Official Study Title: **An innovative proof-of-concept approach to identify age-modulating drugs capable of reversing inflammation and re-setting the epigenetic clock (Topical-RAPA)**

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An innovative proof-of-concept approach to identify age-modulating drugs capable of reversing inflammation and re-setting the epigenetic clock (Topical-RAPA)

Study Chairman or Principal Investigator:
Ellen Kraig, PhD
University of Texas Health Science Center San Antonio
Department of Cell Systems & Anatomy
School of Medicine
7703 Floyd Curl Drive, MC7762
San Antonio, Texas 78229-3900
210-367-3171
kraig@uthscsa.edu

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Dean L Kellogg, Jr, MD, PhD
UT Health Science Center
Department of Medicine – Division of Geriatrics,
Gerontology and Palliative Medicine
7703 Floyd Curl, MC 7885
San Antonio, TX 78229-3900
210-617-5197
kelloggd@uthscsa.edu

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Study Title

An innovative proof-of-concept approach to identify age-modulating drugs capable of reversing inflammation and re-setting the epigenetic clock (Topical-RAPA)

Objectives

AIM 1: Test whether epigenetic changes in skin are elicited by topical treatments with RAPA

AIM 2: Test whether baseline inflammation is affected by topical RAPA

Design and Outcomes

This is a double-blinded pilot treatment study, where each subject provides his/her own control to assess whether rapamycin will stop, slow, or reverse the "epigenetic clock" using DNA extracted from skin and the change in levels of inflammatory mediators measured in blister fluid in healthy subjects aged 65-95 years.

Interventions and Duration

Rapamycin 8% ointment will be applied topically, 0.25ml, to one of the participant's forearms and matching placebo, 0.25ml, to the opposite forearm daily for a total of 6 months. The total participant duration will include a consenting/screening visit (0) will last 60-90 minutes, followed by monthly visits (1-6) of up to 60 minutes, and followed by the final visit (7) of up to 4 hours.

Sample Size and Population

The study aims to recruit 75 subjects to ensure at least 40 completers, approximately half male and half female.

STUDY TEAM ROSTER

Principal Investigator: **Ellen Kraig, PhD**

University of Texas Health Science Center San Antonio
Department of Cell Systems & Anatomy
School of Medicine
7703 Floyd Curl Drive, MC7762
San Antonio, Texas 78229-3900
210-367-3171
kraig@uthscsa.edu

Co-Principal Investigator: **Dean L. Kellogg, Jr, M.D, Ph.D.**

UT Health Science Center
Department of Medicine – Division of Geriatrics, Gerontology and Palliative Medicine
7703 Floyd Curl, MC 7885
San Antonio, TX 78229-3900
210-617-5197
kellogg@uthscsa.edu

SITES:
UT Health San Antonio

1 STUDY OBJECTIVES

1.1 Primary Objective

Test whether epigenetic changes in skin are elicited by topical treatments with RAPA

1.2 Secondary Objectives

Test whether baseline inflammation is affected by topical RAPA

2 BACKGROUND AND RATIONALE

2.1 Background on Condition, Disease, or Other Primary Study Focus

The study is focused on the effects of mTOR antagonism specifically on elderly subjects.

In order to identify drugs and/or interventions with the potential to delay the onset and progression of age-associated pathologies, preclinical studies have used animal models where extension of lifespan could be accurately assessed. Several outstanding candidate therapeutics have been identified over the last decade; these include mTOR inhibitors (like rapamycin), senolytic agents, acarbose, and others. However, translating anti-aging results from animal models into humans has been challenging. For example, measuring extension of lifespan would be impractical in humans, so it is necessary to identify and validate parameters that can serve as surrogate markers of healthy aging. Moreover, the efficacy of age-modulating drugs is often only demonstrable with older individuals where functional deficits are already evident. Thus, testing of potential anti-aging therapeutics should be done in a cohort of human subjects of an advanced age; issues of safe administration would be even more acute in this "at risk" population. To circumvent these limitations, we propose to develop and validate an innovative, minimally invasive, cost-effective assay in human subjects ranging from 65-95 years old. The putative anti-aging test drug will be applied topically to a discrete area of skin on the subject's forearm while a "vehicle only" (control) is applied in parallel to the opposite arm. Thus, each subject will serve as his/her own control. The pharmacodynamic outcomes at the "test" and placebo sites will be compared in both males and females since the effects of age-modulating agents often differ between the sexes. Ascertaining the efficacy of topically-applied therapeutics requires robust biomarkers of aging that can be measured in skin, but will be generalizable to the intact organism. Towards this end, we have opted to use two independent parameters that are known to be modified with aging both locally (in individual tissues) and systemically: i) the DNA methylation pattern or "epigenetic clock" and ii) biomarkers associated with inflammation. To validate the proposed technique, we have chosen a drug, rapamycin (RAPA), which has already been shown to modulate aging in rodents and which has been tested for safe systemic application in humans [1-4]. After a 6 month treatment phase, suction blisters will be generated at each site to allow collection of skin cells (blister flap) for use in the epigenomic analysis (Aim 1) and interstitial (blister) fluid for use in measuring cytokines and other inflammatory regulators (Aim 2). Once validated, our approach can be used to safely and efficiently screen the age-modulating potential of pharmacological agents in humans to identify those agents that warrant large, expensive human clinical trials with systemic agent administration.

2.2 Study Rationale

Demonstrating age modulating benefits of drugs in long-lived humans is challenging. Although there are outstanding animal models used to identify candidate interventions which extend lifespan [2, 3, 13], an efficient and cost-effective approach to test which of these drugs can be used safely and with efficacy in an older human cohort is not available. Thus, we propose to develop and validate an innovative, relatively safe, minimally invasive approach for the initial evaluation of the potential efficacy of purported age-modulating agents in humans prior to moving forward with a large, costly human clinical trial. Our scientific premise is that putative age-modulating agents can be pre-screened for beneficial effects through topical application to the skin of humans. In this way, the drug effects will be limited to the small test area with minimal or no systemic delivery, thereby avoiding serious risks to the human subjects. As summarized in Figure 1, potential agents can be applied to different 'experimental' skin sites in the same person with simultaneous application of 'control' placebo treatments on opposite forearms. This approach will allow each subject to serve as his/her own control. Age-modulating efficacy of the tested agent(s) can be assessed from skin samples obtained from the experimental treated and placebo control sites. Skin samples can be easily obtained with suction blister or small punch biopsy techniques for subsequent testing [14-17]. Ascertaining the age modulating efficacy from these samples will require robust biomarkers of aging that can be measured in skin, but will be generalizable to the intact organism. Towards this end, we have opted to use two parameters that are known to be modified with aging both locally (in individual tissues) and systemically: i) the DNA methylation pattern or "epigenetic clock" and ii) biomarkers associated with inflammation.

To validate the proposed pre-screening approach, we have chosen to initially test a single agent, rapamycin (RAPA), an mTOR pathway antagonist. RAPA has been shown to prolong lifespan in rodents. Improvements in cognition, cardiovascular function, and certain aspects of immunity were also demonstrated in animal models [7, 18-23]. Based on these promising results, we undertook a pilot study of orally-delivered RAPA in older (aged 70-95 yrs) human subjects as a potential anti-aging therapeutic. As described in detail [1], short-term (2 months) RAPA treatment was relatively well tolerated in these older individuals. In addition, we and others have established that RAPA can be efficaciously delivered transdermally [24, 25]. As shown in Figure 2A, easily detectable levels of the drug are obtained locally (in the skin at the application site) within 12 hours of application while there was no detectable level of RAPA in the blood even after 7 days of topical application of 8% RAPA. Moreover, as shown in Figure 2B, 7 days of 8% RAPA led to improved endothelium-dependent vasodilation in older, but not younger persons, demonstrating that this treatment exerted pharmacological effects. In an "N of 1" study, 8 months of topical 8% RAPA treatment decreased the size of keloids (Kellogg, data not shown). Topical RAPA has also been tested clinically and shown to be efficacious in the treatment of psoriasis and tuberous sclerosis [24, 26, 27]. Thus, mTOR inhibition with RAPA should be an excellent model for development and validation of the proposed pre-screening protocol. The 'long-term' significance is that, once validated, our approach can be used to safely and efficiently screen the age-modulating potential of pharmacological agents in humans, in vivo, in a relatively short amount of time in order to identify those agents that warrant large, expensive human clinical trials with systemic agent administration.

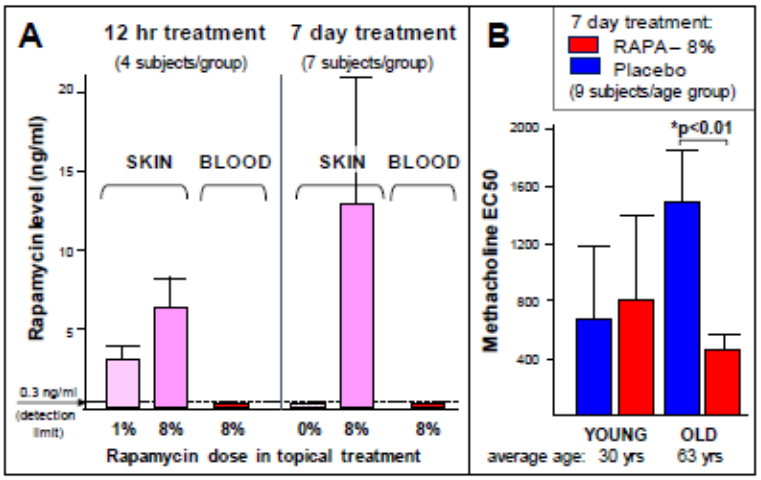

FIGURE 2. Efficacy of topically applied RAPA ointment in human subjects.

Panel A: RAPA levels in skin over time for sites treated twice daily with ointment containing 0% RAPA (vehicle only control), 1% RAPA, or 8% RAPA and in blood for the same time points.

Panel B: Vasodilation was measured in subjects who had treated one skin site with 8% RAPA ointment (red) and a second site with the ointment vehicle only (blue - placebo) for 7 days. The two treatment sites were then instrumented for intradermal microdialysis and increasing doses of a vasodilator, methacholine, were delivered. Skin vascular responses were measured by laser-Doppler flowmetry. EC50s (the concentration of methacholine that elicited half-maximal responses) are shown. RAPA significantly decreased these values on older persons, but had no effect on younger subjects.

b) Innovation:

We will develop a new pre-screening approach that can be safely used for translational studies in humans. The proposed protocol, employing topical application, should both enhance our understanding of underlying mechanisms affected by aging and identify the most promising age-modulating pharmacological therapies for future clinical trials with systemic administration. A priority for translating the pharmacological manipulation of aging from animal models into humans, especially in the geriatric age group, is subject safety. Since these test interventions will be given to essentially healthy persons to treat aging (a normal life process) rather than to mitigate an overt disease process, taking age-modulating drugs must entail no additional risks over those inherent in normal aging. Furthermore, successful agents must act to prolong healthspan or at least delay the onset of age-related pathologies. Ideally, age modulating agents would 'reset' the biological clock and slow or reverse the increase in baseline inflammation; thereby rejuvenating treated individuals. The innovation in this proposal derives from the choice of biomarkers to be followed to assess efficacy of topical treatment. First, we propose to use two independent measures of aging such that simultaneous effects on both outcomes would be highly significant. Second, the "epigenetic clock" defined by Dr. Steve Horvath (a collaborator on this project), is an innovative tool for estimating chronological age of cells based on the DNA methylation at 391 CpG motifs. This test has been previously validated for both skin cells and blood cells, suggesting that the results from topical administration will be directly translatable to systemic dosing. Third, to test for the effect of the topically applied drug on baseline inflammation, we will use a multiplex Luminex array to measure levels of 29 human cytokines (both pro and anti-inflammatory). In addition, ELISAs will be performed on three analytes known to play critical roles in regulating inflammation: IL-6 (interleukin-6), soluble ICAM-1, and RAGE (receptor for advanced glycation end products). Importantly, by using RAPA as the initial test compound, we will be able to directly compare the results with topical application to results from human clinical trials with oral administration. The future implications and potential uses deriving from this "high risk, potentially high reward" R21 are vast.

c) Approach:

Rationale. The NIA Interventions Testing Program (ITP) was developed to assess the potential of drugs to extend lifespan in mice and has identified several promising candidates, including aspirin, rapamycin (RAPA), 17αEstradiol, acarbose, NDGA (nordihydroguaiaretic acid), and Protadim [28] Additional putative age-modulating compounds have been identified through other approaches; these include senolytics, NAD precursors, and Sirtuin activators, among others [2].

Although several of these agents have moved into human clinical trials, such efforts are limited due to: i) high costs; ii) the inability to use "lifespan" as an outcome measure in long-lived humans; and iii) the reluctance to test drugs in an older "at risk" population. This last limitation is often circumvented either by using a younger subject cohort or by targeting individuals with serious illnesses; neither of these options is ideal. For example, some age-modulating drugs only show a treatment effect in older subjects where the loss of function with age changes the baseline allowing an effect to be detected (one such example is shown in Figure 2B where an effect of topical RAPA was seen only in older subjects). Similarly, studies of older subjects with cancer, autoimmunity, or another illness are compromised by associated co-morbidities and other drugs being taken to treat the primary illness. Thus, it is critically important to test putative age-modulating drugs on an older human cohort that is otherwise clinically stable.

The 'gold standard' for testing a drug for its ability to slow the ravages of aging would be a long-term placebo-controlled human clinical trial where changes in lifespan and age-associated pathologies could be assessed. However, this is simply not feasible in humans and is even challenging in relatively long-lived non-human primate models. Thus, we propose to develop and validate an innovative, minimally invasive, safe, and cost-effective technique for initial translational testing of purported age-modulating agents in humans. Our approach utilizes topical application of the intervention being tested to the skin of consenting human subjects. In order to sample the treated skin regions, suction blisters will be generated as a source of skin cells (keratinocytes) and blister fluid to be used in determining efficacy of the putative anti-aging treatment. There are several significant advantages inherent in this new approach. Specifically, the topical administration of agents to small areas of skin in humans will obviate the greater risks of systemic drug administration. As the target area is relatively small, this approach will require significantly less drug than a systemic clinical trial at a much lower cost as well. Moreover, the risk to human participants will be minimized as the agent is only delivered into skin, not systemically. As our proposed approach is safer than systemic administration and less invasive, we anticipate that successful recruitment of volunteer subjects will be much easier which is particularly important when dealing with an older cohort. Lastly, multiple 'experimental' skin sites can be treated simultaneously such that each subject will serve as his/her own control, thereby increasing statistical power and validity of the trial.

3 STUDY DESIGN

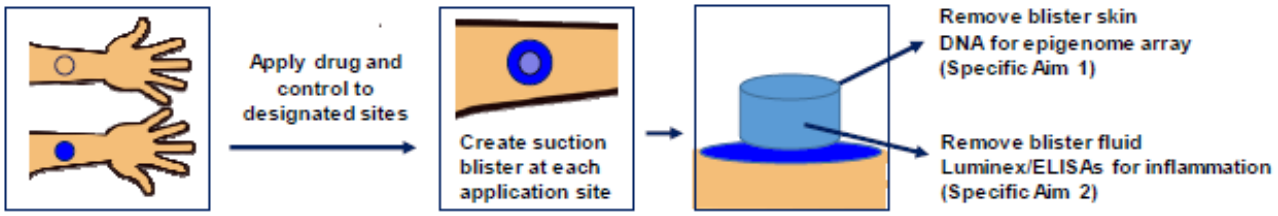

FIGURE 1. Diagrammatic representation of the proposed assay design.

Overall study design.

As shown diagrammatically in Figure 1, consenting subjects would apply the test compound to designated experimental and control sites on their forearms for the study period. As discussed below, we have opted for daily dosing over a 6-month period. At that point, suction blisters

would be generated at each of the sites; this will allow us to harvest both the skin flap and blister fluid. The efficacy of the potential age-modulating drug would then be assessed using two independent parameters: i) the potential to stop, slow, or reverse the "epigenetic clock" (Specific Aim 1) using DNA extracted from the skin and ii) changes in the levels of inflammatory mediators (Specific Aim 2) measured in the blister (interstitial) fluid. The topical approach to drug pre-screening is ideal for several reasons. First, skin is easily accessible and the applications can be handled by the subjects themselves. Second, human skin shows an invariant age-dependent phenotype characterized by decreased epidermal thickness, changes in the vasculature, reduction in the number of melanocytes and dendritic (Langerhans) cells, and lower levels of specific collagen types. Third, it is relatively easy to access the treated skin and fluids therein using the already established suction blister protocol [15, 16]. Lastly, the cost associated with topical application is significantly lower than systemic dosing as one can obtain relatively high concentrations of the test agent when it's being applied to a small area of skin; in this case, 10 cm ^{2}/site.

4 SELECTION AND ENROLLMENT OF PARTICIPANTS

Participants will not be excluded based upon ethnicity or race. For the proposed studies, we will draw on the general population of San Antonio and surrounding area which is more than 50% Hispanic. We suspect that we will have approximately 50% (or slightly more) Hispanic subjects in our proposed study. In our past studies, our volunteer population has been approximately 50% male and 50% female and we anticipate recruiting this equal sex distribution for the proposed trial. All subjects will be in good health, non-smoking persons between the ages of 65 and 95. As outlined previously, all ethnicities, racial groups, and both sexes will be studied. Subjects will be enrolled after confirmation of good health by history and physical exam, clinical laboratory tests (fasting glucose and lipid profile, metabolic profile, complete blood count, Hemoglobin A1C) and 12-lead ECG. The ethnicity of our past study populations generally reflected our local population: 47% Non-Hispanic White, 48% Hispanic, and 5% African American with an equal gender distribution.

Vulnerable Populations: No vulnerable populations will be involved in the proposed protocols.

4.1 Inclusion Criteria

Participants must meet all of the inclusion criteria to participate in this study:

- 65-95 years of age.
- Good health with all chronic diseases (hypertension, coronary artery disease, etc.) clinically stable.
- Selected subjects will be in good health (Per the World Health Organization, good health will be defined as complete physical, mental, and social well-being and not merely the absence of disease or infirmity).
- All diseases or infirmities will be clinically stable whether managed by medications or not.
- CLOX score of 10 or greater

- Women will be postmenopausal
- Postmenopausal women taking hormone replacement will be included if they have been on a stable dose for ≥6 months
- *Participants will have been vaccinated for COVID-19 prior to beginning the treatment course*
- *Participants will live within 20 miles of the UTHSA to facilitate home visits (or may choose to meet in a mutually convenient location within 20 miles of the UTHSA for all home visits)*

4.2 Exclusion Criteria

All candidates meeting any of the exclusion criteria at baseline will be excluded from study participation:

- Diabetes.
- History of skin ulcers or poor wound healing, or keloid formers.
- Smoking (current or within 5 years).
- Current liver disease (acute or chronic).
- Treatment for anticoagulation (coumadin or novel oral anticoagulant).
- Treatment with drugs known to affect cytochrome P450 3A (diltiazem, erythromycin, etc. - due to role in rapamycin metabolism).
- Treatment with an immunosuppressant (prednisone, etc.) within the 6 months.
- History of recent (within 6 months) Myocardial Infarction or active Coronary Disease.
- Hypersensitivity to rapamycin or petrolatum (ointment vehicle).
- Tattoo or scar tissue on the forearm in the application area.

4.3 Study Enrollment Procedures

Healthy human volunteers of normal cognitive function capable of consenting will be consented as study participants. Since the effectiveness of some age-modifying drugs (i.e., RAPA) can become more evident with age (Fig. 1B), we will recruit test subjects ranging from 65 to 95 years old. Subjects will be non-smokers and will be instructed to continue taking any currently prescribed medications. Female subjects will be postmenopausal. All ethnicities and races will be included. Prior to initiating treatment, potential subjects will be screened for exclusions, including serious co-morbidities, by medical history, clinical labs, and physical examination including EKGs. Both male and female subjects will be recruited. This is critically important as the age-modulating drugs often show greater efficacy in one sex. For example, RAPA was shown to extend lifespan to a greater extent in female mice than in males while acarbose showed the reverse [4, 29]. Thus, our goal is to recruit 75 subjects to ensure that there will be 40 "completers", approximately half female and half male. Towards this end, we will use the Subject Recruitment Call Center established and operated by the Research Core-2 (RC2) of the San Antonio OAIC (Pepper Center; Dr. Kellogg, RC2 co-leader). This center has been spectacularly successful in meeting subject recruitment goals for clinical trials of age modulating agents and has registered over 1800 persons who are willing to participate in future human trials.

Other instruments for subject recruitment may also be used. These include: i) the national online registry at https://www.researchmatch.org, ii) UT Health's Find-A-Study webpage at https://vpr.uthscsa.edu/findastudy/; and iii) the Barshop Clinical Trials webpage at https://barshopinstitute.uthscsa.edu/clinical-trials/; iv) ads may be placed in the San Antonio Express News or other local periodicals read by the target population, and v) ads may be placed on Facebook and/or other social media sites.

Once subjects have been appropriately consented and cleared for participation, each individual will be given two Topi-CLICK® containers of ointment and complete instructions on the application protocol. Initially, we will test 1 concentration of the drug with a "vehicle only" (negative control, petrolatum) applied to the opposite arm. Importantly, since each subject will serve as his/her own control, no separate placebo group will be needed.

4.4 Subject Recruitment and Retention

Subject recruitment will take place through the Subject Recruitment Call Center established and operated by the San Antonio Claude D. Pepper Older Americans Independence Center (SA OAIC) Research Core-2 (RC2, Dr. Kellogg Core Co-Leader). RC2 developed the "Call Center and Participant Registry" for efficient subject recruitment. The public is made aware of the Call Center through advertisements placed by the SA OAIC in local newspapers, websites, social media, or other online resources, on broadcast media, and through local presentations to civic groups. The Center has one primary phone line that receives phone calls from potential participants. Potential subjects are asked for permission to enter demographic and preliminary clinical data that are subsequently entered into a REDCap based registry. The Call Center is active 24 hours a day, 7 days a week, and is the main point of entry for participants for aging studies such as the present proposal. RC2 staff determine each caller's study suitability based on the caller's interest, initial screening information, and study eligibility criteria (which can vary among ongoing studies). This subject database currently has records of 1811 persons who are willing trial participants. The REDCap database of potential subjects will be queried for the proposed trial as the major source of recruitment, significantly reducing costs for external advertising. Enrolled subjects will be seen at in-person monthly Visits 1-6 in their homes (or at an alternate convenient site) and contacted bi-weekly by the study team to facilitate retention and to confirm treatment compliance. Visits 4 and 6 may be conducted by phone with those subjects who have demonstrated their strict compliance to protocol (in Visits 2 and 3) and have experienced no adverse effects.

5 STUDY INTERVENTION

5.1 Interventions, Administration, and Duration

The selection of topical application of 8% RAPA in petrolatum is based on our preliminary data. Our preliminary data indicate that topical 8% RAPA ointment:

a) produces local effects (reduced EC50 for endothelium-dependent vasodilator response to methacholine in older, but not younger, persons; it also led to keloid regression);

b) produces detectable intradermal RAPA levels (as measured with intradermal microdialysis);

c) is without local or systemic adverse reactions; and

d) this concentration of RAPA ointment produces no detectable systemic RAPA levels.

These preliminary results support our view that this treatment is safe for use in older humans.

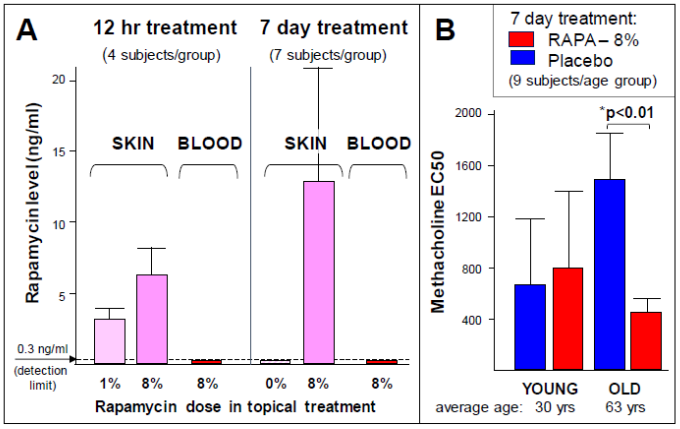

FIGURE 2. Efficacy of topically applied RAPA ointment in human subjects.

Panel A: RAPA levels in skin over time for sites treated twice daily with ointment containing 0% RAPA (vehicle only control), 1% RAPA, or 8% RAPA and in blood for the same time points.

Panel B: Vasodilation was measured in subjects who had treated one skin site with 8% RAPA ointment (red) and a second site with the ointment vehicle only (blue - placebo) for 7 days. The two treatment sites were then instrumented for intradermal microdialysis and increasing doses of a vasodilator, methacholine, were delivered. Skin vascular responses were measured by laser-Doppler flowmetry. EC50s (the concentration of methacholine that elicited half-maximal responses) are shown. RAPA significantly decreased these values on older persons, but had no effect on younger subjects.

To validate the proposed technique, RAPA was chosen as our initial test agent; it has been found to extend lifespan in laboratory animals [4], be transdermally deliverable with topical application (see [24] and Fig. 2), improve epigenetic aging signatures in mice [30, 31], and restore some physiological functions in animal models when systemically delivered [7, 18-23, 32]. Moreover, we and others have shown that RAPA can be used with relative safety in older humans, even with systemic delivery [1, 5, 6]. In preliminary feasibility studies, RAPA ointments (1% or 8%) were applied to forearm skin sites and RAPA delivery into the skin interstitial space was assessed by placing an intradermal microdialysis probe at each site and then measuring the drug levels attained in the collected interstitial fluid by HPLC-mass spec. As shown in Figure 2A, significant RAPA levels (11.5±4.0 ng/ml) were seen with a 7 day course of the 8% RAPA ointment; while a 1% dose yielded much lower values. Simultaneous blood sampling after 1 week of 8% RAPA dosing showed undetectable RAPA in the serum; thus, the treatment should be efficacious locally without having systemic consequences.

5.1.1 Compounded Investigational Product

5.1.1.1 Rapamycin powder (from a supplier to Doyle's pharmacy)

Molecular formula: $C_{51}H_{79}NO_{13}$

Synonyms: Rapamune, sirolimus

PubChem Substance ID 57654583

CAS No. 53123-88-9

An FDA-approved macrocyclic triene antibiotic forms a complex with FKBP12 that binds to and inhibits the molecular target of rapamycin (mTOR). Rapamycin (RAPA) is a potent immunosuppressant and has anticancer activity.

i) Mechanism of Action

The RAPA oral formulation developed by Pfizer (RAPAMUNE, sirolimus) inhibits T lymphocyte activation and proliferation that occurs in response to antigenic and cytokine (Interleukin [IL]-2, IL-4, and IL-15) stimulation by a mechanism that is distinct from that of other immuno-modulators. In cells, sirolimus binds to the immunophilin, FK Binding Protein-12 (FKBP-12). The sirolimus: FKBP-12 complex has no effect on calcineurin activity. This complex binds to and inhibits the activation of the mammalian Target Of Rapamycin (mTOR), a key regulatory kinase. This inhibition suppresses cytokine-driven T-cell proliferation, inhibiting the progression from the G1 to the S phase of the cell cycle. Dosage forms and strengths include: oral solution (60mg/60mL in amber glass bottle) and oral tablets (0.5, 1, and 2mg).

ii) Safety

From oral administration, serious allergic reactions identified by the manufacturer include:

- swelling of face, eyes, or mouth
- trouble breathing or wheezing
- throat tightness
- chest pain or tightness
- feeling dizzy or faint
- rash or peeling of skin

In previous human studies cited earlier in this protocol, topical administration did not produce systemic or serious reactions. Potential Risks due to topical RAPA include local skin rash or irritation; the drug does not reach detectable levels systemically. Participants will be instructed to notify the research team and get help right away if any of the above symptoms of an allergic reaction occur.

iii) Storage

The powder is packaged in a glass bottle with instructions to keep container tightly closed in a dry and well-ventilated place.

5.1.1.2 Petrolatum (Vaseline, vehicle only)

i) Mechanism of Action

Petrolatum is a pale yellow to yellow-colored, translucent, soft unctuous mass. It is odorless, tasteless, and not more than slightly fluorescent by daylight, even when melted. Petrolatum is an inert material with few incompatibilities.

ii) Safety

Petrolatum is mainly used in topical pharmaceutical formulations and is generally considered to be a nonirritant and nontoxic material. Animal studies, in mice, have shown petrolatum to be nontoxic and non-carcinogenic. Although petrolatum is generally nonirritant in humans following topical application, rare instances of allergic hypersensitivity reactions have been reported, as have cases of acne, in susceptible individuals following repeated use on facial skin. However, given the widespread use of petrolatum in topical products, there are few reports of irritant reactions. The allergic components of petrolatum appear to be polycyclic aromatic hydrocarbons present as impurities. The quantities of these materials found in petrolatum vary depending upon the source and degree of refining. Hypersensitivity appears to occur less with white petrolatum and it is often the preferred material for use in cosmetics and pharmaceuticals.

iii) Storage

Petrolatum is an inherently stable material owing to the unreactive nature of its hydrocarbon components; most stability problems occur because of the presence of small quantities of impurities. On exposure to light, these impurities may be oxidized to discolor the petrolatum and produce an undesirable odor. Petrolatum should not be heated for extended periods above the temperature necessary to achieve complete fluidity (approximately 70°C/158°F). When heated to decomposition it emits acrid smoke and irritating fumes.

Petrolatum may be sterilized by dry heat. Petrolatum should be stored in a well-closed container, protected from light, in a cool, dry place.

iv) Regulatory Status

GRAS listed. Accepted for use in certain food applications in many countries worldwide. Included in the FDA Inactive Ingredients Database (ophthalmic preparations; oral capsules and tablets; otic, topical, and transdermal preparations).

5.1.2 Packaging

Drug and placebo will be supplied by the compounding pharmacy in a metered dispenser called the Topi-CLICK® container. The opaque Topi-CLICK® dispenses 0.25 mL of ointment, protects the product from light exposure, and serves to conceal any subtle differences between placebo and investigational product.

Drug will be labeled as an investigational drug as required by law:

"Caution: New **Drug** — Limited by Federal (or United States) law to **investigational** use."

5.2 Handling of Study Interventions

The 8% rapamycin and vehicle-only ointments will be compounded and purchased from Doyle's Professional Pharmacy who have experience compounding rapamycin for topical application in human clinical trials. All the rapamycin ointment and the placebo (petrolatum only) ointment will be provided to participants in Topi-CLICK® containers, a pump bottle for use in clinical trials that dispense 0.25ml per depression of the pump actuator. Potency studies show that the 8% rapamycin in petrolatum retains 96.8% potency after 60 days when stored at room temperature. Dr. Kellogg holds a current IND (#144448) for topical rapamycin ointment used in another trial. The FDA determined that the current study of topical rapamycin was "exempt."

Study drug will be shipped to and stored in a Research Pharmacy and dispensed to participants by the research pharmacy pharmacist. The study team will maintain records of drug accountability.

NOTE: RAPA 8% ointment retains 96.8% potency after 60 days at room temperature, as documented in IND 144448 under Dr. Kellogg.

5.3 Concomitant Interventions

5.3.1 Allowed Interventions

- For female postmenopausal participants, hormone replacement therapy use is permitted if the subject has been on a stable dose for at least 6 months.

5.3.2 Prohibited Interventions

- Systemic steroid or immunosuppressant therapy
- Body lotions, astringents, or other skin applications including cosmetics and especially sunscreen to the targeted skin area(s)
- Other antifungal, antibiotic, or topical preparations

5.3.3 Drug interactions

In previous human studies cited earlier in this protocol, topical administration did not produce systemic or serious reactions. Participants will be instructed to notify the research team and get help right away if any of the above symptoms of an allergic reaction occur.

Drug interactions with systemic use include: Inducers of CYP3A4 and P-gp may decrease sirolimus concentrations whereas inhibitors of CYP3A4 and P-gp may increase sirolimus concentrations. Avoid concomitant use of sirolimus with strong inducers (e.g., rifampin, rifabutin) and strong inhibitors (e.g., ketoconazole, voriconazole, itraconazole, erythromycin, telithromycin, clarithromycin) of CYP3A4 and P-gp. Avoiding consumption of grapefruit juice is also cited.

5.4 Adherence Assessment

Subjects will be contacted monthly either in person or by phone (Visits 2-7) to verify compliance. In addition, subjects will be called approximately 14 days after Visit 1 to ensure that they have been following the application directions and have no questions or potential adverse events. Subjects also receive a drug diary to help record administrations and any notable effects, which supports reporting the participant's experiences at phone follow-ups and clinic visits.

Each Topi-CLICK® container will be weighed before being given out and at collection (after 2 months) to quantify compliance.

6 STUDY PROCEDURES

6.1 Schedule of Activities

VISIT # (Month #)	V0	V1 (0)	V2 (1)	V3 (2)	V4 (3)	V5 (4)	V6 (5)	V7 (6)
Visit goal	**Consent/ Screen**	**Pre-drug**	**RAPA/ Placebo**	**RAPA/ Placebo**	**RAPA/ Placebo**	**RAPA/ Placebo**	**RAPA/ Placebo**	**RAPA/ Placebo**
SCREENING PROCEDURES								
Consent & CLOX1=10+	**X**							
RAPA/CONTROL OINTMENTS								
60 day supplies of study drug		**X**		**X**		**X**		
SAFETY / ADVERSE EVENTS / METABOLIC 9								
Physical Exam	**X**							**X**
ECG/EKG	**X**							
Self-Reported AEs			**X**	**X**	**X**	**X**	**X**	**X**
CBC	**X**							
Metabolic profile, fasting blood, sugar/lipids, HbA1c	**X**							
RAPA blood levels								**X**
SUCTION BLISTER COLLECTIONS								
Blister fluid collection for inflammatory mediators								**X**
Blister roof collection for DNA methylation analysis								**X**

6.2 Schedule of Evaluations

6.2.1 Visit 0 (60 to 90 minutes)

6.2.1.1 Consenting Procedure

Before any screening procedure is performed, informed consent must be obtained. Eligible subjects will be asked to come to the research area to review and sign the consent form. Staff conducting the consent process adhere to local standard operating procedure (SOP) for consent administration. When scheduling consent appointments, staff will ask participants to arrive fasting.

Participants will be asked at each visit whether they would like to continue participation in this study.

6.2.1.2 Screening /Baseline

Following consent and signature, participants will undergo minimal cognitive screen using the CLOX1 test to ensure consent is valid. The CLOX test is a clinical tool widely used in screening for cognitive disorders and dementia, which involves a clock drawing task designed to elicit executive impairment and discriminate it from non-executive failure. Study staff will:

- Obtain vital signs, height and weight, lab work (fasting) to ensure safety and rule out systemic infection (complete blood count [CBC], comprehensive metabolic panel [CMP], lipid panel, HbA1C content)
- Electrocardiogram (ECG/EKG)
- Document medical history, concomitant medication review, physical examination, and
- Perform evaluation of eligibility criteria listed in Sections 4.1 – 4.2 above.

Additional baseline activities and data collection to include:

- Current and recent medications within past 6 months
- Verification of application sites of study drug, RAPA/placebo

6.2.2 Visit 1 (Less than 60 minutes)

- Verify participant is still willing to participate in study
- Explain how to apply medication and possible side effects to monitor.

  Use of the study drug

  Each ointment will be applied to a 10 cm^2 area of skin on the forearm with randomization among subjects of which side (left vs. right) receives RAPA vs. placebo to eliminate any positional bias. Importantly, each subject will be his/her own control. To permit double-blinding while avoiding treatment confusion, dispensers will be color coded and subjects will have the skin sites similarly colored using surgical markers. The initial application will be done for subject education; thereafter, the subjects will apply the compounds daily and refresh the pen outlines as needed. Fresh Topi-CLICK® containers will be given to each subject on a bi-monthly basis.

After ointment application, subjects will cover the site with hypo-allergenic bandage to prevent ointment removal or soiling of clothing. Subjects will be instructed to avoid cross-contamination between forearm sites by washing hands between applications and using new disposable finger cots (glove-like, but cover only the finger) with each application. Subjects will be contacted/visited monthly to verify compliance. Topi-CLICK® containers will be weighed before they are given to participants and at collection (after two months) to quantify compliance and the amounts used.

- Dispense study drug, two color coded Topi-CLICK® containers.
- Phone calls will be made to follow up with subjects regarding adherence or AEs approximately 14 days after Visit 1.

6.2.3 Visit 2 (Less than 60 minutes)

- Verify participant is still willing to participate in study

6.2.4 Visit 3-6 (Each less than 60 minutes)

- Verify participant is still willing to participate in study
- Study drug will be dispensed at Visit #3 and #5 (60-day supply)
- For subjects who have demonstrated outstanding compliance and have experienced no adverse events, Visits 4 and/or 6 may be replaced by phone calls.

6.2.5 Visit 7 (up to 4 hours)

- Verify participant is still willing to participate in study
- Physical exam
- Self-reported AEs
- Rapamycin blood levels
- Suction blister fluid collection
- Suction blister skin flap collection

Process for induction of a suction blister

Blisters are induced at the sites of rapamycin/placebo administration on the subjects by means of a negative pressure cutaneous suction method. The Negative Pressure Instrument System is a commercially available self-contained instrument package that combines all the necessary elements to successfully create suction blisters on a patient's skin. The blisters are created through the use of suction chambers that are attached to the patient's skin. The instrument console contains the power source, vacuum pump, temperature controls and all related controls to operate multiple suction chambers. The suction chambers are connected to the console by a flexible connection. Each of the chambers is controlled by a preset temperature control to provide an optimal skin warming temperature. Both chambers share an adjustable common vacuum source that affects all chambers equally.

1) Assemble the suction device.
2) Disinfect the orifice plate and the skin of the volunteer using alcohol swabs.

3) Attach the suction device within the treatment area on each forearm, by loosely securing the chamber with the provided straps. The chamber will adhere to the skin and maintain its position once negative pressure is applied.
4) Turn the suction device on and adjust the pressure to -20kPa (150mmHg)
5) After 30 minutes, increase the negative pressure to -25kPa (187.5 mm Hg), and then to -30kPa (225 mmHg) after 60 minutes. Keep the pressure at -30kPa (225 mm Hg) until a blister is fully formed. The blister induction phase ranges from 60-180 minutes.
6) Once the blister is fully formed, release the pressure, and carefully remove the suction chamber whilst maintaining integrity of the blister.
7) Note the time taken for blister induction.

Harvesting the suction blister fluid and blister roof

1) Using a sterile 1-3 ml syringe with a 23G-26G needle, insert the needle into the top/lateral side of the blister roof and slowly aspirate the fluid, avoiding touching the floor of the cavity. Withdraw as much fluid as possible, and transfer to a sterile microfuge tube.
2) Remove the blister roof using a sterile scalpel or sterile surgical scissors and store at -80°C for subsequent nucleic acid isolation.
3) Apply an antibiotic ointment (such as Neosporin) and a band aid to the blister, as long as the subject does not have any allergic reaction to the antibiotic ointment. The blister should heal within a week, no anesthesia is required for this procedure.

7 SAFETY ASSESSMENTS

Participant safety will be monitored once an individual is enrolled in the study. Dr. Kellogg will be responsible for ensuring the first-hand monitoring of the data and safety of study participants on a monthly basis. He will be assisted by other members of the study staff.

7.1 Specification of Safety Parameters

Protection against risk is paramount in our research and in the operation of our laboratory. The risks of the proposed studies are extremely small. This is by design and reflects the history and philosophy of the laboratory. All of our proposed studies are reviewed and approved by our IRB prior to any studies in the clinic or laboratory. All volunteers will be in good health, as documented by a medical history and physical examination performed by overseeing clinical staff. Volunteers will be queried using minimally invasive methods. Antibiotic ointment and gauze are used to cover and protect the area of suction blister formation. In the unlikely event of an adverse effect, appropriate medical procedures will be undertaken. There are no known social, legal, or psychological risks. At the time of collection, all data will be de-identified and stored that way so that there is no risk to the privacy of individuals or confidentiality of data. Data are stored in electronic form behind institutional electronic firewalls, or in physically secured laboratory facilities that are patrolled by armed police forces. These procedures have adequately protected our data for decades and we believe that they will continue to be highly effective in the future. To minimize infection risk at suction blister sites, all subjects will have

the sites treated with topical antibiotic ointment and covered with a bandage or gauze. They will be provided with antibiotic ointment and bandages for use at home until the sites have healed. Any participants with an allergy to RAPA and persons with such sensitivity are excluded. Risks are minimized by the monitoring procedures we use, the selection of methods, and by the monitoring of adverse events and by using minimally invasive harvesting techniques for fluid aspiration and blister roof excision.

7.2 Adverse Events and Serious Adverse Events

Adverse Event (AE): In general, AE is used very broadly and encompasses physical and psychological harms and includes:

Any untoward or unfavorable medical occurrence in a human subject, including any abnormal sign (for example, abnormal physical exam or laboratory finding), symptom, or disease, temporally associated with the subject's participation in the research, whether or not it is considered related to the subject's participation in the research.

Intercurrent illnesses or injuries should be regarded as adverse events. Abnormal results of diagnostic procedures are considered to be adverse events if the abnormality:

- results in study withdrawal
- is associated with a serious adverse event
- is associated with clinical signs or symptoms
- leads to additional treatment or to further diagnostic tests
- is considered by the investigator to be of clinical significance

Serious Adverse Event (SAE): Any adverse event that:

- Results in death
- Is life threatening, or places the participant at immediate risk of death from the event as it occurred
- Requires or prolongs hospitalization
- Causes persistent or significant disability or incapacity
- Results in congenital anomalies or birth defects
- Is another condition which investigators judge to represent significant hazards

7.3 Unanticipated Problems Involving Risk to Subjects or Others (UPIRSO)

Any incident, experience, or outcome that meets all of the following criteria:

- Unexpected in nature, severity, or frequency (i.e. not described in study-related documents such as the IRB-approved protocol or consent form, the investigators brochure, etc.)
- Related or possibly related to participation in the research (i.e. possibly related means there is a reasonable possibility that the incident experience, or outcome may have been caused by the procedures involved in the research)
- Suggests that the research places subjects or others at greater risk of harm (including physical, psychological, economic, or social harm).

7.3.1 Adverse Event Reporting Period

The study period during which adverse events must be reported is normally defined as the period from the initiation of any study procedures to the end of the study treatment follow-up. For this study, the study treatment follow-up is defined as 30 days following the last administration of study treatment.

7.3.2 Pre-existing Condition

A preexisting condition is one that is present at the start of the study. A pre-existing condition should be recorded as an adverse event if the frequency, intensity, or the character of the condition worsens during the study period.

At screening, any clinically significant abnormality should be recorded as a preexisting condition. At the end of the study, any new clinically significant findings/abnormalities that meet the definition of an adverse event must also be recorded and documented as an adverse event.

7.3.3 Abnormal Laboratory Values

A clinical laboratory abnormality should be documented as an adverse event if any one of the following conditions is met:

- The laboratory abnormality is not otherwise refuted by a repeat test to confirm the abnormality
- The abnormality suggests a disease and/or organ toxicity

The abnormality is of a clinically significant degree requiring active management; e.g. change of dose, discontinuation of the drug, more frequent follow-up assessments, further diagnostic investigation, etc.

7.3.4 Hospitalization, Prolonged Hospitalization or Surgery

Any adverse event that results in hospitalization or prolonged hospitalization should be documented and reported as a SAE unless specifically instructed otherwise in this protocol. Any condition responsible for surgery should be documented as an AE if the condition meets the criteria for and adverse event.

Neither the condition, hospitalization, prolonged hospitalization, nor surgery are reported as an AE in the following circumstances:

- Hospitalization or prolonged hospitalization for diagnostic or elective surgical procedures for a preexisting condition (e.g., colonoscopy, SCI annual evaluation, respite care).
- Surgery should not be reported as an outcome of an adverse event if the purpose of the surgery was elective or diagnostic and the outcome was uneventful.
- Hospitalization or prolonged hospitalization required to allow efficacy measurement for the study.
- Hospitalization or prolonged hospitalization for therapy of the target disease of the study, unless it is a worsening or increase in frequency of hospital admissions as judged by the clinical investigator.

7.4 Recording of Adverse Events

At each contact with the subject, the investigator or study staff will seek information about adverse events by specific questioning and, if appropriate, by examination. Information on all AEs will be recorded immediately in the source documentation. Site staff will record AEs using the appropriate data collection form in REDCap, which will be exported as an AE Log (See Section 9.3) for periodic review, at least monthly and ad hoc, depending on severity and expected/unexpected nature of the event.

The clinical course of each event will be followed until resolution, stabilization, or until it has been determined that the study treatment or participation is not the cause. SAEs that are still ongoing at the end of the study period will be followed up to determine the final outcome. Any SAE that occurs after the study period and is considered to be possibly related to the study treatment or study participation will be recorded and reported per Institutional policy and according to FDA requirements.

7.6 Reporting of Serious Adverse Events and Unanticipated Problems

SAEs and UPIRSOs will be reported per local IRB policy and procedure.

Each subject is evaluated for any adverse events (AE). Any event that is reported to either the principal investigator or designated research staff by either the subject or medical staff caring for the subject and which meets the criteria will be documented. Any AE reported as serious will necessitate an adverse event report, which will be submitted to the IRB and to the Pepper Center DSMB.

Unanticipated risks to subjects or others (UPIRSO) that are a result of study participation are promptly reported to the IRB and, if deemed appropriate, to the Pepper Center DSMB. The report will include a description of the event, when and how it was reported, as well as any official chart records or documentation to corroborate the event or the reporting of the event. All adverse events will be graded as mild, moderate, or severe. All adverse events will be summarized annually and submitted to the IRB. Any action resulting in a temporary or permanent suspension of this study (e.g. local site IRB actions) will be reported to FDA or drug manufacturer per IRB stipulations.

7.6.1 Investigator Reporting: notifying DSMB

Any study-related SAE-UPIRSO, must be reported to the Principal Investigator by telephone within 24 hours of the event. To report such events, a Serious Adverse Event (SAE) form must be completed and submitted within 24 hours. The investigator will keep a copy of this SAE form on file at the study site.

Report SAEs by email and facsimile to:

Dean L. Kellogg, Jr, MD, PhD
Professor, Medicine-Geriatrics
Email: kelloggd@uthscsa.edu
210-617-5132 fax 210-235-3681 pag

Within the following 48 hours, the Principal Investigator provides further information on the SAE or the UPIRSO in the form of a written narrative. This should include a copy of the completed SAE form, and any other diagnostic information that will assist the understanding of the event.

7.6.2 Investigator reporting: notifying the UTHSCSA IRB

Notifying the IRB and or FDA if SAE or UPIRSO

- ***Within 7 calendar days***
  Any study event that is:
  - associated with the use of the study drug
  - unexpected,
  - fatal or life-threatening, and

- ***Within 15 calendar days***
  Any study event that is:
  - associated with the use of the study drug,
  - unexpected, and
  - serious, but not fatal or life-threatening

    -or-
  - a previous adverse event that was not initially deemed reportable but is later found to fit the criteria for reporting (reporting within 15 calendar days from when event was deemed reportable).

  Any finding from tests in laboratory animals that:
  - suggests a significant risk for human subjects including reports of mutagenicity, teratogenicity, or carcinogenicity.

Additional reporting requirements

Post marketing surveillance and adverse events may be submitted on FDA Form 3500A or in a narrative format. The contact information for submitting safety reports is noted below:

Food and Drug Administration
Center for Drug Evaluation and Research
Division of Dermatology and Dental Products (DDDP)
5901-B Ammendale Road
Beltsville, MD 20705-1266
Phone: (301) 796-2290
Fax: (301) 796-9712

7.7 Medical Monitoring

The Investigator and or Co-PI will review the safety and progress of this study on a monthly basis or when needed if SAE or SAE-UPIRSO occurs.

7.8 Investigator Reporting of Protocol Deviations/Violations

Departures during the conduct of a research study constitute a protocol deviation, violation or exception and as such must be reported to the UTHSCSA IRB.

Tracking and reporting of protocol deviations and violations to the IRB is the responsibility of the PI. To determine whether deviations or violations require prompt reporting or other action, refer to the IRB document entitled "Decision Tree – Evaluating Departures" on the IRB website. Failure to report departures from the protocol according to IRB policy may constitute possible non-compliance, which will require a Prompt Report Form and possible FDA reporting by IRB.

7.8.1 Deviations and violations may be identified in a number of ways including:

- A report by an individual can be made directly to the IRB Office.
- The IRB may learn of event through its continuing review of ongoing research.
- Compliance reviews (audits) conducted by the Office of Regulatory Affairs and Compliance or one of the HSC affiliated institutional compliance offices.
- A report by an individual can be made directly to the Office of Regulatory Affairs and Compliance (Hotline) or one of the HSC affiliated institutional compliance offices.
- A report by another committee, department, institution, or official.
- An audit or report from the study sponsor or sponsor's monitoring entity.

7.8.2 Definitions of Protocol Deviations/Violations

- Protocol deviations – such as out of window visit, missed lab, usually recognized after the fact, etc.
- Protocol violations – enrolling an ineligible participant, using wrong consent version, willful act of not following protocol
- Emergency violations Refer to UTHSCSA IRB Policy website: https://research.uthscsa.edu/irb/policy/deviations for more information

7.9 Safety Monitoring

Data Safety Monitoring Plan (DSMP): Our studies are designed to explore both the responses to topical RAPA on DNA methylation (the Skin Epigenetic Clock) and the altered mechanisms involved in inflammation. Dr. Kellogg will be responsible for ensuring the first-hand monitoring of the data and safety of study participants on a weekly and monthly basis. He will be assisted by other members of the study staff. A REDCap database will be designed to include an inclusion/exclusion checklist that is reviewed and signed by Dr. Kellogg for each subject enrolled before study medications are ordered by the investigators.

The San Antonio Claude D. Pepper Older Americans Independence Center Data Safety Monitoring Board (DSMB) will be responsible for reviewing clinical trial data from the proposed study on an ongoing basis to ensure the safety of study subjects. This DSMB was created by the SA OAIC under the auspices of the SA OAIC Research Core 2 (RC2) to ensure subject safety and data integrity for studies involving older persons Board members are independent, with no vested interest in this study. The DSMB will assign a staff member to conduct quarterly assessments for data quality control/assurance on collected data, which is reviewed on an annual basis by the Pepper Center Regulatory Coordinator and PI at the time of

preparing continuing review documentation for IRB submission. This study will be reviewed by the Pepper Center (DSMB) at least annually. The Pepper Center DSMB meets 3-4 times a year, by teleconference call, to review study progress and participants' safety.

8 INTERVENTION DISCONTINUATION

Should a subject develop a rash during study intervention, RAPA medication will be stopped and their participation will cease.

In the unlikely event that a study-related death or SAE occurs, the decision to stop the trial, either temporarily or permanently, will be the collaborative responsibility of the Pepper Center DSMB and the Principal Investigators.

9 STATISTICAL CONSIDERATIONS

9.1 General Design Issues

The intent of this pilot project is to validate the novel approach of examining age-modulating effects of pharmacological agents in humans, in vivo, through local transdermal drug application thus avoiding the risks of systemic drug administration. We propose to study at least 40 older persons, age 65-95. We will study at least 20 women and 20 men. The test agent for the project will be 8% rapamycin (RAPA) ointment with petrolatum-only ointment as a control agent. Ointment treatments will be for 6 months. The duration derives in part from our unpublished observation that 8 months of topical 8% RAPA ointment caused significant regression of an established keloid in a 63 year old person. At present, there are no other in vivo data relevant to power analyses for our proposed project; however, recently published in vitro work by our collaborator, Dr. Horvath, suggests that our approach may be viable in vivo. In that study, Horvath and colleagues examined the in vitro effects of rapamycin on keratinocytes obtained from 3 different persons and found that rapamycin retarded keratinocyte aging independently from its effects on replicative senescence, proliferation rate or frequency, and differentiation (Horvath, Lu et al. 2019). They also found that rapamycin inhibited cellular senescence, as well as epigenetic aging. Our proposed trial will recapitulate the foregoing in vitro keratinocyte study design with substitution of in vivo treatment of keratinocytes in humans, in lieu of in vitro cell culture approach. Instead of samples from 3 different persons, we will study 50 different persons, half women and half men. Our study uses a "within-subject" design where each person will be his/her own control and thus increase statistical power. In our pilot trial, we are interested in precise estimates of feasibility and acceptability, as well as outcome variability that will aid in the planning of future sufficiently powered efficacy trials.

9.2 Sample Size and Randomization

A sample size of 40 persons with experimental (RAPA) and control samples from each person will allow us to be relatively precise in our conclusions regarding feasibility outcomes.

9.2.1 Treatment Assignment Procedures

Participants are both the active comparator and control arm for the study with one forearm assigned as the active site and the other the control site. Randomization codes will be assigned to each subject for active study drug and placebo. Each participant receives color-coded Topi-CLICK® containers of the study drug, which will be assigned to the same color-coded patch on the corresponding forearm.

9.3 Interim analyses and Stopping Rules

The Sponsor, Dr. Kellogg, has the authority to stop patient participation, suspend accrual or stop the study in its entirety at any time for safety. (in particular any rash or skin reaction will stop that subject's experiment.) Dr. Kellogg will report all events (internal / external) to the UTHSCSA Pepper Center DSMB and IRB per the UPIRSO reporting criteria. UPIRSOs being submitted promptly and non-UPIRSOs reported at minimum annually.

As described above, if an individual event or accumulation of events should occur that warrant stopping the study, this will be done by Dr. Kellogg and DSMB. Dr. Kellogg and DSMB will review safety summary reports and they will be reported to the IRB with progress reports or more promptly if they meet UPIRSO criteria.

9.4 Primary Objective

Test whether epigenetic changes in skin are elicited by topical treatments with RAPA

Methylation of cytosine residues in DNA, particularly when found in CpG islands, is often associated with silencing of downstream genes. Dr. Horvath, a collaborator on this project, has identified a set of 391 DNA methylation marks that change with aging and can be used to assess the age of an individual [34, 35]. His novel “epigenetic clock” has been used to predict “time to death” and parameters of healthy aging. To determine the effects of topical RAPA on the epigenetic clock, DNA will be extracted from the blister skin flaps (keratinocytes) taken after treatment from the two sites on each subject using Qiagen DNeasy kits. The genomic DNA will be sent to the University of Minnesota Genomics Center for analysis on Infinium Methylation EPIC arrays. The data will be analyzed by Dr. Horvath; he will compare the profiles seen with RAPA to the profiles seen at the placebo site for each individual focusing on the methylation marks that comprise the aging clock.

9.5 Secondary Objectives

Test whether baseline inflammation is affected by topical RAPA

In order to examine the effect of topical RAPA (or any future test drug) on the inflammatory milieu in skin, Luminex-based assays will be performed on the interstitial fluid collected from suction blisters at the end of the 6 month treatment period. These allow simultaneous

measurement of ≥29 different human cytokines/chemokines from as little as 30µl. It is anticipated that pro-inflammatory cytokines (i.e., IL-12, IL-6, TNF-α) would be elevated in this older population, so a decrease in their levels upon treatment with RAPA (but not the vehicle) would support an anti-aging potential. Since inflammation underlies many age-associated pathologies, this finding would have broad implications. For this reason, we will also perform sensitive ELISAs for three analytes known to play a functional role in regulating age-associated inflammatory phenotypes. These will include: i) interleukin-6 (IL-6); although present in the Luminex panel, IL-6 detection often requires a more sensitive ELISA [1]; ii) sICAM-1 (soluble intercellular adhesion molecule-1, a biomarker of vascular endothelial cell activation/damage) [36] and iii) RAGE (receptor for advanced glycation end products) [37, 38]. Validated ELISA kits are commercially available for these three analytes and have been tested by us with sera from the subjects in the pilot human trial of oral RAPA (Fig. 4). Sufficient blister fluid will be recovered in order to perform all of the tests outlined including: i) RAPA levels by HPLC-mass spec; ii) Luminex multiplex cytokine arrays; and iii) ELISAs for IL-6, RAGE, and sICAM-1. Remaining fluid will be aliquoted and frozen at -20°C.

9.6 Data Analyses

As pilot studies are preparatory investigations that provide specific information necessary for planning subsequent definitive trials. A major purpose of the present proposal is to perfect the study design, measures, procedures, recruitment criteria, and operational strategies for use in larger and/or longer subsequent trials with rapamycin and/or other purported anti-aging agents.

Our project will provide the means to evaluate the technical aspects of our novel approach of testing topical treatments with simultaneous untreated controls within individual subjects. This pilot project will serve as a platform to generate preliminary data and foster new methodological development. Our pilot 'proof of concept' study is designed so that the information gained can be put to optimal use in future studies that build on scientific evidence of the efficacy and reduced costs of our approach.

Our proposed 'proof of concept' trial will contribute to the development and design of future (larger and/or longer) studies by:

- Refining the research hypotheses
- Identifying barriers to successful study completion
- Evaluating acceptability of methods and instruments to participants
- Estimating the time required for study participation
- Providing estimates of missing data and dropout
- Estimating rates and variability in outcomes for future studies
- Testing mechanistic efficacy/ ‘proof of concept’ for future trials

Regarding analyses of inflammatory mediators:

Assay results will be analyzed by 2 factor ANOVA (RAPA vs Placebo as within factor and sex as second factor); power calculations based on our prior studies with systemically administered

rapamycin indicate that N=9 may be needed for those inflammatory mediators we previously found to decrease significantly with systemic RAPA (ICAM-1 and RAGE). Calculations indicate that as many as 141 samples may be needed for others (IL-6) that we have not found to differ significantly with systemic RAPA. These estimates are based on unpaired comparisons between subjects who received systemic RAPA or placebo for 8 weeks. The design for the proposed trial permits each subject to serve as his/her own control and thus should reduce these estimates.

10 DATA COLLECTION AND QUALITY ASSURANCE

10.1 Data Integrity

Data integrity will be assessed by review of the written/computer (REDCap) documents used to record all collected data on a weekly basis. The representational faithfulness (composed of four essential qualities or core attributes: completeness, currency/timeliness, accuracy/correctness and validity/authorization data) of all data to the true state of the measurement that the information represents will be the responsibility of Dr. Kraig, Dr. Kellogg and study coordinator.

10.2 Data Management

Data integrity will be assessed by review of the written/computer documents used to record all collected data on a weekly basis. The representational faithfulness (composed of four essential qualities or core attributes: completeness, currency/timeliness, accuracy/correctness and validity/authorization data) of all data to the true state of the measurement that the information represents will be the responsibility of the Principal Investigators and the study coordinator.

10.3 Quality Assurance

10.3.1 Training

All study personnel interacting with human subjects or with access to PHI will complete all human subjects training to include Good Clinical Practice training.

10.3.2 Quality Control Committee

We will use the Regulatory Office and Data Safety Monitoring Board operated by RC2 to ensure subject safety and that all regulatory aspects of human trials are met.

10.3.3 Study Monitoring Plan

The Principal Investigators will ensure that the designated regulatory coordinator or other quality assurance reviewer is given access to all the above noted study-related documents and study related facilities (e.g. pharmacy, diagnostic laboratory, etc.), and has adequate space to conduct study monitoring visits as assigned.

10.3.4 Monitoring and Auditing

The Principal Investigators will permit study-related monitoring, audits, and inspections by the IRB, the funding sponsor, the OAIC Pepper Center Data Safety

and Monitoring Board (DSMB), government regulatory bodies, and University compliance groups of all study related documents (e.g. source documents, regulatory documents, data collection instruments, study data etc.). The investigators will ensure the capability for inspections of applicable study-related facilities (e.g. pharmacy, diagnostic laboratory, etc.).
Participation as an investigator in this study implies acceptance of potential inspection by government regulatory authorities and applicable University compliance and quality assurance offices.

11 PARTICIPANT RIGHTS AND CONFIDENTIALITY

11.1 Institutional Review Board (IRB) Review

This protocol and the informed consent document and any subsequent modifications will be reviewed and approved by the IRB responsible for oversight of the study. The IRB responsible for review of this study will be the University of Texas Health Science Center San Antonio Institutional Review Board (UTHSA IRB).

11.2 Informed Consent Forms

Consent forms will be IRB approved and the participant will be asked to read and review the document. The investigator will explain the research study to the participant and answer any questions that may arise. A verbal explanation will be provided in terms suited to the participant's comprehension of the purposes, procedures, and potential risks of the study and of their rights as research participants. Participants will be informed that participation is voluntary and that they may withdraw from the study at any time, without prejudice, and that the quality of their medical care will not be adversely affected if they decline to participate in this study. Participants will have the opportunity to carefully review the written consent form and ask questions prior to signing. The participants will be given a copy of the ICF so that they may discuss the study with their family or surrogates or think about it prior to agreeing to participate. The informed consent process will be conducted and documented in the source document (including the date), and the form signed, before the participant undergoes any study-specific procedures. A copy of the signed informed consent document will be given to the participants for their records.

11.3 Participant Confidentiality

Any data, specimens, forms, reports, video recordings, and other records that leave the site will be identified only by a participant identification number (Participant ID, PID) to maintain confidentiality. All records will be kept in a locked file cabinet. All computer entry and networking programs will be done using PIDs only. Information will not be released without written permission of the participant, except as necessary for monitoring by IRB, the FDA, the NIA, and the OHRP.

11.4 Study Discontinuation

The study may be discontinued at any time by the IRB, the NIA, the OHRP, the FDA, or other government agencies as part of their duties to ensure that research participants are protected.

12 ETHICAL CONSIDERATIONS

This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312) applicable government regulations and Institutional research policies and procedures.

This protocol and any amendments will be submitted to the Institutional Review Board (IRB), in agreement with local legal prescriptions, for formal approval of the study conduct. The decision of the IRB concerning the conduct of the study will be made in writing to the investigator and a copy of this decision will be provided to the funding sponsor before commencement of this study.

All subjects for this study will be provided a consent form describing this study and providing sufficient information for subjects to make an informed decision about their participation in this study.

13 COMMITTEES

The San Antonio Claude D. Pepper Older Americans Independence Center Data Safety Monitoring Board (DSMB) will be responsible for reviewing clinical trial data from the proposed study on an ongoing basis to ensure the safety of study subjects and validity and integrity of the data.

14 PUBLICATION OF RESEARCH FINDINGS

Publication authorship will be determined by the relative contributions of the PIs. It is likely that the first paper from this work will demonstrate validity of the topical age-modulating assay using rapamycin as the test drug. In this case, as in our previous paper (1), Dr. Kraig would likely handle much of the bench data analysis and would be first author, while Dr. Kellogg would have overseen all clinical aspects and comprehensive data analysis, so he would be the appropriate senior author. However, if the epigenomic data were used more extensively in a second paper, then Dr. Horvath might be first and the two PIs would have shared senior authorship. We make it a priority to discuss authorship openly before writing the paper(s).

Dissemination Plan

This trial has been registered in ClinicalTrials.gov; Identifier NCT04608448. All consent documents for the trial will include a specific statement concerning ClinicalTrials.gov. Results will be submitted to ClinicalTrials.gov as required. UT Health San Antonio has an internal policy that ensures that clinical trials registration and results reporting occur in compliance with policy requirements. Open access publication of results will also be made as significant results are obtained.

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16 SUPPLEMENTS/APPENDICES