

Adverse outcome pathway linking nuclear receptor overactivation to feeding disruption

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Background

- Retinoid signaling disruption is an **overlooked** mechanism of **endocrine disruption** [1].
- Exposure to **triazole fungicide** and samples with **retinoid-like activities** from Czech reservoirs has been associated with **teratogenic effect** related to retinoid signaling disruption [2,3,4].
- Only few **Adverse Outcome Pathway (AOP)** related to retinoid signaling disruption [5].
- Lack of data linking ATRA-induced **craniofacial malformation (CFM)** and/or **uninflated posterior swim bladder** to **survival**
- No data linking the prototypical stressor **all-trans-retinoic acid (ATRA)** to **posterior swim bladder (post. SB)** non inflation

Objectives

- Determination of critical **window of sensitivity** for adverse developmental effects (craniofacial malformation and non inflation of posterior swim bladder)
- Linking malformation to **feeding disruption** and **survival**
- For regulatory purpose, determination of a **threshold** as **Retinoid Equivalent (REQ)** above which there is an effect at population level
- Identification of a potential **cross-talk** between **thyroid hormone** and **retinoid signaling disruption**

Methodology

Determination of Molecular initiating event

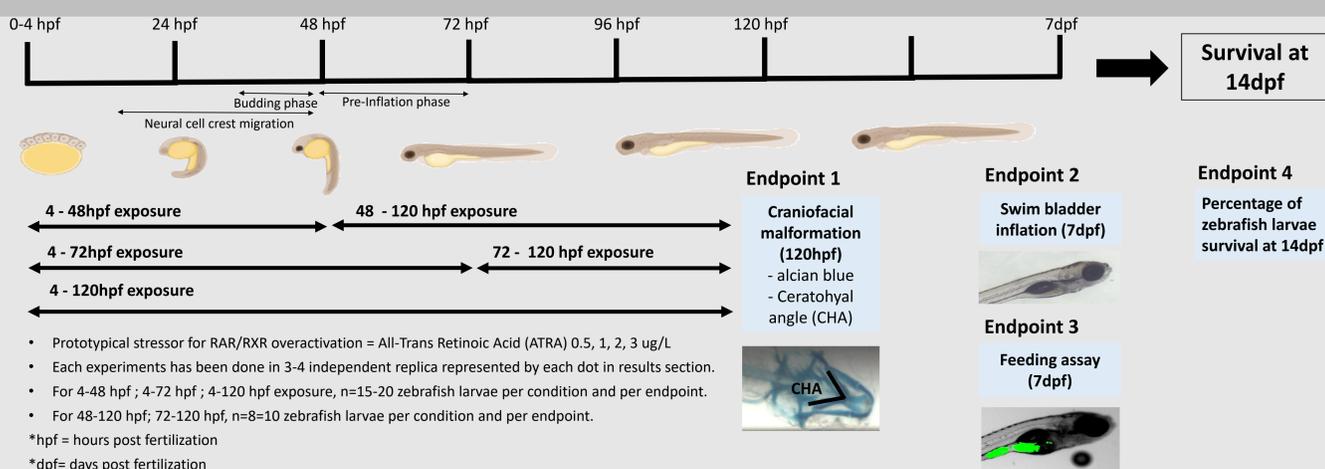
Antagonists of retinoid receptors RAR, RXR and Thyroid hormone Receptor (TR) were used to examine their role in malformation. If co-exposure of ATRA 2ug/L and antagonist **rescue the malformation** (i.e., no more malformation), it means that malformation **occurs through this specific receptor**.

Window of sensitivity

Exposure at different exposure times **inside and outside potential critical periods**. If no malformation occurs outside the hypothesized critical period, it confirms the window of sensitivity

Linking malformations to feeding disruption and survival

Exposure at different ATRA concentrations and exposure times. **Craniofacial malformation (5dpf)**, **posterior swim bladder inflation (7dpf)**, and **feeding (7dpf)** were assessed. **IC50-concentration** inducing 50% inhibition- for feeding assay, was determined. Afterward, **survival** was assessed at a later larval stage (14dpf) using the previous data.



Results

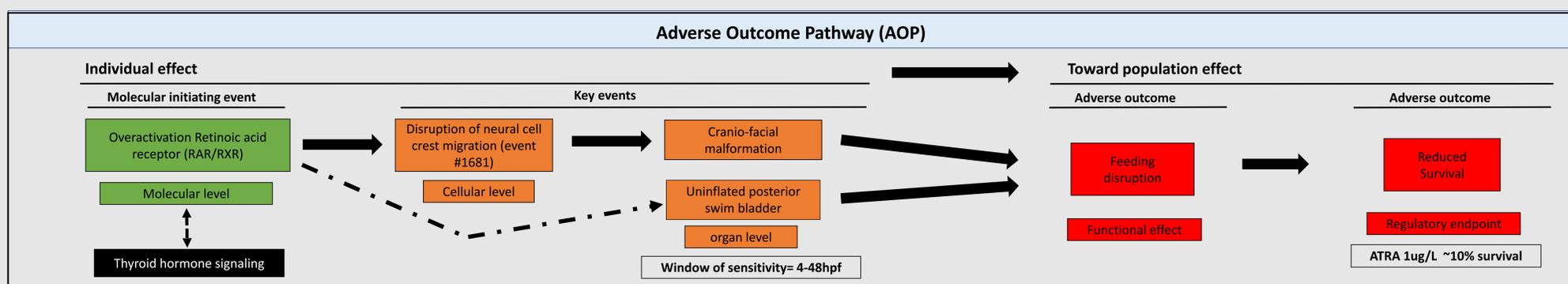
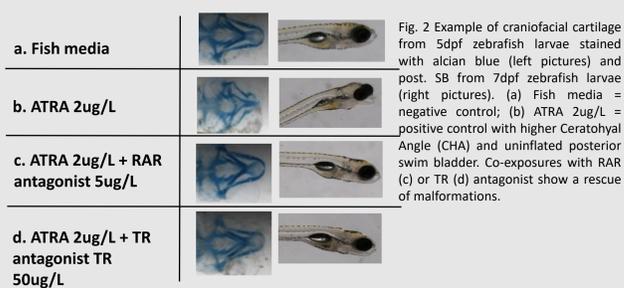


Fig. 1 Putative AOP linking Overactivation of Retinoic Acid Receptor (RAR) / Retinoid X Receptor (RXR) to feeding disruption and reduced survival. Plain arrows indicate moderate to high confidence for the Key Event Relationship (the link between two key events) supported by both scientific literature and the presented experimental data. Dashed arrows indicate low confidence supported by the presented experimental data but with low or no existing scientific literature.

Determination of Molecular initiating event

Craniofacial malformation (CFM) and post. swim bladder (post. SB) non-inflation are **rescued by RAR and TR antagonist**.



Window of sensitivity

For craniofacial malformation and post. SB non-inflation, **4-48hpf is the critical window of sensitivity**.

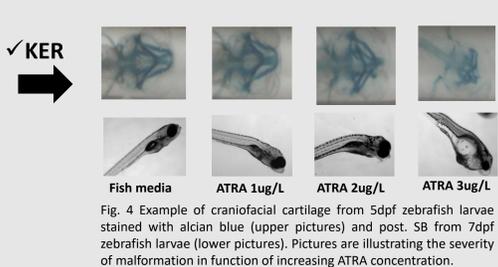


Fig. 4 Example of craniofacial cartilage from 5dpf zebrafish larvae stained with alcian blue (upper pictures) and post. SB from 7dpf zebrafish larvae (lower pictures). Pictures are illustrating the severity of malformation in function of increasing ATRA concentration.

Linking malformations to feeding disruption

IC₅₀ Feeding ≈ EC₅₀ uninflated post. SB ≈ appearance of CHA enlargement ≈ **ATRA 1ug/L**

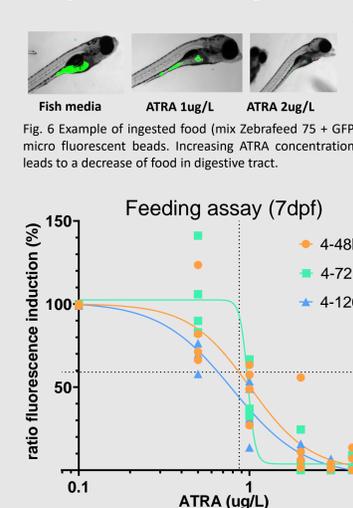


Fig. 5 Each dot represents an independent replica with 15-20 zebrafish larvae (colored curves) and 8-10 larvae (black curve). Concentration-response curves were plotted using Graphpad PRISM 9. Concentration-response curve of (a) measured CHA at 5dpf [Ratio CHA enlargement = CHA(treatment)/CHA (Fish media)] and (b) % Larvae with uninflated post. SB at 7dpf, EC50 was derived as 50 % of larvae affected for non-inflation of post. SB.

Linking feeding disruption to survival

Malformations contributing to feeding disruption at ATRA 1ug/L (7dpf) is translated to **~10% survival at 14dpf**.

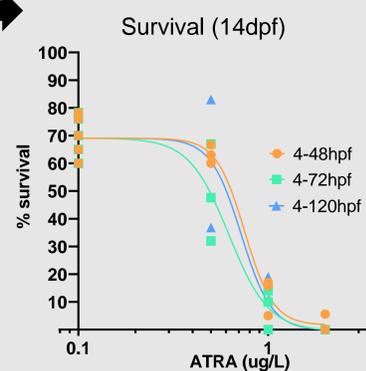


Fig. 6 Each dot represents an independent replica with 15-20 larvae. Ratio fluorescence induction represents the food intake.

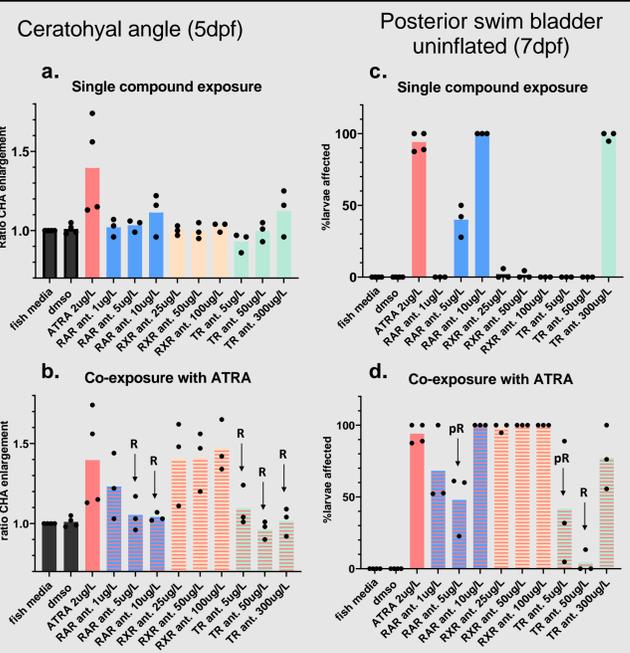


Fig. 3 Each dot represents an independent replica with 15-20 zebrafish larvae. (a-b) Ceratohyal angle (CHA) was measured in 5dpf zebrafish. Ratio CHA enlargement = CHA(treatment)/CHA (Fish media). (c-d) Inflation of posterior swim bladder was assessed as "inflated" and "non-inflated" in 7dpf zebrafish. % Larvae affected is the larvae frequency with uninflated post. SB. The positive control is ATRA 2ug/L (red bar). A decrease of larvae with malformation in co-exposure (b. and d.) indicates a total rescue (R) or partial Rescue (pR). Abbreviation Ant. = Antagonist; RAR = Retinoic Acid Receptor; RXR = Retinoid X Receptor; TR = Thyroid Receptor.

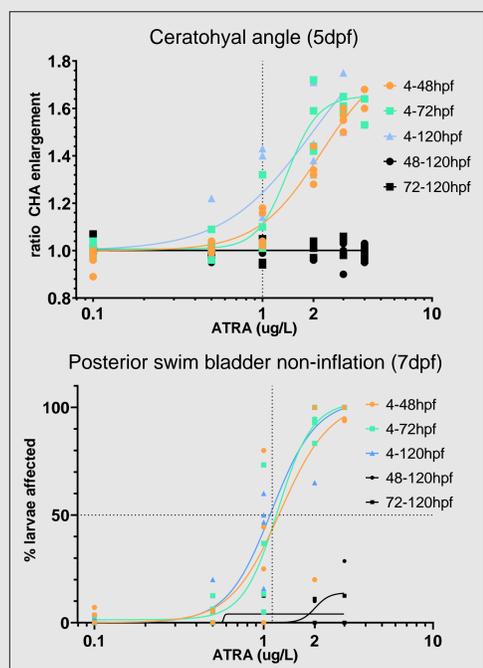


Fig. 5 Each dot represents an independent replica with 15-20 zebrafish larvae (colored curves) and 8-10 larvae (black curve). Concentration-response curves were plotted using Graphpad PRISM 9. Concentration-response curve of (a) measured CHA at 5dpf [Ratio CHA enlargement = CHA(treatment)/CHA (Fish media)] and (b) % Larvae with uninflated post. SB at 7dpf, EC50 was derived as 50 % of larvae affected for non-inflation of post. SB.

Conclusion

- RAR/RXR overactivation leads to
 - craniofacial malformation
 - uninflated posterior swim bladder
- Window of sensitivity for both craniofacial malformation and non-inflation of posterior swim bladder = **4-48hpf**
- AOP : Key Event Relationships (KER) from RAR/RXR overactivation malformation to feeding disruption and survival with quantitative data
- Retinoid Equivalent Threshold for adverse effects = **ATRA 1ug/L**
- Confirmation of cross-talk between thyroid hormone and retinoic acid signaling