UNIVERSITÀ DEGLI STUDI DI MILANO
School of Pharmacy – Laboratory of Toxicology

Un approccio integrato per discriminare i distruttori endocrini dai modulatori endocrini

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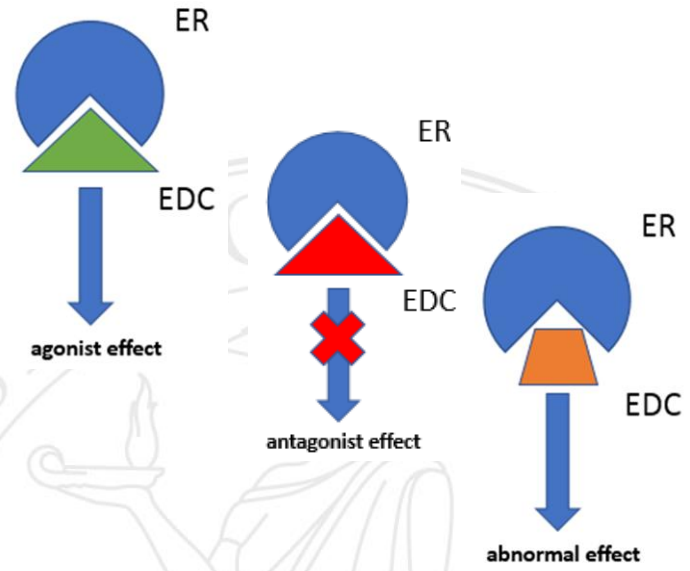
Endocrine Disrupting activity estrogen receptor mediated

Several mechanisms:

Direct :

- Mimicking the biological activity of an endogenous hormone by binding to its specific cellular receptor (agonistic effect)
- Binding to the receptor without any activation and blocking the binding of the natural hormone (antagonistic effect)
- Affecting the production and release of the natural hormones

Indirect: Inflammatory pathway; Oxidative stress pathway



WHO, 2002:

''An endocrine disruptor 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"

AIM

Develop an integrated strategy of testing based on the combination of

- scientific literature review of in vivo data,
- in silico method,
- in vitro and
- in vivo bioluminescence imaging methodologies

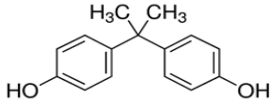
to predict and discriminate the direct endocrine disrupting activity and also the indirect toxicological pathways within the selected chemicals

Putative Endocrine Disrupting Compounds

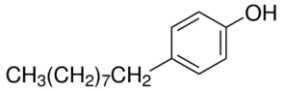
Different group of chemicals such as pesticides, synthetic hormones, plant constituents, food and environmental pollutants

Plasticizers

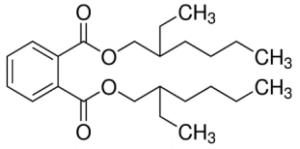
Bisphenol A (BPA)



4-nonylphenol

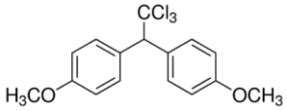


Di(2-ethylhexyl) phthalate (DEHP)

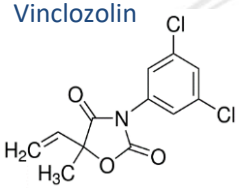


Pesticides

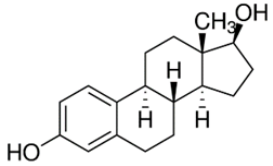
Methoxychlor



Vinclozolin

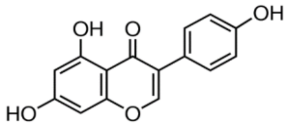


17β-estradiol

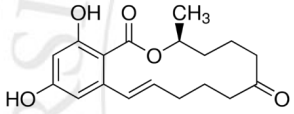


Natural compounds

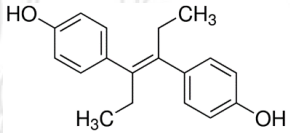
Genistein



Zearalenone



Diethylstilbestrol (DES)



Literature survey

In vivo data from reliable subchronic, chronic, multigenerational toxicity studies, such as U.S. National Toxicological Program studies reports

Organs

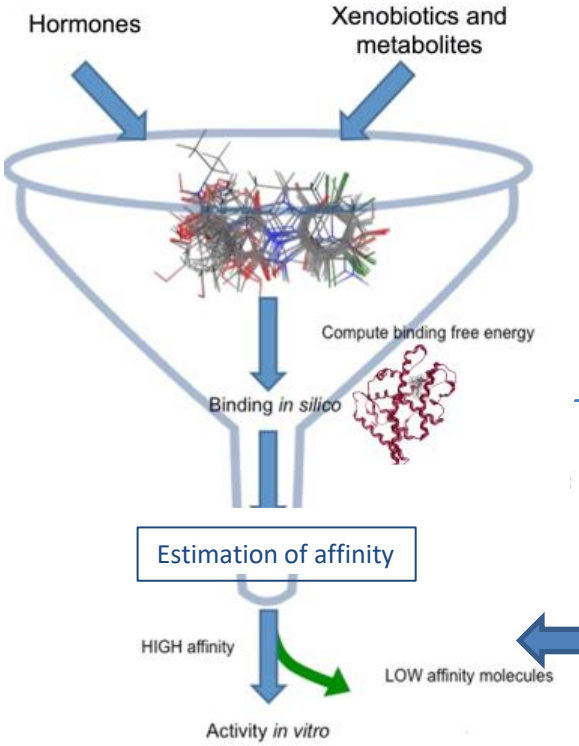
- Testis
- Prostate
- Seminal vescicles

Endpoints

- Weight changes
- Histopathology
- Inflammation
- Tumours

EU- EFSA & ECHA **GUIDANCE** for the identification of Endocrine Disrupting chemicals, 2018

IN SILICO APPROACH



Pipeline to model the interactions of ligand-Ligand Binding Domain (LBD) of human ER alpha bound to estradiol

2°step: In vitro bioluminescence imaging assay

A Computational Approach to Evaluate the Androgenic Affinity of Iprodione, Procymidone, Vinclozolin and Their Metabolites.; Eberini et al., 2014

RESULTS: IN SILICO MODEL

Ligand	XP Glide Score kcal/mol	MMGBSA dG kcal/mol	K_i °(M) (from XP GlideScore
17β – estradiol	-11.3	-78.6	4.6E-09
Zearalenone	-11.3	-57.2	4.8E-09
Diethylstilbestrol	-11.0	-59.1	8.4E-09
Genistein	-9.9	-48.4	4.9E-08
Bisphenol A	-9.7	-47.0	7.6E-08
DEHP	-9.4	n.c.	1.2E-07
Methoxychlor	-9.0	-37.8	2.5E-07
Vinclozolin	-8.3	-43.4	8.1E-07
4-nonylphenol	-7.2	-46.3	5.4E-06

RESULTS: IN SILICO MODEL

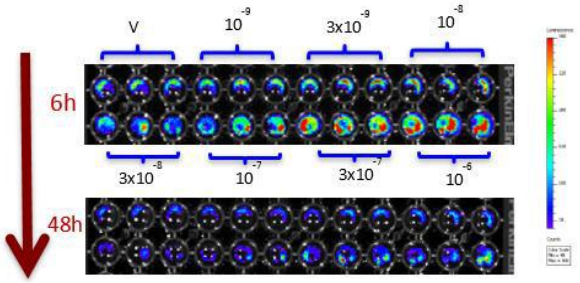
Ligand	K_i °(M) (from XP GlideScore	In vitro Experimental K_i (M)#
17β – estradiol	4.6E-09	1.3E-10
Zearalenone	4.8E-09	8.0E-10
Diethylstilbestrol	8.4E-09	4.0E-11
Genistein	4.9E-08	2.6E-09
Bisphenol A	7.6E-08	1.9E-07
DEHP	1.2E-07	n.a
Methoxychlor	2.5E-07	1.8E-06
Vinclozolin	8.1E-07	n.a
4-nonylphenol	5.4E-06	n.a

 # Kuiper et al., 1998

BIOLUMINESCENCE IMAGING ASSAYS

IN VITRO ASSAY

Model: **ERE-Luc** B17 cells, a clone of the breast cancer cell line **MCF-7** stably transfected with a reporter constituted by the luciferase gene driven by an estrogen-regulated synthetic promoter *.



Example of bioluminescence imaging of photon emission after treatment with different concentrations of Bisphenol A at 6h and 48h.

IN VIVO ASSAY

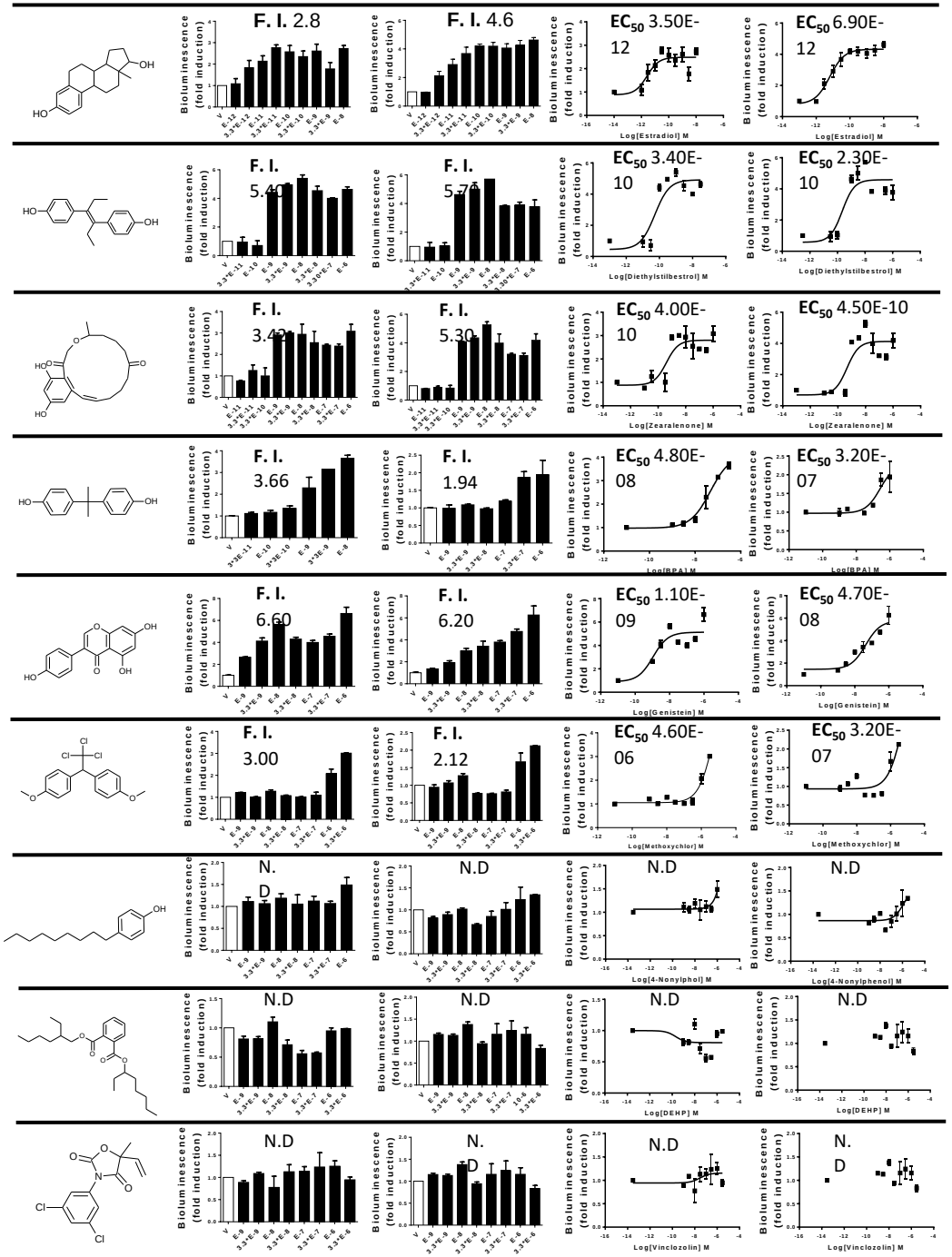
A non-invasive methodology to quantify by a bioluminescence image the biological events (e.g. the activation of receptorial pathway), occurring in the same mouse during the entire time of experiment.

Model: transgenic reporter mice

- **ERE-Luc** reporter mice: activation of **estrogenic pathway***
- **NFkB-Luc** reporter mice: **inflammatory pathway**
- **ARE-Luc** reporter mice: **oxidative stress pathway** response

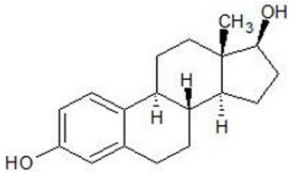
**** Engineering of a mouse for the in vivo profiling of estrogen receptor activity. Ciana P. et al., 2001.***

Selection of regions of interest (ROI) on the bioluminescence image taken by CCD camera, for whole body, genital area, head, chest, abdomen, paw, tail.

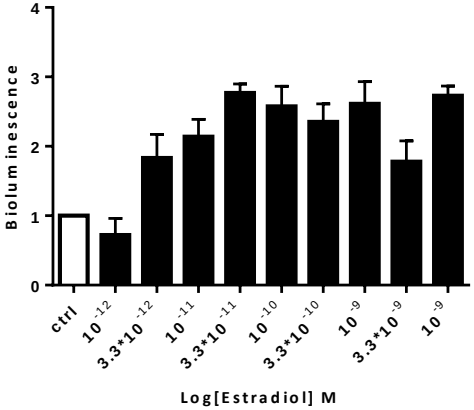

F. I. 2.8
F. I. 4.6
EC50 3.50E-12
EC50 6.90E-12
F. I. 5.40
F. I. 5.70
EC50 3.40E-10
EC50 2.30E-10
F. I. 3.42
F. I. 5.30
EC50 4.00E-10
EC50 4.50E-10
F. I. 3.66
F. I. 1.94
EC50 4.80E-08
EC50 3.20E-07
F. I. 6.60
F. I. 6.20
EC50 1.10E-09
EC50 4.70E-08
F. I. 3.00
F. I. 2.12
EC50 4.60E-06
EC50 3.20E-07
N.D
Bioluminescence (fold induction)
Log[Estradiol] M
Log[Diethylstilbestrol] M
Log[Zearalenone] M
Log[BPA] M
Log[Genistein] M
Log[Methoxychlor] M
Log[4-Nonylphol] M
Log[4-Nonylphenol] M
Log[DEHP] M
Log[Vinclozolin] M

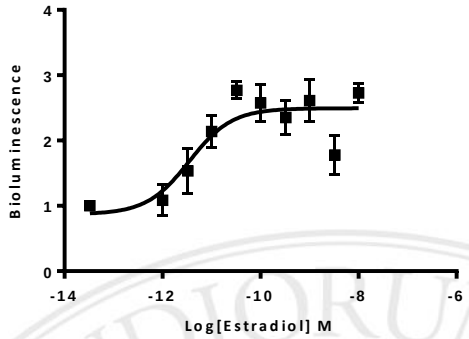
In vitro bioluminescence imaging assay. 1

17β-estradiol



6h





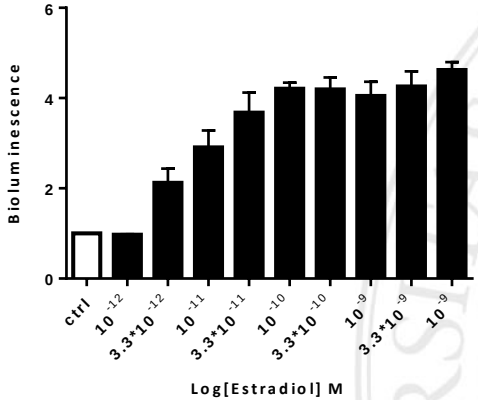
EC_{50} 6h

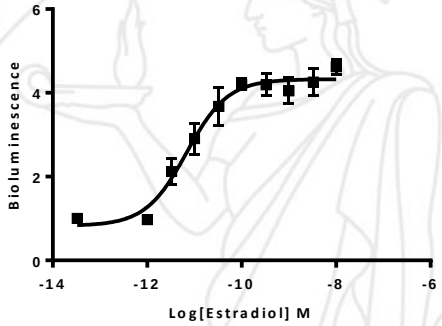
3.50E-12 M

Max Fold Induction 6h

2.80

48h





EC_{50} 48h

6.90E-12 M

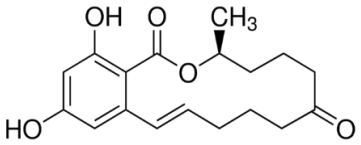
Max Fold induction 48h

4.60

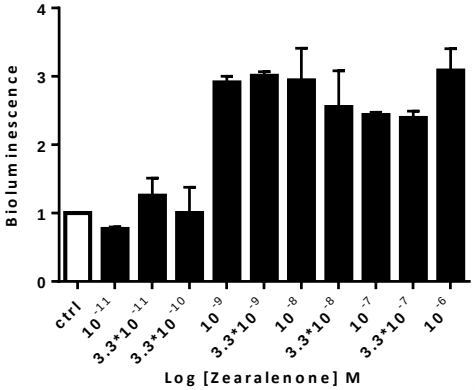
in vitro bioluminescence imaging assay. 2

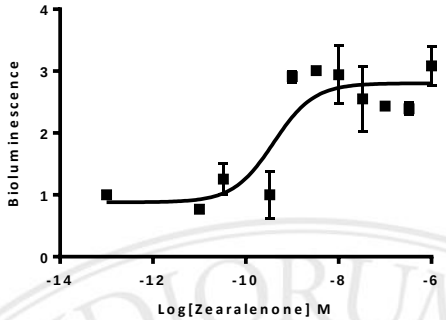
6h

Zearalenone



48h



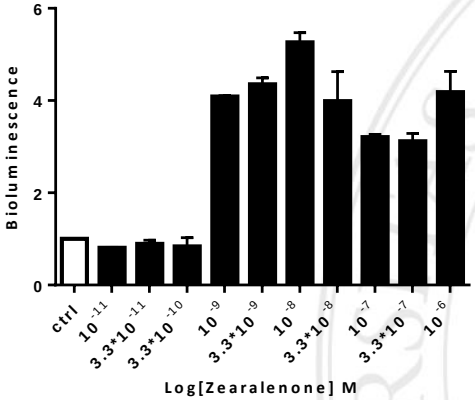


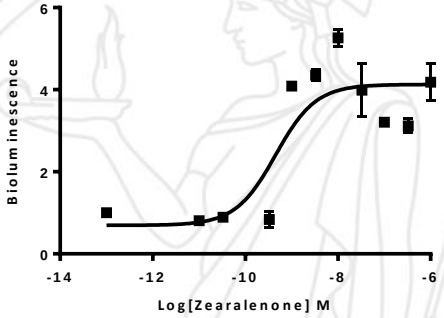
EC_{50} 6h

4.00E-10 M

Max Fold induction 6h

3.42





EC_{50} 48h

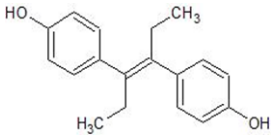
4.50E-10 M

Max Fold induction 48h

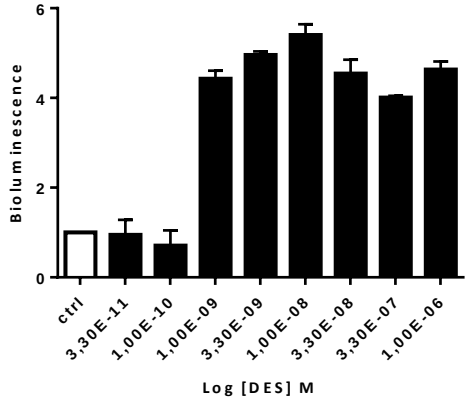
5.30

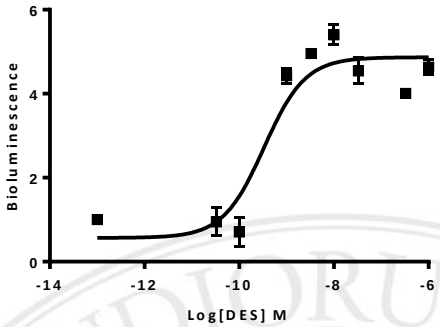
in vitro bioluminescence imaging assay. 4

DES



6h





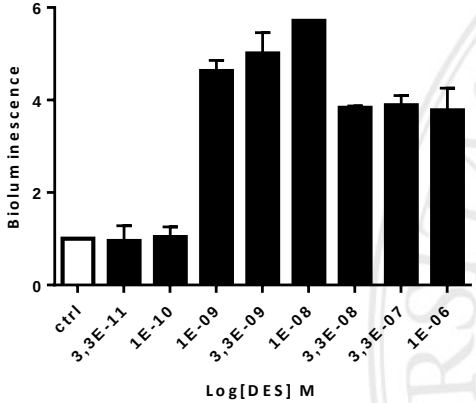
EC_{50} 6h

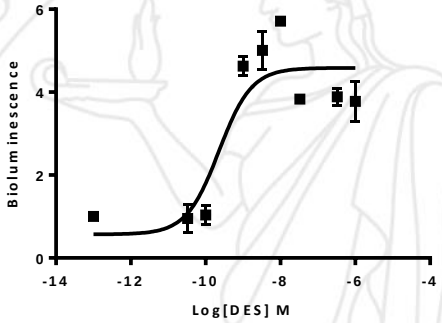
3.40E-10 M

Max Fold induction 6h

5.40

48h





EC_{50} 48h

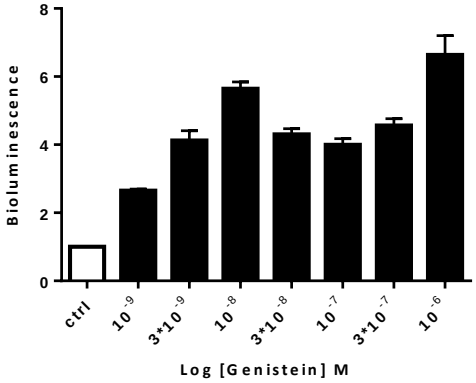
2.30E-10 M

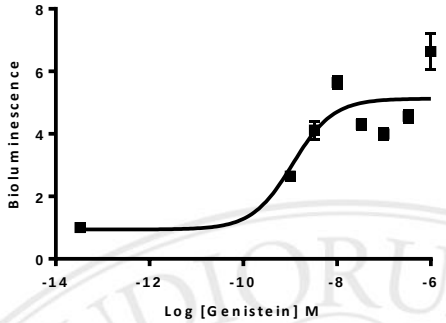
Max Fold induction 48h

5.70

in vitro bioluminescence imaging assay. 5

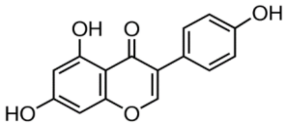
6 h



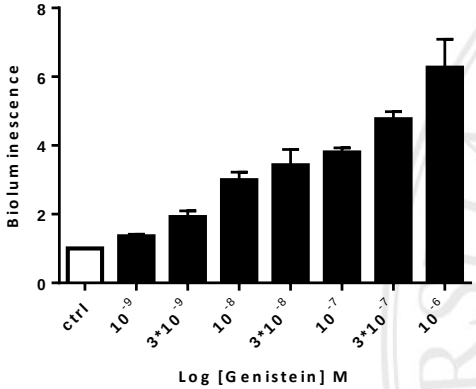


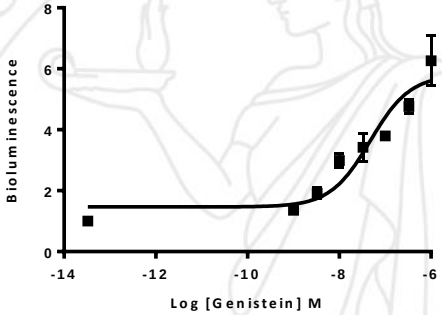
EC_{50} 6h
1.10E-09 M
Max Fold induction 6h
6.60

Genistein



48h



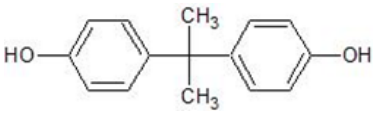


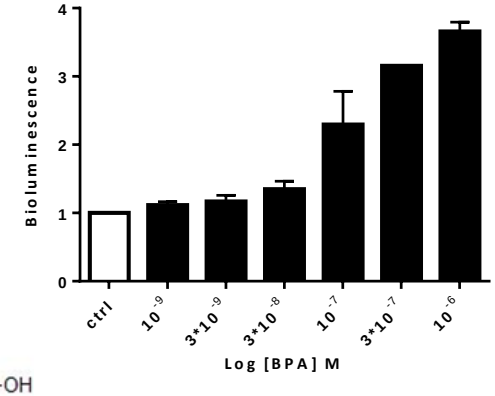
EC_{50} 48h
4.70E-08 M
Max Fold induction 48h
6.20

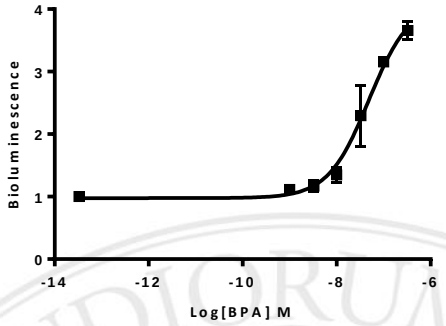
in vitro bioluminescence imaging assay. 3

6h

BPA

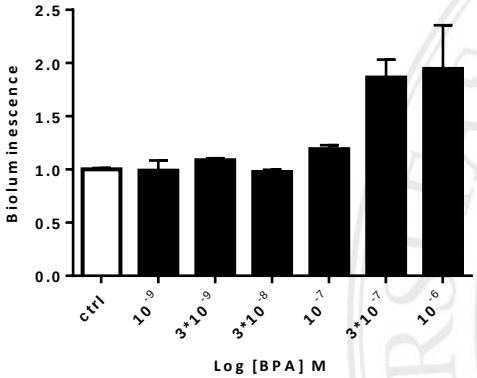


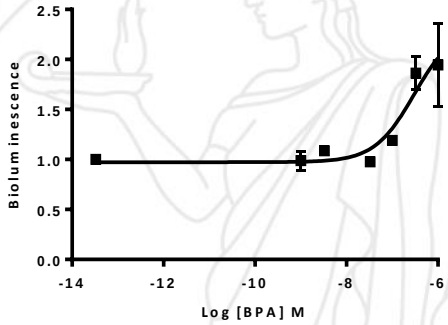




EC_{50} 6h
4.80E-08 M
Max fold induction 6h
3.66

48h



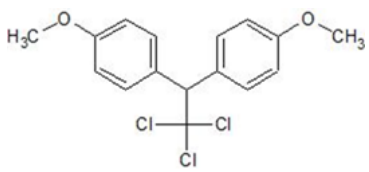


EC_{50} 48h
3.20E-07 M
Max Fold induction 48h
1.94

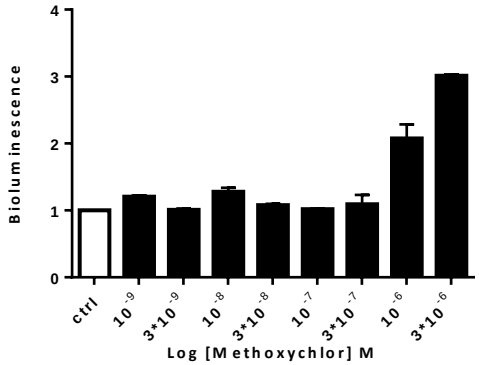
in vitro bioluminescence imaging assay. 6

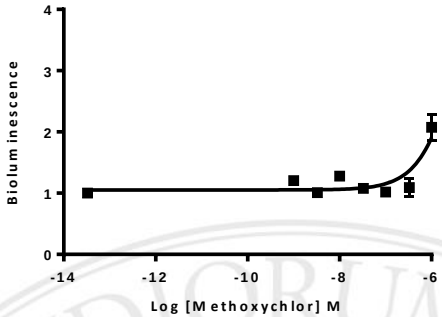
6h

Methoxychlor



48 h



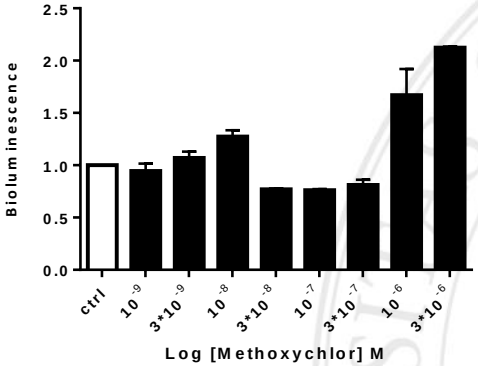


EC_{50} 6h

4.60E-06 M

Max Fold induction 6h

3.00



EC_{50} 48h

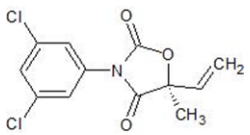
3.20E-07 M

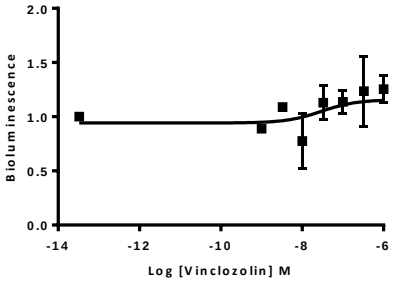
Max Fold induction 48h

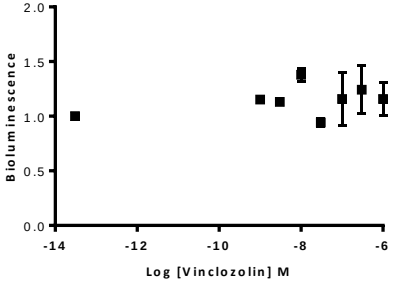
2.12

in vitro bioluminescence imaging assay. 7

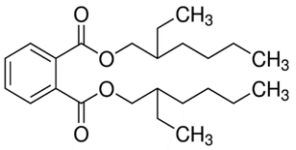
Vinclozolin

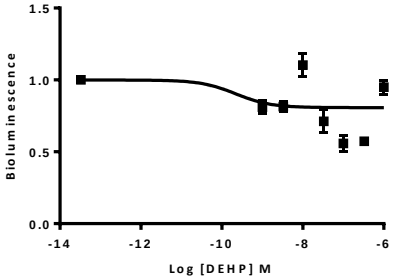


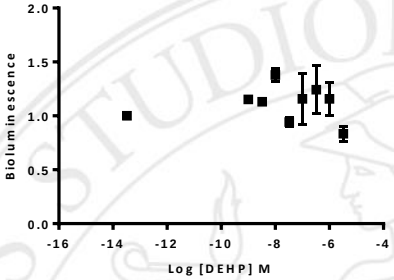




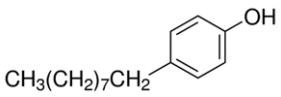
DEHP

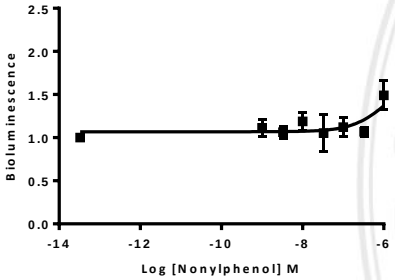


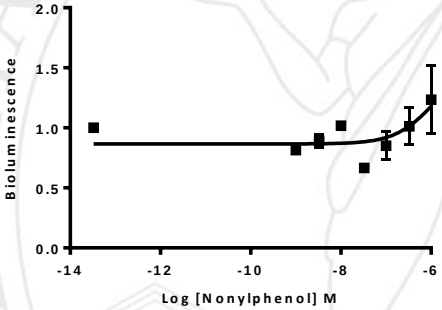




4-nonylphenol







Data at 6h

EC_{50}

Fold induction

Not Detectable

Data at 48h

EC_{50}

Fold induction

Not Detectable

In vitro bioluminescence imaging assay: summary

	POTENCY		EFFICACY*	
COMPOUND	**EC_{50} 6h**	**EC_{50} 48h**	**F.I. 6h**	**F.I. 48h**
17ß - ESTRADIOL	3.50E-12	6.90E-12	2.80	4.60
DIETHYLSTILBESTROL	3.40E-10	2.30E-10	5.40	5.70
ZEARALENONE	4.00E-10	4.50E-10	3.42	5.30
GENISTEIN	1.10E-09	4.70E-08	6.60	6.20
BISPHENOL A	4.80E-08	3.20E-07	3.66	1.94
METHOXYCHLOR	4.60E-06	4.90E-06	3.00	2.12
DEHP	n.d	n.d	n.d	n.d
4-NONYLPHENOL	n.d	n.d	n.d	n.d
VINCLOZOLIN	n.d	n.d	n.d	n.d

*Efficacy = fold induction (F.I.) = maximum response

n.d = not detectable

Results of in vitro bioluminescence imaging assay

Intrinsic activity = (POTENCY RATIO) x (EFFICACY RATIO) x (MAXIMUM EFFICACY VALUE AT 48H)

Classification

COMPOUND	INTRINSIC ACTIVITY
ZEARALENONE	8.17
17ß-ESTRADIOL	7.36
DIETHYLSTILBESTROL	6.12
GENISTEIN	4.76
METHOXYCHLOR	1.49
BISPHENOL A	0.91

Red = endocrine disrupting activity

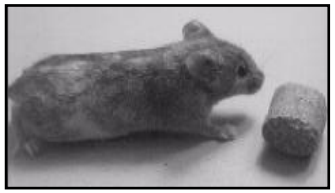
Threshold for no-ED

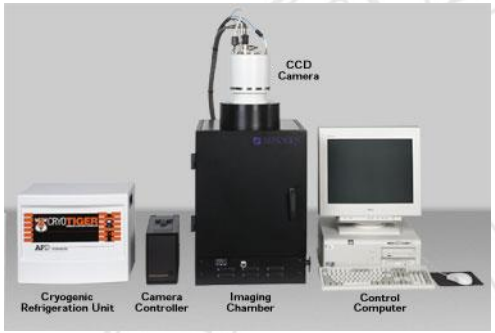
Green = no endocrine disrupting activity

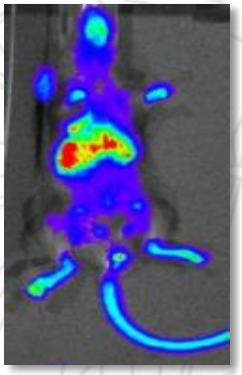
In vivo bioluminescence imaging experiments

Non invasive/dynamic analysis of molecular events

Luciferin administration







CCD- camera monitoring

ZEA (10 and 150 mg/kg/day
BPA (10 and 100 mg/kg/day,
21 days tratment

Ciana et al Nat Med 2003

In vivo bioluminescence imaging

chemicals	In vivo bioluminescence imaging			Literature in vivo data
	Genital area			
	ERE-Luc	NFkB-Luc	ARE-Luc	
ZEA	+/-	Repeated ++ Terminal++ (prostate)		Prostate
BPA				

In vivo bioluminescence imaging			Literature in vivo data
Abdominal area			
ERE-Luc	NFkB-Luc	ARE-Luc	
Acute ++ Repeated+/-	Repeated +/- Terminal ++		Kidney /liver
			Kidney/liver

++ $p < 0.05$ Statistically significant activation

+/- = trend, not statistically significant

No activation

CONCLUSIONS

- The in silico approach allowed a prioritization of the investigated chemicals through the calculation of their **affinity** that well fitted with in vitro competition binding results.
- The in vitro bioluminescence imaging assay identified the potency and efficacy of chemicals in activating the ER, during the time;
- The combination of variation of potency and efficacy, during the time, can be successfully used in **discriminating positive and negative compounds for their endocrine disrupting activity.**
- The reporter mice data offer information on the toxicodynamic of chemicals by a non invasive approach in the same mouse model. It allows the reduction of number of animal used and their suffering. This assay provides an indication of the target organs to be addressed in standard toxicological experiments and of the MoA.
- The use of **tiered approach** based on novel assays as in silico, in vitro and in vivo bioluminescence imaging methodologies **to discriminate EDC, is pivotal to save animal life, time and financial resources**

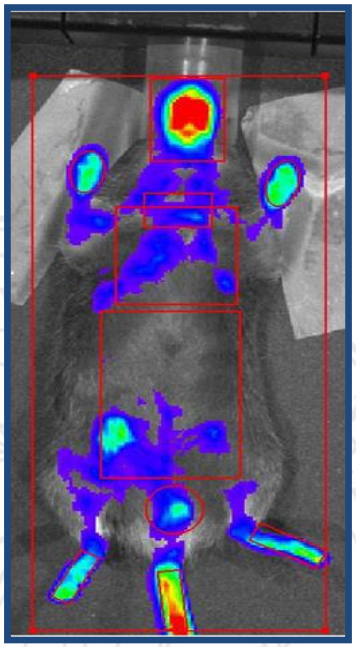
Thank you for the attention and

- Corrado L. Galli
- Paolo Ciana
- Pierpaolo La Fauci
- Ivano Eberini
- Luca Palazzolo
- Chiara Parravicini

for stimulating discussions and practical skills

and **Cariplo Foundation** that supported this study

In vivo bioluminescence imaging experiments



We applied the methodology to 3 different transgenic male mice models which were treated with Zearalenone and BPA in order to investigate:

- ER (ERE-Luc), estrogenic activation
- NFkB (NFkB-Luc), inflammatory pathway and
- ARE-Luc, to investigate the oxidative stress status

Then we focused on the bioluminescence from **genital and abdominal areas** of the same individual transgenic mouse during the entire time of experiment because of these area are considered most sensitive to EDC activity as reported in ECHA and EFSA Guidance;
So, we took into account 4 endpoints of activation status:

I) the acute response at 24 hours
ii) the AUC as repeated activation (along the 21 days)
iii) the end of repeated exposure (day 21)
iv) the ex vivo photon emission measured from the explanted organs.