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# A Chemical Screening System for Glucocorticoid Stress Hormone Signaling in an Intact Vertebrate

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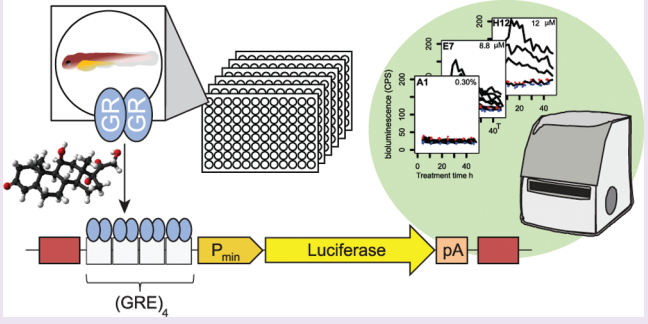
Supporting Information

**ABSTRACT:** Glucocorticoids, steroid hormones of the adrenal gland, are an integral part of the stress response and regulate glucose metabolism. Natural and synthetic glucocorticoids are widely used in anti-inflammatory therapy but can have severe side effects. *In vivo* tests are needed to identify novel glucocorticoids and to screen compounds for unwanted effects on glucocorticoid signaling. We created the **G**lucocorticoid **R**esponsive *In vivo* **Z**ebrafish **L**uciferase activit**Y** assay to monitor glucocorticoid signaling *in vivo*. The GRIZLY assay detects stress-induced glucocorticoid production in single zebrafish larvae, measures disruption of glucocorticoid signaling by an organotin pollutant metabolite, and specifically identifies a compound stimulating endogenous glucocorticoid production in a chemical screen. Our assay has broad applications in stress research, environmental monitoring, and drug discovery.




Glucocorticoids (GCs) bind to the GC receptor (GR), which can stimulate transcription *via* GC response elements (GREs) in target gene promoters (transactivation) or bind to other transcription factors and repress their activity (transrepression).[1] Since the GR ligand type can determine the precise set of target genes activated,[2] novel ligands might activate a target gene spectrum that results in better GC treatment efficiency and tolerability, reducing side effects such as diabetes or glaucoma.[3] Current efforts at identifying novel GCs rely on *in vitro* or cell culture assays.[4] However, ligands do not necessarily exhibit the same effects *in vivo* as in cultured cells.[5] The search for novel compounds with GC activity might thus benefit from *in vivo* chemical screening systems as provided by the zebrafish model,[6,7] since these incorporate whole animal effects such as compound metabolization or manipulation of endogenous GC levels. Importantly, the GR signaling system in zebrafish is more similar to that of mammals than that of other teleosts. Like mammals, zebrafish possess only one GR gene (many teleosts have two), and a GR$\beta$ isoform has also been described.[8−10] Furthermore, 5-day-old zebrafish larvae already possess a functional hypothalamic pituitary adrenal (HPA) axis and increase GC production under stress.[10−12]

Hormonal signaling pathways are also targets for environmental pollutants. So-called endocrine disrupting chemicals (EDCs) fall into multiple compound classes, such as phthalates, dioxins, and organotins, and affect various hormone signaling pathways, including GC signaling.[13,14] While the effects of EDCs on estrogen signaling and the resulting impairment of reproduction and sexual differentiation of aquatic organisms are well studied, relatively little is known about EDCs targeting GC signaling, and tools to evaluate pollutant effects on GC signaling *in vivo* are needed.

Here, we report an assay for measuring GC signaling activity in the living animal *via* GRE driven luciferase expression in a transgenic zebrafish line (GRE:Luc, Figure 1a), which we termed GRIZLY assay (short for **G**lucocorticoid **R**esponsive *In vivo* **Z**ebrafish **L**uciferase activit**Y**). Bioluminescence from single 5 days post fertilization (dpf) transgenic larvae in 96-well microtiter plates was monitored on a luminescence plate reader. GRE:Luc larvae responded to a treatment with dexamethasone (DEX) with an increase in relative luciferase activity (Supplementary Figure 1a). The reporter construct was expressed in many tissues, and GC treatment increased luciferase protein levels at all expression sites (Figure 1b−d, Supplementary Figure 2a,b). GC treatment also induced bioluminescence in adult fin tissue (Supplementary Figure 2c). A zebrafish cell line carrying the GRE:Luc reporter (AB.9 GRE:Luc) showed that GC dose−response characteristics ($EC_{50}$ values) of the zebrafish system were consistent with values reported for mammalian systems (Supplementary Figure 1b−d). Also the GRE:Luc larvae responded in a dose-dependent manner to a challenge with either DEX or cortisol (hydrocortisone, HC) (Figure 1e,f). The compound concen-

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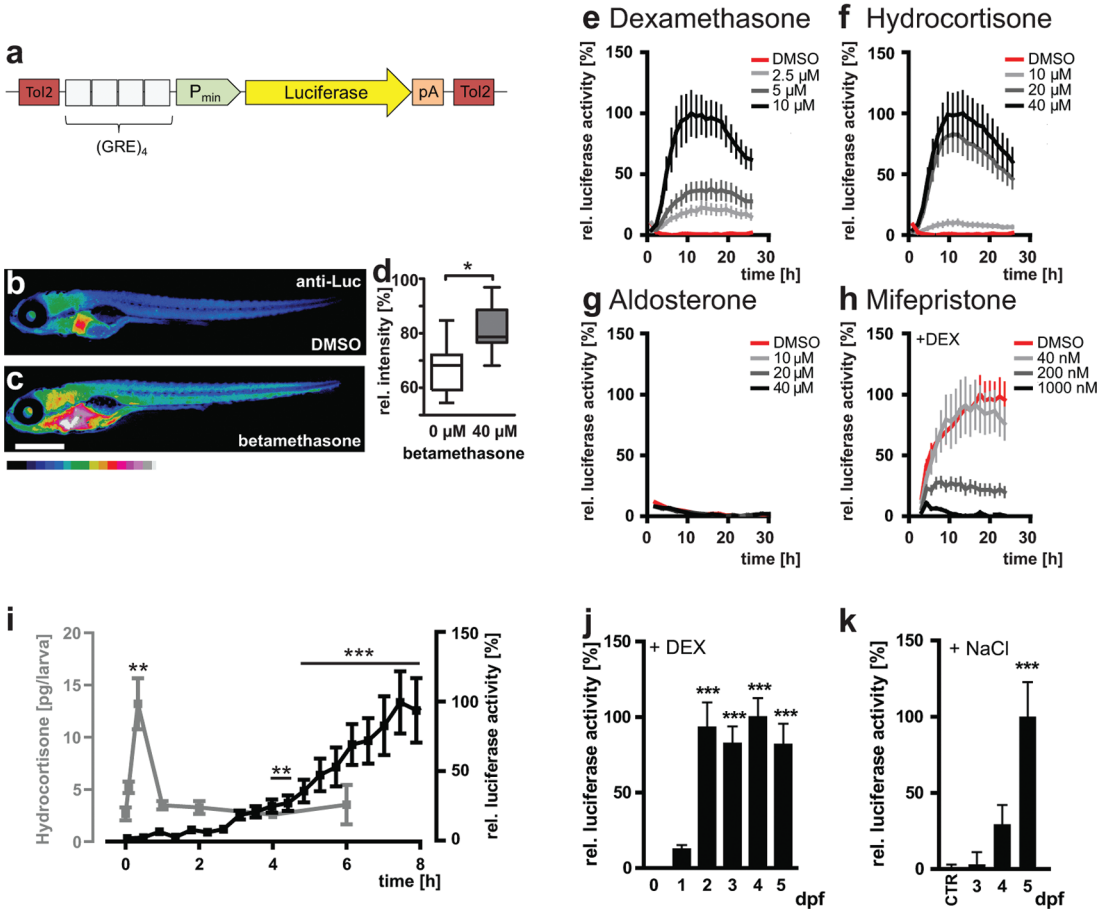


**Figure 1.** Characterization of the GRE:Luc reporter line. (a) Schematic showing the structure of the reporter construct. Tol2, Tol2 transposase sites to facilitate integration into the genome; $(GRE)_4$, four concatemerized Glucocorticoid Response Elements (GRE); $P_{min}$, minimal promoter; pA, polyadenylation site. (b–d) Luciferase expression is ubiquitously upregulated upon GC treatment. (b, c) Immunohistochemistry with an anti-luciferase antibody (anti-Luc) and an Alexa Fluor 488 labeled secondary antibody. Fluorescence intensity is shown color coded. Representative examples of 5 dpf larvae treated with solvent control (0.3% DMSO (b)) or 40 $\mu$M betamethasone (c). Scale bar 0.5 mm. (d) Quantification of fluorescence intensity shows a significant ($p = 0.0115$, $n = 10$) increase in betamethasone treated larvae. (e–h) The bioluminescence response of GRE:Luc larvae ($n = 48$) to GC signaling is specific. Graphs show the relative reporter activity in response to treatment with the indicated concentrations of a GR agonist (dexamethasone (e)), the natural GC cortisol (hydrocortisone (f)), the mineralocorticoid aldosterone (g), and a GR antagonist (mifepristone, in presence of 5 $\mu$M dexamethasone (h)). Red, control treatments. (i) GRE:Luc larvae increase bioluminescence in response to a rise in endogenous cortisol levels under osmotic stress. Upon salt stress, endogenous cortisol levels (gray, left $y$-axis) rise to a peak within 20 min ($p \leq 0.01$, $n = 5$) and a significant increase in bioluminescence (black, right $y$-axis) is observed after 4 h of treatment ($p \leq 0.01$, $n = 22$). (j) Developmental time course of GC signaling activation by DEX. Treatment with DEX leads to a significant rise in bioluminescence starting at 2 dpf (black bars, $p \leq 0.001$, $n \geq 48$). Error bars represent mean values ± SEM. (k) Developmental time course of osmotic stress response. A trend for increased bioluminescence is observed at 4 dpf, which becomes significant at 5 dpf (black bars, $p \leq 0.001$, $n = 288$).

trations added to the water were higher than those necessary in the culture medium to obtain a response, likely reflecting systemic mechanisms such as uptake, metabolism, and excretion dynamics of the compounds. Both transgenic larvae and cells did not respond to treatment with the mineralocorticoid receptor (MR) agonist aldosterone (Figure 1g, Supplementary Figure 1e), whereas the GR antagonist mifepristone blocked the response of the reporter to DEX treatment in both larvae and cells (Figure 1h, Supplementary Figure 1f), further confirming the specificity of the response.

Zebrafish larvae have been reported to increase cortisol production when stressed by various stressors.[15,16] We treated GRE:Luc larvae at 5 dpf with 250 mM NaCl to induce osmotic stress and monitored relative luciferase activity (Figure 1i, black). Cortisol levels were increased in the larvae as early as 20 min after the start of treatment (Figure 1i, gray), and bioluminescence started to significantly increase about 4 h ($p \leq 0.01$) after treatment, showing that the line can monitor stress-induced cortisol production.

To examine when GC signaling activity could first be detected during development, we treated embryos and larvae with DEX at sphere stage (4 h post fertilization (hpf)) as well as on 1–5 dpf and measured relative reporter activity 9 h after the start of the treatments. DEX treatment led to a statistically significant increase of reporter activity already at 2 dpf (Figure 1j). A low-level yet non-significant induction can be observed at 1 dpf. These reporter bioluminescence observations are consistent with the behavior of endogenous GC target genes (Supplementary Figure 3). We also explored when osmotic stress was first able to activate GC signaling activity by submitting the larvae to 250 mM NaCl and measuring relative luciferase activity 9 h after treatment. Salt stress significantly increased bioluminescence at 5 dpf only, with a trend already present at 4 dpf and no response detected at 3 dpf (Figure 1k).


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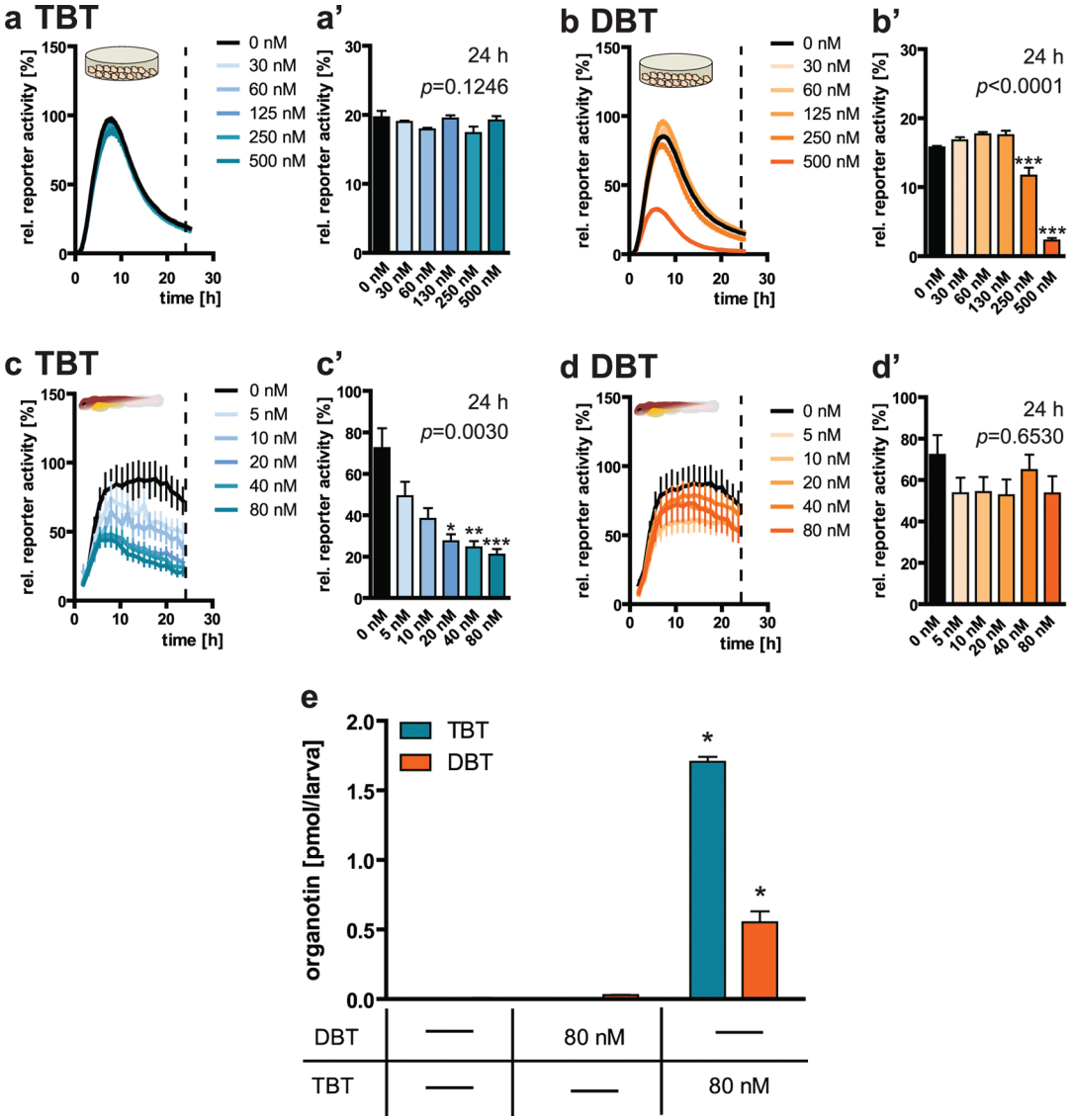


**Figure 2.** *In vivo* detection of organotin metabolism and inhibitory effects on GC signaling. (a–b′) DBT, but not TBT, inhibits GC signaling in cultured zebrafish cells. Relative reporter activity from AB.9 GRE:Luc cells measured for 24 h after treatment with 5 nM dexamethasone and the indicated amounts of TBT ((a, a′) blue) or DBT ((b, b′) orange). GC signaling levels are significantly reduced by treatment with 250 and 500 nM DBT ($p \leq 0.001$, $n = 4$). (c–d′) TBT, but not DBT, is effective in inhibiting GC signaling in GRE:Luc larvae. Relative reporter activity from GRE:Luc larvae measured for 24 h after treatment with 5 $\mu$M dexamethasone and the indicated amounts of TBT (c, c′) or DBT (d, d′). GC signaling levels are significantly reduced ($p < 0.0001$, $n = 96$) starting at 20 nM TBT ($p \leq 0.05$). (e) TBT shows much higher accumulation in the larvae than DBT and is metabolized by the larvae to DBT. Diagram shows levels of TBT and DBT detected by HPLC–MS/MS in larval extracts (100 larvae/extract) after 24 h of treatment with the indicated compounds and concentrations ($n = 3$, $p < 0.05$). Error bars represent mean values ± SEM.

This is in line with previous reports measuring cortisol increases after handling stress.[15] Our results show that receptiveness for stress axis signals is already in place at least 3 days before proper functioning of the stress axis itself.

In order to provide a valid *in vivo* screening tool, the GRE reporter line should be able to pick up effects of drugs or environmental pollutants on GC signaling that cannot be detected with cell culture assays. The environmental pollutant tributyltin (TBT) is an organotin compound that was previously described to be dealkylated in the liver to dibutyltin (DBT), which functions as a GR antagonist.[17−19] Thus, we expected that DBT should affect GC signaling in both larvae and cells, whereas TBT should do so only in larvae after metabolization to DBT by the larval liver. While AB.9 GRE:Luc cells treated with 10 nM of DEX and TBT were indistinguishable from cells treated with DEX alone (Figure 2a,a′), cells treated with DEX and DBT showed statistically significant inhibition of bioluminescence at higher concentrations (Figure 2b,b′, $p \leq 0.001$). In contrast, and consistent with the larval metabolization hypothesis, GC signaling in GRE:Luc larvae was inhibited with concentrations as low as 20 nM TBT (Figure 2c,c′, $p \leq 0.05$), which are environmentally relevant.[20] Surprisingly, and different from the cell culture, DBT treated larvae did not show statistically significant attenuation of GC signaling (although a trend to inhibition is visible, Figure 2d,d′). As the two compounds may accumulate differently in the larvae, we examined levels of TBT and DBT in larval extracts after treatment with 80 nM of either DBT or TBT by HPLC–MS/MS. Indicative of better TBT uptake, TBT levels in the larvae after TBT treatment are much higher


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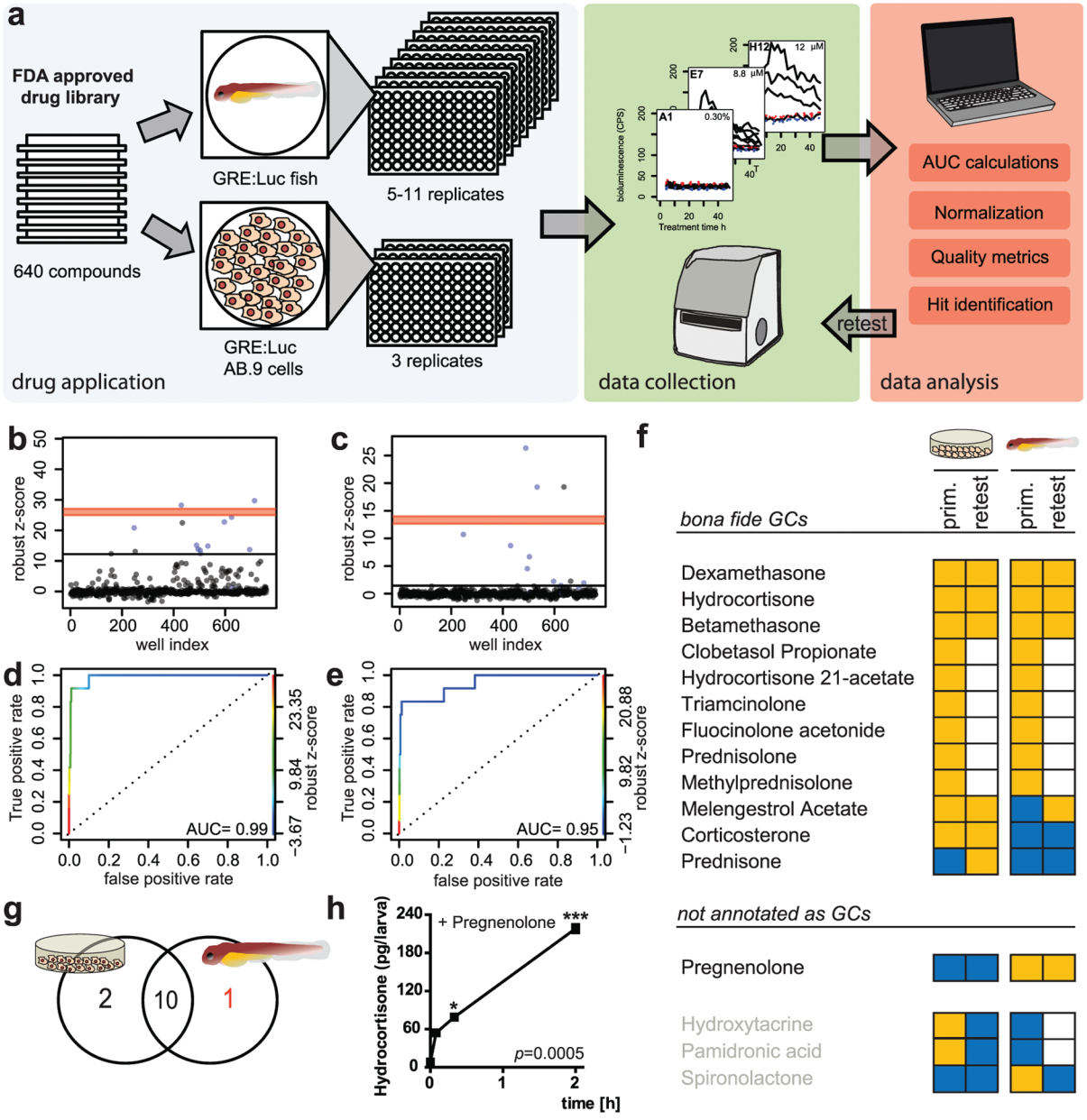


**Figure 3.** A pilot chemical screen with GRE:Luc larvae and cells identifies drugs that affect GC signaling *in vivo*. (a) Scheme of screen design: 640 compounds (distributed in eight 96-well plates) from a FDA approved drug library were applied to 5−11 replica plates with GRE:Luc larvae or to 3 replica plates with GRE:Luc AB.9 cells (drug application, light gray). Bioluminescence was monitored for 48 h (data collection, light green). Bioluminescence traces for each compound were integrated (AUC calculations) and normalized. Quality metrics were assessed to set the threshold for hit identification (data analysis, light red). Hits from the primary screen were retested for dose-dependent effects on GC signaling. (b−e) Determination of the cutoff value for primary screen hits. (b, c) Normalized (robust *z*-score) screen results for all compounds tested in the cell (b) and larvae (c) assay. The horizontal black line indicates the robust *z*-score cutoff based on the ROC curves (see panels d and e) that was used to identify the primary hits. The horizontal red lines indicate the mean value ± SEM for the positive control (DEX) wells. Blue dots, bona fide GCs. (d, e) Receiver operating characteristic (ROC) curves for the cell (d) and larvae (e) assay. ROC curves are calculated by plotting the true positive rate against the false positive rate as robust *z*-score cutoff values are varied (right-hand *y*-axis, color coded). The area under the curve (AUC) values are close to 1 for both cell and larvae assay, indicating good assay performance. (f, g) Primary screen hits and results of retesting. (f) Table showing compounds active (yellow) or inactive (blue) in the GRE:Luc bioluminescence assay in the primary screen (prim.) or after retesting (retest) in cells and fish. *Bona fide* GCs were not retested (white). Of 12 *bona fide* GCs present in the library, 12 were identified as active in the cells and 10 in the larvae. One additional compound not annotated as GC was identified in the larvae, while two false positive hits were found in the cell assay and one in the larvae (gray). (g) Overview of the number of annotated GCs (black) and other compounds (red) identified in cell and larvae assays. (h) Pregnenolone treatment leads to an increase in cortisol (hydrocortisone) levels ($p$ = 0.0005, $n$ = 5). Error bars represent mean values ± SEM.

than those of DBT after DBT treatment (Figure 2e, Supplementary Figure 4). Importantly, we could also detect a substantial amount of DBT in the TBT treated larvae, consistent with degradation of TBT by the larval metabolism. These DBT amounts were much higher than those in DBT treated larvae, likely explaining the higher inhibitory activity of TBT treatment on GC signaling *in vivo*. This example illustrates that the GRIZLY assay can be used to monitor endocrine


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disruptors targeting GC signaling and that it detects biomodification-dependent compound activities that cannot be observed in cell culture systems.

We next conducted a pilot chemical screen for compounds capable of activating GC signaling using a library of Food and Drug Administration (FDA) approved drugs (Figure 3a). Single larvae (5−11 replicates per compound) were treated with the 640 compounds of the library, and the area under the curve (AUC) of the recorded bioluminescence traces was normalized using the robust *z*-score method. The presence of 12 *bona fide* GCs, as annotated in PubChem (http://pubchem.ncbi.nlm.nih.gov), in the library allowed us to calculate a receiver operating characteristic (ROC) curve to estimate the sensitivity and specificity of our assay (Figure 3c, see also Methods section below). On the basis of this curve, we set the robust *z*-score cutoff value for hit identification to 1.49, which identified 9 of the 12 annotated GCs (Figure 3c,f,g). In addition, two more compounds not annotated as GCs were scored as primary screen hits: pregnenolone, a key intermediate in the biosynthesis of GCs, and the MR antagonist spironolactone. In retests, pregnenolone was confirmed to activate the GC signaling pathway *in vivo* (Supplementary Figure 5e′). Two *bona fide* GCs not retained in the primary screen, corticosterone and prednisone, were unable to do so at all concentrations tested (Supplementary Figure 5b′,d′). The inactivity in our *in vivo* assay of corticosterone, which is the major natural GC in rodents, may reflect that in fish, as in humans, cortisol is the main GC hormone.[21] The prodrug prednisone might not be taken up or metabolized well by the fish system. Interestingly, the third missed *bona fide* GC, melengestrol acetate, was found to dose-dependently activate GC signaling in the retest, with a relatively weak response at the concentration used in the screen (Supplementary Figure 5c′). Thus, 10 of 11 compounds active in the larvae assay were already identified in the primary screen, indicating that the screen settings are well suited for *in vivo* detection of GC activity.

To compare the larval test system with more classical cell-based screens and to explore systemic versus cell-autonomous effects of the compounds, we screened the library also with the AB.9 GRE:Luc cell reporter system, analyzing the data as in the larvae screen. Here, examination of the ROC curve led us to a robust *z*-score cutoff of 12.3 that identified 11 of the 12 *bona fide* GCs (Figure 3b,d,f,g), with the prodrug prednisone again being the exception. In retests, prednisone led to a weak activation of GC signaling only at the highest concentration tested (Supplementary Figure 5d). In contrast to the larval assay, corticosterone was able to activate the GC signaling pathway in the cells at the relatively high concentration used in both screens (Supplementary Figure 5b). Two more compounds, pamidronic acid and hydroxytacrine, were false positive hits that could not be confirmed during the rescreen (Supplementary Figure 3 g,h). Importantly, pregnenolone, the confirmed non-GC hit from the larvae screen, did not activate the GC pathway in the cell assay (Supplementary Figure 5e). This might be explained by the fact that the prohormone pregnenolone is metabolized to cortisol in the adrenal/interrenal gland by 3$\beta$-hydroxysteroid dehydrogenase and P450 cytochromes.[21,22] Indeed, pregnenolone treatment led to significantly increased cortisol levels in the larvae already after 20 min (Figure 3h, $p \leq 0.001$). Thus, while identifying known GCs with a sensitivity comparable to that of cell-based screens, the GRIZLY assay can also identify compounds that affect endogenous glucocorticoid biosynthesis. Besides screening for novel ligands capable of activating GR signaling *in vivo*, an additional application of the larvae may be the rescreening of drug candidates for side effects on GC signaling that can be determined only in an intact organism. With different reporter constructs, it might also be possible to apply the GRIZLY assay principle to transrepressive GC signaling activity.

In summary, the GRE:Luc larvae permit the measurement of GC signaling activity upon stimulation with exogenous substances and during the endogenous stress response. Furthermore, the larvae constitute a promising model to study developmental aspects of the stress response and hold great potential for the identification of compounds that regulate various aspects of GC signaling *in vivo*. We present a robust assay for drug discovery that combines relatively cheap running costs, easy handling, and simple readout analysis with the complexity of whole animal studies.

## METHODS

***In Vivo* Bioluminescence Assays.** All bioluminescence measurements were carried out at 28 °C with an Envision Multilabel Plate Reader (PerkinElmer) equipped with enhanced luminescence sensitivity and a plate stacker automation system. To prevent evaporation during measurement, plates with embryos/larvae or cells were sealed with adhesive seals (no. 6005185, TopSeal-A, PerkinElmer).

**Cell Assay.** GRE:Luc cells (35,000 cells/well) were transferred 1 day before measurement into sterile opaque 96-well plates (no. 6005680, PerkinElmer) and were incubated at 28 °C. The next day, the medium was replaced by L-15 medium without phenol red containing 0.5 mM luciferin (L-15 L) before addition of the chemicals to the indicated final concentrations. Organotin experiments were carried out in the presence of 10 nM DEX in 17% (v/v) charcoal treated FCS (no. S3113, Biochrom AG) with either TBT (30−500 nM) or DBT (30−500 nM). Neither DBT nor TBT treatment had any effects on cell viability or on luciferase activity itself (Supplementary Figure 4).

**Larvae Assay.** For larval bioluminescence measurements (GRIZLY = **G**lucocorticoid **R**esponsive ***I**n vivo* **Z**ebrafish **L**uciferase Activit**Y** assay), 4 dpf old larvae were transferred into opaque 96-well plates (no. 6005299, PerkinElmer, one larva per well) containing E3 medium containing 0.5 mM luciferin (E3L). At 5 dpf, larvae were treated with the compounds as indicated. For the developmental studies, eggs were immediately collected after spawning and transferred into E3L medium containing opaque 96-well plates (one egg per well). Every 24 h, a new set of embryos/larvae was treated with 20 $\mu$M DEX or 250 mM NaCl. Viability of the larvae was not affected by GC, organotin or salt treatments, as indicated by their normal swimming behavior after the assays (data not shown).

**Chemical Screen.** The compounds of the FDA approved drug library (#BML-2841, ENZO Life Science) were prediluted into E3 with a robotic liquid handling station (Multiprobe II, PerkinElmer) to a concentration of 40 $\mu$g/mL in 3% DMSO. Cells and larvae were placed into opaque 96-well plates with L-15 L and E3L, respectively, as described above. The prediluted compounds were applied to the cell/larvae plates with a hand-operated 96-channel pipet (Liquidator 96, Steinbrenner) to obtain a final concentration of 4 $\mu$g/mL in 0.3% DMSO. Bioluminescence was measured for 48 h as described above.

**Data and Statistical Analysis.** Data were analyzed with GraphPad Prism 5 (GraphPad Software, Inc.) and in the statistical programming environment R.[23] If not stated otherwise, all statistical tests on experiments with larvae were non-parametric. When two sample groups were compared, a two-tailed Mann−Whitney test was used. Three and more sample groups were analyzed with a Kruskal−Wallis test followed by Dunn's posttest comparing each sample group with the control group. Statistical analyses of repeated measurements were based on Friedman's test, with Dunn's posttest comparing each sample group with the control group. All cell assays and real-time

qPCR measurements were evaluated in a one-way ANOVA followed by Dunnett's or Bonferroni's posttest, respectively. Asterisks in the figures indicate $p$-values of $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

**Dose–Response Curves.** $EC_{50}$ and $IC_{50}$ values of the concentration–response curves were obtained by non-linear regression using the built-in models of GraphPad Prism 5.

**Chemical Screen.** In total, 7,296 luminescence traces from 76 plates for fish and 2,304 luminescence traces from 24 plates for cells were obtained in the FDA compound screen. Each plate contained 8 positive controls (10 $\mu$M DEX) and 8 negative controls (0.3% DMSO). The data were analyzed as follows: The area under the curve (AUC) of the raw data of each well, given as the bioluminescence *versus* incubation time, was approximated with the trapezoidal rule. AUC values were log transformed in order to ensure a higher normality of the data. A robust $z$-score normalization was performed to remove systematic errors from the data, such as plate effects or inter-run variability. To assess the quality of the screen, receiver operating characteristic (ROC) curves were calculated. The AUCs of the ROC curves served as a quality metric of the screen. The resulting estimated true positive rate (TPR) and false positive rate (FPR) were used to define a threshold of the robust $z$-score for hit identification of the compounds. The robust $z$-score with the highest Youden Index (TPR minus FPR) was chosen as the cutoff point. Thus, compounds with a median robust $z$-score greater than 1.49 and 12.3 were identified as hits, yielding 11 and 13 compounds in the primary screen for fish and cells, respectively. Retesting of compounds from a different supplier (see Supplementary Table 1) was performed with the same assay setup and with at least 2 different concentrations.

## ■ ASSOCIATED CONTENT

### Supporting Information

Supplementary methods, figures and table, and references. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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