The *in vitro* assessment of endocrine disruptors/EDCs

**Stefano Lorenzetti**

Istituto Superiore di Sanità (ISS)

Department of Food Safety and Veterinary Public Health

Unit of Food and Veterinary Toxicology

**LIFE-EDESIA workshop on** The role of *in silico* tools

in supporting the application of the substitution principle
**LIFE-EDESIA project: overview**

**LIFE-EDESIA in ACTION(S)**

A. Preparatory actions (if needed)
A1 Identification of potential substitutive chemicals in the literature

B. Implementation actions
B1 Identification of potential substitutive chemicals using *in silico* tools
B2 *In silico* validations
B3 Synthesis of the chemicals (if necessary)

**B4 In vitro validation**
B5 Prototyping and testing for industrial purposes in 3 application domains

C. Monitoring of the impact of the project actions (obligatory)
C1 Monitoring of the project impact

D. Communication and dissemination actions (obligatory)
D1 Communication and dissemination initiatives
D2 Interviews of stakeholders
D3 Web Portal
D4 Brochures, newsletters, layman's report, notice boards
D5 Seminars and workshops
D6 After LIFE communication plan

E. Project management and monitoring of the project progress (obligatory)
E1 Project management
E2 Project monitoring

*Actions in progress*
Endocrine Active Substance / EAS

“a substance having the inherent ability to interact or interfere with one or more components of the endocrine system resulting in a biological effect, but need not necessarily cause adverse effects.”

EFSA Journal 2013;11(3):3132

Endocrine Disruptor / ED

“An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations.”

WHO/IPCS 2002

In other words, ...

Endocrine Disruptors (EDs) (i.e., EASs causing adverse effects mediated by endocrine mechanisms)

Rovida, De Angelis, Lorenzetti. ALTEX 30, 2/13
Endocrine activity...

“endocrine activity as a collection of modes of action, potentially leading to adverse outcomes, rather than a (eco)toxicological hazard in itself.”

EFSA Journal 2013;11(3):3132

... as a sum of different Mode of Actions

“A change in morphology, physiology, growth, reproduction, development or lifespan of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increased susceptibility to the harmful effects of other environmental influences”.

Federal Institute for Risk Assessment (BfR), Berlin workshop 2009”

In vitro only nuclear receptor binding & regulation of gene transcription define an endocrine activity?
In summary, **currently available definitions of “endocrine disrupter”** are **either** neutral in terms of specifying the toxicological relevance of the effects to be described, **or** they introduce the idea of adversity. **The former** is in danger of being insufficiently discriminatory, **the latter** shifts the problem to defining **what adversity should mean in an endocrine context**, which could be too restrictive and not inclusive enough.

At the core of this dilemma is the fact that **“endocrine disruption” cannot presently be anchored to specific assay outcomes in a straightforward way.**

**STATE OF THE ART ASSESSMENT OF ENDOCRINE DISRUPTERS**

[ec.europa.eu/environment/endocrine/.../summary_state_science.pdf](ec.europa.eu/environment/endocrine/.../summary_state_science.pdf)
ENDOCRINE DISRUPTION IN VITRO: A SIMPLE TASK?

A TYPICAL SCHEME OF NR-MEDIATED SIGNALLING

Adapted from Lorenzetti and Narciso, 2012
DOI: 10.1039/9781849735353

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HOW TO SEARCH FOR «NEW» BIOMARKERS OF EFFECT?

Part of a Mechanism of Action

Part of a Mode of Action

DOI: 10.1039/9781849735353

Adapted from Lorenzetti and Narciso, 2012
NRs do not transactivate alone

- Ligand-NR interaction is as important as the presence of dozens of transcriptional coregulators at the DNA binding site within the promoter of NR-target genes.

Indeed, the NR-dependent transcriptional activation (or repression) of NR-target genes depends on specific co-activators (or co-repressors) present within the nucleus of a specific cell in a well determined very short-time period.
How to search for «new» biomarkers of effect?

Part of a Mechanism of Action. Toxicogenomics

Adapted from Lorenzetti and Narciso, 2012
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All substances including endocrine disruptors (EDCs) are subject to registration under REACH (Regulation (EC) No 1907/2006) when they are manufactured or imported into the EU in amounts of, or above, 1 tonnes per year. However, substances with endocrine disrupting properties are subject to the authorisation procedure under REACH only if they are included in Annex XIV as Substances of Very High Concern (SVHC).

Anyway, the test programme does not include specific tests for endocrine disrupting properties because there are no internationally agreed methodologies or criteria available for endocrine disrupting properties (ECHA Guidance for SVHC).

Substances with endocrine disrupting properties are considered as SVHC only on a case-by-case basis and only if scientific evidence and a weight of evidence approach indicate that they are of “equivalent concern“ to CMR, PBT or vPvB substances (see definitions within REACH).

Indeed, EU already adopted a Community Strategy for Endocrine Disrupters that contained, among the short-term actions, the establishment of a priority list of substances
http://ec.europa.eu/environment/endocrine/strategy/substances_en.htm for further evaluation of their role in endocrine disruption.
HOW THE INTERNATIONAL COMMUNITY IS MOVING ON...
3.7 Test method submission related to endocrine disruption

The **Yeast Androgen Screen (YAS) assay** was submitted to EURL ECVAM towards the end of 2013 to be considered for an ESAC peer review. A full validation study, addressing the modular approach, had been carried out with 4 laboratories. **This assay uses yeast cells that are transformed with a human androgen receptor and a lacZ reporter gene.** It measures the response towards chemicals with (anti) androgenic potential. Such assay may have potential to be annexed to a future Performance Based Test Guideline (PBTG) for Androgen Transactivation Assay (ARTAs). The submission dossier has been assessed by EURL ECVAM in early 2014 and feedback was requested from the submitter for some critical procedural aspects prior to continuation of the evaluation.
4. Validation of Alternative Methods

4.1 On-going and finalised validation studies

4.1.1 Endocrine Disruption

At OECD level, there is an ongoing activity towards OECD performance based test guidelines (PBTG) for estrogen receptor transactivation assays (ERTAs) and androgen receptor transactivation assays (ARTAs). PBTG 455 for the detection of estrogen receptor agonists and PBTG 457 for the detection of estrogen agonists and antagonists were both adopted in 2012 while a proposal for a PBTG for the detection of androgen receptor agonists and antagonists was submitted to the OECD and included in the OECD work plan in 2013.

The AR-CALUX method is an androgen receptor transactivation assay (ARTA) for the detection of substances with androgenic properties. Such substances can bind to the androgen receptors and activate (agonist) or block (anti-agonist) cellular responses within the endocrine system. Once validated, this assay in conjunction with other similar assays (e.g. the Japanese EcoScreen assay) will contribute towards an OECD PBTG for androgen receptor transactivation assays. It is based on the use of osteosarcoma cells transfected with the cDNA of a human androgen receptor and the androgen responsive elements (AREs) coupled to a luciferase reporter gene (AR-CALUX®). Response towards substances with androgenic activity is hence easily evaluated via the measurement of emitted light.

The method was accepted to enter a EURL ECVAM validation study in 2012. A multi-study validation trial is scheduled for 2014 and the European Union Network of laboratories for the Validation of Alternative Methods (EU-NETVAL) will be involved in this exercise for the first time. Three test facilities will be selected and after approval by the Member States via the National Contact Points (NCPs), they will participate in the multi-study validation trial.

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4. Validation of Alternative Methods
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4.1.1 Endocrine Disruption

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A validation study on a transactivation assay that detects chemicals with (anti-) estrogenic potential using MELN cells is still ongoing. The cell line used is the MCF-7 human breast cancer cell line, that has been stably transfected with an estrogen responsive reporter system. The MCF-7 cells express endogenously the estrogen receptor. The first part of the validation study was completed in 2012 with an assessment of the reliability (transferability and within and between laboratory reproducibility) for agonist and antagonist manual protocols in three laboratories for 16 chemicals. A second step to assess relevance (predictive capacity) on a wider set of chemicals in one laboratory by transferring the protocol to an inter-plate format on the robotic platform was supported by the OECD's non-animal Validation Management Group. Transfer of the agonist protocol to inter-plate HTS format (robotic platform) for 16 non-coded chemicals followed by the testing of 22 coded chemicals from the PBTG OECD 455 Performance Standards has been completed and good accuracy (i.e. good prediction of negatives and positives) was obtained but the induction factor was low. The next step is to proceed in the same manner for the antagonist protocol using the reference chemicals from the performance standards of the OECD TG 457 (ERTA for antagonist based on BG1 Luc cells), provided that a new batch of cells can be obtained which can respond with a suitably high induction factor.
HOW TO SEARCH FOR <NEW> BIOMARKERS OF EFFECT?

Gene Transactivation

Part of a Mechanism of Action. GENE TRANSACTIVATION

Part of a Mode of Action. FUNCTIONAL ASSAYS

mRNA

protein

mRNA

functional markers

changed cell function

molecular markers

Adapted from Lorenzetti and Narciso, 2012
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The androgen receptor directly regulate development, maturation, functionality and homeostasis of the prostate gland and, in particular, of the prostate epithelium that secrete the prostatic fluid (1/3 in volume of the male ejaculate) an essential component to ensure male fertility.

**PROSTATE and HUMAN HEALTH: TDS as an example**

Testicular Dysgenesis Syndrome (TDS): exposure in utero to environmental factors (anti-androgenic compounds) in Western Europe and USA nei paesi dell’Europa occidentale e negli USA, are responsible of male infertility and associated-diseases/malformations.
LNCaP cells as an *in vitro* tool...

Lorenzetti et al., unpublished data – NOT SHOWN ONLINE
In our experimental culture conditions, only ERβ and AR^{T877A} are expressed whereas ERα is NOT expressed

THUS allowing us to reduce the redundancy between AR and ERα signalling on common target genes

Maranghi et al., 2007
Lorenzetti et al., unpublished data – NOT SHOWN ONLINE
Lorenzetti et al., unpublished data – NOT SHOWN ONLINE
Screening 10 chemicals in a double blind feasibility study:

- 2 confirmed as AR-interfering chemicals (vinclozolin and BPA)
- 1 newly identified as androgen-like chemical
TAKE HOME MESSAGE

- Transactivation o gene reporter assay in *in vitro* models do not characterize an effect as adverse or not, but just indicate the ligand binding in that particular cellular environment (a biomarker of exposure)

- Transactivation assay or, better, any gene expression profiling should be associated to a functional assay (a biomarker of effect) to search for a mode of action

Assessment of adversity is not unique to endocrine related effects. Scientific criteria for assessment of adversity have not been generally defined. In general, but not always, transient, inconsistent and minor fluctuations at the biochemical and molecular level may be considered adaptive, i.e. non-adverse.

Changes at the cell-, organ-, organism-, or (sub)population-level resulting in pathology or functional impairment *in vivo*, as well as altered timing of development, may be considered adverse. It is therefore difficult to propose ED-specific criteria for adversity and expert judgement in a weight-of-evidence approach is needed to assess substances for possible endocrine disrupting properties. EFSA Journal 2013;11(3):313
The AIM

To characterize *in vitro*, in multiple ED-targeted human cells, if the alternatives identified in previous actions are “less toxic” considering their endocrine disrupting properties.

The APPROACH

- Multiple clinical-, physiologically-relevant endpoints will be used to translate the *in vitro* toxicological profile to a suitable prediction for human health.

- Endocrine disrupting properties of the selected alternative substances to phthalates, bisphenols and parabens - in comparison with their reference EDCs - will be assessed by an integrated set of *in vitro* battery test of alternative methods that, although not yet validated, have been so far accepted by the academic community as directly focusing on specific and reliable endocrine endpoints.

- Well characterized human-derived cell lines representing recognized tissue target of the EDCs to be substituted have been selected.
The EXPERIMENTAL MODELS

Endocrine disrupting properties will be tested only in cell lines of human sources, whose employment is well proven, integrating tests representative of the endocrine activities of the following human tissues:

- **prostate**, to investigate ED androgen receptor (AR)-mediated effects on the male reproductive system
  - Lorenzetti *et al*., 2010, Reprod.Toxicol. 30:25-30

- **trophoblast**, to investigate ED estrogen receptor (ER)-mediated effect on the placenta and hence the transgenerational effects on nutrient exchange between mother-child
  - Morck *et al*., 2010, Reprod.Toxicol. 30:131

- **liver**, to investigate multiple ED nuclear receptor (NR)-mediated effects on the programming of the metabolic syndrome.
  - Grasselli *et al*., 2013, Chemosphere. 91(8):1123-9
The METHODS

Within the three model systems will be used in parallel an approach based on the use of three cell-based assays:

a) **cytotoxicity/cell proliferation test** (by MTS assay, a metabolic-based assay relying on mitochondrial functionality) that will assist to distinguish if the changes observed in the other tested endpoints (b. and c.) are cell specific or merely due to cell damages;

b) **assessment of gene expression** (by real time RT-PCR) of a set of nuclear receptors (NRs) known molecular mediators of the actions of parabens, bisphenols and phthalates;

c) **“phenotypic anchoring” by measurements of clinical-, physiologically-relevant endpoints**: to allow the assessment of the physiological relevance of detected change in NR gene expression by the measurement of cell specific cellular biomarkers already employed in clinical practice and well recognized as endocrine endpoints modulated by both endogenous and exogenous hormone-like stimuli.
THE CORE OF ACTION B4 AT A GLANCE

Cell specific endpoint:

**Functional Assay – Phenotypic anchoring**
- prostate: PSA secretion
- trophoblast: βhCG secretion
- liver: intracellular lipid accumulation and AFP secretion

Cell aspecific endpoint:

*Cell Viability* (MTS assay)

Molecular endpoint:

*gene expression of Nuclear Receptors of interest* (qPCR)

Gene reporter assays

*AR-, ER-, PPAR-gene reporter assays*  
(OECD and/or IHCP-JRC guidelines and/or protocols under the validation programme)
Part of a Mechanism of Action. GENE TRANSACTIVATION

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THE CORE OF ACTION B4 AT A GLANCE

**Cell specific endpoint:**
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**Molecular endpoint:**
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Gene reporter assays
- AR-, ER-, PPAR-gene reporter assays
  - OECD and/or IHCP-JRC guidelines and/or protocols under the validation programme

- OECD guidelines for the testing of chemicals [http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm](http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm)
- JRC-IHCP website [http://ec.europa.eu/dgs/jrc/index.cfm](http://ec.europa.eu/dgs/jrc/index.cfm): e.g., the “Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists” (OECD TG 455); the “BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists” (OECD TG 457)

A gene reporter assays do not identify an endocrine disrupting property but only a part of a potential mechanism of action of a chemical whose endocrine disruption property remain to be assessed…! : by a functionally-relevant endpoint (?)

IN THE MEAN TIME... LIFE-EDESIA MEET TIPED
questions are wellcome &... come to visit our website www.iss.it/life