University of Washington
Department of Bioengineering

Qualifying Examination

Arterial Calcification Due to CD73 Deficiency: Investigating Mechanism and Treatment

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1 Abstract and Specific Aims

Vascular calcification in both the intima and media of vessels is associated with increase risk for cardiac events and mortality. Given the significant clinical impact of arterial calcification, the mechanism and genetic basis behind its clinical presentation has been a subject of intense study.

Recently, the human gene NT5E that encodes CD73, the enzyme responsible for converting extracellular AMP to adenosine, has been implicated as a key component behind the metabolic pathway for inhibiting medial vascular calcification. Individuals with mutations in NT5E result in a disease phenotype of arterial calcification and distal joint calcification (ACDC). Preliminary research has suggested that dysfunctional adenosine signaling and pyrophosphate depletion are responsible for the onset of ACDC. However, the lack of systematic and quantitative studies on all the key components involved in the disease pathway has hampered development of effective treatments.

Here we propose research to systematically investigate the roles of key biomolecular players in ACDC pathobiology and employ quantitative modeling to define the biological mechanisms and enable the rational design of treatment options for this disease. The methodology developed through this research is expected to provide insights to the mechanism of vascular calcification and treatment of related diseases.

1.1 Specific Aim 1: Determine Mechanism of Arterial Calcification Due to CD73-Deficiency

An *in vitro* model consisting of vascular smooth muscle cells, will serve as a platform for running molecular biology experiments for mechanistic exploration and model building. Through review of the current literature, several surface bound enzymes, such as ENPP1 and TNAP, were identified as major components contributing to biological processes leading to mineralization of the arterial wall, and will also be incorporated in the working mechanistic hypothesis. A metabolic profile will be created of the ACDC phenotype through quantifying surface enzyme activity and steady state extracellular concentrations of key metabolites. The role of adenosine signaling in the dysregulation of TNAP, a characteristic of ACDC, will be explored through the interrogation of adenosine receptor classes. The combination of metabolic profiling and cell signaling investigation is expected to elucidate the ACDC pathobiology and enable further research on therapeutic interventions.

1.2 Specific Aim 2: Build Pharmacodynamic Models of different Therapeutic Strategies and Evaluate Synergistic Potential for Combination Therapies

Four different theurapeutic strategies, adenosine receptor agonism, pyrophosphate supplementation, adenosine uptake suppression, and TNAP inhibition, will be characterized and quantified in the form of pharmacodynamic (PD) models. The models will be built from *in vitro* experimental data exploring the effects of calcification reduction related to drug exposure. Furthermore, a combination therapy approach will be investigated for the potential to maximize relief of disease burden while minimizing undesired off-target effects. Drug interaction experimentation and modeling will lead to integrative PD models that can serve as the basis for whole animal dose selection and treatment planning.

1.3 Specific Aim 3: Assess Therapeutic Outcomes in an ACDC Mouse Model Guided by Pharmacokinetic-Pharmacodynamic Modeling

A pharmacokinetic (PK) model of drug uptake and clearance will be built from *in vivo* data. The resulting model will be coupled to the PD model established in Aim 2, forming an integrated PK-PD model. The PK-PD model will guide dose selection and therapy planning for whole animal investigation of treatment. *In vivo* assessment of efficacy in devised treatment plans will be performed. The results are expected to be a basis for developing therapies in human ACDC patients.

2 Exam Question

William Gahl, the NIH sleuth who has identified a number of rare diseases, recently found that deletion of the gene NT5E led to calcification in leg arteries, to arterial insufficiency, and to inability to walk. The gene codes for an ecto-5'-nucleotidase, CD73. Write a proposal to support research to define the mechanisms by which the genetic abnormality causes the disease, and to find out how one can treat the disease (St Hilaire et al. 2011).

3 Background

3.1 Significance

NT5E mutations leading to CD73-deficiency and the ACDC condition is a serious disease burden, impairing mobility and causing great pain for those affected, for which there is no accepted treatment option. While the initial discovery and investigations (St Hilaire et al. 2011; Markello et al. 2011) of this disease has provided valuable insights on some of the biomolecular players involved, highly systematic and quantiative studies have been lacking to fully describe the pathobiological mechanism in detail and allow for rational design of therapeutic plans.

Therefore, the purpose of this research is to address the knowledge gaps in the understanding of ACDC progression as well as provide insight on courses of treatment. Molecular biology experimentation and quantative systems modeling will be the tools used in this approach for building understanding of the disease and gaining confidence in reasonable approaches to combat the disease. Furthermore, the biological knowledge gained and modeling approaches used in this work is expected to be useful for understanding other ectopic calcification diseases.

3.2 Vascular Calcification

Vascular calcification is the process in which hydroxyapatite (HA) mineral deposits are formed in the walls of blood vessels. Arterial calcification is a well-defined risk factor in significantly increased patient mortality. (Shaw et al. 2003; Chiu et al. 2010; Blacher et al. 2001; London 2003) While once thought to be a passive process of deposition due to elevated electrolyte imbalances in the blood, vessel calcification has been discovered to be an active process that is similar to bone formation and remodeling (Boström et al. 1993; C.M. Giachelli et al. 1993; Ding et al. 2006).

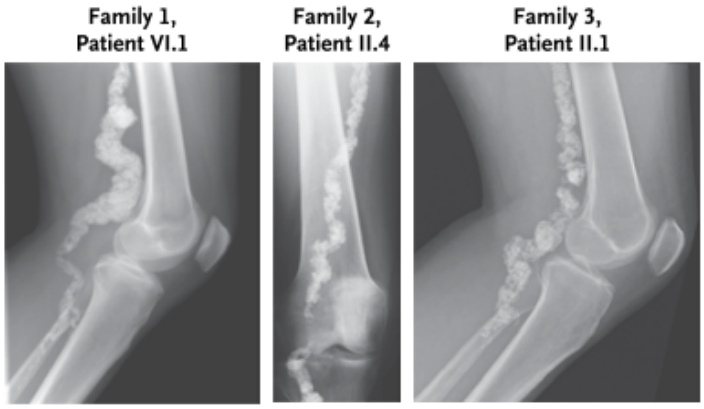

Figure 1: Radiographs of popliteal arteries of individuals from three different families. A similar phenotype is seen from the three distinct NT5E mutation types. ACDC is a painful condition that impairs mobility of the limbs. Symptoms typically begin when patients are in their 20s and progresses with age.

Calcification can occur in either the media (vessel walls) or intima (vessel lumen). Intimal vascular calcification (IVC) is frequently seen in conditions related to atheroschlerosis (S. Nakamura et al. 2009). Medial vascular calcification (MVC), also known as Monckeberg's arteriosclerosis, increases in prevalence for populations with greater age, diabetes mellitus, chronic kidney disease, chronic inflammation, and genetic disorders (Micheletti et al. 2008).

While the subject of arterial calcification is a large and diverse field, the focus of this background will be on the possible biological mechanisms of interest behind the NT5E mutation phenotype. Additional reviews on the pathobiology behind arterial calcification have been published and are referenced here (L. L. Demer and Tintut 2008; Cecilia M. Giachelli 2004).

3.3 Arterial Calcification due to Deficiency of CD73 (ACDC)

Ecto-5'-nucleotidase (CD73) is located on the surface of the plasma membrane and hydrolyzes adenosine monophosphate (AMP) to adenosine (ADO) (Zimmermann 1992). Due to the broad range of AMP and adenosine involvement in biological processes, the CD73 function is implicated in a variety of pathological conditions, including immunodeficiency (Deaglio et al. 2007), inflammation (Colgan et al. 2006), and ectopic calcification (Kathiresan and Srivastava 2012).

Using a genome-wide homozygosity mapping approach, nonsense, missense, and frameshift mutations in the NT5E gene, coding for CD73, have been discovered to cause MVC of the limbs and joints in otherwise healthy individuals. The disease phenontype was exhibited in patients who were homozygous for the defective gene, with the onset of symptoms occuring around age 20 and becoming more severe with age, causing significant pain and discomfort in walking. The study by St Hilaire et al. (2011) demonstrated that cultured fibroblasts from the patients were deficient in the CD73 activity, hydrolyzing AMP into adenosine and inorganic phosphate. It was also shown to be restored in these cells through CD73-deliverying viral vectors.

3.4 Exploring Possible Mechanisms Behind ACDC

In determining the mechanism of ACDC, the literature was reviewed for related enzymes and metabolites that are related to the pathobiology of MVC (Figure 2). The process of bone formation by osteoblasts is closely resembled by the induced osteoblast-like smooth muscle cells that cause MVC (Neven et al. 2011). Furthremore, insight into the pathological mechanism has come from numerous independent genetic

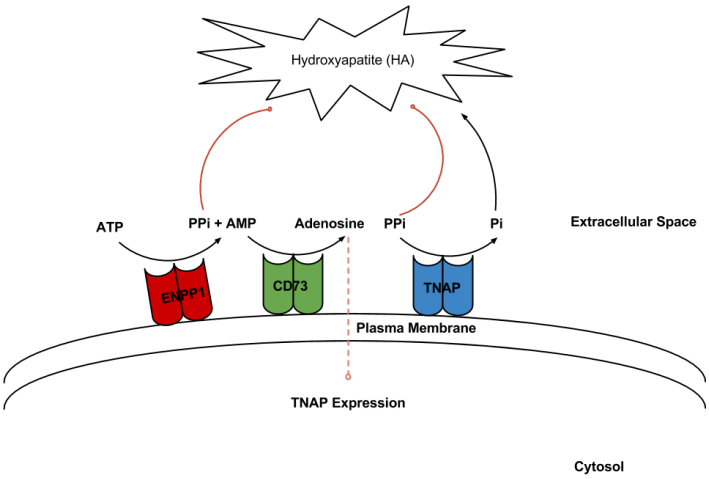

Figure 2: Hypothetical mechanism for calcification regulation by CD73 activity. On the surface of vascular cells, ENPP1 converts ATP to AMP and PP_i, an inhibitor of calcification. Further downstream, CD73 produces adenosine and P_i from AMP. TNAP coverts PP_i to P_i, promoting calcification. Adenosine signaling to the cell is an inhibitor of TNAP expression. Thus, when CD73 activity is deficient, adenosine signaling drops, TNAP inhibition releases, and PP_i levels deplete from TNAP over-activity.

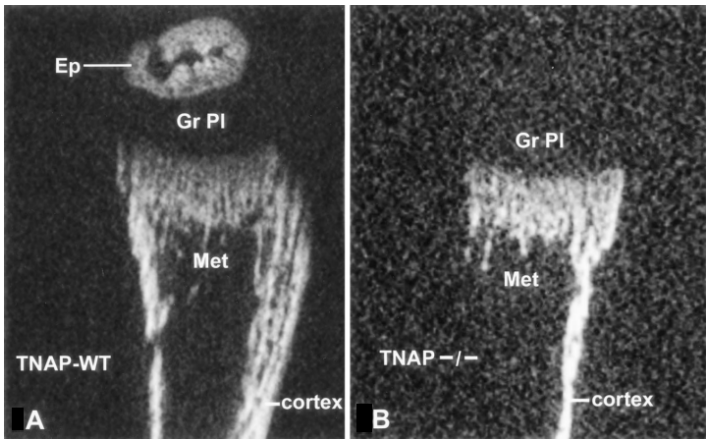

Figure 3: Impaired bone formation in TNAP knockout mice (B) compared to wildtype mice (A). During early development, the lack of TNAP results in excessive extracellular PP_i and inhibits mineralization. Anderson et al. 2004

studies that discovered other biomolecular defects in rare monogenetic disorders (Nitschke and Rutsch 2012).

3.4.1 Tissue-Nonspecific Alkaline Phosphatase (TNAP)

A key enzyme related to calcification, TNAP, was found to be significantly overexpressed on the surface of CD73-deficient cells, which could be reduced to normal levels through adenosine supplementation *in vitro*. TNAP plays a key regulatory role in maintaining proper levels of inorganic pyrophosphate (PP_i) and phosphate (P_i) through the hydrolysis of PP_i into P_i (J. L. Millan 2006). The ratio of PP_i to P_i is crucial in controlling the formation of HA crystals (K. a Lomashvili 2004), one of the primary constituents of bone and product of arterial calcification. PP_i inhibits the deposition of P_i and calcium on to growth sites in HA crystals, however, when PP_i levels fall to abnormal low, ectopic calcification results (Narisawa et al. 2004; Hessle et al. 2002).

The under-expression of TNAP activity also has deleterious effects. In both humans (Henthorn et al. 1992) and mice (Anderson et al. 2004) with defects in TNAP expression, the result is fatality soon after birth. In the TNAP knockout mice, there was normal HA formation containing matrix vesicles that are released by osteoblasts, however, extracellular mineralization was impaired due to elevated PP_i levels (Figure 3).

3.4.2 Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 (ENPP1) and ANK

The tiptoe-walking mouse (*ttw/ttw*) was used for many years as a disease model to study calcification of ligaments. Okawa et al. (1998) showed that the phenotype was due to a mutation in the gene encoding for ENPP1. ENPP1 is a major physiological generator of extracellular PP_i through hydrolyzing ATP. (Kato et al. 2012) The loss in capacity for PP_i to potently inhibit HA formation results in severe MVC, beginning in infancy. ENPP1 knockout mice recapitulated the *ttw/ttw* phenotype. Consequently, defects in ENPP1 for humans were discovered to cause generalized arterial calcification of infancy (GACI). Treatment with bisphosphonates (Ramjan et al. 2009), a PP_i analog, can reverse the course of disease. Interestingly, it has been shown that stopping bisphosphonate supplementation at the age of 2 still allows for healthy development (Edouard et al. 2011), which is perhaps a result of other compensatory mechanisms in maintaining systemic balance of PP_i/P_i.

Mutations in the gene ANKH (ANK in mice) also result in a similar disease phenotype (Nürnberg et al. 2001). Apart from ENPP1, ANKH is the other contributor of PP_i in the extracellular environment by transporting intracellular PP_i.

3.4.3 ATP-binding Cassette Sub-Family C Member 6 (ABCC6) and Role of Adenosine Signaling

St Hilaire et al. (2011) discovered that by supplementing CD73 deficient cells with adenosine, TNAP overexpression can be brought back down to normal levels. Subsequently, the levels of calcification detected *in vitro* were notably reduced. This finding suggests that delivering adenosine, which is the missing product from CD73, may lead to the rescue of the ACDC condition. (Rutsch, Nitschke, and Terkeltaub 2011; Nitschke et al. 2012) Dysfunctional adenosine production was also suggested to be the consequence of ABCC6 mutations (Markello et al. 2011) that cause pseudoxanthoma elasticum (PXE), a disease that shares similar manifestations to ACDC (O. Le Saux et al. 2000). However, later studies (Lefthériotis et al. 2011; Le Saux et al. 2012) showed that ABBC6 does not transport adenosine, and suggests that its role may be more complex. Evidence shows that a yet to be discovered substrate, or substrates, is transported systemically into serum, which prevents ectopic calcification (Le Saux et al. 2006). Interestingly, mutations in ENPP1 or ABCC6 can lead to PXE-like symptoms (Nitschke et al. 2012), which further suggests that the mechanisms behind these diseases are inter-related.

Focusing back on the adenosine-supplementation recovery in CD73-deficient cells, the mechanism in which adenosine apparently suppresses TNAP is not yet known. All adenosine receptors (AR) are G-Protein Coupled Receptors (GPCR) and one of four different pharmacological classes (A_1, A_{2A}, A_{2B}, and A_3), each implicated in a wide range of biological processes in the body (Haskó et al. 2008).

4 Experimental Design and Methods

4.1 Overview

The main focuses of this research plan is to elucidate the mechanism and propose treatment for ACDC using a systematic and quantitative approach (Figure 4). While tremendous work has been already done to discover this disease and provide some preliminary hypotheses on the biological processes involved, detailed studies are still lacking regarding the surface protein expression, extracellular environment, and

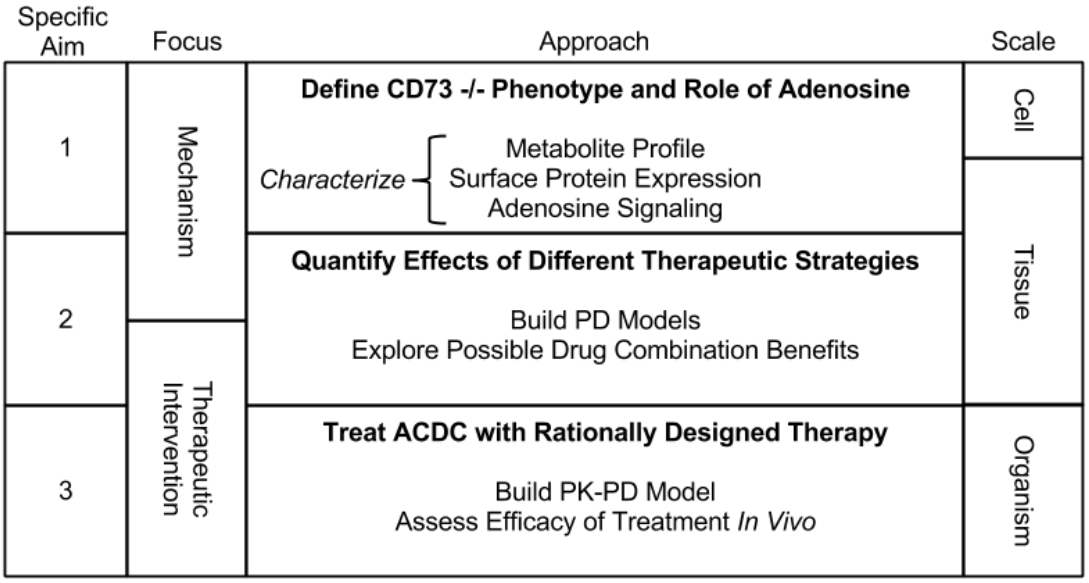

Figure 4: Overview of Specific Aims outlining overall purpose, approach, and focus of each Aim. Scale indicates if research is conducted on the cell, tissue (collections of cells), or organism level.

pathological mechanism of ACDC vascular cells. While the current working hypothesis implicates several surface proteins, such as ENPP1, the expression levels were not measured. Even less known are the extracellular metabolite concentrations. The mechanism of adenosine signaling is also not clear. The current hypothesis suggests that PP_i depletion is the calcification mechanism, however, PP_i levels have not yet been measured in CD73 -/- cells. Aim 1 is expected to provide the missing details to form a more complete picture of ACDC affected cells.

In moving towards treatment development, Aim 2 will build the first catalog of quantiative pharmacodynamic (PD) models using multiple targets for the ACDC pathway. Furthermore, combinatorial PD models will be produced to optimize for treatment potential and highlight any knowledge gaps in the target pathway. Aim 3 proposes to produce the first ACDC animal model and rescue of the ACDC condition through a rationally designed therapy regime devised from pharmacokinetic-pharmacodynamic (PK-PD) modeling.

While to some degree, each of these aims can be performed in parallel, methodology from each Aim will facilitate and strengthen the discoveries of the others. We view it as an advantage that these Aims can be performed partially in parallel, which could lead to faster discovery and course correction of the research plan based on initial findings.

4.2 Specific Aim 1

4.2.1 Strategy and Rationale

While review of the literature has a provided thus far a consistent hypothesis, based on PP_i depletion caused by adenosine signal disruption, Aim 1 will pursue further molecular biology experiments to support or invalidate the working model.

The disease model consisting of the CD73 -/- knockout mouse will be the source of primary VSMCs used for *in vitro* experimentation. This is selection was chosen in order to minimize the inconsistencies between species and different cell types across the experimental plans.

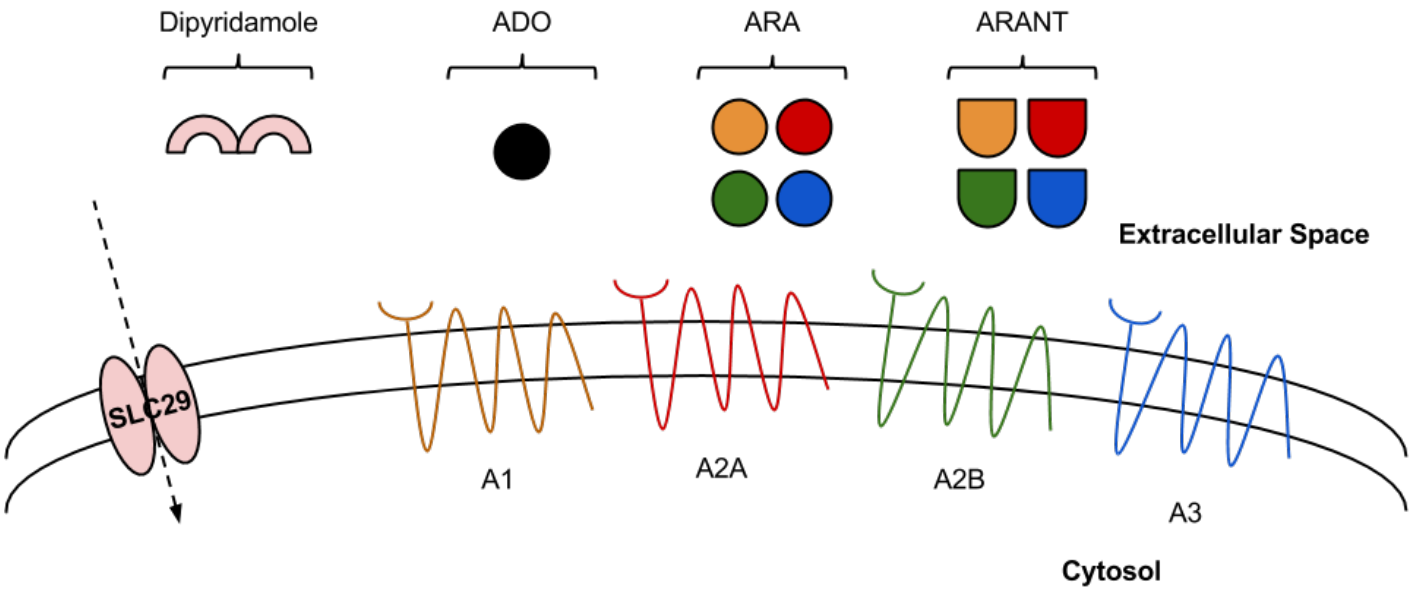

Figure 5: Components in determining ADO signaling mechanism in ACDC. SLC29 is a transport protein that moves extracellular ADO into the cytosol. ADO uptake by SLC29 is inhibited by dipyridamole. A1, A2A, A2B, and A3 are the four major classifications of GPCRs triggered by ADO. Each receptor is triggered by its respective ARA and inhibited by its respective ARANT. These components together allow for experimental probing to pinpoint the exact adenosine signaling mechanism that regulates TNAP expression.

This Aim will first start by characterizing the key metabolic information that is currently missing. While the methodology focused upon in this section are low-throughput and are designed to measure specifically the metabolites of interest, there is a possibility of switching to higher-throughput methods used in metabolomics (Beckonert et al. 2007). This could allow for the profiling of many more metabolites. Similarly, a more proteomic (Sleno and Emili 2008) approach may be used to measure proteins in a higher-throughput manner. However, the underlying strategy for the experimental planning in this proposal is to start with the simplest approaches first and add complexity when needed.

The approach for determining the adenosine (ADO) signaling mechanism, Figure 5, was selected to systematically narrow down the adenosine receptor (AR) of interest. There are four pharmacological classes of (ARs), A_1, A_{2A}, A_{2B}, and A_3. Toward the goal of developing a potential therapeutic through replacement of the missing ADO signal, it is necessary to identify the specific AR which, when activated, inhibits TNAP expression. This also will lead to better understanding of the potential biological repercussions and off-target effects involved. Combinations of AR agonist (ARA) and AR antagonists (ARANT) will pinpoint the receptor class responsible for regulating MVC. Additionally, dipyridamole, a potent inhibitor of ADO uptake by the cell (Hayashi, Maeda, and Shinozuka 1985), will be used to determine if ADO signaling is due to intracellular ADO uptake. Dipyridamole will also be used in Aim 2 as a therapeutic and possible adjuvant therapy.

4.2.2 Experimental Plan

4.2.2.1 Establish *In Vitro* Model of ACDC and Healthy Controls Vascular smooth muscle cells (VSMCs) will be isolated and cultured (Mahabeleshwar, Somanath, and Byzova 2007) from distal arteries of C57BL/6J mice. Medial tissues will be separated from surgically excised vasculature (Jono et al. 2000). Small pieces of tissue (1 to 2 mm^3) will be digested overnight in DMEM supplemented collagenase type I, elastase type III, and soybean trypsin inhibitor at 37° C. The single cell suspensions will then be cultured for several weeks in DMEM supplemented with fetal bovine serum (FBS). The purity of the cultures will be assessed by immunostaining for α-actin and calponin, positive indicators for smooth muscle cells (Liaw et al. 1995). VSMCs will be routinely subcultured in growth medium. For running experiments, VSMCs will be grown to confluency, trypsinized, counted (Invitrogen Automated Cell Counter), and redispersed on to microtiter plates. These cells will serve as the health control VSMCs.

CD73 -/- knockout mice will be purchased from The Jackson Laboratory (http://jaxmice.jax.org/) which contain mutations in the NT5E/CD73 gene coding for the enzyme's catalytic site (Knöfel and Sträter 1999). This same mouse model will be the *in vivo* CD73 -/- model used in Aim 3. VSMCs will be isolated and cultured from this knockout model in a similar procedure as above.

4.2.2.1.1 Expected Results and Proposed Alternatives While VSMC cell cultures have previously been established from *ex vivo* human aorta (Jono et al. 2000), using the similar process in mouse VSMC cell culture may prove difficult due to the drastically reduced amount of "seed" tissue that can be extracted from each specimen to start the colony. Collecting tissue from multiple specimens may be necessary to initiate a sustainable culture. Other cell types may be used for *in vitro* experiments, St Hilaire et al. (2011) used human fibroblasts directly from CD73 -/- patients. However, the selection of mouse VSMCs that come from the same mouse strain is desired because it will likely be the closest extrapolation between cell experiments and whole animal procedures.

4.2.2.2 Quantify Surface Enzyme Expression The relative expression levels of ENPP1, CD73, and TNAP will be determined through Western blot and real-time polymerase chain reaction (qPCR). These measures will create an expression-level profile of healthy and ACDC VSMCs. Cells will be trypsinized, lysed in the presence of protease inhibitor (Roche), centrifuged, and the supernatent protein quantified via the bicinchoninic acid assay (Pierce). A fixed amount of protein will be mixed with SDS protein gel loading solution, run on a polyacrylamide gel, and electrophoresed. After the transfer of proteins, antibodies against ENPP1, CD73, TNAP, and actin (baseline) will be used for quantification.

RNA will be isolated from VSMCs through the use of the RNeasy kit (Qiagen), and complimentary DNA for each of the three enzymes, along with 18S RNA as a qPCR normalizer. Expression levels will be calculated by comparing the cycling threshold compared with the normalizer (PCR Detection System, BioRad).

ENPP1 and TNAP enzyme activity on the cell surface will be assayed using colorimetric substrates, p-nitrophenyl thymidine 5'-monophosphate and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, respectively (Sigma) (Nam et al. 2011). After one hour incubation, the ENPP1 reaction will be stopped by addition of NaOH, and the absorbance through the well measured at 405 nm. The TNAP reaction will be stopped by washing with H_2O and air drying. TNAP activity will be quantified via microscopic densitometry with image analysis software. CD73 activity will be quantified as according to St Hilaire et al. (2011). VSMCs will be washed with 2 mM magnesium chloride, 120 mM sodium

chloride, 5 mM potassium chloride, 10 mM glucose, and 20 mM HEPES. Incubation buffer, consisting of the wash solution supplemented with 2 mM AMP, will be added, and the cells incubated at 37° C for 10 minutes. Inorganic phosphate will be measured with the SensoLyte MG Phosphate Assay Kit (AnaSpec). Inorganic phosphate measurements will be normalized to protein levels.

4.2.2.2.1 Expected Results and Proposed Alternatives The expression levels of TNAP will be expected to be much higher than normal, which was shown in fibroblasts by St Hilaire et al. (2011). Functional CD73 levels should be non-existent in the NT5E mutant cells. The working hypothesis of this proposal is that adenosine signaling is significantly reduced by the lack of CD73 function, which inhibits expression of TNAP. ENPP1 levels have not been reported to change with CD73 mutation, so any differences in expression would be an interesting finding. Different ENPP1 levels may indicate that the excessive extracellular AMP that is left unconsumed by CD73 may play a signaling role as well. While AMP has mostly been reported as merely a precursor to adenosine signaling, orphan GPCRs have been discovered (Inbe et al. 2004) that can respond to both AMP and adenosine.

The enzyme RNA expression levels are expected to correspond with extracellular activity. Discrepancies in these measures may indicate that adenosine signaling has some effect on surface protein recycling or expression. These measurements will be taken again during adenosine agonist addition to further investigate.

In addition to the surface enzymes of primary interest, the levels of proteins and transcription factors related to bone regulation, such as osteopontin, matrix gla protein, Cbfa1/RUNX2, and MSX-2 (Moe and Chen 2008) may also be measured to fully characterize the CD73 -/- phenotype in relation to these other regulator factors. Findings from this work could also lead to additional therapeutic targets.

4.2.2.3 Establish Extracellular Metabolite Profile of ACDC Versus Disease Cells The substrates and products in the hypothesized metabolic pathway involved in ACDC are ATP, AMP, ADO, PPi, and Pi. Steady state metabolite profiles of ACDC and control cell types will provide insight to the pathobiology of this condition. MVC occurs through HA crystal formation, which is dependent on extracellular concentrations of chemical species, especially PP_i.

Cell cultures will be allowed to reach steady state metabolite levels over 24 hours after media change. Sequential readings will be taken to ensure that 24 hours is sufficient time to reach steady state values.

Nucleotides ATP, AMP, and ADO will be measured using high-performance liquid chromatography (HPLC) and enzymatic assays as described in Ciancaglini et al. (2010). PP_i will be measured enzymatically (K. a Lomashvili 2004) by sampling the extracellular media. As in the CD73 activity assay, P_i will be measured using the SensoLyte MG Phosphate Assay Kit (AnaSpec).

4.2.2.3.1 Expected Results and Proposed Alternatives One of the key metabolite levels to be determined is PP_i. The working hypothesis is that ACDC is the result of depressed PP_i due to increased TNAP expression. It has been shown that healthy *ex vivo* rat VSMCs after 3 days of culture generate PP_i endogenously to reach a level of 0.44 μM in medium, without any addition of PP_i (K. a Lomashvili 2004). Depletion of PP_i resulted in spontaneous calcification. In injured aortic cultures, higher levels of pyrophosphate, 5-10 μM was shown to inhibit calcification, although these high levels necessary for calcification inhibition is likely necessary due to increased TNAP expression in injured tissue. In healthy humans, 3.26 +/- 0.17 μM serum levels of PP_i were measured (Koba a Lomashvili, Khawandi, and

O'Neill 2005). Thus, it will be expected that normal VSMCs will equilibrate with the media at around 1 μM PP_i, whereas CD73 -/- VSMCs will be significantly less. Failing to find decreased levels of PP_i could suggest that disrupted regulator of mineralization could be a different HA inhibitor, such as osteopontin (Sapir-Koren and Livshits 2011).

Differences in other metabolite concentrations would suggest other compensatory mechanisms may be upregulated and could direct further experiments to shed light on the discrepancy.

4.2.2.3.2 Combinatorial Probing of Adenosine Receptors with Agonists One of the key goals in this Aim is to elucidate the role of adenosine signaling for regulating TNAP expression. A combinatorial approach of activating a single class of AR, while inhibiting all others, will be used. Four adenosine receptor agonists (ARAs) will be selected (CPA, CGS21680, BAY 60-6583, and IB-MECA) and four adenosine receptor antagonists (ARANTs) (DPCPX, CSC, MRS1754, and MRS1220) will be used to selectively investigate A_1, A_{2A}, A_{2B}, and A_3 effects on TNAP expression, respectively (Haskó et al. 2008). Each ARA will be incubated at its EC95 value on a microtiter plate with three ARANTs onboard, at their IC95 values, for the other AR types. This will be performed for control and CD73 -/- VSMCs. TNAP activity levels will be quantified at time 0, 12 hours, 1 day, 3 days, and 5 days.

4.2.2.3.3 Expected Results and Proposed Alternatives ARs have a wide range of physiological effects when stimulated. Identifying the specific activation site for TNAP repression will greatly aid in the development of a treatment for ACDC. While it is possible that any of the four ARs may be responsible for a signal cascade that regulates TNAP, A_{2B} is probably an unlikely candidate as it a low affinity AR, and is usually only triggered at extremely high physiological levels of adenosine release, such as in the case of cell apoptosis. A_1 and A_3 decrease whereas A_2 increases intracellular cAMP. Tying TNAP expression to cAMP would add it a long list of regulatory functions by cAMP.

Since AR siginaling is an active field of study for many biomedical indications, there is a plethora of ARAs and ARANTs that may be used as alternatives if there are difficulties in eliciting a response measured by TNAP repression. In the event there is no response from ARAs, the next step in the experimental plan is to explore other mechanisms where ADO can affect enzyme expression.

4.2.2.4 Search for Additional Adenosine Signaling Mechanisms If TNAP regulation is controlled by a means other than through the four known ARs, it is possible that either transport of adenosine directly into the cytosol via SLC29 is the mechanism of action or through agonism of a yet to be identified receptor. To explore these possibilities, VSMCs will be incubated with inhibitors of all known ARs at high enough concentrations to reasonably block any ADO binding. Then, cells will be incubated with or without dipyridamole, an inhibitor of ADO uptake, and additional ADO. TNAP expression will be measured at time 0, 12 hours, 1, 3, and 5 days.

4.2.2.4.1 Expected Results and Proposed Alternatives If TNAP suppression is observed in the absence of dipyridamole, and not in its presence, this would suggest that the direct transport of adenosine into the cell is responsible for regulating TNAP. And, that the ultimate fate of CD73 produced adenosine extracellularly is to be transported back into the cell. If TNAP suppression is observed equally withough regard to dipyridamole, it could suggest that another unknown AR is being triggered.

A possibly more exotic result is the lack of any response after blocking the four known AR classes. It could mean that a combination of two or more AR triggers are necessary for TNAP regulation.

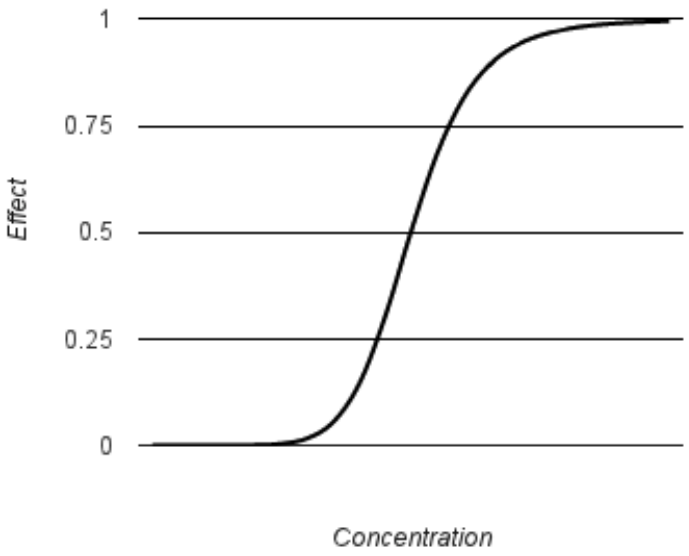

Figure 6: A Hill-type PD curve for modeling dose response from empirical data, based on Equation 1. The parameters that tune the characteristics of this curve are E_{max}, EC_{50}, and n. E_{max} is the concentration that produces half of the maximal effect. E_{max} is the maximal effect for the system. n is the Hill coefficient, which affects the steepness of the curve.

4.3 Specific Aim 2

4.3.1 Strategy and Rationale

The overarching purpose behind this proposed research is to gain enough knowledge on ACDC pathobiology and potential therapies in order to rapidly bring treatment options to patients. This Aim will build from the mechanistic understanding of ACDC obtained from Aim 1 and rationally design potential therapies in a combined experimental and quantitative modeling approach.

Due to the lack of PD data in reversing medial calcification, the task for this Aim is to build PD models from dose-effect experiments using an assortment of strategically selected drug compounds and their use in combination.

While PD models can be built from *in vitro* or *in vivo* experimental data, an *in vitro* approach was selected because of the large number of experimental conditions that need to be tested in order to generate single and combinatorial drug dosing data for PD modeling. Cell-based assays are more amenable to enhancement with high-throughput techniques compared to live animal experiments. Thus, since time needed for data generation is likely to be a bottleneck, PD experiments will be done using the VSMC model built in Aim 1.

PD is the study of the effect a drug has on a biological system, as opposed to PK, which focuses on the effect the body has on the drug. In building a PD model, the general approach will be to design a methodology for deliverying dosages at different concentrations and select a way to measure its effect. One common way to model this relationship between dose and drug effect is using the Hill Equation (Equation 1), where E is the observed drug effect, E_{max} is the maximum observable drug effect, C is the drug concentration, and n is the Hill coefficient (Figure 6).

$$E = \frac{E_{max}^n C^n}{EC_{50} + C^n} \tag{1}$$

Toward the goal of producing treatment options, this study will focus on drugs that are already used for other indications, rather than create an entirely new drug or biologic. Four different pharmacological

strategies will be investigated for ACDC therapy: AR signaling, TNAP inhibition, PP_i supplementation, and adenosine uptake blockage. The ARA identified in Aim 1 will be used to induce the adenosine signaling pathway for the inhibition of TNAP expression. Lansoprazole, an inhibitor of TNAP activity (Delomenède, Buchet, and Mebarek 2009), will be used to bind to the TNAP-PP_i complex and reduce PP_i hydrolysis. PP_i will be supplemented through treatment with bisphosphonates (Fleisch 1998), PP_i analogs that are highly resistant to hydrolysis by TNAP. Adenosine uptake by the cell will be inhibited with dipyridamole (Hayashi, Maeda, and Shinozuka 1985), thus allowing for increased residence time and signaling of extracellular adenosine.

As the case with many complex diseases, such as cancer, often times a one-gene-one-drug target approach is ineffective in perturbing the system enough to get much therapeutic benefit (Fitzgerald et al. 2006). Arterial calcification involves many physiological processes and is characterized by redundancy and homeostasis. Combination therapy may be highly advantageous for 1) lowering the doses of drugs with non-overlapping toxicity and similar efficacy leading to greater safety, 2) sensitize cells to the action of a drug through the use of another drug, and 3) exploiting additive or greater-than-additive effects to achieve higher potency. Thus, PD effects for these four approaches will be studied in combination.

The mechanism of action for these different therapeutic strategies are complementary to each other and additive or *synergistic* (more than additive). Two common models for predicting the additive response of a combination therapy, *Loewe additivity* (Loewe 1953) and *Bliss independence* (Bliss 1956). In Loewe additivity, the relative effect of each compound is added together in equipotent dose ratios. A trivial case of Loewe additivity is when the multiple compounds are actually the same drug. The assumption in Loewe additivity is that the mechanisms of action are mutually exclusive, for example, two ligands that can fit the same receptor, but both can not be bound at the same time. In Bliss independence, the assumption is that the two effects act through independent mechanisms, and thus the combined effect is a union of the two probabilistically independent events. Thus, the relative effects are multiplied. When results are different from either of these two applications, the combined effect is either antagonistic (less than expected) or synergistic (more than expected). Vertical synergy refers to the increased effect given the same relative dosage, and horizontal synergy refers to the decreased dosage necessary to achieve the same effect.

Figure 7 shows a simple simulated example of additivity and synergy, which illustrates the possible results from the proposed combination PD experiments and how they may be analyzed. The simple linear pathway is shown in Figure 7(a,b). Figure 7(c,d) show the effect of two mutually exclusive inhibitors, which is consistent with the Loewe additivity prediction generated from each of the inhibitors acting alone. Figure 7(e,f) shows mutually non-exclusive inhibition, where both inhibitors can bind to the receptor. While Bliss independence might be a reasonable assumption in this case, the result in this case is synergy. Bliss independence treats the double binding as redundant, but the net effect is that enzyme activity is inhibited for longer times due to cooperative suppression by the two inhibitors. Figure 7(g-j) shows the efficacy surfaces from plotting the two dosages. This demonstrates how this synergistic effect can great increase the therapeutic window for efficacy while remaining below toxicity thresholds.

These simulated results illustrates the rationale behind pursuing this combinatorial approach and the methods that will be used to determine the effect of the combination. Furthermore, while the mechanism in the simulated example is explicitly known and our experimental approach is more of a black box, the results from combination experiments can serve to support or refine our current mechanistic understanding.

It is clear that without proper planning, running combination studies can be very labor intensive. Therefore, individual PD experiments will be performed first, and dosages will be evenly sampled from

the dynamic range of the compound, a process resembling the Latin Hypercube method (Marino and Hogue 2008).

4.3.2 Experimental Plan

4.3.2.1 Develop Pharmacodynamic Models of Multiple Drug Targets An *in vitro* system will be used to characterize the PD effects of ARA, lanzoprazole, dipyridamole, and bisphosphonate. Varying concentrations of each drug will be administered on to cell cultures daily. At the end of day 21, cells will be washed with phosphate-buffered saline and fixed with 10% formalin for 10 minutes. After washing with water, calcium phosphate crystals will be stained with alizarin red S and calcification levels will be quantified with using microscopy and image analysis.

The data points of observed calcification levels versus dosage of drug used will be fitted against the parameters found in Equation 1. Since the untreated CD73 -/- cells are expected serve as a high control, data points will be subtracted from the control base level before fitting with nonlinear parameter optimization software (NONMEM).

4.3.2.1.1 Expected Results and Proposed Alternatives The observed drug effects on calcification are expected to form a dose-response curve that will fit a Hill-type equation (Figure 6). The result should be a predictive model of 21-day dosing effects for each of the compounds. If fitting the parameters proves to be a significant challenge, different software packages may be used to perform fitting.

Model fitting could prove difficult if experimental data is far too noisy or unreliable, which would suggest that the experimental protocol should be refined to provide reproducible results that ideally would mimic the onboarding of therapeutic on to VSMCs in live animals. The Latin Hypercube method (Marino and Hogue 2008) will be used to quantify the uncertainty in model predictions. Thus, bounds can be placed on the PD model predictions and experimental reproducibility.

4.3.2.2 Explore Potential Synergistic Effects of Combination Therapy Each of the four therapeutics, ARA, bisphosphonate, dipyridamole, and lansoprazole, will be investigated pairwise for combinatorial PD effects in a manner that is similar to determining the PD for a single compound. The concentrations selected will be evenly sampled from the dynamic range of the single compound PD models.

Drug combination will be evaluated for presence antagonism (negative interaction), additive (no interaction), or synergy (positive interaction) using the *combination index* metric (Equation 2).

$$\text{Combination Index} = \frac{[CI_1]_{X\%}}{[I_1]_{X\%}} + \frac{[CI_2]_{X\%}}{[I_2]_{X\%}} \tag{2}$$

The combination index is the sum where for a given relative effect of $X\%$, I_1 and I_2 are the concentrations needed to individually induce the $X\%$ effect, CI_1 and CI_2 are the combined concentrations required for the same effect. The combination index may be interpreted as less than 1 indicates antagonism, equal to 1 indicates additive, and greater than 1 indicates synergy (Chou and Talalay 1984; Michaud 1996). For the case of Bliss independence, combination index will be determine by the difference between observed effect compared to the multiplied effects from the constituent compounds individually.

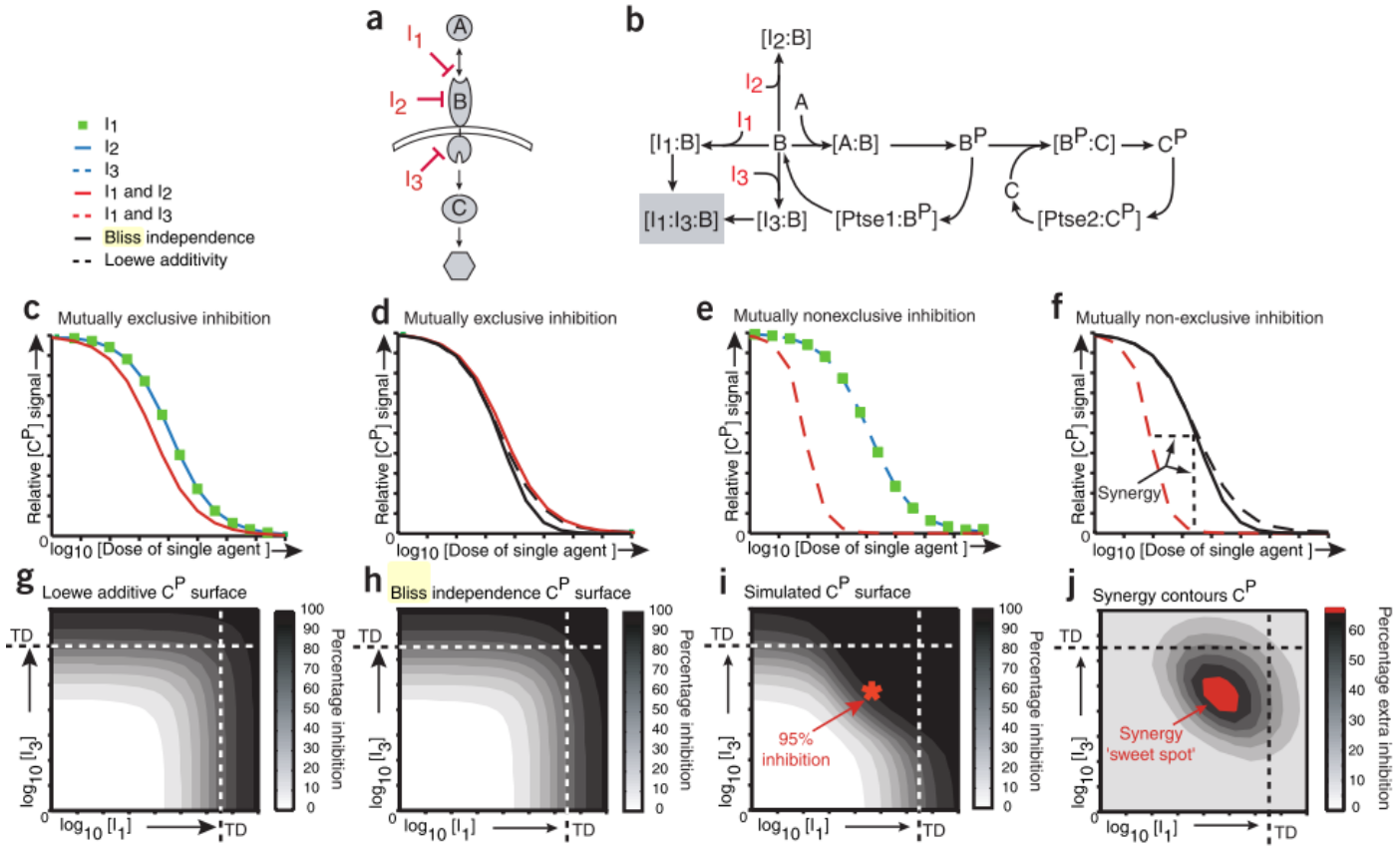

Figure 7: Example of additivity and synergy with dual inhibitors acting on the same pathway. **(a)** Regulatory scheme of a single linear pathway. Inhibitors I_1 and I_2 or I_1 and I_3 act on the same target molecule, receptor B. **(b)** A simplified reaction scheme for **a**. Binding of the second inhibitor either prevents inhibitor I_1 from binding (I_1, mutually exclusive model) or does not affect binding of I_1 (I_3, mutually nonexclusive model). **(c,d)** Simulated dose-response curves, in which C is activated by A in the presence of inhibitor I_1, I_2 or the combination. The mutually exclusive combination follows the Loewe additivity model, confirming the assumption of a similar mode of action. **(e,f)** Simulated dose-response curves, in which C is activated by A in the presence of inhibitor I_1, I_3 or the combination. The mutually nonexclusive combination inhibited activation of C to a greater extent than the expected Bliss independence curve. The enhanced sequestering of B into a long-lasting nonactive triplex [I_1:I_3:B] was responsible for the synergistic effect on activation of C. **(g-j)** Response surfaces for the mutually nonexclusive inhibitors from a checkerboard of 400 dose pairs: Loewe additivity **(g)**, Bliss independence **(h)** and the simulated combination **(i)**. Hypothetical toxic dose (TD) thresholds limit the amount of inhibition the inhibitors can achieve individually. In combination, the inhibitors achieve hypothetical therapeutic efficacy (95% inhibition, marked as *) without reaching the TD. The region of synergistic inhibition was highlighted by subtracting the inhibition in i from the Bliss independence surface, identifying a sweet spot where the extra inhibition exceeded 50% **(j)**. (Fitzgerald et al. 2006)

4.3.2.2.1 Expected Results and Proposed Alternatives While it is difficult to predict when synergy may arise due to the incomplete knowledge of all the pathways involved, the drug compounds are thought to act on the calcification pathway at different levels, thus antagonism is not expected. However, an important aspect of this proposed research is that when results are unexpected, they may be the most fruitful in refining our knowledge of the mechanisms at work.

Dipyridamole combined with ARA is expected to possibly have synergistic effects. Adenosine and adenosine analogs (Zannikos 2001) have been shown to be rapidly be absorbed by the body. Dipyridamole should have little affect alone, since there is expected to be little adenosine production in CD73 -/- cells. However, it can be expected to greatly increase the extracellular half-life of ARA, providing more capacity for adenosine signaling. On the other hand, ARA with lansoprazole may only be additive in combination. ARA should reduce the amount of TNAP expression, leaving lansoprasole with fewer enzymes to inhibit. This mutually exclusive mechanism suggests that a Bliss independence model could be a good fit. However, if synergy is discovered between ARA and any other compound, it may indicate that ARA is actually affecting other calcification regulatory networks, which would help direct future research efforts in elucidating the role of adenosine signaling.

4.4 Specific Aim 3

4.4.1 Strategy and Rationale

This Aim proposes to harness the mechanistic exploration in Aim 1 and Aim 2 to provide results for treating ACDC in a living organism. Leading up to Aim 3, mechanistic knowledge on disease progression and therapeutic strategies will build, thus increasing confidence in the efficacy of the treatments and increasing efficiency of the research plan. For example, it may be discovered that dipyridamole alone has negligible PD effect, but show significant synergy when combined with an ARA. Thus, when the stakes are higher, in terms of experimental resource expenditure, for *in vivo* experimentation, the dipyridamole alone strategy will be lower priority than its use in a combination therapy with ARA.

The major steps in this Aim are to establish a viable animal disease model, optimize a dosing strategy using PK experiment data, and then finally demonstrate and quantify efficacy.

CD73 knockout mice will naturally be the initial choice in building a disease model. While MVC has not been observed in CD73 -/- mice (Castrop and Huang 2004), the initial mouse study was performed several years before CD73 was implicated in ectopic calcification. Significantly elevated levels of serum TNAP were noted in the mice, which is consistent with the hypothesis TNAP overexpression leading to PP_i depletion. Osteoporosis-like trabecular bone weakening has also been reported in CD73 -/- mice (Takedachi et al. 2012), which suggests there may indeed be some dysregulation of mineralization. Furthermore, ENPP1 and ABCC6 knock mice have been confirmed to exhibit phenotypes that mirror the matching conditions in humans. Considering the relatively less severe phenotype associated with CD73 dysfunction, and significantly longer time before onset of symptoms, it is possible that this phenotype was initially overlooked. Therefore, this part of the research will begin by characterizing the ACDC associated phenotype in CD73 knockout mice.

PK modeling will be performed using the animal model, administering dosage of the compound and measuring serum and dialysate levels. Then, nonlinear optimization algorithms will be used to fit parameters to the PK model. The PK model will be used to create an integrated PK-PD model using the PD models developed in Aim 2 (Figure 8).

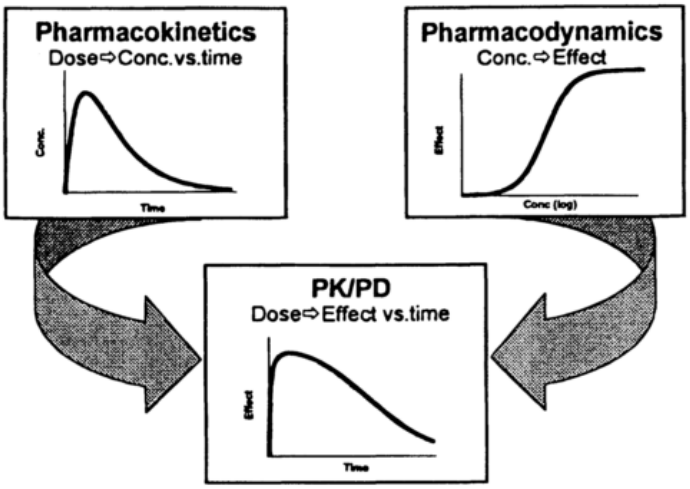

Figure 8: A PK-PD model integrates relationships between where the drug goes the upon entering the body (PK) with the dose dependent effect the drug has on the body (PD).

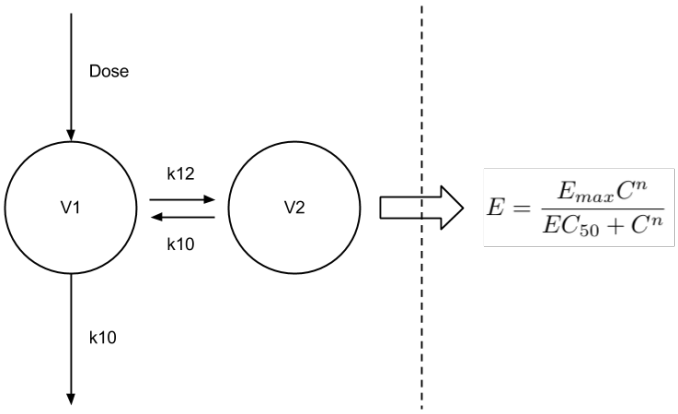

Figure 9: Two-compartment PK model directly linked to PD model. Compartments V1 and V2 represent the circulating serum and the peripheral tissues that the drug is transported in to. Concentration of drug within the effect compartment, in this case V2, is directly fed into the PD model of drug dose response.

This PD dose-effect model will be directly coupled with the concentrations in the effect compartment within a PK model. The general modeling approach that is adopted in this proposal is to start as simple as reasonable and expand in response to new data that suggests a more complicated system would be appropriate. A single compartment PK model could be used as a starting point, however, ACDC symptoms manifest in the peripheral regions of patients, which suggest that perhaps a two-compartment model may be used. This will be pursued if it provides a significantly better fit to the experimental data (Figure 9). The PK-PD model will then be used for dose planning.

One rationale for using a PK-PD model for treatment planning in the animal model is to allow the research to be translatable towards human therapy. Since ACDC is a rare disease, there will be difficulty in finding clinical test subjects. Therefore, a predictive PK-PD model may reduce the number of clinical experiments necessary in humans. Furthermore, the validation of the PK-PD model will also provide further confidence in the combination therapy benefits discovered in Aim 2.

4.4.2 Experimental Plan

4.4.2.1 Establish ACDC Mouse Model CD73 -/- mice will be purchased from The Jackson Laboratory (http://jaxmice.jax.org) and housed until 6 months of age. To detect signs of arterial calcification, the mice will be imaged using high resolution computed tomography scans (micro-CT) (Jorgensen, Demirkaya, and Ritman 1998) and compared to wildtype controls. *Ex vivo* histology and

staining of the vascular cells in the limbs will also be performed to inspect for increased TNAP expression and signs of calcification using similar methodology to the molecular profiling steps proposed in Aim 1.

4.4.2.1.1 Expected Results and Proposed Alternatives Due to the relatively mild phenotype of ACDC compared to other calcification diseases that are present at infancy, several variables may be tuned to induce ACDC symptoms in mice. Mice may be allowed to age longer, for example to 12 months, as ACDC is not found in early life. The diet of the mouse may be modified with higher phosphate and lower vitamin D content (Tani et al. 2007; Shiota et al. 2011) in order to induce calcification. Difficulties in inducing the disease phenotype may indicate that the mice have additional compensatory mechanisms that help to preserve homeostatsis, such as greater endogenous adenosine generation. Calcification inducing conditions applied to the CD73 knockout mice will be repeated with the wildtype controls, ideally finding an optimal condition where MVC is readily observed in the knockout mice but not observed in the control

4.4.2.2 Develop Pharmacokinetic-Pharmacodynamic Model PK models will be built by performing dosing known amounts of compounds into the animals and measuring its rate of clearance from the blood stream. The experimental setup will involve first cannulating the animals for drug infusion and measurements during the procedure (Y.-J. Tsai, Lin, and Tsai 2010). The animals will be hooked up to a microdialysis system, where administration of the compound and sampling of the blood and liver dialysates can be collected quickly. Upon drug infusion, dialysate samples will be collected every ten minutes into a fraction collector. A validated high performance liquid chromatography with photodiode-array detector (HPLC-PDA) will be used to measure drug concentrations in the samples.

The compartmental PK model parameters can then be fit from the experimental data. Uncertainty analysis using the Latin Hypercube method, or a suitable alternative, will be used to determine the uncertainty in the model predictions given the model structure and experimental error.

PK-PD models will be produced by directly linking the effective exposure compartment to the dose response relationship.

4.4.2.2.1 Expected Results and Proposed Alternatives The experimental portion of this step will require many optimization steps along with significant training and experience to be able to conduct the procedure. Validation on the detection system must be performed to ensure that measurements are accurate. The drug infusion procedure will need to be optimized for each drug, and several dosing concentrations will need to be used to gather data for parameter fitting. It is then expected to see exponential-like decay curves in the dialysate concentrations as drug is absorbed and cleared. Difficulty in collecting data may also be addressed through different measurement systems.

In the model fitting, some parameters such as elimination rates may be approximated from literature, since the PK for all the compounds have been previously studied. However, the PK data for our specific disease model and dosing protocol does not exist, thus it is still desirable to measure these parameters experimentally. It would also be advantageous to compare parameter values with other PK models calculated from similar systems.

Building a predictive PK-PD model will likely need to be performed partially in parallel with initial efficacy experiments in order to validate the PK component of this of the model. This is due to that fact that the PK studies may not provide enough information to produce an unambiguous PK model

structure. Additional compartments may be necessary to add in order to reflect any observed delayed responses or reduced exposures. The current PK knowledge of the compounds will also be used in this process. For example, bisphosphonates are typically modeled using a three-compartment PK, due to its rapid absorption by bone and following sustained release (Cremers, Pillai, and Papapoulos 2005; Kimmel 2007).

4.4.2.3 Conduct Dosing Plan on Mouse Model and Evaluate Efficacy Single compound and combination therapy dosing regimes will be devised in accordance to the PK-PD model and evaluated against the experimental efficacy data. Dosing will be performed similarly to the PK studies, however, repeated blood sampling may not be necessary. Similar to the dosing regime in the PD studies, once daily administrations of the drugs will be performed for 21 days. At the end of day 21, the mice will be analyzed using micro-CT (if effective in detecting calcification) and sacrificed to perform *ex vivo* staining of their VSMCs. The effectiveness of the treatment at various dosage levels will be compared to the PK-PD model for further refinement of model structure and parameters. Additional observations, such as dose toxicity in the animals, will be monitored and used to inform the model.

4.4.2.3.1 Expected Results and Proposed Alternatives In the initial rounds of dose planning using the PK-PD model, there is likely to be significant modifications to the model that will be made due to discrepancy in the predictions and simulation. However, through iterative changes in model structure, parameters, and model complexity, the predictive capabilities of the model are expected to be improved over time. Due to the overwhelming number of experimental conditions possible, especially with combination therapies, this iterative model development and feedback will likely prove to be a very useful approach. Experimental procedures may also need to be adjusted, and further *in vitro* experimentation may be needed to explain inconsistencies. If the dose effect itself is difficult to quantify *in vivo*, it may be necessary to revise the PD models to use a measure of efficacy that is more consistent across whole animals and cell culture. For example, levels of PP_i and P_i may serve as a surrogate calcification index. Enough data should be collected in the cell culture PD experiments to be able to re-tune the model to fit a different set of efficacy criteria, but more PD experimentation may be necessary even at this later stage of the research plan.

5 Summary and Future Directions

NT5E mutations leading to defective CD73 protein results in the onset of ACDC, a painful condition of limb and joint calcification. This is a debilitating disease with no known cure. Recent studies have suggested that the CD73 plays a role in providing adenosine signaling which keeps the body's calcification mechanisms in check. However, detailed knowledge on how the signaling mechanism functions and what possible drug treatments may be effective in combating the disease is currently lacking. This proposal outlines the systematic investigation of the biomolecular mechanisms behind the presentation of ACDC and drug interventions to bridge the knowledge gaps. PK-PD modeling, built from *in vitro* and *in vivo* data will be employed to characterize the relationships between drug treatment and efficacy. Furthermore, due to the complex combinatorial control of this disease pathway, a combination drug therapy approach will be investigated for optimizing treatment potency while reducing the risk of high dosages that may lead to safety complications. PK-PD modeling will be used to plan and administer treatment in an animal model, with the aim of ultimately aiding in the personalized treatment planning of affected

patients. Furthermore, the knowledge and methodologies developed in this research proposal may also be applicable to supporting the understanding and treatment of an entire family of ectopic calcification diseases, such as GACI and PXE.

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