Binding Affinity Ranking at the Molecular Initiating Event (BARMIE): An open-source computational pipeline for ecological hazard ranking of endocrine disrupting chemicals.

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Abstract

One of the key challenges in ecological risk assessment lies in identifying the chemicals that pose the greatest threat and determining the species that are most vulnerable to their effects. Computational prediction of protein binding affinity can help in assessing the risk of chemicals to species. In this study we developed and validated an open-source tool called BARMIE (Binding Affinity Ranking at the Molecular Initiating Event) to rank chemical hazards and identify species that are most susceptible based on the binding affinity of the chemical to steroid receptor proteins. As an exemplar of BARMIE's output we focus on 163 teleost fish glucocorticoid receptors (GRs) and the natural ligand cortisol and 10 synthetic glucocorticoid (GCs) drugs and five other potential chemical GR agonists. The hazard ranking is based on the likelihood that the chemicals with the highest binding affinity are likely to outcompete cortisol at the receptor binding site. In this analysis, halcinonide, a GC, was predicted to be the most hazardous based on its binding affinity and the superorder Protacanthopterygii species, including the Esociformes and Salmoniformes, were identified as the most vulnerable. This computational pipeline can be expanded to evaluate more chemicals, species, and proteins as part of an in silico chemical hazard assessment tool.

Introduction

Approximately 350,000 synthetic chemicals are produced globally, yet most have not undergone any human or environmental risk assessment[1]. Consequently, the impact of these novel substances on human and wildlife health is likely underestimated. To protect wildlife, hazard assessors, who evaluate the potential of a chemical to cause harm, and risk assessors, who determine the likelihood of that harm occurring, face the challenge of identifying which of these chemicals are of concern and the species that are most vulnerable to them.

All vertebrates have a similar steroid hormone/receptor based endocrine system that regulates a plethora of physiological and developmental processes. These processes are controlled via the binding of the steroid ligand to their steroid receptor to initiate ligand inducible transactivation or transrepression, or other cellular signalling pathways[2]. Due to their significance the steroid receptors (glucocorticoid, mineralocorticoid, androgen, oestrogen, and progesterone) are highly conserved within the vertebrate subphylum[3]. The importance of this system for health is also reflected in concerns over endocrine active substances that may perturb hormonal actions via these receptors leading to reproductive and metabolic disorders as well as neural development.

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[4,5]. These initially focused on the EATS (estrogen, androgen, thyroid and steroidogenesis) modalities, but in more recent years several New Approach Methodologies (NAMs) utilizing in silico techniques and novel in vitro methodologies have expanded the screening of chemical hazards for non-EATS modalities[6] (e.g. glucocorticoid and progesterone receptor and non-steroidal receptors). NAMs provide valuable insights into a chemical's mode of action (MOA) and its potential effects[7-11], and it is expected that NAMs will be integrated into regulatory frameworks in the future[12]. The majority of NAMs research efforts have concentrated on human risk assessment, which aims to protect the individual. In

contrast, environmental and ecological risk assessments are far more ambitious, seeking to protect numerous species and maintain ecosystem function.

Models such as EcoDrug[3] and Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS)[13] make use of protein conservation across phyla to identify non-target species susceptible to drugs based on the presence of human or veterinary drug targets. Two recent studies have combined the sequence information with empirical toxicity information to provide greater information on the potential environmental impacts of chemicals. 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[14] pathways and points of departure in toxic outcomes[15], 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[16]. However, one issue that is difficult to avoid if the aim is to protect species and ecosystem function, is the lack of species-specific chemical impact data and the models are restricted to data-rich resources for only a few species (e.g. humans, mice, zebrafish). It is known that there are huge differences in the concentrations of a chemical that induce a toxic response between species within a taxon[17] likely due to species specific toxicokinetic and/or toxicodynamic parametres[18]. Within the AOP framework[15] a potential reason for enhanced or decreased chemical sensitivity are differences in binding affinity at the molecular initiating protein. If the mutations in the protein that confer different binding affinity characteristics[19] are conserved within orders or families of species or are species specific, then it is possible to develop a more granular chemical hazard assessment based on interaction at the MIE for groups of species, species or individuals.

This study focuses on glucocorticoid receptors (GRs) in teleost fish as an exemplar of this approach, however examples for other EATS and non-EATS modalities are provided in Supplementary Information (SI 4). The reason for focusing on the GR is two-fold. Firstly,

there are species differences in the binding affinity (K_d), transactivation (EC50) and trans repression activity for both natural and synthetic glucocorticoids (GCs)[20-24], and previous research has indicated a correlation between GR hormone binding affinity and hormone EC50 for ligand-inducible gene transactivation in rainbow trout GR mutants[25]. Secondly, synthetic glucocorticoids, which are commonly used to treat various health conditions, pose growing environmental concerns with 17% of the GCs assessed are predicted to exist at concentrations that pose a risk to fish[16]. The computational pipeline [Binding Affinity Ranking at the Molecular Initiating Event (BARMIE)] ranks chemical binding affinities to GR across a wide range of fish species to determine the binding affinity of synthetic glucocorticoids and other chemicals to identify chemicals and species of concern. This is based on the hypothesis that species with GRs exhibiting the highest binding affinity are more likely to respond to lower concentrations of natural GCs or GR agonists or antagonists in the bloodstream.

Materials and Methods

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, summary of the procedural steps and user instructions are available at https://github.com/ParsaFouladi/Barmie. The pipeline was run on the University of Southampton HPC Iridis 6, and the example provide is specific to teleost fish GRs. The pipeline can be adapted for use with other HPC architecture as well as other species and proteins.

Several steps were necessary in developing BARMIE. Firstly, genome annotation is based on sequence homology to proteins of known function, and the level of validation may vary depending on the database. There are over 250 genomes available from Ensembl and

UniProt provides verified and non-verified protein structures, and it is recommended to use only those verified. Secondly, several receptor isoforms (e.g., splice variants) are predicted in Uniport. Because very few isoforms have been characterized in fish[32] we did not remove these. Thirdly, we only used the Uniport proteins with confirmed structures. However, bespoke structures predicted from amino acid sequence information[33] can be integrated in the pipeline. Fourthly, the absolute orientation of the receptor structure when imported into AutoDock Vina generally differs between structures, making it difficult to automate the definition of the box coordinates to correctly encompass the ligand binding domain. To overcome this, structures are automatically aligned using PyMol scripting, so that the LBD location is aligned for all the proteins. The only manual step in the pipeline is the definition of the box coordinates for docking exploration of the ligand binding pocket. This is set to a reference protein, in this example *Oncorhynchus mykiss* GR AF-P49843, and is identical for all receptor due to the three- dimensional alignment in PyMol. For the GR used in this study, the box size was set to 20, 20, 20 Å, and the coordinates X=5, Y=2, Z=-15.

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) as well as the herbicide atrazine [CHEMBL15063. Unlikely to interact with the GR][34], insecticide glyphosate [CHEMBL95764. Reported interactions with GR] [35], anesthetic sevoflurane [CHEMBL1200694. Reported to affect the immune system but interaction with the GC pathway unknown] [36], cortisol synthesis inhibitor osilodrosat [CHEMBL3099695][37] and antibiotic triclocarban [CHEMBL1076347. A reported GR antagonist][38]. AutoDock Vina has a stochastic algorithm to explore ligand binding poses, and thus, docking searches were run 5 times and an average binding affinity are reported.

Docking simulations were run with exhaustiveness of 8, 32, and 128 to assess consistency of results. Amino acid interaction fingerprint was conducted with the protein/ligand pose in LigPlot^{+} v2.2 (https://www.ebi.ac.uk/thornton-srv/software/LigPlus/).

Results and Discussion

The described pipeline referred to as BARMIE (Binding Affinity Ranking at the molecular initiating event) predicts the binding affinity of 163 fish GRs to the natural ligand cortisol (Figure 1a, for an example of cortisol docking with *Pygocentrus nattereri* AOA3B4D0J0 GR and the amino acid interaction fingerprint), various synthetic GCs, and potential GR agonists. We observed no difference between results with AutoDock Vina at exhaustiveness 8, 32, and 128 (SI 3) and we have reported an average at exhaustiveness of 32. This produces a ranking of binding affinity highlighting those species whose GRs bind with the highest affinity to these chemicals and provides an *in-silico* hazard screening protocol based on binding to the receptor, or in AOP parlance the MIE[39]. As an example of the potential outputs in the top five out of 2822 combinations, we find the GC halcinonide is predicted to bind to 4 GRs with the greatest affinity, and of these 4 GRs belong to the superorder Protacanthopterygii containing the Esociformes and Salmoniformes (Figure 1b) and suggesting in this exercise that the synthetic GC halcinonide may be considered the most significant chemical hazard and the Protacanthopterygii as a group that may be vulnerable. However, the data generated can be used in more nuanced ways by focusing on specific chemicals (Figure 2) and species (Table 1) and for all GCs, we see that 55% of the five most sensitive species belong to the Protacanthopterygii, with the species Northern pike (*Esox lucius*) ranked 1st for 7 of the 11 GCs tested (Table 1). There is limited empirical data on fish GR cortisol binding and transactivation activity, but where this is available, there is a correlation between measured GR hormone binding affinity and hormone EC50 for ligand-inducible gene transactivation for rainbow trout GR[25]. The variance in binding in rainbow trout is due to mutations within the receptors[25], similarly human receptor mutants that cause disease show differences in hormone binding and transactivation characteristics [e.g., 40,41].

Our analysis shows that the Protacanthopterygii, which include the commercially important salmon and trout, are an order of fish particularly susceptible to synthetic glucocorticoids. However, a limitation should be noted that only 86 fish genomes have been annotated out of the potentially 30,000 teleost species[42], and of these genomes, a high proportion, 8%, are Protacanthopterygii. Thus, this order is overrepresented. The number of species genomes sequenced is rapidly expanding for example the Earth BioGenome Project Network[43] has 63 global partners with an ambitious target of "[…] characteriz(ing) the genomes of all of Earth's eukaryotic biodiversity over a period of ten years". This will provide the necessary genetic information for BARMIE to provide a more granular order, family or species chemical risk assessment.

The range of binding affinities is reduced for the non-GC chemicals atrazine, glyphosate, sevoflurane, and osilodrosat (~-5 to ~-6 Kcal/mol,) compared to natural and synthetic GCs (Figure 2 and SI 1). This would suggest that these chemicals would be classed low in an MIE binding affinity-based hazard assessment because they are unlikely to out-compete cortisol at the binding site. However, for risk assessment, the potential to cause harm is based on the exposure concentration as well as the hazard. Thus, if fish accumulate these chemicals to such an extent that this level far exceeds those of the natural ligand, it poses a risk.

Environmental Implications

The challenge for ecological risk assessors is identifying those chemicals of concern amongst the thousands in production and those species most vulnerable. The current pipeline that has been developed enables us to rank chemicals and species based on the receptor's binding affinity. This can be used for hazard assessment because the chemical with a higher binding affinity is likely to out-compete the natural ligand and thus be a more potent agonist or antagonist. The results are an exemplar of what the pipeline can offer, it can be applied to any protein where the binding affinity of a ligand is crucial for its function.

198 This is particularly relevant for all steroid and non-steroid receptors enabling the
199 identification of potent endocrine-disrupting chemicals. BARMIE is a screening tool that
200 allows a toxicity testing program for regulatory purposes focusing on those chemicals and
201 species of concern, reducing costs and the number of animals used for testing.

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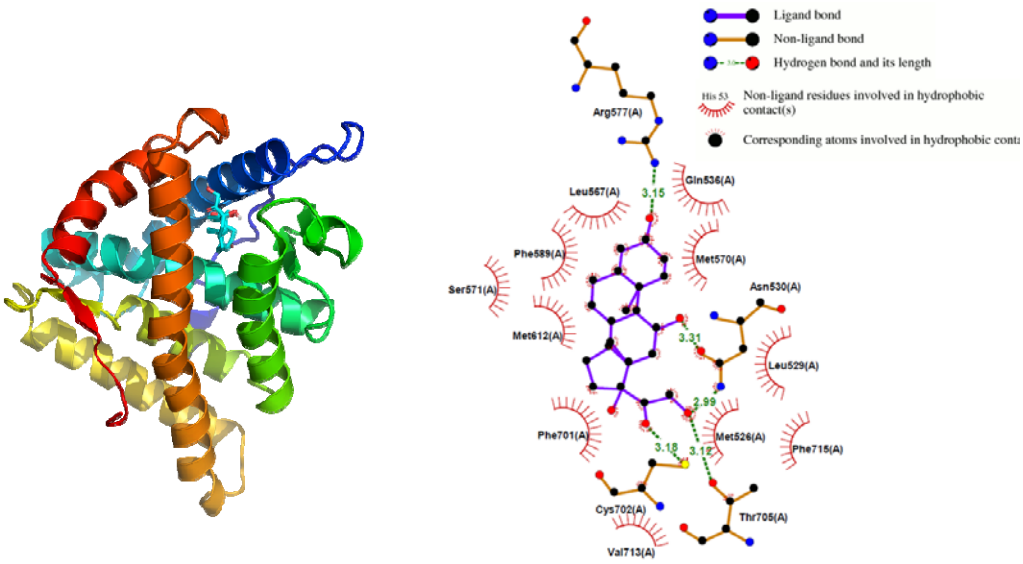
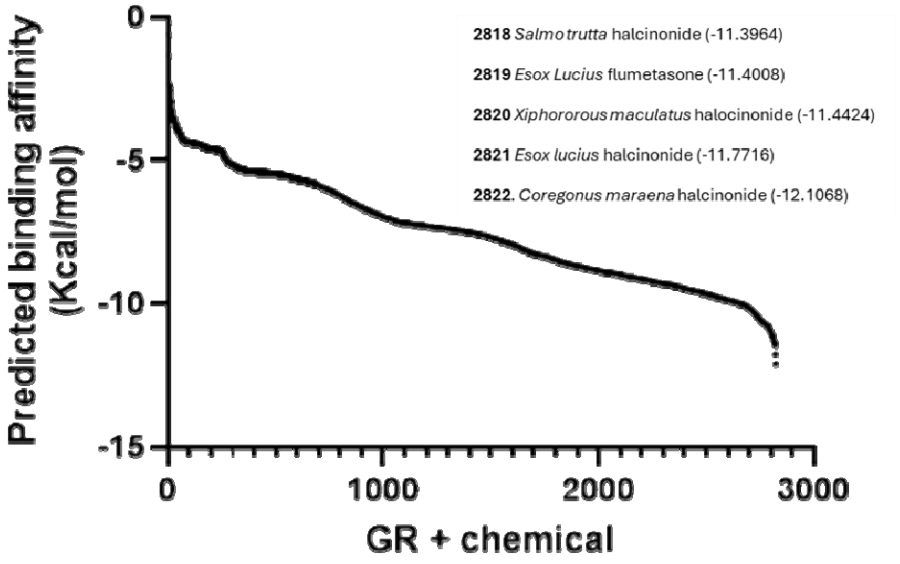
Figure 1. A. Image of cortisol docking into the *Pygocentrus nattereri* AOA3B4D0J0 glucocrticoid receptor with a binding affinity -8.43 Kcal/mol and the corresponding amino acid interaction fingerprint. B. The predicted binding affinity for all chemical and species combinations. Inset, the top 5 species + chemical binding affinities (see supplementary information for values).

A



B



376 **Figure 2.** Binding affinities for the 163 teleost fish glucocorticoid receptors per chemical (see
377 supplementary information for values)

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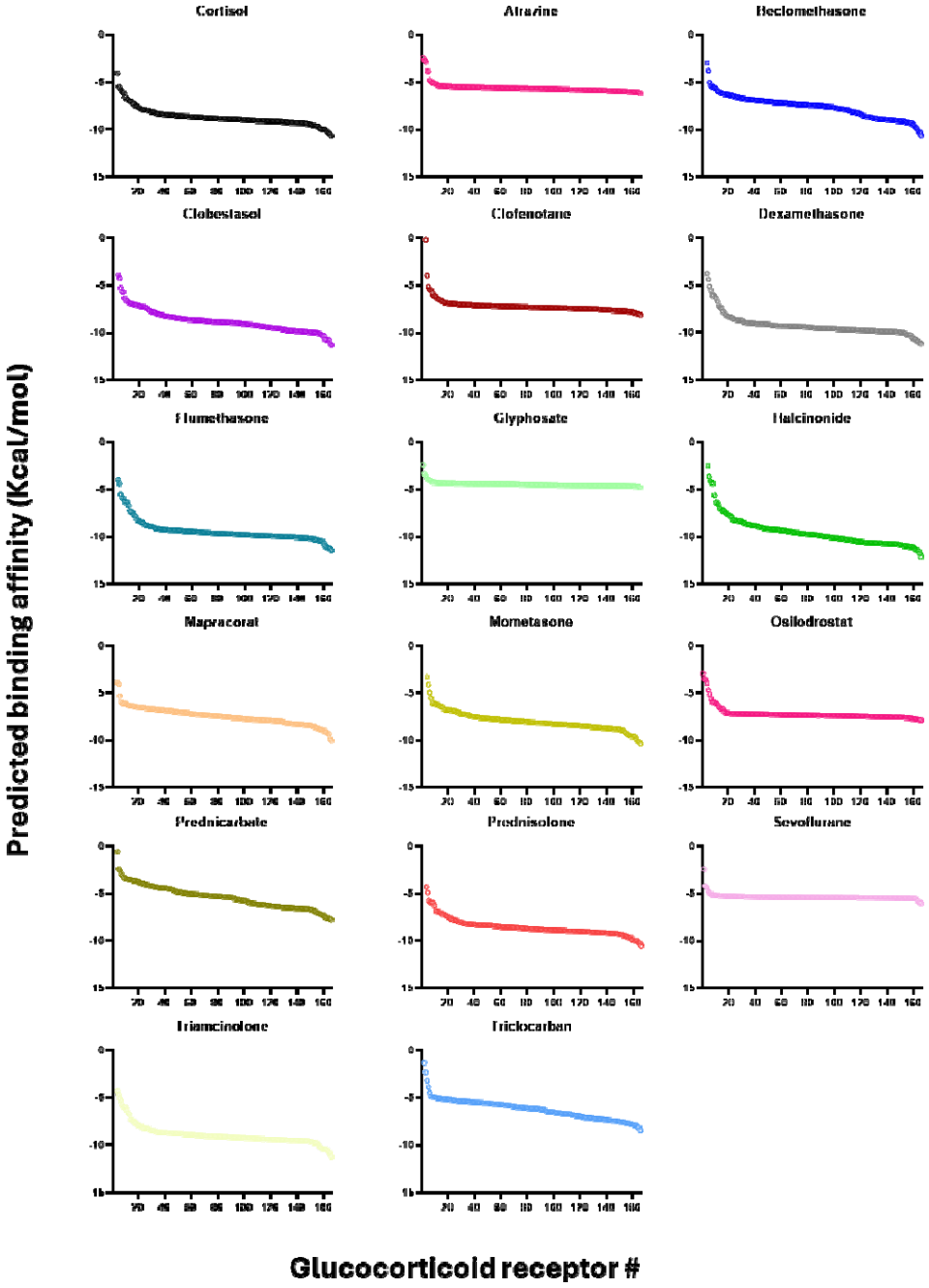

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Table 1 The five species with the highest binding affinity to each chemical.

		Species Rank (*Latin name* and UniProt ID) of 5 most sensitive to each chemical [ΔG (kcal/mol)]				
Chemical	**Class**	**1**	**2**	**3**	**4**	**5**
Cortisol	Natural steroid	*Esox lucius* A0A6Q2WV38 (-10.6856)	*Coregonus maraena* A0A0D6E1Q9 (-10.456)	*Anabas testudineus* A0A7N6BVF0 -10.4278	*Salmo trutta* A0A673WVI2 -10.2504	*Scophthalmus maximus* A0A2U9C6P4 -10.1376
Atrazine	Herbicide	*Anabas testudineus* A0A7N6B9C8 -6.216	*Mastacembulus armateus* A0A7N9ALU0 -6.0674	*Scophthalmus maximus* A0A2U9C6P4 -6.0612	*Poecilia lapipinna* A0A3B3V5Z8 -6.0596	*Xiphorous maculatus* M4AXU0 -6.0576
Beclomethasone	GC	*Esox lucius* A0A6Q2WV38 -10.6038	*Coregonus maraena* A0A0D6E1Q9 -10.3024	*Anabas testudineus* A0A7N6BVF0 -10.1674	*Mastacembulus armateus* A0A7N8X2D0 -9.9236	*Salmo trutta* A0A673WVI2 -9.7614
Clobetasol	GC	*Esox lucius* A0A6Q2WV38 -11.2956	*Coregonus maraena* A0A0D6E1Q9 -11.1776	*Scophthalmus maximus* A0A2U9C6P4 -10.7898	*Anabas testudineus* A0A7N6BVF0 -10.746	*Salmo trutta* A0A673WVI2 -10.742
Clofenotane,	Insecticide	*Oncorhynchus mykiss* Q6RKQ3 -8.1072	*Nothobranchius pienaari* A0A1A8M2D1 -8.0478	*Xiphorous maculatus* M4AXU0 -8.0214	*Scophthalmus maximus* A0A2U9C6P4 -7.9704	*Sinocyclocheilus rhinocerous* A0A673HKV4 -7.91
Dexamethasone	GC	*Esox lucius* A0A6Q2WV38 -11.144	*Coregonus maraena* A0A0D6E1Q9 -11.0824	*Xiphorous maculatus* M4AXU0 -10.9068	*Scophthalmus maximus* A0A2U9C6P4 -10.8816	*Salmo trutta* A0A673WVI2 -10.7178
Flumetasone	GC	*Esox lucius* A0A6Q2WV38 -11.4008	*Coregonus maraena* A0A0D6E1Q9 -11.2524	*Xiphorous maculatus* M4AXU0 -11.1978	*Scophthalmus maximus* A0A2U9C6P4 -11.1266	*Oncorhynchus mykiss* Q6RKQ3 -11.0974
Glyphosate	Herbicide	*Amphilophus citrinellus* A0A3Q0S1C3 -4.7726	*Anabas testudineus* A0A7N6BVF0 -4.7142	*Hucho hucho* A0A4W5NMM7 -4.6994	*Ictalurus punctatus* A0A2D0SEE8 -4.6884	*Electrophorus electricus* A0A4W4EHL7 -4.6826
Halcinonide	GC	*Coregonus maraena* A0A0D6E1Q9 -12.1068	*Esox lucius* A0A6Q2WV38 -11.7716	*Xiphorous maculatus* M4AXU0 -11.4424	*Salmo trutta* A0A673WVI2 -11.3964	*Astatotilapia calliptera* A0A3P8PIE4 -11.3412
Mapracorat	GC	*Electrophorus electricus* A0A4W4EII3 -10.0314	*Stegastes partitus* A0A3B5AC37 -9.8402	*Salarias fasciatus* A0A672FRL6 -9.3478	*Stegastes partitus* A0A3B4ZYN2 -9.2704	*Anabas testudineus* A0A7N6BVF0 -9.1282
Momentasone	GC	*Salarias fasciatus* A0A672FRL6 -10.371	*Coregonus maraena* A0A0D6E1Q9 -10.138	*Esox lucius* A0A6Q2WV38 -10.1016	*Scophthalmus maximus* A0A2U9C6P4 -9.8704	*Anabas testudineus* A0A7N6BVF0 -9.6366
Osilodrosat	Synthesis Inhibitor	*Nothobranchius pienaari* A0A1A8MV28 -7.8638	*Nothobranchius kadleci* A0A1A8C987 -7.8162	*Nothobranchius kuhntae* A0A1A8HYB3 -7.7868	*Scophthalmus maximus* A0A2U9C6P4 -7.7864	*Iconisemion striatum* A0A1A7WDX0 -7.7602
Prednicarbate	GC	*Paralichthys olivaceus* O73673 -7.7606	*Coregonus maraena* A0A0D6E1Q9 -7.7018	*Esox lucius* A0A6Q2WV38 -7.6118	*Electrophorus electricus* A0A4W4EII3 -7.6044	*Salmo trutta* A0A673WVI2 -7.5676
Prednisolone	GC	*Esox lucius* A0A6Q2WV38 -10.5004	*Anabas testudineus* A0A7N6BVF0 -10.2672	*Coregonus maraena* A0A0D6E1Q9 -10.184	*Salmo trutta* A0A673WVI2 -9.9726	*Scophthalmus maximus* A0A2U9C6P4 -9.9648
Sevoflurane	Anaesthetic	*Pundamilia nyererei* A0A3B4GAN3 -6.0414	*Electrophorus electricus* A0A4W4EIA3 -5.9786	*Hucho hucho* A0A4W5NM33 -5.8264	*Hucho hucho* A0A4W5NMM7 -5.7428	Seriola lalandi dorsalis A0A3B4WZ32 -5.5394
Triamcinolone	GC	*Esox lucius* A0A6Q2WV38 -11.198	*Coregonus maraena* A0A0D6E1Q9 -10.8506	*Anabas testudineus* A0A7N6BVF0 -10.7634	*Salmo trutta* A0A673WVI2 -10.5826	*Scophthalmus maximus* A0A2U9C6P4 -10.4684
Triclocarban	Antimicrobial	Hucho hucho A0A4W5NM33 -8.4626	*Hucho hucho* A0A4W5NMM7 -8.1526	*Electrophorus electricus* A0A4W4EII3 -8.1472	*Fundulus heteroclitus* A0A3Q2Q9J2 -7.9158	*Gasterosteus aculeatus* G3QBY4 -7.9082

Species order colour code: Escociformes & Salmoniformes; Pleuronectiformes; Anabantiformes; Synbranchiformes; Cyprinodontiformes; Cichliformes; Cypriniformes; Siluriformes; Blenniiformes; Gymnotiformes; Carangiformes; Scorpaeniformes.