Bibliographical study for selecting test substances for the validation of test methods to characterize endocrine disruptors

1.1 EXPRESSION OF THE NEED

To carry out its work on validation of methods for characterizing endocrine disruptors, Pepper needs lists of substances that can be used in circular blind tests (“test items”).
For this call, two separated lists are needed, one for .

1.2 EXPECTED RESULTS.

a) A list must be established according to the method (parameters it measures and effects it aims to predict).
   - For each one of the methods the list must contain at least 60 substances. Since the expert group will chose 30 among each list.
   - The list of substances should consist of approximately 80% “positive” substances (that should be divided equally for each mode, if relevant) and 20% “negative” substances.
   - This list must be based on observations that can be linked to the method studied: data on other *in vitro* tests, on *in vivo* experiments, observational data in humans...possibly data *in silico*.
   - It must cover all of the parameters measured, cover a wide range of chemical classes/families/structures and cover a wide area of applications/regulations.
   - Details of the substance must be presented (#CAS, solubility, molecular structure). Also details of the exposure conditions must be presented: model, exposure time, concentrations, etc.
   - Availability of substances (delivery time etc.).
   - The elements explaining the choices must be explained in a summarized manner and referenced with documentation provided to allow analysis by an expert committee.

b) A final report must be presented, as a Word document, containing the search strategy used to find all the information from each list and more detailed justifications for the relevance of the substances chosen.

1.3 DATA SOURCES TO INVESTIGATE.

In addition to the literature, databases and suggestions made to PEPPER by its partners will be provided and must be analyzed.

1.4 DEADLINE

2 months for each method.

22 rue Charcot - 75013 PARIS – Tel : +33(0)1 56 59 67 20
E-mail : philippe.hubert@ed-pepper.eu
Association Loi 1901, déclarée à la Préfecture de Police sous le n° W751255371

2 TECHNICAL APPENDIX

2.1 METHODS DESCRIPTION

***In vitro* assay for hepatic triglyceride accumulation**

The *in vitro* assay for hepatic triglyceride accumulation measures the accumulation of triglycerides in human liver cells. This *in vitro* test method determines quantitatively the intracellular accumulation of triglycerides in HepaRG (human hepatocarcinoma) cells. Currently, HepaRG cells are the best known model to study human hepatocytes. This accumulation is measured using a fluorescent dye.

Differentiated HepaRG cells grown on a 96-well plate are treated for 72h (single dose) with the compound at eight different concentrations ranging up to the highest non-toxic concentration. A solvent- and a positive control are included on each plate.

Positive controls:

- The positive control used in this assay is T0901317 (Cas-no.: 293754-55-9, Sigma Aldrich, cat. no. T2320)

References:

- Alarcan, J., de Sousa, G., Katsanou, E.S. et al. Investigating the in vitro steatotic mixture effects of similarly and dissimilarly acting test compounds using an adverse outcome pathway-based approach. Arch Toxicol 96, 211–229 (2022)
- Allard, J., Bucher, S., Massart, J. et al. Drug-induced hepatic steatosis in absence of severe mitochondrial dysfunction in HepaRG cells: proof of multiple mechanism-based toxicity. Cell Biol Toxicol 37, 151–175 (2021). https://doi.org/10.1007/s10565-020-09537-1
- Claudia Luckert, Albert Braeuning, Georges de Sousa, Sigrid Durinck, Efrosini S. Katsanou, Parthena Konstantinidou, Kyriaki Machera, Emanuela S. Milani, Ad A. C. M. Peijnenburg, Roger Rahmani, Andreja Rajkovic, Deborah Rijkers, Anastasia Spyropoulou, Marianna Stamou, Geert Stoopen, Shana Sturla, Bernd Wollscheid, Nathalie Zucchini-Pascal, and Alfonso Lampen. Adverse Outcome Pathway-Driven Analysis of Liver Steatosis in Vitro: A Case Study with Cyproconazole. Chemical Research in Toxicology 2018 31 (8), 784-798
- Dajana Lichtenstein, Almut Mentz, Felix F. Schmidt, Claudia Luckert, Thorsten Buhrke, Philip Marx-Stoelting, Joern Kalinowski, Stefan P. Albaum, Thomas O. Joos, Oliver Poetz, Albert Braeuning. Transcript and protein marker patterns for the identification of steatotic compounds in human HepaRG cells, Food and Chemical Toxicology, Volume 145, 2020, 111690, ISSN 0278-6915
- Dajana Lichtenstein, Claudia Luckert, Jimmy Alarcan, Georges de Sousa, Michail Gioutlakis, Efrosini S. Katsanou, Parthena Konstantinidou, Kyriaki Machera, Emanuela S. Milani, Ad Peijnenburg, Roger Rahmani, Deborah Rijkers, Anastasia Spyropoulou, Marianna Stamou, Geert Stoopen, Shana J. Sturla, Bernd Wollscheid, Nathalie Zucchini-Pascal, Albert Braeuning, Alfonso Lampen. An adverse outcome pathway-based approach to assess steatotic mixture effects of hepatotoxic pesticides in vitro, Food and Chemical Toxicology, Volume 139, 2020, 111283, ISSN 0278-6915
- Dajana Lichtenstein, Alexandra Lasch, Jimmy Alarcan, Almut Mentz, Jörn Kalinowski, Felix F. Schmidt, Oliver Pötz, Philip Marx-Stoelting, Albert Braeuning. An eight-compound mixture but not corresponding concentrations of individual chemicals induces triglyceride accumulation in human liver cells, Toxicology, Volume 459, 2021, 152857, ISSN 0300-483X
- Lasch A, Marx-Stoelting P, Braeuning A, Lichtenstein D. More than additive effects on liver triglyceride accumulation by combinations of steatotic and non-steatotic pesticides in HepaRG cells. Arch Toxicol. 2021 Apr;95(4):1397-1411. doi: 10.1007/s00204-021-02997-2. Epub 2021 Feb 11. PMID: 33575850; PMCID: PMC8032629.

Stably transfected human mineralocorticoid receptor hMR transcriptional activation assay for detection of agonistic and antagonist activity of chemicals towards hMR

To characterize the human mineralocorticoid receptor (hMR) activity of chemical, a stable cell line expressing the ligand binding domain (LBD) of human MR fused to the yeast GAL4 DNA binding domain (DBD) was developed. This reporter cell line was generated by a two-step transfection procedure. U2OS cells are stably transfected by a GAL4 (DBD)-hMR (LBD) plasmid and then by a luciferase plasmid under the control of GAL4 promoter (GAL4-Luciferase). This stable model allows specific and sensitive measurement of hMR ligand activities thanks to the use of luciferase, and is a high-throughput, cell-based screening tool for identifying and characterizing hMR ligands. This test elucidates the Molecular Initiating Event of the activation/inhibition of the MR receptor and should be included in an Integrated Testing Strategy (ITS)/IATA for the regulatory identification of a substance as an endocrine disrupter (figure). The cells are available upon signature of an MTA.

Positive controls:

- Aldosterone is the reference agonist control (Aldo, CAS number 52-39-1)
- Spironolactone is the reference antagonist control (Spiro, CAS number 52-01-7)

References:

- Neale P, Grimaldi M, Boulahtouf A, Leusch F*, Balaguer P*. Assessing species-specific differences for nuclear receptor activation for environmental water extracts. Water Research 2020; 185 : 116247.
- Dellal H, Boulahtouf A, Alaterre E, Cuenant A, Grimaldi M, Bourguet W, Gongora C, Balaguer P*, Pourquier P.* High content screening using new U2OS reporter cell models identifies harmol hydrochloride as a selective and competitive antagonist of the androgen receptor. Cells 2020; 9: 1469.
- Grimaldi M, Boulahtouf A, Toporova L, Balaguer P. Functional profiling of bisphenols for nuclear receptors. Toxicology. 2019, 420: 39-45.
- Leusch FDL, Neale PA, Arnal C, Aneck-Hahn NH, Balaguer P, Bruchet A, Esther BI, Esperanza M, Grimaldi M, Leroy G, Scheurer M, Schlichting R, Schriks M, Hebert A. Analysis of endocrine activity in drinking water, surface water and treated wastewater from six countries. Water Res 2018, 139: 10-18.
- Bellet V, Hernandez-Raquet G, Dagnino S, Seree L, Pardon P, Bancon-Montiny C, Fenet H, Creusot N, Aït-Aïssa S, Cavailles V, Budzinski H, Antignac JP, Balaguer P. Occurrence of androgens in sewage treatment plants influents is associated with antagonist activities on other steroid receptors. Water Res. 2012 ; 46 : 1912-22.
- Escande A, Servant N, Rabenoelina F, Auzou G, Kloosterboer H, Cavaillès V, Balaguer P, Maudelonde T. Regulation of activities of steroid hormone receptors by tibolone and its primary metabolites. J Steroid Biochem Mol Biol. 2009 Aug;116(1-2):8-14.
- Molina-Molina JM, Hillenweck A, Jouanin I, Zalko D, Cravedi JP, Fernandez MF, Pillon A, Nicolas JC, Olea N, Balaguer P. Steroid receptor profiling of vinclozolin and its primary metabolites. Toxicol Appl Pharmacol. 2006; 216: 44-54.

22 rue Charcot - 75013 PARIS – Tel : +33(0)1 56 59 67 20

E-mail : philippe.hubert@ed-pepper.eu

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2.2 EXAMPLE OF EXPECTED LIST CHARACTERISTICS

In this example the substances were to be chosen for a method that assess the estrogenic or antiestrogenic effect in chicken embryo.

S. No.	Substance name	Biological model	References	Link to abstract/fu
18	Norethindrone	Chick embryos; concentrations: 0.01, 0.1 & 0.5 mg; single injection on day 4 of incubation; **Estrogenic effect**: partial retention of Mullerian duct (MD) & feminization of testis in male embryos; partial regression of MD in female	Hutson JM, Donahoe PK, MacLaughlin DT.	https://pubm ed.ncbi.nlm.ni
	Norethindrone acetate	Chick embryo; concentration: 120 µg/g egg; single injection on day 3 of incubation; exposure time: embryonic days E3-E15; **Estrogenic effect**: caused Mullerian duct (MD) deficiencies in female embryos	Stoll R, Faucounau N, Maraud R. Action of	https://pubm ed.ncbi.nlm.ni
19	Propyl pyrazole triol (PPT)	Chick embryo; concentration: 20 µg/g egg; exposure time: embryonic days E4-E18; **ERα agonist**: caused left-side ovotestis formation and retention of the Müllerian ducts in male embryos; caused retention of the right Müllerian	Mattsson A, Olsson JA, Brunström B.	https://pubm ed.ncbi.nlm.ni
20	Tamoxifen	Chick embryo; concentration: 0.1-10 µg/g egg; exposure time: embryonic days E1-E19; **Antiestrogenic effect**: strongly impaired the differentiation of female gonads, led to a significant size reduction of the left ovary and	Jessl L, Lenz R, Massing FG, Scheider	https://www. ncbi.nlm.nih.g
	Tamoxifen	Chick embryos; concentrations: 0.1 mg/egg; single injection on day 3 of incubation followed with testosterone propionate (TP), estradiol benzoate (EB) or dihydrotestosterone (DHT); **Antiestrogenic effect**: significantly	Stoll R, Faucounau N, Maraud R. Influence	https://pubm ed.ncbi.nlm.ni

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