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Computational Estimation of Rainbow Trout Estrogen Receptor Binding Affinities for Environmental Estrogens

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Abstract

Environmental estrogens have been the subject of intense research due to their documented detrimental effects on the health of fish and wildlife, and their potential to negatively impact humans. A complete understanding of how these compounds affect health is complicated because environmental estrogens are a structurally heterogeneous group of compounds. In this work, computational molecular dynamics simulations were utilized to predict the binding affinity of different compounds using rainbow trout (*Oncorhynchus mykiss*) estrogen receptors (ERs) as a model. Specifically, this study presents a comparison of the binding affinity of the natural ligand estradiol-17β to the four rainbow trout ER isoforms with that of three known environmental estrogens 17α-ethinylestradiol, bisphenol A, and raloxifene. Two additional compounds, atrazine and testosterone, were tested that are known to be very weak or non-binders to ERs. The binding affinity of these compounds to the human ERα subtype is also included for comparison. Results of this study suggest that, when compared to estradiol-17 β, bisphenol A binds less strongly to all four receptors, 17α-ethinylestradiol binds more strongly, and raloxifene has a high affinity for the α subtype only. The results also show that atrazine and testosterone are weak or non-binders to the ERs. All of the results are in excellent qualitative agreement with the known *in vivo* estrogenicity of these compounds in the rainbow trout and other fishes. Computational estimation of binding affinities could be a valuable tool for predicting the impact of environmental estrogens in fish and other animals.

Keywords

molecular dynamics; BPA; EE2; raloxifene; atrazine; estrogen; fish

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Introduction

Environmental estrogens comprise a diverse group of human-derived compounds that interact with estrogen receptors (ERs) in vertebrate animals (McLachlan, 1979). Much concern has been raised about environmental estrogens because of their documented detrimental effects on fish and wildlife (Tyler et al., 1998), and potential to impact humans (Toppari et al., 1996). Aquatic animals are particularly targeted because eventually all of these compounds enter the ecosystems they inhabit (Corcoran et al., 2010). Although much is known about the end result of environmental estrogen exposure from both lab and field studies (for reviews see Mathiessen and Sumpter, 1998; Mills and Chichester, 2005), less is known about the precise mechanism(s) of action (Tilghman et al., 2010). A complete understanding is complicated because environmental estrogens, based on their chemical structures, are a heterogeneous group of compounds. Yet all, to varying degrees, appear capable of binding to ERs and initiating an estrogenic response which is their hallmark. The intensity of this estrogenic (biological) response has been equated with the strength of ER binding affinities in target organs (Salum et al., 2008). This provides an important means of comparing different compounds and making predictions on their biological effects.

A key motivation for this study is that purified proteins are often not available to perform assays (as is the case for the rainbow trout ERs studied here), but molecular dynamics (MD) computer simulation can still be used to estimate binding affinities (Oostenbrink et al., 2000; van Lipzig et al., 2004). MD simulations were used in a previous study to determine rainbow trout ER binding affinities for estradiol-17β (E2), the major native estrogen hormone in this fish (Shyu et al., 2010). Currently, use of computational (i.e., *in silico*) methods for modeling the biological consequences of environmental contaminants is not widespread (Kubli-Garfias, 1998; Sugiyama et al., 2009; Novic and Vracko, 2010) in contrast to direct assays (i.e., *in vivo* or *in vitro*) (Klotz et al., 1996). Computational methods such as MD simulation, in which atoms and molecules are allowed to interact consistent with atomistic physics and chemistry based models, provide a view of the motion of the particles. MD simulation permits observation of the dynamics of the individual atoms and, in essence, functions as a virtual microscope with high temporal and spatial resolution (Martínez et al., 2005; Martínez et al., 2006; Sonoda et al., 2007). This unique feature permits the elucidations of the inner workings and atomic interaction of diverse molecules (Allen and Tildesley, 1989; Leach, 2001; Rapaport, 1996).

The purpose of this study was to use MD simulations to determine the binding affinities to rainbow trout ERs for three known environmental estrogens: 17α-ethinylestradiol (EE2), bisphenol A (BPA), and raloxifene (RAL) that differ in chemical structure and reported estrogenicity in fishes (Brian et al., 2005; Caldwell et al., 2008). In addition, the binding affinity of these environmental estrogens with the human ERα subtype was included for comparison. EE2 and BPA are well known environmental estrogens (Arcand-Hoy et al., 1998; Staples et al., 1998). EE2 is a pharmaceutical currently used widely as a human female contraceptive (Shrader and Dickerson, 2008). It is passed from the treated individual in mostly an unconjugated form in the urine and is therefore a prominent estrogen in domestic sewage effluent entering the environment (Kolpin et al., 2002). EE2 stimulates abnormal vitellogenesis (Folmar et al., 2000), causes reproductive problems (Brown et al., 2008), and alters sexual behavior in male fishes (Salierno and Kane 2010). BPA is a component of polycarbonate plastics that leaches from these materials when they degrade (Le et al., 2008). Due to the prevalence of plastics in the environment BPA is a ubiquitous contaminant (Halden, 2010). BPA causes abnormal vitellogenin induction in male fishes (Sohoni et al., 2001). RAL is a pharmaceutical used to prevent bone loss in postmenopausal women and as an anti-estrogen to block breast and uterine cancers (Muchmore, 2000). It is termed a selective estrogen receptor modulator (SERM) that has been reported to almost

exclusively interact with the ERα subtype in mammals. While the negative affects of RAL have yet to be demonstrated in fishes, RAL will be an environmental estrogen with its increasing use by the human population (Teeter and Meyerhoff, 2002).

This study also includes binding affinity results for two additional compounds, atrazine (ATZ) and testosterone (TES). Both ATZ and TES have been reported as very weak or non-binders to fish ERs (Denny et al., 2005; Latonnelle et al., 2002). ATZ is an organic compound widely used as herbicide. It has been shown that ATZ acts as an endocrine disruptor, specifically by altering estradiol signaling via increased aromatase activity (Roberge et al., 2004). Several studies have linked ATZ with the abnormal development of a number of species (Ashby et al., 2002; Wiegand et al., 2001; Dianna et al., 2000). It was suggested that ATZ induced aromatase, the enzyme that controls the rate-limiting step in the conversion of androgens into estrogens and increased the concentration of cyclic adenosine monophosphate (cAMP) in the H295R human adrenocortical carcinoma cell line (Sanderson et al., 2000; 2002).

Materials and Methods

Receptor Structures

While there are no purified proteins available, the amino acid sequence for the complete ER gene family is known for the rainbow trout with two ERα isoforms (i.e., ERα1, ERα2) and two ERβ isoforms (i.e., ERβ1, and ERβ2; Nagler et al., 2007), also known as NR3A1a, NR3A1b, NR3A2a and NR3A2b (Nuclear Receptors Nomenclature Committee, 1999). Since no experimental structures are available, the rainbow trout ER holo structures for the ligand-binding domains were generated by SWISS-MODEL (Arnold et al., 2006; Kiefer et al., 2009) using human ER as template (Protein Data Bank entries 1A52 for both ERα isoforms and 3ERT for both ERβ isoforms). Sequence identities between the rainbow trout and human ligand-binding domains were within the range of 75–85%, and thus we expect that the ER structures determined by SWISS-MODEL are reasonable. Moreover, Shyu et al (2010) have shown that different rainbow trout ER isoforms share a common evolutionary origin, and structure tends to be well conserved. The different estrogenic compound topologies, needed for MD simulations, were generated by the PRODRG server (Schüttelkopf and van Aalten, 2004) with the options of full charges and no energy minimization. Estrogenic compounds were then individually docked into the binding pocket of the receptor holo structures with AutoDock (Morris et al., 1998). The Lamarckian genetic algorithm was employed to search for the most probable binding poses for each estrogenic compound and receptor. The number of genetic algorithm runs was set to 5,000 with a population size of 250,000 individuals and 5,000,000 generations. The number of evaluations was set to 2,500,000 for each individual in the population to ensure thorough exploration of the search space. The mutation rate was set to 0.05 and the crossover 0.8. A two- point crossover was used to generate the offspring at each successive generation. The genetic algorithm automatically preserved the 20 best-fit individuals to the next generation and the 20 least-fit individuals were not used to generate offspring. For this study the five compounds EE2, BPA, RAL, ATZ, and TES, were tested for the best-fit binding pose. Data for E2 was taken from Shyu et al (2010).

Computer Simulation Protocols

The computer simulations were performed using a protocol reported previously by Shyu et al. (2010) to estimate the binding affinity for all rainbow trout isoforms to the compounds. The binding free energy calculations were decomposed into several steps in which the compounds are annihilated (i.e., decoupled) from its bound state in the receptor complex and then made to reappear in solution to complete the thermodynamic cycle (Shyu et al., 2010).

Harmonic restraints were applied to each compound to the receptors in order to accelerate computer simulation convergence and minimize the detrimental effects of end-point singularities commonly reported in computational alchemical simulations (Shirts et al., 2003).

All computer simulations were performed using GROMACS 4.0 (Hess et al., 2008) and the default GROMOS-96 43A1 forcefield (van Gunsteren et al., 1996). For equilibration, the systems were first minimized using 1,000 steps of L-BFGS (Broyden-Fletcher-Goldfarb-Shanno) (Broyden, 1970), followed by 1,000 steps of steepest descent minimization. The system was then subject to 1.0 ns of simulation using isothermal molecular dynamics. This was followed by another 1.0 ns of simulation using isothermal-isobaric molecular dynamics with the Berendsen barostat with a time constant of 1.0 ns. All production simulations were performed with isothermal-isobaric conditions at the temperature of 277 K (van Gunsteren and Berendsen, 1988) and a pressure of 1.0 atm (Liao and Parrinello, 2002). Separate simulations were performed for changes in the Lennard-Jones with 21 values of the scaling parameter λ and the electrostatics with 11 (Shyu et al., 2010). For simulations with only Lennard-Jones, all partial charges were set to zero. Simulations were performed at 2.0 ns for each λ value, and the first 1.0 ns was discarded for equilibration and the last 1.0 ns used to calculate the binding affinity. To minimize the numerical integration errors, we employed the polynomial and regression fitting techniques to calculate the free energy difference (Shyu and Ytreberg, 2009; Shyu and Ytreberg, 2010). The Bennett acceptance ratio approach was used to estimate the free energy associated with the restraints (Bennett, 1976).

It is worth noting that the MD simulations produce an estimate for the free energy of binding (ΔG) in kJ/mol. For this report these results were converted to pK_d for the standard state (i.e., 1 mol/L concentration) p$K_d = -\log_{10} K_d$, where $K_d = e^{-\Delta G/\mathrm{RT}}$ is the dissociation constant, $R = 8.314472\ \mathrm{JK^{-1}mol^{-1}}$ is the universal gas constant, and $T = 277$ K is the absolute system temperature.

Results

A total of 5,000 independent docking trials were performed for each of the four rainbow trout ERs. The best binding pose from each trial was collected and then ranked based on a scoring function. Figure 1 shows the best-fit binding pose for E2, EE2, BPA, RAL, ATZ, and TES in the ERα1 ligand-binding domain. The best-fit binding poses of these six compounds were similar for the other three ERs (i.e., ERα2, ERβ1, ERβ2), and thus are not shown.

Obtaining experimental binding affinity results is not possible for the four rainbow trout ERs because purified proteins are not currently available. Therefore, human ERα was first used to validate the methodology for estimating the binding affinities for the rainbow trout ERs. MD simulations using the human ERα-E2 complex were performed at 300 K and the resulting binding affinity was compared to experimental results reported by Petit et al. (1995). The computational estimate of binding affinity for hER-E2 complex at 300 K was pK_d = 11.0 compared to the experimental value of 9.1. This computational estimate is sufficiently accurate because the discrepancy is within the expected error due to the atomic models (Hess et al., 2008; Shyu et al., 2010). Subsequent MD simulations for human and rainbow trout ERs followed exactly the same procedure as human, beginning with docking the compounds into the ERs.

Table 1 shows results for the computational binding affinities for the various compounds and the rainbow trout ER isoforms and human ERα. Among the known estrogenic compounds tested, the highest binding affinity occurred with EE2 and ERβ1, while the

lowest binding affinity occurred with BPA and ER β1. In general, across all rainbow trout ER isoforms and the human ERα, EE2 had the highest binding affinities, while BPA had the lowest binding affinities. RAL had significantly stronger affinity to the rainbow trout ERα isoforms, compared to the ERβ isoforms. Very similar binding affinities for RAL and the rainbow trout ERα isoforms and human ERα were determined. Our simulation results for ATZ and TES show that the affinity is small or negative, consistent with the fact that these compounds are known to be weak or non-binders to the ERs.

Discussion

To our knowledge this study represents the first MD simulation analysis of ER binding affinities for environmental estrogens in a fish. It provides a unique opportunity to relate computationally derived binding affinities with previous studies in fish that have examined the *in vivo* and *in vitro* estrogenic effects of these compounds (Klotz et al., 1996; Matthews et al., 2000; Parrot and Blunt, 2005; Bjerregaard et al., 2007). A review of the literature on EE2 shows it to be a potent estrogen in rainbow trout and other fishes (Caldwell et al., 2008), with an efficacy greater than E2 (Brian et al., 2005). The results of this study support those findings. For each rainbow trout ER isoform EE2 has a greater estimated binding affinity compared to E2. BPA has been reported to be a weaker estrogen than E2 in fishes, for example (Brian et al., 2005), and the results of this analysis are in line with these reports also. The binding affinities for BPA with ERs from either the rainbow trout or human are the lowest indicated. It is predicted that much more BPA would be required, compared to EE2 or E2, to achieve a similar estrogenic response in the rainbow trout. RAL has not been extensively studied in fishes, but based on the results from this study it would be predicted to have a strong estrogenic response and be more potent than EE2 at equivalent molar concentrations. This suggests that even low levels of RAL release into the environment will pose a threat to fishes.

RAL is a mammalian SERM, acting primarily through the ERα subtype, which is substantiated in this work by comparison between rainbow trout ER subtypes and the human ERα subtype (see Table 1). The binding affinities for RAL with either the rainbow trout ERα isoforms or human ERα subtype are approximately double that of RAL with the ERβ isoforms. Therefore, RAL should be considered a SERM in the rainbow trout, with the majority of its biological activity predicted to function through the ERα a subtype.

There were several instances in which the binding affinities of both rainbow trout ER isoforms within a subtype were very similar for a particular environmental estrogen (see Table 1). For example, both ERα isoforms had nearly identical binding affinities for EE2 or RAL. This was observed for the ERβ isoforms too, with E2 or BPA. The ligand-binding region, within the E-domain of ERs (Tsai and O'Malley, 1994), is the location where ligands interact most intimately with the ER molecule (see Figure 1). The similarity in binding affinities observed reflects the overall similarity in E-domain molecular structure between isoforms within an ER subtype. The very similar RAL binding affinities of the rainbow trout and human ERαs are likely explained by highly convergent molecular structures within the ER ligand-binding region.

In conclusion, MD simulations were used to estimate the binding affinity of three environmental estrogens, EE2, BPA and RAL for the four rainbow trout ERs, and compare them to E2. The results show that BPA binds less strongly to all four receptors, EE2 binds more strongly, and RAL has a very high affinity for the α subtype, but not the β. The results show significant differences between these environmental estrogens that are in concert with their known estrogenic response in biological assays. One test compound, RAL, a known mammalian SERM, is predicted to be highly estrogenic in the rainbow trout, but this will

need to be confirmed by the appropriate assay(s). Finally, it is worth noting that MD simulations successfully predicted that both ATZ and TES are weak or non-binders to ERs. The results of this study suggest that when purified forms of the receptors are not available MD simulation could be a useful technique to predict the estrogenic potential of compounds known to be present in the environment.

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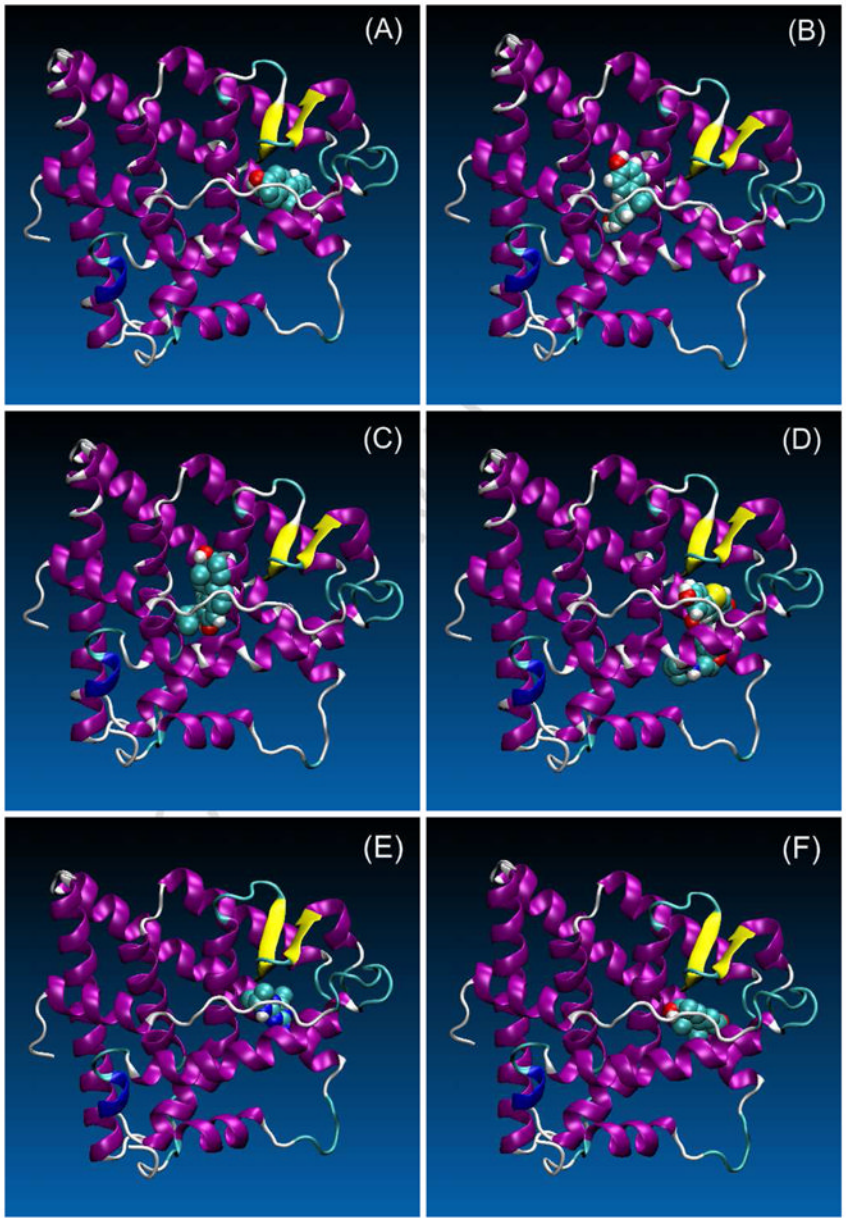

Figure 1.

Computationally determined binding poses for (A) estradiol-17β (E2), (B) 17α-ethinylestradiol (EE2), (C) bisphenol A (BPA), (D) raloxifene (RAL), (E) atrazine (ATZ), and (F) testosterone (TES) in the rainbow trout ERα1 ligand-binding domain. The other rainbow trout isoforms are not shown since the binding poses are similar. Images were rendered using the Visual Molecular Dynamics software (Humphrey et al., 1996). The ligands are drawn based on the chemical elements and receptors the secondary structures of the proteins. Specifically, the ligands are shown as beads representing oxygen (red), carbon (cyan), hydrogen (grey), and sulfur (yellow). For the receptor, helices are shown as coiled ribbons (purple), β-sheets as solid arrows (yellow), π-helix as coiled ribbons (blue), and loop structures as tubes (cyan, gray) (Frishman and Argos, 1995).

Table 1

Binding affinities for estradiol (E2), ethinylestradiol (EE2), bisphenol A (BPA), raloxifene (RAL), atrazine (ATZ), and testosterone (TES) to rainbow trout estrogen receptor (ER) isoforms, ERα1, ERα2, ERβ1, and ERβ2. The affinities are expressed as $pK_d = -\log_{10} K_d$ where K_d is the dissociation constant. Results from the human ERα (hERα) are included for comparison (Shyu et al., 2010). Larger positive pK_d values correspond to stronger binding and negative values indicate that binding will not occur at all. Note that, as expected, ATZ and TES show very weak or negative affinity for all the ERs.

	Receptors				
	ERα1	**ERα2**	**ERβ1**	**ERβ2**	**hERα**
E2	9.6	6.7	8.0	9.4	9.4
EE2	10.0	9.7	13.7	11.0	11.6
BPA	4.3	6.0	3.5	3.9	4.7
RAL	11.7	11.9	6.5	4.5	11.7
ATZ	1.9	2.0	−4.7	0.1	3.2
TES	2.3	−2.2	−1.8	−1.8	−3.7