate, including the blank with no cells.

References

1. Jennings P, Koppelstaetter C, Aydin S, Abberger T, Wolf AM, Mayer G, and Pfaller W. Cyclosporine A induces senescence in renal tubular epithelial cells. *Am J Physiol Renal Physiol*, 2007.
2. Jennings P, Koppelstaetter C, Pfaller W, Morin JP, Hartung T, and Ryan MP. Assessment of a new cell culture perfusion apparatus for *in vitro* chronic toxicity testing. Part 2: toxicological evaluation. *Altex* 21: 61-66, 2004.

Annex 1.10. Cell viability measurements with Resazurin after acute exposure (24h) in differentiated SH-SY5Y cells

Method	SH-SY5Y: Resazurin Assay
Contact	Anna Forsby (Swetox) -> anna.forsby@swetox.se

Standard Operating Procedure (SOP)

1. PURPOSE

The SOP describes how cell viability can be analysed by fluorescence in differentiated SH-SY5Y cells.

2. SCOPE

The cell viability is determined by the number of viable cells in culture. The assay is a modified version of the protocol provided by Paul Jennings, adapted for differentiated SH-SY5Y cells.

3. INTRODUCTION

This method assesses cell viability based on the irreversible reduction of the blue dye resazurin to the pink product resorufin, which is highly fluorescent in red. Although the exact enzyme(s) involved in the reaction have not been identified, the assay is considered to be based on the global REDOX capacity of the cells in culture.

4. RESPONSIBILITY

All personnel performing this assay should follow this SOP.

5. PROCEDURES

METHOD OUTLINE

We used resazurin which is irreversibly reduced into resorufin by live cells producing thereof a die fluorescent in red. The obtained fluorescence intensity correlates to the number of live cells in culture.

For experiments, SH-SY5Y cells are plated in black 96-well plates with clear bottom in EMEM with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids, 100g streptomycin/mL and 100 U penicillin/mL, which is exchanged to differentiation medium with 1 µM RA and without serum (DMEM:F12 [1:1], N2 supplements [diluted 1:100], 1 mM glutamine, 100g streptomycin/mL and 100 U penicillin/mL) after 24 h. Six (6) days differentiated SH-SY5Y cells are exposed to test chemicals and incubated for 24 hours after which the effect on cell viability is measured with resazurin.

DEFINITIONS/ABBREVIATIONS

The following abbreviations are used:

DMEM/F12	Dulbecco's modified medium and Ham's F12 medium (1:1)
DMSO	Dimethyl sulfoxide
EMEM	Minimum essential medium with Earle's salts
RA	All-trans retinoic acid
FBS	Foetal bovine serum
PEST	Penicillin Streptomycin
NEAA	Non-essential amino acids

IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

A. Test Chemicals

The cells were pre-exposed to test chemicals before the viability measurements, simulating acute exposure.

B. Controls

a. Negative control: Cells exposed to N2-RA medium with 0.1% DMSO.

b. Blank: DMEM:F12 cell medium.

MATERIALS

A. Cell Culture Type

Human neuroblastoma SH-SY5Y cell line is cultured and differentiated as described in SOP plating and differentiation.

B. Technical Equipment

a. Tecan® Infinite M200 Pro

b. Tecan® i-control 3.7.3.0 software

c. Resazurin (Sigma, Cat no R7017)

d. PBS, pH 7.4 (Gibco, Cat no 10010056)

e. Syringe filtration unit Filtropur S 0.2 (Sarstedt, Cat no 83.1826.001)

f. Black cell culture 96-well plate with clear bottom (Corning, cat no 3603).

g. 96-well standard plate with flat bottom, solvent resistant polypropylene (Corning, cat no 3364); used as compound plate for storage of lactate samples.

METHOD:

A. Preparation of the resazurin stock

The 880 µM resazurin stock is prepared as follows:

1. Weigh out 0,011g resazurin
2. Add 1000 µL 0,1 M NaOH

3. Stir gently to dissolve
4. Bring to just under 50 mL with PBS
5. Mix
6. Adjust pH from about 11 to 7,8
7. Bring to exactly 50 mL with PBS
8. Sterile filter (0,2 µm) under laminar flow
9. Store at 4 ºC and protected from light (aluminium) for up to 3 years

B. Exposure

For experiments, 10 000 SH-SY5Y cells are plated in black 96-well plates with clear bottom in complete EMEM with 10% foetal bovine serum, which is exchanged to N2-RA differentiation medium after 24 h. After 3 days of differentiation 100 µL of N2-RA differentiation medium are added on top of all wells, the plate is then placed in the incubator and the cells are allowed to differentiate for 3 days more (total differentiation time of 6 days). On the 6 day of differentiation 100 µL of cell medium are removed from the wells containing cells and replaced with 100µL of the assigned treatment.

C. Resazurin cell viability assay

The measurement of resazurin reduction is performed 24 hours after exposure to test chemicals

1. Warm up the 880 µM resazurin stock
2. For one plate, dilute 1000 µL of the resazurin stock in 9000 µL DMEM:F12 to prepare a 2X working solution
3. Remove 150 µL of cell medium from the wells and collect it in a compound plate (supernatant can be used for lactate measurements)
4. Seal the compound plate and store it at -20 ºC
5. Add 50 µL from the rezasurin working solution to all wells including blanks
6. Incubate the plate for 2 hours at 37 ºC
7. Open the Tecan Infinite M200 Pro reader.
8. Measure the resorufin fluorescence:
    a. Excitation wavelength: 540 nM
    b. Emission wavelength: 590 nM
    c. Mode: Fluorescence top reading
    d. Excitation bandwidth: 9 nm
    e. Emission bandwidth: 20 nm
    f. Gain: Calculated from B2
    g. Number of flashes: 25
    h. Integration time: 20 µs

i. Lag time: 0 µs

j. Settle time: 0 ms

k. Z-position: Calculated from B2

l. Temperature: 37 ºC

9. Store the data in the S-disk: S:\Projects\PHC33_EUToxRisk\Case study 4_Mitotox\Resazurin results\24h exposure.

10. Microsoft Excel is used to analyse the data.

11. The average fluorescence intensity of each concentration (triplicates) and standard deviation (SD) are calculated. The average fluorescence intensity value of each concentration is normalised against the mean fluorescence intensity value of control wells (12 wells) and subsequently multiplied by 100 in order to obtain the % of viable cells in each concentration.

IX. HEALTH SAFETY AND ENVIRONMENT

Cell culture procedures take place in a laminar air flow cabinet, class 2. All work with test chemicals is performed in a fume hood. Experiments are planned in order to reduce the exposure time of the laboratory personnel to the test chemical. Wastes of environmentally hazardous compounds are handled according to local regulations.

X. REFERENCES

1. Jennings P, Koppelstaetter C, Aydin S, Abberger T, Wolf AM, Mayer G, and Pfaller W. Cyclosporine A induces senescence in renal tubular epithelial cells. *Am J Physiol Renal Physiol*, 2007.

2. Jennings P, Koppelstaetter C, Pfaller W, Morin JP, Hartung T, and Ryan MP. Assessment of a new cell culture perfusion apparatus for *in vitro* chronic toxicity testing. Part 2: toxicological evaluation. *Altex* 21: 61-66, 2004.

XI. APPENDIX

Summary of the procedure for measurement of cell viability with resazurin in differentiated SH-SY5Y cells:

Day 1

Plating of SH-SY5Y cells, 10 000 cells/well.

Day 2

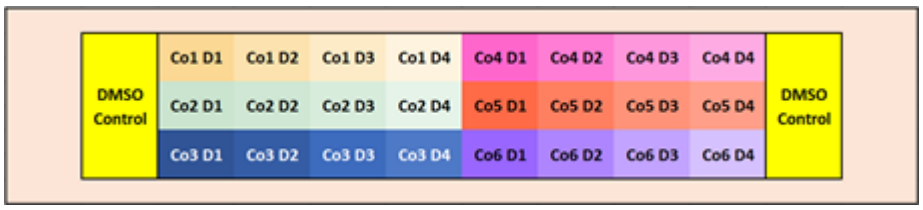
Start differentiation.
Change complete EMEM to N2-RA medium.

Day 3

Addition of fresh N2-RA differentiation medium

Day 6

Exposure to test chemicals.
Remove 100 µL N2-RA medium.
Add 100 µL of the corresponding chemical dilution.



Day 7

Resazurin viability assay (Protocol Cell viability)
Remove 150 µL of supernatant and collect it for lactate measurements.
Add 50 µL of 2X resazurin working solution.
Incubate the plate for 2 hours at 37 °C.
Record the resorufin fluorescence at 540 nM excitation and 590 nM emission with the Tecan Infinite M200 Pro reader.

Annex 1.11. Neurite degeneration measurements in differentiated SH-Sy5Y cells after acute exposure (24 hours) to chemicals

Method	SH-SY5Y: neurite degeneration
Contact	Anna Forsby (Swetox) -> anna.forsby@swetox.se

Standard Operating Procedure (SOP)

1. PURPOSE

The SOP describes how neurite degeneration can be determined by high content fluorescence microscope imaging using calcein AM in combination with a cell viability assay, using propidium iodide exclusion as marker.

2. SCOPE

The cell permeant dye Calcein-AM was used to stain the cytoplasm of differentiated cells allowing the visualisation and quantification of the neurite length. Once inside the cells, calcein-AM is hydrolysed by intracellular esterases producing a green fluorescent dye. Viable cells are visualised under the fluorescence microscope by exclusion of propidium iodide (PI).

3. INTRODUCTION

Neurite degeneration initiated at non-cytotoxic concentrations *in vitro* is an alert for neurotoxicity directed to pathologies in axons and dendrites *in vivo*. Herein, we examine neurite degeneration and cell viability in 6 days differentiated human neuroblastoma SH-SY5Y cells after 24h exposure with chemicals.

4. RESPONSIBILITY

All personnel performing this assay should follow this SOP.

5. PROCEDURES

LIMITATIONS

To be added

METHOD OUTLINE

We used the cell permeant dye calcein AM to stain the cells' cytoplasm and the dye Hoechst for nuclear staining.

For experiments, SH-SY5Y cells are plated in 96-well plates with micro-clear bottom (8 000 cells/well) in EMEM with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids, 100g streptomycin/mL and 100 U penicillin/mL, which is exchanged to differentiation medium with 1 µM RA and without serum (DMEM:F12 [1:1], N2 supplements [diluted 1:100], 1 mM glutamine, 100g streptomycin/mL and 100 U

penicillin/mL) after 24 h. Six (6) days differentiated SH-SY5Y cells are exposed to test chemicals and incubated for 24 hours after which the effect on neurite degeneration is measured by fluorescent microscopy.

DEFINITIONS/ABBREVIATIONS

The following abbreviations are used:

DMEM/F12	Dulbecco's modified medium and Ham's F12 medium (1:1)
DMSO	Dimethyl sulfoxide
EMEM	Minimum essential medium with Earle's salts
RA	All-trans retinoic acid
FBS	Foetal bovine serum
PEST	Penicillin Streptomycin
NEAA	Non-essential amino acids

IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

A. Test Chemicals

The cells were pre-exposed to test chemicals before the neurite degeneration measurements, simulating acute exposure.

Dilutions:

An initial screening was performed by testing all chemicals at 10 µM, 2 µM and 0.4 µM. For those chemicals showing effect at the lowest tested concentration during the screening, extra 5X dilution steps were performed until a no effect concentration was reached. If no effect was observed during the screening, the experiment was repeated only a second time.

Table 1. A typical pipetting scheme for preparation of the dilution series for test chemicals dissolved in DMSO.

Dilution step	Conc. In ep-tube (µM)	Test chemical (µl)	Medium + 0.2% DMSO (µl)	Conc. In wells (mM)
D1	2,00E+01	2	998	1,00E+01
D2	4,00E+00	200 of D1	800	2,00E+00
D3	8,00E-01	200 of D2	800	4,00E-01
D4	1,60E-01	200 of D3	800	8,00E-02
D5	3,20E-02	200 of D4	800	1,60E-02
D6	6,40E-03	200 of D5	800	3,20E-03
D7	1,28E-03	200 of D6	800	6,40E-04
D8	2,56E-04	200 of D7	800	1,28E-04
Controls	0	0	1000	0

B. Controls

a. **Negative control:** Cells exposed to N2-RA with DMSO 0.1%.

b. **Positive control:** Rotenone 10 µM.

MATERIALS

A. Cell Culture Type

Human neuroblastoma SH-SY5Y cell line is cultured and differentiated as described in SOP plating and differentiation.

B. Technical Equipment

a. ImageXpress® Micro (Molecular Devices, UK)
b. MetaXpress® Software (Molecular Devices, UK)
c. Calcein (Life technologies, Cat no C1430)
d. Hoechst 10 mL (ThermoFisher, cat no H3570), 10mg/mL.
e. Black cell culture microplate, 96-well plate with microclear bottom (Greiner, No 655090)

METHOD:

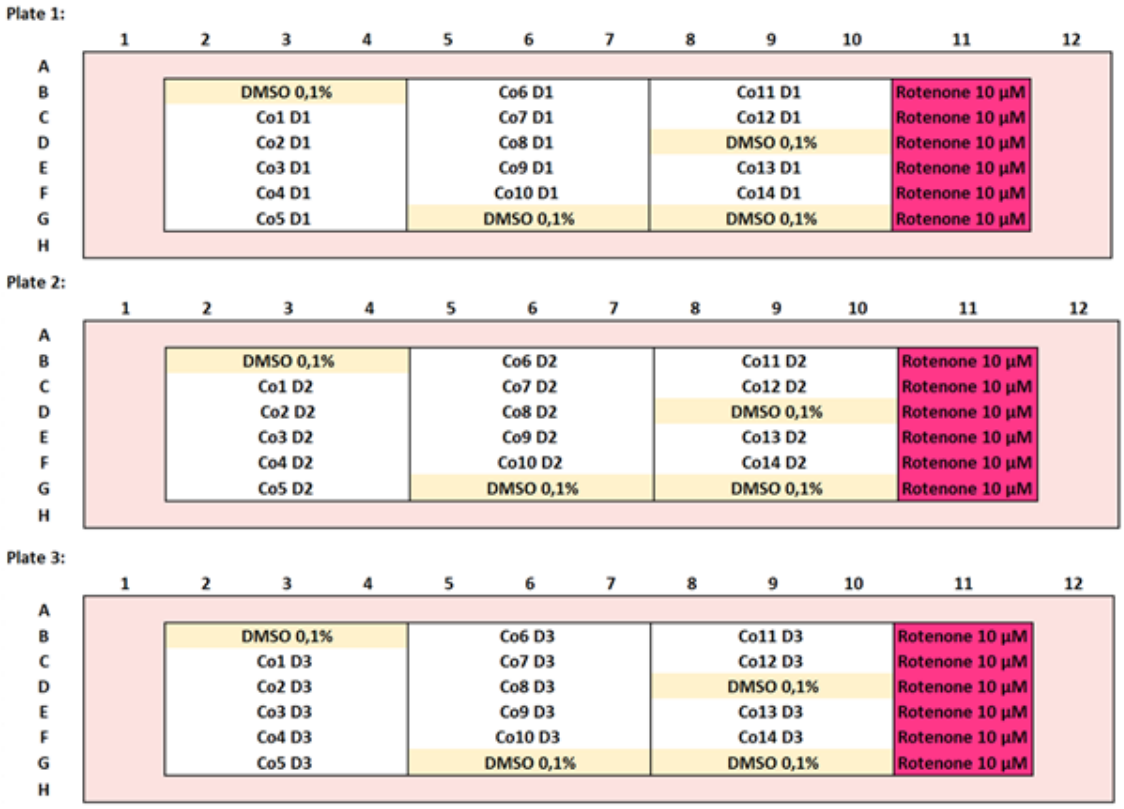
A. Preparation of Calcein stocks

1. Dilute the calcein powder (1mg) in 1 mL 100% DMSO
2. Warm up the diluted calcein to ensure proper dilution
3. Prepare 20 µL Aliquots
4. Store at 4 ºC

B. Exposure

For experiments, 8 000 SH-SY5Y cells are plated in black microclear bottom 96-well plates in complete EMEM with 10% foetal bovine serum, which is exchanged to N2-RA differentiation medium after 24 h. After 3 days of differentiation 100 µL of N2-RA differentiation medium are added on top of all wells, the plate is then placed in the incubator and the cells are allowed to differentiate for 3 days more (total differentiation time of 6 days). On the 6 day of differentiation 100 µL of cell medium are removed from the wells containing cells and replaced with 100µL of the assigned treatment.

Figure 1. Plate lay out for screening of CS4 test chemicals. Six days differentiated SH-SY5Y are exposed to 10 µM (D1), 2µM (D2) and 0.4µM (D3) in an initial screening.



C. Neurite staining

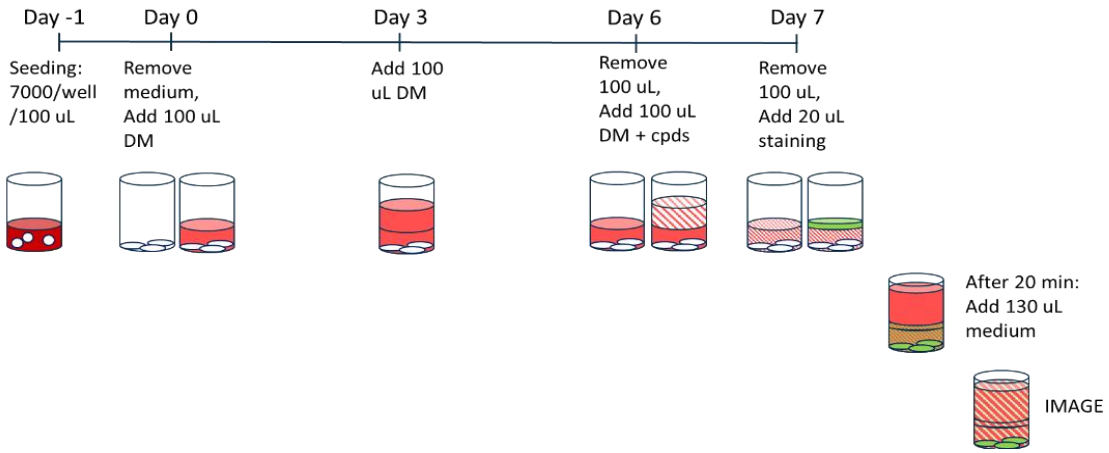
The measurement of neurite degeneration is performed 24 hours after exposure to test chemicals

1. Be very careful not to keep plates out of incubator for longer time than necessary, and be extremely gentle with pipetting
2. Take one plate at a time out, carefully remove 100 µL of medium from the wells
3. Put the plate back in the incubator as fast as possible, do not let it cool down
4. Prepare and warm up 22 mL of DMEM:F12 medium for later dilution of the staining
5. Ultrasonicate Hoechst-H33342 stock solution in ultrasonic water bath for 15 min
6. Prepare dye solution (enough for two plates):
    a. 4 mL of DMEM/F-12
    b. 12 µL Hoechst 33342 stock 10 mg/mL (16,2 mM) [final conc: 5 µg/mL]
    c. 24 µL Calcein-acetoxymethylester (CAM) stock of 1 mg/mL (1 mM) [final conc: 1 µM]
    d. Vortex

e. Protect from direct light (switch off light in the sterile hood)

7. Put dye solution into a reservoir
8. Add 20 µL of dye solution to all wells in one plate, quickly but gently pipet up and down twice in each row (without changing tips for the same treatment)
9. Incubate the plate for 20 min at 37°C, 5% CO_2
10. Put 11 mL of warm DMEM/F-12 into a reservoir
11. Take the plate out
12. With a 12-channel pipette, quickly but gently, add 130 µL of DMEM:F12 in each well
13. Immediately image the plate (calcein in FITC channel, H33342 in DAPI channel, 4 sites/well)

Figure 2. Overview of the experimental set up used for imaging of neurite degeneration on six days differentiated SH-SY5Y cells.

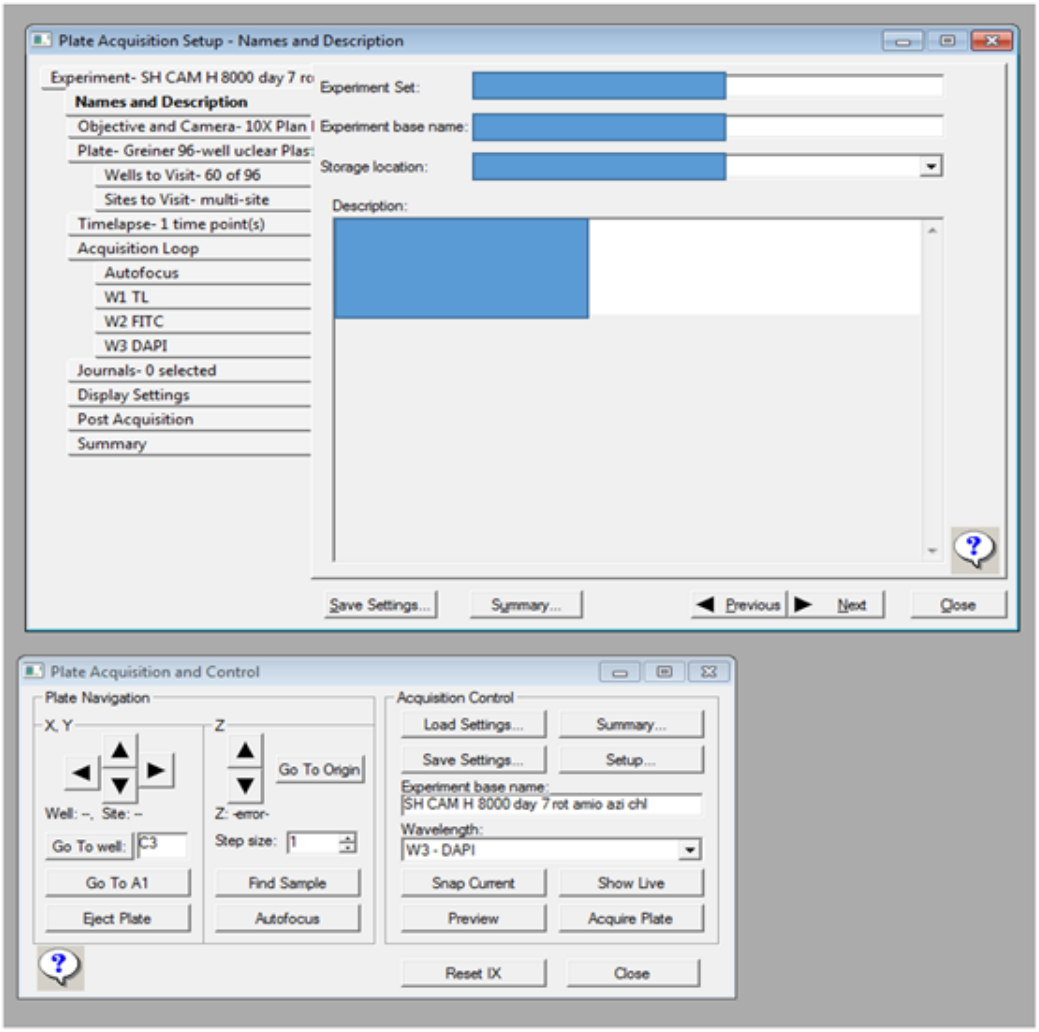


D. Neurite degeneration imaging

ImageXpress® Micro (Molecular Devices, UK) is used to obtain images of the exposed cells. Four images per well are taken with three channels: Transmitted light (TL, yellow arrow), Hoechst in DAPI channel (nuclear staining, blue arrow), and calcein in FITC Channel (cytoplasm/neurite staining, green arrow).

1. Turn on the machine 30 minutes before starting the measurements.
2. Open MetaXpress®
3. On the top menu of the MetaXpress® software, select screening → plate acquisition set up.
4. On the top menu of the MetaXpress® software, select screening → plate acquisition and control.
5. Once the plate acquisition set up and plate acquisition and control windows are opened, start by writing the name of the experiment and a description under the "Names and description" tab (see below, Figure 3).

Figure 3. "Names and description tab"



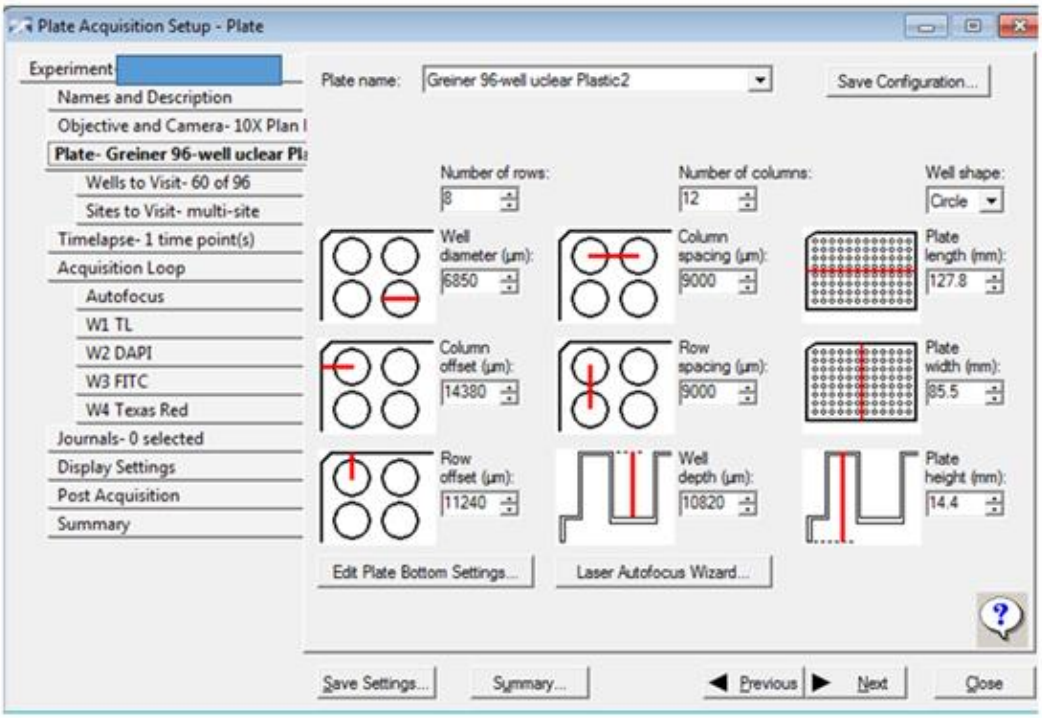
6. Under the "objective and camera" tab, select a 10X magnification, camera binning 1 and gain 1. These settings should only be modified by the instrument's responsible person or technician.

7. Under the "Plate" tab use the settings optimised for 96 well black microclear bottom cell culture plates (Greiner, No 655090). Figure 4 contains a description of the settings used for 96 well plates in this instrument, these settings should only be modified by the instrument's responsible person or technician.

   [Note: different batches of plates can have differences in the well depth within the same plate, this will affect the sharpness of the image taken during the testing of the settings but it will not affect the final images]

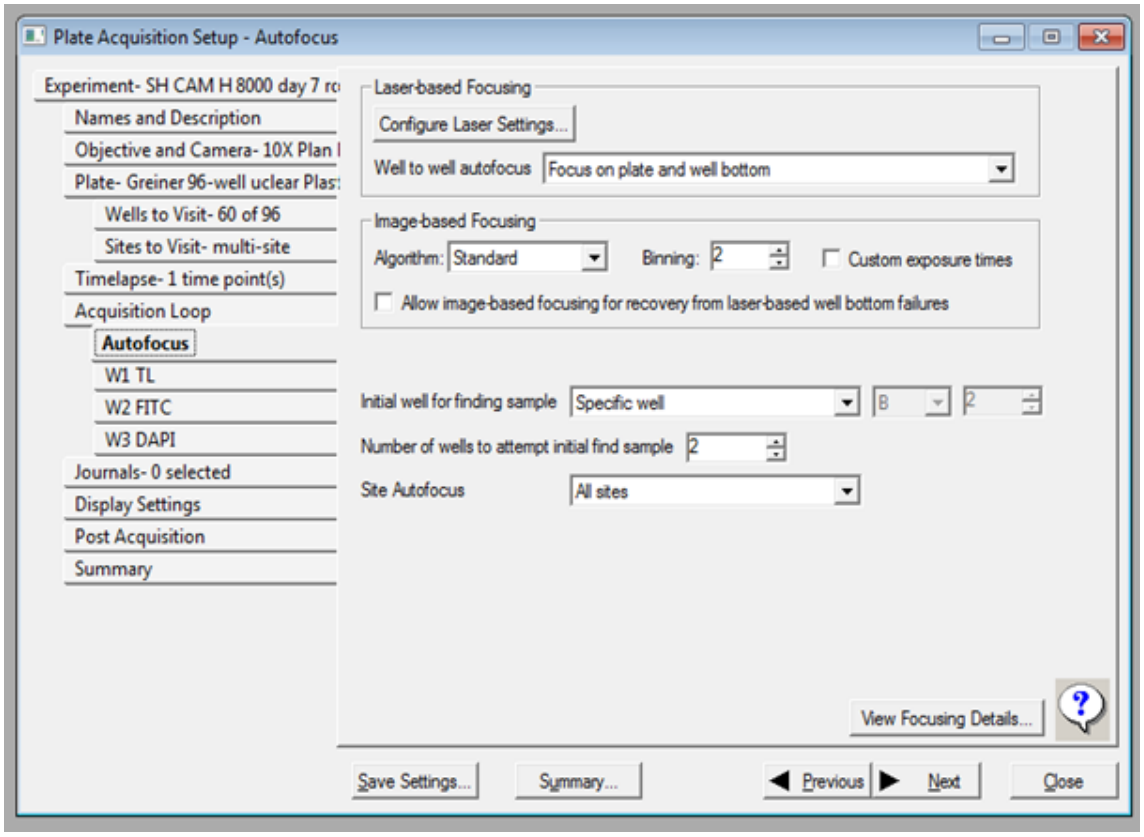
Figure 4. "Plate" tab including the setting that have been standardised for measurements in 96 well plates.

These settings should only be modified by the person responsible for the instrument.



8. Under the "wells to visit" tab, select the 60 innermost wells for analysis. Make sure that the selected wells are showed in a green colour.
9. Under the "sites to visit" tab select 2 columns and 2 rows to obtain 4 images/well.
10. Under the "timelapse" tab select number of endpoints = 1.
11. Under the "autofocus" tab (see Figure 5) select:
    a. Well to well autofocus: focus on plate and well bottom.
    b. Imaged based focusing:
        i. Algorithm: standard
        ii. Binning: 2
    c. Initial well for finding sample: specific well
    d. Site autofocus: all sites.

Figure 5. "Autofocus" tab with the settings used for measurements in 96 well plates.



12. Under the tabs W1 to W3 the setting for image acquisition are specified:

Table 2. Settings used for the acquisition of images with MetaXpress®.

The specific exposure time, target maximum intensity and Z- offset set for each of the three wavelengths used to measure neurite degeneration in differentiated SH-SY5Y cells are described.

Illumination setting	TL	DAPI	FITC
Exposure time (ms)	8	100	120
Target max intensity	16960	33000	34464
Z-offset (µm)	8.96	2.92	10.44
Calculate Offset (use Z stack and Custom range)	Range: 50 um Step: 1.5 um	Range: 50 um Step: 1.5 um	Range: 50 um Step: 1.5 um

13. Insert the plate in the imager (see Figure 6)

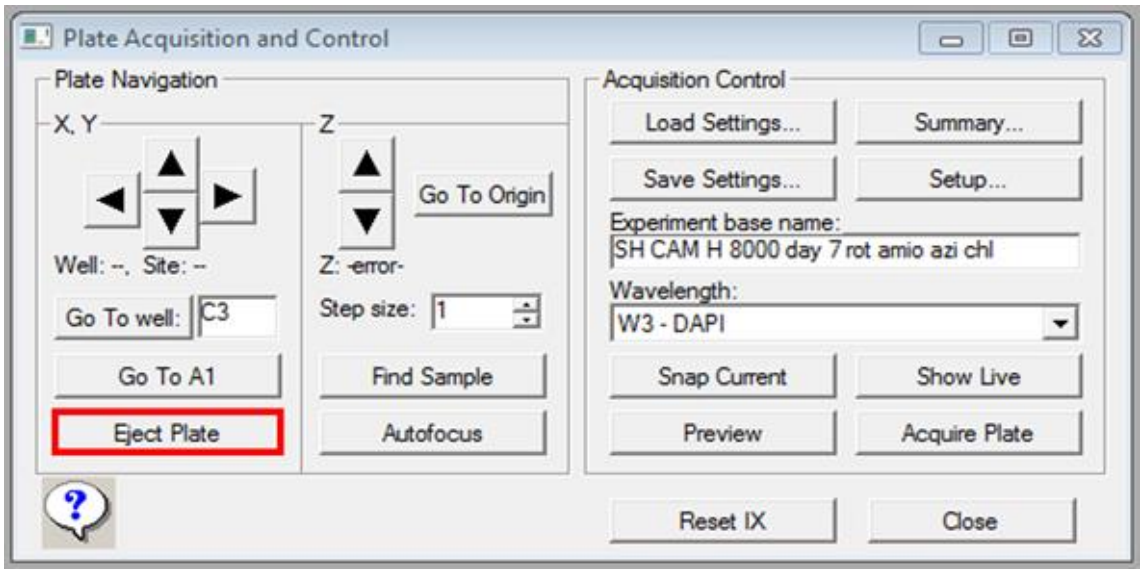
    a. In the plate acquisition and control menu choose eject plate

    b. Place the plate inside the imager

    c. In the plate acquisition and control menu choose load plate

Figure 6. Plate acquisition and control window.

To insert the plate in the imager press "eject plate".



14. With the mouse, right click button select one of the 4 sites and one control well, select the DAPI channel to evaluate the nuclear staining with Hoechst. To be able to see the cells click on "find sample" in the plate acquisition and control window.
15. With the mouse right click button select one of the 4 sites and one control well, select the FITC channel to evaluate the calcein staining. To be able to see the cells click on "find sample" in the plate acquisition and control window.
16. Click under the "summary" tab → acquire plate.
17. All obtained images are automatically stored in MetaXpress® in the D disk of the computer. The SOP "procedure for backing up old experiments from the imager" describes the steps to follow in order to create back up files.

E. Image analysis

1. Open MetaXpress®
2. On the top menu of the MetaXpress® software, select screening → Review plate data [DB].
3. Select the plate for analysis.
4. Select all the wells to be analysed.
5. On the "Run analysis" tab select:
   a. Analysis: <Neurite Outgrowth>
6. Settings: Press configure settings, and select:
   a. Neurite image: FITC
   b. Illumination: Fluorescence
   c. Cell bodies:
      i. Approximate max width: 75 µm = 115 pixels

ii. Intensity above local background: 100 graylevels

iii. Minimum area: 90 µm^2 = 213 pixels

d. Nuclear stain:

i. Nuclear image: DAPI

ii. Approximate min width: 5 µm = 8 pixels

iii. Approximate max width: 13 µm = 20 pixels

iv. Intensity above local background: 80 graylevels

e. Outgrowths:

i. Maximum width: 2 µm = 3 pixels

ii. Intensity above local background: 15 graylevels

iii. Minimum cell growth to log as significant: 15 µm = 23 pixels

7. In the configure settings window press "test run" to visualise the correct identification of cell bodies and outgrowths in the software (Figure 7 and 8)

Figure 7. "configure settings tab".

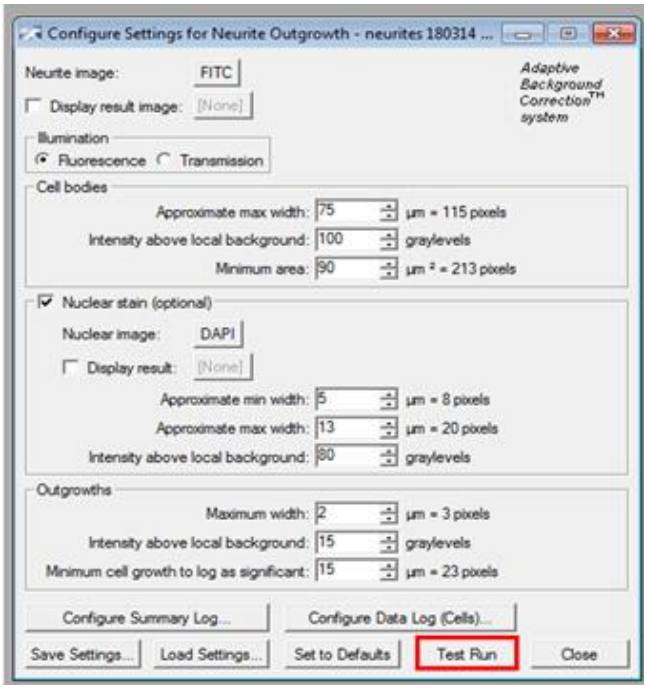
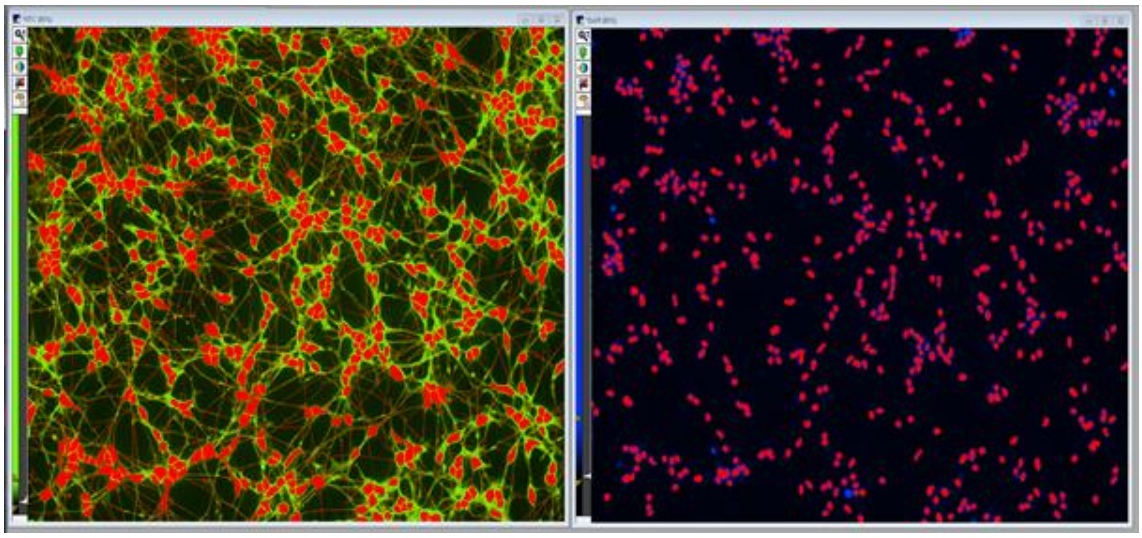


Figure 8. Analysis of neurite length in differentiated SH-SY5Y cells.

Left: staining with calcein in FITC channel, Right: nuclear staining with Hoechst in DAPI channel. Live cells are recognised as structures with both calcein and Hoechst staining.



Note: variations in the intensity of the staining can lead to inappropriate identification of cells bodies and/or neurites. If needed, the settings in the "configure settings" tab should be modifies to assure proper identification of most of the imaged structures.

8. Analyse the plate by pressing "run analysis" on the "Review plate data" window.
9. After analysis, the data can be exported to an excel file.
10. Store the data in the S-disk: S:\Projects\PHC33_EUToxRisk\Case study 4_Mitotox\Neurite degeneration\Compilation of results 24h.
11. Microsoft Excel is used to analyse the data.

F. Data analysis

1. The differences in the mean process length (cell: mean process length) used for stablishing the effect of the test compounds on the neurites (neurite degeneration).
2. Normalise the mean process length value of each well against the mean of all negative controls:

$$ND\ (\%) = \frac{\text{Mean process length exposed cells}}{\text{Mean process lenght negative controls}} * 100\%$$

3. Take the mean of the effect in the three technical replicates of each concentration.
4. Plot the values sin GraphPad Prism.

HEALTH SAFETY AND ENVIRONMENT

Cell culture procedures take place in a laminar air flow cabinet, class 2. All work with test chemicals is performed in a fume hood. Experiments are planned in order to reduce the exposure time of the laboratory personnel to the test chemical. Wastes of environmentally hazardous compounds are handled according to local regulations.

X. REFERENCES

To be added

XI. APPENDIX

Summary of the procedure for measurement of neurite degeneration in differentiated SH-SY5Y cells:

Day 1

Plating of SH-SY5Y cells, 8 000 cells/well.

Day 2

Start differentiation.
Change complete EMEM to N2-RA medium.

Day 3

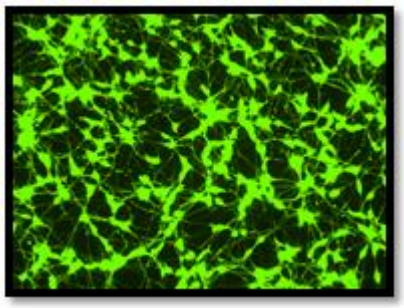
Addition of fresh N2-RA differentiation medium
Add 100 µL N2-RA to all wells

Day 6

Exposure to test chemicals.
Remove 100 µL N2-RA medium.
Add 100 µL of the corresponding chemical dilution.

Day 7

Neurite degeneration imaging
Remove 100 µL of supernatant.
Add 20 µL/well of Calcein + Hoechst staining solution.
Incubate the plate for 20 minutes at 37 °C.
Add 130 µL DMEM:F12.
Image the plate (Calcein in FITC channel, H33342 in DAPI channel, 4 sites/well)



Annex 1.12. NeuriTox/UKN4 assay in galactose conditions

Method	LUHMES: Neuron outgrowth in galactose medium
Contact	Johannes Delp (UKN) -> johannes.delp@uni-konstanz.de

Introduction

Aim

This protocol describes the analysis of how to assess the impairment of neurite outgrowth of live LUHMES cells (human neuronal) in galactose conditions, which make the cells more dependent on mitochondrial metabolism.

Purpose

In standard cell culture conditions, cells can use only glycolysis to meet their metabolic needs since glucose is highly abundant (e.g. 18 mM). Additionally, many cells don't have energetic requirements except from proliferation. Our biological model system are non-proliferating neurons, so they have low energy needs and don't need to be electrically active, thus they can afford to use mainly glycolysis. Galactose metabolism yields the same energy per molecule as glucose does, but has much slower kinetics, thus cells become more dependent on mitochondrial metabolism, which resembles the *in vivo* situation of neurons much better. Since mitochondria are then stronger involved in homeostasis, the hazard potential of mitochondrial inhibitors can be assess better.

Limitations

Galactose is a non-normal substrate for neuronal cells, therefore general cell viability and functionality parameters have to be controlled to ensure healthy, but metabolically tuned cells.

Method outline

Cells are cultured normally according to established DM-ALM protocol for UKN4/NeuriTox. The only difference is that as differentiation and centrifugation medium, medium with 18 mM galactose instead of 18 mM glucose is used.

Method description

Chemicals and buffers

- Medium: Thermo A2494301 (Silac AdvDMEM/F12 no glucose) to be supplemented with galactose, lysine and arginine to have the same composition as the medium mentioned in the DB-ALM protocol
- No other changes to the DB-ALM protocol

Preparation upfront

- Prepare the medium

- No other changes to the DB-ALM protocol

Experimental procedure

- Cells are cultured normally according to established DM-ALM protocol for UKN4/NeuriTox. The only difference is that as differentiation and centrifugation medium, medium with 18 mM galactose instead of 18 mM glucose is used
- No other changes to the DB-ALM protocol

Data analysis

- No changes to the DB-ALM protocol

Annex 1.13. Biokinetic Modelling Description

Method	Biokinetic Modelling
Contact	Iain Gardner (Certara) -> Iain.Gardner@certara.com

Introduction to Biokinetic Modelling

Effective concentrations determined in *in vitro* toxicological assays are routinely based on a range of nominal treatment concentrations. However, the use of the nominal treatment concentration as the driving concentration for observed toxicity *in vitro* does not account for factors that reduce the free concentration within the assay and determine the true effective concentration. For cell based assay systems, this will dictate the concentration available for distribution into the cell, and subcellular compartments, and so determine the driving concentration at the target site mediating toxicity. In order to more accurately translate concentration driven toxicity from *in vitro* to *in vivo*, it is necessary to correct the nominal effect concentration, accounting for the distribution of the compound within the assay system. Factors to be considered in modelling this *in vitro* distribution include binding to the plastics used in assays, exchange at the interface between culture media and the air in the culture vessel, and binding to components that may be included in the culture media (e.g. lipids and proteins originating from foetal bovine serum, FBS); the modelling of these processes is termed biokinetics (1).

A number of biokinetic models have been published that account for some or all of the factors listed above in cell based assays. Armitage and colleagues (2) published a model framework to predict intracellular concentrations, correcting for some of the distribution factors in monolayer cell culture. This steady-state framework assumes instantaneous partitioning between media, headspace, serum-lipids, serum-proteins, dissolved organic material, and the cultured cell volume. However, a critical assumption of the Armitage model is that the test compounds are neutral or not significantly ionised under the conditions of the *in vitro* assay. This assumption of neutrality was, to some degree, addressed in the model developed by Fischer *et al* (3) where the authors adopted the same steady-state assumption, but excluded the partitioning of compound into the headspace. The proposed model incorporated separate partition constants for both the ionised and unionised fraction of test compound, determining the fraction ionised assuming a uniform pH=7.4 throughout the test system. This neglects the differential ionisation potential between the culture media and intracellular water resulting from their differing pH. Furthermore, the interior of the cell itself is not a uniform environment, the microenvironment of specific organelles being maintained at pH specific to their function (i.e. lysosomes (pH≈4.5), mitochondria (pH≈8), cytosol (pH≈7). Indeed, the differential ionisation of compounds between organelles and intracellular water can result in the preferential sequestration of compounds within organelles; a phenomena commonly known by the misnomer ‘ion-trapping’(4). It is also critical to note that differences in the intrinsic permeability of the unionised/ionised form are not the only factors determining the distribution of ionised compound into cells. The potential difference maintained across the cell membrane, membrane potential (mV), can actively promote the uptake or exclusion of ionised compounds from the cell interior and can vary significantly between cell types.

Steady-state Biokinetic Model 2D Monolayer Cell Culture

Given that compounds in this read-across are monoprotic acids, significantly ionised at physiological pH, the assumption of neutrality or uniform ionisation is not applicable. As such, an alternative model revising the relevant assumptions of the published approaches was used to predict the intracellular concentrations in 2D monolayer test systems. Based on partition coefficients between different mediums, and physical volumes, we can predict an apparent volume of distribution in the *in vitro* system. Given this volume, we can calculate the unbound medium concentrations, $C_{medium,u}$.

$$C_{medium,u} = \frac{C_{nominal} \cdot fu_{FBS,dilu} \cdot V_{medium}}{V_{medium} + k_{air} f_{ui} V_{air} + k_{cell} V_{totalcell} + k_{plastic} SA_{medium} \cdot 10^3}$$

Where $C_{nominal}$ is the nominal concentration, V_{medium} (L) is the volume of culture medium, V_{air} (L) the volume of air in the headspace above the media, and $V_{totalCell}$ (L) is the total volume of cultured cells at the time of the assay, SA_{medium} (m^2) is the surface area of plastic in direct contact with cuture medium, $fu_{FBS,dilu}$ is the fraction unbound in foetal bovine serum accounting for the dilution of FBS in culture (where FBS is not included in the culture medium $fu_{FBS,dilu}$ =1), the partition coefficients between culture medium and air, cells and plastic are k_{air}, k_{cell}, and $k_{plastic}$, respectively, and are defined below. The fraction unionised, f_{ui}, is calculated based on the Henderson Hasselbalch equation using the compound specific pKa and the compartment relevant pH.

$$f_{ui} = \frac{1}{1 + Y}$$

$$Y_{neutral} = 0$$

$$Y_{acid} = 10^{(pH - pKa)}$$

Binding to Serum Components

The predominant binding protein present in untreated foetal bovine serum (FBS) is albumin. FBS also contains lipids and free fatty acids. The lipids within FBS are diverse and not individually characterised or quantified routinely. However, the neutral lipid triacylglyceride (TAG) is routinely quantified and reported in the certificate of analysis, as is the concentration of albumin. Assuming albumin and TAG to be the most significant binding components in FBS and complete cell culture medium, we can predict the fraction unbound in FBS, fu_{FBS}.

$$fu_{FBS} = \frac{1}{1 + K_{protein} f_{protein} + \frac{P_{nl} f_{nl,FBS}}{1 + Y_{FBS}}}$$

Where $f_{protein}$ (v/v) is the fraction of FBS comprised of protein, $K_{protein}$ is the albumin:water partition coefficient, P_{nl} is the neutral lipid partition coefficient (defined below), and f_{nl} is the fraction of FBS comprised of neutral lipid. Assuming that the fraction of albumin in the FBS is representative of the total protein fraction responsible for protein binding in the FBS, this can be calculated from the mass of albumin reported in the certificate of analysis for a batch of FBS.

$$f_{protein} \approx f_{alb,FBS} = \frac{mass\ albumin \cdot PSV_{albumin}}{1000}$$

Where $PSV_{albumin}$ is the partial specific volume of albumin (0.73 mL/g (5)). The albumin to water partition coefficient, $K_{protein}$, can be determined experimentally or can be calculated based on a previously described relationship with the octanol to water partition coefficient (6).

if $logP_{ow} < 4.5$

$$logk_{albumin} = 1.08 \cdot logP_{ow} - 0.7$$

if $logP_{ow} \geq 4.5$

$$logk_{albumin} = 0.37 \cdot logP_{ow} + 2.56$$

In much, the same way the volumetric fraction of neutral lipid in FBS can be calculated, using TAG as a surrogate for neutral lipid content. TAG concentration is routinely determined using an enzymatic assay and so reported as a molar concentration.

$$f_{nl,FBS} \approx f_{TAG} = \frac{[TAG] \cdot 10^{-3} \cdot MW_{TAG} \cdot PSV_{TAG}}{1000}$$

Where PSV_{TAG} is the partial specific volume of TAG (1.09 mL/g (7)) and the molecular weight of TAG is taken to be 885.453 g/mol; specifically, this corresponds to the molecular weight of trioleate, a TAG molecule comprising a glycerol backbone and three oleic acid residues.

A dilution factor, D, can then be calculated to correct fu_{FBS} for the volumetric fraction of media comprising serum, f_{serum}, and so $fu_{FBS,dilu}$ can be calculated.

$$D = \frac{1}{f_{serum}}$$

$$fu_{FBS,dilu} = \frac{fu_{FBS}}{\frac{1}{D} \cdot (1 - fu_{FBS}) + fu_{FBS}}$$

Calculation of Partition Coefficients

The partition coefficient between the culture medium and air is derived from the test compounds Henry's Law constant, which may be determined experimentally or predicted using a variety of *in silico* tools. The dimensionless air medium partition coefficient is determined:

$$k_{air,u} = \frac{k_H}{RT}$$

Where K_H is the Henry's Law constant expressed in SI units (Pa m^3 mol^{-1}), R is the universal gas constant (8.314 Pa m^3 K mol^{-1}), and T is the reference temperature at which Henry's Law constant has been defined (K).

The plastic to medium partition coefficient is predicted based on the octanol to water partition coefficient using a linear relationship established by Kramer (8) and used in the

model published by Comenges *et al* (9). Note that this partition-coefficient is not dimensionless and has units (m).

The partition coefficient between cells and culture medium is based on an adaption of the published approach of Rodgers and Rowland for predicting the partitioning of different compound classes between plasma and tissues, based on composition (10, 11). We derive $K_{cell\ uu,uu}$ and $K_{iw\ uu,uu,\ organelle}$ from the steady-state Fick-Nernst-Planck equation to describe the passive permeation of electrolytes across the cell and organelle membranes, respectively, as well as the passive permeation of neutral molecules,

$$k_{cell\ uu,uu} = \frac{1 + \frac{P^{unbound,ionised}}{P^{unbound,unionised}} \frac{N}{e^{N_1} - 1} Y_{ew}}{1 + \frac{P^{unbound,ionised}}{P^{unbound,unionised}} \frac{N}{e^{N_1} - 1} e^{N} Y_{iw}}$$

$$k_{IW_{organelle}^{uu,uu}} = \frac{1 + \frac{P^{unbound,ionised\ 1}}{P^{unbound,unionised}} \frac{N_1}{e^{N_1} - 1} Y_{iw}}{1 + \frac{P^{unbound,ionised\ 1}}{P^{unbound,unionised}} \frac{N_1}{e^{N_1} - 1} e^{N_1} Y_{organelle}}$$

$$N_{neutral} = 0$$

$$N_{monoacid} = -\frac{\emptyset F}{RT}$$

Where P is the permeability coefficient of either the ionised or unionised moiety. Using a ratio of the permeability coefficient between the unionised and ionised species, we can describe the differential permeability of the two molecular forms. It has previously been assumed that the permeability coefficient of the ionised species is 3-4 log units lower than that of the neutral form (12). Here we assume ionised, unionised permeability coefficient ratio of 3.3 log units for the monoprotic anion. F is the Faraday constant (96484.56 C mol^{-1}) and ϕ is the cell membrane potential (V).

$$k_{cell,u} = \frac{C_{cell}}{C_{media_{unbound}}} = \begin{pmatrix} \left(1 - f_{lyso+}f_{mito}\right)\left(f_{iw}(1 + Y_{iw}) + P_{nl}f_{nl} + P_{np}f_{np}\right) \\ +f_{lyso}\left(f_{iw}(1 + Y_{lyso}) + P_{nl}f_{nl} + P_{np}f_{np}\right)K_{iw\ lyso}^{uu,uu} \\ +f_{mito}\left(f_{iw}(1 + Y_{mito}) + P_{nl}f_{nl} + P_{np}f_{np}\right)K_{iw\ mito}^{uu,uu} \end{pmatrix} \frac{1}{1 + Y_{ew}} k_{cell\ uu,uu}$$

Incorporating $K_{cell\ uu,uu}$ and $K_{iw\ uu,uu,\ organelle}$ we adapt the original Rodgers and Rowland approach where f_{iw}, fl_{yso}, f_{mito}, f_{nl}, f_{np} denote the fractional cellular volumes of the intracellular water, lysosomes, mitochondria, neutral lipids, and neutral phospholipids, respectively. P_{nl} and P_{np} describe the partitioning of the compound between intracellular water and neutral lipids and neutral phospholipids, respectively. Where the olive oil to water ($P_{vo:w}$) and octanol to water ($P_{o:w}$) partition coefficients are used as surrogates, respectively.

This revises a fundamental assumption of the published Rodgers and Rowland approach, and previously published biokinetic models that only unionised molecular species can passively traverse biological membranes (2, 9-11). The approach also expands on previously published biokinetic models by describing distribution into two subcellular organelle compartments (lysosome and mitochondria). Based on the approach described above, total intracellular concentrations corresponding to nominal effect concentrations determined experimentally can then be calculated.

$$C_{cell} = k_{cell,u} \cdot C_{media,dissolved,u}$$

It should be noted that while this approach can be used to model ampholytes, monoprotic and diprotic acids and bases, the equations above are described in forms with specific to neutral compounds and monoprotic acids, relevant to the compounds investigated in this read across. A description of this model has been presented previously (13), and is currently submitted for peer-review.

Summary of Model Assumptions

The model described above, like those previously published (2, 3), is a steady-state approximation of multiple dynamic processes. Critically, it assumes a closed system such that the loss of test compounds within the *in vitro* system is assumed to be negligible with no metabolic clearance or instability. When that is not the case, the steady-state assumption may lead to overestimation of intracellular concentrations of the parent molecule, particularly for highly metabolised chemicals. In line with the assumption of a closed system, the culture system is assumed to be hermetically sealed, such that the air above the culture medium is a defined volume. For volatile compounds, tested in unsealed systems, this could also result in an overprediction of intracellular compounds, since distribution into the air will act as a clearance mechanism. As part of the steady-state assumption, the model assumes that the volume of cultured cells is constant with no significant increase or decrease over the course of the test assay. Finally, the model assumes that all binding and partitioning processes are non-saturable, with the distribution of test compound into the cell mediated through passive diffusion. The model assumes that there are no active uptake or efflux transport processes relevant to the partitioning of the test compound within the test system.

Alternative Biokinetic Predictions

An assumption of the biokinetic model used to predict intracellular concentrations is that cells are cultured as a 2D monolayer. Permeability into cells cultured in multi-layer, three dimensional systems may not be well described based on the approach detailed above; particularly permeability into cells that may not be in direct contact with the culture medium (i.e sandwiched between adjacent cells). Thus, we adopt a simplified approach to predict the corresponding concentration of free drug in test medium, as if the determined effective concentration was made up in complete culture medium prior to cell treatment. This predicted unbound effective concentration can then translated to an unbound plasma concentration *in vivo*; such approaches have been described previously (14).

As described above, the predominant binding components present in untreated FBS are albumin and neutral lipids. In order to predict the free concentration of test compounds in treatment medium it is necessary to account for binding to these media components. Here we assume that the binding to albumin and lipid (TAG) in complete culture (treatment) medium, are the only significant processes limiting the availability of test compound for distribution into subsequently treated cells. Thus, loss of compound due to volatility, or the binding to the plastics used in cell culture are not accounted for in this approach.

If we consider the threshold for significant volatility to be an air-water partition coefficient ($K_{air} < 0.03$), as previously assumed by Fischer *et al*[1], then the assumption that volatility has no significant impact on the freely dissolved media concentration holds for all of the compounds investigated here. Polymer-water partition coefficients have been shown to be

significantly lower than octanol-water partition coefficients (P_{ow}) (2), here used to determine the partitioning to TAG and albumin, as described below. As such, we assume here that binding to plastics used in the handling and preparation of culture medium have no significant impact on the free concentration of test compound. Finally, assuming that the maximal tested concentration does not exceed the solubility of the compound in complete culture medium, and taking the binding to protein and lipid in culture media to be linear across the tested concentration range, we can calculate an unbound fraction of test compound given the composition of the medium and the P_{ow} of the test compound.

$$fu_{media} = \frac{1}{1 + K_{albumin} f_{albumin} + \frac{P_{vow} f_{nl}}{1 + Y}}$$

$$Y_{neutral} = 0$$

$$Y_{monoprotic\ acid} = 10^{(pH - pKa)}$$

$$logK_{vow} = 1.115 \cdot logK_{ow} - 1.35\ ^{4}$$

Where $f_{albumin}$ is the volumetric fraction of medium comprised of protein, $K_{albumin}$ is the albumin-water partition coefficient, K_{vow} is the olive oil-water partition coefficient (derived from P_{ow}), f_{nl} is the volumetric fraction of medium comprised of neutral lipid (TAG), and Y is the ratio of the ionised to unionised concentrations in the culture medium calculated using the Henderson-Hasselbalch equation. As above, the binding of test compound to neutral lipids is assumed to be limited to the unionised fraction of the solubilised compound in medium. The volumetric fractions of albumin and TAG in the culture medium can calculated using an analogous approach to that described above using the partial specific volumes of albumin and TAG.

$$f_{albumin} = \frac{[albumin] \cdot PSV_{albumin}}{1000}$$

$$f_{nl} \approx f_{TAG} = \frac{[TAG] \cdot PSV_{TAG}}{1000}$$

It should be noted that this relationship assumes that the neutral and the ionised fractions of compound partition equally into the hydrophobic phase and so $logK_{albumin}$ is not influenced by the ionisation state for ionisable compounds. Here, both the concentration of albumin and TAG are taken to be in units of mg/mL.

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Annex 1.13.1. PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELLING TO SUPPORT THE STROBIN READ ACROSS CASE STUDY

REPORT OBJECTIVE

To provide

a) a description of methods and relevant equations and

b) additional information and context

for the physiologically based pharmacokinetic (PBPK) models constructed for pyraclostrobin, kresoxim methyl, azoxystrobin, picoxystrobin, trifloxystrobin and antimycin A in the rat and human for subsequent use in the read across assessment for these compounds within the framework of the EU_TOX_RISK project.

In this context of use, a PBPK model is not intended to precisely characterise the PK processes but to represent an interpretation of the available data by addressing the relationships between an external dose and internal tissue, blood or excretion dose (WHO publication Harmonisation Project Document No. 9. CHARACTERIZATION AND APPLICATION OF PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELS IN RISK ASSESSMENT).

In constructing, the PBPK models for the strobin read across study the following aspects were considered as suggested in the WHO PBPK guidance.

1) Although from *in vitro* studies there are some potential signals of neurotoxicity *in vivo* the critical effects observed for these compounds is liver effects and a decreased bodyweight. Some of the strobilurins have been tested in guideline compliant neurotoxicity studies (OECD TG 424) with no sign of neurotoxicity reported. **The specific aim of this case study was to use NAM data to justify waiving of an OECD TG 424 study for azoxystrobin**.

2) Parent compound was assumed to be the toxic moiety i.e. plasma and tissue levels of formed metabolites were not considered in the PBPK model.

3) Based on experimental observations metabolism is thought to be the major clearance pathway of the compounds in this read across case study and the clearance in human subjects was predicted using an *in vitro – in vivo* extrapolation approach using intrinsic clearance data generated in human hepatocytes.

4) The physiology (i.e. tissue weights and blood flow rates) of the species of interest were the default values in the Simcyp rat and human simulator (V17).

Specific aims of the modelling exercise were

1) To develop PBPK model to predict comparative human exposure of the compounds using *in vitro* to *in vivo* extrapolation approaches i.e. to answer the question whether at a given dose is the internal exposure to the compounds comparable.

2) To determine if allometric extrapolation of the predicted human pharmacokinetics allows recovery of the available pharmacokinetic data in the rat.

3) To assess how well the human and rat PBPK models can simulate published tissue exposure data in the brain.

4) To examine the inter-individual variability in the simulated exposure of compounds in humans.

PBPK model assumptions

The following assumptions have been made in the development of PBPK models for the strobin case study:

1) The metabolism of the compounds is assumed to be linear (i.e. not to saturate) over the range of doses simulated.

2) The clearance in rat can be predicted from the simulated human clearance using an allometric approach where the clearance of free (i.e. not bound to plasma proteins) compound scales across species based on differences in body weight scaled with an exponent of 0.75.

3) There is no intestinal metabolism or metabolism in any of the tissues in the PBPK model apart from in the liver.

4) Potential cleavage of the compounds in the blood was not considered in the models.

ABBREVIATIONS

AAG - alpha-1 acid glycoprotein

ADAM – advanced dissolution, absorption and metabolism

ADME – absorption, metabolism, distribution and excretion

AUC – area under the plasma drug concentration-time curve

BCRP – breast cancer resistance protein

BSA – body surface area

B/P – ratio of concentration of drug in blood to plasma

C_{max} – maximum plasma concentration

C_{ss} – plasma drug concentration at steady state.

CL_{int} – intrinsic metabolic clearance

CL_{uint} – unbound intrinsic metabolic clearance

CL – intravenous clearance

CL_R - renal clearance

CV – coefficient of variation

CYP – cytochrome P450

E_G - gut extraction ratio

E_H - hepatic extraction ratio

E:P – erythrocyte : plasma ratio

fa - fraction absorbed

fm – fraction metabolised

fu – fraction unbound in plasma

fu_{EW} – unbound fraction in the extracellular water

fu_{hep} – unbound fraction in the *in vitro* hepatocyte incubation.

fu_{IW} – unbound fraction in the intracellular water

fu_{mic} – fraction of unbound substrate or inhibitor in a microsomal incubation

GI – gastrointestinal tract

HC – haematocrit

HBD – number of hydrogen bond donors

HPGL – hepatocellularity per gram of liver

HV – healthy volunteer

HLM – human liver microsomes

HSA – human serum albumin

IVIVE – *in vitro in vivo* extrapolation

J_{max} – maximum rate of transporter mediated uptake or efflux

K_a - absorption rate constant

K_m – Michaelis constant

logP – log octanol/buffer partition coefficient (neutral species)

MPPGL – microsomal protein per gram of liver

MSR – maximum supersaturation ratio

NEC – North European Caucasian

Papp – apparent *in vitro* permeability

$P_{eff,man}$ – *in vivo* effective permeability in humans

P-gp – Permeability-limiting glycoprotein, encoded by the multidrug resistance 1 (MDR1) gene

PBPK – physiologically based pharmacokinetic

PK – pharmacokinetic(s)

pK_a – acid dissociation constant

PRC – precipitation rate constant

PSA – polar surface area

$P_{T:p}$ – partition coefficient of drug between tissues and plasma

Q_H - hepatic blood flow

QSPR - Quantitative Structure-Property Relationship

RAF/REF – relative activity or expression factor

rhCYP – recombinantly expressed human CYP enzyme

t_{max} – time at which the maximum plasma concentration of drug is reached

V_{max} – maximum metabolic rate

V_C - volume of central compartment

V_{ss} – volume of distribution at steady state

1. Summary

Human and rat PBPK models were constructed for all of the strobin case study compounds. There was limited observed pharmacokinetic data available for comparison with the simulated results but the models for azoxystrobin, picoxystrobin, trifloxystrobin and kresoxim methyl were consistent with available data. For antimycin-A there was no data available and for pyraclostrobin the simulated concentrations in the rat were not consistent with the observed data and so should not be used for the read across exercise.

The distribution of the chemicals into tissues was predicted to be similar regardless of the tissue composition used to make the predictions. The brain concentrations for the compounds were predicted to be slightly higher than the plasma concentrations for the majority of compounds. The brain concentrations were simulated in the PBPK model and can be compared to the concentrations exerting effects in the *in vitro* assays used in the EU_TOX RISK project.

2. Introduction

The synthetic strobilurin fungicides are derived from the naturally occurring fungal metabolites strobilurin A and B. The strobilurins bind to the quinol oxidation site of cytochrome b of mitochondrial complex 3, disturbing electron transfer and causing a perturbation in NADH oxidation and ATP production.

As part of the strobin read across case study PBPK models were constructed for pyraclostrobin, kresoxim methyl, azoxystrobin, picoxystrobin, trifloxystrobin and antimicin-A. The available information to construct the PBPK models was physicochemical information for the compounds (pKa, molecular weight, log P), *in vitro* metabolism data in cryopreserved human hepatocytes, measured human blood:plasma ratios (B/P) and *in silico* and measured values for the fraction unbound in human (fu).

Due to the constraints of the EU-TOX-Risk project, it was not possible to measure the *in vitro* metabolism, B/P or fu in rat tissues and so these parameters in the rat were extrapolated from the data in humans as described below.

3. Methods and Model Development

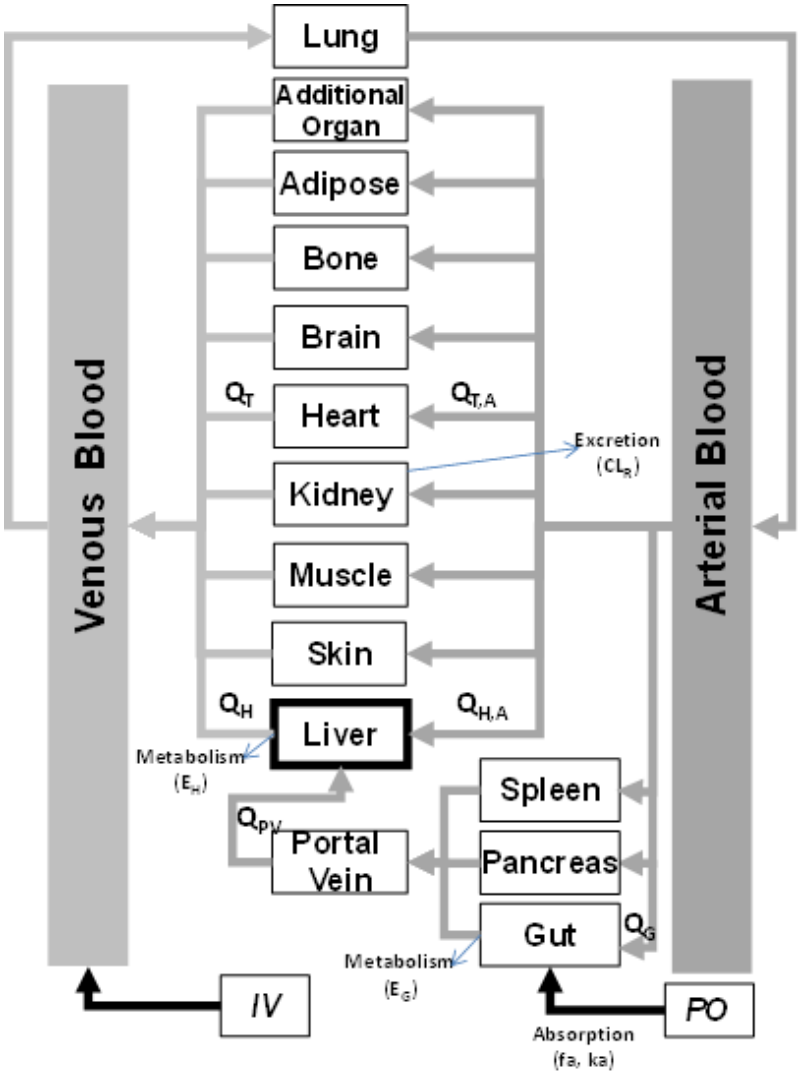
PBPK models for the compounds were constructed in the rat and human Simcyp Simulator (V17) (www.simcyp.com).The Simcyp simulator has been extensively tested and used by a consortium of industry, regulatory and academic scientists. Lists of known bugs within the code of the Simcyp simulator are maintained on our website (https://members.simcyp.com/account/softwareIssues/). The QA system used to support the production of each version of the Simcyp simulator has been described in detail (1).

3.1.1 Physiologically based pharmacokinetic models

An aim of the read across case study was to estimate the concentrations of the compounds in different tissues of the body including the brain which is the target organ for toxicity of these agents in the AOP under investigation. To accomplish this a full body physiologically based pharmacokinetic (PBPK) model (Figure 1) was used. In the human simulator, the ability to add further specific organs as an additional organ is available, but in the simulations for the strobin case study, this functionality was not used.

Figure 1. A physiologically based pharmacokinetic model.

Q_H, $Q_{H,A}$, Q_{PV}, Q_G, $Q_{T,A}$ and Q_T are blood flows in the hepatic vein, hepatic artery, hepatic portal vein, gut and blood flows into and out of the other tissue (T) compartments, respectively; E_G and E_H are the fractions undergoing first pass metabolism in the gut and liver, respectively; CL_R is the renal clearance; fa and ka are the fraction absorbed and the first order absorption rate constant, respectively.



3.1.2 Distribution

In the human simulator, inter-individual variability in tissue distribution is accounted for through relationships between tissue volume and age, sex, weight and height (2). In both the human and rat PBPK models the *in vivo* volume of distribution at steady state (V_{ss}) is predicted using Equation 1 from Sawada *et al.* (3):

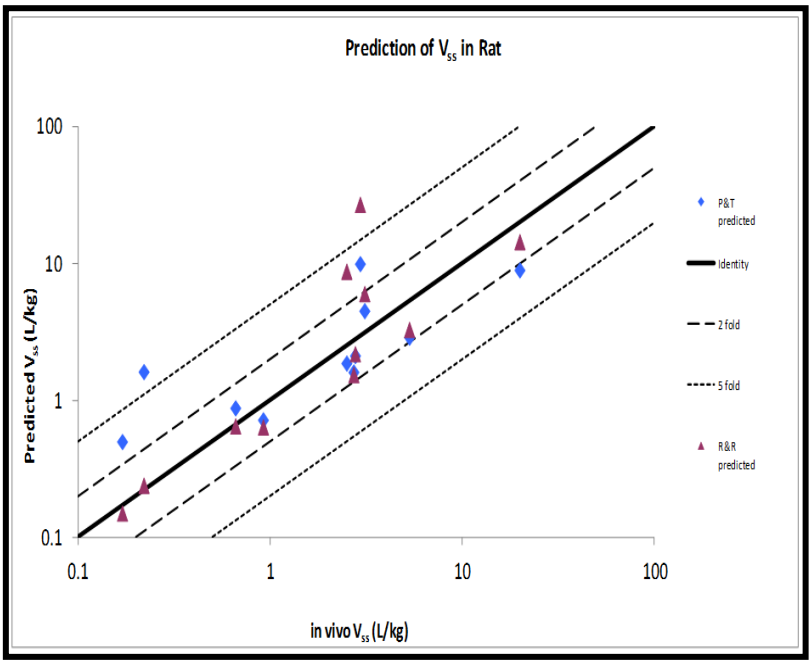
$$\boldsymbol{V_{ss}} = \left(\sum \boldsymbol{V_T} \times \boldsymbol{P_{T:p}}\right) + (\boldsymbol{V_e} \times \boldsymbol{E}\boldsymbol{:}\boldsymbol{P}) + \boldsymbol{V_P} \qquad \text{Equation 1}$$

Where V is the fractional body volume (L/kg) of a tissue (T), erythrocytes (e), and plasma (p), E:P is the erythrocyte: plasma drug concentration ratio and $P_{T:p}$ is the partition coefficient of drug between tissues and plasma components. Three methods are available for prediction of $P_{T:p}$. The first method was reported by Poulin and Thiel (4) as corrected by Berezhkovskiy (5) (Method 1). Method 1 uses the physicochemical properties of the compounds (pKa and log P) together with *in vitro* information (B/P and fu) to predict partitioning into the tissues with the assumption that tissues and plasma are mixtures of lipids, water and proteins with a global pH of 7.4.

The second method was developed by Rodgers and Rowland (6) (Method 2). The latter splits the tissue water volume into intra- and extracellular components, along with the addition of a tissue acidic phospholipid fraction. These equations take explicit account of the extent of ionisation of a compound at the pH of the compartment concerned and have been shown to improve the prediction of tissue:plasma partition coefficients, and consequently V_{ss}, for strong bases. The Rodgers and Rowland method was further extended by the science team at Simcyp to account for the effect of differences in membrane potential in the different tissues on compound distribution (Method 3). Method 3 is only available in the human PBPK models and also allows for the distribution of compounds into specific subcellular organelles to be modelled. Method 3 also forms the basis for the biokinetic model developed within the EU-TOX-Risk project (7). These mechanistic predictions assume non-saturating conditions prevail for all binding processes, drug transport is via passive processes (i.e. no active transport), and each tissue has a well-stirred distribution limited by blood perfusion (i.e. tissues are considered as perfusion limited not permeability limited).

Performance verification for both Method 1 and Method 2 in the rat with a wide range of compounds (molecular weight of 192-1202kDa, 19% acidic, 46% basic, 9% neutral, 27% ampholyte) is shown in Figure 2. The range of *in vivo* V_{ss} for the studied compounds was 0.17-19.9 L/kg and for Method 1 and Method 2, 64% and 82% of predictions were within 2-fold of observed values, respectively.

Figure 2. Performance verification of Vss predictions in Rat for Method 1 (P&T, blue diamonds) and Method 2 (R&R, purple triangles)



3.1.2.1 Perfusion and permeability limited distribution

Lipophilic drugs diffuse rapidly across the capillary membrane into tissue interstitial fluid such that blood flow to the tissue is the rate-limiting step in uptake. This is described as perfusion-limited distribution and is implemented in all tissues represented in the PBPK model (Figure 1). In addition, an option is provided within the Simcyp Simulator to allow for permeability-limited uptake, simulating both passive diffusion in parallel with active uptake and efflux in specific organs such as the liver, kidney (human only), intestine and brain. In these models, the tissue is divided into compartments representing vascular, extracellular and intracellular fluid spaces - with distribution between these spaces defined as a dynamic process. The 'permeability-limited' models in the liver, brain and kidney are only available when Method 2 or 3 are selected to predict $P_{T:p}$. Given the lipophilic nature of the compounds in the strobing read across case study perfusion, limited models were used for all tissues in the simulations presented here.

3.1.3 Oral Absorption

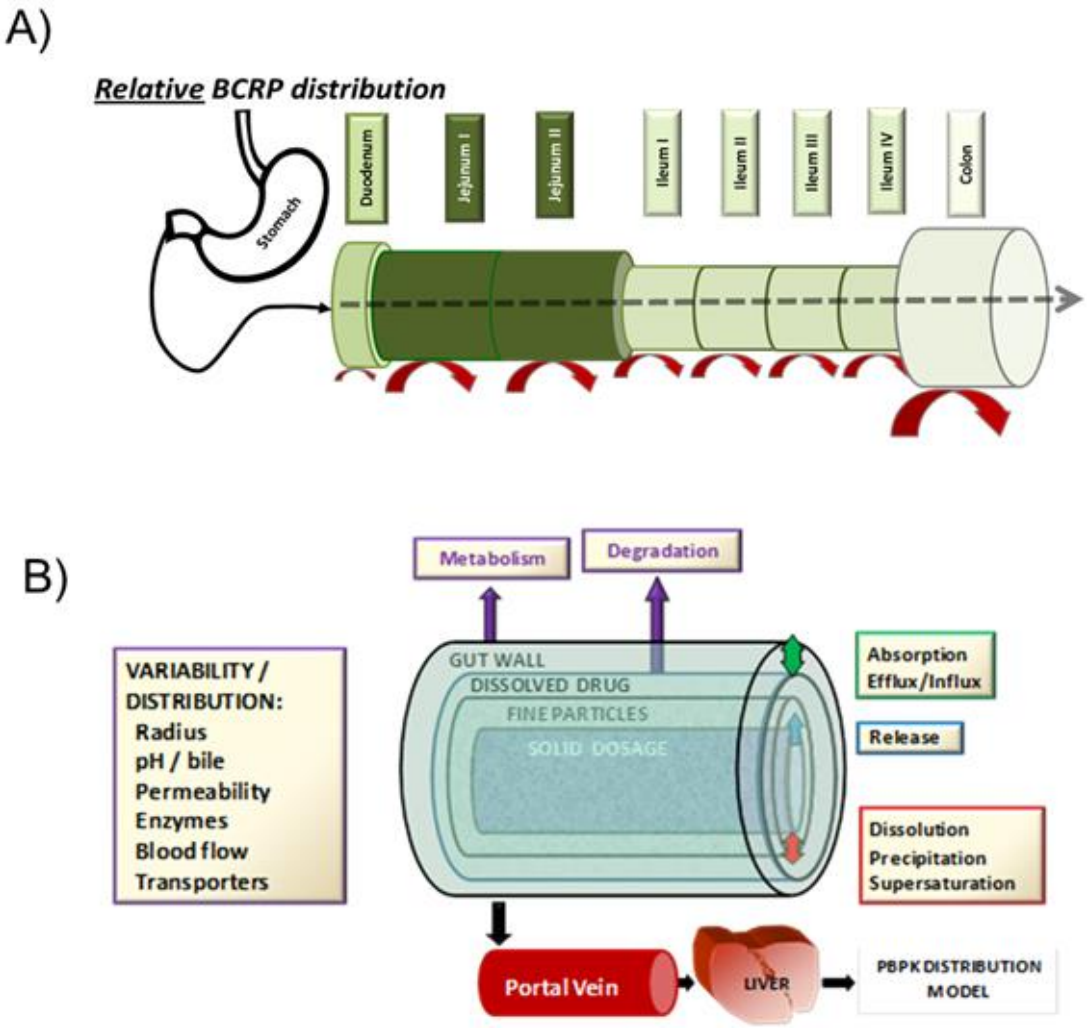
For drugs in solution, several absorption models are available within the Simcyp Simulator including a first-order absorption model, a compartmental absorption transit (CAT) model (8) and the advanced dissolution absorption metabolism (ADAM) model (9). Simulation of the absorption of drugs from solid dosage forms requires use of the ADAM model. The ADAM model, as implemented in the Simcyp Simulator, divides the gastro-intestinal tract (GIT) into nine anatomically defined segments from the stomach through the intestine to the colon (Figure 3). Drug absorption from each segment is described as a function of release from the formulation, dissolution, precipitation, luminal degradation, permeability, metabolism, transport and transit from one segment to another. It is assumed that absorption from the stomach is insignificant compared with that from the small intestine, and that movement of liquid and solid drug through each segment of the GIT may be described by

first-order kinetics. Dissolution rate from solid dosage forms is calculated from information on drug aqueous solubility and particle size using diffusion layer models (DLM) (10, 11).

Figure 3

(A): Structure of the ADAM model in which the GI tract is divided into 9 sections with segregated blood flows to each section. The abundance of various enzymes and transporters in each segment varies non-monotonically along the intestine as indicated by the varying intensity of the colour for each section (BCRP distribution is indicated) (12-14)

(B): Segment of the small intestine indicating the various processes that can be simulated (from (15)).

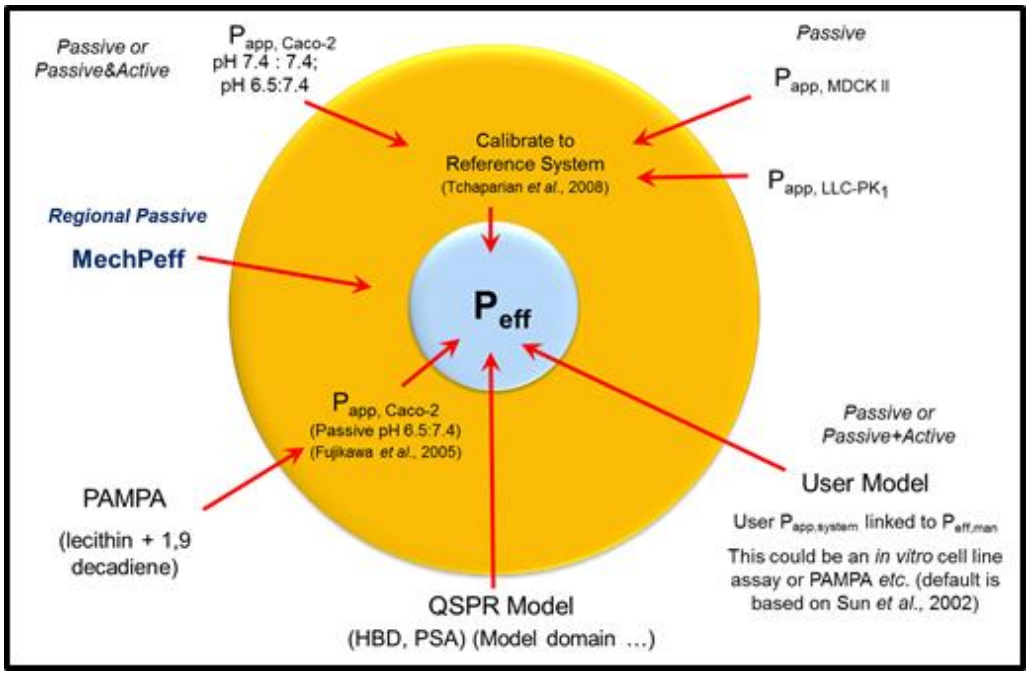


The effective permeability in humans, $P_{eff,man}$ (jejunal), can be measured using the Loc-i-gut methodology and can be used in simulations to describe the absorptive processes in the intestine (16). For novel investigation drugs and many marketed agents and all industrial chemicals measured values of $P_{eff,man}$ (jejunal) are not available and therefore several methods can be used within the Simcyp Simulator to predict $P_{eff,man}$ (jejunal). These are based on data obtained with cell lines (such as Caco-2, MDCK-II or LLC-PK1 cells) (17), PAMPA or from a QSPR model based upon physicochemical properties (PSA and HBD,(18)) or by using the mechanistic permeability (MechPeff) model (Figure 4). The regional permeability (seven small intestine segments plus colon) for all of the methods

(apart from MechPeff) is assumed to be the same by default but can be modified by the user. The regional distributions of drug metabolising enzymes and efflux transporters such as P-gp and BCRP are also incorporated, allowing simulation of the effects of efflux transport and metabolism on drug absorption (Figure 3A).

Figure 4. Input options to predict P_{eff} and subsequently absorption within the Simcyp Simulator.

The physicochemical based QSPR (HBD and PSA) and MechPeff models were used in the simulations in this study.



The MechPeff Model is a fully mechanistic model that predicts passive intestinal permeability using a compound's physicochemical parameters such as LogP, pKa, molecular weight and compound type. The MechPeff model within Simcyp is adapted from the original structure described by Sugano (19). In brief, the model considers:

1) Intrinsic transcellular permeability according to the pH-partition hypothesis (default setting) but allows the user to select an additional model permitting transcellular ion permeation if desired;

2) Paracellular permeability based on the chemical's molecular size in relation to paracellular pore sizes (modelled via a Renkin function). In addition pore charge-charge interactions are considered (ionic species as well as neutral species can pass through the paracellular pathways)

3) The luminal Unstirred Boundary Layer (UBL) which may be the rate limiting barrier for otherwise highly permeable compounds.

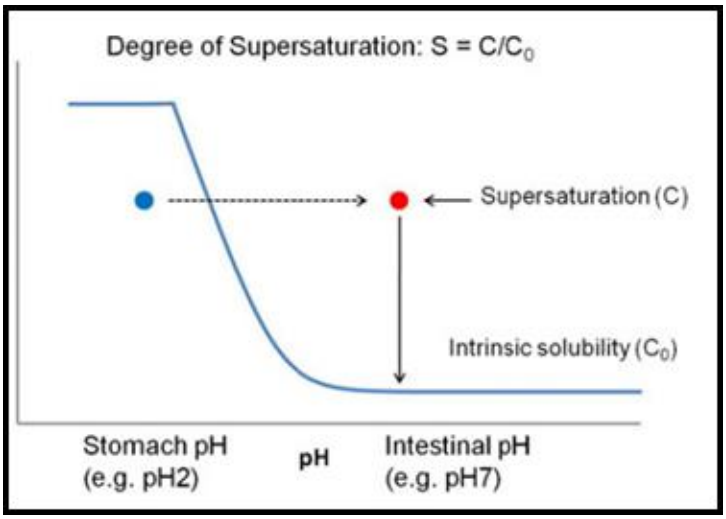
A scaling procedure is used to account for the impact of the varying regional GI morphology and physiology along the rat small intestine upon P_{eff}. A further scaling of effective available absorptive surface area, according to epithelial permeability, is provided. This accounts for the fact that highly permeable compounds may be almost entirely absorbed at the villi tips while less permeable compounds will tend to diffuse down

the inter-villus spaces and thus be exposed to a larger effective surface area. This scaling procedure is based upon the model described by Oliver *et al.* (20). The model also considers the impact of partitioning of the compound into bile salt micelles upon P_{eff}.

3.1.4 Supersaturation and Precipitation

Supersaturation describes the phenomenon whereby in the intestine some chemicals can remain in solution at concentrations above their thermodynamic equilibrium solubility and can be particularly important for poorly soluble compounds. Supersaturation is often observed for ionised compounds when there are abrupt pH changes. For example the pH change when moving from the stomach (pH 1.5-3.5) to the duodenum (pH 5-7) can promote precipitation of weakly basic compounds, (pKa values 5 to 8) (21). Dependent on the rate of precipitation of the chemical and the relative solubilities of the ionised and unionised forms of the chemical this can lead to a supersaturated solution being formed (21) (Figure 5). When compounds can supersaturate, the maximal extent and duration of supersaturation is very much compound-dependent and whilst this can be measured experimentally (21), it cannot currently be (accurately and consistently) predicted.

Figure 5. Schematic of supersaturation phenomenon for a weakly basic drug which is soluble at low pH (e.g. stomach) and subsequent formation of the less soluble species at higher pH (e.g. intestine).



Within Simcyp the extent and duration of supersaturation is specified by two parameters the maximum supersaturation ratio (MSR) and a first order precipitation rate constant (PRC). The MSR specifies the maximal allowable extent of supersaturation of a compound in solution in the gut lumen defining the maximum ratio of kinetic solubility to equilibrium solubility. When luminal compound concentrations exceed this maximum kinetic solubility, (MKS) precipitation occurs. When the concentration is reduced to (or below) the MKS the precipitation rate is defined by a first order rate constant (PRC). Precipitation no longer occurs once the luminal drug concentration is equal to, or less than, the equilibrium solubility (22).

The PRC is applied to total dissolved compound concentration including that in the ionised state and compound partitioned into bile micelles. Thus it is assumed that the inter-conversion of the compound between neutral and ionised states and the partition/departition of these monomers into bile micelles are not rate limiting for precipitation. For a given pH

and bile salt concentration it is assumed that the proportions of compound in the neutral, ionised and bound (within micelles) states are constant. The default PRC of 4 h^{-1} is equivalent to a mean duration of 15 minutes for the supersaturated state thus increasing the time available for the dissolved compound to be absorbed.

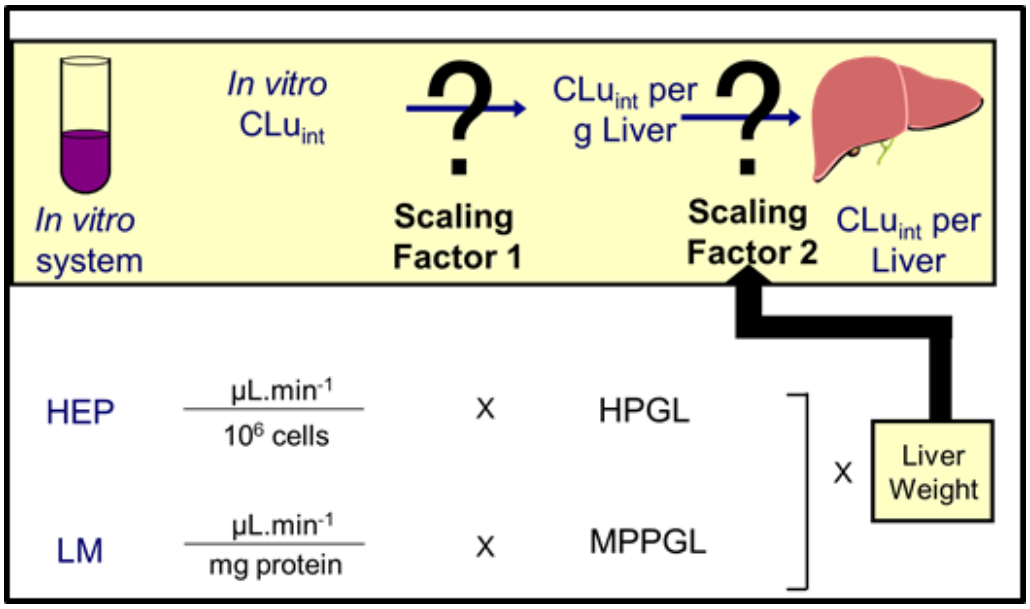
3.1.5 Metabolic Clearance

Elimination of a compound can be characterised by various inputs of clearance such as intravenous or oral clearance (CL_{iv} or CL_{po}), whole organ metabolic clearance via hepatocytes (CL_{int}; µL/min/106 cells), liver and intestinal microsomes (CL_{int}; µL/min/mg) or incubation with intestinal slices (CL_{int}; µL/min/g of intestine).

3.1.5.1 Hepatic metabolic clearance

On a general basis the *in vivo* hepatic metabolic clearance is predicted using *in vitro-in vivo* extrapolation (IVIVE) as shown schematically in Figure 6 followed by scaling for the specific metabolising tissue blood flow (liver in this case) and fraction of unbound drug in blood.

Figure 6. Schematic of IVIVE for scaling of *in vitro* intrinsic clearance available for the Simcyp Simcyp simulator.



For the human simulator clearance was predicted using metabolic intrinsic clearance (Clint, hep) data generated in cryopreserved human hepatocytes. *In vitro* $CL_{int,hep}$ was scaled up to the *in vivo* Clint ($Clint_{u,H}$) according to Equation 4.

$$CLint_{u,H} = \frac{CL_{int,hep}}{fu_{hep}} \times uptake \times HPGL\ Scailing\ Factor \times Liver\ Weight \times 10^{-6} \times 60$$

Equation 4

Where fu_{hep} is the fraction unbound of the compound in the hepatocyte incubation, HPGL is the number of hepatocytes per gram of liver, uptake was assumed to be only be due to passive processes in these simulations (set =1), and 10^{-6} and 60 are to adjust units from µl/min per 10^6 cells to L/h in the whole liver.

Correction for non-specific protein binding is important for IVIVE (23, 24). $Fu_{,hep}$ was predicted from an estimate of the fraction unbound in microsomal incubations ($fu_{,mic}$) as described by Kilford *et al* (25). $Fu_{,mic}$ may be determined experimentally by equilibrium dialysis, ultrafiltration or ultracentrifugation methods (26). Measured values of fu_{mic} were not available for the compounds studied in this exercise, and therefore in order to account for non-specific binding of the compound in the incubation, fu_{mic} was predicted using the QSAR model implemented within the Prediction toolbox in the Simcyp Simulator (V17). The Simcyp fu_{mic} prediction is based on a dataset of both human and rat liver microsomes. It has been shown that there are no species differences in microsomal binding once corrections for any differences in protein content in the incubation are accounted for (27). The fu_{mic} prediction model with Simcyp uses separate models for fully ionised acids, bases and neutral compounds (28). The fu_{mic} for a partially ionised compound is calculated as described by Gao *et al.* (29).

$$fu_{hep} = \frac{1}{1 + \frac{K_{HM}}{K_{mic}} \times \frac{V_R}{P} \times \left(\frac{1 - fu_{mic}}{fu_{mic}}\right)} \qquad Equation\ 5$$

Where K_{mic} represents microsomal protein binding affinity, P the microsomal protein concentration (mg/ml), K_{HM} the hepatocyte/medium concentration ratio, and V_R a V_{cell}/V_{inc} ratio, where V_{cell} is the cell volume and V_{inc} the incubation volume. A K_{HM}/K_{mic} ratio of 125 was assumed (25). V_R is 0.005 at the cell concentration of 10^6 cells/ml (25), and was normalised for P of 1 mg/ml.

The hepatic clearance (CL_H) in humans was calculated from the whole liver scaled *in vivo* Clintu using the well stirred model (equation 6)

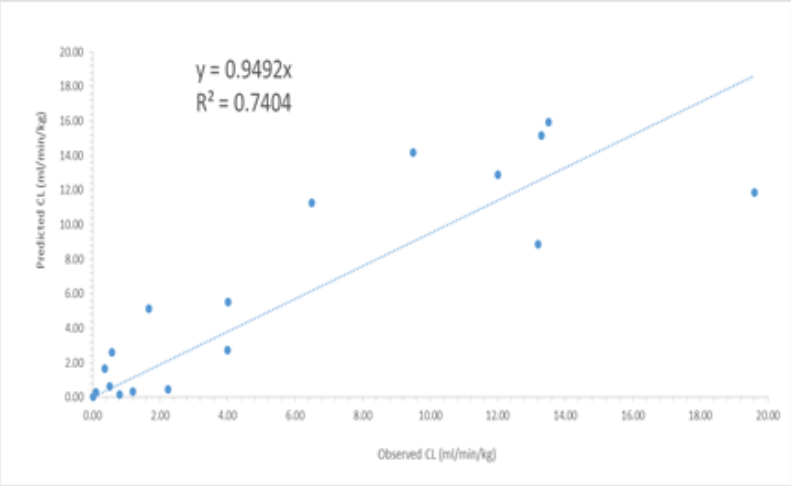
$$CL_H = \frac{Q_H * Clint_{u,h} * fu_B}{Q_H + \left(Clint_{u,h} * fu_B\right)} \qquad Equation\ 6$$

Where Q_H = hepatic blood flow, $Clint_{u,h}$ is the *in vivo* hepatic intrinsic clearance and fu_B = fu/(B/P).

The accuracy of this *in vitro – in vivo* approach to predict human clearance from human hepatocytes using data generated in human hepatocytes under the same experimental method as used for the strobin read across case studies is shown below (figure 7). The range of LogP values for these compounds was -0.07 to 4.8 although the highest log P for a neutral compound was 2.82.

Figure 7. Relationship between predicted and observed human Clearance for a series of 18 compounds.

The predicted clearance was made using IVIVE approaches described above based on the *in vitro* intrinsic clearance generated in human hepatocyte incubations by Cyprotex.



3.1.5.1.1 Intestinal Clearance

Intestinal unbound intrinsic metabolism can be directly scaled from *in vitro* intestinal microsomal (IM) incubations using a microsomal protein per intestine (MPPI) scaling factor Equation 7.

$$CL_{int,u,G} = CL_{int,u,IM} \cdot MPPI \qquad \text{Equation 7}$$

Where $CL_{int,u,IM}$ is in units of L/h/mg intestinal microsomal protein and $CL_{int,u,G}$ is in units of L/h. $CL_{int,u,G}$ can also be expressed as $Vmax_G/Km_u$. Where Vmax is the maximum rate of metabolism and Km_u is the unbound Michaelis constant.

Alternatively, when the enzyme(s) responsible for metabolic elimination are known along with their absolute abundance, intestinal metabolism can be transformed to a rate per unit enzyme either by normalising observed intrinsic clearance in liver microsomes (LM) (Equation 8) and subsequently scaled on the basis of intestinal absolute enzyme abundance in the intestine ($Abundance_{Ge}$), or directly scaled from recombinant enzyme data (Equation 9). Metabolism via multiple enzymes can be accounted for by the summation of the respective enzyme contributions (Equation 10).

$$CL_{int,u,enz} = \frac{CL_{int,u,LM}}{Abundance_{Le}} \qquad Equation\ 8$$

$$CL_{int,u,G,e} = Cl_{int,u,enz} \cdot Abundance_{Ge} \cdot MPPI \quad Equation\ 9$$

$$CL_{int,u,G=\sum_{e=1}^{n} CL_{int,u,G,e}} \qquad Equation\ 10$$

Where $CL_{int,u,enz}$ is in units of L/h/pmol enzyme and $CL_{int,u,G}$ is in units of L/h.

When the first order absorption models are used the metabolism in the intestine is estimated using the Q_{gut} model. The Q_{gut} model (Equation 11) can be used to predict intestinal metabolism under the assumption of a homogenous intestinal compartment. Whilst this dramatically simplifies the regional differences in metabolism and transport expression and activities, the Q_{gut} model has been shown a useful approach to predict intestinal metabolism

of compounds (30). The model considers the impact of Q_{gut}, a hybrid parameter consisting of both Q_{villi} (villus blood flow) and cellular permeability through the enterocyte (CL_{perm}) (Equation 12) and the net intrinsic metabolic clearance in the gut based on unbound drug concentration ($CL_{uint,G}$) to drive prediction of F_G (30). Utilisation of this strategy provided reasonable predictions for low to medium intestinal extraction drugs using the assumption of fu_G (the fraction of drug unbound in the enterocyte) is 1 (i.e. all unbound) (30, 31). However, the predictions were less accurate for drugs with moderate to high intestinal extractions (F_G <0.5) (31).

$$F_G = \frac{Q_{GUT}}{Q_{GUT} + fu_{GUT} \times CLu_{int,G}} \qquad Equation\ 11$$

$$Q_{GUT} = \frac{Q_{villi} \times CL_{perm}}{Q_{villi} + CL_{perm}} \qquad Equation\ 12$$

The Advanced Dissolution, Absorption and Metabolism (ADAM) module of the Simcyp Population-Based Simulator® represents the GI tract as seven compartments based upon their physiological and anatomical attributes. The blood flows to each anatomical region of the GI tract are defined separately and the metabolism takes place in various regions of the intestine separately, assuming that each segment is a well-stirred compartment. The regional intestinal metabolism contributions for each compartment of the ADAM model can be calculated by accounting for the distribution of enzymes along the GI tract (Equation 13). Total intestinal metabolism is calculated from the sum of these segmental (Equation 14) and total enzyme contributions (Equation 10).

$$CL_{int,u,G,e,i} = CL_{int,u,enz} \cdot Abundance_{G,e,i} \cdot distribution_{G,MPPI} \cdot MPPI \quad Equation\ 13$$

$$CL_{int,u,G,e} = \sum_{i=1}^{7} CL_{int,u,G,e,i} \qquad Equation\ 14$$

Where $CL_{int,u,G,e,i}$ = intrinsic clearance per pmol enzyme

Abundance $G_{e,i}$ = abundance per segment (i) in pmol/mg micoromal protein

$Distribution_{G,MPPI}$ = % of microsomal protein per intestine (MPPI)

3.1.5.2 Clearance inputs in the rat models

Equivalent *in vitro* metabolic data in rat hepatocytes could not be generated as part of the EU-TOX-RISK project therefore to predict the exposure in the rat the following approach was taken. The *in vivo* clearance predicted in the human simulator was scaled to predict a clearance in rat using a single species scaling approach with an exponent of 0.75 as described by Hosea *et al.* (32) (equation 15).

$$CLu_{human} = CLu_{rat} * \left(\frac{W_{human}}{W_{rat}}\right)^{0.75} \qquad Equation\ 15$$

Where CLu = unbound clearance after intravenous dosing, W = body weight

3.1.6 Excretion

It is possible to account for the excretion of unchanged drug in the kidney or via the biliary system in the PBPK models, but in the models developed here metabolism was considered as the only route of clearance.

3.1.7 General description of equations for eliminating and non-eliminating organs

Each compartment within the full body PBPK model was initially described as a perfusion limited model with representative equations for an eliminating and non-eliminating organ as shown below. The equations describing the behaviour of the compound within the intestine following oral absorption are as described by Jamei *et al* (9). The basic principles for a PBPK model outlined on P19 of the WHO guidance on PBPK modelling were adhered to. Namely

1) the mixing of the chemical in the effluent blood from the tissues is instantaneous and complete;
2) blood flow is unidirectional, constant and non-pulsatile; and
3) the presence of chemicals in the blood does not alter the blood flow rate

In non-eliminating tissues the compound concentration (C) at a given time (t) is defined as follows

$$\frac{dC_{tissue}}{dt} = \frac{Q_{tissue}}{V_{tissue}}\left(C_{ab} - \frac{C_{tissue}}{P_{tissue:p}/_{BP}}\right)$$

Where Q = blood flow to the tissue, V = tissue volume, C_{ab} = arterial blood concentration, BP = blood to plasma ratio and $P_{Tissue:p}$ – partition coefficient of drug between tissues and plasma

In the liver the following equation is applied

$$\frac{dC_{liver}}{dt} = \frac{1}{V_{liver}}\left((Q_{liver} - Q_{pv})C_{ab} + Q_{pv}C_{pv} - \frac{Q_{liver}C_{liver}}{P_{liver:p}/_{BP}} - \frac{f_u}{BP} * \frac{Clu_{int}}{P_{liver:p}/_{BP}} * C_{liver}\right)$$

Where Q_{liver} = sum of blood flow to the liver by the hepatic artery and hepatic portal vein, Q_{pv} = hepatic portal blood vein flow, C_{pv} = hepatic portal compound vein concentration, fu = fraction unbound in plasma, Clu_{int} = intrinsic clearance

Within each simulated animal or human subject the sum of the tissue blood flow rates (excluding the lung) are equal to cardiac output. In line with accepted mammalian physiology the lung also receives a blood flow equal to total cardiac output. Tissue volumes and blood flow rates are within the documented range for each species and age group (paediatric versus adult) considered (2, 33, 34).

Within each simulated animal or human subject the sum of the tissue blood flow rates (excluding the lung) are equal to cardiac output. In line with accepted mammalian physiology the lung also receives a blood flow equal to total cardiac output. Tissue volumes and blood flow rates are within the documented range for each species considered (2, 34).

3.1.8 Population data

Predictions of plasma drug exposure, clearance and other parameters such as fraction metabolised by a particular pathway were made for virtual populations of healthy volunteers. Each population is generated using values and formulae describing demographic, anatomical and physiological variables. Thus, in order to assess clearance

predictions in a specific population, data are required for the population variables as well as for the *in vitro* metabolism/transport of the test drug and its observed clearance in the population of interest. The parameter values within the Simcyp Simulator for creating a virtual healthy volunteer population (population, physiological parameters including liver volume and blood flows, enzyme abundances) have been described previously (2).

3.1.9 Physiology data used in the healthy human population PBPK populations

Table 1. Range of physiology data used in the human population PBPK simulations.

A virtual population of 100 individuals aged 20-50 with 50% of the subjects being female was used for the simulations.

Parameter	Mean value	Range
Age (y)	29.6	20 - 48
Weight (kg)	72.45	45 - 118
Height (cm)	168.4	149 - 189
Cardiac output (L/h)	321.8	252 - 416
Serum albumin (g/L)	45.7	38 - 60

4. Data for simulation of the *in vivo* kinetics of pyraclostrobin, kresoxim methyl, azoxystrobin, picoxystrobin, trifloxystrobin and antimycin-A.

Data and the corresponding source and/or reference used in the compound files are shown in Table 2 to 7 below. A summary of the *in vivo* information available for these compounds is presented in section 9. All of the plasma/blood and tissue concentration data in the public domain is based on measurements of total radioactivity and so cannot be used to verify the performance of the PBPK models that only simulate the exposure to parent compound.

Table 2. Initial input parameter values used to simulate the kinetics of pyraclostrobin

Parameter	Value	Method / Comment	Source/Reference
MW [g/mol]	387.8		https://pubchem.ncbi.nlm.nih.gov/compound/6422843#section=Chemical-and-Physical-Properties
logP	4.1 3.99	Predicted Experimental	Pubchem
Compound Type	Neutral		
TPSA (Å^2)	65.8		Pubchem
Hydrogen bond donors	0		
fu	0.00152 0.00158 (0.018-0.045)	Experimental Experimental Predicted	Cyprotex data (CYP1440-R7B) (35) ISTMN model (EU TOX RISK)
B/P ratio	0.708	Measured	Cyprotex data (CYP1440-R7B)
fa	0.99 1.00	Predicted using HBD and PSA Predicted using mech peff model	(18, 36) (19)
ka (h^{-1})	2.34	Predicted Simcyp Simulator V17	
fu_{Gut}	1	Assumed	
Clint (μl/min/10^6 cells)	23.4 53.9 16.1	Experimental Experimental (1 μM) Experimental (10 μM)	Cyprotex data (CYP1440-R7B) (35)
Microsomal binding (fu_{mic})	0.306	Predicted	Simcyp simulator
Hepatocyte binding (fu_{heps})	0.414	Calculated from fu_{mic}	(25)
Solubility (mg/mL)	0.0019	Experimental	Pubchem

Table 3. Initial input parameter values used to simulate the kinetics of kresoxim methyl

Parameter	Value	Method / Comment	Source/Reference
MW [g/mol]	313.35		https://pubchem.ncbi.nlm.nih.gov/compound/6112114#section=Chemical-and-Physical-Properties
logP	4.1 3.4	Predicted Experimental	Pubchem
Compound Type	Neutral		Pubchem
TPSA (Å^2)	57.1		Pubchem
Hydrogen bond donors	0		
fu	0.0276 (0.023 – 0.047)	Experimental Predicted	Cyprotex data (CYP1440-R7B) ISTMN model EU-TOX-RISK
B/P ratio	0.68		Cyprotex data (CYP1440-R7B)
fa	1.00 1.00	Predicted using HBD and PSA Predicted using mech peff model	(18, 36) (19)
ka (h^{-1})	2.92	Predicted	Simcyp Simulator v17
fu_{Gut}	1	Assumed	
Clint (μl/min/10^6 cells)	355	experimental	Cyprotex data (CYP1440-R7B)
Microsomal binding (fu_{mic})	0.473	Predicted	Simcyp simulator
Hepatocyte binding (fu_{heps})	0.59	Calculated from fu_{mic}	(25)
Solubility (mg/mL)	0.002	Experimental	Pubchem

Table 4. Initial input parameter values used to simulate the kinetics of azoxystrobin

Parameter	Value	Method / Comment	Source/Reference
MW [g/mol]	403.39		https://pubchem.ncbi.nlm.nih.gov/compound/3034285#section=Chemical-and-Physical-Properties
logP	3.7 2.5	Predicted Experimental	https://pubchem.ncbi.nlm.nih.gov/compound/3034285#section=Chemical-and-Physical-Properties
Compound Type	Neutral		Pubchem
TPSA (Å^2)	104		Pubchem
Hydrogen bond donors	0		
fu	0.103 (low recovery 67%) 0.048 (0.038 – 0.039)	Experimental Experimental Predicted	Cyprotex data (CYP1440-R7B) (35) ISTMN model EU_TOX_RISK project
B/P ratio	0.76		Cyprotex data (CYP1440-R7B)
fa	0.92 1	Predicted using HBD and PSA Predicted using mech peff model	(18, 36) (19)
ka (h^{-1})	0.89	Predicted	Simcyp Simulator v17
fu_{Gut}	1	Assumed	
Clint (μl/min/10^6 cells)	32.5 32.4 14.3	Experimental Experimental (1 μM) Experimental (10 μM)	Cyprotex data (CYP1440-R7B) (35)
Microsomal binding (fu_{mic})	0.726	Predicted	Simcyp simulator
Hepatocyte binding (fu_{heps})	0.809	Calculated from fu_{mic}	(25)
Solubility (mg/mL)	0.006		Pubchem

Table 5. Initial input parameter values used to simulate the kinetics of picoxystrobin

Parameter	Value	Method / Comment	Source/Reference
MW [g/mol]	367.32		https://pubchem.ncbi.nlm.nih.gov/compound/11285653#section=Chemical-and-Physical-Properties
logP	3.6 3.68	Predicted Experimental	Pubchem PICOXYSTROBIN (258) First draft prepared by Dr Samuel Margerison, Australian Pesticides and Veterinary Medicines Authority, Canberra, Australia
Compound Type	neutral		
TPSA (Å^2)	57.6		Pubchem
Hydrogen bond donors	0		Pubchem
fu	0.0127 (low recovery 69%) (0.047 – 0.102)	Experimental Predicted	Cyprotex data (CYP1440-R7B) ISTMN model EU_TOX_RISK project
B/P ratio	0.71		Cyprotex data (CYP1440-R7B)
fa	1.00 1.00	Predicted using HBD and PSA Predicted using mech peff model	(18, 36) (19)
ka (h^{-1})	2.89	Predicted	Simcyp Simulator v17
fu_{Gut}	1	Assumed	
Clint (μl/min/10^6 cells)	31.3	Experimental	Cyprotex data (CYP1440-R7B)
Microsomal binding (fu_{mic})	0.391	Predicted	Simcyp simulator
Hepatocyte binding (fu_{heps})	0.507	Calculated from fu_{mic}	(25)
Solubility (mg/mL)	0.00325		PICOXYSTROBIN (258)

Table 6. Initial input parameter values used to simulate the kinetics of trifloxystrobin

Parameter	Value	Method / Comment	Source/Reference
MW [g/mol]	408.4		https://pubchem.ncbi.nlm.nih.gov/compound/11664966#section=Chemical-and-Physical-Properties
logP	4.9 4.5	Predicted Measured	Pubchem
Compound Type	Neutral		Pubchem
TPSA (Å^2)	69.5		Pubchem
Hydrogen bond donors	0		Pubchem
fu	0.00159 (0.0079 – 0.061)	Experimental Predicted	Cyprotex data (CYP1440-R7B) ISTMN model EU_TOX_RISK project
B/P ratio	0.713		Cyprotex data (CYP1440-R7B)
fa	0.99	Predicted using HBD and PSA Predicted using mech peff model	(18, 36) (19)
ka (h^{-1})	2.14	Predicted	Simcyp Simulator v17
fu_{Gut}	1	Assumed	
Clint (μl/min/10^6 cells)	236	Experimental	Cyprotex data (CYP1440-R7B)
Microsomal binding (fu_{mic})	0.193	Predicted	Simcyp simulator
Hepatocyte binding (fu_{heps})	0.277	Calculated from fu_{mic}	(25)
Solubility (mg/mL)	0.0006	experimental	Pubchem

Table 7. Initial input parameter values used to simulate the kinetics of antimycin A

Parameter	Value	Method / Comment	Source/Reference
MW [g/mol]	548.63		https://pubchem.ncbi.nlm.nih.gov/compound/14957#section=Chemical-and-Physical-Properties
logP	5.3 4.2	Predicted	https://pubchem.ncbi.nlm.nih.gov/compound/14957#section=Chemical-and-Physical-Properties; https://www.ebi.ac.uk/chembl/compound/inspect/CHEMBL211501 (ACD log P)
Compound Type	Acid (pKa 7.28)		Chembl
TPSA (Å^2)	157		Pubchem
Hydrogen bond donors	3		Pubchem
fu	0.00085 (result is questionable) (0.05 – 0.15)	Experimental Predicted	Cyprotex data (CYP1440-R7B) ISTMN model EU_TOX_RISK project
B/P ratio	0.556	Experimental	Cyprotex data (CYP1440-R7B)
fa	0.106 1.00	Predicted using HBD and PSA Predicted using mech peff model	(18, 36) (19)
ka (h^{-1})	0.034	Predicted	Simcyp Simulator v17
fu_{Gut}	1	Assumed	
Clint (μl/min/10^6 cells)	86.1	Experimental	Cyprotex data (CYP1440-R7B)
Microsomal binding (fu_{mic})	0.278	Predicted	Simcyp simulator
Hepatocyte binding (fu_{heps})	0.381	Calculated from fu_{mic}	(25)
Solubility (mg/mL)	0.000089		

4.1.1 Sensitivity Analyses on uncertain parameters

For some parameters within the PBPK model, there is uncertainty as to what is the correct input value to use. For these parameters, a sensitivity analysis was conducted and the impact on the simulation results (plasma exposure as judged by the area under the plasma concentration time profile) was evaluated.

Table 8. Parameter sensitivity and uncertainty table.

		UNCERTAINTY		
		High	Medium	Low
SENSITIVITY	High			
	Medium			
	Low			

5. Results

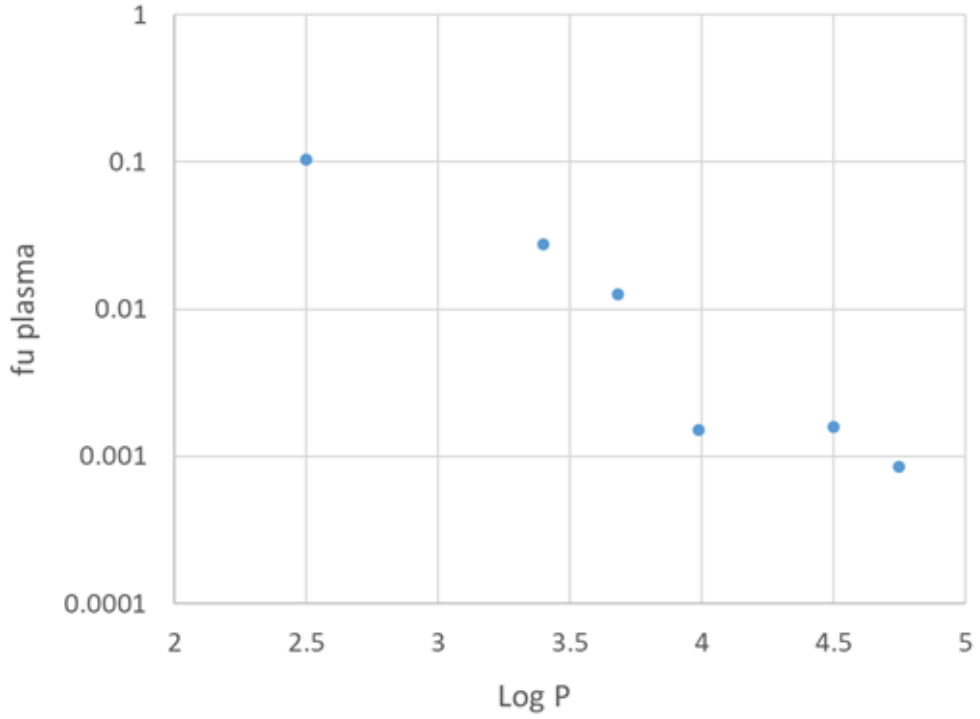
5.1.1 Physicochemical and blood binding data

The compounds utilised in the read across exercise have a range of experimentally determined lipophilicity values. All of the compounds with experimental log P values have a preference for partition into octanol compared to water (Azoxystrobin log P = 2.5; kresoixm methyl Log P = 3.4; Picoxystrobin log P = 3.68; pyraclostrobin log P = 3.99; trifloxystrobin log P = 4.5) and are neutral. Antimycin-A is an acidic compound (pKa 7.25) and an average of 2 different predicted log P values (4.75) was used in the PBPK simulations.

All of the compounds distribute preferentially into plasma compared to red blood cells (BP ratio <1) and were measured to be highly bound (fu 0.103 – 0.00085) to plasma proteins in

the determinations made at Cyprotex for the EU_TOX_Risk project. The protein binding increased (fu decreased) as the lipophilicity of the compounds increased. Azoxystrobin is the least lipophilic of the compounds and has the highest fu value.

Figure 8. The relationship between fu in plasma and log P for the strobin case study compounds.



5.1.2 Absorption

Using the physicochemical properties (HBD and PSA) of the compounds to predict the fraction of oral absorption (fa) resulted in high predicted values for the fraction absorbed following a 100 mg oral dose in humans (fa ≥ 0.99 for all compounds except azoxystrobin predicted fa = 0.92 and antimycin-A predicted fa = 0.106). The low predicted fa for antimycin-A is due to the compound having a much higher PSA and more hydrogen bond donors than the other compounds.

Predictions for the permeability and absorption of the compounds was similar using the mechanistic permeability model for all of the compounds except antimycin-A where the mechanistic permeability model predicted almost complete absorption of the compound in the absence of solubility limitations. As the physicochemical properties of Antimycin-A are outside the range of properties used to construct the PSA and HBD QSAR model simulations were conducted with this compound using the mechanistic permeability model to predict permeability across the intestine.

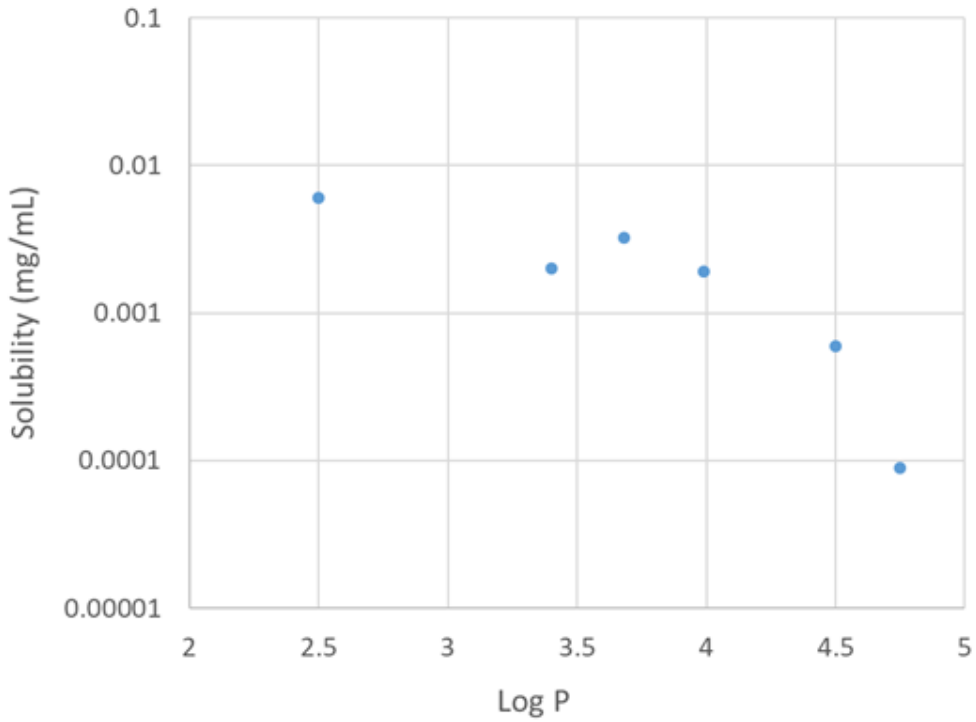
5.1.3 Solubility

Although the compounds are predicted to have reasonable intestinal permeability, they also have low solubility that is likely to limit their exposure following oral dosing. Indeed

incomplete absorption and less absorption of total radioactivity at higher doses was observed for several of the compounds in *in vivo* studies in the rat (section 9).

The measured solubility for the strobin case study compounds ranged between 0.002 and 0.000089 mg/mL and a trend for decreasing solubility with increasing log P was observed (figure 9).

Figure 9. The relationship between solubility (mg/mL) and log P for the strobin case study compounds



Simulations were run at a 1 or 10 mg/kg dose of the case study compounds to examine the effect of including solubility on the predicted fraction absorbed. These simulations were run using the default values for supersaturation ratio (1000) and precipitation rate constant (4 h^{-1}) in the Simcyp simulator V17 in the absence of compound specific measured values for these parameters (table 9).

At the lower simulated dose (1 mg/kg) all of the compounds were predicted to be well absorbed apart from antimycin-A. At the higher simulated dose (10 mg/kg) only azoxystrobin and picoxystrobin were predicted to be well absorbed.

Table 9. Simulated fraction absorbed (median and 5th and 95th percentiles) for the strobin case study compounds after oral administration at a dose of 100 mg in a population of 100 fasting human subjects (aged 20 – 50; 50% female).

Simulations were conducted twice once looking only at the effect of permeability on absorption (with the assumption that all of the dose was in solution) and then again where the solubility/precipitation of the compounds was considered in the simulation (solution with precipitation). Simulations incorporating information on solubility were conducted using default values for precipitation rate constant (4 h^{-1}) and supersaturation ratio (1000) in the absence of any compound specific values.

Compound	Fraction absorbed		
	Solution dose (solubility not considered)	Incorporating solubility/precipitation (1 mg/kg)	Incorporating solubility/precipitation (10 mg/kg)
Pyraclostrobin	0.94 (0.82 – 0.98)	0.94 (0.78 – 0.98)	0.36 (0.19 -0.86)
Kresoxim-methyl	0.97 (0.87- 0.99)	0.97 (0.87 – 0.99)	0.39 (0.22-0.97)
Azoxystrobin	0.97 (0.89 – 0.99)	0.97 (0.89 – 0.99)	0.96 (0.63 – 0.99)
Picoxystrobin	0.95 (0.84 – 0.98)	0.95 (0.84 – 0.98)	0.76 (0.34 – 0.98)
Trifloxystrobin	0.93 (0.79 – 0.98)	0.80 (0.48-0.98)	0.23 (0.14 – 0.45)
Antimycin-A	0.91 (0.76 – 0.97)	0.25 (0.15 - 0.45)	0.15 (0.08 – 0.25)

5.1.4 Distribution

The volume of distribution at steady state (V_{ss}) was predicted for each compound using Methods 1, 2 and 3 in the human simulator and methods 1 and 2 in the rat simulator using the measured fu values. See the section above (3.1.2) for description of the methods.

The results are shown in Table 10 and 11 below. In humans, the three methods of predicting Vss and tissue distribution gave similar values of predicted V_{ss} for each of the compounds using the input parameters listed in Tables 2-7. The predictions for a given compound were within 1.6-fold of each other with the different methods showing that all of the prediction methods are giving comparable outputs with the same input data for this group of compounds.

For the rat, no measured values of protein binding have been found so the following approach was taken to predict fu in rat plasma. The binding affinity for serum albumin (K_D) was calculated in the Simcyp human simulator and assumed to be the same in rat and human and this was assumed to be the only binding protein for rotenone or deguelin. The K_D value was then used to predict the fraction unbound in rat plasma accounting for the abundance of albumin in rat plasma (average value = 31.1 mg/mL). Blood:plasma ratio was also assumed to be the same as in humans.

The predicted brain:plasma concentration ratios at steady state for all of the strobin case study compounds in human and rat are shown in table 12. The distribution methods 2 for rat and 3 for human were used to make these predictions. Quantitatively similar values were obtained in the human simulator using method 2 to make the predictions (data not shown). For all of the compounds except antimycin-A the distribution into the brain was predicted to be similar in human and rat. For all of the comp0unds except pyraclostrobin in human and antimycin-A in rat the total concentration in the brain was predicted to be higher than in the plasma at steady state.

Table 10. Predicted V_{ss} (L/kg) for the strobin case study compounds in humans

Compound	Predicted V_{ss} (L/kg)		
	Method 1	Method 2	Method 3
Pyraclostrobin	0.94	0.80	0.64
Kresoxim methyl	2.60	3.05	2.29
Azoxystrobin	1.39	1.36	0.99
Picoxystrobin	2.47	2.79	2.14
Trifloxystrobin	2.43	2.65	2.13
Antimycin-A	1.60	1.18	0.97

Table 11. Predicted V_{ss} (L/kg) for the strobin case study compounds in rats

Compound	Predicted rat plasma fu	Predicted V_{ss} (L/kg)	
		Method 1	Method 2
Pyraclostrobin	0.0022	0.88	0.67
Kresoxim methyl	0.039	2.31	2.56
Azoxystrobin	0.142	1.34	1.24
Picoxystrobin	0.0183	2.17	2.34
Trifloxystrobin	0.0023	2.02	2.07
Antimycin-A	0.00123	0.75	0.35

Table 12. Predicted brain:plasma ratio for the strobin case study compounds in humans and rats

Compound	Predicted Brain:plasma ratio	
	Rat	Human
Pyraclostrobin	1.24	0.98
Kresoxim methyl	3.92	4.36
Azoxystrobin	1.91	2.10
Picoxystrobin	3.5	3.85
Trifloxystrobin	2.91	3.25
Antimycin-A	0.38	1.38

5.1.5 Metabolic Clearance

The clearance of the strobin case study compounds was simulated in a population of 100 healthy human subjects (aged 20-50; 50% female) using the available *in vitro* metabolism data and blood binding data as inputs to the model. The predictions of clearance assume linear clearance with increasing concentrations of compound (i.e. no saturation occurs).

Table 13. Predicted human clearance of the strobin case study compounds.

Data are shown as median values from the population and the 5th and 95th percentiles of the population to give an idea of the simulated inter-individual variability

Compound	Median CL (L/h)	5th and 95th percentiles
Pyraclostrobin	0.81	0.42 – 1.50
Kresoxim methyl	43.89	34.71 – 56.96
Azoxystrobin	24.39	15.57 – 37.37
Picoxystrobin	4.71	2.33 – 7.82
Trifloxystrobin	10.65	6.04 =18.24
Antimycin-A	1.77	0.93 – 3.23

The strobin case study chemicals have a wide range of values of predicted human clearance. Figure 10 (total concentration) and 11 (unbound concentration) show the predicted mean exposure of the compounds following a 10 mg/kg intravenous bolus dose to a population of 100 healthy human subjects (age 20- 50; 50% female). Azoxystrobin has the shortest predicted half-life of the compounds and is eliminated from the body to a significant extent over the duration of the simulation (72 hours).

Figure 10. Predicted mean exposure (total concentration) of the strobin case study chemicals in human plasma following a 10 mg/kg intravenous dose to a population of 100 healthy human subjects (age 20-50; 50% female).

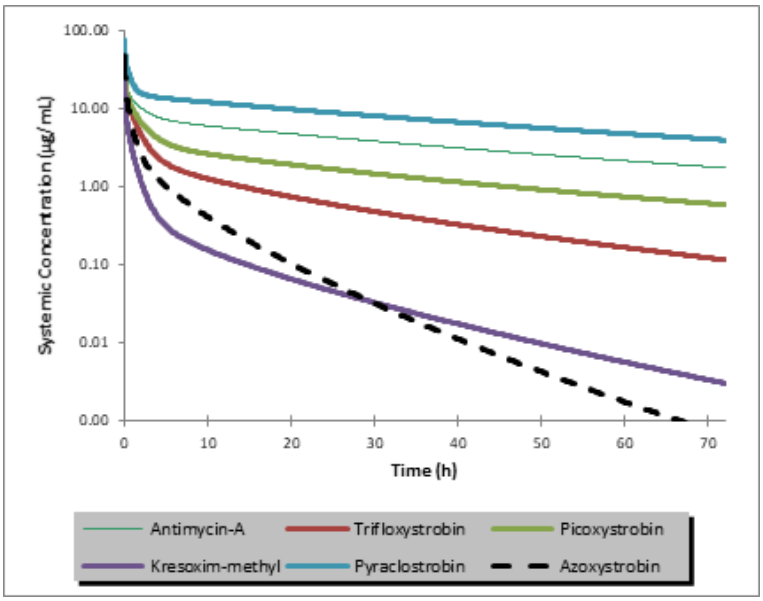
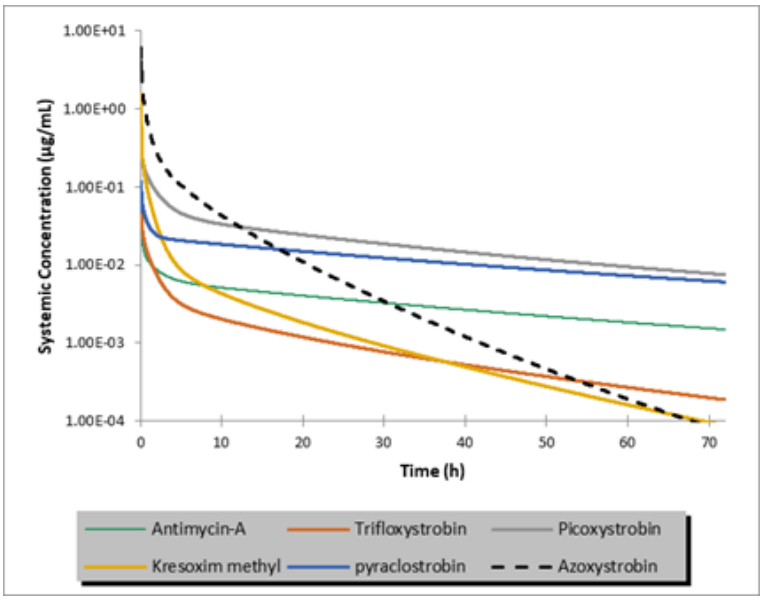


Figure 11. Predicted mean exposure (unbound concentration) of the strobin case study chemicals in human plasma following a 10 mg/kg intravenous dose to a population of 100 healthy human subjects (age 20-50; 50% female).



The clearance in the rat was extrapolated from the values obtained in humans (Table 14).

Table 14. Predicted metabolic clearance of strobin case study compounds in the rat

Compound	Predicted CL	
	Human (L/h)	Rat (ml/min)
Pyraclostrobin	0.81	0.26
Kresoxim methyl	43.89	13.92
Azoxystrobin	24.39	7.55
Picoxystrobin	4.71	1.53
Trifloxystrobin	10.65	3.46
Antimycin-A	1.77	0.58

5.1.6 Excretion

In line with the available data in rats (summarised in section 9) metabolism was considered to be the only mechanism of elimination for the parent compound when each of the strobin case studies were dosed to humans or rats. Biliary and renal excretion of unchanged drug was set to 0 in the PBPK models.

5.1.7 Simulating oral exposure of the stobin case study compounds in the rat.

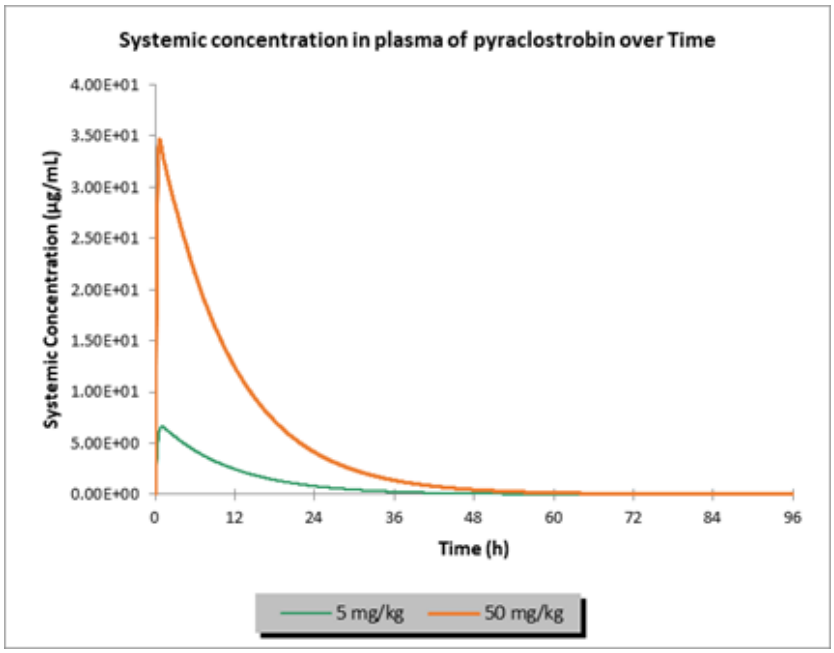
5.1.7.1 Pyraclostrobin

The pharmacokinetics of pyraclostrobin were predicted in the rat using the predicted clearance and volume of distribution data (method 2). The compound was considered to be dosed as a solution with precipitation being possible. Intestinal permeability was predicted using the mechanistic permeability model and available solubility data was incorporated into the model. As in the human model default values were used for the supersaturation ratio (1000) and precipitation rate constant (4 h^{-1}). Under these conditions, the fraction absorbed was predicted to be 1 at a dose of 5 mg/kg and 0.51 at a dose of 50 mg/kg. The time of the maximum plasma concentration for pyraclostrobin occurred between 0.7 and 1 hour post dosing. The fraction absorbed (at the higher dose) and T_{max} data (at both doses) are consistent with the findings from the radiolabelled studies with pyraclostrobin. The predicted plasma concentration time profile following dosing with pyraclostrobin are shown below in figure 12. Consistent with the radiolabelled data the majority of the compound is eliminated within the first 48 hours post dose. The simulated half-life for pyraclostrobin in the rat was 10 hours at the 5 mg/kg dose and 20 hours at the 50 mg/kg dose which is in line with the reported half-life for total radioactivity. The simulated AUC of pyraclostrobin at the highest dose is about 4-fold higher than the AUC reported for total radioactivity in plasma after dosing 50 mg/kg orally to rats. This suggests that the clearance of pyraclostrobin in the rat is underpredicted using the technique of back extrapolation from human and may suggest that the rate of metabolism is much faster in rats than humans or that the protein binding of pyraclostrobin in the rat is lower than the predicted value used in the simulation. Unfortunately, it is not possible to generate metabolism data in rat hepatocytes or protein binding in rat plasma under the auspices of the EU_TOX_Risk project to distinguish these hypotheses. Using a retrograde calculation approach the intrinsic clearance of pyraclostrobin in rat hepatocytes was calculated to be about 130 µl/min/10^6 hepatocytes.

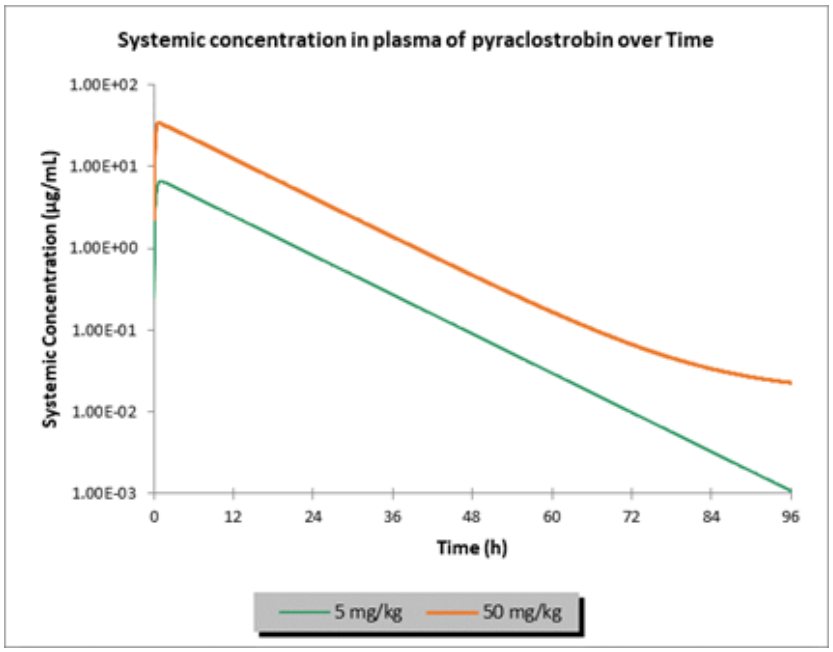
Figure 12. Predicted plasma concentration versus time profile for pyraclostrobin following oral doses of 5 or 50 mg/kg dosed to the rat.

Data is shown (A) in normal scale and (B) with concentration data on a log scale.

(A)



(B)



5.1.7.2 Picoxystrobin

The pharmacokinetics of picoxystrobin was predicted in the rat using the predicted clearance and volume of distribution data (method 2). The compound was considered to be dosed as a solution with precipitation being possible. Intestinal permeability was predicted

using the mechanistic permeability model and available solubility data was incorporated into the model. As in the human model default values were used for the supersaturation ratio (1000) and precipitation rate constant (4 h^{-1}). Under these conditions, the fraction absorbed was predicted to be 1 at a dose of 10 mg/kg and 0.51 at a dose of 100 mg/kg. The time of the maximum plasma concentration for pyraclostrobin occurred between 0.5 and 1 hour post dosing. The fraction absorbed at the higher dose was a little bit lower than observed in the 100 mg/kg study but otherwise the predictions were reasonable. Sensitivity analysis showed that the fraction absorbed was sensitive to the value used for the precipitation rate constant in the model (figure 13). As experimental data showed that 100% of the dose was absorbed the simulations were repeated using a precipitation rate constant of 1.5 h^{-1}. Using this value of PRC the fraction absorbed was predicted to be 1 at 10 mg/kg and 0.73 at 100 mg/kg the concentrations of picoxystrobin at these doses are shown in figure 14. The compound was rapidly absorbed and eliminated with the vast majority of the dose predicted to be excreted within 48 hours at both doses.

Figure 13. Predicted fraction absorbed of picoxystrobin at various different values of the precipitation rate constant.

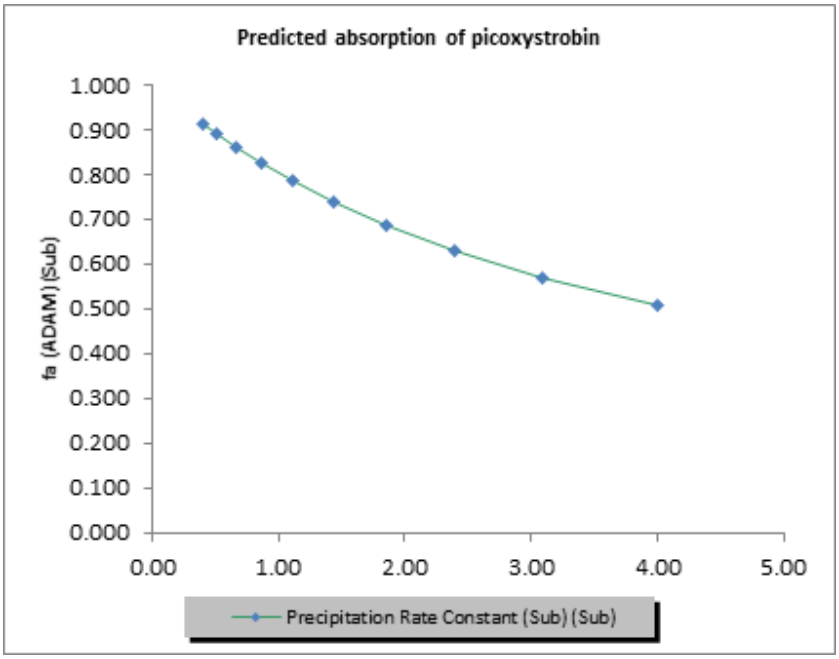
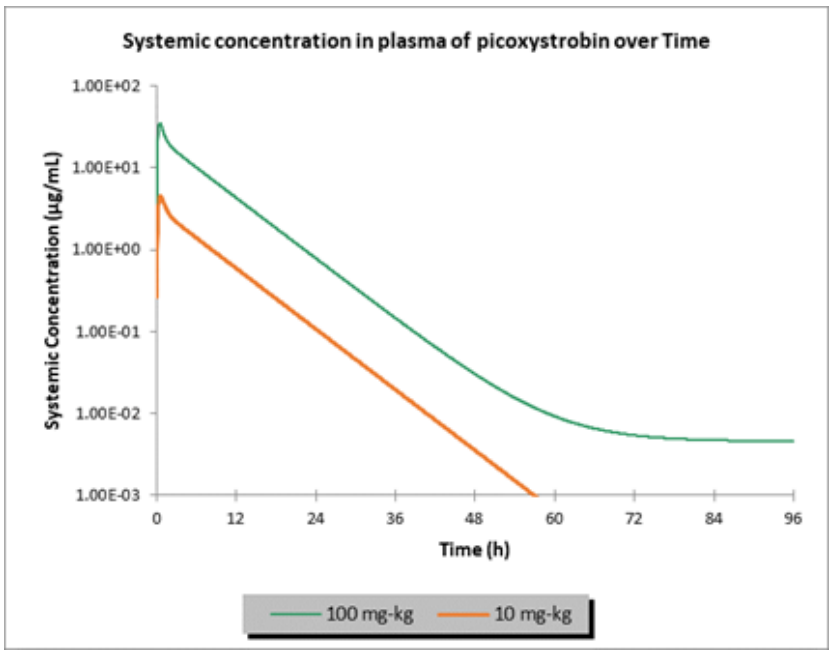


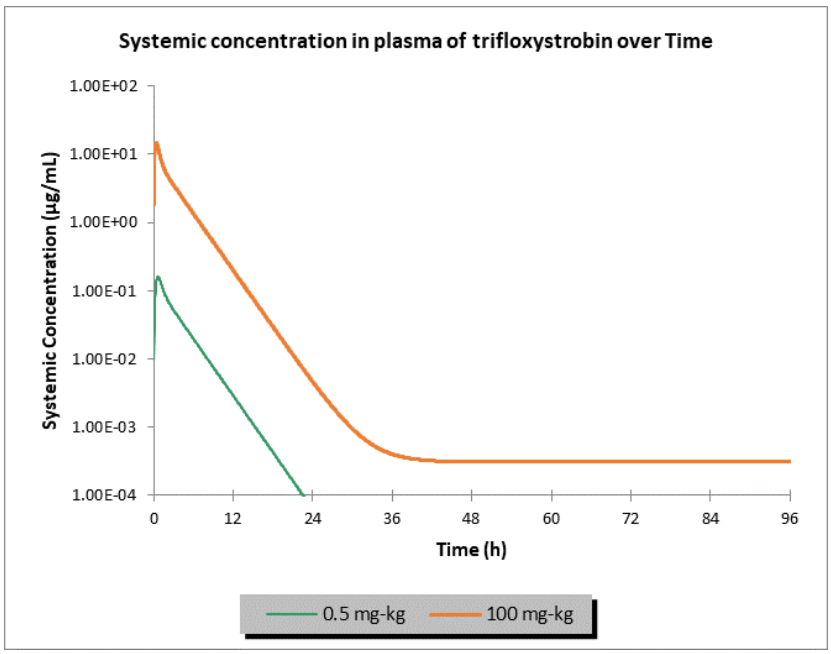
Figure 14. Predicted plasma concentrations of picoxystrobin at doses of 10 and 100 mg/kg.



5.1.7.3 Trifloxystrobin

The pharmacokinetics of trifloxystrobin was predicted in the rat using the predicted clearance and volume of distribution data (method 2). The compound was considered to be dosed as a solution with precipitation being possible. Intestinal permeability was predicted using the mechanistic permeability model and available solubility data was incorporated into the model. As in the human model default values were used for the supersaturation ratio (1000) and precipitation rate constant (4 h^{-1}). Under these conditions, the fraction absorbed was predicted to be 1 at a dose of 0.5 mg/kg and 0.37 at a dose of 100 mg/kg. The fraction absorbed was overpredicted at the low dose but at the higher dose, the predicted fa was within the range of reported values. The predicted concentration of trifloxystrobin after dosing of 0.5 or 100 mg/kg are shown in figure 15. The vast majority of the absorbed drug is eliminated within the first 48 hours post dosing. The predicted AUC at the two doses is lower than the observed AUC for total radioactivity meaning that the predictions from the PBPK model are plausible, as the AUC of total radioactivity measured in the study should be larger than the predicted AUC due to parent compound.

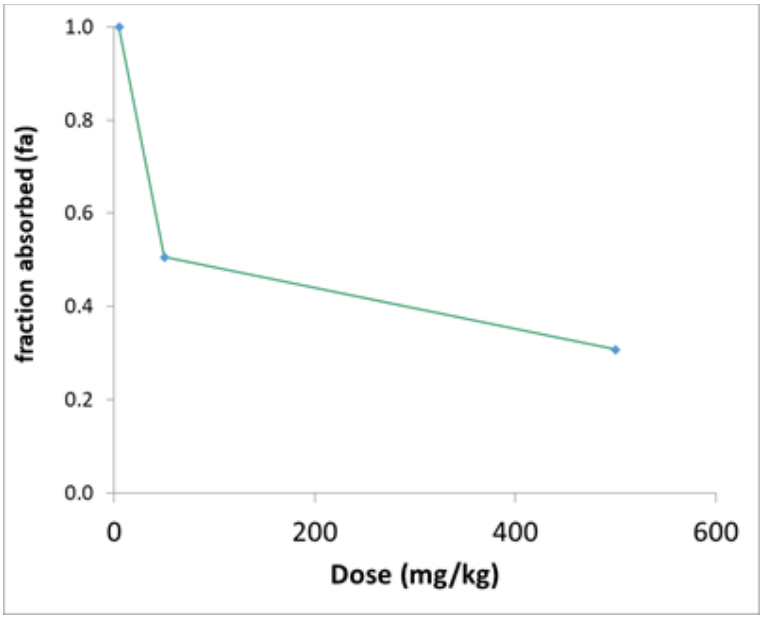
Figure 15. Predicted plasma concentrations of trifloxystrobin at doses of 0.5 and 100 mg/kg.



5.1.7.4 Kresoxim methyl

The predicted fraction absorbed for Kresoxim methyl across the oral dose range 5 to 500 mg/kg is shown in figure 16. The predicted oral absorption of Kresoxim methyl is consistent with the change in absorption observed in rats as the dose of Kresoxim methyl was increased from 5 to 500 mg/kg (see section 8).

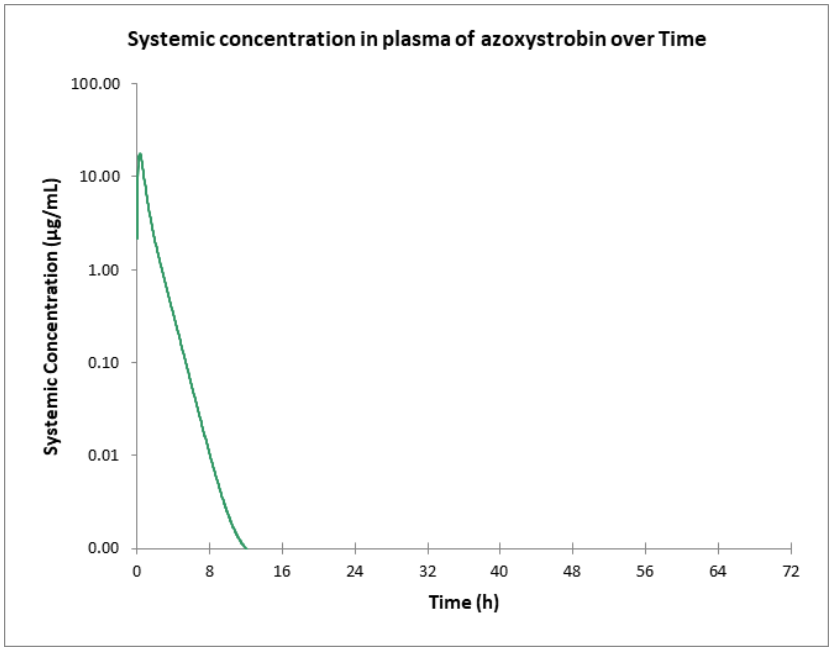
Figure 16. Predicted fraction absorbed of Kresoxim methyl after oral doses of 5, 50 and 500 mg/kg to rats.



5.1.7.5 Azoxytrobin

The pharmacokinetics of azoxystrobin was predicted in the rat using the predicted clearance and volume of distribution data (method 2). The compound was considered to be dosed as a solution with precipitation being possible. Intestinal permeability was predicted using the mechanistic permeability model and available solubility data was incorporated into the model. As in the human model default values were used for the supersaturation ratio (1000) and precipitation rate constant (4 h^{-1}). Under these conditions, the fraction absorbed was predicted to be 0.51 at a dose of 100 mg/kg. A sensitivity analysis was undertaken to examine the effect of changing the precipitation rate constant on the fraction absorbed. A PRC value of 1 gave a predicted fa of ~0.8 in the rat at a dose of 100 mg/kg which was in line with the experimental data. The simulated plasma concentration of azoxystrobin at a 100 mg/kg dose are shown in figure 17. The majority of the absorbed compound is predicted to be eliminated within the first 48 hours of the simulation consistent with experimental data.

Figure 17. Predicted plasma concentrations of azoxystrobin at doses of 100 mg/kg.



The plasma concentrations of all compounds was simulated at oral doses of 10 and 100 mg/kg/day for 14 days and the predicted exposure of the total and unbound concentration for each compound at each of the two doses is shown below.

Figure 18. Predicted exposure of the strobin case study chemicals in the rat following multiple doses of 10 mg/kg.

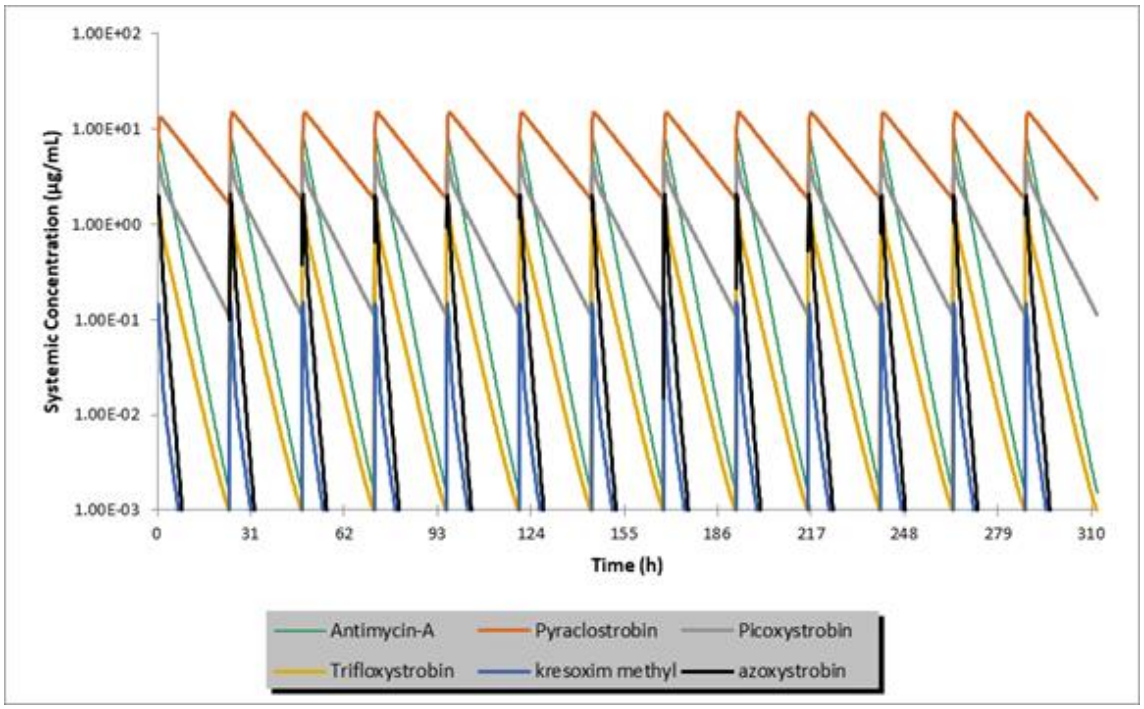


Figure 19. Predicted unbound exposure of the strobin case study chemicals in the rat following multiple doses of 10 mg/kg.

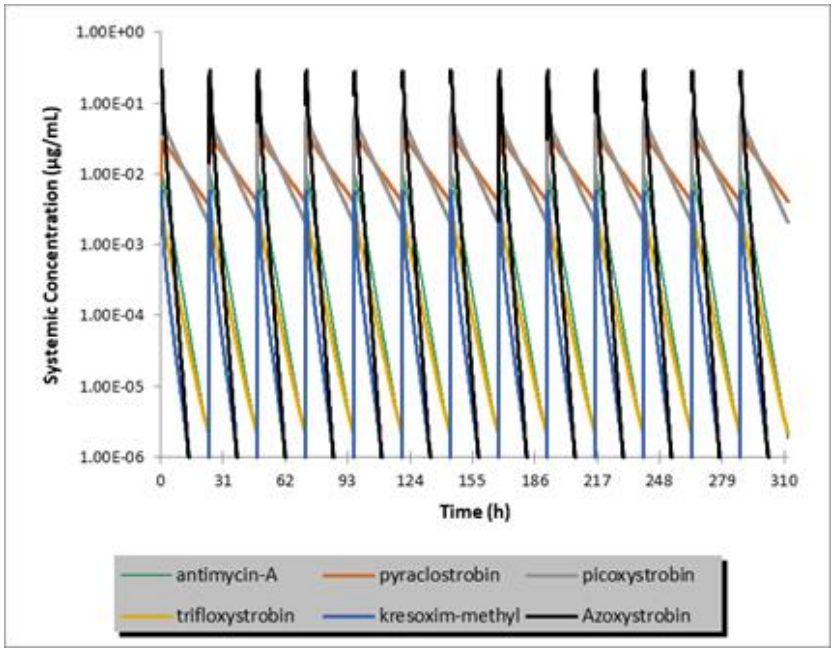


Figure 20. Predicted unbound exposure of the strobin case study chemicals in the rat following multiple doses of 100 mg/kg.

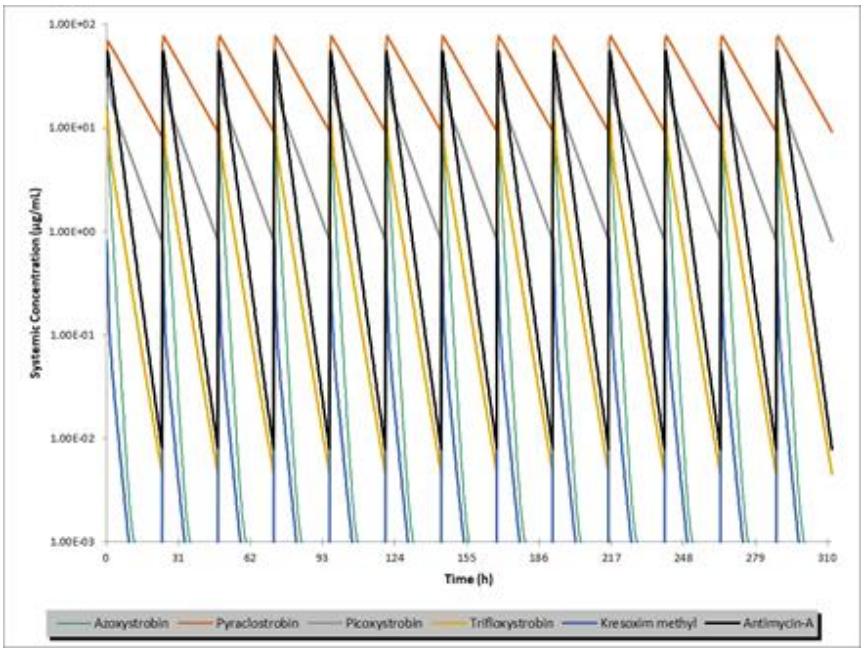
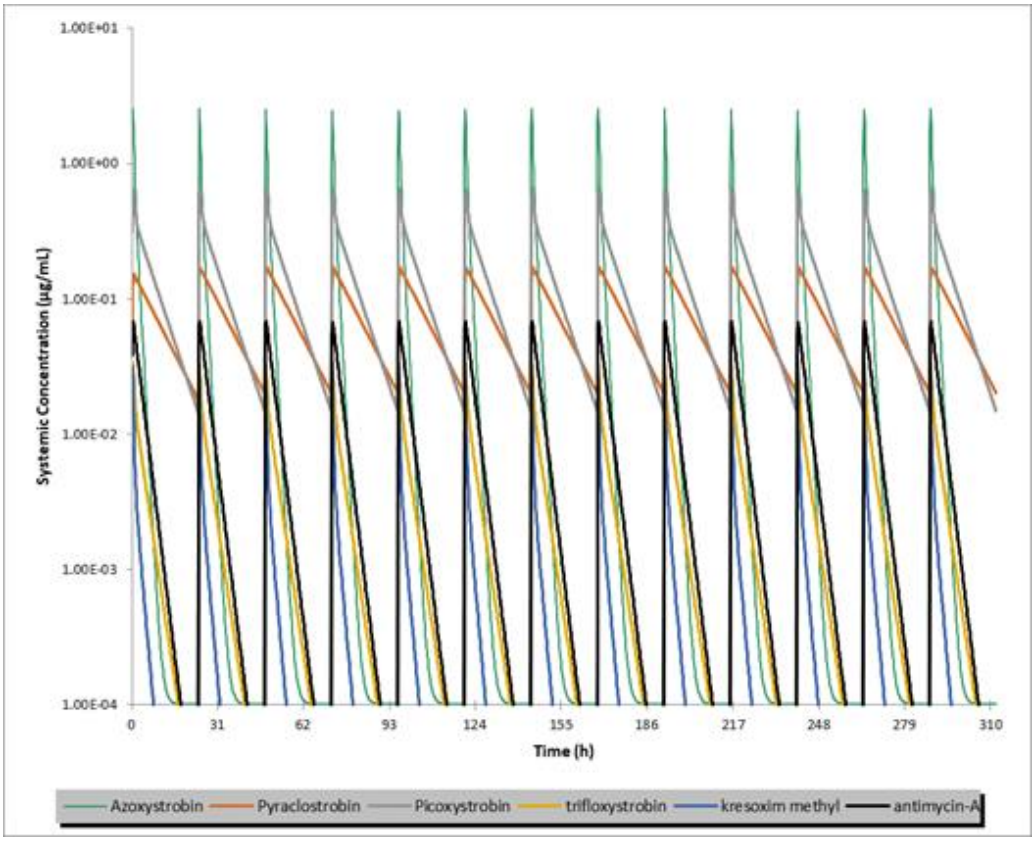


Figure 21. Predicted unbound exposure of the strobin case study chemicals in the rat following multiple doses of 100 mg/kg.



The predicted average steady state exposure (total and unbound) for the strobin case study compounds at doses of 10 and 100 mg-kg are presented in tables 15.

Table 15. Predicted average steady state exposure (total and unbound) for the strobin case study compounds at an oral doses of 10 and 100 mg-kg.

Compound	10 mg/kg		100 mg/kg	
	Cav_{ss} (μg/ml)	Cav_{ss} unbound (nM)	Cav_{ss} (μg/ml)	Cav_{ss} unbound (nM)
Pyraclostrobin	6.54	37.11	32.98	187.1
Kresoxim methyl	0.0071	0.89	0.029	3.64
Azoxystrobin	0.095	33.33	0.76	268
Picoxystrobin	1	50.04	7.35	366.3
Trifloxystrobin	0.19	1.09	1.36	7.64
Antimycin-A	1.07	2.4	6.90	15.5

A sensitivity analysis to look at the predicted exposure of Azoxystrobin and Picoxystrobin over a dose range of 10-2000 mg/kg/day for 13 doses was conducted. The caveats to these simulations were that clearance and fraction unbound were unchanged with dose (i.e. no saturation of metabolism or binding occurred) and that absorption was not linear with dose due to solubility/precipitation limitations at higher doses.

Table 16. Predicted exposure (total and unbound) to azoxystrobin at oral doses between 10 and 2000 mg-kg.

Dose (mg/kg)	Cav_{ss} (μg/ml)	Cav_{ss} unbound (nM)	C_{max} (μg/ml)	C_{max} unbound (nM)
10.00	0.09	33.3	2.1	722
18.02	0.17	60.1	3.7	1300
32.46	0.31	108.2	6.7	2343
58.48	0.55	194.9	12.0	4220
105.36	0.80	282.4	18.9	6658
189.82	1.38	486.4	33.0	11612
342.00	2.34	823.1	56.3	19833
616.16	4.21	1480.9	101.4	35700
1110.09	7.55	2659.4	182.6	64290
2000.00	13.61	4789.8	328.9	115788

Table 17. Predicted exposure (total and unbound) to picoxystrobin at oral doses between 10 and 2000 mg-kg.

Dose (mg/kg)	Cav_{ss} (μg/ml)	Cav_{ss} unbound (nM)	C_{max} (μg/ml)	C_{max} unbound (nM)
10.00	1.00	50.0	4.6	230
18.02	1.81	90.2	8.3	414
32.46	3.26	162.4	15.0	745
58.48	4.31	214.9	21.0	1048
105.36	7.74	385.8	37.9	1887
189.82	12.53	624.4	62.5	3113
342.00	21.67	1079.8	109.9	5475
616.16	35.45	1766.3	180.4	8985
1110.09	62.78	3128.1	323.2	16104
2000.00	113.01	5630.4	582.0	28993

6. Discussion

Using available physicochemical and *in vitro* data PBPK models were constructed for the strobin case study compounds in humans. The physicochemical data and an allometrically scaled clearance (from the predicted human value) were used to construct PBPK models for the rat. Simulations of exposure following various oral doses of the strobin case study compounds were made.

The available observed data for comparison with the simulations were limited or in some cases (antimycin A) missing. In this case, a worse case assumption for absorption due to permeability was made. All of the compounds have limited aqueous solubility and where possible values for supersaturation ratio and precipitation rate constant were calibrated to make the rat models consistent with available experimental data.

Is there any human data for comparison e.g. biomonitoring or anything?

Predictions in humans

The compounds have a range of lipophilicity values and azoxystrobin is the least lipophilic of the compounds. The measured human protein binding showed a trend for fraction unbound to increase with Log P with azoxystrobin having the lowest protein binding (highest fu). In the absence of solubility limitations all of the compounds were predicted to have reasonable passive membrane permeability and to have almost complete absorption (fa for all compounds >0.9). There was a relationship between Log P and solubility for the strobing compounds with azoxystrobin having the highest solubility. Using default values for precipitation rate constant and supersaturation ratios, the human absorption of the strobin compounds was predicted. At a dose of 10 mg/kg only azoxystrobin and picoxystrobin were predicted to have high absorption. Although these is some uncertainty about the correct values to use for these parameters the overall conclusion from these analyses is that azoxystrobin and picoxystrobin are likely to have higher oral absorption at high doses in humans than the other case study compounds.

The predicted distribution of all of the compounds was consistent across the different prediction methods tested. All of the compounds had moderate predictions of volume of distribution that were higher than total body water (0.6 L/kg) indicating that the compounds distribute into the tissues. The distribution was also predicted to be similar in the rat. The predicted steady-state brain to plasma distribution ratio was predicted to be 0.4 to 3.9 in the rat across the series of compounds and between 0.98 and 4.4 in humans. Azoxystrobin was in the middle of the range in both species with the brain concentration being predicted to be about 2-fold of those in plasma in both rat and human.

In vitro-in vivo extrapolation of the human metabolic clearance of this series of compounds resulted in a wide range of predicted clearance values (table 13). Azoxystrobin has the shortest predicted half-life of all of the compounds and was simulated to be significantly removed from the body over 72 hours after an intravenous dose of 10mg/kg. When the unbound concentrations were considered azoxystrobin has the highest initial plasma concentrations but due to the short predicted half-life the concentrations are the lowest by the end of the simulation period.

Simulated rat pharmacokinetics

The clearance of the strobin case study chemicals was predicted in the rat using an allometric approach to scale down the human clearance. This leads to a wide range of

predicted clearance values in the rat. These values were used as input in the PBPK model in the rat and simulations of oral doses were made and compared to any available pharmacokinetic data in the rat.

For pyraclostrobin the fraction and rate of absorption were consistent with data obtained dosing 5 or 50 mg/kg to the animals. Consistent with the radiolabel data the majority of the compound is eliminated within 48 hours however the predicted plasma exposure in the rat is higher than the observed radiolabel AUC. This suggests that the clearance of pyraclostrobin in the rat is underpredicted using the technique of back extrapolation from human and may suggest that the rate of metabolism is much faster in rats than humans or that the protein binding of pyraclostrobin in the rat is lower than the predicted value used in the simulation. Unfortunately, it is not possible to generate metabolism data in rat hepatocytes or protein binding in rat plasma under the auspices of the EU_TOX_Risk project to distinguish these hypotheses. Regardless the discrepancy between simulated and observed data described herein suggests that the simulated pyraclostrobin data should be used with caution in the read across exercise.

For picoxystrobin, trifloxystrobin, kresoxim methyl, azoxystrobin and picoxystrobin the rat PBPK models were consistent with available pharmacokinetic data. For antimycin-A no observed pharmacokinetic data in the rat was found in the literature. The pharmacokinetics of all of the case study chemicals were predicted at oral doses of 10 and 100 mg/kg the total concentrations of azoxystrobin were within the range of simulated concentrations of the other compounds however when the free plasma concentrations were considered azoxystrobin has the highest unbound C_{max} but due to the rapid clearance and short half-life the steady state concentrations were lower than but similar to those of picoxystrobin.

With the stated assumptions around linearity of clearance and fraction unbound, the concentrations of azoxystrobin and picoxystrobin were compared across a range of doses up to 2000 mg/kg. The average steady state total plasma concentration of azoxystrobin were lower and the unbound steady state plasma concentrations were comparable to those of picoxystrobin. The C_{max} (total and unbound) concentrations of azoxystrobin were higher than those of picoxystrobin.

7. Conclusion

Model Evaluation Aspect	Observation
Biological basis	The structure of the human and rat PBPK models are consistent with known physiology. The tissue volumes and blood flow are consistent with measured values from the literature.
Model simulations of data	There is no observed human data available for comparison. The PBPK models developed in the rat were consistent with available pharmacokinetic data for kresoxim methyl, azoxystrobin, picoxystrobin and trifloxystrobin although the observed data in somewhat limited. There was no data for antimycin-A available for comparison with model predictions. For pyraclostrobin the predicted plasma concentrations were inconsistent with the observed data making it difficult to use the data for this compound in the read across exercise.
Reliability (model testing, uncertainty and sensitivity)	The clearance of the strobing compounds *in vivo* in humans was predicted from *in vitro* metabolism data in human hepatocytes from this the clearance in rat was extrapolated based on allometric principles. The compounds have low solubility and oral absorption was simulated accounting for supersaturation and precipitation. Where available observed data was used to help calibrate the models. Although *in vivo* data for the compounds was limited PBPK models that were consistent with the observed data were developed using a physiologically plausible model structure for azoxystrobin, picoxystrobin, trifloxystrobin, and kresoxim methyl and mainly *in vitro/in silico* input data.

8. References

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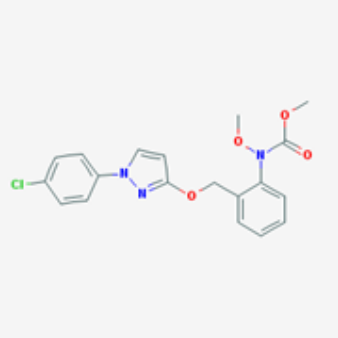
9. Summary of *in vivo* data

Pyraclostrobin

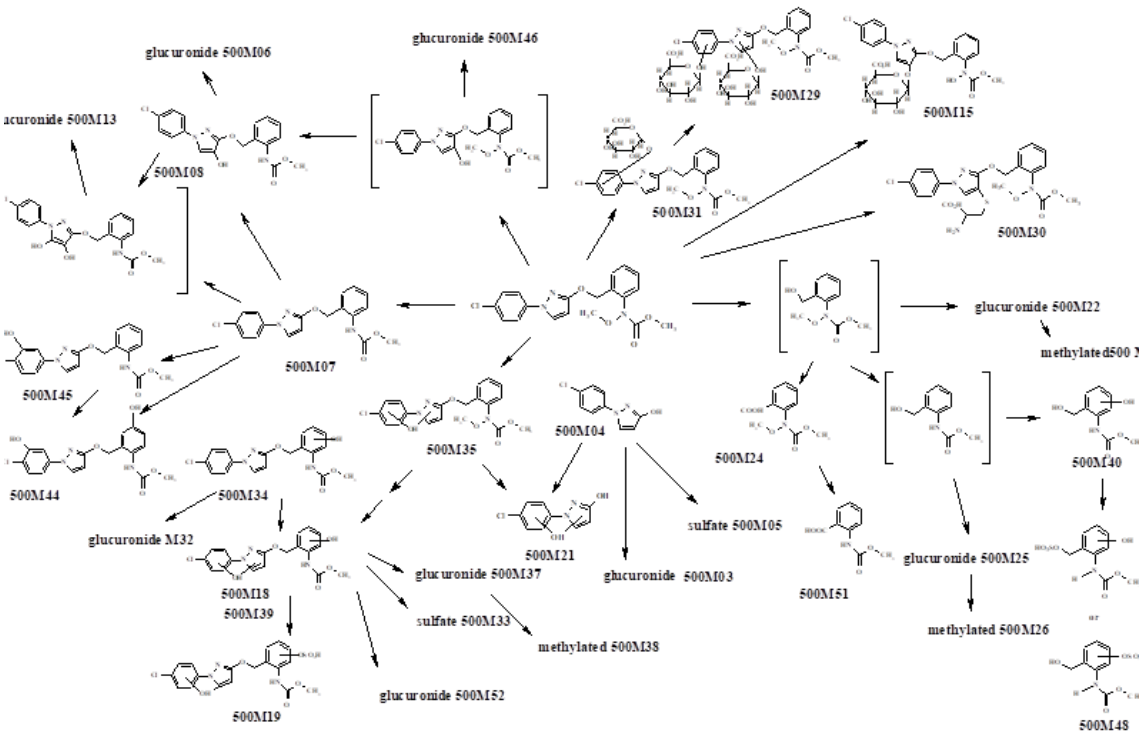


Excretion of radiolabel studied at oral doses of 5 or 50 mg/kg. Peak levels of radioactivity were seen after 0.5 to 1 hour indicating rapid absorption of the material although there was evidence of a second radioactive peak suggesting that enterohepatic circulation of radiolabelled material occurred. In the first 48 hours post dose urinary recovery was 11-15% of dose whilst faecal recovery was 81-92% of the dose. 35-38% of the dose was recovered in the bile of bile duct cannulated rats suggesting that absorption was about 50% of the dose. The initial half-life of radioactivity excretion was 10 hours at the low dose (5 mg/kg) and about 20 hours at the 50 mg/kg dose, and the radioactivity AUC increased proportionately with dose. The clearance of total radioactivity was calculated to be in the range 2-3 ml/min.

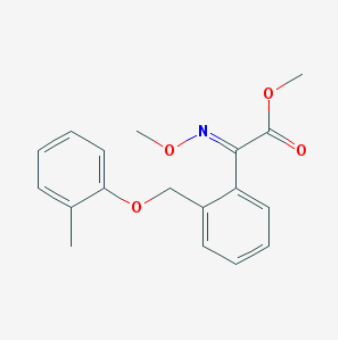
No unchanged parent compound was found in the bile or urine of rats indicating that the absorbed drug was extensively metabolised and that metabolism would be the major clearance mechanism for pyraclostrobin. In the faeces, only small amounts of parent drug were observed indicating either that pyraclostrobin was degraded by the microbiota in the rat intestine or that more of the parent compound was absorbed and some metabolites were directly excreted into the intestinal lumen (i.e. not excreted via bile). A metabolite with demethoxylation of the methoxycarbamate group was found in the faeces but not in bile supporting that one of these two mechanisms occurs but the data provided is not sufficient to distinguish between the two different possibilities.

The identified metabolites of pyraclostrobin are shown below

Figure 22. Identified metabolites of pyraclostrobin



Kresoxim Methyl

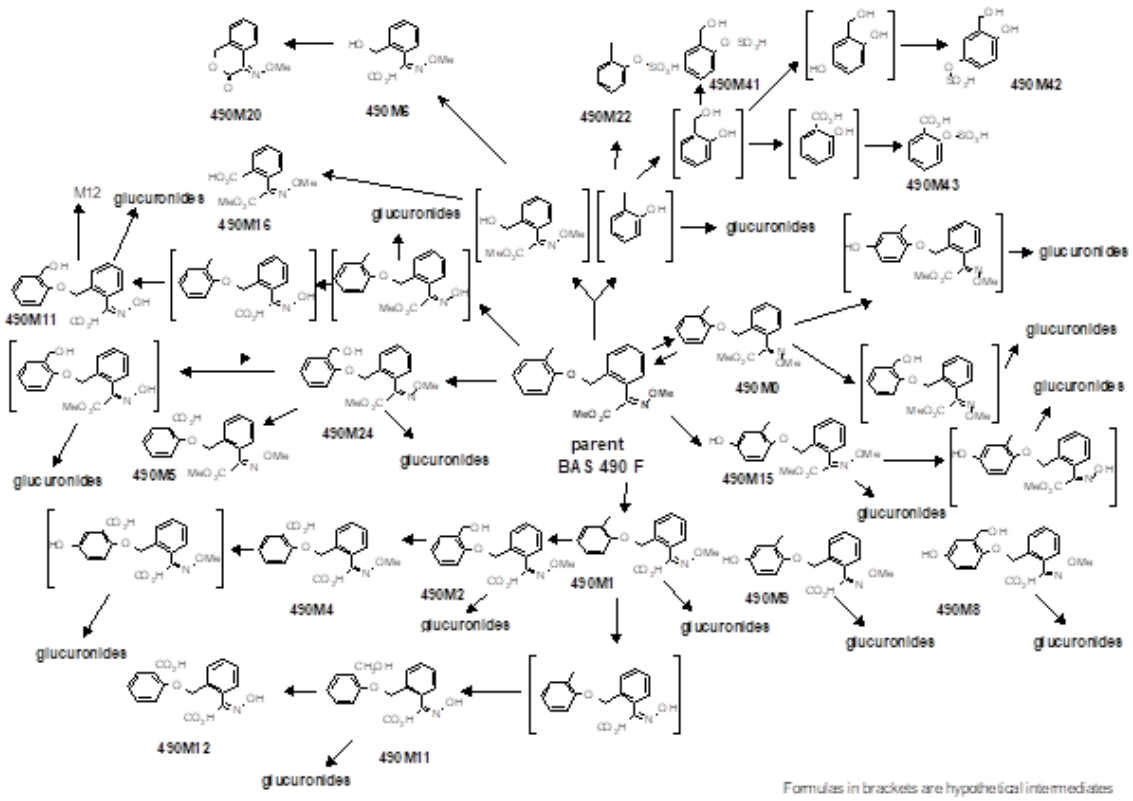


Dosed by gavage at 50 or 500 mg/kg or as a single IV bolus (5 mg/kg). The clearance of total radioactivity was ~ 6 ml/min at the lowest dose and ~25 ml/min at the highest dose. The non-linearity in clearance probably reflects lower absorption at the higher dose.

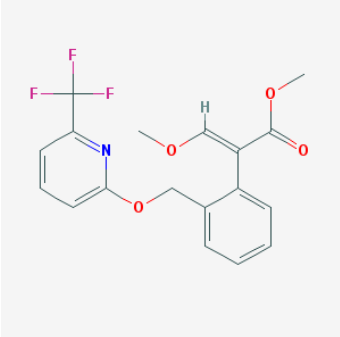
The recovery of parent compound in faeces was 7% at a dose of 5 mg/kg, 35-50% of the dose at 50 mg/kg and 32-75% of the dose at 500 mg/kg. This suggests that absorption is >90% at the lowest dose ≥505 at 50 mg/kg and > 25% at the highest dose (500 mg/kg).

Metabolism studies showed that Kresoxim methyl is extensively metabolised in the rat with a number of different metabolites being detected. The phase I biotransformation of the compound in rats comprised the cleavage of the ester, the oxime ether and the benzyl ether bonds, hydroxylation of ring A in para position to the existing oxygen substituent, and it subsequent oxidation to the corresponding carboxylic acid. The resulting OH-groups underwent conjugation with glucuronic acid or sulphate.

Figure 23. Metabolism of Kresoxim methyl in the rat

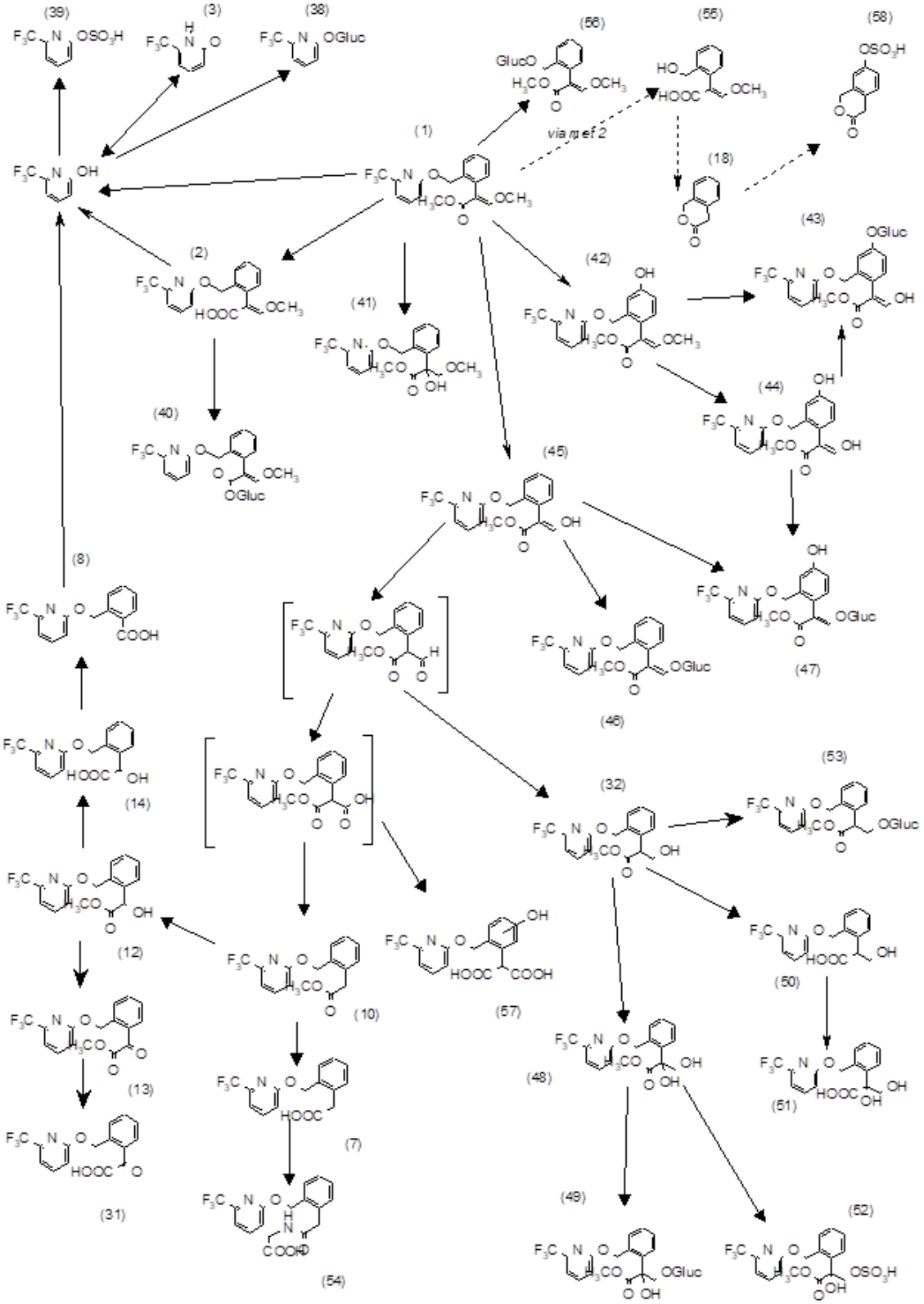


Picoxystrobin

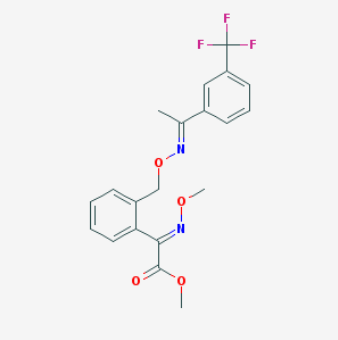


Peak radioactivity seen at 2 and 12 hours after dosing of 10 or 100 mg/kg picoxystrobin by oral gavage to rats. At the higher dose, at least 70% of the dose was detected in urine (20 – 30% of the dose) and bile (40-50% of dose) indicating significant absorption of the compound. Excretion of radioactivity is essentially complete within 120 hours post dose. Metabolism was extensive with over 30 different metabolites identified. The metabolism scheme of picoxystrobin in the rat is presented below.

Figure 24. Metabolites of picoxystrobin identified in the rat.

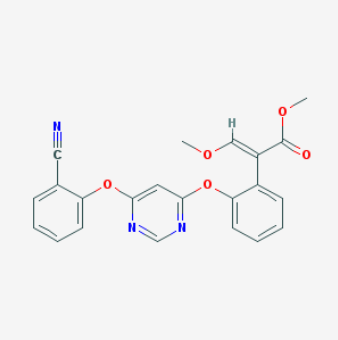


Trifloxystrobin



At a dose of 0.5 mg/kg absorption was incomplete (56-65%) with 41-47% of the dose (i.e. almost all of the absorbed radioactivity) being excreted into the bile. At the high dose (100 mg/kg), 25 – 45% of the radiolabelled material was absorbed with 19 – 35% being recovered in the bile. Urinary recovery at the high dose was 10-12% of the dose in males and 27% in female rats. Radioactivity in blood had two peaks 0.5 and 12 hours at the low dose and 12 and 24 hours at the high dose. Excretion of radiolabel was rapid with 85-96% of the dose being excreted into urine in the first 48 hours post dose. There is some evidence of enterohepatic recirculation of radiolabel. At the low dose, where absorption was highest, parent compound excreted in faeces accounted for only 4-7% of the dose indicating that the absorbed compound is extensively metabolised and that this is the major clearance mechanism of the compound. The half-life of total radioactivity in blood was long with values in the range of 30-82 hours being observed. Unchanged drug in the faeces was about 4-7% at the low dose (0.5 mg/kg) and 31-47% at the high dose (100 mg/kg).

Azoxystrobin



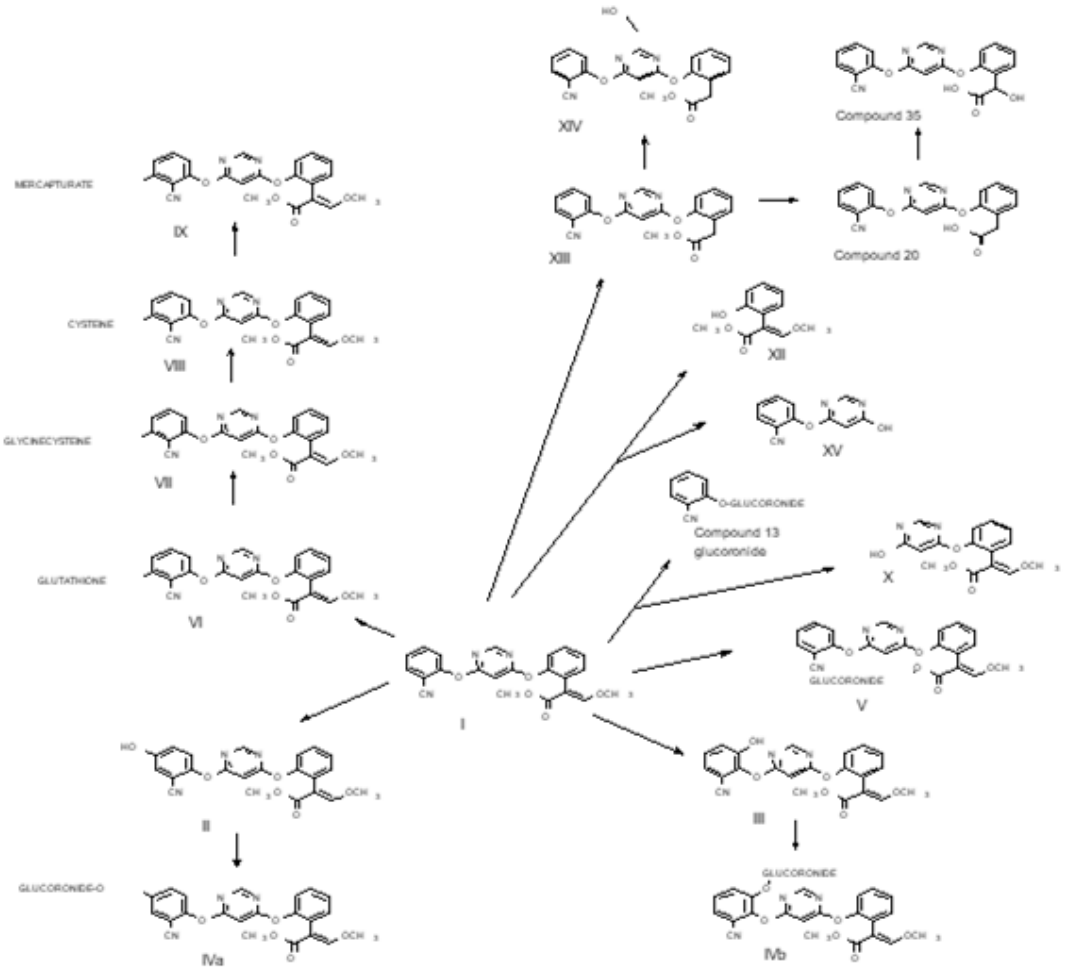
In rats dosed with 1 mg/kg for 14 days then a single dose of radiolabel the majority of the dose was excreted in faeces (87 (f) and 89 (m)%) with relatively small amounts of radioactivity in urine (17 (f) and 13 (m)%). Excretion was fast with > 96% of the dose being excreted in the first 48 hours. At this low dose, absorption was almost complete.

In bile duct cannulated animals given a dose of 100 mg/kg radiolabelled (14)C-pyrimidinyl - azoxystrobin the majority of the radioactivity was recovered in the bile (74%(m) and 81% (f)) over the first 48 hours post dose suggesting that the compound is well absorbed following oral exposure. Changing the position of the radiolabel changed the amount of radioactivity recovered in the bile although this route still accounted for the majority of the dose (> 56%). Parent azoxystrobin was not detected in the bile indicating significant metabolism of the absorbed compound was occurring.

There is some evidence of incomplete absorption of azoxystrobin at the 100 mg/kg dose with 19-26% of the dose being detected in faeces as parent compound.

The metabolic scheme of azoxystrobin in the rat is shown below

Figure 25. Metabolic scheme of Azoxystrobin in the rat.



Annex 1.14. Multiplex measurement of neurite degeneration, PI staining, ATP content.

Method	SH-SY5Y: repeat dosing
Contact	Anna Forsby (Swetox) -> anna.forsby@swetox.se

Standard Operating Procedure (SOP)

1. PURPOSE

The SOP describes the procedures for multiplexing measurements of neurite degeneration and cell viability determined by high content fluorescence microscope imaging together with quantification of ATP content determined by luminescence in differentiated SH-SY5Y cells after repeated exposure.

2. SCOPE

A multiplex assay for neurite degeneration, cell viability and ATP measurement has been developed to be able to estimate all three endpoints in the same plate of exposed human neuroblastoma SH-SY5Y cells. The cell permeant dye Calcein-AM was used to stain the cytoplasm of differentiated cells allowing the visualisation and quantification of the neurite length. Once inside the cells, calcein-AM is hydrolysed by intracellular esterases producing a green fluorescent dye. Viable cells are visualised under the fluorescence microscope by exclusion of propidium iodide (PI). After monitoring calcein and PI staining in a fluorescence microscope imaging system, the ATP content in each well was analysed by the ATP luminescence assay, using the CellTiter Glo kit.

3. INTRODUCTION

Neurite degeneration initiated at non-cytotoxic concentrations *in vitro* is an alert for neurotoxicity directed to pathologies in axons and dendrites *in vivo*. Furthermore, imbalances in the energy metabolism and ATP deficiency is a key event for neurotoxicity. Herein, we have combined assays for neurite degeneration, cell viability and ATP measurements in the same plates with SH-SY5Y cells after repeated exposure with chemicals.

4. RESPONSIBILITY

All personnel performing this assay should follow this SOP.

5. PROCEDURES

LIMITATIONS

To be added

METHOD OUTLINE

Neurite degeneration and cytotoxicity were evaluated by fluorescent microscopy with the dyes calcein-AM (for neurite staining) and propidium iodide (PI) (for early apoptotic/necrotic cell staining). In addition, ATP content was quantified after imaging by luminescence with the CellTiter-Glo® assay (Promega).

For experiments, SH-SY5Y cells were plated in 96-well plates with micro-clear bottom (8 000 cells/well) in EMEM with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids, 100g streptomycin/mL and 100 U penicillin/mL, which is exchanged to differentiation medium with 1 µM RA and without serum (DMEM:F12 [1:1], N2 supplements [diluted 1:100], 1 mM glutamine, 100g streptomycin/mL and 100 U penicillin/mL) after 24 h. The cells are exposed to test chemicals after three and six days from the start of differentiation. The total exposure time is 120 hours. Neurite degeneration and cytotoxicity are measured by fluorescent microscopy with the dyes calcein-AM (for neurite staining) and propidium iodide (for staining of late apoptotic/necrotic cells). In addition, ATP content is quantified after imaging by luminescence using the CellTiter-Glo® 2.0 assay (Promega).

The Promega's CellTiter-Glo® assay system which employs the properties of a thermostable luciferase to enable reaction conditions that generate a stable "glow-type" luminescent signal while simultaneously inhibiting endogenous enzymes released during cell lysis (e.g., ATPases).The luminescence signal is recorded by luminescence reader (Tecan Infinite M200 Pro) for 300ms per well.

DEFINITIONS/ABBREVIATIONS

The following abbreviations are used:

DMEM:F12	Dulbecco's modified medium and Ham's F12 medium (1:1)
DMSO	Dimethyl sulfoxide
EMEM	Minimum essential medium with Earle's salts
RA	All-trans retinoic acid
FBS	Foetal bovine serum
PEST	Penicillin Streptomycin
PI	Propidium iodide
NEAA	Non-essential amino acids

IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

A. Test Chemicals

The cells were pre-exposed to test chemicals for 120 h before the measurements, simulating repeated exposure.

Dilutions:

All chemicals were tested at 10 µM, 2 µM, 400 nM, 80 nM and 16 nM. For those chemicals showing effect at the lowest tested concentration during the screening, extra 5X dilution steps were performed until a no effect concentration was reached.

Table 1. A typical pipetting scheme for preparation of the dilution series for test chemicals dissolved in DMSO.

Dilution step	Conc. In ep-tube (µM)	Test chemical (µl)	Medium + 0.2% DMSO (µl)	Conc. In wells (mM)
D1	2,00E+01	2	998	1,00E+01
D2	4,00E+00	200 of D1	800	2,00E+00
D3	8,00E-01	200 of D2	800	4,00E-01
D4	1,60E-01	200 of D3	800	8,00E-02
D5	3,20E-02	200 of D4	800	1,60E-02
D6	6,40E-03	200 of D5	800	3,20E-03
D7	1,28E-03	200 of D6	800	6,40E-04
D8	2,56E-04	200 of D7	800	1,28E-04
Controls	0	0	1000	0

B. Controls

b. **Negative control:** Cells exposed to N2-RA with DMSO 0.1%.

c. **Positive control:** Rotenone 400 nM.

MATERIALS

A. Cell Culture Type

Human neuroblastoma SH-SY5Y cell line is cultured and differentiated as described in SOP plating and differentiation.

B. Technical Equipment

a. ImageXpress® Micro (Molecular Devices, UK)

b. MetaXpress® Software (Molecular Devices, UK)

c. Tecan® Infinite M200 Pro

d. Tecan® i-control 3.7.3.0 software

e. Calcein (Life technologies, cat no C1430)

f. Hoechst 10 mL (ThermoFisher, cat no H3570), 10mg/mL.

g. Propidium iodide solution (Sigma, cat no P4170)

h. CellTiter-Glo® Assay (Promega, cat no G7571 or G9242)

i. Black cell culture microplate, 96-well plate with microclear bottom (Greiner, No 655090)

METHOD:

A. Preparation of Calcein stocks

a. Dilute the calcein powder (1mg) in 1 mL 100% DMSO

b. Warm up the diluted calcein to ensure proper dilution

c. Prepare 20 µL Aliquots

d. Store at 4 ºC

B. Preparation of propidium iodide (PI) stock

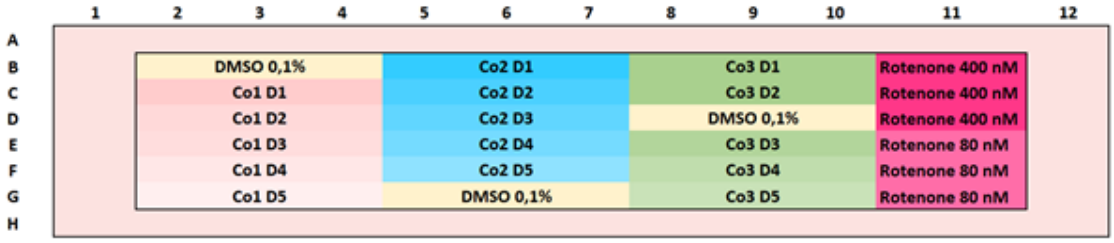
1. Prepare a 5 mg/mL (7.48 mM) stock solution by diluting 10 mg PI in 2 mL PBS. Store at 4˚C protected from light.

2. Prepare a 1 mM PI working solution by diluting 134 µL of the 5 mg/mL PI stock solution in 866 µL PBS. Store at 4˚C protected from light.

C. Exposure

For experiments, 8 000 SH-SY5Y cells are plated in black microclear bottom 96-well plates in complete EMEM with 10% foetal bovine serum, which is exchanged to N2-RA differentiation medium after 24 h. After 3 days of differentiation 100µL of the assigned treatment (test chemical dilution) are added on top of the wells. The plates are incubated with the test chemicals for 3 days. On the 6 day of differentiation 100 µL of cell medium are removed from the exposed cells and 100 µL of the same treatment are added on top of each well. The cells are placed in the incubator for 2 days more, the total time of exposure is 120 hours.

Figure 1. Plate lay out for multiplex assays (Neurite degeneration, propidium iodide and ATP content) of CS4 test chemicals.



D. Neurite and propidium iodide staining

The measurement of neurite degeneration is performed 120 hours after exposure to test chemicals

1. Be very careful not to keep plates out of incubator for longer time than necessary, and be extremely gentle with pipetting
2. Take one plate at a time out, carefully remove 100 µL of medium from the wells
3. Put the plate back in the incubator as fast as possible, do not let it cool down
4. Prepare and warm up 22 mL of DMEM:F12 medium for later dilution of the staining
5. Ultrasonicate Hoechst-H33342 stock solution in ultrasonic water bath for 15 min
6. Prepare dye solution (enough for two plates):
   a. 4 mL of DMEM/F-12
   b. 24 µL Hoechst 33342 stock 10 mg/mL (16,2 mM) [final conc: 5 µg/mL]

c. 24 µL Calcein-acetoxymethylester (CAM) stock of 1 mg/mL (1 mM) [final conc: 1 µM]

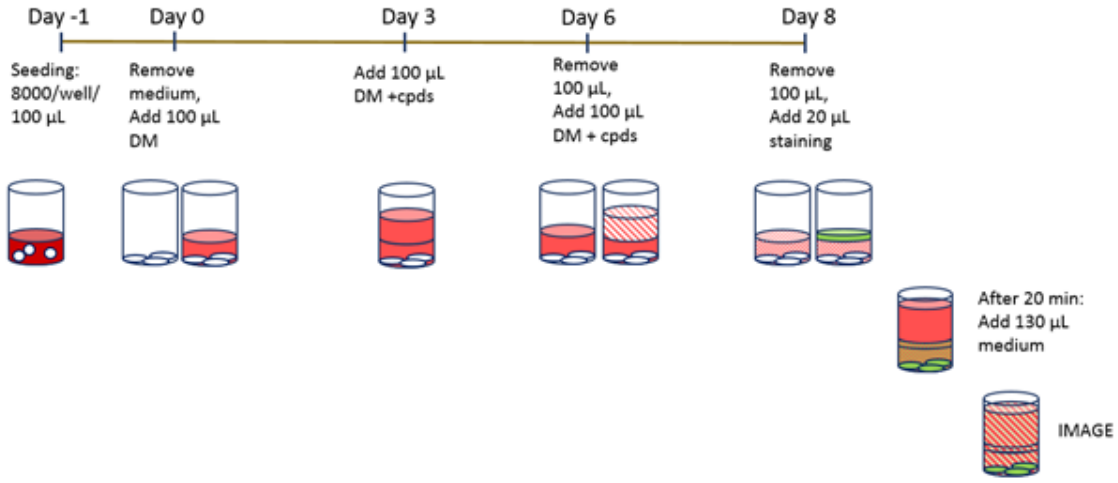
d. 24 µL PI 1 mM [final conc: 5 µM]

e. Vortex

f. Protect from direct light (switch off light in the sterile hood)

7. Put dye solution into a reservoir
8. Add 20 µL of dye solution to all wells in one plate, quickly but gently pipet up and down twice in each row (without changing tips for the same treatment)
9. Incubate the plate for 20 min at 37°C, 5% CO_2
10. Put 11 mL of warm DMEM/F-12 into a reservoir
11. Take the plate out
12. With a 12-channel pipette, quickly but gently, add 130 µL of DMEM:F12 in each well
13. Immediately image the plate (calcein in FITC channel, H33342 in DAPI channel, PI in Texas red channel, 4 sites/well)

Figure 2. Overview of the experimental set up used for imaging of neurite degeneration and cytotoxicity on differentiated SH-SY5Y cells after repeated exposure to test chemicals.



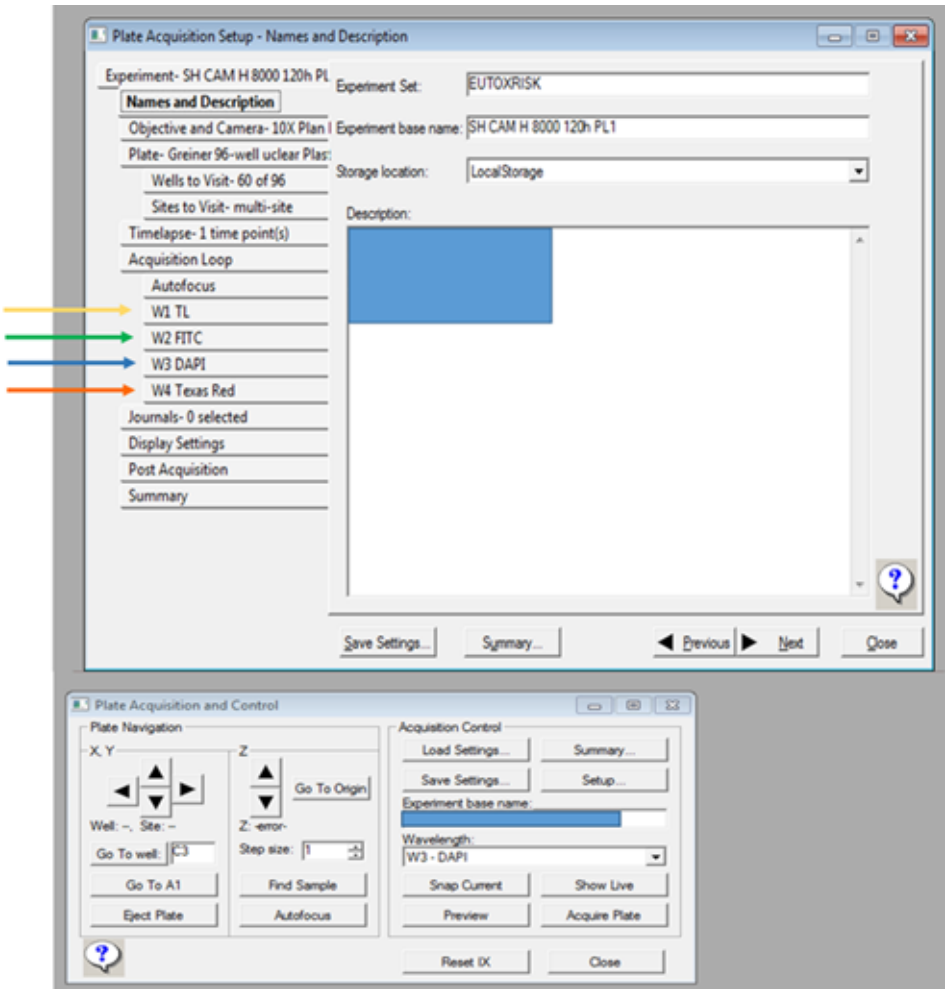
E. Neurite degeneration imaging

ImageXpress® Micro (Molecular Devices, UK) is used to obtain images of the exposed cells. Four images per well are taken with three channels: Transmitted light (TL, yellow arrow), Hoechst in DAPI channel (nuclear staining, blue arrow), calcein in FITC Channel (cytoplasm/neurite staining, green arrow) and PI in Texas red channel (cytotoxicity, orange arrow).

1. Turn on the machine 30 minutes before starting the measurements.
2. Open MetaXpress®

3. On the top menu of the MetaXpress® software, select screening → plate acquisition set up.
4. On the top menu of the MetaXpress® software, select screening → plate acquisition and control.
5. Once the plate acquisition set up and plate acquisition and control windows are opened, start by writing the name of the experiment and a description under the "Names and description" tab (see below, Figure 3).

Figure 3. "Names and description tab"

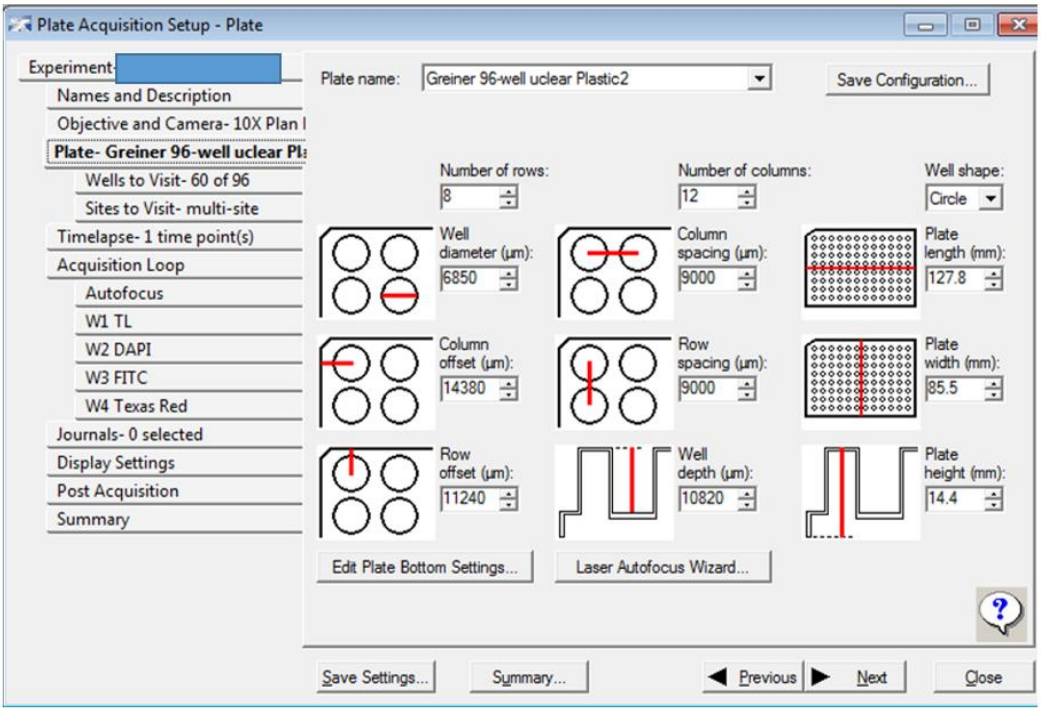


6. Under the "objective and camera" tab, select a 10X magnification, camera binning 1 and gain 1. These settings should only be modified by the instrument's responsible person or technician.
7. Under the "Plate" tab use the settings optimised for 96 well black microclear bottom cell culture plates (Greiner, No 655090). Figure 4 contains a description of the settings used for 96 well plates in this instrument, these settings should only be modified by the instrument's responsible person or technician.

[Note: different batches of plates can have differences in the well depth within the same plate, this will affect the sharpness of the image taken during the testing of the settings but it will not affect the final images]

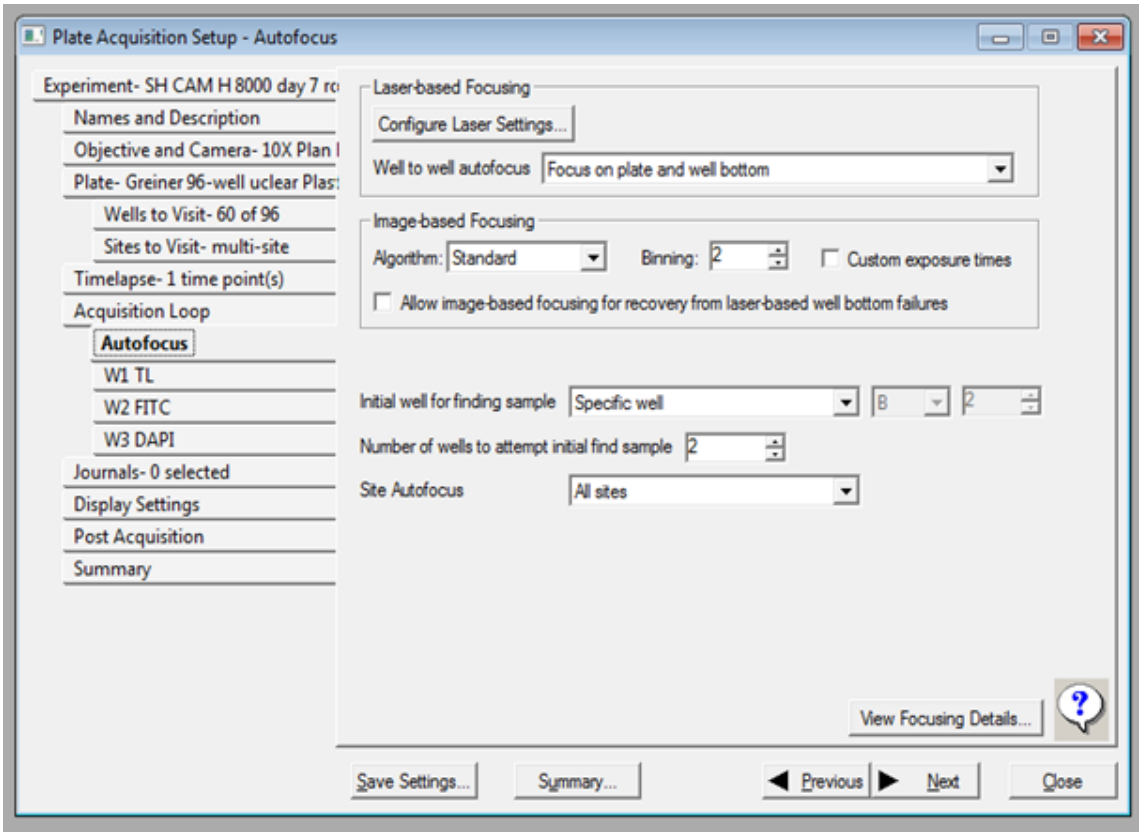
Figure 4. "Plate" tab including the setting that have been standardised for measurements in 96 well plates.

These settings should only be modified by the person responsible for the instrument.



8. Under the "wells to visit" tab, select the 60 innermost wells for analysis. Make sure that the selected wells are showed in a green colour.
9. Under the "sites to visit" tab select 2 columns and 2 rows to obtain 4 images/well.
10. Under the "timelapse" tab select number of endpoints = 1.
11. Under the "autofocus" tab (see Figure 5) select:
    a. Well to well autofocus: focus on plate and well bottom.
    b. Imaged based focusing:
        i. Algorithm: standard
        ii. Binning: 2
    c. Initial well for finding sample: specific well
    d. Site autofocus: all sites.

Figure 5. "Autofocus" tab with the settings used for measurements in 96 well plates.



12. Under the tabs W1 to W3 the setting for image acquisition are specified:

Table 2. Settings used for the acquisition of images with MetaXpress®.

The specific exposure time, target maximum intensity and Z- offset set for each of the three wavelengths used to measure neurite degeneration in differentiated SH-SY5Y cells are described.

Illumination setting	TL	DAPI	FITC	Texas Red
Exposure time (ms)	8	100	120	400
Target max intensity	16960	33000	34464	33000
Z-offset (µm)	8.96	2.92	10.44	4.2
Calculate Offset (use Z stack and Custom range)	Range: 50 um Step: 1.5 um	Range: 50 um Step: 1.5 um	Range: 50 um Step: 1.5 um	Range: 50 um Step: 1.5 um

13. Insert the plate in the imager (see Figure 6)

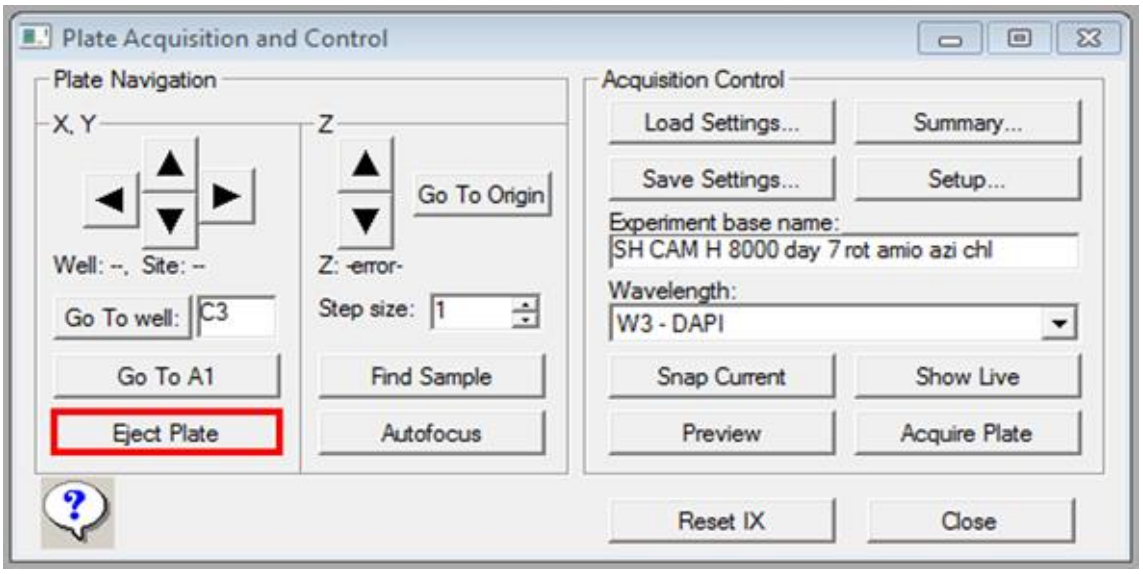
    a. In the plate acquisition and control menu choose eject plate

    b. Place the plate inside the imager

    c. In the plate acquisition and control menu choose load plate

Figure 6. Plate acquisition and control window.

To insert the plate in the imager press "eject plate"



14. With the mouse, right click button select one of the 4 sites and one control well, select the DAPI channel to evaluate the nuclear staining with Hoechst. To be able to see the cells click on "find sample" in the plate acquisition and control window.
15. With the mouse right click button select one of the 4 sites and one control well, select the FITC channel to evaluate the calcein staining. To be able to see the cells click on "find sample" in the plate acquisition and control window.
16. With the mouse right click button select one of the 4 sites and one control well, select the Texas Red channel to evaluate the PI staining. To be able to see the cells click on "find sample" in the plate acquisition and control window.
17. Click under the "summary" tab → acquire plate.
18. All obtained images are automatically stored in MetaXpress® in the D disk of the computer. The SOP "procedure for backing up old experiments from the imager" describes the steps to follow in order to create back up files.

F. ATP content (CellTiter-Glo cell viability assay)

The measurement of ATP content is performed after imaging the cells:

1. Thaw the CellTiter-Glo® 2.0 (Promega, cat no G9242) and make 10 ml aliquots (for 2 plates). Store aliquots at -20°C until use.
2. Thaw the CellTiter-Glo® 2.0 and equilibrate to room temperature prior to use. For convenience the CellTiter-Glo® 2.0 (Promega, cat no G9242) may be thawed and stored at room temperature for up to 48 hours prior to use.
3. Alternatively, thaw the CellTiter-Glo® buffer (Promega, cat no G7571) and transfer 10 mL into the bottle containing CellTiter-Glo® substrate. Mix by gently vortexing.
4. Remove 200 µl from each well and add 50 µl of Reagent.
5. Put the plates on an orbital shaker for 2 minutes to induce cell lysis.

6. Allow the plate to incubate at room temperature for 15 minutes to stabilise luminescent signal.
7. Record the luminescence for 300 ms per well by using the Tecan Infinite M200 Pro reader.
8. Store the data in the S:\Projects\PHC33_EUToxRisk\Case study 4_Mitotox\Repeated exposure folder.

G. Image analysis

Neurite degeneration

1. Open MetaXpress®
2. On the top menu of the MetaXpress® software, select screening → Review plate data [DB].
3. Select the plate for analysis.
4. Select all the wells to be analysed.
5. On the "Run analysis" tab select:
   a. Analysis: <Neurite Outgrowth>
6. Settings: Press configure settings, and select:
   a. Neurite image: FITC
   b. Illumination: Fluorescence
   c. Cell bodies:
      i. Approximate max width: 40 µm = 62 pixels
      ii. Intensity above local background: 100 graylevels
      iii. Minimum area: 30 μm^2 = 71 pixels
   d. Nuclear stain:
      i. Nuclear image: DAPI
      ii. Approximate min width: 5 µm = 8 pixels
      iii. Approximate max width: 13 µm = 20 pixels
      iv. Intensity above local background: 10 graylevels
   e. Outgrowths:
      i. Maximum width: 2 µm = 3 pixels
      ii. Intensity above local background: 10 graylevels
      iii. Minimum cell growth to log as significant: 15 µm = 23 pixels
7. In the configure settings window press "test run" to visualise the correct identification of cell bodies and outgrowths in the software (Figure 7 and 8)

Figure 7. ”configure settings tab”.

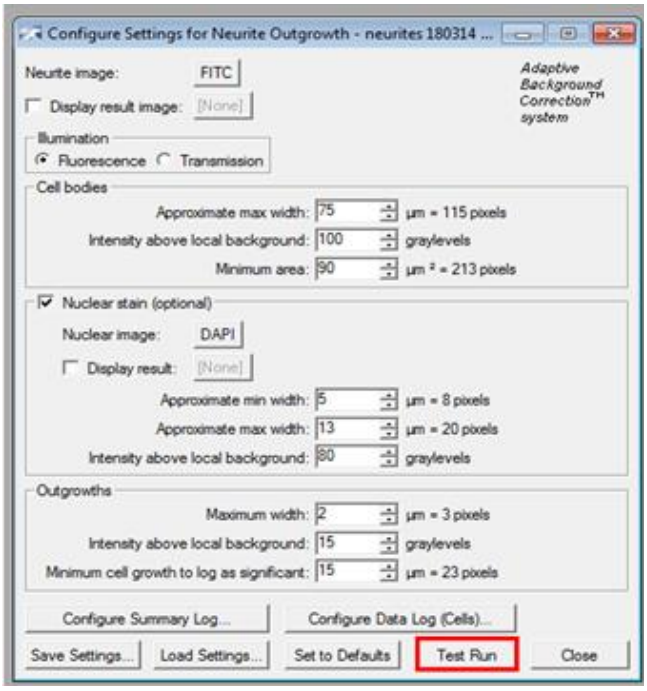
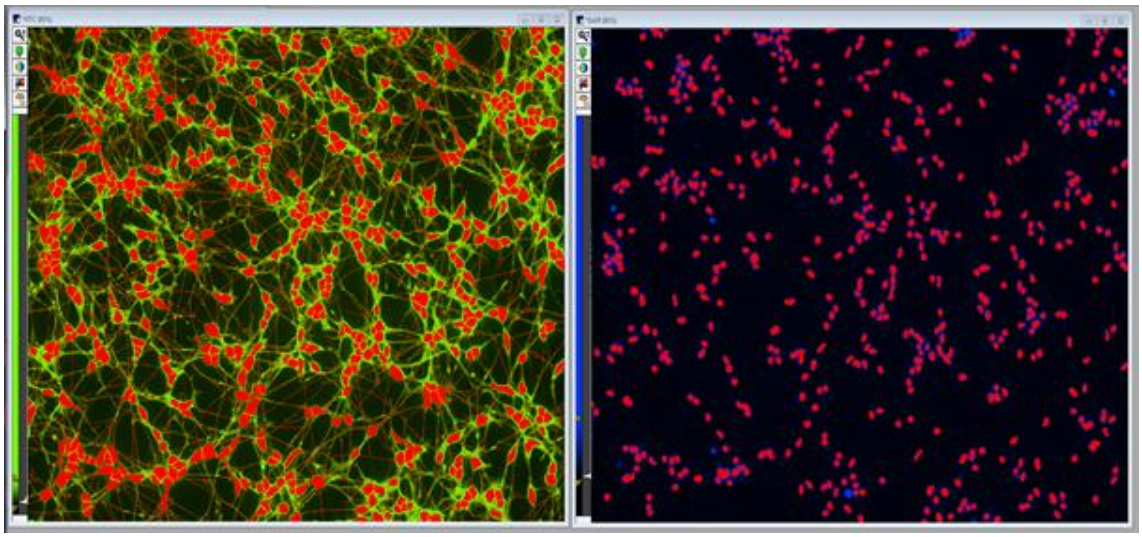


Figure 8. Analysis of neurite length in differentiated SH-SY5Y cells.

Left: staining with calcein in FITC channel, Right: nuclear staining with Hoechst in DAPI channel. Live cells are recognised as structures with both calcein and Hoechst staining.



Note: variations in the intensity of the staining can lead to inappropriate identification of cells bodies and/or neurites. If needed, the settings in the “configure settings” tab should be modifies to assure proper identification of most of the imaged structures.

8. Analyse the plate by pressing “run analysis” on the “Review plate data” window.
9. After analysis, the data can be exported to an excel file.
10. Store the data in the S-disk: S:\Projects\PHC33_EUToxRisk\Case study 4_Mitotox\Neurite degeneration\Compilation of results 24h.

11. Microsoft Excel is used to analyse the data.

Cytotoxicity (PI staining)

1. Open MetaXpress®
2. On the top menu of the MetaXpress® software, select screening → Review plate data [DB].
3. Select the plate for analysis.
4. Select all the wells to be analysed.
5. On the "Run analysis" tab select:
   a. Analysis: <transfluor HT>
6. Settings: Press configure settings, and select:
   a. Check the box "Nuclear stain"
   b. Nuclear image: Texas Red
      i. Approximate min width: 2 µm = 3 pixels
      ii. Approximate max width: 8 µm = 12 pixels
      iii. Intensity above local background: 10 graylevels
7. In the configure settings window press "test run" to visualise the correct identification of PI stained cells. **Note:** variations in the intensity of the staining can lead to inappropriate identification of cells bodies. If needed, the settings in the "configure settings" tab should be modified to assure proper identification of most of the imaged structures.
8. Analyse the plate by pressing "run analysis" on the "Review plate data" window.
9. After analysis, the data can be exported to an excel file.
10. Store the data in the S-disk: S:\Projects\PHC33_EUToxRisk\Case study 4_Mitotox\Neurite degeneration\Compilation of results 24h.

H. Data analysis

Neurite degeneration

1. The differences in the mean process length (cell: mean process length) used for stablishing the effect of the test compounds on the neurites (neurite degeneration).
2. Normalise the mean process length value of each well against the mean of all negative controls:

$$ND\ (\%) = \frac{\text{Mean process length exposed cells}}{\text{Mean process lenght negative controls}} * 100\%$$

3. Take the mean of the effect in the three technical replicates of each concentration.
4. Plot the values in GraphPad Prism.

Cytotoxicity (PI staining)

1. Add the total number of imaged cells per well to the number of PI stained cells in the same well to obtain the total number of imaged cells.
2. Calculate the % of viable cells per well as follows:

$$Cell\ viability\ (\%) = 100 - [\frac{\text{Number of PI stained cells}}{\text{Total number of imaged cells}}] * 100\%$$

3. Take the mean of the effect in the three technical replicates of each concentration.
4. Plot the values in GraphPad Prism.

ATP content

1. Microsoft Excel is used to analyse the data.
2. The average of luminescence of each concentration (triplicates) and standard deviation (SD) are calculated. The average luminescence value of each concentration is normalised against the mean luminescence value of control wells (6 wells) and subsequently multiplied by 100 in order to obtain the % of viable cells in each concentration.
3. Plot the values in GraphPad Prism.

IX. HEALTH SAFETY AND ENVIRONMENT

Cell culture procedures take place in a laminar air flow cabinet, class 2. All work with test chemicals is performed in a fume hood. Experiments are planned in order to reduce the exposure time of the laboratory personnel to the test chemical. Wastes of environmentally hazardous compounds are handled according to local regulations.

X. REFERENCES

To be added

XI. APPENDIX

Summary of the procedure for multiplex analysis of neurite degeneration, cytotoxicity and ATP content in differentiated SH-SY5Y cells:

Day 1

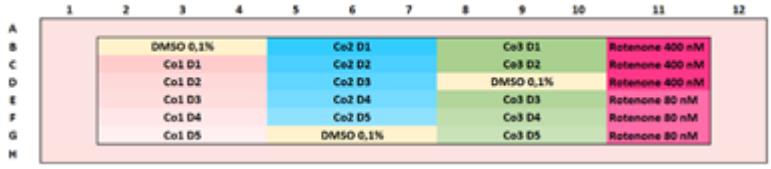
Plating of SH-SY5Y cells, 8 000 cells/well.

Day 2

Start differentiation.
Change complete EMEM to N2-RA medium.

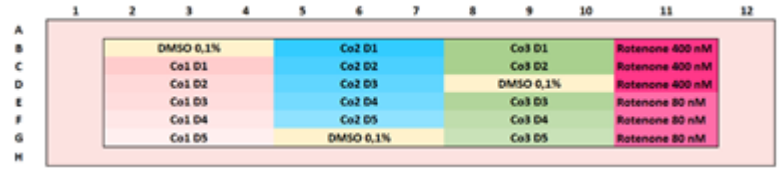
Day 3

First exposure to test chemicals.
Add 100 µL of the corresponding chemical dilution.



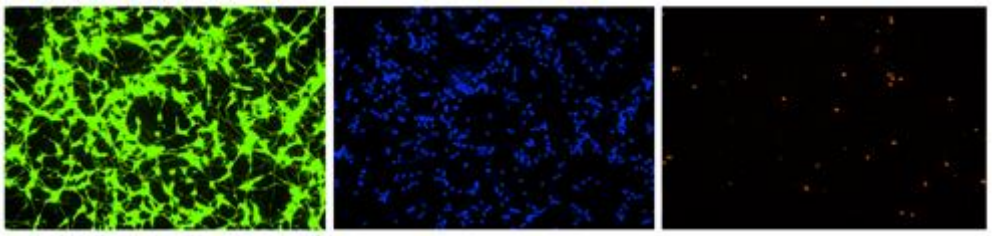
Day 6

Second exposure to test chemicals.
Remove 100 µL from all wells.
Add 100 µL of the corresponding chemical dilution.



Day 8

Multiplex
Remove 100 µL of supernatant.
Add 20 µL/well of Calcein + Hoechst + PI staining solution.
Incubate the plate for 20 minutes at 37 °C.
Add 130 µL DMEM:F12.
Image the plate (Calcein in FITC channel, H33342 in DAPI channel, PI in Texas red channel 4 sites/well).
After imaging, measure ATP content with CellTiter-Glo® 2.0 (Promega)

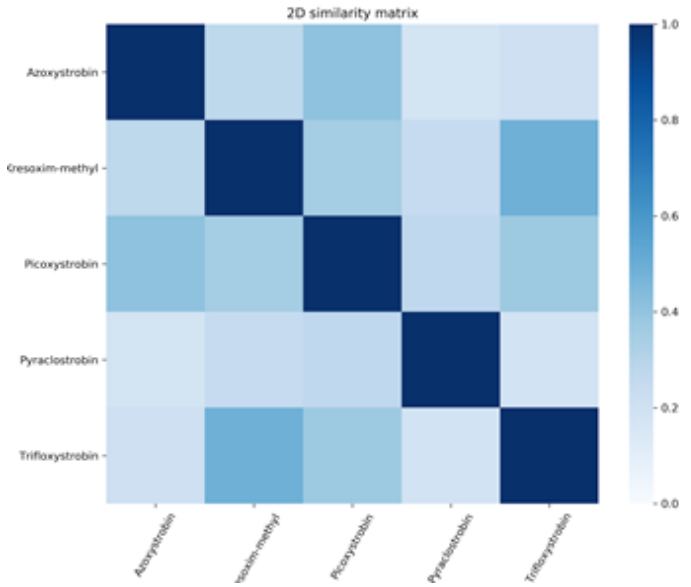


Annex 1.15. Strobilurins SMARTS: Common substructures and structural similarity

Method	SMARTS calculations
Contact	Jose Carlos -> josecarlos.gomez@upf.edu

2D similarity

2D structural similarity was calculated using RDkit Morgan fingerprints (ECFP, radius=2) and tanimoto score. Structures were extracted from the Eu-ToxRisk case study data provided by EMBL-EBI.



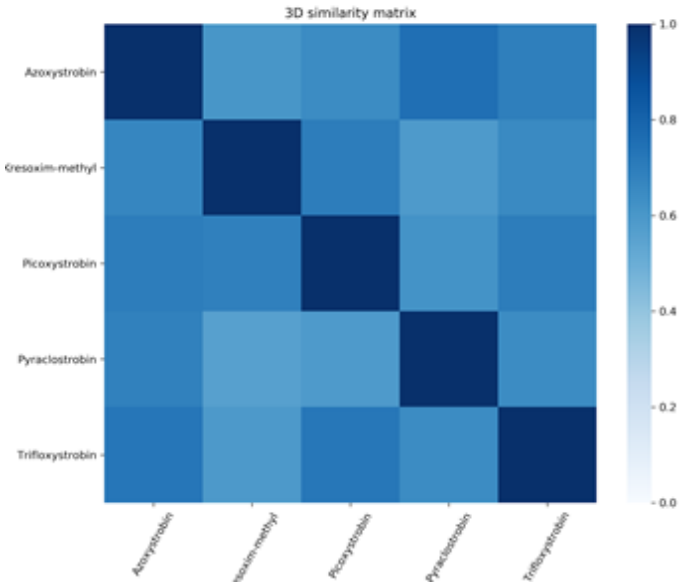
	Azoxystrobin	Kresoxim-methyl	Picoxystrobin	Pyraclostrobin	Trifloxystrobin
Azoxystrobin	1	0.28	0.41	0.18	0.21
Kresoxim-methyl	0.28	1	0.35	0.25	0.49
Picoxystrobin	0.41	0.35	1	0.27	0.38
Pyraclostrobin	0.18	0.25	0.27	1	0.19
Trifloxystrobin	0.21	0.49	0.38	0.19	1

Is spite of all compounds share the same toxicophore their 2D structural similarity is rather low.

3D-similarity.

In cases like the present, where the mechanism of action depends on the specific interaction of the compounds with a defined protein binding site (mitochondrial complex III), the 3D properties of the compound structure play a major role. Therefore, molecular descriptors able to characterise the 3D shape can have advantages over 2D descriptors.

3D shape descriptors with atom types *USR-CAT* were calculated using 10 conformers obtained from RDKit (http://www.rdkit.org). Results are shown in the below heatmap and table.



	Azoxystrobin	Kresoxim-methyl	Picoxystrobin	Pyraclostrobin	Trifloxystrobin
Azoxystrobin	1	0.61	0.65	0.76	0.7
Kresoxim-methyl	0.67	1	0.71	0.59	0.65
Picoxystrobin	0.7	0.69	1	0.62	0.7
Pyraclostrobin	0.69	0.56	0.59	1	0.64
Trifloxystrobin	0.73	0.59	0.72	0.65	1

It must be noted that the use of this 3D descriptors represents better the internal similarity of the compounds of interest with values ranging 0.61-0.76 for Azoxystrobin (compared with the 2D similarity ranging 0.3-0.69)

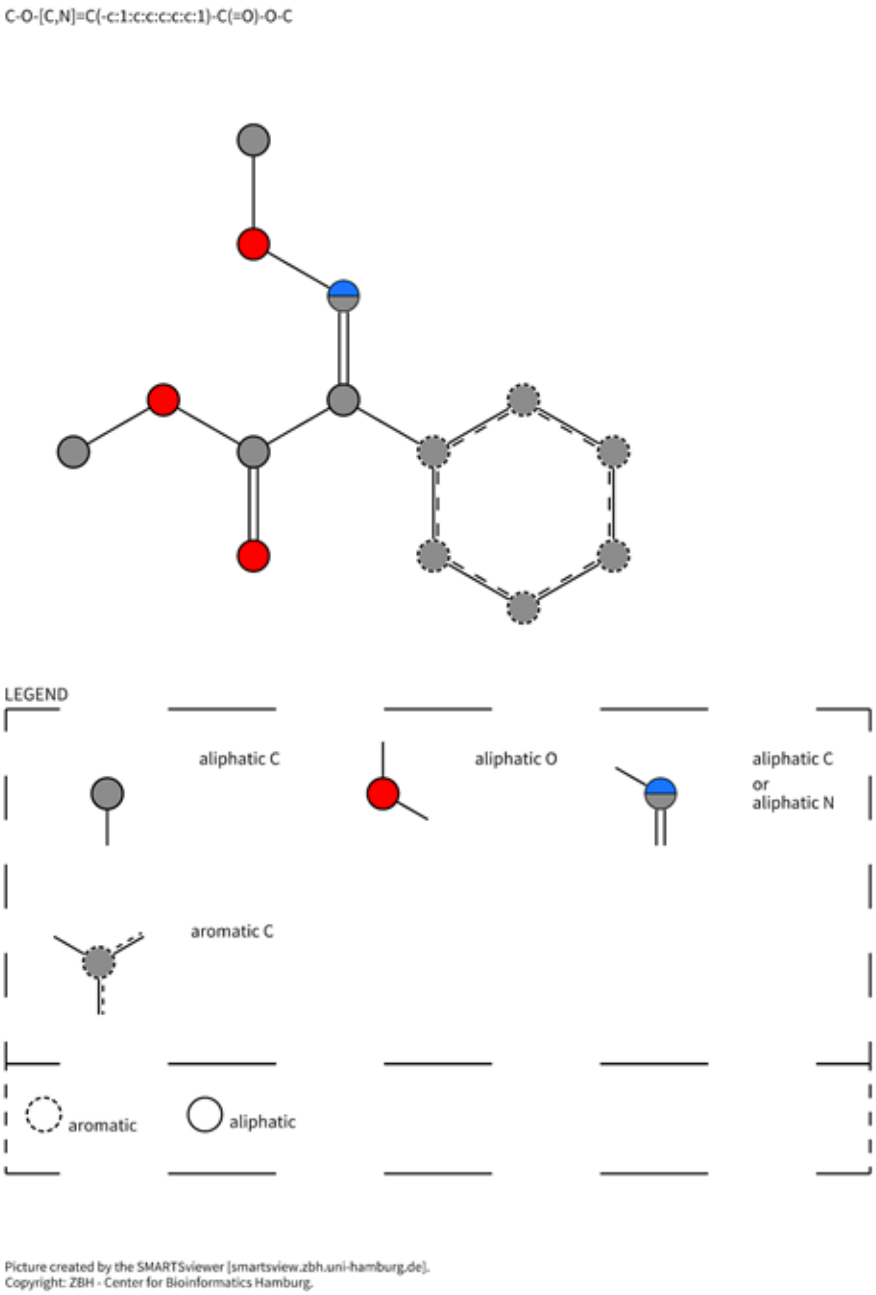
Common patterns SMARTS characterisation

A different strategy to characterise the compound similarity is the use of SMARTS patterns *(SMARTS - A Language for Describing Molecular Patterns, http://www.daylight.com/dayhtml/doc/theory/theory.smarts.html*), which overcomes some of the limitations of using Tanimoto and molecular fingerprints similarity. SMARTS search returns only compounds having the same structural patterns. Three SMARTS patterns, matching all the compounds of interests have been defined, as it is shownbelow:

Group1

Azoxystrobin, Picoxystrobin, Kresoxim-methyl,Trifloxystrobin.

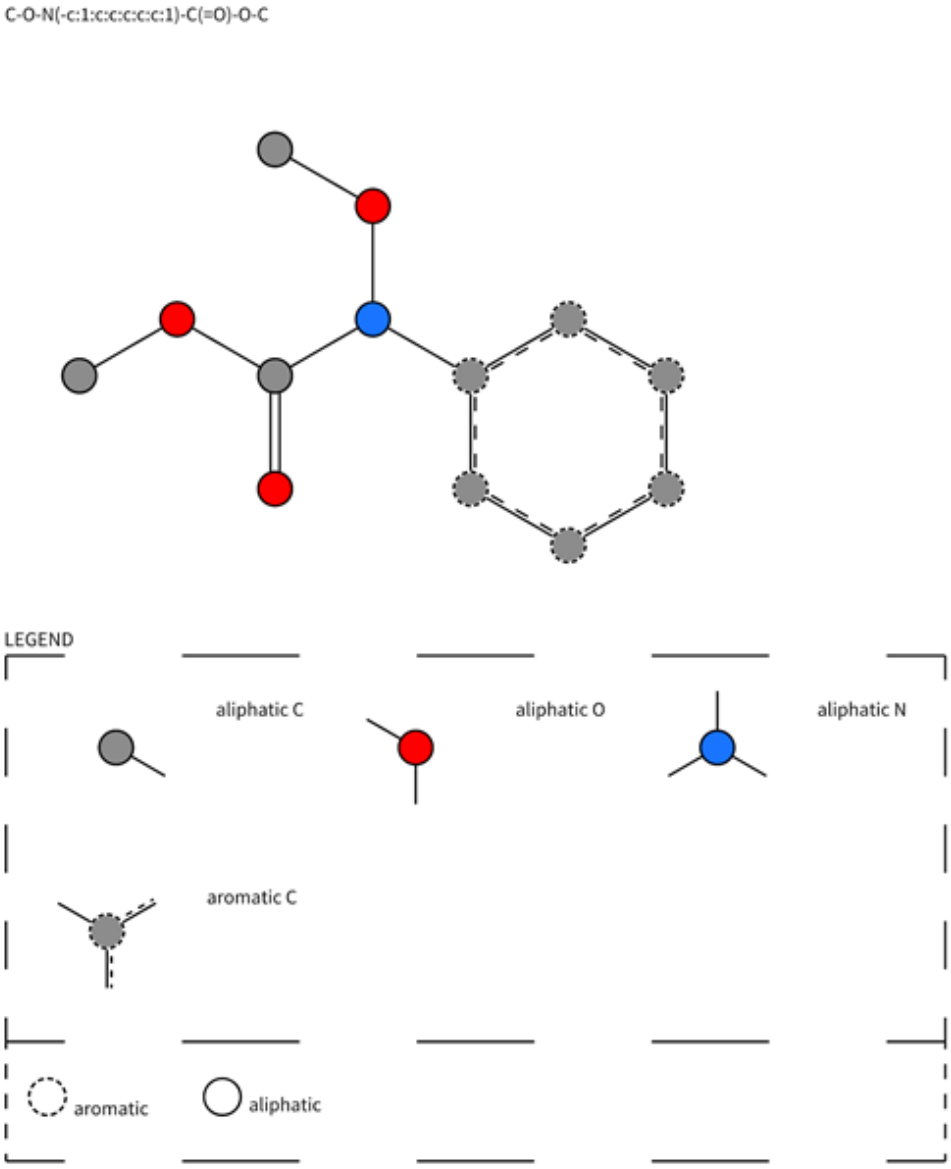
SMARTS: C-O-[C,N]=C(-c:1:c:c:c:c:c:1)-C(=O)-O-C



Group 2

Pyraclostrobin

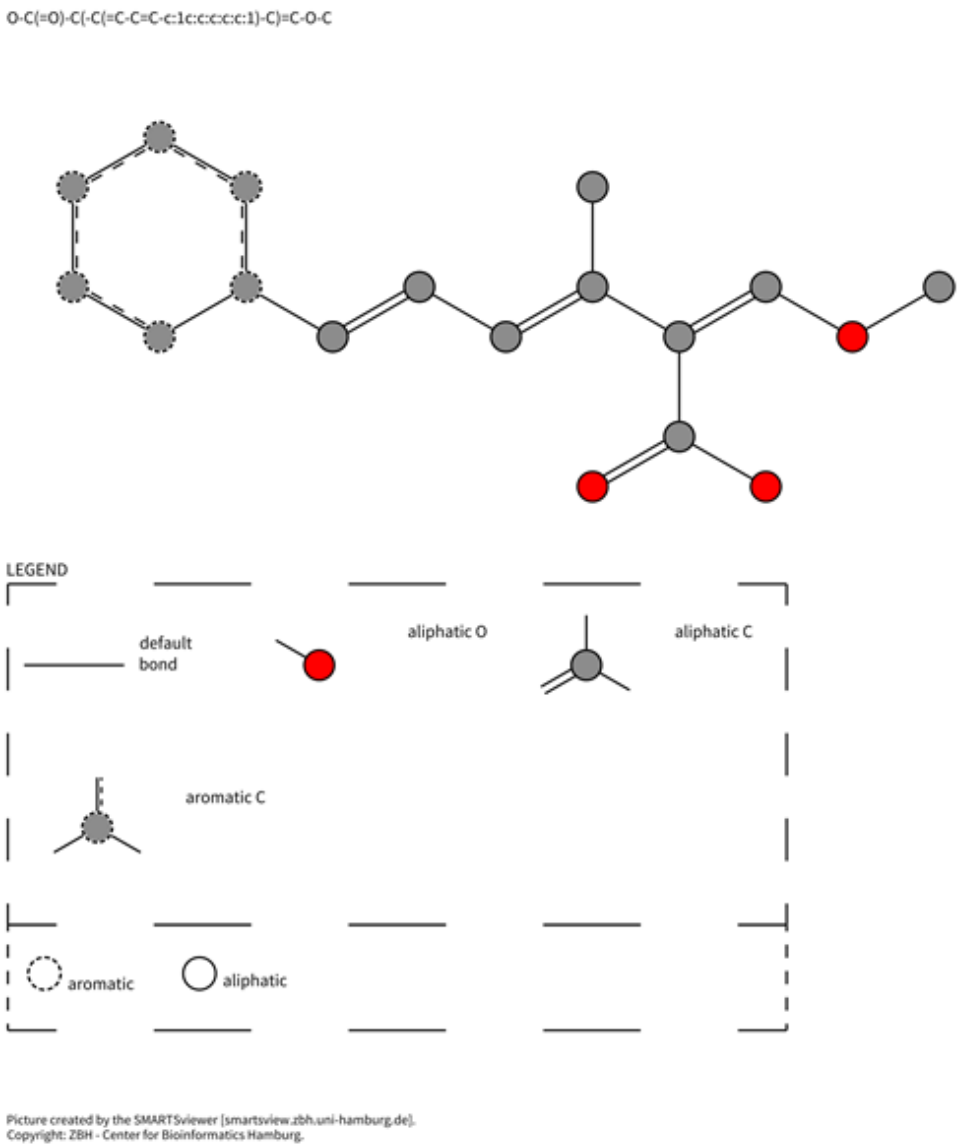
SMARTS: C-O-N(-c:1:c:c:c:c:c:1)-C(=O)-O-C



First generation strobilurins

Strobilurin A, K, H, N, etc…

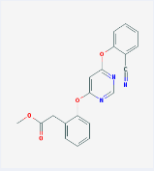
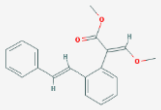
SMARTS: O-C(=O)-C(-C(=C-C=C-c:1c:c:c:c:c:1)-C)=C-O-C



Correspondence SMARTS-compound table.

	SMARTS		
Compound	C-O-[C,N]=C(-c:1:c:c:c:c:c:1)-C(=O)-O-C	C-O-N(-c:1:c:c:c:c:c:1)-C(=O)-O-C	O-C(=O)-C(-C(=C-C=C-c:1c:c:c:c:c:1)-C)=C-O-C
Azoxystrobin			
Picoxystrobin			
Kresoxim-methyl			
Trifloxystrobin			
Pyraclostrobin			
Strobilurins (A…)			

Comparison between similarity search and SMART search.

Type of search	Compounds retrieved	methoxyacrylate group	Actives	Most similar/first match
Similarity	238	101	1 (Azoxystrobyn)	
SMARTS [CH3]-O-[C,N]=C(-c:1:c:c:c:c:c:1)-C(=O)-O-[CH3] (More restricted than the proposed before)	3132	3132	38	

Annex 1.16. Assessment of structural similarity

Method	structural similarity assessment (Tanimoto similarity)
Contact	Susanne Hougaard -> suhou@mst.dk

Structural similarity was calculated by using a workflow integrated into a KNIME analytic platform (https://www.knime.com/knime-software). We used smiles codes as input parameter.

Smiles codes were extracted from high quality open source databases. In this case the majority comes from the US EPA chemical dashboard (https://comptox.epa.gov/dashboard) and one from CHEMID Plus (https://chem.nlm.nih.gov/chemidplus/, Table 1). Prior to the calculation of structural fingerprints, the smiles codes for all analogues were quality controlled, a correction was not needed.

We used a fingerprint method, which involves encoding the structural information within a molecule as a bit string in which each bit indicates the presence of ("1") or absence ("0") of a particular molecular feature. Molecular fingerprints per compound were calculated with RDKIT using a MACCS keys (Molecular ACCess System). MACCS keys (Durant JL *et al.* 2002) are a classical fingerprint in chemoinformatics consisting of a dictionary of 166 structural fragments, as well as UNITY fingerprints (Patterson DE *et al.*) that assemble atom pathways of pre-defined lengths.

In order to assess structural similarity between compounds we used the Tanimoto coefficient (Gillet *et al.* 2003). The Tanimoto coefficient (S) calculates similarity comparing pairs of compounds, e.g. A and B (Equation 1). It uses three values, the number of bits set to 1 in both fingerprints (value a and b) and the number of shared bits set to 1 between the two compounds (value c, equation 1).

$$\text{Equation 1: } S(A, \boldsymbol{B}) = \frac{c}{a + b - c}$$

Gillet VJ and Leach AR (2003) An Introduction to Chemoinfromatics. Kluwer Academic Publishers Drdrecht, The Netherlands, 103.

Durant JL, Leland BA, Henry DR, *et al.*: Reoptimization of MDL keys for use in drug discovery. *J Chem Inf Comput Sci*. 2002;42(6):1273–1280. 10.1021/ci010132r

Patterson DE, Cramer RD, Ferguson AM, *et al.*: Neighborhood behavior: a useful concept for validation of "molecular diversity" descriptors. *J Med Chem*. 1996;39(16):3049–3059. 10.1021/jm960290n

Table 1. Input parameter for structural similarity calculation

name	SMILES	MW	structure_source
Pyrimidifen	CCOCCc1ccc(OCCN=c2[nH]cnc(CC)c2Cl)c(C)c1C	377,916	EPA
Picoxystrobin	CO/C='C('/C(=O)OC)c1ccccc1COc1cccc(C(F)(F)F)n1	367,323	EPA
Tebufenpyrad	CCc1nn(C)c(C(=O)NCc2ccc(C(C)(C)C)cc2)c1Cl	333,863	EPA
Cyazofamid	Cc1ccc(-c2c(Cl)nc(C#N)n2S(=O)(=O)N(C)C)cc1	324,793	EPA
Fenazaquin	CC(C)(C)c1ccc(CCOc2ncnc3ccccc23)cc1	306,409	EPA
Thifluzamide	Cc1nc(C(F)(F)F)c(C(O)=Nc2c(Br)cc(OC(F)(F)F)cc2Br)s1	528,066	EPA
Azoxystrobin	CO/C='C('/C(=O)OC)c1ccccc1Oc1cc(Oc2ccccc2C#N)ncn1	403,394	EPA
Fenpyroximate	Cc1nn(C)c(Oc2ccccc2)c1/C='N'/OCc1ccc(C(=O)OC(C)(C)C)cc1	421,497	EPA
Antimycin A	CCCCCC[C@H]1C(=O)O[C@H](C)[C@H](NC(=O)c2cccc(NC=O)c2O)C(=O)O[C@@H](C)[C@@H]1OC(=O)CC(C)C	548,633	ChemIDplus
Trifloxystrobin	CO/N='C('/C(=O)OC)c1ccccc1CON=C(C)c1cccc(C(F)(F)F)c1	408,376	EPA
Kresoxim-methyl	CO/N='C('/C(=O)OC)c1ccccc1COc1ccccc1C	313,353	EPA
Fenamidone	CSC1=N[C@@](C)(c2ccccc2)C(=O)N1Nc1ccccc1	311,41	EPA
Pyraclostrobin	COC(=O)N(OC)c1ccccc1COc1ccn(-c2ccc(Cl)cc2)n1	387,823	EPA
Fenfuram	Cc1occc1C(=O)Nc1ccccc1	201,225	EPA
Capsaicin	COc1cc(CNC(='O)CCCC'/C='C'/C(C)C)ccc1O	305,418	EPA
Deguelin	COc1cc2c(cc1OC)[C@@H]1C(=O)c3ccc4c(c3O[C@@H]1CO2)C=CC(C)(C)O4	394,423	EPA
Carboxin	CC1=C(C(=O)Nc2ccccc2)SCCO1	235,308	EPA
Mepronil	Cc1ccccc1C(=O)Nc1cccc(OC(C)C)c1	269,344	EPA
Flutolanil	CC(C)Oc1cccc(NC(=O)c2ccccc2C(F)(F)F)c1	323,314	EPA
Hydramethylnon	CC1(C)CNC(=NN='C('/C='C'/c2ccc(C(F)(F)F)cc2)/C='C'/c2ccc(C(F)(F)F)cc2)NC1	494,483	EPA
Rotenone	C=C(C)[C@H]1Cc2c(ccc3c2O[C@@H]2COc4cc(OC)c(OC)cc4[C@@H]2C3=O)O1	394,423	EPA
Pyridaben	CC(C)(C)c1ccc(CSc2cnn(C(C)(C)C)c(=O)c2Cl)cc1	364,942	EPA

Annex 1.17. CS4 read across SH-SY5Y method description (Swetox)

Method	SH-SY5Y: Lactate, Resazurin, Rho, Calcein, ATP, neuron degeneration
Contact	Anna Forsby (Swetox) -> anna.forsby@swetox.se

Cell Model:

SH-SY5Y is a thrice-cloned sub-line of the bone marrow biopsy-derived line SK-N-SH (Biedler *et al.*, 1973). The cells are characterised as sympathetic cells with high expression of muscarinic acetylcholine receptors and voltage operated Ca^{2+} channels, among other receptors and ion channels. The cells grow in monolayer cultures and consist of a neuronal phenotype (90-100%) and sometimes an epithelial-like phenotype (0-10%). The cultures shall not be used as models for neurons when the epithelial cells exceed 10% of the cell population, since these cells do not display any neuronal characteristics (Ciccarone *et al.*, 1989). The cells can be differentiated by all-trans retinoic acid (RA), thereby the cells develop into a phenotype with neuronal structural and functional properties and transcriptional profiles (Cheung *et al.*, 2009; Korecka *et al.*, 2013; Krishna *et al.*, 2014; Xicoy *et al.*, 2017). Herein, viability is studied after exposure of differentiated cells with test chemicals.

The cells can be purchased from ATCC (cat# CRL-2266), ECACC (cat# 94030304) or from other labs, e.g. Uppsala University or Stockholm University (Sweden). The native Stockholm cells display a more developed neuronal phenotype as compared to the cells obtained from ECACC.

Routine cell culture procedures:

The cells are maintained in culture in 75 cm^2 flasks and fed with MEM supplemented with 10% foetal bovine serum, 2 mM glutamine, 1% non-essential amino acids, 100g streptomycin/mL and 100 U penicillin/mL (named complete EMEM). The cells are subcultured every 7^{th} day and the medium is replaced on the 3^{rd} to 4^{th} day after subcultivation.

Cell splitting and differentiation for experiments:

For experiments, SH-SY5Y cells are plated in white 96-well plates complete, which is exchanged to differentiation medium with 1 µM RA and without serum (DMEM:F12 [1:1], N2 supplements [diluted 1:100], 1 mM glutamine, 100g streptomycin/mL and 100 U penicillin/mL) after 24 h. The cells are allowed to differentiate for 6 days before the exposure to the test chemicals. On the third day of differentiation, half of the cell medium in the wells is removed and replaced by fresh differentiation medium. The cell density used varies between assays, 10 000 cells/well were used for viability (resazurin), lactate, mitochondrial membrane potential and propidium iodide staining. For measurements of neurite degeneration, a cell density of 8 000 cells/well was used.

Experimental exposure to test chemicals:

After arrival, all complex inhibitors were dissolved in Dimethyl Sulfoxide (DMSO) in a concentration of 10, 50 or 100 mM. All test chemicals were then diluted to obtain a 10mM stock which is subsequently aliquoted and stored at minus 20°C.

Six days differentiated SH-SY5Y cells were exposed to the test chemicals at fixed concentrations of 10 µM, 2µM, 400nM, 80nM and 16nM. Further testing at lower concentrations was performed for chemicals yielding an effect at 16 nM. For the exposure, the DMSO concentration in cell medium was 0.1 % (v/v) in all samples.

Endpoints:

A separate SOP is provided for each assay, a general description of the procedures is found below.

1. Cell viability (resazurin reduction) and lactate quantification:

For experiments, SH-SY5Y cells were plated in black 96-well plates with clear bottom (10 000 cells/well) in complete EMEM, which was exchanged to differentiation medium after 24 h. Six (6) days differentiated SH-SY5Y cells were exposed to the test chemicals and incubated for 24 hours after which the effect on cell viability was measured with resazurin.

Procedure: After the 24 hours incubation, the supernatant was collected and stored at -20 ºC for lactate measurements. An 88µM resazurin working solution was then added on top of the cell medium in the wells yielding final concentration of 44 µM resazurin. The plates were incubated for two hours, afterwards the resorufin fluorescence was measured at 540 nm excitation and 590 nm emission.

The amount of lactate in the collected supernatant was quantified by a colorimetric assay. The lactate assay is based on the conversion of lactate to pyruvate by the enzyme lactate dehydrogenase (LDH), reducing the co-factor NAD to NADH (Babson *et al*). In the assay, NADH reduces PMS to PMSH which reduces INT to INTH. INTH is the reagent measured by colorimetry. The samples are incubated with a reagent mix for 7 minutes, the absorbance is then measured at 490 nm.

2. Mitochondrial membrane potential with Rhodamine 123 and cytotocixity assessment with propidium iodide:

Mitochondrial membrane potential (MMP) is correlated with the cells' ability to produce ATP through oxidative phosphorylation. Acute changes in MMP are monitored by measuring the changes in fluorescence intensity after loading the cells with Rhodamine 123 and exposing them to a selection of chemicals for 24 hours.

For experiments, SH-SY5Y cells were plated in 96-well plates with micro-clear bottom (10 000 cells/well) in complete EMEM, which was exchanged to differentiation medium after 24 h. Six (6) days differentiated SH-SY5Y cells were stained with 1 µM Rhodamine123 for 45 minutes before the exposure with test chemicals. After 24h, incubation with the test chemicals Hoechst and propidium iodide were added to the wells in order to stain the nuclei for cytotoxicity measurements. The effect of the test chemicals on the mitochondrial membrane potential was reflected as an increased (hyperpolarisation) or decreased (depolarisation) Rhodamine 123 fluorescence intensity.

3. Neurite degeneration assessed by calcein staining:

The cell permeant dye Calcein-AM was used to stain the cytoplasm of differentiated SH-SY5Y cells allowing the visualisation and quantification of the neurite length. Once inside the cells, calcein-AM is hydrolysed by intracellular esterases producing a green fluorescent dye. For experiments, SH-SY5Y cells were plated in 96-well plates with micro-clear bottom (8 000 cells/well) in complete EMEM, which was exchanged to differentiation medium after 24 h. Six (6) days differentiated SH-SY5Y cells were exposed and incubated with the test chemicals for 24 hours. After incubation, the cells were stained with calcein and imaged. All test chemicals were initially screened at 10 µM, 2 µM and 0.4 µM. For those chemicals showing effect at the lowest tested concentration during the screening, extra 5X dilution steps were performed until a no effect concentration was reached. If no effect was observed during the screening, the experiment was repeated only a second time.

4. Neurite degeneration, cytotoxicity and ATP content after repeated exposure:

Neurite degeneration and cytotoxicity were evaluated by fluorescent microscopy with the dyes calcein-AM (for neurite staining) and propidium iodide (PI) (for early apoptotic/necrotic cell staining). In addition, ATP content was quantified after imaging by luminescence with the CellTiter-Glo® assay (Promega). For experiments, SH-SY5Y cells were plated in 96-well plates with micro-clear bottom (8 000 cells/well) in complete EMEM, which was exchanged to differentiation medium after 24 h. Three days after the start of differentiation the cells were exposed to the test chemicals, the exposure was repeated on the day six of differentiation. On the eighth day of differentiation (120h after the first exposure to test chemicals), the cells were stained with calcein-AM, PI and Hoechst and imaged. After imaging, the ATP content is measured with the Promega's CellTiter-Glo® assay system which employs the properties of a thermostable luciferase to enable reaction conditions that generate a stable "glow-type" luminescent signal while simultaneously inhibiting endogenous enzymes released during cell lysis (e.g., ATPases).The luminescence signal is recorded by luminescence reader (Tecan Infinite M200 Pro) for 300ms per well.

Normalisation of asymptotes for BMR calculations:

The values obtained from all assays are expressed as percentage of 0.1% DMSO controls. The data is transfer to GraphPad prism where the concentrations (expressed in M) are transformed to the logarithm (log 10). The data is then analysed by fitting a nonlinear regression with least squares fitting method and without constrains to obtain the best fit values for Top. If the lowest tested concentration is not exactly 100% of control (no effect), the asymptotes are normalised by calculating a factor as follows:

Factor = (100 / best fit values for Top as a factor)

Each obtained data point is then multiplied by the previously calculated factor.

If after the normalisation the values in the lowest tested concentration still don't fall in 100% of control, the mean of the two lowest concentrations is calculated and used to replace the best fit values for Top for calculation of the factor.

After normalisation of asymptotes, the BMR values were calculating using the online tool "BMC, Bench mark concentration" provided by AG Leist, University of Konstanz

(http://invitrotox.uni-konstanz.de:3838/BMC/). If the reported BMR value was higher than the highest tested concentration, the BMR were reported as >highest tested concentration.

Exceptions:

Mitochondrial membrane potential: Azoxystrobin produced hyperpolarisation (values over 100%) at some of the lower tested concentrations. To provide a proper fit for these values, the data points were not normalised, instead the BMR for both effects (depolarisation and hyperpolarisation) were calculated.

Calculation of depolarisation: the values were uploaded in the online tool "BMC, Bench mark concentration" and the BMR were calculated as usual.

Calculation for hyperpolarisation: the section of the dose-response curve corresponding to the hyperpolarisation effect was inverted as follows:

Hyperpolarisation effect= 100 – (data point – 100)

The obtained values correspond to an inhibition curve, these values were then uploaded in the online tool "BMC, Bench mark concentration" and the BMR were calculated as usual.

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Annex 1.18. CS4 read across HepG2 method description (LU)

Method	HepG2: Lactate, Resazurin, Rho123/PI
Contact	Wanda van der Stel (LU) -> w.van.der.stel@lacdr.leidenuniv.nl

Cell Model

The assay is performed in HepG2 (ATCC® HB8065™).

Routine cell culture procedures

The cell are maintained in 75 cm^2 flasks using DMEM supplemented with 10% foetal bovine serum, 25g streptomycin/mL and 25U penicillin/mL.

Cell splitting

For routine culture the cells are splitted every 3 à 4 days. The medium is replaced during the procedure of cell splitting. For the CS4 experiments, cells are seeded in 384wells black unclear plates at a density of 10.000 cells per well. The seeded cells were used for the various assay 2 days after seeding.

Experimental exposure to mitotoxicants

After arrival, all complex inhibitors were dissolved in Dimethyl Sulfoxide (DMSO) in a concentration of 100 mM. All test chemicals were then diluted to obtain a 10mM stock which is subsequently aliquoted and stored at minus 80°C. Aliquots used for experiments were stored at minus 20°C.

Endpoints

A separate SOP is provided for each assay, a general description of the procedures is found below.

All assays are performed using the same cells in the following order:

1. Mitochondrial functioning based on mitochondrial membrane potential (rho123) and Cell death based on PI staining

With the Rhodamine123 mitochondrial membrane potential sensitive dye the presence of the membrane potential can be monitored over time using confocal imaging systems. 75min before compound exposure the cells are treated with 0,5uM Rho123 and 0.1ug/ml Hoechst (by adding 2x concentration solution on top of the cells). Subsequently the medium is replaced by a mixture of Rho123 (final conc = 0.1uM) and PI (final conc = 100nM), followed by the various chemicals. The Hoechst, rho123 intensity and PI intensity were monitored using a Nikon eclipse system with an automated stage, incubator, A1 confocal system and either equipped with photon multiplier (PMT) or Gasp detector (Hoechst = 408nM, Rho123 = 488nM and PI = 561nM). Using an in house pipeline (including CellProfiler and R scripts) the intensity and the localisation of the various signals were quantified. For the mitochondrial membrane, potential assessment the intensity of the dye

is used and for the assessment of viability, the fraction of PI positive nuclei is represented. All values are percentages of 0.1% DMSO controls.

2. Glycolysis based on lactate concentrations

The concentration of excreted lactate can be used to assess the glycolytic status of a cell system. To do so, the medium was collected after 24h of exposures (when the imaging was completed). The concentration of lactate is assessed using a colorimetric assay. The change in 490 nm absorption upon the reduction of INT to INTH (see SOP for complete description) can be assessed as a measure for the lactate concentration. All values are percentages of 0.1% DMSO controls.

3. Viability based on resazurin reduction

Cellular viability was finial quantified by assessing the reduction of resazurin in to resorufin, which is considered to be a measure for global REDOX capacity of the cell. Upon collection of the medium for the lactate assay, a mixture of 44 uM resazurin in medium was applied to the cells for 2h. Subsequently, the Fluorescence of the resofurin is read at excitation 540 nm/emission 590 nm. All values are percentages of 0.1% DMSO controls.

Re-normalisation of datasets for BMR calculations:

1. Supernatant lactate

Values of at least two no effect concentrations were forced to upper asymptote exactly at 100 %, data sets were re-normalised as percentage of no effect concentrations. Curve fitting and ECs values couldn´t be generated using the *in vitro* toxicology on-line tool provided by AG. Leist, University of Konstanz (http://invitrotox.uni-konstanz.de:3838/BMC/) as the algorithm is suitable only for inhibition curves. Lactate production increase with increase of the insult leading to activation curve, therefore curve fitting and ECs values are generated using point-to-point extrapolation in GraphPad Prism.

2. Resazurin reduction

Values of at least two no effect concentrations were forced to upper asymptote exactly at 100 %, data sets were re-normalised as percentage of no effect concentrations. Curve fitting and BMRs values are generated using the *in vitro* toxicology on-line tool provided by AG. Leist, University of Konstanz

3. MMP with Rho123

An in house script was used to determine the maximal value of the concentration response curve. This value was forced to upper asymptote exactly at 100. Curve fitting and BMRs values are generated using the *in vitro* toxicology on-line tool provided by AG. Leist, University of Konstanz

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- BMC modelling
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