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 spawnsings 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:MC 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 cta gaa caa aat gta cca gaa caa aat gta ccg gta cat ttt gtt cta gaa caa aat gta cca -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 mMMESSAGE 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 \textit{b-actin} controls. Primer sequences were: \textit{b-actin} \textit{fw}: gcctgaeggaaggtcat, \textit{rv}: aecgegctagttcatc; \textit{fkbp5} \textit{fw}: ttecacacetgtggtcgaga, \textit{rv}: acegatecacatctctgtgctgtct; \textit{pck1} \textit{fw}: tgacgtcctggaagaacca, \textit{rv}: gcgtacagggacggaggt

\textbf{Cell viability assay}

Cell viability assays were carried out as described\cite{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).

\textbf{In vitro bioluminescence assay}

The \textit{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\cite{12} in the presence of different concentrations of either DBT or TBT (0-500 nM, Suppl. Fig. 4).

\textbf{Caudal fin bioluminescence assay}

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

\textbf{HPLC-MS/MS detection of organotin compounds}

100 larvae per sample were raised up to 5 dpf in 75 cm$^2$ 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\cite{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 N2-cooled tissue homogenizer
(prechyls24, 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 EC50 and goodness of fit values indicated (R2) 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 ≤0.01; ROI 2, p ≤0.001; ROI 3, p ≤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 ≤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 ≤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).
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

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suppl. fig. 1

(a) +5 μM DEX

(b) +10 nM DEX

(c) Dexamethasone
   EC50 = 13 nM
   R² = 0.9673

(d) Hydrocortisone
   EC50 = 25 nM
   R² = 0.9809

(e) Aldosterone

(f) Mifepristone
   IC50 = 2.0 nM
   R² = 0.9627
   + 10 nM DEX
rel. induction

BM

ROI

a

b

time after induction [h]

rel. reporter activity [%]

suppl. fig. 2
suppl. fig. 3

a  *fkbp5*

b  *pck1*

|        | 1 dpf | 5 dpf |
|--------|-------|-------|
| *fkbp5* |       |       |
| *pck1*  |       |       |

**Graphs:**

- **fkbp5**
  - 1 dpf: DMSO, 20 µM DEX
  - 5 dpf: DMSO, 20 µM DEX

- **pck1**
  - 1 dpf: DMSO, 20 µM DEX
  - 5 dpf: DMSO, 20 µM DEX

**Significance Levels:**

- ***: p < 0.001
suppl. fig. 4

a no treatment

b 80 nM DBT

c 80 nM TBT
suppl. fig. 5

a  Betamethasone

b  Corticosterone

c  Melengestrol acetate

d  Prednisone

e  Gestrinone

f  Pregnenolone

g  Spironolactone

h  Pamidronic acid

i  Hydroxytacrine
suppl. fig. 6

(a) Graph showing the effect of organotin concentration on cell viability. The x-axis represents the organotin concentration (0 nM, 125 nM, 250 nM, 500 nM) and the y-axis represents the percentage of cell viability.

(b) Bar chart showing the relative luciferase activity for TBT (0 nM, 125 nM, 250 nM, 500 nM) compared to the control (no Luciferase). The bars are arranged from left to right: 0 nM TBT, 125 nM TBT, 250 nM TBT, 500 nM TBT, no Luciferase. The relative luciferase activity is indicated on the y-axis. The bars are shaded in different colors and a symbol *** indicates a significant difference.

(c) Bar chart showing the relative luciferase activity for DBT (0 nM, 125 nM, 250 nM, 500 nM) compared to the control (no Luciferase). The bars are arranged from left to right: 0 nM DBT, 125 nM DBT, 250 nM DBT, 500 nM DBT, no Luciferase. The bars are shaded in different colors and a symbol *** indicates a significant difference.
### 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    |
| TMT                               | Sigma-Aldrich    | T50202   |
| DBT                               | Sigma-Aldrich    | 205494   |
| Luciferin potassium salt          | Biosynth         | L-8220   |
| Thiazolyl Blue Tetrazolium Bromide (MTT) | Sigma-Aldrich | M5655   |