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In Silico Study of *Haematococcus pluvialis* Biomarker Compound as Supplement to Fish Bone Remodelling

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ABSTRACT

This study aims to determine the docking predictions for RAR, RXR, and ROR in the bone remodeling pathway using Haematococcus, which is the highest carotenoid-producing microalgae. Furthermore, it determines the projection of using this carotenoid-producing alga in bone development. Carotenoids include provitamin A and non-provitamin A, which are predicted to replace vitamin A in bone control. It is also required *in silico* proof of carotenoids' function of bone remodeling control. Furthermore, molecular and visualization docking validation was conducted using PyRx and Discovery Studio Visualizer software respectively. According to binding affinity and RMSD value, each biomarker compound had particular binding sites on RARα, RARβ, RORβ, and RORγ. Astaxanthin was the only compound with binding sites on all four receptors. Through enzymatic action, provitamin A carotenoids can serve as a precursor to retinol, allowing them to act as a native RXR ligand. Therefore, the biomarker compound used in *Haematococcus pluvialis* can replace the role of vitamin A in the regulation of fish bones. The prediction of bone regulation in biomarker compounds through the RAR-RXR pathways inhibited osteoblast and osteclast. Otherwise, VDR-RXR pathways regulated osteoclast maturation and osteoblast mineralization.

1. Introduction

The idea behind a culture is to exploit manipulated environmental factors and mimic natural conditions to keep fish larvae and seeds alive and healthy. However, there are challenges with the advancement of fish farming technologies, one of which is the high prevalence of bone malformations. These bone complications are common in cultivated fish, including grouper (Nagano *et al.* 2007), sea bream (Andrades *et al.* 1996), snapper (Chatain 1994; Fraser and De Nys 2005), fish flounder (Gavaia *et al.* 2009), and a variety of other species. Since 1982, the incidence of bone malformations in cultured fish has been discovered in zebrafish (Danio rerio) (Newsome and Piron 1982). The problem of bone malformation is still a major concern, particularly in types of fish consumption. Furthermore, losses are caused by the high proportion of bone malformations, and this is attributed to the high mortality caused by disrupted natural larval movement, which affects the rate of predation and normal larval growth (Başaran *et al.* 2009).

Vitamin A is an essential nutrient highly required in bone growth. By regulating chondrocyte activity, cell maturation, and proliferation, vitamin A regulates skeletogenesis and cartilage growth (Kochhar 1973; Harada *et al.* 1995; Koyama *et al.* 1999). There are some inconsistencies in the administration of vitamin A to the body. Since the deficiency may interrupt organogenesis (See *et al.* 2008) and excess may induce deformity in fish (Haga 2002), vitamin A should be supplied in a controlled dosage.

The administration of a single compound in the treatment of illness is very successful, but it has side effects when used for an extended period. A dietary solution based on raw materials containing some active ingredients can affect the body's processes. Carotenoids are vitamin A precursors needed in the visual cycle and gene control of many developmental and physiological processes (Grune *et al.* 2010). Furthermore, carotenoids are C40 terpenoids, that are part of the hydrocarbon class synthesized by all photosynthetic species (Bacteria, fungi, and plants) (Nisar *et al.* 2015). Under stressful conditions,

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Haematococcus pluvialis is a freshwater microalga from the Chlorophyceae family that produces carotenoids (Shah *et al*. 2016). Also, the carotenoids with high concentrations in *H. pluvialis* include lutein, zeaxanthin, violaxanthin, carotene, and carotene (Rangga *et al* 2009). Astaxanthin is the most abundant in *H. pluvialis*, accounting for 70%, 30%, and 4% of the monoester, diester, and free structure respectively (Johnson and An 1991). Astaxanthin has been widely used as an antioxidant and radical scavenger (Naguib 2000; Dose *et al.* 2016). However, the involvement of astaxanthin or other carotenoid compounds in bone modulation has not been studied.

Retinoic acid receptor (RAR) and retinoic X receptor (RXR) are nuclear receptors that bind to DNA, regulating cellular functions such as growth, differentiation, and metabolism. Furthermore, both RAR and RXR have 3 subtypes α, β, γ (Le Maire *et al.* 2019; de Almeida and Conda-Sheridan 2019). Vitamin A activation is regulated by binding with retinoic acid (RARs) and X receptors (RXRs). They are activated by two stereoisomers of retinoic acid, both trans and cis retinoic acids. RXR and RAR act as transcription regulators. RAR-agonist activation was conducted through heterodimerization with RXR (Zhang *et al*. 2011). Meanwhile, RXR can act as a homodimer on its own (Menéndez-Gutiérrez and Ricote 2017), and through heterodimerization with other nuclear receptors, it regulates osteoclastogenesis and bone remodeling (bone formation) (NR). Furthermore, the RXR forms heterodimers with RAR, VDR, and thyroid hormone receptors (TR). These interactions that occur are permissive to RXR, and it does not react to the ligand (Forman *et al*. 1995) but runs up to a certain context for the ligand partner (Li *et al*. 2002; Castillo *et al*. 2004).

In addition to RAR and RXR, retinoids can bind to retinoid-related orphan receptors (RORs), which do not form heterodimers with RXR. Furthermore, ROR controls gene expression by forming monomers that bind to the reaction elements (ROREs) in the promoter region (Jetten 2009). RORs are also made up of three isoforms α, β, γ, and are believed to bind to retinoids. ROR β in cultivated murine osteoblasts has been shown to eliminate mineralization and to decrease osteocalcin and osterix mRNA expression (Roforth *et al.* 2012). ROR is also linked to the metabolism of osteoblasts, and mice lacking it have irregular bone growth (Benderdour *et al.* 2011).

The function of carotenoids in bone regulation can be illustrated *in silico*. Furthermore, the docking of the compounds on *H. pluvialis* was accomplished using RARs (α, β, γ), RXRα (receptors in the liver and kidney), and RORs (α, β, γ). This study aims to predict the docking relationship between *H. pluvialis* biomarker compounds (astaxanthin, lutein, zeaxanthin, violaxanthin, carotene, and carotene) and RARs (α, β, γ), RXR (receptor in liver and kidney), RORs (α, β, γ). Also, it determines the projection of using Haematococcus in bone development.

2. Materials and Methods

2.1. Molecule Structure Preparation

RARα (NDB: 3KMR), RARβ (NDB: 4DM8), RARγ (NDB: 2LDB), RXRα (NDB: 4ZSH), RORα (NDB: 1N83) RORβ (NDB: 1N4H), and RORγ (NDB: 5K38) were used as macromolecules (Figure 1). Furthermore, water and congenital ligan should be eliminated before docking. All trans (PubChem CID: 444795) and 9 cis retinoic acid (PubChem CID: 449171) were use as native ligan (Figure 2). Astaxanthin (PubChem CID: 5281224), lutein (PubChem CID: 5281243), zeaxanthin (PubChem CID: 5280899), violaxanthin (PubChem CID: 448438), α carotene (PubChem CID: 6419725), β carotene (PubChem CID: 5280489) were also used as ligan candidate (Table 1). The open babel integrated into the Pyrx program was used to convert the SDF format of the ligand structure to a PDB file. It was also used in minimizing free energy in the ligand structure.

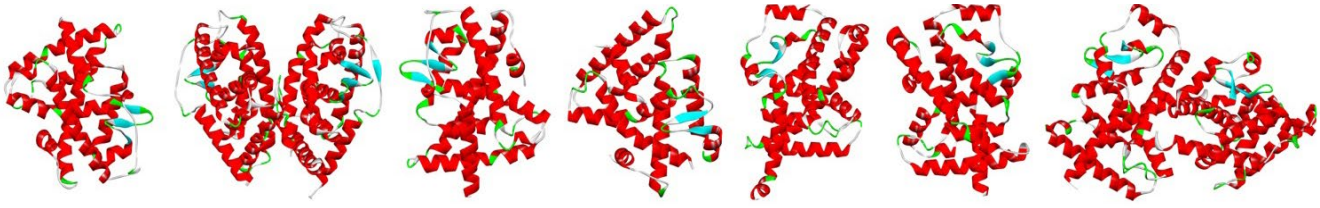


Figure 1. Molecule structure of retinoic acid receptor (RARs), retinoic X receptor (RXR) and retinoid-related orphan receptors (RORs). Crystal structure of RARα (A), RARβ (B), RARγ (C), RXRα (D), RORα (E), RORβ (F), and RORγ (G)

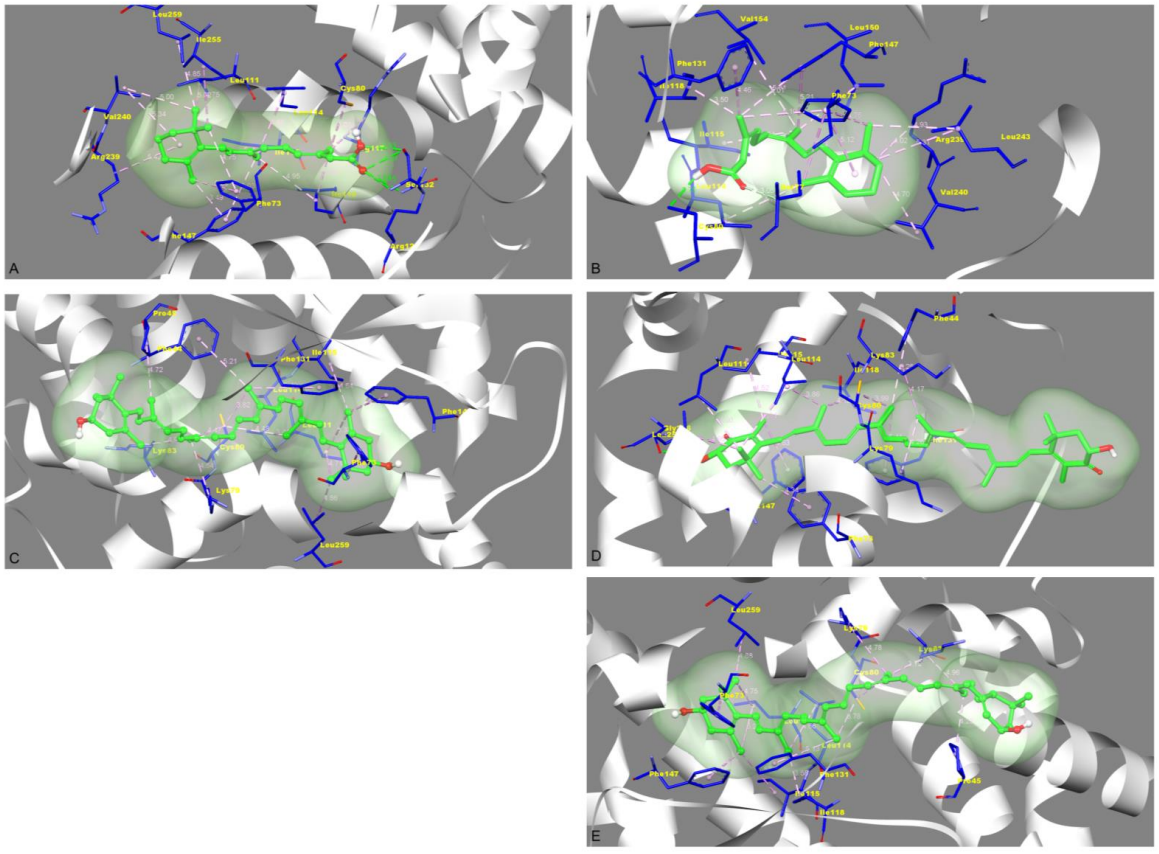
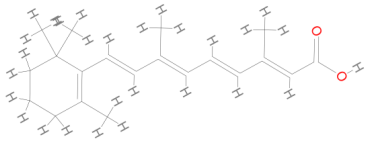
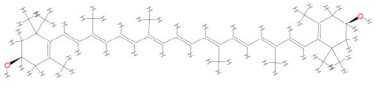
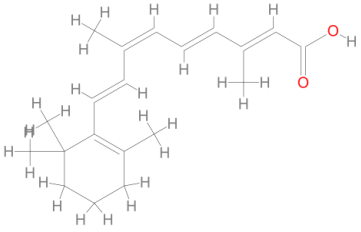
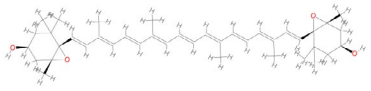
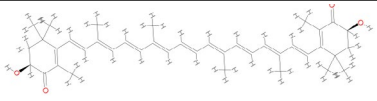
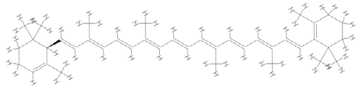
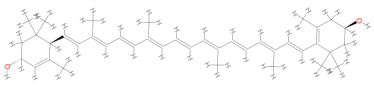
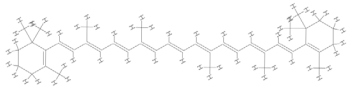


Figure 2. Molecular docking interaction of RARα-predicted ligand candidate (zeaxanthin (C), astaxanthin (D) and lutein (E)) and native ligand (all trans retinoic acid (A) and 9 cis retinoic acid (B)) as comparison. Note: ---: Alkyl bound type, ---: hydrogen bound type.

Table 1. The structure of native and targeted ligand candidate. Native ligand structure of all-trans retinoic acid (A) and 9 cis retinoic acid (B). Candidate targeted ligand structure of astaxanthin ($C_{40}H_{52}O_4$) (C), lutein ($C_{40}H_{56}O_2$) (D), zeaxanthin ($C_{40}H_{56}O_2$) (E), violaxanthin ($C_{40}H_{56}O_4$), α carotene ($C_{40}H_{56}$), β carotene ($C_{40}H_{56}$)

Ligand name	Ligand structure	Ligand name	Ligand structure
All-rans retinoic acid		Zeaxanthin	
9 cis retinoic acid		Violaxanthin	
Astaxanthin		α carotene	
Lutein		β carotene	

2.2. Molecular Docking Validation

Molecular and visualization docking validation were conducted using PyRx and Discovery Studio Visualizer software. The docking validation result was confirmed using RMSD scoring and was approved when the RMSD scoring was less than or equal to two (Kartasasmita *et al.* 2009).

3. Results

Astaxanthin is the only compound with active binding sites on RARα, RARβ, RORβ, and RORγ based on binding affinity energy and RMSD value (Table 2). Some substances have active binding sites on specific receptors. Furthermore, biomarkers, such as astaxanthin, zeaxanthin, lutein, and carotene, need a lot of energy to dock with RARγ, RXRα, and RORα. In contrast, some do not have active binding sites on RARγ, RXRα, and RORα. Therefore, carotenoid compounds lack an active site in RARγ, RXRα, and RORα.

Zeaxanthin, astaxanthin, and lutein domain site prediction in RAR is in the B domain (Figure 2), and the forms of alkyl and pi-alkyl bonds in each compound control the binding to amino acid residues (Table 3). In addition, hydrogen bonds are only found in native ligands and astaxanthin compounds.

The domain sites of violaxanthin and astaxanthin compounds in RAR are predicted to be A and B. Meanwhile, zeaxanthin compounds are found in the site A domain, while carotenoids are found in site B (Figure 3, Table 4). The native ligand of all-trans retinoic acid domain sites is on B. Alkyl and pi-alkyl bonds dominate the relations between amino acid residues and biomarker compounds (Table 4). The amino acid residues in astaxanthin, zeaxanthin, and native ligand, which are all-trans retinoic acid compounds, contain hydrogen bonds.

Astaxanthin, α carotene, and β carotene domain site prediction in RORβ is in A site (Figure 4, Table 5). Alkyl and pi-alkyl bonds dominate the bonds formed between the amino acid residue and the ligand. All the amino acid residues in astaxanthin and all-trans retinoic acid compounds contain hydrogen bonds, and a pi-sigma bond was in the residue of β carotene–RORβ.

Table 2. The binding affinity of native ligand (all trans retinoic acid and 9 cis retinoic acid) and *H. pluvialis* biomarker (astaxanthin, lutein, zeaxanthin, violaxanthin, α carotene, β carotene)

Receptor	Parameter	Native ligand		Biomarker					
		All trans retinoic acid	9 cis retinoic acid	Astax.	Zeax.	Viola.	Lutein	α carotene	β carotene
RARα (3KMR)	Binding Affinity	-10.3	-9.8	-8.5*	-8.6*	-6.6	-8.5*	-6.5	-9.3
	RMSD/UB	2.377	2.512	2.544*	2.776*	20.777	2.222*	3.931	17.904
	RMSD/LB	1.083	1.93	1.959*	2.103*	11.713	1.64*	1.62	1.317
RARβ (4DM8)	Binding Affinity	-11.1	-7.9	-7.8*	-8*	-8.5*	-7.4	-7.8*	-8.3
	RMSD/UB	2.432	4.044	2.71*	2.687*	2.05*	3.969	2.787*	38.499
	RMSD/LB	1.087	2.32	1.425*	1.717*	1.403*	2.283	1.679*	33.008
RARγ (2LBD)	Binding Affinity	-11.2	-8	-4	-4	-	-4.9	-	-2.8
	RMSD/UB	2.284	2.149	19.135	19.135	-	2.273	-	18.271
	RMSD/LB	0.724	0.99	0.984	0.984	-	1.457	-	1.636
RXRα (4ZSH)	Binding Affinity	-8.5	-8.9	10.1	-	-	11.9	-	-
	RMSD/UB	2.249	2.51	1.655	-	-	1.428	-	-
	RMSD/LB	0.924	0.94	0.519	-	-	1.204	-	-
RORα (1N83)	Binding Affinity	-8.7	-8.5	0.7	-	-	-2.4	-3.8	-4.5
	RMSD/UB	2.862	2.223	1.941	-	-	2.082	17.857	17.894
	RMSD/LB	1.618	1.534	0.868	-	-	1.57	1.128	0.641
RORβ (1N4H)	Binding Affinity	-9.4	-8.6	-7.6*	-7.5	-7.5	-7.9	-7.7*	-7.3*
	RMSD/UB	1.946	4.156	2.624*	6.113	18.716	26.789	2.035*	2.408*
	RMSD/LB	1.248	2.15	1.921*	3.028	1.084	20.519	1.082*	1.381*
RORγ (5K38)	Binding Affinity	-7.3	-6.6	-8*	-8	-8*	-7.8*	-8.1	-7.9
	RMSD/UB	5.213	2.341	2.488*	4.306	2.533*	2.078*	14.294	7.494
	RMSD/LB	4.528	1.845	1.458*	2.093	1.613*	1.307*	7.296	6.101

Astax: Astaxanthin, Zeax: Zeaxanthin, Viola: Violaxanthin, *: value close to that of the natural ligand

Table 3. Domain site, amino acid residu and bound type of RARα and predicted ligand candidate interaction (zeaxanthin, astaxanthin, lutein)

Molecule interaction	Domain site	Amino acid residu	Bound type
RARα-Lutein	B	LYS79, CYS80, LYS83, PRO45, LYS83, CYS80, LEU114, ILE118, LEU111, LEU259, ILE115	Alkyl
		PHE73, PHE131, PHE147	Pi-Alkyl
RARα-Astaxanthin	B	GLY236	Hydrogen bond
		CYS80, LEU114, ILE118, LEU111, LEU114, LEU259, ILE115, LYS79, LYS83	Alkyl
		PHE44, PHE73, PHE131, PHE147	Pi-Alkyl
RARα-zeaxanthin	B	CYS80, LEU114, LEU111, LEU259, ILE115, LYS79, CYS80, LYS83, PRO45	Alkyl
		PHE44, PHE73, PHE131, PHE147	Pi-Alkyl
RARα-9 cis retinoic acid	B	CYS80:SG	Hydrogen bond
		SER77	Carbon hydrogen bond
		ILE115, LEU150, VAL154, ARG239, VAL240, LEU243, LEU114, ILE118	Alkyl
		PHE73, PHE131, PHE147	Pi-Alkyl
RARα-all trans retinoic acid	B	ARG121, SER132	Hydrogen bond
		ARG239, VAL240, LEU114, ILE115, ILE118, ILE255, LEU111, VAL240, LEU259, CYS80, ARG117, ILE118	Alkyl
		PHE73, PHE147	Pi-Alkyl
RARα-all trans retinoic acid	B	ARG121, SER132	Hydrogen bond
		ARG239, VAL240, LEU114, ILE115, ILE118, ILE255, LEU111, VAL240, LEU259, CYS80, ARG117, ILE118	Alkyl
		PHE73, PHE147	Pi-Alkyl

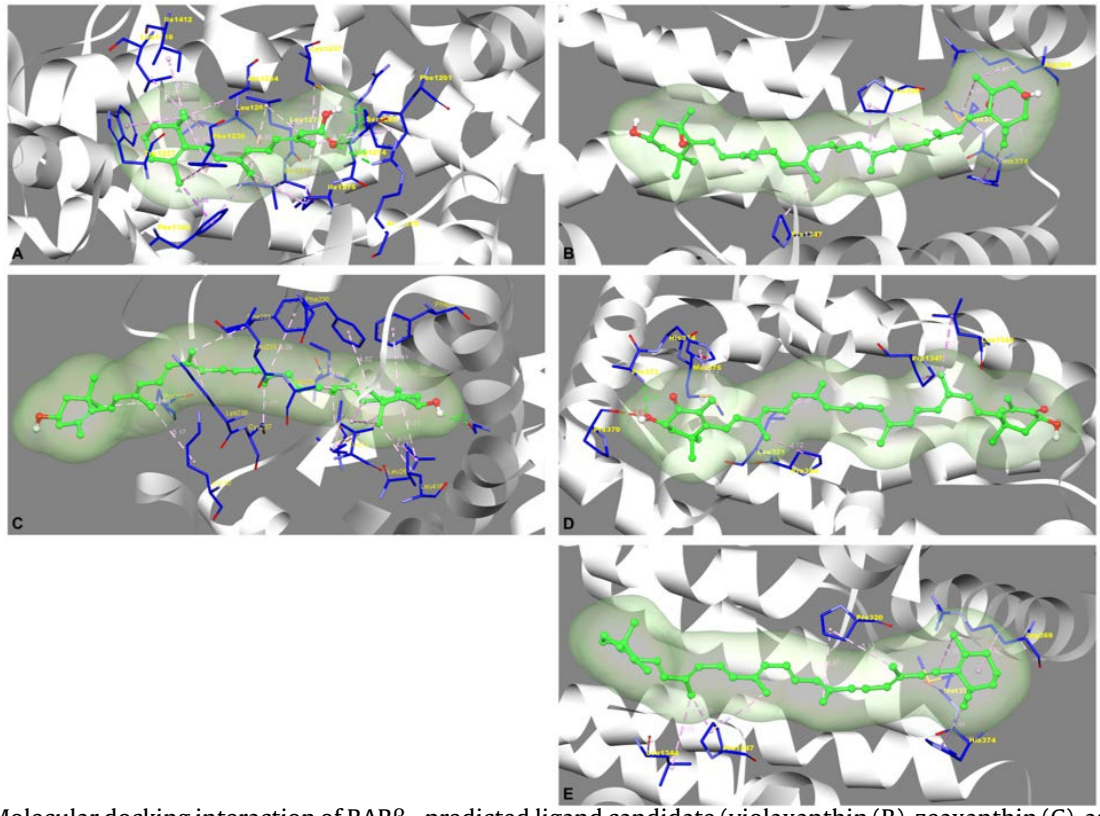


Figure 3. Molecular docking interaction of RARβ - predicted ligand candidate (violaxanthin (B), zeaxanthin (C), astaxanthin (D), and α carotene (E)) and native ligand (all trans retinoic acid (A)) as comparison. Note: ---: Alkyl bound type, ---: hydrogen bound type, ---: unfavorable bump

Table 4. Domain site, amino acid residue and bound type of RARβ and predicted ligand candidate (violaxanthin, zeaxanthin, astaxanthin, α carotene).

Molecule interaction	Domain site	Amino acid residu	Bound type
RARβ-α carotene	B	ARG369, PRO1347, LEU1344, PRO1347, PRO320, MET375 HIS374	Alkyl Pi-Alkyl
RARβ-astaxanthin	A B A A	PRO373 LEU1344, PRO1347 LEU321, PRO320, MET375 HIS374	Hydrogen Bond Alkyl Alkyl Pi-Alkyl
RARβ-zeaxanthin	A	GLY393 ALA234, LEU233, LYS236, PRO202, LYS240, CYS237, LEU271, ILE275, LEU268, LEU271, ILE412, LEU416, ILE412, LEU268 PHE230, PHE288, PHE304	Hydrogen Bond Alkyl Pi-Alkyl
RARβ-violaxanthin	A B A	PRO320, ARG369, MET375 PRO1347 HIS374	Alkyl Alkyl Pi-Alkyl
RARβ-all trans retinoic acid	B	ARG1278, SER1289 PHE1304 ALA1234, LEU1271, ILE1272, ILE1275, ILE1412, LEU1268, LEU1416, CYS1237, ARG1274, PHE1201, TRP1227, PHE1230, PHE1304	Hydrogen Bond Pi-Sigma Alkyl Pi-Alkyl

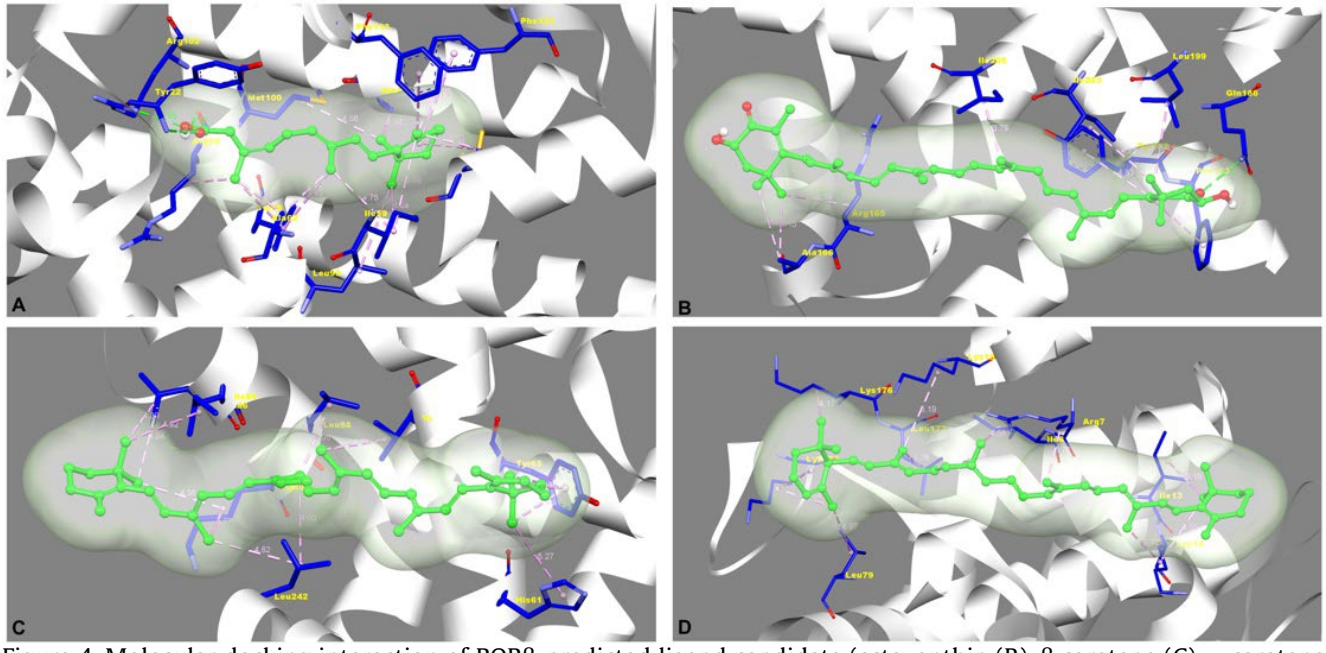


Figure 4. Molecular docking interaction of RORβ-predicted ligand candidate (astaxanthin (B), β carotene (C), α carotene (D) and native ligand (all trans retinoic acid (A)) as comparison. Note: ---: Alkyl bound type, ---: hydrogen bound type

Astaxanthin, lutein, and violaxanthin domain site prediction in RORγ was in A and B (Figure 5, Table 6), and the domain site of 9 cis retinoic acids (native ligands) was only in A. Furthermore, alkyl and pi-alkyl bonds dominated the relations between amino acid residues and biomarker compounds (Table 4). Hydrogen bonds were found in some of the amino acid residues of astaxanthin, violaxanthin, and 9 cis retinoic acid compounds. Also, a carbon-hydrogen bond was found in an amino acid residue of ROR-astaxanthin.

Table 5. Domain site, amino acid residu and bound type of RORβ and predicted ligand candidate (astaxanthin, β carotene, α carotene)

Molecule interaction	Domain site	Amino acid residu	Bound type
RORβ-Astaxanthin	A	GLN186 ALA166, ARG165, ILE206, ILE203, LEU199 TYR182, PHE183	Hydrogen Bond Alkyl Pi-Alkyl
RORβ-βcarotene	A	TYR65 VAL67, LEU88, LEU242, LYS89, ILE85, LEU86, LYS89 HIS61, TYR65	Pi-Sigma Alkyl Pi-Alkyl
RORβ-αcarotene	A	LYS173, ILE8, LEU177, LYS180, LYS176, LEU79, ARG7, LYS14, ILE13	Alkyl
RORβ-all trans retinoic acid	A	TYR22, ARG102, ARG99 CYS55, ALA62, ALA135, ILE59, VAL96, LEU93, MET100, CYS55, ARG99 PHE113	Hydrogen Bond Alkyl Pi-Alkyl

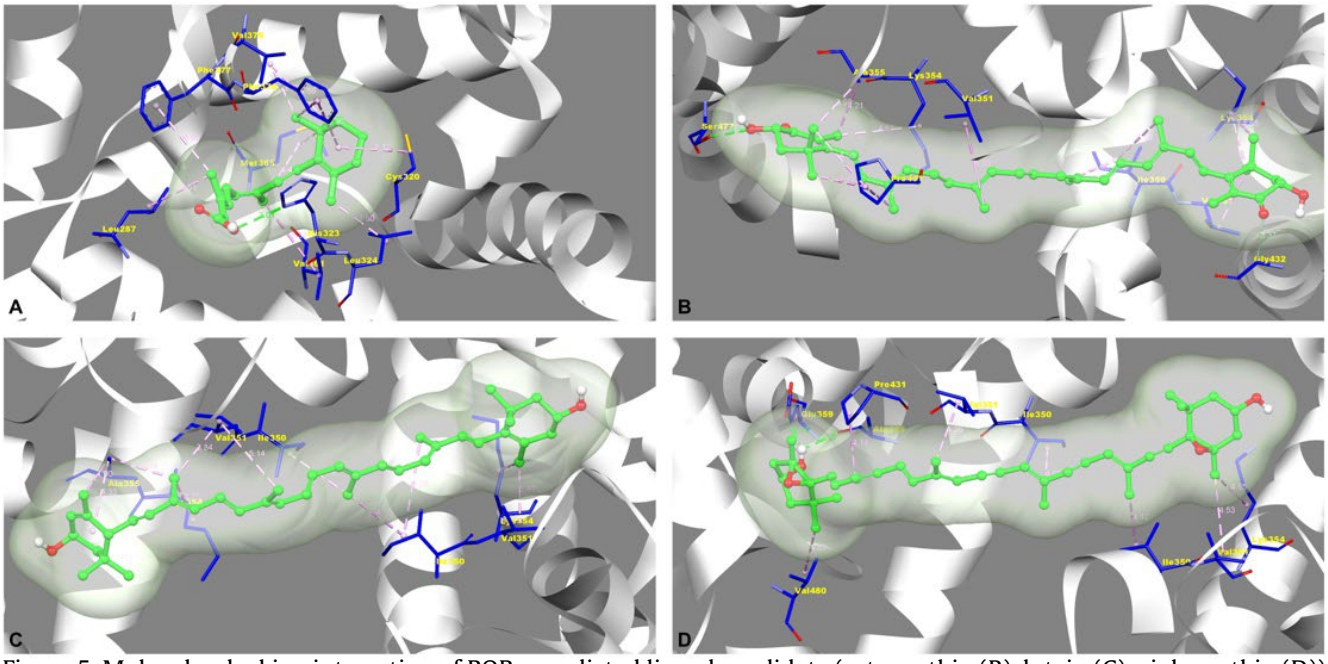


Figure 5. Molecular docking interaction of RORγ-predicted ligand candidate (astaxanthin (B), lutein (C), violaxanthin (D)) and native ligand (9 cis retinoic acid (A)) as comparison. Note: ---: Alkyl bound type, ---: hydrogen bound type

4. Discussion

Table 7 showed that biomarker compounds bind to specific receptors. The forming of dimers with RXR activates biomarkers-RARs. Since no compounds bind to RXR, only all-trans retinoic acid or 9 cis retinoic acid can be used as a ligand to activate the RXR-RAR heterodimer. Meanwhile, carotenoids are classified into provitamin A and non-provitamin A. The enzymes BCO1 convert provitamin A to all-trans retinoic acid (Figure 6A). Therefore, Haematococcus extract can be administered without the addition of vitamin A. The biomarkers of astaxanthin, zeaxanthin, lutein, violaxanthin, and α carotene as RAR ligands control heterodimer by silencing RXR activity and regulate transcription of their ligand response. Furthermore, RXR serves as a silent partner in this situation, and cannot respond to its ligand in RAR-containing heterodimers unless its heterodimeric partner is bound to an agonist (Le Maire *et al.* 2019). Heterodimers RAR-RXR function as transcription factors and activating retinoic acid respond elements

Table 6. Domain site, amino acid residu and bound type of RORγ and predicted ligand candidate (astaxanthin, violaxanthin, lutein)

Molecule interaction	Domain site	Amino acid residu	Bound type
RORγ-Astaxanthin	A	GLY432	Carbon Hydrogen Bond
	B	SER477	Hydrogen Bond
	A	ILE350, VAL351, LYS354	Alkyl
	B	ALA355, VAL351, PRO431, LYS354	Alkyl
RORγ-Violaxanthin	A	ALA355, GLU359	Hydrogen Bond
	A	VAL351, PRO431, VAL480,	
	B	ILE350	Alkyl
		ILE350, VAL351, LYS354	Alkyl
RORγ-Lutein	A	ILE350, VAL351, LYS354	Alkyl
	B	ALA355, VAL351, LYS354, ILE350	Alkyl
RORγ-9 cis retinoic acid	A	HIS323	Hydrogen Bond
		CYS320, VAL361, MET365, VAL376, LEU324, LEU287	Alkyl
		PHE377, PHE378	Pi-Alkyl

Table 7. Biomarker related specific receptor

Receptor	Biomarkers					
RARα	Astaxanthin	Zeaxanthin	-	Lutein	-	-
RARβ	Astaxanthin	Zeaxanthin	Violaxanthin	-	α carotene	-
RORβ	Astaxanthin	-	-	-	α carotene	-
RORγ	Astaxanthin	-	Violaxanthin	Lutein	-	β carotene

(RAREs) in the target genes promoter (Figure 6B) (Conaway *et al*. 2013). This type of RXR heterodimer is referred to as a non-permissive (de Almeida and Conda-Sheridan 2019), and the VDR-RXR pair is the case formed. RORβ and RORγ have binding sites for certain biomarkers (Table 7). The majority of the nuclear receptors have well-defined natural ligands, while some are labeled as an orphan since natural ligands are not well understood (Zhang *et al*. 2015). In addition, RORs regulate gene transcription by binding as monomers to ROR response elements (ROREs) in target genes and constitutively activate gene transcription (Jetten and Joo 2006). The tendency of retinoids and carotenoid compounds to bind with RORs makes the bone remodeling regulatory mechanism more complex.

In silico docking of the Haematococcus carotenoid biomarker as a replacement compound for vitamin A, the RARs, and ROSs signaling pathways was shown. There were 3 predictable roles of carotenoids in bone regulation. First, Carotenoids and vitamin A, serve as agonists while RAR and RXR serve as an inhibitor of osteoblasts (bone formation) and osteoclasts (bone resorption) (bone resorption) (Figure 7A). Agonist-RAR serves as an inhibitor of osteoclast regulation by inhibiting receptor activator of nuclear factor -κB ligand (RANKL) forming. RANKL ligand stimulates the development of monocytes (osteoclast progenitors) into mature osteoclasts (Duong and Rodan 2001). Agonist-RAR also inhibits bone mineralization in osteoblasts by blocking the expression of osterix, osteocalcin, and Runx2 (Yee *et al.* 2021). Furthermore, osteocalcin (Oc) is a bone-specific protein gene stimulated by osteoblasts in the final step of differentiation during the mineralization of extracellular matrix development (Perwad and Portale 2011). Runt-related transcription factor 2 (Runx2) is involved in osteoblast differentiation and chondrocyte maturation (Komori 2011). Second, carotenoid (provitamin A and non-provitamin A) stimulates the differentiation of osteoblasts (Figure 7A and B) (Yee *et al.* 2021). Carotenoid improves the expression of alkaline phosphatase (ALP), osteopontin, and Runx2. Also, ALP expression is often used as a marker to assess osteoblast development, but not for gene analysis associated with the differentiation (Green *et al.* 2016). The secreted phosphoprotein osteopontin (OPN) belongs to the small integrin-binding ligand N-linked glycoprotein (SIBLING) class of cell-matrix. It participates in a variety of biological activities

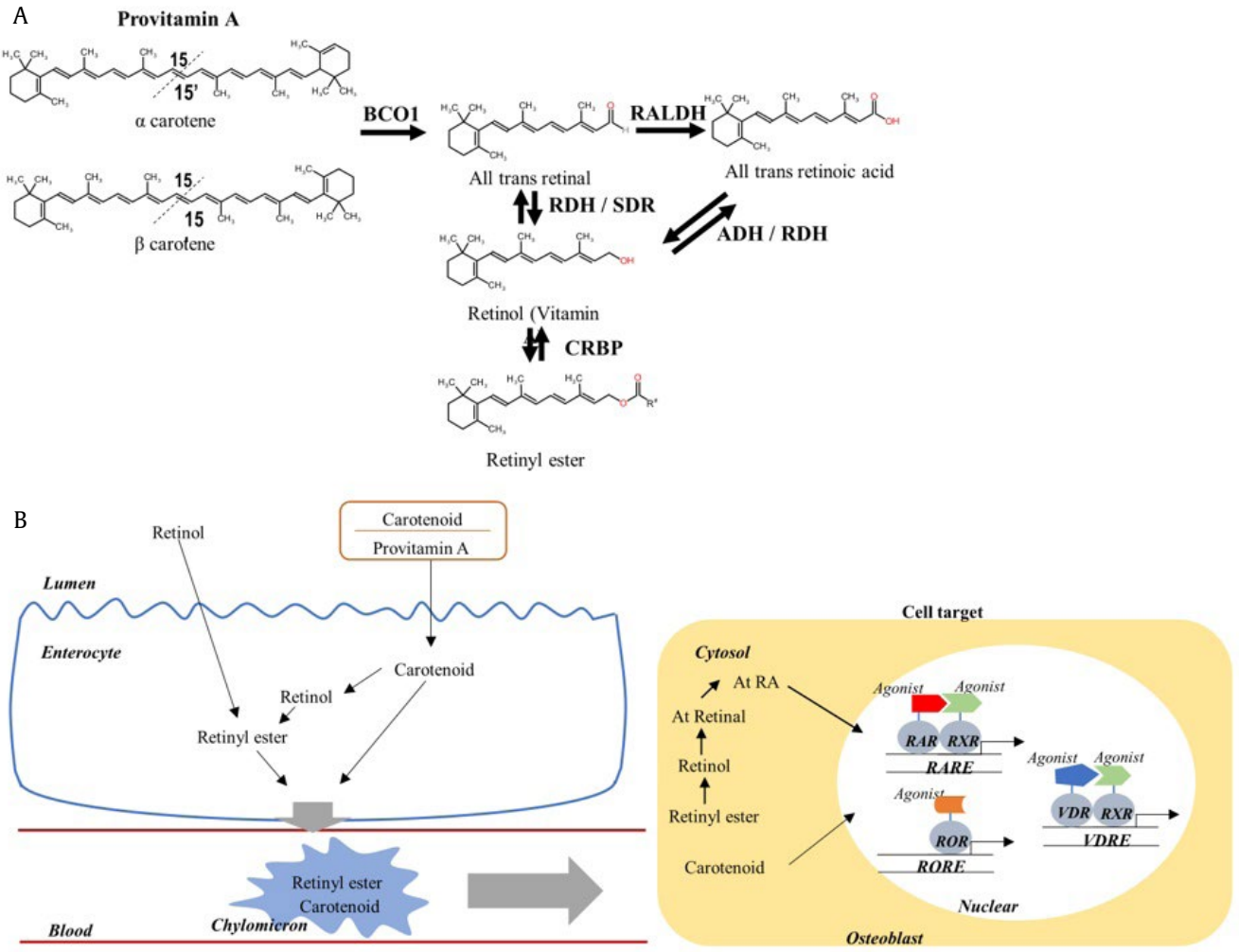


Figure 6. Provitamin A conversion and agonist biomarker compound schematic. Conversion of provitamin A carotenoid into all-trans retinoic acid (A). BCO1's enzymatic activity aids in the formation of an all-trans retinal by splitting the double bond chain at 15.15'. Meanwhile, RALDH is involved in the conversion of all-trans retinal to all-trans retinoic acid. All trans retinal can also be converted into retinol by RDH/SDR. Retinol is then converted into trans retinoic and retinyl ester, by ADH/RDH and CBRP facilities, respectively. Intracellular signaling of agonist biomarker compound is in the complex of RAR-RXR, ROR, and VDR-RXR (B). In the lumen, retinol and carotenoids (provitamin A) are converted into retinyl ester, which cannot be formed from non-provitamin A. The chylomicron transports retinol and carotenoids into the bloodstream to target cells, and once retinyl enters the target cells, it is converted into ATRA. Carotenoids and ATRA serve as agonists in the RAR-RXR, ROR, and VDR-RXR complexes. Abbreviations: BCO1: enzyme β-carotene 15,15′-oxygenase 1. RALDH: retinal dehydrogenase. RDH: retinol dehydrogenase. SDR: short-chain dehydrogenase/reductase. ADH: either alcohol dehydrogenase. CRBP: cellular retinol-binding protein, ATRA: all-trans retinoic acid

and plays a significant role in bone metabolism and homeostasis. Third, carotenoids serve as RXR agonists in osteoblast control, forming complex heterodimers with VDR (Lemon *et al.* 1997; Meyer *et al.* 2006). Vitamin D3 (VD3) is a VDR agonist that works directly on osteoblasts to stimulate bone cell development in the skeletal system, prevent proliferation, control differentiation, and regulate extracellular matrix mineralization (Sutton 2005). Furthermore, VDR-RXR formation regulates osteoblasts and osteoclasts by increasing RANKL expression (Figure 7C) (Takahashi *et al.* 2014), and also plays a role in mineralization. VDR stimulates osteocalcin expression. (Macdonald *et al.* 1993) dan ALP. VDR stimulates ALP significantly during the osteoblast differentiation and (Anderson 1995) enhances mineralization (Woeckel *et al.* 2010). The role of retinoids as ROR-agonists in osteogenesis is poorly understood. In a mouse cell culture experiment, the agonist-ROR decreased the expression of osteocalcin and osterix genes

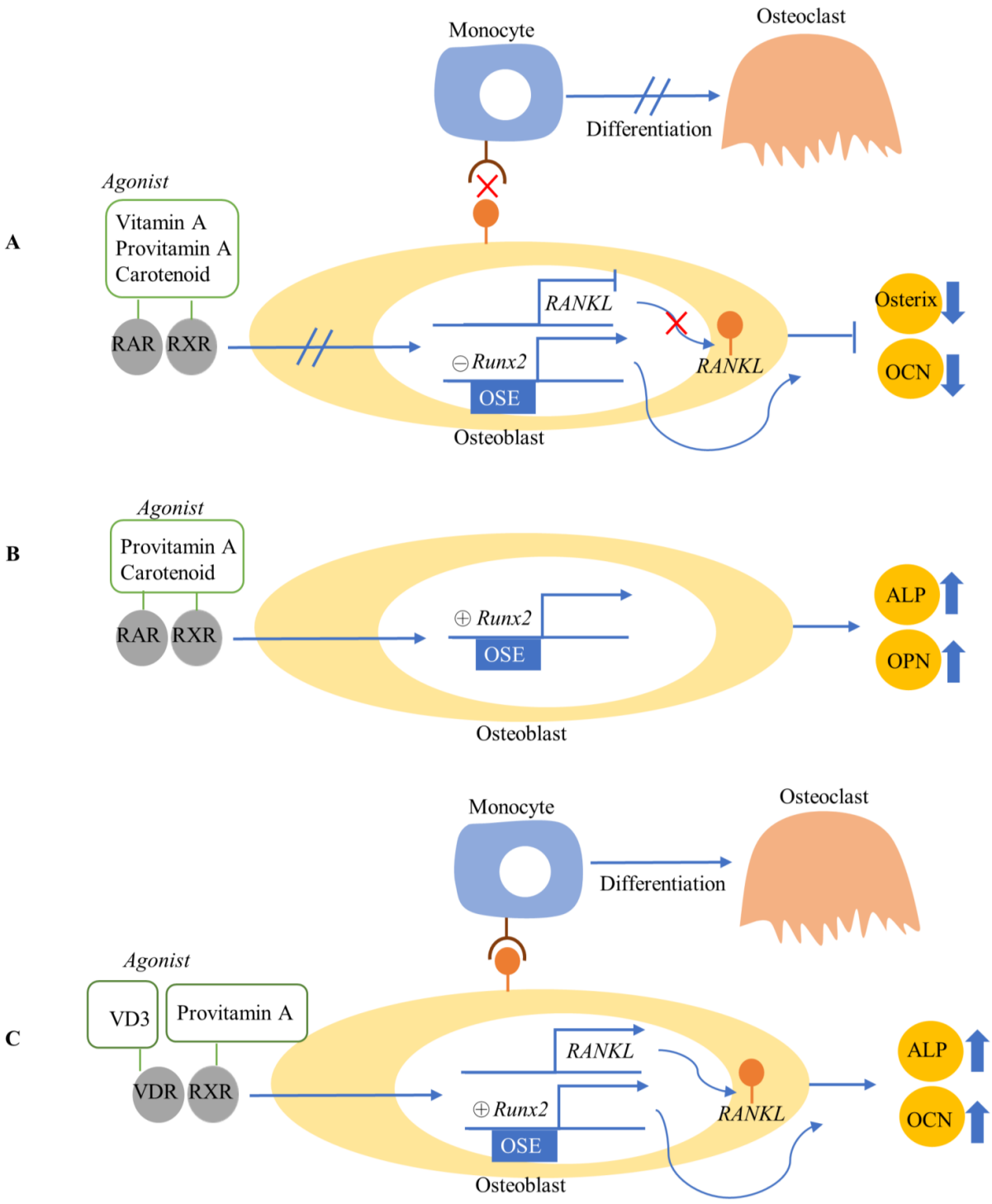


Figure 7. Biomarker compound regulation prediction in bone remodeling. RAR-RXR agonists are involved in the downregulation of RANKL and bone mineralization marker genes (A). Downregulation of RANKL prevents monocyte activation through the binding of RANK and RANKL with an effect on osteoclast maturation. By decreasing Runx2 expression, RAR-RXR agonists suppress the expression of osterix and osteocalcin (OCN) genes. Furthermore, biomarkers act as activators in the regulation of bone mineralization in osteoblasts (B). By increasing the expression of runx2, biomarker compounds affect the upregulation of alkaline phosphatase (ALP) and osteopontin (OPN). The agonists in the VDR-RXR complex regulate osteoclasts and osteoblast bone mineralization (C). VDR-RXR agonists play a role in osteoclast control by upregulating RANKL and stimulating monocyte cells through RANKL and RANK binding. In addition, the active monocyte cells then bind together with 2-50 other monocytes to form osteoclasts. The VDR-RXR agonists stimulate the transcription pathway in osteoblast control by increasing Runx2 expression, which results in the upregulation of ALP and OCN

(Roforth *et al*. 2012). Therefore, it is reasonable to state that agonist-RORs control downregulates bone mineralization.

Carotenoids serve as RXR agonists in osteoblast control, forming complex heterodimers with VDR (Lemon *et al.* 1997; Meyer *et al.* 2006). Vitamin D3 (VD3) is a VDR agonist that works directly on osteoblasts to stimulate bone cell development in the skeletal system, prevent proliferation, control differentiation, and regulate extracellular matrix mineralization (Sutton 2005). VDR-RXR formation not only regulates osteoblasts but also regulates osteoclasts by increasing RANKL expression (Figure 7C) (Takahashi *et al*. 2014). VDR-RXR has a role in osteoblast mineralization. VDR stimulates osteocalcin expression. (Macdonald *et al*. 1993) and ALP. VDR stimulates ALP significantly during the osteoblast differentiation period (Anderson 1995), providing a means of enhancing mineralization (Woeckel *et al.* 2010).

The role of retinoids as ROR-agonists in osteogenesis is poorly understood. In a mouse cell culture experiment, however, agonist-ROR actually decreased the expression of osteocalcin and osterix genes (Roforth *et al*. 2012). As a result, it can be inferred that agonist-RORs control downregulation bone mineralization.

The complex structure of bone tissue results in a balance between the activity of osteoblasts to form matrix and osteoclasts (Crockett *et al*. 2011). Malformations may result when the equilibrium is broken, and such cases in fish were discovered from the larval stage before adulthood. During the growth season, Haematococcus can be used as a fish meal additive to replace vitamin A. Since haematococcus carotenoids inhibit RANKL, they should be used in combination with VD3. The VDR-RXR heterodimer can stimulate bone mineralization in osteoblasts and RANKL, which promotes osteoclast maturation. The Haematococcus administration dosage should also be taken into consideration since excessive doses potentially prevent the formation of VDR-RXR heterodimers (Thompson *et al.* 1998). This is linked to the agonist's role in promoting the formation of RXR homodimers.

In conclusion, each Haematococcus biomarker compound had certain binding sites on RAR, RAR, ROR, and ROR following the docking results. The only compound with binding sites on these four receptors is astaxanthin. RAR and ROR agonists with carotenoids serve as inhibitors of osteoclast control. In the binding pathway with ROR, the expression of genes involved in bone mineralization control is reduced, and in the pathway with RAR, the formation of RANKL is inhibited. Furthermore, Beta and alpha carotenoids are provitamin A that can be converted to all-trans retinoic acid using enzymes (native ligand RAR and RXR). Haematococcus can be used in conjunction with vitamin D in the formation of the VDR-RXR heterodimer complex in normal fishbone growth. Meanwhile, the VDR-RXR heterodimers can activate the regulatory pathways of both osteoblasts and osteoclasts. The dosage and duration of Haematococcus administration are issues that should be addressed for optimal outcomes.

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