Discovery and Development of a Novel Drug for the Treatment of Hypertrophic Cardiomyopathy

by Jonathan Glaser

Summary

Hypertrophic cardiomyopathy (HCM) is characterized by a growth in the interventricular septum in the heart, therefore leading to an obstruction of the left ventricular outflow tract. Although beta blockers, calcium channel blockers, and other antiarrhythmic drugs are useful in temporarily relieving HCM patients of various symptoms associated with the disease, it seems that there is no treatment that has been shown to prevent or reverse the disease entirely. Thus, there is a medical need for a drug to be developed that targets the underlying mechanisms responsible for the phenotype of HCM.

The ubiquitin proteasome system (UPS) is important in that it is necessary for regulating the synthesis and degradation of proteins in many organs, including the heart. In recent years, it has become increasingly recognized that UPS function plays an important role in the pathogenesis of various cardiac diseases, including HCM. A study conducted by Liu et al. found that the deubiquitinating enzyme USP14 is responsible for positively regulating cardiac hypertrophy (17). As a result, inhibition of USP14 would make for a desirable pharmaceutical target. While Liu et al. have demonstrated that the compound IU1 is capable of inhibiting USP14 for *in vitro* and *in vivo* animal models, a drug has not yet been developed that produces similar results in humans. Development of NCE5 built upon

the structure of IU1 in order to maximize binding with human USP14, and minimize binding with toxic targets such as CYP, hERG, AngII, and RPN11.

To test for *in vivo* efficacy of NCE5, the drug was injected at various quantities into DBA/2J mice, which express mutations causing HCM. Key biomarkers included heart weight, left ventricular posterior wall size, as well as blood concentrations of β-MHC, ANP, BNP, and α1-actin. The results from this study confirmed the ability of NCE5 to successfully inhibit USP14 *in vivo*. Furthermore, animal toxicology studies were performed in mice and rhesus monkeys in order to determine the no observed adverse effect level (NOAEL), maximum tolerated dose (MTD), and maximum recommended starting dose (MRSD).

The information obtained from the animal studies provided the basis for the design of the Phase 1 clinical trial. Beginning with the calculated MRSD, dosage was escalated based on a traditional 3+3 design. The frequency and severity of side effects were recorded, and pharmacokinetics were evaluated. Based on these results, three favorable doses were then selected for the Phase 2 trial in order to obtain preliminary data on the effectiveness of NCE5 in treating HCM. The most effective dose was then selected for the Phase 3 trial in order to further confirm the safety and effectiveness of NCE5 in treating HCM. After a successful Phase 3 trial, a New Drug Application was filed with the FDA.

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1. Hypertrophic Cardiomyopathy Symptoms, Treatment, and Epidemiology

Hypertrophic cardiomyopathy is a heart disease in which there is an abnormal increase in cardiac muscle size, resulting in abnormal cardiovascular function. The disease is typically caused by an autosomal dominant missense mutation, in which the nucleotide sequence that codes for sarcomere proteins is flawed by the incorrect replacement of a single nucleotide. This mutation most commonly takes place in the genes that code for the beta myosin heavy chain, but it can also occur in the myosin binding protein C and troponin T. The genetic aberration causes the interventricular septum to protrude into the left ventricle, thereby decreasing the amount of blood that is able to enter that space, and obstructing ventricular outflow. The characteristic stiffening of the heart muscle combined with the low stroke volume can lead to dyspnea, and ultimately diastolic heart failure. Additionally, the growth in the interventricular septum gets in the way of the left ventricular outflow tract during systole, leading to an increase in blood velocity. This pulls the anterior leaflet of the bicuspid valve towards the septum, causing a systolic heart murmur. Furthermore, since cardiac muscles are large, they require more oxygen than normal. A decrease in blood flow can cause the heart to become ischemic, which leads to exertional chest pain and dangerously fast arrhythmias. Other signs of hypertrophic cardiomyopathy include “disruption of the electrical system running through the abnormal heart muscle, lightheadedness, fatigue, fainting (called syncope) and sudden cardiac death” (1).

In order to diagnose the disease, a physician reviews the patient’s medical and family history in combination with a variety of physical examination tests. In particular, the tests are used to evaluate the state of the left ventricular wall, heart valves, and rate of blood flow. Common and reliable methods of diagnosis include echocardiogram (either

transthoracic or transesophageal), electrocardiogram, treadmill stress test, holter monitor, cardiac catheterization, and cardiac MRI. Although a large majority of patients with hypertrophic cardiomyopathy don't exhibit any symptoms, they are advised to avoid intense exercise or other strenuous activities. For symptom relief, a variety of medications can be used in order to relax and slow down the heart so that the heart is able to pump more efficiently. These include beta blockers, calcium channel blockers, and medications that regulate heart rhythm (2). A variety of each of these drugs exist on the market. Consumer Reports outlines the beta and calcium channel blockers that are available on the market, and sorts them in terms of effectiveness on specific heart conditions (Appendix 1 and 2). Almost all are sold under both the generic brand names (3). If medications don't relieve symptoms, surgical procedures such as septal myectomy, septal ablation, or implantation of a cardioverter-defibrillator might be viable options (2).

A paper published by the American Heart Association called "Prevalence of Hypertrophic Cardiomyopathy in a General Population of Young Adults" by Maron et al. details a study conducted on the epidemiology of the disease. Out of 4111 men and women between the ages of 23 and 35, evidence of mild hypertrophic cardiomyopathy was discovered in 7 subjects. From this, it was estimated that the disease is present in approximately 0.2% of the population, and is about 2.9 times more common in men than women, and 2.4 times more common in blacks than whites (5). Another study conducted by Maron et al. focused on the nature of hypertrophic cardiomyopathy patient deaths. The authors discovered that while most of the examined patients who died from sudden death were adolescents and young adults, "such catastrophes were not confined to patients of

these ages and extended to later phases of life" (6). However, deaths related to heart failure and stroke were more consistent in older patients.

2. Comparative Analysis of Current Drugs to Treat Hypertrophic Cardiomyopathy

Beta blockers, calcium channel blockers, and antiarrhythmic drugs are three medications which are most commonly used to treat hypertrophic cardiomyopathy. Beta blockers work by competitively binding to the receptor sites for adrenaline on adrenergic beta receptors (8). Adrenaline raises blood pressure by increasing heart rate, increasing heart muscle contraction, and constricting arteries throughout the body (3). In hypertrophic cardiomyopathy patients, adrenaline can cause severe strain on the circulatory system. This leads to symptoms such as dyspnea, angina, and heart palpitations (1). The use of beta blockers can therefore relieve patients of these symptoms. In their comparison of the beta blockers that are currently available on the market, Consumer Reports highlights specific ones which are most effective in mitigating symptoms associated with hypertrophic cardiomyopathy (3). For example, since the authors assert that no beta blocker has been proven to be more effective than the other in treating high blood pressure and angina, they suggest that it makes most sense to choose the cheapest option (either metoprolol, atenolol, or propranolol). They also specifically recommend using metoprolol in treating heart failure, and support this by highlighting the fact that it's been proven to reduce deaths by approximately 30 percent. Based on the total amount of money made from sales in 2014, metoprolol seems to be the most popular beta blocker for managing these heart symptoms (7).

Calcium channel blockers help with cardiovascular disease by disrupting calcium movement into the muscle cells of blood vessel walls and the heart. As a result, blood flows more easily since blood vessels become relaxed. Calcium channel blockers are also able to reduce heart rate by interfering with the nerve impulse conduction that is responsible for

making the heart contract. Like beta blockers, calcium channel blockers relieve patients of symptoms such as dyspnea, angina, and heart palpitations (4). However, this class of drugs has been shown to be associated with more side effects (9). On the basis of "proof of effectiveness, dosing convenience, and cost," Consumer Reports highly recommends diltiazem and verapamil for treatment of an abnormal heart rate. The authors also state that these two drugs are useful in treating high blood pressure and angina.

Disopyramide and amiodarone are two commonly used antiarrhythmic drugs that operate through different mechanisms. Whereas disopyramide is a class I agent which interferes with sodium channels, amiodarone is a class III agent which interferes with potassium channels (10). Highly recommended by the American Heart Association in its treatment of hypertrophic cardiomyopathy, disopyramide is widely considered the most potent agent available for relieving symptoms associated with the disease (11).

A medical need exists for the development of a new drug

Although beta blockers, calcium channel blockers, and other antiarrhythmic drugs are useful in temporarily relieving hypertrophic cardiomyopathy patients of various symptoms associated with the disease, it seems that there is no treatment that has been shown to prevent or reverse the disease entirely. They do not possess the ability to target the underlying mechanisms that are responsible for the phenotype of hypertrophic cardiomyopathy (12). According to work conducted by Marian at the University of Texas, "cardiac hypertrophy in [hypertrophic cardiomyopathy] is a secondary phenotype due to activation of pro-growth signaling molecules instigated by the functional defects imparted by the mutations and, hence, it is potentially reversible and preventable" (13). Thus, a new

drug that might ultimately prevent or reverse the disease could directly target the pro-growth signaling molecules that are responsible for its pathogenesis.

3. Molecular Target and Medical Hypothesis

The ubiquitin proteasome system (UPS) is important in that it is responsible for regulating the synthesis and degradation of proteins in many organs, including the heart. UPS allows for the body to exist in a constant flux in which misfolded or damaged proteins are removed, so that they can be replaced. It consists of a multilayered protein degradation mechanism which marks proteins for destruction through ubiquitination by 20S proteasome subunits when assembled into the 26S proteasome complex (14). As illustrated in Figure 1, the enzymes E1, E2, and E3 are responsible for activating, conjugating, and ubiquitinating proteins that are specific to E3. In general, proteasome function can be regulated by "altered proteasome composition (i.e. association of the 20S proteolytic core with different regulatory complexes, such as the 19S or 11S) or by post-translational modifications (i.e. – phosphorylation, oxidation) that affect proteasome assembly, stability and activity. Proteasome regulation thus has the potential to provide highly dynamic responses to cellular signals and stresses." (15). Depending on whether or not UPS is activated or inhibited ultimately determines the extent to which muscle can grow in a particular environment.

In recent years, it has become increasingly recognized that UPS function plays an important role in the pathogenesis of various cardiac diseases, including hypertrophic cardiomyopathy (HCM). Studies on the relationship between the disease and UPS suggest that the sarcomere mutant protein expression that causes HCM contributes to proteasome dysfunction (15). This allows for the increase in cardiac muscle growth that is characteristic of HCM. While the specific cause of proteasome dysfunction in hypertrophic cardiomyopathy requires confirmation by further research, it is known that deubiquitinating

enzymes (DUBs) have the ability to inhibit proteasome function (16). A study conducted by Liu et al. verified that "USP14, a 19S proteasome associated DUB, positively regulate[s] cardiac hypertrophy by promoting GSK-3B phosphorylation" (17). Phosphorylation deactivates GSK-3B, an enzyme that negatively regulates cardiac hypertrophy. As a result, inhibition of USP14 would make for a desirable pharmaceutical target. The compound 1-[1-(4-fluorophenyl)-2,5-dimethylpyrrol-3-yl]-2-pyrrolidin-1-ylethanone, also known as IU1, has been shown to act as an inhibitor of USP14 for *in vitro* and *in vivo* animal models. In their study, Liu et al. demonstrated that IU1 was able to successfully diminish myocardial hypertrophy, and reduce the induced GSK-3B phosphorylation (17).

I therefore hypothesize that this inhibition of USP14 could potentially be translated to human patients for treatment of HCM. Since there are not any published studies on the effect that IU1 has in the clinic, it is difficult to predict whether it would have the same inhibitory effect in humans. A comprehensive investigation on the physiological effects that IU1 might have on the heart and the rest of the body is necessary in determining its efficacy as a drug. Additionally, further analysis in the clinic would allow researchers to make modifications to IU1 while examining the extent to which it is successful at reducing hypertrophic cardiomyopathy while also minimizing side effects. The current drugs available on the market only provide temporary relief for symptoms associated with HCM. A drug that targets USP14 has the potential to be more effective in providing treatment for HCM patients because it interferes with the underlying mechanism of the disease.

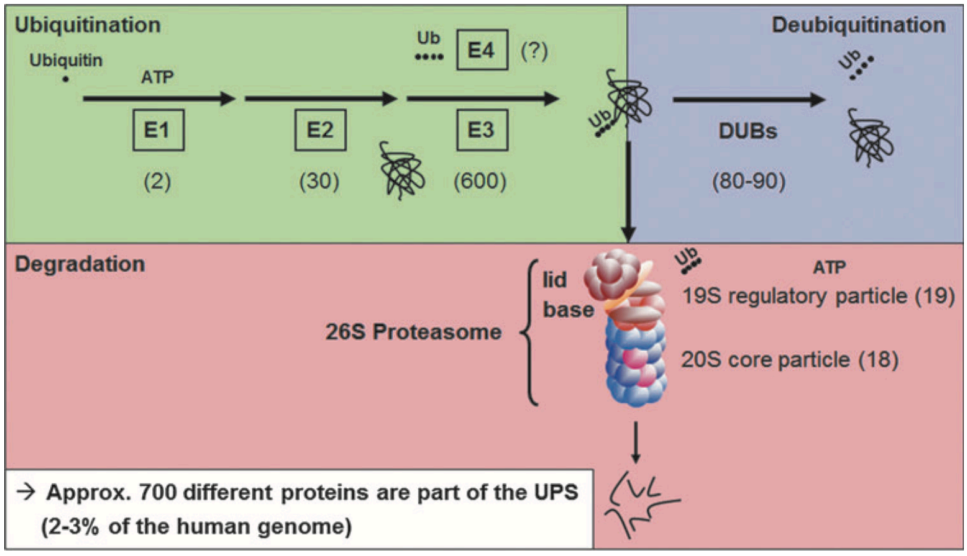
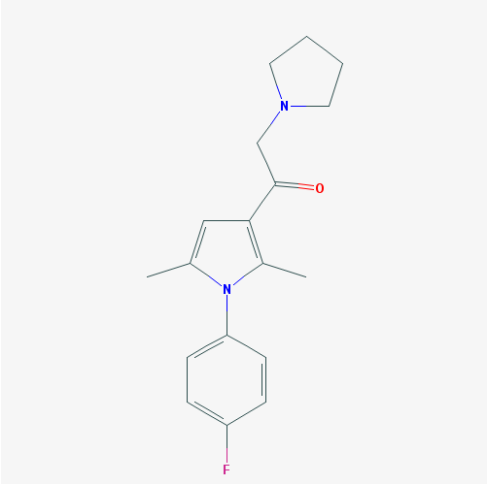


Figure 1: "The UPS is a multilayered machinery - E1 enzymes activate, E2 conjugate, and E3 ligate ubiquitin proteins to target substrate proteins for proteasomal degradation by 26S proteasomes. If E4 enzymes aid in the process of ubiquitination, multiple ubiquitins are transferred in the form of ubiquitin chains (100). Ubiquitination is counteracted by DUBs, but deubiquitination at the 19S regulatory particle is also required for efficient degradation and ubiquitin recycling. The 26S proteasomes are composed of 19S regulatory and 20S core particles. Association of 19S with 20S proteasomes and substrate unfolding via the 19S proteasome are ATP dependent. The numbers in parentheses indicate the number of distinct genes encoding for proteins of the corresponding layer. It should be noted that not all UPS proteins are required simultaneously. A single E3 enzyme or 14 different 20S proteasome subunits are sufficient for ubiquitin ligation or a functional 20S proteasome, respectively. Furthermore, many E3 genes have been identified by homology only. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars" (16).

4. Structures of Lead Compound and Target

IU1 (USP14 Inhibitor)

Molecular Weight: 300.377 g/mol
LogP: 2.69
Hydrogen Bond Donor Count: 0
N + O = 3
IC_{50} = 4–5 μM

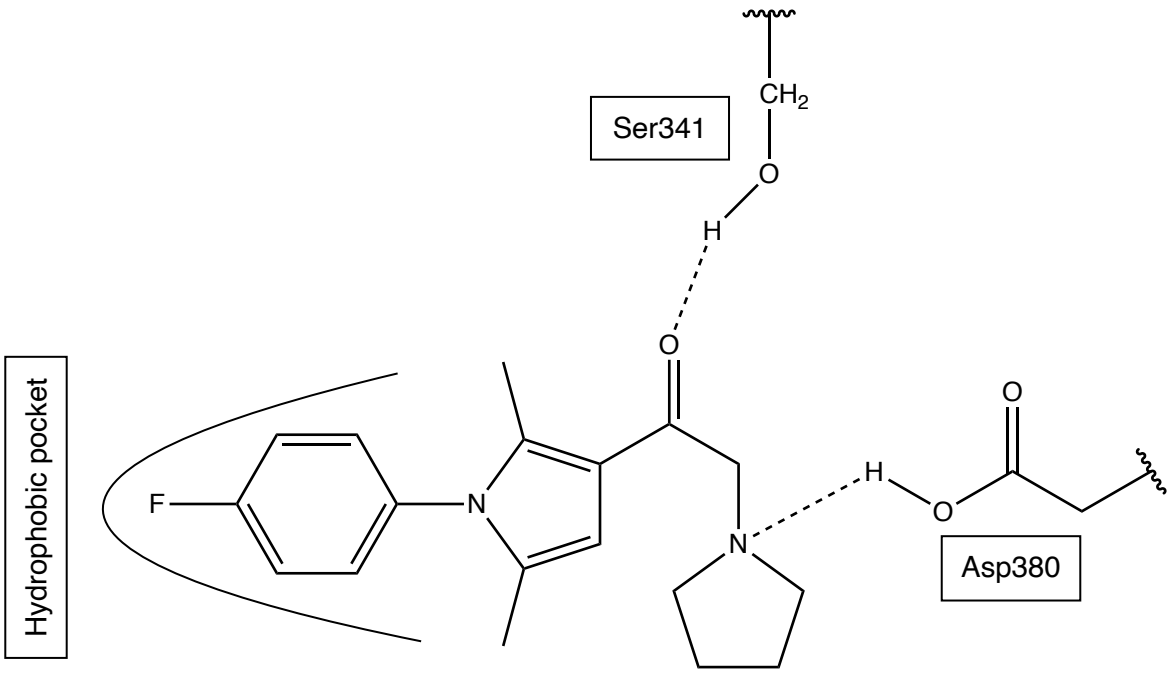


(19)

According to the study conducted by Liu et al., IU1 is able to selectively inhibit USP14, diminish chain ubiquitin chain trimming, and enhance proteasomal degradation. This was proven to be the case both in vitro and in vivo. These findings, combined with the fact that IU1 follows the rule of four, make IU1 a suitable lead for the inhibition of USP14 (17).

The overall structure of my target, human USP14, consists of 494 amino acids, including a 45-kDa catalytic domain, and a 9-kDa domain at its N-terminus. The catalytic domain consists of three main components as shown in Figure 2: the fingers, palm, and thumb. The active site is located between the palm and the thumb (20). The enzyme functions as a cysteine protease, and cleaves the polypeptide bonds that hold ubiquitin together when situated on a protein designated for destruction by the proteasome. When USP14 cleaves ubiquitin, the proteasome is no longer able to recognize and destroy that protein (21). A study conducted by Hu et al. analyzed the structure of various deubiquitinating enzymes, including USP14, when complexed with ubiquitin aldehyde. This

allowed them to deduce the amino acid residues that are responsible for binding with ubiquitin on the active site. When ubiquitin binds to the active site, “Gln2, Lys48, Glu64, and Thr66 make two hydrogen bonds each to…Lys378 and Asp380, Asp305 and Glu308, Ser341 and Tyr379, and Glu345 and Lys391, respectively" (22). Additionally, a hydrophobic interactions occur between Phe4 of ubiquitin and “a hydrophobic pocket formed by Ile332, Leu373, Tyr379, and the aliphatic portion of the side chain of Lys391” (22). It is likely that the mechanism by which IU1 inhibits USP14 involves binding to several of these residues in the active site, thereby causing the protein to undergo a conformational change, and preventing ubiquitin from interacting with USP14. For example, the pyrrolidine group can hydrogen bond with Asp380, and the carbonyl oxygen can hydrogen bond with Ser341. In addition, the fluorophenyl group can be inserted into the hydrophobic pocket.



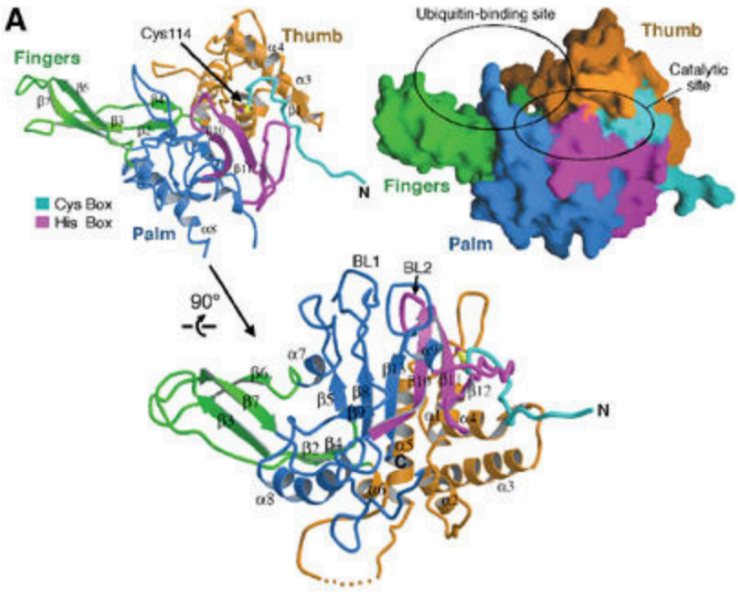


Figure 2: Structure of the catalytic domain of USP14 (20). The catalytic domain of the enzyme consists of the fingers (green), palm (blue), and thumb (purple), and the active site is located between the palm and the thumb.

5. Target and Cell Assays

My initial assay would be designed to directly measure the extent to which my lead is able to inhibit the deubiquitinating enzyme (DUB) USP14. The process of proteasomal protein degradation in the cell involves the initial step of ubiquitinating an unwanted protein as a way of marking it for destruction. DUB's are known to reduce proteasomal degradation by cleaving ubiquitin, so that fewer proteins are eliminated. A common and effective method of measuring DUB activity involves the use of a single ubiquitin attached to the N-terminus of phospholipase A_2 (PLA_2). When the ubiquitin is cleaved by USP14, the free PLA_2 is able to cleave the 2-acyl linkage of a fluorescent 3-*sn*-phosphoglyceride, leading to fluorescence (23). Thus, in the presence of a successful USP14 inhibitor, fewer ubiquitin-PLA_2 complexes are expected to be cleaved, resulting in minimal fluorescence. Fluorescence is measured in relative fluorescent units (RFU). As described by Tian et al., "The increase in fluorescence intensity over time [is] determined on a Perkin Elmer Envision fluorescence plate reader with excitation and emission filters corresponding to the fluorescence resonance energy transfer peptide utilized. Unless stated otherwise, net relative fluorescence units (RFUs) [are] determined by subtracting the blank RFU value (20 nM EKL substrate I or 100 nM EKL substrate II in isopeptidase assay buffer) from each data point" (24). IC_{50} can be determined by plotting RFU against the logarithm of lead concentrations at various intervals (41).

In addition to ubiquitin, there are several ubiquitin-like (UbL) proteins, such as SUMO3 and NEDD8, that have similar function but are involved in other cellular processes. As a result, it might be also be favorable to test my lead's inhibitive ability in the presence of deSUMOylation and deNEDDylation proteins as well (24). This would allow for

comprehensive analysis on the extent to which my lead is specific for USP14. Examining my lead's selectivity for USP14 over other proteases would enable me to modify it accordingly. Thus, I would expand on the proposed assay involving ubiquitin-PLA_2 complexes in order to include the other UbL's. As described in Tian et al.'s study, SUMO3 can be fused with enterokinase light chain (EK_L), and NEDD8 with granzyme B (GZMB). Combining these assays in a multiplex format is highly favorable in that it would allow for the simultaneous detection of protease activities in the presence of a USP14 inhibitor (Figure 3).

A cell-based assay that I would consider involves the stable transfection of H9C2 cardiomyocyte cells with a reporter that consists of a sixteen amino acid CL1 degron fused with green fluorescent protein (GFP) (23). In their study regarding IU1's ability to inhibit USP14, Liu et al. used a neonatal rat cardiomyocytes model, thereby making the similarly derived H9C2 cell line favorable for my own analysis (17). In general, degrons are proteins that have the ability to regulate protein degradation rates. Ubiquitin-dependent degrons, such as CL1, can increase protein degradation by causing more ubiquitination (25). Cells that are tranfected with the CL1-GFP complex (GFP^u) immediately begin to degrade this reporter at the proteasome leading to a decrease in fluorescence. However, in the presence of USP14, an increase in fluorescence should indicate that proteasomal degradation does not occur. I hypothesize that exposing cells to my lead would increase proteasomal degradation by inhibiting USP14. This would be indicated by a decrease in fluorescence (Figure 4). As with my target assay, IC_{50} can be extrapolated from a graph of log[lead] versus RFU.

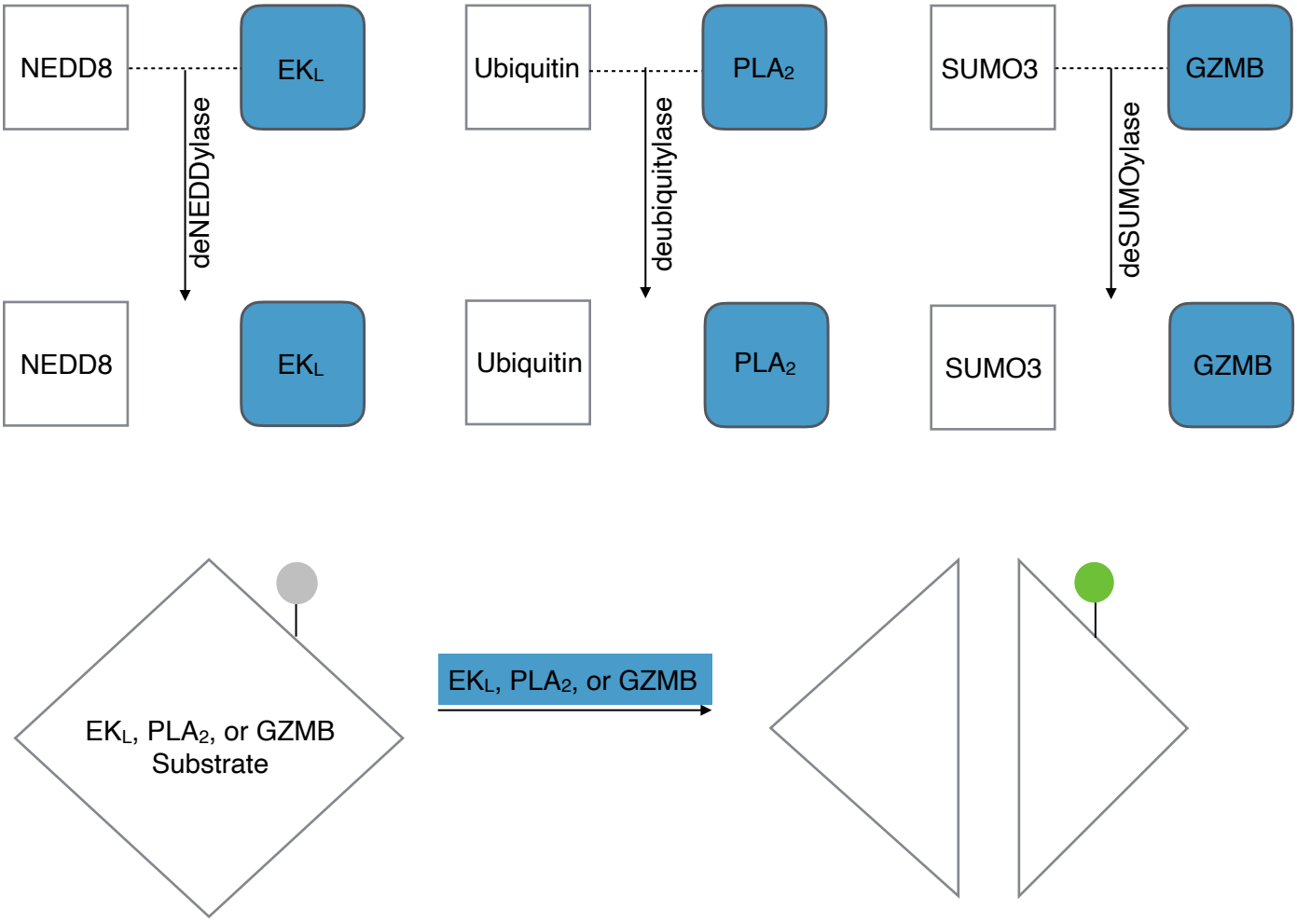


Figure 3: NEDD8, Ubiquitin, and SUMO3 can be fused with either EK$_L$, PLA$_2$, or GZMB. When either NEDD8, ubiquitin, or SUMO3 are cleaved by their respective deubiquitinating enzyme, the free EK$_L$, PLA$_2$, or GZMB are able to activate a fluorescent marker. Successful inhibition of the DUB leads to minimal fluorescence.

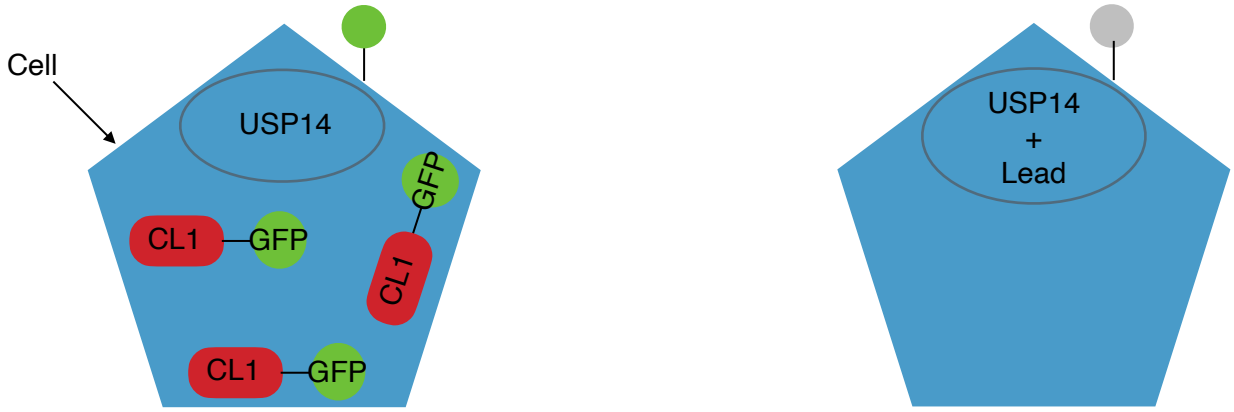
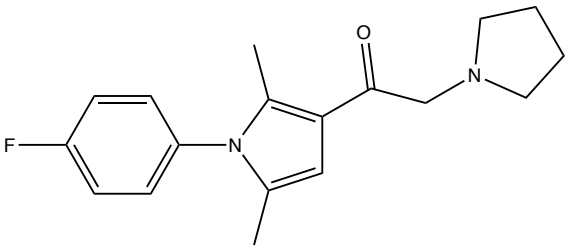
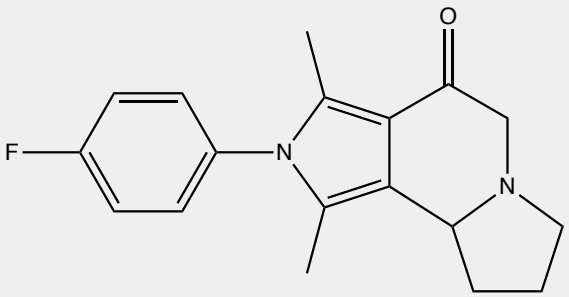
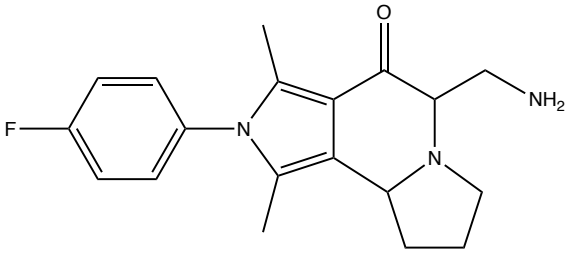
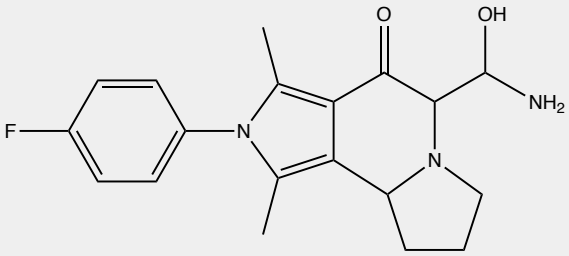
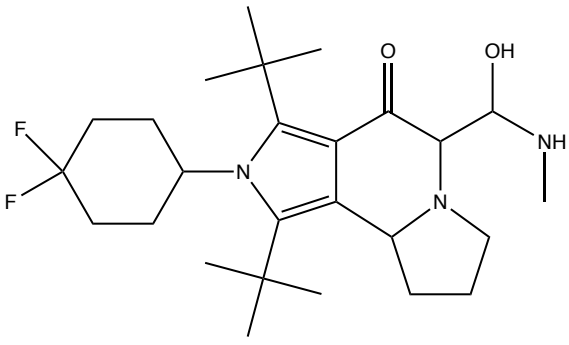


Figure 4: On the left, H9C2 cardiomyocyte cells that overexpress USP14 are tranfected with the CL1-GFP complex. USP14 prevents the CL1-GFP complex from proteasomal degradation, leading to an increase in fluorescence. On the right, similar H9C2 cardiomyocyte cells are transfected with the CL1-GFP complex. Addition of the lead inhibits USP14, thereby allowing for proteasomal degradation to occur. This results in minimal or no fluorescence.

6. From Lead to NCE

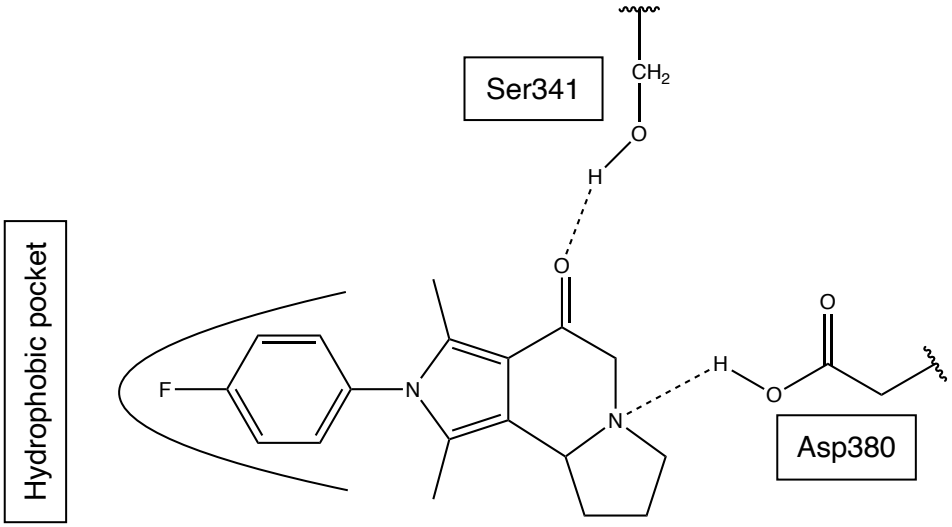
Compound	Structure
NCE 1	
NCE 2	
NCE 3	
NCE 4	
NCE 5	

NCE	MW (g/mol)[a]	LogP[a]	ΔG Compared to NCE 1 (kcal/ mol*K)	IC_{50}
1	300.38	1.28	-	4.7μM [b]
2	298.36	0.65	0.7	1.4 μM
3	327.40	-0.1	2.1	130 nM
4	343.40	-0.17	3.5	12 nM
5	465.63	2.32	4.9	1.2 nM

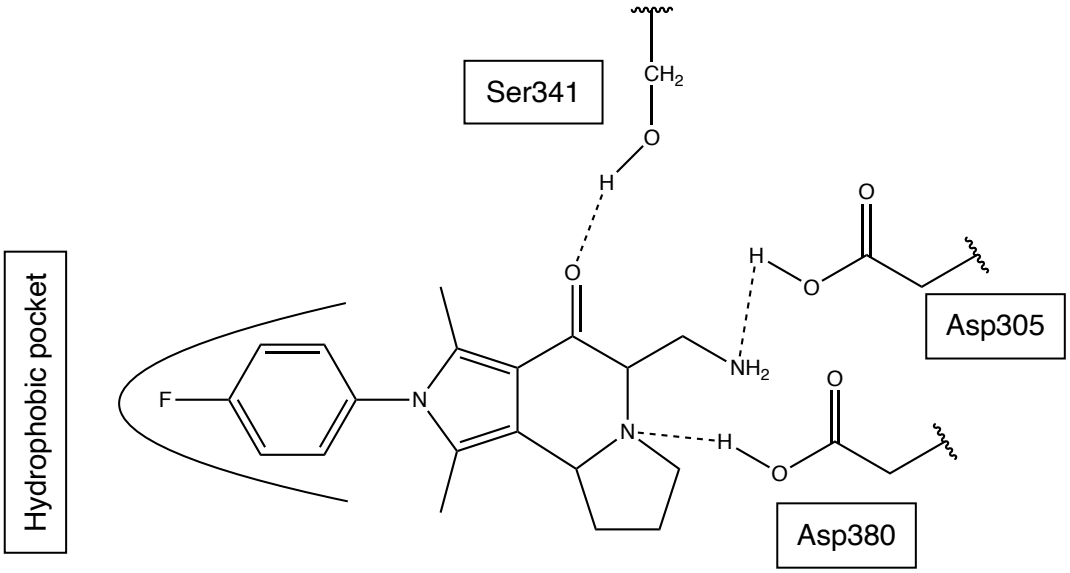
[a] Values obtained from ChemDraw Professional 16.0
[b] Value obtained from Byung-Hoon Lee et al. (18)

NCE2 is a variation on my lead compound in that it increases the rigidity of the pyrrolidine functional group. I predict that this would lead to an increase in affinity for USP14 relative to the lead compound since it would decrease the likelihood that this important functional group would interact with other receptors. Addition of an amino functional group in NCE3 is intended to imitate the interaction that normally occurs between Lys48 on ubiquitin and Asp305 on USP14. This would therefore increase hydrogen bonding in the active site, resulting in a decreased IC_{50}. Affinity for USP14 is further increased with the addition of a hydroxyl group in NCE4, which is intended to resemble ubiquitin's Glu64 interaction with Tyr379 on the active site. Furthermore, I predict that addition of two tertiary butyl groups would increase interaction with the hydrophobic pocket formed by Ile332, Leu373, Tyr379, and the aliphatic portion of the side chain of Lys391 (22).

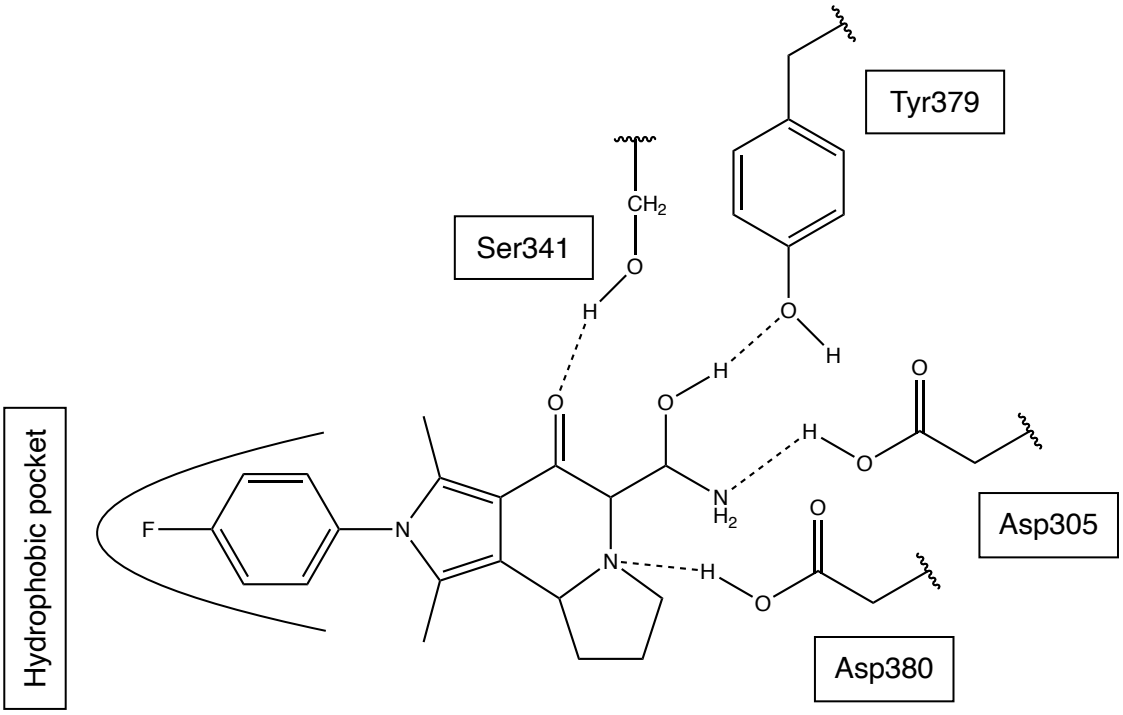
NCE2


Ser341
CH2
O
H
O
Hydrophobic pocket
F
N
N
H
O
O
Asp380

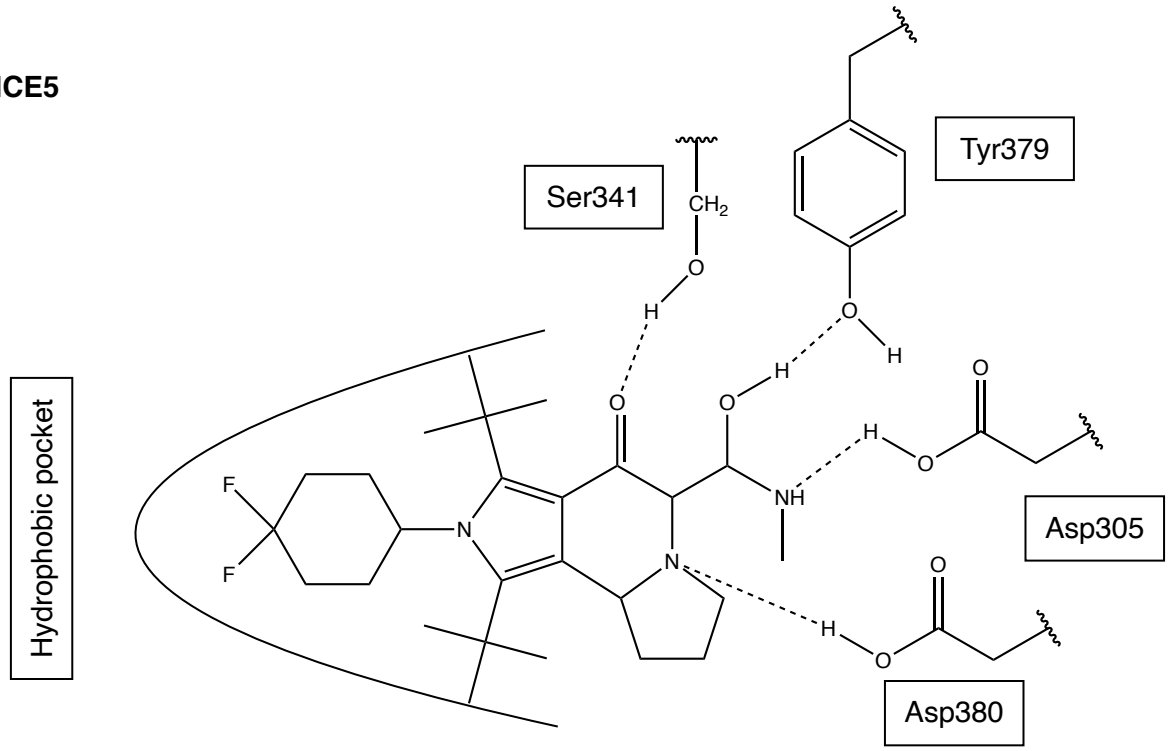
NCE3


Ser341
CH2
O
H
O
Hydrophobic pocket
H
O
O
Asp305
F
N
NH2
N
H
O
O
Asp380

NCE4


Tyr379
Ser341
Hydrophobic pocket
Asp305
Asp380

NCE5


Tyr379
Ser341
Hydrophobic pocket
Asp305
Asp380

7. On-Target and Off-Target Toxicity

My target, USP14, is one of three deubiquinating enzymes (DUBs) associated with the mammalian proteasome. It is known to inhibit proteasomal degradation in eukaryotic cells by removing ubiquitin chains from proteins which were originally targeted for destruction. Thus, increased USP14 activity leads to the accumulation of misfolded or damaged proteins within the cell. It has been shown by Liu et al. that the use of IU1, a USP14 specific inhibitor, can help restore normal proteasomal activity, and reduce the excess cellular protein accumulation that is characteristic of diseases such as hypertrophic cardiomyopathy (17). USP14 plays an important role in neuromuscular junctions where it acts to stabilize ubiquitin pools at synaptic terminals by facilitating proteasomal ubiquitin recycling. Mice deficient of USP14 exhibit severe tremors, extensive muscle wasting, and paralysis (26). These symptoms are the result of synaptic transmission defects in both the peripheral and central nervous systems, as described by Kowalski and Juo (26). At the peripheral neuromuscular junction, USP14-deficient mice have a presynaptic deficiency in neurotransmitter release. Additionally, these mice exhibit defects in synaptic plasticity at central synapses. This was revealed by reduced measurements of paired-false facilitation and posttetanic potentiation in the hippocampus. I therefore predict that the use of a USP14 inhibitor would result in similar toxicities in adult human patients. While inhibition of USP14 can be beneficial in reversing the effects of hypertrophic cardiomyopathy, it's necessary to consider this on-target toxicity that may occur on the function of the peripheral and central nervous system.

When designing a USP14 inhibitor, measures should be taken to avoid inadvertently targeting the isozyme RPN11. Unlike USP14, which acts independently of the proteasome,

RPN11 is a DUB that exists as a subunit on the 19S component of the proteasome. This enzyme exhibits a similar function as USP14, but is only capable of cleaving long ubiquitin chains that are able to reach the proteasome (28). It is important to consider the fact that direct proteasomal inhibition, such as inhibition of RPN11, can lead to an increase in susceptibility to viral infection (29). Basler et al. have shown that mice treated with bortezomib, a proteasome inhibitor, resulted in a reduced expansion of $CD8^+$ T-cells, and an increase in viral titers. As a result, off-target toxicity of a USP14 specific inhibitor might be indicated in the form of viral symptoms. In a study conducted by Perez et al., it was discovered that 8-thioquinoline displays strong inhibition of RPN11 (30). Since addition of another sulfur-containing functional group increased the compound's affinity for RPN11, it would probably be favorable to avoid adding similar components when designing a USP14 inhibitor. A review by Kisselev et al. also reveals that RPN11 can be inhibited by interaction between a ketone and the active site threonine (40).

8. Profile of NCE5

NCE	MW	LogP	Target Assay IC_{50}	Cell Assay IC_{50}	hERG Assay IC_{50}	CYP Assay IC_{50}	AngII Assay IC_{50}	RPN11 (Off-target) IC_{50}
5	465.6	2.3	1.2 nM	120 nM	24 μM	15 μM	30 μM	2 μM

CYPs are a category of enzymes that play an important role in the metabolism of drugs. It is necessary to determine whether or not a particular drug has the ability to inhibit a CYP because the administration of multiple compounds may result in preventing the metabolism of the others. This could potentially lead to toxicity or adverse drug reactions. In order to test for CYP inhibition, I've adapted the method described by Lin et al (31). In their approach, the extent to which the drug being tested is capable of inhibiting the five CYP isoforms is determined. LC/MS is used to measure the signal of each CYP before and after incubation of the drug with human liver microsomes. 10 μM of the drug is typically used since it is the industry standard for initial CYP screening. A reduction in the LC/MS signal following addition of the drug indicates inhibition of CYP activity. If the compound is measured to exhibit an IC_{50} of less than 10 μM, it is considered a potent CYP inhibitor.

hERG is a potassium voltage-gated channel that is necessary for coordinating the depolarization and depolarization of the heart. Inhibition of hERG causes QT interval prolongation, which could result in a fatal ventricular arrhythmia called Torsade de Pointes. The hERG inhibition assay described by Cyprotex, uses a high throughput single cell planar patch clamp approach (32). First, the hERG gene is transfected into Chinese hamster ovary cells. Perforated patch clamping is then used to measure hERG tail current prior to the addition of the compound being tested. The compound is then added at increasing concentrations, and the hERG current is recorded again at each interval. IC_{50} is determined

from a plot of inhibitor concentration against post-compound hERG currents expressed as a percentage of pre-compound currents.

I would also consider performing an additional assay in order to determine angiotensin II (AngII) interference. According to the review by Whitebread et al., possible adverse drug reactions caused by AngII inhibition include increased blood pressure, cell proliferation and migration, and tubular Na^+ resorption. I would test for interaction between my two NCEs and AngII through a scintillation proximity assay. This technique involves a radiolabelled molecule which is in close proximity to a fluomicrosphere bead. If the bead becomes bound to the radiolabelled molecule, light emission is stimulated. A photomultiplier tube can then be used to detect emitted photons (35). AngII is an important enzyme to avoid inhibiting, particularly when the desired target is involved in the cardiovascular system. In the study conducted by Liu et al., it was observed that AngII induced USP14 expression (17). While it does play a role in exacerbating diseases such as hypertrophic cardiomyopathy, it would be more efficient to target enzymes such as USP14 which are more directly involved in the pathogenesis of the disease. Additionally, it might be favorable to avoid targeting AngII in order to avoid the widespread cardiovascular malfunction that AngII inhibition might impart.

The hydrophobic bulk of NCE5, contributed by its two tert-butyl groups, make it least likely to inhibit CYP. In their review, Sevrioukova and Poulos point out that potent inhibitors often act through “direct ligation to the heme iron via nitrogen provided by imidazole, pyridine or primary amino groups” (36). This prevents the oxygen binding and electron flow necessary for the proper metabolism of other drugs. NCE 5 does not contain the lone pair of electrons on its nitrogen that is necessary for this interaction. However, I predict that the

existence of a secondary amine might make the IC_{50} of NCE5 for CYP close to the threshold of 10 μM (approximately 15 μM). According to Price et al., inhibition of hERG activity "could be avoided most effectively with strong hydrogen bonding groups" (37). As the authors discuss in the optimization of maraviroc, the inclusion of a difluorocyclohexyl group is likely to decrease the possibility that NCE 5 would inhibit hERG (37).

9. Animal Model for Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy can most commonly be attributed to mutations in sarcomere protein genes such as Mybpc3 and Myh7 (38). In the study conducted by Zhao et al., it was shown that mice from the DBA/2J (D2) strain which have sequence variants in Mybpc3 and Myh7 demonstrate the key hallmarks of the disease. The investigators also used C57BL/6J (B6) mice as a reference strain. The use of D2 and B6 mice would thus make for a suitable and comprehensive model for my animal study.

Key biomarkers measured by Zhao et al. throughout their study include increased heart weight, left ventricular posterior wall size, "as well as elevated markers for cardiac hypertrophy including β-myosin heavy chain (MHC), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and skeletal muscle alpha actin (α1-actin)" (38). Expression of mRNA for β-MHC, ANP, BNP, and α1-actin are known to increase under conditions of cardiac hypertrophy, and are commonly used as markers for the disease (38). Thus, heart weight and left ventricular posterior wall size would serve as valid surrogate biomarkers for my study. These measurements provide a direct evaluation of NCE5's ability to reverse the most prominent feature of the disease: increased cardiomyocyte mass along the left ventricular wall. In addition, measurements of β-MHC, ANP, BNP, and α1-actin would serve as valid efficacy biomarkers since they're more indirectly indicative of NCE5's success.

While heart weight, left ventricular posterior wall size, and expression of β-MHC, ANP, BNP, and α1-actin are appropriate surrogate and efficacy biomarkers, it is important that this efficacy study also takes into account a safety biomarker. For example, it is possible to test for RPN11 off-target toxicity.

A duration of 42 days was chosen for the treatment length in keeping with other similar hypertrophic cardiomyopathy therapy studies such as the one conducted by Lim et al. (39). Over the course of the 42 days, I would inject each group of 10 mice with either saline, 1.1, 3.3, 10.8, 32.4, or 108 μg/kg. At the end of the study, I would euthanize the mice, and harvest their hearts and blood. I would then compare heart weight, left ventricular posterior wall size, and expression of β-MHC, ANP, BNP, and α1-actin. As mentioned by Zhao et al., left ventricular posterior wall size can be measured using an image quantitative digital analysis system (38). Additionally, I'd measure the expression of β-MHC, ANP, BNP, and α1-actin through the RNA sequencing method discussed by Zhao et al. (38). This involves extracting the RNA from the blood using an RNeasy kit. The extracted RNA can then be amplified to generate sufficient material for sequencing (38). The data obtained from each dose group would be compared to the B6 reference strain in order to determine the effect of my NCE dosage on HCM.

I predict that the IC_{50} of NCE5 will be approximately 1.2 nM. Since its molecular weight is 451.60 g/mol, then I would need 1.2 nmol/kg * 451.60 ng/nmol = 541.92 ng/kg = 0.54 μg/kg. Taking into account the assumption that the bioavailability of my drug is 50%, and 90% of the remaining drug is protein bound, I would need to increase this dose by a factor of 20 (10.8 μg/kg). This implies that a 1.1 μg/kg dose would lead to IC_{10}, and a 108 μg/kg dose would lead to IC_{90}. Additionally, NCE5 was found to have an even volume of distribution and a half-life of 24 hours.

Dose (µg/kg)	Number of Animals	Primary Endpoints / Surrogate Biomarkers (at 42 days)	Efficacy Biomarkers
Saline	10	HW/BW[a] = 6.7 mg/g Left Ventricular Posterior Wall Thickness = 0.96 mM	β-MHC = 72 RPKM[b] α1-Actin = 330 RPKM ANP = 216.3 RPKM BNP = 19 RPKM
IC_{10} = 1.1	10	HW/BW = 6.6 mg/g Left Ventricular Posterior Wall Thickness = 0.92 mM	β-MHC = 66.8 RPKM α1-Actin = 301.5 RPKM ANP = 198.4 RPKM BNP = 18.2 RPKM
IC_{30} = 3.3	10	HW/BW = 6.28 mg/g Left Ventricular Posterior Wall Thickness = 0.87 mM	β-MHC = 56.4 RPKM α1-Actin = 244.5 RPKM ANP = 162.6 RPKM BNP = 16.6 RPKM
IC_{50} = 10.8	10	HW/BW = 6 mg/g Left Ventricular Posterior Wall Thickness = 0.84 mM	β-MHC = 46 RPKM α1-Actin = 187.5 RPKM ANP = 126.9 RPKM BNP = 15 RPKM
IC_{70} = 32.4	10	HW/BW = 5.72 mg/g Left Ventricular Posterior Wall Thickness = 0.80 mM	β-MHC = 35.6 RPKM α1-Actin = 130.5 RPKM ANP = 91.1 RPKM BNP = 13.4 RPKM
IC_{90} = 108	10	HW/BW = 5.4 mg/g Left Ventricular Posterior Wall Thickness = 0.75 mM	β-MHC = 25.2 RPKM α1-Actin = 73.5 RPKM ANP = 55.3 RPKM BNP = 11.8 RPKM

[a]HW/BW = Heart weight/body weight

[b]RPKM = Reads Per Kilobase of transcript per Million mapped reads

In their study, Zhao et al. compare the heart weight, cardiomyocyte diameter, and expression of cardiac hypertrophy markers of D2 and B6 mice (38). The data that the authors obtained for each parameter allowed me to calculate my endpoints for each dose. For example, the average HW/BW for D2 and B6 mice were 5.3 mg/g and 6.7 mg/g, respectively. An IC_{10} dose leads to a 10% decrease in the difference between the HW/BW of D2 and B6 mice (6.7 mg/g - 5.3 mg/g)*0.9+5.3 mg/g = 6.6 mg/g.

10. Animal Toxicology Study

Mouse

Dose (μg/kg)	Number of Mice	Adverse Effects	$CD8^+$ T Cell Count (cells/μL)
1.1	10	None	10
3.3	10	None	10
10.8	10	None	10
32.4	10	None	10
108	10	Average of 20% weight loss (3/10 mice) Prolonged exhaustion (3/10 mice) Mortality (7/10 mice)	4

[a]Weight of average DBA/2J mouse = 0.029 kg
[c]Blood volume of average mouse = 79 mL/kg
*Study conducted for 6 months

Safety endpoints: $CD8^+$ T cell count, myocardial infarction, liver failure, mortality

NOAEL = 32.4 μg/kg

HED = 32.4 x (3/37) = 2.63 μg/kg

MTD = 108 μg/kg

MRSD = 0.1 x 2.63 μg/kg = 0.263 μg/kg

Rhesus Monkey

Dose (μg/kg)	Number of Monkeys	Adverse Effects	$CD8^+$ T Cell Count (cells/μL)
1.1	4	None	30
3.3	4	None	30
10.8	4	None	30
32.4	4	None	30
108	4	Average of 20% weight loss AND Prolonged exhaustion (3/4 monkeys) Mortality (1/4 monkeys)	17

[b]Weight of average rhesus monkey = 7.7 kg
[c]Blood volume of average rhesus monkey = 54 mL/kg
*Study conducted for 6 months

Safety endpoints: $CD8^+$ T cell count, myocardial infarction, liver failure, mortality

Monkey NOAEL = 32.4 μg/kg

HED = 32.4 x (12/37) = 10.5 μg/kg

MTD = 108 μg/kg

MRSD = 0.1 x 10.5 μg/kg = 1.05 μg/kg

11. Investigational New Drug Application

I. First-In-Human

A. Introductory Statement and General Investigational Plan

In recent years, it has become increasingly recognized that UPS function plays an important role in the pathogenesis of various cardiac diseases, including hypertrophic cardiomyopathy (HCM). Studies on the relationship between the disease and UPS suggest that the sarcomere mutant protein expression that causes HCM contributes to proteasome dysfunction (15). This allows for the increase in cardiac muscle growth that is characteristic of HCM. While the specific cause of proteasome dysfunction in hypertrophic cardiomyopathy requires confirmation by further research, it is known that deubiquitinating enzymes (DUBs) have the ability to inhibit proteasome function (16). A study conducted by Liu et al. verified that "USP14, a 19S proteasome associated DUB, positively regulate[s] cardiac hypertrophy by promoting GSK-3B phosphorylation" (17). Phosphorylation deactivates GSK-3B, an enzyme that negatively regulates cardiac hypertrophy. As a result, inhibition of USP14 would make for a desirable pharmaceutical target. The compound 1-[1-(4-fluorophenyl)-2,5-dimethylpyrrol-3-yl]-2-pyrrolidin-1-ylethanone, also known as IU1, has been shown to act as an inhibitor of USP14 for *in vitro* and *in vivo* animal models. In their study, Liu et al. demonstrated that IU1 was able to successfully diminish myocardial hypertrophy, and reduce the induced GSK-3B phosphorylation (17).

I therefore hypothesize that this inhibition of USP14 could potentially be translated to human patients for treatment of HCM. The goal of my investigation is to develop a NCE that is capable of producing a similar effect in humans. The current drugs available on the market only provide temporary relief for symptoms associated with HCM. A drug that

targets USP14 has the potential to be more effective in providing treatment for HCM patients because it interferes with the underlying mechanism of the disease.

B. Investigator's Brochure

Investigator's brochure was submitted to the FDA.

C. Phase 1 Clinical Protocol

The objective of this study is to develop an NCE that can inhibit USP14 in order to reverse the physiological effects of hypertrophic cardiomyopathy (HCM). It is hypothesized that inhibition of USP14 can restore normal proteasomal function in the heart, thereby eliminating unnecessary proteins in myocardial cells. This is expected to result in reduced left ventricular mass, reduced concentration of biomarkers (β-MHC, α1-Actin, ANP, and BNP), and improvement of the symptoms associated with the disease.

This Phase 1 trial will recruit healthy patient volunteers in order to assess dose safety and pharmacokinetics. By strategically escalating dosage, the goal is to determine the ideal dose range for which the NCE is tolerated with the fewest side effects. As part of the inclusion criteria, patients must must be between 18 and 75 years of age, have a BMI of 20-35, and be willing to avoid consumption of grapefruit, grapefruit juice and Seville oranges. Exclusion criteria include competitive exercisers, current smokers, those consuming > 2 drinks/day, and those with a history of a bleeding disorder, or history of significant cardiac, renal, hepatic, gastrointestinal, pulmonary, neoplastic, biliary or endocrine disorders such as uncontrolled thyroid disease, or uncontrolled hypertension or diabetes.

In this double-blinded study, 27 patients will be assigned to take either tablets containing the NCE or a placebo twice a week for 1 month. Patients will be checked every week over the course of the study. Blood pressure, heart rate, and ECG will be closely monitored. Investigators will also assess respiratory rate, tidal volume, hemoglobin oxygen saturation, motor activity, behavioral changes, coordination, motor reflex responses, and body temperature. In addition, $CD8^+$ T cell count will be monitored in order to determine the extent to which RPN11 inhibition occurs in humans.

The starting dose for this trial will be 15.6 μg (0.263 μg/kg). The dose escalation schedule will follow the traditional 3+3 design in which the first cohort of three patients is treated with the starting dose. If none of the three patients experience a dose-limiting toxicity (DLT), another three will be given the next highest dose. If no points of concern are reached, the dosage will be doubled. Once considerable adverse effects are encountered, the increase in dosage between each cohort will follow Fibonacci increments (i.e. the dose first increases by 67%, 50%, 40%, and 33% thereafter). However, if one patient experiences a dose limiting toxicity (DLT), the next cohort will be treated with the same dose. If 2 or 3 patients experience a DLT, 6 patients will be treated at the previous dose level to determine the MTD. Out of the 27 patients enrolled in the study, only one cohort will be provided with the placebo.

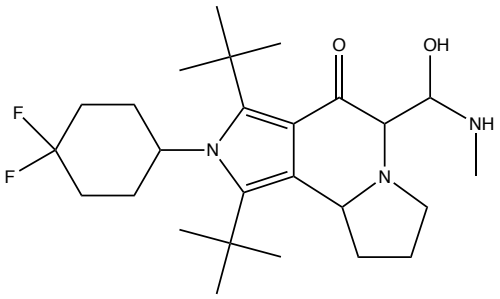
Primary endpoints: Side effects associated with increasing the dose; determine safe dose range and schedule; establish maximum tolerated dose

Secondary endpoints: Assessment of pharmacokinetics (bioavailability, Cmax, and half-life)

Safety endpoint: $CD8^+$ T cell count

D. Chemistry, Manufacturing, and Control Information (CMC)

NCE5:



1,3-di-*tert*-butyl-2-(4,4-difluorocyclohexyl)-5-(hydroxy(methylamino)methyl)-7,8,9,9a-tetrahydro-2*H*-pyrrolo[3,4-*g*]indolizin-4(5*H*)-one

Molecular Weight: 465.32 g/mol
LogP: 2.32
Boiling Point: 1083.69 K
Melting Point: 753.12 K
Gibbs Free Energy: 43.71 kJ/mol

Biological activity: NCE5 inhibits deubiquitination by USP14, demonstrating an IC_{50} value of 1.2 nM

Purity: ≥98% by HPLC

Format: Crystalline solid

NCE5 will be sold in pill form. 100 pills (125 µg each) will be provided in each bottle. NCE5 was verified through NMR and LCMS to be stable for approximately 3 months when placed in packaging at 25°C. The prevalence of impurities was found to be negligent.

E. Pharmacology and Toxicology

NCE5 is designed to inhibit USP14 by imitating the interaction that normally takes place between the enzyme and ubiquitin. The drug is expected to fit in the active site by binding with the Asp305, Asp380, Ser341, and Tyr379 residues that are normally responsible for ubiquitin binding. NCE5 is predicted to bind to USP14 with an IC_{50} of 1.2 nM. It exhibits minimal hERG, CYP, and AngII binding (IC_{50}s of 24, 15, and 30 µM, respectively). In mice, an increase in dosage from IC_{10} to IC_{90} corresponded with a concomitant decrease in heart-weight/body-weight ratio (HW/BW), left ventricular wall size, and concentration of biomarkers (β-MHC, α1-Actin, ANP, and BNP). Since the IC_{50} of NCE5 for RPN11 is approximately 2 µM, toxicity was observed in both mice and rhesus

monkeys when provided with a dosage of 108 µg/kg daily over the course of 42 days. This toxicity was manifested in the form of severe viral symptoms.

In preparation for Phase 1, an animal toxicology study was conducted for NCE5. Mice and rhesus monkeys were provided with various doses twice a week over the course of 42 days. NOAEL, MTD, and MRSD were determined to be 32.4 µg/kg, 108 µg/kg, and 0.263 µg/kg, respectively. The drug's bioavailability was determined to be 50% with an even volume of distribution, and plasma protein binding was determined to be 90%. The half-life of NCE5 was 24 hours.

The initial safety pharmacology core battery studies included an assessment of NCE5's effects on the cardiovascular, respiratory, and central nervous systems at the dose levels proposed to be used for Phase 1. Blood pressure, heart rate, and ECG were closely monitored, and resulted in normal findings. Investigators also assessed respiratory rate, tidal volume, hemoglobin oxygen saturation, motor activity, behavioral changes, coordination, motor reflex responses, and body temperature, and found no concerning results. In addition, genotoxicity tests were conducted in order to ensure that NCE5 is not a possible mutagen. Both the Ames test and the *in vitro* mouse lymphoma tk assay concluded in negative results.

Therapeutic Index = 32.4 µg/kg / 10.8 µg/kg = 3

II. Pre-Phase 2 IND Update

A. Phase 1 Summary

In Phase 1, we recruited 27 healthy volunteers in order to determine dose safety, tolerability, and pharmacokinetics. The subjects were provided with NCE5 at various doses twice a week over the course of 1 month, and the resulting adverse effects were recorded. In addition, $CD8^+$ T cell count was monitored in order to determine the extent to which RPN11 inhibition occurs in humans.

B. Phase 1 Results

Dose[a]	Number of Subjects	Adverse Effects	$CD8^+$ T Cell Count (cells/μL)
Placebo	3	None	700
15.6 μg (0.26 μg/kg)	3	None	700
31.2 μg (0.52 μg/kg)	3	None	700
62.4 μg (1.04 μg/kg)	3	None	700
125 μg (2.08 μg/kg)	3	None	700
250 μg (4.17 μg/kg)	3	None	700
500 μg (8.33 μg/kg)	3	Mild nausea (1/3 subjects)	700
834.6 μg (13.91 μg/kg)	3	Mild nausea, vomiting, dizziness (2/3 subjects)	700
1250 μg (20.86 μg/kg)	3	Severe nausea, vomiting, dizziness, tremor (2/3 subjects)	700

[a]Assumes average human weight of 60 kg

Beginning with the starting dosage of 15.6 µg, additional cohorts received double the previous dosage. Once it was discovered that a considerable number of patients experienced mild nausea, subsequent doses were increased in Fibonacci increments. The value of the hMTD for NCE5 is 834.6 µg. The drug is well-tolerated between the range of 15.6 - 500 µg, in which there are mild adverse effects, but no dose-limiting toxicities. In addition, $CD8^+$ T cell count remained static despite an increase in dosage, therefore showing that RPN11 inhibition does not occur. Doses of 125, 250, and 500 µg are chosen for Phase 2 because they are the highest doses that do not cause significant adverse effects in the majority of subjects.

Bioavailability and plasma protein binding were determined to be 50% and 90%, respectively. The c_{max} for the 125, 250, and 500 µg doses were determined to be 0.012, 0.024, and 0.048 µg/mL respectively. Additionally, the half-life of NCE5 was approximately 24 hours, indicating that it should be provided to patients in Phase 2 on a once-daily basis.

C. Pre-Phase 2 CMC Update

NCE5 was verified through NMR and LCMS to be stable for approximately 12 months when placed in packaging at 25°C. The prevalence of impurities was found to be negligent.

D. Pre-Phase 2 Toxicology Update

In a follow-up animal toxicology study for NCE5, mice and rhesus monkeys were provided with various doses twice a week over the course of 6 months. NOAEL, MTD, and MRSD were determined to be 32.4 µg/kg, 108 µg/kg, and 0.263 µg/kg, respectively.

Additionally, the *in vivo* test for chromosomal damage in rodent hematopoietic cells concluded in negative results. An *in vivo* evaluation of potential for delayed ventricular re-polarization also eliminated the possibility that NCE5 could induce QT prolongation.

E. Investigator's Brochure

Investigator's brochure was updated and submitted to the FDA.

F. Phase 2 Clinical Protocol

This Phase 2 trial will recruit patients diagnosed with HCM in order to obtain preliminary data on the effectiveness of NCE5 in treating the disease. It will also seek to further assess tolerability and the proper dosing regimen. As part of the inclusion criteria, patients must must be between 18 and 75 years of age, have a left ventricular wall thickness greater than or equal to 20 mm as measured in any left ventricular segment by MRI, and show severe symptoms refractory to medical treatment (New York Heart Association functional class III or IV). Exclusion criteria include coronary artery disease, chronic atrial fibrillation, pregnancy or breast-feeding, lactating women, use of immunosuppressives or steroids, renal impairment, inability to estimate left ventricular wall thickness, and/or a condition that prevents a patient from performing an MRI test. Phase I inclusion and exclusion criteria also apply here.

In this double-blinded study, 200 patients will be assigned to take either a placebo or tablets of NCE5 containing 125, 250, or 500 µg once a day throughout the duration of the 6 month study. Use of a placebo is necessary in order to compare the effects of NCE5 to a control group, therefore allowing for a determination of the drug's efficacy. At the beginning

of the trial, patients will undergo MRI, treadmill exercise test, cardiac catheterization, and echocardiogram to determine baseline measurements. $CD8^+$ T cell count and blood concentration levels of β-MHC, α1-Actin, ANP, and BNP will also be recorded. In order to measure the blood concentration expression of the efficacy biomarkers, RNA would be extracted from blood samples using an RNeasy kit, and sequenced for analysis. At the end of the trial, patients will repeat all measurements conducted at the start of the trial in order to determine the effects of the drug on their heart condition. Patients will also be monitored for any other unsafe cardiovascular, respiratory, and nervous system patterns every two weeks as described in the Phase 1 clinical protocol.

Primary endpoint: A reduction in left ventricular wall thickness (as determined by MRI) to approximately 6-10 mm.

Secondary endpoints: Reduction or elimination of dyspnea, angina, and/or heart palpitations during normal physical activity.

Safety endpoints: $CD8^+$ T cell count, myocardial infarction, mortality

Efficacy biomarkers/Exploratory endpoints: concentration of β-MHC, α1-Actin, ANP, and BNP

III. Pre-Phase 3 IND Update

A. Phase 2 Summary

In Phase 2, we recruited 200 hypertrophic cardiomyopathy patients, and provided them with varying doses of NCE5 in order to obtain preliminary data on the drug's effectiveness in treating the disease. The subjects were provided with 125, 200, and 500 µg doses each day over the course of 6 months. Left ventricular wall thickness was determined by MRI. In addition, $CD8^+$ T cell count and blood concentration levels of β-MHC, α1-Actin, ANP, and BNP were measured.

B. Phase 2 Results

Dose	Number of Subjects	Adverse Effects	Left Ventricular Wall Thickness at 6 Months (mm)	Efficacy Biomarkers	$CD8^+$ T Cell Count (cells/µL)
Placebo	50	None	22	β-MHC = 404.8 RPKM α1-Actin = 1650 RPKM ANP = 1120 RPKM BNP = 132 RPKM	700
125 µg (2.08 µg/kg)	50	None	18	β-MHC = 253 RPKM α1-Actin = 1031 RPKM ANP = 698 RPKM BNP = 82.5 RPKM	700
250 µg (4.17 µg/kg)	50	None	15	β-MHC = 196 RPKM α1-Actin = 718 RPKM ANP = 501 RPKM BNP = 73.7 RPKM	700
500 µg (8.33 µg/kg)	50	Mild nausea (33% of subjects)	10	β-MHC = 138.6 RPKM α1-Actin = 404 RPKM ANP = 304 RPKM BNP = 64.9 RPKM	700

The doses of 125, 250, and 500 µg were chosen because they are below the value of the hMTD for NCE5 of 834.6 µg as determined in Phase 1 trials. It was discovered that increasing the dosage led to a decrease in readings of the efficacy biomarkers. This confirms the effectiveness of NCE5 in reversing hypertrophic cardiomyopathy in these patients. Similar to Phase 1, 33% of patients receiving 500 µg doses experienced mild nausea. However, these patients also demonstrated the lowest average readings of β-MHC, α1-Actin, ANP, and BNP, therefore making this dose most favorable for Phase 3 trials. In addition, $CD8^+$ T cell count remained static despite an increase in dosage, therefore showing that RPN11 inhibition does not occur.

C. Pre-Phase 3 CMC Update

NCE5 was verified through NMR and LCMS to be stable for approximately 24 months when placed in packaging at 25°C. The prevalence of impurities was found to be negligent.

D. Pre-Phase 3 Toxicology Update

In a follow-up animal toxicology study for NCE5, mice and rhesus monkeys were provided with various doses twice a week over the course of 24 months. NOAEL, MTD, and MRSD were determined to be 32.4 µg/kg, 108 µg/kg, and 0.263 µg/kg, respectively.

E. Investigator's Brochure

Investigator's brochure was updated and submitted to the FDA.

F. Phase 3 Clinical Protocol

This Phase 3 trial will recruit patients diagnosed with HCM in order to confirm the efficacy and safety of NCE5 in treating the disease. Phase 1 and 2 inclusion and exclusion criteria also apply here.

In this double-blinded study, 1000 patients will be assigned to take either a placebo or 500 μg tablets of NCE5 once a day throughout the duration of the 24 month study. Relevant cardiac measurements taken before and after the study will be compared as described in the Phase 2 clinical protocol. Patients will also be monitored for any other unsafe cardiovascular, respiratory, and nervous system patterns every two weeks as described in the Phase 1 clinical protocol.

Primary endpoint: A reduction in left ventricular wall thickness (as determined by MRI) to approximately 6-10 mm.

Secondary endpoints: Reduction or elimination of dyspnea, angina, and/or heart palpitations during normal physical activity.

Safety endpoints: $CD8^+$ T cell count, myocardial infarction, mortality

III. Post-Phase 3 IND Update

In Phase 3, we recruited 1000 hypertrophic cardiomyopathy patients in order to further confirm NCE5's effectiveness and safety. 250 patients were provided with a placebo and 750 were provided with 500 µg of NCE5 each day over the course of 24 months. Left ventricular wall thickness was determined by MRI. In addition, $CD8^+$ T cell count was measured.

A. Phase 3 Results

Dose	Number of Subjects	Adverse Effects	Left Ventricular Wall Thickness at 24 months (mm)	$CD8^+$ T Cell Count (cells/ µL)
Placebo	250	None	22	700
500 µg (8.33 µg/kg)	750	Mild nausea (33% of subjects)	10	700

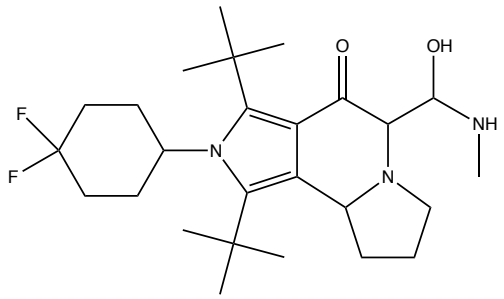
The observed ability for a 500 µg dose of NCE5 to reduce the average left ventricular wall thickness to approximately 10 mm demonstrates the effectiveness of the drug in reversing HCM. Additionally, $CD8^+$ T cell levels remained static indicating that a lack of inhibition with RPN11. Taken together, these results provide strong evidence that NCE5 is suitable for taking to the market with the suggested dose of 500 µg daily.

B. Investigator's Brochure

Investigator's brochure was updated and submitted to the FDA.

12. New Drug Application

NCE5



1,3-di-*tert*-butyl-2-(4,4-difluorocyclohexyl)-5-(hydroxy(methylamino)methyl)-7,8,9,9a-tetrahydro-2*H*-pyrrolo[3,4-*g*]indolizin-4(5*H*)-one

It has become increasingly recognized that UPS function plays an important role in the pathogenesis of various cardiac diseases, including hypertrophic cardiomyopathy (HCM). In particular, studies conducted by investigators such as Liu et al. have demonstrated that overactivity of USP14 plays an important role in the pathogenesis of HCM. NCE5 is designed to inhibit USP14 by imitating the interaction that normally takes place between the enzyme and ubiquitin. The drug is expected to fit in the active site by binding with the Asp305, Asp308, Ser341, and Tyr379 residues that are normally responsible for ubiquitin binding. While there are drugs on the market that are able to temporarily suppress the symptoms of HCM, there is no treatment currently available that eliminates the disease itself. Approval of NCE5 by the FDA would address this current medical need.

13. Appendix

1.

Table 2. Effectiveness of Beta-Blockers Against Specific Heart Conditions				
Generic Name	**Brand Name(s)**	**Treating Angina**	**After a Heart Attack**	**Treating Heart Failure**
Acebutolol	Sectrol	Yes		
Atenolol	Tenormin	Yes	Yes	
Betaxolol	Kerlone			
Bisoprolol	Zebeta			Yes
Carvedilol	Coreg		Yes	Yes
Labetalol	Normodyne, Trandate			
Metoprolol succinate	Toprol XL	Yes		Yes
Metoprolol tartrate	Lopressor	Yes	Yes	
Nadolol	Corgard	Yes		
Penbutolol	Levatol			
Pindolol	Visken			
Propranolol	Inderal	Yes	Yes	
Timolol	Blocadren		Yes	

(1)

2.

Table 2. Summary of Evidence on the CCBs					
Generic Name (Daily Dose – Range)	**Brand Name(s)**	**Proven to Lower Blood Pressure?**	**Proven to Reduce Angina?**	**Proven to Reduce Heart Rate?**	**Approved for Treatment of:**
Amlodipine 5 to 10 mg	Norvasc	Yes	Yes	No	■ HBP[1] ■ Angina
Diltiazem 180 to 240 mg	Cardizem, Cartia XT, Dilacor XR, Taztia, Tiazac	Yes	Yes	Yes	■ HBP[1] ■ Angina ■ Fast irregular heart rhythms
Felodipine 5 to10 mg	Plendil	Yes	Likely[2]	No	■ HBP[1]
Isradipine 5 to 10 mg	DynaCirc	Yes	Likely[2]	No	■ HBP[1]
Nicardipine 60 to 120 mg	Cardene	Yes	Yes	No	■ HBP[1] ■ Angina
Nisoldipine 10 to 40 mg	Sular	Yes	Likely[2]	No	■ HBP[1]
Verapamil 120 to 240 mg	Calan, Covera-HS, Verelan	Yes	Yes	Yes	■ HBP[1] ■ Angina ■ Fast irregular heart rhythms

(2)

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