Progress at PHE and overview on ongoing activities in the field.

Abigail Dalzell, Tim Gant

22nd March 2017, Antwerp

01 Background and purpose

Background

In relation to the present category, ECHA took note of the generic compilation of compositional information that was submitted by the Registrant in the updated category justification document, following the request of ECHA within the draft decision previously notified. However, while this generic data reveals structural similarity to some degree among the category members, ECHA stresses several deficiencies.

Firstly, contrary to the explicit requirement of Annex XI, 1.5, the Registrant does not define the category based on the structural similarity of the substances concerned, but persists in relying exclusively on manufacturing processes and performance characteristics to justify the grouping approach.

Secondly, the Registrant does not sufficiently qualify the compositional variability of the substances concerned by the category in order to justify that the compositional variability would not be such as to affect the determination of the actual hazard of the substances concerned.

Thirdly, the generic compositional data submitted only refers to the average carbon numbe distribution and average relative mass (%) of four major hydrocarbon classes. However, in the absence of detailed compositional information on the substances concerned by the category, including representative ranges of hydrocarbon classes content, ECHA considers that the respective hazards of these substances cannot be identified in a representative way which does not underestimate the hazard.

Consequently, ECHA considers that the category '*Bitumens*' does not fulfil the requirement defined in Annex XI, 1.5. and does not allow the Registrant to meet the objective pursued by the REACH Regulation. As a result and based on the information analysed by ECHA,

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these substances cannot be considered as a group, or category of substances under the REACH Regulation, irrespective of the status of these substances under other legal systems.

- Compositional variability not sufficiently addressed to justify determination of hazard (via read across)
- Category or grouping not accepted

Background : Purpose

1. Low Boiling Point Naphthas (Gasolines)
2. Kerosines
3. **Straight-run Gas Oils**
4. Cracked Gas Oils
5. **Vacuum Gas Oils, Hydrocracke**
6. **Other Gas Oils**
7. Heavy Fuel Oil Components
8. Unrefined / Acid Treated Oils
9. Other Lubricant Base Oils
10. Highly Refined Base Oils
11. Foots Oils
12. Paraffin and Hydrocarbon Waxe
13. Slack Wax
14. Petrolatum
15. Untreated Distillate Aromatic E
16. Treated Distillate Aromatic Extr
17. **Residual Aromatic Extracts**
18. **Bitumen**

Name	EINECS definition	CAS	EINECS
Asphalt	A very complex combination of high molecular weight organic compounds containing a relatively high proportion of hydrocarbons having carbon numbers predominantly greater than C25 with high carbon-to-hydrogen ratios. It also contains small amounts of various metals such as nickel, iron, or vanadium. It is obtained as the non-volatile residue from distillation of crude oil or by separation as the raffinate from a residual oil in a deasphalting or decarbonization process.	**8052-42-4**	232-490-9
Residues (petroleum), vacuum	A complex residuum from the vacuum distillation of the residuum from atmospheric distillation of crude oil. It consists of hydrocarbons having carbon numbers predominantly greater than C34 and boiling above approximately 495°C (923°F).	**64741-56-6**	265-057-8
Residues (petroleum), hydrodesulfurized vacuum	A complex combination of hydrocarbons obtained by treating a vacuum residuum with hydrogen in the presence of a catalyst under conditions primarily to remove organic sulfur compounds. It consists of hydrocarbons having carbon numbers predominantly greater than C34 and boiling approximately above 495°C (923°F).	**64742-85-4**	265-188-0
Residues (petroleum), thermal cracked vacuum	A complex combination of hydrocarbons obtained from the vacuum distillation of the products from a thermal cracking process. It consists predominantly of hydrocarbons having carbon numbers predominantly greater than C34 and boiling above approximately 495°C (923°F).	**92062-05-0**	295-518-9

In addition CONCAWE has prepared the joint parts of the Registration Dossier for the following stand-alone substances:

- MK1 diesel fuel (EC number 931-250-7),
- **Oxidised Asphalt (EC number 265-196-4)**
- Sulfur (EC number 231-722-6)

To link together UVCB categories using a biologically based measure (gene expression) of hazard rather than use a chemical grouping.

02 What is required?

What is required: the system

- A UVCB
- Reference compounds
- A biological system
- A measure of gene expression
- An algorithm
- Phenotypic outcome measures

What is required: The cell

The Ideal

- Metabolic competency
- Expression of the proteins necessary for the MoA to be active (necessary proteins expressed)
- Genetically stable
- Easy to culture
- Able to execute the phenotypic end points
- Available
- Inexpensive

It does not really matter for the purposes here what the cell is, or from what species. It is simply acting as a chemical system. The reality though is that a panel of cells is likely to be necessary to capture all possible interactions because cells of different types express different proteins and pathways.

What is required: Introducing the cells

Cell type	Organ	Advantages	Disadvantages
A549	Lung	Common, widely used, lung	Redox resistant (active NRF2)
MCF7	Breast	Common, widely used, ER receptor	Unstable. Many clones exist.
HepaRG	Liver	Metabolically competent	Need to be differentiated
HLMVEC	Lung Microvascular Endothelial	Represents target area	
HMePC	Mammary Epithelial Cell	A normal human mammary cells	
A375	Skin melanoma	Commonly used	Hypertriploid (62 chromosomes)

What is required: Introducing the cells

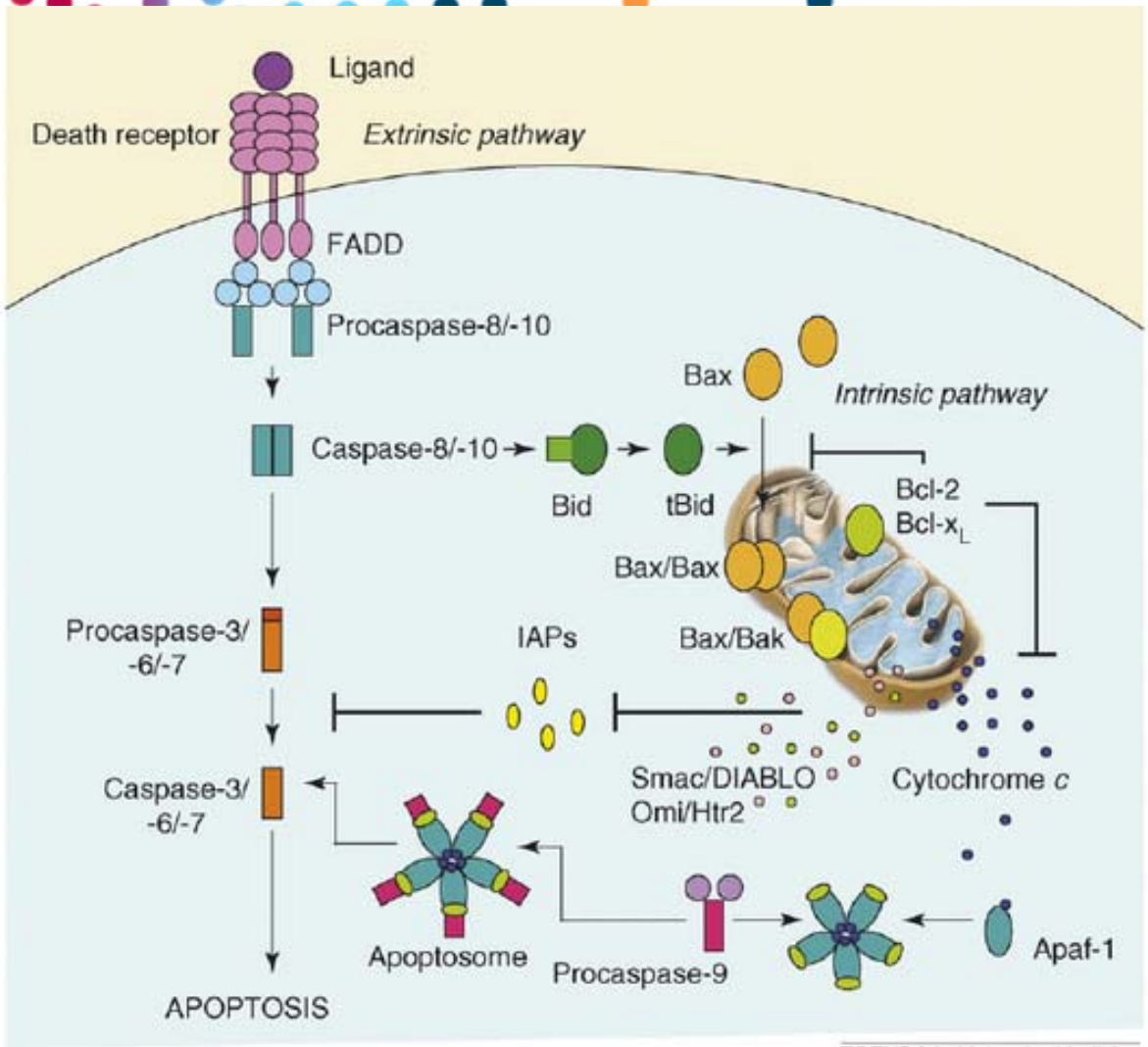
Cell	Organ	Advantages	Disadvantages
HEK10205f	Epidermal Keratinocytes	can differentiate into a stratified squamous epithelium	2n karyotype (46 chromosomes)
HT29	Colon	Commonly used; sensitive to drugs	
HepG2	Hepatocytes	Not tumorogenic and expressed some differentiate markers. Commonly used	Re-arranged chromosome 1 and unstable karyotype
LN229	Brain Glia	Sensitive to apoptosis and protein synthesis inhibitors	Mutant P53
SH-SY5Y	Neurones	Can form neurites with adrenergic action	Form two phenotypes

03 Phenotypic Reference compounds

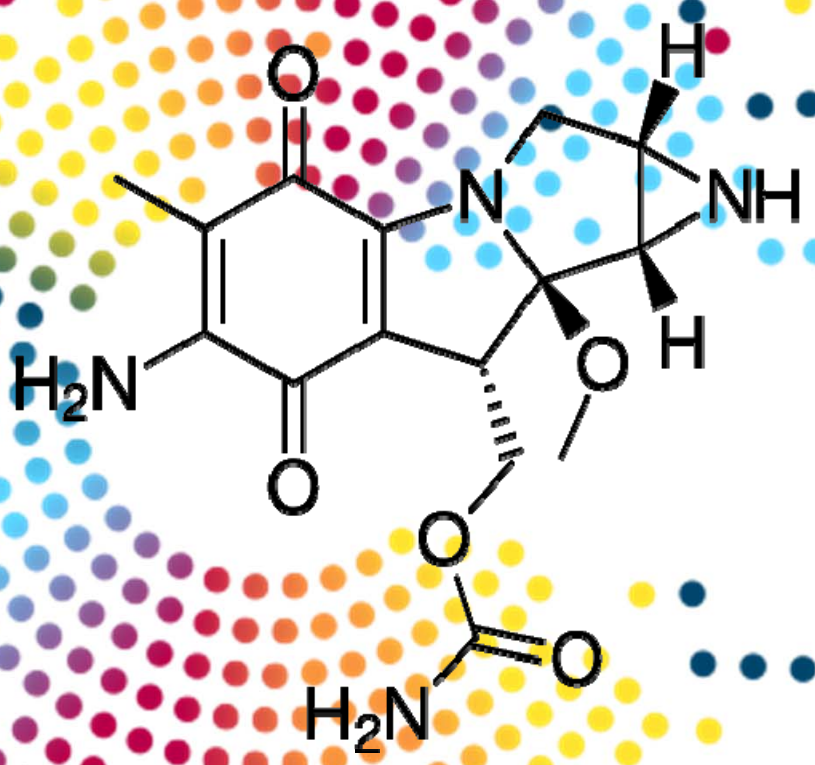
We have chosen phenotypic reference compounds rather than cell specific reference compounds. The rationale for this is that many MoA for UVCB will not necessarily be cell type specific.

Phenotypic assays: Apoptosis

Apoptosis is a fundamental response to cell damage and therefore represents a pertinent end point for indicating cellular stress: Activation of caspase 3/7 is a final step in the biochemical pathway to apoptosis.



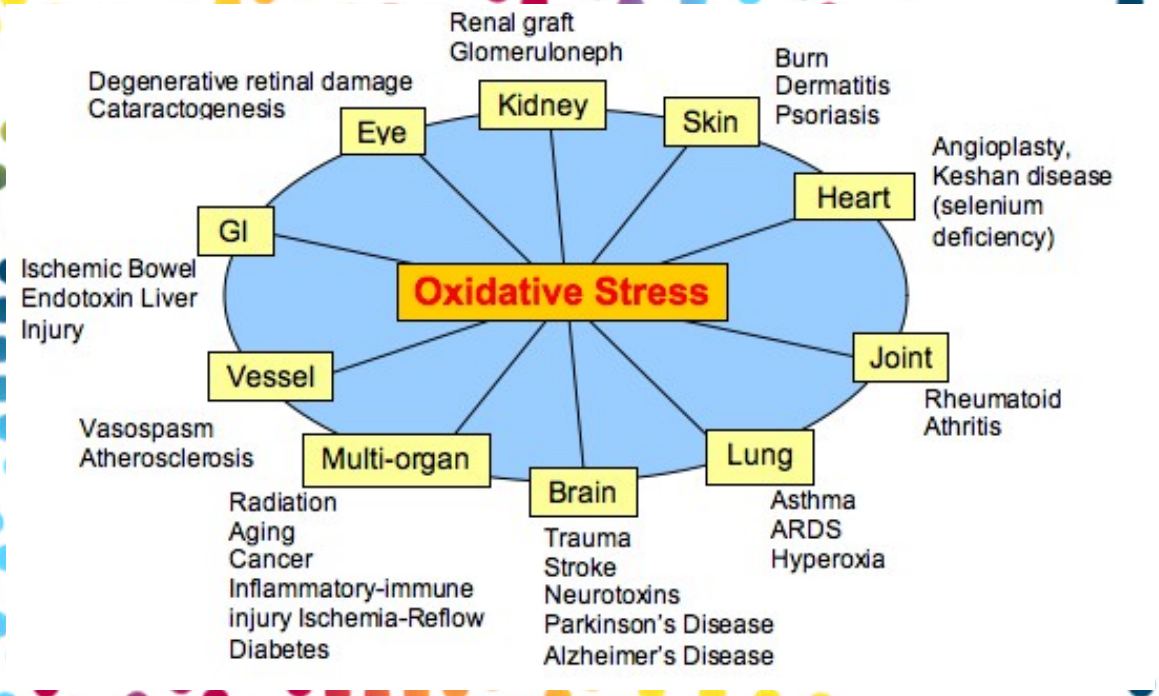
Mitomycin C

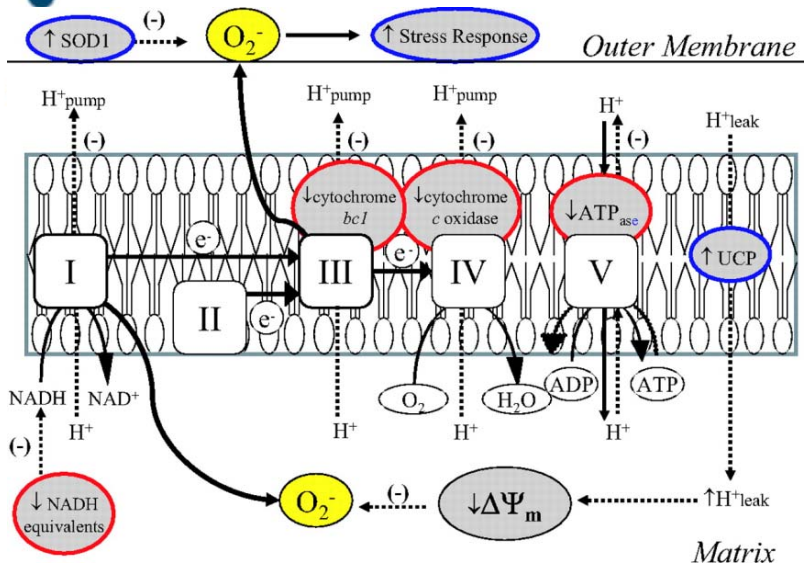


- Intercalates and alkylates DNA
- Inhibits DNA synthesis
- Produces ROS

Hypothesis: Apoptosis is a generic end point to toxicity is likely to be relevant to the toxicity of oil products and UVCBs in particular.

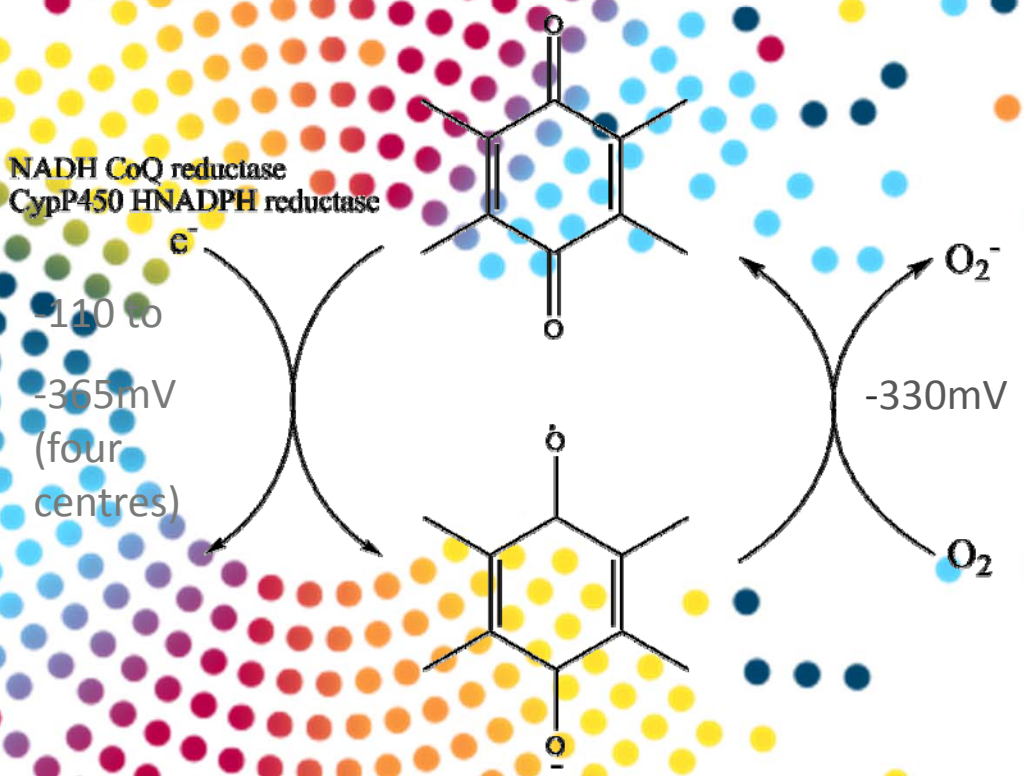
Phenotypic assays: oxidative stress

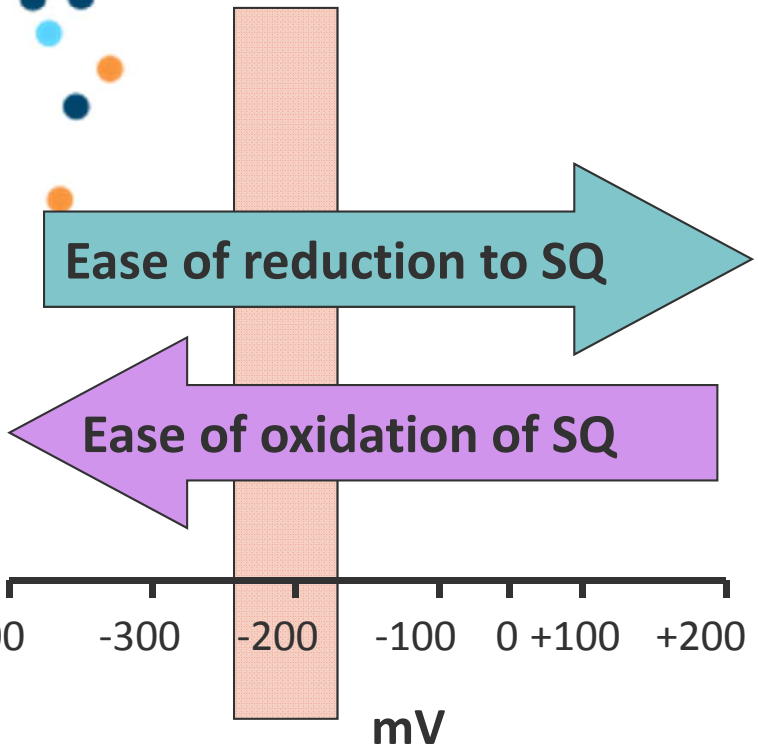




Hypothesis : that compounds within oil products, or more likely the metabolic reaction products, will cause redox stress in the cell

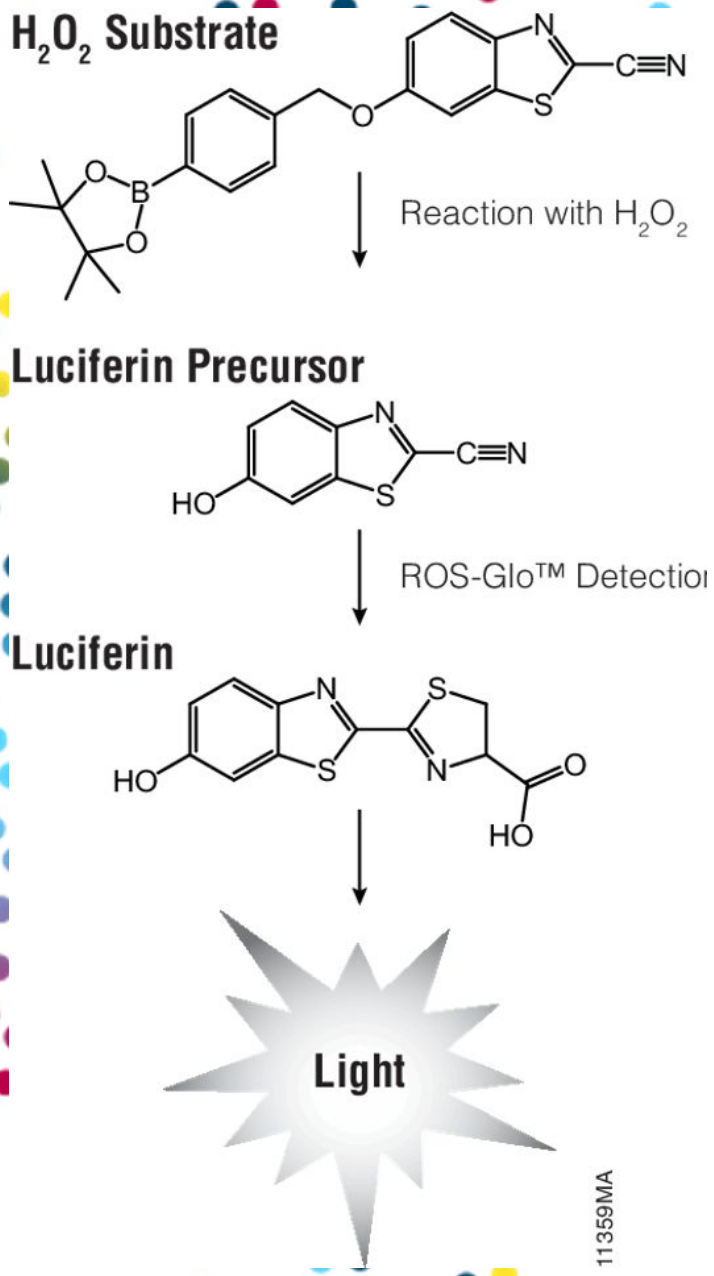
Phenotypic assays: oxidative stress

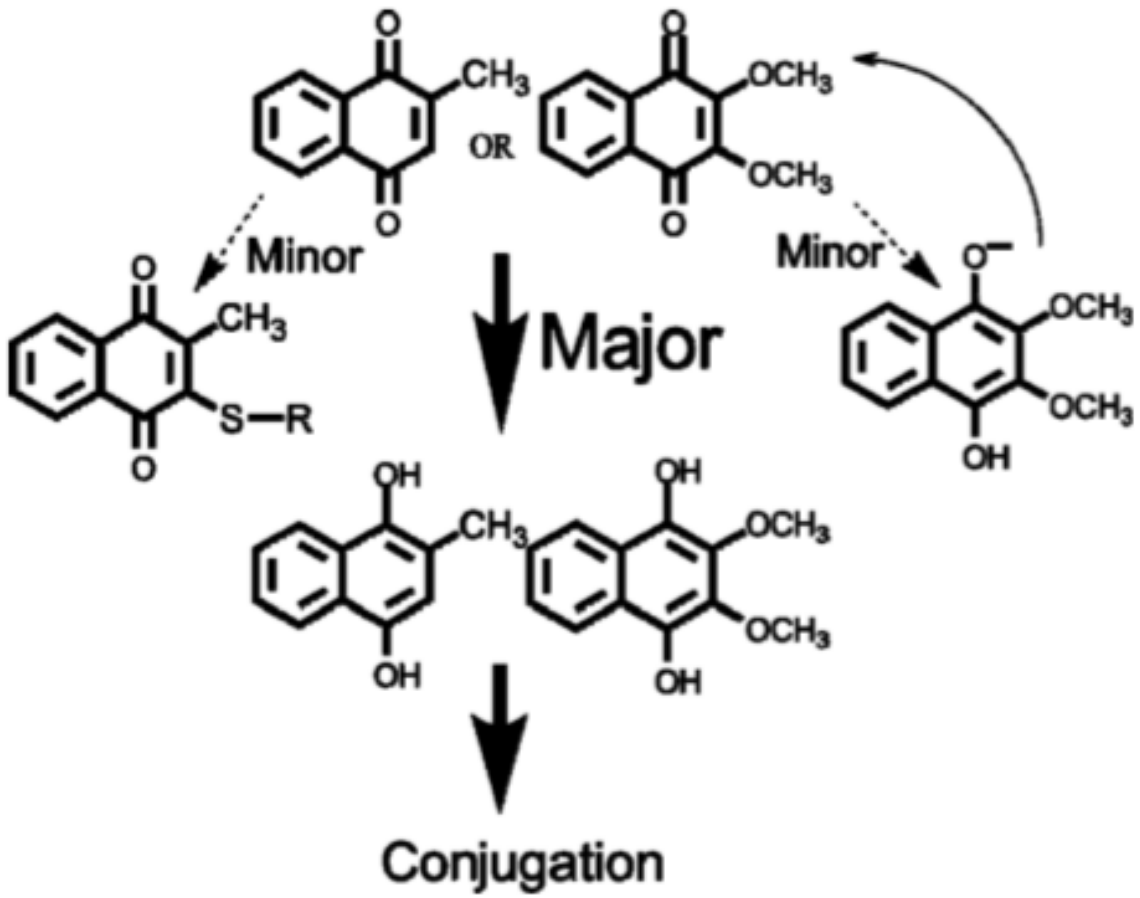




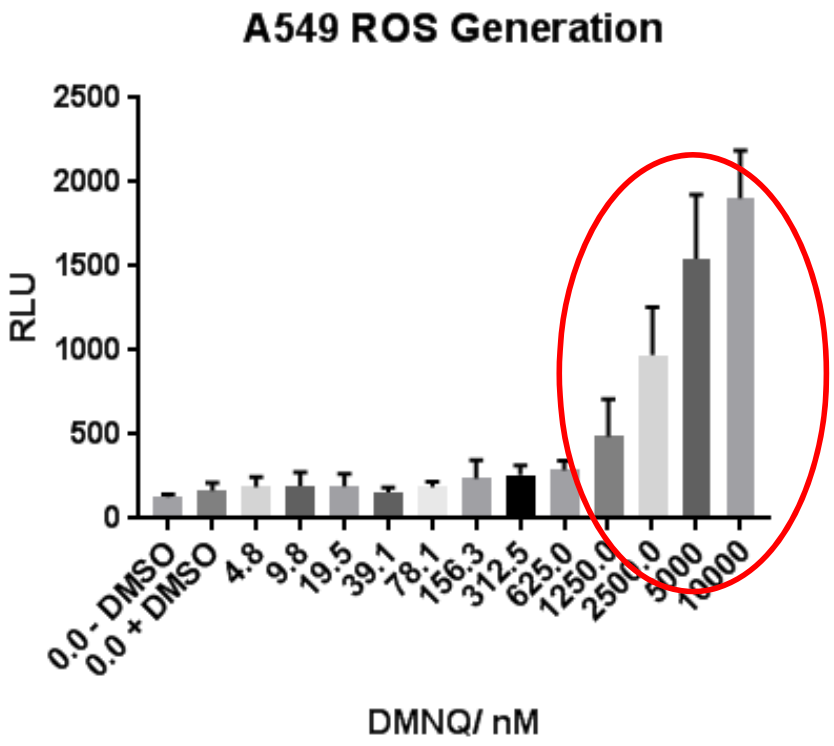
- Oxidative stress has been cited as the cause of many potentially xenobiotic induced disease type.
- There are some compounds that can genuinely redox cycle producing reactive oxygen
- For most though it is likely to be a secondary effect of mitochondrial damage.

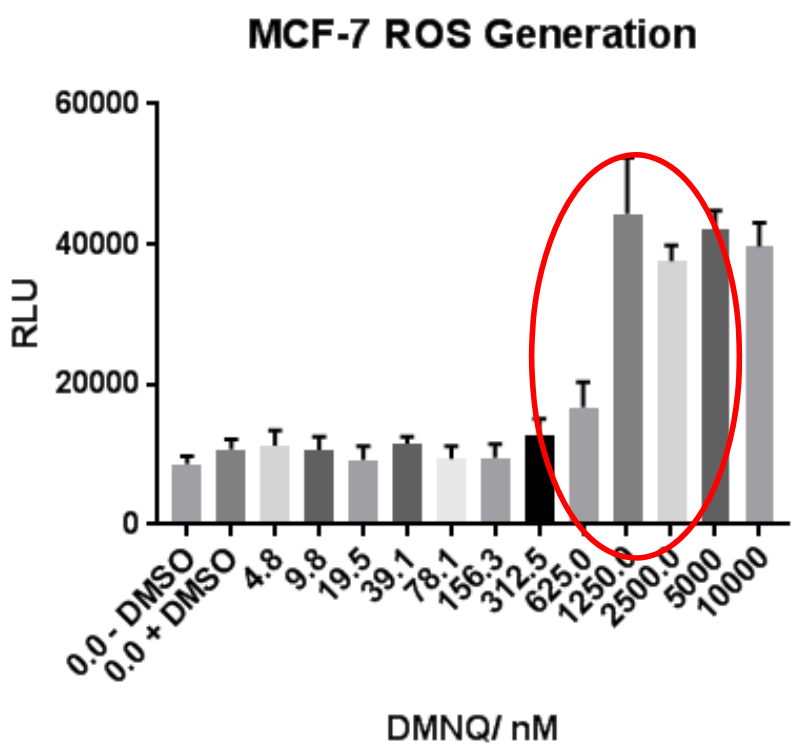
Phenotypic assays: oxidative stress



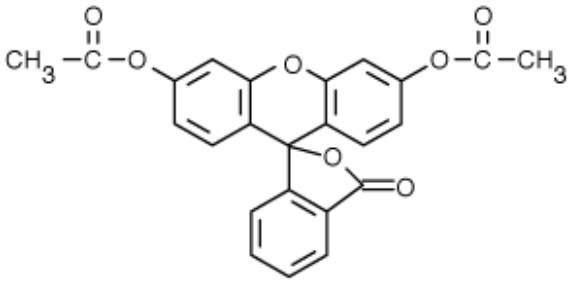


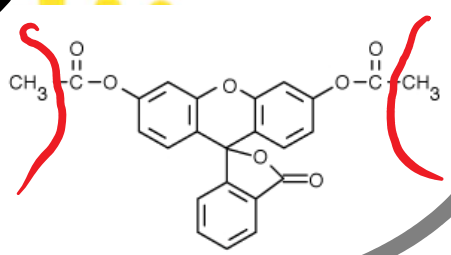
DMNQ – ROS Generation

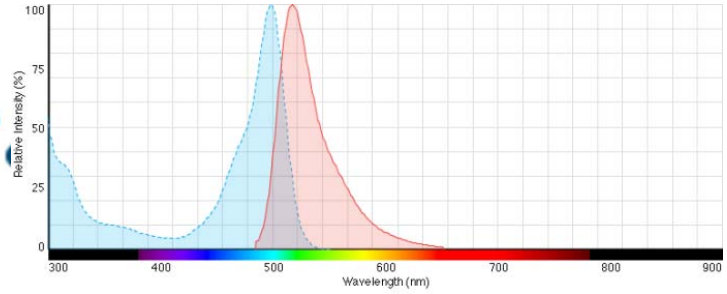




Phenotypic assays: loss of cell membrane integrity



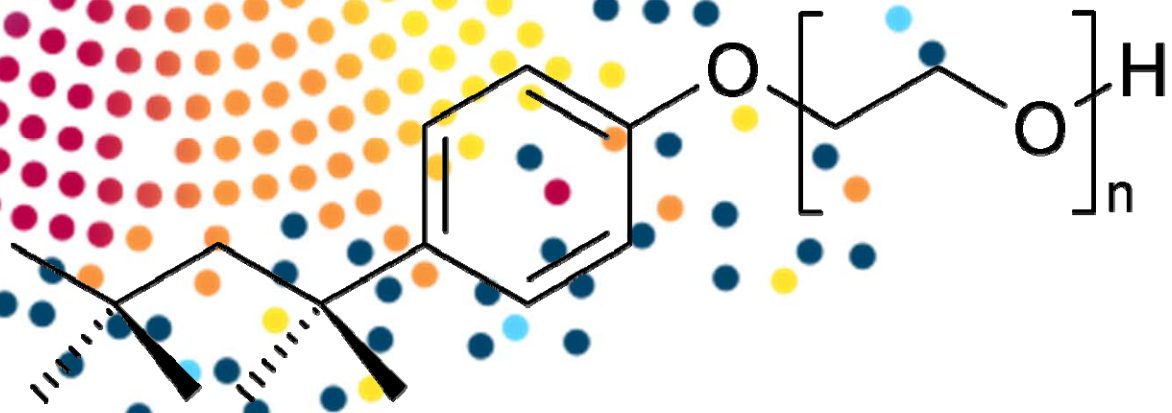




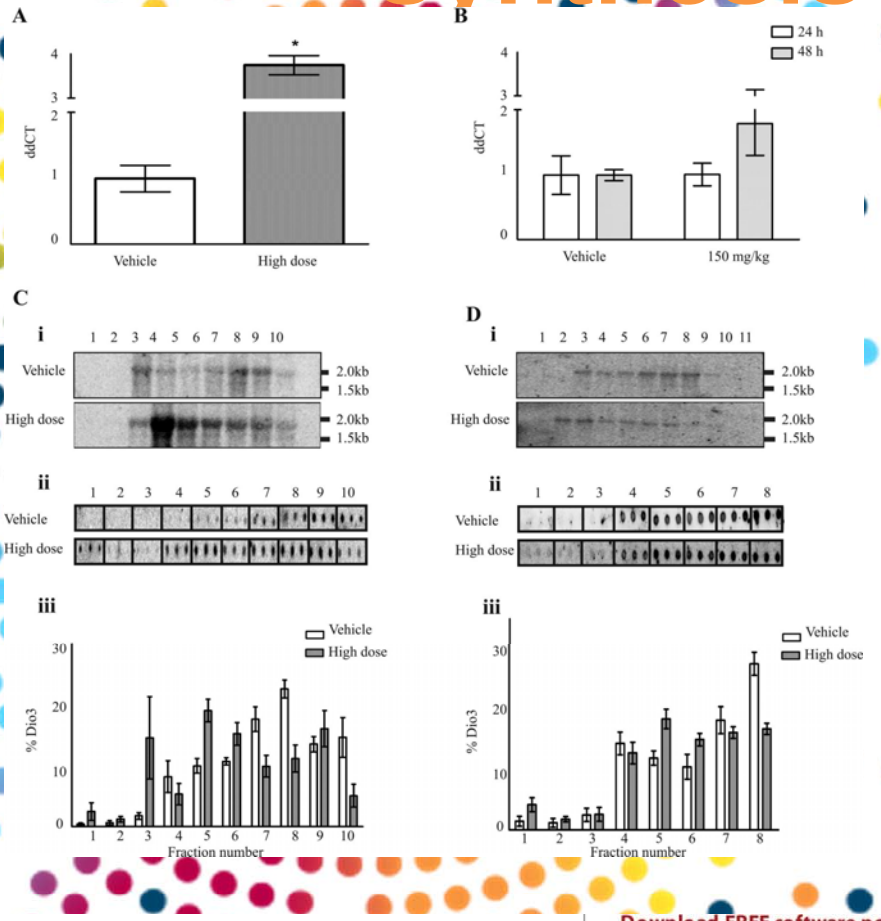
Hypothesis : Oil products many of which have detergent capabilities will be able to disrupt cell membranes and give a measure of general viability.

Phenotypic assays: loss of cell membrane integrity

Cell membrane detergent Triton X100



Phenotypic assays: protein synthesis inhibition



Decreased protein synthesis is not a universal response to toxicity – some proteins are excepted but overall there is generally a decrease that can be a sensitive indicator of toxicity

Biochem. J. (2013) **453**, 71–82 (Printed in Great Britain) doi:10.1042/BJ20130049

online data

Decreased translation of *Dio3* mRNA is associated with drug-induced hepatotoxicity

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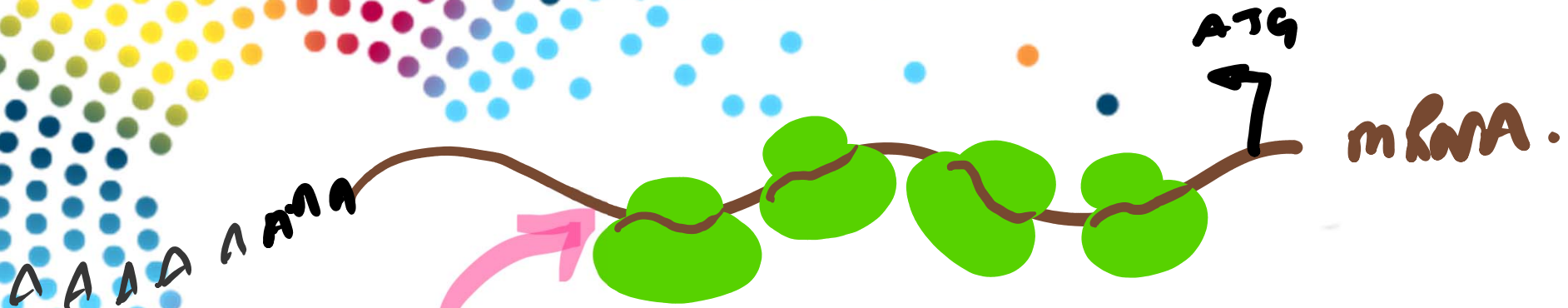
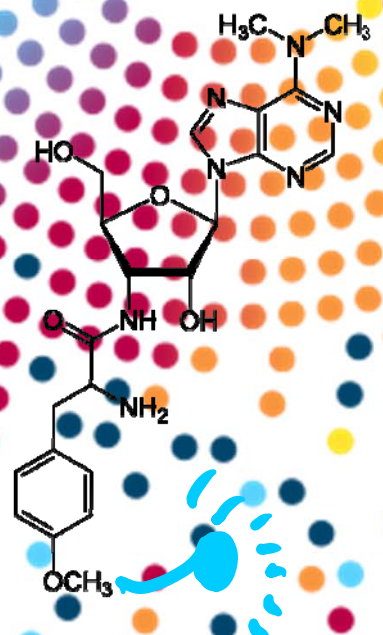
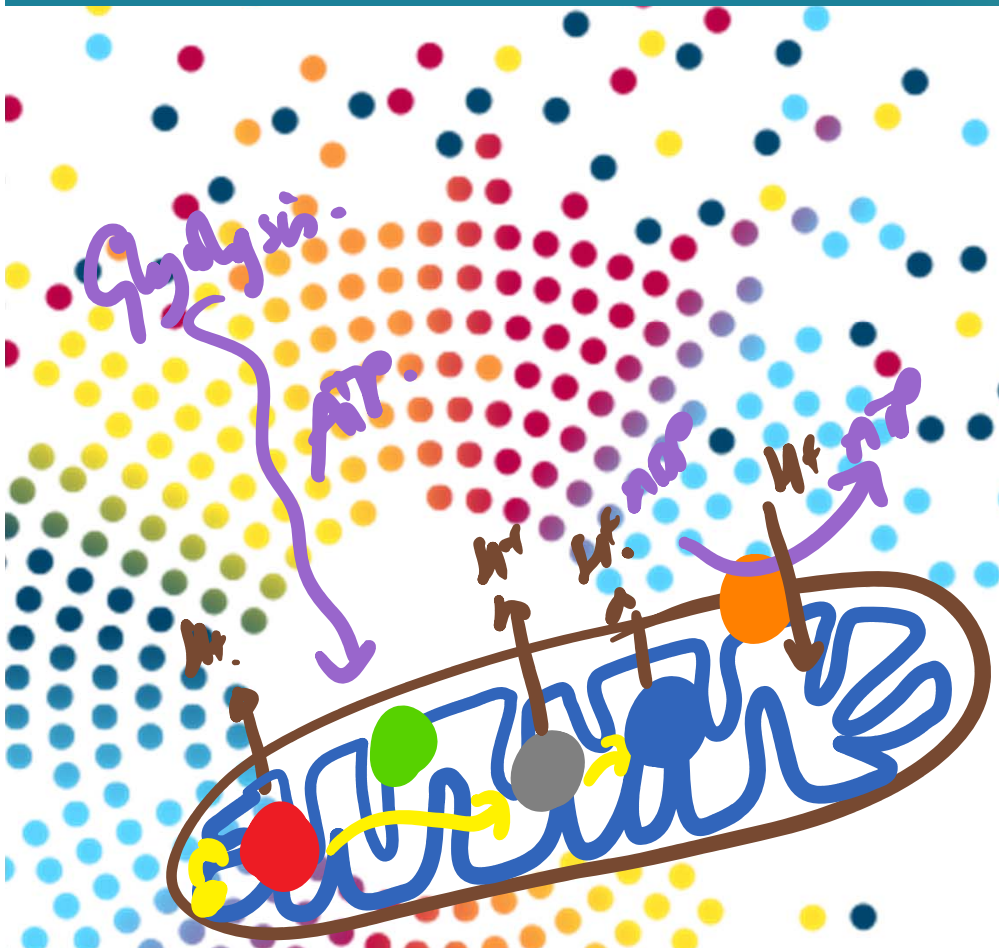
Phenotypic assays: protein synthesis inhibition



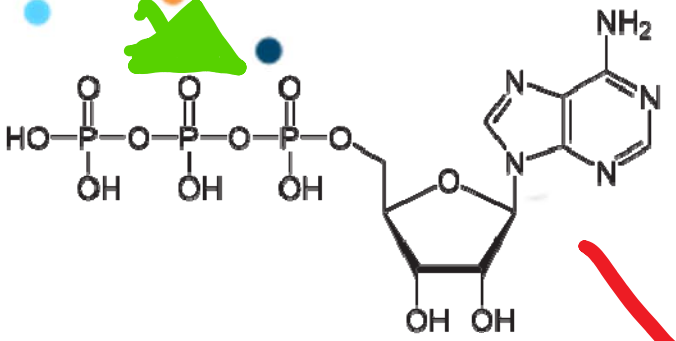


- Fluorescent labelled puromycin incorporated into the growing polypeptide chain.
- This stops the chain elongation thus labelling new chains
- Polypeptide synthesis can then be measured using fluorescent methods.

Phenotypic assays: loss of ATP



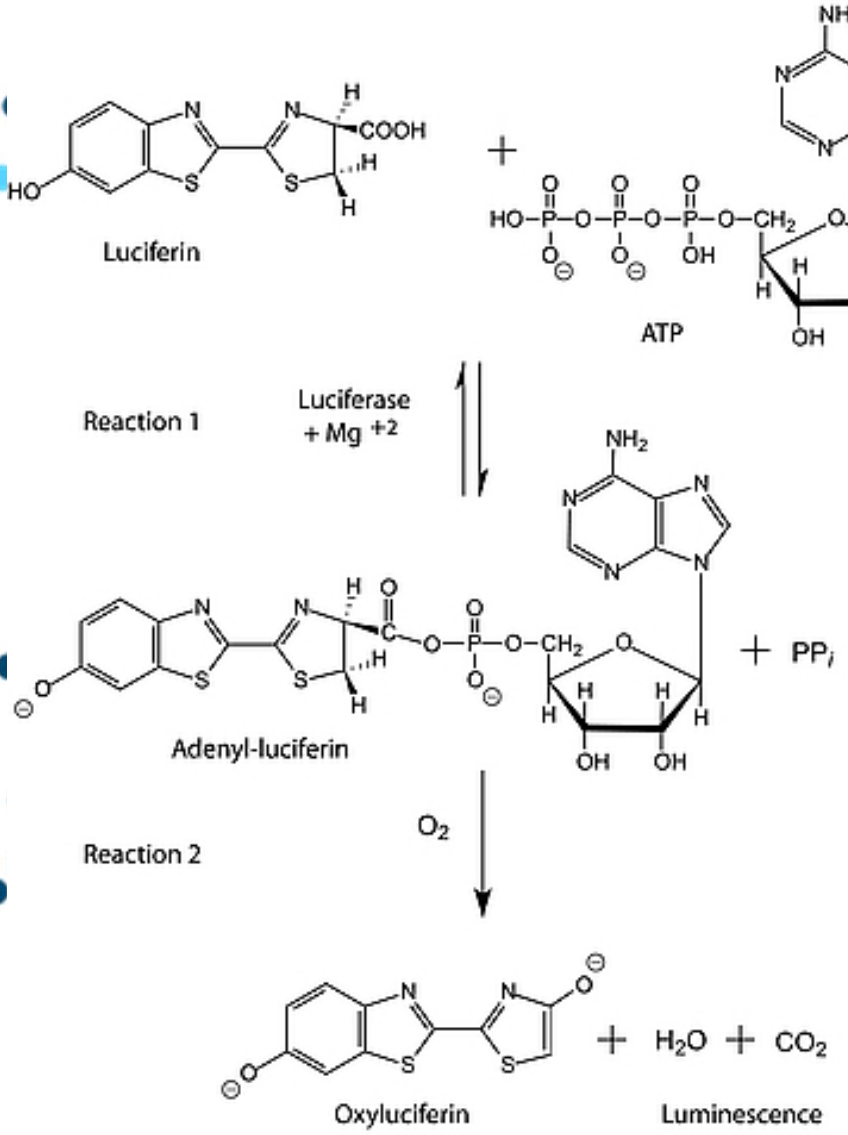
Production



Oxidation

- Any effect on mitochondria or glucose oxidation, or increases in energy use in the cell, will have a profound effect on energy balance in the cell.
- This will be reflected on the ATP level in the cell

Phenotypic assays: loss of ATP



04 Our data and assay designs for the oil derived UVCBs

Will be presented by Abi tomorrow

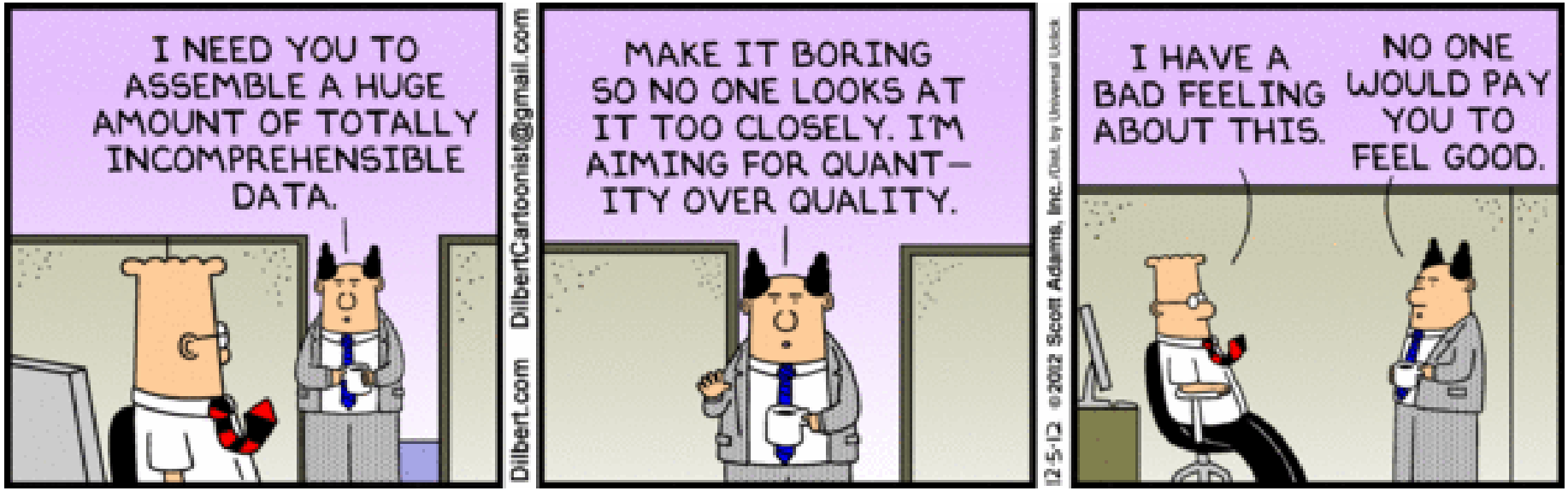
05 Public Engagement

Public Engagement : New Scientist Excel Event

Public Engagement : New Scientist Excel Event

2017

- We are getting more ambitious.
- Stand size – 8 by 3 metres in the centre of the exhibition (2016 was 2 by 3 m). 4x bigger
- Our stand will be opposite the BP stand
- We will have a variety of science on display and will include UVCBs.


I NEED YOU TO ASSEMBLE A HUGE AMOUNT OF TOTALLY INCOMPREHENSIBLE DATA.
MAKE IT BORING SO NO ONE LOOKS AT IT TOO CLOSELY. I'M AIMING FOR QUANT-ITY OVER QUALITY.
I HAVE A BAD FEELING ABOUT THIS.
NO ONE WOULD PAY YOU TO FEEL GOOD.
DilbertCartoonist@gmail.com
Dilbert.com
12-5-12 ©2012 Scott Adams, Inc./Dist. by Universal Uclick

Overview

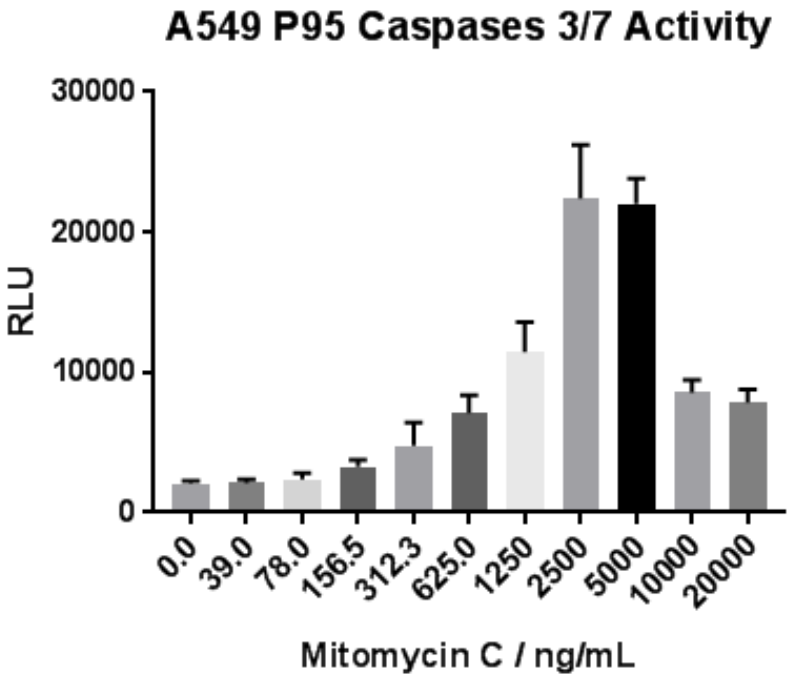
03

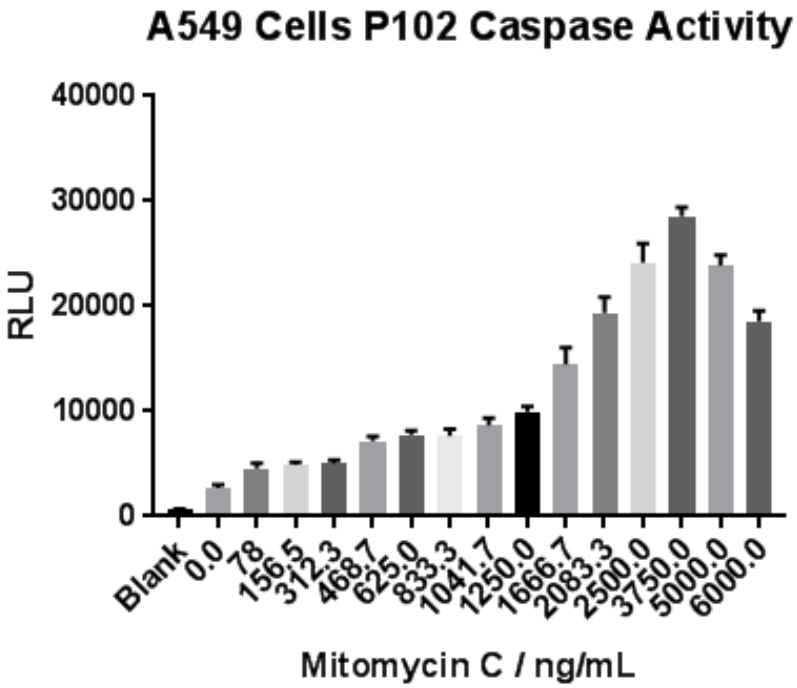
- Cell types, main characteristics
- Assays selected
- Reference compounds
- Dose-response setup data
- Plate design
- Upcoming work

Reference Compounds

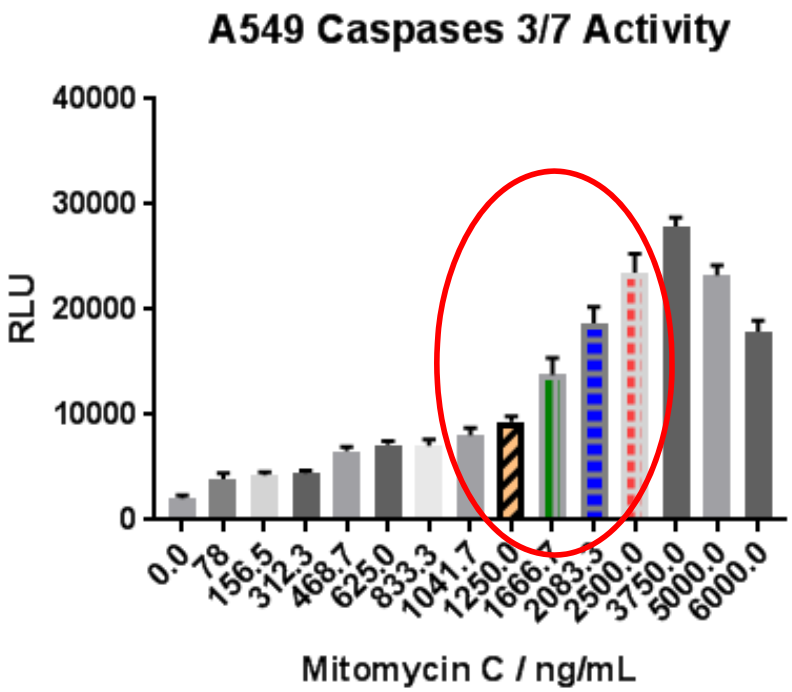
- Mitomycin C
- DMNQ
- Triton-X-100
- Hygromycin B
- Xxxxx???
- Xxxxx???
- Xxxxx???

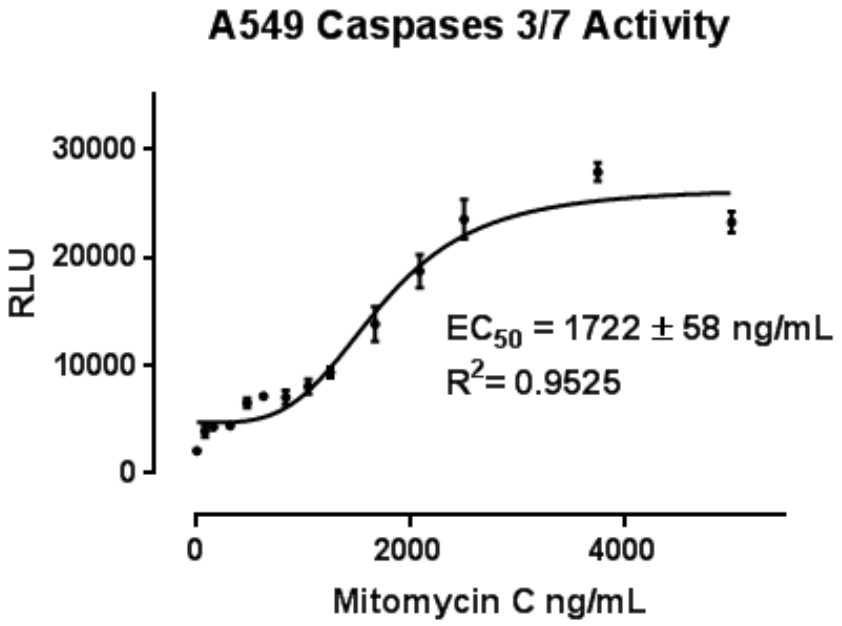
Mitomycin C – Caspase Activity



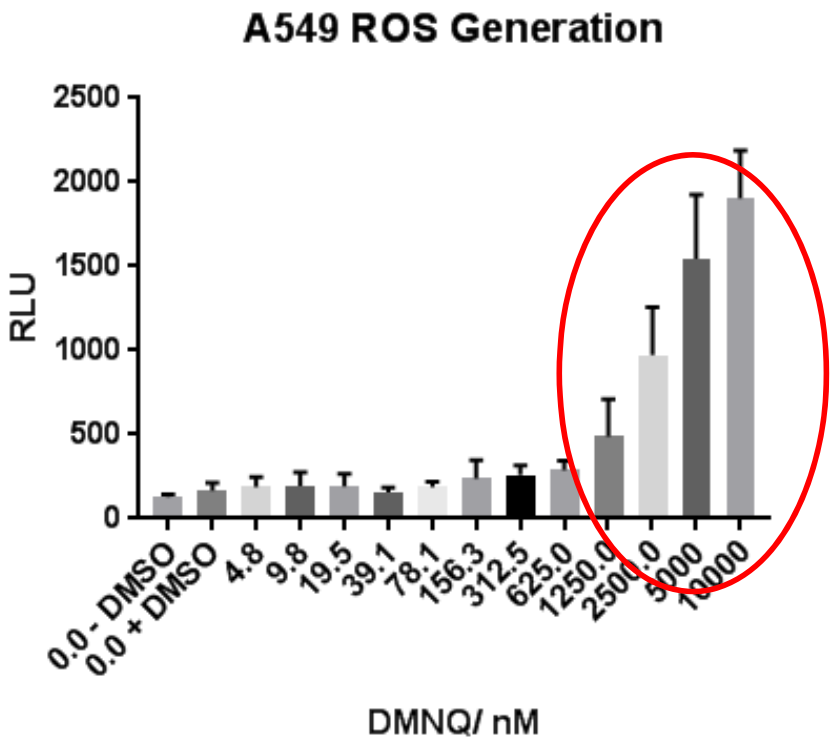


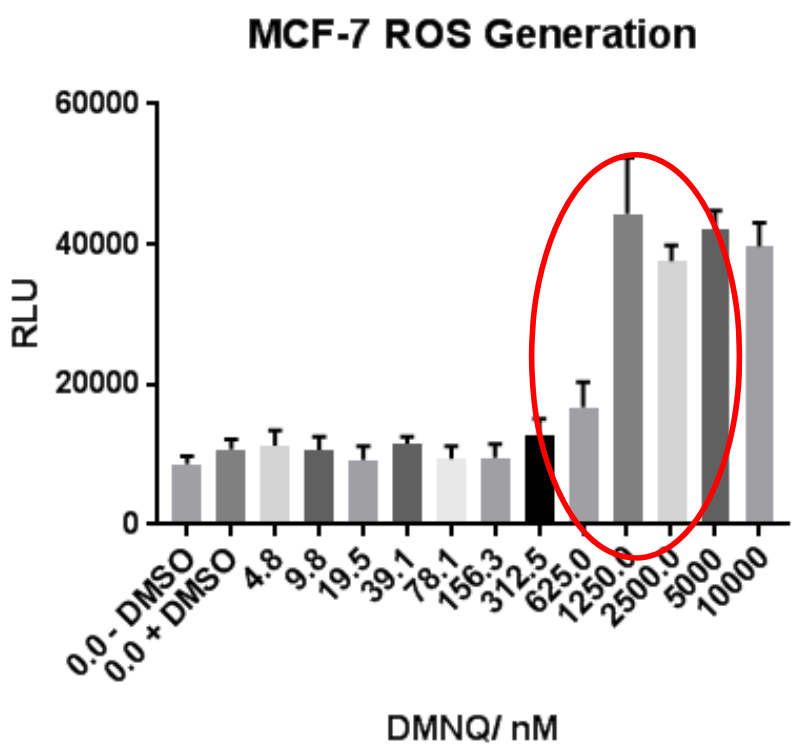
Mitomycin C – Caspase Activity



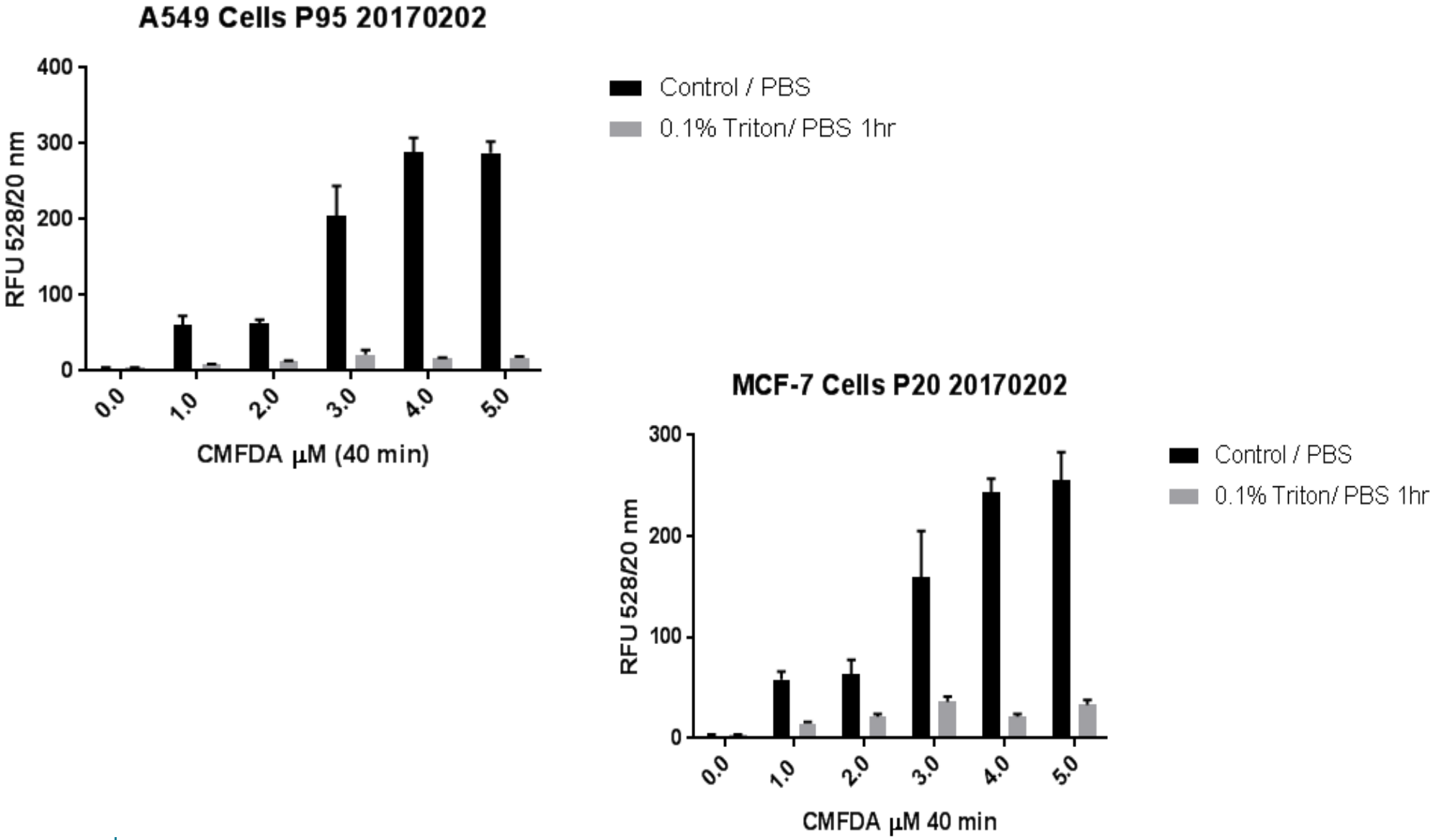


DMNQ – ROS Generation

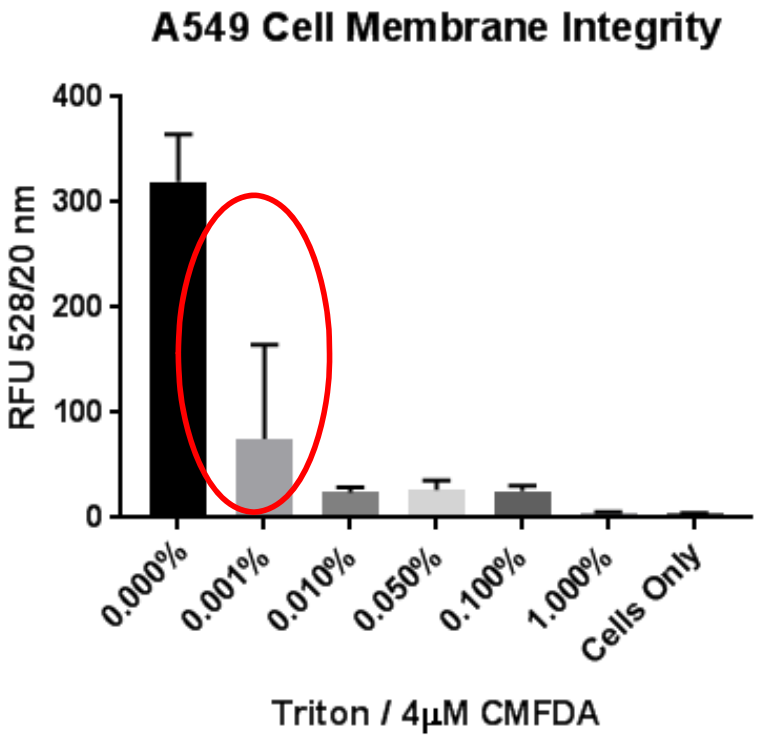


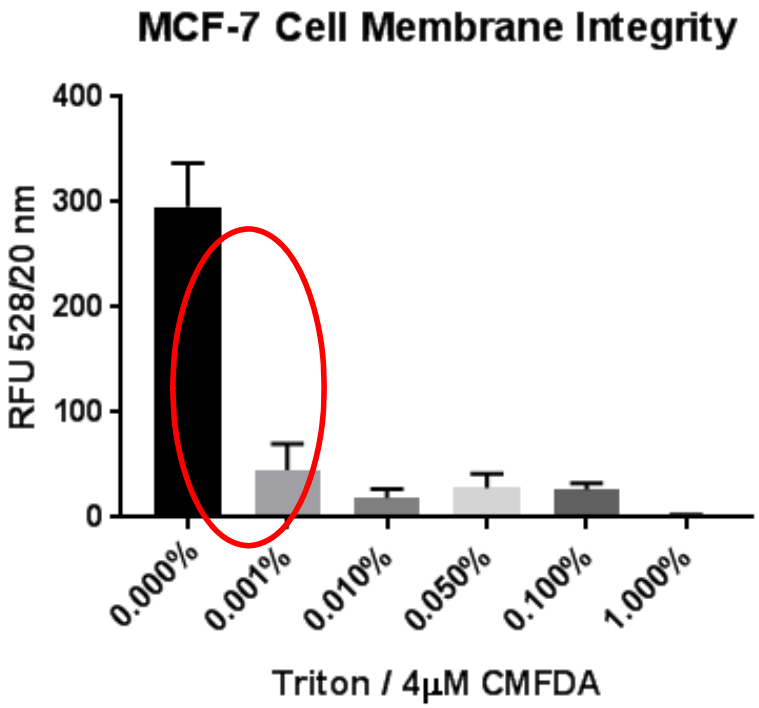


Triton-X-100 – Cell membrane integrity



Triton-X-100 – Cell membrane integrity





Overview

04

- Cell types, main characteristics
- Assays selected
- Reference compounds
- Dose-response setup data
- Plate design
- Upcoming work

Plate Design for Chemical Exposures

Mitomycin C 0; 625; 1250; 2500; 5000 ng/mL

Triton-X-100 0, 0.001, 0.0005, 0.0001, 0.00005, 0.00001 %

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B		1	2	3	4	5	6	7	8	9	10	extraction method blank (DMSO with traces of cyclohexane)		11	12	13	14	15	16	17	18	19	20	
C		21	22	23	24	25	26	27	28	29	30			31	32	33	34	35	36	37	38	39	40	
D		ASSAY SPECIFIC CONTROLS							MED					DMSO (pure)			ASSAY SPECIFIC CONTROLS							
E		R1	R2	R3	R4	R5	R6	R7	R8	R9	R10			41	42	43	44	45	46	47	48	49	50	
F		51	52	53	54	55	56	57	58	59	60			61	62	63	64	65	66	67	68	69	70	
G		ASSAY SPECIFIC CONTROLS							MED					DMSO (pure)			ASSAY SPECIFIC CONTROLS							
H		71	72	73	74	75	76	77	78	79	80			R11	R12	R13	R14	R15	R16	R17	R18	R19	R20	
I		Repl1				Repl2				Repl3				Repl3		Repl4				Repl5				
J		81	82	83	84	85	86	87	88	89	90			91	92	93	94	95	96	97	98	99	100	
K		ASSAY SPECIFIC CONTROLS							MED					DMSO (pure)			ASSAY SPECIFIC CONTROLS							
L		R1	R2	R3	R4	R5	R6	R7	R8	R9	R10			41	42	43	44	45	46	47	48	49	50	
M		101	102	103	104	105	106	107	108	109	110			111	112	113	114	115	116	117	118	119	120	
N		ASSAY SPECIFIC CONTROLS							MED					DMSO (pure)			ASSAY SPECIFIC CONTROLS							
O		121	122	123	124	125	126	127	128	129	130			131	132	133	134	135	136	137	138	139	140	
P																								

Hygromycin B 0; 10; 20; 40; 55; 67.5; 80; 100 µM

DMNQ 0; 625; 1250; 2500; 5000 nM

Overview

05

- Cell types, main characteristics
- Assays selected
- Reference compounds
- Dose-response setup data
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Upcoming Work

1. Complete ROS curves, Hygromycin B preliminary data
2. Chemical exposures of A549 and MCF-7 cells
3. Establish HepaRG Cells & expose to chemical
4. Compare A549 and MCF-7 data with primary lung and breast cells (HLMVEC; HMePC)
5. Alongside – prepare TempO Seq plates and set up sequencing at PHE.
6. Set up ATP activity assay.
7. Further Cell line order of use to be confirmed.

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