Integrated Exposure and Effects Assessment (IEEA)

11-12 April 2017
Norman workshop Amsterdam

Integrated approaches to investigate the effect of progestins in fish and their occurrence in the aquatic environment

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EDCs in aquatic systems

Endocrine Disrupting Chemicals (EDCs)
in aquatic systems

↓

Disruption of development, sexual differentiation, reproduction

↓

Risks for aquatic species

Natural & synthetic estrogens
Xeno-estrogens

↓

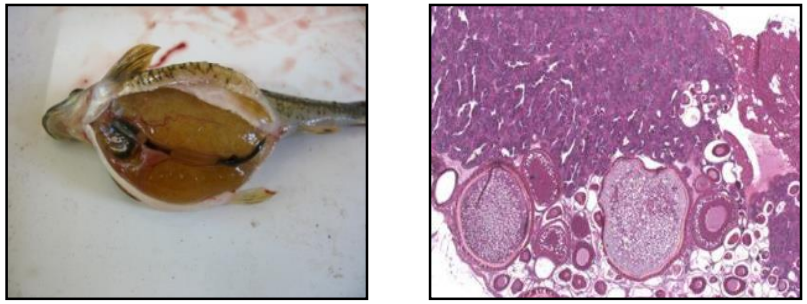
Environmental estrogens can act at low concentrations on various biological levels
(from molecular to population level)

↓

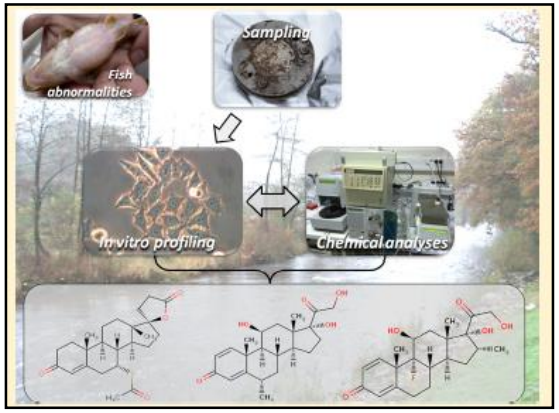
Derivation of EQS for EE2, E2, E1
(Watchlist of the WFD)

Environmental hazards associated with other steroidal compounds ?

• Evidence of the occurrence of other natural and synthetic steroidal compounds in the aquatic environment released from WWTPs effluents



Disruption of vitellogenesis & gonadal histology
(Sanchez et al., 2011 *Env. Int*.)



Hormonally active pharmaceuticals identified
but no steroidal estrogens identified !
(Creusot et al., 2014 *ES&T*)

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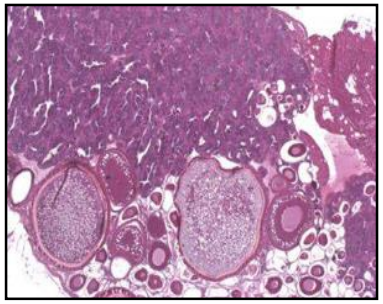

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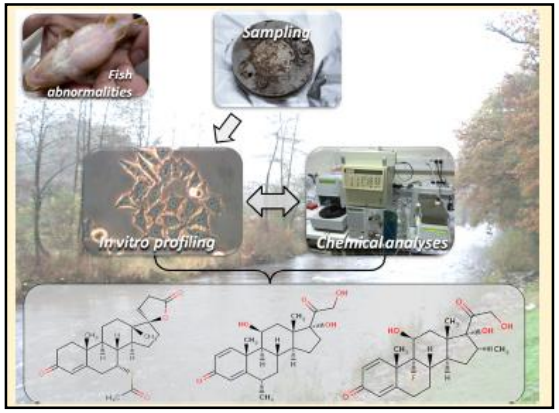

Hormonally active pharmaceuticals identified
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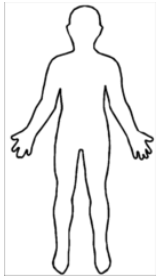
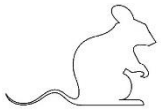
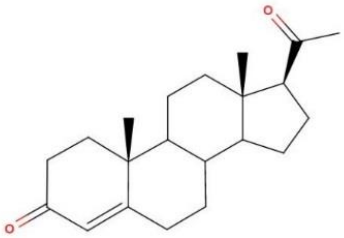
Need to increase knowledge on the effects of steroidal pharmaceuticals (other than estrogens) on aquatic organisms

Progestagenic sex steroid hormones in vertebrates





Progesterone (P4)

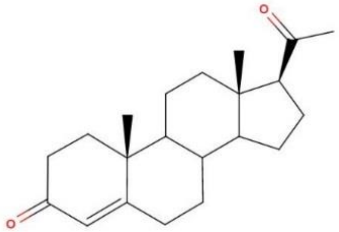


Progesterone (P4)

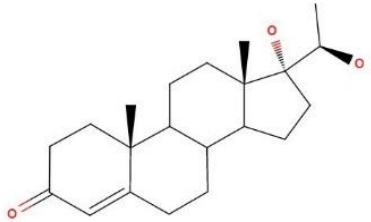
17α, 20β-dihydroxy-4-pregnen-3-one (DHP),

17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S)

P4



DHP



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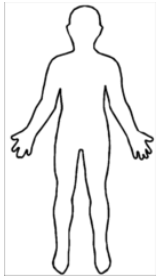

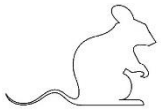

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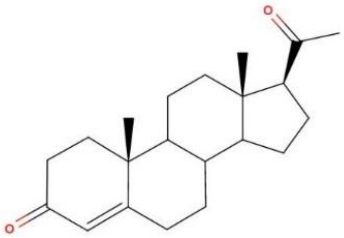

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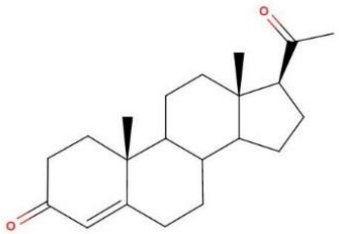

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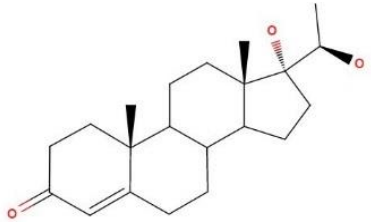
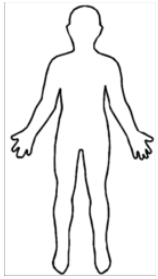
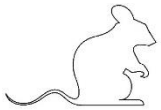


Membrane (mPR) , Nuclear progesterone receptor (nPR)

Strong expressions in brain and gonads

Progestagenic sex steroid hormones in vertebrates





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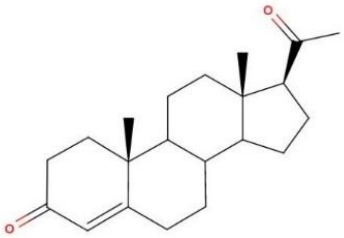

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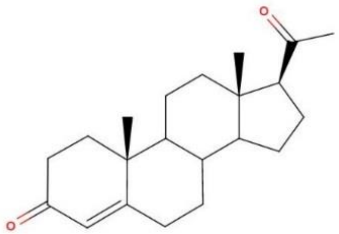

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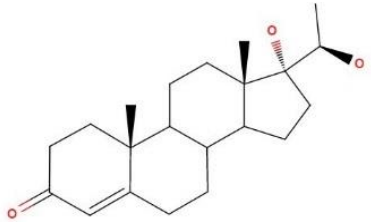


Membrane (mPR) , Nuclear progesterone receptor (nPR)

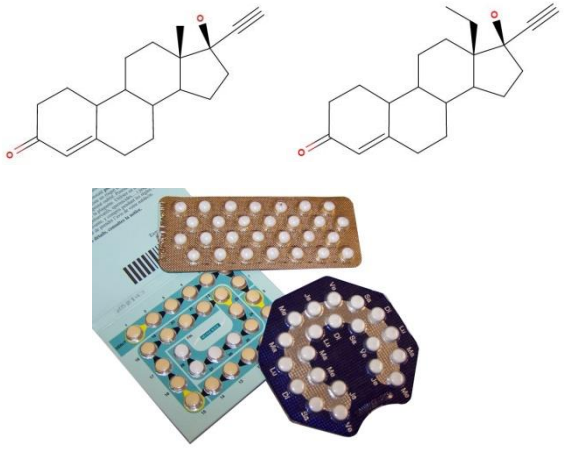
Strong expressions in brain and gonads

P4 = neurosteroid involved in different brain functions

Critical roles in reproduction in females and males

Environmental risks related to progestins ?

Widely used as pharmceuticals



Present in effluents and surface waters

Low ng/L range
Up to µg/L

Can alter fish development and reproduction

Fent (2015) for review

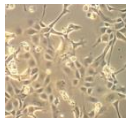
We need to investigate the mechanism & effect of progestins in fish and their occurrence in the aquatic environment to bring data (if not proofs!) to assess their hazards and risks to aquatic species and environment

AGENCE NATIONALE DE LA RECHERCHE ANR PROOFS

An integrated approach to investigate the effects of progestins in fish and their occurrence in the aquatic environment

Environmental hazard posed by environmental ligands of the progesterone receptor to aquatic species ?

in vitro & *in vivo* species-specific mechanism-based bio-assays



Substance

Mixture

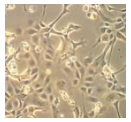
24 selected synthetic progestins

An integrated approach to investigate the effect of progestins in fish and their occurrence in the aquatic environment

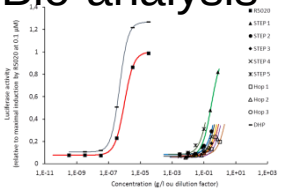
Environmental hazard posed by environmental ligands of the progesterone receptor to aquatic species ?

Occurrence of (anti-)progestagenic activities & identification of substances in aquatic systems ?

in vitro & *in vivo* species-specific mechanism-based bio-assays



Bio-analysis



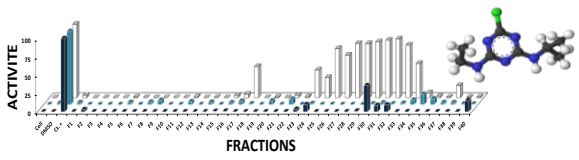
Substance

Mixture

Environmental matrices

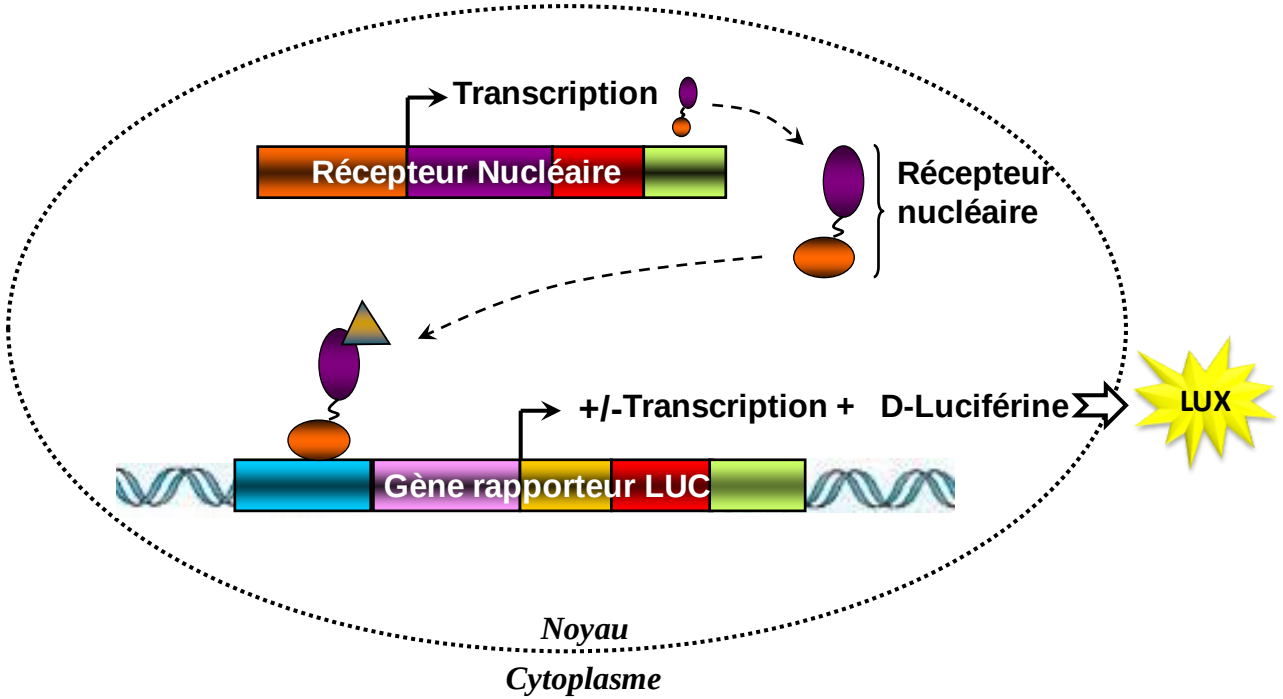
24 selected synthetic progestins

Effect-directed analysis



Interaction of progestins with nuclear steroidal receptors ?

Human cell lines stably expressing human or zebrafish NRs



- hPRB
- zfPR

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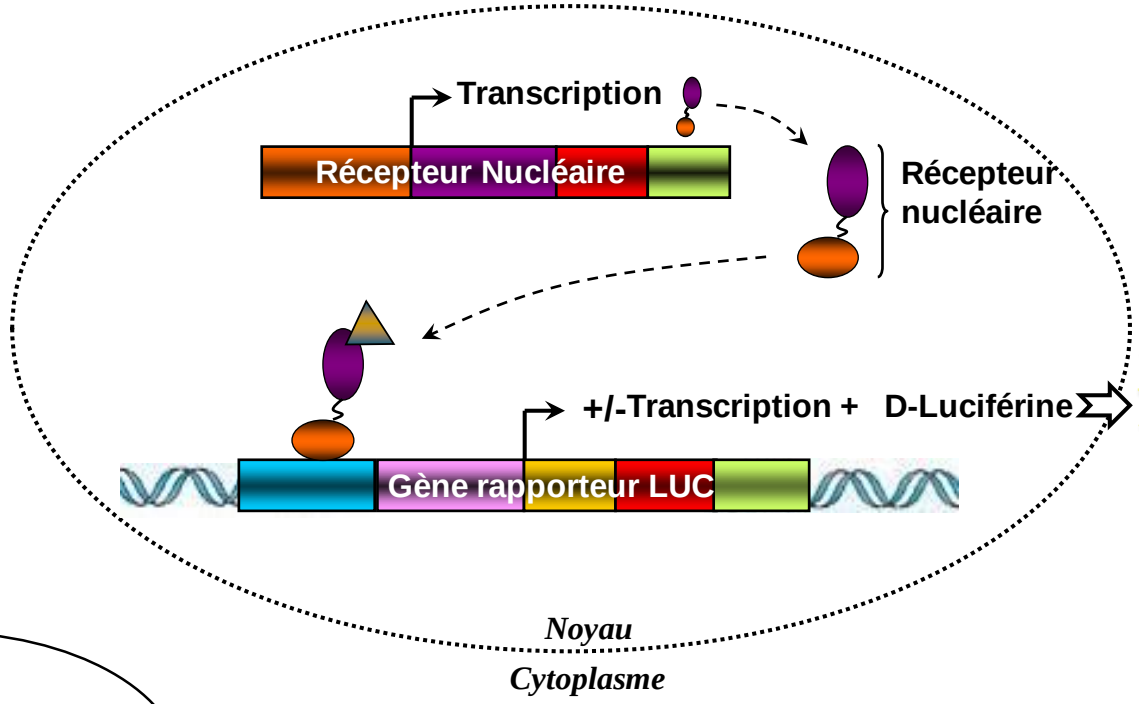


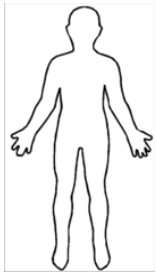
- hAR
- zfAR

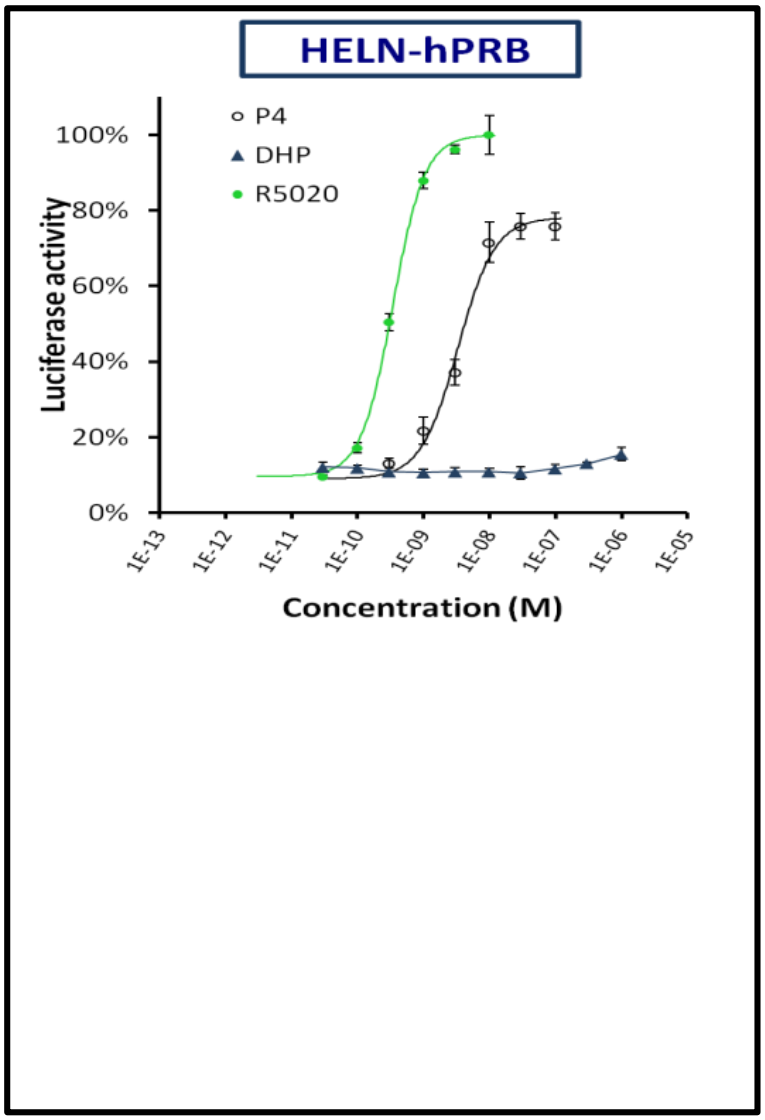
- hERα
- zfER α, β1,β2

- hPRB
- zfPR

- hGR
- zfGR

Activity of reference ligands towards hPR and zfPR





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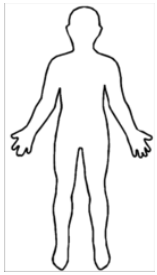

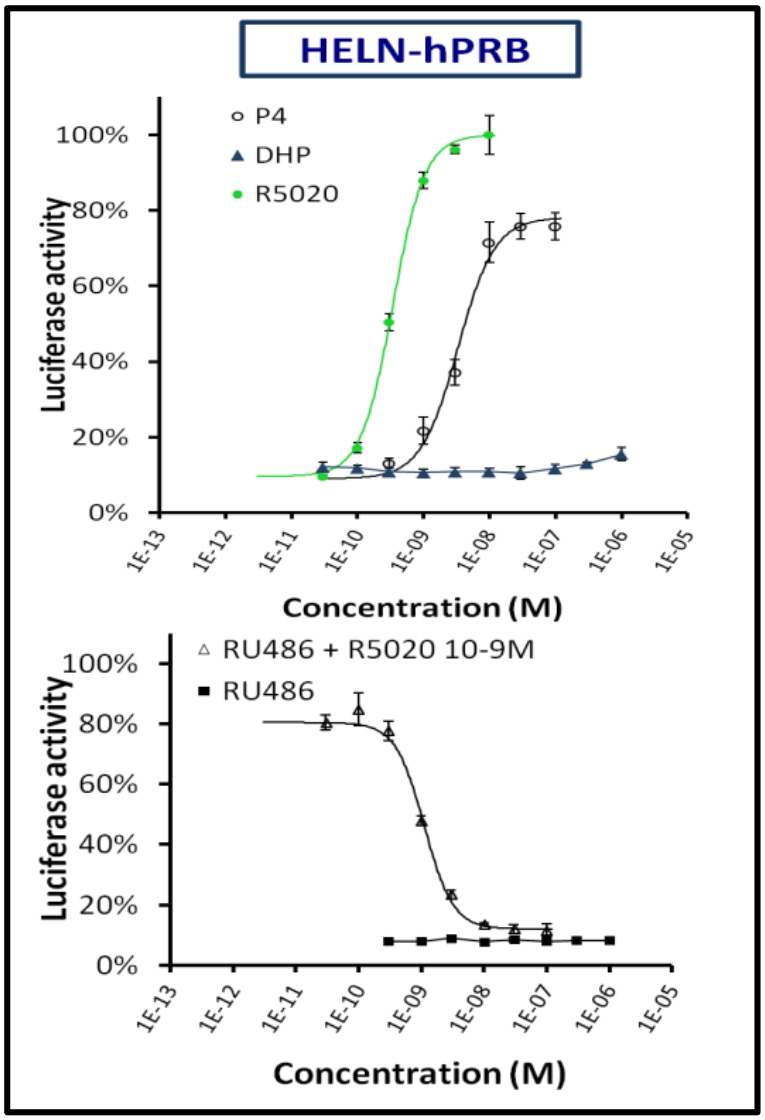

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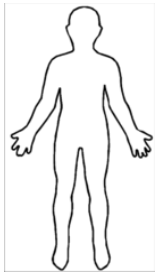

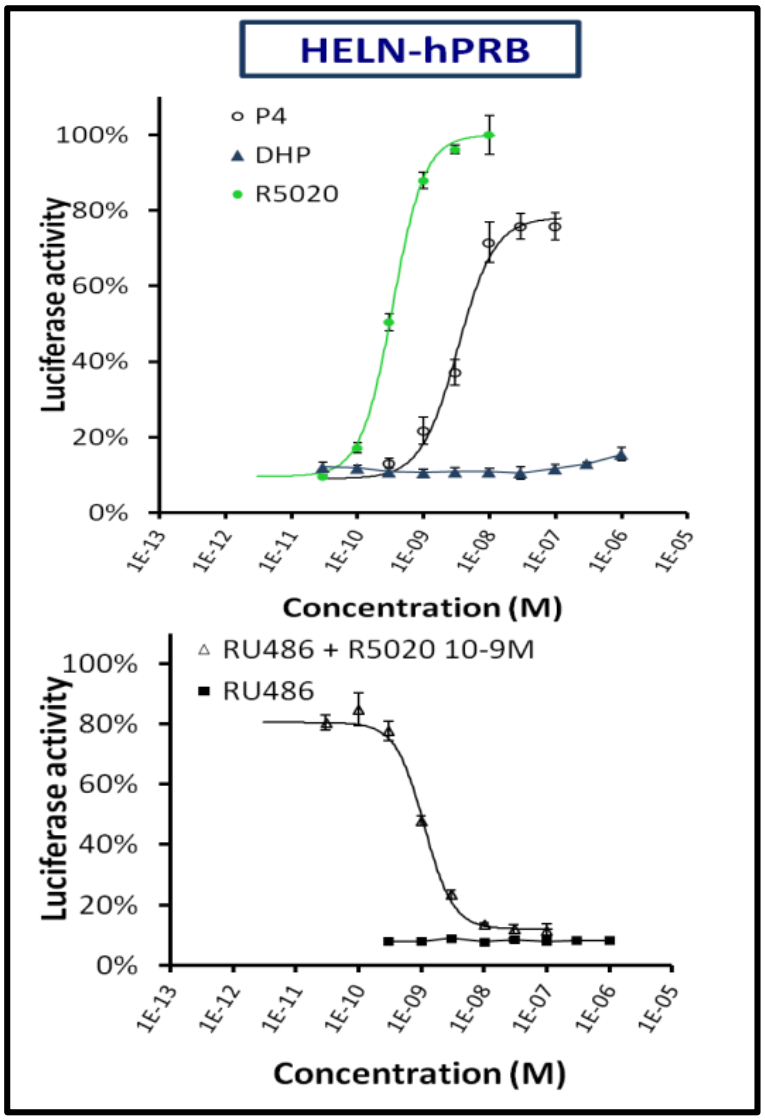

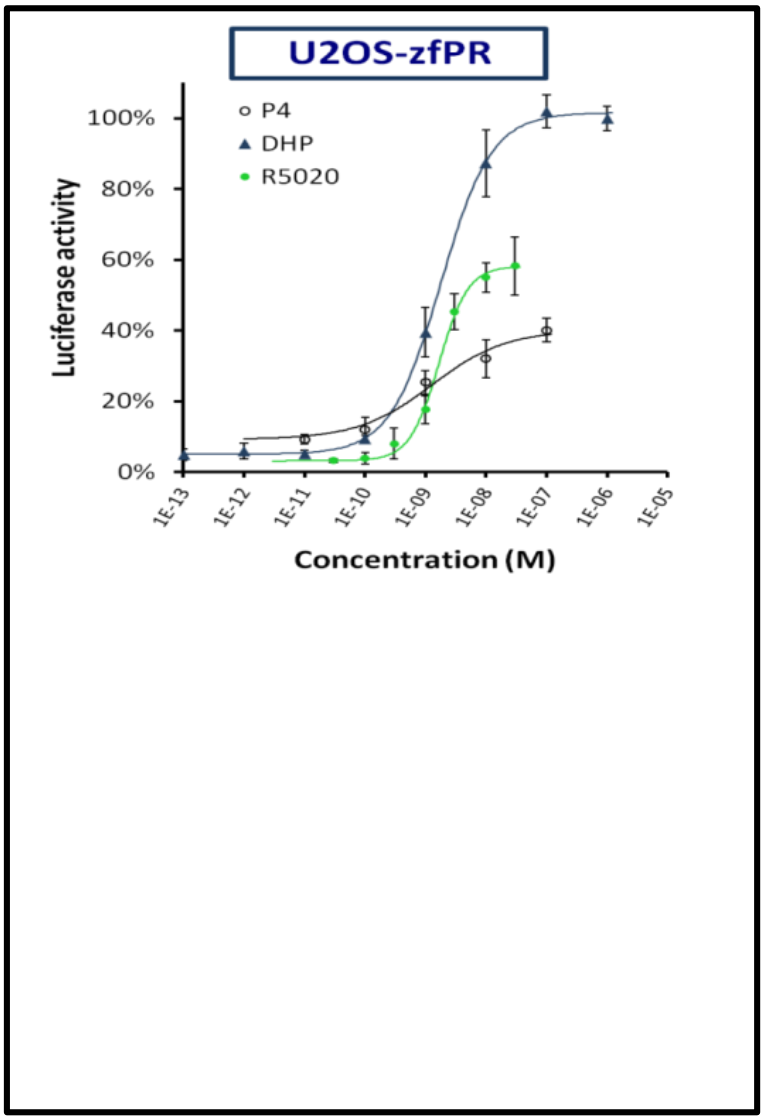

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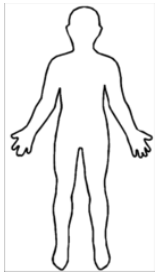
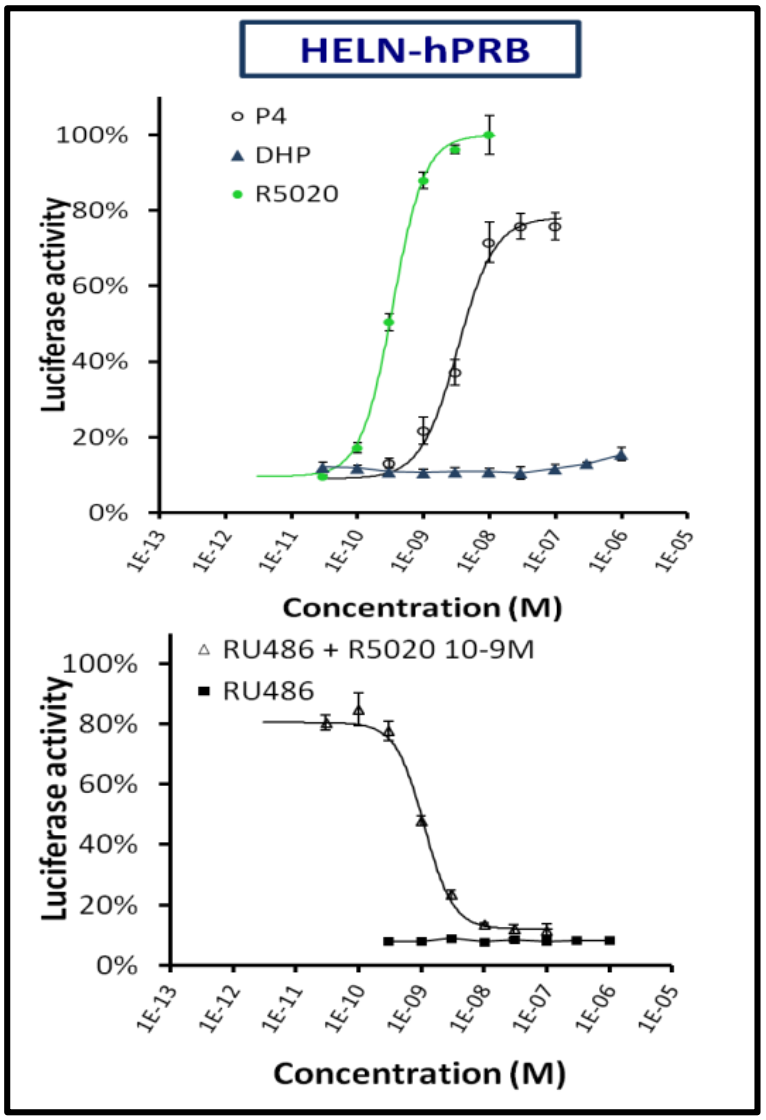
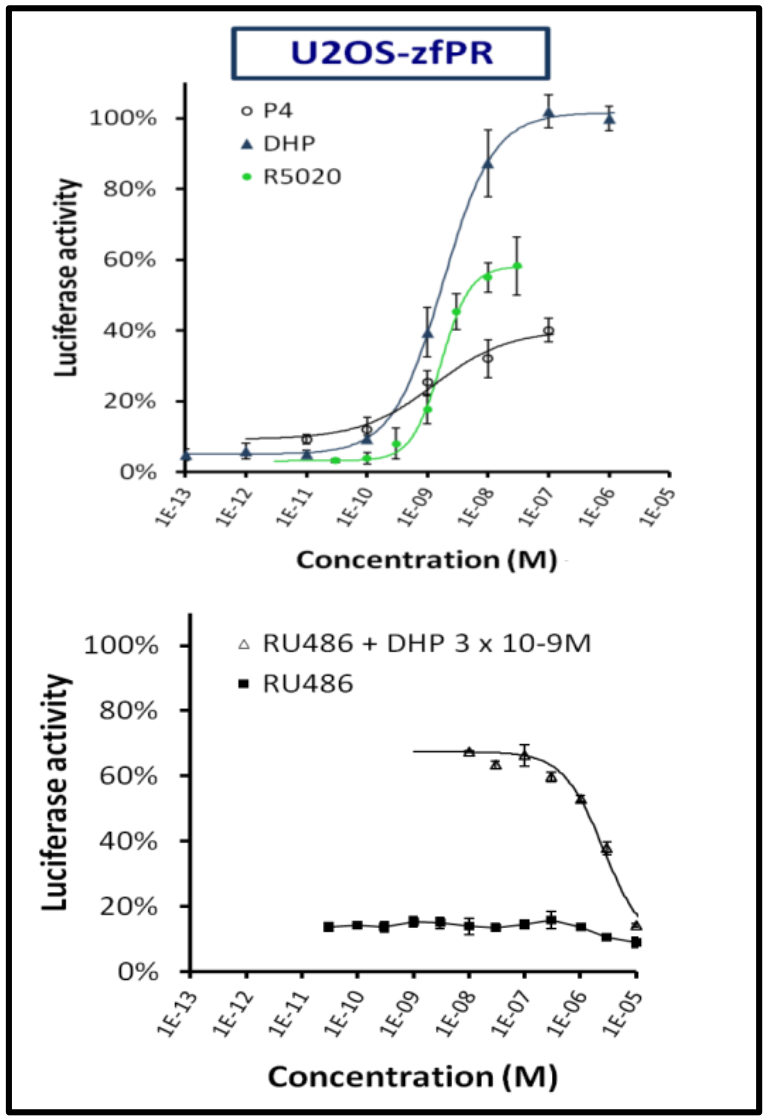




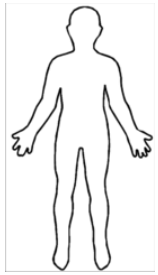


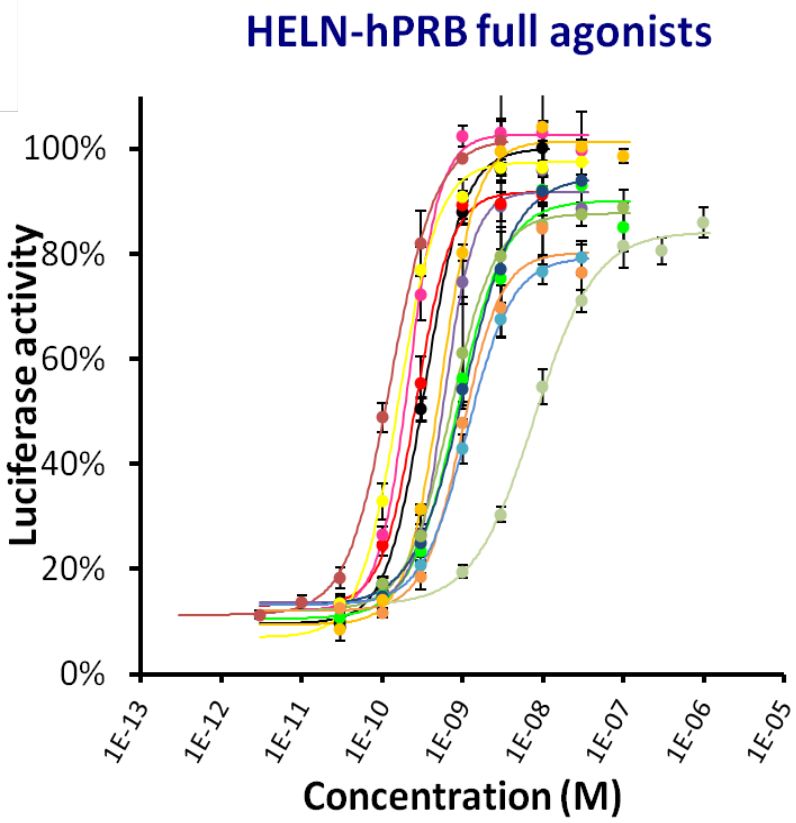
The luciferase activities are PR-dependent and PR-specific

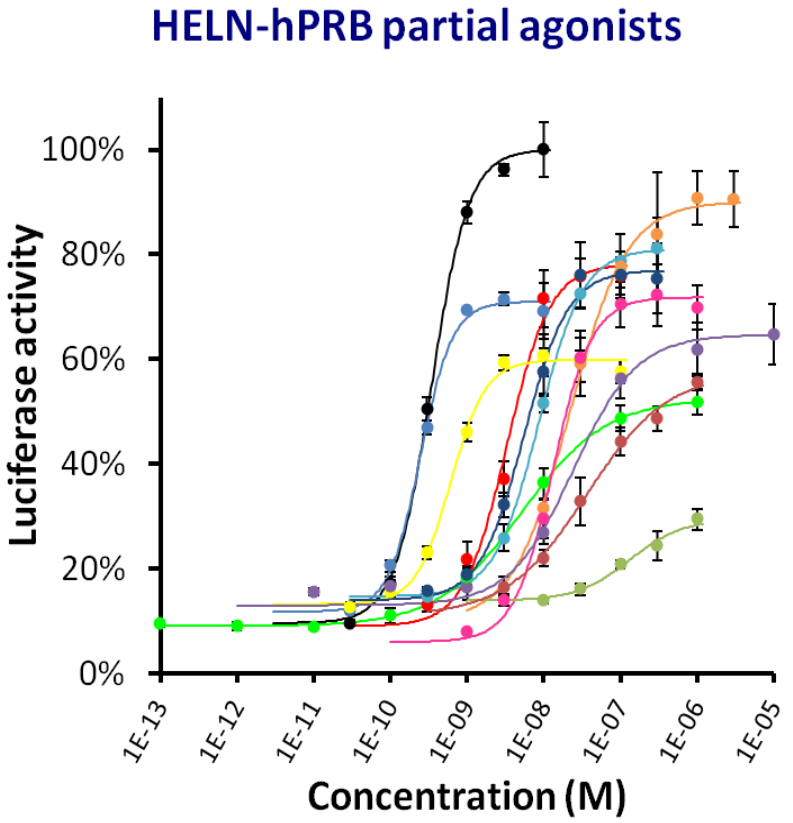
Selected ligands: 26 pharmaceuticals progestins

Classification		Compound
Natural estrogen		17β-estradiol
Natural progestin		Progesterone
Synthetic progestin retroprogesterone		Dydrogesterone
Progestins structurally related to progesterone	Derived from 17α-hydroxyprogesterone	Medroxyprogesterone Medroxyprogesterone acetate Megestrol acetate Chlormadinone acetate Cyproterone acetate
	Derived from 19-norprogesterone	Promegestone Nestorone Nomegestrol acetate
	Derived from 17α-hydroxy-19-norprogesterone	Gestonorone
Progestins structurally related to testosterone: derived from 19-nortestosterone	Estranes	Ethisterone Ethynodiol diacetate Lynestrenol Norethindrone acetate Norethindrone Tibolone
	Gonanes	Desogestrel Etonogestrel Gestodene Levonorgestrel Norgestimate Norgestrel
Progestin structurally related to spironolactone		Drospirenone
PR antagonist		Mifepristone

All the synthetic progestins act as agonist of hPR



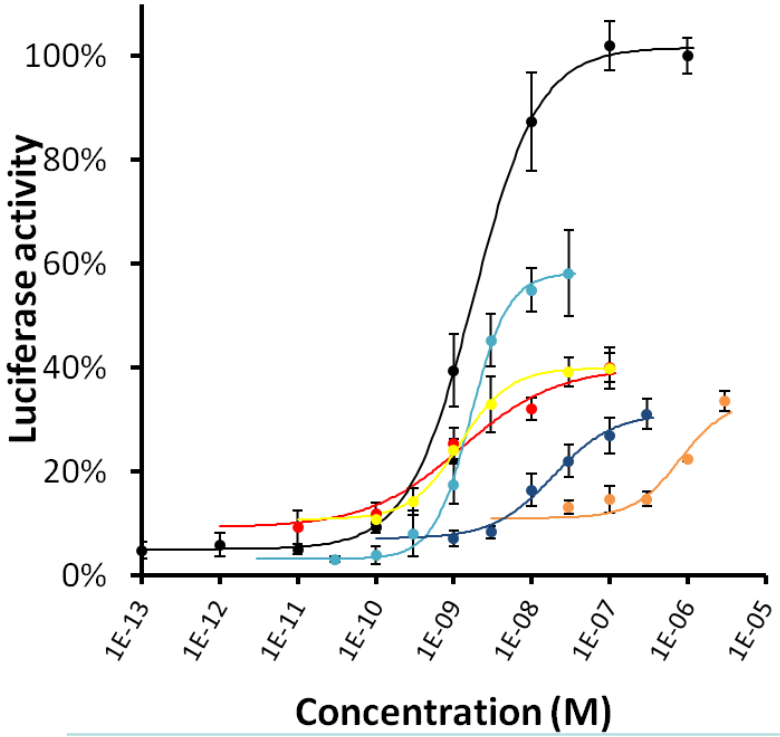




- EC_{50} ranging from 0.33 nM (R5020) to 9.97 nM (TIB=Tibolone).

- EC_{50} ranging from 0.20 nM (medroxyprgesterone acetate) to 249 nM Medroxyprogesterone).

Most of the progestins act as zfPR antagonists



	EC_{50} ± sd (nM)
P4	1.97 ± 0.57
DHP	1.87 ± 0.44
R5020	1.93 ± 0.60
Nestorone	1.41 ± 0.41
Drospirone	14.1 ± 4.49
Spironolactone	674 ± 300

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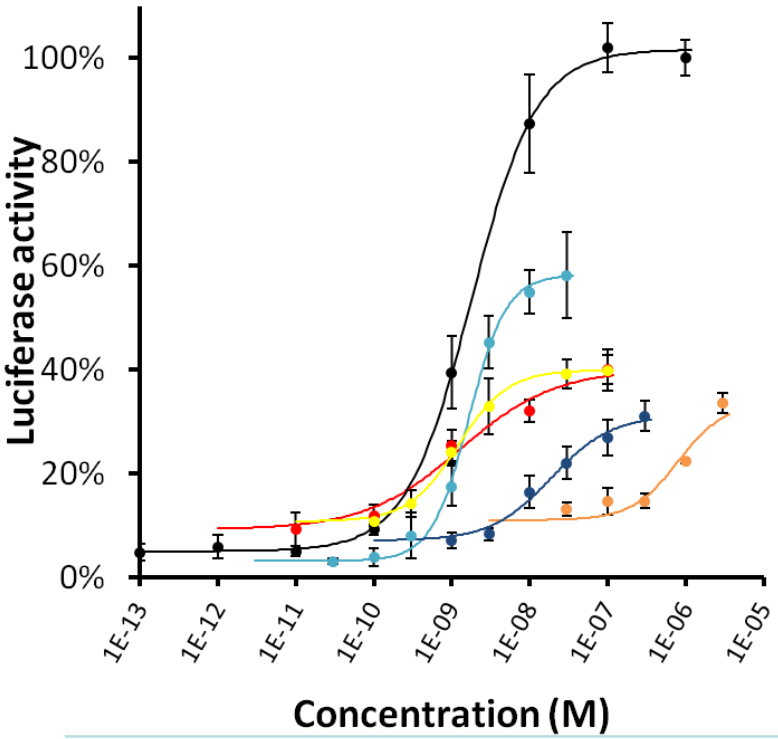

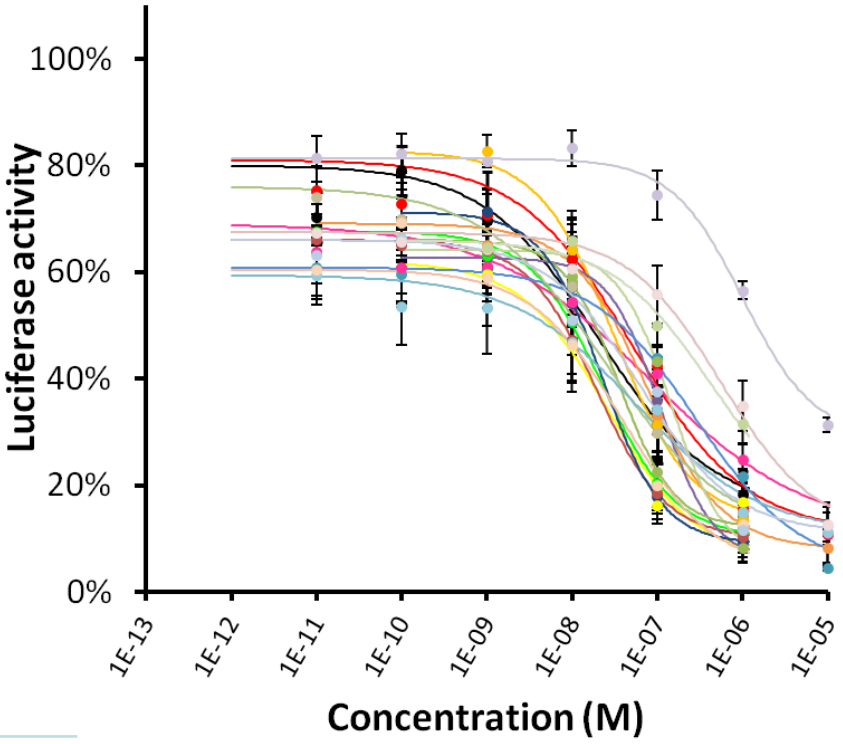

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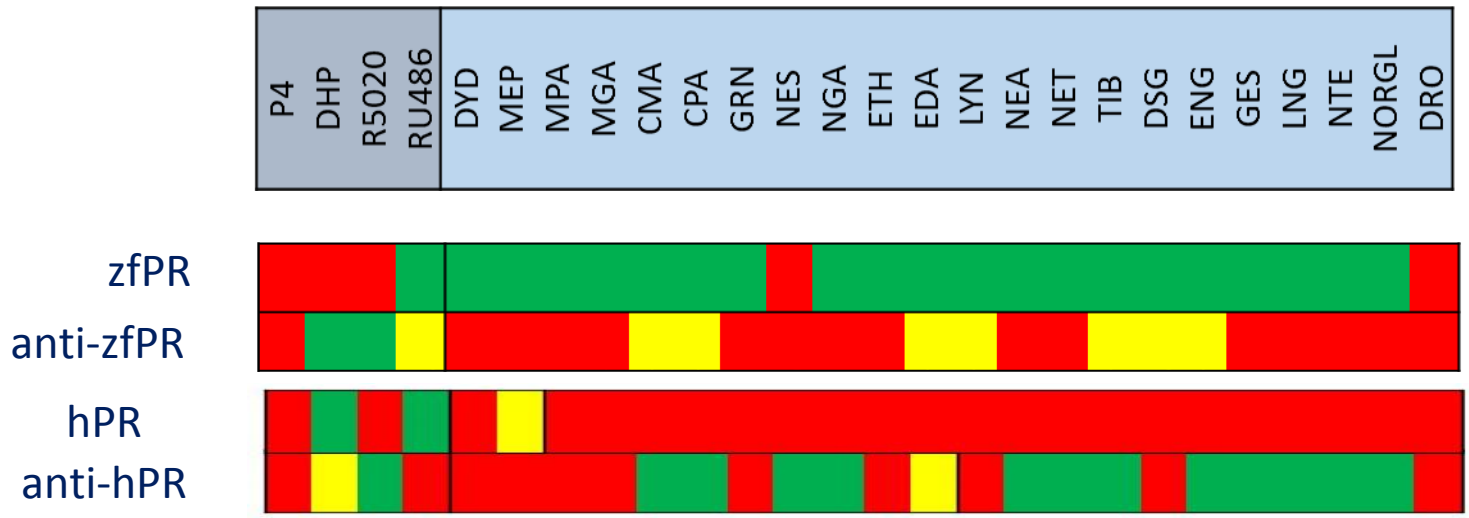
Spironolactone	674 ± 300

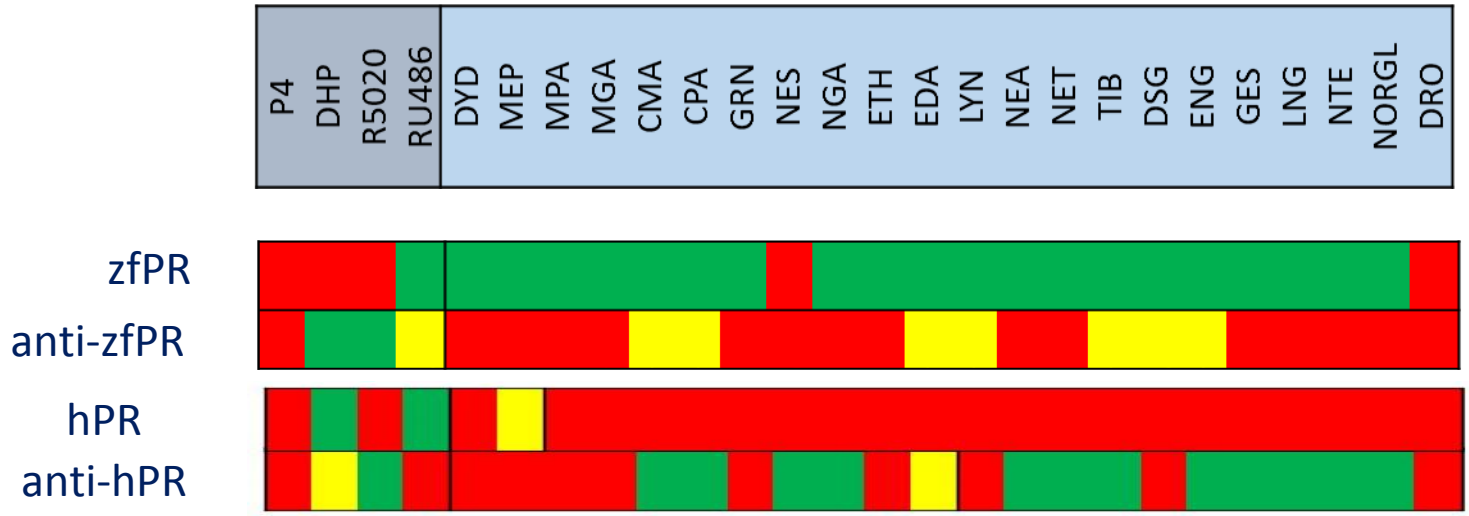
- IC_{50} ranging from 14 nM (levonorgestrel) to 1000 nM (desogestrel).

Complex toxicological profiles of progestins towards zfNRs



n.a.	> 10 µM
3E-05	0.1 - 10 µM
1E-08	< 0.1 µM

Complex toxicological profiles of progestins towards zfNRs



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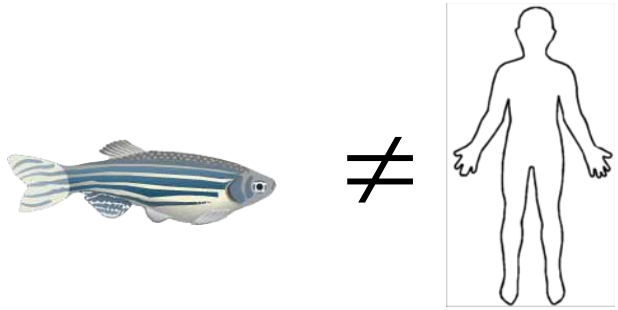

•**hAR**
•**zfAR**

Need to characterize the interactions of progestins towards zf-nuclear receptors

•**hGR**
•**zfGR**

•**hERα**
•**zfER α, β1,β2**

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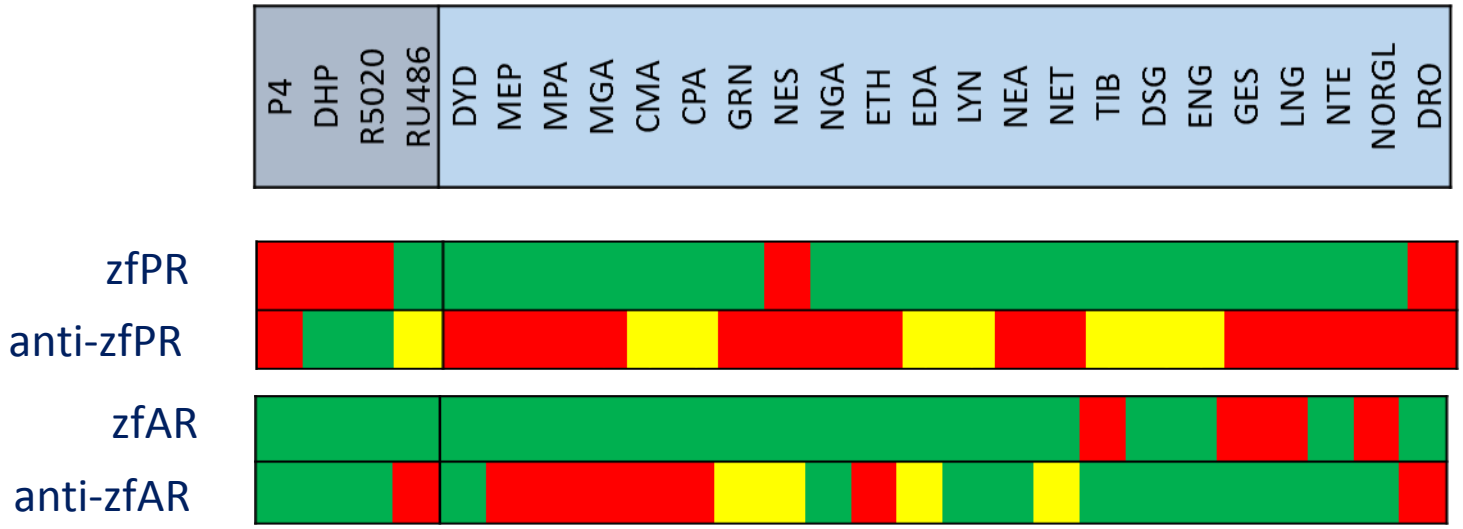
maîtriser le risque pour un développement durable

Complex toxicological profiles of progestins towards zfNRs



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Some are potent androgenic compounds
Levonorgestrel is the most potent one
(EC50= 4 nM)

But most of progestins elicit anti-androgenic activity (EC50= 30 - 600nM)

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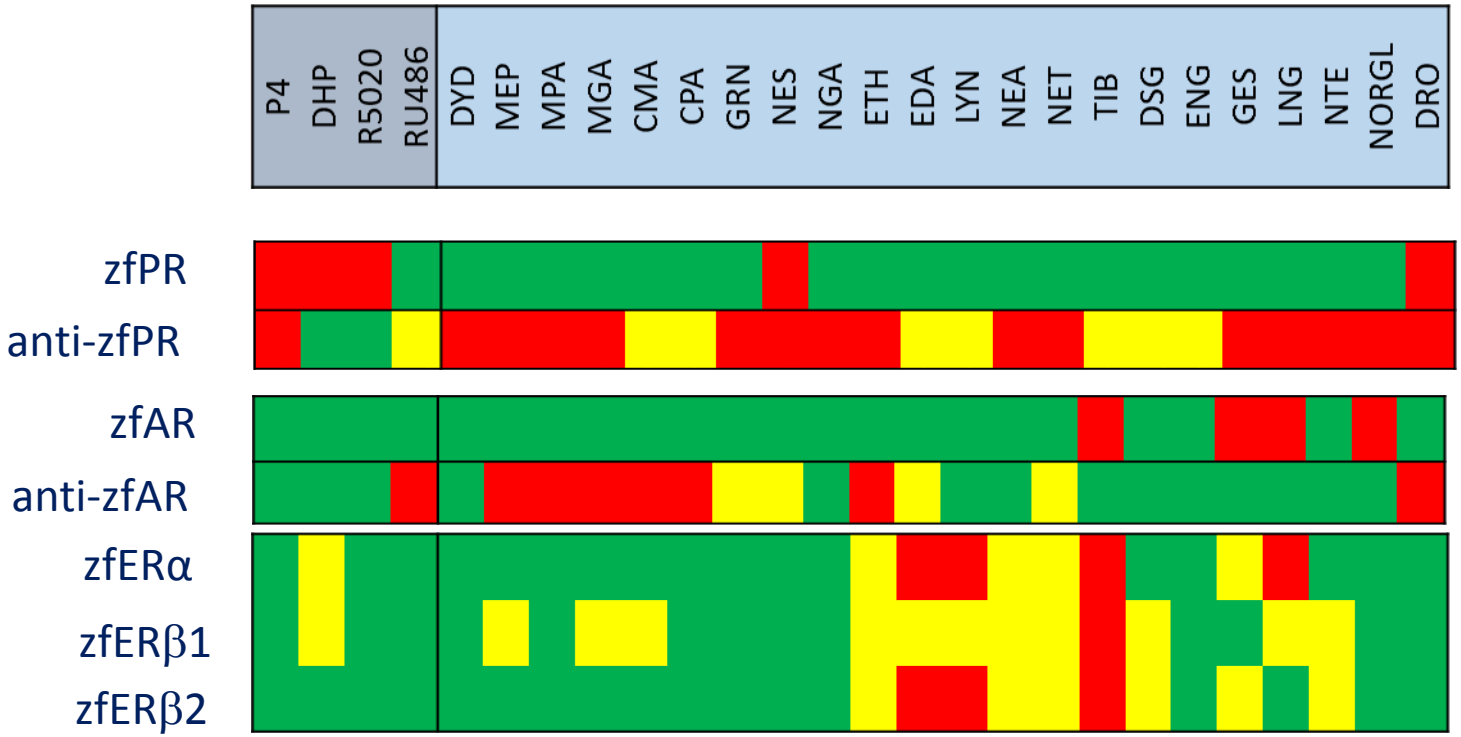
pour un développement durable

Complex toxicological profiles of progestins towards zfNRs

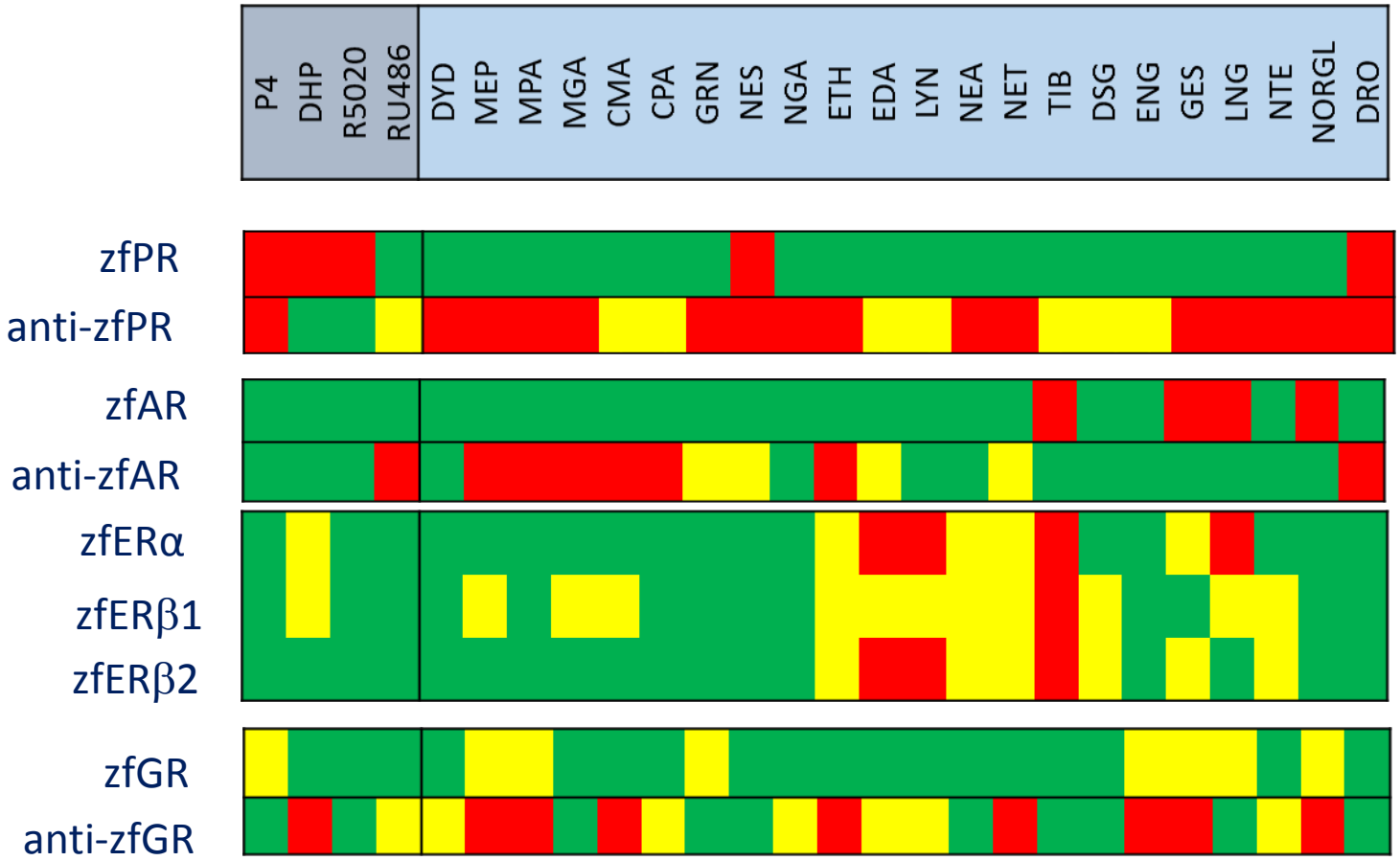


n.a.	> 10 µM
3E-05	0.1 - 10 µM
1E-08	< 0.1 µM

Progestins derived from 19-nortestosterone (estrane and gonane) elicit estrogenic activity towards the 3 zfERs subtypes

INERIS
maîtriser le risque pour un développement durable

Complex toxicological profiles of progestins towards zfNRs



n.a.	> 10 µM
3E-05	0.1 - 10 µM
1E-08	< 0.1 µM

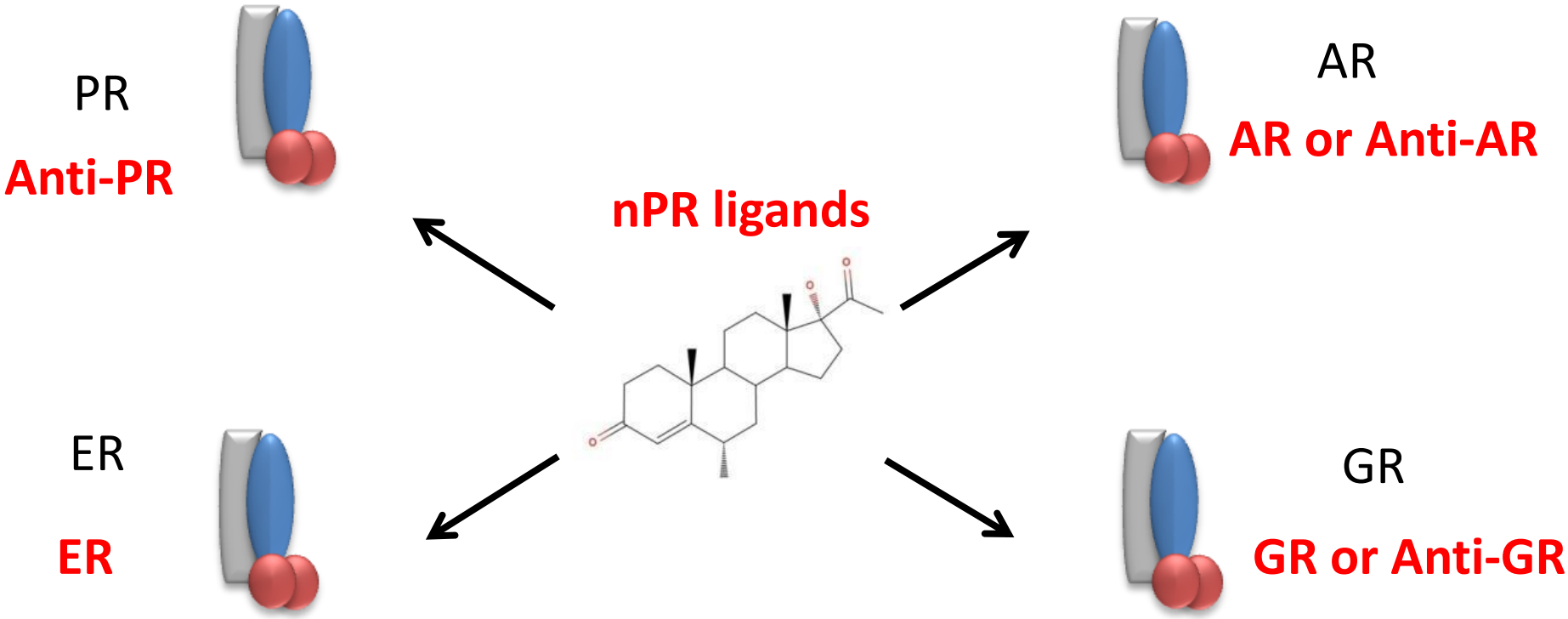
Interaction of progestins with zfGR

Most elicit strong anti-zfGR activities

INERIS
maîtriser le risque pour un développement durable

Interactions of progestins towards zf nuclear steroidal receptors

Complex toxicological profiles
Zebrafish-specific responses



Interactions of progestins towards zf nuclear steroidal receptors

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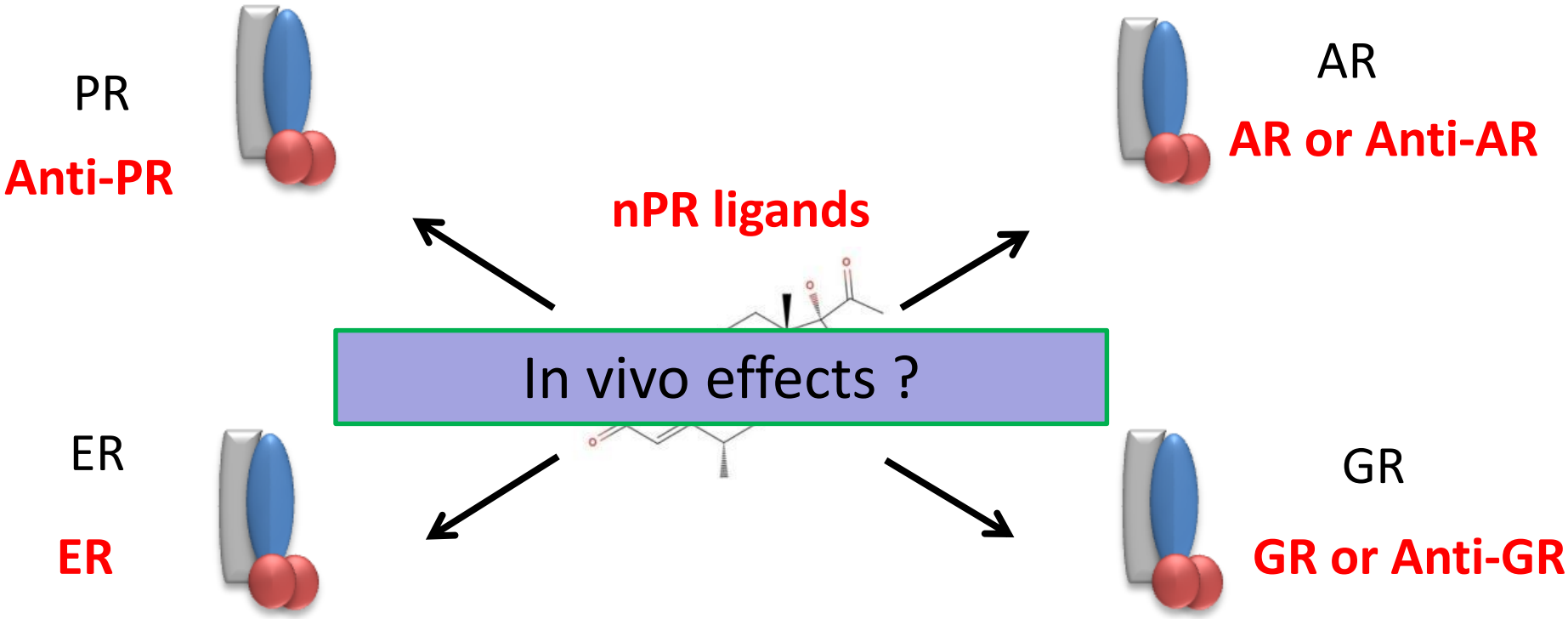
Zebrafish-specific responses



Interactions of progestins towards zf nuclear steroidal receptors

Complex toxicological profiles
Zebrafish-specific responses

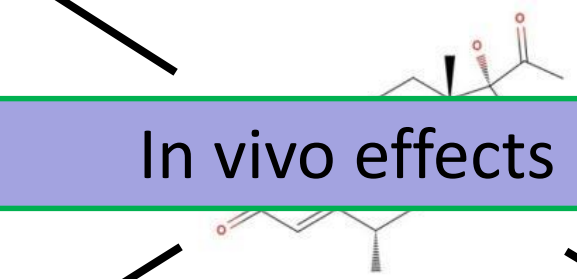
PR

Anti-PR

AR

Strong andogenic effect of levonorgestrel in stickleback (Svensson et al., 2013, 2014)

nPR ligands



In vivo effects ?

ER

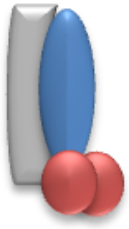
ER

GR

GR or Anti-GR

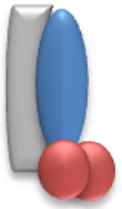
Interactions of progestins towards zf nuclear steroidal receptors

Complex toxicological profiles
Zebrafish-specific responses



PR

Anti-PR



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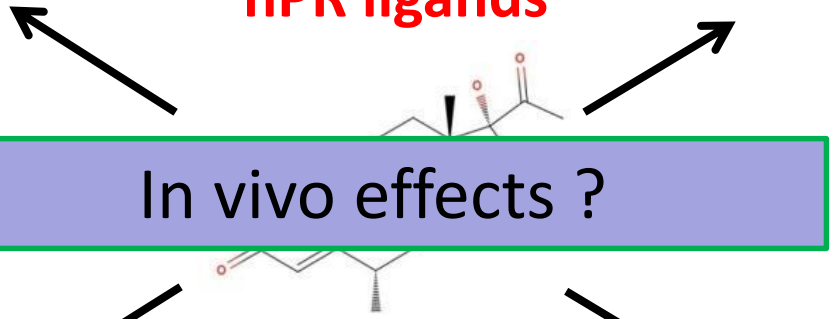
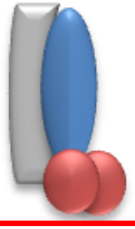
nPR ligands



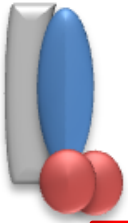
In vivo effects ?



ER

ER

No data



GR

GR or Anti-GR

No data

Interactions of progestins towards zf nuclear steroidal receptors

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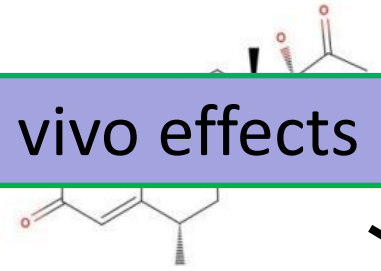


In vivo effects ?

ER

ER

No data

GR

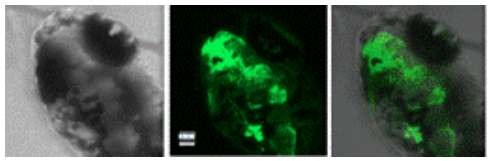
GR or Anti-GR

No data

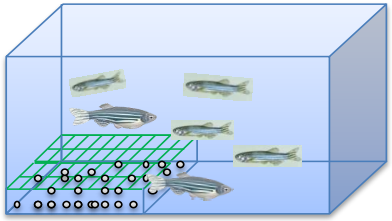
In vivo zebrafish mechanism-based assays using transgenic models

In vivo screening estrogenic activity of 24 selected progestins

Detection of **E**ndocrine **A**ctive **S**ubstance, acting through estrogen receptors, using transgenic cyp19a1b-GFP **Z**ebrafish Embr**Y**os **(EASZY)**

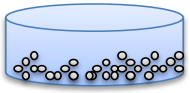


① **Spawning**

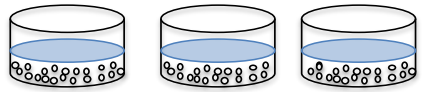


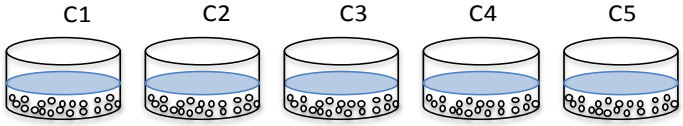
Mass spawning in 100L aquaria with 60-100 male: female fish (ratio from 1:1 to 2:1)

② **Collecting,**
Cleaning eggs.
Selection of fertilized eggs
with normal development



③ **N= 20 eggs per test condition & Start of exposure** (< 4 hours hpf)



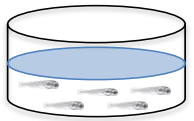


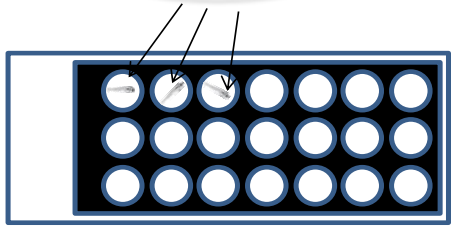
Substance

Crystallizer (25 ml of water)

④ **96 hrs of exposure**

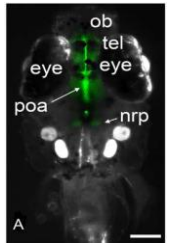
Incubator 27± 1°C,
14 hrs light photoperiod



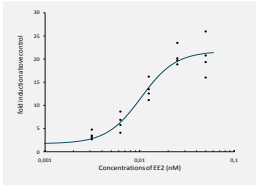


Transfer of post-hatched larvae in multi-well Fluorescence slides

⑤ ***In vivo*** **Fluorescence imaging**



⑥ **Image Analysis and data processing**

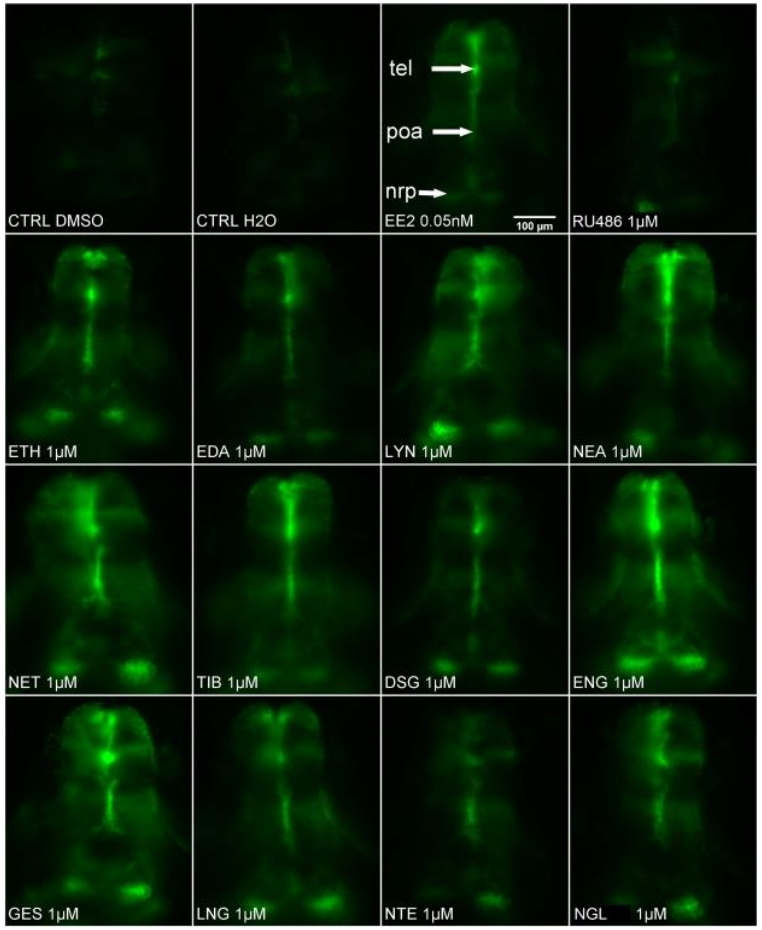


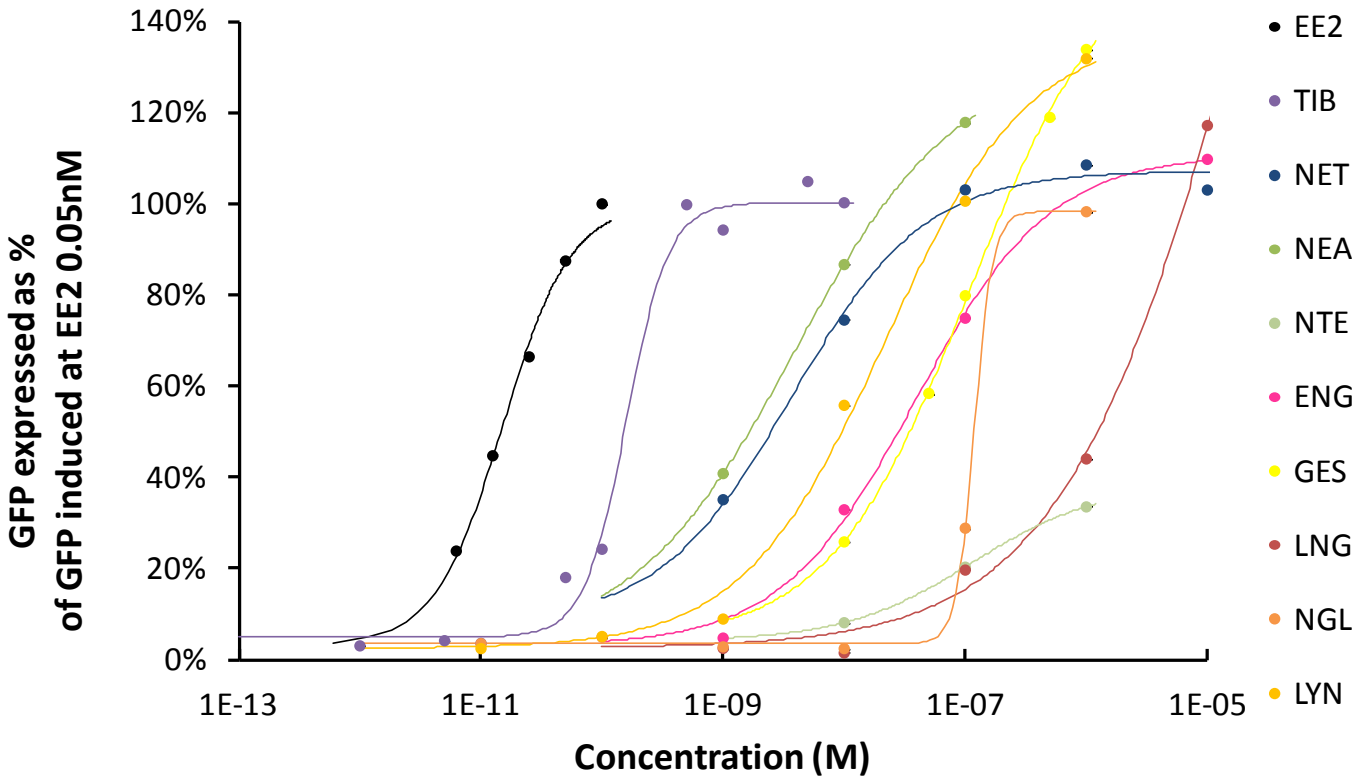
Brion et al., 2012
Plos One

Effects of progestins on the ER-regulated brain aromatase expression

- No effect of progesterone and progesterone derivative

- All progestins derived from testosterone tested so far are estrogenic to fish





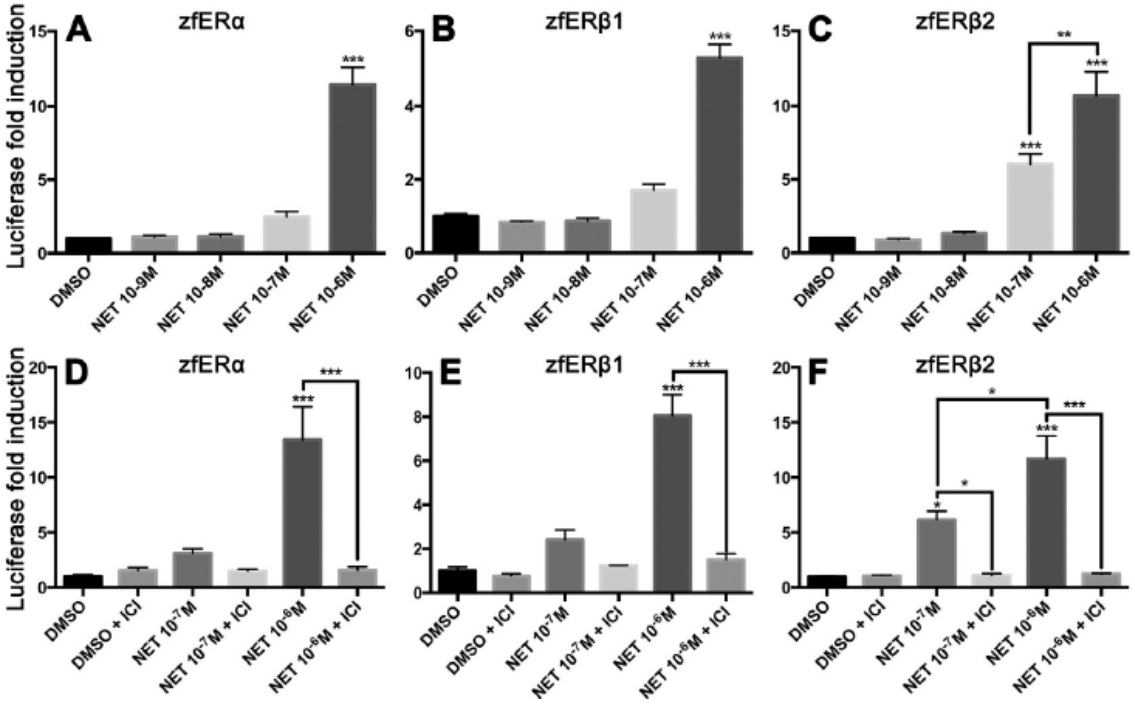
Cano-Nicolau, Garoche *et al.,* 2016 *Tox. Appl. Pharmacol*.

Effects of progestins on the ER-regulated brain aromatase expression

Compound	Abbreviation	Effect in EASZY	EC_{20} (nM)	EC_{50} (nM)	REP
17β-estradiol	E2	+	0.62	1.7	1
Testosterone	T	+	337	505	0.003
Pregnenolone (progesterone precursor)	P5	+	105	826	0.002
Progesterone	P4	ne	–	–	–
Dydrogesterone	DYD	ne	–	–	–
Medroxyprogesterone	MEP	ne	–	–	–
Medroxyprogesterone acetate	MPA	ne	–	–	–
Megestrol acetate	MGA	ne	–	–	–
Chlormadinone acetate	CMA	ne	–	–	–
Cyproterone acetate	CPA	ne	–	–	–
Promegestone	R5020	ne	–	–	–
Nestorone	NES	ne	–	–	–
Nomegestrol acetate	NGA	ne	–	–	–
Gestonorone	GRN	ne	–	–	–
Ethisterone	ETH	+	5.5	40	0.04
Ethynodiol diacetate	EDA	+	34	53	0.03
Lynestrenol	LYN	+	3.5	16	0.1
Norethindrone acetate	NEA	+	0.4	2.4	0.7
Norethindrone	NET	+	1	4.0	0.4
Tibolone	TIB	+	0.1	0.3	5.7
Desogestrel	DSG	+[a]	nc	nc	nc
Etonogestrel	ENG	+	7	39	0.04
Gestodene	GES	+	41	222	0.008
Levonorgestrel	LNG	+	39	89	0.02
Norgestimate	NTE	+	65	126	0.01
Norgestrel	NGL	+	73	184	0.009
Drospirenone	DRO	ne	–	–	–
Mifepristone	RU486	+[a]	nc	nc	nc

Cano-Nicolau, Garoche *et al.*, 2016 *Tox. Appl. Pharmacol.*

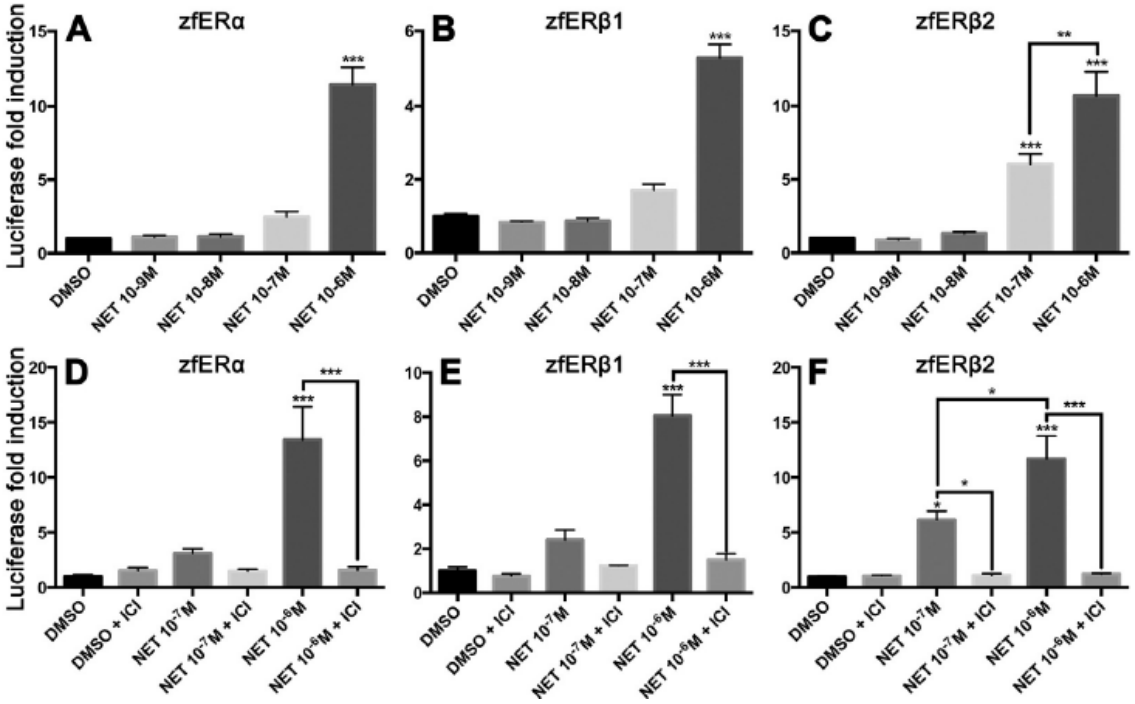
Functional zfERs are required to induce cyp19a1b expression



- Induction of luciferase activities in U251 MG cells transfected with zfERs and cyp19a1b-luc

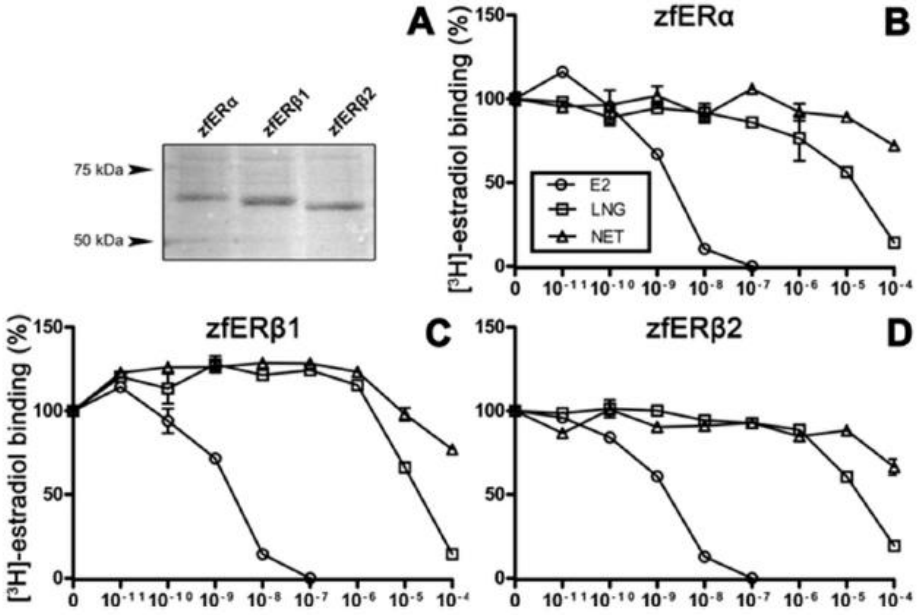
Cano-Nicolau, Garoche *et al.,* 2016 *Tox. Appl. Pharmacol*.

Functional zfERs are required to induce cyp19a1b expression



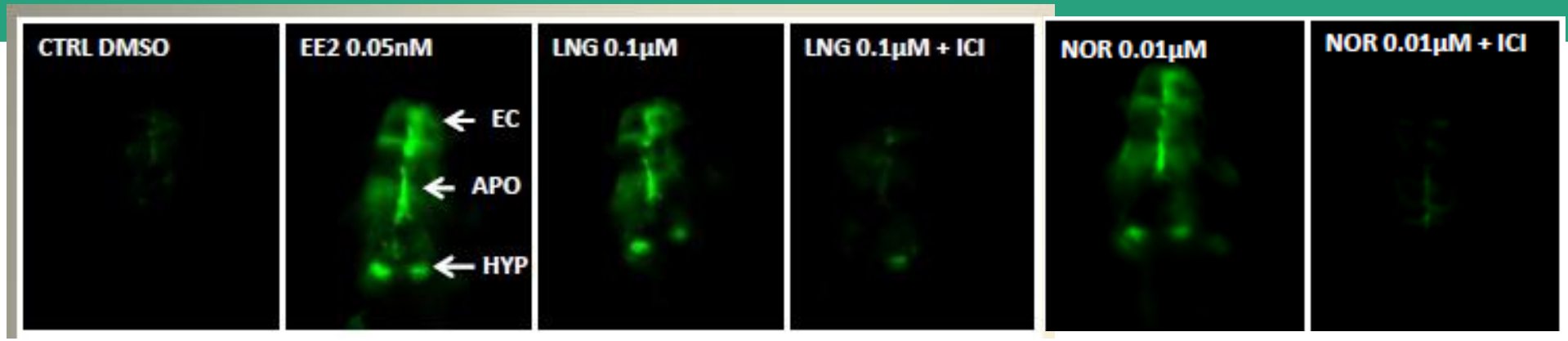
- Induction of luciferase activities in U251 MG cells transfected with zfERs and cyp19a1b-luc

- Very low binding affinity of levonorgestrel and norethindrone towards zfERs

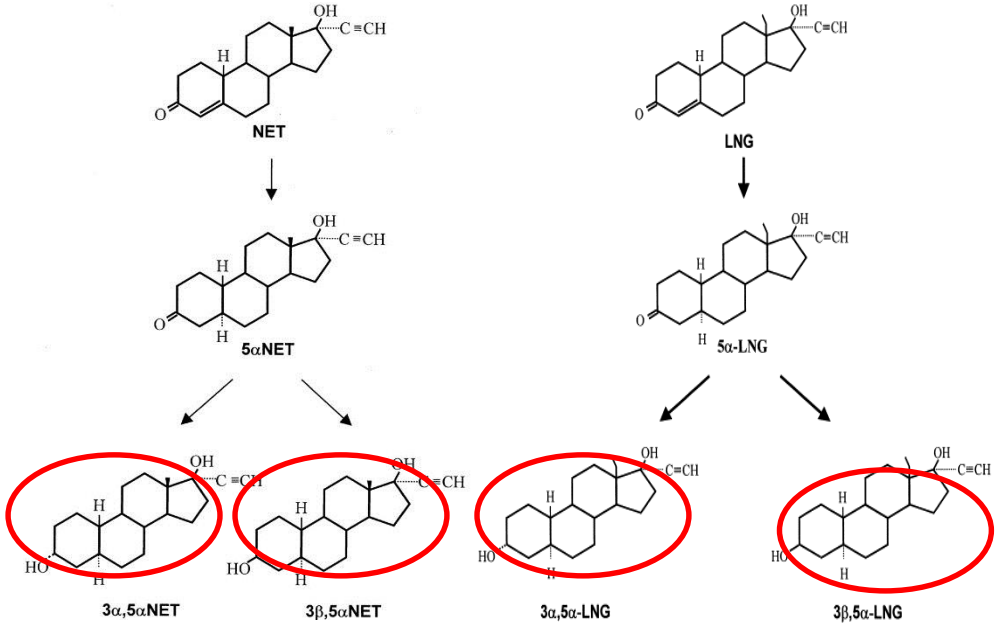


Cano-Nicolau, Garoche *et al.,* 2016 *Tox. Appl. Pharmacol*.

Biotransformation of progestins into estrogenic metabolites is likely required



NOR= Norethindrone LGN= Levonorgestrel

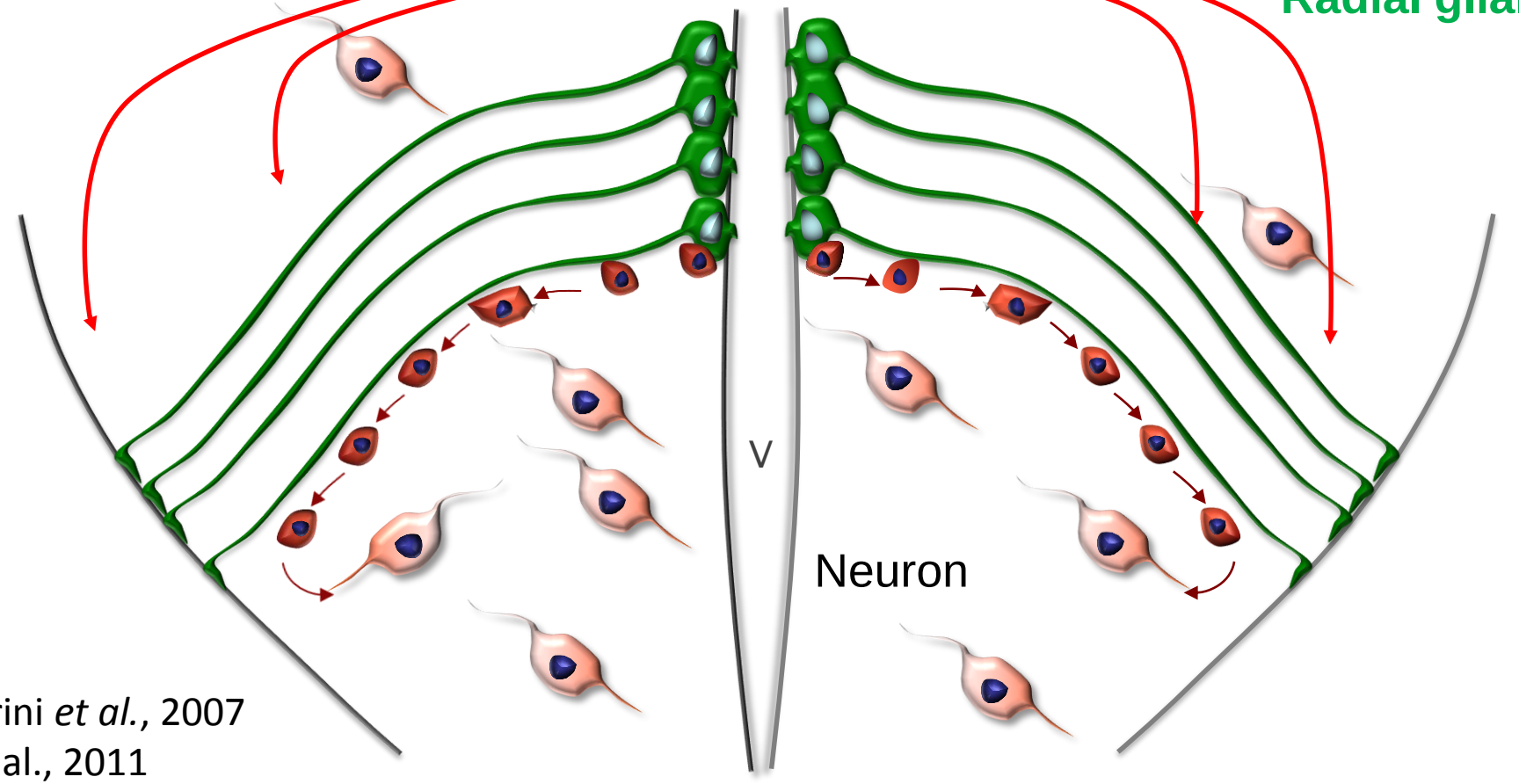


3α,5α- and 3β,5α-tetrahydro derivated of NET and LNG are estrogenics in mammalian models (Larrera et al., 2001 , Garcia-Becerra et al., 2002)

Induction of the glial cell-specific expresson of the brain aromatase

19-nortestosterone derived progestins
(pro-estrogenic substances)

estrogenic metabolites



From Pellegrini *et al.*, 2007
Kah et al., 2011

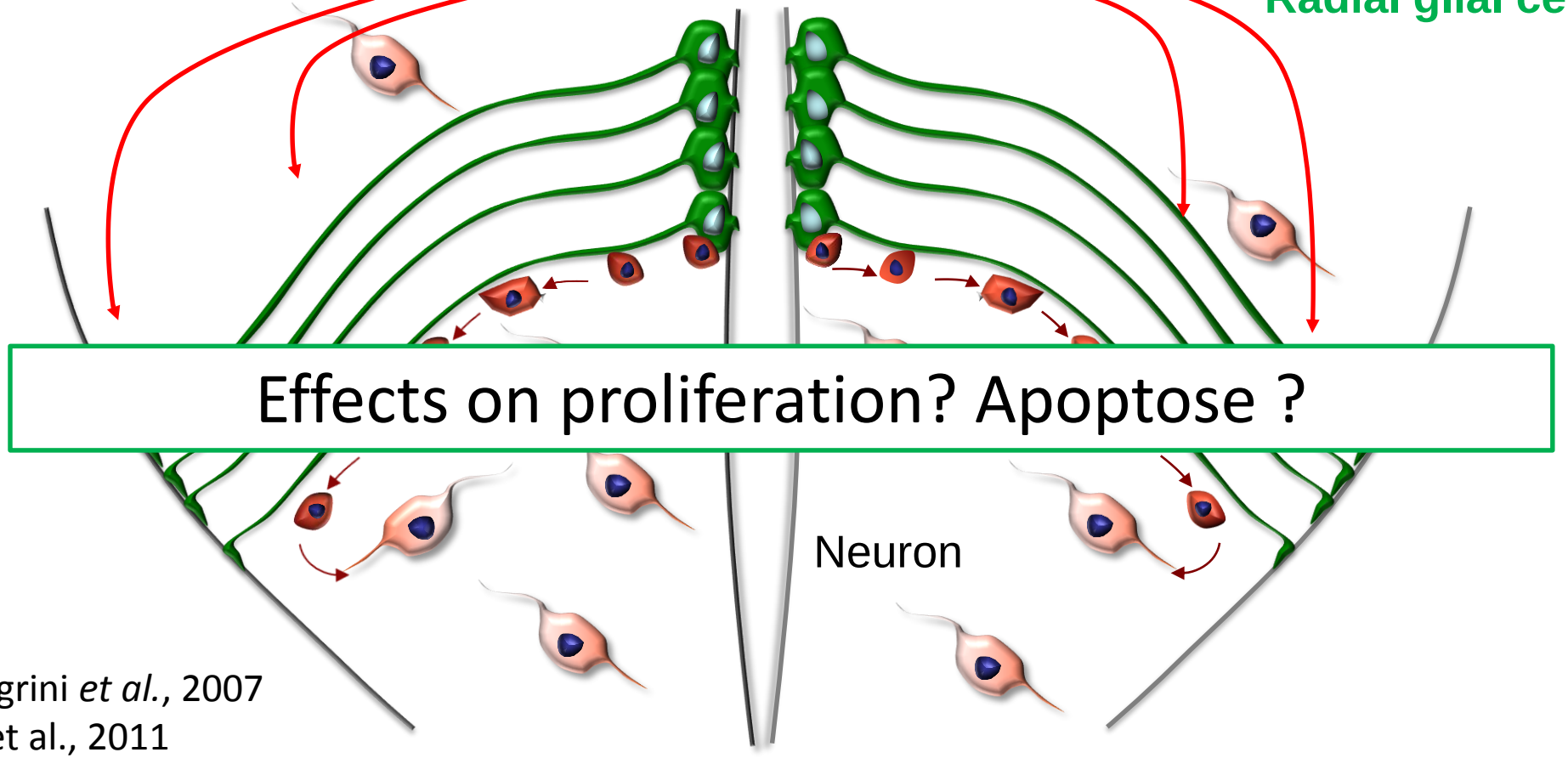
Radial glial cells = progenitor cells of neurons

Induction of the glial cell specific expresson of the brain aromatase

19-nortestosterone derived progestins
(pro-estrogenic substances)

estrogenic metabolites

Radial glial cells



From Pellegrini *et al.*, 2007
Kah et al., 2011

Radial glial cells = progenitor cells of neurons

Effect on apotosis and cell proliferation within the brain

	nM	Poliferation	Apoptosis
Progesterone	0.1	=	=
	1	=	=
	10	=	=
Norethindrone	0.1	=	=
	1	=	+
	10	=	+
17β-estradiol	0.1	=	=
	1	-	=
	10	-	=

Pro-apoptotic effects seen in different brain regions in zebrafish larvae (Hypothalamus++)

Anti-proliferative effect of E2.

Similar to adult zebrafish treated wiht E2 (Diotel et al., 2011)

Pellegrini *et al.*, in prep

In vitro and In vivo mode of action of progestins in fish

Complex toxicological profiles
Zebrafish-specific responses

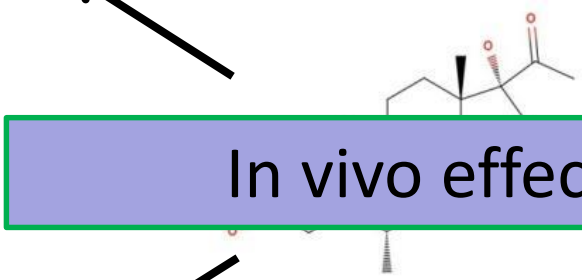
PR

Anti-PR

nPR ligands

AR

Strong andogenic effect of levonorgestrel in stickleback (Svensson et al., 2013, 2014)



In vivo effects ?

ER

GR

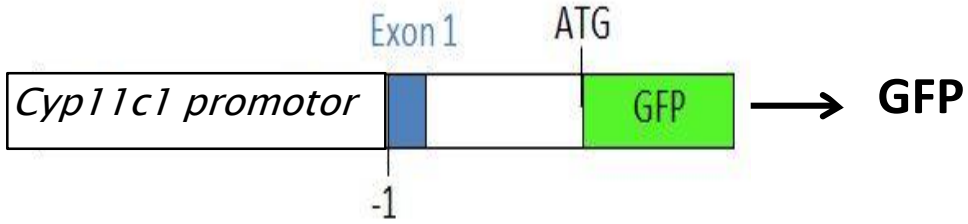
GR or Anti-GR

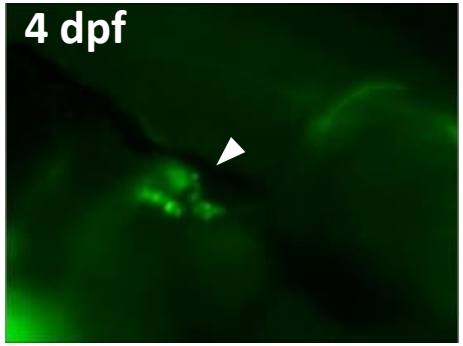
No data

??

Testosterone-derived progestins affect the estrogenic signalling pathway and brain development in zebrafish embryos (Cano-Nicolau, Garoche et al., TAAP, Pellegrini et al., in prep)

Novel transgenic cyp11c1-GFP zebrafish model



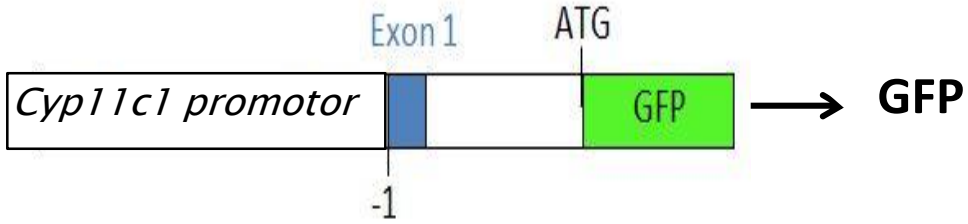


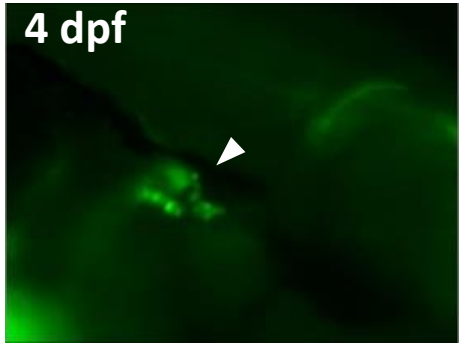
Interrenal cells expressing GFP

- ***cyp11c1*** codes for **11β-hydroxylase** the enzyme esponsible for the biosynthesis of cortisol the principal corticosteriod in fishes

11 deoxcortisol —**11β-hydroxylase**→ **cortisol**

Novel transgenic cyp11c1-GFP zebrafish model



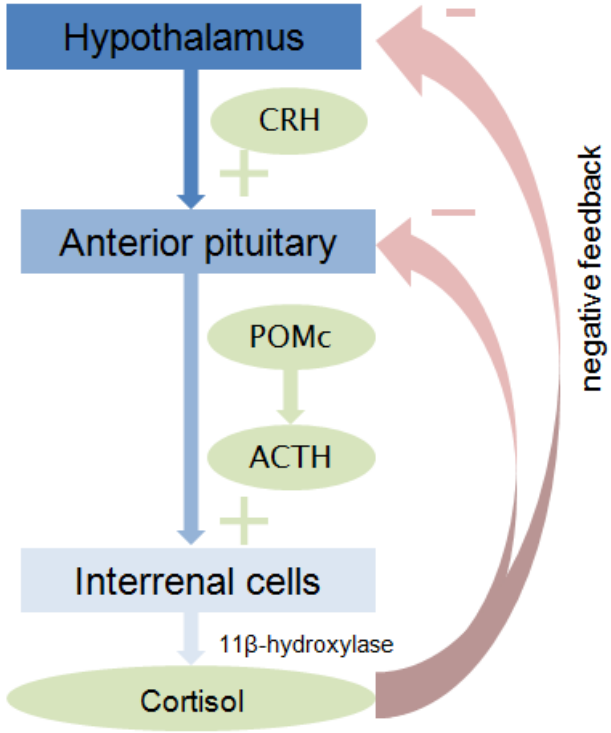


Interrenal cells expressing GFP

- ***cyp11c1*** codes for **11β-hydroxylase** the enzyme esponsible for the biosynthesis of cortisol the principal corticosteriod in fishes

11 deoxcortisol —**11β-hydroxylase**→ **cortisol**

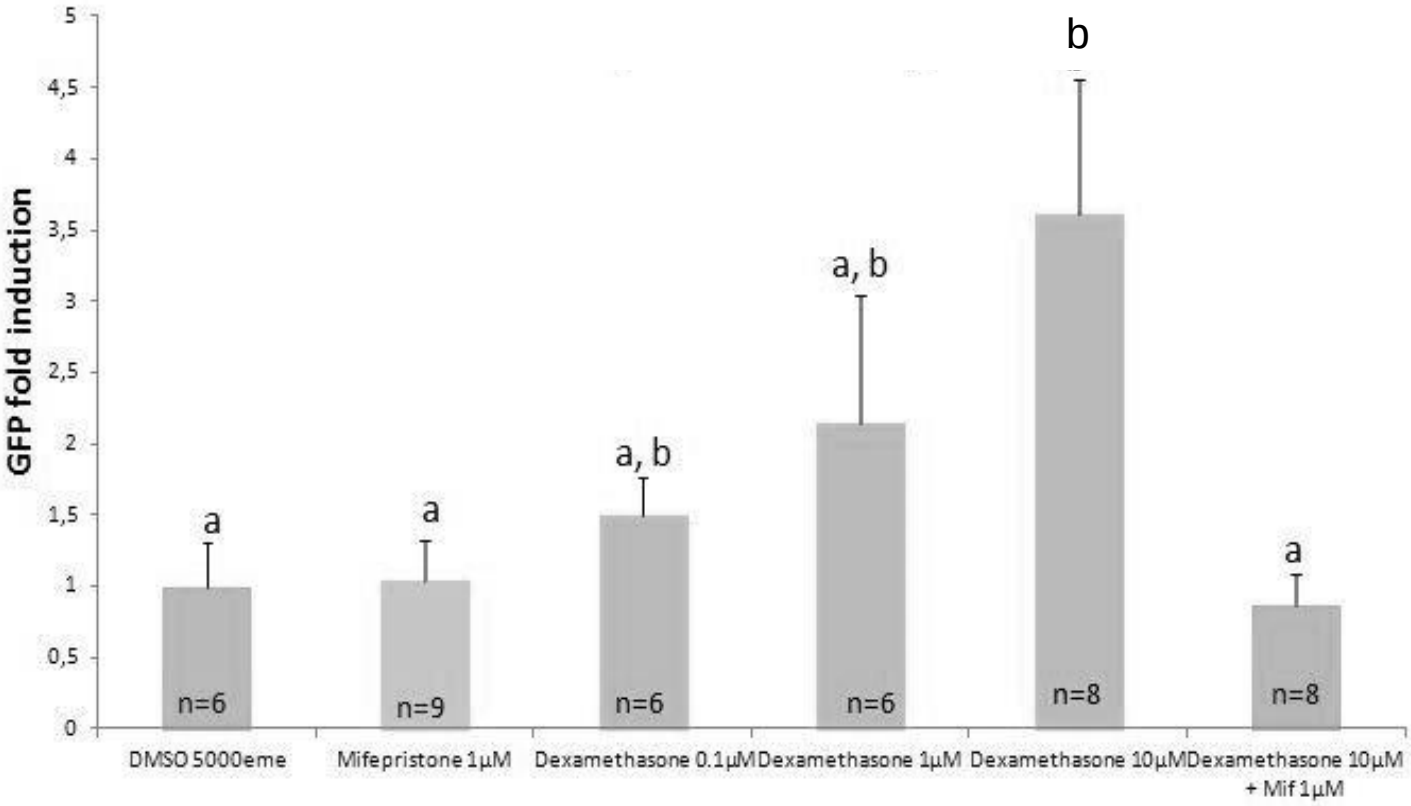
- ***cyp11c1*** is integral of the Hypothalamus-Pituitary-Adrenal axis (HPA) which is functional from 4dpf-old zebrafish

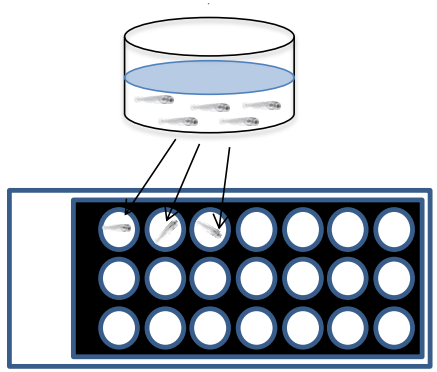


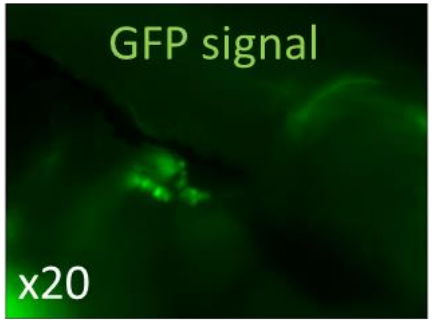
To 2006, Liu 2007, Alsop 2008

Effect of GR agonist and antagonist ligands

- **Regulation of *cyp11c1* expression in zebrafish** larvae

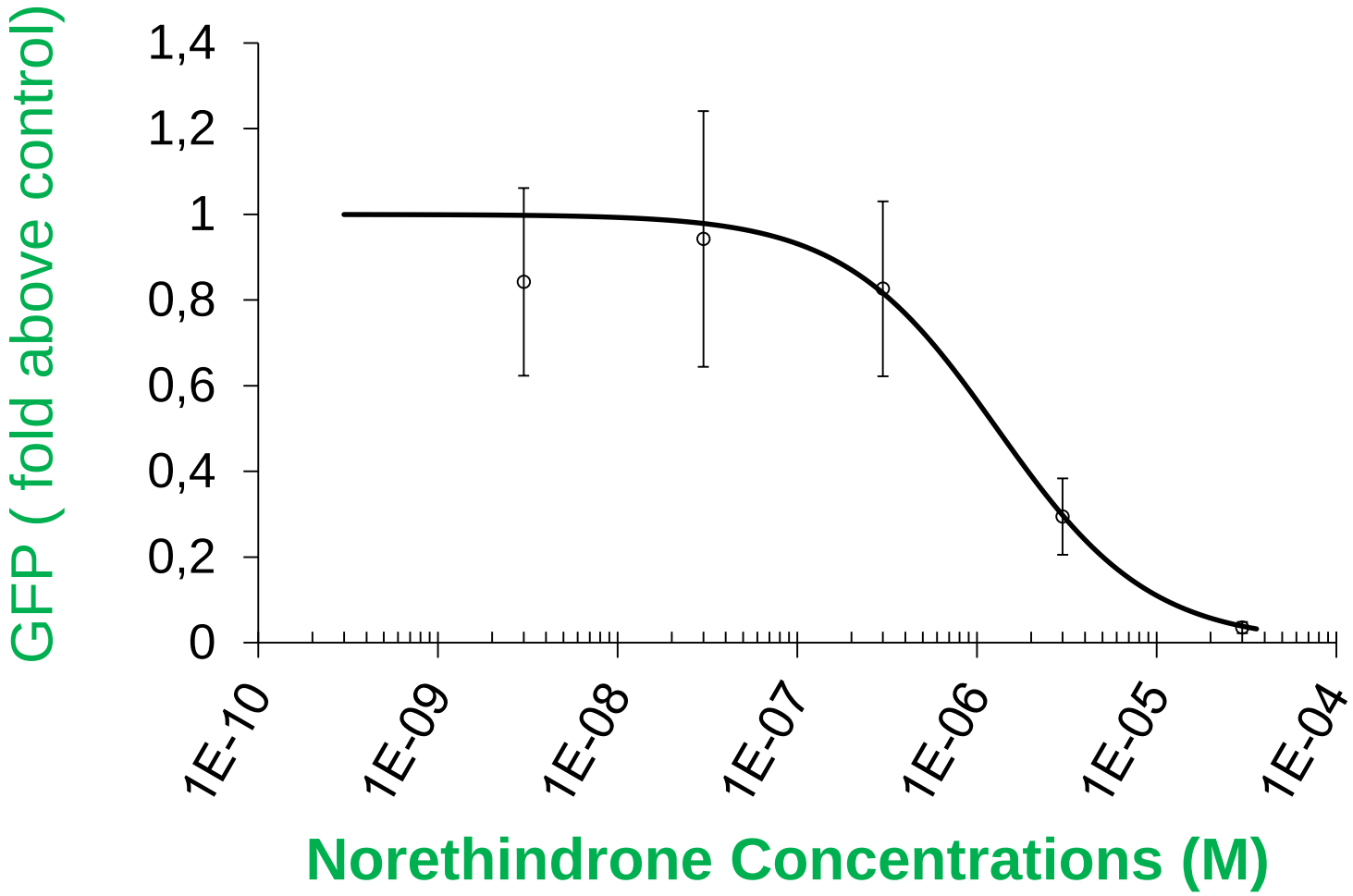






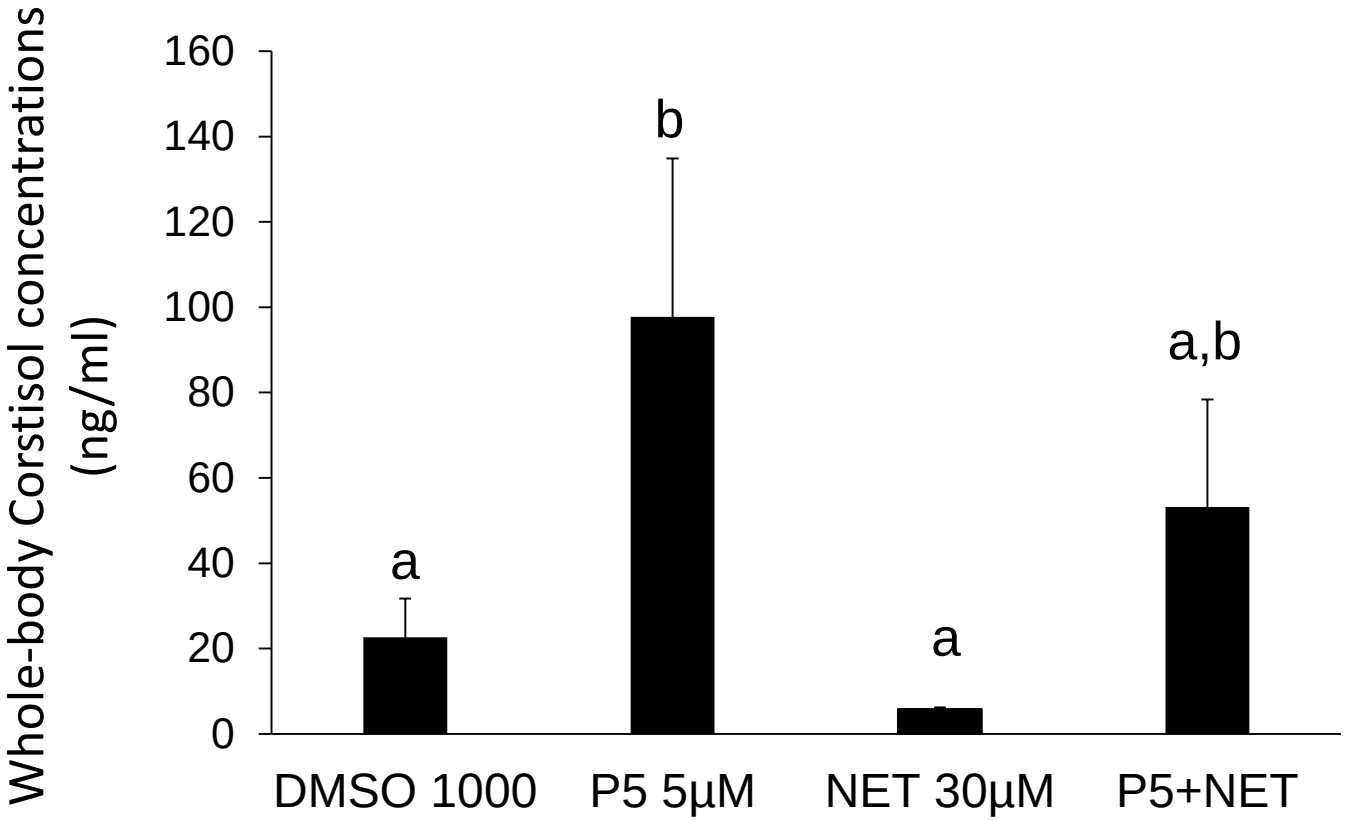
- GFP intensity is induced by DEX.
- GFP-induced GFP is block mifepristone
- Putative regulation of cyp11c1 by GR

Effect of norethindrone on GFP in cyp11c1-GFP zebrafish larvae



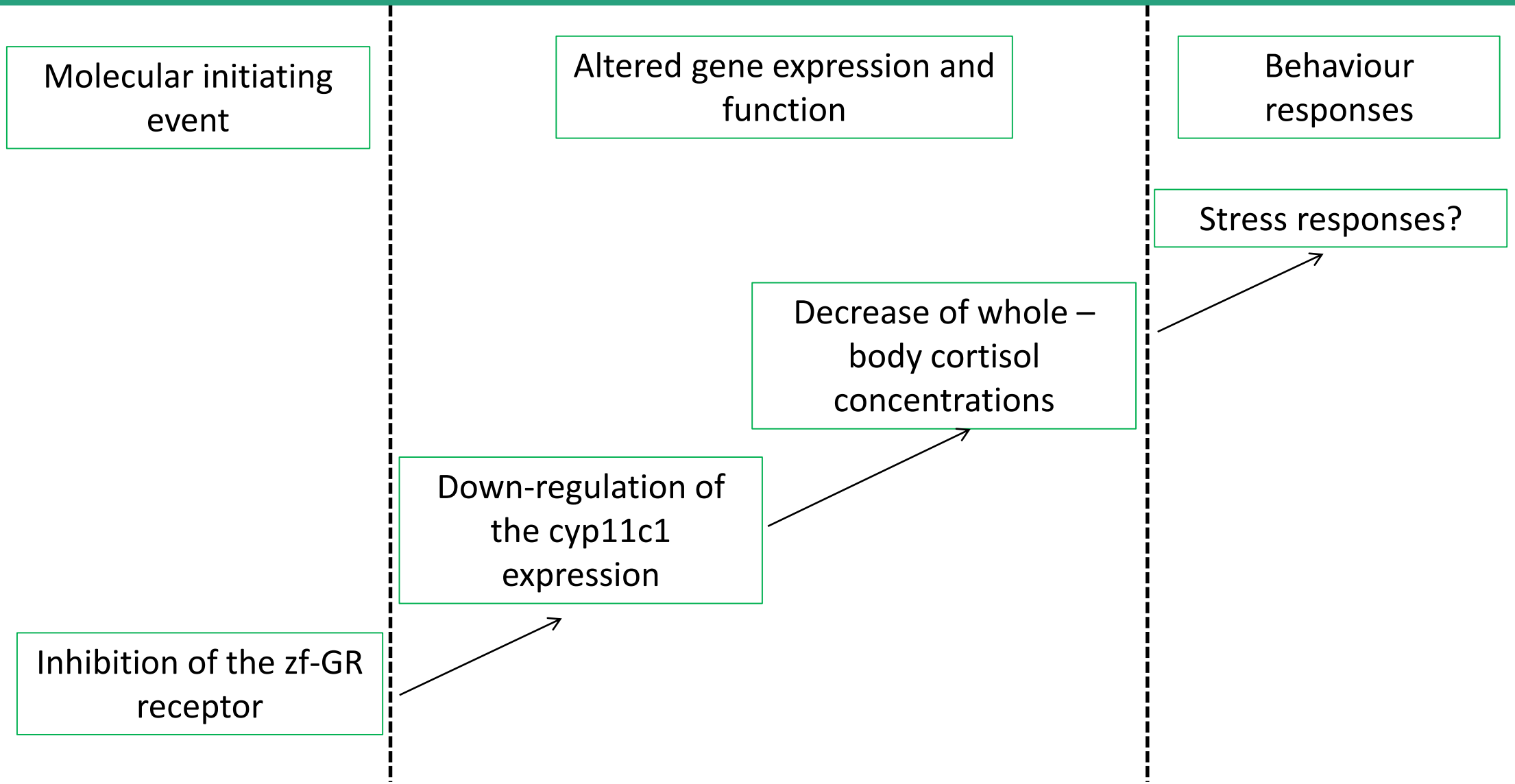
NET down-regulates GFP intensity in a concentration-dependent manner in interrenal cells.

Effect of norethindrone on cortisol concentrations



NET lowered whole-body cortisol concentrations and inhibited Pregnenolone-induced cortisol.

Summary of Norethindrone-induced effects in zebrafish



Norethindrone induce a suite of molecular events which supports the disruption of GR signaling pathways in zebrafish larvae

In vitro and *In vivo* modes of action of progestins in zebrafish

Complex toxicological profiles
Zebrafish-specific responses

PR

Anti-PR

nPR ligands

AR

Strong andogenic effect of levonorgestrel in stickleback (Svensson et al., 2013, 2014)

In vivo effects

ER

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GR or Anti-GR

Testosterone-derived progestins affect the estrogenic signalling pathway and affect the brain development in zebrafish embryos (Cano-Nicolau, Garoche et al., TAAP, Pellegrini et al., in prep)

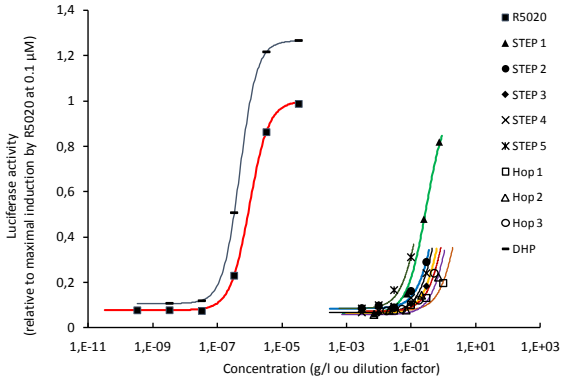
Norethindrone can disrupt corticosteroidogenesis in zebrafish larvae Garoche et al., in prep)

zfPR ligands in aquatic systems ?

Bioanalysis and Effect-Directed-Analysis

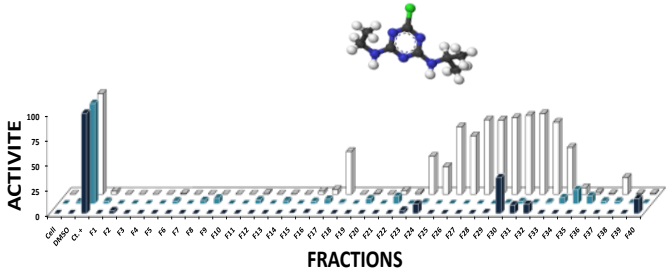
1. Screening of a bank of >100 archived samples, incl. sediments, surface and wastewaters from different contexts (urban, industrial, hospital…)

➔ *In vitro Bio-TEQs using hPR and zfPR bioassays*



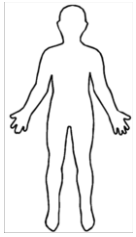
2. Identification of PR ligands at selected active sites

➔ *Effect-directed analysis*



PR activities: marked inter-species differences

42 active samples (out of 100) in either human (33) or zebrafish (13) cellular models



- **hPR** (6): urban WWs
- **anti-hPR** (27): surface waters, sediments, urban and hospital WWs

- **zfPR** (13): urban WWs
- **anti-zfPR** (0): not detected

≠ sites

As for pure synthetic ligands, marked differences are reported for h and zf progestagenic activities in environmental matrices

Identity of Zf-specific PR agonist ligands?

New sampling of WW from urban plants

New sampling of urban WWs

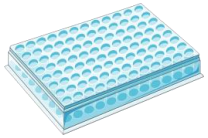
Wastewaters

(1 L)

SPE

(HLB™ phase)

In vitro bioassays

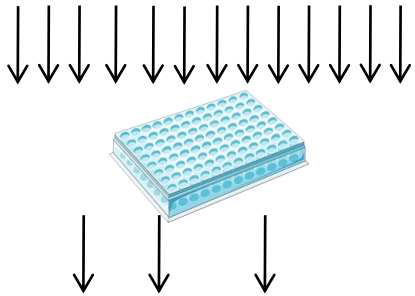


WWTPs *(infl.)*	**zfPR** *µg R5020-EQ/L*	**hPR** *µg R5020-EQ/L*
La Teste	16.5	n.d.
Cantinolle	1.3	n.d.
Biganos	4.3	n.d.
Blank	n.d.	n.d.

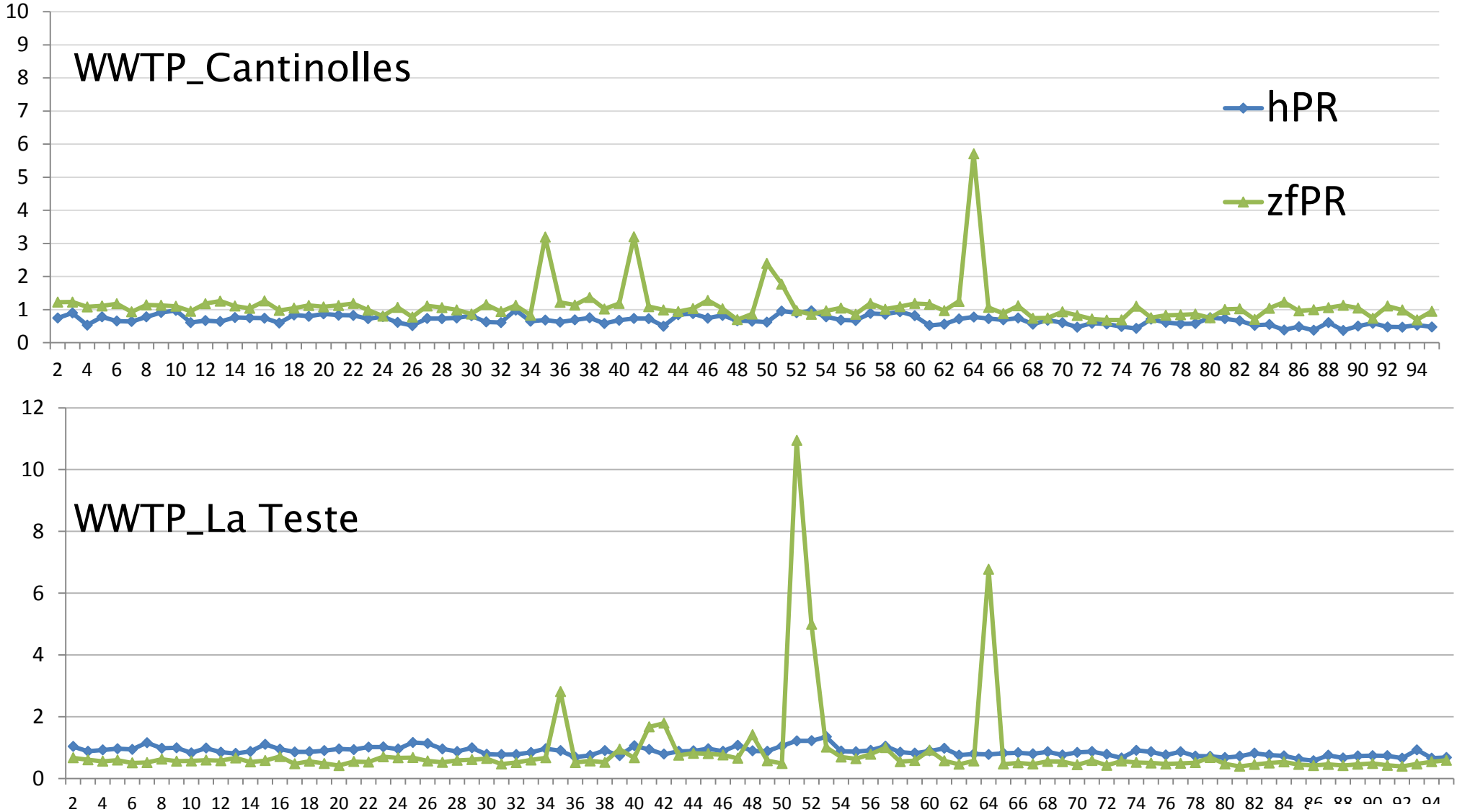
96-w fractionation (ACN/H2O)

RP-HPLC

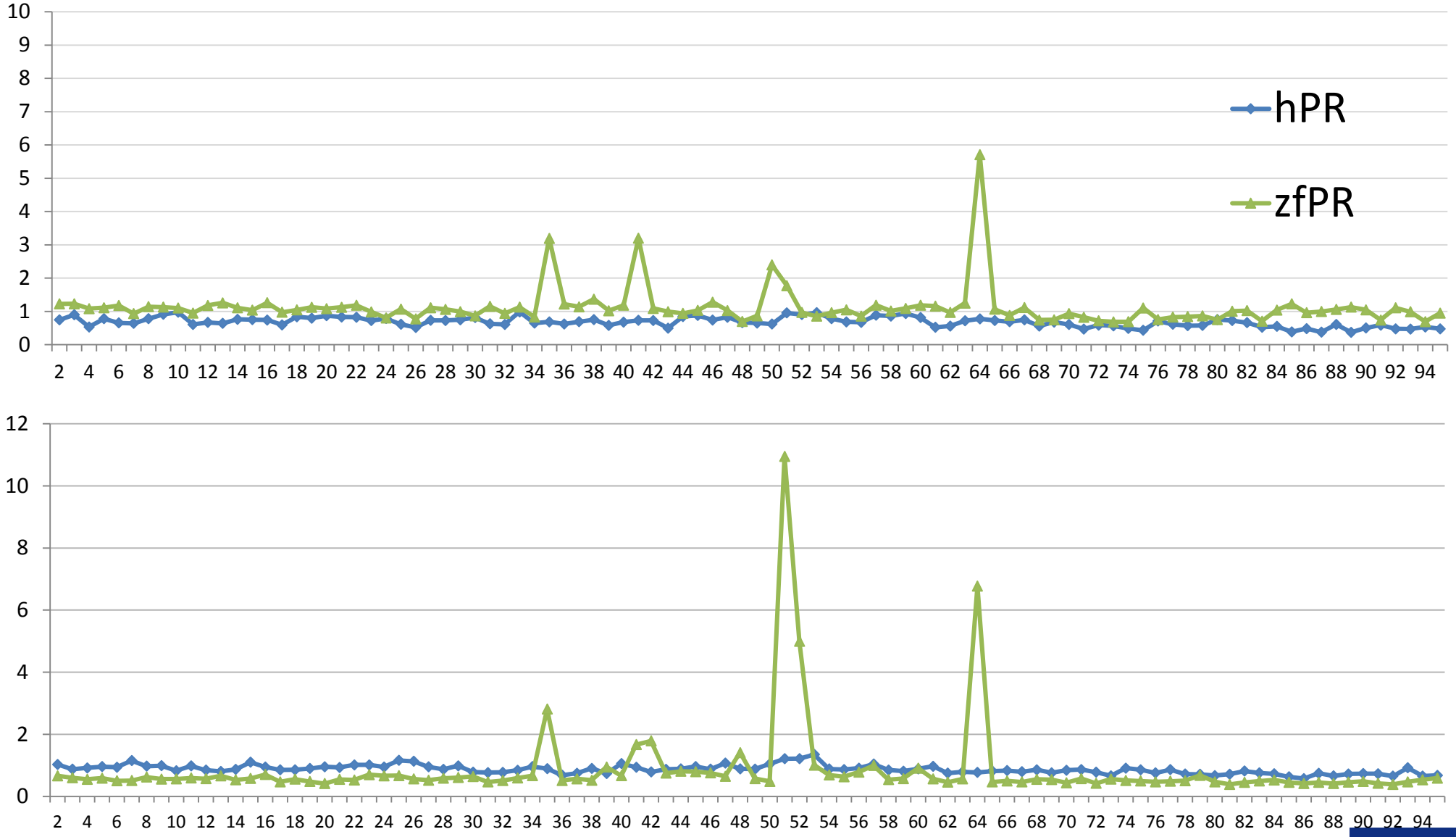
(C18)



RP-HPLC profiles of urban WWs



RP-HPLC profiles of urban wastewater influents



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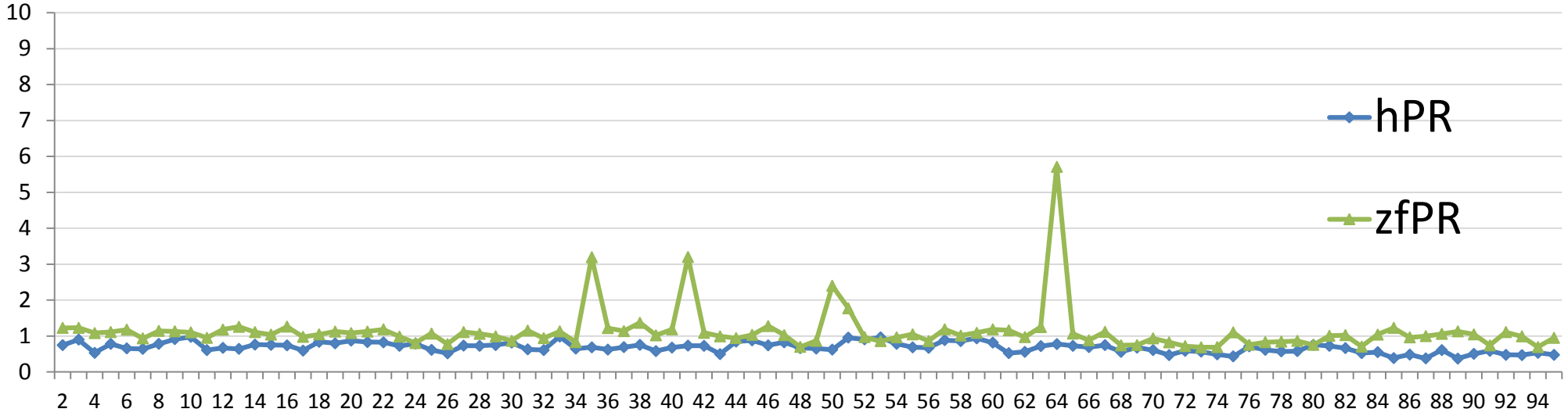
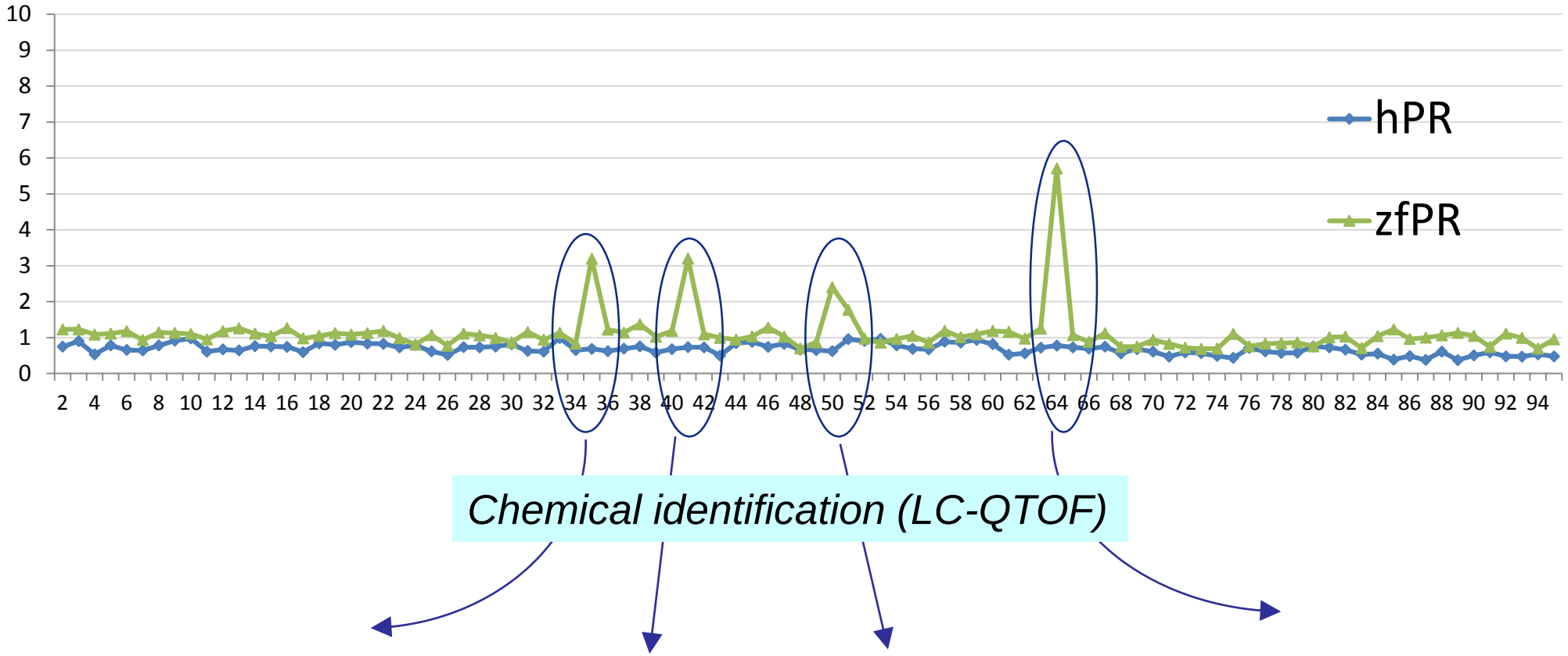
RP-HPLC profiles of urban wastewater influents



Chemical identification (LC-QTOF)

RP-HPLC profiles of urban wastewater influents

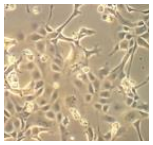


Hdyroxymetabolite of a synthetic porgestin

Drugs; non-progestagenic Pharmaceuticals

Conclusions

in vitro & *in vivo* species-specific mechanism-based bio-assays



Progestins

Environmental samples

- Complex toxicological profiles
- Zebrafish-specific responses
- Tissue-specific disruption of hormono-regulated genes

- (anti)progestagenic activities
- Zebrafish-specific responses
- Substances identified (fish-specific ligands of PR)?

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