STRUCTURAL STUDIES OF THE HUMAN RETINOIC ACID RECEPTOR GAMMA LIGAND-BINDING DOMAIN IN COMPLEX WITH ANTI-CANCER HETEROAROTINOIDS

By

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Finally, I would like to dedicate my work to my husband and parents for their encouragement and selfless love.

ABBREVIATIONS

ACR	Acyclic retinoid
AF-1	Ligand-independent transcriptional activation function
AF-2	Ligand-dependent activation function
AML	Acute myelogenous leukemia
APL	Acute promyelocytic leukemia
BCIP	5-bromo-4-chloro-3'-indolyphosphate *p*-toluidine salt
BMS184394	6-[hydroxy-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-naphtalen-2-yl)-methyl]-naphtalene-2-carboxylic acid
cDNA	Complementary DNA
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
9C-RA	9-*cis* retinoic acid
Cys	Cysteine
DBD	DNA-binding domain
DTT	1,4-dithiothreitol
EK	Enterokinase
Flex-Het	Flexible heteroarotinoid
gDNA	Genomic DNA
His	Histidine

4-HPR	All-trans-N-(4-hydroxyphenyl) retinamide
hRAR	Human retinoic acid receptor
HRE	Hormone response elements
HNSCC	Head and neck squamous cell carcinoma
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB medium	Luria-Bertani medium
LBD	Ligand-binding domain
LBP	Ligand-binding pocket
MW	Molecular weight
NBT	Nitro-Blue tetrazolium chloride
ODC	Ornithine decarboxylates
PCR	Polymerase chain reaction
PIPES	1,4-Piperazinediethanesulfonic acid
PML	Promyelocytic leukemia
RA	Retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RXR	Retinoid X receptor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHetA2	1-(2,2,4,4-tetramethylthiochroman-6-yl)-3-(4-nitrophenyl) thiourea
SHetC2	1-(2,2,4,4-tetramethylthiochroman-6-yl)-3-(4-nitrophenyl) urea
TOC	Tracheal organ culture

T-RA	All-*trans* retinoic acid
VAD	Vitamin A deficiency

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

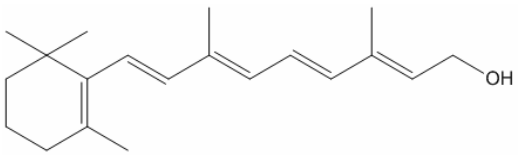
CHAPTER I

BACKGROUND AND REVIEW OF LITERATURE

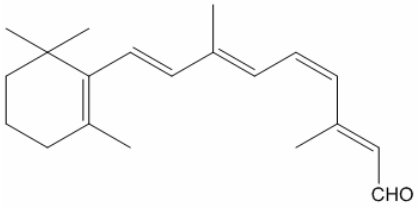
Since the mid 1980s, structure-based drug design has been greatly enhanced to guide the development of more effective compounds. Taking advantage of X-ray crystallography and computer modeling, the crystal structure of a receptor in complex with a drug candidate can be solved. The study of structure reveals active sites of receptors, such as the positions of the hydrogen bonding donors and acceptors in a receptor binding pocket as well as cavities in the binding site into which substituents might be placed in a drug candidate to increase its binding affinity for the receptor compounds.[1]

In the field of molecular biology, a receptor is a protein that binds to a ligand and initiates a cellular response. The retinoic acid receptors (RARs) bind retinoids and mediate both organismal and cellular effects.[2] The term "retinoid" includes both natural forms of vitamin A, which is a generic term for any compound possessing the biological activity of retinol, and the many synthetic analogs of retinol, such as arotinoids and heteroarotinoids, with or without biological activity.[3] Vitamin A, found in foods from animal sources, exists in forms of retinol (alcohol) [**1**], retinal (aldehyde) [**2**] and retinoic acid (acid) [**3, 5, 6**] (see Figure 1-1). Provitamin A carotenoids, including α-carotene, β-carotene and β-cryptoxanthin, found in foods from plant sources can be converted into

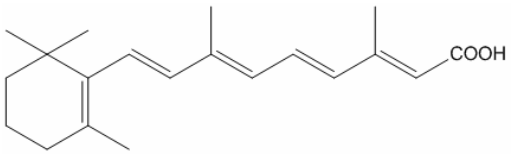
retinol in the body. Among these carotenoids, β-carotene [**4**] (see Figure 1-1) is converted into retinol with the highest efficiency.[4]



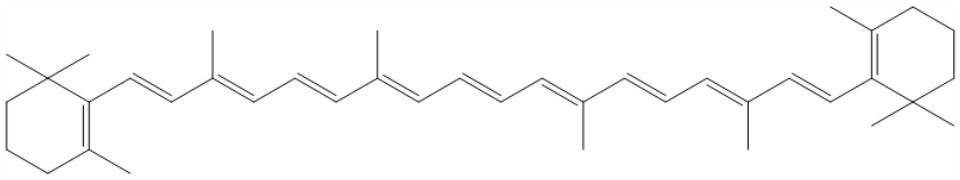
1 All-*trans* retinol



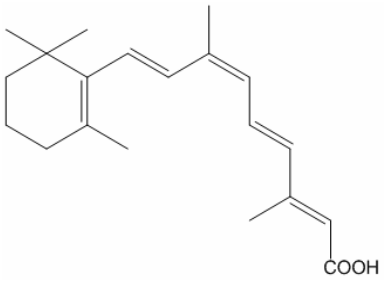
2 11-*cis*-retinal



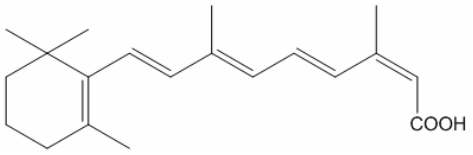
3 All-*trans* retinoic acid



4 β-carotene



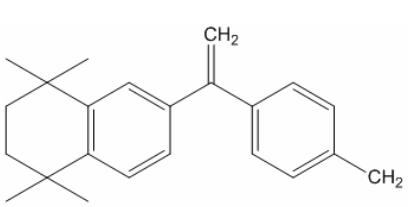
5 9-*cis* retinoic acid



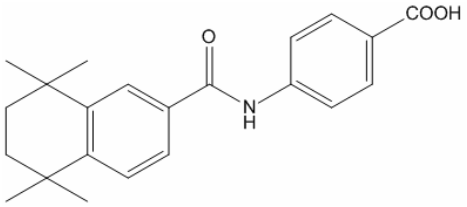
6 13-*cis* retinoic acid

Figure 1-1. Naturally occurring forms of retinoids

The basic structure of natural retinoid molecules is composed of a cyclic end group, a polyene side chain and a polar end group[5] (see Figure 1-1). The end groups and the conjugated system with alternating C=C double bonds in the polyene side chain create dramatic modification of retinoids.[5] Both arotinoids [**7, 8**] (see Figure 1-2) and heteroarotinoids [**9a, 9b, 10a, 10b**] (see Figure 1-3) have at least one aromatic group in their basic structure, but the latter has a heteroatom in the cyclic ring.

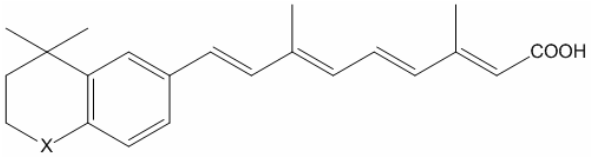


7 Am 580

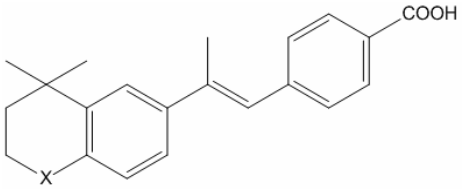


8 TARGRETIN, LGD 1069

Figure 1-2. Examples of arotinoids



9a X=O; **9b** X=S



10a X=O; **10b** X=S

Figure 1-3. Examples of heteroarotinoids

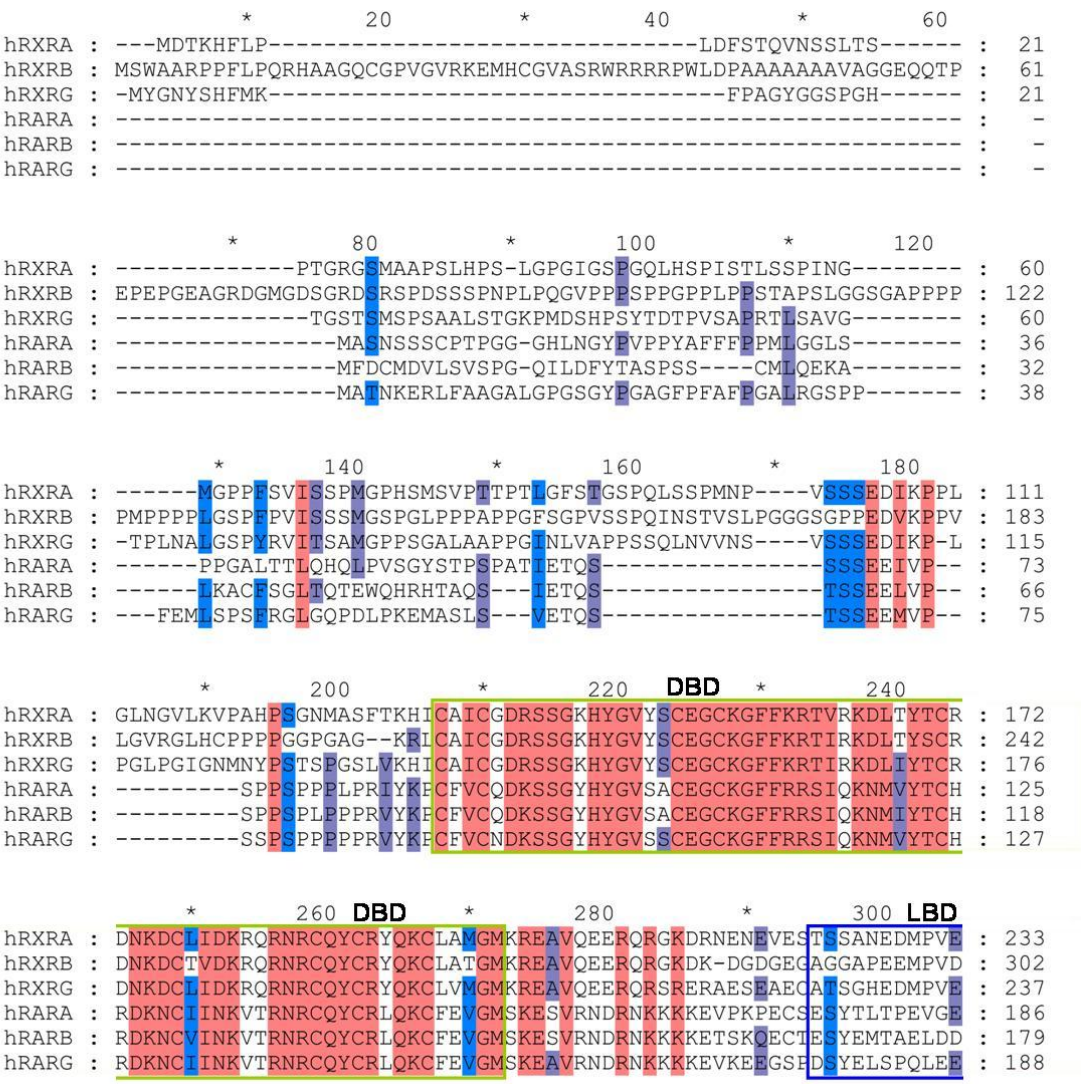
The three retinoic acid receptor (RAR) and three retinoid X receptor (RXR) proteins (α, β and γ, and their isoforms), which specifically bind retinoids, belong to the nuclear receptor (NR) superfamily. The activity of those proteins is similar to the steroid hormone receptors which can enter the cell nucleus and modulate gene expression by interacting with specific DNA sequences upstream of the target genes in response to the

activation by the ligand.[6] This regulation functions through a receptor conformational change, which is induced by binding of the ligand and later results in physiological changes that induce the biological activities of the ligands.[7,8] RARs are activated by two common isomers of retinoic acid (RA), namely all *trans*-retinoic acid (T-RA [**3**]) and 9-*cis*-retinoic acid (9C-RA [**5**]), but RXRs are activated only by 9C-RA [**5**]. When binding T-RA [**3**], an endogenous retinoid, the RARs become transcriptionally active. RXRs function as common partners to many other NRs, but the actual biological role of RXRs is still an enigma.[9]

Nuclear receptors exhibit a modular structure consisting of six regions (A through F).[10] The A-B region (N-terminal) shows ligand-independent transcriptional activation functionality (AF-1). Each of the three isotypes of RAR and RXR contains isoforms that differ only in their A-region. RARα and RARγ have two major isoforms (α1 and α2; γ1 and γ2), but RARβ has four major isoforms (β1, β2, β3 and β4) and a truncated isoform (β').[11] There are two major isoforms in each of the RXR isotypes, i.e., RXRα (α1 and α2), RXRβ (β1 and β2) and RXRγ (γ1 and γ2)[9]. The DNA-binding domain (DBD; region C), which contains two zinc fingers, is highly conserved and binds specific DNA sequences[12] called hormone response elements (HRE) but, in the case of the RARs, it is called the retinoic acid response element (RARE).[13] The ligand-binding domain (LBD; region E), which is highly conserved in both structure and sequence between the RAR and isotypes, contains a ligand-binding pocket (LBP) and a ligand-dependent activation function (AF-2)[12] which suggests different responses for binding of ligands.[13] The highly conserved regions of DBD and LBD in both receptors are indicated in Figure 1-4. RARs

and RXRs only share 29% homology in their LBD.[14] The hinge domain (region D) connects the LBD to the DBD. The sequence of the undefined C-terminus (region F) varies among the different nuclear receptors.

Nuclear receptors typically bind to their target sequences as dimers.[3] In addition to ligand binding, the ligand-binding domain of nuclear receptors participates in the activity of homo- and/or heterodimerization. The LBD of a NR contains a dimerization interface and a surface to interact with co-activator and co-repressor proteins. The RAR/RXR heterodimer has much more efficiency of binding to cognate response elements than either RAR or RXR homodimers.[10] In the absence of ligand, RAR can heterodimerize with RXR and bind to RARE in complex with a co-repressor protein.[3] Binding of an agonist to RAR induces a conformational change so that the receptor structure becomes more compact.[8] This results in dissociation of the co-repressor and recruitment of a co-activator protein for transcriptional regulation.[3] AF-2 is responsible for this conformational change.[15] In contrast, antagonists could interfere with the formation of the active receptor conformation, and then the interaction with co-activators.[8]



(Figure 1-4. Continues on next page)

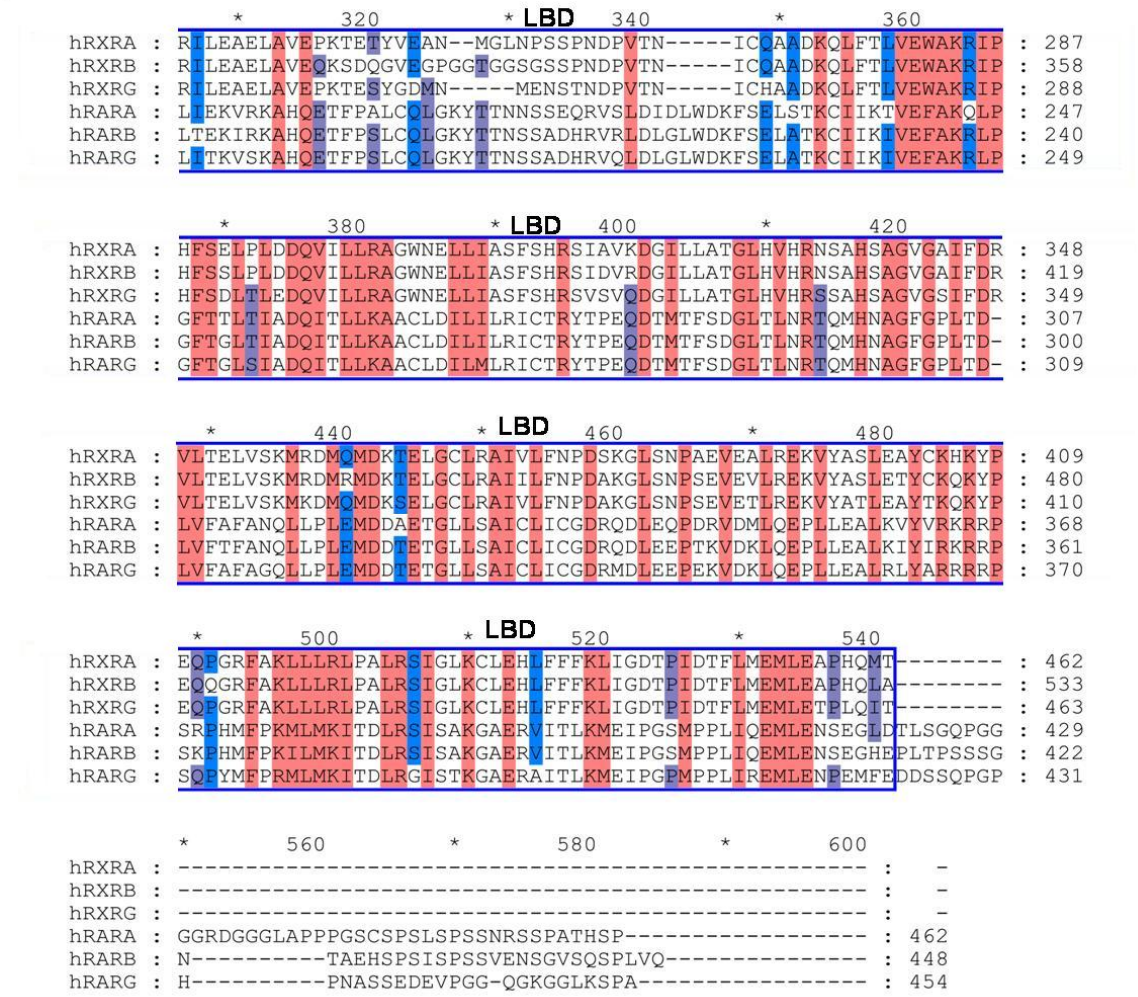


Figure 1-4. Full sequence alignment of the three isotypes of hRARs (hRARα, β and γ or hRARA, B and G) and the three hRXRs (hRXRα, β and γ or hRXRA, B and G). The numbering scheme along the top of the sequences shows every tenth (*) and twentieth (number) space. The register for the individual sequences is shown at the right side of a row. The DNA-binding domain (DBD) is boxed in green and the ligand-binding domain (LBD) is boxed in blue. The conserved sequences are highlighted in dark pink (completely conserved; 6 out of 6), blue (highly conserved; 5 out of 6) and dark purple (less conserved; 4 out of 6). A gap in the alignment is shown with a dash (-). The alignment was performed with the program Clustal X[16] and displayed with GeneDoc[17].

The different isotypes of retinoic acid receptor correspond to different drug targets and perform specific functions. For example, acute promyelocytic leukemia (APL), a subtype of acute myelogenous leukemia (AML), is associated with

chromosomal translocations involving RARα. This translocation produces the PML (promyelocytic leukemia)-RARα fusion protein. The expression of this protein then results in the induction of APL.[18,19] RARβ acts as a potential tumor suppressor[2] in growth of different cell types and might be a key factor in the anti-proliferative effect of retinoids.[12,20] RARγ is involved in skin photo-aging and carcinogenesis, and in skin diseases like psoriasis and acne.[21] A sequence alignment (see Figure 1-5) shows all but three residues in the ligand-binding pockets (LBPs) of RARs are conserved.[21] This implies that the three different residues of the LBP, which are located in α-helices H3, H5 and H11,[12] are involved in differentiating the specificity of the RARs between retinoids (see Table 1-1). (The numbering system for the α-helices of RARs was named according to the similarity to the crystal structure of RXRα.[13]) Among retinoic acid receptors, RARγ occurs in nearly all tissues and is the most important RAR for regulation of the rate of retinoic acid turnover and retinoic acid induced growth inhibition.[22]

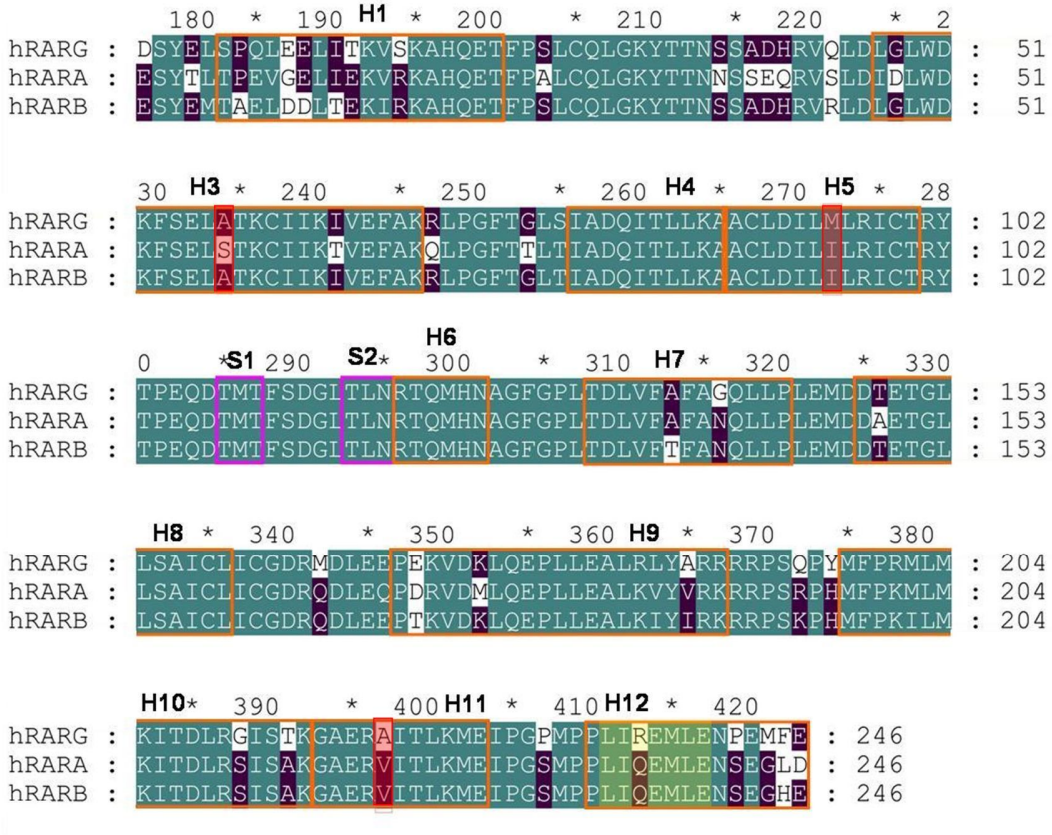


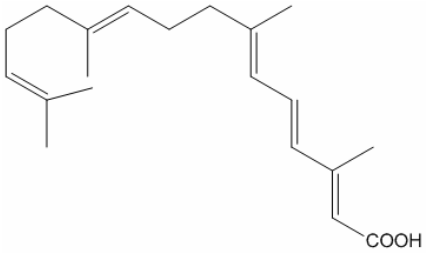
Figure 1-5. Sequence alignment of the LBD of hRARα (hRARA; amino acids 154-462), hRARβ (hRARB; amino acids 146-448) and hRARγ (hRARG; amino acids 178-423). Amino-acid numbering in the alignment is for hRARγ, which means amino acids located at the same column may not have the same number in the other isotypes. The labeled α-helices and β-strands are boxed in orange and purple, respectively. The three hRAR isotype-specific residues in the LBP are indicated with red highlight (Table 1-1). The activation domain of AF-2 is highlighted in yellow-green. The dark green shadowed sequence shows completely conserved sequences (3 out of 3); The dark purple highlighted sequence shows highly conserved sequences (2 out of 3); The sequence column with the white background indicates non-conserved sequences. The alignment was performed with the program Clustal X[16] and displayed with GeneDoc[17].

RAR Isotype	α-helix 3 (H3)	α-helix 5 (H5)	α-helix11 (H11)
RARα	Ser232	Ile270	Val395
RARβ	Ala225	Ile263	Val388
RARγ	Ala234	Met272	Ala397

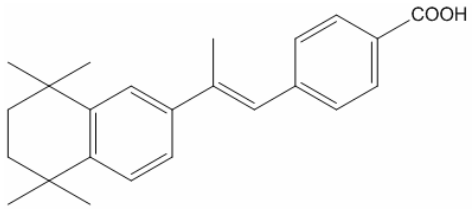
Table 1-1. Comparison of residues located at α-helices H3, H5 and H11 (see residues highlighted in red for the alignment in Figure 1-4) among different RAR isotypes.

Retinoids are commonly used in medicine since they exert pleiotropic effects on regulating biological processes such as cell growth, differentiation, apoptosis, embryo development, angiogenesis, metastasis and immune function.[23] Vitamin A deficiency (VAD) can cause eye disorders, skin disease, growth retardation in children and vulnerability to urinary and respiratory infections. Furthermore, retinoids play an important role in the therapy and prevention of cancer by interacting with RARs and RXRs, which are ligand-dependent transcription regulators. Retinoids are well known as one of the most promising classes of cancer chemoprevention agents. Generally, apoptosis is not induced by single retinoid agents but can be enhanced by retinoids combined with DNA damaging agents.[23] The most impressive example of anticancer activity of retinoids is the treatment of APL by T-RA [**3**].[12,24] Taking advantages of receptor specificity, synthetic retinoids have been developed to enhance their anti-proliferative activity and have shown promise as chemotherapy agents. For example, polyprenoic acid [**11**] (see Figure 1-6), an acyclic retinoid (ACR), can inhibit the growth of hepatoma cells *in vitro* and *in vivo*[25] and also prevent the development of recurrent or second primary hepatocellular carcinoma.[26] Another example is fenretinide (4-HPR) [**12**] (see Figure 1-6), which has shown reduction of secondary breast cancers[27] and primary ovarian cancers.[28] (The term "second primary" in cancer refers to a new primary cancer in a person with a history of cancer.[29] The term "secondary cancer" is used to describe either a new primary cancer or cancer that has spread from the place in which it originated to other parts of the body.[29])

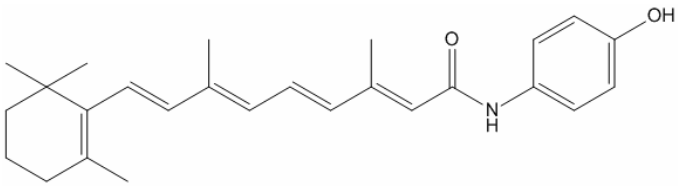
However, the clinical effectiveness of natural retinoids and some synthetic retinoids is limited by their significant toxicities.[30,31] The term "hypervitaminosis A" refers to the toxicities that accumulate with chronic retinoid treatment, and its effects are observed in skin, hair, eyes, liver, and bones of the body. Early research from the 1980s to develop retinoid analogs, like arotinoids, focused on structure modifications of the polyene side chain with inclusion of an aryl ring. The compound TTNPB [**13**] (see Figure 1-6) is a representative example. TTNPB [**13**] is 10-fold more potent than T-RA [**3**] in the tracheal organ culture (TOC) differentiation assay and equally effective in the ornithine



11 Polyprenoic acid



13 TTNPB

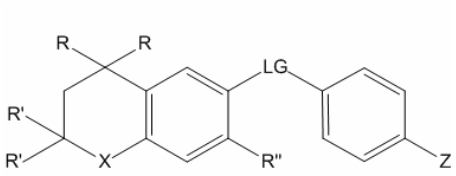


12 Fenretinide (4-HPR)

Figure 1-6. Example of synthetic retinoids as chemoprevention agents

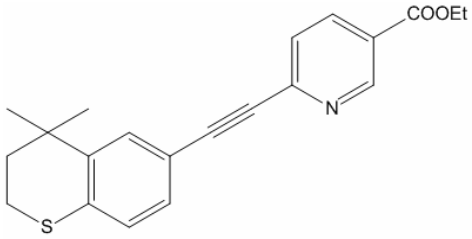
decarboxylates (ODC) induction assay.[23] But in clinical application, this compound is prohibited because of its 10,000-fold greater toxicity than T-RA [**3**].[23] To reduce the

toxicity of arotinoids, an oxygen or sulfur heteroatom was added into the cyclic ring to block metabolic oxidation of the compounds.[23] This is how the heteroarotinoids [**14**] (see Figure 1-7) were designed to improve the therapeutic ratio (efficacy/toxicity) as anti-cancer agents. Tazarotene [**15**] (see Figure 1-7), a heteroarotinoid with a sulfur heteroatom in the cyclic ring, has shown promise in clinical applications with a good safety profile.



R=R'=R''=H, Alkyl, OAlkyl
X=N-Alkyl, O, S
Z=NO_2, CO_2Alkyl, CO_2H, SO_3Alkyl
LG=Linking Group

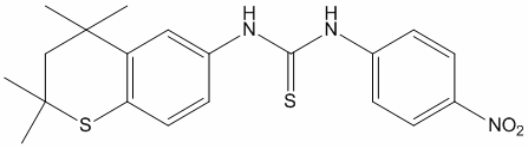
14 Typical heteroarotinoid with biological activity



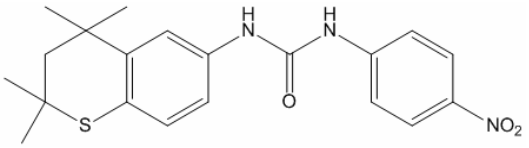
15 Tararotene

Figure 1-7.

A new class of Flexible Heteroarotinoids (Flex-Hets) (see Figure 1-8), which contains a 3-atom thiourea [**16**] or urea [**17**] linker, exhibited improved therapeutic ratios as anti-cancer agents in comparison with natural retinoids.[31,32] The heteroarotinoid with a thiourea or urea linker becomes more flexible and was better accommodated in the RARγ receptor ligand-binding domain.[33] In addition to regulating growth and differentiation similar to RA, Flex-Hets induced potent apoptosis in ovarian cancer and in head and neck squamous cell carcinoma (HNSCC) through a mitochondria-dependent pathway.[24-27]



16 SHetA2



17 SHetC2

Figure 1-8. Flex-Hets: SHetA2 and SHetC2

Among Flex-Hets, the lead compound SHetA2 [**16**] (1-(2,2,4,4-tetramethyl-thiochroman-6-yl)-3-(4-nitrophenyl) thiourea) exhibited significantly potent activity and lack of toxicity across all cell lines tested *in vitro* and *in vivo*.[34] The latest research shows the optimal structure of Flex-Hets for enhancing potency is made up of a urea linker, a NO_2 substitution and a flexible thiochroman unit.[33] Compared to SHetA2, a new Flex-Het compound SHetC2 [**17**] (1-(2,2,4,4-tetramethylthiochroman-6-yl)-3-(4-nitrophenyl) urea), which contains a urea linker instead of a thiourea linker, exhibited increased activity with a similar mechanism of induction of apoptosis while keeping the same therapeutic ratio.[33] The compound structures of SHetA2 [**16**] and SHetC2 [**17**] are specifically designed and synthesized for hRARγ selectivity. The goal of this research is to obtain recombinant hRAR LBD and determine the X-ray crystal structures of the LBD in complex with SHetA2 [**16**], SHetC2 [**17**], and other heteroarotinoids and explore how these synthetic heteroarotinoids interact with hRARγ. This information will be used to enhance the understanding of the binding pocket of the hRAR LBD and how it can adapt to different drug targets. Atomic resolution of the heteroarotinoid interactions with the receptor will aid in optimization of the drugs conformation and functional groups. To approach this goal, a highly pure and concentrated protein solution is inevitably necessary. The development of molecular cloning techniques allows almost any protein-

encoding gene to be isolated from its parent organism and to be expressed at high levels in a microorganism.[1] The recombinant protein, which accumulates to high levels and constitutes a high percentage (up to 40%) of the total cell protein, is far easier to isolate than it would be from its parent organism, in which it may occur in small or trace amount.[1] We have successfully cloned the gene of hRARγ LBD and have efficiently obtained the recombinant protein expressed with a poly-histidine tag. The preliminary crystallization trials of hRARγ LBD in complex with T-RA [**3**] are underway to check the integrity of the construct and to establish a lead crystallization condition. Those experimental details are reported in Chapter II (Methodology). So far, small crystals have been obtained and await characterization by X-ray diffraction.

CHAPTER II

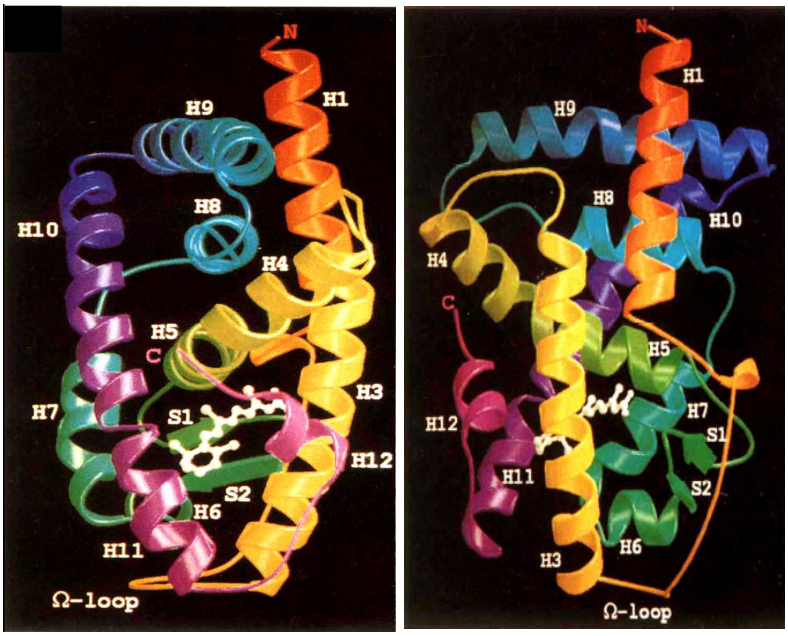
METHODOLOGY

2.1 Introduction

The retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) belong to the nuclear receptor family, which act as transcriptional factors. The regulation of gene expression by RARs or RXRs is ligand-dependent. The crystal structure of the hRARγ LBD bound to T-RA has been determined at a resolution of 2.0 Å by Renaud *et al.* in 1995[9] (Protein Data Bank (PDB) code: 2LBD; see Figure 2-1).

The LBD of hRARγ is made up of eleven α-helices (H1, H3-H12), two β-strands (S1 and S2) and two Ω-loops.[9] The lack of α-helix H2 in the structure of the hRARγ LBD is due to consideration of the numbering method in comparison with the crystal structure of hRXRα[9] where there is an extra helix between the H1 and H3 helices as revealed when the similar structures are superimposed. The hRARγ LBD structure exhibits an anti-parallel α-helical sandwich[8] in which the ligand-binding pocket (LBP) is formed for ligand binding. The α-helices H4, H5, H6, H8 and H9 stay in the center of this three-layer structure with H1 and H3 on one side, and H7, H10 and H11 on the other

side.[9] The ligand-dependent activation function 2 (AF-2) is located on H12[12] and determines the binding of different ligands. The crystal structure shows that the binding



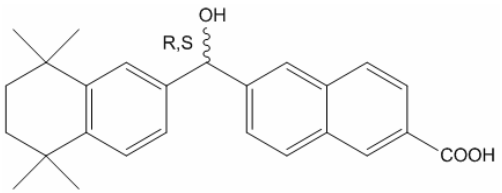
(a) **(b)**

Figure 2-1. A ribbon diagram of the hRARγ LBD bound to all-trans retinoic acid.[9] The LBD model is colored in a rainbow gradient from the N-terminus (red) to the C-terminus (violet). T-RA is shown as a ball-and-stick model in white, the α-helices are depicted as coils, and the β-strands as arrows. The α-helices, β-strands and Ω-loop are labeled. (a) A view of the hRARγ LBD structure. (b) The view at +150° rotation of (a).

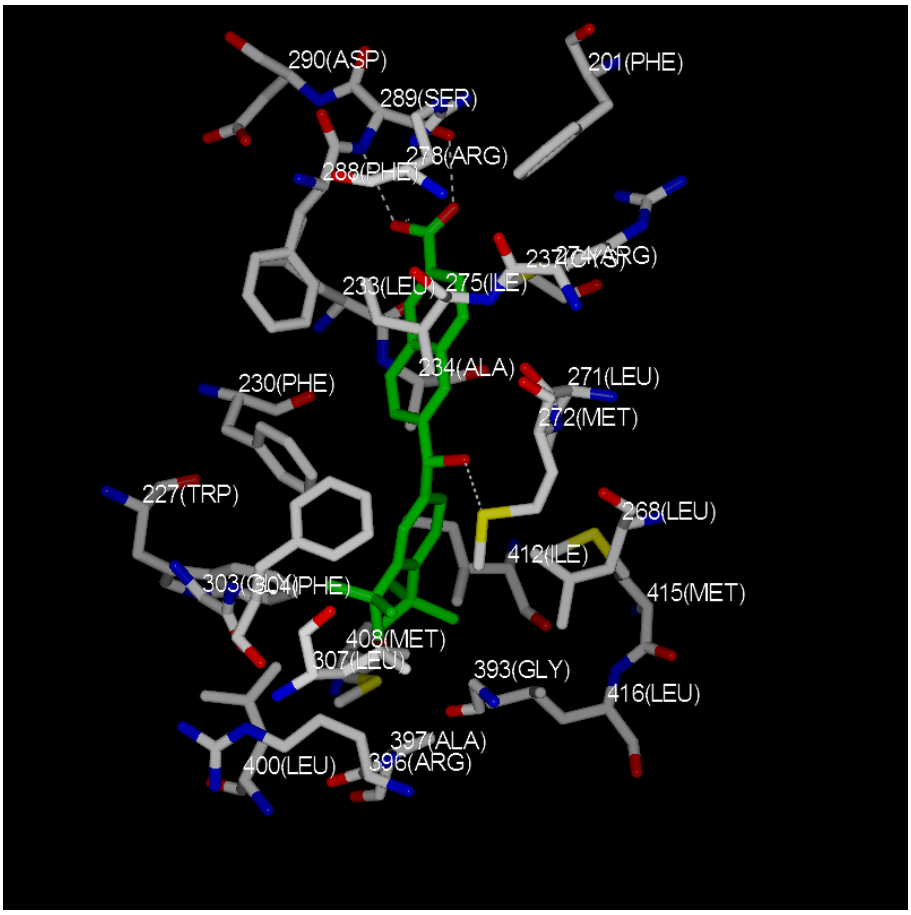
of T-RA [**3**] can be explained by the electrostatic guidance mechanism proposed by Renaud *et al.*[9] The negatively charged ligand is attracted into the LBP by a positively charged amino acid cluster[8] in hRARγ. The ligand-induced conformational change of hRARγ LBD occurs with the refolding of the Ω-loop and α-helix H12.[8] The highly selective hydrophobic and hydrophilic regions in the hRARγ LBP makes possible the maximum activation by a ligand.[35] The hRARγ LBD is monomeric in solution, with or without the presence of a ligand.[9]

Among the RAR isotypes, the sequences are highly conserved. In the LBP there are three residues that are changed between the three isotypes.[21] This suggests the mechanism for the different selectivity among the RAR isotypes. For example, formation of hydrogen bonds between an amino group in the RARα selective ligands and the RARα-specific Ser232 residue favors RARα selectivity.[12] This is because no such hydrogen bond forms in RARβ or γ LBPs, which consist of an Ala instead of a Ser.[12] The prerequisite for RARγ selectivity is that the hydroxyl group of RARγ selective retinoids forms a hydrogen bond to the sulfur atom of Met272 in RARγ.[21] The hydroxyl group reduces the affinity of ligands, but the formation of this hydrogen bond helps regain some of the binding.[21] Figure 2-2 shows an example of hydrogen bonds between RARγ and a synthetic retinoid molecule BMS184394 [**18**] (6-[hydroxy-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-naphtalen-2-yl)-methyl]naphtalene-2-carboxylic acid) from the co-crystal structure of this compound with the LBD (PDB code: 1fcx).[21] Note the hydrogen bond between the hydroxyl group of BMS184394 and Met272 of RARγ LBD.

RARγ plays an essential role in mediating topical retinoid efficacy and irritation associated with the use of retinoids in animal models.[36] RARγ in human epidermis preferentially traps T-RA [**3**] as its ligand rather than 9C-RA [**5**] under physiological and pharmacological conditions.[37] T-RA [**3**] will be used as the ligand in the preliminary co-crystallization trial to check the integrity of the construct through examination of the complex by X-ray analysis.



(a) 18 BMS184394



(b)

Figure 2-2. (a) Synthetic retinoid BMS184394. (b) A view of the RARγ LBP in complex with BMS184394. The formation of a hydrogen bond between the hydroxyl group (red) of BMS184394 (green backbone) and the sulfur atom of Met272 (yellow) of RARγ (white backbone) is shown by a white dotted line.

2.2 Materials and Methods

2.2.1 Genetic engineering of hRARγ LBD

The general experimental outline of genetic engineering includes three major steps: isolation, insertion into a vector and transformation.[38] Usually, a gene of interest (a segment of DNA) can be obtained from cDNA (complementary DNA) or gDNA (genomic DNA) libraries and amplified using polymerase chain reaction (PCR) techniques. The isolated gene is cloned into a vector, such as a plasmid or a viral vector. The construct then is used to transform target organisms (such as virus, prokaryotes and eukaryotes), which depends on the type of vector used. Finally, the recombinant gene which is isolated from the transformant culture will be expressed into functional gene product, i.e., protein. Experimentally, a sterile environment is an extremely important factor to guarantee correct results. Before carrying out the experimental protocols, we always sterilized all experimental bench space, instruments, appliances and DI water by Amphyl disinfectant (Reckitt Benckiser), ethanol, bleach or autoclaving (Electric Pressure Steam Sterilizer 25X, maximum temperature: 126°C; maximum pressure: 20 psi; All-American). For safety, Amphyl disinfectant, ethanol or bleach was used for different waste handling.

2.2.1.1 Isolation and PCR

The full-length hRARγ cDNA (Clone ID IOH14052, corresponding to GenBank NM000966.3, Invitrogen) was obtained as a part of a pENTR221 plasmid in *Escherichia coli* (see Figure 2-3). The plasmid (with a kanamycin-resistant gene for selection) was

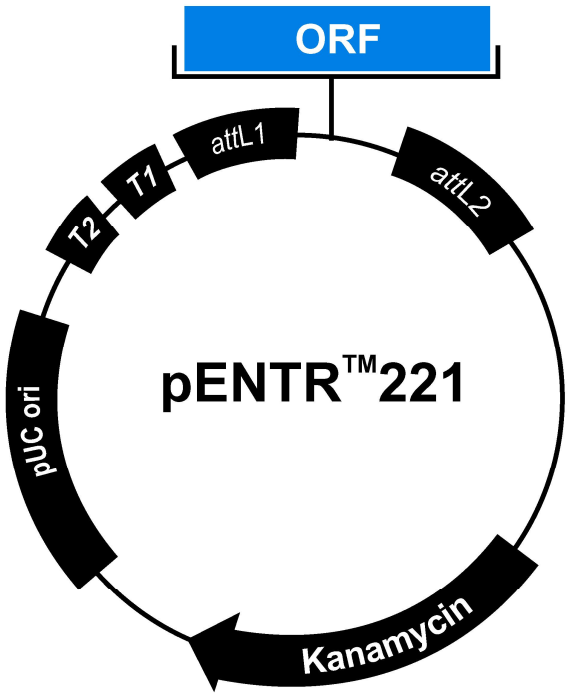


Figure 2-3. The gene map of the pENTR221 plasmid (Invitrogen). A cDNA containing the full-length hRARγ gene (blue region) was obtained as part of pENTR221 plasmid.

isolated at room temperature by the Wizard *Plus* SV Miniprep DNA Purification System (Promega). The procedure was to take a fresh Luria-Bertani (LB) agar plate with 50 μg/ml kanamycin and streaking it with frozen *E. coli* stock which contains the pENTR221 plasmid. The *E. coli* colonies were produced on the plate, which was placed in an inverted position, with overnight incubation at 37°C. A 10 ml volume of LB medium containing 50 μg/ml kanamycin was placed in a 50 ml conical tip tube and inoculated by a single, well-isolated *E. coli* colony (selected using a sterile toothpick) and grown with shaking (200 rpm, Max Q 500 shaking incubator, Barnstead Lab-line) for 16 hours at 37°C in a vertical position. The cells were harvested by centrifugation (Allegra X-15R Benchtop Centrifuge, Beckman Coulter) of the bacterial culture at 4500 rpm for 10 min at 4°C. The supernatant was discarded. The cell pellet was resuspended

completely in 250 μl Cell Resuspension Solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA, and 100 μg/ml RNase A) by vortexing. Afterwards, the resuspended pellet was transferred into a 1.5 ml microcentrifuge tube and mixed with 250 μl Cell Lysis Solution (0.2 M NaOH and 1% SDS) by inverting the tube 4 times. Partial clearing of the lysate was observed after 5 min incubation. To quench the endonucleases and other proteins released during the lysis, Alkaline Protease Solution (10 μl) was added into the lysate, mixed by inverting the tube 4 times and incubated for 5 min. After incubation, the cell solution was mixed with 350 μl Neutralization Solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, and 2.12 M glacial acetic acid) by inverting the vial 4 times for solution neutralization. The mixture was centrifuged (Marathon Microcentrifuge, Fisher Scientific) at maximum speed for 10-30 min until the cell debris was pelleted. The cleared lysate then was decanted into a Spin Column, which was inserted into a 2 ml Collection Tube for the plasmid DNA purification. The contents in the Spin Column were centrifuged at maximum speed for 1 min, and the flowthrough from the Collection Tube was discarded. The Column Wash Solution (750 μl, 60% ethanol, 60 mM potassium acetate, 8.3 mM Tris-HCl, 0.04 mM EDTA) was added into the Spin Column and centrifuged at maximum speed for 1 min, and the flowthrough was discarded. Ethanol will precipitate the plasmid DNA, which was collected by the resin of the Spin Column. The wash was repeated using 250 μl Column Wash Solution and centrifuged for 2 min at maximum speed. The Spin Column was transferred to a new 1.5 ml microcentrifuge tube. By adding 100 μl Nuclease-Free water into the column with 1 min centrifugation at maximum speed, the plasmid DNA was eluted into the 1.5 ml

microcentrifuge tube. The Spin column was discarded, and the plasmid DNA samples were stored at -20°C until processed.

The fragment of human retinoic acid receptor consisting of the hRARγ LBD was amplified from the full-length hRARγ cDNA (Invitrogen) by PCR (AccuPrime Taq DNA Polymerase System, Invitrogen; PTC-150 Minicycler, MJ Research) with primers (Invitrogen) RARG 1F 5'-CAC CGA CAG CTA TGA GCT GAG CCC-3' and RARG 1R 5'-CTC AAA CAT TTC AGG GTT CTC C-3'. Each of the primers was diluted to the concentration of 20 μM before PCR setup. In each 100 μl PCR mixture (10 μl of 10X AccuPrime PCR buffer II, 1 μl of 20 μM forward primer, 1 μl of 20 μM reverse primer, 1 μl of Taq DNA polymerase solution, 2 μl or 4 μl of the isolated plasmid), after 2 min initial denaturation at 94°C, each of 30 PCR cycles included the following steps: 30 seconds denaturation at 94°C, 30 seconds annealing at 55°C and 1 min extension at 68°C. The final extension was allowed to go for 5 min at 68°C.

The PCR product (738 bp) was loaded onto a 1% agarose gel (total of 4 lanes) containing 0.5 μg/ml ethidium bromide and verified through agarose gel electrophoresis (100 V, 2 hrs, Sub-Cell GT agarose gel electrophoresis, BioRad). The bands corresponding to the correct size of the PCR product were visualized under UV light. To purify the PCR product, the Invitrogen PureLink Quick Gel Extraction Kit was used. This purification procedure removes primers, nucleotides, enzymes, mineral oil, salts, agarose, ethidium bromide, and other impurities from DNA samples. The bands containing the PCR product were cut from the gel and divided into two 1.5 ml microcentrifuge tubes to

dissolve the gel material. The Gel Solubilization Buffer (644.7 μl) was added into the tube which contained 214.9 mg of the excised gel slice. The other tube contained 246.5 mg of gel slice so that 739.5 μl of Gel Solubilization Buffer was added. The tubes were incubated at 50°C for 15 min with mixing every 3 min. An additional 5 min incubation at 50°C was done to ensure that the gel completely dissolved. The solutions were pipetted respectively into two Quick Gel Extraction Columns, which were placed onto the Wash Tubes, and centrifuged in the microcentrifuge for 1 min at maximum speed. The flowthrough was discarded. Wash Buffer (700 μl) containing ethanol was added into each of the Spin Column. After 5 min of incubation at room temperature, the solutions were centrifuged for 1 min at maximum speed, and the flowthrough was discarded. After one more round of centrifugation to assure removal of all liquid, the columns were transferred onto new 1.5 ml Recovery Tubes. The Nuclease-Free water (50 μl) was then added into each of the columns, and the PCR products bound to the resin of the columns were eluted into the Recovery Tubes. This gel-purified PCR product will be used for the cloning step or stored at -20°C. According to the band intensity comparison between the PCR product and the DNA mass ladder (Invitrogen), it was known that the concentration of the gel-purified PCR product is between 10 ng/μl and 14 ng/μl.

2.2.1.2 TOPO cloning reaction of PCR product

Following PCR amplification of the gene, the hRARγ LBD PCR product with the "CACC" sequence at the 5'-end was directionally cloned into TOPO pET100/D vector (Champion pET Directional TOPO Expression kits, Invitrogen) in frame with the N-terminal tag (see Figure 2-4). The "CACC" sequence gave the PCR product an end that

was complementary to the "sticky end" of the plasmid, whereas the other end was blunt without sequence complementarity. This was designed to increase the chances that the insert was cloned in the correct orientation. The pET100/D vector contained an ampicillin-resistant gene for selectivity and provided expression of recombinant protein with a poly-histidine (His) tag and an enterokinase (EK) recognition site in the N-terminus (see Figure 2-5). The poly-His tag would allow for purification of the fusion protein with a more convenient and efficient method, i.e. nickel(II) chelate affinity chromatography, followed by gel filtration chromatography. The EK recognition site would provide the option to remove the N-terminal tag with enterokinase.

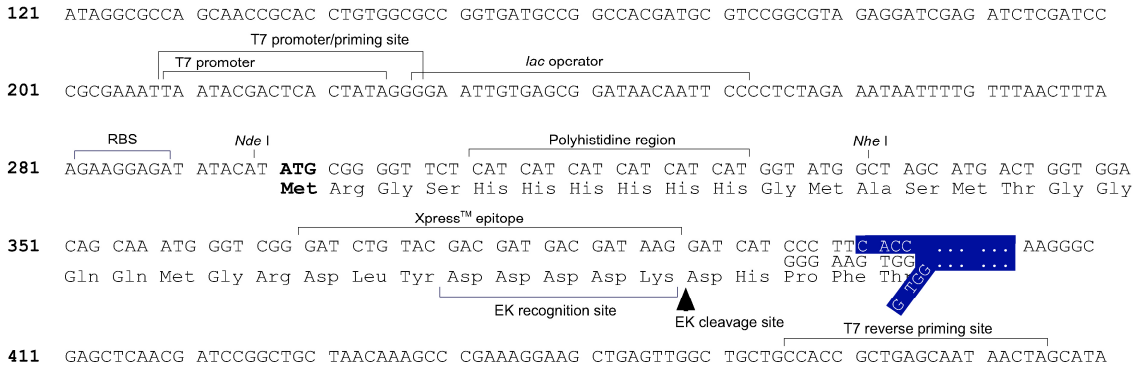


Figure 2-4. A PCR product with a "CACC" 5' end (blue highlight) was inserted into a vector of pET00/D by TOPO directional cloning (Invitrogen).

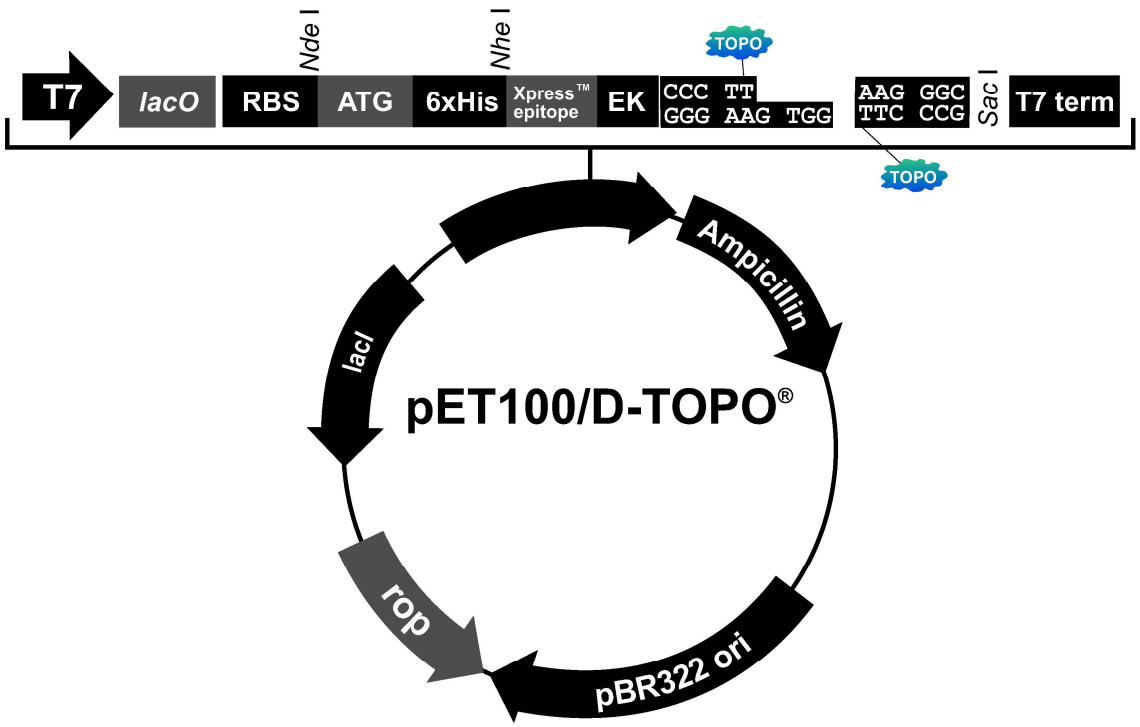


Figure 2-5. The gene map of the pET100/D-TOPO vector (Invitrogen). The plasmids are linearized by Topoisomerase I and become circularized upon insertion of a PCR product containing a 5' CACC overhang.

To perform the TOPO cloning reaction, 4 μl of fresh PCR product, 1 μl salt solution and 1 μl TOPO pET100/D vector were added into a 0.5 ml microcentrifuge tube, mixed gently, and incubate for 30 min at room temperature. The reaction was then placed on ice to proceed to transformation. The hRARγ LBD was propagated on either *E. coli* strain TOP10 (Invitrogen) or *E. coli* BL21 Star DE3 cells (Invitrogen) in LB plates or medium containing 200 μg/ml ampicillin for selection of transformants and expression experiments. The optimal growth temperature was 37°C. The hRARγ LBD/pET100/D construct (4 μl of TOPO cloning reaction) was added into one vial of competent TOP10 cells (Invitrogen) and gently mixed. The solution was incubated on ice for 30 min. The cells were heat-shocked for 30 seconds at 42°C without shaking and transferred back on

ice immediately. The room temperature SOC medium (250 μl) was added into the vial to provide sufficient nutrition to help cell propagation. The vial was capped tightly and incubated with shaking (200 rpm) at 37°C for 1 hour. Then, using a bent glass rod, 150 μl of the transformant was spread on a pre-warmed (37°C) agar plate containing 200 μg/ml ampicillin and was incubated overnight at 37°C. Into each of six 15-mL conical bottom vials, 12 ml of pre-warmed LB medium was added, and each was inoculated with a single, well-isolated colony picked from the overnight incubated plate and grown with shaking (200 rpm) overnight at 37°C. An aliquot (1.5 ml) of the overnight cell culture was mixed gently with 0.5 ml of a sterile 60% glycerol solution in a 2 ml cryo-vial and stored at -80°C for future propagation and maintenance of gene construct. The remaining media from the cultures were isolated by the Wizard Plus SV Miniprep DNA Purification System as previously described.

To pick the right plasmid DNA with the correct insertion, the isolated plasmid DNA (hRARγ LBD/TOPO pET100/D) (~10 ng/ml) was digested by the restriction enzyme *SacI* (Promega). In each of the 0.5 ml microcentrifuge tubes (total of six), 14.8 μl of sterile water, 2 μl of RE 10x buffer, 0.2 μl acetylated BSA, 4 μl isolated plasmid DNA and 1 μl of the *Sac* I solution were added and incubated at 37°C for 2 hrs. After running the protease-treated samples through agarose gel electrophoresis with the undigested plasmid DNA as a negative control, the integrity of the plasmid DNA was checked to confirm that the hRARγ LBD gene was cloned into the pET100/D vector with the correct size and orientation. The DNA fluorescent sequencing with T7 promoter and terminator primers, which control the expression from the plasmid, was performed by the

Recombinant DNA/Protein Resource Facility of the Biochemistry and Molecular Biology department to confirm the construct was in frame with the appropriate N-terminal fusion tag. Later, the correct isolated plasmid DNA (3 μl) with a concentration of 10 ng/μl was transformed into BL21 Star DE3 cells using the same protocol as that of transforming TOP10 cells. Without plating, the transformant was directly used for cell culturing.

2.2.2 Protein expression and purification

A 10-ml sample of LB medium with 200 μg/ml ampicillin was inoculated with hRARγ LBD/pET100/D TOPO-transformed *E. coli* BL21 Star (DE3). The cell culture was grown overnight at 37°C with shaking (225-250 rpm) until an optical absorbance of the culture, measured at 600 nm (A_{600}), was equal to between 1 and 2. The entire overnight culture was used to inoculate 200 ml LB media containing 200 μg/ml ampicillin. Cells were grown at 37°C with shaking (225-250 rpm). Once the absorbance (A_{600}) was about 0.6 (about 3 h), the incubator's temperature was changed from 37°C to 25°C. Then the expression of the recombinant protein was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma), and the bacteria were incubated with shaking for an additional 3 to 4 h at room temperature until a final absorbance of $A_{600} = 1.3$. Cells were harvested by centrifugation (Allegra X-15R Benchtop Centrifuge, Beckman Coulter) at 4500 rpm for 10 min at 4°C. The supernatant was discarded and the pellets were frozen and kept at -80°C until needed.

Part of the hRARγ LBD purification and crystallization protocol followed the procedure described by Natacha Rochel, *et al*.[39] It was modified to find the most

appropriate conditions for this particular construct. The cell pellet from the 400-ml culture was fully resuspended in 10 ml ice-cold buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris HCl pH 8.0). After addition of 100 mg/ml of lysozyme, the cells were incubated for 30 min on ice and 5 min at 42°C. The lysate was sonicated (Sonifier 150 Liquid Processor, Branson) for 1 min at analog speed setting of "3" (about 540 rpm) followed by cooling on ice for 1 min. The sonication/cooling steps were repeated twice more until the lysate was no longer viscous. During the optimization for the expression of the hRARγ LBD recombinant protein, it was discovered that the protein was soluble. Using the uninduced bacteria as a negative control, a protein band from induced bacteria corresponding to the expected size (~30 kDa) of hRARγ LBD was seen on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels using coomassie brilliant blue staining (see Figure 2-8 and 2-10). Electrophoresis was carried out using a Mini-PROTEAN 3 Cell (BioRad). After sonication, the cell debris was pelleted by centrifugation at 4500 rpm for 30 min at 4°C in an Allegra X-15R Benchtop Centrifuge (Beckman Coulter). The supernatant was transferred into a 10 ml conical tip centrifuge tube and centrifuged 3 or more hours until a clear crude extract was obtained.

To confirm that the overexpressed band was the desired protein, a Western blot was performed using an alkaline phosphatase (AP)-conjugated antibody (Anti-HisG-Ap, Invitrogen). This antibody specifically detects the 6x histidine tag. The samples of crude extract were run through an SDS-PAGE gel (15%), and then soaked in cold running buffer (20% methanol, 25 mM Tris pH 8.3, 192 mM glycine) for 15 min at 4°C. A sandwich containing a fiber pad, filter paper, the SDS-PAGE gel, a nitrocellulose

membrane, a second piece of filter paper, and a second fiber pad was assembled. The sandwich was placed in the Western blot apparatus with an ice block, a stir bar, and cold transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% (v/v) methanol) filled container. The protein was transferred onto the nitrocellulose membrane electrophoretically at 100 V for 1 hour. Stirring was continued during the run to evenly mix the solution. After electrophoresis, the nitrocellulose membrane was removed from the sandwich and soaked in 10 ml of blocking solution (5% nonfat dry milk mixed with PBS solution containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7\ H_2O$ and 1.4 mM KH_2PO_4, pH 7.5) with gentle agitation on a rocker platform for 1 hour. For best results, the following steps were all done with gentle agitation. The membrane was washed with 20 ml TBST (20 mM Tris-HCl, 140 mM NaCl, pH 7.5, 0.1% Tween-20) for 5 min and repeated two more times. The membrane was then transferred into a 10 ml dilution buffer (TBST solution with 1% nonfat dry milk and 2 μl of the AP-conjugated antibody) and incubated overnight. The membrane was moved into 20 ml of TBST and washed for 5 min and repeated three additional times. The membrane was washed in 20 ml of TBS (20 mM Tris-HCl, 140 mM NaCl, pH 7.5) for 5 minutes to remove detergent. After another two rinses in 20 ml alkaline phosphate buffer (100 mM diethanolamine, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5), the membrane was transferred to 10 ml of the alkaline phosphate buffer containing 33 μl of a 50 mg/ml BCIP (5-bromo-4-chloro-3'-indolyphosphate *p*-toluidine salt) solution and 330 μl of a 10 mg/ml NBT (Nitro-Blue tetrazolium chloride) solution, and the color was allowed to develop. The recombinant protein was shown on the membrane as purple bands at the correct size (see Figure 2-9).

The color development was stopped by washing with DI water, and the membrane was air-dried.

The Biologic Duoflow Chromatography System (BioRad) was used for purification of hRARγ LBD recombinant protein. The nickel(II) chelate affinity chromatography was performed as the first step of the purification protocol because the engineered protein contained a poly-His tag in the N-terminus. Since proteins without this tag do not bind to the nickel metal ion, or bind only weakly, they were eluted first. Therefore, it is possible to separate proteins with the tag from those without the tag by gradually increasing the imidazole concentration during the elution step.

By the use of ampicillin antibiotics, β-lactomase, which was encoded in the ampicillin resistant gene, was produced in substantial amounts during culturing. The β-lactomase could not be washed out of the column by using the same condition as most of the other non-specific proteins in the crude extract were. The stepwise purification protocol was optimized after a purification run using a linear gradient method. A nickel(II) chelate column with a 5-ml bed volume (HisTrap HP, GE Healthcare) was equilibrated with 10 column volumes of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris HCl pH 8.0) at a flow rate of 5 ml/min. The clear crude extract (10 ml) from 400 ml of cell culture was preloaded into the column at 1 ml/min, followed by washing with 10 volumes of elution buffer I (50 mM imidazole, 500 mM NaCl, 20 mM Tris HCl pH 8.0) and 12 volumes of elution buffer II (500 mM imidazole, 500 mM NaCl, 20 mM Tris HCl

pH 8.0) at 5 ml/min. The protein of interest, hRARγ LBD (~30 kDa), was eluted with 200 mM or 500 mM imidazole but β-lactomase was eluted with 50 mM imidazole.

After the fractions that contained the LBD were concentrated (Allegra X-15R Benchtop Centrifuge, Beckman Coulter, at 3000 rpm, 4°C) to 1 ml using a 20 ml centricon with 10,000 MW cut-off (Vivaspin, Vivascience), the hRARγ LBD sample was loaded onto a Superdex 200 10/300GL column for gel filtration chromatography. Adding reducing agents, such as 1,4-dithiothreitol (DTT), can prevent the hRARγ LBD protein, which contains free Cys residues, from oxidization and degradation.[40] The buffer containing 10 mM DTT, 500 mM NaCl, 10 mM Tris-HCl pH 8.0, and 0.15 mM n-dodecyl-β-D-maltoside[39] was run through the column at a flow rate of 0.25 ml/min for protein washing and elution (see Figure 2-15). The purified recombinant protein hRARγ LBD was concentrated (2000 rpm, 4°C, time varies), and the final concentration was determined by the Bradford assay using bovine serum albumin as the standard (see Figure 2-6). In this method, Coomassie Brilliant Blue G-250 will bind to proteins and then obtain absorbance in the visible region (blue color; 600 nm). The protein concentration is proportional to the absorbance and can be calculated based on a calibration curve of absorbance *vs.* concentration.

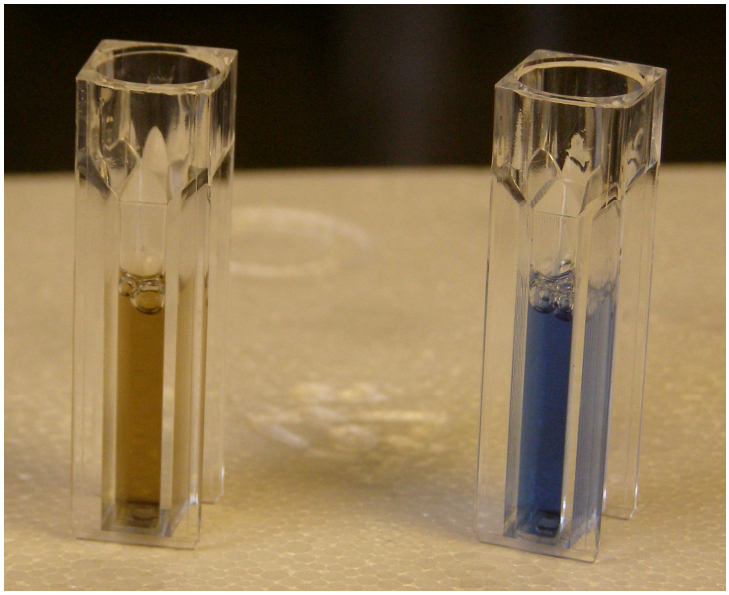


Figure 2-6. Bradford assay. The left solution (brown) without protein acts as a blank. The right solution containing hRARγ LBD turns blue after 5 minutes incubation at room temperature. The darker blue the solution becomes, the more concentrated the protein solution.

2.2.3. Crystallization

The process of exploring the three-dimensional structure begins with obtaining large, single protein crystals which are suitable for X-ray diffraction. Crystals that diffract well and are larger than 100 μm are needed for this purpose.[41] A single crystal then is mounted in a loop or a capillary tube and placed in an X-ray beam. The diffraction pattern is collected (at cryo-temperatures in the case of mounting in the loop) and analyzed to obtain the structure of the protein. But it is very difficult to predict the optimal condition for protein crystal growth because a number of environmental variables, such as protein purity, pH, protein concentration, temperature, precipitants, etc., have to be considered.[42] A highly purified protein with at least 90-95% purity is a prerequisite for the formation of sufficient homogeneity during crystallization. A buffer helps stabilize the pH value of the crystallization condition. The addition of precipitants

is necessary to promote crystallization of protein from solution. A change of temperature makes protein more or less saturated.

In this research, the hanging-drop vapor diffusion method was used (see Figure 2-7), which is the most frequently reported method for crystallization. In a vacuum grease-sealed space, the well solution usually consisted of a mixture of buffer, precipitant and additives. The droplet hanging on the coverslip is composed of both protein solution and well solution. Since the droplet contained precipitate with less concentration than that in the well solution, water vapor diffused from the droplet and was transferred to the reservoir in the closed space. When the droplet becomes supersaturated,[40] spontaneous nucleation of the crystals occurs. If the right condition was achieved, which means a metastable region (a condition between solubility and supersolubility), the crystal would keep growing.[40] Since the system would be in equilibrium, the optimum conditions would be maintained until the crystallization was complete.[42] Usually, crystal screening (Hampton Research) is the first step to find the possible conditions for crystallization. It is a method of sparse matrix sampling proposed by Jancarik *et al.*[43] The solution and precipitate conditions are empirically derived based on known or published crystallization conditions of various proteins in the past.[43] Once the appearance of micro-crystals, polycrystalline aggregates, thin plates, etc., has been observed for the protein[40] in the screening, the crystallization conditions should be optimized by small adjustments of pH, temperature alteration, or changes in concentration of precipitant and additives.

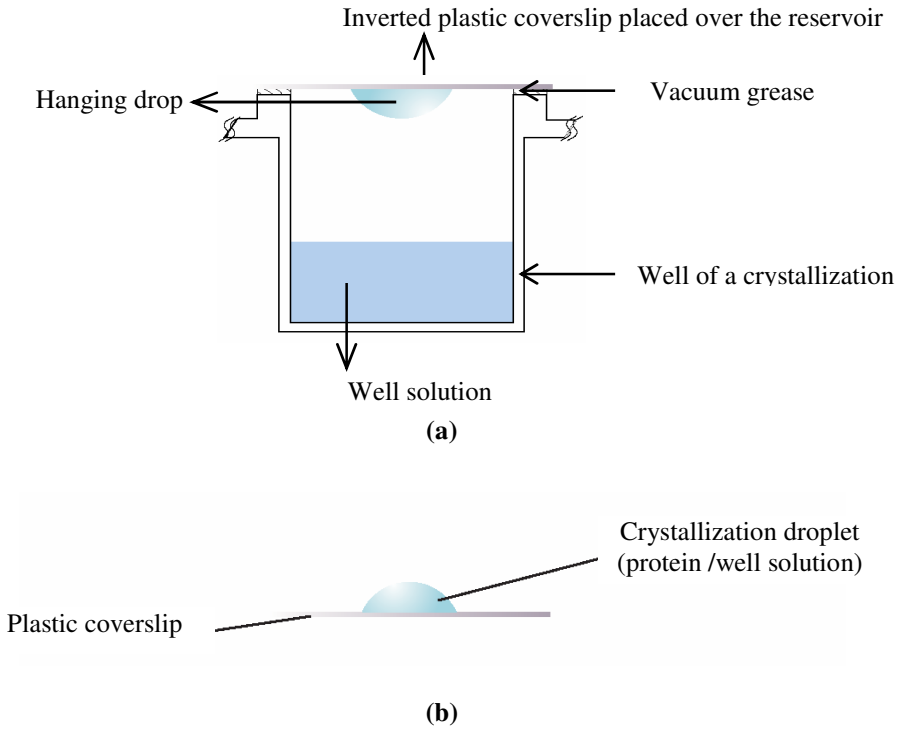


Figure 2-7. The demonstration of the hanging drop vapor diffusion method (Hampton Research). (a) Vacuum grease-sealed experimental setup with a droplet hanging on the inverted coverslip. (b) hanging drop, which is usually an equal amount of protein solution and well solution.

The purified protein of hRARγ LBD with a poly-His tag (~30 kDa) was used for crystallization trials. The co-crystallization of hRARγ LBD and T-RA was used as the starting point to check the integrity of our construct against the published crystallization condition by Rochel *et al.*[39]and Klaholz *et al.*[21] and to establish a methodology and comparison point for the investigation of the LBD bound to heteroarotinoids. The poly-His tag was designed with an EK recognition sequence so it could be removed, if necessary, by EK digestion. However, Klaholz *et al.* observed that the protein became unstable after removal of the poly-His tag.[21] After purification, the protein was concentrated to 2-3 mg/ml and was incubated with 2 equivalents of T-RA (Sigma) (20

mM ethanolic stock solution) overnight at 4°C. Then the complex of hRARγ LBD and T-RA was centrifuged for 30 min at maximum speed (Marathon Microcentrifuge, Fisher Scientific) to remove any precipitate before the crystallization setup. The final protein solution contained 10 mM DTT, 500 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.15 mM n-dodecyl-β-D-maltoside, 2 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) and 4% glycerol. Since the reducing agent DTT only remained active for 2-3 days in the protein solutions,[30] crystallization trials were carried out within three days of gel filtration. Crystallization trials were performed at room temperature and 4°C using the hanging-drop vapor diffusion method in 24-well tissue culture plates.

The 500 μl crystallization well solution was composed of a combination of 100 mM 1,4-piperazinediethanesulfonic acid (PIPES) (pH 6.5-8.5), 1.0-1.7 M sodium acetate, and DI water. Hanging drops (10 μl) were typically formed by pipetting 3, 4, 5, 6, or 7 μl of well solution into 7, 6, 5, 4, or 3 μl of protein solution on coverslips, respectively. Hanging drops with a volume of 5 μl or 6 μl were also explored. Meanwhile, the crystal screen kits (Hampton research) were tried to find new leads to optimize the crystallization conditions.

2.3 Results and discussion

Finally, the highly purified and concentrated protein solution of hRARγ LBD has been obtained and crystallization trials are underway. But it took a long time to get the cloning right. The problem arose with the plasmid containing the sequence in the incorrect register, which was caused by the use of a plasmid with, as the company

(Invitrogen) explained, “some background”. We obtained a new plasmid. Transformation was a very crucial step to guarantee expression of the desired recombinant protein. Therefore, to avoid contamination from other non-specific plasmids, the use of gel-purified PCR product and a sterile experimental environment should be considered at all times during cloning.

The fragment of hRARγ LBD contains 738 bp. The molecular weight of the recombinant protein fused with a histidine tag is around 30 kDa. The sequence of the construct of hRARγ LBD/pET100/D-TOPO matches that of the known DNA of hRARγ from the NCBI database completely with the exception of one nucleotide, which causes a silent mutation that still produces Gln (the “G” was substituted by “A”: CAG→CAA; see Appendices I and II). This change does not affect the constructed LBD amino acid sequence.

During the bacterial culturing stage, the addition of 1% or 5% glucose or sucrose[21] was done to assess production levels in comparison with an experiment without sugar added. In these experiments, there did not seem to be more bacteria produced in the culture with the addition of sugar. The recombinant protein with a poly-His tag has been detected and verified by Western Blot analysis with alkaline phosphatase (AP)-conjugated antibodies (see Figure 2-8 and 2-9).

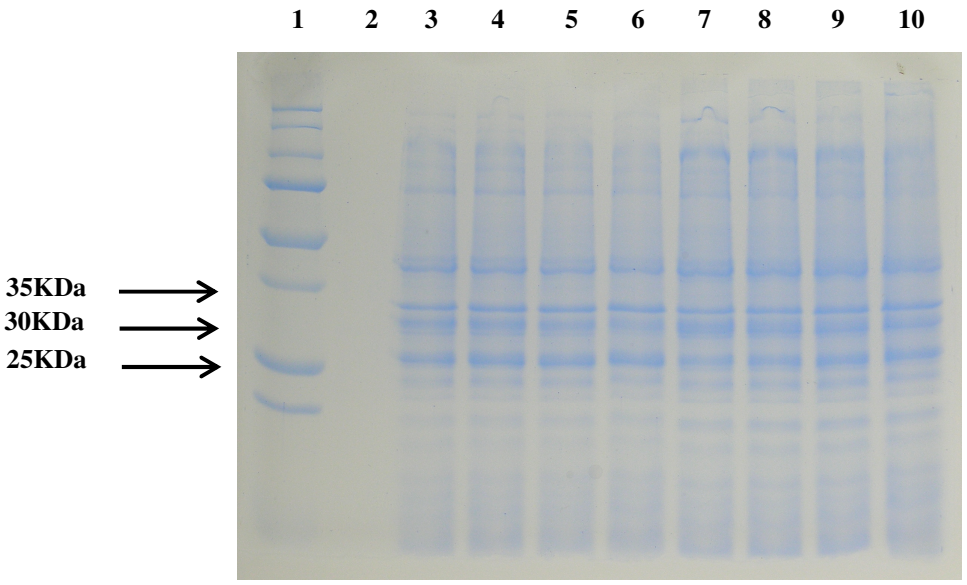


Figure 2-8. SDS-PAGE gel shows protein expression of hRARγ LBD over time after IPTG induction. There are 10 lanes on the gel. The first lane shows the size marker. The second lane was loaded with blank. From the third to tenth lane, the lanes with odd number were loaded by the sample from the culture without sugar addition; the lanes with even number were loaded by the samples from the culture with addition of 1% sucrose. There were 4 time points. Samples were taken every one hour (two lanes) after induction.

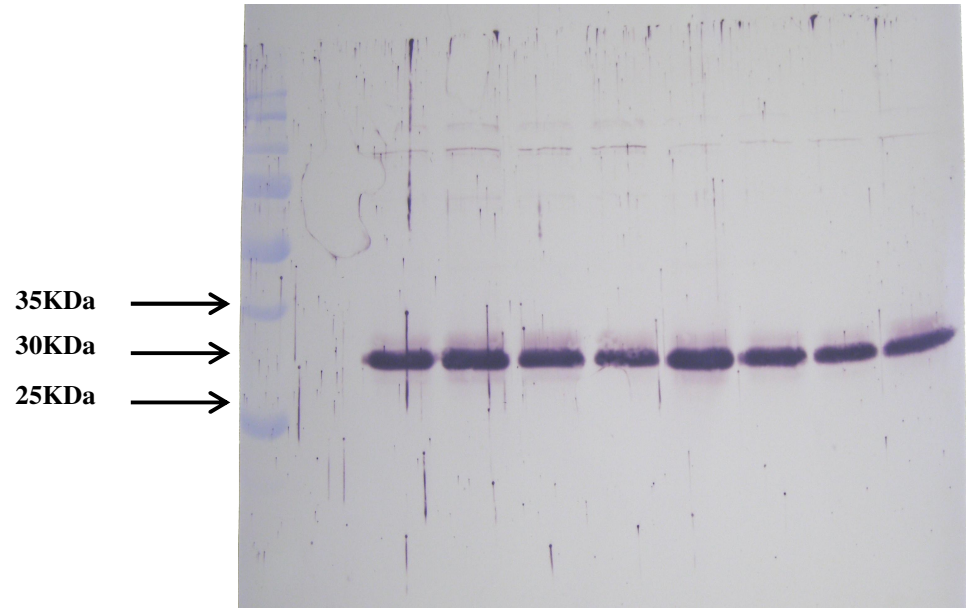


Figure 2-9. Verification of the recombinant protein of hRARγ LBD on a nitrocellulose membrane by Western blot technique. The Western blot was obtained from an identically run, but unstained, SDS-PAGE gel as in Figure 2-8.

Each stage of expression and purification of hRARγ LBD was analyzed by SDS-PAGE gel electrophoresis (see Figure 2-10). The protein analysis through MALDI-TOF mass spectroscopy confirmed the desired protein obtained (see Figure 2-11).

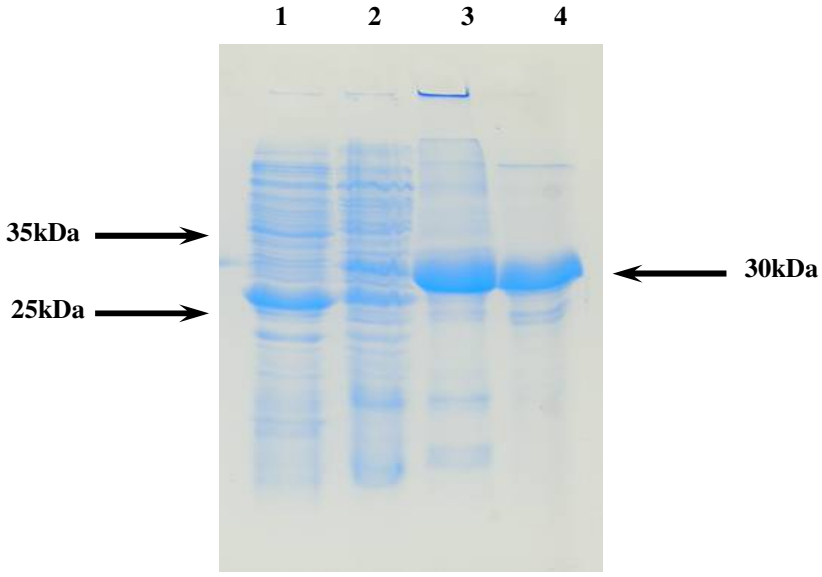


Figure 2-10. Coomassie blue-stained SDS-PAGE gel of $(His)_6$-hRARγ LBD at each stage of purification. Lane 1: crude extract from non-induced bacteria; Lane 2: crude extract from IPTG-induced bacteria; Lane 3: $(His)_6$-hRARγ LBD eluted from the nickel(II) chelate affinity chromatography column; Lane 4: $(His)_6$-hRARγ LBD after gel-filtration chromatography (final stage).

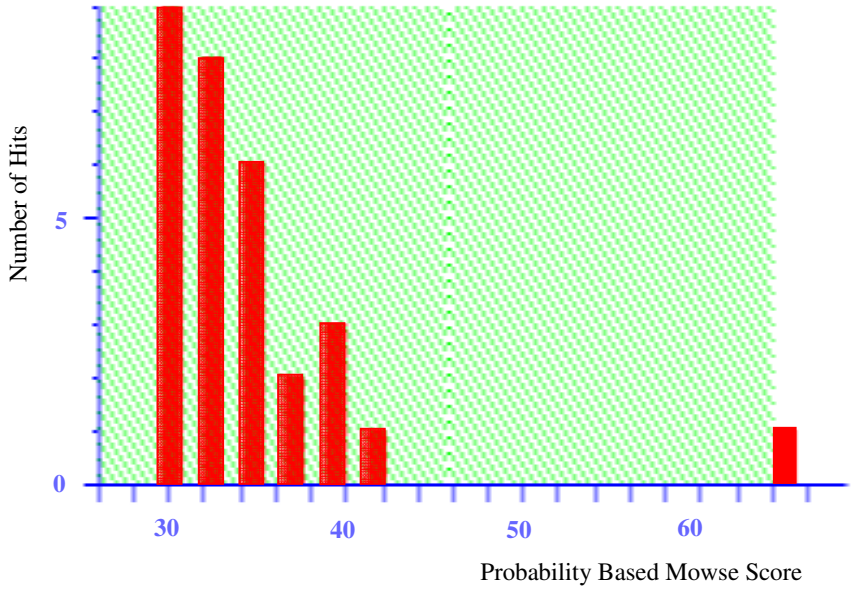


Figure 2-11. Result of MALDI-TOF mass spectroscopic analysis for recombinant hRARγ LBD. The sample was cut from the band on the fourth lane of the SDS-PAGE gel (Figure 2-10). Top score is 65 for human retinoic acid receptor gamma LBD (ligand-binding domain, residues 178-423). Protein scores greater than 64 are significant ($p<0.05$).

The following chromatograms (Figures 2-12 through 2-15) show the protein purification at different stages with different protocols. In each chromatogram, the relationship of absorbance (280 nm; blue) *vs.* time (min) or conductivity (mS/cm; red) *vs.* time (min) is displayed. The top horizontal scale shows fraction numbers of the eluted solution collected. The change in concentration of imidazole is indicated by the black line. The purple line shows the change of pH value during separation. To obtain more concentrated protein solution after nickel column, 500 mM imidazole was directly used without the elution step with 200 mM imidazole after β-lactomase elution. The chromatograms of Figure 2-14 and 2-15 correspond to the protocols currently in use for protein purification. In Figure 2-14, the recombinant protein of hRARγ LBD was purified with 500 mM imidazole through a nickel(II) column and collected from the fractions 38 to 48. In Figure 2-15, the protein eluted from gel-filtration without imidazole, was

purified and collected from fractions 15 to 17. During the gel-filtration step (see Figure 2-15), no protein was detected in the third peak by SDS-PAGE electrophoresis. This peak should correspond to imidazole elution since imidazole will absorb at 280 nm. Thus, without the protein dialysis step, which was used in the purification that Rochel, *et al.* described,[39] imidazole can be removed at the gel-filtration stage so that the purified protein was ready for crystallization.

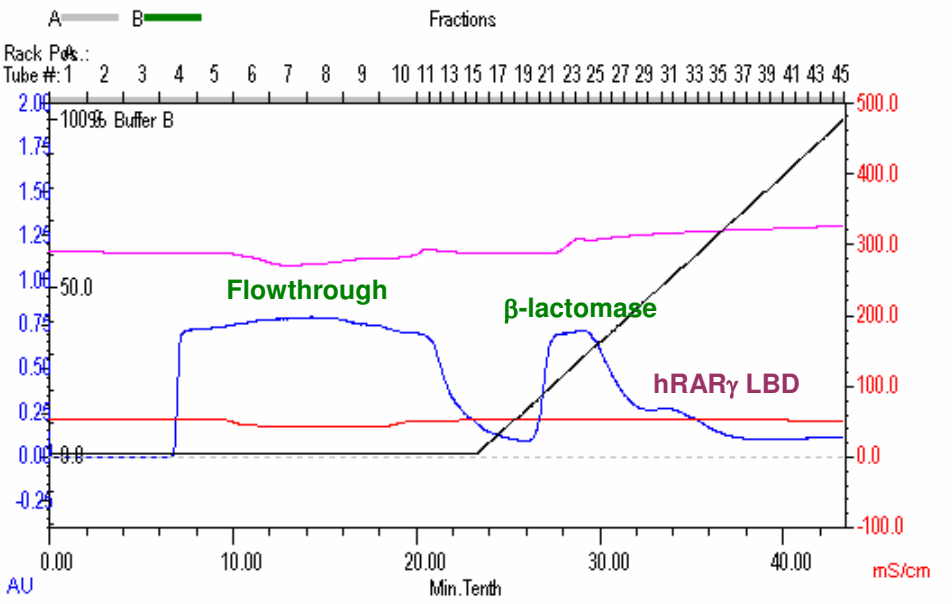


Figure 2-12. Purification of the hRARγ LBD through nickel(II) chelate affinity chromatography using a linear gradient method where the imidazole concentration is gradually varied from 5 to 500 mM.

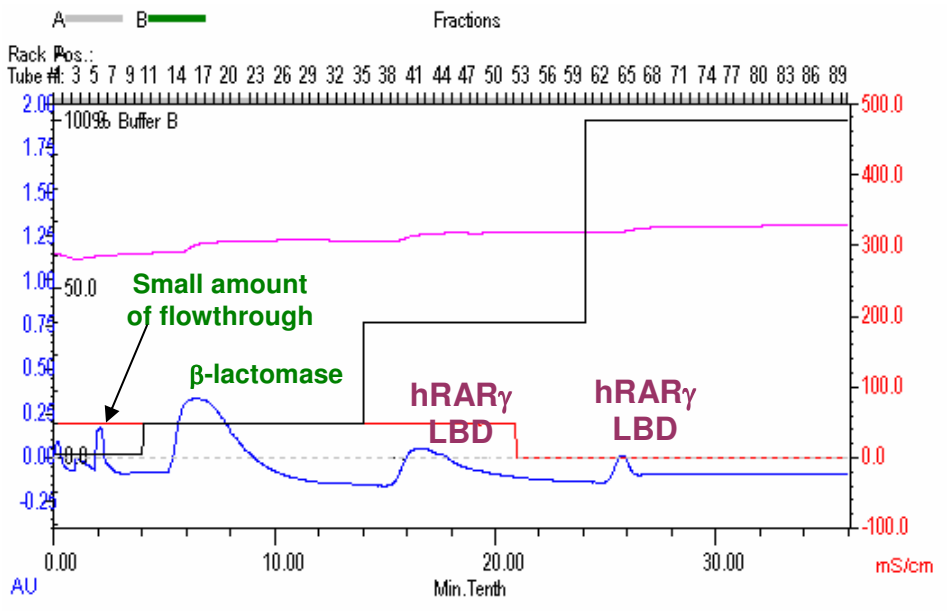


Figure 2-13. Purification of the hRARγ LBD through nickel(II) chelate affinity chromatography by a stepwise purification method. The crude extract was preloaded and washed with 5 mM imidazole. The target protein was eluted with 200 mM and 500 mM imidazole.

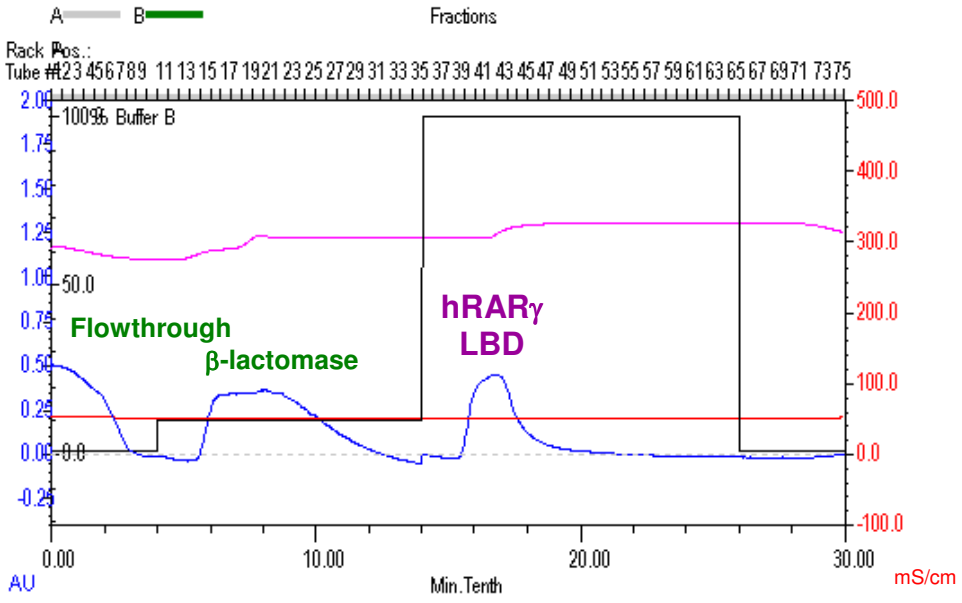


Figure 2-14. Purification of the hRARγ LBD through nickel(II) chelate affinity chromatography by a stepwise purification method. The target protein was eluted only with 500 mM imidazole.

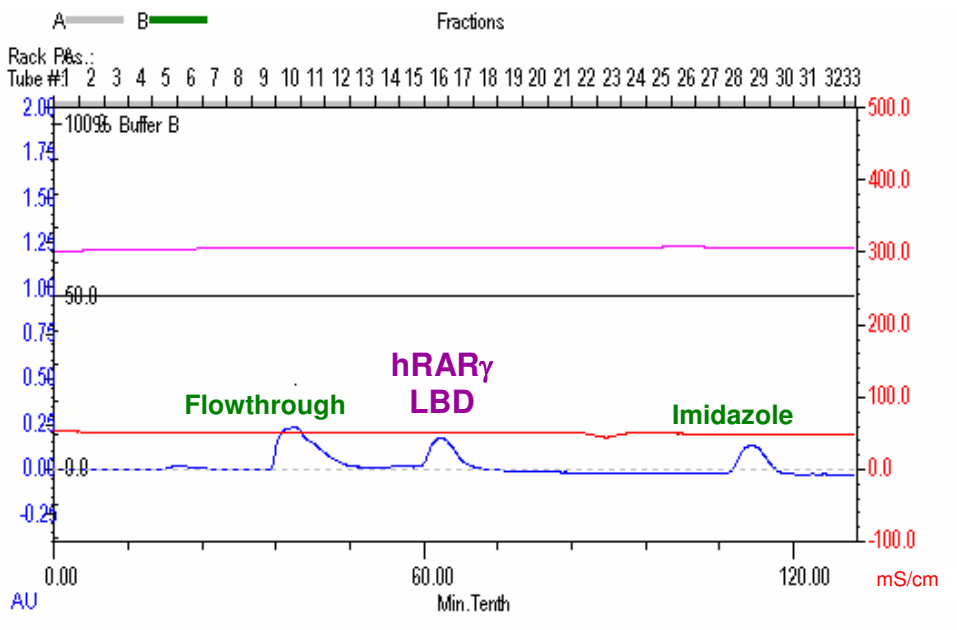


Figure 2-15. Purification of hRARγ LBD by gel-filtration chromatography. In the flowthrough solution, there are non-specific proteins with small amount of the protein hRARγ LBD which detected by SDS-PAGE.

The yield of the purified recombinant protein is measured by the Bradford method. For a 750-ml culture, about 4 mg of purified protein is obtained and ready to be used in crystallization. After gel-filtration, the protein of the hRARγ LBD is about 4/5 less than that of the one out of the nickel (II) chelate resin. This is not only because of the removal of impurities during the gel-filtration stage, but also because of two more reasons. Part of the recombinant protein becomes denatured during purification and has to be removed by centrifugation due to its loss of biological activity. Also, some protein sticks to the centricon membrane in the concentration procedure so that not all of the protein is completely recovered. Those factors should be considered to optimize the purification condition and improve the yield of purified protein with biological activity.

The crystal screening was done by using the screen kits HR2-110 containing 50 conditions, HR2-112 containing 48 conditions, and Index containing 96 conditions (Hampton Research). A few very small crystals of hRARγ LBD complexed with T-RA with good three-dimensional structure and sharp edges were observed by using a well solution containing 0.1 M cadmium chloride hydrate, 0.1 M sodium acetate trihydrate (pH 4.6) and 30% v/v polyethylene glycol 400 (No. 34, Crystal Screen 2, HR2-112) at room temperature. From the crystallization condition optimized from the literature,[39] some larger but thinner crystals always grew in 2-3 days under pH 7.0 at either 4°C or room temperature. None of the crystals diffracted X-rays at medium to high resolution to warrant the collection of data or even to obtain the crystal space group. To obtain crystals with high quality, there are three ways that could be helpful. First, increase the concentration of protein (such as 5 mg/ml) for crystallization because proteins possibly form aggregates more easily in dilute solution making it hard to crystallize.[40] Second, optimization of crystallization conditions based on the screen results need to be done by small adjustments of pH and concentration of salts or precipitant. Third, microseeding techniques are worth exploring to improve the quality of the crystals as well as promote growth of single crystals. For the future work, improved crystals of hRARγ LBD in complex with T-RA [**3**] are desired. If the integrity of the hRARγ LBD construct can be proven through structural analysis based on X-ray crystallography, the construct should be used to co-crystallize with SHetA2, SHetC2, and other heteroarotinoids to better understand the interaction between hRARγ and retinoid ligands so as to help improve drug design.

CHAPTER III

CONCLUSION

The hRARγ plays an essential role in the modulation of the retinoid-induced therapeutic benefits in the skin and the retinoid-induced skin irritation.[36] Flex-Hets with a flexible amide linker and a sulfur atom fused ring are very important for RARγ receptor activation and for biological activity. Recent research shows that heteroarotinoids exhibit very low toxicity while keeping their therapeutic promise in tissue and in animal models.[23,31-33] This is very critical for drug design. X-ray analyses of single crystals of various heteroarotinoid-RARγ complexes will provide more information and details of the RARγ selectivity and on the impact of ligands in the RARγ LBP. Taking advantage of the binding properties of the protein will lead to additional and improved drug development. This is the ultimate goal of our research.

The hRARγ LBD fragment (residues 178-423) has been successfully cloned into pET 100/D vector and overproduced with high efficiency using the TOPO *E. coli*/T7 system. This work demonstrates the feasibility of production of recombinant hRARγ LBD fusion construct by the TOPO system. The highly pure recombinant protein of hRARγ LBD with a poly-His tag was obtained via nickel (II) chelate affinity chromatography, followed by gel-filtration chromatography. The formation of small

crystals of hRARγ LBD in complex with T-RA in the preliminary crystallization trials shows promising crystallization possibilities. Further efforts will focus on improving the quality of single crystals co-crystallized by the complex of hRARγ LBD and T-RA or heteroarotinoids.

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APPENDICES

I. DNA Sequencing Results for Recombinant hRARγ LBD

hRARγ LBD/T7 promoter primer:

TGTAGTGGCGTACATTCCCCTCTGAATAATTTTGTTTAACTTTAAGAAGGAGA
TATACATATGCGGGGTTCTCATCATCATCATCATCATGGTATGGCTAGCATGA
CTGGTGGACAGCAAATGGGTCGGGATCTGTACGACGATGACGATAAGGATCA
TCCCTT**CACCGACAGCTATGAGCTGAGCCCTCAGTTAGAAGAGCTCATCA**
CCAAGGTCAGCAAAGCCCATCAGGAGACTTTCCCCTCGCTCTGCCAGCT
GGGCAAGTATACCACGAACTCCAGTGCAGACCACCGCGTGCAGCTGGAT
CTGGGGCTGTGGGACAAGTTCAGTGAGCTGGCTACCAAGTGCATCATCA
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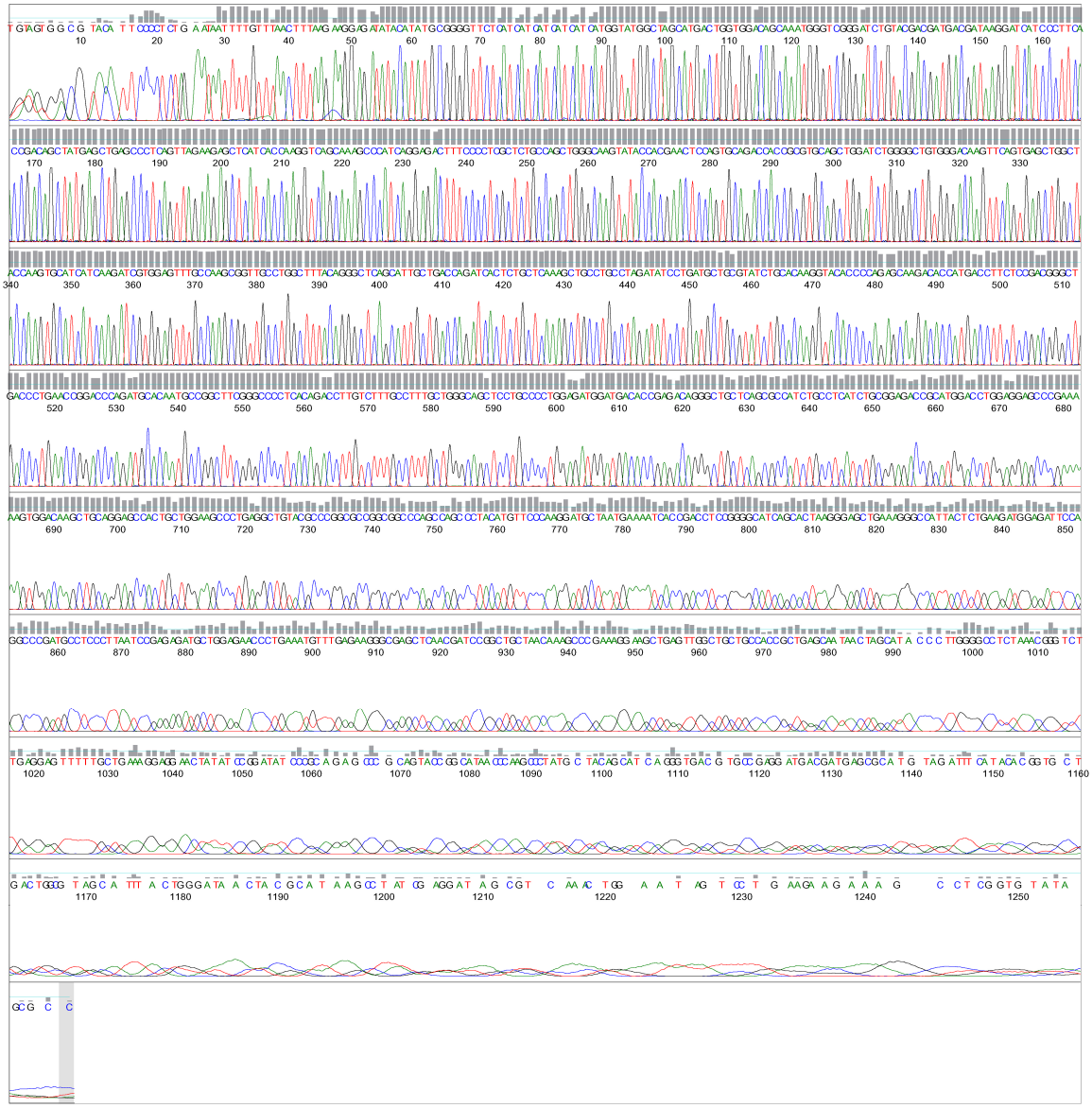
GGTGCTGACTGGCGTAGCATTTACTGGGATAACTACGCATAAGCCTATCGAG
GATAGCGTCAAACTGGAATAGTCCTGAAGAAGAAAGCCTCGGTGTATAGCGC

File: F04_5A3-1-T7PRO_011_copy.ab1

Geospiza
www.geospiza.com

Sample Name:	5A3-1-T7PRO	Signal Strengths:	A = 4891, C = 3345, G = 4867, T = 3722
Mobility:	KB_3730_POP7_BDTv1.mob	Lane/Cap#:	11
Spacing:	15.4103	Matrix:	n/a
Comment:	Xinyi	Direction:	Native



hRARγ LBD/T7 terminator primer:

GGTCAACACAATCGCTTCTTTCGGCTTTGTTAGCAGCCGGACGTTGACCTCGC
CCTT**CTCAAACATTTCAGGGTTCTCCAGCATCTCTCGGATTAAGGGAGGC**
ATCGGGCCTGGAATCTCCATCTTCAGAGTAATGGCCCTTTCAGCTCCCTT
AGTGCTGATGCCCCGGAGGTCGGTGATTTTCATTAGCATCCTTGGGAAC
ATGTAGGGCTGGCTGGGCCGCCGGCGCCGGGCGTACAGCCTCAGGGCT
TCCAGCAGTGGCTCCTGCAGCTTGTCCACTTTTTCGGGCTCCTCCAGGTC
CATGCGGTCTCCGCAGATGAGGCAGATGGCGCTGAGCAGCCCTGTCTCG
GTGTCATCCATCTCCAGGGGCAGGAGCTGCCCAGCAAAGGCAAAGACAA
GGTCTGTGAGGGGCCCGAAGCCGGCATTGTGCATCTGGGTCCGGTTCAG
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GTGCAGATACGCAGCATCAGGATATCTAGGCAGGCAGCTTTGAGCAGAG
TGATCTGGTCAGCAATGCTGAGCCCTGTAAAGCCAGGCAACCGCTTGGC
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GGTATACTTGCCCAGCTGGCAGAGCGAGGGGAAAGTCTCCTGATGGGCT
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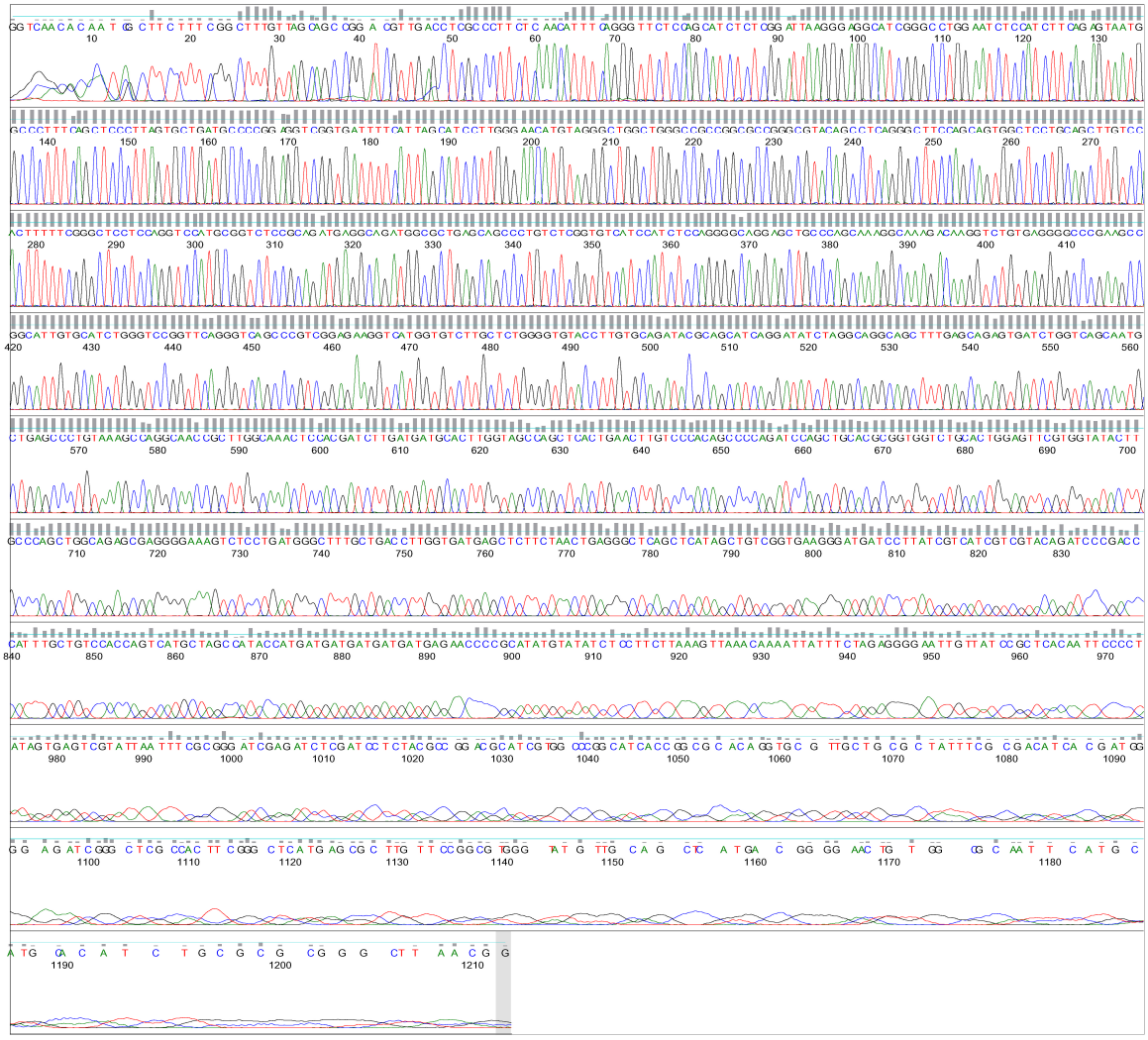
GTCGGTGAAGGGATGATCCTTATCGTCATCGTCGTACAGATCCCGACCCATT
TGCTGTCCACCAGTCATGCTAGCCATACCATGATGATGATGATGATGAGAAC
CCCGCATATGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGG
AATTGTTATCCGCTCACAATTCCCCTATAGTGAGTCGTATTAATTTCGCGGGA
TCGAGATCTCGATCCTCTACGCCGGACGCATCGTGGCCCGGCATCACCGGCG
CACAGGTGCGTTGCTGCGCTATTTCGCGACATCACGATGGGGAGATCGGGCT
CGCCACTTCGGGCTCATGAGCGCTTGTTCCGGCGTGGGTATGTTGCAGCTCAT
GACGGGGAACTGTGGCGCAATTCATGCATGCACATCTGCGCGCGGGCTTAA

File: B11_5A3-T7TERM_047.ab1

Geospiza
www.geospiza.com

Sample Name:	5A3-T7TERM	Signal Strengths:	A = 853, C = 1013, G = 1951, T = 1093
Mobility:	KB_3730_POP7_BDTv1.mob	Lane/Cap#:	47
Spacing:	16.0129	Matrix:	n/a
Comment:	Xinyi	Direction:	Native



Note:

The bold sequence comes from the gene of hRARγ LBD, and the rest of sequence is from the plasmid pET100/D-TOPO.

ATG: Start codon;

CATCATCATCATCATCAT: 6xHis tag;

GATCTGTACGACGATGACGATAAG: EK recognition site;

CACC: Overhang addition to the primer

CAg (in complementary sequence, it is "cTG"): The "A" was substituted by "g" in the cloned sequence. This is a silent mutation. Both "CAA" and "CAG" are translated into "Gln".

The chromatograms opened by software of FinchTV (Finch Trace Viewer, Geospiza) show the nucleotide sequence data of the construct hRARγ LBD/pET100/D- TOPO. Each of the trace peaks correspond to a base that is displayed on the top of the chromatograms. The peaks are generated in a fluorescence spectrum. Each of the base types is shown with different colors. The reliability of base identification is dependent on the peak height of the traces. The greater the peak height, the more reliable the base identification.

VITA

XINYI PENG

Candidate for the Degree of

Master of Science

Thesis: STRUCTURAL STUDIES OF THE HUMAN RETINOIC ACID RECEPTOR GAMMA LIGAND-BINDING DOMAIN IN COMPLEX WITH ANTI-CANCER HETEROAROTINOIDS

Major Field: Chemistry

Biographical:

Personal Data: Born in Jianyang, Sichuan, P. R. China, 1975.

Education: Received Bachelor of Science degree in July, 1997, from Chongqing Normal University, Chongqing, P. R. China; Completed the requirements for the Master of Science with a major of analytical chemistry at Oklahoma State University, Stillwater, Oklahoma in December, 2007.

Experience: Research chemist and instructor of General Chemistry, Analytical Chemistry and Physical Chemistry in Chengdu Textile College, Sichuan, P. R. China (1997-2003);
Teaching/research assistant in the Department of Chemistry, Oklahoma State University, Oklahoma (2004-present).

Name: XINYI PENG Date of Degree: December 2007

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: STRUCTURAL STUDIES OF THE HUMAN RETINOIC ACID RECEPTOR GAMMA LIGAND-BINDING DOMAIN IN COMPLEX WITH ANTI-CANCER HETEROAROTINOIDS

Pages in Study: 55 Candidate for the Degree of Master of Science

Major Field: Chemistry

Scope and Method of Study: Retinoids play an important role in the therapy and prevention of cancer by interacting with the retinoic acid receptors (RARs) and the retinoid receptors (RXRs), which are ligand-dependent transcription regulators. Human RARγ is involved in skin photo-aging and carcinogenesis. The ultimate goal of this study is to obtain X-ray crystal structures of the hRARγ ligand-binding domain (LBD; residues 178-423) in complex with novel flexible heteroarotinoids (Flex-Hets), which are made up of a thiourea or urea linker, a NO_2 substitution and a flexible thiochroman unit and have been shown to be effective anti-cancer agents against certain human cancer cell lines. Atomic resolution of the heteroarotinoids interactions with the protein will aid in optimization of the compounds.

Findings and Conclusions: The hRARγ LBD has been successfully cloned with an *N*-terminal tag containing six histidines and over-expressed by TOPO *Escherichia coli*/T7 system. The highly pure protein solutions of the hRARγ LBD with the poly-His tag are effectively purified via nickel(II) chelate chromatography followed by gel filtration chromatography. Currently, preliminary crystallization trials of the hRARγ LBD in complex with all-trans retinoic acid (T-RA) are underway to check the integrity of the construct and establish a lead crystallization condition. The formation of small crystals shows promising crystallization possibilities. Further efforts will focus on the quality improvement of single crystals co-crystallized by the complex of hRARγ LBD T-RA or novel Flex-Hets.

ADVISER'S APPROVAL: Dr. Stacy D. Benson