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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.

Keywords

Docking; Dynamics; Glucocorticoids; *In vivo*; Regeneration; SAR; Tissue; Zebrafish

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)

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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 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 $\times$ magnification on 2% agarose plates after anesthetizing the embryos using tricaine.

2.2. Chemical exposures

Amputated larvae (2 dpf) were exposed to 1 μ M dexamethasone (DEX) (D1756, Sigma-Aldrich, St Louis, MO, USA), beclomethasone dipropionate (BDP) (B3022, Sigma), beclomethasone (Beclonide) (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.

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 μ g 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 listed in supplemental Table S1. Each sample was normalized to endogenous β -actin quantity. Agarose gel electrophoresis and melt curve analysis confirmed expected PCR product formation. Statistical significance of differences in mRNA abundance was determined by one-way ANOVA on log₁₀ transformed data with a post test using Tukey's method ($p<0.05$) (Sigmastat Software).

2.4.1. Oligonucleotides—The primers used for qRT-PCR were synthesized by MWG-Biotech (Alabama, USA). Oligotech and Primer blast programs were used to design the primers listed in supplemental Table S1. Forward and antisense reverse primers are prefixed with F and R accordingly.

2.5. Morpholinos

Fluorescein tagged zebrafish GR (zf GR) morpholino (5'-CGGAAC-CCTAAAATACATGAAGCAG-3') designed to target the splicing of exons 7 and 8 was used to knockdown GR expression. Standard control morpholino (Gene Tools) (5'-CTCTTACCTCAGTTACAATTTATA 3') was injected at matching concentration. The morpholinos were diluted to a stock concentration of 3 mM in 1 $\times$ Danieau's solution (58

mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca (NO₃)₂, 5 mM HEPES, pH 7.6) (Nasevicius and Ekker, 2000) and approximately 2 nl of control and zf GR morpholino was injected in 1-2 cell stage embryos. The injected embryos were screened for uniform fluorescence at 24 hpf to confirm uniform distribution of the morpholino. At 2 dpf, caudal fins of morphants were amputated, followed by exposure to chemical.

2.6. Sequence alignment and homology modeling

The GR ligand binding domain (LBD) sequences in FASTA format for human and zebrafish were retrieved from the NCBI database. Sequence alignment was performed online with the LALIGN program [http://www.chembnet.org/software/LALIGN_form.html]. We used the X-Ray crystal structure of the human GR-LBD bound to dexamethasone (DEX) in the agonist conformation available in the Protein Data Bank (PDB ID: 1P93) as the 3D coordinate template for the homology modeling of the zebrafish GR-LBD. The model was energetically refined using the internal coordinate space with Molsoft ICM v3.5-1p (Abagyan et al., 1994; Cardozo et al., 1995). Geometrical quality assessments of the pdb and homology models, were performed using PROCHECK (Laskowski et al., 1993).

2.7. Molecular docking

The energy terms were based on the all-atom vacuum force field ECEPP/3 with appended terms from the Merck Molecular Force Field to account for solvation free energy and entropic contribution (Abagyan et al., 1994). Modified intermolecular terms such as soft van der Waals and hydrogen-bonding as well as a hydrophobic term were added. Conformational sampling was based on the biased probability Monte Carlo (BPMC) procedure, which randomly selects a conformation in the internal coordinate space and then makes a step to a new random position independent of the previous one according to a predefined continuous probability distribution. It also has been shown that after each random step, full local minimization improves the efficiency of the iterative docking procedure. In the ICM-VLS (Molsoft ICM v3.5-1p) screening procedure, the ligand scoring was optimized to obtain maximal separation between binders and non binders (Totrov and Abagyan, 1997, 2001).

The 3D coordinates of human GR-LBD-DEX complex in the agonist conformation was taken from crystal structures (PDB ID:1P93) (Kauppi et al., 2003). BDP was manually inserted into the GR-LBD binding pocket by matching the orientation of the 3-C=O ketone oxygen from the A-ring of DEX in the crystal structure involved in HB interactions with residues Gln570 (α 3) and Arg611 (α 7) (Kauppi et al., 2003).

2.8. Molecular dynamic simulations

The prep files of DEX and BDP were performed using the program ANTECHAMBER (AMBER 10), (Case et al., 2008). GR-LBD-DEX and GR-LBD-BDP complex models were immersed in a box of water molecules (TIP3P model) and Na⁺ counter ions were added to the solvent bulk of the protein/water complexes to maintain neutrality of the system using program LEAP AMBER10, (Case et al., 2008). Periodic boundary conditions were applied. The AMBER force field (Case et al., 2008) all atom parameters (parm03) were used for the protein and the Na⁺ ions. The total number of atoms for the GR-LBD-DEX and GR-LBD-BDP water boxed complexes is 34,926 and 34,942, respectively. The minimization protocol consisted of 1000 cycles of steepest descent followed by conjugate gradient method until the root-mean square deviation (rmsd) of the Cartesian elements of the gradient reached a value smaller than 0.15 Å. The dynamic protocol consisted of three steps: MD1, MD2 and MD3. The initial temperature for MD1, MD2 and MD3 were set at 0, 150, and 300 K respectively. During all dynamic steps the reference temperature of the system was fixed at 300 K according to Berendsen's coupling algorithms (Di Nola et al., 1994). The initial velocity of

the beginning of simulation is taken from Maxwellian distribution set at the desired temperature. The time step for all three dynamic procedures was 0.002 picosec (ps). For minimization and molecular dynamics, the primary cutoff distance for non bonded interaction was set at 9 Å. Regarding the molecular dynamic protocol used, the first (MD1) aimed the equilibration of water molecules and ions of the water boxed and charge neutralized model. An initial velocity was given to the system and trajectories were allowed to evolve in time according to Newtonian laws keeping the model protein fixed. The number of dynamic steps was 7500 corresponding to 15 ps. Next, 15 ps of constant volume dynamic (MD2) was performed on the entire system to adjust density to a value of one (g/cm³). In the third step, a 1000 ps (GR-LBD-Dex) and 1500 ps (GR-LBD-BDP) constant pressure dynamic (MD3) at 1 atm was applied without any constraint to assess conformational stability. The energy minimization, molecular dynamics and the corresponding analyses were performed using program AMBER10. Geometrical quality assessments of the pdb models, were performed at different time points using PROCHECK (Laskowski et al., 1993).

2.9. Statistical analysis

All experiments comprised a sample size of n = 12. All statistical calculations were performed using Sigmaplot v. 11 (Systat Software Inc., San Jose, CA, USA) and p-values of <0.05 were considered statistically significant.

3. Results

3.1. Glucocorticoids elicit differential regenerative responses

Previous chemical genetic screening revealed GCs such as BDP as novel modulators of tissue regeneration in a zebrafish model. We followed this screen with a dose response analysis, and pursued mechanistic studies with BDP (Mathew et al., 2007). Since BDP inhibits regeneration even at low nanomolar concentrations, we performed further experiments at a screening concentration of 1 µM to understand how BDP modulates the regenerative process. Our results demonstrated that activation of GR is necessary for these GCs to inhibit tissue regeneration. Mechanistic data revealed that over-expression of Cripto-1, an inhibitor of Activin signaling, mediates BDP impaired tissue regeneration (unpublished data). Since all the members of the glucocorticoid family act as ligands of the GR, we reevaluated the results of our screen that contained a total of thirty three GCs. While seven GCs of the library inhibited tissue regeneration, twenty-one had no effect. The list of these twenty-one GCs composed of well-known GCs such as Dexamethasone (Dex), Hydrocortisone (HC) and beclomethasone (beclo). A representative group of chemical structures is illustrated in Fig. 1. We further validated the results of DEX, HC and beclo by purchasing these chemicals from commercial sources and repeating the regeneration assays. The results confirmed that the chemicals did not inhibit regeneration at the screening concentration. We adopted a SAR approach to understand this differential response of members of the glucocorticoid family based primarily on their effects on fin regeneration. There is a strong correlation between GCs that inhibit regeneration and their ability to induce Cripto-1 expression. Unlike BDP, DEX, HC and beclo did not result in elevated Cripto-1 expression (Fig. 2) and they had no impact on regeneration. We then aimed to understand this differential response of the GC through SAR.

3.2. Inappropriate activation of GR is requisite for inhibiting tissue regeneration

All of the chemicals selected for the study are GR ligands known to modulate downstream GR target genes such as Annexin a1b and FKBP506. Annexin a1b (*anxa1b*) is one of the transcripts repressed by activated GR at 24 h post amputation following BDP exposure. In order to evaluate whether these ligands activated GR in our system irrespective of their

effect on regeneration, we performed qRT-PCR and evaluated *anxa1b* expression following ligand exposure. DEX, HC, Beclo and BDP exposure suppressed *anxa1b* expression at 1 μ M at 24 h post exposure, indicating activation of GR by exposure to these ligands (Fig. 3). However, among the above ligands only BDP inhibits regeneration. The fact that DEX, HC and Beclo activate GR and modulate *anxa1b* expression similar to BDP, yet are unable to inhibit regeneration or elevate Cripto-1 expression indicate differential activity of GR upon binding by BDP compared to DEX, HC or Beclo. We hypothesized that specific GR conformation triggered by certain ligands inhibits tissue regeneration.

3.3. Molecular docking studies revealed a conformational difference induced by ligand binding

To understand the difference in activated forms of GR we performed molecular docking studies with the human GR-LBD, as the crystallographic structure of zebrafish homologue is not available. The human and zebrafish GR-LBD share 72% sequence identity and majority of the residues directly involved in the binding to DEX such as Gln 570 (α 3), Arg 611 (α 7), Gln 642 (α 8) and Thr 739 (α 11) are conserved between the two species (Supplemental Fig. 1). The homology model of zebrafish GR-LBD was then built using the available 3D coordinate of the human GR-LBD bound to DEX (PDB ID: 1P93) and energetically refined as described in the Material and methods section.

3.4. Molecular docking

Selected steroidal GR agonists that evoked differential impact on regeneration were docked into the human and zebrafish GR-LBD models. Docking results were similar for both species. Most of the ‘active’ GR ligands that inhibit regeneration did not dock into the GR-LBD binding pocket, while most of the ‘inactive’ GR ligands that did not inhibit regeneration did dock into GR-LBD (Table S2). This suggests that docked steroidal GR ligands are stable in GR-LBD-DEX agonist conformation, while steroidal GR ligands like BDP do not stably fit into the binding pocket. In order to bind and stabilize the agonist 3D tertiary structure of the GR-LBD, active compounds induce conformational changes involving either residue side chains or secondary structure portions of the protein. Indeed, strong steroidal GR agonists deacetylcortivazol (DAC) and fluticasone furoate (FF) were co-crystallized into the human GR-LBD (PDB ID: 3BQD and PDB ID: 3CLD, respectively) (Suino-Powell et al., 2008) (Supplemental Fig. 2).

To identify the conformational changes of the GR-LBD upon active ligand binding, we ran 1.01 ns and 1.51 ns molecular dynamic (MD) simulations with the human GR-LBD complexed with ligands DEX and BDP, respectively. DEX is a known inactive compound (with respect to inhibition of tissue regeneration) and for this reason the X-Ray crystal structure (PDB ID: 1P93) was considered as the 3D structure reference for steroidal GR agonists unable to inhibit regeneration. BDP was instead manually inserted into the human GR-LBD binding pocket as described in the Material and methods section.

3.5. Molecular dynamic simulations

By looking at the Root-Mean-Square-Deviation (RMSD) as a function of time of all models, the GR-LBD–DEX complex reached a plateau during 1.01 ns MD (Fig. 4A), representing stable conformation over time. On the other hand, GR-LBD–BDP complex reached stability after adding 500 ps for a total of 1.51 ns MD (Fig. 4A). This is mainly due to the instability of BDP in the binding pocket during the simulation time (Fig. 4B). DEX crystallographic orientation with the hydrogen bonding (HB) network remained stable over time with a low RMSD of 0.75 Å, while BDP revealed considerable instability especially in the range between 0.3 and 0.5 ns MD (Fig. 4B). Overall, the low RMSD of both GR–LBD complexes at equilibration between 2.5 and 2.85 Å and structural comparison along the simulation time

indicates that the starting X-Ray structure represents a stable conformation and the MD protocol is suited to assess the stability of the models.

Distances (Å) between atoms of specific residues were calculated and analyzed over the simulation time applied. The stability of the crystallographic orientation of DEX in the GR-LBD over time was confirmed (Fig. 4C–E). The HB network involving the side chains of residues Gln 570 (Fig. 4C), Arg 611 (Fig. 4D) and Gln 642 (Fig. 4E) is critical for the stability of DEX in the GR-LBD agonist conformation with an average calculated distance of 3 Å. This was not the case for BDP. (Fig. 4F–H) The stability of the human GR-LBD–DEX complex during simulation time is proved by superimposing DEX and the side chains of residues Gln 570, Arg 611, Gln 642 and Thr 739 from the pdb structure of the complex at initial (t = 0 ns) and final (t = 1.01 ns) MD time (Fig. 5A). No 3D significant differences were detected for the residues and the ligand over time (Fig. 5A). During 1.51 ns MD the GR-LBD–BDP complex was instead very unstable (Fig. 4A,B). As a matter of fact, the calculated inter-atomic distances between BDP and key residue side chain atoms showed that conformational changes are occurring over time in order to stabilize the ligand–protein complex in the agonist conformation (Fig. 4F–H).

BDP was inserted manually into the GR-LBD binding pocket by positioning the 3-C=O keton oxygen in the vicinity of the side chains of Gln 570 and Arg 611 to maintain the energetically favorable HB network observed with DEX and other GR agonists. During the simulation; however, these HB interactions were unstable due to residue side chain conformation changes, which produced significant fluctuations in the calculated inter-atomic distances (Fig. 4F–H). For a better understanding of these fluctuations we superimposed BDP and amino acids Gln 570, Arg 611 and Gln 642 from the PDB structure of the complex at initial (t = 0 ns) and final (t = 1.51 ns) MD time (Fig. 5B,C). During 1.51 ns MD (Figs. 4F, 5B) the side chain of Gln 570 rotated increasing the distance between the amidic N–H of the side chain of Gln 570 and BDP from 3.3 Å (t = 0) to 4.52 Å (t = 1.51 ns), and thus excluding the formation of any HB interaction. In the case of Arg 611, there was only a change in the orientation between the two N–H atoms of the primary amino group of the side chain of Arg 611. Hence, that HB interaction with BDP remained stable with an inter-atomic distance between the two functional groups of less than 3 Å for the entire period of simulation (Figs. 4G, 5B). The analysis of the inter-atomic distances over time between BDP and the side chain of Gln 642 produced the most interesting results (Fig. 4H). Significant conformational changes involving this residue and the ligand are taking place. We calculated the inter-atomic distance between the side chain of Gln 642 and the two carbonyl oxygen atoms C-2=O (C-17-*endo*-propionate ester) and C-4=O (C-17-*exo*-propionate ester) of BDP (Fig. 1). From the graphic shown in Fig. 4H we observed that the distance during simulation time between the side chain of Gln 642 and C-2=O of BDP remains more or less stable around 3.5 Å, whereas the distance between Gln 642 and C-4=O of BDP is unstable (some stability is reached after 1 ns MD) proving the C-17-*exo*-propionate ester moiety of BDP is moving in a considerable way. We then analyzed the superimposition of residue Gln 642 and BDP from the pdb structure of the complex at initial (t = 0 ns) and final (t = 1.51 ns) MD time (Fig. 5C). The side chain of Gln 642 shifts towards the binding cavity to stabilize BDP in the binding pocket. As a consequence, the C-17-*exo*-propionate ester moiety of BDP (Fig. 1) is moving towards a hydrophobic pocket surrounded by residues Trp 600, Leu 732, Leu 733 and Ile 757 (Fig. 5C).

3.6. Novel ligands identified based on molecular docking results confirm the importance of C17 substitution

To further validate the effect of the C17-substitution on in vivo regeneration inhibition, we selected and acquired several steroidal compounds commercially available from Sigma (Diflorasone diacetate (DFDA)), and R198897 TimTec (ST 075183 and ST 075178) (Fig.

6). Initially, the compounds were docked into DEX-GR-LBD and none of them docked suggesting potential inhibitory properties. Based on the *in silico* results, dose dependent *in vivo* larval regeneration analysis was performed with the new compounds revealing that all of them inhibit regeneration at the 1 μ M concentration (Table S3) (Supplemental Fig. 3). Thus, we identified novel GR ligands that kept the cortisol backbone, but varied in C17 substitution size with the presence of sterically hindered esters or chlorine atoms (Fig. 6). In addition, QRT-PCR studies demonstrated induced *fkbp5* expression in embryos exposed to the novel ligands. In the absence of GR, this induction was diminished. Finally, *Cripto-1* expression (Fig. 6) is elevated following exposure to these inhibitory ligands in a GR dependent manner.

4. Discussion

Ligand dependent response of nuclear receptors has led to structure activity predictions and eventually understanding the biology of the nuclear receptors. Since the GR is the major target of the most widely used class of drugs, understanding how this receptor responds to varying structures of ligands is crucial for further drug development. However, majority of the ligands are evaluated *in vitro*. Numerous lead compounds that demonstrate excellent results *in vitro* are withdrawn from the market due to either acute *in vivo* toxicity or the inability to replicate cell based results. This can be avoided by utilizing *in vivo* models that are amenable to screening to identify new GR ligands with differential activities. We previously reported that inappropriately activated GR modulates tissue regeneration (Mathew et al., 2007). This has opened avenues for the potential use of GR ligands for regenerative medicine. However, further studies are required not only to understand the role of activated GR in tissue regeneration, but also to explore how GC structure dictates regenerative outcome.

So far, chemical genetic approaches have identified numerous modulators of stem cell differentiation and stem cell fate (Shi et al., 2008a, 2008b; Li et al., 2009). The recent characterization of fluorinated GCs as modulators of stem cell activity underlines the requirement for better understanding of structure function relationship amongst the GR ligands (Wang et al., 2010). Since there are numerous commercially available structural analogs of cortisol, we exploited existing drugs. This allowed us to bypass the requirement of synthesizing new analogs to modulate regeneration.

Our previous results demonstrate that GR activation inhibited tissue regeneration; however, not all ligands that activate GR inhibit regeneration (Mathew et al., 2007). Previous studies demonstrated that ligand chemistry dictates biological response by activated receptor. The best examples are the estrogen receptor ligands estradiol and tamoxifen that invoke different gene expression profile as well as different function in different cell type (Jordan, 2004; Kian Tee et al., 2004). The striking difference in ligand structures suggests complicated correlation between chemical structures and biological response.

It has been reported that differences in ligand chemistry can give rise to a host of functionally distinct GR-containing regulatory complexes (Wang et al., 2006) and hence impact different set of genes. Since we have evaluated the role of ligand-dependent GR activation in the *in vivo* regeneration model, the phenotypic assay served as an initial read out of differential response of the ligands. This was further confirmed by GR-dependent activation of *Cripto-1*, which was required to induce a block in early stages of blastema formation (unpublished data). However, we observed induction of GR target genes such as FKBP506 upon exposure to the GR ligands irrespective of their effect on regeneration. Lack of *Cripto-1* induction by DEX, HC or Beclo supports previous reports that the hosts of genes affected by these ligands are different and not critical for inhibition of tissue regeneration

(Croxtall et al., 2002; Brichetto et al., 2003; Sengupta Sumitra et al., 2011) The differential regulation of genes by the GR is likely due to the recruitment of distinct co-regulators by the GR upon binding to different ligands.

In order to initiate a SAR study we performed docking studies against human and zebrafish GR-LBD models with the database of known steroidal GR agonists previously tested in the regeneration assay (Table S2). The conformational changes of residues observed with BDP during 1.51 ns MD are similar to the reported data (Biggadike et al., 2008; Suino-Powell et al., 2008). These residues in the GR-LBD influence ligand binding directly and are flexible enough to expand the binding pocket volume to accommodate large ligands. This allows the helical tertiary structure of the GR-LBD agonist conformation to stay intact. This also suggests that these residues might play a role in the thermodynamic equilibrium between the inactive (no effect on regeneration) and the active (inhibit regeneration) GR-LBD conformation. Active ligands possess sterically hindered ester moieties or chlorine atoms as substituents at C-17 position and *exo*-Me-stereochemistry at C-16 position (*endo*-Me-stereochemistry also works for few non-regenerating compounds like Triamcinolone Diacetate). From the SAR analysis, substitutions at C-3, C-9 and C-11 positions do not play a role in the inhibitory activity. Molecular Docking runs showed that active GR agonists are unable to dock into the human GR-LBD-DEX binding pocket (inactive conformation) (Table S2) and this is primarily due to the size of ligands.

For our study we have utilized an *in vivo* system and thus, metabolism and uptake might play a role in differential response. Metabolism of BDP involves hydrolysis to beclomethasone monopropionate (17-BMP) and finally beclomethasone (Becllo). Unfortunately, we could not evaluate the effect of 17-BMP in tissue regeneration due to commercial unavailability of the compound. However docking studies revealed comparable results for 17-BMP and BDP (Table S2). This offers a potential possibility that even if BDP is metabolized to 17-BMP *in vivo*, according to our *in silico* predictions it induces the same conformation as of BDP. Since we are unable to characterize the metabolism of GR ligands in zebrafish, the fact that, exposing all these ligands induces suppression of *anxa1b* expression, confirms both the activation of GR upon ligand binding and the uptake of the ligands.

5. Conclusions

Regenerative medicine is an emerging field, while major contributions in terms of therapeutic approach have been made by stem cell biology, recently established larval zebrafish regeneration model has the potential to further advance the field. This study demonstrates the power of early life stage regeneration model in not only elucidating signaling molecules involved in regeneration, but also in correlating ligand structure with functional preference. *In silico* and experimental studies revealed that type and size of substitutions at C-17 position of the steroidal backbone of corticoids influence GR activation and tissue regeneration. Our results demonstrated the new potential of GCs in regenerative biology. It is expected that in upcoming years novel synthetic steroidal and non-steroidal glucocorticoid molecules will provide new tools for regenerative medicine.

This is one of the first GC SAR studies performed in vertebrates. *In vivo* zebrafish SAR models will remain an attractive tool for drug development in forthcoming years to help medicinal chemists improve drug-likeness properties of compounds and to get a better understanding of the role of specific protein targets in desired phenotypic responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

dpf	Days post fertilization
dpa	Days post amputation

GR-LBD	GR ligand binding domain
DEX	dexamethasone
BDP	Beclomethasone dipropionate
Becl	beclomethasone
PDB	Protein data bank
RMSD	Root mean square deviation
HB	hydrogen bonding

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.

Fig. 2.

Differential response of glucocorticoids in larval regeneration model. Caudal fin of 2 dpf larvae was amputated (dotted lines mark the plane of amputation) and continuously exposed to A) beclomethasone dipropionate (BDP), B) beclomethasone (Beclomethasone) (Beclomethasone) (Beclomethasone), C) dexamethasone (Dex) and D) hydrocortisone (HC) at 1 μ M concentration for three days for regeneration assay. Images were acquired at 3 dpa (10 $\times$) and RNA was collected at 1 dpa from whole embryos for cDNA synthesis and qRT PCR for *Cripto-1* expression. The abundance of *Cripto-1* transcript at 1 dpa is elevated on BDP exposure. However, there was no difference in expression on dex, beclomethasone or HC treatment. The respective values represent the mean $\pm$ SEM and the asterisks indicate statistical significance (One way ANOVA, n = 3) ($p < 0.05$).

Fig. 3.

Activation of GR by different ligands irrespective of their effects on regenerative response. 2 dpf larvae were exposed to 1 μ M dex, beclo, HC, BDP and DMSO following amputation. The abundance of *anxa1b* transcript estimated by qRT PCR at 1 dpa in the whole embryo indicate significantly reduced expression in the exposed larvae indicating GR activation. The respective values represent the mean $\pm$ SEM and the asterisks indicate statistical significance (One way ANOVA, n = 3) ($p < 0.05$).

Fig. 4.

Molecular dynamic simulations reveal instability and residue side chain conformational changes in the GR-LBD when bound to BDP. RMSD graphics (all atoms plotted) *versus* time (picoseconds) of A) GR-LBD (pdb 1p93) in complex with dexamethasone, Dex (red) during 1.01 ns MD and beclomethasone di-propionate, BDP (black) during 1.51 ns MD and B) Dex (red) (pdb 1p93) during 1.01 ns MD and BDP (black) during 1.51 ns MD. Evolution of interatomic intramolecular distance during MD in the complex between C, D, E) Dex and F, G, H) and BDP and GR-LBD, respectively. Initial time (t=0 ps) is measured after minimization stage (see Material and methods). Color code: C) black, NH2 R611—O1=C Dex; D) black, NE2 Q570—O1=C Dex; E) black OE1 Q642—HO3-C Dex; F) black, NE2 Q570—O6=C BDP; G) black, NH1 R611—O6=C BDP, red, NH2 R611—O6=C BDP; H) black, OE1 Q642—O4=C BDP, red, OE1 Q642—O2=C BDP.

Fig. 5.

GR-LBD residue side chain conformational changes allow binding of bigger size GR agonist BDP for regeneration inhibitory activity. Residual side chain and ligand shift of GR-LBD (pdb 1p93) in the complex between A) Dex during 1.01 ns MD and B,C) BDP during 1.51 ns MD. The ligands are colored by atom type with carbon atoms in white (Dex) and in yellow (BDP) at initial time ($t = 0$ ps) D) and in magenta (1015 ps MD for Dex and 1515 ps MD for BDP) and displayed as sticks. Residues are colored in orange (Initial time, $t = 0$ ps) and green (1015 ps MD for Dex and 1515 ps MD for BDP) and displayed as sticks (ICM v3.5-1p).

Fig. 6.

Novel ligands identified based on C17 substitution inhibit tissue regeneration. Structures of novel GR ligands B) R198897, C) ST75183, D) DFDA and E) ST75178 identified based on cortisol backbone and C17 substitution. GR splice variant MO transiently knocked down GR compared to standard control morpholino injected embryos. GR and control morphants were amputated at 2 dpf and exposed to DMSO or the novel GR ligands. Regenerative progression was assessed and images were acquired after three days of exposure. The abundance of FKBP506 and Cripto-1 estimated by qRT PCR at 1 dpa in the whole embryo indicates significantly elevated expression in the control morphants and significantly reduced expression in the corresponding GR morphants when exposed to the novel GR ligands. The respective values represent the mean $\pm$ SEM and the asterisks indicate statistical significance (One way ANOVA, $n = 3$) ($p < 0.05$).