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FAKULTETA ZA FARMACIJO

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THE STUDY OF SELECTED LIGAND BINDING TO THE RXR-RAR NUCLEAR RECEPTOR HETERODIMER IN THE PRESENCE OF THE NCoR5 CO-REPRESSOR AND THE ATTEMPT TO CRYSTALLIZE THE RXR-RAR-NCoR5 COMPLEX

PREUČEVANJE VEZAVE IZBRANIH LIGANDOV NA HETERODIMER JEDRNIH RECEPTORJEV RXR-RAR V PRISOTNOSTI KOREPRESORJA NCoR5 TER POSKUS KRISTALIZACIJE KOMPLEKSA RXR-RAR-NCoR5

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All research work was carried out at the Centre for Structural Biochemistry (CBS - Centre de Biochimie Structurale) in Montpellier, France under the working supervision of dr. Albane le Maire CR, and the mentorship of assist. prof. dr. Matjaž Jeras.

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And last but not least to Yi for always being there for me…

Magistrsko nalogo posvečam mamama in se vama zahvaljujem za vse!

Statement

I declare that I have made this thesis/work independently, under the working supervision of dr. Albane le Maire CR, and the mentorship of assist. prof. dr. Matjaž Jeras.

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ABSTRACT

Receptors are macromolecules expressed in the cell or on its surface, which selectively bind specific ligands, such as endogenous substances, bioactive molecules, etc. Such interactions generate specific physiological responses. Based on their structures, receptors are classified into four main groups: ion channel, G-protein-coupled, kinase-linked, and intracellular receptors. Members of intracellular receptors, i.e. nuclear receptors (NRs), bind steroid hormones, thyroid hormones and retinoids. Their response time is measured in hours or days and is much slower than that of membrane-bound receptors.

When their cognate ligands are not present, some nuclear receptors can act as repressors of transcription by engaging corepressor complexes (CoR) in order to target genes. This kind of repression is quite imperative in reproduction of metazoan, cell development and homeostasis. However, its specific molecular determinants are still not fully understood (1).

The mechanism, by which the retinoid acid receptor (RAR), in a physiologically relevant form of heterodimer, interacts, together with corepressors, with the retinoid X receptor (RXR), is still poorly understood. Additionally, the formation of their ternary complex can be modulated with different classes of RAR- and RXR- selective ligands. Agonists enhance the engagement of coactivators and destabilize the interaction with corepressors, thereby enabling transcriptional activity. While pure agonists inhibit CoR interactions, the inverse agonists stabilize the interaction with corepressors thereby increasing the receptor-mediated gene silencing (2).

Our first goal was to express RAR, RAR and NCoR (Nuclear receptor co-repressor) in *E. coli*, then form the RXR-RAR-NCoR complex, and finally try to obtain its crystal structure. The second goal was to analyse the effects of ligands on NCoR interaction with the expressed RXR and the pre-formed heterodimer RXR-RAR, with fluorescence anisotropy.

Crystallization of the complex was not successful. On the other hand we obtained promising results by analysing the effects of ligands with anisotropy. They imply, that there is a significant dissimilarity in NCoR binding affinity to RXR-RAR in the presence of the two tested ligands, i.e. AM580 (agonist) and BMS493 (inverse agonist). We were able to prove that BMS493 indeed acts as inverse agonist, which

stabilizes the interaction of RXR-RAR with NCoR. On the other hand the activity of AM580 was not significant enough to confirm its agonistic effect.

Keywords: nuclear receptor, ligand, heterodimer, anisotropy, crystallization

RAZŠIRJENI POVZETEK

Receptorji so makromolekule prisotne na površini ali v notranjosti celice, ki selektivno vežejo različne biogene snovi (endogene, eksogene učinkovine), kar sproži specifične fiziološke odzive. Poznamo štiri tipe receptorjev, in sicer ionske kanale, z G-proteinom sklopljene receptorje, receptorje, povezane s kinazami ter znotrajcelične ali citoplazemske receptorje. Slednji vežejo steroidne in tiroidne hormone ter retinoide, njihov fiziološki odziv pa merimo v urah in dnevnih, kar je veliko kasneje kot po vezavi ligandov na membranske receptorje.

Nekateri jedrni receptorji v odsotnosti ligandov delujejo z vezavo korepresorskih kompleksov, in sicer kot transkripcijski represorji tarčnih genov. Represija genov je ključnega pomena pri živalski reprodukciji ter razvoju in homeostazi organizmov. Kljub temu specifične molekularne determinante ostajajo nejasne.

Mehanizem interakcije jedrnega receptorja za retinojsko kislino (RAR) s korepresorjem (CoR), v povezavi s fiziološko pomembnejšim retinoidnim receptorskim heterodimerom (RXR) še ni v celoti razjasnena. Na nastanek tega heterodimera lahko vplivamo z vezavo specifičnih ligandov. Agonisti povečajo vezavo koaktivatorja (CoA) in s tem destabilizacijo interakcije s koreporesorjem, to pa vodi v transkripcijsko aktivnost. Inverzni agonisti pa stabilizirajo interakcijo s koreporesorjem, posledica česar je utišanje genov.

V okviru magistrske naloge smo si zadali dva cilja. Najprej smo želeli izolirati RAR, RXR, NCoR (jedrni korepresor) v *E. coli* in tvoriti kompleks RXR-RAR-NCoR5, nato pa ga še kristalizirati. S pridobljenimi kristali bi nato skušali določiti 3D-strukturo tega kompleksa. S pomočjo poznavanja 3D-strukture bi prispevali k boljšemu razumevanju delovanja kompleksa in s tem k načrtovanju nadaljnjega razvoja zdravil. Naš drugi cilj pa je bil preučevanje vpliva vezave dveh ligandov, in sicer AM580 in BMS493 na heterodimer jedrnih receptorjev RXR-RAR ob prisotnosti korepresorja NCoR5. Oba liganda se lahko vežeta na RAR in s tem povzročita spremembo njegove konfiguracije, pri čemer deluje AM580 kot agonist, ki naj bi z vezavo na heterodimer destabiliziral interakcijo med NCoR5 in heterodimerom, BMS493 pa kot inverzni agonist, ki naj bi stabiliziral omenjeno interakcijo ter tako povzročil utišanje tarčnih genov.

Po vnosu genskih konstruktov, ki so jih pripravili v Centru za strukturno biokemijo (CBS) v Montpellier-ju, v seve *Escherichie coli*, je sledila izolacija

proteina. Izolacija je potekla s pomočjo Ni-kolone, kjer se proteini, ki vsebujejo polihistidinske repe, vežejo na Ni ione priostne v koloni. Te nato izločimo ob prisotnosti imidazola. Po čiščenju na Ni-koloni smo najprej odstranili polihistidinske repe in proteinske molekule ločili z gelsko filtracijo, glede na njihovo velikost. Tako pripravljene vzorce smo nato še koncentrirali. Na ta način smo izolirali dva kompleksa, in sicer RXR-RAR-NCoR5 in heterodimer RXR-RAR.

Za preučevanje vplivov vezave ligandov na heterodimer RXR-RAR v prisotnosti NCoR5 smo uporabili fluorescenčno anizotropijo. Oba izolirana liganda, AM580 in BMS493, so pripravili v CBS-u, prav tako tudi NCoR5, ki je bil označen s fluoroforom ATTO 647 N.

Tvorba kompleksa RXR-RAR-NCoR5 ni bila povsem uspešna, saj je bil v njem prisoten še neznan kontaminant, ki smo ga izolirali skupaj z NCoR5. Predpostavili smo, da sta razloga za to lahko bodisi človeška napaka ali pa kontaminacija kolone.

Poskus kristalizacije je bil neuspešen, saj nismo uspeli pridobiti kristalov, čeprav smo uspeli tvoriti kompleks RXR-RAR-NCoR5. Vzroka za to bi lahko bila prisotnost prej omenjenega kontaminanta in prenizka koncentracija vzorca. Priložnosti za ponovitev eksprimenta pa zaradi okvare kristalizacijskega robota žal nismo imeli.

Poskus s fluorescenčno anizotropijo je bil uspešnejši, saj smo uspeli dokazati inverzno agonističen vpliv liganda BMS493 na heterodimer RXR-RAR v prisotnosti NCoR5. Agonističnega vpliva liganda AM580 pa na podlagi dobljenih rezulatov nismo mogli potrditi, in sicer zato, ker je bilo odstopanje pri rezultatih preveliko. Predvidevamo, da je bil ligand pri sobni temperaturi nestabilen. Z meritvami koncentracije AM580 pred vsakim poskusom smo ugotovili, da se ta po vsakem odmrzovanju zmanjša. Zato bi bilo smiselno, da bi za pripravo anizotropnih ploščic uporabili robota in tako zmanjšali negativen vpliv temperature ter se hkrati tudi izognili potencialnemu vplivu človeških napak.

Med oziroma po izvedbi naših poskusov smo prišli do nekaterih koristnih ugotovitev in dobili nekaj idej za bodoče delo, ki bi lahko doprinesle k boljšim rezultatom:

- tvorba heterodimera RXRd12-RAR (veriga H12 na RXR ni prisotna);
- tvorba policistroničnega vektorja NCoR5-RXR-RAR, v katerem bi bilo hkrati prisotnih več genov, s čimer bi se izognili potrebi po tvorbi kompleksa;

- uporaba kromatografije SEC-MALS (kromatografija ločevanja na podlagi velikosti sipanja svetlobe pod večimi koti angl. size exclusion chromatography with inline multi-angle light scattering), kjer sipanje svetlobe in koncentracijo analita merimo neposredno v vsaki eluirani frakciji, s čimer bi se izgonili potencialnim napakam, katerih vir je sama kolona;
- uporaba metode SAXS (sipanje X-žarkov pod majhnim kotom (op.p small-angle X-ray scattering), ki bi nam omogočila popolno strukturno opredelitev interakcije heterodimera RXR-RAR z domeno NID NCoR5.

Čeprav z našimi poskusi nismo bili popolnoma uspešni, pa menimo, da smo z dobljenimi rezultati kljub temu doprinesli k boljšemu razumevanju delovanja jedrnih receptorjev in tako naredili pomemben korak v smeri, ki vodi v uspešno kristalizacijo preučevanega receptorskega kompleksa v prihodnosti.

Ključne besede: jedrni receptor, ligand, heterodimer, anizotropija, kristalizacija

LIST OF ABBREVIATIONS

A

AF – activation function

AM580 - 4-[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2 naphthalenyl) carboxamido] benzoic acid

AR- androgen receptors

APS - Ammonium persulfate

B

BMS 493 - 4-[(1E)-2-[5,6-Dihydro-5,5-dimethyl-8-(2-phenylethynyl)-2-naphthalenyl] ethenyl] benzoic acid

C

CAR - Constitutive androstane receptor

CBS – Centre for structural biochemistry in Montpellier

CoA – coactivator

CoR – corepressor

COUP-TF - chicken ovalbumin upstream promoter-transcription factor

CV – column volume

D

DAX – dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene

DNA – Deoxyribonucleic acid

DBD – DNA binding domain

E

EC50 - half maximal effective concentration

ER – estrogen receptor

ERR – estrogen related receptor

F

FR – flow rate

FXR - farnesoid X receptor

G

GCNF – germ cell nuclear factor

GR – glucocorticoid receptor

H

HAT – histone acetyltransferase

HDAC – histone deacetylase

HNF – hepatocyte nuclear factor

HRE – hormone response element

HSP - heat shock proteins

I

IPTG – Isopropyl β-D-1-thiogalactopyranoside

IRCM - Institut de Recherche en Cancérologie de Montpellier (The institute of cancer research Montpellier)

L

LB – lysogen broth

LBD – ligand biding domain

LHR - liver receptor homolog-1

LXR - liver X receptor

M

mAU – miliabsorbance units

MR – Mineralocorticoid receptor

N

NCoR – nuclear receptor co-repressor

NGFIB - Nerve Growth factor IB

NOR - neuron-derived orphan receptor

NURR - Nuclear receptor related

NR – nuclear receptor

NTD – N-terminal domain

P

PAGE -polyacrylamide gel electrophoresis

PNR - photoreceptor cell-specific nuclear receptor

PPAR-peroxisome proliferator-activating receptor

PR – progesterone receptor

PXR - pregnane X receptor

R

RA – retinoic acid

RE – response elements

RAR - retinoic acid receptor

PNR - photoreceptor cell-specific nuclear recepto

ROR - RAR-related orphan receptor

RXR - retinoid X receptor

S

SDS – sodium dodecyl sulfate

SF - steroidogenic factor

SHP - small heterodimer partner

SHR - steroid hormone receptors

SERM – specific estrogen receptor modulator

T

TBP – TATA-binding protein

TEMED - Tetramethylethylenediamine

TFIIB – Transcription factor IIB

TGS - Tris/Glycine/SDS

TLX - homologue of the drosophila tailless gene

TR- thyroid hormone receptor

V
VDR – vitamin D receptor

1 INTRODUCTION

Receptors are important macromolecules located on and within the cell that selectively bind specific biogenic substances resulting specific physiological response.

Based on their structures, receptors are classified into following four groups:

- ✓ ion channel receptors;
- ✓ G-protein-coupled receptors;
- ✓ kinase-linked receptors;
- ✓ intracellular receptors.

Among receptors there are around 50 intracellular ones that are particularly important for direct regulation of gene transcription (3). Nuclear receptors (NRs) are important therapeutic targets, ideal for drug discovery. Not only do they control a countless biological and disease processes, but are also regulated by small lipophylic molecules and can be easily switched on/off with a specific drug. For example, ideal selective estrogen receptor (ER) modulator (SERM) would be able to specifically activate ER within in the bone to fight osteoporosis, but not in breast or endometrial tissue, which might lead to cancer (2).

Tamoxifen which activates estrogen receptors in breast cancer, and thiazolidinedione activating peroxisome proliferator-activated receptor – gamma (PPAR-gamma) in type 2 diabetes, are two important examples of currently used prescription drugs (Rx). By some estimations, NR ligands represent 10-20 % of the worldwide pharmaceuticals market (1).

In a form of heterodimers, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) are leading regulators of human gene expression, and therefore relevant drug targets. They play a crucial role as transcription factors, controlled by ligands, regulating a number of gene networks involved in cell growth, differentiation, survival and apoptosis (4).

The role of RAR modulation in the active healing of acute promyelocytic leukemia (APL) has caused increase of interest for the improvement of currently

available modulators of RAR and RXR. This has been additionally supported by recent progress in knowledge regarding biological functions of RARs and RXRs, and consequently in construction of their selective modulators that might be more effective than the currently available ones (5).

1.1 NUCLEAR RECEPTORS

Receptors are proteins that are undoubtedly by far the most important drug targets in medicine (1). This class of proteins found within the cells of metazoan organisms are naturally activated by hormones. Hormones are produced by special organs, from where they are transported by blood to target organs, where they can carry out their effects after binding to their specific receptors. Most of these receptors are expressed on the cell surface, but not all of them. Some are located within the cells, and are known as intracellular receptors, nuclear hormone receptors or nuclear transcription factors (NRs). The chemical messengers for NRs include steroid hormones, thyroid hormones and retinoids, which must first pass through the cell membrane in order to reach their receptors, and therefore they have to be hydrophobic in nature (3). NRs are known for their significant roles in intracellular signalling. Namely, they are converging different intracellular and extracellular pathways, thereby regulating different genetic programs.

They are known as transcription factors, that:

I. respond directly following their physical association to a diversity of hormonal and metabolic ligands;
II. integrate various signalling pathways as they are themselves targets of post-translational modification;
III. regulate the activities of other major signalling cascades (6).

In humans there are currently 48 known NRs that can directly regulate the expression of adjacent genes, by binding to DNA. This unique property differentiates them from other classes of receptors, therefore they are classified as transcription factors. NRs have an almost direct role in regulating the expression of hormone-response genes, thereby importantly impacting development, homeostasis, and

metabolism of the organism. These regulatory capacities of NRs are a consequence of their ability to identify specific promotor sequences of their target genes. They also have the capability to directly interact with genomic DNA, and to regulate its expression, thereby playing an important role in embryonic development and adult homeostasis (7).

The regulation of gene expression by NRs can only occur in the presence of appropriate ligands, i.e. small lipophylic molecules. A ligand is a molecule that affects the NR behaviour. Namely, when it specifically binds to its target NR, signals are generated that trigger gene program via conformational changes of receptor, which in turn lead to change of gene expression (up-regulation or down-regulation) (7).

1.1.1 THE LIST OF HUMAN NRS (8)

I. <u>Thyroid Hormone Receptor – like:</u>

a) Thyroid Hormone Receptor
   - TRα
   - TRβ

b) Retinoic Acid Receptor (RAR)
   - RARα
   - RARβ
   - RARγ

c) Peroxisome Proliferator-activating receptor (PPAR)
   - PPARα
   - PPAR-β/δ
   - PPARγ

d) Rev-ErbA
   - Rev- ErbAα
   - Rev-ErbAβ

e) RAR-related orphan receptor
   - RORα
   - RORβ
   - RORγ

f) Liver X receptor - like
   - LXRα
   - LXRβ
   - FXR

g) Vitamin D receptor (VDR) - like
   - VDR
   - PXR
   - CAR

h) NRs with two DNA binding domain
   - 2DBD-NRα
   - 2DBD-NRβ
   - 2DBD-NRγ

II. Retinoid X Receptor (RXR) -like

a) Hepatocyte nuclear factor-4 (HNF)
   - HNF4Aα
   - HNF4γ

b) Retinoid X receptor (RXR)
   - RXRα
   - RXRβ
   - RXRγ

c) Testicular Receptor (TR)
   - TR2
   - TR4

d) TLX/PRN
   - TLX
   - PNR

e) COUP/EAR
   - COUP-TFI
   - COUP-TFII
   - EAR-2

III. Estrogen receptor-like

a) Estrogen receptor (ER)
   - ERα

- ERβ

b) Estrogen - related receptor (ERR)
   - ERRα
   - ERRβ
   - ERRγ

c) 3-ketosteroid receptors
   - Glucocorticoid receptor (GR)
   - Mineralocorticoid receptor (MR)
   - Progesterone receptor (PR)
   - Androgen receptor (AR)

IV. Nerve Growth Factor IB-like

a) NGFIB/NURR1/NOR1
   - NGFIB
   - NURR1
   - NOR1

V. Steroidogenic Factor-like

a) SFL/LRH1
   - SF1
   - LRH-1

VI. Germ Cell Nuclear Factor-like (GCNF)

a) GCNF
   - GCNF

VII. Miscellaneous

a) DAX/SHP
   - DAX1
   - SHP

1.1.2 THE NR STRUCTURE

Molecular mass of a typical NR is usually between 50 and 100 kDa. All of them also have quite alike structures, as they contain a single protein with a ligand-binding domain at the C-terminal end and a DNA binding domain near the centre of the molecule (1). In general, according to general conserved sequences and functions

of NRs, their structure can be divided into six domains (A-F), based on their construction and/or function (Figure 1).

A/B – The N-terminal domain (NTD) harbouring a ligand-independent, cell- and promoter-specific transactivation (also termed activation) function (AF-1), is also known as the modulator A/B domain (6). A/B domains are highly variable between NRs and their subtypes, regarding their amino acid sequences and lengths (4). The most frequent sites of alternative splicings and secondary starts are found within this region. It also contains a variety of kinase recognition sequences. All of this suggests that NTD sequences may be responsible for specific receptor effects, its species- and cell-specific type, and also for promoter-dependent properties of NR transactivation. Unfortunately, the precise NTD structural data are still unavailable (6).

C - The central DNA binding domain (DBD) is a vastly conserved structural region, containing nine cysteine residues, eight of which are involved in binding two zinc ions (3). These two ions maintain the overall DBD core fold, which encompasses a total of 66 amino acid residues (4). The zinc ions therefore play a crucial function in determining and stabilizing the conformation of this structural region. The stretches of this structural form of the protein are called zinc finger domains. They bind to hormone response elements (HRE), which are specific sequences of DNA (3). Within the DBD domain two zinc fingers are present. The first contains a proximal box (P-box) region, consisting of a α-helix that enables high-affinity recognition of the "core half-site" within the HRE. The second one contains a distal box (D-box), which is also a α-helix, lying upright to the P-box. This is the site that mediates receptor dimerization and determines spacing between the half-sites (4). NRs bind to HREs within their target promoters, via DBD, either as monomers or dimers, depending on the class of a particular NR engaged (9).

D – The function of the hinge region or the D domain is not well defined. It consists of carboxy-terminal extension (CTE) of DBD, and is therefore considered to be involved in recognizing the 5’extended end of HRE. It appears that this domain allows for conformational changes after binding of ligand (4). Additionally, some data suggests that it may contain nuclear localization signals and protein-protein interaction sites.

E – The E domain, better known as the ligand binding domain (LBD), is conserved to a smaller extent. This structural region is involved in ligand binding, dimerization and ligand-dependent transactivation function (AF-2) (1). LBD amino acid sequences are quite diverse between NRs. However, a common structure of 12 α-helices located around a hydrophobic cavity or pocket is present in all NRs. The binding pockets have volumes from 0 to 1.500 $Å^3$, and are unique in their size and characteristics for each NR (4). Amino acid residues within this cavity predispose LBD's properties regarding binding of different ligands (steroid hormones, retinoid compounds, xenobiotics). This region also provides nuclear localization signals, protein interactions with dimerization motifs for heat shock proteins (HSP), coregulators and other transcription factors (10).

F- The C-terminal domain is highly variable and not present in all NRs (4).

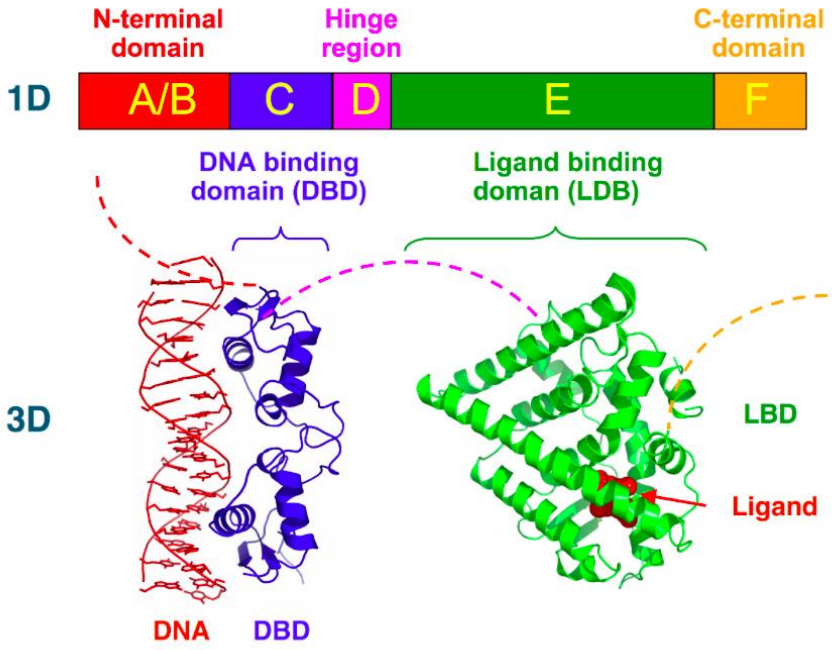


Figure 1: Schematic structure of a nuclear receptor (13).

1.1.3 MECHANISMS OF ACTION

NRs bind specific DNA elements located within the regulatory regions of genes, via highly conserved DBDs, as well as specific ligands, by other highly conserved LBD domains, that consist of a series of approximately 12 α-helices, forming a

hydrophobic pocket (2). Once the chemical messenger, i.e. ligand, crosses the cell membrane, it seeks out its specific receptor and binds to it at the ligand-binding site (pocket). This in turn leads to dimerization of the ligand-receptor complex (3). The resulting dimer binds to coactivator and finally the whole complex is coupled to a particular region of cell's DNA (3).

The binding of ligand into the pocket induces conformational changes of receptor (induced fit), thereby affecting the recruitment of coregulatory molecules (cofactors) that either stimulate (binding of co-activators) or repress (binding of corepressors) transcription, usually via modifications of chromatin and interactions with the basal transcription machinery (2).

Allosteric conformational changes, which are generated upon ligand binding either promote or repress the following receptor-coregulator interactions:

- coactivator recruitment with receptor binding an agonist;
- coactivator dissociation with receptor binding an antagonist;
- corepressor stabilization with receptor binding an inverse agonist (11).

A specific hormone-nuclear receptor complex may exert various effects in different tissues. In case of cortisol, which is secreted by adrenal glands and then transported via blood stream, it regulates gene expression in inflamed tissues, so that the anti-inflammatory proteins are stimulated and pro-inflammatory ones are inhibited. On the other hand, in the liver, the cortisol-NR complex can reduce the levels of blood sugar by controlling the transcription of involved proteins. While some NRs are inhibited or activated by hormones, others are affected by fatty or bile acids or vitamins. In some cases, the natural signalling molecule (ligand) is unknown. By designing drugs that bind to NRs, gene expressions can be affected, thereby potentially targeting a number of diseases and pathological medical conditions (12).

NRs are divided in two classes, based on mechanisms of their action and binding to response elements (RE). These are short sequences of DNA found within promoter regions of genes, having the ability to bind specific transcription factors that regulate transcription of associated genes (13). Hormones can bind to NRs that are located either in cytosol (type 1) or nucleus (Type II). Hormone binding to NRs

causes their conformational changes, which in turn trigger a signalling cascade, resulting in changes of gene expression, i.e. its up or down regulation. Furthermore, there are two more classes of NRs, i.e. Type III which derives from Type I, and Type IV, which have ability to bind to DNA as monomers (13).

1.1.3.1 Type I

Type I NRs, also called steroid hormone receptors (SHRs), include the following members: ER, AR, PR and GR. Steroid hormone ligands of this subgroup of NRs are transported from their respective endocrine gland producing sites via blood, bound to steroid binding globulin.

Activation of some SHRs is triggered when their specific ligands bind to them within the cytoplasmic compartment. The ligand-receptor complexes then move into the nucleus where they, upon homodimerization, bind to HREs within the promoter of a target gene. Binding of acetyltransferases as co-activators and the general transcription paraphernalia (TBP, TFIIB, and RNA polymerase II) to NR transactivation domain, being responsible for interactions within the promoter, results in activation of transcription (14).

In case when the ligand is absent, the transcriptionally inactive SHRs, i.e. MR, PR, GR, AR and ER, can be sequestered into a large complex consisting of receptor and heat shock, as well as other proteins. The SHRs are present as inactive complexes within the cytoplasm and nucleus. However, it was confirmed that SHRs are cytosolic in the un-liganded form. Binding of a specific hormone to a given SHR evokes a substantial conformational change in receptor structure, which points out the initiation of the signal transduction process. Dissociation of heat shock proteins (HSPs), followed by release of monomeric receptor from the complex and its bind to specific HRE is a typical consequence of hormone binding to the 3-ketosteroid receptor subfamily members (GR, AR, MR, PR). Type I NRs bind to HREs that consist two half-sites, divided by a volatile length of DNA, and with the second half-site containing a sequence that is inverted compared to the first one (inverted repeat) (15). It was confirmed by genetic analyses and *in vitro* protease digestion experiments, that the changes of receptor conformation induced by agonists are comparable, but distinct from those caused by antagonists. However, it appears that both conformations are incompatible with the HSP binding (10).

1.1.3.2 Type II

Type II non-steroid NRs, i.e. TR, RAR, VDR, and PPARγ, do not often interact with HSPs, but can use RXRs as partners for heterodimerization. Receptor heterodimers, which are located in the nucleus, as components of complexes formed with histone deacetylases (HDACs) and other corepressors (CoR), are keeping target DNA in a compactly bound conformation, thereby minimizing the risk of its exposure to transactivation factors. Only upon binding of a specific ligand, HDAC dissociates, chromatin is de-repressed and transcription, via RNA polymerase induction is activated. Some of ligand-free NRs may act as repressors of transcription when interacting with DNA. It is believed that this is a consequence of their collaboration with co-repressor proteins. It seems that "the switch" of NRs activation is a hormone-induced conformational change, which happens after binding of ligand (16).

1.1.3.3 Type III

These NRs are known as Dimeric Orphan Receptors. They bind to DNA as homodimers, and are similar to type I NRs. Unlike classical type I NRs which bind to inverted repeat HREs, type III NR's bind to direct repeat HREs (13).

1.1.3.4 Type IV

Type IV NRs, Monomeric or Tethered Receptors, bind to DNA either as monomers or dimers. However, only a single DBD of NR binds to a single half site of HRE, regardless of any dimerization the receptor may undergo (17).

1.1.4 RXR

The retinoid X receptor (RXR) belongs to the family of NRs II, is mobilized by the 9-cis retinoic acid and acts as a ligand-dependent transcription factor. RXR exists in three variants, as RXR-α, RXR-β, and RXR-γ, which are encoded by the RXRA, RXRB and RXRG genes, respectively.

Their heterodimers can be formed with members of the NR I subfamily, including CAR, FXR, LXR, PPAR, PXR, RAR, TR, and VDR (18).

RXR contains two specific regions, typical for NR family proteins, i.e. DBD and LBD, and takes its role in cellular differentiation, hematopoiesis, reproduction,

and bone development, as well as in patterning mechanism during embryogenesis. The loss of RXR function proved to be lethal in embryogenesis (19). Its involvement in some pathological conditions, such as neoplastic formation, makes it a promising therapy target (20).

As in case of the rest of type II NRs, its heterodimerization takes place in the absence of ligands. Namely, RXR binds into a complex with HRE and a corepressor (CoR) protein. When agonist ligands bind to the RXR it dissociates from CoR and recruits a co-activator (CoA) protein. This cascade promotes transcription of the downstream target gene into mRNA and eventually the production of a specific protein (21).

1.1.5 RAR

The retinoic acid receptor (RAR) belongs to a family of hormone NRs, which can act as transcription factors. It is mobilized by the 9-cis retinoic acid and all-trans retinoic acid. There are 3 subtypes of RAR receptor, RAR-α, RAR-β, and RAR-γ, which are encoded by the RARA, RARB and RARG genes, respectively. Additionally, each subtype has few splice forms, i.e. 2 for RAR-α, 4 for RAR-β, and 2 for RAR-γ (22).

Like the rest of type II NRs, it heterodimerizes with RXR in the absence of ligand. Newly formed dimers then bind to HREs, known as retinoic acid response elements (RAREs), complexed with CoR proteins.

The agonist binding to RAR, causes its dissociation from CoR and recruitment of CoA, leading to transcription of a downstream target gene into mRNA and ultimately the production of protein (23).

Agonistic ligands make direct contact to H12 residues and maintain the helix in its position, which, together with other secondary structural elements, creates a surface with increased affinity for LxxLL (x stands for any amino acids) motifs that are located on the NR-interacting surface of CoA proteins (24).

Both, RXR and RAR receptors exercise their effects by binding to particular sequences within promoter regions of target genes as homo- or heterodimers, and thereby regulate their transcription. The proteins encoded by these genes belong to the steroid and thyroid hormone receptor superfamily of transcription factors. When there

is no ligand present, the RXR-RAR heterodimers connect with multiprotein complexes containing transcription CoRs. This causes histone acetylation, chromatin condensation and suppression of transcription. When a ligand binds, the CoR dissociates from receptor, which then in turn associates with the CoA, leading to its transcriptional activation (11).

1.1.6 COREGULATORY PROTEINS

NRs, bound to HRE, recruit a significant number of other proteins facilitating or inhibiting associated target gene transcription into mRNA. The functions of coregulators vary. These include remodelling of chromatin or a bridging function to stabilize other coregulatory protein binding. NRs may specifically interact with several coregulator proteins, thereby directly, as well as indirectly, influencing cellular mechanisms of signal transduction (24).

1.1.6.1 Coactivators (CoA)

Binding of an agonist to NR generates a conformational change of receptor, which induces preferential binding of CoA proteins. These frequently possess intrinsic histone acetyltransferase (HAT) activity, which lowers the histone to DNA binding affinity, thereby promoting gene transcription (25).

1.1.6.2 Corepressors (CoR)

Binding of an antagonistic ligand to NR generates such conformational change of receptor, which induces preferential binding of CoR proteins that in turn, recruit HDACs. This then boosts the affinity of histone to DNA association, thereby repressing gene transcription (25).

1.1.6.2.1 NCOR

The nuclear receptor co-repressor (NCoR) is a transcriptional co-regulatory protein, containing only few NR interacting domains. It has an autonomous repression domain and interacts with ligand-free non-steroid receptors, as well as with antagonist-bound steroid receptors (26).

NCoR mediates repression of transcription, exerted by particular NRs. It is a part of a complex that promotes histone deacetylation and formation of chromatin repressive structures, which can obstruct access of basal transcription factors (Figure 2) (27).

NCoR is also known as thyroid-hormone- or RAR-associated CoR protein that is encoded by the NCoR gene in humans (27).

Additionally, it seems to be responsible for recruitment of histone deacetylases to DNA promoter regions. Hence, NCoR helps NRs in down regulation of expressed DNA. Significant strength and power increase of muscles in mice were observed upon the loss of function of this protein (28).

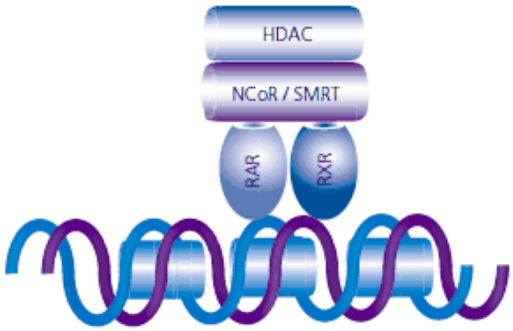


Figure 2: The apo-RAR-RXR heterodimer recruits NCoR and other CoRs having HDAC activity, which increases condensation of chromatin and inhibits gene transcription (29).

1.1.7 NUCLEAR RECEPTOR AGONISM/ANTAGONISM

The effects of NRs, induced by various ligands, can be agonistic, antagonistic or inversely agonistic. These effects are depending on the type of receptor involved, chemical structure of its specific ligands and the tissue that is being affected. The state when no ligand is bond to NRs is called the apo state (24).

Primary determinants of H12 helix position are ligand's interactions with H11 and H12 helices or amino acid residues in their proximity. Structural analysis indicates that H12 can, besides active or inactive position, adopt also several intermediary states and dynamics (Figure 3) (4).

Allosteric conformational changes, which are generated upon ligand binding, either promote or repress the following receptor-coregulator interactions:

- CoA recruitment with receptor binding an agonist;

- CoA dissociation with receptor binding an antagonist;
- CoR stabilization with receptor binding an inverse agonist (11).

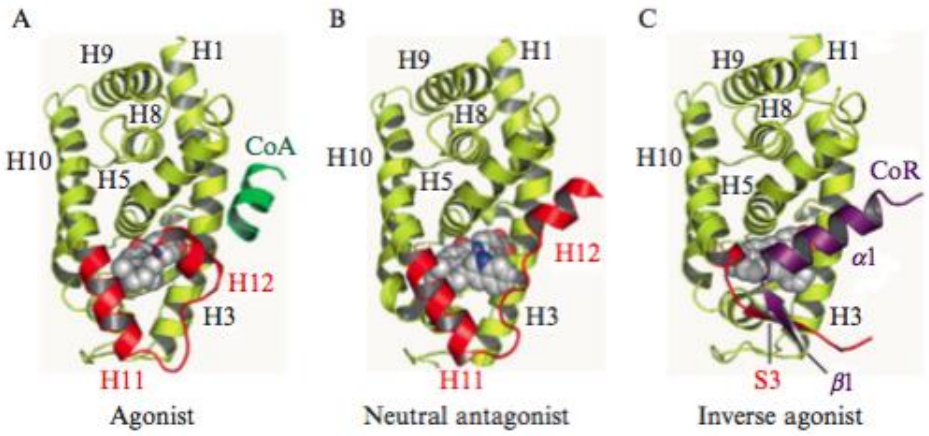


Figure 3: Crystal structures of RAR in different functionally active states (24).

1.1.7.1 Agonists

The agonist's activity following its binding to NR up regulates gene expression. Certain synthetic ligands exist, that can mimic agonistic effects of endogenous hormones. Agonists enhance the recruitment of CoAs and destabilize NR interactions with CoRs, thereby enhancing their transcriptional activity. In other words, agonists induce receptor conformation that favours CoA binding (30).

1.1.7.1.1 AM580 (THE AGONIST OF RAR)

AM580 acts as a selective agonist of RARα, which means it is an analog of retinoic acid (Table 1). Its EC50 values are: 0.3 nM for RARα, 8.6 nM for RARβ and 13 nM for RARγ. The studies showed that it significantly induces production of IL-4, IL-5 and IL-13, while inhibiting that of IL-12 and IFNγ. *In vitro,* AM580 also induces cell differentiation with a 7-times higher activity than retinoic acid. Additionally, AM580 decreases binding affinities of all CoRs to RAR (31).

1.1.7.2 Antagonists

Compounds with antagonistic activity can be split into two groups, according to mechanism by which they counteract the function of agonists. While classical antagonists inhibit both interactions needed for agonistic activity, the inverse agonists

stabilize the interaction of NR with CoRs, thereby increasing the receptor-mediated gene silencing (24, 30).

1.1.7.3 Classical / neutral antagonists

Classical antagonists exert their effects via competitive binding to same NR interaction sites that are also being used by agonists. As already mentioned, antagonistic ligands induce conformational change of NR, which prevents CoA, and promotes CoR binding (23).

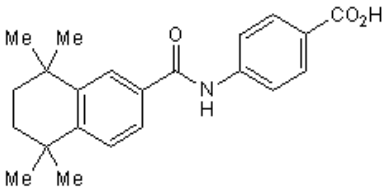
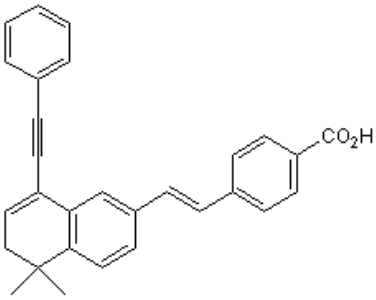
1.1.7.4 Inverse agonists

An inverse agonist induces opposite pharmacological response when binding to the same NR as would an agonist do. Inverse agonists are less effective in preventing CoA binding to NRs, but on the other hand have the potential to strengthen the interactions of NRs with CoRs (24).

1.1.7.4.1 BMS493 (THE INVERSE AGONIST OF RAR)

BMS493 is a pan-retinoic acid receptor (pan-RAR) inverse agonist (Table 1). Its binding causes analogous conformational changes in all RAR types (RARα, RARβ and RARγ). BMS493 shows lower efficacy in preventing the recruitment of CoA. However, it strongly enhances CoR-NR interaction, by preserving high binding affinity, thereby stabilizing the RAR-NCoR complex (32).

Table 1: Chemical structure of AM580 and BMS493 (31, 32).

Ligand	Structure	Action
AM580		Agonist
BMS 493		Inverse agonist

2 THE AIM OF THE WORK

Our work will consists of two parts.

First we plan to form the RXR-RAR-NCoR5 complex, obtain its crystals and define their 3D structure, which is still not available.

Constructs will be expressed in Rosetta strain of *E. coli*, harvested and then purified using adequate methods (affinity chromatography, gel filtration, …). Formation of the complex will be performed in 2 steps. In the first step we plan to isolate the formed RXR-RAR complex and NCoR5, separately. In the second one we will form the RXR-RAR-NCoR5 complex.

We think that 3D structure of formed complex would provide new insights into the wide spectrum of molecular interaction and for design of more specific drugs that would have a great potential in preventing and treatment of cancer.

The second part of our work will consist of forming RXR-RAR heterodimers and analysing the effects of AM580 and BMS493 ligand binding to them, on NCoR5 affinity. We expect that AM580 will decrease RXR-RAR interaction with NCoR5, while on the other hand BMS493 will increase it.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 CONSTRUCTS

The NCoR5 construct was prepared by the CBS's collaborator, l'Institut de recherche en cancérologie de Montpellier (engl. Institute of Cancer research Montpellier, IRCM). Both, RXR and RAR constructs, prepared at the CBS Montpellier, are being used in the lab since a long time. RAR and NCoR5 were tagged with His-tag (polyhistidine tag, His6 tag), so that Ni-column can be used for separation.

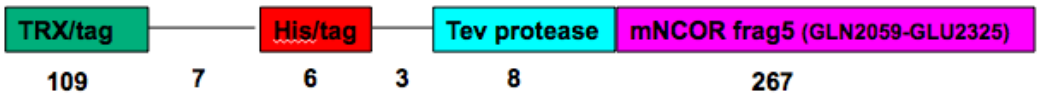


Figure 4: The NCoR5 fragment.

3.1.2 SMALL LABORATORY MATERIALS

- ✓ Tubes: 250 µl, 500 µl, 1 ml, 10 ml, 25 ml, 100 ml (Thermo Fischer);
- ✓ Single use plastic pipettes and pipetting devices: Pipetman ultra (Gilson Medibase);
- ✓ Laboratory glassware: graduated cylinders, jars, erlenmeyer flasks, beakers (SciLabware);
- ✓ Syringe needles (BD);
- ✓ Kit for acryl gel preparation: plastic holder, glass plate, comb, silicon plates (Pierce);
- ✓ Magnetic stir/stirrer (Gilson);
- ✓ Filters – brown 50 µm and yellow 0.45 µm (Minisart, Sartorius);
- ✓ 384 well NBS low V microplates (Corning);
- ✓ Microtiter plates, 1:4, 96 well (Corning).

3.1.3 EQUIPMENT

- ✓ pH meter: Inolab WTW SERIES pH720 (Inolab);

- ✓ Automatic purifier for Chromatography (ÄKTA™);
- ✓ Gel filtration column: Hiload tm 26/60 superdex 75 pergrade, average particle size 13 μm (Sigma Aldrich);
- ✓ Spectrophotometer Nanodrop 2000 (Thermo Scientific);
- ✓ Centrifuge Megafuge (10 Hereaus) (Thermo Scientific);
- ✓ Centrifuge Sorvall elution RC (Thermo Scientific);
- ✓ Centrifuge KR 25i (Jouan);
- ✓ Centrifuge 54174R (Eppendorf);
- ✓ Crystallization robot Tecan genesis RSP 100 (Tecan);
- ✓ Dispensing system (Cartesian);
- ✓ Electrophoresis kit (Pierce);
- ✓ Heat-shock device (Gilson);
- ✓ HisTrap HP colomn, 5 x 1 ml (GE Healthcare Life Science);
- ✓ HiLoad 26/60 Superdex 75 pg column, 1 × 320 mL (GE Healthcare Life Science);
- ✓ Safire microplate reader (TECAN);
- ✓ GraphPad Prism 4.0 (Graphpad Software, San Diego).

3.1.4 CHEMICALS AND BUFFERS

- ✓ Buffer for resuspending cell pellets: 50 mM Tris-HCl, 150 mM NaCl, pH=7.4;
- ✓ Ni column Buffer A: 50 mM TRIS, 150 mM NaCl, pH=7.4;
- ✓ Ni column Buffer B: 50 mM Tris, 300mM NaCl, 500 mM imidazole, pH=7.4;
- ✓ GF buffer: 50 mM TRIS, 150 mM NaCl, 5 mM DTT, pH=7.5;
- ✓ Buffer for anisotropy: 20 mM TRIS, 150 mM NaCl, 5 mM DTT, 1 mM EDTA, 5% glycerole, pH=7.7;
- ✓ Tris/Glycine/SDS (TGS) running buffer: 250 mM Tris, 192 mM glycine, 0.1% SDS, pH=6.8;
- ✓ **Lysogeny broth (LB) for agar plates:** into at least 2 L beaker:
    - 10 g tryptone, 5 g yeast extract, 5 g NaCl, 800 mL of distilled water;
    - stir until everything is completely dissolved;
    - add 400 μL of 5 M NaOH and adjust to pH to 7.5,

- bring the liquid level up to 1.000 ml with distilled water;
- add 15 g of granulated agar and stir until it is dissolved;
- remove the stirring bar, cover the flask with aluminium foil and autoclave for 20 min using the liquid cycle.

✓ **Lysogeny broth (LB)** - a nutritionally rich medium for culturing and maintaining of recombinant Rosetta strains of *E. coli*. LB generally contais peptides and casein peptones, vitamins, trace elements and minerals. Preparation of 1 L medium:

- 10 g tryptone;
- 5 g yeast extract;
- 10 g NaCl;
- suspend the solids in ~800 ml of distilled or deionized water;
- add further distilled or deionized water, using measuring cylinder to ensure accuracy, to make a total of 1 L;
- adjust pH to 7.5 with 1 M NaOH and autoclave at 121 °C.

✓ Ampicillin (AMP);

✓ TEV protease (Sigms Aldrich);

✓ Ammonium persulfate (ASP) (Sigms Aldrich);

✓ Tetramethylethylenediamine (TEMED);

✓ Stacking solution: 0.5 M Tris-NaCl, pH=6.8, 10% (w/v) SDS, Acrylamide 30% (w/v), 10% (w/v) APS;

✓ Crystallization media (Molecular Dimensions Limited);

✓ Thrombine from bovine plasma (Sigma Aldrich);

✓ Ligands: 10^{-2} M AM580 and 10^{-2} M BMS493 (CBS);

✓ NCoR5-atto 647N FP, 10^{-2} M (CBS).

3.2 METHODS

3.2.1 WORKING PROCEDURES

To summarize, our work will be divided into following steps:

1.) Preparation of host/production cells
   - ✓ preparation of Petri dishes for cell cultures;
   - ✓ insertion of constructs into Rosetta strains of *E. coli*;

- ✓ preparation of glycerol stock solution;
- ✓ preparation of pre-cultures;
- ✓ preparation of cell cultures;
- ✓ protein harvesting from host cells.

2.) Purification of the protein:

- ✓ sonication of host cells;
- ✓ preparation of the Ni column;
- ✓ equilibration of the chromatography apparatus;
- ✓ affinity chromatography (His Trap - Ni-column);
- ✓ overnight cleavage of fusion proteins;
- ✓ calibration of the column for gel filtration;
- ✓ gel filtration (GF);
- ✓ concentration of isolated proteins by amicon cell under pressure and tubes via centrifuge;
- ✓ characterization of proteins (Ɛ - molar extinction coefficient and MW – molecular weight);
- ✓ separation of protein with SDS-page electrophoresis.

3.) Crystallization of the purified protein.

4.) Anisotropy.

3.2.2 PRODUCTION OF THE PROTEIN

3.2.2.1 List of constructs

For our experiments three constructs were prepared:

- RXR inserted into the peT3a plasmid;
- RAR inserted into the peT15b plasmid, and
- NCoR pet TEV mNCoR5 gln 2059, gly 2395.

All these constructs will be expressed in Rosetta strain of *E. coli*, harvested and then purified using adequate methods (affinity chromatography, gel filtration, …)

3.2.2.2 Expression screening of the construct

In our previous work screenings were carried out for all 3 plasmids containing constructs, being transfected into Rosetta cells.

The Rosetta host cell strains are also BL21 derivatives, designed to enhance the expression of eukaryotic proteins that contain codons, rarely used in *E. coli*. These strains supply tRNAs for AGG, AGA, AUA, CUA, CCC, and GGA codons on a compatible chloramphenicol-resistant plasmid. Thus Rosetta strains provide for "universal" translation, which is otherwise limited by the common codon usage of E. coli (33).

3.2.2.2.1 IPTG INDUCED EXPRESSION OF THE PROTEIN

IPTG, i.e. Isopropyl-Thio-2-D-Galactopyranoside is used for inducing protein expression via the lac promoter and its variants. This molecule mimics allolactose, a lactose metabolite that triggers transcription of the lac operon. Unlike allolactose, the IPTG's sulphur (S) atom of IPTG creates a chemical bond with lac operon, which cannot be hydrolyzed by the cell, thereby preventing it from degrading the inducer. As a consequence the IPTG concentration remains constant. IPTG binds to the lac repressor and releases the tetrameric repressor from the lac operator in an allosteric manner. This allows for the transcription of genes within the lac operon, such as the gene coding for beta-galactosidase, an enzyme that catalyzes the hydrolysis of β-galactosides into monosacharides.

In cloning experiments, colonies that have been transfected with recombinant plasmid, rather than those with a non-recombinant one, need to be identified. For this purpose X-gal is used, as it is metabolised by beta-galactosidase to a blue colored product. Thus all the cells expressing beta-galactosidase grown in the presence of X-gal and IPTG as the inducer of expression, will turn blue. Where a DNA fragment is inserted into the LacZ (one of the genes coding for beta-galactosidase) the X-gal will not be degraded and the cells will not turn blue, thus identifying only those that carry recombinant plasmid rather than the non-recombinant one.

The lac operon is required for the transport and metabolism of lactose in *E. coli* and some other enteric bacteria. It consists of three adjacent structural genes,

lacZ, lacY and lacA. The lac operon is regulated by several factors including the availability of glucose and lactose (34).

3.2.2.3 Preparation of LB agar gel in Petri dishes

A flask of pre-prepared LB agar gel mixture was heated in a microwave oven until all the ingredients were completely liquefied, after which the content was poured into sterile Petri dishes in aseptic conditions. The agar plates were solidified after approximately 20 minutes. Restrictive AMP solution (0.1 M) was placed onto all gel surfaces in order to enable the selection of Rosetta cells containing the plasmids. After that the plates were stored in a refrigerator at 4 °C.

3.2.2.4 Transfer of plasmids into E. coli

Two aliquots of Rosetta cells were taken from refrigerator and marked accordingly. We always used one aliquot as a control and the other one as a working sample. They were left on ice for 10 min to thaw. After adding 1 μL of a specific concentrated plasmid, i.e. either RXR, RAR or NCoR5, which were prepared by the CBS and IRCM and stored in refrigerator at -70 °C, to the working sample, we continued the incubation on ice for 30 min. Subsequently both, control and working sample, were transferred to 42 °C for 45 s in order to heat-shock the cells, thereby allowing the entrance of plasmid into bacteria. Then the cells were put on ice for additional 5 min. To each sample 950 μL of the LB was added and after gentle mixing at 240 rpm, the cells were incubated for 1 hour at 37 °C. After that, both samples were mixed and centrifuged at 7.500 rpm for 4 min, and then 950 μL of LB was poured away. Both, the control and the working sample were then transferred with a pipette near the open flame onto two separate sterile LB agar plates. Both samples were dispersed on the whole plate surface. Petri dishes were marked accordingly, and put in an incubator (37 °C) over night. First 15 min they were incubated with the agar layer being on the top, so that the plates could dry, and then with the agar layer on the bottom. The next day, after the non-growth of non-transfected cells on the control plate was checked and confirmed, the plates were sealed and put in the refrigerator at 4 °C.

3.2.2.5 Glycerol stock solution

In order to store the cells at -80 °C for further analyses, we prepared a stock freezing solution by mixing 700 µL of the LB pre-culture medium with antibiotic and 700 µL of 50% glycerol, in each cryovial. Then we transferred the cells from agar plates into appropriate cryovials in the vicinity of an open flame. The cryotubes were marked accordingly and stored at -80 °C until use.

3.2.2.6 Preparation of pre-cultures

For the preparation of pre-cultures, 1 M AMP solution was added to the LB. Then, the cells, taken either directly from agar plates or from thawed stocks that were kept at -80 °C, were used to prepare pre-cultures. The whole procedure was carried out in the vicinity of an open flame, so that the cell cultures were kept sterile. Subsequently the pre-cultures were incubated at 37 °C overnight, under constant shaking at 220 rpm.

An illustrative example:

To 100 mL of LB*, 100 µL of the 1 M AMP stock solution was added. The resulting mixture was split evenly into 3 erlenmeyer flasks and then either the thawed cells from the frozen stock (-80 °C) or those taken directly from Petri dishes that were stored in a refrigerator at 4° C, were added to start their pre-culture growth.

*100 mL medium LB
+ 100 µL of the 1 M AMP stock solution
+ ROS(A) cells, either thawed of or directly from a Petri dish

3.2.2.7 Preparation of cell cultures

Cell cultures were prepared according to the method used for the cell growth induction with IPTG.

IPTG culture

For the preparation of an IPTG culture, a pre-culture, and 1 M AMP solution were added into erlenmayer flask containing 700 ml of LB medium*. The cultures

were put in the incubator at 37 °C with continuous shaking at 225 rpm, for 4 hours, and then the growth of Rosetta cells was checked by measuring the absorbance at 600 nm. If the absorbance was between 0,8 – 1,2, 350 μL of 1 M IPTG solution was added to the culture (final concentration of 0.5 mM IPTG) to induce the expression of designed protein. The cell culture was then additionally incubated overnight for approximately 20 hours, at 20 °C.

*700 mL LB medium
+ 700 μL of 1 M AMP stock solution
+ 15 mL of pre-culture
+ 350 μL of 1 M IPTG solution when A_{600nm} = 0,8 – 1,2

3.2.2.8 Harvesting of Rosetta host cells

When cell growth in cultures was successful, the cells were transferred into special centrifuge tubes and centrifuged for 20 min at 6.000 rpm and 4 °C. In the mean time cryotubes were prepared, each pre-filled with 25 mL of resuspending buffer, containing 50 mM Tris-HCl, 150 mM NaCl, pH=7.4 and put on ice. After centrifugation the supernatants were poured off and the cell pellets transferred and re-suspended into the resuspending buffer containing cryotubes that were labelled and stored at -80 °C. This freezing step already made the cells fragile and facilitated the isolation of target recombinant protein.

3.2.3 PURIFICATION OF PROTEINS

3.2.3.1 Sonication of Rosetta host cells

Cryovials containing frozen cells, which were kept at -80 °C, were taken out of the freezer and put into not too warm water for a couple of minutes for thawing. In each thawed sample, ½ of an EDTA free crushed tablet containing antiprotease was added. In order to additionally limit the protease activity, all the tubes were kept on ice during the whole procedure. The next step was the sonication of cells. Each sample was sonicated 3 times for 3 minutes at the amplitude of 60%, with 1 s intervals in between. After that each sample was divided into two tubes, which were

kept on ice. Their contents were balanced and then centrifuged at 18.000 x g for 40 min and 4 °C. The supernatants, containing recombinant proteins were carefully removed and filtered through production filters (minisart yellow - 50 μm and brown – 0.45 μm) into a measurement cylinder.

3.2.3.2 HisTrap (Ni column) protein purification

HisTrap is a pre-packed, ready-to-use column for preparative purification of His-tagged recombinant proteins by immobilized metal affinity chromatography, using a pre-charged Ni Sepharose High Performance column. The special design of the column, together with its high performance matrix, provides fast, simple and easy separations in a convenient format. HisTrap HP columns can be operated with a syringe, peristaltic pump or liquid chromatography system, such as ÄKTA or HPLC (35).

Polyhistidines bind strongly to divalent metal ions, such as nickel or cobalt, therefore all untagged proteins pass through the column freely. Bound proteins can then be eluted for example with imidazole (in our case), which competes with polyhistidine tag for binding to the column, or by decreasing pH, which diminishes the affinity of the tag for the resin.

3.2.3.3 Preparation of the Ni column

We used the HisTrap pre-packed 5 mL column. The column was washed at a slow flow rate using a syringe.

The washing protocol:

1. Wash with 50 mL purified H_2O.
2. Wash with 20 mL EDTA (16 mL of buffer A + 4 mL of 1 M EDTA).
3. Wash with 100 mL purified H_2O.
4. Wash with 10 mL 0.1 M $NiSO_4$ (2 mL $NiSO_4$ + 8 mL H_2O).
5. Wash with 20 mL purified H_2O.

3.2.3.4 Equilibration of the chromatography apparatus

Before the ÄKTA chromatography apparatus was used, all its tubes and loops were first washed with purified water. Then, after the buffers were prepared, its equilibration was started. The pump A1 was put into a loading buffer and the pump B1 into elution buffer. On display we chose the following: run → show details →type of column to be used (5 mL) → flow rate 5 mL/min → start concentration of 4% → direct sample 0→step10.
The equilibration lasted about 30 minutes.

3.2.3.5 HisTrap chromatography apparatus

The Äkta apparatus allows automatic recombinant protein purification. When it was equilibrated and washed, the protein detection and separation procedure could be started with the use of a Ni column. As already described, each protein sample obtained from host production cells was filtered and collected, usually in a measuring cylinder, which was attached to the apparatus holder with a sample loop linked to it. The loop A1 was then put into the buffer A (loading buffer) and the loop B1 into the buffer B (elution buffer). The red loop, also named the "flow through" or F3, was placed in a separate bottle to collect the flow through. On the display, the following running orders were chosen: → show details → choose a column → no pump wash (washing was manual) → start concentration 4% → volume of the sample→ "wash out" unbound sample, 20,00 mL. Then the protein containing sample was loaded on a column. After it was fully loaded, the apparatus automatically started with filling the column with the buffer A to wash out the unbound proteins.

The elution of the bound protein was then performed using the imidazole gradient, i.e. buffer B. For RXR and RAR: 4% of buffer B – 1 CV, 10% of buffer B - 20 CV, 50% of buffer B – 20 CV. Fraction sizes were of 8 mL till 50 % of buffer B, and after that point the fractions were of 2 mL. These eluted fractions were diluted with 5 mL of buffer A, due to instability of the protein in imidazole. For purifying NCoR5 we used linear gradient, i.e. 100% of buffer B in 20 CV.

3.2.3.6 Protein cleavage

In order to obtain cleaved proteins, thrombine, TEV protease and beta mercaptoethanole were used accordingly. After pooling the fractions obtained with the Ni-column purification method, the appropriate substances for cleaving the target protein were added.

For RAR fractions:
+ Thrombine: 2 U/µL of protein

For NCoR5 fractions:
+ TEV protease, final dilution in collected fractions: 1/400
+ Beta mercaptoethanole, final dilution in collected fractions: 1/1000

After adding the protein cleaving substance to eluted fractions, they were placed on ice and kept in the cooling room overnight.

3.2.3.7 Gel filtration (GF) chromatography

Gel filtration chromatography separates proteins, peptides or oligonucleotids on the basis of their size. Molecules move through a bed of porous beads, diffusing into them to a greater or lesser degree, depending on their sizes. Smaller molecules diffuse further into the bead pores and therefore move through the bed more slowly, while the larger ones hardly enter the pores or not at all and thus move through the bed more quickly. Both, the molecular weight and the three-dimensional shape contribute to this selective retention. Gel filtration chromatography may be used for the analysis of molecular size and separation of different components in a mixture, as well as for salt removal or buffer exchange from a preparation of macromolecules (36). For our experiments we used Superdex 75 (Figure 5).

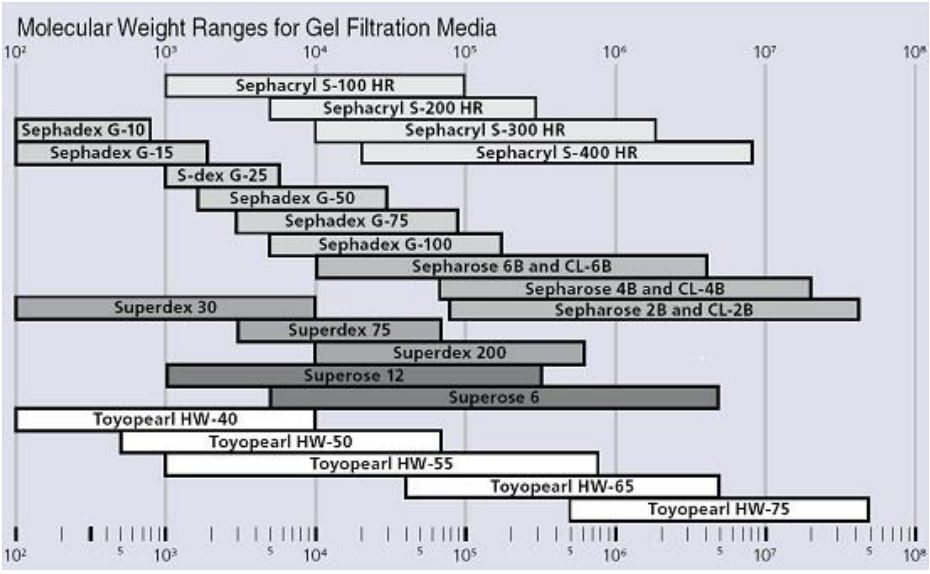


Figure 5: Molecular weight ranges of different gel filtration media (36).

3.2.3.8 Calibration of the GF column

In order to make sure that the GF column was appropriate, calibration of the gel filtration process was performed once per 2 months. First the loop was washed with the calibration buffer (20 mM Tris, 500 mM NaCl). Usually, the column was washed using the amount of buffer that was 3 times the volume of the loop. The loop and the column (S75 26/60 superdex) were then attached to the chromatography apparatus. To check for possible dysfunctions, the volume of the loop was injected into the apparatus. The sample that was used for the calibration of the column was a mixture of 4 different components:

Ovalbumin Mw 43.000 Da, 3 mg,
Conalbumin, Mw 75.000 Da, 3 mg
Ribonuclease A, Mw 13.700 Da, 3 mg
Chymotrypsinogen A, Mw 25.000 Da, 3 mg

All of them were dissolved in calibration buffer (20 mM Tris, 500 mM NaCl). On the apparatus display we chose the type of the column used, adjusted the parameters and started the run. After the first step of equilibration (washing) was finished the sample was transferred to injector and injected into the loop manually,

after which it was automatically proceeded into the column. The flow rate was 2.5 mL/min.

3.2.3.9 Protein purification by gel filtration (GF)

For gel filtration purification the protein samples eluted from the Ni-column were used. The GF column was first calibrated with the working buffer, and then the sample was injected manually into the chromatography apparatus.

After each protein purification we decided which elutes to pool for next analyses. This decision was based on the graphically displayed results, and on the measurements of protein concentrations in eluted samples, using the nanodrop spectrophotometer.

3.2.3.10 Concentration of target proteins

The protein concentration process was the last step in preparation of target protein sample before performing fluorescence anisotropy and crystallization. This can be carried out in special centrifuge tubes by centrifugation or in amicon cells, under pressure.

When the first method was applied, special tubes with 5.000 MWCO (molecular weight cut-off) were usually used. Each sample was concentrated by centrifugation at 3.000 x g, for 3 minutes. Between each centrifugation the protein pellet was resuspended using a pipette. As proteins are very sensitive and can coagulate easily if there are air-bubbles present, this was done with extreme caution.

The second method for concentration of target proteins is based on the use of amicon cells, and is carried out under pressure. Amicon stirred cells provide high flow rates of solutions with up to 10% macrosolute concentration. Gentle magnetic stirring minimizes concentration polarization and shear-inducing denaturation. After the targeted concentration of protein was achieved, the amicon cell membrane was washed with a small volume of buffer that was used for GF, in order to thoroughly wash out the product.

3.2.3.11 Characterization of proteins (Ɛ, MW)

Target recombinant proteins that were isolated, purified and concentrated were RAR, RXR, and NCoR5. The ratios of their molecular weights (MW) and the $\frac{1}{12}$ of the mass of the carbon-12 (12C) were calculated and represent their dimensional numbers in kDa. These data were then put into a nanodrop system in order to measure precise absorbances of concentrated protein solutions. User-selected values for molar extinction coefficients Ɛ (M^{-1} cm^{-1}) and molecular weights (MW) in kDa of respective proteins are presented in Table II.

Table 2: Characterization constants of target proteins, following different steps of their purification.

	RXR-RAR (after Ni)	RXR-RAR (after GF)	NCoR5 (after Ni)	NCoR5 (after GF)	RXR-RAR-NCoR5 (after GF)
Ɛ (M^{-1} cm^{-1})	29,0	29,0	19,9	4,5	33,5
MW (kDa)	56,7	54,8	43,1	28,9	83,7

3.2.3.12 Separation of proteins using SDS page gel

Sodium dodecyl sulphate (SDS-page) gels were cast in order to check for the presence and the purity of specific target proteins. For that purpose 15% polyacrylamide gels were prepared for electrophoresis.

The protocol for preparation of 6 gels:

- Separating phase

Acrylamid 30%	15.6 mL
Tris 1,5 M, pH 8.8	47.8 mL
Purified H_2O	7.2 mL
SDS 15%	642 µL
TEMED	30 µL
APS 10%	300 µL

Temed and APS 10% were added at the end. Once this solution was prepared, it was poured between two glass plates, and filled with isopropanol on top of it, to offset the line.

- Stacking phase

Stacking solution	9.8 mL
TEMED	9 µL
APS 10%	90 µL

Before inserting the stacking phase, isopropanol was completely removed. When stacking phase was inserted, the combs were put on the top of the gel. After 30 minutes the gels were ready. They were wrapped up in wet towels and then stored in a cooling room until being used.

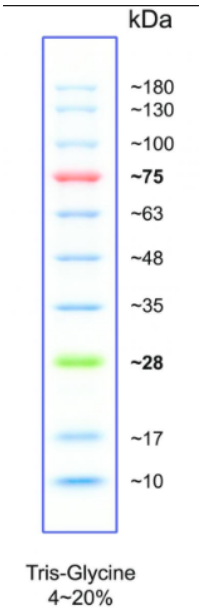


Figure 6: The scale of protein size markers used in SDS-PAGE analysis.

To run a gel we put it on a holder and sunk it into the TGS running buffer (25 mM Tris, 192 mM, 0.1% SDS, pH=6.8). The samples to be analysed were heated for 5 min at 95 °C. Each gel loading "pocket" received 20 µL of a sample, while the last one was loaded with 2 µL of the protein size marker (Figure 6). Electrophoresis was performed at 160 V for first 10 min and then at 200 V. After approximately 45 min the gel was removed, washed in water and then put into the staining instant blue solution for about 1 hour. After that it was transferred onto the light table to be checked for the presence and positions of target proteins.

3.2.3.13 *Measurement of protein concentrations*

3.2.3.13.1 THE NANODROP SPECTROPHOTOMETRY

To measure concentrations of proteins at 280 nm, we used the Nanodrop 2000 spectrophotometer (Thermo Scientific). It enables the input of protein molar and mass extinction coefficients by choosing "Other protein (Ɛ & MW)" for calculation of its concentration from absorption (see Table II). We used 2 μL of buffer A as a blank. After a zero line was set up, we measured the concentration of particular protein in each 2 μL sample. There was no need to dilute the samples, as concentrations up to 50 mg/ml can be measured by this technology (37).

3.2.4 FLUORESCENCE ANISOTROPY

Fluorescence anisotropy is a phenomenon where the light emitted by a fluorophore has unequal intensities along different axes of polarization (34).

Measurement of fluorescence anisotropy is a powerful tool in biochemical research and medical testing. Upon excitation with polarized light the emission spectra of many samples are also polarized. Substances exhibiting nonzero anisotropies are said to display polarized emission. The origin of anisotropy is the existence of transition moments for absorption and emission, that are located along specific directions within the fluorophore structure. In a homogeneous solution the ground-state fluorophores are all randomly oriented. When exposed to polarized light, those of them, that have their absorption transition moments oriented along the electric vector of the incident light, are preferentially excited. Hence the excited-state population becomes partially oriented. The fact is that significant fractions of excited molecules have their transition moments oriented along the electric vector of the polarized exciting light (38).

The effect of different ligands on the NR recruitment of coregulators can be easily and rapidly monitored by measuring the affinities (Kd) of fluorescin-labeled peptides, derived from CoAs and CoRs in their various ligation states (24).

3.2.4.1 The fluorescence anisotropy protocol

Step 1:

1. Transfer the protein into eppendorf tube with the final concentration of 40 μM.
2. Add anisotropy buffer until the final volume of 60 μL, and gently mix with a pipette.
3. Transfer 30 μL of mixture into a microplate well.
4. Add 30 μL of buffer into eppendorf tube and gently mix with the pipette.

- Repeat the 3rd and the 4th step until the 15th well of a microplate is filled.

Step 2, performed in the absence of direct light:

1. Prepare 500 μL 2 nM solution of fluorescein + 2 eq of ligand, as compared to the concentration of target protein, and gently mix with the pipette.
2. Transfer 30 μL of mixture into 15 wells of the microplate prepared in step 1.

Into the 16t^{h} well place 60 μL of anisotropy buffer, and in the 17th 60 μL of purified H_2O. Microplate and buffer should be placed on ice to minimize the effects of warming and the process of decay. Step 2 has to be performed in the absence of direct light due to sensitivity of fluorescein.

Step 3:

1. Determine anisotropy values on the Safire microplate reader with the excitation wavelength set at 470 nm and emission measured at 647 nm.
2. Repeat measuring, so that results reported are given as average of 3 measurements.
3. Fit binding data and determine K_d using the sigmoidal dose-response model, and nonlinear regression (GraphPad Prism software).

3.2.5 CRYSTALLIZATION OF TARGET PROTEIN

The phase diagram of protein crystallization represents how the protein and its precipitant concentrations are related. Protein crystals can only form in supersaturated solutions. As shown in Figure 7, low protein and/or precipitant concentrations will cause undersaturation and consequently no crystal formation will be induced.

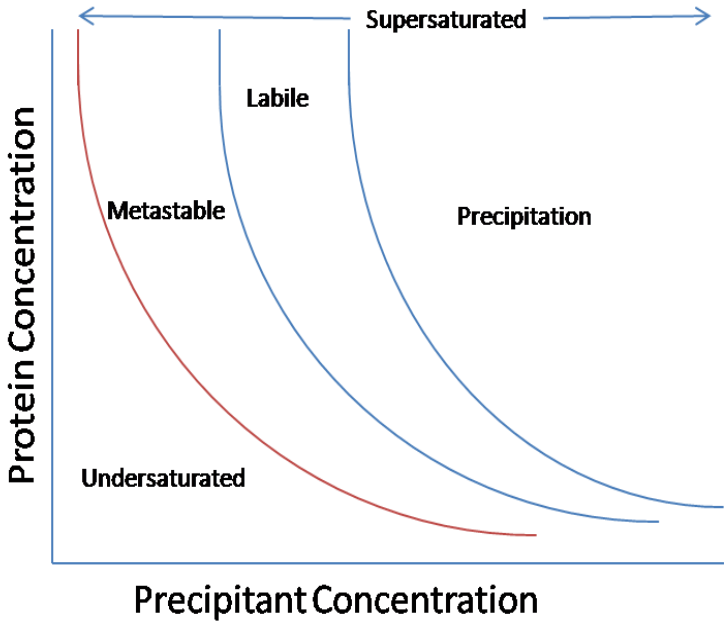


Figure 7: A schematic graphical display of correlations between protein and precipitant concentrations (39).

The red curve in Figure 7 that separates the undersaturated conditions from the supersaturated ones is known as the solubility curve. A clear benefit of determining this curve is, that it can help to guide us when analysing particular protein crystal growth conditions. The crystallization setup which is undersaturated or in the metastable phase will appear clear, however, the latter phase has the potential of crystal growth only if seeded (spiked) with crystals. Precipitation occurs when the protein comes out of solution as an aggregate, but this is not suitable for crystallographic studies. The labile or nucleation zone is important, since this is the point, where the crystal nucleation and its initial growth occurs. As crystals form, the protein concentration will diminish thereby causing condition transition from labile to metastable zone (40).

3.2.5.1 Protein crystallization

The goal of crystallization is to obtain well ordered crystals, lacking contaminants and being large enough to provide a diffraction pattern when hit by x-rays. The procedure of protein crystallization is inherently difficult because of the fragile nature of crystals. Namely, the surfaces of proteins are irregularly shaped, which results in formation of large channels within any protein crystal. Therefore, the

non-covalent bonds that hold together the lattice must often be formed through several layers of solvent molecules. In addition to overcoming the problem with inherent fragility of protein crystals, a successful production of crystals that can be analysed by x-rays, depends upon a number of environmental factors. Because so much variation exists among proteins, each individual one requires unique conditions for its successful crystallization. Therefore, attempting to crystallize a given protein without a reliable and highly optimized protocol can be very tedious. Some of the environmental factors that require consideration are protein purity and concentration, pH, temperature and addition of precipitants. To reach sufficient homogeneity, the protein should be at least 97% pure. The pH conditions are also very important, as different pH values can result in different packing orientations. Therefore high capacity buffers, such as Tris-HCl, are often needed to maintain the optimal pH value. Precipitants, such as ammonium sulphate or polyethylene glycol, are compounds that are used for protein precipitation out of its solution (41).

The optimal concentration range for our purified protein crystallization was around 10 mg/mL. After the protein was isolated, the preparation of its sample for crystallography was started. First it was centrifuged at 14.000 rpm and 4 °C for 10 minutes.

Crystallization was performed by using two different robots, i.e. Tecan genesis RSP 100 and Cartesian dispensing system. First, the water container was washed with purified water in order to clean all the loops of the apparatus. Then several crystallization media were used: miDAS, Classic I, Classic II, PEG I, PEG II, Cations, PGA screen, pH clear, pH clear II, AMSO4, clev I, clev II, JSCG II, procomlex A, JSCG+, JSCG 1, JSCG 2, JSCG 3 and JSCG 4.

Before the experiment they were kept in a cooling room and centrifuged just before being used. A selected medium was placed into the holder 1 of the Tecan robot and a new empty plate (Corning 1:4, 96 well) was inserted into the holder 2. The apparatus divided the applied media automatically by transferring 70 μL aliquots in each well. The microtiter plate was then labelled and the medium, which was not needed any more, was placed back into the cooling room. In-between the Cartesian dispensing apparatus was prepared. The water-containing bottle was changed for a new one, which was first sonicated for 5 minutes and then carefully attached to the apparatus, so that there were no air bubbles present and the apparatus was washed. A pre-prepared plate containing appropriate medium was inserted on position 2, while

the plate containing the NR protein, i.e. 10 μL of the protein complex in wells 12B and 12F was put on position 1. The apparatus then dispensed 100 nL drops of our target protein solution and 100 nL drops of medium. When the preparation of microplate was finished, a plastic cover was put on it, because drops are very small and can dry easily if not protected. The plates were then stored in the refrigerated closet, equipped with a camera, taking pictures of drops every 24 hours, and documenting them.

4 RESULTS AND DISCUSSION

4.1 EQUILIBRATION OF THE SUPERDEX 75 COLUMN

The equilibration of the S75 column was essential in order to achieve better separation of target proteins by GF. The results presented graphically in Figure 8, show the expected size-based distributions of different control peaks.

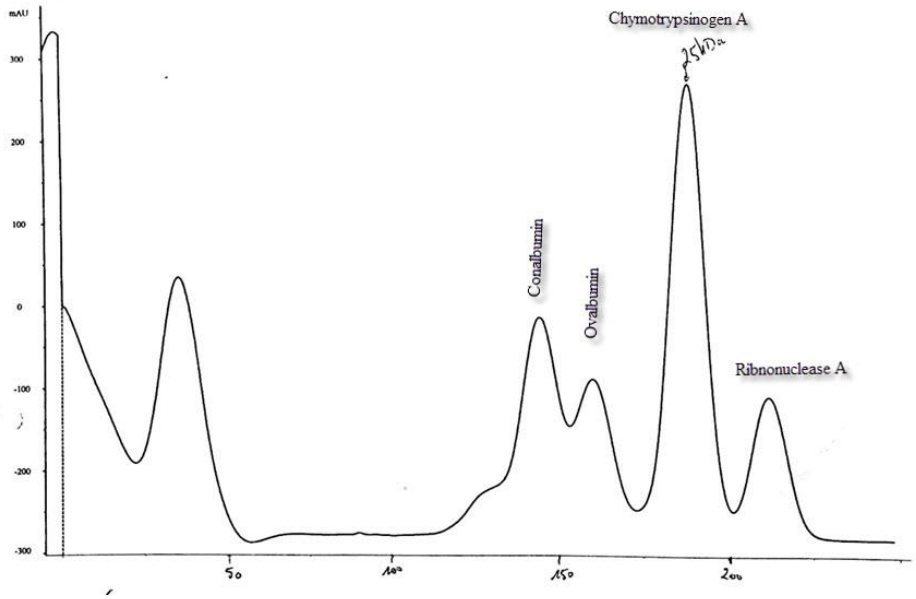


Figure 8: The equilibration curve of superdex 75 column, using a four-component standard (ovalbumin, conalbumin, ribonuclease A, chymotrypsinogen). Conditions: FR 2.5 mL/min, UV detector. Legend: y= mAU- miliabsorbance units; x=volume of eluted buffer.

4.2 THE 1ST TRIAL - FORMATION AND ISOLATION OF NCoR5, AND RXR-RAR

4.2.1 ISOLATION OF NCoR5

For the first trial we used a frozen sample containing NCoR5 that was expressed in Rosetta cells (a strain of *E. coli*) and induced by IPTG. Two cryotubes were thawed, each of them harvested from 700 mL LB culture. The contents of both were combined and after sonication and filtration the sample was purified by hisTrap chromatography, using Ni-column (42).

Figure 9 shows typical chromatograph obtained with the linear gradient. The first peak represents the elution phase, and the second one our target recombinant

protein, NCoR5. The two peaks are not fully separated, as some fronting is observed, but we can nevertheless specify and define the area of the specific peak. Before sample pooling, the following fractions were selected for the SDS-page gel analysis: 4, 6, 8, 10, 12, 13, 15, and 17.

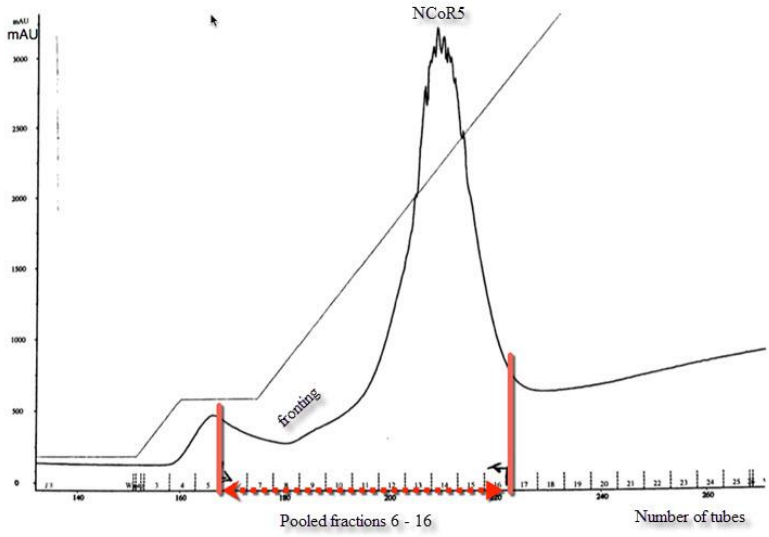


Figure 9: HisTrap NCoR5 purification in LB medium using Ni column. Conditions: min FR 0.1 mL/min, start concentration 4%, sample volume 70 mL, wash out volume 10 CV. UV detector. Legend: y= mAU-miliabsorbance units; x=volume of eluted buffer and number of collection tubes.

After we carried out the HisTrap separation on the Ni-column, we diluted the 1 to 35 eluted fractions by adding of 3 mL of buffer A to each one, and then, based on the electrophoresis results finally decided to pool the fractions from 6 to 16, as marked in the Figure 9. The total pooled volume was 55 mL, and it contained 2,178 mg/mL (MW=43.1 kDa, ε=19.94) of target protein. So, altogether we finished with 119,79 mg of NCoR5. We divided the pooled volume into two tubes, each containing 27,5 mL and marked them with A and B. Into each tube we added 100 μL of TEV (1/400 dilution of TEV/sample) and 27 μL of β-mercaptoethanole (1/1000 dilution β-mercaptoethanole/sample), and left them in the cool room over night. After approximately 16 hours we ran the SDS-page gel with the following samples: M (marker/ladder), A (before addition of TEV), B (before addition of TEV), fractions 4, 6, 8, 10, 12, 13, 15, and 17, as well as A (after the addition of TEV), B (after the addition of TEV).

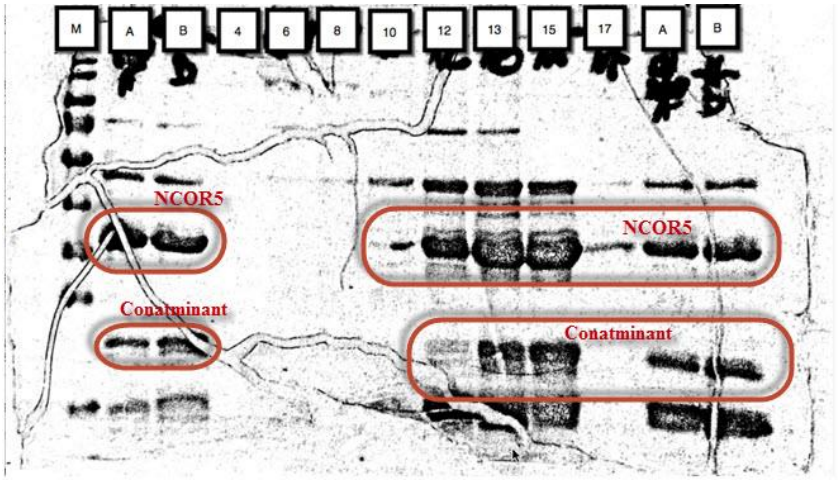


Figure 10: The SDS-page electrophoresis results of selected His-trap eluted fractions and pooled samples, before and after the addition of TEV.

The results of SDS page electrophoresis show that the NCoR5 is spotted as a dark line with a size near 43 kDa. The fractions containing the most of NCoR5 were 12-15. Based on these results, we assumed that TEV cleavage was successful. The last electrophoresis lines spotted in samples A and B (after the addition of TEV) are darker. Around 26 kDa we spotted a contaminant and assumed, that we would be able to get rid of it with the GF purification method.

We started the concentration process of the protein in Amicon cells, until we reached the final volume of 10 mL. This sample was then centrifuged and loaded onto the GF column.

The chromatograph of the GF purification step shows, that the resolution of peaks was not as expected (Figure 11). We pooled the eluted fractions from 59 to 80, as marked in Figure 11. The concentration of NCoR5 was 1,932 mg/mL (MW=28.86 kDa, ε=4.47) with the final volume of 25 mL, which seemed quite promising.

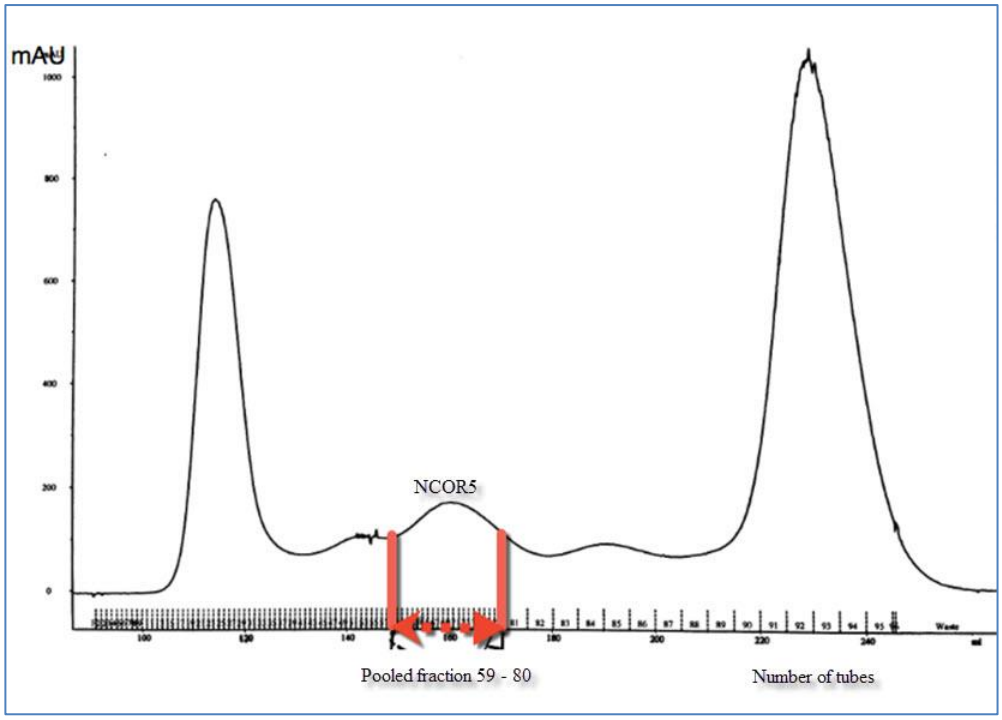


Figure 11: The results of GF of the NCoR5 separation after the addition of TEV. Conditions: FR 5 mL/min, UV detector. Legend: y= mAU-miliabsorbance units; x=volume of eluted buffer and number of collecting tubes.

Then we checked, by using SDS page electrophoresis, whether the contaminant was still present. As seen in the Figure 12, there was still an additional line present with a size of approximately 26 kDa. Despite of the presence of this contaminant we decided to use the NCoR5 for formation of the RAR-RXR-NCoR5 complex, and its subsequent crystallization.

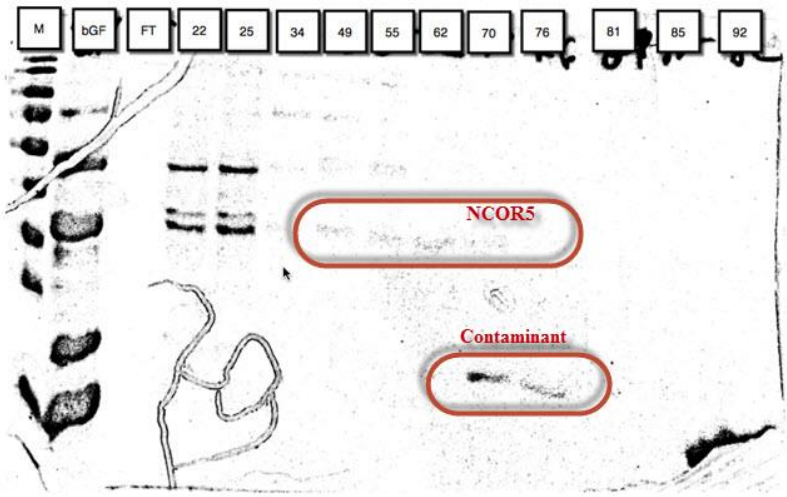


Figure 12: The results of SDS-page electrophoresis of the GF purified fractions.

4.2.2 FORMATION AND ISOLATION OF THE RAR-RXR HETERODIMERS

For the first trial we used frozen samples containing RXR and RAR that were expressed in Rosetta cells, and induced by IPTG. Three cryotubes of RAR and two of RXR were thawed each of them containing proteins harvested from a 700 mL LB culture. The contents of all were pooled and after sonication and filtration the sample was purified by the HisTrap chromatography, using Ni column.

Figure 13 presents a typical HisTag chromatograph with all three procedural phases, i.e. loading (L), washing (W), and elution (E) (42). The second peak represents the RXR-RAR heterodimer. The resolutions of the two peaks were not ideal, as the tailing of the second one was observed. Once the fractionation was finished, we diluted the eluted fractions from 10 to 40, each with 5 mL of buffer A, in order to prevent destabilization of the RXR-RAR complex.

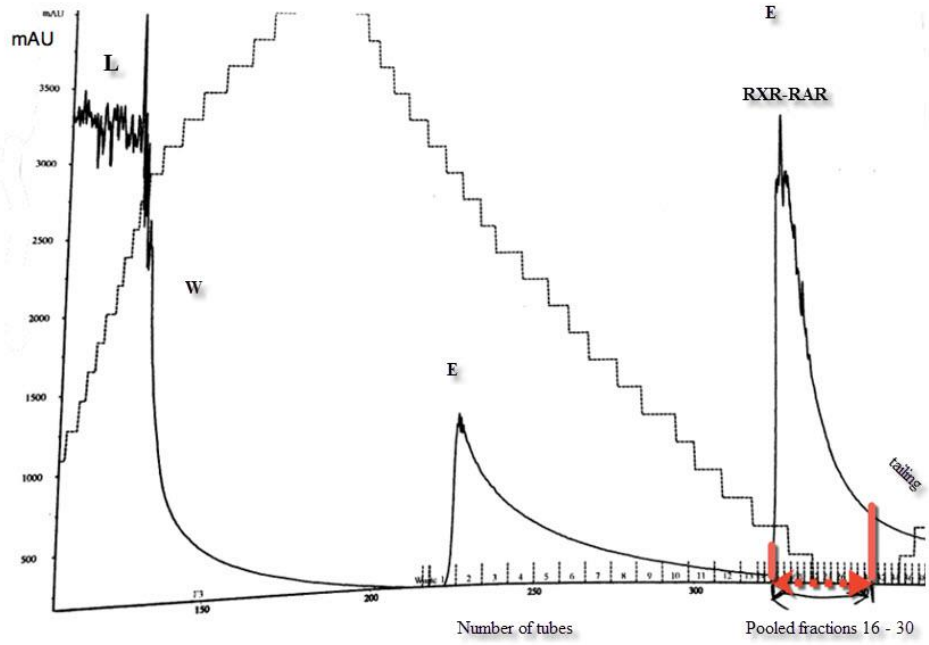


Figure 13: The results of the HisTrap RXR-RAR purification in LB medium on Ni column. Conditions: FR min 0.1 mL/min, starting concentration c. 4%, sample volume 125 mL, wash out with 20 CV, UV detector. Legend: y= mAU-miliabsorbance units; x=volume of eluted buffer and number of collecting tubes.

After the HisTrap separation on the Ni-column, we pooled the eluted fractions from 16 to 30, as marked in the Figure 13. The total pooled volume was 25 mL and it contained 3,58 mg/mL (MW=56.7 kDa, ε=29.0) of RXR-RAR. So, altogether we finished with 89,5 mg of heterodimer. Then we added 172 U of thrombine (2 U/mg),

and left the sample in the cool room over night. After approximately 16 hours we started the concentration of the purified protein in an amicon cell, until its volume of was reduced to 12 mL.

Subsequently we proceeded with the GF purification step.

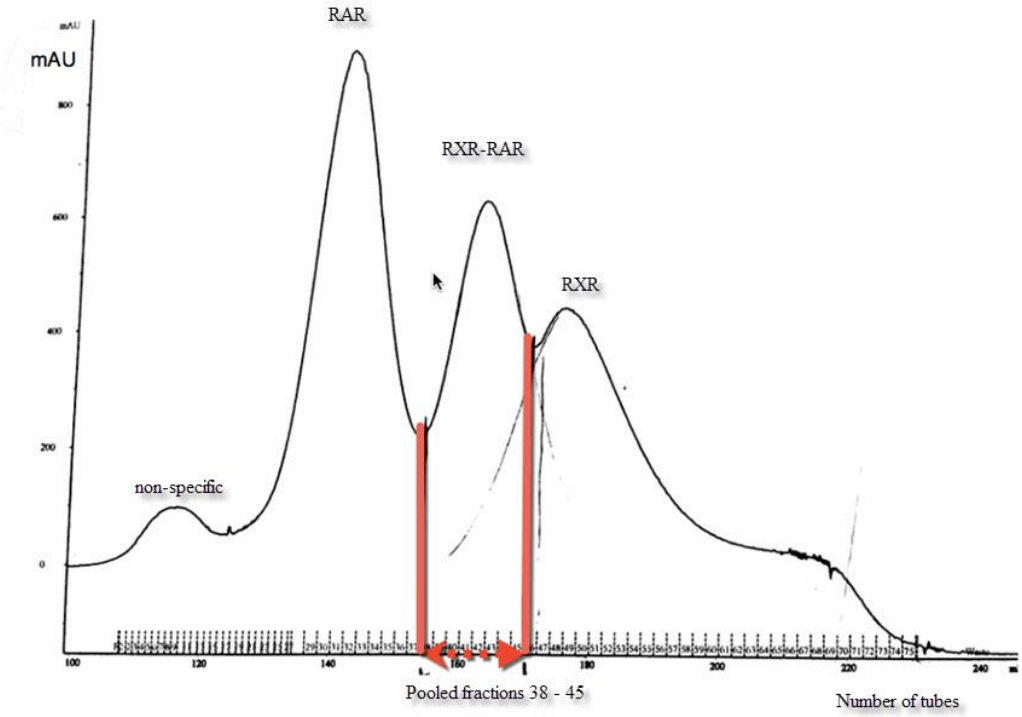


Figure 14: GF purification of RXR-RAR after the addition of thrombin. Conditions: FR 5 mL/min, UV detector. Legend: y= mAU-miliabsorbance units; x=volume of eluted buffer and number of collecting tubes.

Figure 14 displays the results of the GF purification procedure, showing that the resolution of peaks was not as we would like it to be. The first peak was not specific, the second represents RAR alone, while the third one is that of RXR-RAR and the fourth of the RXR alone. In order to spot the target protein we sampled the fractions 8, 31, 34, 36, 39, 41, 43, 45, 46, 47, 48 and 51, and run the SDS-page gel, together with the marker (M) and the sample before GF (bGF) (Figure 15). Based on the electrophoresis results we decided to pool the fractions from 38 to 45, finishing with 2,75 mg/mL (MW=54.8 kDa, ε=29.0) of the RXR-RAR complex in the total volume of 16 mL.

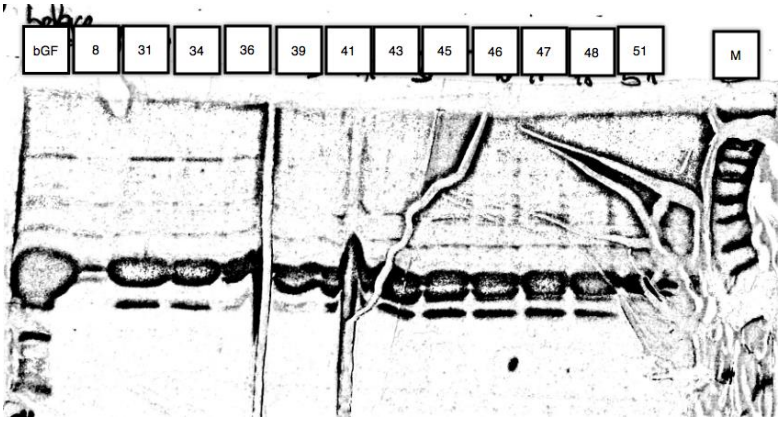


Figure 15: The results of SDS-page electrophoresis of GF purified fractions.

4.2.3 FORMATION OF THE RXR-RAR-NCoR5 COMPLEX

In the order to form the RXR-RAR-NCoR5 complex we decided to combine 10 mL of purified RAR-RXR and 10 mL of NCoR5 containing an unknown contaminant, and left the mixture in the cool room over night. After approximately 16 hours we started to concentrate the sample in an amicon cell, until its volume was reduced to10 mL, and then proceeded to GF purification.

The results obtained and presented in Figure 15 were not as good as expected, as the peak eluted at 220 mL was the contaminant. However, the peak at 140 mL was promising.

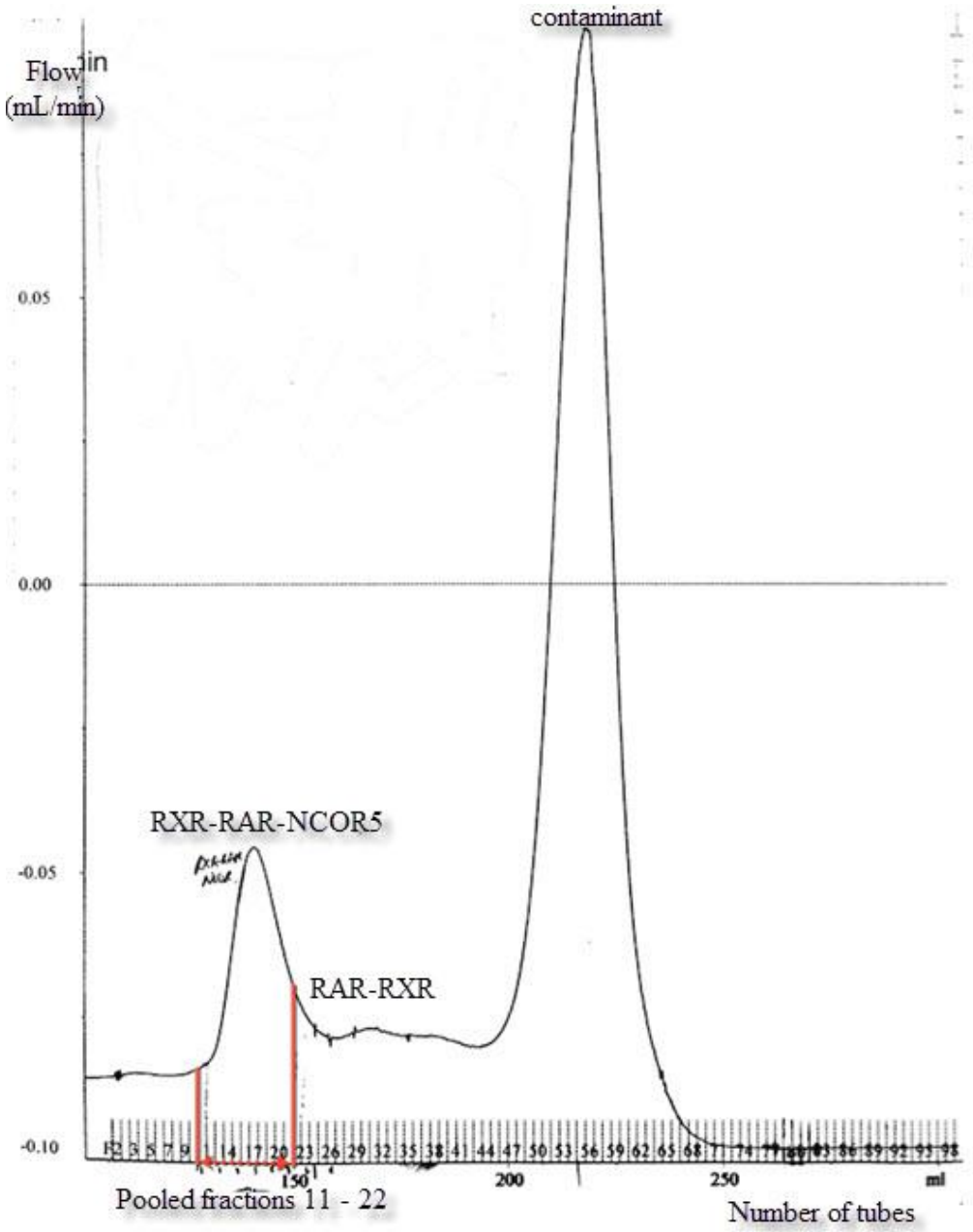


Figure 16: The results of GF purification of the RXR-RAR-NCoR5 complex. Conditions: FR 5 mL/min, UV detector. Legend: y= mAU-miliabsorbance units; x=volume of eluted buffer and number of collecting tubes.

Subsequently we ran SDS-page analysis with samples taken from fractions 56, 52, 26, 24, 21, 19, 17, and 11, as well as with the marker (M), the complexes RXR-RAR-NCoR5 (C) and RXR-RAR (RR), as well as NCoR5 alone. Based on the results presented in the Figure 17 we pooled the fractions from 11 to 22. The concentration of the RXR-RAR-NCoR5 complex in the pooled 22 mL sample was 0,599 mg/mL (MW=83.7, kDa, ε=33.5).

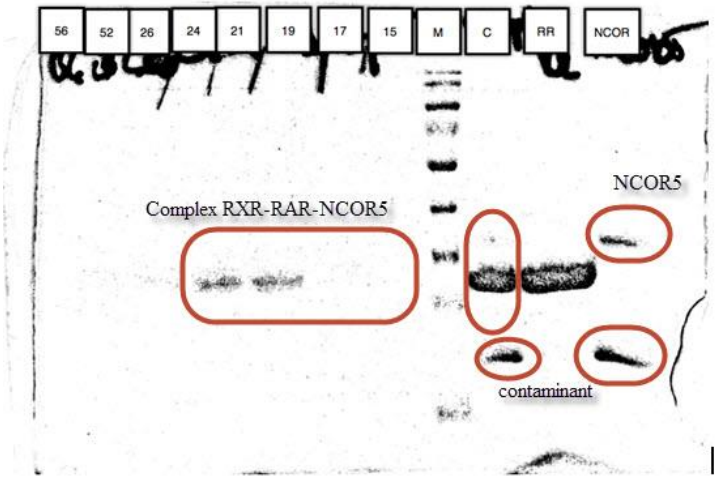


Figure 17: The results of SDS-page electrophoresis of GF purified fractions of the RXR-RAR-NCoR5 complex.

4.2.4 CRYSTALLIZATION OF THE RXR-RAR-NCoR5 COMPLEX

We first concentrated the RXR-RAR-NCoR5 sample until its volume was reduced to 750 µL, and its concentration was 9,078 mg/mL (MW=83.7, kDa, ε=33.5). After we started the crystallization process, we left the plates for certain amount of time. They were checked for crystal formations after 1, 3, 7, 10, 14, 21 and 28 days. Unfortunately, the crystallization was unsuccessful, as we did not obtain any crystals, not even the amorphic ones. Most of the samples were clear all the time and only out of few the proteins precipitated.

4.3 THE 2ND TRIAL - FORMATION AND ISOLATION OF RXR-RAR HETEROIMERS

4.3.1 FORMATION AND ISOLATION OF RAR-RXR HETERODIMERS

For the second trial we once again used frozen samples containing RXR and RAR, expressed in Rosetta cells and induced by IPTG. Three cryotubes of RAR and 2 of RXR were thawed, each of them harvested from 700 mL LB culture. The contents of all were combined and following sonication and filtration, the pooled sample was purified by HisTrap chromatography using Ni column (42).

The second peak in Figure 18 represents the RXR-RAR heterodimer. The area of the peak can easily be specified. Once again, as in the first trial, the tailing of RXR-

RAR peak was observed. We diluted the fractions from 15 to 30, each with 5 mL of buffer A.

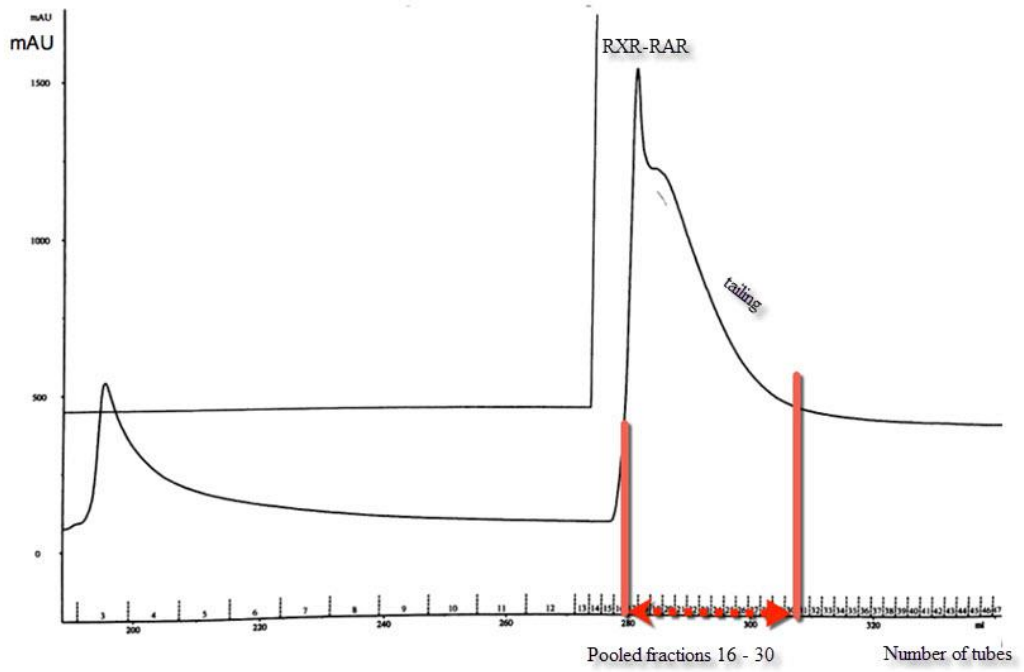


Figure 18: the results of the HisTrap RXR-RAR purification in LB medium using Ni column. Conditions: FR min 0.1 mL/min, starting concentration 4%, sample volume 125 mL, wash out volume 20 CV, UV detector. Legend: y= mAU-miliabsorbance units; x=volume of eluted buffer and number of collecting tubes.

Based on the SDS-page electrophoresis results presented in Figure 19, we observed a very low concentration of RXR (the second line in fractions 17-19). Therefore we decided to pool fractions from 16 to 30 (100 mL), and concentrate them in Amicon tube until reaching the volume of 50 mL.

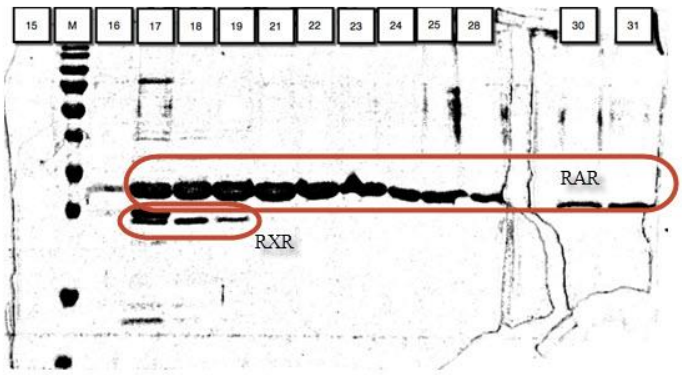


Figure 19: The results of SDS-page electrophoresis of Ni-column purified RXR-RAR heterodimer samples.

In order to increase the quantity of RXR, we decided to redo the Ni-column purification step after adding one additional thawed RXR containing sample.

As seen in Figure 20, we could easily specify the area of the RXR-RAR peak. The resolution of the two peaks in the chromatogram was however not completely satisfactory, as the tailing of the second was present. Once the fractionizing was finished, we diluted each of the eluted fractions from 13 to 30 with 5 mL of buffer A, to prevent destabilization of the RXR-RAR complex.

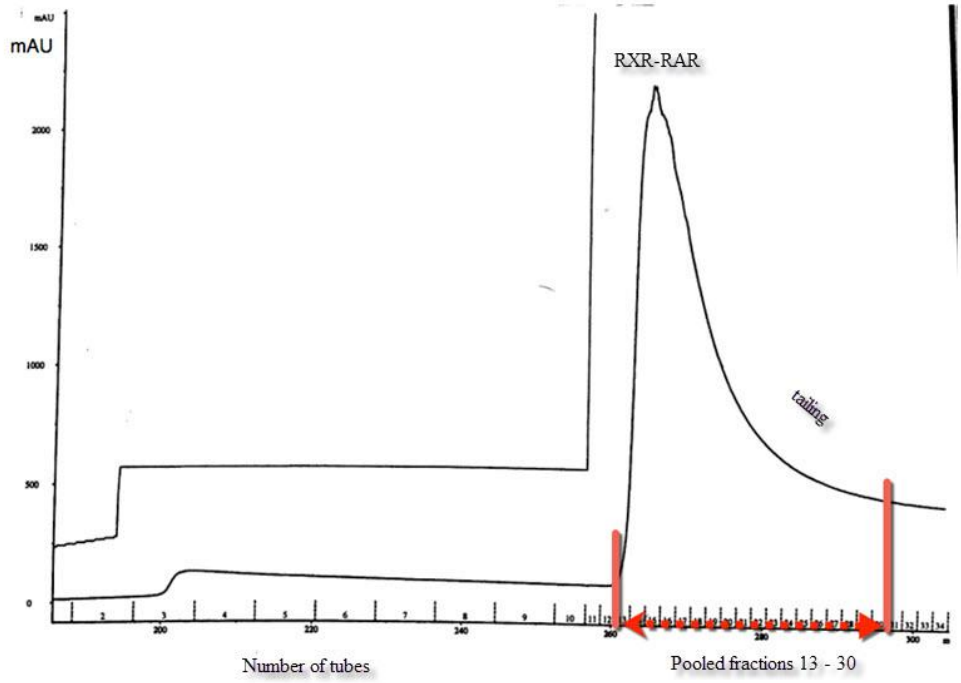


Figure 20: The results of repeated RXR-RAR purification in LB medium on the Ni column. Conditions: FR min 0.1 mL/min, starting concentration 4%, sample volume 125 mL, wash out volume 20 CV, UV detector. Legend: y= mAU-miliabsorbance units; x=volume of eluted buffer and number of collecting tubes.

In order to check whether the quantities of expressed and purified RXR-RAR protein were sufficient we ran SDS-page electrophoresis with used the following samples: fraction before repeated purification (B), marker (M), fractions 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 24, 27 and 30.

The results of the SDS-page electrophoresis, presented in the Figure 21, were very promising. The quantities of RXR and RAR were almost equal. Therefore we decided to pool the fractions from 14 to 27, and concentrate them in the Amicon cell until the volume was reduced to 12 mL. The final concentration of the RXR-RAR complex was 4,90 mg/mL (MW=56.7 kDa, ε=29.0).

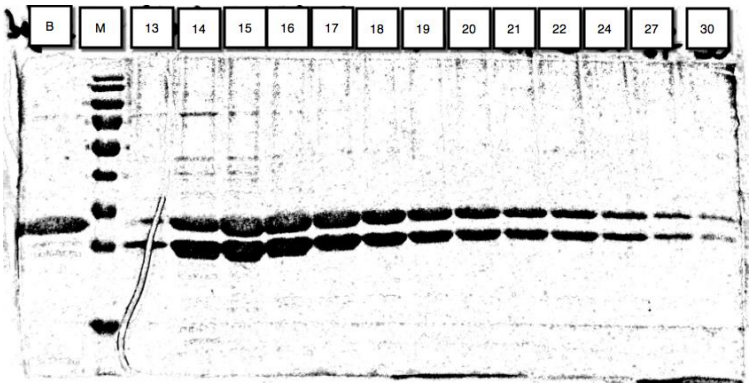


Figure 21: The results of SDS-page electrophoresis of Ni-column purified fractions containing the RXR-RAR heterodimer (the repeated purification).

Then we added 140 U of thrombine (2 U/mg), and left the sample overnight in a cool room. After approximately 16 hours we started the GF purification of the pre-centrifuged sample.

The resolution of peaks presented in Figure 22 was very promising. The first peak is not specific, the second one represents RXR-RAR, and the third one is RXR alone, as a part of tailing.

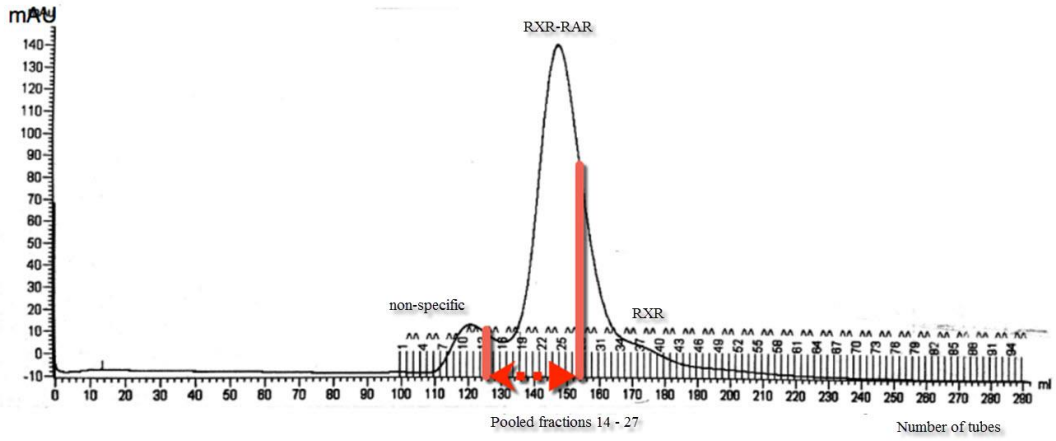


Figure 22: The results of the GF purification of RXR-RAR, following the addition of thrombin. Conditions: FR 5 mL/min, UV detector. Legend: y= mAU-miliabsorbance units; x=volume of eluted buffer and number of collecting tubes.

In order to spot the protein, we prepared the following samples: before GF (A), marker (M), fractions 10, 13, 16, 19, 22, 25, 28, 30, 32, 34, 37, 40 and 46, and ran the SDS-page gel (Figure 23).

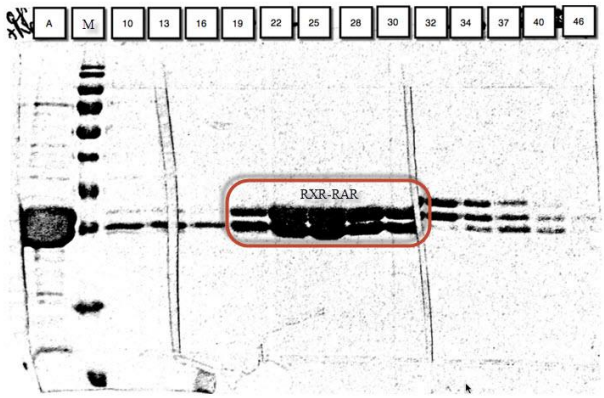


Figure 23: The results of SDS-page electrophoresis of GF purified fractions containing RXR-RAR heterodimer.

Based on the results presented in Figure 23 we decided to pool the fractions from 19 to 32. The final RXR-RAR concentration was 1,03 mg/mL (MW=54.3 kDa, ε=29.0) in a total volume of 28 mL. The pooled sample was stored at -80º C.

4.4 FLUORESCENCE ANISOTROPY OF THE RXR-RAR HETERODIMER WITH FLUORESCEIN NCoR5-ATTO IN THE PRESENCE OF SELECTED NR LIGANDS

For each anisotropy analysis we took 5 mL of the RXR-RAR sample obtained after GF purification, containing 1,03 mg of the complex/mL (MW=54.3 kDa, ε=29.0), and concentrated it down to 750 µL.

4.4.1 THE 1ST ANISOTROPY TRIAL

The concentration of RXR-RAR in the concentrated sample was 4,62 mg/mL. The curve that represents the activity of NCoR5 in the presence of ligand AM580 (green) was shifted above the APO curve (red), as expected (Figure 24). However we anticipated a bigger shift than observed.

We also expected a bigger shift in case of BMS593 (black curve), but this was only seen at its high concentration area.

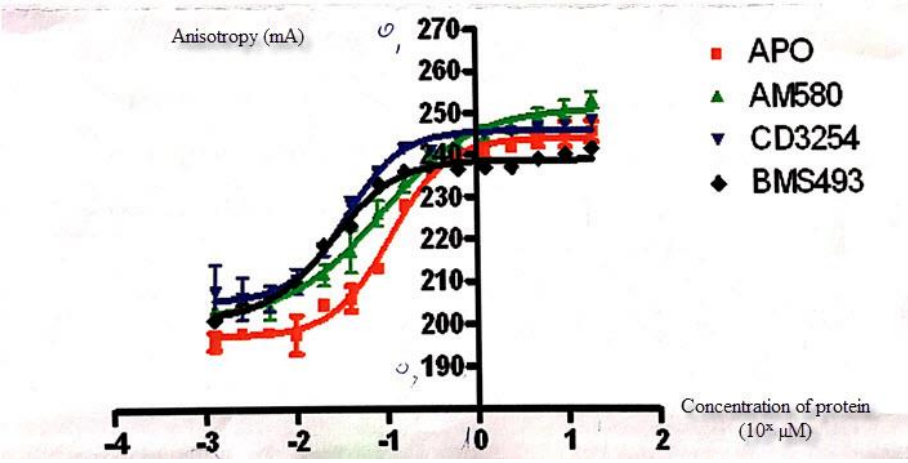


Figure 24: The results of the first anisotropy trial with the RXR-RAR and tagged fluorescein NCoR5-atto, in the presence of AM580 and BMS493 ligands. Legend: x=concentration of the RXR-RAR (10^x µM), y=mA-miliAmpers.

4.4.2 THE 2ND ANISOTROPY TRIAL

This time, the concentration of the concentrated RXR-RAR sample was 3,14 mg/mL. Based on results of the 1st trial we decided to perform the 2nd one with the use of BMS493 ligand. However, we have once again observed the expected shift exclusively at high ligand concentrations (Figures 24 and 25).

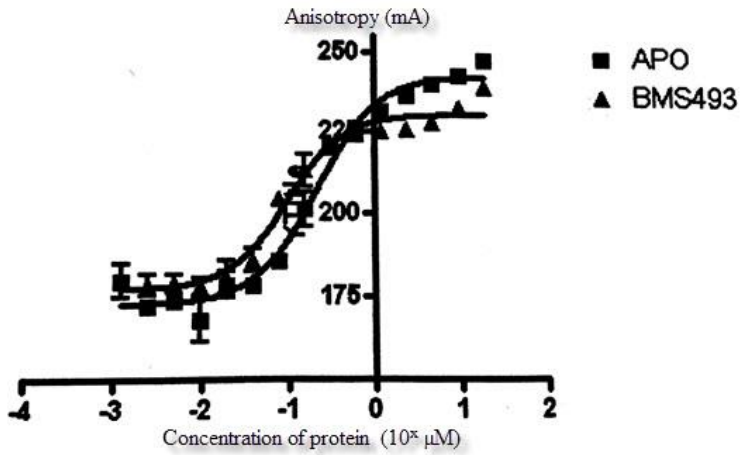


Figure 25: The results of the 2nd anisotropy trial with the RXR-RAR and tagged fluorescein NCoR5-atto in the presence of the BMS493 ligand. Legend: x=concentration of RXR-RAR (10^x µM), y=mA-miliAmpers.

4.4.3 THE 3RD ANISOTROPY TRIAL

The concentration of the concentrated RXR-RAR sample was 3,31 mg/mL. The curve that represents the activity of NCoR5 in the presence of AM580 ligand (green) was not shifted above the APO curve (red), as we were expecting. However the shift of the BMS593 curve (black) is nicely visible and it is as anticipated.

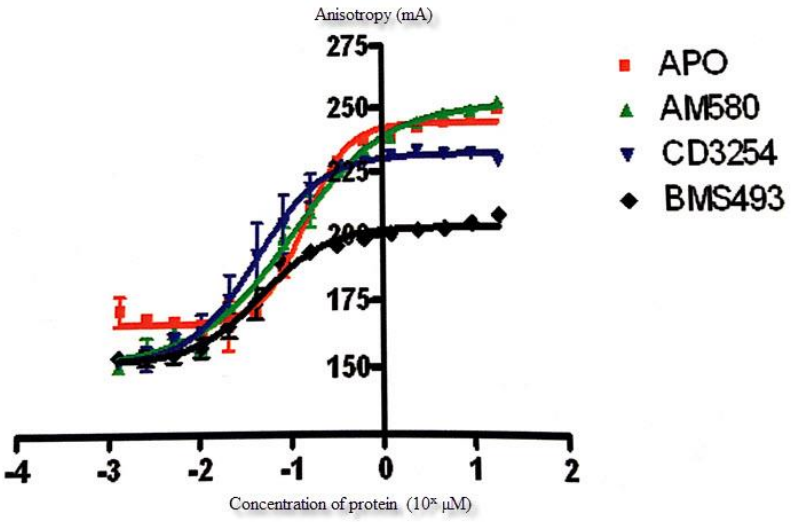


Figure 26: The results of anisotropy with RXR-RAR and tagged fluorescein NCoR5-atto, in the presence of AM580 and BMS493 ligands. Legend: x=concentration of RXR-RAR (10^x µM), y=mA-miliAmpers.

Table 3: The EC50 values obtained in all three anisotropy trials.

EC_{50} Values			
	APO state	Ligand AM580	Ligand BMS494
1st trial	0.271	0.322	0.097
2nd trial	0.217	/	0.066
3rd trial	0.137	0.103	0.046

We have shown that both ligands affect the NCoR5 affinity. The BMS493 increased interaction of RXR-RAR with NCoR5, while the AM580 should have decreased it; however our data are too inconsistent to confirm that (Table 3).

5 CONCLUSION

The formation of the RXR-RAR-NCoR5 complex was not completely successful, as we were not expecting to have contaminant present following the isolation and purification of NCoR5. Its reason is not obvious, but we can assume human errors or contaminated columns. In order to check this, the ternary complex formation should be repeated with re-washed and re-calibrated columns, but unfortunately, due to the lack of time, we were not able to do this.

The crystallization was not successful either and the possible reasons for that could be: the presence of contaminant in NCoR5 and/or low protein concentration in the complex. We assume that the results of crystallization ratio of clear vs. precipitated protein solutions should be around 50% : 50%. Our results may suggest that the concentration of the RXR-RAR-NCoR5 complex was too low, therefore the crystallization step should be repeated using higher amounts of protein. Additionally, this experiment was quite unfortunate for us, as the crystallization robot broke down, and therefore it was not at our disposal during the time that we had at our disposal.

The results of fluorescence anisotropy are promising, as they suggest that there is a significant difference in NCoR's binding affinity to RXR-RAR in the presence of AM580 and BMS493 ligands. We were able to confirm that BMS493 works as an inverse agonist, stabilizing the interaction of RXR-RAR with NCoR5. The activity of AM580 was not significant enough for reliable confirmation of its agonistic effect. During our work we also had problems with the RXR-RAR concentration. Namely, the isolated protein complex was highly unstable at room temperature, where it dissociated rather quickly. One of solutions would be to use an automated apparatus for preparation of anisotropy plates in order to avoid potential human mistakes. We also still do not know the precise effects/consequences of repeated freezing/thawing on RXR-RAR concentration. Based on our results we can only say that under such conditions its concentration diminishes. We would also suggest preparing new stocks of ligands in smaller quantities in order to avoid the impact of re-freezing.

During our experiments we came up with potential improved approaches regarding the future work:

a) formation of heterodimer RXRd12-RAR, with the H12 chain on the RXR being absent;
b) formation of the polycistronic NCoR5-RXR-RAR complex, were multiple genes are present, and we would avoid the need of making the complex;
c) the use of SEC-MALS (Size Exclusion Chromatography – Multi Angle Laser light Scattering) where light emission and concentration are measured directly in eluted fraction would enable avoiding potential mistakes caused by column;
d) the use of SAXS (Small-Angle X-ray Scattering) would enable full structural determination of RXR-RAR heterodimer interaction with the domain NID NCoR5.

Even though we have not successfully completed our experiments, we think that we have made an important contribution to the field, and that our results might help others to reach our goals in future.

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