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Absence of 11-keto reduction of cortisone and 11-ketotestosterone in the model organism zebrafish

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Abstract

Zebrafish are widely used as model organism. Their suitability for endocrine studies, drug screening and toxicity assessements depends on the extent of conservation of specific genes and biochemical pathways between zebrafish and human. Glucocorticoids consist of inactive 11-keto (cortisone, 11-dehydrocorticosterone) and active 11β-hydroxyl forms (cortisol, corticosterone). In mammals, two 11β-hydroxysteroid dehydrogenases (11β-HSD1, 11β-HSD2) interconvert active and inactive glucocorticoids, allowing tissue-specific regulation of glucocorticoid action. Furthermore, 11β-HSDs are involved in the metabolism of 11-oxy androgens. Since zebrafish and other teleost fish lack a direct homologue of 11β-HSD1, we investigated whether they can reduce 11-ketosteroids. We compared glucocorticoid and androgen metabolism between human and zebrafish using recombinant enzymes, microsomal preparations and zebrafish larvae. Our results provide strong evidence for the absence of 11-ketosteroid reduction in zebrafish. Neither human 11β-HSD3 nor the two zebrafish 11β-HSD3 homologues, previously hypothesized to reduce 11-ketosteroids, converted cortisone and 11-ketotestosterone (11KT) to their 11β-hydroxyl forms. Furthermore, zebrafish microsomes were unable to reduce 11-ketosteroids, and exposure of larvae to cortisone or the synthetic analogue prednisone did not affect glucocorticoid-dependent gene expression. Additionally, a dual role of 11β-HSD2 by inactivating glucocorticoids and generating the main fish androgen 11KT was supported. Thus, due to the lack of 11-ketosteroid reduction, zebrafish and other teleost fish exhibit a limited tissue-specific regulation of glucocorticoid action, and their androgen production pathway is characterized by sustained 11KT production. These findings are of particular significance when using zebrafish as a model to study endocrine functions, stress responses and effects of pharmaceuticals.

Introduction

The zebrafish (*Danio rerio*) has emerged as a powerful model organism to study disease mechanisms and for drug discovery and toxicity assessment (Hill, et al. 2005; Kari, et al. 2007; McGrath and Li 2008; Peterson and Macrae 2012; Rubinstein 2006; Stern and Zon 2003; Zon and Peterson 2005). They are also frequently used to study steroid hormone action and to assess endocrine disrupting chemicals (Dang 2016; Schoonheim, et al. 2010; Segner 2009; Tokarz, et al. 2013a). The small size of zebrafish, their high fecundity and rapid development makes them ideal for efficient screening of small bioactive molecules. Compounds can be easily administered; they are readily absorbed by the embryo and early-stage larvae through the skin and gills, and by later-stage animals through the digestive system. Most organs present in mammals start developing in zebrafish at embryonic or early larval stage, allowing the use of larvae for experimentation. Sequencing of the zebrafish genome revealed that 70% of human genes have at least one direct zebrafish counterpart (Howe, et al. 2013). While *in vivo* studies utilizing small mammals allow a comprehensive insight into systems biology and toxicology, the associated ethical and financial burden limits their use in early-stage drug candidate and toxicology screening. On the other hand, investigations in cellular and *in vitro* models are limited regarding complexity. Thus, zebrafish represent a suitable alternative, allowing medium- to high-throughput investigations into systemic effects of chemicals. Nevertheless, for translational relevance of results, the limitations of zebrafish as a model organism need to be better characterized.

The present study characterized the metabolism of 11-oxy glucocorticoids and androgens in zebrafish compared to human. Glucocorticoids regulate a vast number of physiological functions, ranging from metabolism and development to immune system, neuronal and cardiovascular function. Glucocorticoids are produced in a circadian rhythm, and their levels highly increase under stress in order to maintain homeostasis. The stress response pathway is under the control of the Hypothalamus-Pituitary-Adrenal (HPA) axis in human and the functionally equivalent Hypothalamus-Pituitary-Interrenal (HPI) axis in fish (Alsop and Vijayan 2009; Alsop and Vijayan 2008). The main glucocorticoid in human is cortisol. In other animals, including amphibians, reptiles, birds and most rodents it is corticosterone (Palme, et al.

2005). Interestingly, teleost fish utilize cortisol as a major stress hormone, which modulates embryonic and larval development, osmoregulation, regulation of metabolism and circadian rhythmicity (Dickmeis, et al. 2007; Hillegass, et al. 2007, 2008; Kumai, et al. 2012; Nesan, et al. 2012; Nesan and Vijayan 2013; Wendelaar Bonga 1997).

Glucocorticoids exist in an active 11β-hydroxyl form (cortisol, corticosterone) and an inactive 11-keto form (cortisone, 11-dehydrocorticosterone). In mammals, amphibians and birds two 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes control the cell- and tissue-specific interconversion of active and inactive glucocorticoids, thereby tightly controlling glucocorticoid receptor (GR)- and mineralocorticoid receptor (MR)-mediated signaling pathways (Baker 2010; Odermatt and Kratschmar 2012). Cells expressing the receptors in the absence of 11β-HSDs are dependent on extracellular cortisol levels, with altered responses during circadian rhythm and stress. Cells expressing 11β-HSD1 produce cortisol from the inactive cortisone pool, thereby stimulating receptor-dependent signaling, and possibly also affecting neighboring cells in a paracrine manner. 11β-HSD1 is important in metabolically active cells including adipocytes, myocytes and hepatocytes, but also in immune cells including macrophage and dendritic cells (Odermatt and Kratschmar 2012). The importance of 11β-HSD1 is highlighted by the potent anti-inflammatory effects of cortisone and its synthetic analogue prednisone and their use to treat autoimmune diseases, allergic reactions and chronic inflammatory diseases (Barnes 2006; Baschant, et al. 2012). Despite their potent clinical effects, both steroids are inactive and do not bind to GR and MR or any other known nuclear receptor. However, they are rapidly converted by 11β-HSD1 to their active forms cortisol and prednisolone (Diederich, et al. 2002). Conversely, cells expressing 11β-HSD2 exhibit low sensitivity towards cortisol. MR and GR are thought to be activated by cortisol in such cells only during stress where 11β-HSD2 can be saturated (Odermatt, et al. 2001).

In zebrafish and several other fish species 11β-HSD2 has been identified and found to control cortisol levels (Jiang, et al. 2003; Kusakabe, et al. 2003; Meyer, et al. 2012; Miura, et al. 1991). Upon oxidation by 11β-HSD2, the formed cortisone can be further metabolized in zebrafish by 20β-hydroxysteroid dehydrogenase type 2 (20β-HSD2) to 20β-hydroxycortisone, which is subsequently excreted (Tokarz, et

al. 2012; Tokarz, et al. 2013b). 11β-HSD2 has also a key role in fish by producing the major androgen 11-ketotestosterone (11KT) (Jiang et al. 2003; Kusakabe et al. 2003; Miura et al. 1991). Thus, 11β-HSD2 exerts a dual role in fish by inactivating glucocorticoids and activating androgens. A role for 11β-HSD2 in the production of potent androgen receptor (AR) ligands has also been proposed in human, with relevance to prostate cancer (du Toit, et al. 2016; Storbeck, et al. 2013). Besides the classical pathway of Δ4-androstene-3,17-dione (A4) conversion by testicular 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3) to testosterone and further metabolism by 5α-reductase to the most potent androgen 5α-dihydrotestosterone (DHT), recent studies showed that 11-keto-DHT (11KDHT) was as potent a human AR ligand as was DHT at 1 nM, while 11KT was a partial agonist (Storbeck et al. 2013). At 10 nM all 11-oxy testosterone derivatives were found to be as potent as DHT. The human adrenals produce both A4 and 11β-hydroxy-A4 (11OHA4) (Swart, et al. 2013), and adrenal vein sampling revealed higher 11OHA4 than A4 levels, i.e. 811 nM and 585 nM, respectively (Rege, et al. 2013). Although 11OHA4 is considered to be inactive, in peripheral tissues it can be converted to the more potent androgen 11OHT and upon further metabolism by 5α-reductase to 11β-hydroxy-DHT (11OHDHT). In human, 11β-HSD1 and 11β-HSD2 interconvert in a cell- and tissue-specific manner the 11β-hydroxyl and 11-keto forms of these androgens (Figure 1) (Swart et al. 2013).

Zebrafish possesses 11β-HSD2 that converts 11OHA4 and 11OHT to 11KA4 and 11KT (de Waal, et al. 2008; Meyer et al. 2012). Both human and zebrafish 17β-HSD3 convert 11KA4 to 11KT (Mindnich, et al. 2005). Whether 17β-HSD3 can additionally contribute to 11KT production through conversion of 11OHA4 to 11OHT remained controversial. One report suggested that both human and zebrafish 17β-HSD3 are able to perform this reaction (Mindnich et al. 2005), whereas a more recent study did not detect this activity (Storbeck et al. 2013). Due to the importance of 11KT as an AR ligand, especially in fish, we aimed to elucidate whether this enzymatic reaction is present.

Unlike human and higher mammals, the zebrafish genome does not possess a direct homologue of 11β-HSD1 (Baker 2010). The gene encoding 11β-HSD1 (SDR26C1) is absent in teleost fish but present in amphibians and birds, and it likely first appeared in shark (Baker 2010). Earlier studies provided evidence

for the existence of a third 11β-HSD isoform (Ge, et al. 1997; Gomez-Sanchez, et al. 1997; Huang, et al. 2009; Ohno, et al. 2013), namely 11β-HSD3, exhibiting high-affinity $NADP^+$-dependent cortisol oxidation activity, for example in rat Leydig cells (Ge et al. 1997), pig testis (Ohno et al. 2013) and sheep kidney (Gomez-Sanchez et al. 1997). Phylogenetic analyses revealed 11β-HSD3 (SDR26C2, SCDR10B) as a close relative of 11β-HSD1 (Baker 2004, 2010). Human 11β-HSD3 was found to oxidize cortisol to cortisone, but not the reverse reaction, albeit at high substrate concentrations and indirectly measured (Huang et al. 2009). In contrast to human, two genes exist in zebrafish, 11β-HSD3a and 11β-HSD3b, and it has been suggested that the 11β-HSD3 enzymes have 11-oxidoreductase activity (Baker 2004, 2010; Huang et al. 2009). However, this has not been confirmed by functional data.

In this study, we investigated whether zebrafish are able to reduce 11-ketosteroids and whether 11β-HSD3 would possess such activity. Additionally, we studied the role of zebrafish 11β-HSD2 and 17β-HSD3 in the formation of 11KT, compared to the human enzymes. For this purpose, we expressed recombinant human and zebrafish enzymes in human and zebrafish cell lines for activity evaluation. To further establish whether 11-ketosteroid reduction exists, we used zebrafish homogenates for enzyme activity analysis and implemented *in vivo* studies in zebrafish larvae. Our results strongly support the absence of an enzymatic activity for cortisone to cortisol and 11KT to 11OHT conversion in zebrafish, and support the pivotal role of 11β-HSD2 in glucocorticoid and androgen metabolism.

Materials and Methods

Steroids and other reagents

Steroids were purchased from Steraloids (Newport, RI), and all other reagents from Sigma–Aldrich (Buchs, Switzerland).

Molecular cloning and generation of expression plasmids

Human 11β-HSD3 (NM_198706.2) was PCR-amplified from a donor vector and subcloned into the pcDNA3 vector (Life Technologies, Zug, Switzerland) between the *Bam*HI/*Xba*I restriction sites. A nine

nucleotide linker sequence was added after the coding sequence, followed by the sequence encoding for the FLAG epitope. For cloning of zebrafish 11β-HSD3a (NM_200323.2) and 11β-HSD3b (XM_696067.3), total RNA from zebrafish was reverse transcribed to cDNA using SuperScript® II (Invitrogen, Carlsbad, CA). The nucleotide sequence encoding FLAG was added downstream of the coding sequence. 11β-HSD3a was cloned between the *Bam*HI/*Xho*I and 11β-HSD3b between the *Bam*HI/*Xba*I sites of pcDNA3 (for primer sequences see Supplementary Table 3). Human 11β-HSD1, 11β-HSD2 and 17β-HSD3 FLAG-tagged at the C-terminus, FLAG-tagged zebrafish 11β-HSD2 and 17β-HSD3 were described earlier (Engeli, et al. 2016; Meyer et al. 2012; Mindnich et al. 2005; Tsachaki, et al. 2015).

Cell culture and transfection

Human embryonic kidney (HEK-293) cells (ATCC, Manassas, VA, USA) were cultivated as described earlier (Tsachaki et al. 2015). Zebrafish embryonic fibroblast cells ZF4 (LGC Promochem, Wesel, Germany) were cultivated at 28°C in a humidified 5% CO_2 atmosphere in DMEM:F12 medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin. HEK-293 cells were transiently transfected with the calcium phosphate method [44] and ZF4 cells using Fugene HD (Promega AG, Switzerland) (Arends, et al. 1999). Cells at passage number below 30 were used in this work.

Zebrafish strain and treatment of larvae

Zebrafish (*Danio rerio*, ABC strain) were cultured using standard procedures. Eggs were collected after spawning and kept in E3 medium at 28°C (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, pH 7.2). At 3 days post fertilization (dpf) they were treated for 24 h with the cortisol, cortisone, prednisone, or prednisolone at a final concentration of 80 μM. The stock solutions of the compounds were prepared in DMSO, which did not exceed 1% (v/v) in the larval medium. 15 larvae were pooled per treatment and immediately transferred to tubes containing 1 ml Trizol reagent (Invitrogen). RNA isolation,

cDNA synthesis and qPCRs are described earlier (Chantong, et al. 2012). Relative gene expression was determined by the ddCt method using RLP13a as an internal control (for primer sequences see Supplementary Table 4).

GRIZLY assay

The GRIZLY assay was performed as described previously (Weger, et al. 2013; Weger, et al. 2012) (http://pubs.acs.org/doi/suppl/10.1021/cb3000474).

Protein extraction and Western blotting

Transfected cells were lysed 48 h post-transfection using RIPA buffer, followed by Western blot (Engeli et al. 2016). For detection of the FLAG epitope tag, the mouse monoclonal M2 antibody from Sigma–Aldrich (Buchs, Switzerland) was used, and for detection of Cyclophilin A (PPIA) the rabbit polyclonal antibody ab41684 (Abcam, Cambridge, UK), both used at 0.5 µg/ml final concentration.

Enzyme activity assay using cell lysates

Assessment of enzyme activity in cell lysates was described previously (Meyer et al. 2012). Briefly, 48 h post-transfection, cell lysates were incubated for 1 h at 37°C or 28°C in a total volume of 500 µl of TS2 buffer (100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM $MgCl_2$, 250 mM sucrose, 20 mM Tris–HCl, pH 7.4) containing 1 µM substrate (cortisone, cortisol, 11KT or 11OHT) in the presence of 500 µM co-factor (either NADH, NADPH, NAD^+, or $NADP^+$), and 0.05% of detergent Brij ®58 for microsome permeabilization. For liquid-liquid extraction, each sample was mixed with 500 µl acetonitrile that contained deuterated cortisol (9,11,12,12-D4-cortisol) or testosterone (1,2-D2-testosterone) as internal standards. The samples were incubated for 10 min at 4°C with shaking and centrifuged at 16'100 × g for 10 min at 4°C. The supernatants were evaporated to dryness and 500 µl methanol was added to each sample, followed by incubation for 10 min at 4°C (1300 rotations/min). Samples were centrifuged at

16'100 × g for 10 min at 4°C, evaporated to dryness, reconstituted in 100 µl methanol, and stored at -20°C until analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Enzyme activity assay in intact cells

At 24 h post-transfection, cells grown in 24-well plates were incubated in 500 µl of charcoal-treated culture medium containing 100 nM of the respective steroid. Following incubation for 24 h for 11β-HSD3, 1 h for 11β-HSD1 and 11β-HSD2, and 4 h for 17β-HSD3 isoforms, the supernatants were collected and an equal volume of acetonitrile was added. Sample preparation was performed as described for cell lysates.

Isolation of zebrafish microsomes and enzyme activity assay

Microsomes were prepared from three pooled samples of adult whole zebrafish or freshly isolated adult liver, brain or testis as described earlier (Meyer, et al. 2013), yielding 1.5 mg/ml for full body microsomes, 0.015 mg/ml for liver microsomes, 0.8 mg/ml for brain microsomes and 0.12 mg/ml for testis microsomes. Microsomes were incubated for 1 h at 28°C in TS2 buffer containing 1 µM substrate (cortisone, cortisol, 11KT) in the presence of 500 µM co-factor (NADPH, NAD^+). Steroid extraction with acetonitrile was performed as described above for cell lysates.

Steroid quantification by LC-MS/MS

All analytes were measured simultaneously by ultra-pressure liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using an Agilent 1290 UPLC instrument (for details see supplementary tables 1 and 2 and the Supplementary materials and methods).

Results

Assessment of possible 11-ketosteroid reduction activity of 11β-HSD3

Because of the absence of a direct homologue of 11β-HSD1 in zebrafish and previous reports suggesting that 11β-HSD3 might exert this function in fish (Baker 2004, 2010; Huang et al. 2009), we transiently expressed human 11β-HSD3 (*Hs*11β-HSD3) or the two zebrafish isoforms *Dr*11β-HSD3a and *Dr*11β-HSD3b in HEK-293 cells and tested for the presence of cortisone reduction activity. 24 h post-transfection the cells were incubated with 1 μM cortisone for 24 h, followed by determination of the cortisol formed. We did not detect any formation of cortisol for either the human or zebrafish enzymes upon incubation of the cells at 37°C (Table 1). We also examined possible oxidation activity by incubating the cells with 1 μM cortisol for 24 h; however, no activity was observed despite proper expression of the corresponding proteins as verified by Western blotting (Fig. 2). *Hs*11β-HSD1 and *Hs*11β-HSD2 showed the expected activities by efficiently metabolizing glucocorticoids (Table 1). To exclude that *Dr*11β-HSD3a and *Dr*11β-HSD3b were inactive due to their expression in a human cell line, we also transiently expressed these proteins in ZF4 zebrafish cells and tested for cortisone reduction and cortisol oxidation activity upon incubation for 24 h at 28°C. None of these enzymes showed any activity towards cortisone and cortisol (not shown). Next, we tested the human and zebrafish 11β-HSD3 enzymes for activity towards 11KT and 11OHT (Table 1). Similar to glucocorticoids, 11KT and 11OHT did not serve as substrates of the 11β-HSD3 isoforms, whereas *Hs*11β-HSD1 and *Hs*11β-HSD2 catalyzed the expected reactions. Also, neither *Hs*11β-HSD2 nor *Dr*11β-HSD2 was able to catalyze 11-keto reduction, but exclusively catalyzed oxidation. Additionally, we performed assays in cell lysates from ZF4 cells expressing *Dr*11β-HSD3a or *Dr*11β-HSD3b. The lysates were incubated for 2 h at 28°C in the presence of cortisone or 11KT and NADH or NADPH. Also, the reverse reactions using cortisol or 11OHT and NAD^+ or $NADP^+$ were tested, without detecting any activity under the conditions used.

Qualitative assessment of possible 11-ketosteroid reduction activity using zebrafish microsomes

Because the above results exclude a role for *Hs*11β-HSD3, *Dr*11β-HSD3a, and *Dr*11β-HSD3b in the 11-keto reduction of steroids, we next tested whether such an enzymatic activity is present at all in zebrafish. For this purpose, we isolated microsomes from zebrafish liver, since this tissue exhibits high 11β-HSD1

activity in mammals including human (Tannin, et al. 1991). To exclude the presence of such an activity in other tissues, we isolated microsomes from brain, testis and full body of adult zebrafish. The microsomes were incubated for 1 h at 28°C with 1 μM of cortisone or 11KT as substrates in the presence of 500 μM NADPH as cofactor. No conversion of cortisone to cortisol or 11KT to 11OHT could be detected under any of the above reaction conditions (Table 2). However, we observed an efficient reduction of cortisone to 20β-hydroxycortisone, a reaction catalyzed by 20β-HSD2 (Tokarz et al. 2012; Tokarz et al. 2013b), in full body and brain microsomes (protein amount used: 50 μg and 40 μg, respectively). This reaction was also confirmed in testis (6 μg) and liver microsomes (1 μg), although only low amounts of microsomes could be isolated from these tissues. These data suggested that 11-ketosteroid reduction activity is absent in zebrafish and that cortisone is further metabolized to 20β-hydroxycortisone. Upon incubation with 1 μM cortisol and 500 μM NAD^+, most efficient conversion to cortisone was found in testis, followed by full body and brain microsomes. No such activity could be detected using liver microsomes; however, the yield of liver microsomes was low and existence of low 11β-HSD2 activity cannot be excluded from these results. Together, the above qualitative results confirmed the presence of oxidative 11β-HSD2 and reductive 20β-HSD2 activity in zebrafish microsomes; however, the 11-ketosteroid reduction activity was absent.

Cortisone and prednisone fail to activate the GRE-dependent reporter expression in the GRIZLY assay

To confirm the inability of zebrafish to convert the inactive glucocorticoid cortisone to the active cortisol *in vivo* we employed the GRIZLY assay (Weger et al. 2012), in which transgenic zebrafish express a GR-dependent luciferase reporter (GRE:luc). 5 dpf zebrafish larvae were incubated with cortisone or cortisol, and luciferase reporter activation was followed up to 30 h (Fig. 3A). Whereas cortisol induced the GRE-driven luciferase expression in a time-dependent manner, cortisone failed to activate the reporter. The same observation was made for the inactive synthetic glucocorticoid prodrug prednisone, which in human is efficiently converted to its active form prednisolone by 11β-HSD1 (Fig. 3B). The potent synthetic

glucocorticoid dexamethasone was used as a positive control in this experiment. The above results suggest that in 5 dpf zebrafish larvae cortisone and prednisone cannot be converted to their active forms by an enzymatic activity equivalent to that of human 11β-HSD1.

Expression of GR target genes after treatment of zebrafish larvae with glucocorticoids

To further confirm the absence of 11-ketosteroid reduction activity *in vivo*, we treated 3 dpf zebrafish larvae with cortisol, cortisone, prednisone or prednisolone at a final concentration of 80 μM for 24 h, and examined the mRNA levels of three GR target genes GILZ, FKBP5 and 11β-HSD2 (Mathew, et al. 2007; Schaaf, et al. 2009; Wilson, et al. 2013). We observed an increase in mRNA levels of all three genes upon treatment with cortisol and prednisolone, but no change in expression upon treatment with cortisone and prednisone (Fig. 4). These results further support the absence of 11-ketosteroid reduction activity in zebrafish.

Androgen metabolism by 11β-HSD2 and 17β-HSD3 in human and zebrafish

Whilst our results show that 11-ketosteroid reduction activity is absent in zebrafish, 11β-HSD2 seems to have an important dual role by inactivating cortisol and also converting 11OHT to the main fish androgen 11KT (Meyer et al. 2012). 11β-HSD2 has also been proposed to convert 11OHA4 to 11KA4 (de Waal et al. 2008; Swart and Storbeck 2015). The latter can be further converted by 17β-HSD3 to 11KT (Mindnich et al. 2005). To verify these previously reported observations, we transiently expressed *Hs*11β-HSD2 or *Dr*11β-HSD2 in HEK-293 cells, and incubated the cells for 1 h at 37°C with 100 nM 11OHT or 11OHA4, followed by quantification of the steroids present in the supernatant by LC-MS/MS. Both human and zebrafish 11β-HSD2 were able to convert 11OHT to 11KT and 11OHA4 to 11KA4 (Fig. 5). The lower activity observed for *Dr*11β-HSD2 compared with *Hs*11β-HSD2 may be attributed to the lower expression levels of this construct, as suggested by Western blot analysis (Fig. 2). Expression of the zebrafish enzyme in a human cell line may provide an additional explanation for its lower activity. Although 11KA4 is converted to 11KT by 17β-HSD3, contributing to the main androgen pool in zebrafish, it has been a

matter of debate whether 17β-HSD3 can also convert 11OHA4 to 11OHT (Mindnich et al. 2005; Storbeck et al. 2013). To address this question, we incubated HEK-293 cells expressing *Hs*17β-HSD3 at 37°C or ZF4 cells expressing *Dr*17β-HSD3 at 28°C for 4 h with A4, 11OHA4 or 11KA4, and measured the formation of the corresponding 17β-hydroxylated product by LC-MS/MS. Whereas A4 and 11KA4 were metabolized by both 17β-HSD3 enzymes, 11OHA4 was not accepted as substrate (Fig. 5). Also, prolonged incubation of the cells with 11OHA4 for 24 h did not result in the formation of any product (not shown).

Discussion

In the present study, we show that zebrafish do not catalyze 11-ketosteroid reduction. As a consequence, exposure to cortisone or prednisone did not affect GR-dependent gene expression. This finding is particularly important for understanding glucocorticoid action in fish and needs to be considered when using zebrafish as a model to study endocrine functions, stress and cardio-metabolic pathways. In zebrafish and other teleost fish lacking 11-ketosteroid reduction, the cortisol produced upon HPI axis activation acts on GR in peripheral tissues prior to inactivation by 11β-HSD2 and 20β-HSD2 to cortisone and 20β-hydroxycortisone, respectively. Due to the lack of 11β-HSD1 in zebrafish, cortisone cannot be recycled, is further metabolized, conjugated and excreted, and a new cortisol molecule must be synthesized to maintain glucocorticoid signaling.

Furthermore, our comparative data of recombinant *Hs*11β-HSD1, *Hs*11β-HSD2 and *Hs*11β-HSD3 exclude a role of the latter in the metabolism of cortisone and cortisol. Also, we showed that the two related zebrafish enzymes *Dr*11β-HSD3a and *Dr*11β-HSD3b, previously suggested to play a role in glucocorticoid metabolism (Baker 2004, 2010), do not convert cortisone to cortisol. Back conversion by another enzyme endogenously expressed in HEK-293 or ZF4 cells can be excluded because cortisol was neither oxidized by untransfected cells nor by cells expressing the human and *Dr*11β-HSD3 isoforms. Thus, a substrate and physiological function of the human and zebrafish 11β-HSD3 isoforms still needs to be uncovered. Importantly, our results suggest the complete absence of 11-oxosteroid reduction in

zebrafish. This is supported by measurements in whole body microsomes and microsomes of different tissues, as well as *in vivo* experiments evaluating GR-dependent luciferase reporter expression in 5dpf zebrafish larvae. Of note, the stress axis is fully developed in larvae of this developmental stage (Alsop and Vijayan 2008; Weger et al. 2012; Wilson et al. 2013). In accordance, we observed that treatment of larvae with cortisol and prednisolone, but not with cortisone and prednisone, led to the activation of GR target genes.

NCBI BLAST searches identified homologues of the human 11β-HSD1 protein in many mammals but also in amphibians and birds ((Baker 2010), Supplementary Figures 1 and 2). From the absence of 11β-HSD1 in teleost fish, which belong to Osteichthyes (bony fish), one cannot conclude that this is also the case for Chondrichthyes (cartilaginous fish). The genome of *Callorhinchus milli* (commonly known as elephant shark), belonging to the Holocephali subclass of Chondrichthyes, was recently sequenced and analyzed (Venkatesh, et al. 2014). That study showed that the elephant shark has the most slowly evolving genome compared to all known vertebrates, which makes it an emerging model for phylogenetic studies. Surprisingly, 271 genes of the elephant shark were found to be lost from the teleost lineage compared to only 34 in the tetrapod lineage. BLAST of human 11β-HSD1 protein with the predicted proteins of the elephant shark (http://esharkgenome.imcb.a-star.edu.sg) yields a protein (SINCAMP00000014194) with 56% identical amino acids (and 76% identical and similar residues) compared to the human enzyme. Another predicted protein of the elephant shark (SINCAMP00000008284) shares high similarity with the human 11β-HSD3 (SCDR10B) and zebrafish 11β-HSD3a and 11β-HSD3b (52-57% identical and 66-78% identical and similar residues; Supplementary Figure 2). Whether the elephant shark protein resembling human 11β-HSD1 indeed has 11-ketoreduction activity, and whether the second protein represents a functional homologue of 11β-HSD3 remains to be investigated.

The absence of 11β-HSD1, which besides 11-ketosteroids such as cortisone and prednisone, also catalyzes the carbonyl reduction of 7-oxy oxysterols and several non-steroidal chemicals (Odermatt and Klusonova 2015), may limit the translational relevance of results obtained from zebrafish studies. Furthermore, recent evidence suggested that 11β-HSD enzymes in human are also involved in androgen metabolism (Storbeck

et al. 2013; Swart et al. 2013). The 11β-hydroxyl and 11-keto forms of A4 and testosterone can be interconverted by 11β-HSD1 and 11β-HSD2 (Fig. 1). Interestingly, as shown in Table 1, the 11-oxy testosterone metabolites appear to be better substrates than cortisone and cortisol, corroborating earlier observations by Swart et al. (Swart et al. 2013). Also, 11OHT was found to be a better substrate than 11OHA4 for both *Hs*11β-HSD2 and *Dr*11β-HSD2 (Figure 5).

In contrast to human, where DHT is the main androgen produced from testosterone by 5a-reductase activity, the main androgen in fish is 11KT. Interestingly, although 11KT has been shown to be the dominant androgen circulating in blood in teleost fish (Koldras M 1990), studies in the African catfish showed that the most highly produced androgen in testis is 11OHA4, emphasizing the importance of 11OHA4 conversion to 11KT in extra-testicular tissues (Cavaco 1997). As would be expected from the absence of 11-ketosteroid reduction, we found that zebrafish cannot convert 11KT to 11OHT, thereby preventing the inactivation of 11KT in tissues where 11β-HSD2 is active. Of note, it has been demonstrated that in zebrafish 17β-HSD2, the enzyme converting testosterone back to A4 in human, seems to be expressed but is functionally inactive (Mindnich, et al. 2007). In addition, we showed that neither human nor zebrafish 17β-HSD3 are able to convert 11OHA4 directly to 11OHT, in line with observations by Storbeck et al on human 17β-HSD3 (Storbeck et al. 2013). Collectively, our results indicate that 11KT in zebrafish can be produced via two different pathways: A4 → testosterone → 11OHT → 11KT, or A4 → 11OHA4 → 11KA4 → 11KT (Fig. 1).

At present, it is not clear whether the lack of 11-ketosteroid reduction constitutes an evolutionary advantage for zebrafish and other teleost fish. The absence of cortisone conversion to cortisol might facilitate the functional interactions of glucocorticoids and androgens in these species. Cortisol was shown to promote spermatogonial mitosis in the Japanese eel by increasing 11KT production (Ozaki, et al. 2006), which is likely mediated through upregulation of 11β-HSD2 expression. However, excess levels of cortisol, which may competitively inhibit the formation of 11KT, abolished proliferation of spermatogonia. Thus, a tight regulation of the glucocorticoid/androgen balance is essential.

In conclusion, the current study reveals important species-specific differences between human and zebrafish glucocorticoid and androgen metabolism. The existence of two 11β-HSD enzymes in human that catalyze opposite reactions allows a tightly regulated tissue-specific glucocorticoid action, with the energetic advantage that the steroid molecule can be recycled, thereby prolonging its half-life and sparing energy to produce a new molecule. In contrast, due to the absence of 11-ketosteroid reduction zebrafish, and likely most teleost fish, exhibit a limited tissue-specific regulation of glucocorticoid action and *de novo* cortisol production is indispensable once cortisol is converted to the inactive cortisone. Furthermore, the loss of 11-ketoandrogen reduction activity, in combination with the inability of 17β-HSD2 to metabolize androgens, renders the androgen production pathway of zebrafish highly unidirectional towards sustained 11KT production. Our results emphasize that an in-depth dissection of molecular and metabolic pathways will improve the use of zebrafish as a model organism with translational relevance for human.

Declaration of interest

The authors declare no conflict of interest and have nothing to disclose.

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Author contributions

MT performed experiments and wrote the manuscript. AM, HGB and BW performed experiments. TD contributed materials and wrote the manuscript. DVK performed the LCMS/MS analysis. JT, JA and MA wrote the manuscript. AO wrote the manuscript and supervised experiments.

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580

Figure legends

Figure 1. Glucocorticoid and androgen metabolism pathways that involve the function of 11β-HSD1, 11β-HSD2 and 17β-HSD3. The main glucocorticoid cortisol is produced from 11-deoxycortisol by CYP11B1. Cortisol can be oxidized to the inactive steroid cortisone by 11β-HSD2, and cortisone can be re-activated through the reductive activity of 11β-HSD1. Upon synthesis, the androgen Δ4-androstene-3,17-dione (A4) can be converted to testosterone by 17β-HSD3. The reverse reaction is catalyzed by 17β-HSD2. A fraction of A4 and testosterone can be 11β-hydroxylated by CYP11B1. These metabolites can be further converted by 11β-HSD2 to their 11-keto forms 11KA4 and 11KT. The opposite reaction is catalyzed by 11β-HSD1. 11KA4 is converted to 11KT by 17β-HSD3. It remained controversial whether the same enzyme can accept 11OHA4 as a substrate to produce 11OHT. The dotted rectangles depict pathways involving 11β-HSD1 or 17β-HSD2 activities, which seem to be absent from zebrafish. Production of DHT, 11OHDHT and 11KDHT is catalyzed by 5α-reductase activity.

Figure 2. Verification of protein expression after transfection of different constructs. HEK-293 cells were transfected with pcDNA3, *Hs*11β-HSD3 (coding for a protein of 286 residues), *Dr*11β-HSD3b (336 residues) or *Dr*11β-HSD3a (287 residues) (A), and with pcDNA3, *Hs*11β-HSD2 (405 residues) or *Dr*11β-HSD2 (414 residues) (B). All expressed proteins contain a C-terminal FLAG epitope. Western blots of cell extracts were analyzed using the anti-FLAG antibody M2. In (B) due to the high intensity of the signal produced by *Hs*11β-HSD2, two different exposure times (2 seconds and 2 minutes) are shown for the same blot. Membranes were reprobed for PPIA as a loading control.

Figure 3. GRIZLY assay for the evaluation of GRE activation by glucocorticoids in zebrafish larvae. GRE:luc larvae (n=48) were treated with the glucocorticoids indicated, and the bioluminescence response was monitored over time. The relative reporter activity, based on luminescence measurements, is

shown over time (left) or after 24 h (right). The steroids used to treat larvae were cortisol or cortisone (A), and prednisone or dexamethasone (B) at the indicated final concentrations.

Figure 4. Effect of various glucocorticoids on GR-target gene expression. 3dpf zebrafish larvae were treated for 24 h with the steroids indicated, and the expression of three GR-target genes was measured with qPCR from cDNA generated from 15 larvae per sample. The genes tested were GILZ, FKBP5 and 11β-HSD2. The relative quantification and fold change calculation were performed in relation to the RPL13a house keeping gene expression using the ddCt method. The results represent mean ± standard deviation from three independent experiments.

Figure 5. Androgen metabolism by 11β-HSD2 and 17β-HSD3. (A) HEK-293 cells were transfected with plasmids for *Hs*11β-HSD2 or *Dr*11β-HSD2. At 24 h post-transfection cells were incubated with 11OHT or 11OHA4 for 1 h and formation of 11KT and 11KA4, respectively, was measured by LC-MS/MS. (B) HEK-293 cells were transfected with a plasmid for *Hs*17β-HSD3, and ZF4 cells with a plasmid for *Dr*17β-HSD3. At 24 h post-transfection cells were incubated for 4 h with A4, 11OHA4 and 11KA4, followed by determination of the generation of testosterone, 11OHT and 11KT, respectively, by LC-MS/MS. Results are presented as mean of three independent experiments, and error bars represent standard deviation.

Table 1. Enzyme activities of recombinant 11β-HSDs in intact HEK-293 cells

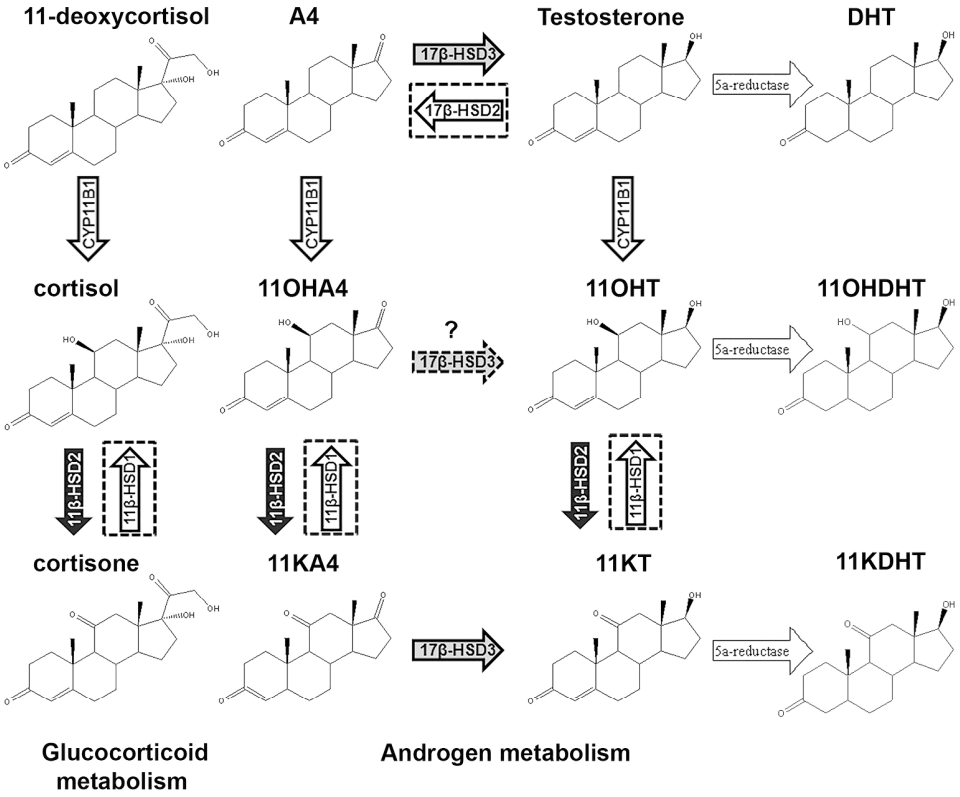
Enzyme	Cortisone to cortisol	Cortisol to cortisone	11KT to 11OHT	11OHT to 11KT
***Hs*11β-HSD3**	n.d.	n.d.	n.d.	n.d.
***Dr*11β-HSD3a**	n.d.	n.d.	n.d.	n.d.
***Dr*11β-HSD3b**	n.d.	n.d.	n.d.	n.d.
***Hs*11β-HSD1**	23 ± 9*	38 ± 7*	39 ± 16*	45 ± 12*
***Hs*11β-HSD2**	n.d.*	68 ± 10*	n.d. *	>90*

HEK-293 cells were transiently transfected with plasmids for the expression of the respective 11β-HSD enzyme. Cells were incubated for 24 h at 37°C, followed by determination by LC-MS/MS of the percentage of product formed from 1 µM of initially supplied substrate. * Incubation time of 1 h; n.d.: not detectable. Data represent mean ± SD from three experiments.

Table 2. Qualitative assessment of reduction of cortisone and 11KT in zebrafish microsomes

Type of microsomes (protein amount used)	Cortisone to cortisol (% conversion)	11KT to 11OHT (% conversion)	Cortisone to 20β-hydroxycortisone (% conversion)	Cortisol to Cortisone (% conversion)
Full body (50 µg)	n.d.	n.d.	28 ± 2	7 ± 0.4
Liver (1 µg)	n.d.	n.d.	2 ± 0.6	n.d.
Brain (40 µg)	n.d.	n.d.	6 ± 0.3	4 ± 0.2
Testis (6 µg)	n.d.	n.d.	1 ± 0.3	27 ± 4

Microsomes were incubated for 1 h at 28°C with 1 µM of the respective substrate and 500 µM of NADPH for reduction reactions or 500 µM NAD^+ for oxidation reactions, followed by determination of the percentage of product formed by LC-MS/MS. n.d.: not detectable. Data represent mean ± SD from three activity measurements performed with one microsome preparation.



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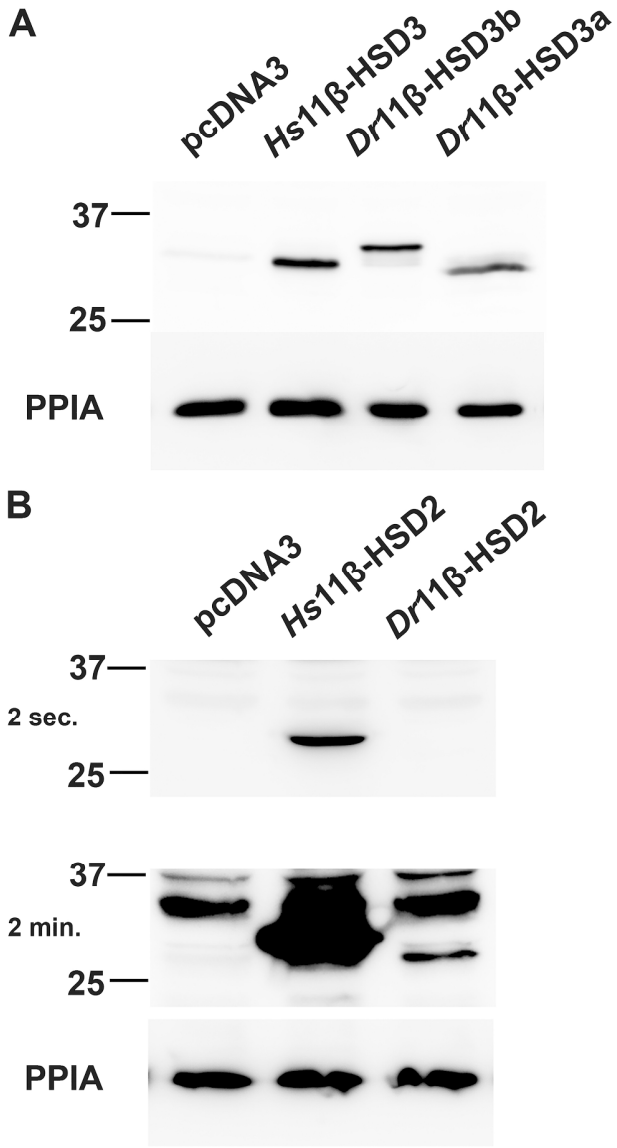

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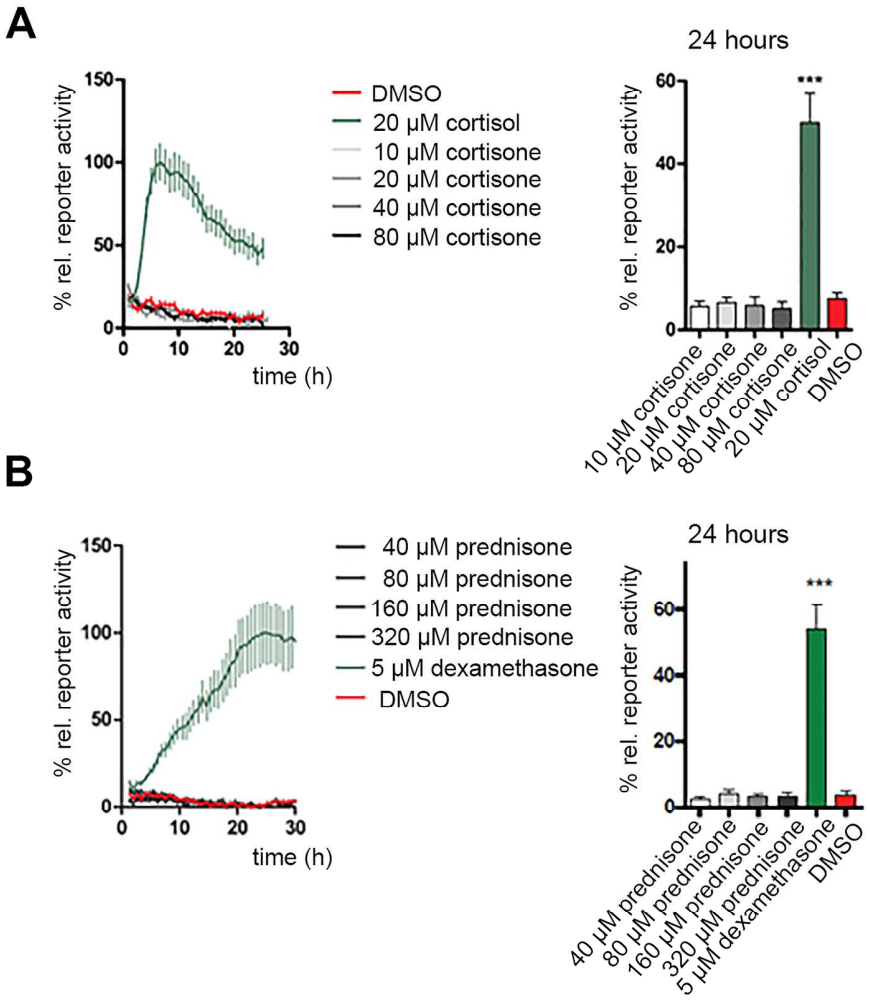

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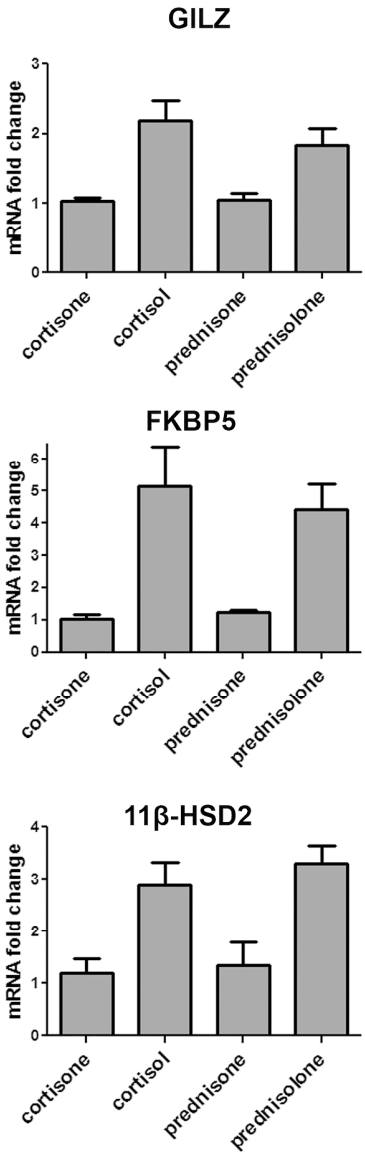

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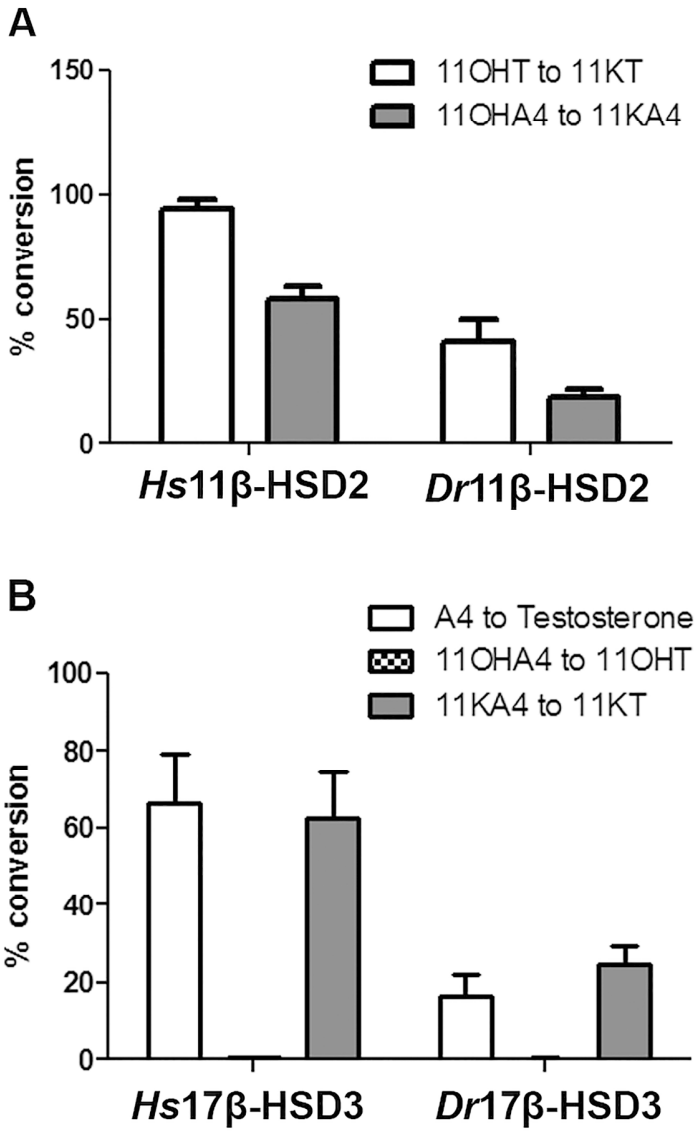

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94x150mm (600 x 600 DPI)