University of Washington
Department of Bioengineering

Qualifying Examination

A Quantitative Systems Approach for Studying and Treating Arterial Calcification Due to CD73 Deficiency

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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 *NT53* 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). As of the writing of this proposal, there is no standard treatment or therapy for alleviating this condition.

This proposal will outline research to determine the biological mechanism and investigate possible therapeutic interventions for ACDC. Work will involve *in vitro* and *in vivo* experimentation along with pharmacokinetic (PK) and pharmacodynamic (PD) modeling in order to rapidly support the design of a treatment plan for this disease

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, primarily ENPP1 and TNAP, were identified as major components contributing to pyrophosphate depletion, leading to mineralization of the arterial wall, and will also be incorporated in the working mechanistic hypothesis. Metabolic profile will be created of the ACDC phenotype, through quantification of surface enzyme activity and steady state extracellular concentrations of key metabolites. The role of adenosine signaling in the dysregulation of TNAP, characteristic of ACDC, will be explored through the interrogation of adenosine receptor classes.The combination of metabolic profiling and cell signaling investigation will elucidate the ACDC pathobiology and enable further research for therapeutic interventions.

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

Three different theurapeutic strategies, adenosine receptor agonism, pyrophosphate supplementation, 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, an combination therapy approach will be investigated for potential to maximize amelioration 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 of 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 mouse model drug uptake and clearance will be built from *in vivo* data. The resulting model will be coupled with the PD model establised in Aim 2 to form an integrated PK-PD model. The PK-PD model will guide dose selection and therapy planning for whole animal model investigation of treatment efficacy. *In vivo* assessment of the efficacy in devised treatment plans will be performed. The results can be a basis for developing therapies to ease disease burden 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 C, Ziegler SG, Markello TC, Brusco A, Groden C, Gill F, Carlson-Donohoe H, Lederman RJ, Chen MY, Yang D, Siegenthaler MP, Arduino C, Mancini C, Freudenthal B, Stanescu HC, Zdebik AA, Chaganti RK, Nussbaum RL, Kleta R, Gahl WA, and Boehm M. NT5E mutations and arterial calcifications. New Eng J Med 364: 432-442, 2011.)

3 Background and Significance

In this section, the scientific context of this biological problem will be introduced. While the subject of arterial calcification is a large and diverse field, the focus of this background will be on the biological mechanisms of interest behind the NT5E mutation phenotype in designing this disease model. Additional reviews on the pathobiology behind arterial calcification have been published. (L. L. Demer and Tintut 2008; Cecilia M. Giachelli 2004)

3.1 Vascular Calcification

Vascular calcification is the process in which hydroxyapatite 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); @Chiu2010; @Blacher2001; @London2003] 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)

Calcification can occur in either the media (within vessel walls) or intima (vessel lumen interior). Intimal vascular calcification (IVC) is frequently seen in conditions related to atheroschlerosis [@Nakamura2009]. Medial vascular calcification (MVC), also known as Monckeberg's arteriosclerosis, increases in prevalence in populations with increased age, diabetes mellitus, chronic kidney disease, chronic inflammation, and genetic disorders. (Micheletti et al. 2008)

3.2 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. (Zimmermann 1992) Due to the broad range of AMP and adenosine involvement in biological processes, CD73 function is implicated in a variety of pathological conditions, including immunodeficiency (Deaglio et al. 2007), inflammation (Colgan et al. 2006), and ectopic calcification.

Using a genome-wide homozygosity mapping approach, a popularizing technique in cardiovascular research (Kathiresan and Srivastava 2012), nonsense, missense, and single-nucleotide insertion frameshift mutations in the NT5E gene, coding for CD73, have been discovered to cause MVC of the limbs and joints in otherwise healthy individuals. (St Hilaire et al. 2011) 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 CD73 activity of hydrolyzing AMP into adenosine and inorganic phosphate, which was shown to be restored through CD73-deliverying viral vectors.

3.3 Exploring Possible Mechanisms Behind ACDC

In determining the mechanism of ACDC, the literature was reviewed for related enzymes and metabolites that are related in the pathobiology of MVC (Figure 3). (Rutsch, Nitschke, and Terkeltaub 2011) 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) Insight into the pathological mechanism has come from numerous independent genetic studies that discovered molecular defects in rare monogenetic disorders. (Nitschke and Rutsch 2012)

3.3.1 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 PP_i. (J. L. Millan 2006) The ratio of PP_i to P_i is crucial in controlling the formation of hydroxyapatite (HA) crystal (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, when PP_i levels fall to abnormal levels, 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 shortly after birth. In the TNAP knockout mice, there was normal formation HA containing matrix vesicles that are released by osteoblasts, however, extracellular mineralization was impaired due to elevated PP_i levels (Figure 1).

3.3.2 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. @Okawa1998 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 by hydrolyzing ATP. (Kato et al. 2012) The loss the capacity for PP_i to potently inhibit HA formation results in severe MVC beginning in infancy. ENPP1 knockout mice recapitulated the *ttw/ttw* phenotype and Consequently, defects in ENPP1 for humans were discovered to cause generalized arterial calcification of infancy (GACI). Treatment with bisphosphonates (Ramjan et al. 2009), a pyrophosphate analog, can reverse the course of disease. Interestingly, it has been shown that stopping bisphosphonate supplementation at the age of 2 in infants still allows for healthy development [@Edouard2011], which is perhaps a result of other compensatory mechanisms for maintaining systemic balance of PP_i/P_i.

Mutations in the gene ANKH (ANK in mice) also result in a similar disease phenotype [@Nurnberg2001]. ANKH is the other contributor of PP_i in the extracellular environment, through the transport of intracellular PP_i.

(Hotton et al. 1999): Differential Expression and Activity of Tissue-nonspecific Alkaline Phosphatase (TNAP) in Rat Odontogenic Cells In Vivo Phosphate (Jono et al. 2000): Phosphate Regulation of Vascular Smooth Muscle Cell Calcification - in vitro assays

3.3.3 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, and subsequently the levels of calcification

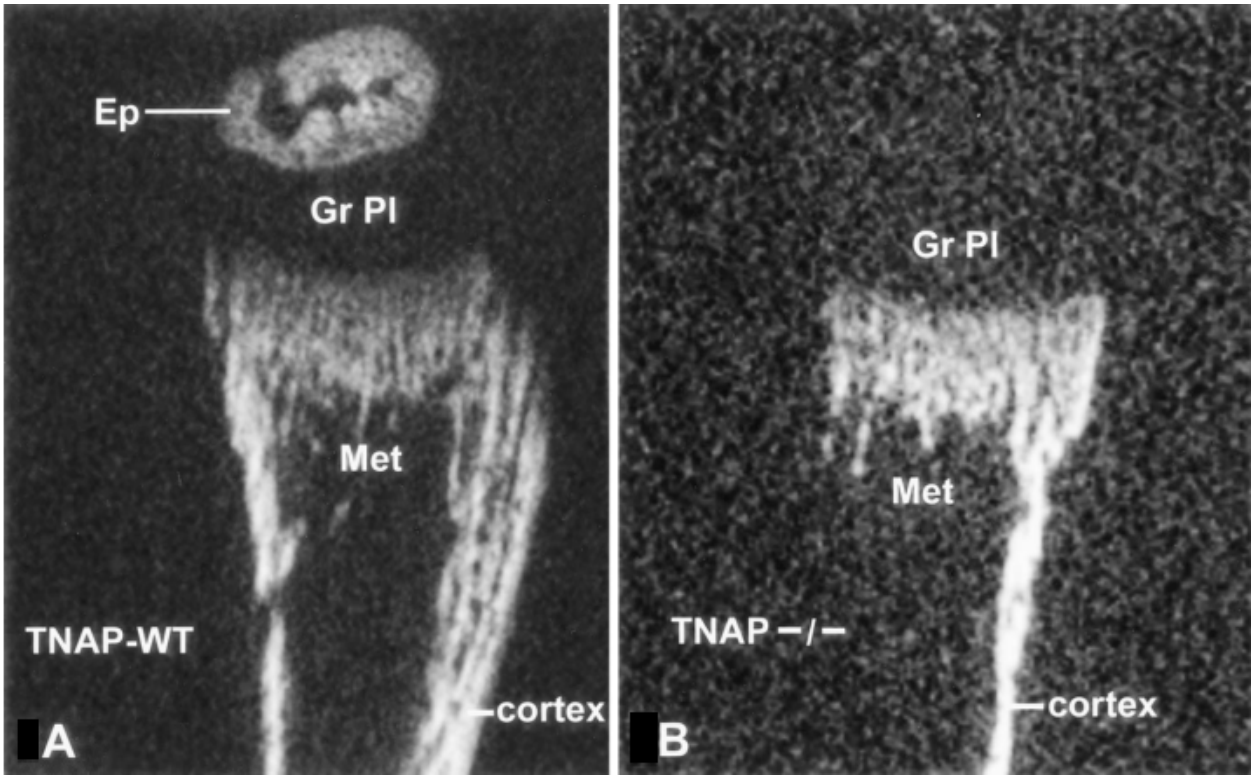

Figure 1: Impaired bone formation of TNAP knockout mice (B) compared to wildtype mice (A). (Anderson et al. 2004)

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 ATP-binding Cassette Sub-Family C Member (ABCC6) mutations (Markello et al. 2011) that cause pseudoxanthoma elasticum (PXE), a disease that shares similar manifestations to ACDC [@LeSaux2000]. 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 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 are G-Protein Coupled Receptors (GPCR), are one of four different pharmacological classes (A_1, A_{2A}, A_{2B}, and A_3), and have a wide range of effects on the body. (Haskó et al. 2008)

(Gutenkunst et al. 2007): Systems biology models are universally "sloppy", meaning they that they contain many insensitive parameters and their behaviors are determined by relatively few number of stiff parameters.

4 Experimental Design and Methods

4.1 Overview

Figure 2 shows an overview of the specific aims.

Specific Aims 1 and 2 will serve to explore the mechanism behind ACDC and will be performed in parallel. The results from the *in vitro* and *in silico* modeling will be used to inform each process.

Specific Aims 2 and 3 will serve to intelligently select and design for a therapeutic intervention for treating the disease. Analysis of the optimized *in silico* model will enable the selection of the best therapeutic intervention to be tested in a CD73 -/- mouse model.

4.2 General Protocols

4.3 Specific Aim 1

4.3.1 Strategy and Rationale

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

Proposed mechanism shown in Figure 3.

4.3.2 Experimental Plan

4.3.2.1 Establish *In Vitro* Model of ACDC and Healthy Controls Vascular smooth muscle cells (VSMCs) will be isolated and cultured [@mahabeleshwar2007methods] 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

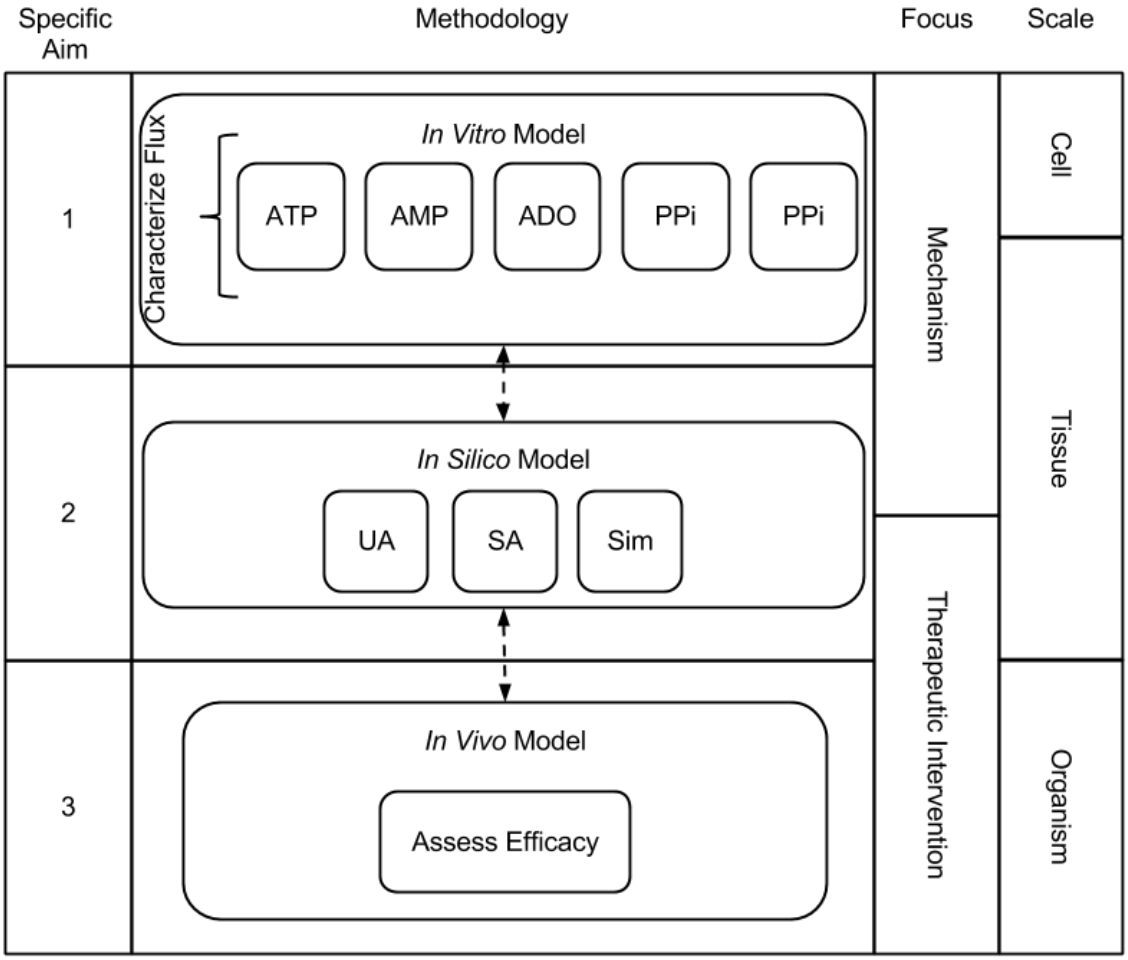

Figure 2: Overview of Aims

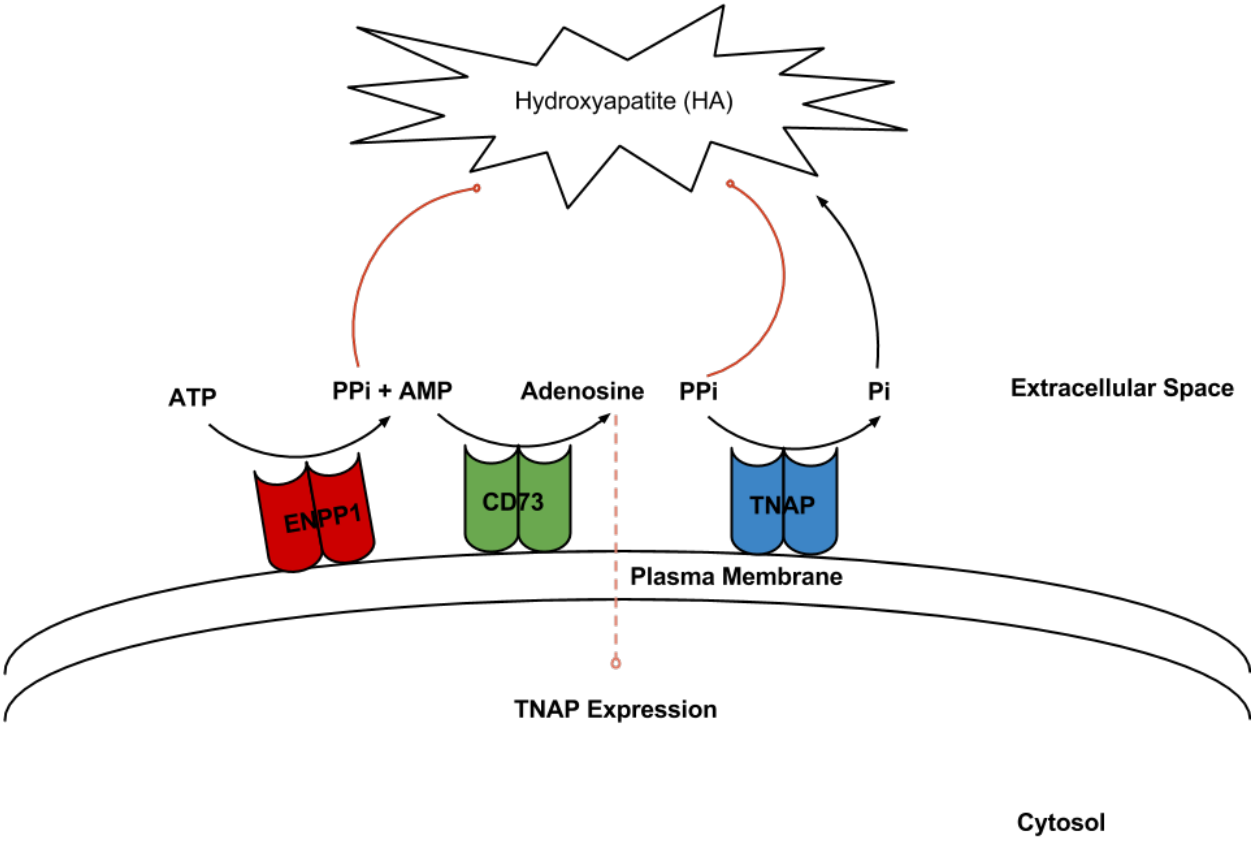

Figure 3: Proposed biological pathway involved in arterial calcification caused by ACDC, GACI, and PXE.

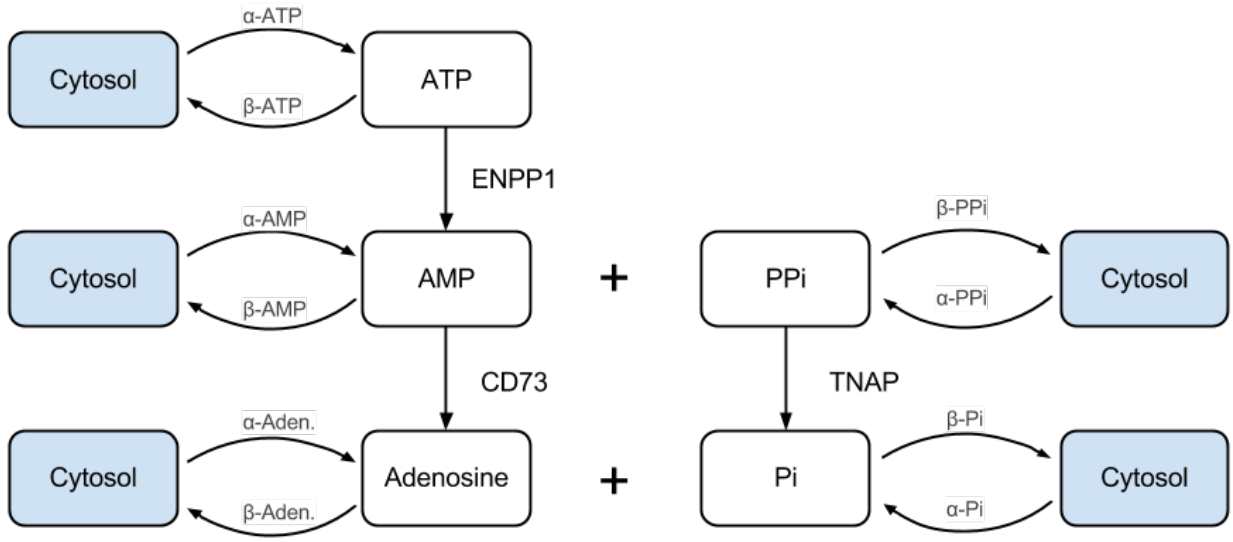

Figure 4: Pathway to be modeled.

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. [@Liaw1995] 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. [@Knofel1999] 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.

4.3.2.1.1 Expected Results and Proposed Alternatives While VSMC cell cultures have previously been established from *ex vivo* human aorta (Jono et al. 2000), the similar process in mouse VSMC cell culture may be difficult due to the drastically smaller 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 relationship between cell dish experiments to whole animal across the experimental aims.

4.3.2.2 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 4-20% polyacrylamide gel, and electrophoresed at 120V for 1.5 hours. 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). [@Nam2011] 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.

4.3.2.2.1 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 expression inhibition of TNAP. ENPP1 levels have not been reported to change with CD73 mutation, so any differences in expression would be an interesting finding, as it may indicate that the excessive extracellular AMP that is left unconsumed by CD73 may play a role signaling role as well. While AMP has mostly been reported

as merely a precursor to adenosine signaling, orphan GPCRs have been discovered [@Inbe2004] 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 may be taken again during adenosine agonist addition to further investigate.

4.3.2.3 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, namely 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) as described in @Ciancaglini2010. PP_i will be measured enzymatically (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).

4.3.2.3.1 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. (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. [@Lomashvili2005] 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 are upregulated and could direct further experiments to shed light on the discrepancy.

4.3.2.3.2 Combinatorial Probing of Adenosine Receptors with Agonists One of the key goals in this Aim is to elucidate the adenosine signaling mechanism for regulating TNAP expression. There are four pharmacological classes of adenosine receptors (ARs), A_1, A_{2A}, A_{2B}, and A_3. Toward the goal of developing a potential therapeutic by replacement of the missing adenosine signal, and to understanding the biological repercussions and potential off-target effects, it is necessary to identify the specific AR which, when activated, inhibits TNAP expression.

A combinatorial approach of activating a single type of adenosine receptor, 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 EC90 value in a microtiter plate with three ARANTs onboard, at their IC90 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.

4.3.2.3.3 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 will add it a long list of regulatory functions of 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.

4.3.2.4 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 adenosine binding to them. Then, cells will be incubated with or without dipyridamole, an inhibitor of adenosine uptake. TNAP expression will be measured at time 0, 12 hours, 1, 3, and 5 days.

4.3.2.4.1 Expected Results and Proposed Alternatives If TNAP suppression is observed in the absence of dipyridamole, and not in its presence, this would suggest that it is the direct transport of adenosine into the cell that is the ultimate fate of CD73 produced adenosine in regulating TNAP. If TNAP suppression is observed equally withough regard to dipyridamole, it would suggest that another unknown adenosine receptor is being triggered.

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

4.4 Specific Aim 2

4.4.1 Strategy and Rationale

This Aim will guide the rational design of three potential therapies for treating ACDC. The strategy that will be adopted in this aim is to create useful PK-PD models that encompass three different pharmacological approaches to reducing MVC. The three different drug approaches are AR signaling, TNAP inhibition, PP_i supplementation with bisphosphonates. A combination of *in vitro* assays to determine drug PD along with *in vivo* measurements to determine PK.

This modeling approach will be immensely useful for simulating treatment plan efficacy of these three different drug classes. Furthermore, experimentation will be done to to create interaction PK-PD models to evaluate the efficacy of combination therapies. Since these three drug classes have different mechanism of actions, it is hopeful that a combination therapy may yield a synergistic effect on efficacy while minimizing safety risk due to different disposal pathways.

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 cooperativeity coefficient.

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

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 would 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 if it significantly fits the experimental data better (Figure 5).

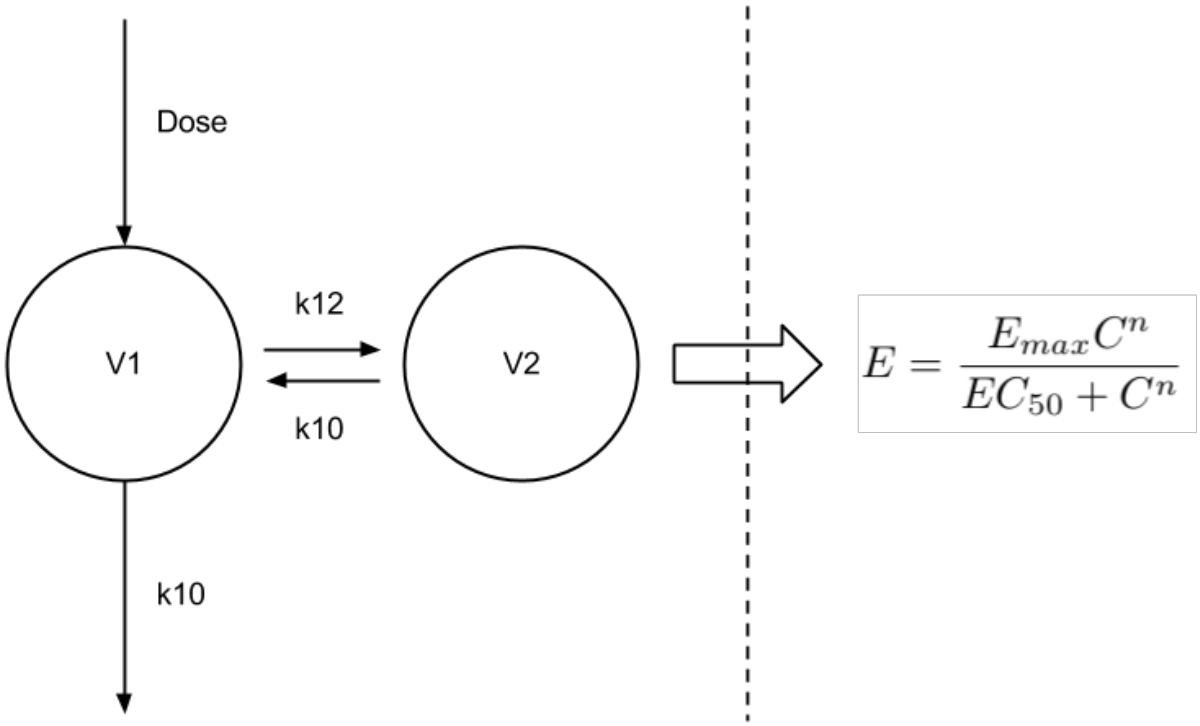

Figure 5: Two-compartment PK model directly linked to PD model. Compartments V1 and V2 represent the circulating serum and the peripheral tissues that the drug is transported in to. Concentration of drug within the effect compartment, in this case V2, is directly fed into the PD model of drug effects.

4.4.2 Experimental Plan

4.4.2.1 Develop Pharmacodynamic Models of Multiple Drug Targets An *in vitro* system will be used to characterize the PD effects of ARA, lanzoprazole, 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).

4.4.2.1.1 Expected Results and Proposed Alternatives The observed drug effects on calcification are expected to form a dose-response curve that will fit in a Hill-type equation (Figure 6). The result should be a predictive model of 21-day dosing effects for each of the three compounds. The fitting of the parameters is not expected to be a significant challenge, however, different software packages may be used to perform fitting if the need arises.

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.

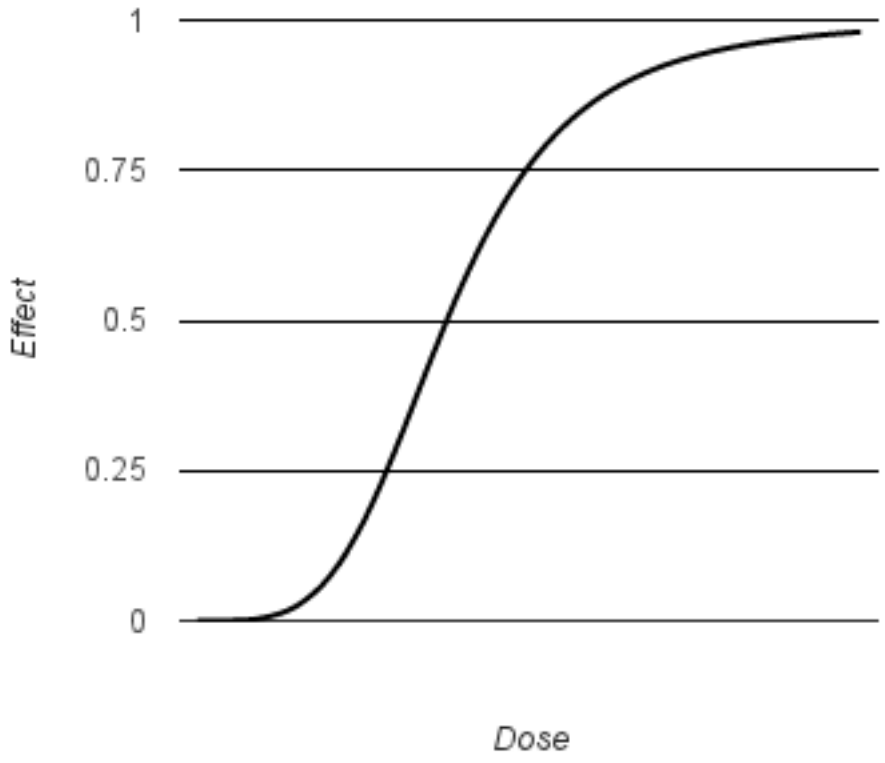

Figure 6: Typical PD curve for drug effects.

4.4.2.2 Explore Potential Synergistic Effects of Combination Therapy

4.4.2.2.1 Expected Results and Proposed Alternatives

4.5 Specific Aim 3

4.5.1 Strategy and Rationale

4.5.2 Experimental Plan

4.5.2.1 Establish ACDC Mouse Model

4.5.2.1.1 Expected Results and Proposed Alternatives

4.5.2.2 Develop Pharmacokinetic Model

4.5.2.2.1 Expected Results and Proposed Alternatives

4.5.2.3 Develop Pharmacokinetic-Pharmacodynamic Model

4.5.2.3.1 Expected Results and Proposed Alternatives

4.5.2.4 Conduct Dosing Plan on Mouse Model and Evaluate Efficacy

4.5.2.4.1 Expected Results and Proposed Alternatives

5 Summary and Future Directions

Treatment of CD73-defiency with bisphosphonates may also have an added advantage for reducing the effects of osteoporosis-like trabecular bone weakening, which has been found in CD73 -/- mice (Takedachi et al. 2012). However, this has not yet been reported in humans.

6 References

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