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Su-Wei Lee April 12, 2022

The Structural and Biochemical Studies of the Mineralocorticoid Receptor Ligand Binding Domain Complexed with Vamorolone

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Abstract

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By Su-Wei Lee

Duchenne muscular dystrophy (DMD) is a genetic disorder that shows chronic and progressive damage to skeletal and cardiac muscle ultimately resulting in premature death in boys due to end-stage heart failure. Heart failure is often treated with drugs that lower blood volume and pressure, decreasing functional demand on the heart, such as mineralocorticoid receptor (MR) antagonists, spironolactone and eplerenone. Furthermore, the current standard of care for DMD, anti-inflammatory corticosteroids, alleviates DMD progression but drives adverse side effects such as bone mineral density loss and growth stunting through interactions with glucocorticoid receptors (GR). Through subtle modification to a steroidal backbone, vamorolone, a recently developed drug, appears to decrease muscle inflammation, stabilize muscle strength, and suppress cardiomyopathy without significant side effects due to its interactions with both MR and GR. Although the interaction between vamorolone and GR has been extensively studied, how vamorolone antagonizes MR remains poorly understood. In this study, we determined the crystal structure of MR LBD vamorolone complex and through biochemical studies identified that vamorolone could be acting as a potential passive antagonist. Our result sheds light to the possible similarities between vamorolone and known MR passive antagonists, spironolactone and eplerenone, guiding future studies to focus on the local conformational dynamics and coregulator binding patterns. Collectively, this work advances our understanding of vamorolone's mechanism of action and efficacy of cardiac muscle protection.

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Chapter 1: Introduction

Mineralocorticoid Receptor (MR), a ligand-dependent transcription factor belonging to the Nuclear Receptor superfamily, plays a key role in the regulation of electrolyte balance and blood pressure. In the distal nephrons, aldosterone binds to the MR receptor, forming a complex that regulates sodium and potassium balance by stimulating the activity of the epithelial sodium channel (ENaC) and the Na+-K+-ATPase [1]. In addition to its effects in kidneys, MR is also found in the heart, vasculature, brain, and adipose tissue and is responsible for vasoconstriction and the increase of blood pressure [2].

Duchenne muscular dystrophy (DMD), a X-linked degenerative disorder caused by the mutation to the dystrophin gene, is one of the most severe and common types of muscular dystrophy with an incidence rate of 1 in 5000 boys. The absence of the functioning dystrophin proteins in muscle cells weakens and disrupts the integrity of muscle fibers. Early manifestation includes general skeletomuscular weakness and atrophy. As DMD progresses, cardiac muscular degeneration will eventually be observed, resulting in an average life expectancy lower than 30 years. End stage heart failure induced by DMD is the deciding factor contributing to patient mortality. MR antagonists, spironolactone and eplerenone, have been used over years to treat heart failure by lowering blood volume and pressure thereby decreasing functional demand on the heart [3].

In addition, the inflammatory response to the degeneration of muscle tissues is critical in the exacerbation of the disease. Activation of the proinflammatory nuclear factor-κB (NF-κB) pathway observed in muscles of patients with DMD is believed to cause chronic inflammation in muscles that contributes to disease onset and progression. The current standard of care for DMD is corticosteroid

treatment such as deflazacort and prednisolone. Both FDA-approved drugs bind to glucocorticoid receptor (GR) and repress the transcription of pro-inflammatory genes to exert anti-inflammatory effects, thereby reducing fibrosis and stabilizing muscle strength [3]. However, like other corticosteroid drugs, both deflazacort and prednisolone have debilitating side effects such as loss of bone mineral density, stunting, and muscle wasting in the limbs under long-term treatment. Furthermore, similar to aldosterone, prednisolone simultaneously acts as a MR agonist and increases left ventricular volume, heart fibrosis, and early dilatation in the muscular dystrophy mouse model of DMD [4].

Vamorolone is a corticosteroid derivative recently developed by ReveraGen BioPharma Inc. and is currently in a phase 3a clinical trial for DMD. It is characterized by a double bond between C9 and C11 on the conventional steroidal ring and lacks an oxygen moiety at the C11 position [5] (Figure 1). Like both deflazacort and prednisolone, vamorolone binds to GR and elicits anti-inflammatory effects. However, long term treatment of vamorolone has shown no signs of most corticosteroid side effects in boys with DMD [6]. In addition, exhibiting similar effects as spironolactone and eplerenone, vamorolone inhibits aldosterone's action by reducing blood pressure, and preventing the increase of heart fibrosis, low fractional shortening, and high left ventricular wall thickening [7]. These results suggest that vamorolone may also treat DMD-related cardiomyopathy as a MR antagonist.

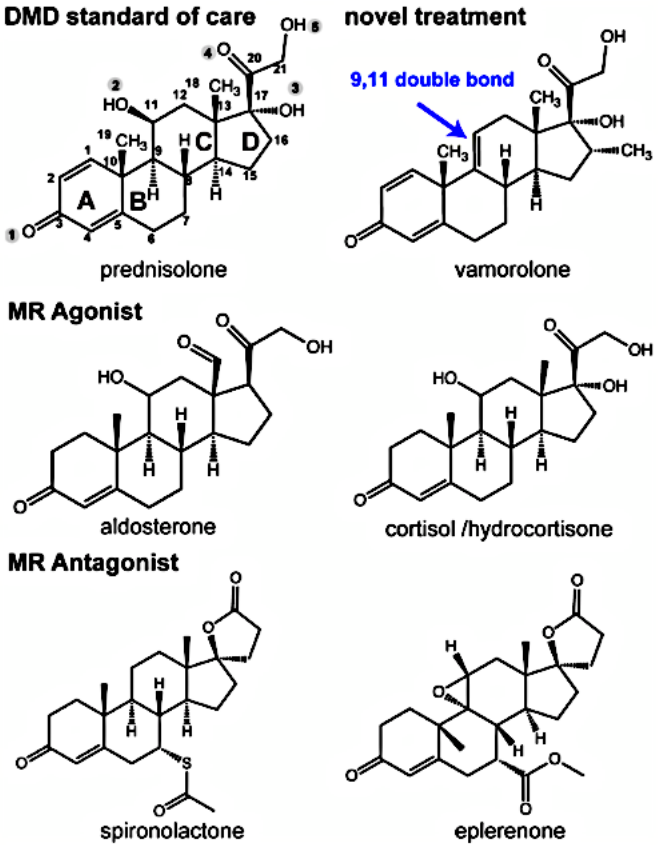

Figure 1. Chemical structures of various steroids. Canonical carbon and oxygen atom (in gray circle) numbering is shown on the structure of prednisolone. The character defining double bond between C9 and C11 of vamorolone is pointed out by the blue arrow. Known MR agonists and antagonists are shown in the middle and bottom row, respectively.

Vamorolone stands out from the MR antagonists spironolactone and eplerenone by interacting with GR and demonstrating anti-inflammatory effects. Additionally, spironolactone induces side effects such as gynecomastia due to its off-target interaction with androgen receptor (AR), and eplerenone causes hyperkalemia due to its high dosage levels to maintain consistent drug exposure [8]. Unlike spironolactone and eplerenone, vamorolone treatment has shown no evidence of hyperkalemia or cross interaction with AR. Moreover, in comparison to the other corticosteroids, vamorolone decreases muscle inflammation, stabilizes muscle strength, and suppresses

cardiomyopathy with little or no side effects thanks to its dual interactions with GR and MR (Table 1) [7].

Steroids	Effects	Side Effects
Deflazacort, Prednisolone	anti-inflammatory (GR agonistic)	bone mineral density loss, stunting, muscle wasting
Spironolactone, Eplerenone	aldosterone-inhibitory (MR antagonistic)	gynecomastia, hyperkalemia
Vamorolone	All the above	None

Table 1. Properties of various steroids. Effects and side effects of known steroids are shown in juxtaposition with vamorolone, the focus of the study.

Although the leading causes of death for DMD are hypertrophic and dilated cardiomyopathy and heart failure, current research and therapeutics are mostly focusing on skeletomuscular dystrophy. There remains a specific need for treatments targeting cardiac muscles to either prevent or prolong the development of DMD symptoms. Vamorolone has demonstrated functional and pathological improvements in cardiac muscle in muscular dystrophy mice while exhibiting little or no side effects commonly seen among corticosteroids in clinical trials [7]. However, how vamorolone antagonizes MR remains poorly understood. It is hypothesized that vamorolone binds to MR ligand binding domain (LBD), alters the LBD's conformational dynamics by allosteric communication, which ultimately antagonizes MR-mediated gene transcription. The identification of key interactions between vamorolone and MR will bridge the gap necessary to integrate our knowledge of the molecular mechanisms of vamorolone to the data from clinical studies and advance our understanding of its efficacy of protection in cardiac muscles.

Goals

The goals of this research project are to structurally and biochemically characterize the binding between vamorolone and MR.

This will be achieved by:

1) Expressing and purifying C808S S810L MR LBD complexed vamorolone as well as C808S MR LBD complexed with other agonists.

2) Determining the structure of C808S S810L MR LBD complexed with vamorolone through X-ray crystallography.

3) Employing biophysical and biochemical approaches such as thermal shift assays and fluorescence polarization peptide coregulator binding assays.

Chapter 2: Purifying MR LBD with various ligands

Expression and purification of MR LBD C808S complexed with vamorolone was difficult as vamorolone acts as a MR antagonist, destabilizing the MR LBD C808S-vamorolone complex. Crystallization of the unstable MR LBD C808S-vamorolone complex would also be improbable. Therefore, an additional mutation S810L was introduced to MR LBD to facilitate a more stable protein expression and purification and to acquire stable MR LBD-vamorolone complex crystal structures.

The MR S810L mutation was identified in pregnant women who were complicated by marked exacerbation of hypertension, low serum potassium levels and undetectable aldosterone levels without signs of preeclampsia [9]. The S810L mutation lies in the MR LBD and leads to antagonist ligands switching to become agonists. Known MR antagonists such as progesterone (high in pregnant women) and typical hypertension medications (eplerenone and spironolactone) activate transcription via MR carrying the S810L mutation [9]. In lieu of the hydrophilic serine residue, the S810L mutation establishes hydrophobic interactions with the Gln776 residue and with the antagonist ligands such as progesterone, spironolactone, and eplerenone. The presence of these hydrophobic interactions provides more stabilization to the ligand within the binding pocket. It is thus observed that known wild-type MR antagonists such as progesterone, spironolactone, and eplerenone exhibit agonistic effects in the presence of the S810L mutation in MR LBD [9].

Human MR LBD (amino acids 735-984) was cloned into pSmt3 vector with an N-terminal, 6xHis-SUMO tag with a complementary C808S mutation to increase soluble expression and a S810L mutation for MR LBD-vamorolone complex stabilization. E. coli strain BL21 (DE3) cells transformed with the pSmt3 vector were grown in Lysogeny broth (LB) medium until OD_{600} reached

0.6 AU. Expression of protein was subsequently induced by 0.15 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) and 100 μM vamorolone, facilitating the correct folding of MR for 16 hours at 16°C. E. coli cells were harvested by centrifugation at 4000 rpm for 20 minutes and subsequently lysed in a buffer containing 20 mM Tris pH 8.0, 25mM imidazole, 200mM NaCl, 5% glycerol, phenylmethylsulfonyl fluoride (PMSF), 2-mercaptoethanol, protease inhibitor, and 10 μM vamorolone by sonication for 15 minutes, followed by high-speed centrifugation at 16000 rpm for 40 minutes to separate the soluble lysates.

The expressed protein was purified via nickel (Ni) affinity chromatography with a 3-step elution profile (5%, 50%, and 100% of 250 mM imidazole) in a buffer consisted of 200 mM NaCl, 25 mM imidazole, 20 mM Tris pH 8.0, and 20 μM vamorolone. Most of the protein eluted was collected at 50% of 250 mM imidazole. (Figure 2). The His-SUMO tag was removed by Ulp1 proteolysis overnight and separated from the MR LBD by a second Ni column. The eluted protein was collected, concentrated, and further purified through size exclusion chromatography using a HiLoad 16/60 Superdex 200 column in a sizing buffer containing 20 mM HEPES, 150 mM NaCl, 5% glycerol, and 20 μM vamorolone (Figure 3). MR LBD was eluted from the size exclusion chromatography at 18 mL, falling between 17.3 mL (44 kDa) and 20.4 mL (17 kDa) and corresponded to the expected molecular weight (29.1 kDa). Gel electrophoresis was used to confirm that the peaks during Ni affinity chromatography and size exclusion chromatography corresponded to the expected molecular weight (42.7 kDa and 29.1 respectively) and that the SUMO tag was successfully cleaved during Ulp1 proteolysis (Figure 4).

As for the protein purification for MR LBD complexed with other ligands (prednisolone (PRED), hydrocortisone (HCY), or dexamethasone (DEX), the human MR LBD C808S mutant strain

was induced with 50 μM ligands following the exact same protein purification procedures.

Although MR LBD C808S S810L complexed with vamorolone is stable enough to crystallize and undergo X-ray crystallography to determine its structure, vamorolone is acting as an MR LBD agonist due to the S810L mutation. In order to test the stability and peptide coregulator binding of MR LBD that shares identical functions with MR LBD under physiological conditions, MR LBD C808S complexed with vamorolone is required and obtained through ligand exchange dialysis.

Purified MR LBD C808S complexed with dexamethasone was concentrated to 10 μM in a buffer containing 20 mM HEPES (pH 7.4), 200 mM NaCl, and 5 % glycerol and placed in a dialysis bag with 80 μM vamorolone. The bag was dialyzed in buffer consisted of 20 mM HEPES, 150 mM NaCl, 2.5 mM DDT, and 60 μM vamorolone for a total of 3 days at 4°C. Buffer was refreshed daily.

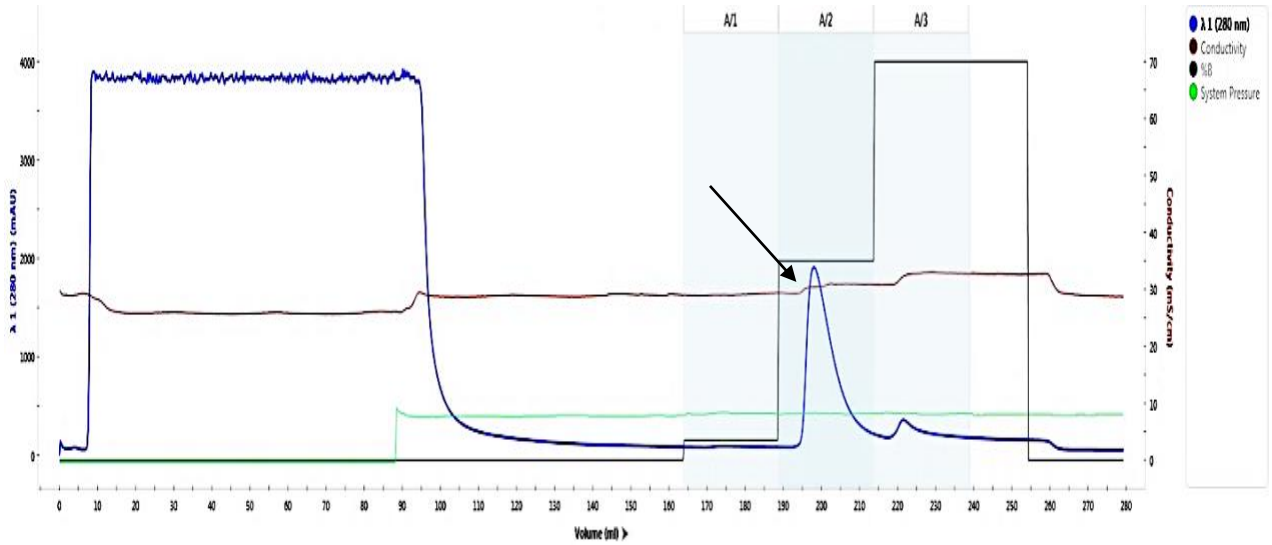

Figure 2. MR LBD C808S S810L complexed with vamorolone Ni affinity chromatography. The blue line represents the UV absorption at 280 nm. The black line represents the percent of Buffer B (250 mM imidazole). The three steps were set to 5%, 50%, and 100% of 250 mM of imidazole. The arrow represents where the protein was eluted and collected. Most of the protein eluted was collected at 50% Buffer B.

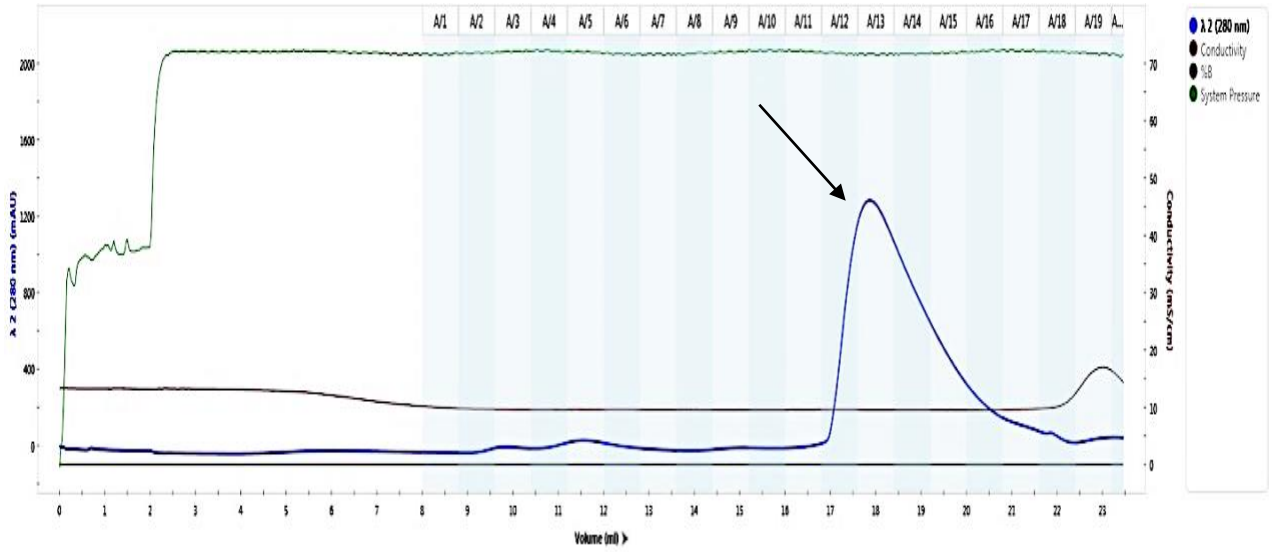

Figure 3. MR LBD C808S S810L complexed with vamorolone size exclusion chromatography. The blue line represents the UV absorption at 280 nm. The protein eluted at 18 mL, falling between 17.3 mL (44 kDa) and 20.4 mL (17 kDa) and corresponded to the expected molecular weight (29.1 kDa) of MR LBD. The arrow represents where the protein was eluted and collected.

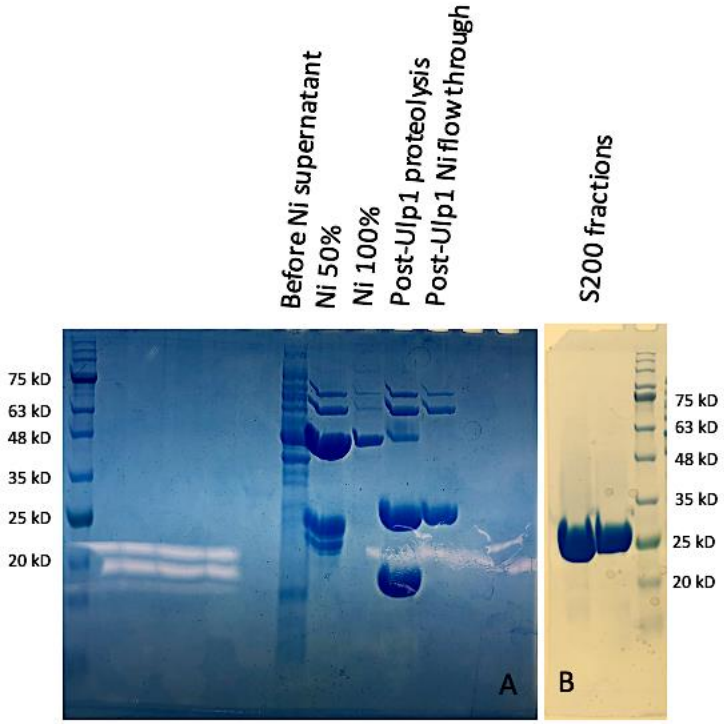

Figure 4. MR LBD C808S S810L complexed with vamorolone SDS-PAGE gel electrophoresis. SDS-Page protocols were followed. (A) The thick protein band observed in Ni 50% was not observed at the same molecular weight after Ulp1 proteolysis. Instead, two bands (~25 kDa and ~15 kDa) in post-Ulp1 proteolysis were observed, suggesting the Ulp1 proteolysis successfully cleaved the SUMO tag. The expected molecular weight of MR LBD (29.1 kDa) and SUMO tag (13.6 kDa) matched the molecular weight of the bands, respectively. (B) Size exclusion chromatography reconfirmed the molecular weight of MR LBD at ~25 kDa.

Chapter 3: MR LBD-vamorolone structure via X-ray crystallography

To gain insight into the antagonist mechanism of vamorolone, the X-ray crystal structure of MR LBD C808S S810L-vamorolone complex was obtained through X-ray crystallography. MR LBD C808S S810L complexed vamorolone along with MR LBD C808S complexed with other ligands (hydrocortisone and prednisolone) were concentrated to 4-6mg/mL in a buffer containing 20 mM HEPES (pH 7.4), 200 mM NaCl, and 5 % glycerol for crystallization screen on Intelli-Plate 96-3. Crystals were further optimized around the conditions (buffer pH, salt and Poly-ethylene glycol (PEG) concentration) identified from the initial screens, using the hanging-drop vapor diffusion method at 16 °C. Crystals were formed in 0.2 M sodium malonate and 12% PEG 3350 and were flash-frozen in liquid nitrogen after soaking for 5 seconds in mother liquor containing 25% glycerol (Figure 5).

X-ray diffraction data were collected on the Southeast Regional Collaborative Access Team (SER-CAT) beamline 22-ID at the Advanced Photon Source (APS) at Argonne National Laboratories at 100 K and processed using HKL-2000 [10]. The structures were determined by molecular replacement using the structure of C808S S810L MR LBD with bound spironolactone (PDB: 2AB2) as an initial search model with program PhaserMR [11]. Repeated rounds of manual refitting and crystallographic refinement were performed using PHENIX (v1.19) and COOT [12]. One round of PDB_REDO server was used to conclude the crystallographic refinement [13]. PyMOL (v1.8.2) was used to perform alignments and generate figures (Schrödinger, LLC) (Figure 6 and 7).

The overall crystal structure of MR LBD complexed with vamorolone is consisted of 12 α-helices (including the activation function helix) and four β-strands and is folded into the classical steroid receptor LBD structure with three-layered α-helical sandwich that fully encloses vamorolone

in the ligand binding pocket (Figure 6). Residues in the ligand binding pocket form extensive hydrogen bonds with vamorolone. Glu776 and Arg817 form a hydrogen bond network with carbonyl O_1 on the A ring of vamorolone. Residue Asn770 hydrogen bonds with the hydroxyl O_4. Residue Thr945 makes two hydrogen bonds with both atoms O_3 and O_4 on the D ring. Residue Met845 hydrogen bonds with the hydroxyl O_2. As a result of the S810L mutation, the hydrophilic serine is replaced by a hydrophobic leucine, which exhibits an additional hydrophobic interaction with vamorolone further stabilizing the ligand binding (Figure 7).

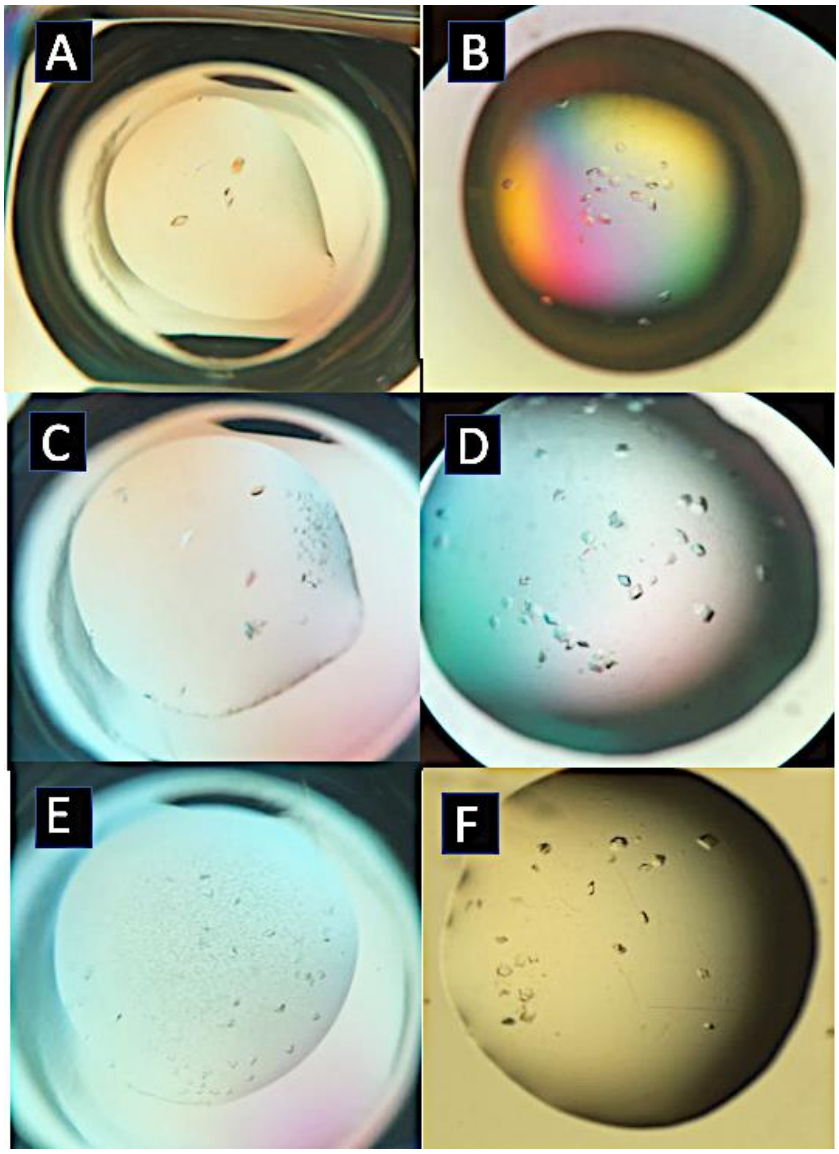

Figure 5. Crystals of MR LBD complexed with ligands. (A, C, E) Crystals formed in the 96-well screening plate in 0.1 M MIB buffer pH 8.0, 0.2 M potassium thiocyanate, and 0.2 M sodium malonate. (B, D, F) Crystals formed in optimized hanging drop vaporization (0.1 M bis tris propane pH 6.5, 0.1 M MES buffer pH 6.5, and 0.2 M sodium malonate pH 6.0).

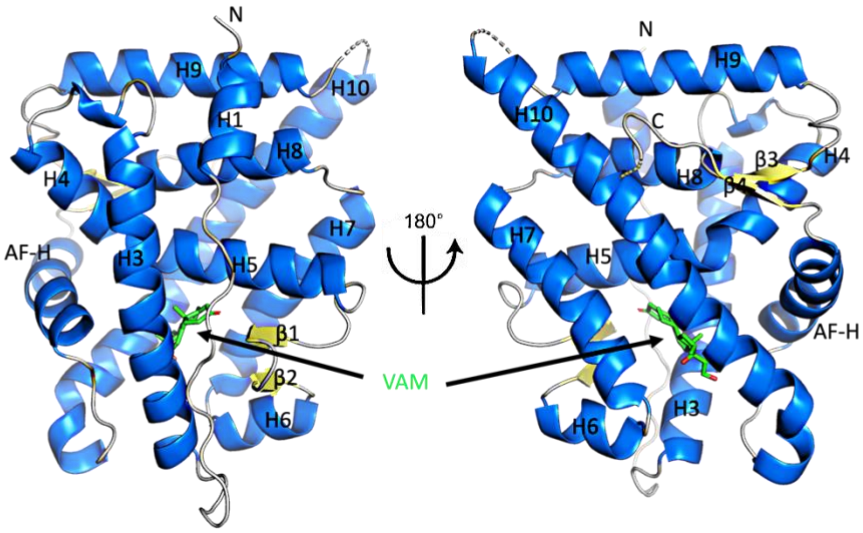

Figure 6. Overall structure of MR LBD complexed with vamorolone. Overall structure of MR LBD C808S S810L-vamorlone complex (green) with α-helices shown in marine blue, β-strands in yellow, and loops in gray.

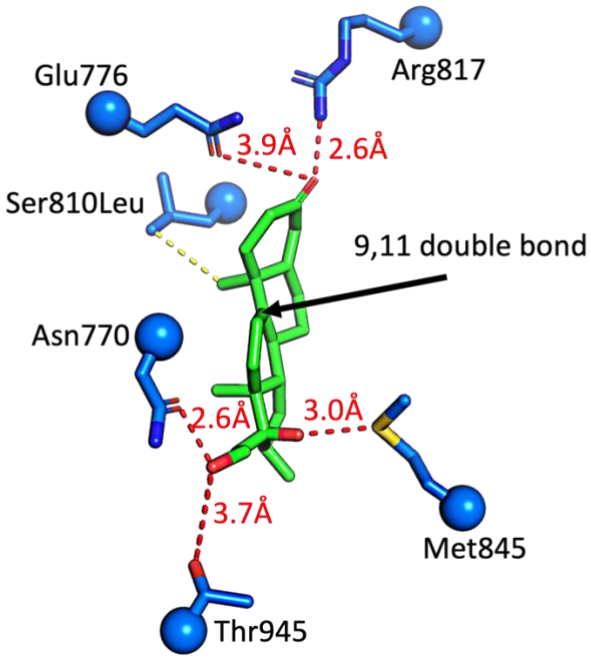

Figure 7. Hydrogen bonds with vamorolone. Extensive hydrogen bonds (red) are formed between MR LBD C808S S810L and vamorolone with bond distance labeled. The S810L mutation establishes an additional hydrophobic interaction between Leu810 and vamorolone.

Chapter 4: Biophysical and biochemical assays with MR LBD C808S

To gain insight into the difference in protein conformational dynamics among MR LBD complexed with agonists and antagonists, thermal shift assay was used to characterize the stability of MR LBD C808S complexed with vamorolone, the product of ligand exchange dialysis, along with MR LBD C808S complexed with hydrocortisone, prednisolone, and dexamethasone. Protein thermal shift assays were performed using the using a StepOne Plus Real Time PCR System. MR LBD C808S in 20 mM HEPES (pH 7.4), 150 mM NaCl, and 5% glycerol at 10 mM was incubated with 50 μM ligands (hydrocortisone, prednisolone, dexamethasone, and vamorolone) and Sypro® Orange at a final 1:1000 dilution. All measurements included a negative control containing no protein. The incubation was started at 25 °C for 2 min, followed by temperature increase with a ramp rate of 1 °C/min to 80 °C. Three technical replicates and three biologic replicates were conducted. The data were normalized and fit to a two-state model with a single transition between native and denatured protein using Boltzmann sigmoidal curve.

The measured T_m of MR LBD C808S-vamorolone complex was lower (~40°C) than the average T_m (~46°C) of the other three MR-ligand complexes (MR-hydrocortisone, MR-dexamethasone, and MR-prednisolone), suggesting MR LBD is less stable in the presence of vamorolone and that the ligand exchange dialysis successfully switched out dexamethasone with vamorolone (Figure 8).

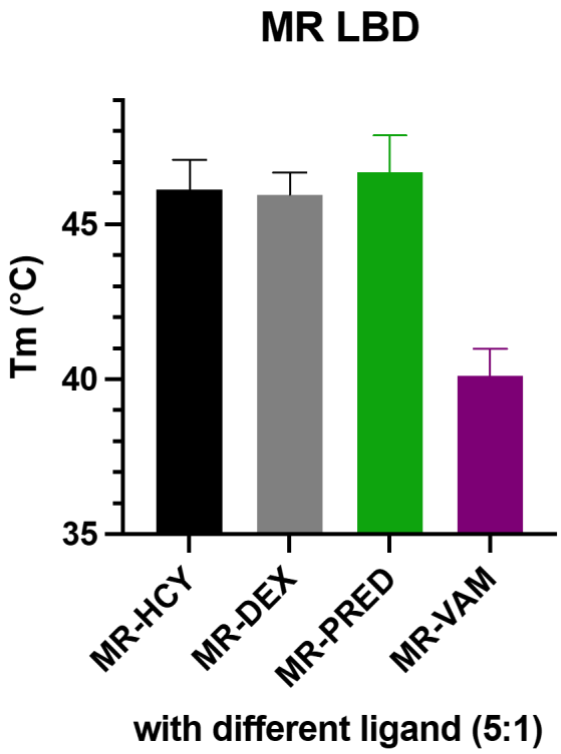

Figure 8. MR LBD C808S thermal shift assay. MR-hydrocortisone (HCY), MR-dexamethasone (DEX), MR-prednisolone (PRED) were directly from protein purification while MR-vamorolone (VAM) was a product of ligand exchange dialysis. The T_m of MR-vamorolone complex was lower than the other three MR complexes, suggesting MR LBD is less stable in the presence of vamorolone and that the ligand exchange dialysis successfully switched out dexamethasone with vamorolone.

To determine the mechanism of action of vamorolone binding to MR LBD C808S, fluorescence-labeled coregulator peptides (PGC1α, NCoR, and SHP) were used at a final concentration of 50 nM in the assay buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, and 5% glycerol. Increasing concentration of MR LBD C808S complexed with vamorolone was added into labeled peptides with fluorescence polarization signal monitored using a Neo plate-reader (BioTek Instruments, Winooski, VT) at an excitation and emission wavelength of 485 and 528 nm,

respectively. Three technical replicates and three biological replicates were conducted. Binding data were fit with a one-site specific binding curve [Y = Bmax * X / (Kd + X)] in GraphPad Prism.

The coregulator binding assay revealed that MR LBD-VAM has a higher affinity to coactivators (PGC1α and SHP) compared to the corepressor (NCoR), suggesting that vamorolone acts as a passive antagonist when it binds to MR LBD (Figure 9). As opposed to active antagonists that permit the LBD to recruit transcriptional corepressors, passive antagonist refers to the stabilization of nonproductive conformations of key residues in the ligand-binding pocket, thereby disfavoring the activated conformation and lead to the stabilization of inactive conformation. [14].

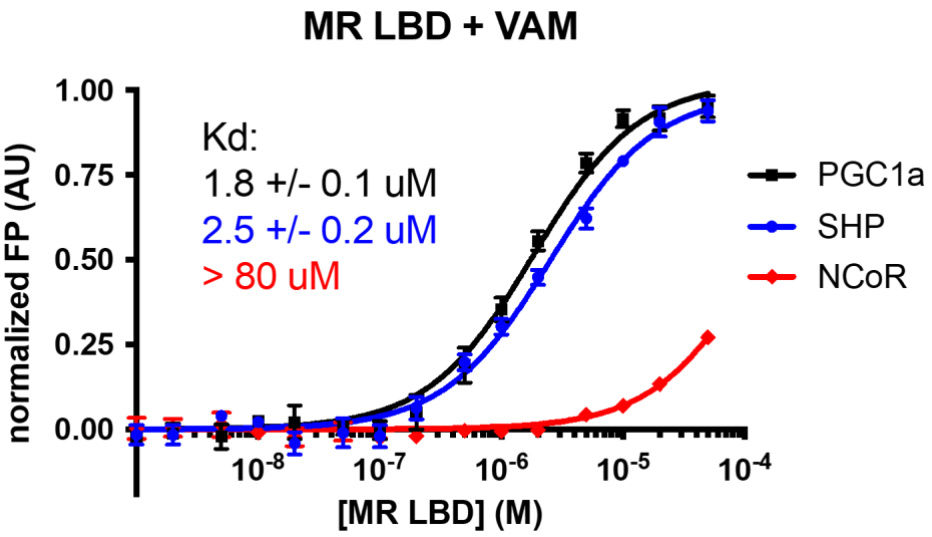

Figure 9. MR LBD C808S complexed with vamorolone peptide coregulator binding assay. In the presence of vamorolone, MR LBD has a lower affinity to corepressor (NCoR) than coactivators (PGC1α and SHP), suggesting that vamorolone acts as a passive antagonist.

Chapter 5: Discussion and future directions

MR is the most common drug target for numerous cardiovascular diseases ranging from chronic coronary artery disease to severe DMD related cardiomyopathy such as end stage heart failure [15]. Chronic treatment of these diseases with MR antagonists is effective, yet the associated side effects such as hyperkalemia and acute deterioration of renal function can create additional physiological burdens for the patients, especially boys already suffering from DMD [16]. Vamorolone is not only a first-in-class steroidal anti-inflammatory drug but also an effective MR antagonist targeting DMD and its related cardiomyopathy with little or no side effects [17]. Animal studies have shown that vamorolone acts as an equally effective MR antagonist as spironolactone and eplerenone, standard of care drugs for hypertension and heart failure [7]. Vamorolone treatment has shown normal bone growth and muscle strength maintenance without evidence of hyperkalemia or cross interaction with AR, side effects of spironolactone and eplerenone [6, 18].

Vamorolone is also known to target glucocorticoid receptor (GR), the closest paralog of MR, for the treatment of chronic and dysregulated inflammation found in a variety of diseases such as asthma, arthritis, atherosclerosis, neurodegeneration, and DMD-induced muscular dystrophy [19]. Vamorolone lacks a C11 -OH group, which establishes a crucial hydrogen bond interaction with human GR2 N564 that stabilizes the ligand-dependent transcription factor binding [5]. The MR LBD N770 residue is analogous to hGR N564, indicating that the lack of specific hydrogen bond interaction between N770 and C11 -OH in the MR LBD ligand binding pocket destabilizes vamorolone binding. In addition, the lack of -OH group at C11 is shared among other MR antagonists such as spironolactone and progesterone. This could be a possible explanation for the antagonistic effect that vamorolone exhibits through its interaction with MR LBD.

To understand how vamorolone acts as an antagonist to MR LBD, the MR-vamorolone structure was determined and the hydrogen bond network in the binding pocket was identified. The additional hydrophobic interaction from residue L810 showed how the S810L mutation provides stability to ligand binding pocket by replacing the hydrophilic S810 residue. Thermal shift assays revealed the lower stability of MR LBD C808S-vamorolone complex relative to the other MR LBD C808S-ligand complexes, verifying the destabilizing effect of vamorolone. Furthermore, fluorescence polarization coregulator binding assays revealed that vamorolone may potentially act as a passive antagonist. This study obtained, to our knowledge, the first crystal structure of MR LBD complexed with vamorolone and provided insight to potential mechanisms by which vamorolone might antagonize MR LBD.

Spironolactone and eplerenone, known passive antagonists, bind to and prevent MR LBD from achieving a stable activated conformation, interfering with recruitment of transcriptional coregulators [14]. To further elucidate the antagonistic mechanism of vamorolone, stability could be determined via thermal shift assay and compared between MR LBD complexed with vamorolone and MR LBD complexed with spironolactone and eplerenone to identify any similarities in the overall protein conformational dynamics.

Moreover, local conformational dynamics, particularly near the coregulator binding site, contributes to the response of nuclear receptors [20]. To gain insight to how vamorolone affects MR LBD's local conformational dynamics in comparison to known antagonists, solution hydrogen deuterium exchange-mass spectrometry (HDX-MS) could be utilized. Regions where hydrogens are replaced more readily with deuterium show greater conformational flexibility. HDX-MS can reveal local conformational dynamics of MR LBD that cannot be detected through X-ray crystallography.

The types of coregulators recruited by MR ultimately decide its transcription activity. Binding MR as passive antagonists, spironolactone and eplerenone would facilitate MR to exhibit a relatively lower affinity to corepressors compared to the relatively higher affinity induced by active antagonist binding. By exposing MR complexed with vamorolone along with MR complexed with spironolactone and eplerenone to a large amount of distinct peptide coregulators, their coregulator association pattern can be compared. Therefore, using a high-throughput format to profile binding patterns of coregulators with MR complexed with vamorolone and other ligands in the future would help identify the antagonistic mechanism of vamorolone.

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