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Cancels & replaces the same document of 24 September 2020

Annex II: In vivo data and in vitro experiments WITHIN CASE STUDY ON THE USE OF INTEGRATED APPROACHES TO TESTING AND ASSESSMENT FOR PREDICTION OF A 90 DAY REPEATED DOSE TOXICITY STUDY (OECD 408) FOR 2-ETHYLBUTYRIC ACID USING A READ-ACROSS APPROACH FROM OTHER BRANCHED CARBOXYLIC ACIDS

Series on Testing and Assessment
No. 324

The corresponding monograph to this annex is available under the following cotes: ENV/JM/MONO(2020)20.

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1. *In vivo* repeated dose toxicity data

Three source compounds have repeated dose toxicity studies with oral and interperitoneal exposure. The data were gathered from literature and available databases like Fraunhofer RepDose database (www.fraunhofer-repdose.de). A summary compound is given in the following.

2-Ethylhexanoic acid (2-EHA):

1) Reference: Juberg, D. R. et al. (1998) Food and Chemical Toxicology, Vol 36, 429-436.

Study design: Fischer rat (F334), dietary exposure, 90 days + 28 days recovery period; purity of test compound = 99.9%; conducted according to TSCA Health Effects Testing Guidelines for Subchronic Oral Toxicity Studies (TSCA, 1992).

Tested doses: m: 61/303/917 mg/kg bw/d; f: 71/360/1068 mg/kg bw/d

Dose (mmol/kg bw/d)	Effect	Comment
0.5 mmol/kg bw/d	cholesterol increased	reversible in recovery period
	urine volume decreased	incidental observation
2 mmol/kg bw/d	Liver hypertrophy	
	Rel. weight changed in liver/kidney and testes	
6 mmol/kg bw/d	Body weight decr./rel brain wt decreased Food consumption decr.	some incidental changes in haematology

2) Reference: Juberg, D. R. et al. (1998) Food and Chemical Toxicology, Vol 36, 429-436.

Study design: B6C3F1 mouse, dietary exposure, 90 days + 28 days recovery period; purity of test compound =99.1%; conducted according to TSCA Health Effects Testing Guidelines for Subchronic Oral Toxicity Studies (TSCA, 1992).

Tested doses: m: 180/885/2728 mg/kg bw/d; f: 205/1038/3139 mg/kg bw/d

Dose (mmol/kg bw/d)	Effect	Comment
6 mmol/kg bw/d	cholesterol increased	Reversible in recovery period
	Liver hypertrophy; relative weight increased	
7 mmol/kg bw/d	Absolute weight adrenals decr. relative weight kidney increased; body weight decreased Bilirubin and triglycerides decreased in plasma	adrenals not considered to be biological relevant/kidney weight changes persistent after recovery
19 mmol/kg bw/d	Albumin and ALAT increased	Indicator of potential liver toxicity
	kidney	Basophilia
	liver	Eosinopenia

Valproic Acid (VPA):

Pharmacological Mode of Action

VPA is used as antiepileptic drug. VPA dissociates to the valproate ion in the gastrointestinal tract and then binds to and inhibits GABA transaminases. The drug's anticonvulsant activity may be related to increased brain concentrations of gamma-

aminobutyric acid (GABA), an inhibitory neurotransmitter in the CNS, by inhibiting enzymes that catabolise GABA or block the reuptake of GABA into glia and nerve endings. Valproic acid may also work by suppressing repetitive neuronal firing through inhibition of voltage-sensitive sodium channels. It is also a histone deacetylase inhibitor. Valproic acid has also been shown to be an inhibitor of an enzyme called histone deacetylase 1 (HDAC1). HDAC1 is needed for HIV to remain in infected cells. Patients treated with valproic acid in addition to highly active antiretroviral therapy (HAART) showed a 75% reduction in latent HIV infection.

Toxicological data

VPA as well as some of its metabolites induce microvesicular liver steatosis *in vivo*.

Adverse reactions have been reported in clinical epilepsy trials. Minor elevations of transaminases (e.g. SGOt and SGPT) and LDH are frequent and appear to be dose related. Occasionally also an increase of bilirubin in serum and abnormal changes in other liver functions are reported. These results may reflect potentially serious hepatotoxicity (FDA approval package, NDA 22-152, 2008).

It has been demonstrated that VPA and its metabolite 4-en valproate (4-en VPA) form complexes with CoA and arrest the electron transport chain (ETC) and ATP synthesis. This impairment of mitochondrial function leads to microvesicular steatosis and cirrhosis (Patel and Sanyal 2013, Kassahun and Abbott 1993). For VPA an impairment of mitochondrial β-oxidation of long-, medium-, and short-chain natural fatty acids by depletion of the intramitochondrial pool of CoA (Fromenty and Pessayre 1995, 1997) is discussed. Also oxidative stress is discussed to be one mechanism by which VPA induce hepatotoxicity. These effects have been recently reviewed by Chang and Abbott (Chang and Abbott 2006). The authors conclude that overall based on the evidence from *in vitro* and *in vivo* studies, it appears that VPA treatment is associated with the development of oxidative stress in rats and in humans.

Several *in vivo* studies were found in literature, some of them having a special focus on liver toxicity (Table 1, study quality D). One study donated by the etox consortium has a full scope of examination (GLP study, 10 day exposure). Table 2 summarises the main findings per study, dose level and gender.

Table 1. Overview on available animal studies testing VPA, study quality D indicates a special focus on liver toxicity in the study, A indicates a GLP guideline study.

Name	Duration (days)	Route	Study_id	Animal age	Quality	species	dosing_mgkg	Author	Journal	year
Valproic acid CAS 99-66-1	4	ip	5577		D	rat	0;500	Tong, V. *et al*	Toxicological Sciences	2005
	4	ip	5578	10d	D	rat	0;160;320;500;650	Espandiari, P. *et al*	Journal of Applied Toxicology	2008
	4	ip	5579	25d	D	rat	0;160;320;500;650	Espandiari, P. *et al*	Journal of Applied Toxicology	2008
	4	ip	5580	40d	D	rat	0;160;320;500;650	Espandiari, P. *et al*	Journal of Applied Toxicology	2008
	4	ip	5581	80d	D	rat	0;160;320;500;650	Espandiari, P. *et al*	Journal of Applied Toxicology	2008
	7	ip	5576		D	rat	0;500	Tong, V. *et al*	Toxicological Sciences	2005
	7	ip	5597	42-44d	D	rat	0;500	Sugimoto, T. *et al*	Epilepsia	1987
	7	ip	5605		D	rat	0;750	Löscher, W.	Epilepsy Research	1992
	10	gavage	etox	9-12w	A	rat	0; 100 to 1000	Novartis, GLP study	Contribution from etox database	
	10	ip	5575		D	rat	0;500	Tong, V. *et al*	Toxicological Sciences	2005
	14	gavage	8564		D	rat	0;500	Abdel-Dayem, M. A.	Drugs R D	2014
	14	oral	5583		D	mouse	0;100	Knapp, A. C.	J Pharm Exp Ther	2008
	14	ip	5574		D	rat	0;500	Tong, V. *et al*	Toxicological Sciences	2005
	15	ip	5594		D	mouse	0;25	Ibrahim, M.A.	Life Science Journal	2012
	30	ip	5595		D	mouse	0;25	Ibrahim, M.A.	Life Science Journal	2012
	30	ip	5596		D	mouse	0;25	Ibrahim, M.A.	Life Science Journal	2012

Table 2. VPA- observed effects in animal studies with repeated oral or interperitoneal exposure (study IDs refer to Table 1);

effect LOELs above study LOELs are indicated in blue.

Study_ID	affected organ	observed effect	effect_additional	affected gender	Effect_LOEL	Study LOEL	Study NOEL
5574	clinical chemistry	changed enzyme activity		male	500	500	
5574	clinical symptoms	mortality	increased; 3/16	male	500	500	
5574	liver	increased LPO and TBARs		male	500	500	
5574	liver	changes in cellular structures	scarring of capsule surface	male	500	500	
5574	liver	inflammation	mild; surface	male	500	500	
5574	liver	necrosis	massive	male	500	500	
5575	clinical chemistry	changed enzyme activity	sign. increased	male	500	500	
5575	liver	changes in cellular structures	scarring of capsule surface	male	500	500	
5575	liver	inflammation	mild; surface	male	500	500	
5575	liver	other	sign. increased LPO	male	500	500	
5575	liver	steatosis	incidence 80%; zonal distribution	male	500	500	
5576	clinical chemistry	changed enzyme activity	sign. increased	male	500	500	
5576	liver	increased LPO		male	500	500	
5576	liver	changes in cellular structures	scarring of capsule surface	male	500	500	
5576	liver	inflammation	mild; surface	male	500	500	
5576	liver	necrosis	mild	male	500	500	
5576	liver	steatosis	incidence 50%; zonal distribution	male	500	500	
5577	clinical chemistry	changed enzyme activity	sign. increased	male	500	500	
5577	liver	changes in cellular structures	scarring of capsule surface	male	500	500	
5577	liver	inflammation	mild; surface	male	500	500	
5577	liver	necrosis	mild	male	500	500	
5577	liver	steatosis	incidence 25%; zonal distribution	male	500	500	
5578	clinical chemistry	changed enzyme activity	decreased ALAT only 160 mg/kg bw/d; AP dose-dependent	male & female	160	160	
5578	haematology	leukocytes	decreased WBC	male & female	160	160	
5578	haematology	platelets	decreased platelet count and	male & female	160	160	
5578	spleen	weight decreased	rel. wt.	male & female	160	160	
5578	body weight	weight decreased		male & female	320	160	
5578	clinical chemistry	urea/nitrogen	sign. increased; blood; BUN	male & female	320	160	

5578	liver	inflammation	mild to moderate (10/13)	male & female	320	160	
5578	liver	oedema	mild (3/13)	male & female	320	160	
5578	liver	haemorrhage	mild (3/13)	male & female	320	160	
5578	liver	vacuolisation	mild (3/13); cytoplasmic	male & female	320	160	
5578	spleen	atrophy	moderate; in T-cell area	male & female	320	160	
5578	clinical symptoms	mortality	increased; 90%	male & female	500	160	
5579	haematology	platelets	decreased platelet count; increased RBC (650 mg/kg bw/d)	male & female	160	160	
5579	spleen	atrophy	moderate; in T-cell area	male & female	320	160	
5579	body weight	weight decreased		male & female	500	160	
5579	spleen	weight decreased	rel. wt.	male & female	500	160	
5579	caecum	swelling	and packed with food	male & female	650	160	
5579	clinical symptoms	mortality	increased; 40%	male & female	650	160	
5579	haematology	leukocytes	decreased WBC	male & female	650	160	
5579	haematology	RBC parameters changed	decreased	male & female	650	160	
5579	liver	necrosis	small foci of central lobular; (1/5)	male & female	650	160	
5579	liver	vacuolisation	(5/5)	male & female	650	160	
5579	liver	haemorrhage	mild	male & female	650	160	
5579	liver	oedema		male & female	650	160	
5579	stomach	swelling	and packed with food	male & female	650	160	
5579	other					160	
5580	body weight	weight decreased		male & female	160	160	
5580	spleen	weight decreased	rel. wt.	male & female	500	160	
5580	spleen	atrophy	severe; in T-cell area	male & female	500	160	
5580	haematology	RBC parameters changed		male & female	650	160	
5580	haematology	leukocytes	decreased WBC	male & female	650	160	
5580	liver	necrosis	central lobular (1/5)	male & female	650	160	
5580	liver	vacuolisation	(5/5)	male & female	650	160	
5580	liver	inflammation	mild	male & female	650	160	
5580	clinical chemistry					160	
5581	spleen	weight decreased	rel. wt.	male & female	320	320	160
5581	body weight	weight decreased		male & female	500	320	160
5581	clinical chemistry	changed enzyme activity	decreased ALAT and AP	male	500	320	160
5581	haematology	leukocytes	decreased WBC	male & female	500	320	160

5581	haematology	platelets	decreased; not dose-dependent; only at 500 mg/kg bw/d	male & female	500	320	160
5581	spleen	atrophy	severe; in T-cell area	male & female	500	320	160
5581	clinical chemistry	total protein	increased	male	650	320	160
5581	liver	necrosis	(5/5); more severe in central lobule than in periportal or midzonal	male & female	650	320	160
5581	liver	haemorrhage	(2/5); with destruction of endothelial and sinusoidal cells	male & female	650	320	160
5583	liver	weight increased	rel. wt.; jvs+/- strain only	no data	100	100	
5583	liver	changed enzyme activity	increase; jvs+/- strain only	no data	100	100	
5583	liver	other	oxidation rates decreasd: L-glutamate, succinate (jvs+/- only), Palmitoyl-CoA, beta oxidation of C14 palmitic palmitate	no data	100	100	
5583	clinical chemistry	palmitate oxidation	increased; jvs+/- strain only	no data		100	
5583	clinical chemistry	changes in carnitine content	changes in plasma carnitine content (free, SCA, TAS, LCA, SCA/TAS, total), jva+/-: SCA not significant	no data		100	
5583	liver	steatosis	fat accumulation; slight increase in wt, more increase in jvs+/- strain	no data		100	
5583	liver	changes in carnitine content	free, SCA, TAS, LCA, SCA/TAS, total; wt only SCA, LCA and SCA/TAS significant	no data		100	
5583	other	changes in skeletal muscle carnitine content	changes in skeletal muscle carnitine content; wt: LCA; jvs+/-: free, TAS, total	no data		100	
5583	urine analysis	changes in carnitine excretion	changes in carnitine excretion (free, total, SCA); renal clearance, free carnitine: jvs+/- only	no data		100	
5594	clinical chemistry	total protein	mild to moderate	female	25	25	
5594	liver	changes in cellular structures	partial distortion	female	25	25	
5594	liver	necrosis		female	25	25	
5594	liver	vacuolisation	vacuolar; focally	female	25	25	
5594	liver	inflammation	focal aggregates; portal areas and between hepatocytes	female	25	25	
5594	liver	congestion	portal vein	female	25	25	
5594	liver	enlargement	portal vein	female	25	25	
5595	clinical chemistry	total protein		female	25	25	
5595	liver	changes in cellular structures	markedly distorted; variation in nuclei (size/shape)	female	25	25	
5595	liver	hypertrophy	nuclei; fragmented chromatin	female	25	25	
5595	liver	necrosis	multifocal areas	female	25	25	
5595	liver	vacuolisation		female	25	25	
5596	clinical chemistry	total protein	reversible	female	25	25	
5596	liver	vacuolisation		female	25	25	
5596	liver	necrosis	mild; focal	female	25	25	

5596	liver	congestion	dilated central vein	female	25	25
5597	clinical chemistry	glucose		male	500	500
5597	clinical chemistry	other	beta OH butyrate, carnitine in serum and free fatty acids	male	500	500
5597	clinical chemistry	other	lactate, acylcarinitine, ammonium (herpannimia) increased	male	500	500
5597	liver	steatosis	microvesicular	male	500	500
5605	body weight	weight decreased	from day 2 on	male	750	750
5605	clinical chemistry	other	ammonia increased	male	750	750
5605	clinical chemistry	changed enzyme activity	ALAT decreased	male	750	750
5605	clinical symptoms	poor general conditions	sedation; flat body posture, shortly after exposure	male	750	750
5605	FOB	muscle tone	decreased,shortly after exposure	male	750	750
5605	liver	steatosis	(1/10), microvesicular. intracytoplasmic lipid droplets, not claerly sign as also higher level observed in control group	male	750	750
5605	clinical symptoms	ataxia	marked (score 3-4/6), shortly after exposure	male		750
8564	clinical chemistry	changed enzyme activity	other: 4.2-fold increase in myeloperoxidase (MPO) activity in liver homogenates	male	500	500

8564	clinical chemistry	albumin	-40%	male	500	500
8564	clinical chemistry	other	5-fold increase of Tumor necrosis factor-α (TNF-α) levels in liver and 6.1-fold in serum	male	500	500
8564	clinical chemistry	other	3-fold rise in malondialdehyde (MDA) levels	male	500	500
8564	clinical chemistry	glutathione	35% reduction of Glutathione	male	500	500
8564	liver	enlargement		male	500	500
8564	liver	weight increased		male	500	500
8564	liver	discoloration	pale	male	500	500
8564	liver	foci	multiple foci of focal lytic necrosis; replacement inflammatory cells, cellular degenration	male	500	500
8564	liver	steatosis	macrovesicular and microvesicular	male	500	500
8564	liver	degeneration		male	500	500
8564	liver	necrosis	focal pericentral necrosis	male	500	500
etox	adrenals			male	1000	
etox	clinical sign			male	1000	
etox	FOB	reduced		male	1000	
etox	hematology	reduced		male	1000	
etox	hematology	increased		male	1000	
etox	hematology	increased		male	1000	
etox	hematology			male	1000	

Pivalic acid:

1) Reference: IUCLID, Shell Netherlands (1990)

Study design: Fischer rat (F334),gavage exposure, GLP, 28 days, purity of test compound not given. Conducted according to OECD guideline 307. Vehicle control: water/polyethyleneglycol 50/50 (v/v). Samples prepared once per week.

Tested doses: male and female 10/30/100/300 mg/kg bw/d

Dose (mg/kg bw/d)	Dose (mmol/kg bw/d)	Effect	Comment
10	0.098	Nothing observed	
30	0.29	Alkaline phosphathase (f) increased	
		Cholesterol increased (f)	slight
100	0.98	Dark discharge from nose (m+f)	immediately after exposure, accompanied with shaking of heads and sneezing
		Bilirubin increased (m+f)	slight
		Alkaline phosphathase (m) increased	
		Cholesterol increased (m)	
300	2.9	Body weight decreased (f)	slight, uncertain relation to treatment
		Creatinine increased (f)	
		Kidney and liver weight increased	

References:

Chang, T. K. H., and F. S. Abbott

2006 Oxidative stress as a mechanism of valproic acid-associated hepatotoxicity. Drug Metabolism Reviews 38(4):627-639.

Fromenty, B., A. Berson, and D. Pessayre

1997 Microvesicular steatosis and steatohepatitis: role of mitochondrial dysfunction and lipid peroxidation. J Hepatol 26 Suppl 1:13-22.

Fromenty, B., and D. Pessayre

1995 Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity. Pharmacol Ther 67(1):101-54.

Kassahun, K., and F. Abbott

1993 *In vivo* formation of the thiol conjugates of reactive metabolites of 4-ene VPA and its analog 4-pentenoic acid. Drug Metabolism and Disposition 21(6):1098-106.

Patel, V., and A. J. Sanyal

2013 Drug-Induced Steatohepatitis. Clinics in Liver Disease 17(4):533

2. Calux assay

Protocol name

Automated CALUX reporter gene assay procedure - Effects on Endocrine System (DB-ALM 197) Combined DB-ALM/OECD GD 211 template for reporting individual (nonguideline) *in vitro* methods

Abstract

This protocols describes a reporter gene assays for the detection of nuclear hormone receptor modulation, or the activation of cell signalling pathways, using high throughput screening.

Résumé

The current protocol describes the performance of BioDetection Systems Chemically Activated LUciferase eXpression (BDS CALUX) reporter gene assays in automated high throughput screening of pure compounds. It has been published before in the context of several screening studies (Piersma *et al.* 2013, van der Burg *et al.* 2013, van der Burg *et al.* 2015a, van der Burg *et al.* 2015b, van Vugt-Lussenburg *et al.* 2018).

Experimental description

Biological endpoint and endpoint measurement:

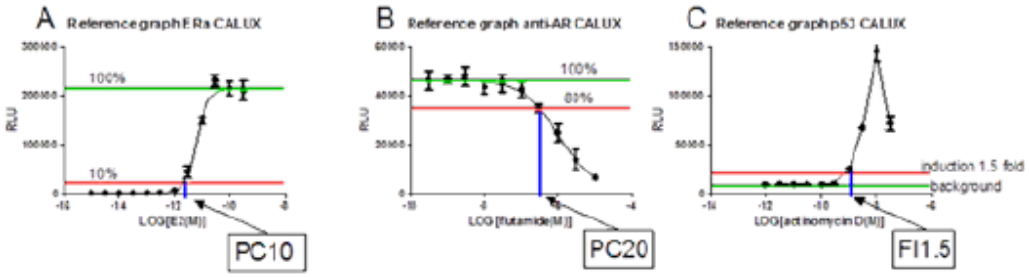
Receptor agonism, antagonism or cell signalling pathway activation (see the section Annexes, Table 1), assessed by luminescence.

Endpoint value:

Reported values are lowest effect concentrations (LEC) in Log(M). Depending on the assay, LECs are defined as:

- the concentration where the test compound causes an activation/agonist effect equal to 10% of the maximum effect elicited by the test's reference compound (PC_{10}, figure A);
- the concentration where the test compound causes an antagonist effect equal to 20% of the maximum effect elicited by the test's reference compound (PC_{20}, figure B);
- the concentration where the test compound elicits pathway activation 1.5-fold above background (FI 1.5, figure C)

Figure. Definition of lowest effect concentrations in mechanistically different CALUX assays.



Experimental system:

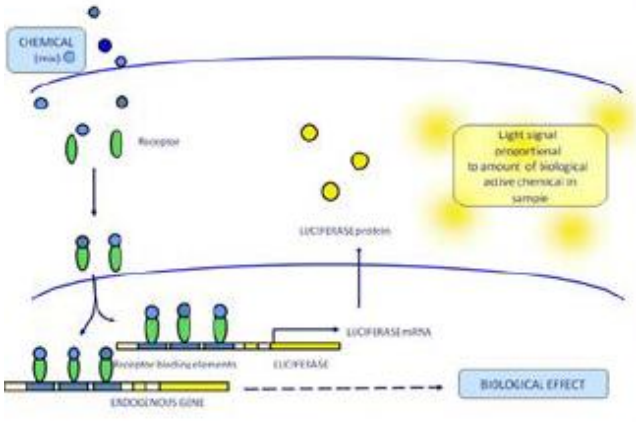
Assays are based on the human osteosarcoma cell line U2-OS (ATCC HTB-96), or the rat hepatoma H-4-II-E (ATCC CRL-1548) (AhR CALUX only).

From OECD GD 211

2.1 Purpose of the test method

2.2 Scientific principle of the method

The CALUX panel consist of 26 cell based reporter gene assays. Each cell line measures the activation or inhibition of one specific nuclear hormone receptor or cell signalling pathway (figure). The assay results can be used to identify compounds activating similar MIE's, and is therefore well suited for read across studies. Furthermore, the results provide clues for the MoA of the different compounds. Therefore, a direct link with an AOP (if available) can be made.



Cell line	Endpoint
Ela CALUX (ago/anta)	Estrogen receptor (ant)agonists
AR CALUX (ago/anta)	Androgen receptor (ant)agonists
PR CALUX (ago/anta)	Progesterone receptor (ant)agonists
GR CALUX (ago/anta)	Glucocorticoid receptor (ant)agonists
Tlb CALUX (ago/anta)	Thyroid receptor (ant)agonists
RAR CALUX	Retinoic acid receptor agonists
LXR CALUX	Liver X receptor agonists
PER CALUX	Pregnane X receptor agonists
PPARa CALUX	Peroxisome proliferator activated receptor agonists
PPARg2 CALUX	Peroxisome proliferator activated receptor agonists
PPARd/CALUX	Peroxisome proliferator activated receptor agonists
AhR CALUX	Aryl Hydrocarbon receptor agonists
Hif1a CALUX	Chemical hypoxia response
TCF CALUX	wnt/TCF pathway activation
AP-1 CALUX	AP1 pathway activation / cell cycle control
ERE CALUX	Endoplasmic reticulum stress
NFkB CALUX	Activation of NF-kB pathway (immune response)
Nrf2 CALUX	Oxidative stress
p21 CALUX	Transcription of p21 inhibitor of cell cycle progression
p53 CALUX	p53-dependent pathway activation / genotoxicity
Cytotox CALUX	Cytoxicity

2.4 Metabolic competence of the test system

Main metabolic genes (p450s, GSTs, UGTs, SULTs) have been assessed using both activity measurements and RT-qPCR analysis; no metabolic activity could be detected using either method (van Vugt-Lussenburg *et al.* 2018).

2.8 Known technical limitations and strengths

Strengths:

Since the U2-OS cells have very low expression levels of endogenous receptors, no cross-talk with other receptors can occur and therefore the assay is highly specific and responsive.

Also, U2-OS cells have no or little metabolic capacity, enabling the specific analysis of the test (parent) compound, rather than a mixture of parent compound plus metabolites. If information on bio(in)activation is required, a metabolic module can be added to the CALUX assay to study the activity of metabolites.

The CALUX panel consists of 26 assays, covering a broad range of nuclear receptors and cell signalling pathways, all in the same cellular background. This makes the results more easily comparable to each other than when using assays with several different cellular backgrounds.

Limitations:

Several sources of variation are known and are carefully controlled at BDS:

- Serum
- Plastics / labware
- Cell passage number

Compounds with high volatility or low water solubility are less suitable to test; it should be taken into account in those cases that the nominal (dosed) concentration is much higher than the actual bioavailable concentration. PBK-models can be used to identify compounds where bioavailability-issues may arise, and correction factors can be derived.

Compounds that aspecifically influence the expression, activity or stability of the luciferase reporter gene can result in false- positive or false-negative signals. Therefore, a cell line constitutively expressing luciferase (Cytotox CALUX) is used as a control for aspecific effects for all test compounds.

4.4 Scope and limitations of the assay, if known

See also section 2.8 above.

Scope of the assay panel:

It has been shown to be applicable for a wide range of applications including, but not limited to, chemicals, cosmetic ingredients, hormones and pharmaceuticals, pesticides, either alone or in complex (environmental) mixtures, like surface and waste water, body fluids, and tissue extracts.

Discussion

- The method described here is an adaptation of BDS CALUX reporter gene assays, some of them already formally validated. Although the validated assays are identical to the assays used in the current protocol, the experimental protocol used is slightly different, namely on the type of cell culture plates used (384-wells instead of 96-wells plates). Details like detection limits and acceptable standard deviations may therefore

differ, while the overall performance of the cell lines (robustness, stability, predictivity) remains the same.

- No ethical issues arise from the use of these immortalised cell lines.
- Equipment: absolute requirements are a laminar flow cabinet, a cell culture incubator and a luminometer.
- For the automated procedure, as used for the EU-ToxRisk case studies, additionally a cell washer-dispenser, a liquid handling machine and a stacker coupled to the luminometer are required.
- The cell lines are genetically modified organisms (GMOs). Therefore, a GMO permit is required (Dutch regulations: safety level Microbiologisch Laboratorium Klasse I (ML-I)).
- Training sessions consist of 1 or 2 weeks, depending on the prior experience of the trainee.
- CALUX assays are performed over the duration of three days; day 1: seeding; day 2: exposure; day 3: harvesting and measurement.
- The CALUX assays are very sensitive; especially the nuclear receptor hormone assays and the aryl hydrocarbon receptor assay can suffer from background signals caused by e.g. plasticisers leaching from plastics, or hormones present in foetal calf serum. Therefore it is recommended to use only BDS-approved brands of plastic consumables, and to use only BDS-approved dextran coated charcoal (DCC)-stripped foetal calf serum.

From OECD GD 211

4.1 Robustness of the method

CALUX assays are licensed world-wide by BioDetection Systems BV, Amsterdam, The Netherlands. This company also provides world-wide training and support. The assays can be easily transferred to and performed at other labs all over the world; training generally takes 1-2 weeks. Examples for inter- and intralab validations can be found in the following references, where cv values on EC_{50}/IC_{50}'s were 1-30% (intralab), and standard deviations on EC_{50}/IC_{50}'s were 6-13% (interlab) (van der Burg *et al.* 2010b); cv values on EC_{50}/IC_{50}'s 3-20% (intralab), and standard deviations on EC_{50}/IC_{50}'s 18-26% (interlab) (van der Burg *et al.* 2010a).

4.2 Reference chemicals/chemical libraries, rationale for their selection and other available information

The CALUX panel described here consists of 26 different assays; specific information on reference compounds and performance per assay can be found at https://eu-toxrisk.douglasconnect.com/test-methods. A table of all assays and their reference compound is also given in the section Annexes, Table 1. In general, the assays have been validated with relevant known positive and negative controls for the specific endpoint (nuclear receptor or cell signalling pathway) (Gijsbers *et al.*, Garrison *et al.* 1996, Sonneveld *et al.* 2005, Sonneveld *et al.* 2006, van der Burg *et al.* 2010b, van der Burg *et al.* 2010a, Gijsbers *et al.* 2011, Sonneveld *et al.* 2011, Gijsbers *et al.* 2013, van der Linden *et al.* 2014, OECD 2016).

4.3 Performance measures/predictive capacity (if known)

Dose-response curves are analysed in Prism (four parameter sigmoidal, variable slope).

QA/QC criteria / typical performance:

Z-factor >0.6

Negative control within set limits (relative induction <10%)

Positive control: induction factor within set limits (differs per assay); EC_{50} value within set limits (differs per assay)

Standard deviation on triplicates within 15%

Status

In development:

All assays have passed their developmental phase and have already been used in several high throughput screening programs (van der Burg *et al.* 2010b, Sonneveld *et al.* 2011, Piersma *et al.* 2013, van der Burg *et al.* 2015b).

Known laboratory use:

CALUX assays are marketed by BDS. As such, many assays are in use in other laboratories (see the section Annexes, Table 1, column 'external use'). The method has been used in the EU-ToxRisk context.

Participation in evaluation study:

All assays have undergone internal validation with respect to robustness and stability, and predictiveness with respect to known positive controls.

Participation in validation study:

Various CALUX assays have undergone validation studies, and/or participated in inter-laboratory ring studies (see the section Annexes, Table 1, column 'validation').

Currently, two assays are in OECD Test Guideline 455 (OECD 2016), four more assays are being evaluated according to OECD-guidelines, and one assay is ISO-certified.

Although the validated assays are identical to the assays used in the current protocol, the experimental protocol used is slightly different: the current protocol describes automated 384-wells screening, while the validation was performed manually on 96-wells plates. Details like detection limits and acceptable standard deviations may therefore differ, while the overall performance of the cell lines (robustness, stability, predictivity) remains the same.

Regulatory accepted:

Currently, two assays are in OECD Test Guideline 455 (OECD 2016), four more assays are being evaluated according to OECD-guidelines, and one assay is ISO-certified.

Proprietary and/or confidentiality issues

The name CALUX is a registered trademark; CALUX assays are marketed by BioDetection Systems (BDS; http://www.bds.nl/).

Health and safety issues

General precautions:

Standard laboratory procedures apply. Always use gloves and labcoat; when working with dangerous or unknown compounds, always work in a fume hood. If the dilution/exposure work is performed by a liquid handling machine, the risk for the employee is greatly reduced.

MSDS information:

In addition to the safety measures regarding the compounds in use, there are no safety measures needed for the performance of this method.

Abbreviations and definitions

ATP: Adenosine triphosphate

AhR: Aryl hydrocarbon receptor

BDS: BioDetection Systems bv

CALUX®: Chemically Activated LUciferase eXpression

CDTA: Trans-1,2-diaminocyclohexane-N,N,N'N'-tetra acetic acid monohydrate

DCC: dextran coated charcoal

DCC-Stripped FCS: active charcoal-stripped FCS (a process to remove steroids from the serum)

DMEM/F12: Dulbecco's modified eagle medium supplemented with F12

DMSO: Dimethyl sulfoxide

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

FCS: Foetal calf serum

GMOs: Genetically modified organisms

NADPH: b-Nicotinamide adenine dinucleotide phosphate tetrasodium salt

PBS: Phosphate buffered saline

PC: Positive Control

Trypsinate: Enzymatic treatment of cells with trypsin to remove the intercellular and surface attachment resulting in single rounded cells

Subculture: The transfer of a cell suspension into a new culture flask

Last update

15-January-2018

Part B. Technical description

Procedure details, latest version:

15-January-2018

Protocol name:

Automated CALUX reporter gene assay procedure

Contact person:

Bart van der Burg
Innovation Unit
BioDetection Systems BV
Science Park 406
Amsterdam 1098 XH
Netherlands
email: bart.van.der.burg@bds.nl
telephone: +31-20-4350750

Barbara van Vugt-Lussenburg
Innovation Unit
BioDetection Systems BV
Science Park 406
Amsterdam 1098 XH
Netherlands
email: Barbara.lussenburg@bds.nl
telephone: +31-20-4350750

Materials and preparations

Assays are based on the human osteosarcoma cell line U2-OS (ATCC HTB-96), or the rat hepatoma H-4-II-E (ATCC CRL-1548) (AhR CALUX only).

Each cell line is stably transfected with a reporter gene plasmid containing luciferase under control of the responsive elements of a specific receptor (e.g. oestrogen receptor) or cell signalling pathway (e.g. NFκB). Activation of the transfected pathway or receptor by a test chemical results in luciferase production, which can be measured as light production by adding the substrate luciferin. Since most nuclear hormone receptors are not endogenously expressed in U2-OS cells, these receptors have been co-transfected when applicable (see the section Annexes, Table 1).

Equipment

Fixed Equipment

- Cell culture incubator (37°C, 5% CO_2)
- Cell washer-dispenser (BioTek EL406)

- Centrifuge
- Freezers (-20 and -80 and liquid nitrogen)
- Inverted phase contrast Microscope
- Laminar flow cabinet
- Liquid handling machine (Hamilton STARlet)
- Luminometer with stacker

Consumables

Several CALUX assays are sensitive to background signals arising from contaminations present in, or leaching from, reagents and consumables. When a specific brand and order number is quoted below, the advised brand and type is preferred. In cases where the brand and order numbers are only indicative, it is preceded by 'e.g.'

- 15 ml tubes (Greiner, 188271)
- 384 wells plates (Greiner, 781080)
- 50 ml tubes (Greiner, 210261)
- 96-deepwell plates (Greiner, 780261)
- Adhesive Plate Seal (e.g. Thermo Fisher, 11524794)
- BreathEasy plate seals (Molecular Devices, E1005)
- Brown autosampler Glass conical vials (VWR, BROW153810)
- Cell culture flasks (75 cm2, e.g. Greiner, 658 175)
- Cryovials (e.g. Greiner, 121261)
- Filter units pore size 0.2 micron (e.g. Whatman 10462200)
- Mr FrostyTM container (e.g. Thermo Fisher, 10110051)
- Pipetting tips 1000, 300 and 50 µl (Hamilton, 235904, 235902 and 235966)
- Sterile reservoirs (50ml) (e.g. VWR, 613-1184)
- Sterile serological pipettes 5ml, 10ml and 25ml (Greiner, 606180, 607180 and 760180)

Media, reagents, sera, others

- 1M HCl solution (pH meter) (e.g. Sigma, H1758)
- 1M NaOH solution (pH meter) (e.g. Sigma, S5881)
- Adenosine triphosphate (ATP) (e.g. Ducheva Biochemie BV, A 1335.0010)
- Alcohol 70% (e.g. BioSolve, 5210502)
- b-Nicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH) (e.g. Applichem, A1395,0500)
- Dithiothreitol (DTT) (e.g. Ducheva Biochemie BV, D1309-0025)
- D-Luciferine (e.g. Resem bv, D-luciferin 500mg)

- Dulbecco's modified eagle medium supplemented with F12 (DMEM/F12) with phenol red as pH-indicator (e.g. Thermo Fisher, 11514436)
- Dulbecco's modified eagle medium supplemented with F12 (DMEM/F12) without phenol red (e.g. Thermo Fisher, 11580546)
- Ethylenediaminetetraacetic acid (EDTA) (e.g. Acros, 147855000)
- Foetal calf serum (FCS) (South American origin, Thermo Fisher, 11573397)
- Glucose-6-phosphate (e.g. Biosynth, G-3340)
- Glucose-6-phosphate dehydrogenase type VII (e.g. Sigma, G7877)
- Glycerol (e.g. Baker, 7044)
- Magnesium carbonate hydroxide (e.g. VWR, AAA18070-0B)
- Magnesium sulphate(e.g. Sigma, M7506)
- $MgCl_2$ hexahydrate (e.g. Sigma, M2393)
- Non-essential amino acids (100x) (e.g. Thermo Fisher, 11140-035)
- Penicillin-streptomycin (e.g. Thermo Fisher Scientific, 15070-063; 5000 penicillin units per ml / 5000 µg streptomycin per ml)
- Phosphate buffered saline pH 7.2 (PBS, e.g. Thermo Fisher, 20012-019)
- Rat liver S9 (e.g. MolTox Trinova, 11-105.5)
- Trans-1,2-diaminocyclohexane-N,N,N'N'-tetra acetic acid monohydrate (CDTA) (e.g. Sigma, 32869)
- Tricine (e.g. Sigma, T0377)
- Tris (e.g. Sigma, T1378)
- Triton X-100 (e.g. Sigma, T8787)
- Trypsin (e.g. Thermo Fisher, 27250-042)

Preparations

Media and endpoint assay solutions

Cell Culture Medium (all solutions should be sterile and handled in a sterile environment):

Take a new bottle of DMEM/F12 with phenol red in the laminar flow cabinet.

Add a tube of FCS (41 ml) to the flask DMEM/F12 (7.5%).

Add 5 ml of non-essential amino acids (100x NEAA).

Add 1 ml of penicillin-streptomycin solution.

Store at 4 °C for maximum 2 months.

Assay Medium (all solutions should be sterile and handled in a sterile environment):

Open a 500 ml bottle of DMEM/F12 without phenol red in the laminar flow cabinet

Add 26.6 ml DCC-FCS (5% v/v).

Add 5 ml of non-essential amino acids (100x NEAA).

Add 1 ml of penicillin-streptomycin solution.

Store at 4 ºC for maximum 2 months.

Trypsin solution:

Trypsin solution should be diluted with PBS containing 0.2 g/L EDTA until the trypsin solution has a concentration of 0.05% trypsin (g/L). Filter-sterilise using a 0.2 micron pore size filter. Store the tubes of trypsin at -20 o C until use. After thawing, store at 4 o C for maximum 2 months.

Freezing medium (all solutions should be sterile and handled in a sterile environment):

Open a 500 ml bottle of DMEM/F12 with phenol red in the laminar flow cabinet.

Remove and discard 143ml of DMEM/F12 medium.

Add 5.5 ml of non-essential amino acids (MEM 100x).

Add 100 ml of FCS.

Add 37.5 ml of DMSO.

Mix gently and distribute as 40 ml aliquots in 50 ml Greiner tubes.

Label the plastic tubes containing the freezing medium (preparation date; expiring date (1 year)).

Store freezing medium at -20 °C.

Triton Lysis Buffer:

Dissolve Tris (25mM), DTT (2 mM) and CDTA (2 mM) in demineralised water.

Add 10% (v/v) glycerol and 1% (v/v) Triton® X-100.

Adjust the pH to 7.8 using 1 M HCl and/or 1 M NaOH solutions.

Transfer aliquots of approximately 40 ml into 50 ml tubes.

Store at -20°C for a maximum of 1 year or at 4°C for a maximum of 1 month.

BDS Illuminate Mix:

Dissolve in demineralised (demi) water: tricine (20 mM), magnesium carbonate hydroxide (1 mM), magnesium sulphate (2.7 mM), EDTA (0.1 mM), DTT (1.5 mM) and D-luciferine (539 µM).

After adding the D-Luciferine, the BDS illuminate-mix should be kept in the dark and further preparation may last no longer than 0.5h due to the instability of the compounds used.

Add ATP (5.49 mM).

Adjust the pH to 7.8 using 1 M HCl and/or 1 M NaOH solutions.

Divide the BDS illuminate mix into 100 ml portions in HDPE bottles.

Close and label the bottles.

Store at -20°C for a maximum of 3 months or at -80°C for one year.

Test compounds

1. Prepare a 0.1M stock of the test compound in DMSO (if not soluble: go down in 0.5 log unit increments until dissolved).
2. Divide into 100 µl aliquots and store at -20°C.
3. Use glass vials only (Brown autosampler vials)!
4. Before use: thaw one aliquot.
5. The liquid handling robot prepares a dilution series in a 96-deep-well plate; the following 14 concentrations (M) with 0.5Log increments are prepared:

   1.0E-04; 3.0E-05; 1.0E-05; 3.0E-06; 1.0E-06; 3.0E-07; 1.0E-07; 3.0E-08; 1.0E-08;

   3.0E-09; 1.0E-09; 3.0E-10; 1.0E-10; 0
6. The compound is exposed by the Hamilton STARlet; the deep-well plate containing the dilutions is discarded.
7. If the compound is tested again later (as a duplicate measurement, or on different assays), a fresh aliquot is used. EXCEPTION: if a compound is only very scarcely available, the deep-well plates with dilution series will be re-used, and stored at -20 o C, covered with a plate lid.

Note. For volatile compounds, the 96w-dilution plates are covered with a non-breathable seal until exposure. The assay plates containing the cells exposed to volatile compounds are covered with a breathable seal immediately after exposure.

Note 2. Compounds insoluble in DMSO cannot be tested using the routine automated HTS-method, and have to be tested separately. For compounds dissolved in water, PBS or medium, a method is available. Compounds in EtOH, MeOH or other solvents will have to be tested manually on 96-wells plates.

Note 3. No specific protocol for very viscous compounds is in place; they may have to be diluted until they can be pipetted by the robotic system. However, within the EU ToxRisk case studies, compounds with such high viscosity have not been encountered yet (CS1 to 5).

Positive Control(s)

For the positive controls (see the section Annexes, Table 1), aliquots of 1000x stock solutions in DMSO are frozen at -20oC. For each experiment, the relevant positive controls are thawed, and diluted by the liquid handling machine together with the test compounds (see the section Annexes, Table 1).

Negative Control(s)

DMSO is used as a negative control for all cell lines. A fresh aliquot of DMSO is used for each experiment.

Method

Experimental system procurement

Freezing of cells:

- Prepare 75 cm2 culture flasks with cells (start 1.5 week in advance).
- Check whether the flasks are approximately 90% confluent before starting the freezing procedure.
- Trypsinise all cells according to the procedure described in the subculturing of cells section (below) including the removal of trypsin.
- Suspend the cells in 10ml growth medium per flask.
- Pool and count the cells from the different flasks. Calculate how many cryovials can be prepared at a concentration of 1.5 x 10^6 cells/vial.
- Divide the cells over 50 ml Greiner tubes and centrifuge (250 g, 5 minutes).

Note. After the centrifugation step, keep the vials and 50 ml Greiner tubes on ice at all times and work as quickly as possible to minimise the toxic effect of DMSO at room temperature!

- Discard medium and re-suspend the cells in freezing medium. The volume in ml of freezing medium to be used equals the number of vials that can be prepared. Each cryovial should contain 1 ml of cell suspension.
- Divide the re-suspended cells in freezing medium over the number of cryovials calculated (1 ml per cryovial) and close the cryovials.
- Put the cryovials in the Mr. Frosty freezing containers and place it at -80 °C (only overnight) to achieve acooling rate of 1 °C/minute.
- Transfer cryovials to liquid nitrogen for storage.

Thawing of cells:

- Heat water bath to 37°C.
- Take a flask of growth medium from the refrigerator and heat it in the water bath.
- Retrieve a cryovial of CALUX cells from the liquid nitrogen.

Note. Wear safety glasses and protective gloves.

- Thaw the cells quickly by gently moving the vial in a water bath of 37°C until the ice has almost melted.
- Clean the outside of the cryovial with 70% alcohol.
- Pipette 0.5 ml of growth medium from the culture flask into the cryovial using a sterile pipette.
- Transfer cells to a 15 ml sterile plastic tube.
- Add drop wise 10 ml of cold cell culture medium and mix gently (4°C).
- Spin down the cells in the centrifuge at approximately 250x g, 5 minutes.

- Remove the medium.
- Resuspend the pellet in 10 ml of culture medium (RT to 37°C), transfer to a culture flask (75 cm2), and transfer to the CO_2-incubator. Indicate the type of cells, date of preparation, name and passage number.

Routine procedures

Subculturing of cells:

- Subculture preferably every Monday and Friday.
- Thaw a tube of trypsin.
- Take a flask growth medium from the refrigerator.
- After pre-warming the solutions in a 37ºC water bath, place the trypsin tube and the growth medium and a PBS flask in the safety-cabinet and open them.
- Take a culture flask (75 cm^2) with cells to be subcultured from the CO_2 incubator.
- Transfer the growth medium from the culture flask into the waste bottle by sterile pipetting.
- Carefully pipette 5 ml of PBS into the culture flask by sterile pipetting. Place the tip of the pipette just below the neck of the culture flask. Ensure that the pipette-tip does not to touch the neck of the culture flask.
- Swirl the culture flask approximately 5 times.
- Transfer the PBS from the culture flask into the waste bottle using a sterile 10 ml pipette.
- Rinse again with 5 ml PBS.
- Pipette 2 ml of trypsin into the culture flask by sterile pipetting.
- Swirl the culture flask approximately 5 times.
- Transfer all the trypsin from the culture flask into the waste bottle by sterile pipetting.
- Gently slap the bottom of the culture flask against the palm of your hand after 5 minutes to see if the cells are detaching from the bottom of the culture flask.
- Pipette sterile 10 ml of growth medium supplemented with FCS in the culture flask using a new sterile pipette.
- Swirl the culture flask 5 times allowing the cells to go into suspension. Ensure the cells just below the neck of the flask are in suspension too.
- Re-suspend the cells by performing 10 careful up and down cycles in growth medium using 10 ml pipette. Ensure no flocks of cells are visible anymore.
- Transfer the proper amount of cell suspension into the new culture flask (75 cm^2). The amount of cells to be transferred depends on the growth-rate of the cells and the number of days until the day it is intended to subculture them. Generally, a ratio of 1:6 or 1:8 is appropriate.

- After transfer of cell suspension, fill up the new culture flasks to a final volume of 10 ml with growth medium.
- Label the culture flasks. Indicate the type of cells, date of subculturing, passage number, dilution factor and your name.
- Place the culture flask in the CO_2 incubator.

Test material exposure procedures

- Prepare a cell suspension of 1×10^5 cells/ml in white assay medium (see above), and seed white 384-wells plates with 30 µl cell suspension/well using an automatic multidispense multichannel pipette.
- 24 h after seeding, (or, in the case of assays BDS21a and BDS21b, 48h after seeding, see below), prepare exposure medium: prepare a dilution series in 0.5log unit increments of each test compound (in DMSO), in 96W-deepwell plates, and add 1 µl of each concentration to a 96-wells plate containing 500 µl assay medium (or, in the case of assays BDS21a and BDS21b, 10 µl of each concentration, see below).
- Of this exposure medium, add 30 µl, in triplicate, to the assay plates containing the CALUX cells (final DMSO-concentration: 0.1% (or 1% in the case of assays BDS21a and BDS21b, see below)).
- Additionally, prepare DMSO blanks and a full dose-response curve of the relevant reference compounds in similar fashion (for reference compounds and concentrations, see the section Annexes, Table 1).
- At BDS, the preparation of the compound dilution series as well as the exposure of the cells are performed on a Hamilton STARlet liquid handling robot coupled to a Cytomat incubator. This automated CALUX assays have been described in the publication (van der Burg *et al.* 2015a).

Note 1. In order to be able to detect receptor antagonism, six CALUX assays were also performed in antagonistic mode. The assay procedure was as described above, with the only exception that the cells were supplemented with an EC_{50} concentration of the reference agonist before exposure (for concentrations and compounds: see the section Annexes, Table 1, column 'EC_{50} agonist').

Note 2. The assays BDS21a and BDS21b have a slightly modified protocol to ensure good performance. They are seeded 48 h before exposure instead of 24 h; and they are exposed at 1% (DMSO v/v) instead of 0.1%.

Additionally, immediately after exposure 6 µl/well of a 10x concentrated S9 mix is added to BDS21b_Geno_RGA_p53S9_act_24h in order to allow metabolic (in)activation by hepatic enzymes. 10x S9 mix consisted of: 3 mg/ml PB-BNF induced Sprague-Dawley rat liver S9, 2 mM NADPH, 30 mM glucose-6-phosphate, 50 mM MgCl2, and 3 units/ml glucose-6-phosphate dehydrogenase. After 3 h, the exposure medium is replaced by assay medium, and cells were allowed to recover for 21 h. After a total of 24 h, cells are harvested and analysed as described in the following paragraph.

Endpoint measurement

- After 24 h exposure, remove the exposure medium, e.g. using an EL406 cell washer-dispenser (Biotek).

- Add 10 µl/well triton lysis buffer.
- Measure the luminescent signal in a luminometer (e.g. InfinitePro coupled to a Connect Stacker (both TECAN)), by injecting BDS Illuminate Mix and measuring light output.
- Machine settings:
  - Injection speed: 200 µl/second.
  - Injection volume: 35 µl/well.
  - Measurement time (luminescence): 1 sec/well.
  - Temperature settings: none (ambient).

Acceptance criteria

Acceptance criteria have been established for triplicate datapoints as well as for the entire 384 wells plates. First, determine for each triplicate on the plate whether it meets the acceptance criteria; then, determine for the entire plate if it meets the acceptance criteria.

- Discard triplicates with a standard deviation >15%
- Discard datapoints where the compound is cytotoxic (<80% cell viability as determined using a separate cytotoxicity assay)
- Using only datapoints that passed the two first criteria: negative control sample (DMSO) should be within set limits (relative induction <10%)
- Using only datapoints that passed the two first criteria: positive control sample (reference compound) maximum induction factor and EC_{50} value should be within set limits (relative induction >30%; EC_{50} +/- 0.5 Log units; for EC_{50} values, see the section Annexes, Table 1)

Perform two analyses as independent (biological) duplicates. If the results (PC_{10} or PC_{20} values) of the first and second experiment deviate for >0.5 log unit, perform a third replicate.

From OECD GD 211

2.7 Quality/acceptance criteria

- All data, both raw and calculated, is saved on our servers; data is backed-up daily. Raw data consists of text files (.txt or .asc) containing 384 numbers (24 columns x 16 rows), which are the Relative Light Units (RLUs) measured by the luminometer. Calculated data is reported in % activity compared to reference, and induction factor above blank, calculated by and stored in a custom database tool on the BDS servers. Calculated datafiles also contain information on test compound names, concentrations, and test compound stock solution numbers (internally assigned).

- The database tool performs baseline subtraction and recalculates test compound data into '% of reference compound' and 'fold induction above background'. EC_{10}, EC_{50}, PC_{10}, NOEL and LOEL and maximum induction (fold, %) are calculated and summarised in a results-table. Quality control calculations are also performed (% SD on triplicates).

- Each plate contains a suitable reference compound (assay-specific, see the section Annexes, Table 1), and a negative control (DMSO); plates are rejected if the following

criteria are not met: negative control within set limits (relative induction <10%); positive control: induction factor within set limits (differs per assay); EC_{50} value within set limits (differs per assay). Individual datapoints are discarded if standard deviation on triplicates >15%.

Data analysis

Data is uploaded into our custom designed database tool. This tool performs baseline subtraction and recalculates test compound data into '% of reference compound'. EC_{10}, EC_{50}, PC_{10}, NOEL and LOEL and maximum induction (fold, %) are calculated based on a sigmoidal fit (four parameters, variable slope) and summarised in a results-table.

Prediction model

The prediction model differs per assay, and can be found in detail on https://eu-toxrisk.douglasconnect.com/test-methods. In short, the CALUX assays measure transactivation of a specific receptor, or activation of a cell signalling pathway. Since the U2-OS cells have very low expression levels of endogenous receptors, no cross-talk with other receptors can occur and therefore the assay is highly specific and responsive. A compound is considered a 'hit' if it gives a response that equals 10% of that of the positive control reference compound, 20% of that of the positive control reference compound, or 1.5-fold induction above background (DMSO). See also the picture in the section 'Experimental description' and in the section Annexes, Table 1, column 'reported value'.

Annexes

Table 1. CALUX assay characteristics

Name	Endpoint	EC50 or max	(Stock) (M)	Reported value	Reference compound	EC50 agonist (M)	Validation	Ext. use
B.05a_ER_KBA_ERa_ago_24h	Estrogen receptor alpha agonism	5.0E-12	1.0E-06	PC10	estradiol		TG455 / van der Burg (2010b)	yes
B.05b_ER_KBA_ERa_anta_24h	Estrogen receptor alpha antagonism	1.4E-06	1.0E-05	PC10	tamoxifen	Estradiol 6.6E-12	TG455 / van der Burg (2010b)	yes
B.05a_ER_KBA_ERb_ago_24h	Estrogen receptor beta agonism	1.6E-10	1.0E-06	PC10	estradiol		internal	yes
B.05b_ER_KBA_ERb_anta_24h	Estrogen receptor beta antagonism	5.0E-06	1.0E-05	PC10	tamoxifen	Estradiol 2.5E-10	internal	yes
B.06a_AR_KBA_AR_ago_24h	Androgen receptor agonism	1.6E-10	1.0E-06	PC10	dihydrotestosterone (DHT)		OECD in progress / van der Burg (2010a)	yes
B.06b_AR_KBA_AR_anta_24h	Androgen receptor antagonism	5.4E-07	1.0E-02	PC10	flutamide	DHT7E-10	OECD in progress / van der Burg (2010a)	yes
B.06a_PR_KBA_PR_ago_24h	Progestin receptor agonism	3.6E-10	1.0E-05	PC10	progesterone		bonnevald (2011)	yes
B.06b_PR_KBA_PR_anta_24h	Progestin receptor antagonism	9.0E-11	1.0E-05	PC10	RU486	Org 2058 5E-10	bonnevald (2011)	yes
B.07a_GR_KBA_GR_ago_24h	Glucocorticoid receptor agonism	1.7E-09	1.0E-04	PC10	dexamethasone		internal	yes
B.07b_GR_KBA_GR_anta_24h	Glucocorticoid receptor antagonism	1.4E-06	1.0E-05	PC10	RU486	Dexamethasone 2E-9	internal	yes
B.08a_TR_KBA_TRb_ago_24h	Thyroid receptor beta agonism	6.3E-10	1.0E-06	PC10	3,5',3-Triiodo-L-thyronine (T3)		OECD in progress	yes
B.08b_TR_KBA_TRb_anta_24h	Thyroid receptor beta antagonism	4.0E-07	1.0E-02	PC10	diiodothyronine	T3 5E-10	OECD in progress	yes
B.09a_PPARa_KBA_PPARa_ago_24h	Retinoic acid receptor agonism	2.0E-07	1.0E-02	PC10	retinoic acid		internal	no
B.09a_LXRa_KBA_LXRa_ago_24h	Liver X receptor agonism	1.0E-07	1.0E-02	PC10	GW3965 hydrochloride		internal	no
B.09a_PXR_KBA_PXR_ago_24h	Pregnane X receptor agonism	2.6E-07	1.0E-02	PC10	rifampicine		internal	no
B.10a_PPARa_KBA_PPARa_ago_24h	PPAR alpha receptor agonism	2.5E-09	1.0E-04	PC10	GW7647		internal	yes
B.11a_PPARg_KBA_PPARg_ago_24h	PPAR gamma receptor agonism	5.7E-06	1.0E-02	PC10	troglitazone		internal	yes
B.12a_PPARd_KBA_PPARd_ago_24h	PPAR delta receptor agonism	1.0E-07	1.0E-02	PC10	L-165041		internal	yes
B.13a_AhR_KBA_AhR_ago_24h	Aryl hydrocarbon receptor agonism	1.0E-11	1.0E-06	PC10	2,3,7,8-Tetrachlorodibenzo-p-dioxin		SID	yes
B.14a_Hypoxia_KBA_Hif1a_act_24h	Hif1a pathway activation	5.2E-04	3.7E-01	PC10	cobalt(II) chloride		internal	no
B.15a_Wnt_KBA_TCF_act_24h	Wnt pathway activation	6.3E-03	1.3E-01	Fold>1.5	lithium chloride		internal	no
B.16a_Nrf2_KBA_ARE_act_24h	Activation Protein 1 pathway activation	7.0E-10	1.0E-05	PC10	Phorbol 12-myristate 13-acetate		internal	no
B.17a_Stress_KBA_ESRE_act_24h	Stress pathway activation	5.0E-06	1.0E-05	PC10	curcumin		internal	no
B.18a_NFkB_KBA_NFkB_act_24h	NFkB pathway activation	5.0E-10	1.0E-05	PC10	Phorbol 12-myristate 13-acetate		internal	no
B.19a_Oct1_KBA_Nrf2_act_24h	Nrf2 pathway activation	1.0E-05	1.0E-01	Fold>1.5	curcumin		van der Linden (2014)	no
B.20a_Sens_KBA_p21_act_24h	p21 Pathway activation	1.0E-05	1.0E-04	PC10	actinomycin D		van der Linden (2014)	no
B.21a_Sens_KBA_p53_act_24h	p53 pathway activation	1.0E-05	1.0E-04	Fold>1.5	actinomycin D		van der Linden (2014)	yes
B.22a_Sens_KBA_p53SA_act_24h	p53 pathway activation (-S9)	5.2E-04	1.0E-01	Fold>1.5	cyclosporin A		van der Linden (2014)	yes
B.23a_Tox_KBA_cytotox_act_24h	Cytotoxicity	1.0E-07	1.0E-02	PC10	Tributyltinchloride		van der Linden (2014)	no

Legend.

Name: name of the assay.

Endpoint: receptor or pathway addressed.

EC_{50} / max: concentration where half-maximal activation is achieved (for assays showing a full dose-response curve), or concentration where maximum activation is achieved (for assays not showing a full dose-response curve).

Stock (M): stock concentration of the reference compound.

Reported: the output parameter of the assay (PC_{10}, PC_{20} or FI).

Reference compound: name of the reference compound used for the assay.

EC_{50} agonist (M): for antagonist assays, an agonist is added to the assay in order to be able to detect repression of this agonist. The column shows the name and concentration of this agonist.

Validation: how is the assay validated (+ reference)?

External (Ext.) use: is the assay used by third parties in other labs yes/no?

Downloads

Section linked to the protocol and made available through DB-ALM independently of the file containing Part A and Part B. Serves to accommodate supporting content that cannot be adequately reproduced in a PDF version of the protocol. May include:

- templates for data storage and analysis used in the laboratories,
- data samples,
- scripts or source code used for analysis,
- tabulated lists of reference substances and/or results,
- high quality graphs and figures,
- other supplementary documents.

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3. GFP assay

Part A. Protocol Introduction

Protocol Name: LU HepG2 BAC-GFP reporter cell line assay

Abstract:

This protocol describes how to culture HepG2 BAC-GFP reporter cells, how to perform compound exposures, how to perform imaging of the reporters and how to perform image analysis.

Résumé

With the HepG2 BAC GFP reporter platform the activation of cellular stress pathways can be accurately measured over time on a single cell level. The platform includes HepG2 reporters for oxidative stress (KEAP1, NRF2, SRXN1, HMOX, NQO1), ER stress (ATF4, XBP1, CHOP, BIP), inflammation (IkBa, RelA, ICAM, A20), DNA damage (53BP1, P53, P21, BTG2), Heat shock (HSF-1/2, HSP70), Heavy metal (MTF1) and hypoxia (VHL, HIF-1a, HUMMR). The combination of a protein based reporter system with confocal imaging has proven to be a powerful tool in drug safety testing as well as mechanistic research in cellular stress responses. See references.

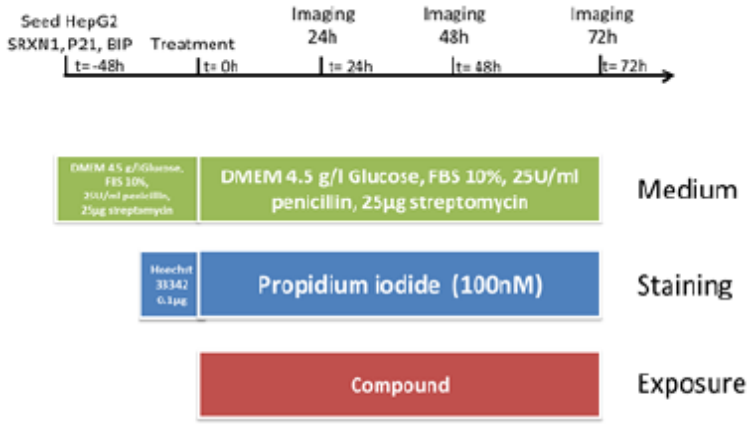
Experimental Description

Biological Endpoint and Endpoint Measurement:

The endpoints of our toxicity test system include:

Activation of responsive part of the cellular stress response pathway of interest by GFP signal intensity in the nucleus or cytoplasm

Cytotoxicity based on Propidium Iodide (PI)



The acquisition of microscopic pictures can be done at specific timepoints, i.e. 24h/48h/72h, or through live imaging up to 72h. The imaging interval during the live

imaging is usually one hour, but can be further reduced, if necessary. The smallest possible imaging interval is depending on the amount of measurements necessary during the imaging interval.

For each time point three independent experiments were conducted consisting of three biological replicates. Per cell line, nine plates were measured in total.

Endpoint Value:

The number of cells is quantified using Hoechst 33342 staining.

Upregulation of the EGFP-tagged protein of interest is determined based on the fluorescence signal in the EGFP-channel. Proteins which are located in the nucleus is quantified by the integration of pixels in the single cell.

Single cell location is based on the nuclear Hoechst 33342 staining and automated high content image analysis.

The cytotoxicity measurement is based on the percentage of PI positive nuclei.

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:

HepG2 BAC-GFP reporter cell lines are based on HepG2 (ATCC® HB8065™) cells which were transfected with a bacterial artificial chromosome containing an EGFP-tagged-gene including the flanking regulatory domains. After G418 selection, a monoclonal cell lines were generated and after experimental confirmation (western blot, siRNA and time lapse microscopy), functional clones were selected (see also Wink *et al.* 2014).

Basic procedure

The HepG2 reporter cell lines are seeded in multi-well imaging plates, stained with Hoechst 33342 and 633 and propidium iodide, and treated with different concentration of testing compounds. The cells are then imaged live on a confocal laser scanning microscope.

From OECD GD 211

2.1 Purpose of the test method

The purpose of the GFP reporter assay is to follow the expression and localisation of proteins of interest upon compound exposure. Combined with cell death markers this high throughput test method can map the dynamics of proteins of stress pathway responses and thereby may predict toxicity. Key events as oxidative stress, unfolded protein response, inflammation and DNA damage can be investigated with the matching reporters.

2.2 Scientific principle of the method

The GFP reporter assay consist of more than 60 cell based reporter gene assays, some of which are still in optimisation phase. In this read across 3 reporters are used, namely SRXN1-GFP, P21-GFP and BIP-GFP. Each cell line expresses the GFP tagged protein cell upon compound exposures, reflecting the corresponding signalling pathway. The assay results can be used to screen for KE activation, and is therefore well suited for read across studies.

2.3 Tissue, cells or extracts utilised in the assay and the species source

See experimental system: HepG2 BAC-GFP reporter cell lines are based on HepG2 (ATCC® HB8065™) cells which were transfected with a bacterial artificial chromosome containing an EGFP-tagged-gene including the flanking regulatory domains. After G418 selection, a monoclonal cell lines were generated and after experimental confirmation (western blot, siRNA and time lapse microscopy), functional clones were selected (see also Wink *et al.* 2014).

2.4 Metabolic competence of the test system

Phase I, II and III drug metabolism enzymes have been assessed using RT-qPCR analysis; these are generally lower expressed than in HepG2 3D systems and the liver *in vivo*. (Ramaiahgari *et al.*,manuscript in preparation).

2.5 Description of the experimental system exposure regime

See EXPERIMENTAL SYSTEM PROCUREMENT

2.6 Response and Response Measurement

GFP is detected with fluorescence microscopy. See Endpoint measurement for more details.

2.7 Quality / Acceptance criteria

See Endpoint measurement and Positive, Negative control(s)

2.8 Known technical limitations and strengths

Strengths:

The HepG2 GFP reporter assay is a high throughput cell based system. Furthermore, by imaging the GFP signal, not only the amount of protein expressed but also the localisation of the protein can be mapped. Additionally, by imaging over time (e.g. every hour one picture) the dynamics of protein expression can be investigated.

Limitations:

Limitations are similar as in the CALUX assay: Compounds with high volatility or low water solubility are less suitable to test; it should be taken into account in those cases that the nominal (dosed) concentration is much higher than the actual bioavailable concentration. PBK-models can be used to identify compounds where bioavailability-issues may arise, and correction factors can be derived.

Furthermore, compound with auto fluorescence are less suitable to test, since they might interfere with the GFP, Hoechst or PI signals.

See also applicability domain.

4.1 Robustness of the method

The HepG2 GFP reporter assay is only used in Leiden University. Several reporter screens have been performed with positive and negative controls showing the same results. (Di *et*

al, 2012, Hiemstra *et al.*, 2016, Niemeijer *et al.*, 2018, Wink *et al.*, 2015, Wink *et al.*, 2016, Wink *et al.*, 2018).

4.2 Reference chemicals/chemical libraries, rationale for their selection and other available information

See Positive Controls

4.3 Performance measures/predictive capacity (if known)

See acceptance criteria, Dose response analysis and Point of Departure (PoD) modelling

4.4 Scope and limitations of the assay

See 2.8

Discussion

This bioassay is based on the HepG2 cell line and the genetic modification is done with at biosafety level 1, therefore no further management of ethical issues nor higher biosafety standards are required. Depending on the test compounds, further safety measures might be necessary. For image acquisition a confocal laser scanning microscope including a cell culture incubator is required.

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 affects the image acquisition with a confocal laser scanning microscope and the single nuclei segmentation during the high content image analysis.

Status

In Development:

Several drug and knockdown screens have been performed with the HepG2 GFP reporter assays. The reporters used in this read across have passed developmental phase (Wink *et al.*, 2014, Wink *et al.*, 2016). However, analysis pipelines are continuously in development, as for modelling (Point of Departures) and calculating cut off values (e.g. EC values)

Known Laboratory Use:

The laboratory history of HepG2 BAC-GFP reporter so far is limited to the group of Bob van de Water at the University of Leiden.

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. However, the method is validated in several publications.

Regulatory Accepted:

There are no relevant guidelines available.

Proprietary and/or Confidentiality Issues

The use of HepG2 BAC-GFP reporter technology is not restricted by patents. 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

CDDO-me… 2-Cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oic acid methyl ester

DEM…Diethyl maleate

DMEM…Dulbecco modified eagle medium

DMSO…Dimethyl sulfoxide

EDTA… Sodium ethylenediaminetetraacetate

EGFP…Enhanced green fluorescent protein

FBS…foetal bovine serum

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

Protocol Name: LU HepG2 reporter cell line assay

Contact person

Bob van de Water
Research Division of Drug Discovery and Safety / Leiden Cell Observatory High Content Imaging Screening Facility
Leiden Academic Centre for Drug Research (LACDR) / Leiden University
Einsteinweg 55 / P.O. Box 9502 / 2300 RA LEIDEN / The Netherlands
E-mail: b.water@lacdr.leidenuniv.nl

MATERIALS AND PREPARATIONS

CELL OR EXPERIMENTAL SYSTEM

The original HepG2 wildtype cells were obtained from ATCC (ATCC® HB-8065™). Using bacterial artificial chromosome (BAC) recombination a copy of the biomarker protein with at least 10kb upstream of the promoter region and at least 10kb downstream of the gene was inserted together with GFP was transfected in HepG2, resulting in a C-terminally tagged stable cell line. Multiple clones per biomarker protein were picked and the clone was selected that showed endogenous regulation of the reporter-GFP fusion protein. The created cell lines have been biobanked and stored at -80°C for short term storage and -150°C for long term storage.

EQUIPMENT

Fixed Equipment

Material/Equipment	Supplier	Catalogue No
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		
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,	
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

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	D8537
Trypsin EDTA	In house	NA
Hoechst 33342, Trihydrochloride, Trihydrate	Life technologies	H1399
Propidium iodide	In house	NA
Diethyl maleate	Sigma Aldrich	D97703
Tunicamycin	Sigma Aldrich	T7765
Etoposide	Sigma Aldrich	E1383-25MG

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.
Penicillin Streptomycin aliquots	Thaw out Penicillin Streptomycin bottle, prepare aliquots of 2 ml in sterile 15 plastic tubes, frozen again and stored at -20°C.
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 Na2HPO4*2 H20; 0.25 gr KHP2PO4. 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)
Hoechst staining solution	200ng/ml in complete DMEM by diluting from Hoechst 33342 aliquot (1:5000).
Propidium iodide aliquots	Stock solution 100µM in sterile distilled water, sterile filtered
Propidium iodide staining solution	200 nM in complete DMEM by diluting from Propidium iodidealiquot (1:500).
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.
Freezing medium	20% DMSO in FBS
Sterilizing CellTrics® filters	Celltrics® filters are placed in a jar and sterilised with dry heat (110°C) overnight.
Test compound stock solution	
DMSO stock	DMSO stocks are created by weighing a test compound in an sterile micro tube 1.5ml and dissolving it 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).

Test compounds

The test compounds were tested at 9 different concentrations between 62.5 µM and 16 mM nominal concentration. All treatment solutions had a concentration of 0.1 % (v/v) of DMSO. All test compounds were prepared freshly for every experiment.

Positive Control(s)

Positive controls should induce GFP expression. Therefore, positive controls differ per BAC-GFP reporter cell line.

HepG2 BAC-GFP reporter cell lines	Positive control compound	Concentration
SRXN1	DEM	100 µM
BIP	Tunicamycin	12 µM
P21	Etoposide	50 µM

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 (cm²)	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	150	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 BAC-GFP reporter 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% CO_2 and 95% humidity).
5. After one further passaging step, the cells can be used in an assay.

Splitting of HepG2 BAC-GFP reporter 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 withTC20™ 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 BAC-GFP reporter 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 BAC-GFP reporter 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.

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 staining solution	100µl	30µl
PI staining solution	50µl	25µl
Compound solution	50µl	25µl

1. Add Hoechststaining 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 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.

ENDPOINT MEASUREMENT

For the laser and filter setup consult table 1Fluorescence 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 reporters respective positive control compound. 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

ACCEPTANCE CRITERIA

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

A test is discarded when:

More than 15% of the cells are PI-positive in the solvent control (DMSO)

To determine if the control provides the expected result, we determine the robust Z score per plate.

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.

$$Estimated\ Z-factor = 1 - \frac{3(\sigma_P - \sigma_n)}{|\mu_P - \mu_n|}$$

At least 4 negative controls (complete DMEM), solvent control (complete DMEM + 0.1% (V/V) DMSO), and 4 positive controls should be taken along.

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).

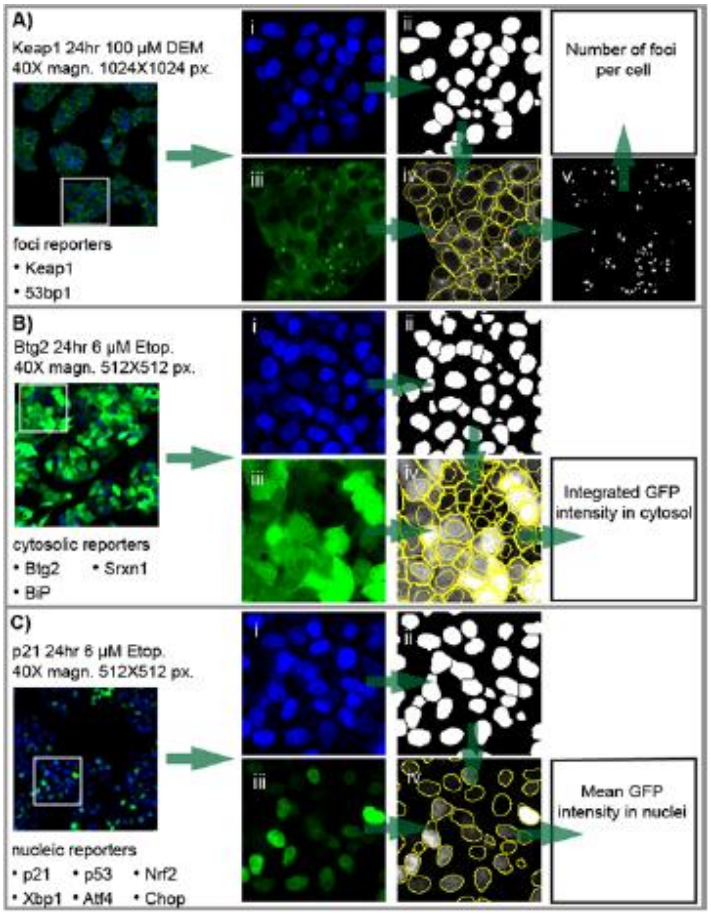
The exported tiff files were then sorted into image location specific folders (i.e. B02_1, meaning well B02 first imaging location). These were used while reading in the images in cell profiler to associate the images location_ID.

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, GFP intensity is determined in the nuclei. If the reporter protein is located in the cytoplasm, the cytoplasm is identified as a tertiary object based on the cell's nucleus and the intensity of the GFP channel picture.
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.

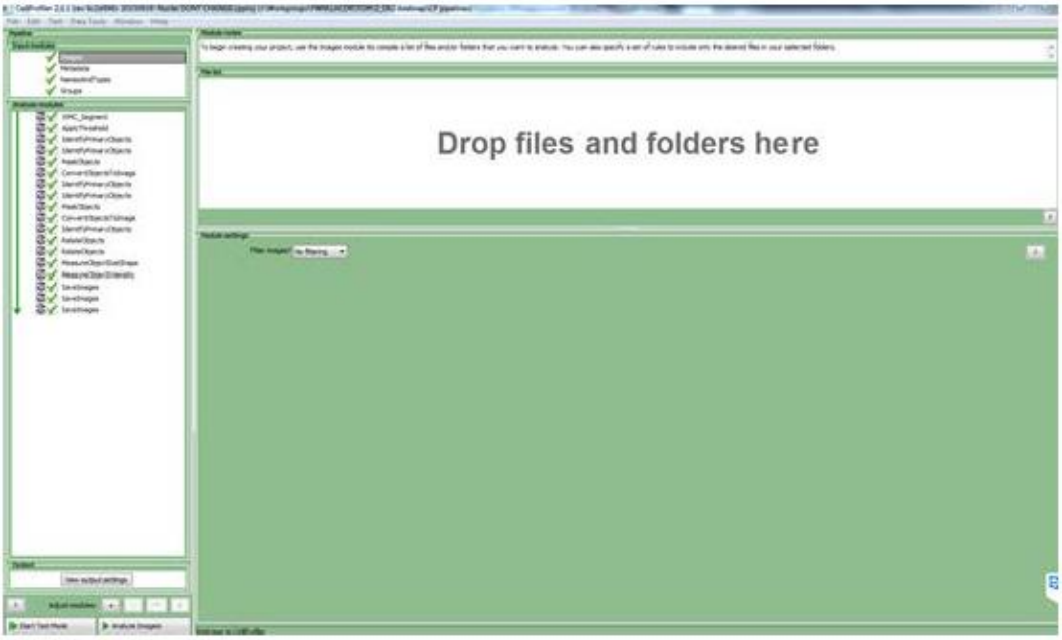
Figure 1. See also Wink *et al* 2014



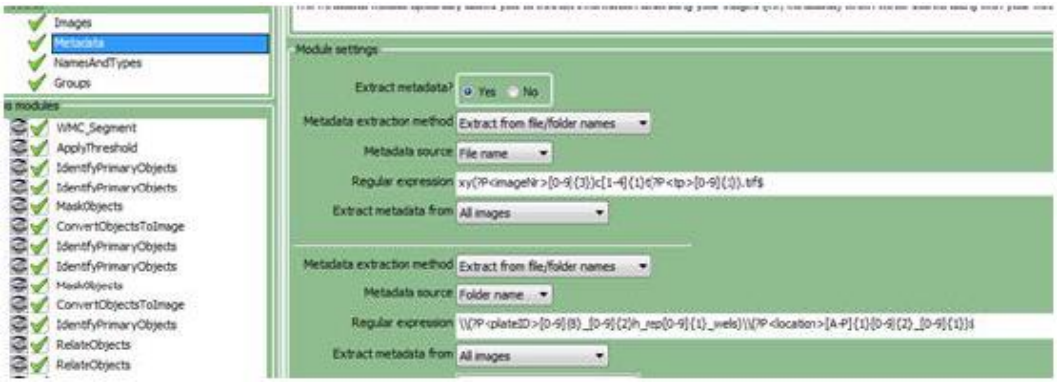
Cell Profiler analysis > set Input modules

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 (which channel is Hoechst or GFP) in the “names and types” module. Click on update

OPTIONAL: set groups

Cell Profiler analysis > set Analysis modules

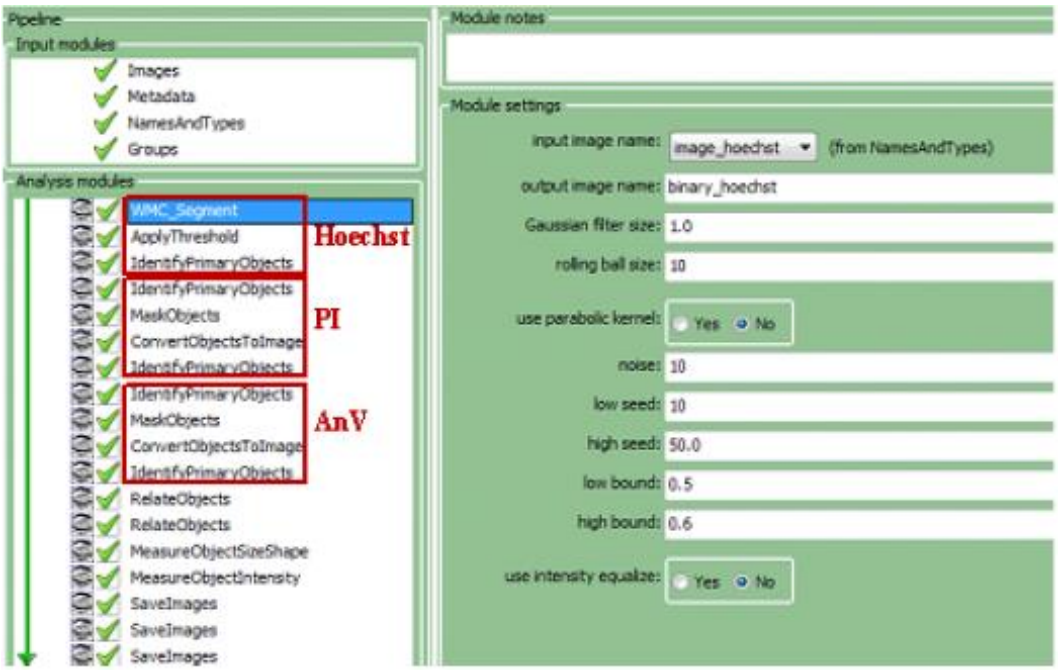
Start test mode (bottom left corner)

Nucleus identification

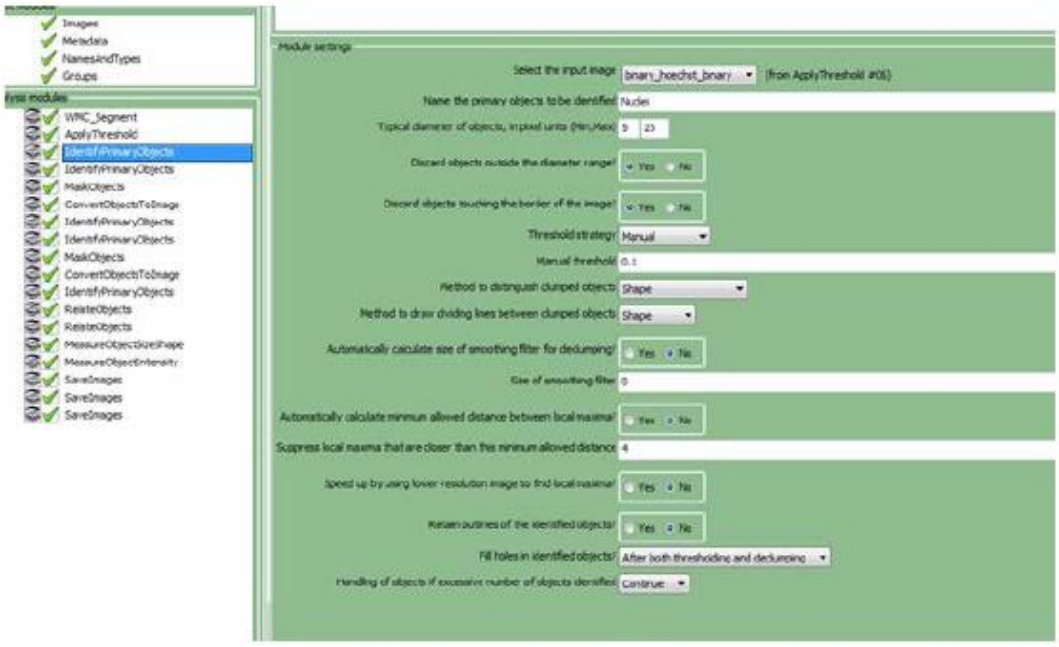
Input = Hoechst Channel

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”

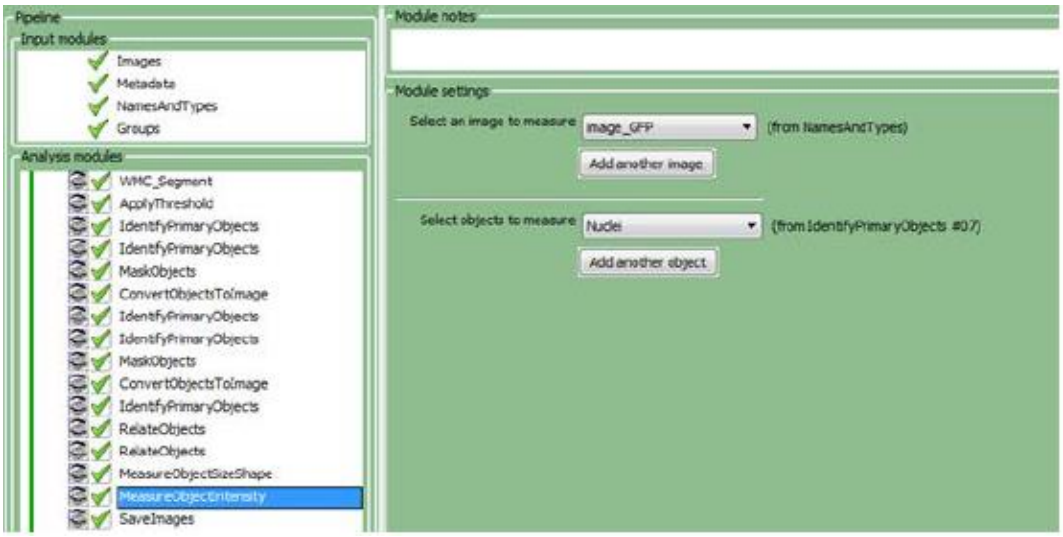


*GFP signal identification > in the nucleus (**p21, CHOP**)*

Input = GFP channel

For monitoring of GFP intensity in the nucleus, you do not need to create extra objects

MeasureObjectIntensity => Measure GFP intensity in the earlier identified Hoechst-based nuclear objects



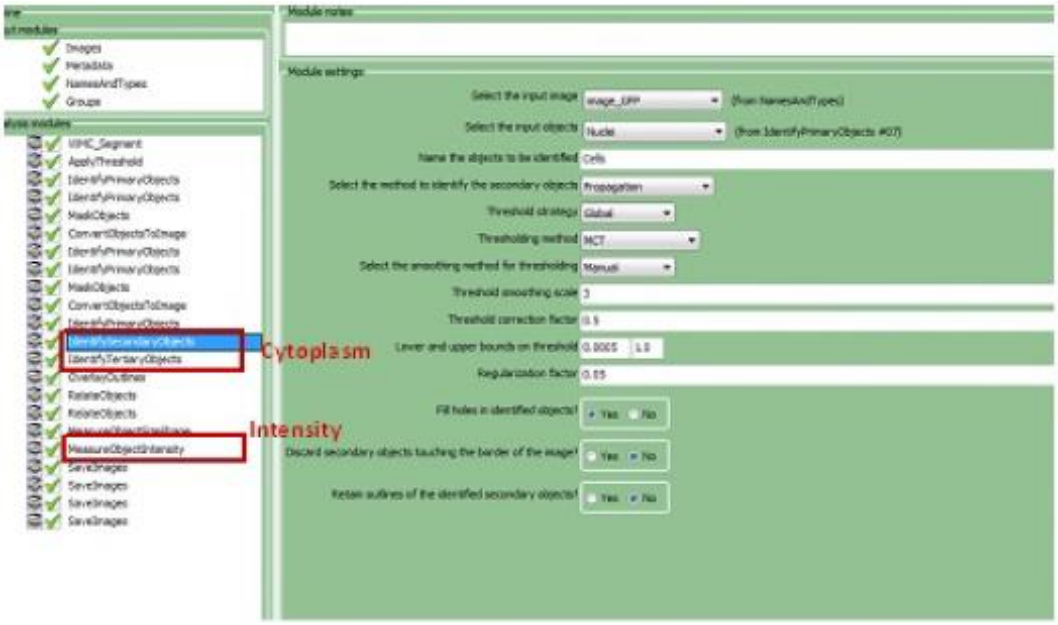
*GFP signal identification > in the cytoplasm (**Srxn1, BiP, Btg2, HMOX1**)*

Input = GFP channel

When identification of the cytoplasm is desired (for: Srxn1, BiP, Btg2, HMOX1), 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)



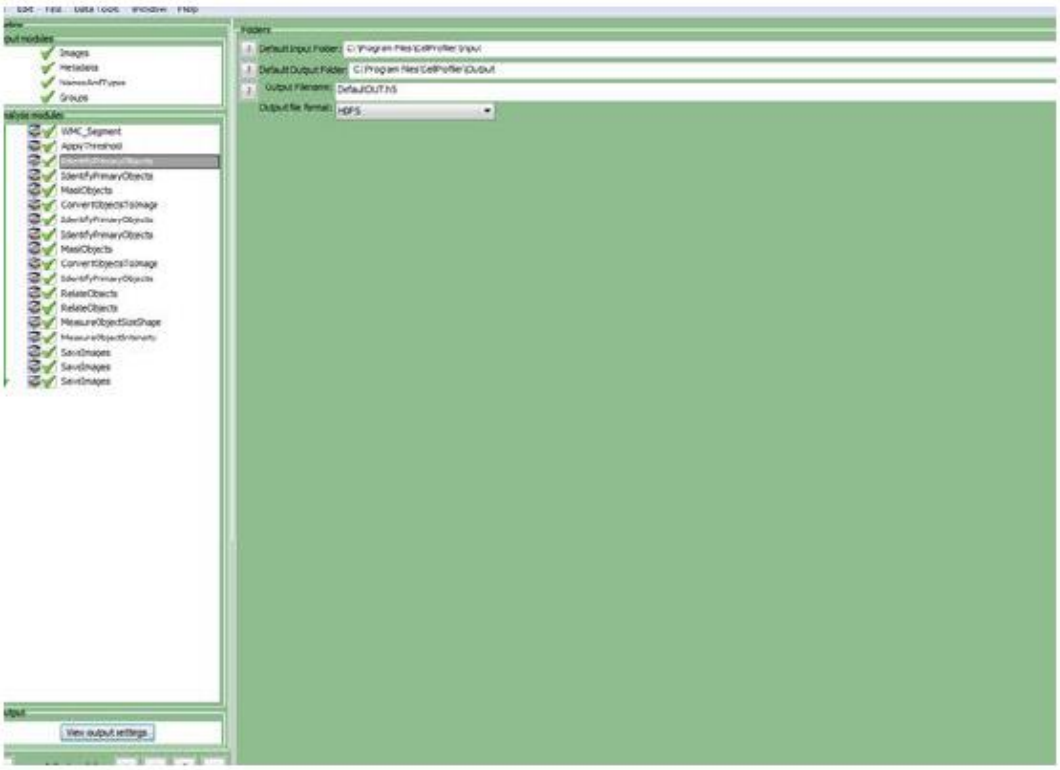
PI and AnV staining

Input = PI/AnV image

See SOP: PI-AnV staining for the analysis

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)
- Mean GFP intensity for reporter with GFP signal in the nucleus (Nuclei_Intensity_MeanIntensity_image_GFP)
- Integrated GFP intensity for reporter with GFP signal in the cytoplasm.(Nuclei_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

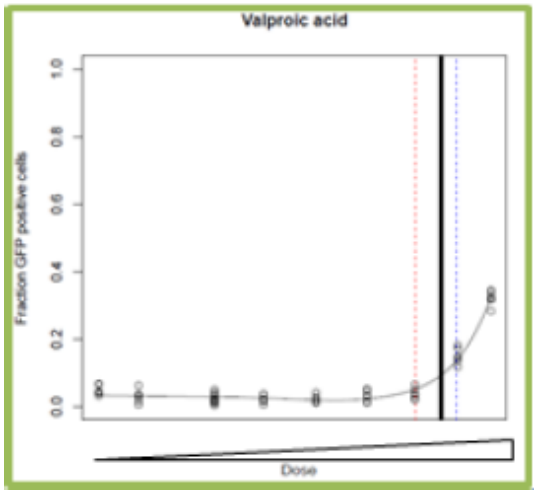
  (count_PI_masked_primaryID_AreaShape_Area.DIV.Nuclei_AreaShape_Area_larger_0.1_)
- Annexin V positive cells

Dose response analysis

The dose response modelling was performed using the R package from Christian Ritz, ritz@bioassay.dk, with the four parametric log logistic function. An in house automation script created by Steven Wink (s.wink@lacdr.leidenuniv.nl) was used to analyse the curves, which can be found on https://github.com/Hardervidertsie/drc_wrapper.

Point of Departure (PoD) modelling

The PoD modelling is based on fitting non mechanistic piece wise natural cubic spline function. The PoD is calculated as the concentration where the effect level equals the control mean plus two times the total response standard deviation. The total response standard deviation is the combined regression standard deviation from the fit and raw data control standard deviation (as the root of the summed squares). The upper and lower limit concentration levels are calculated as the PoD response level +/- 2 times the total response level standard deviation. The method is applied using an in house R script created by Steven Wink (s.wink@lacdr.leidenuniv.nl). The script depends on the R packages splines and ggplot2.



Shown is the PoD model for Valproic acid and measured cell line SRXN1 after 24h compound stimulation. Black line = PoD, red line= lower value, blue line = upper value, which represent the 95% confidence intervals.

Prediction Model

Rationale of your test method (= scientific purpose)

This HepG2 GFP reporter panel test system enables us to visualise and quantify the induction of various adaptive cellular stress responses in a systematic approach. This panel includes specific GFP reporters for the oxidative stress, ER stress, inflammation, DNA damage or heat shock response; heavy metal stress and hypoxia. For every stress response, an upstream regulator, transcription factor and target gene has been tagged in order to follow the activation of the whole signalling cascade. By combining these reporters with high content confocal imaging, perturbations of specific stress response signalling cascades by chemicals can be observed in a high-throughput manner including time dynamics of each key event. On single cell level, the induction and localisation of GFP can be observed also revealing the heterogeneity between cells in stress response induction. In this way, this test system can reveal the molecular mechanisms of toxicity. By combining these reporter with PI staining, the activation of these stress pathways can be related to induction of cell death to reveal the tipping point at which the activation of adaptive pathways are insufficient to overcome stress.

Prediction model

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

Stress response activation

Protein expression and/or translocation of stress response pathway's proteins display the onset, extent and dynamic of pathway activation. This can be described with the following readouts

The fraction of cells which are higher than two times the average GFP-level of cells exposed to DMSO are considered GFP positive. When a compound induces the number of GFP positive cells with 20% it is being considered as a stress response activator.

Cytotoxicity

The threshold of cytotoxicity is determined by dose response modelling or point of departure modelling of the PI and AnV readout.

Prediction model setup

The options for the use of this cell system as a prediction model are under investigation.

Dose response modelling can be done via BMDExpress.

IVIVE *In vitro – in vivo* extrapolation

The BAC GFP-reporter cell system can be linked to *in vivo* data by comparing the data with rat liver and hepatocytes gene expression microarray data from TG-GATEs (Igarashi Y *et al*, Nucleic Acids Res. 2015). Up or downregulation of similar networks can be verified and support the translation to the *in vivo* situation. Models for the *in vitro* kinetics will be developed soon but don't exist so far. Up until now the test system has not been used for accurate IVIVE determination.

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 the respective cellular stress response.

Incorporation in test battery

The HepG2 GFP reporter cell line can be used in combination with the other reporter cell line for comprehensive testing of a broad range of stress response pathway activation.

Strengths	Weaknesses
High-content and –throughput	Correct handling is crucial for HepG2 cells
Live time dynamics	Lack of metabolic capacity in HepG2 cell system
Identification of AOP while cell death measures can be observed	The need of a powerful confocal microscopy system including heater and CO_2 for live cell imaging
Stand-alone test system	
Single cell measurements	
Protein based reporter with endogenous regulation	

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Wink, S. *et al.*, 2014. Quantitative high content imaging of cellular adaptive stress response pathways in toxicity for chemical safety assessment. *Chemical Research in Toxicology*, 27(3), pp.338–355.

Wink, S. & Water, B. Van De, 2015. A High Throughput Microscopy Toxicity Pathway Reporter Platform for Chemical Safety Assessment. *Toxicology Letters*, 238(2), p.S38.

Wink, S. *et al.*, 2016. High-content imaging-based BAC-GFP toxicity pathway reporters to assess chemical adversity liabilities. *Archives of Toxicology*, pp.1–17.

Wink *et al.*, 2018. Dynamic imaging of adaptive stress response pathway activation for prediction of drug induced liver injury. *Archives of Toxicology* 92:1797–1814

4. HEPG2- assay

Protocol name

High-content imaging for the detection of drug-induced steatosis

Institute: HULAFE

Abstract

This protocol describes an *in vitro* assay for the detection of drug-induced steatosis and the mechanisms implicated using high-content imaging. The protocol is based on the measurement of different endpoints that are indicative of hepatotoxicity and specifically steatosis by means of high-content imaging and it has been previously used and published in the context of drug-induced hepatotoxicity ((Donato *et al.*, 2017; Donato *et al.*, 2012; Tolosa *et al.*, 2015a; Tolosa *et al.*, 2015b; Tolosa *et al.*, 2017; Tolosa *et al.*, 2016; Tolosa *et al.*, 2013; Tolosa *et al.*, 2012))

Experimental description

Biological endpoint and endpoint measurement:

Lipid overaccumulation, phospholipidosis, cell viability, GSH depletion, mitochondrial superoxide production and MMP assessed by HCI.

Endpoint value:

Reported values are minimum effective concentration (MEC) and EC_{50}/IC_{50} values in µM. Depending on the assay, MECs are defined as:

- the concentration where the test compound causes an increase equal to 20% respect to control cells (i.e. lipid overaccumulation)
- the concentration where the test compound causes a decrease equal to 20% respect to control cells (i.e. viability or GSH depletion)

Experimental system:

Assays are based on the human hepatoma cell line HepG2 (ATCC HB-8065)

1. Description of the general features of the test system

1.1 Cellular system component(s)

HepG2 cell line

HepG2 are non tumorogenic cells with high proliferation rates and an epithelial-like morphology that perform many differentiated hepatic functions.HepG2 is the human hepatoma that is most commonly used in drug-metabolism and hepatotoxicity studies.

They are usually cultured in a 2D monolayer but they can be also grown into 3D structures ((Huang *et al.*, 2016)).

1.2 Definition of cells used

- ATCC number: Hep G2 [HEPG2] (ATCC® HB-8065TM); cells were provided by University of Leiden in order that all the labs working with HepG2 cells in EU ToxRisk project use the same cells.

Origin: HepG2 was derived from a liver hepatocellular carcinoma of a 15 year old Caucasian male.

Gender: male

Morphology: epithelial

Culture properties: adherent

Doubling time: approx. 14 -20 h (depending on passage)

Expression: Cells express alpha-fetoprotein (alpha fetoprotein); albumin; alpha2 macroglobulin (alpha-2-macroglobulin); alpha1 antitrypsin (alpha-1-antitrypsin); transferrin; alpha1 antichymotrypsin; (alpha-1-antichymotrypsin); haptoglobin; ceruloplasmin; plasminogen; complement (C4); C3 activator; fibrinogen; alpha1 acid glycoprotein (alpha-1 acid glycoprotein); alpha2 HS glycoprotein (alpha-2-HS-glycoprotein); beta lipoprotein (beta-lipoprotein); retinol binding protein (retinol-binding protein).

1.3 Maintenance culture protocol

Cells are maintained in DMEM + P/S + 10 % FBS. Cells are normally passaged twice/week and experiments for HCI are seeded in 96-well plates at 5000 cells/well.

2. Definition of the test system

2.1 Principles of the culture protocol

Cells are maintained in DMEM + P/S + 10 % FBS. Cells are normally passaged twice/week and experiments for HCS are seeded in 96-well plates at 5000 cells/well.

HepG2 cultures are exposed to exogenous lipids, as previously described [1, 2]. Briefly, cells are incubated with 62µM of free fatty acid mixture (2:1 ratio of oleate and palmitate) in culture medium containing 1% bovine serum albumin (BSA) for 14-16 hrs, followed by a change to standard culture medium containing different concentrations of the test compounds and exposed for the required time period.

2.2 Endpoints

Control under microscope: morphology and doubling time.

2.3 Analytical endpoints

Actually used method: Microscopical visualisation

2.4 Acceptance criteria for test system

Qualitative evaluation

- medium not orange/yellow
- appropriate cell density

2.5 Features relevant for cytotoxicity testing

Cells are sensitive to many toxicants. We have different positive controls according to the final goal of the experiment.

2.6 Metabolic capacity of the test system

Important cytochrome P-450 (CYP) enzymes involved in phase I drug oxidations in the liver, such as CYP3A4, CYP2C9, CYP2C19, CYP2A6 or CYP2D6, are lacking or barely detectable in HepG2 cells. The abundance of most expressed drug-metabolizing CYP genes is markedly lower (<10%) than in primary hepatocytes (Guo *et al.*, 2011). This poor expression of CYP enzymes in HepG2 cells has been related to their altered pattern of the so-called liver-enriched transcription factors, a group of transcription regulatory proteins that control the hepatic expression of different genes, including CYPs (Jover *et al.*, 1998; Rodriguez-Antona *et al.*, 2002). Although the overall expression levels of phase II enzymes UDP-glucuronosyltransferase, glutathione S-transferase (GST), sulfotransferase or N-acetyltransferase (NAT) are lower in HepG2 cells than in human hepatocytes, the observed differences are even less marked than for CYPs (Guo *et al.*, 2011).

3. Test method description

3.1 Test documentation in published literature

Donato, M. T., Gomez-Lechon, M. J., and Tolosa, L. (2017). Using high-content screening technology for studying drug-induced hepatotoxicity in preclinical studies. Expert Opin Drug Discov 12, 201-211.

Donato, M. T., Tolosa, L., Jimenez, N., Castell, J. V., and Gomez-Lechon, M. J. (2012). High-content imaging technology for the evaluation of drug-induced steatosis using a multiparametric cell-based assay. J Biomol Screen 17, 394-400.

Tolosa, L., Carmona, A., Castell, J. V., Gomez-Lechon, M. J., and Donato, M. T. (2015a). High-content screening of drug-induced mitochondrial impairment in hepatic cells: effects of statins. Arch Toxicol 89, 1847-1860.

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Tolosa, L., Gomez-Lechon, M. J., and Donato, M. T. (2015b). High-content screening technology for studying drug-induced hepatotoxicity in cell models. Arch Toxicol 89, 1007-1022.

Tolosa, L., Gomez-Lechon, M. J., and Donato, M. T. (2017). A Multi-Parametric Fluorescent Assay for the Screening and Mechanistic Study of Drug-Induced Steatosis in Liver Cells in Culture. Curr Protoc Toxicol 72, 14 15 11-14 15 11.

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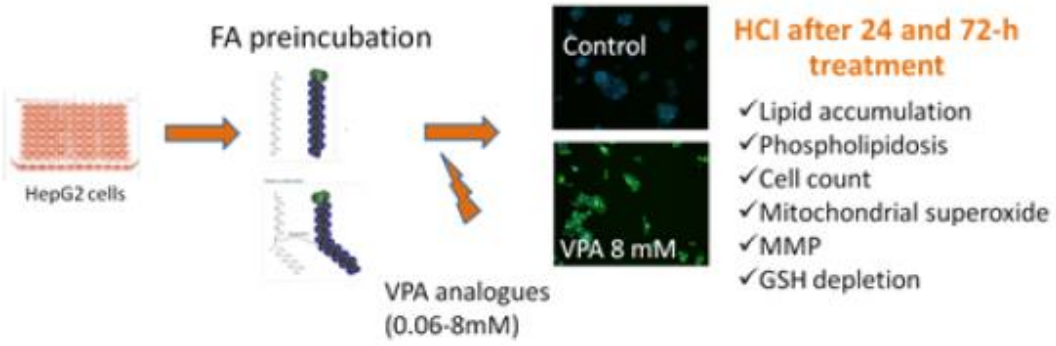
3.2 Exposure scheme for toxicity testing



3.3 Test method endpoint

24-h or 72-h after compound's incubation, medium is removed and cells are incubated with a mixture of fluorescent probes indicative of pre-lethal or lethal changes in the cells:

Table 1. Selection of fluorescent probes and endpoints for the study of drug-induced steatosis (HULAFE).

Endpoint	Fluorescent probe	Excitation /emission wavelengths
Cell count	Hoechst 33342	361/486
Viability/ Apoptosis	PI	536/617
Oxidative stress	CellROX mBCl (GSH)	644/665 390/478
Mitochondrial injury	MitoSOX Red TMRM	510/580 549/576
Lipids staining	BODIPY493/503 LipidTOX™ Red Phospholipidosis	493/503 595/615

3.4 Analytical endpoint(s) overview

Following treatments, cells are simultaneously loaded with 1a combination of fluorescent probes. After a 30-min incubation at 37°C with the culture media containing fluorescent probes, cells are imaged using the iNCELL Analyser 6000 (GE Healthcare), as previously described (Tolosa *et al.*, 2012). Fluorescent dyes were combined according to their optical compatibility in a standard flow cytometer (Donato *et al.*, 2009). The 20X objective was used to collect images for the distinct fluorescence channels. Nine fields per well were imaged and analysed by using INCELL6000 module which allows simultaneous quantification of subcellular inclusions that are marked by different fluorescent probes and measures fluorescence intensity associated with predefined nuclear and cytoplasmic compartments.

The cell count is generated from the number of Hoechst 33342-stained nuclei. This probe also allowed the detection of condensed pycnotic nuclei in apoptotic cells. Cell viability is determined by PI exclusion. Since PI is not permeant to live cells, it is also commonly used to detect dead cells in a population. This allows not only the direct quantification of cytotoxicity, but also the exclusion of dead cells from the HCI, thus restricting further functional determinations to the live-cell population in each sample. Intracellular fat deposits were detected by measuring BODIPY 493/503 fluorescence; phospholipids accumulation was determined by the quantification of intracellular LipidTox red

inclusions; cellular mitochondrial membrane potential (MMP) is defined as the TMRM fluorescence intensity in the punctuate cytosolic regions around the nucleus.

Reactive oxygen species (ROS) production is measured as an increase in CellROX fluorescence intensity in the cytoplasm. GSH depletion is defined as a decrease in mClB intensity fluorescence. Each measure is performed in individual cells; the values for the same treatment (e.g., triplicate wells) are averaged and then normalised by the average value from the untreated cells.

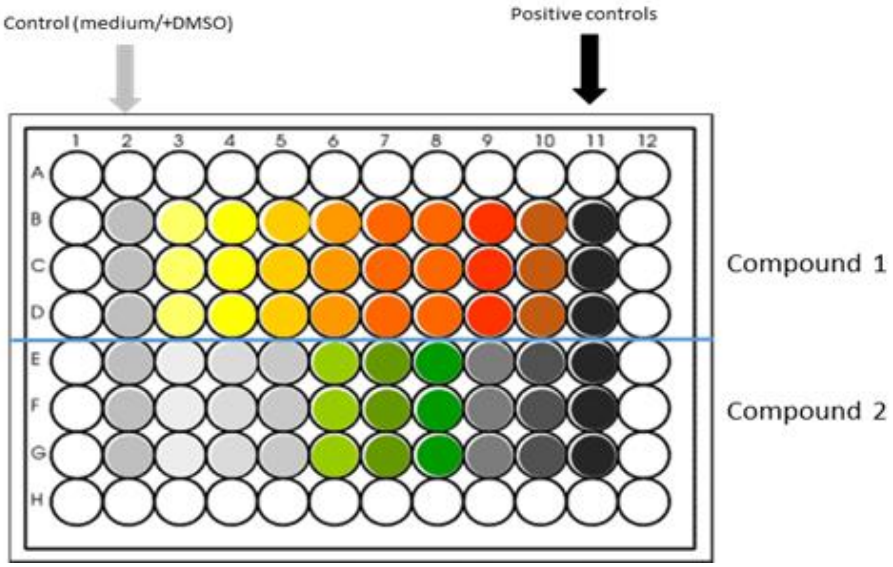
3.6 Acceptance criteria

Differences about 10% between replicates let us to discard an experiment.

3.7 Throughput estimate

Data point = three biological replicates; each concentration/condition of a compound counts as data point

Typical plate



3.8 Concentration settings

As default a serial dilution 1:2 is used.

3.9 Special instrumentation

The method requires a HCI system (in our case INCELL Analyser 6000, GE Healthcare) that may not be present in a standard lab.

Health and safety issues

General precautions:

Standard laboratory procedures apply. Always use gloves and labcoat; when working with dangerous or unknown compounds, always work in a fume hood. If the dilution/exposure

work is performed by a liquid handling machine, the risk for the employee is greatly reduced.

MSDS information:

In addition to the safety measures regarding the compounds in use, there are no safety measures needed for the performance of this method.

Contact person:

Laia Tolosa, PhD
Unidad Hepatología Experimental.
FUNDACIÓN PARA LA INVESTIGACIÓN DEL HOSPITAL LA FE.
Avinguda de Fernando Abril Martorell 106, Hospital U. y P. La Fe, Torre A, planta 6ª. Lab 6.06
46026 Valencia, SPAIN
email: laiatolosa@hotmail.com
Tel: +34961246622

Materials and preparations

Assays are based on the human hepatoma cell line HepG2 (ATCC HB-8065).

Equipment

Fixed Equipment

- Cell culture incubator (37°C, 5% CO_2)
- Centrifuge
- Freezers (-20 and -80 and liquid nitrogen)
- Inverted phase contrast Microscope
- Laminar flow cabinet
- Liquid handling machine (Hamilton STARlet)
- INCELL Analyser 6000 (GE Healthcare)

Reagents

Sodium oleate (Sigma O7501)

Sodium palmitate (Sigma P9767)

Methanol

PBS

Bovine Serum Albumin solution 30% Fatty acid free (Sigma A-9205)

DMEM (Gibco)

FCS: (Hyclone Research Grade FBS South American origin. Batch RAB35926)

Lipid depleted serum (Biowest S181L)

0.25% trypsin/0.02% EDTA (Gibco BRL, Paisley, UK)

Fluorescent probes (Table 1)

PROTOCOL

1. Seed of the cells

HepG2 cells were cultured in DMEM (Gibco BRL, Paisley, UK) supplemented with 5% newborn calf serum, 50 U penicillin/mL and 50 µg streptomycin/mL. For subculturing purposes, cells were detached by treatment with 0.25% trypsin/0.02% EDTA (Gibco BRL, Paisley, UK) at 37ºC. For steatosis-inducing studies, HepG2 cells were seeded into 96-well culture plates at a density of 5000 cells/well. External files and columns are left without cells but contain culture media in order to protect the culture. Before treatments, cells were allowed to grow and equilibrate for 24 hours.

2. Dissolution of free fatty acids

- Dissolve sodium oleate at 50 mM in PBS. Aliquot and storage at -20ºC until use.
- Dissolve sodium palmitate at 50 mM in methanol. Aliquot and storage at -20ºC until use.

3. Preparation of culture medium containing FA mixture

- Prepare the FA medium as follows:

HepG2 medium (DMEM-10% lipid depleted FBS)* 98 ml

BSA 30% (FA free) 2 ml

Palmitate 50 mM 41.3 µl

Oleate 50 mM 82.6 µl

*FBS contains approximately 4% of albumin

4. Pre-loading with FA

24 h after seeding remove culture medium and add the culture media containing the FA mixture*.

*add FA last thing the day before dosing in order to incubate with FA for 14-16 h.

5. Incubation with test compounds

14-16 h after adding the FA-medium, remove medium and change to lipid-free culture medium containing different concentrations of the test compounds the required period.

6. High-content imaging assay

- Remove the cell media and incubate the cells with a combination of fluorescent probes as follows:

Panel 1: 3.75 ng/ml BODIPY 493/503, 1X LipidTOX phospholipidosis and 1.5 µg/ml Hoechst 33342

Panel 2: 75 µg/ml TMRM, 2 µg/ml mClB, 1.5 µg/ml PI

Panel 3: 5 µM MitoSOX and 1.5 µg/ml Hoechst 33342) + 5 µM CellROX +1.5 µg/ml Hoechst 33342

- Incubate for 30 min at 37ºC
- Remove the combination of probes and add pre-warmed medium
- Adquire images with INCELL Anlyser 6000
- Analyse the images with specific protocol for quantification

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5. HepaRG 3D Spheroid HCI Assay

5.1 General information

5.1.1 Assay name

High content imaging of HepaRG 3D Spheroids to detect drug-induced steatosis

5.1.2 Summary

This protocol describes an *in vitro* assay for the detection of drug-induced using high-content imaging in a three-dimensional (3D) spheroid model using HepaRG cells. The prediction of drug-induced steatosis is based on the measurement of different endpoints that are indicative of hepatotoxicity and specifically changes in lipid accumulation after a long-term exposure of the 3D spheroid model to test compound. The use of 3D HepaRG spheroid model in high-content imaging (Ramaiahgari *et al.* 2017) as well as the use of HepaRG to predict steatosis (Tolosa et. al 2016) have been published previously.

5.1.3 Date of method description

N.A.

5.1.4 MD authors and contact details

Dr. Paul Walker, Cyprotex Discovery Ltd
No. 24 Mereside,
Alderley Park,
Macclesfield,
Cheshire,
UK.
SK10 4TG
+44.(0)1625.505151
p.walker@cyprotex.com;

5.1.5 Date of MD updates and contacts

29.04.2019, Dr. Caroline Bauch, c.bauch@cyprotex.com

5.1.6 Assay developers/laboratory details

Toxicology team, Cyprotex Discovery Ltd.,
No. 24 Mereside,
Alderley Park,
Macclesfield,
Cheshire,
UK.
SK10 4TG

5.1.7 date of assay development and/or publication

N.A.

5.1.8 References to main scientific papers

See Bibliography

5.1.9 availability of information about assay in relation to proprietary elements

N.A.

5.1.10 availability of information about throughput of the assay

96 or 384 well plate format.

5.1.11 Status of method development and uses:

N.A.

5.1.12 Abbreviation and Definitions

3D – three dimensional

MEC – minimum effective concentration

AC_{50} - concentration with 50% maximum effect

HCS/HCI – high content screening/high content imaging

DMSO – dimethyl sulfoxide

ATP - Adenosine triphosphate

PHH – primary human hepatocytes

5.2. Test Method Definition

5.2.1 Purpose of the test method

Drug-induced steatosis is determined by measuring cell health markers including the accumulation of specific fluorescent lipids and cellular ATP in HepaRG spheroids following 14 days of repeated compound exposure. *In vitro* toxicity assays traditionally utilise cells restricted to a two dimensional (2D) single cell type arrangement not suitable for long-term exposure studies. The environment created by a 3D model allows reconstitution of the natural cellular physiology by promoting the complex cell-cell and cell matrix network communications found *in vivo* allowing improved toxicity prediction. Confocal high content screening (HCS) allows the simultaneous detection of spheroid size and accumulation of lipids in combination with a measure of cellular ATP. The test compound is considered positive to cause steatosis when a fluorescent dye indicates accumulation of lipids.

5.2.2 Scientific principle of the method:

Drug induced steatosis is assessed in 3D HepaRG spheroids by measuring the accumulation of lipids in each spheroid upon test compound treatment using fluorescent dyes. Cellular ATP indicates viability of the cells and is assessed as ATP content of each corresponding spheroid using a biochemical assay.

5.2.3 Tissue, cells or extracts utilised in the assay and the species source:

Cryopreserved HepaRG cells purchased through Thermofisher

5.2.3 Metabolic competence of the test system:

HepaRG are considered to be a metabolically complete system. Over the formation period and dosing period the cells are induced with DMSO to maintain the metabolic competency of the cells.

5.2.5 Description of the experimental system exposure regime:

HepaRG cells are seeded into ultra-low adhesion microplates in appropriate culture media containing DMSO. The cells are left at 37 ºC and 5% CO_2 to from 3D spheroids. After 4 days of culture, the media is replenished with appropriate media (Bell *et al.* 2016) containing DMSO. Dosing of test compounds commences at day 7 after seeding. Test compound is diluted in culture medium at 0.5% DMSO and added to spheroids at time points described in the table below for 10-day repeat dose. Each compound is tested as an 8 point dose response curve with n=3 replicates per concentration. The spheroids are stained with Hoechst 33342 (sigma) and HCS LipidTOXTM Red Neutral Lipid Stain (ThermoFisher). The spheroid count, spheroid size, DNA structure and Steatosis measurements are determined using an Arrayscan XTi/CX7 and the cellular ATP is determined at the end of the assay (CellTitre-glo® 3D cell viability assay, Promega) and read using a luminescence reader (Biotek).

Table 2. Dosing regimen for HepaRG 10 day repeat dose.

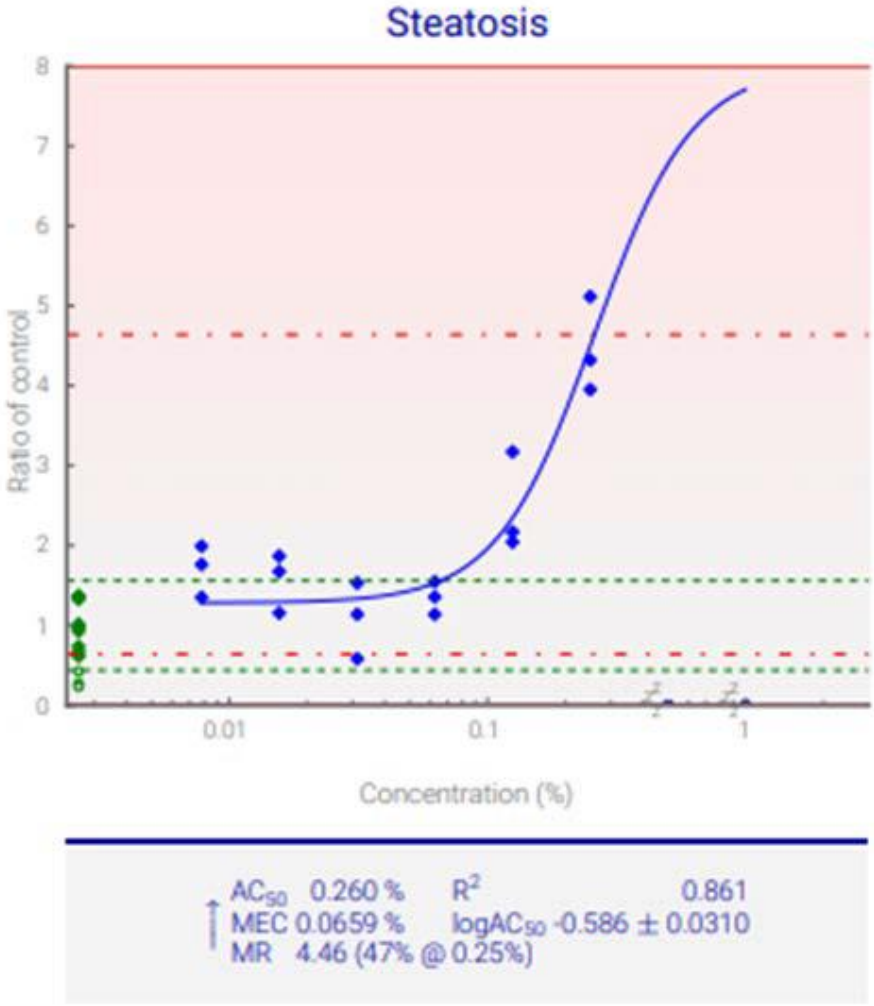
10 Days Rpt Dose	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Dose	Dose			Dose			Dose			Dose	
Endpoint/Assay											Assay

5.2.6 Response and Response Measurement:

Reported values are minimum effective concentration (MEC), AC_{50}, $logAC_{50}$, Maximum Response (MR), and R^2. MEC is the concentration at which the dose response curve crosses the vehicle control threshold. AC_{50} is the concentration at which 50% maximum effect (based on historical assay data) is observed for each cell health parameter. MR is the maximum response of each endpoint as a ratio of control.

Figure 1: Green dashed lines: Significant cut-off from vehicle control (used to calculate the MEC).

Filled blue diamonds: Mean data points for each concentration (plus or minus standard deviation). Blue x: Data points excluded from plot due to precipitate in well. Open blue circles: Data points excluded from plot due to data plateau, or other reasons. Points lying outside y-axis limits are annotated with small arrows. Open blue squares: Data points excluded from plot due to cell loss or nuclear size. Red solid lines: Historical maximum and minimum responses, used to calculate AC_{50}.



5.2.7 Quality / Acceptance criteria:

Quality and acceptance criteria for HepaRG cells: viability >70% after thawing, consistent cell count compared to historical cell counts

Quality and acceptance criteria for HepaRG 3D spheroids: spheroid size (circumference) and cellular ATP levels of negative controls compared to historical data.

Positive controls: sertraline is a known steatosis-inducing compound

5.2.8. Known technical limitations and strengths:

HepaRG™ cells have expression levels of key metabolic enzymes and nuclear receptors consistent with levels observed in PHH, and therefore, are more suitable to assess the metabolic stability of candidate compounds (Guillouzo *et al.* 2007)

HepaRG™ cells are a metabolically competent system and tolerant of long culture periods (i.e. =22 days). In addition, they are well suited for *in vitro* determinations of acute and chronic toxicity resulting from intrinsic and/or metabolism-based mechanisms. (Damell *et al.* 2012, Gunnes *et al.* 2013, Leite *et al.* 2012)

HepaRG™ cells lack donor variability, and lot sizes are not limited by donor tissue availability, ensuring an indefinite and consistent supply of cells. Thus experimental results can be reproduced over long term studies.

5.2.9 Other related assays that characterise the same event as in 5.2.1:

5.3. Data interpretation and prediction model

5.3.1 Assay response(s) captured in the prediction model:

N.A.

5.3.2 Data analysis:

The assay measurements are spheroid count, spheroid size, DNA structure, Steatosis and Cellular ATP.

Data was normalised to vehicle control and for each compound dose-response curves were defined and evaluated with the following equations:

$$\xi(C; c;\ \omega) \equiv {(\ln(C) - c)}/{\omega},$$

$$t(\xi) \equiv {(1 + \tanh(\xi))}/{2};$$

$$R(t; R0; R^{\infty}) \equiv R0(1 - t) + R^{\infty}t;$$

In which C represents the test compound concentration and R0, R^{∞}, c, and ω are fitting parameters. The final response at a given concentration C is expressed as R(t(ξ(C; c; ω));R0;R∞). It was restricted such that ω > 0, which implies R ≡ R0 as C ≡ 0 and R ≡ R∞ as C ≡ ∞. The lowest concentration exceeding the vehicle control limits (0.85 – 1.15 of the vehicle control values) were defined as the minimum effective concentration (MEC). The concentration, which results in 50% response, was defined as an AC_{50} value and calculated according to historical data for maximal responses. The coefficient of determination (R^2) was calculated for each compound and each feature assessed. Responses with a R^2 value smaller than 0.65 were rated as non-significant and were not considered a true response.

5.3.3 Explicit prediction model:

N.A.

5.3.4 Software name and version for algorithm/prediction model generation:

In house LIMS system

5.4. Test Method Performance

N.A.

5.4.1 Robustness of the method

N.A.

5.4.2 Reference chemicals/chemical libraries, rationale for their selection and other available information:

See positive controls

5.4.3 Performance measures/predictive capacity (if known):

N.A.

5.4.4 Scope and limitations of the assay, if known:

N.A.

5.5. Potential Regulatory applications

N.A.

5.5.1 Context of use:

N.A.

5.6. Bibliography

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5.7. Supporting information

N.A.

6. Primary human hepatocytes

6.1 Acute cytotoxicity assay in primary human hepatocytes

IFADO – Prof. Dr. Jan Hengstler

All study compounds were tested in cryopreserved primary human hepatocytes from three different donors. The hepatocytes were obtained from BioIVT.

Protocols:

Thawing and plating of cyropreserved primary human hepatocytes

Materials needed:

Cryopreserved human hepatocytes (IVT Bioreclamation),

Rat tail collagen 0.25 mg/ml in 0.2% Acetic acid,

96 well cell culture plates, PBS, FBS, PHH cultivation medium, KHB buffer (Invitrogen)

Cut tips (1ml and 200µl), 0.2% Tryptanblue, Neubauer counting chamber

Preparations:

PHH cultivation medium:

Table 1. PHH cultivation medium

Component	Volume
William's E medium (PAN Biotech P04_29510)	500 ml
Penicillin/ Streptomycin (PAN Biotech P06-07100)	5ml
Gentamicin (PAN Biotech P06-13001)	500µl
Dexamethason (Sigma-Aldrich D4902)	20 µl 2.5mM Stock in ethanol
Stable L – Glutamine (PAN Biotech P04-82100)	5ml
Insulin supplement (Sigma 3146)	5µl

Plating medium:

Table 2. Plating medium

Component	Volume
PHH cultivation medium	90 ml
FBS (SeraPlus Pan Biotech 3702-P103009)	10ml

The 96 well plates are coated rat tail collagen. 100µl 0.25 mg/ml rat tail collagen are added to each well and are immediately removed again. Afterwards the plates are left drying at room temperature for at least 2h with open lids or overnight with closed lids. Prior to thawing and plating of the cells the plates are washed 3 times with PBS. The medium or PBS can be removed by tipping the cell culture plates on folded paper towels.

200 µl of warm plating medium are pipetted in each well. Our experience shows that is beneficial not to use the wells on the border of the plate but to fill them with PBS instead.

For each donor a 50ml tube with 5ml of plating medium per vial is prepared.

Thawing and plating of PHH:

The primary hepatocytes are quickly thawed in a 37°C water bath. As soon as the first liquid is visible the cells are added to the tubes with plating medium by inverting the cryovials. Any remaining cells in the cryovials are collected by transferring 1 ml of cell suspension into the cryovial and pipetting the entire volume back into the tube with a cut-tip 1ml pipette tip.

The cell suspension is mixed well by gently inverting the tubes. For counting the cells a 1:10 dilution is prepared (350µl medium or buffer – we use KHB buffer + 100µl 0.2% tryptan blue + 50µl cell suspension. The cell suspension is added to the dilution with a cuttip 200µl pipette tip. The dilution is mixed by pipetting gently pipetting up and down.

Both chambers of the Neubauer counting chamber are filled with the dilution and all 8 squares are counted under the microscope. Afterwards the number of vital cells/ square, dead cells/square; cells/square and vitality are calculated. To determine the number of vital cells/ml the number of vital cells/ square is multiplied with 10 ^5. For each well 50 000 vital cells are needed. Therefore the volume for 50 000 vital cells is calculated and divided by the vitality.

Before and during the plating of cells the cell suspension is frequently mixed. For plating the cells cut tip 200µl pipette tips are used. 50 000 vital cells/ well are added to the prepared 96-well plates with 200 µl of plating medium / well.

The cells are left to attach for 3 hours at 37°C and 5% CO_2. For the first 30 minutes the plates are gently moved in north- south and east – west direction every 5-10 minutes.

After attachment the cells are washed 3 times with warm PBS and 200 µl/well PHH cultivation medium is added. After 16-20 hours of culture at 37°C and 5% CO_2 the experiments start. The medium or PBS can be removed by tipping the cell culture plates on folded paper towels.

Experimentally induced steatosis in primary hepatocytes

Materials needed:

Plated human hepatocytes, PHH cultivation medium, 30% fatty acid free BSA in saline (Sigma A9205), 50mM Sodium oleate in PBS, 50mM sodium palmitate in methanol.

Preparations:

Preparation of fatty acid stock solutions:

For preparing the 50mM sodium oleate stock solution 15.22 mg of sodium oleate (Sigma 07501) are dissolved in 1ml of PBS by vortexing and warming the solution in a 70-80°C water bath for approximately 20 minutes.

For preparing the 50mM sodium palmitate stock solution 13.92 mg of sodium palmitate (Sigma P9767) are dissolved in 1ml of methanol by vortexing and warming the solution in a 70-80°C water bath for approximately 1 hour.

The aliquots are stored at -20 °C.

Preparation of the media:

The fatty acid and BSA media are freshly prepared each day.

BSA medium:

Table 3. BSA medium

Component	Volume
PHH cultivation medium	9.8ml
30% BSA solution	0.2ml

Fatty acid media:

Table 4. Fatty acid media

	62µM fatty acid medium	558µM fatty acid medium
PHH cultivation medium	4.9 ml	4.9 ml
30% BSA solution	100 µl	100µl
50mM palmitate	2.07 µl	18.63µl
50mM oleate	4.13µl	37.17µl

Pre-incubation with fatty acids:

16-20 hours after plating the cells the pre-incubation with fatty acid is started.

The medium is removed is by tipping the cell culture plates on folded paper towels.

Afterwards the wells for steatosis treatment receive 200µl/well of fatty acid medium (Table 4). As an additional control some wells receive 200µl/well of BSA medium (Table 3).

The control wells receive 200µl of PHH cultivation medium. Afterwards the cells are incubated for 24 hours at 37°C and 5% CO_2.

Cytotoxicity testing in experimentally induced steatotic primary hepatocytes:

Materials needed:

Plated human hepatocytes, PHH cultivation medium, 30% fatty acid free BSA in saline, 50mM Sodium oleate in PBS, 50mM sodium palmitate in methanol, PBS, Cell Titer Blue Assay (Promega G8081), plate reader (Infinity M200 Pro plate reader – Tecan), black 96 well cell culture plates (Sigma Aldrich M0562).

Preparations:

Treatment solutions:

For the cytotoxicity assay treatment solutions are prepared. In general 5 concentrations and vehicle matched controls are prepared for each compound applying a dilution factor of 3.16.

For each concentration and condition 2-3 wells are required. Medium soluble compounds are freshly solved in the corresponding media and are sterile filtered. For DMSO solvable compounds stock solutions (200x or 1000x) can be prepared, which are freshly diluted in media on the day of the experiment.

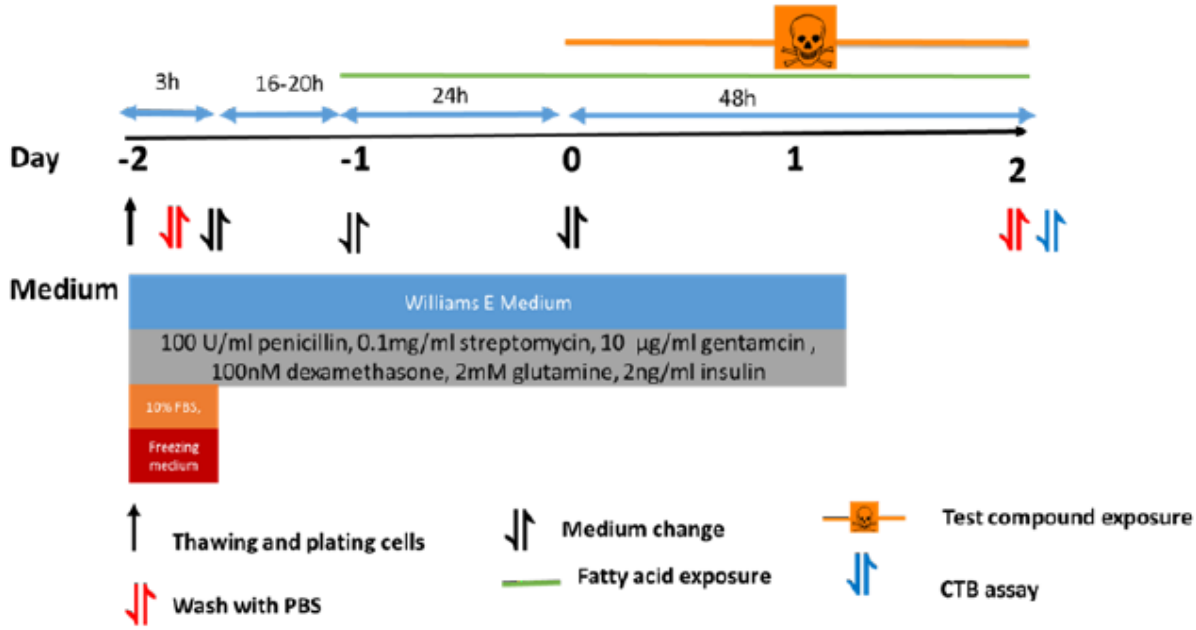
Cytotoxicity testing in experimentally induced steatotic primary hepatocytes:

After 24 hours of pre-incubation with the fatty acid media the medium is removed by tipping the cell culture plates on folded paper towels. The control wells and half of the pre-

incubated wells receive 200µl/well treatment solutions in PHH cultivation medium, the BSA pre-incubated wells receive 200µl/well treatment solution in BSA medium and half of the pre-incubated wells receive 200µl/well treatment solution in fatty acid medium (please refer to figure 2 for an example).

After incubation for 48 hours at 37°C and 5% CO_2 the cells are washed 3 times with warm PBS and 100µl/well of diluted Cell Titer Blue reagent (80µl PHH cultivation medium + 20 µl Cell Titer Blue reagent are pipetted in each well. The cells are incubated for 2-4 hours at 37°C and 5% CO_2 until the Cell Titer Blue reagent in the vehicle control wells is metabolised (turned pink). Afterwards the supernatant is quickly transferred into a black 96-well plate using a multipipette. The plate with the supernatant is kept in the dark and is read out in the plate at reader at excitation 540nm and emission 590nm. Be careful to include some wells of diluted Cell Titer Blue reagent as control for background substraction.

Figure 1. Exposure scheme



Concentrations tested;

Compound	Control [mM]	C1 [mM]	C2 [mM]	C3 [mM]	C4 [mM]	C5 [mM]
(S)- (+)-2-Methylbutyric acid	0	0.316	1	3,16	10	31.6
2,2- dimethyl-pentenoic acid	0	0.316	1	3,16	10	31.6
2-ethyl1-hexanol	0	0.1	0.316	1	3,16	10
2-ethylbutyric acid	0	0.316	1	3,16	10	31.6
2- Ethylhepatanoic acid	0	0.316	1	3,16	10	31.6
2-Ethylhexanoic acid	0	0.316	1	3,16	10	31.6
2-Ethylpentenoic acid	0	0.316	1	3,16	10	31.6
2- methylhexanoic acid	0	0.316	1	3,16	10	31.6
2- methylpentenoic acid	0	0.316	1	3,16	10	31.6
2-n-propylhexanoic acid	0	0.316	1	3,16	10	31.6
2-n-propylhepatanoic acid	0	0.316	1	3,16	10	31.6
4-ene VPA	0	0.316	1	3,16	10	31.6
4-pentenoic acid	0	0.316	1	3,16	10	31.6
Hexanoic acid	0	0.316	1	3,16	10	31.6
Octanoic acid	0	0.316	1	3,16	10	31.6
Pivalic acid	0	0.316	1	3,16	10	31.6
Propionic acid	0	0.316	1	3,16	10	31.6
Valproic acid	0	0.316	1	3,16	10	31.6

Data analysis:

The Background corrected fluorescence values were used for curve fitting to obtain EC_{20} values.

6.2 Lipid droplet accumulation in primary human hepatocytes

Institute: IFADO

For the compounds valproic acid, pivalic acid and 2- propylhexenoic acid the lipid accumulation was investigated in primary human hepatocytes from three different donors. The primary hepatocytes were obtained from BioIVT. The lipid accumulation was tested after 1 day and 5 days of treatment with the study compound in medium containing 62µM fatty acids. The cells were cultivated utilizing a gel on the top approach with a thin collagen layer on the coverslip and a thick collagen layer on top of the cells.

Protocols:

Thawing and plating of human primary hepatocytes:

Preparations:

24-Well plates (6 x 1/2) are assembled with glass cover slides and covered with collagen monolayer gel. Immediately aspirated the gel and leave the plates lid-opened for drying for 2 h. Afterwards the plates are washed 3x with sterile PBS and 500µl of plating medium is added to each well.

For plating medium:

please refer to acute toxicity testing in primary human hepatocytes.

Before and during the plating of cells the cell suspension is frequently mixed. For plating the cells cut tip 200µl pipette tips are used. 250, 000 vital cells/ well are added to the prepared 24-well plates with 500 µl of plating medium / well.

The cells are left to attach for 3.5 hours at 37°C and 5% CO_2. For the first 30 minutes the plates are gently moved in north- south and east – west direction every 5-10 minutes.

After attachment the wells are washed three times with warm PBS and a thick layer of collagen is added on top of the cells.

Preparation of the collagen:

For 10 ml of 1mg/ml collagen dissolve 10mg of ratail collagen in 9ml of 0.2% acetic acid. Add 1ml of 10x DMEM and titrate dropwise with sodium hydroxide until a colourshift to pink is observed.

Add 100µl of 1mg/ml collagen to each well and distribute well. Afterwards the collagen is left to polymerise at 37°C and 5% CO_2 for 5 minutes. After polymerisation 0.5 ml of cultivation medium is added to each well (please refer acute toxicity testing in primary human hepatocytes).

After 16 to 20 hours the cells can be treated with study compounds.

Treatment:

The cells were treated with the compounds dissolved in medium containing 62µM fatty acids.

For preparation of the medium please refer to acute toxicity testing in primary human hepatocytes. The cultivation medium is aspirated and 0.5ml of treatment solution is added to each well. The cells were incubated for 1 day and 5 days with the compounds. During the 5 day incubation period the medium was exchanged each day with new treatment solution.

The primary human hepatocytes were incubated with the following concentrations of compounds:

Compound	Control [mM]	C1 [mM]	C2 [mM]	C3 [mM]
Valproic acid	0	1	5	10
Pivalic acid	0	0.1	0.5	1
2- Propylhexenoic acid	0	1	5	10

Fixation:

After 1 day or 5 days of exposure the treatment solution was aspirated and the cells were washed 2x with PBS for 5 minutes and fixated with 0.5ml/ well 4% PFA at 7°C for 20 minutes. Afterwards the cells were washed three times for 5 minutes with PBS and stored at 4°C in PBS.

Staining:

After fixation the cells were stained with bodipy (lipid droplets), phalloidin (actin) and DAPI (cell nuclei). First the cells were permeabilised for 10 minutes with 0.5ml of 0.3% Triton X in PBS at room temperature. After permabilisation the cells were washed two times with PBS for 5 minutes with PBS at room temperature. A phallodin solution was prepared by adding 40µl phalloidin to 20mlm of 0.3% BSA solution in PBS. The cells were incubated with 0.25ml of phalloidin solution for 2 hours in the dark at room temperature, while shaking. Afterwards the cells were washed three times for 5 minutes with PBS at room temperature. For staining of the lipid droplets a bodipy solution was prepared by

adding 600µl of a 1mg/ml bodipy solution to 11.4ml PBS. The cells were incubated with 0.15ml of the bodipy soluition for 45 minutes at room temperature in the dark, while shaking. Afterwards the cells were washed three times with PBS at room temperature.

For staining of the nuclei a DAPI solution was prepared by adding 2µl DAPI to 20ml PBS. The cells were incubated with 0.25m of DAPI solution for 10 minutes at room temperature on the shaker in the dark, before washing three times at room temperature for 5 minutes with PBS.

Afterwards the cells were washed for 5 minutes with distilled water at room temperature, the cover slides were removed from the plate and fixated on slides. The samples were stored in the dark at 4°C.

Imaging and analysis:

The slides were imaged for all three dyes utilizing a whole slide scanner. The exposure time was set for each dye and slide manually. The generated images were evaluated manually for the accumulation of lipid droplets. The minimal effect concentration at which lipid droplets occur compared to control were used in the case study.

7. RPTEC/TERT1 assay

RPTEC/TERT cells were in addition tested to the three independent liver models in this read-across investigation. The outcome so far is cell viability. As the viability of kidney cells did not give any additional information, this outcome was finally not used for read-across.

Measured endpoints are:

Resazurin reduction is used as a widely utilised cell viability assay (Limonciel *et al.* 2011 PMID: 21635945). The water soluble resazurin crossed the plasma membrane of cells where it is converted by NADH/NADPH dependent reduction to a highly fluorescent product, resorufin. Resorufin can passively diffuse out of the cells, vs a concentration gradient and thus can be easily detected in the supernatant membrane. This test method is used to quantify cell viability.

Supernatant lactate production is a sensitive endpoint to monitor cellular stress in various cell types (Limonciel *et al.* 2011 PMID: 21635945). Lactate is produced by the cells from glucose and is extruded from living cells, thus making it measurable in collected cell culture supernatants. The method uses a combination of dyes and enzyme that render a stoichiometric production of yellow dye, quantified with a microplate readerRPTEC/TERT1 culture, resazurin reduction and lactate measurement as in Limonciel *et al.* 2011. Deviation #1: the same DMEM/F-12 base medium was used, but with the addition of 0.5% FBS.

7.1 Cellular system component(s)

RPTEC/TERT1

Human Renal Proximal Tubular Cells. Telomerase immortalised, non cancerous (Wieser *et al.*, 2008). These cells are cultured in DMEM/F-12 supplemented with ITS, glutamax, hydrocortisone, EGF, pen/strep and 0.5% FBS in 2D cultures. They can be cultured on plastic support (apical exposure only) or microporous filter inserts (exposure possible apically and/or basolaterally). Here, we focus on the culture on plastic plates in a 96-well plate format. The cells undergo a spontaneous differentiation into contact-inhibited, primarily oxidative cells after 7 days of confluence and remain differentiated for several weeks.

Definition of cells used

ATCC: CRL-4031 or via Evercyte GmbH. The RPTEC/TERT1 cells used in MUI were acquired from Evercyte and are used up to passage 100.

Origin: human renal cortex, immortalised by transfection with the gene for human telomerase.

Gender: male

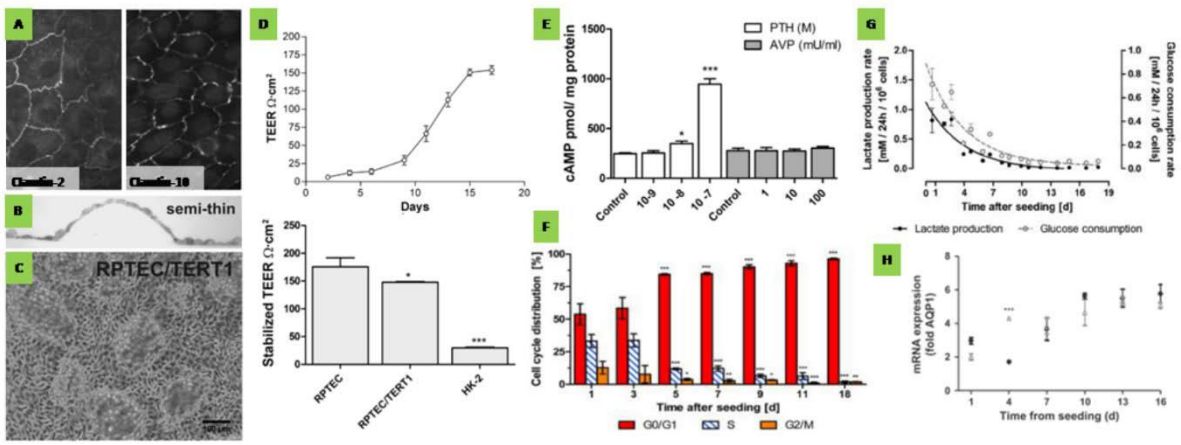
Morphology: monolayer made of cobblestone-shaped cells (upon time differentiation of 7 days after confluence used for this test system). Prior to differentiation, the cells are more bundle-shaped. Differentiated cells transport water and solutes across the monolayer, causing the formation of "dome" structures on plastic (Figure 1B-C).

Phenotype: proximal tubule (PT) phenotype (see Figure 1) – The cells express claudins 2 and 10 and transport water (proximal characteristics) (Figure 1A-C). Like primary PT cells, they build a trans-epithelial electrical resistance (TEER) of 100-150 Ω.cm² when cultured on filter inserts (Figure 1D). RPTEC/TERT1 cells exhibit parathyroid hormone (PTH) sensitivity (proximal characteristic) and lack of vasopressin (AVP) sensitivity (distal characteristic) (Figure 1E). The cells are arrested in G0/G1 phase (quiescent, Figure 1F). Proliferating RPTEC/TERT1 cells are highly glycolytic (Figure 1G). Differentiation into a primarily oxidative phenotype occurs spontaneously as the cells reach confluence (Figure 1G), concomitantly with the acquisition of other PT cells characteristics, including the expression of numerous transporters (Aschauer *et al.*, 2015a; Aschauer *et al.*, 2013). Figure 1H shows the increase in ABCB1 (P-glycoprotein) mRNA over time differentiation.

Expression: tight junction proteins claudin-2 and claudin-10, BBOX1 (last step of L-carnitine synthesis), active p53 (Aschauer *et al.*, 2013).

Figure 1. Characteristics of RPTEC/TERT1 cells during and after time differentiation.

APT-specific combination of tight junction proteins is expressed at the periphery of differentiated cells (A). Semi-thin section (B) and phase contrast picture (C) show dome formation. When cultured on filter inserts, the cells build a TEER as they get confluent and build tight junctions (D, top) and reach a level similar to that of primary PT cells in culture (D, bottom). The cells have PT-specific responses to hormonal stimulation (E). After time differentiation, the cells become quiescent and stabilise in G0/G1 phase (F). Reliance on glycolytic metabolism (glucose consumption and lactate production) decreases during differentiation (G). The cells increase their expression of numerous transporters upon differentiation both at the mRNA and protein levels (e.g. in H: mRNA level of ABCB1 / P-glycoprotein over differentiation time). A, B, C, D, E from Wieser *et al*, 2008; F, G from Aschauer *et al*, 2013; H from Aschauer *et al*, 2015.



Culture/differentiation protocol

Seeding density only influences the time to reach confluence, not the quality of differentiation. However, the **seeding must be homogenous** so as to avoid discrepancies in the initiation of differentiation within a given cell population (i.e. use a single cell suspension evenly spread around the dish/well/filter surface). No special coating is needed.

After day 7, cells are **stably differentiated for several weeks**. Seeding: onto 96-well plastic culture plates (Greiner Bio-One CELLSTAR 655 180).

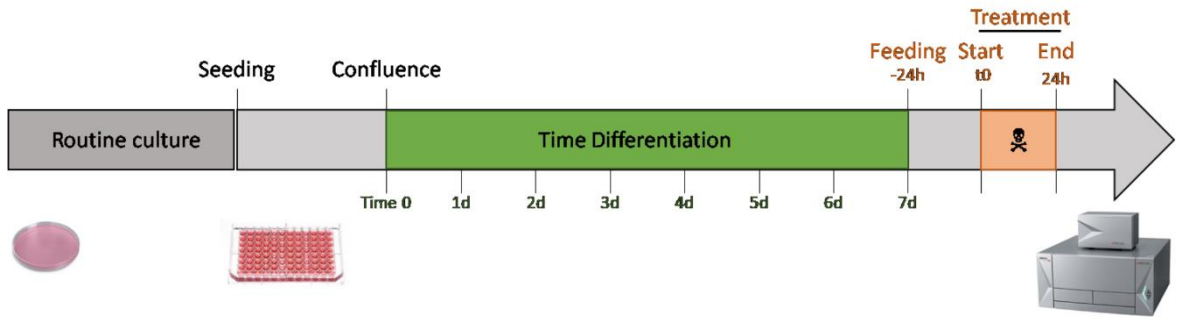
Time 0 of differentiation: Start counting time for differentiation when confluence is reached. Cells are differentiated after 7 days of confluence. For differentiation, cell culture medium is renewed every 2-3 days.

Feeding: cells are fed with fresh medium 24h prior to exposure to ensure synchronicity of the cells and comparability of the experiments.

Exposure: Test chemicals are applied at time 0 of exposure and removed exactly 24h later. Some endpoints are measured directly in the wells after removal of the test chemical and a 1.5h incubation with dyes (resazurin). Some are measured from sample preparations made at the 24h time point (supernatant lactate).

Figure 2. Culture, differentiation and treatment protocol timeline for RPTEC/TERT1 cells grown on solid plastic support for 24h bolus exposure.

RPTEC/TERT1 cells are cultured routinely in 10 cm dishes and split shortly before reaching confluence. For experiments, a single cell suspension is seeded homogenously onto the plastic support. A time differentiation process starts when the cell population has reached confluence. After 7 days, the cells are ready for chemical exposure. All cells are fed fresh medium on day 7 (24h before exposure at t0). For this system, cells are treated for 24h before cell viability and cellular stress assays are performed.



Endpoints

Differentiation of the cells prior to chemical exposure is controlled using non-invasive endpoints. Microscopic examination is critical to assess RPTEC/TERT1 differentiation. After confluence is established, the cells switch from a bundle-like to a cobblestone-like morphology. For cells cultured on plastic, the formation of domes attests of **vectorial transport** across the monolayer. Low levels of supernatant lactate (< 8 mM within 24h in 96-well plates) can also be used to confirm the **metabolic switch** in differentiated cells.

In addition, depending on the analytical endpoints used in the experiment, mRNA and or protein levels of claudin 2, claudin 10, BBOX1 and transporters can be checked in control cells to ensure that the cell batch was adequately differentiated – note: this is not used routinely but can be studied when the data is available.

Analytical endpoints

Additional endpoints can include the measurement in differentiated cells of tight junction proteins (claudins 2 and 10), adherents junction proteins (E-cadherin), transporters (ABC family, organic anion and cation transporters, Na/K ATPase), metabolism-related proteins (BBOX1). We have previously shown that these proteins and their respective mRNAs are differentially expressed in proliferating and differentiated RPTEC/TERT1 cells and are thus good indicators of cell differentiation (Aschauer *et al*, 2013). However, their measurement often requires lysis of the cells.

Acceptance criteria for test system

Qualitative criterion: **presence of domes (on plastic) attests of the readiness of the cells.** Quantitative criterion, on plastic: **24h supernatant lactate < 8 mM in controls in 96-well plates.** Quantitative criterion, on filter inserts: stable TEER in the range of 100-200 ohm.cm².

Variability and troubleshooting

Any deviation from the cell culture medium constituents (different company or catalogue number) can result in differences in RPTEC/TERT1 differentiation and phenotype; e.g. collaborators have used DMEM/F12 with 10mM glucose instead of 5mM, resulting in higher glucose consumption of the cells. The cells have higher supernatant lactate levels in 96-well compared to 6-well plates. Quality control cutoff is **8 mM for 96-well** but 3 mM for 6-well.

Omics characterisation of the test system

We have extensively characterised the differentiation of these cells over time and compared it to the current PT cell gold standard: primary human PT cells (Aschauer *et al.* 2013). Over time, and after contact inhibition, the cells switch their energy metabolism, increase expression of PT-specific mRNAs, proteins and transporters. The transcriptomic data is available from DiXa.

From OECD GD 211

7.2 Test method definition

7.2.1 Resazurin assay

Purpose of the test method:

Resazurin reduction is used as a widely utilised cell viability assay (Limonciel *et al.* 2011 PMID: 21635945). The water soluble resazurin crossed the plasma membrane of cells where it is converted by NADH/NADPH dependent reduction to a highly fluorescent product, resorufin. Resorufin can passively diffuse out of the cells, vs a concentration gradient and thus can be easily detected in the supernatant membrane. This test method is used to quantify cell viability.

Detailed protocol:

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 and adjust pH from about 11 to 7.8. Bring to exactly 50 mL with PBS. Filter sterilise (0.2 µm) under laminar flow and 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 and

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.

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

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.

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Scientific principle of the method:

This method assesses cell viability based on the reduction of non-fluorescent resazurin to the highly fluorescent resorufin.

Tissue, cells or extracts utilised in the assay and the species source:

Human Renal Proximal Tubular Cells. Telomerase immortalised, non-cancerous (Wieser *et al.*, 2008) as explained in 7.1.

Metabolic competence of the test system:

The complete metabolic activity of RPTEC/TERT1 cells have not yet been fully explored. Although they are known to have a no. of renal phase I and phase II metabolism enzymes, including gamma-glutamyl transferase.

Description of the experimental system exposure regime:

Cells are fed with fresh medium 24h prior to exposure to ensure synchronicity of the cells and comparability of the experiments. Test chemicals are applied at time 0 of exposure and removed exactly 24h later. Endpoint is measured directly in the wells after removal of the test chemical and a 1.5 h incubation with dyes (resazurin).

Quality/Acceptance criteria:

Differentiated RPTEC/TERT1 cells have a lowered glycolytically rate compared to undifferentiated cells (less than 4 mM lactate / 24 hour under standard surface area to medium volume ratio). Their morphology can be used to determine the quality of the cultures, the cells compact with time and become contact inhibited. When culture on plastic for example the cells exhibit dome formation.

Known technical limitations and strengths:

Strengths: Unlike other viability assays like MTT, resazurin can be measured without lysing the cells as the dye permeable to the plasma membrane and will follow a concentration gradient.

Limitations: Some treatments increase the rate of resazurin reduction, potentially due to Nrf2 and NQO1 induction.

Test Method performance:

The assay is linear with regard to cell no. and typically produces a sigmoidal concentration response.

Robustness of the method:

The assay is robust, but we need to provide inter and intra-laboratory values.

7.2.2 Lactate Assay

Purpose of the test method:

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.

Scientific principle of the method:

Supernatant lactate production is a sensitive endpoint to monitor cellular stress in various cell types (Limonciel *et al.* 2011 PMID: 21635945). Lactate is produced by the cells from glucose and is extruded from living cells, thus making it measurable in collected cell culture supernatants. The method uses a combination of dyes and enzyme that render a stoichiometric production of yellow dye, quantified with a microplate reader.

Detailed protocol:

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

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-Methyl**p**henazonium **m**ethyl **s**ulphate) – Sigma P9625, FW=306.3
INT (p-**i**odo**n**itro**t**etrazolium 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

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
- 3.97 g $MgCl_2$ anhydrous

Bring to 1 L with ddH_20
pH to 7.5
Store at 4°C for up to 12 months.

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 bottle
Discard if light yellow colour darkens towards red

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 (Limonciel *et al*, 2011).

Reagent mix

Concentrations: 326 µM PMS, 790 µM INT, 3.37 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 (108 mM Triethanolamine HCl, 10.7 mM EDTA.Na_2, 42 mM $MgCl_2$)
2 mL colour reagent (1.63 mM PMS, 3.95 mM INT, 35% ethanol, 2% Triton-X-100)
22.4 mg β-NAD
3.3 µL LDH

Final: 86 mM Triethanolamine HCl, 8.6 mM EDTA.Na_2, 34 mM $MgCl_2$, 326 µM PMS, 790 µM INT, 7 % ethanol, 0.4 % Triton-X-100, 4 U/ml Lactate Dehydrogenase).

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.

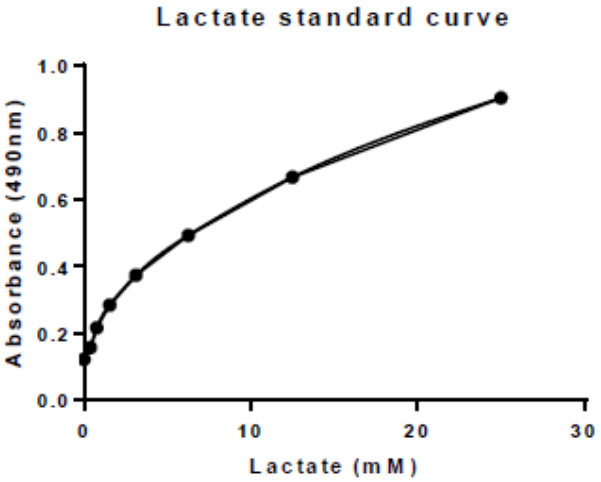
If necessary (e.g. there is no close access to the plate reader), the assay can be stopped with 50 µL 1N HCl.

Stopped assay should be light protected and read within 1 h;

Read optical density (absorbance) at 490 nm (no need for 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.



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Tissue, cells or extracts utilised in the assay and the species source:

Human Renal Proximal Tubular Cells. Telomerase immortalised, non cancerous (Wieser *et al.*, 2008) as explained in 7.1.

Metabolic competence of the test system:

The complete metabolic activity of RPTEC/TERT1 cells have not yet been fully explored. Although they are known to have a no. of renal phase I and phase II metabolism enzymes, including gamma-glutamyl transferase.

Description of the experimental system exposure regime:

Cells are fed with fresh medium 24h prior to exposure to ensure synchronicity of the cells and comparability of the experiments. Test chemicals are applied at time 0 of exposure and removed exactly 24h later. Supernatant lactate is measured from sample preparations made at the 24h time point.

Quality/Acceptance criteria:

Exclude if:

(1) Control lactate values are > 5 mM / 24h as this indicates RPTEC/TERT1 are not differentiated, or contaminated.

(2) A 24h exposure to15 µM CsA does not increase lactate production (>= 1.5 fold over control) as this likely indicates the samples were not collected appropriately, or the lactate assay was not performing.

Known technical limitations and strengths:

Strengths: Sensitive and non-invasive global marker of compound induced cellular stress.

Limitations: Supernatant lactate generally increases when cells are sub-lethally injured (Limonciel et. al. and Jennings et. al.). When there is a significant loss of viable cells supernatant lactate will decrease, so the assay should be used in conjunction with a viability assay such as resazurin. If the incubation time is excessive, values are too high to be read by the spectrophotometer. Incubation should be monitored by eye based on the colour of the highest lactate standard concentration (dark red). Yellow to red gradient should be visible by eye at the moment of measurement. Reaction should be protected from direct light, as the reagents will eventually all be consumed to dark red, even in conditions with low lactate. If sample values are above highest standard concentration, the samples should be measured again with a broader range of standard concentrations.

Negative, positive controls:

Vehicle control: Default: 0.1% DMSO unless other vehicle/% needs to be used.

Positive controls: Cyclosporine A 15 µM is a heavy inducer of glycolysis in RPTEC/TERT1 cells.

Uncertainties, Problems:

Under SOP conditions, the RPTEC/TERT1 cells provide very reproducible results between experiments for many endpoints, including the ones described here. The cells should be time differentiated as described above in Culture/Differentiation protocol.

Prediction model:

Cytotoxicity threshold: IC_{20}s (80% of control) and PODs of resazurin decrease based on one-way ANOVA.

Cellular stress threshold: IC_{20}s (120% of control) and PODs of lactate increase based on one-way ANOVA.

Reference chemicals/chemical libraries, rationale for their selection and available information:

Inducers: Cyclosporine A, Chloracetaldehyde, Ifosfamide, Cadmium chloride (chronic), amiodarone, cephaloridine. PMID: 25450743, 21635945.

References

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