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Alternate glucocorticoid receptor ligand binding structures influence outcomes in an *in vivo* tissue regeneration model

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ABSTRACT

Since their characterization, glucocorticoids (GCs), the most commonly prescribed immunomodulatory drugs, have undergone numerous structural modifications designed to enhance their activity. *In vivo* assessment of these corticosteroid analogs is essential to understand the difference in molecular signaling of the ligands that share the corticosteroid backbone. Our research identified a novel function of GCs as modulators of tissue regeneration and demonstrated that GCs activate the glucocorticoid receptor (GR) to inhibit early stages of tissue regeneration in zebrafish (*Danio rerio*). We utilized this phenomenon to assess the effect of different GC analogs on tissue regeneration and identified that some GCs such as beclomethasone dipropionate (BDP) possess inhibitory properties, while others, such as dexamethasone and hydrocortisone have no effect on regeneration. We performed *in silico* molecular docking and dynamic studies and demonstrated that type and size of substitution at the C17 position of the cortisol backbone confer a unique stable conformation to GR on ligand binding that is critical for inhibitory activity. In the field of tissue regeneration, our study is one of the first Structure Activity Relationship (SAR) investigations performed in vertebrates demonstrating that the *in vivo* tissue regeneration model is a powerful tool to probe structure function relationships, to understand regenerative biology, and to assist in rational drug design.

1. Introduction

Glucocorticoids are the most commonly used anti-inflammatory and immunosuppressive agents highly efficacious in the treatment of disease, but they are also associated with serious side effects. Hence, improvements in the therapeutic profile of these drugs are needed. To date, the majority of the structural modifications of glucocorticoid receptor (GR) ligands were designed to eliminate side effects. There are approximately 20 topically active anti-inflammatory corticosteroids on the market (http://www.hfs.illinois.gov/pharmacy/topical.html). Development of non-steroidal dissociated ligands such as AL438 (Einstein et al., 2004; Schacke et al., 2007; Xu et al., 2009) suggested that ligand structures can be manipulated to induce differential and more desired biological activity. Recent reports of distinct ligand guided responses by GR have opened new avenues in the field of drug discovery and development. While most of the studies have been either *in silico* or in cultured cells, an *in vivo* model to evaluate ligand dependent responses of GR is lacking.

In the last few years, the use of *in vivo* zebrafish (*Danio rerio*) model in scientific research is rapidly growing. Initially, it was a popular model to study vertebrate development because the zebrafish embryos rapidly develop externally from the mother and are nearly transparent (Hao et al., 2010). The current use of zebrafish in early drug discovery and lead optimization phases covers a wide range of applications: screening of lead compounds, target identification, target validation, morpholino oligonucleotide screens, assay development for drug discovery, physiology based drug discovery, quantitative structure–activity relationship (QSAR) and drug toxicity assays (Chakraborty et al., 2009). The zebrafish model is also useful to identify compounds with favorable physiochemical properties and excellent drug-likeness with the aim of speeding up the drug development process.

While GCs are mostly used as anti-inflammatory agents, the newly identified role of GCs as modulators of tissue regeneration has opened a new paradigm in the field of regenerative medicine. In our group, we identified GCs as modulators of tissue regeneration utilizing an early life stage model. A two-day post fertilization (dpf) zebrafish completely regenerates its caudal fin three days post amputation (dpa) by a process known as epimorphic tissue regeneration. Since mammals have limited capacity for epimorphic regeneration of complex structures, larval zebrafish offers a unique alternative model to

Abbreviations: dpf, Days post fertilization; dpa, Days post amputation; GR-LBD, GR ligand binding domain; DEX, dexamethasone; BDP, Beclomethasone dipropionate; Beclo, beclomethasone; PDB, Protein data bank; RMSD, Root mean square deviation; HB, hydrogen bonding.

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identify therapeutic strategies that can promote optimal healing and replacement of tissue damaged by trauma, disease, or congenital defects. We combined this early life stage regeneration model with chemical genetics to identify modulators of regeneration. The guiding hypothesis is that a chemical that inhibits regeneration must have impacted a molecular target critical for the regenerative process. Identification of such chemical targets will allow a better understanding of the regeneration promoting pathways, paving a path for enhanced mammalian regeneration. As a proof of concept, we screened a 2000 member library of FDA approved drugs that contained thirty-three GCs. The GCs that inhibited regeneration rendered characteristic 'V' shaped architecture to the caudal fin upon exposure (Mathew et al., 2007). We performed further studies with beclomethasone dipropionate (BDP) as a representative GC and determined that activation of GR is necessary for the GCs to block the earliest stages of tissue regeneration. The activated GR functions as a ligand dependent transcriptional regulator and GCs exert a wide range of physiological effects following binding. We aimed to explore the structure activity relationship (SAR) of the known GR ligands in the context of tissue regeneration in order to identify a pharmacophore backbone that dictates regenerative response as well as reveal novel facts about ligand dependent responses of GR in an *in vivo* model.

2. Material and methods

2.1. Zebrafish husbandry and imaging

Zebrafish (*Danio rerio*) embryos (5D strain) (Hillwalker et al., 2010) were obtained from a breeding colony and raised using standard husbandry procedures for all the experiments (Westerfield, 1993, 2000). Caudal fins of 2 day post fertilization (2 dpf) larvae were amputated as previously described (Poss et al., 2002; Andreasen et al., 2006; Mathew et al., 2006) and chemical screening was performed based on our previously described *in vivo* larval regeneration assay protocol (Mathew et al., 2007). All experimental groups consisted of sample size n = 12. Images were captured under bright field using a Nikon SMZ1500 microscope at 10× magnification on 2% agarose plates after anesthetizing the embryos using tricaine.

2.2. Chemical exposures

Amputated larvae (2 dpf) were exposed to 1 μM dexamethasone (DEX) (D1756, Sigma-Aldrich, St Louis, MO, USA), beclomethasone dipropionate (BDP) (B3022, Sigma), beclomethasone (Beclo) (B0385, Sigma), or hydrocortisone (HC) (H4001, Sigma) as shown in Fig. 1. R198897 (21-Cl-9-α-F-17-α-HO-16-β-me-pregna-1,4-diene-3,11,20-trione butanoate), was also purchased from Sigma, and ST075178 (2 S,10 S,11 S,13 S,15 S,17 S,1R,14R)-1-fluoro-17-hydroxy-14-(2-hydroxyacetyl)-2,13, 15-trimethyl-5-oxotetracyclo[8.7.0.0<2,7>.0<11, 15>]heptadeca-3,6-dien-14-yl pentanoate, and ST075183(2-((2 S,10 S, 11 S,15 S,17 S,1R,13R,14R)-1-fluoro-14,17-dihydroxy-2,13,15-trimethyl-5-oxotetracyclo[8.7.0.0<2,7>.0<11,15>]heptadeca-3,6-dien-14-yl)-2-oxoethyl acetate) were purchased from TimTec (Newark, DE, USA) as shown in Fig. 6. All chemicals were resuspended in DMSO and exposures were performed in zebrafish embryo buffer. DMSO concentration was maintained at less than 1% and controls used for each chemical were maintained at matched DMSO concentration.

2.3. RNA isolation

The caudal fins of 2 dpf embryos were amputated, and embryos were placed individually in wells of 96-well plates with exposure solutions of dimethyl sulfoxide (DMSO, vehicle control) or chemical. Twelve embryos were pooled for each of the three replicates per treatment and RNA was isolated from the whole embryos using Tri Reagent from Sigma, as per manufacturer's instruction.

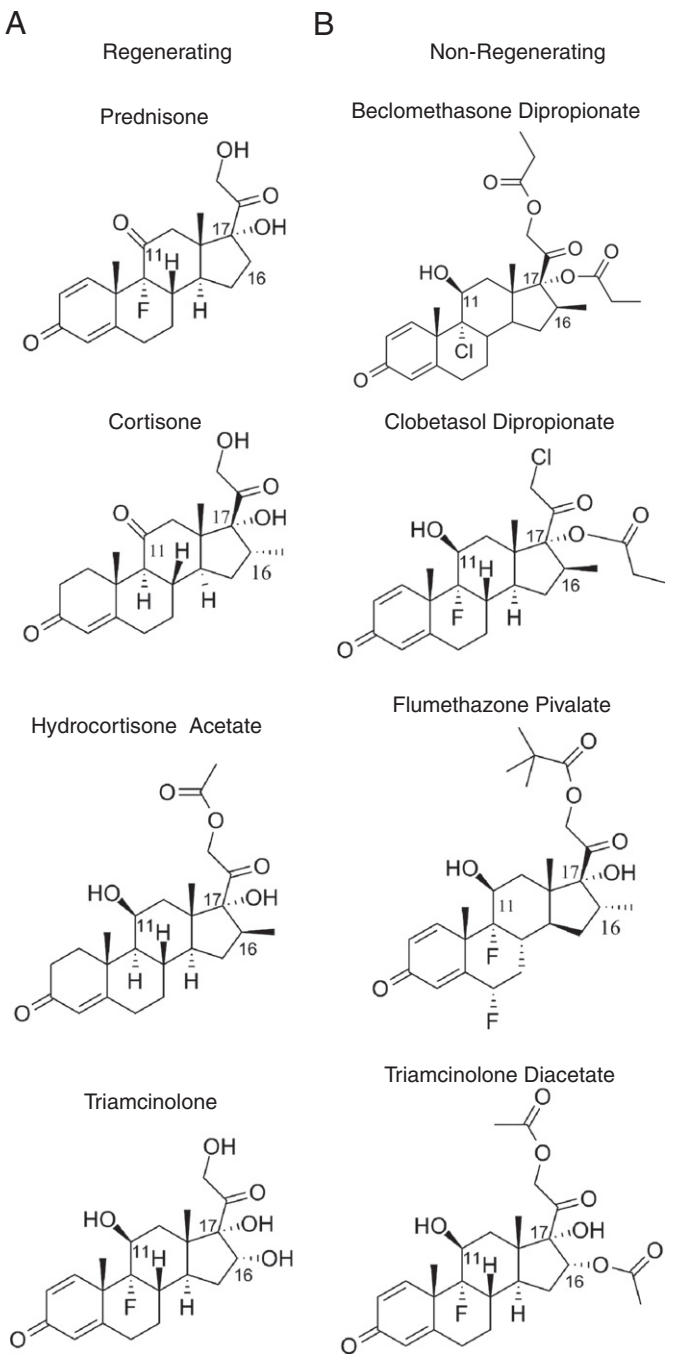

Fig. 1. Structure of selected glucocorticoids. Chemical structure of selected chemicals from the 2000 member FDA approved library that permitted regeneration (cortisol, prednisone, hydrocortisone acetate, triamcinolone) or inhibited tissue regeneration (beclomethasone dipropionate, clobetasol dipropionate, flumethazone pivalate, triamcinolone diacetate) response.

2.4. Quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated from whole embryos. Each treatment comprised three replicates with an n = 12 embryos per replicate and cDNA was synthesized from 1 μg of total RNA isolated from each group using Superscript II (Life Technologies) with oligo(dT) primers followed by RNaseH treatment to eliminate RNA contamination following manufacturer's instructions. QRT-PCR was performed on the Opticon 2 real time PCR detection system (MJ Research) using SYBR green qPCR detection kits (Finnzymes). Gene specific primers are