cyprotex AN EVOTEC COMPANY

In vitro **Toxicology**

Cell Stress Panel:

An *in vitro* Next Generation Risk Assessment (NGRA) Tool

Background Information

'Next Generation Risk Assessment (NGRA) is defined as an exposure-led, hypothesis driven risk assessment approach that integrates *in silico*, *in chemico* and *in vitro* approaches,'

[1] Dent M *et al.*, (2018) *Comput Toxicol* **7**; 20-26

- Current *in vitro* preclinincal strategies require ethical and regulatory consideration of animal use (3R's) and require human relevant data. This expedites new approach methodologies (NAMs) aimed to accurately predict *in vivo* toxicities at therapeutically relevant *in vivo* concentrations[1, 2].
- Despite *in vitro* two-dimensional cellular assays lacking the complexity of the *in vivo* microenvironment, they provide a fast economical readout for high throughput analysis[3,4].
- 36 biomarkers are measured (figure 1) that represent 12 key cellular stress mechanisms including mitochondrial toxicity, gene regulation and inflammation. In addition, general cell health are monitored utilising a variety of *in vitro* assays including the Seahorse platform, Promega kits and ELISA's, however, predominantly high content imaging (HCI) is used.
- It has been shown that the cellular stress panel can be used, together with other new approach methodologies, to identify chemical exposures that are protective of consumer health[5].

Protocol

Cell Line
HepG2 (human hepatoblastoma) cell line*

Analysis Platform
Confocal Cellomics ArrayScan® (Thermo Scientific), Seahorse XF^{e}96 extracellular flux analyser and Intellicyt iQue® Screener PLUS.

Test Compound Concentrations
8 point dose response curve with top concentration based on 100x Cmax or solubility limit. 3 replicates per concentration.*

Compound Requirements
200 µL of a solution to achieve 100x Cmax (200 x top concentration to maintain 0.5% DMSO) or equivalent amount in solid compound.

Time Points
24 hour*

Quality Controls
Negative control: 0.5% DMSO (vehicle)
Positive controls: 2 compounds per mechanism

Data Delivery
Dose response curve, MEC and AC_{50} value for each measured parameter.
Cell count, nuclear size, DNA structure, ER stress panel 1 (ER integrity, BiP, XBP1), ER stress panel 2 (ATF4, PERK, CHOP), AhR translocation, DNA damage (pH2AX, p53), inflammation & pH (ICAM-1, intracellular pH, HIF1α, IL-1β, IL-6, IL-8, IFN-γ), mitochondrial oxidative stress (mitochondrial ROS, PGC1α, TNFAIP3), oxidative stress (NRF2, HMOX1, SRXN1), osmotic & heat shock (NFAT, hsp70), metal stress (metallothionein, MTF1), apoptosis & necrosis (caspase 3/7, NFkB, cell membrane permeability), phospholipidosis & steatosis, glutathione (GSH) content, reactive oxygen species (ROS), mitochondrial membrane potential, mitochondrial mass, cellular ATP & LDH release, Seahorse assay (oxygen consumption rate (OCR), extracellular acidification rate (ECAR), reserve capacity)*

*Other options available on request

To find out more contact **enquiries@cyprotex.com**

Figure 1

Cell stress panel overview: summarising the 12 cell stress mechanisms and their associated molecular markers detected within the cell stress panel assay.

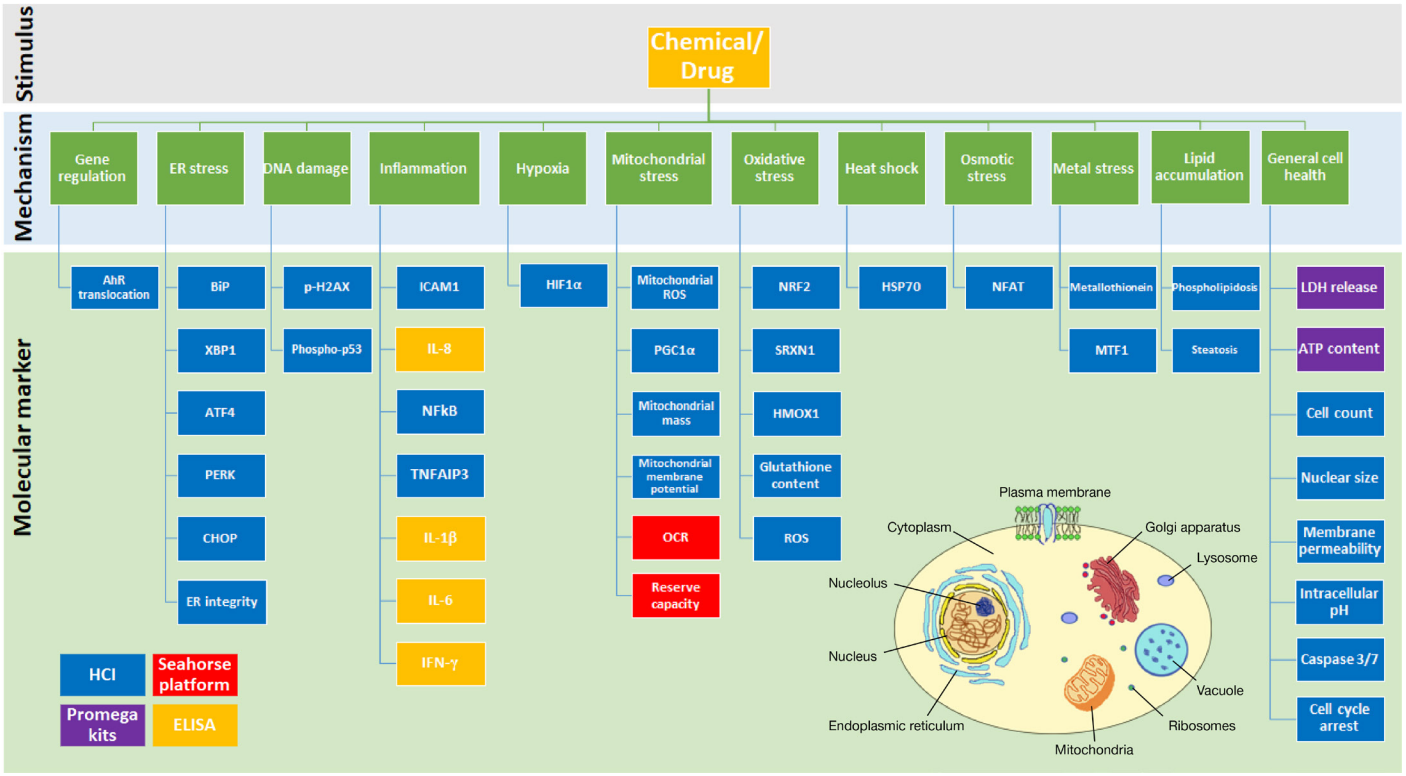


Figure 2

Representative HCS images of the endoplasmic reticulum (ER) stress panel assay showing HepG2 cells labelled with Hoechst (blue, nuclei), ER tracker (green, an ER integrity dye), BiP (orange, an ER chaperone with high affinity for misfolded proteins) and CHOP (magenta, a transcription factor activated via PERK) following 24 hour exposure to 10 µM of known ER toxin, tunicamycin.

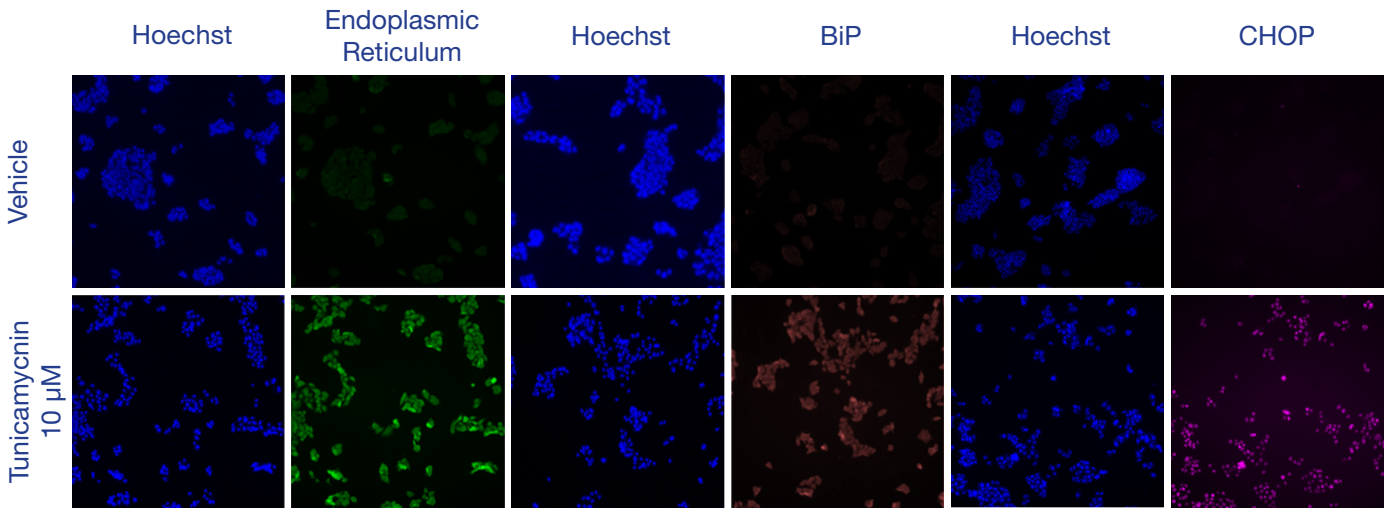
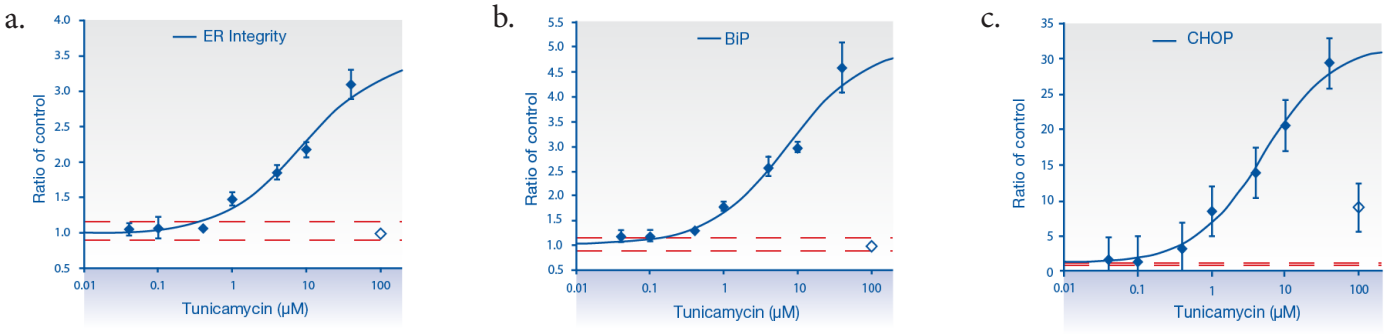


Figure 3

Representative dose response curves of the endoplasmic reticulum (ER) stress panel assay showing (a) ER integrity, (b) BiP and (c) CHOP in HepG2 cells following 24 hour exposure to known ER toxin, tunicamycin.



An increase in endoplasmic reticulum (ER) integrity staining (figure 2 & 3a) suggests increased ER dyshomeostasis with a higher abundance of unfolded and/or misfolded proteins requiring BiP (figure 3b) binding and thus increased CHOP activation (figure 3c) following PERK receptor targeting by BiP bound proteins.

Figure 4

Representative HCS images of the mitochondrial oxidative stress panel assay showing HepG2 cells labelled with Hoechst (blue, nuclei), MitoSox (orange, mitochondrial ROS), PGC1α (red, transcription factor co-activator in mitochondrial biogenesis and metabolic homeostasis), and TNFAIP3 (green, cytoplasmic protein regulator involved in inflammation and immunity) following 24 hour exposure to 0.1 and 40 µM of a known mitochondrial toxin, rotenone.

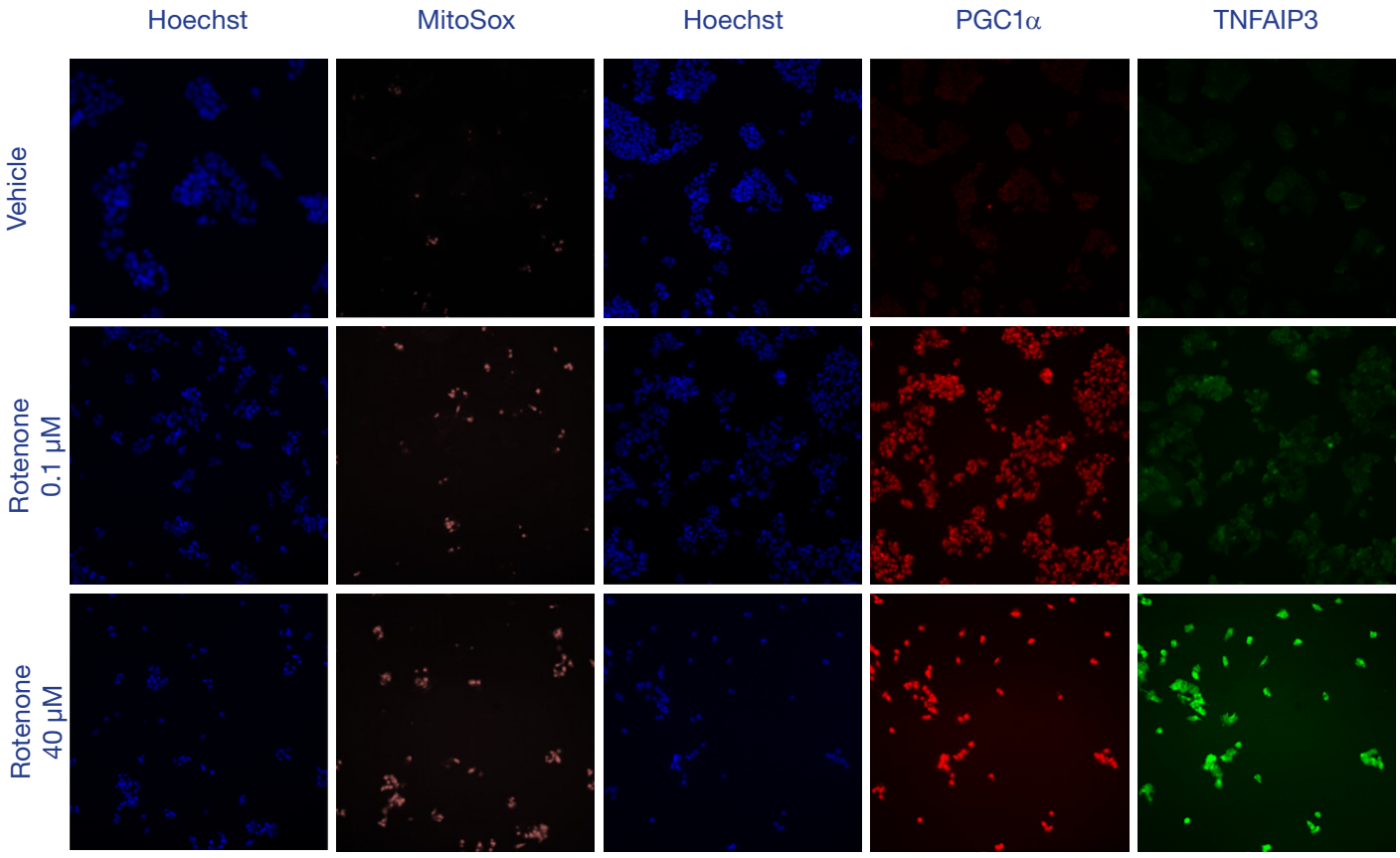
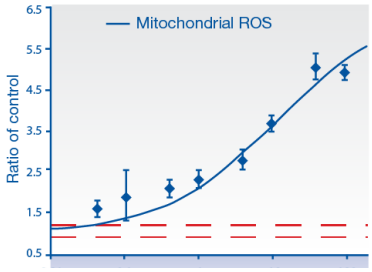


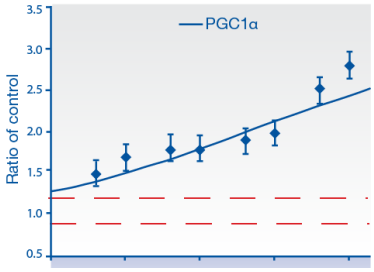
Figure 5

Representative dose response curves of the mitochondrial oxidative stress panel showing (a) mitochondrial ROS formation, (b) PGC1α activation and expression and (c) TNFAIP3 expression in HepG2 cells following 24 hour exposure to known mitochondrial toxin, rotenone

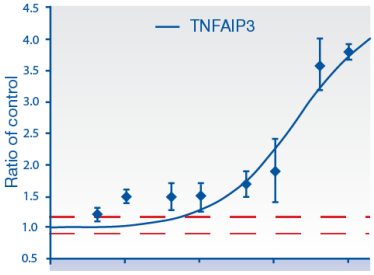
a.



b.



c.

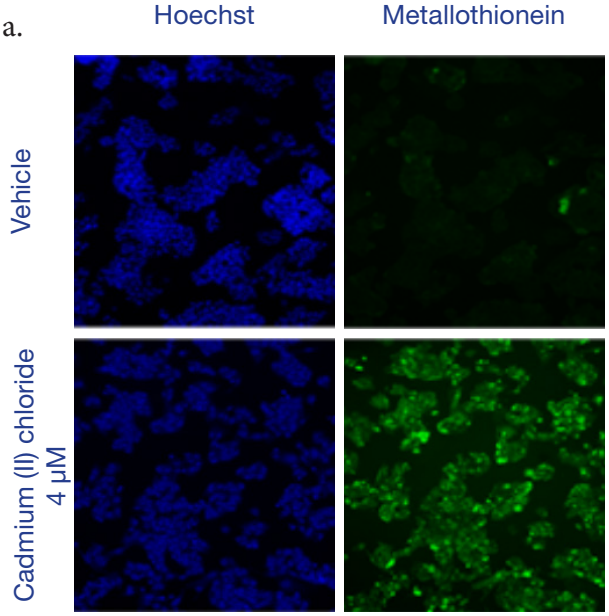


An increase in mitochondrial ROS (reactive oxygen species) (figure 5a) activates PGC1α (figure 5b) which signals the increased transcription of TNFAIP3 (figure 5c) and the induction of an inflammatory response while also activating a redox pathway.

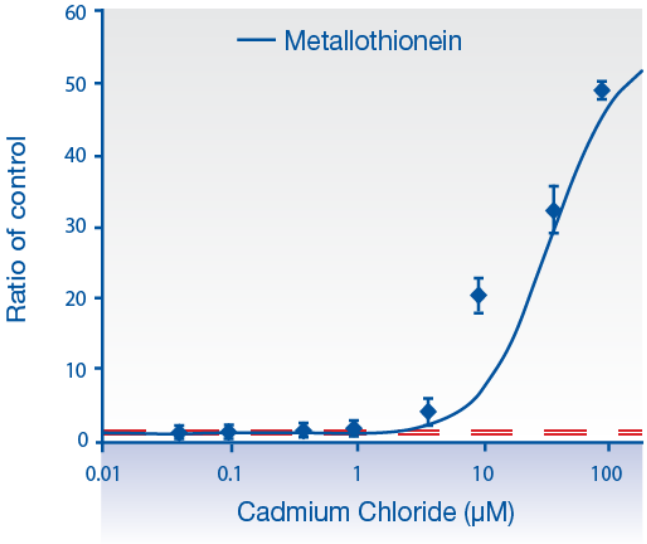
Figure 6

(a) Representative HCS imaging of the metal stress assay showing HepG2 cells labelled with Hoechst (blue, nuclei) and metallothionein (green, a protein induced by transcription factor MTF-1) following exposure to 4 µM of known metal toxicant, cadmium chloride. (b) Representative dose response curve showing the cadmium chloride induced metallothionein response in HepG2 cells following 24 hour exposure.

a.



b.



Metallothionein protein is regulated by MTF1 in order to bind and sequester toxic heavy metals, therefore an increase in this molecular marker indicates metal stress (figure 6a & 6b). Hatherell *et al* (2020) describe the initial validation of the cell stress panel whereby this predominantly high content imaging (HCI) strategy has the potential to improve our understanding of chemical exposure outcomes using point of departure (PoD) in relation to C_{max} with a set of 13 benchmark substances[5]. In combination with other cellular assays and in silico approaches this panel could provide a powerful NGRA tool to use in non-animal safety decision making.

References

[1] Dent M *et al*. (2018) Principles underpinning the use of new methodologies in the risk assessment of cosmetic ingredients. *Comput Toxicol* **7**: 20-26

[2] Middleton A *et al.* (2017) Case studies in cellular stress: defining adversity/adaptation tipping points. *Appl In Vitro Toxicol* **3(2)**: 199-210

[3] Campbell JL *et al*. (2012) Physiologically based pharmacokinetic/toxicokinetic modeling. *Methods Mol Biol* **929**: 439-499

[4] Moxon TE *et al.* (2020) Application of physiologically based kinetic (PBK) modelling in the next generation risk assessment of dermally applied consumer products. *Toxicol In Vitro* **63**: 104746

[5] Hatherell S *et al*. (2020) Identifying and characterizing stress pathways of concern for consumer safety in next generation risk assessment. *Toxicol Sci* 10.1093/toxsci/kfaa054