Binding Affinity Ranking at the Molecular Initiating Event (BARMIE): An open-source computational pipeline used to identify novel fish glucocorticoid receptor chemical agonists and antagonists

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ABSTRACT. A challenge in ecological risk assessment is identifying the chemicals that pose the greatest threat and determining which species are most vulnerable to them. To help address this, the study has developed an open-source tool called BARMIE (Binding Affinity Ranking at the Molecular Initiating Event) to rapidly predict the chemical binding affinity of steroid receptor proteins to synthetic steroids, and, when combined with an in vitro transactivation assay, to identify novel endocrine-disrupting chemicals (EDCs). BARMIE was used to screen 163 teleost fish glucocorticoid receptors (GRs) for binding to the natural ligand cortisol and to 10 synthetic glucocorticoid drugs (GCs) designed to interact within the ligand-binding pocket (LBP) of GRs. GC halcinonide was predicted to have the most significant binding affinity, and species from the superorder Protacanthopterygii were identified as having high-affinity GRs. BARMIE was then used to screen the binding profiles to compounds of the Medicine for Malaria Venture Global Health Priority Box to rainbow trout GRs (rtGR1 and rtGR2). Of the 178 compounds, 24 and 36 bound within the LBP with an affinity ≥ -7.5 kcal/mol to the rtGR1 and rtGR2, respectively. For 30 of these compounds, transactivation activity was assessed at 1μM in the presence or absence of 1μM cortisol. Compound #18, a 1,2,4-oxadiazole, a compound known to have biological activities, significantly stimulated both rtGR1 and rtGR2, as well as enhanced the activity in the presence of cortisol, and compound #14, a predicted IL-1R-associated kinase four inhibitor (IRAK4) inhibitor, had antagonist properties in both receptors. Neither has been shown to affect fish GR functioning previously.

INTRODUCTION

Approximately 350,000 synthetic chemicals are produced globally, yet most have not undergone any human (HRA) or ecological risk assessment (ERA)[1]. Consequently, the impact of legacy and novel substances on human and wildlife health is likely underestimated. The evaluation of chemical ecological risk requires information on impacts on mortality, growth, and reproduction, derived from toxicity tests, as well as on environmental fate and bioaccumulation properties. These toxicity tests are costly and time-consuming, and when fish are used, there are ethical concerns. Given the vast number of chemicals, it is difficult to determine whether we can rapidly generate sufficient toxicity data using current methods. In recent years, there has been a move to identify novel ways to provide the necessary toxicological information for ecological hazard and risk assessments that use rapid, high-throughput in silico and in vitro methods, termed New Approach Methodologies (NAMs) [2-6].

All vertebrates have a similar steroid hormone/receptor-based endocrine system that regulates a plethora of physiological and developmental processes. These processes are primarily controlled by the binding of the natural steroid ligand to its receptor to initiate ligand-induced transactivation, transrepression, or to stimulate other cellular signaling pathways[7]. These steroid receptor proteins (glucocorticoid, mineralocorticoid, androgen, estrogen, and progesterone) are highly conserved within the vertebrate subphylum[8].

The importance of the endocrine system is reflected in concerns about industrially produced chemicals that are endocrine-active substances that perturb hormonal actions. In response to this concern, national and international governments implemented testing programs over 10 years ago to elucidate the endocrine-disrupting potential of synthetic and natural compounds [9, 10].

These initially focused on the EATS (estrogen, androgen, thyroid, and steroidogenesis) modalities, but in more recent years, this has expanded to include non-EATS modalities[11] (e.g., glucocorticoid and progesterone receptors and non-steroidal receptors). The majority of NAMs' research in the field of EDC efforts has focused on HRA, with numerous in vitro and in silico tools available for the EATS modalities[11]. HRA aims to protect the individual; in contrast, ERA is far more ambitious and complex, seeking to protect numerous species and maintain ecosystem function.

A good example of ERA NAMs for EDCs is models such as EcoDrug[8] and Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS)[12], which use protein conservation across phyla to identify non-target species susceptible to drugs based on the presence of human or veterinary drug targets. When these approaches are combined with other toxicological information, they yield a more comprehensive picture of the potential environmental impacts of chemicals. For example, SeqAPASS in combination with Genes to Pathway – Species Conservation Analysis (G2P-SCAN) uses network analysis, based on Adverse Outcome Pathway (AOP) information, to identify conserved Reactome[13] pathways and points of departure in toxic outcomes[14], and RASRTox (Rapidly Acquire, Score, and Rank Toxicology data) links the sequence information with toxicological databases (ECOTOX, ToxCast21) and QSAR models to develop tool for ranking chemicals for hazard assessment[15]. It is known that there are significant differences in the concentrations of chemicals that induce a toxic response between species within a taxon[16,17], thus a challenge is to increase confidence in predictive tools to identify sensitive species and chemicals of concern. One approach for EDC is to investigate the protein structural reasons for species differences in the interaction between chemicals and their

receptors[18]. The hypothesis is that species with receptors with high binding affinity are likely to be affected at much lower concentrations of a chemical.

To develop in silico tools to identify chemicals and species of concern, the study focuses on teleost fish glucocorticoid receptors (GRs). The reason for focusing on the GRs is threefold. Firstly, synthetic glucocorticoids, which are commonly used to treat various health conditions, are a group of drugs of growing environmental concerns, with 17% of the GCs being at concentrations in the aquatic environment predicted to pose a risk to fish[19]. Secondly, species differences in the binding affinity (Kd) and transactivation (EC50) activity for both natural and synthetic glucocorticoids (GCs) have been reported[20-25]. Thirdly, the GRs are vital for a wide range of physiological and developmental processes, and identifying novel GR EDCs is essential for both human and wildlife RA. The approach uses drug-discovery methodology [26] applied to environmental toxicology. The first stage uses computational docking tools for high-throughput screening of the binding affinity of steroid receptors from 100s of species to known agonists. This tool was used to screen a chemical library of compounds with unknown EDC potential to identify leads for further in vitro transactivation assays.

MATERIALS AND METHODS

Computational model construction

The novel computational pipeline, Binding Affinity Ranking at the Molecular Initiating Event (BARMIE), uses open-source database APIs and software (UniProt[27], Chembl[28], OpenBabel[29], PyMol[30], and AutoDock Vina[31]). The code to estimate receptor binding affinities, a summary of the procedural steps, and user instructions are available at https://github.com/ParsaFouladi/Barmie, and a training video is provided at www.burylabs.co.uk. The pipeline was run at the University of Southampton HPC Iridis 6, and the example provided is specific to teleost fish GRs. The pipeline can be adapted for use with other HPC architectures and other species and proteins.

BARMIE overview

BARMIE uses the verified fish protein receptor structures derived from AlphaFold[32] available in UniProt. These structures, in turn, are derived from the annotated fish genomes within the Ensembl database. Ensembl contains receptor isoforms (e.g., splice variants), and because very few isoforms have been characterized in fish[33], we did not remove these from our final analysis. When individual receptor structures are imported into AutoDock Vina, their orientations differ, and the box coordinates encompassing the ligand-binding pocket (LBP) for the docking experiment must be defined for each structure. To enable docking assessment for a large number of receptors and chemicals, the structures are automatically aligned using PyMol scripting so that the LBP location is in the same orientation for all proteins. The only manual step in the pipeline is defining the box coordinates for docking exploration of the LBP. This is set to a single reference protein, in this example, Oncorhynchus mykiss GR1 (accession # P49843), and is used

within the BARMIE script for analysis of all receptors due to the three-dimensional alignment in PyMol. For the GRs utilized in this study, the docking box size was set to 20, 20, 20 Å, and the coordinates encompassed the LBP at X=5, Y=2, Z=-15.

Predicted docking binding affinity were estimated for 163 teleost GR proteins (SI 1 contains the Uniprot ID codes) in complex with the natural ligand cortisol (CHEMBL389621), the synthetic glucocorticoids beclomethasone (CHEMBL1586), clobetasol (CHEMBL1201362), dexamethasone (CHEMBL 384467), flumetasone (CHEMBL1201392), halcinonide (CHEMBL1200845), mapracorat (CHEMBL2103876), mometasone (CHEMBL1201404), prednicarbate (CHEMBL1200386), prednisolone (CHEMBL131), and triamcinolone (CHEMBL1451). AutoDock Vina uses a stochastic algorithm to explore ligand binding poses; thus, docking searches were run 5 times, and the average binding affinity is reported (SI 2 for cortisol). Docking simulations were run with exhaustiveness levels of 8, 32, and 128 to assess consistency of results; however, we observed no difference among the exhaustiveness levels and report results from exhaustiveness 32 (SI 3). Amino acid interaction fingerprint was conducted with the protein/ligand pose in LigPlot+ v2.2 (https://www.ebi.ac.uk/thornton-srv/software/LigPlus/) and visualization of structure via PyMol.

Screening of the Medicines for Malaria Venture Global Health Priority Box compounds.

The Medicines for Malaria Venture (MMV) Global Health Priority Box (GHPB) chemical library was kindly supplied by Bristol-Myers Squibb Company and IVCC. BARMIE was used to predict the binding affinity of the 178 compounds in the GHPB to rainbow trout GR1 (rtGR1) and GR2 (rtGR2) (SI 4). Thirty compounds (#1 to #30), with a binding affinity ≥ -7.5 kcal/mol and 10 compounds predicted to either bind with low affinity or outside of the LBP (#31 to #40), were also evaluated in an in vitro transactivation assay25 for agonist and antagonistic activity.

Briefly, COS-7 cells were seeded into 96-well plates at a density of 10,000 cells per well and grown o/n in DMEM (Gibco) media containing 5% v/v FBS (Gibco) at 5% CO2 atmosphere and 37 °C. The following day, the cells in each well were transfected with 44.5 ng of the reporter plasmid PF31-LUC and 0.5 ng of the NanoLuc® control vector, PNL1.1. TK[Nluc/TK] (Promega) and five ng of the expression vector pcDNA 3.1(+) containing rainbow trout GR1 (rtGR1, UniProt # P49483) and GR2 (rtGR2, UniProt Q6RKQ3) supplied by Genscript Biotech using Escort™ IV Transfection reagent (Merck). The Global Health Priority Box compounds were resuspended in DMSO at a concentration of 10mM. After 24 hr, duplicate wells were incubated with either 1µM of each of the GHPB compounds or 1µM of GHPB compound and 1µM of cortisol in DMEM/F12 containing 5% charcoal-stripped FBS (Gibco) with a final concentration of 0.01% (v/v) DMSO. The cells were incubated for a further 24h. Then, firefly and Nluc luciferase activity was measured using Promega Glomax Navigator, following the methods described in the Dual-Glo protocol (Promega cat~ N1630). Firefly luciferase activity was normalized to the Nluc activity and expressed as fold induction of the DMSO controls. The experiment was conducted in triplicate except for #36 to #40, which were performed in duplicate.

RESULTS AND DISCUSSION

Output from BARMIE

BARMIE predicted the binding affinity of 163 fish GRs to the natural ligand cortisol as well as synthetic GCs that are designed to interact within the LBP of GRs (Figure 1). From this screen, halcinonide (Figure 1) may be considered the most potent GC to interact with the fish GRs, whereas prednicarbate (Figure 1) is the least potent. For all GCs, the screen shows that species in the order Protacanthopterygii (Esox lucius, Salmo trutta, Coregonus mareana) possess GRs with high binding affinity for the synthetic GC (Figure 1A). In addition, 55% of the five most sensitive GRs in species belong to the Protacanthopterygii, with the species Northern pike (Esox lucius) ranked 1st for 7 of the 11 GCs tested (Table SI 1). A caveat is that only 86 fish genomes have been annotated out of the potentially 30,000 teleost species34, and of these genomes, a high proportion, 8%, are Protacanthopterygii. Looking forward, the number of species genomes sequenced is rapidly expanding with the Earth BioGenome Project Network35 setting an ambitious target of "… characterizing the genomes of all of Earth's eukaryotic biodiversity over a period of ten years". This has the potential to provide a wealth of genetic information for in silico approaches in ecotoxicology.

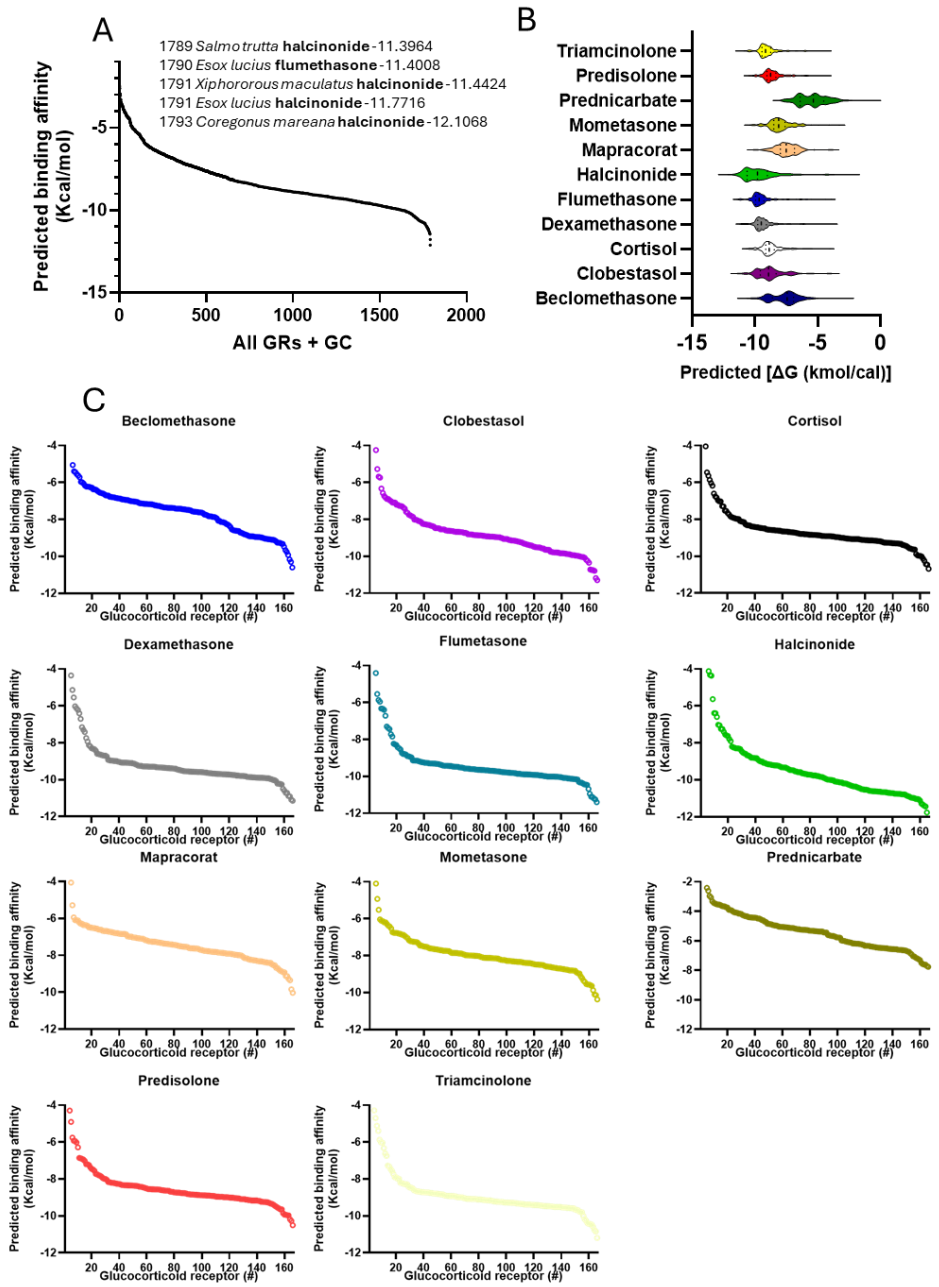


Figure 1. A. The predicted binding affinity for all chemical and species combinations, the inset list provides the top 5 species + chemical binding affinities. B Violin plot showing spread of binding affinities per GC. C. Binding affinities for the 163 teleost fish glucocorticoid receptors per glucocorticoid (see supplementary information for further species values).

Screening the Medicines for Malaria Venture Global Health Priority Box chemical library

BARMIE provided docking information for GCs designed with a steroid backbone comprising 17 carbon atoms arranged in 4 rings (Figure 1) to interact with the LBP of GRs. Industrial chemicals may not have this structure, but they may nonetheless dock into the LBP or interact

with other parts of the receptor to act as receptor agonists or antagonists, disrupting endocrine signaling. The binding of a compound to the LBP is the first step in identifying a compound's potential to exert an effect, but binding may not equate to a biological action. Communication between the LBP and the transactivation function site, termed AF2 in helix 12 of GRs, determines the recruitment of the transcriptional machinery and gene expression[36]. The signaling pathway between the LBP and the AF2 site begins with hydrogen bond formation between the ligand and key amino acids within LBP[37] (Figure 3), which induces the necessary protein conformational changes for signal transduction and transcription factor recruitment. Despite the acknowledged complexity of the structural changes essential for receptor function, in-silico tools are often used in the initial stages of a drug discovery pipeline to identify promising leads and reduce the number of compounds to be tested in downstream assays[26]. Taking this approach, BARMIE was used to screen the 178 GHPB compounds and identified 24 and 36 of these that bind within the LBP of rtGR1 and GR2 with an affinity of ≥ -7.5 kcal/mol, with the highest binding affinity of -8.908 and -9.953 kcal/mol for GR1 and GR2, respectively. For comparison, BARMIE predicts cortisol binding to rtGR1 and GR2 as -9.383 kcal/mol and 9.98 kcal/mol, respectively. From these, 30 lead compounds were selected to assess agonist and antagonist behavior at a concentration of 1μM in the presence or absence of 1μM cortisol, as well as 10 compounds that were predicted not to bind to the receptors. Only a handful of compounds showed significant agonist activity (Figure 3B and D), and activity was substantially lower than the 100-fold increase observed with cortisol (Figure 3C and E). The reason may be that the one μM concentration is too low to elicit a response in many of these compounds, as they are not designed to mimic the structure of endogenous cortisol. The concentration was chosen because it reflects elevated plasma cortisol levels in fish[38] and also corresponds to the maximum rtGR

transactivation activity[20,25]. To fully evaluate a hazard, a higher concentration may be required, and to identify a risk, further toxicokinetic experiments to determine or predict chemical plasma concentrations are necessary.

Several compounds from the screen are of significant interest: #4 and #5 stimulate GR1 activity in the presence of cortisol; #18 significantly stimulates both rtGR1 and rtGR2 in the absence and presence of cortisol; #19 stimulates rtGR1; and #14 inhibits the transactivation activity of 1 μM cortisol in both receptors. Compounds #4 (2-fluoro-N-[(4-hydroxy-2,3-dihydrochromen-4-yl)methyl]benzenesulfonamide) and #5 (5,7-dichloro-3-hydroxy-3-(2-methylimidazo[1,2-a]pyridin-3-yl)-1H-indol-2-one) have been synthesized with potential bioactivity, but their precise actions are unclear. #18 (3-(4-methylphenyl)-5-phenyl-1,2,4-oxadiazole) and #19 (3-(3-methyl-2-pyridinyl)-5-phenyl-1,2,4-oxadiazole) are 1,2,4-oxadiazole compound known to have biological activities, including estrogen receptor antagonism[39], were as #14 (6-(6-chloroimidazo[1,2-a]pyridin-3-yl)-N-pyrrolidin-3-ylpyridin-2-amine) is an IL-1R-associated kinase 4 inhibitor (IRAK4) inhibitor[40,41]. None have been reported as GR agonists or antagonists in fish, and we are not aware of any reports of their concentrations in the environment.

Understanding the reasons for non-steroidal chemical agonism or antagonism is complex. For example, from the amino acid interaction plots, compounds #18 and #19 both form hydrogen bonds with Asn539 of rtGR1(Figure 4), an essential amino acid in the docking of the natural ligand cortisol into the LBP[37] (Figure 1), and is involved in the initiation of the signal to enable recruitment of the transcriptional machinery. However, #18 also stimulates rtGR2, and there is no predicted hydrogen bond formation with those amino acids known to interact with cortisol. For #14, the compound interacts with known amino acids essential for cortisol binding within the LBP (Gln545 in rtGR1 and Asn456 in rtGR2 (equivalent to Asn539 in rtGR1)). Still, in this instance,

the outcome is antagonism, not agonistic behavior. Further work is required to understand better the link between non-steroidal chemical interactions with fish steroid receptors and ED agonist or antagonistic behavior, and to predict their impact.

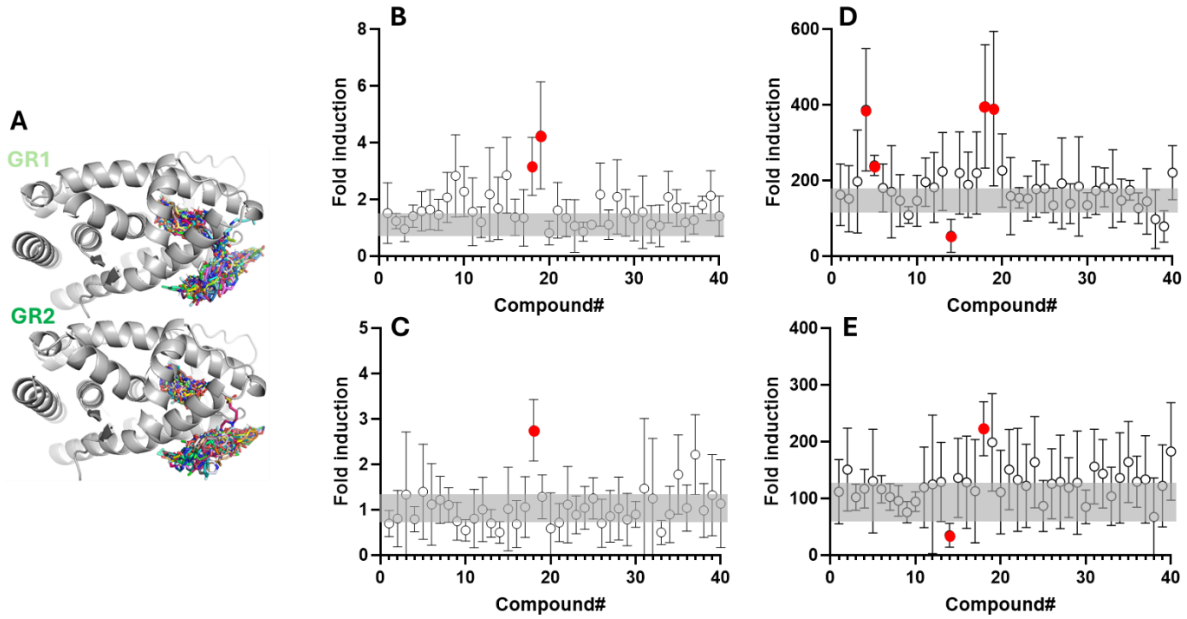
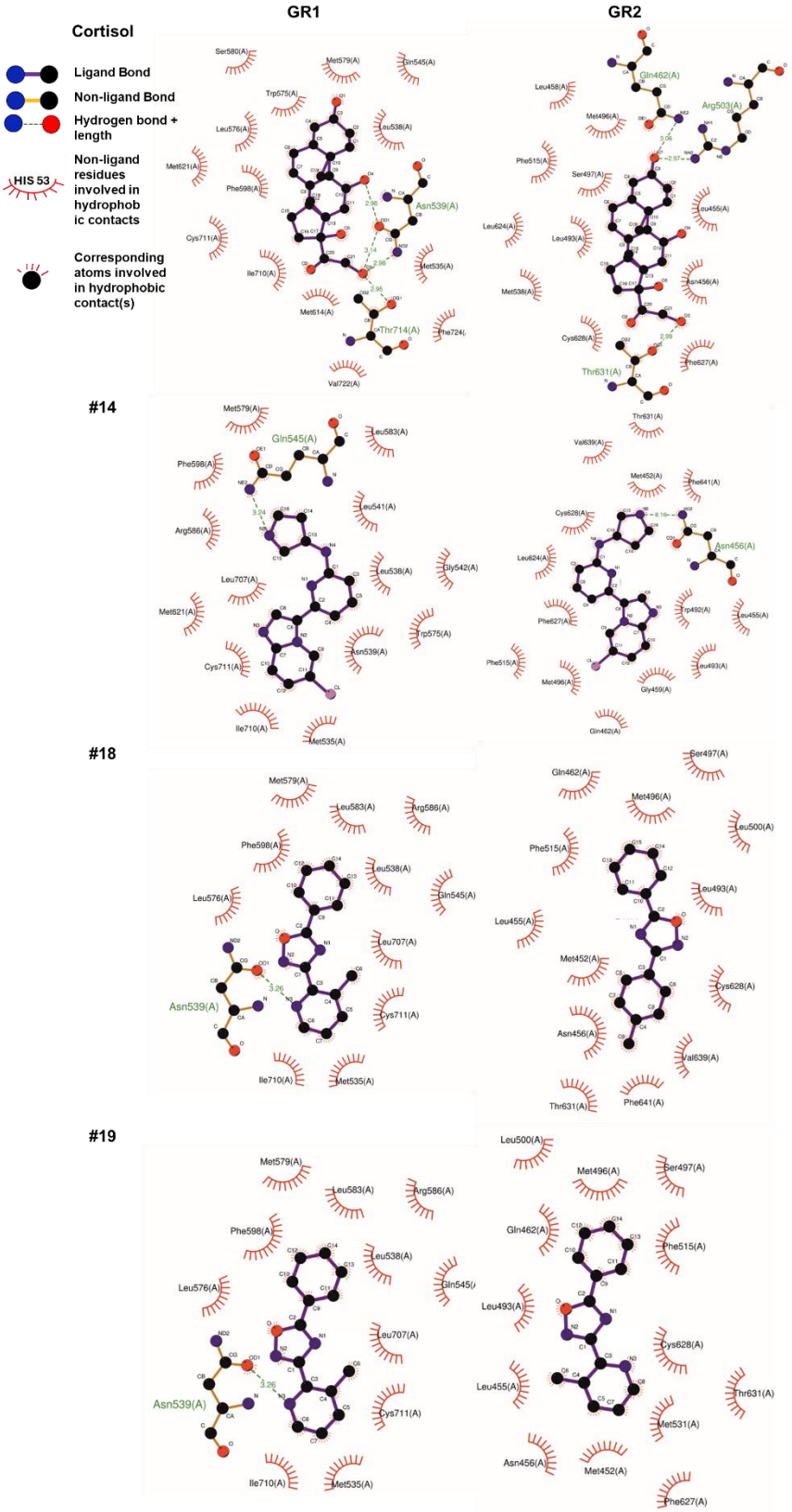


Figure 3. A. a composite image of the ligand binding profiles of the MMV Global Health Priority Box from BARMIE. B – E. Fold induction (B & D) and rtGR2 (C & E) relative to DMSO controls for compounds #1 to #30 predicted to bind with the ligand bind pocket (LBP) with a binding affinity of ≥-7.5 kcal/mol and #30 to #40 for those compounds predicted not to bind with the LBP in absence (B & C) or presence of 1µM cortisol (D & E). The grey rectangles represent the range (average ± SD) of values from the DMSO controls (B & C) and 1µM cortisol alone (D & E). The red circle indicates those compounds whose SD are outside of the range of the controls. Values represent average of 3 separate experiments ± SD, except #36 to #40 where the values represent repeats. See SI 4 for list of compounds and predicted binding affinities.

ENVIRONMENTAL IMPLICATIONS

BARMIE provides an open-access tool to rapidly screen synthetic drug ligands and industrial chemical binding affinities to proteins. The current study used glucocorticoid receptors as an example, but this can be expanded to other steroid and non-steroid receptors, as well as other proteins at the molecular initiating event (MIE) of an Adverse Outcome Pathway (AOP)42. This in silico approach is used in drug discovery, but only as a first screen for potential leads to be further investigated. When applied to environmental toxicology, BARMIE should be used similarly, by providing a list of species with proteins with high binding affinity for known synthetic ligands and identifying chemicals that interact with the active sites of proteins, warranting further investigation. For example, we screened the MMV Global Health Priority Box chemical library and identified 30 of 178 compounds for additional testing. Of these, we identified compounds with novel GR agonist and antagonist activity in a transactivation assay. The next phase would be to conduct more in-depth dose-response curves, widen the screen to include other fish species to identify if there is a pattern in sensitivity, use this data to understand the chemical/protein interactions that govern agonistic or antagonistic behaviors, and focus on these compounds in bioaccumulation studies to identify the risk these chemicals pose.

Figure 4. The amino acid interactions between compound #14,18,19 (See SI …) and cortisol with rainbow trout GR1 and 2, as predicted by LigPlot.



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SUPPORTING INFORMATION.

Supplementary Information 1 – Excell spreadsheet with predicted binding affinity of various synthetic glucocorticoids and cortisol to fish glucocorticoid receptors.

Supplementary Information 2 – Five repeat runs with BARMIE for cortisol.

Supplementary Information3 – Summary of exhaustiveness 8,16 and 32 results.

Supplementary Information4 – Summary of predicted binding affinities to the MMV GHPB compounds to rainbow trout GR1 and GR2.

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