A chemical screening system for glucocorticoid stress hormone signaling in an intact vertebrate

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Supplementary Material

Supplementary Materials and Methods:

Chemicals

Chemicals were purchased from different suppliers as indicated in Suppl. Table 1. Dexamethasone (DEX), hydrocortisone (HC), betamethasone (BM), aldosterone (AD) mifepristone (MIF), and dibutyltin dichloride (DBT) were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. Drugs used for the screen retest were dissolved in DMSO at a concentration of 2 mg/ml. Luciferin potassium salt was dissolved in water and stored at -80°C.

Cell culture maintenance

AB.9 cells (ATCC, CRL-2298™) were maintained in Leibovitz's (L-15) medium (Invitrogen) containing 17% (v/v) FCS (Biochrom AG) and antibiotics (100 U/ml penicillin/100 mg/ml streptomycin, 50 mg/ml gentamicin [#15750060, Life Technologies]) at 28°C, and were passaged once every week at a ratio of 1:7.

Animal husbandry

Adult zebrafish (AB line, University of Oregon; Eugene, USA) were raised, bred and crossed according to standard methods(*1*). All zebrafish husbandry and experimental procedures were performed in accordance with the German animal protection standards and were approved by the Government of Baden-Württemberg, Regierungspräsidium Karlsruhe, Germany. Fertilized eggs were collected within 2 h of laying and transferred into petri dishes (10 cm diameter) containing E3 medium(*2*) and the fungicide methylene blue (1 mg/ml). E3 medium was changed regularly. Eggs from different spawnings were pooled prior to the experiments.

Generation of stable luciferase cell reporter lines

Stable luciferase cell reporter cell lines were obtained as previously described(*3*). For transfection and integration, the PathDetect® pGRE-Luc cis-Reporter plasmid (#240133, Agilent Technologies) and the pGL3-Control vector (#E1741, Promega) were linearized with ScaI and XmnI, respectively.

Cloning of the pT2Luci:GRE construct

A fragment consisting of the multiple cloning site (MCS), the minimal TATA promoter and the luciferase reporter gene was obtained from the pLucMCS vector (Agilent Technologies) by double digestion with BamHI and XhoI. This luciferase cassette was inserted into the BglII and XhoI sites of the Tol2 plasmid pT2KXIGΔin(*4*) to obtain the pT2Luci:MCS vector with Tol2 transposase sites flanking the luciferase cassette. Next, 5' phosphate modified oligonucleotides containing a GRE element adapted from the PathDetect® pGRE-Luc cis-Reporter plasmid were annealed as described(*5*) and subcloned into the XhoI site of pT2Luci:MCS to generate the pT2Luci:GRE construct (Fig. 1a). The GRE sequence is based on the consensus sequence published by Jantzen et al.(*6*) Oligonucleotide sequences: sense: 5'- tcg atg gta cat ttt gtt cta gaa caa aat gta ccg gta cat ttt gtt ctg gta cat ttt gtt cta -3'; antisense: 5'- tcg ata gaa caa aat gta cca gaa caa aat gta ccg gta cat ttt gtt cta gaa caa aat gta cca -3'.

Generation of transgenic GRE fish reporter line

The transgenic pT2Luci:GRE fish line was generated following the procedure outlined in (*7*). Briefly, pCS-TP was linearized with NotI and transcribed into capped *tol2 transposase* RNA with the mMESSAGE mMACHINE Sp6 Kit (#AM1340, Ambion). A mixture containing *tol2* RNA (5 ng/µl), pT2Luci:GRE plasmid (20 ng/µl) and phenolred (0.1%) was injected into fertilized eggs at the single cell stage with a gas-driven microinjector (Femtojet express, Eppendorf)(*8*). Larvae were grown to 5 days post fertilization (dpf) and transferred into 96 well plates (one larva/well) with E3 medium containing 0.5 mM luciferin (E3L medium), treated with 20 µM dexamethasone (DEX) and screened for increased luciferase activity (see below). About 40% of larvae showed DEX responsiveness in these transient expression

conditions. 63 larvae exhibiting DEX induced expression were raised as potential founders (F0). They were outcrossed into wildtype fish and the progeny (F1) was again tested for DEX induced bioluminescence. Larvae from one founder exhibiting increased luciferase activity upon DEX treatment were raised to adulthood, yielding the F1 generation of stable transgenic GRE reporter fish. All experiments were carried out with offspring from the F1 and F2 generations.

Whole mount immunohistochemistry (WIHC)

For WIHC, GRE:Luc larvae (5 dpf) were treated with 40 µM betamethasone for 8 h prior sampling. Luciferase protein expression in the larvae was visualized as described(*9*), with the primary antibody rabbit anti-luciferase (#PM016, MBL; 1:4000 dilution) and the secondary antibody Alexa Fluor® 488 goat anti-rabbit (A-11034, Life Technologies; 1:1000 dilution).

Cortisol-ELISA

30 larvae were raised at a constant temperature of 28°C and treated at 5 dpf with 250 mM NaCl for the indicated time periods. Cortisol was measured as described(*10*) with minor modifications. Briefly, larvae were extracted with ethylacetate and extracts measured with a Cortisol Saliva Elisa Kit (#RE52611, IBL) using a VersaMax ELISA Microplate Reader (Molecular Devices).

Real-time qPCR

Triplicates of 20 embryos/larvae (1 dpf and 5 dpf) were treated with 20 µM DEX or 0.03% DMSO for 9 h, then were sampled in 1ml TRIzol (#15596-018, Life Technologies) and homogenized with micropistilles (#0030120.973, Eppendorf). RNA extraction was carried out as recommended by the manufacturer. RNA (1 µg) was reverse transcribed using random primers (#48190-011, Life Technologies) and SuperScriptIII reverse transcriptase (#18080-044, Life Technologies). mRNA levels of *fkbp5* and *pck1* were determined by real-time qPCR (StepOne Plus, Applied Biosystems), following the manufacturer's instructions. First-strand cDNA aliquots from each sample served as templates in a PCR reaction consisting of master mix, SYBR Green I fluorescent dye (Bio-Rad), and 500 nM gene-specific

primers. Copy numbers were normalized using *b-actin* controls. Primer sequences were: *b-actin* fw: gcctgacggacaggtcat, rv: accgcaagattccataccc; *fkbp5* fw: ttccacactcgtgttcgaga, rv: acgatcccaccatct tctgt; *pck1* fw: tgacgtcctggaagaacca, rv: gcgtacagaagcgggagtt

Cell viability assay

Cell viability assays were carried out as described(*11*) with minor modifications: 35,000 cells were seeded into a well of a 96-well plate and incubated over night at 28°C. After treatment with the indicated concentrations (Suppl. Fig. 4) of DBT or TBT for 24 h, MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to a final concentration of 1 mg/ml to the cells and incubated for 6 h at 28°C. Absorbance at 570 nm and reference absorbance at 630 nm were measured with a VersaMax ELISA Microplate Reader (Molecular Devices).

***In vitro* bioluminescence assay**

The *in vitro* bioluminescence assay was performed with luciferase extracts. Lysates from AB.9 cells constitutively expressing luciferase (pGL3-Control) were prepared with Lysis Buffer (#E3971, Promega) following the instructions of the manufacturer. Luciferase activity was assessed as described(*12*) in the presence of different concentrations of either DBT or TBT (0-500 nM, Suppl. Fig. 4).

Caudal fin bioluminescence assay

Caudal fin biopsies were carried out according to (*1*). Fin clips were transferred to opaque 96-well plates containing L-15L medium and measured as described for the *in vivo* cell assay.

HPLC-MS/MS detection of organotin compounds

100 larvae per sample were raised up to 5 dpf in 75 cm² cell flasks containing 30 ml E3 medium at 28°C, and then treated with either 80 nM DBT or TBT (3 flasks per treatment condition) for 24 h. Sampling was carried out as described(*10*). For extraction and analysis of TBT and DBT a procedure

similar to that described by Jones-Lepp et al.(*13*) was used. Briefly, flash frozen larvae were lyophilized (Alpha 2-4, Christ) overnight and were homogenized the next day in homogenization tubes (#91-PCS-CK14, peqLab) containing 1.5 ml acetonitril/0.1% tropolone (w/v) with a N_2-cooled tissue homogenizer (precellys24, peqLab, (4x15 sec, 6000 rpm)). After centrifugation of the homogenates, supernatants were dried in a gentle stream of nitrogen and residues were redissolved in methanol/ammonium acetate (50 mM)/acetic acid (80/19.9/0.1, v/v/v). TBT and DBT levels were examined using a API 4000™ turbo ion spray source tandem mass spectrometer (Applied Biosystems/ MDS SCIEX) coupled to a 1100 Series HPLC system (Agilent). The organotins were separated on a ZORBAX 300-SCX (5 µm, 4.6 mm x 250 mm) separation column. The isocratic mobile phase was methanol/ammonium acetate (50 mM)/acetic acid/tropolone (80/19.9/0.1/0.09, v/v/v/w). For monitoring TBT and DBT in the positive mode the mass transitions m/z 290.9 to m/z 234.8 and m/z 354.9 to m/z 240.8 were used, respectively.

Supplementary Figure Legends

Suppl. Fig.1: Characterization of *in vivo* response kinetics and dose response curves of the GRE:Luc system in the zebrafish. (a)-(b) Mean of relative reporter activity from (a) 48 larvae upon stimulation with 5 µM dexamethasone (DEX) and from (b) AB.9 GRE:Luc cells (n=8 wells) upon stimulation with 10 nM DEX for 40 h. **(c)-(f)** The increase in bioluminescence in GRE:Luc AB.9 cells upon treatment with GCs is specific. Relative reporter activity at the peak after treatment is plotted against the treatment dose. Dose response curves for dexamethasone (c) and cortisol (hydrocortisone) (d) are shown, with EC_{50} and goodness of fit values indicated (R^2) in the graph. (e) Treatment with the mineralocorticoid aldosterone does not elicit a response at all concentrations tested. (f) GC signaling is dose-dependently inhibited by treatment with the GR antagonist mifepristone. Cells were co-treated with

10 nM dexamethasone and the indicated mifepristone concentrations. The IC_{50} and goodness of fit values are indicated in the panel. Error bars represent mean values ± s.e.m.

Suppl. Fig.2: Luciferase expression is upregulated by GC treatment to a similar extent in three distinct regions of the larva. (a) "Regions of interest" (ROI) of the larva selected for region specific quantification of immunohistochemistry fluorescence**. (b)** Quantification of fluorescence intensity shows a significant increase in all examined regions of the betamethasone (BM) treated larvae (ROI 1, $p \leq 0.01$; ROI 2, $p \leq 0.001$; ROI 3, $p \leq 0.05$; n=10). **(c)** Mean of relative reporter activity from tail fin biopsies after treatment with 20 µM DEX. Error bars represent mean values ± s.e.m. (n=6, $p \leq 0.001$).

Suppl. Fig.3: Real-time qPCR quantification of transcript levels of endogeneous GC target genes upon GC induction during development. (a) *fkbp5* and **(b)** *pck1* levels in control treated embryos/larvae (DMSO, white bars) and embryos/larvae treated with 20 µM DEX (black bars) at 1 and 5 dpf. Error bars represent mean values ± s.e.m,($p \leq 0.001$, n= 3).

Suppl. Fig.4: Detection of organotins in zebrafish larvae by HPLC-MS/MS. Shown are extracted ion chromatograms (XIC) using the transitions m/z 290.9 to m/z 234.8 for tributyltin (TBT, red) and m/z 354.9 to m/z 240.8 for dibutyltin (DBT, green). (a), untreated larvae, (b), larvae treated with 80nM DBT, (c), larvae treated with 80 nM TBT.

Suppl. Fig.5: Rescreen results for compounds selected from the primary screen. GRE:Luc AB.9 cells (a-h) and GRE:Luc larvae (a'-f') were treated with the indicated concentrations of the selected compounds (grey-black) or with DMSO (red) as control. Shown are relative luciferase reporter activity traces over 48 h of treatment time (n=48 larvae, cells n=8 wells). Error bars represent mean values ± s.e.m.

Suppl. Fig.6: TBT and DBT treatment do not affect cell viability or luciferase activity. (a) MTT test for cell viability. Results for different concentrations of DBT (Green squares) and TBT (red squares) are shown. Values do not differ significantly between all treatment conditions ($p>0.05$, n=4).

(b),(c) *In vitro* bioluminescence test with different concentrations of TBT (b) or DBT. (c) Luciferase activity values are indistinguishable between the different treatment conditions and only differ from the control (no luciferase) ($p \leq 0.001$, n=4). Error bars represent mean values ± s.e.m.

Supplementary Table Legends

Suppl. Table 1: Source of chemical compounds.

Supplementary References

1. Westerfield, M. (2007) *The zebrafish book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), 5th Edition*, University of Oregon Press, Eugene.
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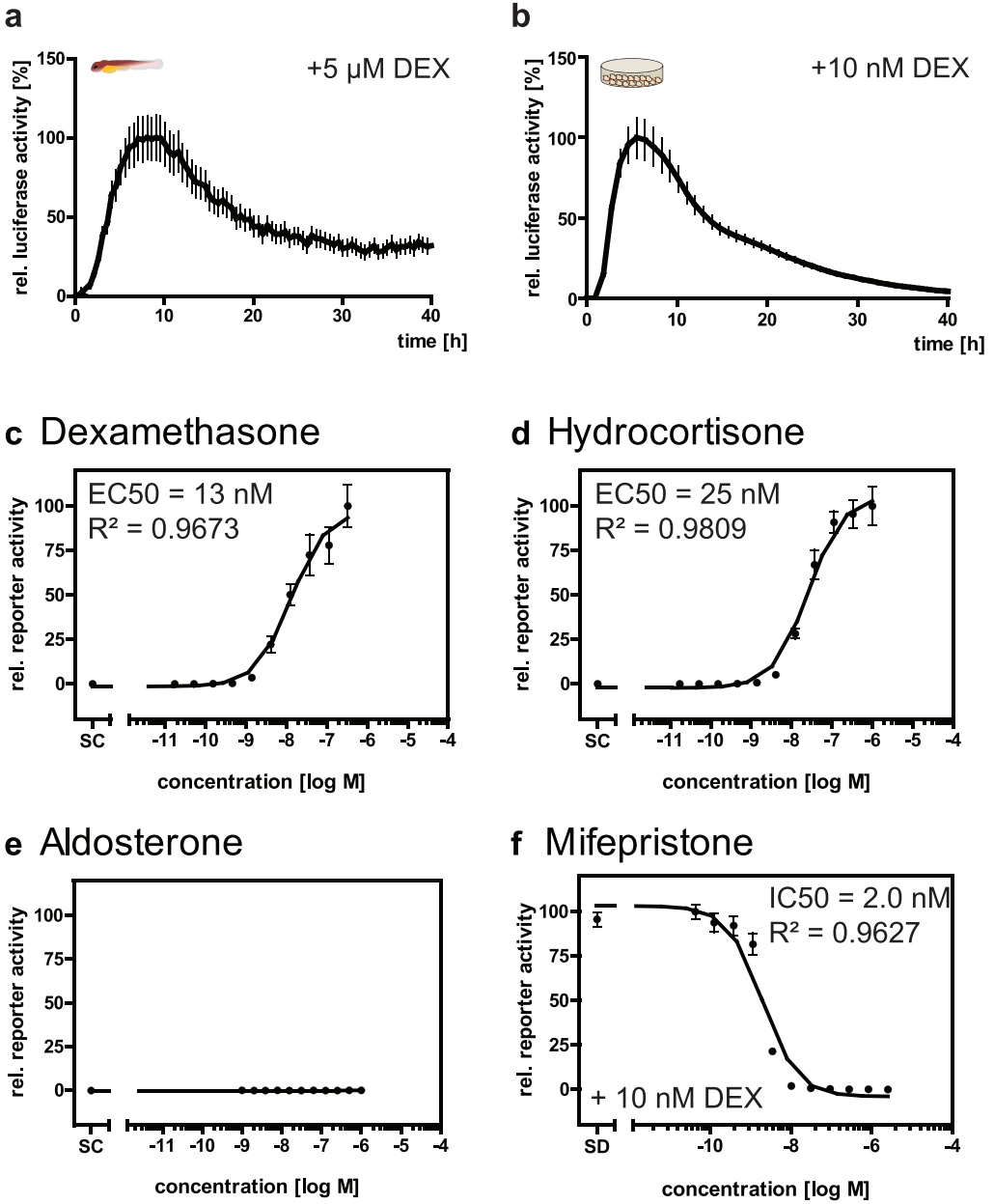
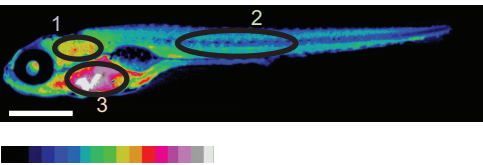
suppl. fig. 1

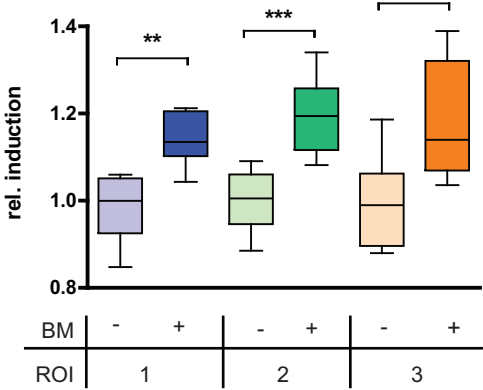

a
+5 µM DEX
rel. luciferase activity [%]
150
100
50
0
0
10
20
30
40
time [h]
b
+10 nM DEX
rel. luciferase activity [%]
150
100
50
0
0
10
20
30
40
time [h]
c Dexamethasone
EC50 = 13 nM
R² = 0.9673
rel. reporter activity
100
75
50
25
0
SC
-11
-10
-9
-8
-7
-6
-5
-4
concentration [log M]
d Hydrocortisone
EC50 = 25 nM
R² = 0.9809
rel. reporter activity
100
75
50
25
0
SC
-11
-10
-9
-8
-7
-6
-5
-4
concentration [log M]
e Aldosterone
rel. reporter activity
100
75
50
25
0
SC
-11
-10
-9
-8
-7
-6
-5
-4
concentration [log M]
f Mifepristone
IC50 = 2.0 nM
R² = 0.9627
+ 10 nM DEX
rel. reporter activity
100
75
50
25
0
SD
-10
-8
-6
-4
concentration [log M]

suppl. fig. 2

a



b



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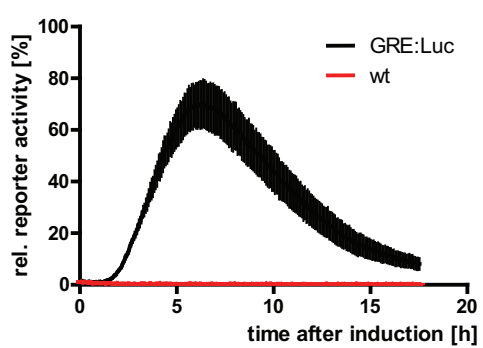
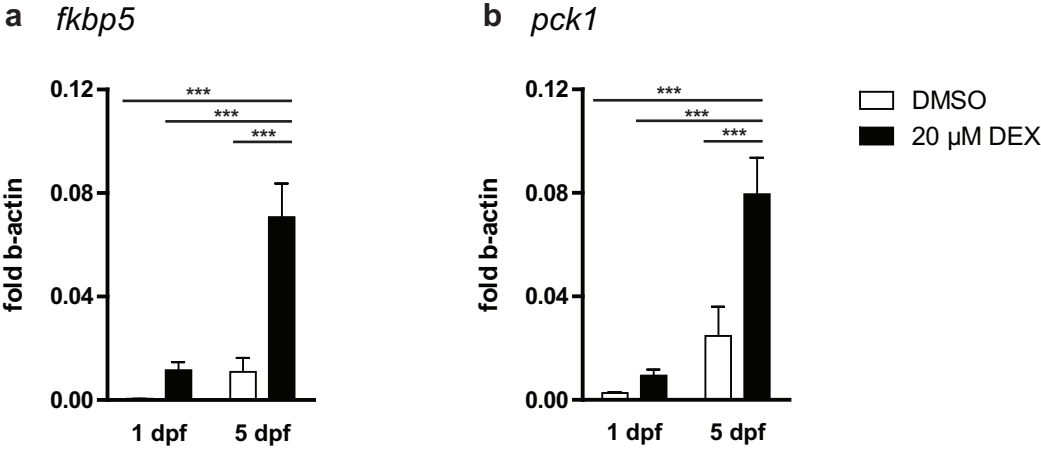


suppl. fig. 3


a
fkbp5
b
pck1
0.12
0.08
0.04
0.00
fold b-actin
***
***
***
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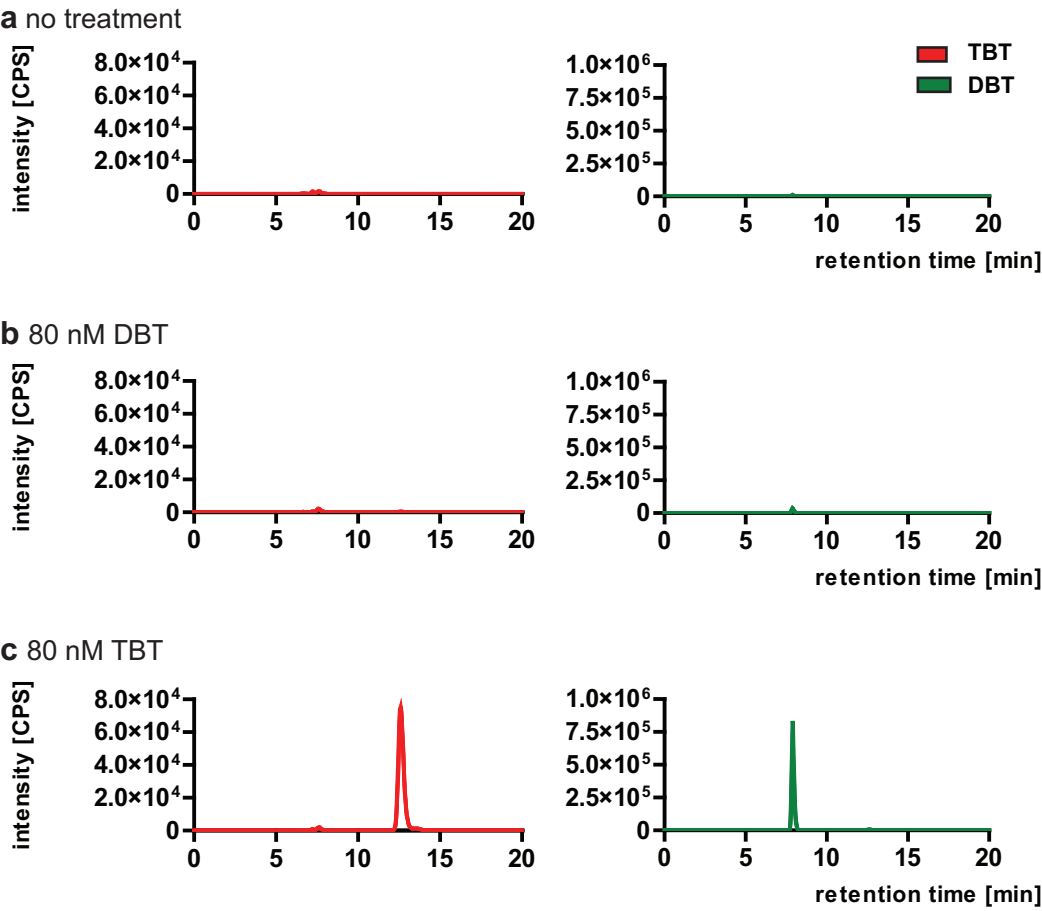
5 dpf
DMSO
20 µM DEX

suppl. fig. 4


a no treatment
b 80 nM DBT
c 80 nM TBT
intensity [CPS]
8.0×10^4
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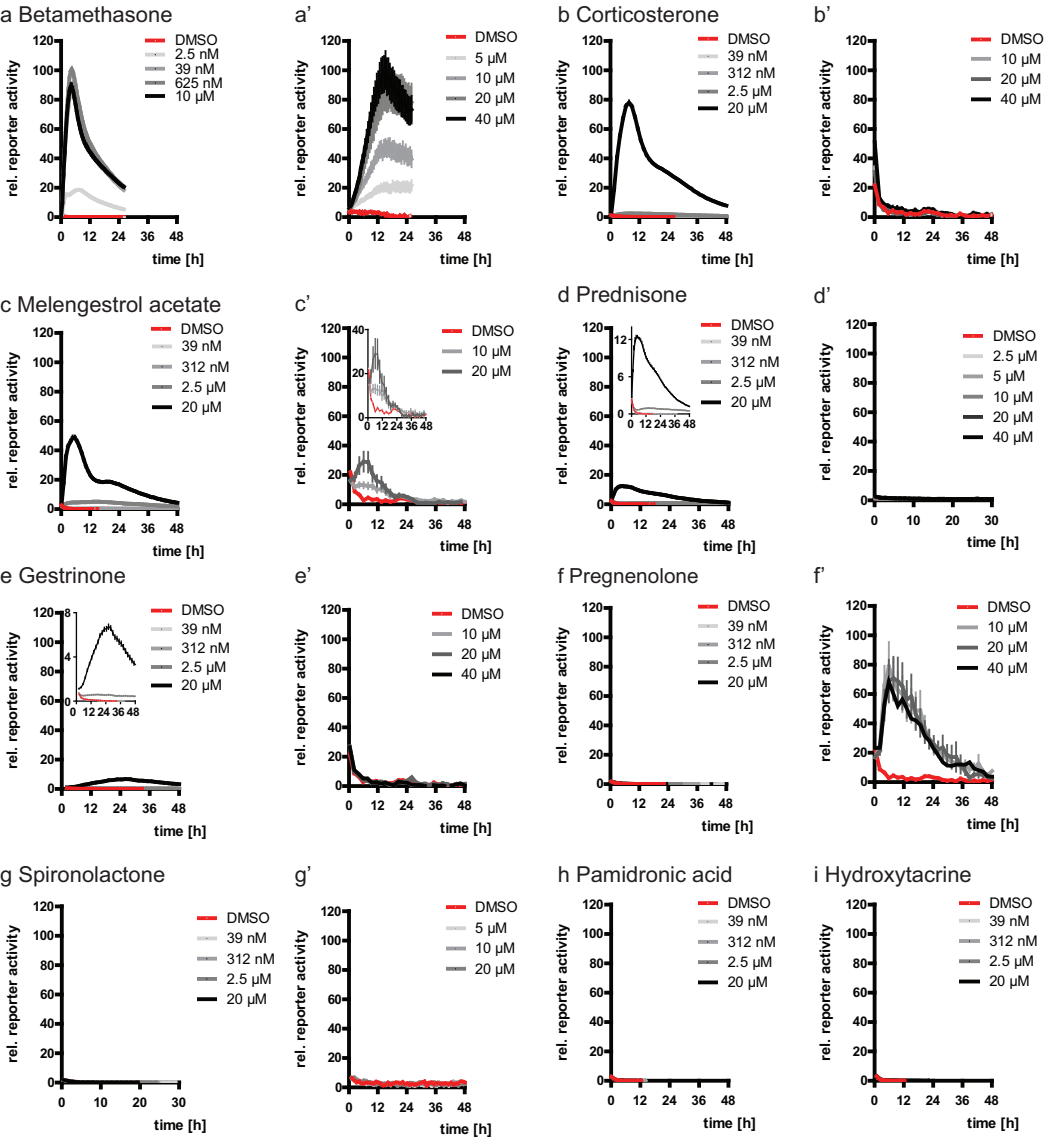
4.0×10^4
2.0×10^4
0
1.0×10^6
7.5×10^5
5.0×10^5
2.5×10^5
0 5 10 15 20
retention time [min]
TBT
DBT

suppl. fig. 5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DMSO
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39 nM
625 nM
10 µM
a'
DMSO
5 µM
10 µM
20 µM
40 µM
b Corticosterone
DMSO
39 nM
312 nM
2.5 µM
20 µM
b'
DMSO
10 µM
20 µM
40 µM
c Melengestrol acetate
DMSO
39 nM
312 nM
2.5 µM
20 µM
c'
DMSO
10 µM
20 µM
d Prednisone
DMSO
39 nM
312 nM
2.5 µM
20 µM
d'
DMSO
2.5 µM
5 µM
10 µM
20 µM
40 µM
e Gestrinone
DMSO
39 nM
312 nM
2.5 µM
20 µM
e'
DMSO
10 µM
20 µM
40 µM
f Pregnenolone
DMSO
39 nM
312 nM
2.5 µM
20 µM
f'
DMSO
10 µM
20 µM
40 µM
g Spironolactone
DMSO
39 nM
312 nM
2.5 µM
20 µM
g'
DMSO
5 µM
10 µM
20 µM
h Pamidronic acid
DMSO
39 nM
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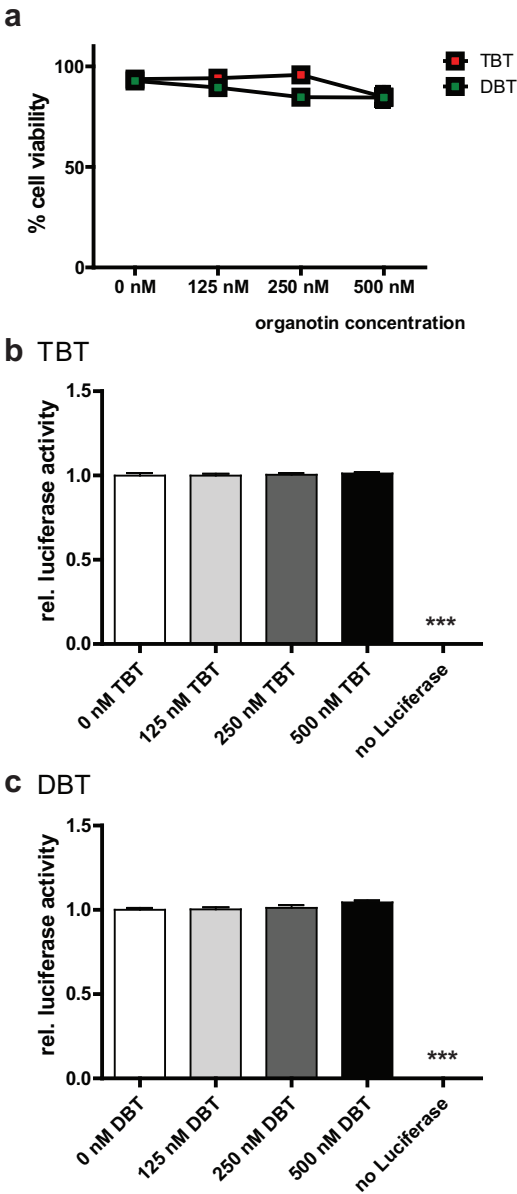
2.5 µM
20 µM
rel. reporter activity
time [h]

suppl. fig. 6


a
100
50
0
% cell viability
TBT
DBT
0 nM
125 nM
250 nM
500 nM
organotin concentration
b TBT
1.5
1.0
0.5
0.0
rel. luciferase activity
***
0 nM TBT
125 nM TBT
250 nM TBT
500 nM TBT
no Luciferase
c DBT
1.5
1.0
0.5
0.0
rel. luciferase activity
***
0 nM DBT
125 nM DBT
250 nM DBT
500 nM DBT
no Luciferase

Suppl. table 1

Chemicals	Supplier	order No
Dexamethasone	Sigma-Aldrich	D4902
Betamethasone	Sigma-Aldrich	B7005
Hydrocortisone	Sigma-Aldrich	H0888
Corticosterone	Sigma-Aldrich	27840
Melengestrol acetate	Sigma-Aldrich	73248
Prednisone	Sigma-Aldrich	P6254
Pregnenolone	Sigma-Aldrich	P9129
Pamidronic acid	Chemos	239642
Hydroxytacrine	Chemos	146092
Spironolactone	Sigma-Aldrich	S3378
Mifepristone	Sigma-Aldrich	M8046
Aldosterone	Sigma-Aldrich	A9477
TBT	Sigma-Aldrich	T50202
DBT	Sigma-Aldrich	205494
Luciferin potassium salt	Biosynth	L-8220
Thiazolyl Blue Tetrazolium Bromide (MTT)	Sigma-Aldrich	M5655