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Differential ligand selectivity of androgen receptors α and β from Murray–Darling rainbowfish (*Melanotaenia fluviatilis*)

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ABSTRACT

Androgen receptors (ARs) mediate the physiological effects of androgens in vertebrates. In fishes, AR-mediated pathways can be modulated by aquatic contaminants, resulting in the masculinisation of female fish or diminished secondary sex characteristics in males. The Murray–Darling rainbowfish (*Melanotaenia fluviatilis*) is a small-bodied freshwater teleost used in Australia as a test species for environmental toxicology research. We determined concentration–response profiles for selected agonists and antagonists of rainbowfish ARα and ARβ using transient transactivation assays. For both ARα and ARβ, the order of potency of natural agonists was 11-ketotestosterone (11-KT) > 5α-dihydrotestosterone > testosterone > androstenedione. Methyltestosterone was a highly potent agonist of both receptors relative to 11-KT. The relative potency of the veterinary growth-promoting androgen, 17β-trenbolone, varied by more than a factor of 5 between ARα and ARβ. The non-steroidal anti-androgen bicalutamide exhibited high inhibitory potency relative to the structurally related model anti-androgen, flutamide. The inhibitory potency of the agricultural fungicide, vinclozolin, was approximately 1.7-fold relative to flutamide for ARα, but over 20-fold in the case of ARβ. Fluorescent protein tagging of ARs showed that the rainbowfish ARα subtype is constitutively localised to the nucleus, while ARβ is cytoplasmic in the absence of ligand, an observation which agrees with the reported subcellular localisation of AR subtypes from other teleost species. Collectively, these data suggest that *M. fluviatilis* ARα and ARβ respond differently to environmental AR modulators and that *in vivo* sensitivity to contaminants may depend on the tissue distribution of the AR subtypes at the time of exposure.

1. Introduction

Classical vertebrate androgen receptors (ARs) are members of the nuclear receptor (NR) superfamily (Mangelsdorf et al., 1995) that function as ligand-activated transcription factors regulating the expression of a large number of target genes via androgen response elements (AREs) usually located in the proximal promoter region (Bolton et al., 2007). Signalling pathways mediated by androgen receptors (ARs) are essential for sexual differentiation in vertebrates (MacLean et al., 2008; Matsumoto et al., 2005; Ogino et al., 2011) and are required for early development of the male reproductive system (Murashima et al., 2011).

In teleost fishes, androgens mediate male sexual differentiation, spermatogenesis, and reproductive behaviour (reviewed in Borg (1994)). Contaminants with androgenic or anti-androgenic activity occur in aquatic environments impacted by human activity and can alter sexual dimorphism in fish. For example, male secondary sex characteristics can develop in female fish exposed to pulp mill effluent (Parks et al., 2001) or cattle feedlot runoff (Orlando et al., 2004), while male behavioural traits are attenuated in fish exposed to environmental anti-androgens such as some pesticides and fungicides (Aït-Aïssa et al., 2010; Bayley et al., 2002; Jolly et al., 2009; Sebire et al., 2009). Some agricultural fungicides such as vinclozolin (VIN) can competitively bind to ARs and inhibit receptor activation by natural androgens (Molina-Molina et al., 2006).

In contrast to most vertebrate genomes, which contain a single *AR* gene, two distinct *AR* genes or transcripts have been identified in a number of teleosts (Douard et al., 2008; Ogino et al., 2009). Like many paralogous gene pairs present in teleost genomes, the duplicated *AR* genes are thought to have arisen from an ancestral teleost-specific whole genome duplication (WGD) that occurred around 300 million years ago and contributed strongly to species diversification (Chiu et al., 2004; Hoegg et al., 2004; Jaillon et al., 2004). In some lineages such as the otomorphata, one copy of the *AR* gene was subsequently lost, while in others the duplicated

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copies were maintained. In salmonids, it has been postulated that one copy of the duplicated *AR* gene was lost but the other duplicated again in an additional round of WGD (Douard et al., 2008), which has been dated to approximately 60 million years ago (Crête-Lafrenière et al., 2012). In the acanthomorphata, two *AR* genes were maintained and the receptor subtypes are now most commonly referred to as ARα and ARβ. The subtype designated ARβ is more closely related to AR from mammals, birds and reptiles, while ARα exhibits sequence divergence indicative of possible functional differentiation (Douard et al., 2008; Ogino et al., 2009). Although differences in transactivation potency have been reported in response to androgens (Ogino et al., 2009) differential roles for the subtypes *in vivo* are yet to be confirmed.

The binding potency of natural or synthetic ligands has been characterised for some fish ARs, and transactivation of reporter genes in response to androgens has been experimentally confirmed for ARs from a few fish species. Reporter gene transactivation assays are preferable for determining the potency of receptor agonists, since the activation of gene expression by nuclear receptors relies on a number of events occurring downstream of receptor binding. An AR from three-spined stickleback was the first receptor to be confirmed to be preferentially activated by natural fish androgen, 11-ketotestosterone (11-KT) (Olsson et al., 2005). The authors showed that various steroids bound the receptor with different affinities using binding competition analysis, but the use of transactivation assays revealed that stickleback AR was more potently activated by 11-KT than other androgens. The affinity of various agonists and antagonists to fathead minnow and rainbow trout ARs was determined by performing binding competition studies using the synthetic androgen R1881 and compared to the human AR (Wilson et al., 2007). While these studies showed that the relative binding affinities of the selected ligands, including agonists and antagonists, correlates well between ARs from different species, the potency of the ligands was not determined using transactivation assays. Ogino et al. (2009) compared ligand potency in transactivation assays using ARs from teleosts, basal ray-finned fishes and a shark species. This study revealed differences in transactivation responses not only between ARs from different fish lineages, but also between different subtypes from the same species (e.g. western mosquitofish ARα and ARβ). Interestingly, mosquitofish ARα, which has diverged considerably from teleost ARβ and tetrapod AR in terms of amino acid sequence, displayed markedly higher transactivation potential than ARβ in the assays employed in the study (Ogino et al., 2009). Antagonism of teleost ARs by the non-steroidal anti-androgen, flutamide (FLT), was also demonstrated (Ogino et al., 2009), however, quantitative measures of ligand potency such as median effect concentrations (EC50s) were not reported for either agonists or antagonists.

The Murray–Darling rainbowfish (*Melanotaenia fluviatilis*) has been established as a laboratory test species for toxicological endpoints including non-specific narcosis due to hydrocarbon exposure (Pollino and Holdway, 2002a,b), estrogenic endocrine disruption (Pollino et al., 2007; Woods and Kumar, 2011; Woods et al., 2009), reproductive toxicity (Bhatia et al., 2013; Bhatia et al., 2014b) and anti-androgenicity (Bhatia et al., 2014a). Biomarkers for estrogen exposure have been validated in *M. fluviatilis*, including two estrogen receptors (Woods et al., 2009), and vitellogenin (Woods and Kumar, 2011). Gene expression assays for androgen receptors have also been developed based on partial cDNA sequences (Bhatia et al., 2014a). However, no sex steroid receptors from this species have yet been characterised in terms of ligand selectivity.

In the present study, we cloned complete protein-coding regions for two AR subtypes from *M. fluviatilis* and assigned them as ARα and ARβ according to a phylogenetic analysis conducted using representative teleost ARs. We established concentration–response relationships for six androgens and three anti-androgens using receptor transactivation assays. The results indicate variability in the sensitivity of ARα and ARβ in response to environmental AR modulators.

2. Materials and methods

2.1. Isolation and cloning of rainbowfish androgen receptor coding sequences

The entire protein coding region of AR cDNAs were amplified by RT-PCR using the primers for ARα, RF-ARα-ATG-S1: 5′-ATGG CCTTTCACTCGAGCTTGTC-3′ and RF-ARα-TGA-R1: 5′-CTAGGCTG-TATTGTGGAA AAGGATTGG-3′, for ARβ, RF-ARβ-ATG-S1: 5′-ATGAGCCAAACTGACCGACAGTTATC-3′ and RF-ARβ-TGA-R1: 5′-CTATTTGTGGAACAAGATTGGTTTAGCC-3′. The entire protein coding region of AR cDNAs lacking the terminal codon were amplified using the following reverse primers, RF-ARα-TGA-R3: 5′-GGCTGTATTGTGGAAAAGGATTGGCTT-3′ for ARα and RF-ARβ-TGA-R3: 5′-TTTGTGGAACAAGATTGGTTTAGCCAAACC for ARβ. To generate the expression plasmids encoding AR and DsRed monomer fusion protein, amplified cDNAs were first cloned into pGEM-T Easy vector (Promega, Tokyo, Japan) and the expression vectors subsequently constructed by inserting AR coding sequences into the CMV-driven expression vector, pCS2 + MT (Rupp et al., 1994), or pDsRed monomer-N1 (Life Technologies) via EcoRI sites. The *M. fluviatilis* ARα and ARβ coding sequences have been deposited in NCBI GenBank under accession numbers KM485682 and KM485683, respectively.

2.2. Phylogenetic analysis

To confirm the rainbowfish AR subtypes as ARα and ARβ, a phylogenetic analysis of representative teleost ARs was performed on amino acid sequences aligned using CLUSTALW (Thompson et al., 1994), excluding positions with a gap. Sequences selected for the analysis were restricted to full-length ARα and ARβ amino acid sequences from teleost species used as laboratory or field test organisms in aquatic toxicology, or from ancestral fish lineages used in prior phylogenetic studies of fish ARs (Ogino et al., 2009). Molecular phylogeny was inferred by the neighbor-joining method (Saitou and Nei, 1987) based on Jukes–Cantor distances (Jukes and Cantor, 1969). Confidence in the phylogeny assessed by bootstrap resampling of the data (x1000) (Felsenstein, 1985). Names of the species used in these analyses and their accession numbers retrieved from GenBank were shown in Supplemental Table S1.

2.3. Transactivation assays

AR transactivation assays were conducted essentially as previously described (Ogino et al., 2009). Briefly, COS-7 cells were seeded into the wells of 24-well plates at a density of 10,000 cells per well in 0.5 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dextran-coated charcoal-stripped fetal bovine serum (DCC-FBS; HyClone/GE, Tokyo, Japan), and incubated for approximately 72 h at 37 °C under a humidified 5% CO_2 atmosphere. Cells were then co-transfected with an expression construct encoding rainbowfish ARα or ARβ, a firefly luciferase reporter construct driven by four AREs (Matsui et al., 2002), and a *Renilla* luciferase reporter under the control of a constitutive promoter (pRL-SV40; Promega, Tokyo, Japan) as an internal control. Transfection complexes were prepared in serum-free DMEM (Sigma–Aldrich, Tokyo, Japan) using TransFast® reagent (Promega, Tokyo, Japan) and 0.2 mL was added directly to each well after removal of the growth medium. After incubating for 1 h, 1 mL