COVFIS-HOME: A PHASE 2 PLACEBO-CONTROLLED PILOT STUDY IN COVID-19 OF FISETIN TO ALLEVIATE DYSFUNCTION AND DECREASE COMPLICATIONS IN AT-RISK OUTPATIENTS

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Table of Contents

PROTOCOL SYNOPSIS
TITLE: COVFIS-HOME: A PHASE 2 PLACEBO-CONTROLLED PILOT STUDY IN COVID-19 OF FISETIN TO ALLEVIATE DYSFUNCTION AND DECREASE COMPLICATIONS IN AT-RISK OUTPATIENTS

INDICATION: Reduction of complications in patients with COVID-19 infection.

OBJECTIVES: The primary objective of this study is to determine whether short-term treatment with Fisetin reduces the rate of death and long-term complications related to COVID-19. The secondary objective is to determine the safety of treatment with Fisetin in this patient population. The exploratory objective is to evaluate links between aging biomarkers and treatment effects.

PATIENT POPULATION: Males and females, at least 18 years of age, who have been diagnosed with a COVID-19 infection and have at least one high-risk criterion.

Inclusion Criteria:

- Males and females, at least 18 years of age, capable and willing to provide informed consent;
- Patient must have received a diagnosis of COVID-19 infection within the last 10 days;
- Outpatient setting (not currently hospitalized);
- Patient must possess at least one of the following high-risk criteria: 60 years or more of age, obesity (BMI $\geq$ 30 kg/m^2), diabetes mellitus, uncontrolled hypertension (systolic blood pressure $\geq$150 mm Hg), known respiratory disease (including asthma, chronic obstructive pulmonary disease, or present or past smoking), known heart failure, known coronary disease, fever of $\geq$38.4°C within the last 48 hours, dyspnea at the time of presentation, the combination of high neutrophil count and low lymphocyte count;
- Female patient is either not of childbearing potential, defined as postmenopausal for at least 1 year or surgically sterile, or is of childbearing potential and practicing at least one method of contraception and preferably two complementary forms of contraception including a barrier method (e.g. male or female condoms, spermicides, sponges, foams, jellies, diaphragm, intrauterine device (IUD)) throughout the study and for 30 days after study completion;
- Patient or their caregiver must be able and willing to comply with the requirements of this study protocol.

Exclusion Criteria:

- Patient currently hospitalized or under immediate consideration for hospitalization;
- Patient currently in shock or with hemodynamic instability;
- Patient with severe hepatic disease (as *per* clinical judgement) or liver enzymes >2x the upper limit of normal;
- Female patient who is pregnant, breast-feeding, or is considering becoming pregnant during the study or for 1 day after the last dose of study medication;
- Patient currently taking Sirolimus, Tacrolimus, or other mTOR inhibitors for other indications (mainly chronic indications represented by organ transplantation or autoimmune diseases);
- On Warfarin therapy; Subjects taking any of the medications listed in Appendix C may participate if they are otherwise eligible AND the medication can be safely held during the following times:

  - Immediately before the 1st IP administration (Day 0) until at least 10 hours after the 2nd IP administration (Day 1)
  - Immediately before the 3rd IP administration (Day 8) until at least 10 hours after the 4th IP administration (Day 9)
- Patient with a history of an allergic reaction or significant sensitivity to Fisetin;
- Patient undergoing chemotherapy for cancer;
- Patient is considered by the investigator, for any reason, to be an unsuitable study candidate.
- History of diverticulitis or diverticulosis with GI bleeding, as per clinical judgement.

STUDY DESIGN: This will be a randomized, double-blind, placebo-controlled study. Following signature of the informed consent form, approximately 300 subjects meeting all inclusion and no exclusion criteria will be consented with a goal of randomizing 150 subjects to receive either Fisetin or placebo (1:1 allocation ratio) on Days 0 and 1 and 8 and 9 (4 doses over 10 days). Follow-up phone contacts will occur at 15 and 30 days following randomization for evaluation of the occurrence of any trial endpoints or other adverse events, with unblended follow-up contacts at Days 60.

OUTCOMES: The primary endpoint will be the rate of complications related to COVID-19 (CoV) disease using a 7 point score adapted from the WHO Ordinal Scale for Clinical Improvement of CoV (Appendix A) in the 30 days following randomization. The secondary endpoint is to determine safety and tolerability of treatment with Fisetin in this patient population. Exploratory objectives are to evaluate links between soluble and genetic markers of senescent cell abundance, SASP factors, inflammation, viral load, and treatment effects and if Fisetin treatment during acute CoV infection decreases Long-hauler syndrome at Days 60.

STATISTICAL RATIONALE AND ANALYSIS: All main analyses in COVFIS-HOME will be conducted on an intention-to-treat basis. Assuming 75 subjects/ group with a significance level of 0.05, the study will have 80% power to detect an odds ratio of 2.24 comparing the placebo- to Fisetin-treated group, using the 7-point severity score at Day 30 as the endpoint in an ordinal logistic regression model.

A fully independent 5-member Data and Safety Monitoring Board (DSMB) will be established and will review unblinded safety data as detailed in the DSMB charter. An interim analysis is planned after approximately 50% of randomized patients have completed 30 days of follow-up.

The DSMB charter will pre-specify the methods of interim efficacy analyses and the rules for early study termination, approved by all board members. The stopping rules for efficacy will be based on the O'Brien-Fleming α-spending function or on a similar conservative approach so that the impact on the final alpha level will be negligible. Futility will be assessed by computing conditional power. The DSMB will have the option of recommending early study termination because of overwhelming efficacy, early termination for futility, or continuation of the trial as planned.

The primary analysis of efficacy will be based on the intent-to-treat principle. The primary endpoint will be compared between the two treatment groups using a chi-square test. Secondary endpoints will be analyzed similarly.

ANTICIPATED TOTAL NUMBER OF RANDOMIZED PATIENTS: Approximately 150 patients in total will be randomized in this study.

STUDY LOCATION: Mayo Clinic Minnesota.

GLOSSARY

AE	Adverse Event/Adverse Experience
AFFIRM	Alleviation by Fisetin of Frailty, Inflammation, and Related Measures Trial
ALT	Alanine Aminotransferase
ARDS	Acute Respiratory Distress Syndrome
AST	Aspartate Aminotransferase
BAP	Biospecimens Accessioning and Processing Laboratory
CFR	Code of Federal Regulations
CK	Creatine Kinase
CoV	Coronavirus-19
CRF	Case Report Form
CTCAE	Common Terminology Criteria for Adverse Events
CVA	Cerebrovascular Accident
D	Dasatinib
DAMP	Damage-Associated Molecular Profile
DSMB	Data and Safety Monitoring Board
ECMO	Extra-Corporeal Membrane Oxygenation
eGFR	Estimated Glomerular Filtration Rate
ESR	Erythrocyte Sedimentation Rate
FDA	Food and Drug Administration
γ-H2.AX	γ-Histone 2.A DNA Damage Foci
GCP	Good Clinical Practice
GCSF	Granulocyte Colony Stimulating Factor
GMP	Good Manufacturing Practice
HIPAA	Health Insurance Portability and Accountability Act
HUVEC	Human Umbilical Vein Endothelial Cell
IB	Investigator's Brochure
IL	(*e.g.*, IL-6) Interleukin (*e.g.*, Interleukin-6)
IND	Investigational New Drug Application
IP	Investigational Product
IPF	Idiopathic Pulmonary Fibrosis
IRB	Institutional Review Board
LAR	Legally Authorized Representative
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemoattractant Protein-1
MHV	Mouse Hepatitis Virus, a Mouse β-Coronavirus
MIP1-α	Macrophage Inflammatory Protein 1-α
miRNA	Micro-RNA
MMP	Matrix Metallopeptidase
MSC	Mesenchymal Stem Cell
NME	Normal Microbial Experience
PAMP	Pathogen-Associated Molecular Profile
PHI	Protected Health Information
PI	Principal Investigator
Q	Quercetin

SA-βGal	Senescence-Associated β-Galactosidase
SAE	Serious Adverse Event/Serious Adverse Experience
SASP	Senescence-Associated Secretory Phenotype
SCAP	Senescent Cell Anti-Apoptotic Pathway
sHLH	Secondary Hemophagocytic Lymphohistiocytosis
SOP	Standard Operating Procedure
TAF	Telomere-Associated DNA Damage Foci
TNF-α	Tumor Necrosis Factor-α
U of M	University of Minnesota
UPIRSTO	Unanticipated Problems Involving Risk to Subjects or Others
WCBP	Women of Childbearing Potential

TIME PERIOD AND NUMBER OF PATIENTS:

A. Anticipated Starting Date of Study: 1Q21
B. Anticipated Completion Date: 4Q22
C. Anticipated Number of Patients per Site: Centralized recruitment and follow-up
D. Anticipated Number of Sites: Mayo Clinic Minnesota. Centralized recruitment and follow-up.

Generic Name	Strength and Dosage Form	Therapeutic Classification
Fisetin	100 mg capsules, 20 mg/kg body weight/ day, as intact capsules or opened with powder mixed into room temperature or chilled drinks or food (*e.g.*, pudding, apple sauce, rice pudding)	Senolytic
	Placebo to match 100 mg capsules	NA

1 INTRODUCTION

1.1 Background

Coronavirus-19 (CoV): Coronaviruses constitute the subfamily *Orthocoronavirinae*, in the family *Coronaviridae*, order *Nidovirales*, and realm *Riboviria*. They are enveloped viruses with a positive-sense single-stranded RNA genome and a nucleocapsid of helical symmetry. They are the largest among known RNA viruses. As of May 5, 2020, there have been at least 25,486 confirmed deaths and more than 3,646,206 confirmed cases in the β-coronavirus-19 (CoV) pandemic. CoV can cause physical dysfunction (frailty) and death from hyper-inflammation, cytokine storm, and associated acute respiratory distress syndrome (ARDS) and myocarditis with troponin leak, particularly in older or chronically-ill individuals[8]. Mouse hepatitis virus (MHV) is a β-coronavirus that is related to CoV, which causes an epidemic murine illness with high mortality, especially among colonies of laboratory mice[9]. We previously found that cellular senescence leads to physical dysfunction (frailty) and hyper-inflammation (cytokine storm), factors that contribute to morbidity and mortality from CoV. We are currently conducting a clinical trial of Fisetin in elderly subjects for these indications, AFFIRM (Alleviation by Fisetin of Frailty, Inflammation, and Related Measures in Older Adults; ClinicalTrials.gov identifier NCT03430037; IND 134052).

Longhauler Syndrome: Initial reports from WHO-China in February described clinical recovery from time of onset of COVID symptoms to be between 2-6 weeks, the latter being patients with more severe cases (https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200306-sitrep-46-covid-19.pdf?sfvrsn=96b04adf_4). As more information becomes available, patients are coming forward with experiences of longer recovery times and lingering symptoms, Long Covid, Long-haul Covid, or Long-haulers[10-12]. Though largely undefined, "Long-haulers" denotes patients who experience persistent symptoms after having acute COVID-19. While the full impact of long COVID is widely unknown, some sources are reporting upwards of 10% of patients experiencing symptoms lasting greater than 3 weeks[12]. An earlier study done in Italy showed that 87% of hospitalized patients reported at least one ongoing symptom 60 days after onset of first COVID-19 symptoms, with 55% of patients reporting 3 or more ongoing symptoms[13]. These long lasting symptoms can occur in adults across all age groups, regardless of pre-existing conditions[14]. The long term sequelae of the virus are largely unknown, but early reports of significant lung damage[15,16] and persistent myocardial inflammation[17] are concerning, making the discovery of long term impact at the mechanistic level all the more urgent.

Cellular Senescence: Cellular senescence contributes to age-related dysfunction and multiple diseases throughout the lifespan. Senescent cell burden is very low in young individuals but increases with aging in several tissues. These include adipose tissue, skeletal muscle, heart, kidney, brain, bone, and skin[5,18-24]. Senescent cells accumulate at sites of pathology in multiple chronic diseases, including, among others, the lungs in chronic obstructive pulmonary disease, asthma, idiopathic pulmonary fibrosis (IPF), and smoking, adipose and other tissues in diabetes/ obesity, the brain in Alzheimer's and other dementias, and blood vessels and the heart in cardiovascular disease.

Senescent cells were discovered in 1961[25] when serially-subcultured human embryonic fibroblasts were shown to lose replicative capacity, yet remain viable. This prompted work to test if aging leads to accumulation *in vivo* of pre-senescent cells, which have limited remaining replicative potential, and senescent cells, which cannot replicate but are metabolically active. In 1979, this was reported to be true for fibroblasts in skin biopsied from older than younger subjects[6] and by us in 1990, for primary adipocyte progenitors (preadipocytes) cloned from adipose tissue of rats across the age

spectrum that had been raised under controlled conditions[7]. Senescent cells appear at pathogenic sites of many major diseases, including chronic lung disease, diabetes/ obesity, cardiovascular diseases, Alzheimer's disease, cancers, osteoporosis, renal disease, and cirrhosis in preclinical species and humans[5,21,23], conditions associated with physical dysfunction (frailty), inflammation, and CoV morbidity and mortality.

Senescence is essentially a cell fate, like differentiation, proliferation, apoptosis, or necrosis. External and internal signals can contribute to driving a cell into senescence. These are generally cell or tissue damage-related, including DNA alterations (dysfunctional telomeres, strand breaks, *etc*.), metabolic dysfunction (reactive oxygen species [ROS], high glucose, bioactive lipids, mitochondrial dysfunction), protein alterations (aggregates, misfolding, failed autophagy), inflammatory signals, mechanical/shear stress, pathogen-associated molecular pattern factors (PAMPs), including viruses, bacterial/fungal proteins, lipopolysaccharide (LPS), *etc*., damage-associated molecular pattern factors (DAMPs; tissue damage signals [extracellular nucleotides, *etc*.]), cellular damage signals (DNA damage, telomeric dysfunction, protein aggregates, mitochondrial dysfunction), oncogenes, and mitogens (insulin-like growth factor-1, *etc*.). Once initiated, senescence takes 10 days to 6 weeks to become established through transcription factor cascades (that may, but do not always, include $p16^{INK4a}$/ retinoblastoma protein and/or $p53/p21^{CIP1}$), causing extensive changes in gene expression, histone modifications, altered organelle function (*e.g*., mitochondria, endoplasmic reticulum, nucleolus, nuclear envelope), elevated protein production due to increased mTOR and decreased autophagy, and profound morphological and metabolic shifts[21,26]. After drug-induced removal, senescent cells take from 10 days to 6 weeks to re-occur if cellular senescence inducers remain present, at least *in vitro*.

There are no fully sensitive or specific markers for identifying senescent cells. These cells are generally large, may have high $p16^{INK4a}$ and/or $p21^{CIP1}$ (but not always), and exhibit DNA damage foci, particularly in telomeres (telomere-associated DNA damage foci [TAFs]), senescence-associated distention of pericentromeric satellite DNA, and increased senescence-associated β-galactosidase (SA-βGal)[21]. Like cancer cells, senescent cells frequently are metabolically-shifted from fatty acid utilization toward glycolysis, resulting in ROS generation, lipid accumulation, lipotoxicity, and dysdifferentiation into adipocyte-like but insulin-resistant "MAD" (mesenchymal adipocyte-like default) cells[27]. Senescent cells are resistant to apoptosis[28].

Senescent Cell Characteristics: Cellular senescence leads to inflammation, fibrosis, DNA damage, mitochondrial dysfunction, ROS generation, NAD^+ depletion, protein aggregation, failed autophagy, lipotoxicity, and stem and progenitor cell dysfunction. In turn, cellular senescence can be caused by other fundamental aging processes, including DNA damage, low NAD^+, mitochondrial dysfunction, ROS, protein aggregates, and lipotoxicity. Thus, from our and others' data, fundamental aging processes appear to be interlinked, findings that led us to formulate a "Unitary Theory of Fundamental Aging Processes", which posits that interventions, such as senolytics, targeting any one of these processes, such as cellular senescence, will affect many of the rest of the aging processes that predispose to aging phenotypes, frailty, hyper-inflammation, chronic diseases, and morbidity and mortality from acute insults, including viral infection. Effects of the study agent in our clinical trial will be ascertained not only on senescent cell abundance and effects, but also on these other fundamental aging processes.

Senescence-Associated Secretory Phenotype (SASP): Accumulation of senescent cells can cause local and systemic inflammation, tissue destruction, immune system inhibition, and stem and progenitor cell dysfunction due to their SASP[3,4,21,29,30]. Generally 30-70% of senescent cells develop

a SASP that entails release of pro-inflammatory, pro-apoptotic cytokines (TNFα, IL-1α, IL-6, IL-7, IL-8, IFNγ, and many more), chemokines (*e.g*., MCP-1, MIP1-α, RANTES, RARRES2) that attract, activate, and anchor immune cells (including macrophages, dendritic cells, T-lymphocyte subsets, and neutrophils), tissue-destroying proteases (MMP-3, -9, -12, *etc*.), pro-coagulant factors (PAI-1, *etc*.), factors that impede stem cell/ progenitor function (Activin A, TGF-β-related proteins), ferritin, growth factors (*e.g*., GCSF), bioactive lipids (bradykines, saturated ceramides, prostanoids), micro-RNA's (miRNA's), tissue-damaging non-coding nucleotides (including release of mitochondrial DNA, which attracts, activates, and anchors dendritic cells[31]), microvesicles including exosomes, and other factors that cause tissue necrosis, systemic inflammation, stem cell/ progenitor dysfunction, and spread of senescence to non-senescent cells. These SASP factors include those that result in frailty, hyper-inflammation/ cytokine storm/ ARDS/ myocarditis with troponin leak in CoV patients[8].

Likely due to their SASP, only a small number of senescent cells can cause considerable dysfunction. In pre-clinical experiments, transplanting senescent cells around the knee joints of young mice was sufficient to induce an osteoarthritis-like phenotype, while transplanting non-senescent cells did not[32]. Transplanting 10^6 radiation- or chemotherapy-induced senescent autologous ear fibroblasts or syngeneic adipocyte progenitors intraperitoneally into lean, adult mice, so that fewer than 1/10,000 of all cells in the transplanted mice were senescent, induced impaired physical function (frailty) and premature death due to early onset of the same age-related diseases that cause death in naturally-aged mice[32]. This also occurred after human senescent (*vs*. non-senescent) cells were transplanted into immune-deficient (SCID) mice. When labelled senescent cells are transplanted intraperitoneally, they remained within the peritoneum, yet the transplant recipients developed non-labeled senescent cells in their limbs, demonstrating that senescence can spread from cell to cell, even at a distance[32].

We established that adipose tissue from obese individuals have an increased burden of senescent adipocyte progenitors[33]. Furthermore, these senescent cells have a potent pro-inflammatory SASP. This leads to the hypothesis that an older or obese individual or any other individual with an increased burden of senescent cells could be predisposed to frailty, hyper-inflammation/ cytokine storm/ ARDS/ myocarditis with troponin leak after CoV infection.

Clearance of Senescent cells by the Immune System: Senescent cells are usually cleared by the immune system[34]. They can attract, activate, and anchor immune cells, including macrophages, dendritic cells, T-lymphocytes, and neutrophils through such SASP chemokines as MCP-1, MIP1-α, RANTES, and RARRES2, cytokines such as IL-6, and TNFα, extracellular mitochondrial DNA, possibly miRNAs, and other factors[31,32,34,35]. However, above a threshold burden, senescent cells interfere with the immune system and its ability to remove them. For example, IL-6, a SASP component, interferes with macrophage migration, MMPs can cleave FAS ligand and other immune cell surface proteins, senescent cells cause fibrosis (impeding immune cell infiltration and trapping immune cells within inflammatory foci), and senescent cells can express "don't eat me" signals[34]. Consistent with this, *senescent cells can drive cancer initiation and progression, which senolytics are highly effective at preventing, with substantial delays in death from multiple forms of cancer in mice[5,32,36]. Fisetin, which selectively clears senescent cells, delays death of older mice, 50% of which die from cancer, by over 17%[36].*

Threshold Theory of Senescent Cell Burden: Based on the above, we formulated a "Threshold Theory of Senescent Cell Burden", which holds that once senescent cell abundance is sufficient to cause spread of senescence that exceeds capacity of the immune system to keep up with clearing

these cells, an accelerated aging-like state ensues[32,34]. Consistent with this: 1) the number of senescent cells that needs to be transplanted to cause frailty, limit healthspan, and cause premature death, including from cancer, is higher in young than old mice and in young lean than young obese mice. Old mice or young, obese mice have more pre-existing senescent cells than young, lean mice[32]. 2) Senescent cell abundance remains low in skin until early old age in humans, followed by an upward inflection in senescent cell burden in subjects who are in their late 60's through the mid-70's[37]. This precedes the age-related increase in multi-morbidity[38]. 3) The lag between induction of senescence by chemotherapy and development of age-related morbidities is longer in childhood cancer survivors than adults who received higher chemotherapy doses in preparation for bone marrow transplantation[39,40]. These findings support the hypothesis that there is a threshold above which senescent cell burden due to spread of cellular senescence becomes self-amplifying. This causes increased risk for senescence- and age-related phenotypes, physical dysfunction/ frailty, hyper-inflammation, and diseases, perhaps contributing to age-related multi-morbidity[38]. Potentially, attraction, activation, and anchoring of immune cells is accelerated after senescent cells exceed this threshold, predisposing to excessive inflammation in frailty and during viral infection, plus continuing and exacerbating production of the SASP factors that underlie frailty, hyper-inflammation/ cytokine storm, ARDS, and cardiomyopathy, as occurs in fatal CoV cases, as well as subsequent fibrosis, *e.g.*, of the lungs.

Pathogen-Associated Molecular Profile Factors (PAMPs): Of note, the SASP is modifiable: drugs, hormones, and especially the PAMPs associated with infections such as coronaviruses, can modify the SASP. We found that PAMPs can exacerbate the SASP in previously relatively quiescent human senescent cells by multiple fold (much greater than the effect of cytokines on the SASP), with greatly increased secretion of factors underlying cytokine storm (IL-6, IL-8, MCP-1) and increased attraction and activation of innate immune cells, potentially driving the frailty, hyper-inflammation, cytokine storm, and ARDS caused by coronavirus (Fig. 1).

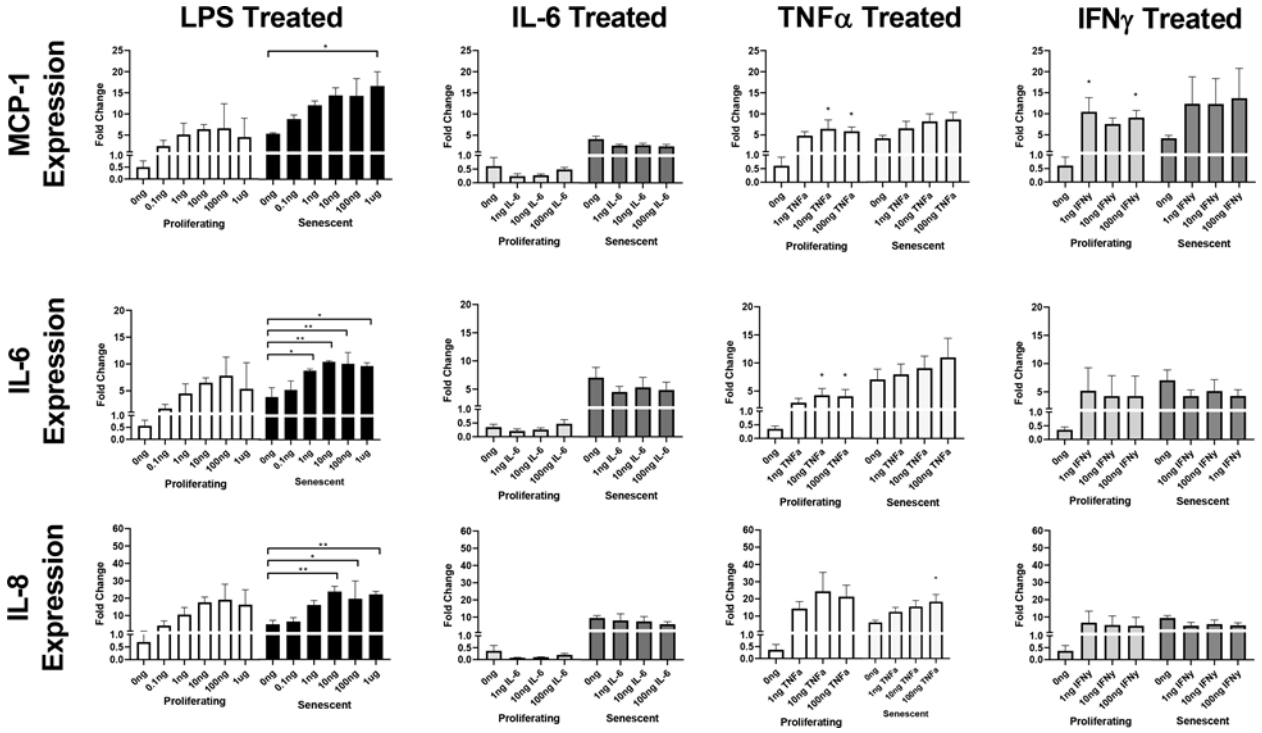


Fig. 1. Senescent cells challenged with PAMPs highly express SASP factor mRNAs. Human subcutaneous adipocyte progenitors from different subjects were irradiated or not to generate senescent or

proliferating cells, respectively (n=3). Then cells were treated with different concentrations of the prototype PAMP, LPS, for 3 hours before mRNA isolation. One-way ANOVA and Dunnett multiple comparison post-tests were performed against the control (0 ng) for each condition (proliferating or senescent). * = p<0.05, ** = p<0.01. Human non-senescent and radiation-induced senescent preadipocytes were treated with human recombinant IL-6, TNF-α, and IFN-γ for 3h.

Similar results were obtained *in vivo* when progeroid mice, which have senescent cells to the same extent and in the same tissues as aged mice, were challenged with the PAMP, LPS (Fig. 2). In two tissues examined (kidney and liver), levels of senescence markers ($p16^{Ink4}$, $p21^{Cip1}$) and the SASP (*Il1*□, *Il6*, *TNF*□□ and *Mcp1*) were greater in the progeroid mice compared to age-matched controls, illustrating an amplification of the response to PAMPs in organisms with increased senescent cell burden. In another study focused on neuroprotection in the face of LPS challenge, rats were administered Fisetin 20 i.p. mg/kg/day x 14 days, while being challenged with LPS[41]. In Fisetin-treated animals, inflammation and neurodegeneration were suppressed, while memory improved.

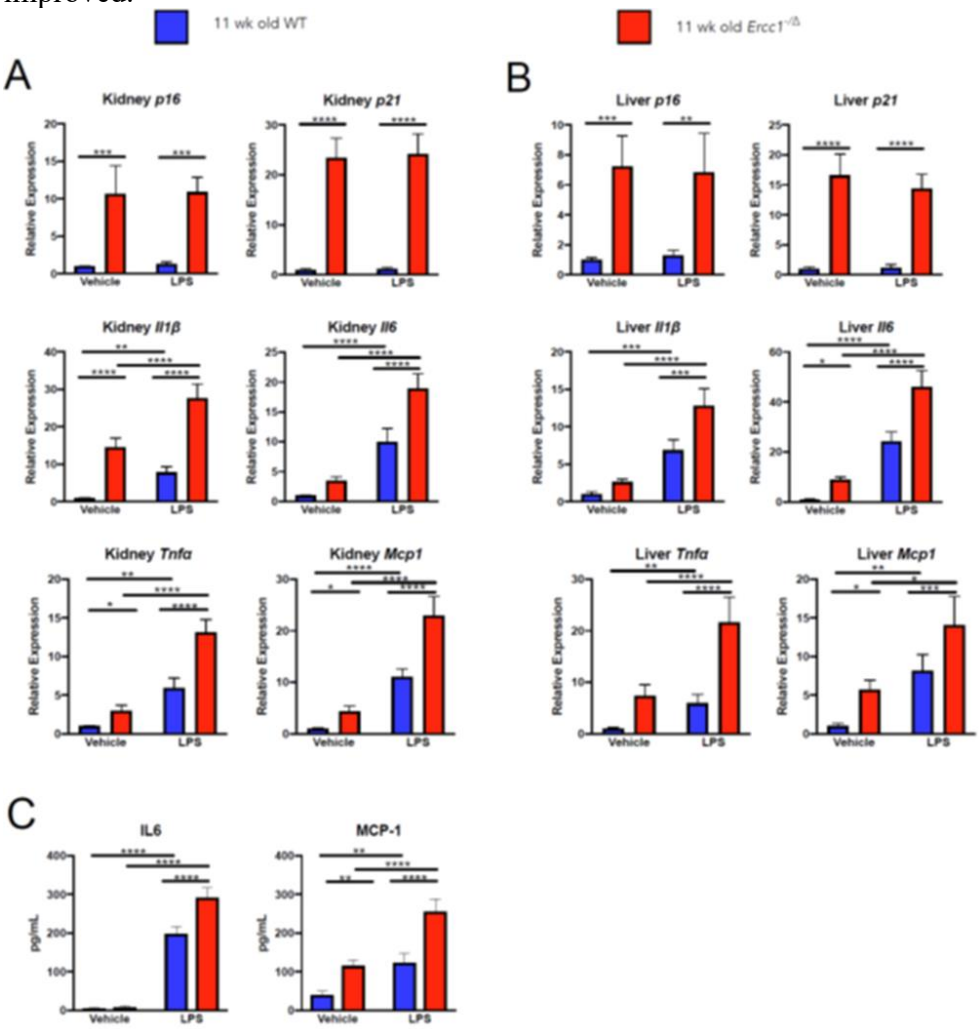


Fig. 2. Pre-existing senescence causes an exaggerated response to pathogens *in vivo*. Eleven-week-old wild-type (blue) and progeroid *Ercc1*$^{-/□}$ (red) mice were injected with 500 ng/kg of LPS or vehicle (PBS) i.p. (N=4/ group). Twenty-four hours post-injection, mice were euthanized and tissues were collected. Total RNA was isolated from tissues and senescence and SASP marker expression were quantified in the (**A**) kidney and (**B**) liver by real-time qPCR using the ΔΔCt method. *Gapdh* was used as a control for gene expression analysis. (**C**) Circulating levels of IL-6 and MCP-1 were quantified in serum by ELISA. All values represent

the mean ± SD. Statistical significance was determined by two-way ANOVA. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001. (unpublished data, M. Yousefzadeh, B. Hughes, P. Robbins, L. Niedernhofer).

Amplifier/ Rheostat Hypothesis of Cellular Senescence: The SASP depends on the type of cell that became senescent, how senescence was induced, and its *milieu*. The SASP changes over time in response to internal (*e.g.*, transposons) and external cues (*e.g.*, PAMPs, DAMPs, glucocorticoids, SASP inhibitors such as rapamycin). PAMPs are particularly effective at exacerbating the SASP (Figs. 1&2), perhaps accounting for severe morbidity in previously highly-functioning elderly individuals with an increased (but quiescent) senescent cell burden that is activated by infections such as CoV or septicemia. Based on these considerations, we advanced an "Amplifier/ Rheostat Hypothesis" of cellular senescence: that the increased senescent cell burden in elderly and chronically ill patients contributes to their excessive, frailty/morbidity/mortality-inducing hyper-inflammatory (cytokine storm) response to infections that have much less cytokine-inducing effects in younger, previously healthy people (Fig. 3).

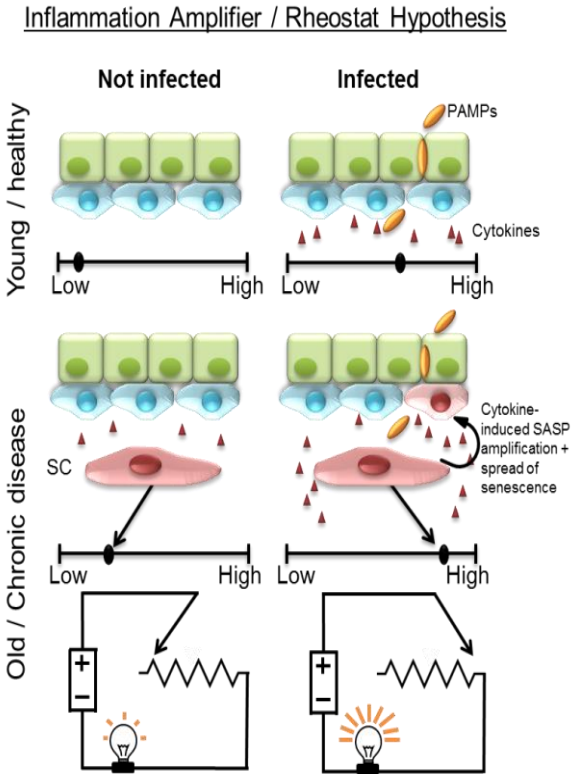


Figure 3: Amplifier/Rheostat hypothesis

Fig. 3. SASP Amplifier/Rheostat Hypothesis. Among the 10 leading causes of mortality are influenza, pneumonia, genitourinary infections, and septicemia. The elderly and patients with cellular senescence-associated diseases (*e.g.*, diabetes, dementias, smoking, asthma) are more susceptible to adverse outcomes in response to infections (*e.g.*, ARDS, cytokine storm, myocardial damage with troponin leak, delirium, hypotension, thrombosis, delayed recovery) and develop a more exaggerated inflammatory state than young patients without pre-existing chronic diseases. These adverse outcomes of infections in the elderly or patients with senescence-associated chronic diseases have generally been attributed to immune system dysfunction or to chronic inflammation. Our "Amplifier/ Rheostat" Hypothesis is that PAMPs (*e.g.*, LPS, viral antigens) cause a shift in the SASP of pre-existing senescent cells (SC) into a highly inflammatory, pro-apoptotic, pro-fibrotic SASP. This may not only exacerbate acute systemic inflammatory responses and cytokine release by innate immune system cells (macrophages, dendritic cells, T lymphocyte subsets, neutrophils), but could also amplify spread of senescence to other cells. The resulting additional senescent cells may act to exacerbate and prolong inflammation, attenuate or delay recovery, lead to persistent frailty, cause long term tissue fibrosis if the afflicted individual survives long enough, and contribute to multi-organ failure (L. Langhi, T. Tchkonia, J.L. Kirkland, October, 2018; unpublished).

ß-Coronavirus Causes Infected Non-Senescent Cells to become Senescent: We found that mice infected with a mouse ß-coronavirus closely related to human COVID-19 develop increased senescent cell burden (Fig. **4**). Similarly, human cells exposed to COVID-19 Spike-1 antigen or infected with an attenuated SARS-COVID-19 virus become senescent, as manifest by increased cellular SA-βGal (Fig. **5**).

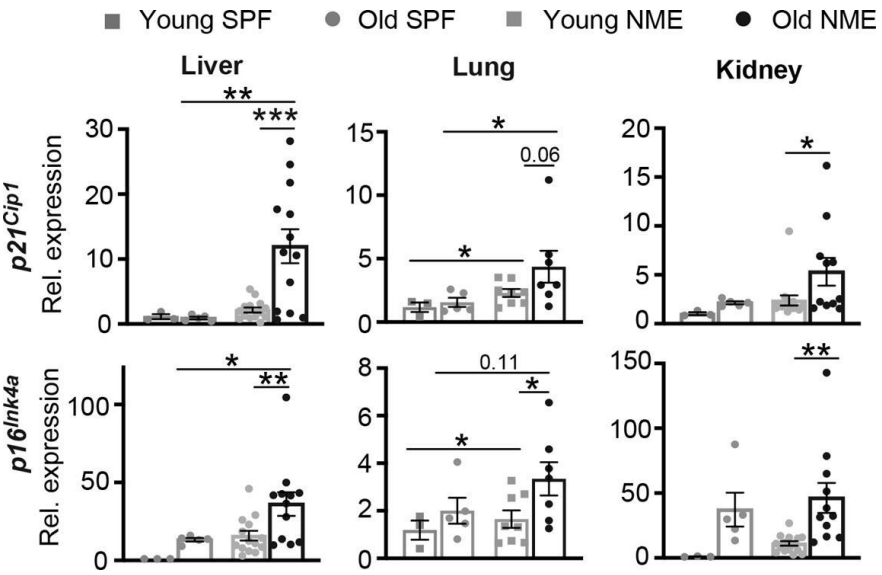


Fig. 4: Specific pathogen-free (SPF) mice exposed to a normal microbial experience (NME) that includes acute mouse ß-coronavirus infection develop an increased abundance of senescent cell. Young (3-month-old) and old (20-24-month-old) male and female WT mice were exposed to NME bedding produced from pet store mice for 7 days. Abundance of p16Ink4a and p21Cip1 senescent cells was assayed in the liver, lung, and kidney of SPF or NME young and old mice. The ß-coronavirus-infected old mice had a particularly marked increase in senescent cell burden, with trends evident even in younger mice. Two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001.

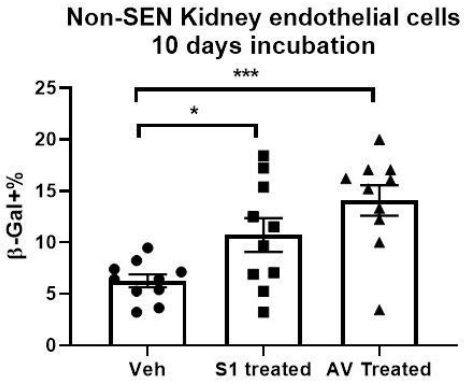


Fig. 5: Both COVID-19 Spike-1 antigen and attenuated SARS-CoV cause non-senescent human kidney endothelial cells to become senescent within 10 days as manifested by increased cellular SA-βGal. Two-way ANOVA. *p<0.05, ***p<0.001.

Senolytics: In 2004 Sharpless *et al*.[42] showed that senescent cell accumulation is delayed in Ames dwarf mice (with pituitary hormone deficiencies) and calorically-restricted mice, models with increased healthspan and lifespan. This was critical in prompting us to test the hypothesis that targeting senescent cells may alleviate multiple age-related disorders. Our efforts to discover senolytics, drugs that selectively eliminate senescent cells, began in 2004/5, with initial attempts to

create fusion proteins comprising a senescent cell surface-binding domain coupled to a toxin, high throughput compound library screens for candidates that eliminate senescent but not non-senescent cells, and other approaches. These traditional approaches were not initially successful, so we turned to a hypothesis-driven discovery paradigm to discover the first senolytic drugs[21,43,44]. We hypothesized that since senescent cells kill cells around them and damage tissues due to their inflammatory, apoptotic SASP, yet these senescent cells do not die, they must have pro-survival networks that defend them against their own SASP: senescent cell anti-apoptotic pathways (SCAPs)[5,43-45].

From proteomic/transcriptomic databases and using bio-informatics methods, we discovered the SCAP network[43]. Through RNA interference analyses, we demonstrated that transiently disabling this SCAP network leads to apoptosis of senescent cells, while normal cells without a SASP are not affected, the "Achilles' heel" of senescent cells. We next used bioinformatics approaches to identify drugs and natural products that act on critical SCAP nodes. Among these are Fisetin, Quercetin (Q), Dasatinib (D), and now over 20 others. We found these agents selectively eliminate senescent cells in mice and humans. They alleviate multiple conditions in mice, including diabetes and its complications, cardiovascular disorders (including heart failure and vascular dysfunction), dementias, pulmonary dysfunction (including bleomycin-induced lung disease), diabetes and its complications, frailty, kidney and liver damage or dysfunction, osteoporosis, osteoarthritis, and delay cancer and extend healthspan and lifespan[5,21-24].

We intentionally selected drugs for further development from among those identified as being senolytic. We focused on drugs that: 1) were already FDA-approved or were natural products with a history of safe human use, 2) could be administered orally, and 3) have a short elimination half-life (T½). Fisetin met these criteria. It is a natural product with a favorable safety profile, is effective orally, and has an elimination T½ ~3 hours, meaning it is cleared within a half day.

The target of senolytics is senescent cells, not a single receptor, enzyme, or biochemical pathway. By targeting pro-survival networks instead of single molecules, specificity for senescent cells can be increased, side-effect profiles flattened, and off-target effects on non-senescent cells reduced. Drugs that have single or limited targets, such as Navitoclax (ABT-263), which we discovered is senolytic at the same time as another group[46,47], or Nutlin3a, have off-target apoptotic effects on multiple non-senescent cell types, making them "panolytic". Navitoclax, a BCL-2/ BCL-w/ BCL-xL inhibitor, eliminates only a restricted range of senescent cells, *e.g.*, BCL-2 inhibitors are not senolytic against human adipocyte progenitors, one of the most abundant senescent cell types in aged humans or people with diabetes and obesity. Our strategies for developing senolytics have more in common with approaches for developing antibiotics than the conventional one-target/one-drug/one-disease drug development approach.

Description of the population to be studied. Adult women and men with PCR positive SARS-CoV-2 infection, will be screened for inclusion in this study. Subjects who meet screening criteria will be randomized to receive Fisetin 20 mg/kg/day orally (capsules or powder dissolved in water or added to apple sauce, pudding, or yogurt) or by nasogastric (NG-tube) or transabdominal duodenal tube (D-tube; powder dissolved in water within 30 minutes, to achieve transient, high tissue Fisetin levels in a "hit-and-run" approach or placebo for 2 days (Days 0-1 and 8-9) and will be followed until study Day 30 or death. Subjects will continue to be followed for 2 months. The primary outcome is need for hospitalization and death with COVID-19. Secondary outcomes will be assessment for long hauler syndrome. Buccal swabs, urine and stool tests will be conducted before and after initiating treatment with Fisetin or placebo serially.

1.1 Investigational Product (IP)

An IP is defined as a pharmaceutical form of an active substance or placebo being tested or used as a reference in a clinical study, including products already with a marketing authorization but used or assembled (formulated or packaged) differently than the authorized form, or used for an unauthorized indication, or when used to gain further information about the authorized form. The IP will be stored in a secure area according to local regulations. Investigators will have responsibility for ensuring that IP is only dispensed to study subjects. The IP (Fisetin and placebo) will be dispensed only by authorized personnel according to regulations that apply at Mayo Clinic Rochester. In this protocol, the IP are Fisetin and placebo.

Fisetin: Fisetin [redacted] is a member of the flavonoid family, a family of naturally-occurring polyphenolic compounds. As a natural product, it is widely sold, including by Amazon, from which it is available in containers of 30 capsules of 100 mg Fisetin/ capsule at <$20 per container. The structure of Fisetin is similar to that of Q, which is one of the first two senolytic drugs discovered[43] (Fig. 6). Fisetin, a high Trolox-equivalent antioxidant, is present in many fruits and vegetables such as apples, persimmon, grapes, onions, and cucumbers, with the highest concentration found in strawberries (160 µg/g)[48,49] (Fig. 7). The average dietary intake of naturally-occurring Fisetin was approximately 0.8 mg/day in an earlier Japanese study[50,51]. However, the average total intake of polyphenols, comprised mainly of flavonoids, is around 1 gram per day, although it may be quite variable[52-54]. Fisetin has little or no odor or taste.

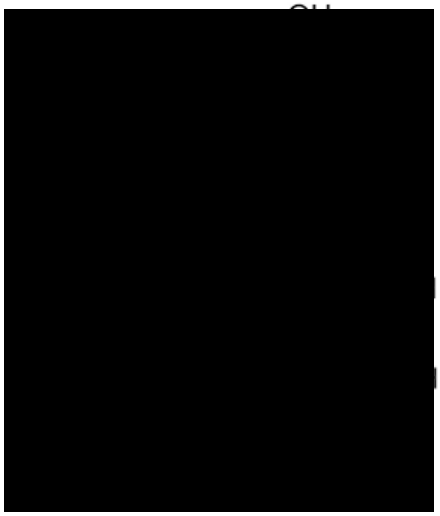


Fig. 6. Structure of the senolytic flavonol, Q. Quercetin, one of the first two senolytic drugs identified (along with Dasatinib [D]), and which is present in apple peels, has a bitter taste and is less bioavailable and has a longer elimination T½ than Fisetin.

Fig. 7. Structure of Fisetin. Fisetin differs from Q by an hydroxyl group. It has little or no odor or taste. Its terminal T½ in mice is ~3.1 hrs[1]. This short half-life makes Fisetin ideal as a senolytic drug to ablate pro-inflammatory, pro-apoptotic, non-dividing senescent cells in a "hit-and-run" fashion[5].

Due to its being a hydrophobic compound, Fisetin easily penetrates cell membranes, entering into cells to exert antioxidative and other effects[55,56]. Other promising biological activities include anti-hyperlipidemic[56-58], anti-inflammatory[57], neurotrophic, apoptotic[59], and antiproliferative effects[36].

There are reports of genotoxicity due to some flavonols, but Fisetin may exert little or no genotoxic effects *in vivo*[60]. Indeed, Fisetin has been noted to be anticarcinogenic[48,61,62]. We found that chronic Fisetin administration delays cancer in mice and increases remaining lifespan in old mice by over 17%[63]. Since up to 50% of most mouse strains die from cancers, this suggests that the beneficial effects of Fisetin on such pro-carcinogenic factors as senescent cell burden and tissue chronic inflammation appear to more than counteract any theoretical effects of flavonol-associated genotoxicity. In the same mouse study, Fisetin was administered to a murine model of a human progeroid syndrome[63]. These mice are profoundly sensitive to genotoxic agents[64], yet their health and lifespan were improved by long-term, high dose Fisetin[63].

Fisetin is available as a dietary supplement. Other flavonols such as Q have been safely used in clinical trials in amounts up to 500 mg twice daily for up to 8 weeks. To date, no reports of significant toxicity for Fisetin *in vivo* have been made. Since Fisetin along with other flavones and flavonoids, has been shown to inhibit cytochrome P450 2C9, CYP3A4, and others (Section 3.9.4), precaution will be taken to avoid significant drug-drug interactions, *e.g*., with warfarin[65].

We demonstrated that Fisetin is a senolytic agent and alleviates multiple senescence-associated disorders in mice, decreases physical dysfunction if administered to old mice, attenuates age-related tissue damage and pathology, and extends remaining lifespan by 17%[44,63]. We have not found any adverse effects in mice (at up to 25 times the dose used in the mouse effectiveness studies [below] or the dose for humans proposed here), old *Rhesus* monkeys (at up to 5 times the dose for humans proposed here), or humans so far. We obtained a full IND for Fisetin from the FDA and have a GMP drug supply handled by Mayo Research Pharmacy.

Fisetin 100 mg capsules or Fisetin (with cellulose) powder (in water, other chilled or room-temperature drinks, apple sauce, pudding, yogurt, or enteral hydration/ nutrition solutions) will be administered orally for 2 consecutive days on study Days 0 and 1. To reduce senescent cell abundance by a "hit-and-run" approach, the full daily doses of Fisetin will be administered within 30 minutes. The investigational supplies will be obtained from Vital Nutrients, a contracted GMP manufacturer. Fisetin capsules supplied by Vital Nutrients are opaque blue in color. Fisetin powder is a green yellow color. We established stability parameters for Fisetin in collaboration with Mayo Clinic's Department of Laboratory Medicine and Pathology. We acquired an IND for Fisetin after establishing botanical sourcing, GMP isolation by Vital Nutrients, assurance of lack of contaminating pesticides, heavy metals, and solvents, and stability, degradation, and accelerated toxicity testing.

1.2 Preclinical Data

In our article about the first senolytics discovered, we demonstrated D+Q reduces senescent cell burden in multiple tissues and improves function in naturally-aged animals[43]. Since then, D+Q, Fisetin (which is closely related to Q), Navitoclax, and other senolytics have been shown to alleviate multiple diseases and disorders in animal models (Table 1). Among effects are: restoration of mesenchymal stem cell (MSC) function; improved cardiac ejection fraction, fractional shortening, and regeneration in old mice; enhanced vascular reactivity in old mice, decreased vascular calcification and restored vascular reactivity in hypercholesterolemic, high fat-fed *ApoE*$^{-/-}$ mice; decreased loss of intervertebral disc glycosaminoglycans and spondylosis in progeroid *Ercc1*$^{-/\Delta}$ mice; decreased gait disturbance in mice following radiation damage to a leg and hematological dysfunction caused by whole body radiation; decreased age-related changes of skin; improved pulmonary function and reduced pulmonary fibrosis in mice with bleomycin-induced lung damage, a model of IPF, plus decreases in: liver fibrosis in *Mdr*$^{-/-}$ cirrhotic mice; insulin resistance, hepatic steatosis, and renal dysfunction in high fat-fed mice; neuropsychiatric dysfunction and impaired neurogenesis in high fat-fed mice; neuro-inflammation, impaired neurogenesis, microvascular impairment, and cognitive dysfunction in mouse models of dementia; age-related osteoporosis; uterine fibrosis; physical frailty; and muscle weakness in naturally-aged, progeroid, radiated, and senescent cell-transplanted young mice as well as delayed cancer and increased healthspan and lifespan in old mice[5,20-23,32,35,43,47,66-73].

Condition	References
Diabetes/ Obesity	35,71,74-77
Cardiac Dysfunction	66,67,78
Vascular Hyporeactivity/ Calcification	67
AV Fistulae	79
Frailty	32,43,63,68
Age-Related Muscle Loss (Sarcopenia)	80
Chemotherapy Complications	32,40,47,68,81
Radiation Complications	82
Cancers	32
Bone Marrow Transplant Complications	40
Organ Transplantation Complications	31,83
Myeloma/ MGUS	84
Age-Related Cognitive Dysfunction	85
Alzheimer's Disease	86,87
Parkinson's Disease	88
Amyotrophic Lateral Sclerosis	89
Ataxia	43
Obesity-Related Neuropsychiatric Dysfunction	90
Renal Dysfunction	91,92
Urinary Incontinence	43
Osteoporosis	93-95
Osteoarthritis	96
Age-Related Intervertebral Disk Disease	43,97
Idiopathic Pulmonary Fibrosis	68,98
Hyperoxic Lung Damage	99
Chronic Obstructive Pulmonary Disease	100
Tobacco	101
Hepatic Steatosis	70
Cirrhosis	69
Primary Biliary Cirrhosis	102
Progerias	43,63
Pre-eclampsia	103
Macular Degeneration	104,105
Glaucoma	106-108
Cataracts	109
Prostatic Hypertrophy	110-112
Psoriasis	113
Healthspan	32,43,63
Lifespan	32,43,63

Table 1. Conditions with emerging evidence for a causal contribution of cellular senescence or that are alleviated by senolytic drugs. Cellular senescence contributes to age-related dysfunction and diseases throughout the lifespan. Senescence can contribute to local and systemic inflammation, dysfunction, and spread of senescence through release of cytokines, chemokines, proteases, ferritin, stem cell/progenitor toxins, reactive metabolites, miRNA's, mitochondrial DNA, other nucleotides, and extracellular vesicles, the SASP[2-4]. Pre-senescent cells, which have limited remaining replicative potential, and senescent cells, which cannot replicate, accumulate to a greater extent *in vivo* in skin biopsies from older than younger subjects[6]. Senescent and pre-senescent adipocyte progenitors cloned from adipose tissue increase with aging in rats raised under controlled conditions[7]. Senolytics have been shown to alleviate many of these conditions by us (**bold**) and others.

Most studies of senolytics for various disease states, including in humans, have been with D+Q since D and Q were the first senolytics. However, while D+Q is relatively safe, it is likely not as safe as Fisetin for very ill patients. Unlike Fisetin, D cannot be administered safely to patients with a prolonged QTc (>450 msec). Many elderly and chronically-ill patients have a prolonged QTc, meaning they would be excluded from studies involving treatment with D. Additionally, there are multiple other drug interactions with D that would result in fewer patients being able to be treated than with Fisetin. Navitoclax has a poorer safety profile than Fisetin or D+Q: unlike D, which was approved by the FDA in 2006, Navitoclax has failed to gain FDA approval so far as a generally-available prescription drug, largely because of extensive and unpredictable induction of apoptosis in non-senescent cell types. For example, Navitoclax can cause life-threatening neutropenia or hemorrhage due to platelet depletion. Fisetin so far has had an exceptional safety profile in our clinical trials for elderly patients with multi-morbidity. Therefore, we decided to analyze Fisetin here rather than D+Q or Navitoclax.

"Hit and run": D+Q or Fisetin are effective if administered once every few days or weeks since they do not need to be continuously present to occupy a receptor or interfere with an enzyme, reducing off-target effects. Brief disruption of pro-survival pathways is sufficient to kill senescent cells in mouse and human cell cultures, *in vivo* in mice and monkeys, and in human adipose explants freshly-isolated from obese subjects[21,43,63,68]. In mice, monthly D+Q is as effective as daily administration to alleviate age-related osteoporosis[95], reducing potential side-effects. These points, together with satisfying a modified set of Koch's postulates (Table 2), show that senolytics alleviate

dysfunction by removing senescent cells, not other off-target mechanisms. Unlike microbes or cancer cells, since senescent cells do not divide, they are unlikely to acquire replication-dependent drug resistance. Fisetin is well-suited for a "hit-and-run" administration protocol since it has an elimination T½ of ~3 hours, we demonstrated that it takes only 30 minutes of exposure of human tissues for senolytics to initiate the apoptosis process[32], and senescent cells take over 1 week to re-accumulate. Of note, no steady state levels for Fisetin need to be achieved and the goal is to eliminate senescent cells in a "hit-and-run" fashion, not to achieve a steady state blood level. The critical outcome here is target engagement, *i.e*., do we kill senescent cells?, not do we achieve a particular drug or tissue level? Therefore, as in our other trials, traditional blood pharmacokinetic parameters will not be assayed. Rather, the critical pharmacodynamic measure, senescent cell abundance, will be assayed. This approach has been tacitly agreed to by the FDA regarding our other clinical trials of senolytics, including with Fisetin.

Table 2. A Modified Set of Koch's Postulates

To establish causation:

- Are senescent cells present in animals or humans with the disorder?
- Do individuals without senescent cells have the disorder?
- Is the disorder reproduced by inducing local accumulation of senescent cells (*e.g*., by transplanting senescent cells, focal irradiation, or tissue-specific genetic approaches)?
- Does removing these transplanted or induced senescent cells prevent or alleviate the disorder?
- Does targeting naturally-occurring senescent cells alleviate the disorder?
- Does administering the potentially senolytic candidate have few or no effects related to the disorder being tested in individuals with few or no senescent cells (*e.g*., young mice)?
- Does the potentially senolytic candidate alleviate the condition if given intermittently, at intervals longer than the drug's half-life, since senescent cells can take 10 days to 6 weeks to re-accumulate (at least in culture)? In the case of D+Q, senolytic drugs are as effective if administered monthly as continuously, at least in the case of age-related osteoporosis[95].
- Does the candidate alleviate multiple age-related conditions? (If a candidate is truly senolytic and the Geroscience Hypothesis is true, then it should alleviate multiple age- and chronic disease-related disorders).

The above criteria have been met for senolytic drugs for frailty, diabetes, and age-related osteoporosis and many have been met for osteoarthritis and neurodegenerative diseases in mice.

Fisetin selectively causes apoptosis of human cultured senescent cells: Fisetin reduces viability of 30-70% of senescent human umbilical endothelial cells (HUVECs) *in vitro*[44] (Fig. 8). Note that 30-70% elimination of senescent cells was achieved in our *INK-ATTAC* mice by activating the suicide gene expressed only in $p16^{Ink4a+}$ cells (many or most of which are senescent) in these mice[114]. This extent of elimination was sufficient to alleviate multiple age-related conditions in chronologically-aged and progeroid mice[20,67,68,114].

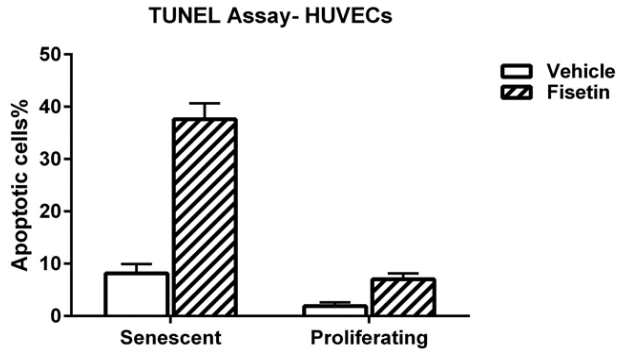


Fig. 8. Fisetin reduces human senescent cell viability through apoptosis. Senescent and proliferating non-senescent cultured HUVEC's were treated with Fisetin 20 μM or vehicle, respectively, for <12 hours and the percentage of apoptotic cells was measured by TUNEL assay.

Fisetin selectively eliminates senescent cells from mice: Fisetin reduces age-related senescent cell accumulation in mice (Figs. 9 & 10).

Fig. 9. Fisetin reduces senescent cell abundance in mouse adipose tissue. Inguinal adipose tissue from a 27 month old mouse that had been treated with Fisetin as daily pulse doses (tissue above) for 2 consecutive days once a month for 3 months (from age 24 months) was assayed for SA-βGal activity 4 days after the last dose of Fisetin. Adipose tissue from a control 27 month-old littermate treated with vehicle is shown below. Fisetin also attenuated age-related lipodystrophy, as is evident by the larger adipose depot in the treated mouse. SA-βGal makes adipose tissue appear blue. Representative of 3 pairs of Fisetin and vehicle-treated littermates. A 27 month-old mouse is equivalent to an 80-90 year old human.

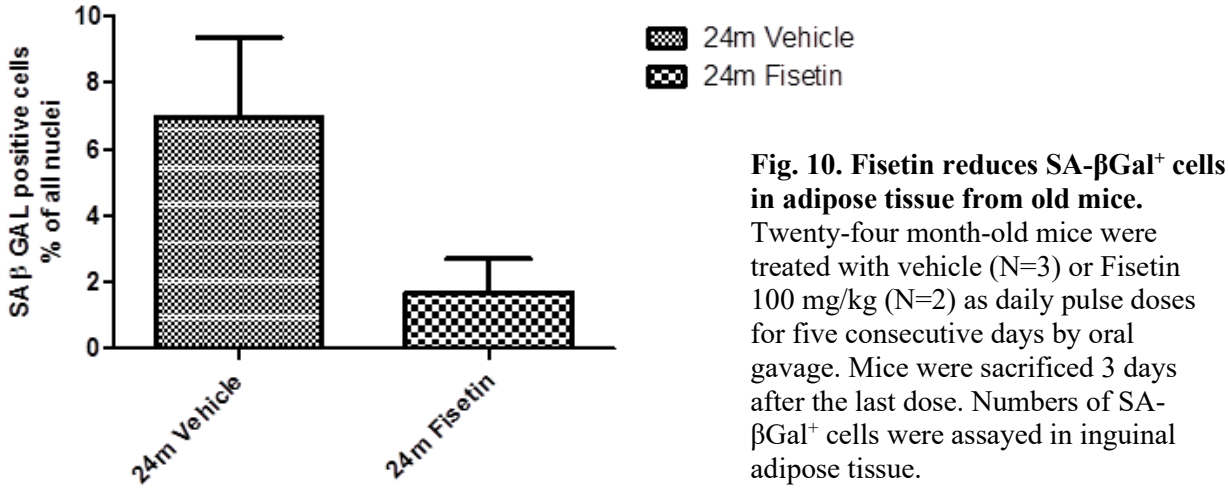


Fig. 10. Fisetin reduces SA-βGal$^+$ cells in adipose tissue from old mice. Twenty-four month-old mice were treated with vehicle (N=3) or Fisetin 100 mg/kg (N=2) as daily pulse doses for five consecutive days by oral gavage. Mice were sacrificed 3 days after the last dose. Numbers of SA-βGal$^+$ cells were assayed in inguinal adipose tissue.

Fisetin selectively eliminates senescent cells from monkeys: Fig. 11.

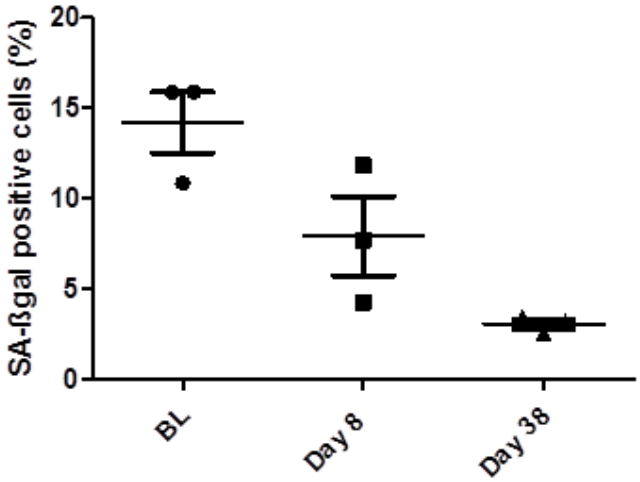
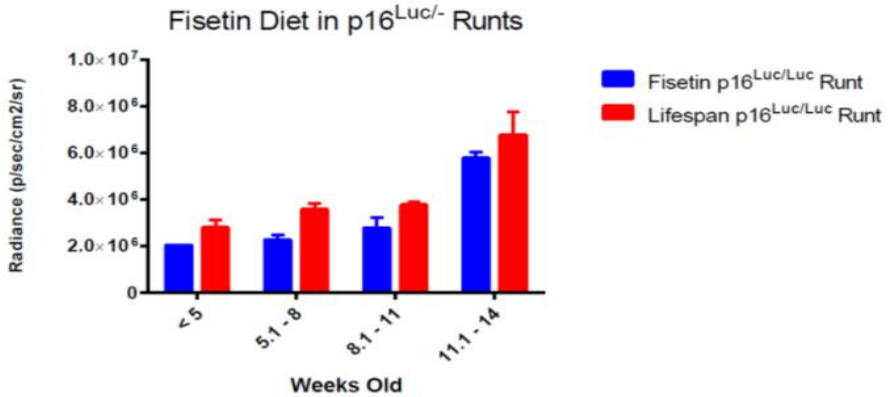


Fig. 11. SA-βGal$^+$ cell number in subcutaneous adipose tissue biopsies of *Rhesus* macaques at baseline and after treatment with pulse doses of Fisetin. There is a greater decrease of senescent cells in the adipose tissue of *Rhesus* macaques at day 38 than at day 8 after the first dose of Fisetin 100 mg/kg. Animals were treated with Fisetin as daily pulse doses for 3 consecutive days. Biopsies were done at baseline, 8 days, and 38 days after the first dose of Fisetin.

Fisetin alleviates cellular senescence-related phenotypes and extends healthspan and lifespan in mice: *Ercc1*$^{-/\Delta}$ mice, which model human XFE progeria, have features resembling accelerated aging, with a lifespan of 6 months[115]. They develop progressive age-related chronic degenerative diseases[116] and accumulate senescent cells in the same tissues as aged wild-type mice, albeit more rapidly[117]. To track senescent cell abundance *in vivo*, *Ercc1*$^{-/\Delta}$ mice were crossed with *p-16-luc* transgenic mice. *p-16-luc* mice express luciferase under the control of the *p16*^{Ink4a} promotor (p16^{Ink4a} is increased in many or most senescent cells). After administering luciferin i.v., p16^{Ink4+} cells expressing the transgene emit light and can be seen and quantified *in vivo* using a light-detection box. The light signal (radiance) indicates the intensity and location of p16^{Ink4+} cells. Note that oral exposure to Fisetin at this dose, which is higher than that proposed for our human studies, does not cause toxicity even in these frail mice. There were no adverse effects on mobility, feeding, appearance, or weight.

Fisetin decreased senescent cell burden in *Ercc1*$^{-/\Delta}$*; p16-luc* mice as determined by decreased radiance (Fig. 12). *Ercc1*$^{-/\Delta}$*; p16-luc* mice on C57Bl/6 Albino:FVB background at 3-4 weeks of age were fed *ad libitum* with a standard Teklad 2020 chow diet ("Lifespan" diet; N=15) or the standard Teklad 2020 chow diet containing Fisetin 500 ppm (N=2). Fisetin 60 mg/kg/day was consumed by each mouse based on Fisetin 500 ppm (500 mg/kg) being in the diet and 3-5 g chow eaten/ day. Senescent cell burden was measured at baseline and biweekly in control (lifespan) and Fisetin-treated mice. Imaging of luciferase activity indicates a significant decrease in senescence signal.



	P16 L/-;Ercc1 -/? Fisetin			P16 L/-;Ercc1 -/? Lifespan			
Weeks Old	Mean	Std Dev	n	Mean	Std Dev	n	p-Value
< 5	2.03E+06		1	2.79E+06	6.46E+05	4	0.3718
5.1 - 8	2.26E+06	3.91E+05	3	3.56E+06	1.05E+06	15	0.0557
8.1 - 11	2.76E+06	6.60E+05	2	3.76E+06	4.83E+05	10	0.028
11.1 - 14	5.77E+06	352639	2	6.76E+06	4072220	17	0.7405

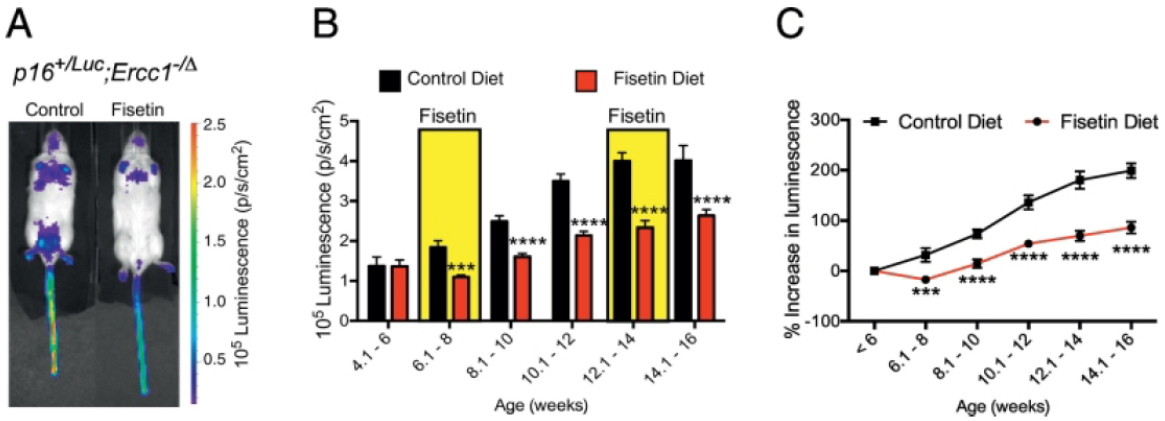


Fig. 12. Fisetin reduces age-related senescent cell accumulation in *p16$^{Luc/-}$;Ercc1$^{-/\Delta}$* mice. Intermittent treatment of progeroid mice with Fisetin reduces senescent cell burden. (**A**) Representative image of age-matched, 12 week-old male *p16^{+}/Luc;Ercc1$^{-/\Delta}$* mice fed a diet containing Fisetin 500 ppm (500 mg/kg), or drug-free control diet. (**B**) Luciferase signal was measured biweekly in *p16^{+}/Luc;Ercc1$^{-/\Delta}$* mice fed either control chow or chow containing Fisetin 500 ppm, N = 4–10 mice/ group and time point. The Fisetin was administrated intermittently for two weeks at a time (yellow bars). Otherwise the mice were fed a control diet. (**C**) The same data as seen in (B), but plotted as the percent change in luciferase signal as the animals aged. Values represented as the mean ± SEM. Two-tailed unpaired Student's t-test. ***p < .001, ****p < .0001.

Fisetin increased skin hydration and elasticity and increased healthspan in *Ercc1*$^{-/\Delta}$ mice (Figs. 13-15).

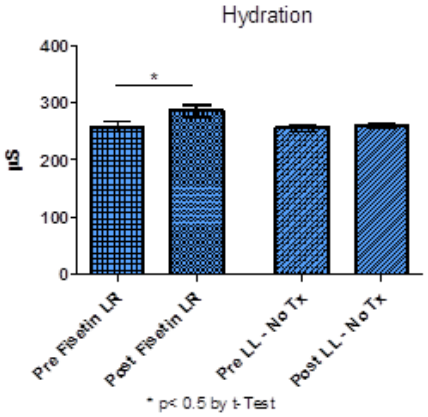


Fig. 13. Skin hydration decreases with aging. Fisetin alleviated age-related loss of skin hydration. Hairless *Shk*$^{-/-}$*;K14-Cre*$^{+/-}$*;Ercc1*$^{-/fl}$ mice lack the DNA repair protein ERCC1 in the epidermis, so that they are photosensitive. Mice were UV-irradiated 3X/ week at a dose that provides the equivalent of 30 years of photo-aging in humans. Mice (N=3) were treated topically with Fisetin 5 mg/ml in a gel (100 µL), 3X/ week. No Tx = UV + Vehicle only. Fisetin caused a significant increase in skin hydration.

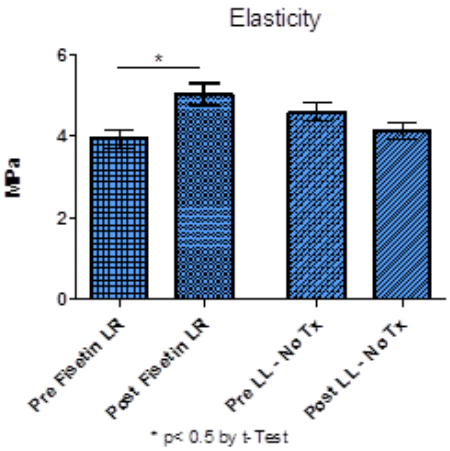


Fig. 14. Fisetin alleviated the age-related decline in skin elasticity. Mice (N=3) were treated topically with Fisetin 5 mg/ml in a gel (100 µL), 3X/ week. No Tx = UV + Vehicle only. Fisetin caused a significant increase in skin elasticity.

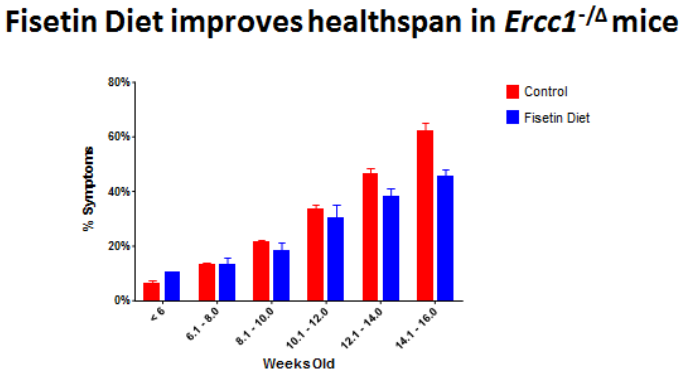


Fig. 15. Fisetin extends healthspan measured by a composite frailty score in the *Ercc1*$^{-/\Delta}$ mouse model of accelerated aging. At 3-4 weeks of age, *Ercc1*$^{-/\Delta}$ mice on a *C57Bl/6:FVB* background were fed *ad libitum* with a standard Teklad 2020 chow diet (N=5) or standard Teklad 2020 chow diet containing Fisetin 500 ppm (N=5). Fisetin 60 mg/kg/day is consumed by each mouse based on Fisetin 500 ppm (500 mg/kg) in the diet and 3-5 g of chow eaten/ day. The composite frailty score for mice includes assays of mobility, cognition, appearance, continence, tremor, gait disorder, and other variables. This score was measured in both groups at baseline and biweekly by an investigator blinded as to the treatment group.

Fisetin increases remaining lifespan in old mice, consistent with a delay in cancers, which account for 50% of deaths in mice, and other causes of death (Fig. 16).

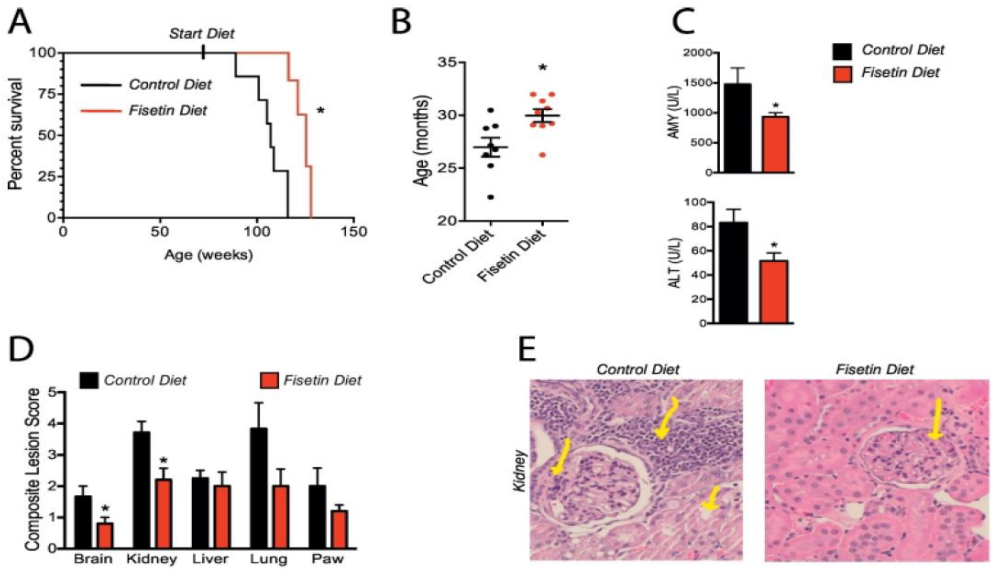


Fig. 16. Fisetin increases survival in chronologically-aged wild-type mice. Late-life intervention with Fisetin in aged wild-type mice extends health span and lifespan. (**A**) At 85-weeks of age (>20 months; equivalent to age >70 years in humans), male and female mice were administered a diet containing Fisetin 500 ppm (500 mg/kg) or fed a control diet with no drug. Lifespan was measured. N = 8–9 mice/ group. Log rank (Mantel-Cox) test. (**B**) Median lifespan of the same cohort of mice. Each dot represents an individual animal. Black bars indicate the mean ± S.E.M. Two-tailed unpaired Student's t-test. (**C**) Clinical chemistry of blood from the above mice to measure markers of liver (alanine aminotransferase [ALT]) and pancreatic (amylase [AMY]) dysfunction. N = 3–6 mice/ group. Two-tailed unpaired Student's t-test. (**D**) Composite lesion scores for aged-related pathologies in multiple tissues determined by histopathologic analysis according to the criteria of the Geropathology Grading Platform[118]. N = 3–8 mice/ group. Two-tailed unpaired Student's t-test. (**E**) Representative images of the kidney of a mouse fed control chow or Fisetin chow. In the control mouse, arrows (from left to right) indicate increased cellularity at a segment of the glomerular capsule border, moderate levels of lymphoid aggregates, and tubular cell vacuolization. In the Fisetin-treated mouse, the arrow indicates only mild segmental cellularity at the glomerular capsule border and a few scattered lymphoid cells near the glomerulus (200× magnification). *p < .05.

Idea generation: The generation of this study began with our Amplifier/ Rheostat Hypothesis (Fig. 3), which we validated in September, 2018 by testing if PAMPs exacerbate the SASP (Fig. 1). Together with the finding that coronavirus causes deaths from cytokine storm/ ARDS, we formulated the hypothesis that the senolytic drug, Fisetin (Fig. 7), could alleviate complications of coronavirus due to an excessive inflammatory reaction in February, 2020. This led us to search for a mouse model of coronavirus. Our collaborators at the U of M had an infected colony and had done an experiment in December, 2019 showing that young mice survive, but old mice die beginning 7 days after exposure to the mouse β-coronavirus, Mouse Hepatits Virus (MHV; Fig. 4) and develop high circulating IL-6, unlike infected young mice (Fig. 5). We administered Fisetin *vs*. vehicle to old mice and sent them to the MHV-infected colony at U of M (Fig. 17). We and our collaborators discovered that Fisetin substantially increased survival of β-coronavirus-infected old mice (Figs. 18, 20, &22).

Aged, infected prevention results: We conducted an experiment in which 10 male and 10 female mice >24 months old, equivalent to humans aged >80 years, were administered Fisetin 20 mg/kg as daily pulse doses by oral gavage for 2 days and 10 control >24 month old male and 10 female mice were administered vehicle at Mayo Clinic. After 2 more days, the mice were moved from Mayo

Clinic to the mouse β-coronavirus-contaminated facility at U of M. Since they fight, the males were placed in bedding in which infected male mice had been housed. The females were placed directly in contact with contaminated female mice, and so had a much higher exposure than the males. Similarly to the male experiment, in a second experiment, 10 female mice pretreated with Fisetin and 10 female mice with vehicle at Mayo Clinic were placed in bedding in which infected female mice had been housed. Those mice pretreated with Fisetin or vehicle by gavage before being transferred from Mayo Clinic to the infected colony at U of M were administered Fisetin 500 ppm or vehicle, respectively, in food while they were in the contaminated facility (Fig. 17).

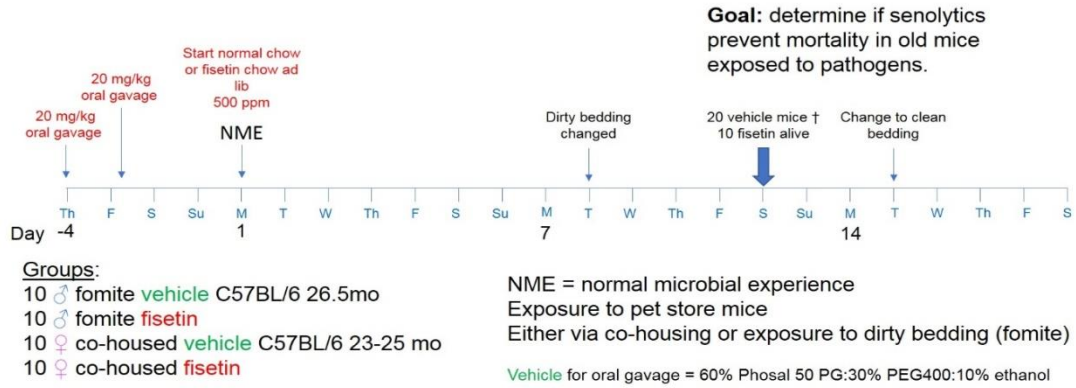


Fig. 17. Mouse coronavirus Fisetin experiment design.

All of the 20 vehicle-treated mice in the first experiment died within 12 days of pathogen exposure. However, 5 of the 20 Fisetin-treated bedding-exposed male ($p<0.0001$) and infected mouse-exposed female mice ($p=0.2$) were still alive after 15 days of continuous infection exposure plus a further 12 days after being switched to clean bedding at Day 16 (Fig. 18). In the second experiment (Fig. 20), all of the 10 vehicle-treated bedding-exposed female mice died within 10 days of infection exposure. However, 7 of the 10 Fisetin-treated female mice were still alive ($p<0.0002$) after 13 days of continuous infection exposure. Thus, Fisetin prevents mortality in old mice caused by viral exposure that includes the mouse β-coronavirus. All mice tested had blood test-proven mouse β-coronavirus infection. Tests for multiple other pathogens were negative.

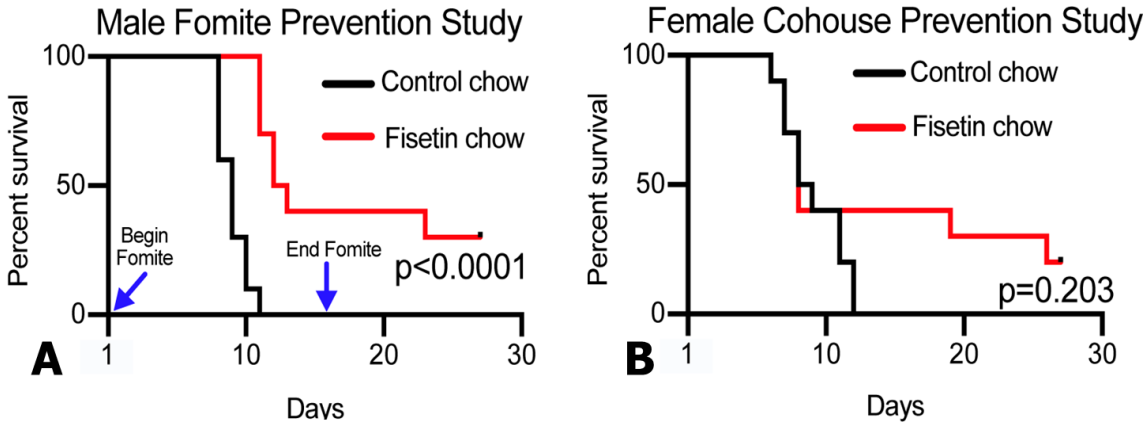


Fig. 18. Survival of male mice exposed to bedding contaminated with mouse β-coronavirus is increased substantially by Fisetin. (A) Old male mice (age>24 months; N=10) were administered Fisetin 20 mg/kg/day as daily pulse doses, and 10 control old male mice vehicle, by oral gavage beginning 4 days before being transported from Mayo Clinic to a contaminated facility at U of M, where they were exposed to bedding from β-coronavirus-infected mice. Males cannot be exposed directly to infected mice because they fight. At the U of M facility, the Fisetin-treated mice were maintained on a Fisetin 500 ppm diet and the vehicle-treated mice were fed regular chow. Male mice treated with Fisetin had a significant improvement in

survival *vs*. vehicle-treated old male mice in this prevention study ($p < 0.0001$; log-rank Mantel Cox test). **(B)** Old female mice (age >24 months; N=10) were also administered Fisetin at Mayo Clinic beginning 4 days before being transported to U of M and exposed directly to infected female mice (likely resulting in a much higher viral load than in the males described above that were exposed only to infected bedding). The females exposed directly to infected female mice tended to have better survival *vs*. vehicle-treated old female mice (N=10; p=0.2). Of note, by day 11, when the vehicle-treated mice had died, the only pathogen that the female co-housed mice had antibodies against was the mouse β-coronavirus, MHV (Fig. 19).

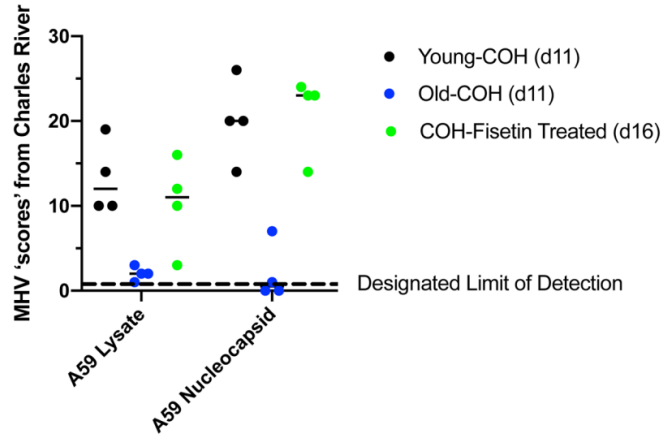


Fig. 19. Fisetin increases the antibody response of old female mice co-housed with mouse β-coronavirus-infected mice. Old female mice (24 months) had less antibody response against coronavirus than young mice by 11 days after both groups were co-housed with mice infected with mouse β-coronavirus. However, at Day 16, the 24-month old female mice treated with Fisetin (as in Fig. 17) had an antibody response similar in magnitude to the young mice. All old female mice treated with vehicle had died by Day 16. Thus, Fisetin treatment does not prevent antibody response of old mice to coronavirus infection and allows time for the older mice to develop an antibody response similar to that of young mice.

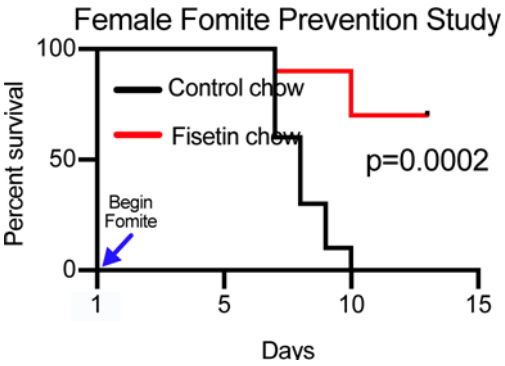


Fig. 20. Fisetin increases survival of female mice exposed to bedding contaminated with mouse β-coronavirus. As for males in the above experiment (Fig. 20), old female mice (age >24 months; N=10) were administered Fisetin 20 mg/kg/day as daily pulse doses or vehicle (N=10) by oral gavage beginning 4 days before being transported from Mayo to the contaminated facility at U of M, where they were continuously exposed to bedding from β-coronavirus-infected mice. At the U of M facility, Fisetin-treated mice were fed Fisetin 500 ppm chow and vehicle-treated mice were fed regular chow. Like males, female mice treated with Fisetin had significant improvement in survival *vs*. vehicle-treated mice in this prevention study ($p < 0.0002$; log-rank Mantel Cox test).

Aged, infected treatment results: In this experiment, we tested if Fisetin intervention in old mice after they have been infected and as they become symptomatic slows death is underway. Old mice (24 months) were transferred from Mayo Clinic to the contaminated facility at U of M and, 3 days after introduction to that facility from Mayo Clinic (study Day 0), were treated for 3 consecutive days with Fisetin 20 mg/kg/day as daily pulse doses *vs*. vehicle by gavage on study Days 3 to 5, 10 to 12, and 17 (Figs. 21&22). After each series of high daily Fisetin doses by gavage, they were maintained on Fisetin 500 ppm in food or regular chow, respectively, on study Days 6 to 9 and 13 to 16.

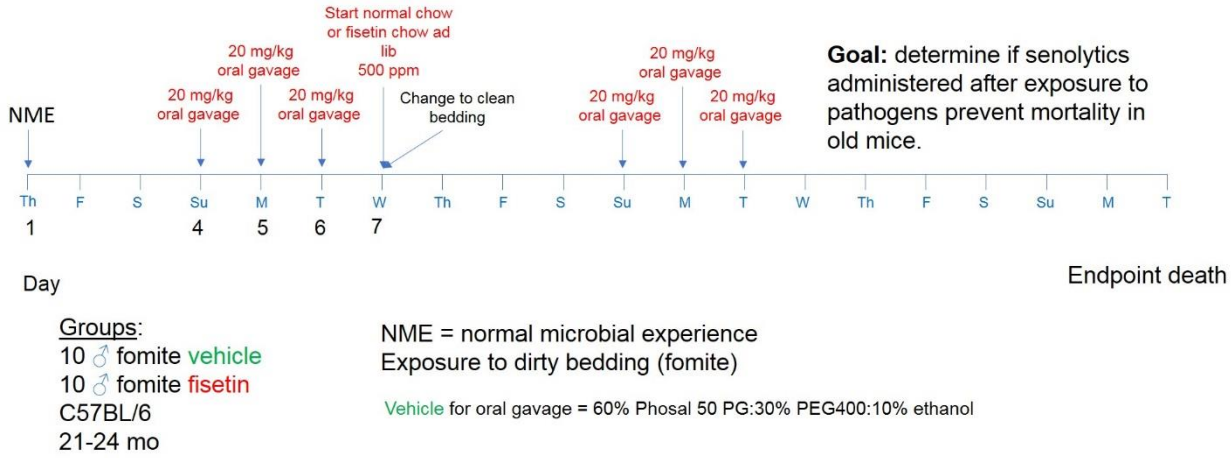


Fig. 21. Experiment to determine if senolytics administered after exposure to β-coronavirus slows mortality in old mice.

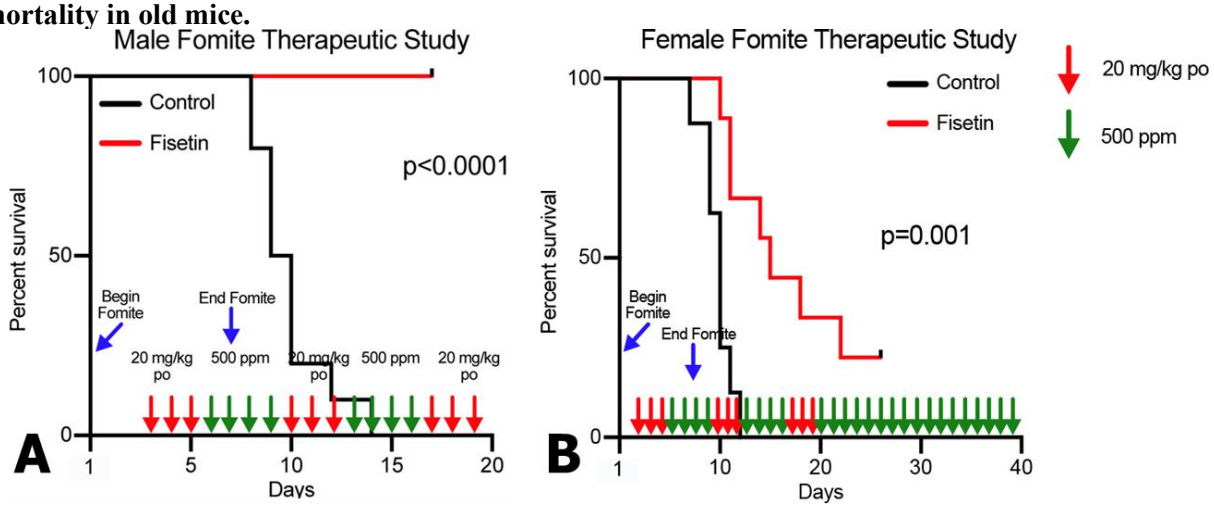


Fig. 22. Fisetin treatment slows death of old mice infected with β-coronavirus. (A) Male 24 month old mice were transported from Mayo Clinic to the mouse β-coronavirus contaminated colony at U of M, depicted here as Day 0. All mice were exposed to bedding used by infected male mice. Beginning 3 days after transfer from Mayo Clinic, they were treated for 3 days with Fisetin 20 mg/kg/day as daily pulse doses *vs*. vehicle by oral gavage (N=10 mice/ group) on Days 4 to 6. On Days 7 to 9, the Fisetin-treated mice were fed with chow containing Fisetin 500 ppm and the vehicle-treated mice were fed chow only. On Days 10 to 12 and 13 to 16, animals were treated with Fisetin 20 mg/kg as daily pulse doses *vs*. vehicle by oral gavage on Days 10 to 12 and 13 (all vehicle-treated mice were dead at Day 14). On Day 17 animals were treated with Fisetin 20 mg/kg as daily pulse doses. **(B)** Female 24 month old mice were treated in the same way. Fisetin significantly increased survival of β-coronavirus-infected mice (males: $p < 0.0001$; females: $p < 0.001$; log-rank Mantel Cox test).

Fisetin treatment of old, β-coronavirus-infected mice slowed death in an experiment close to the clinical study design planned here (Fig. 22). Of note, in separate, parallel studies in which animals were euthanized after cohousing or exposure to dirty bedding, transmission of MHV was 100% and antibodies against the virus were detected by day 11 (Fig. 19). No antibodies to other common mouse pathogens were detectible.

1.3 Clinical Data to Date

Senescent cell clearance from humans: In the first clinical study published about senolytics, a

pilot, open-label study in 14 patients with IPF, we found that 9 pulse-doses of oral D+Q over 3 weeks led to improved 6-minute walk distance, walking speed, ability to rise from a chair, and short physical performance battery by 5 days after the final dose[119]. These results led to initiation of a Phase IIb randomized, placebo-controlled, double-blind trial that is currently underway.

Interim results of a Phase 1, open-label, clinical trial of D+Q for subjects with diabetic kidney disease (DKD) underway at Mayo (ClinicalTrials.gov Identifier: NCT02848131) showed a 3 day oral course of D+Q administered as pulse doses in 9 subjects with DKD reduced adipose tissue senescent cell burden assayed by $p16^{INK4a+}$ and SA-βGal^{+} cells by 11 days after the last dose, compared to biopsies before D+Q administration[76]. A composite score of 10 circulating SASP factors was significantly decreased 11 days after completing the 3 day D+Q intervention. Furthermore, activated $CD68^{+}$ macrophage tissue infiltration was significantly decreased. This trial is continuing (goal=30) to test effects of senolytics on adipose tissue and skin senescent cell abundance and urine SASP factors, metabolic and renal function, inflammation, quality of life, safety (drug toxicity), and tolerability. No serious drug side effects have emerged so far. Evidence continues to show clearance of senescent cells. Each subject will be followed for 4 months after the single course of D+Q to provide data for a larger Phase IIb randomized, placebo-controlled, double-blind trial of senolytics for DKD.

The senolytic drug to be used in our study here is Fisetin. We found that frailty in elderly women is associated with senescent cell burden and the associated inflammation in adipose tissue biopsies[98]. Furthermore, senolytics alleviated frailty in the subjects in our IPF trial[120]. Therefore, a Phase IIb double-blind, placebo-controlled clinical trial of Fisetin to reduce senescent cell burden and alleviate frailty and inflammation, AFFIRM, has commenced. Oral Fisetin 20 mg/kg/day for 2 days *vs*. placebo is being administered as daily pulsed doses for frailty (gait speed <0.8 m/sec) and inflammation in elderly women with multi-morbidity. This trial at Mayo Clinic is FDA-approved (ClinicalTrials.gov Identifier: NCT03430037) and has enrolled over 20 subjects who have received Fisetin or placebo. The trial is not yet unblinded. No participants have had severe or serious adverse events so far at the same doses of Fisetin as proposed here for the same indications, clearing senescent cells and alleviating physical dysfunction and inflammation (in CoV patients).

1.4 Dose Rationale

Others have treated mice with Fisetin 50 mg/kg/day for weeks without evidence of toxicity[121]. We treated 4 male and 4 female 8 month old C57/Bl6 mice with Fisetin 500 mg/kg/day (25-fold higher than the dose to be used in this clinical trial) by oral gavage for 2 consecutive days compared to 4 male and 4 female mice treated with vehicle. We found no evidence of substantial toxicity, as assessed by monitoring activity, food intake, and respiratory quotient in metabolic cages (Comprehensive Laboratory Animal Monitoring System, Columbus Instruments) for 48 hours after the last Fisetin dose. We administered 100 mg/kg/day for 2 consecutive days by mouth as daily pulse doses, 5 times the dose proposed in in this clinical trial, to 2 older *Rhesus* monkeys (ages 19 and 28 years, which is old for this species) at the National Primate Center. In over 1 month of close monitoring, neither monkey had adverse effects such as reduced appetite, altered activity, vomiting, or diarrhea. Acknowledging the higher rates of drug metabolism in mice compared to humans and differences in physiology between non-human primates and humans, in an effort to avoid side effects, a daily dose of ~20 mg/kg was selected here. We verified that this dose is sufficient to reduce senescent cell burden in mice. Our studies in very old coronavirus-infected mice demonstrated that this dose of Fisetin dramatically enhances survival (Figs. 18, 20, and 22).

1.5 Risks and Benefits

There are appear few if any risks of Fisetin at the doses to be used that we have evidence for, provided the subjects meet the exclusion criteria in the protocol regarding medications that Fisetin could interfere with. We have not seen serious or severe adverse events so far in elderly frail women enrolled in our AFFIRM studies from Fisetin 20 mg/ kg/ day for 2 consecutive days in the over 20 subjects randomized to Fisetin or placebo. We did not see adverse effects such as altered food intake, weight, or behavior in old *Rhesus* macaques given 5 times the dose proposed here or in mice given 25 times the dose.

Based on our studies in mice, there may be decreased morbidity and mortality due to β-coronavirus infection after Fisetin administration. All subjects in this study will be elderly (and/or chronically-ill) and hospitalized with mild or moderate CoV infection that could progress to severe or critical disease. The potential benefit to individual subjects in this trial and other people might be large.

In this study, we will examine the effect of oral/ enteral Fisetin as a senolytic agent in humans, which may have immense potential for and reducing frailty/ physical dysfunction and inflammation and alleviating morbidity and mortality from CoV or other infections in older subjects and those with other conditions and diseases associated with accumulation of senescent cells. As an additional, advantageous, off-target effect, removal of senescent cells may allow for functional improvement of neighboring or distant tissues and cells, including MSC's, accelerating recovery. We found that senolytics restore function of MSC's[20,73]. Our collaborator, G. Ellison, working with investigators in China, noted that transplanting normal MSC's can alleviate symptoms and signs in severely ill CoV-infected patients[122]. Thus, an additional benefit of senolytics for CoV could be alleviation of stem cell and progenitor dysfunction.

Based upon review of the published literature and our pre-clinical data, we do not anticipate the occurrence of serious adverse events with the prescribed regimen in this study. We have identified a variety of drug-drug interactions (*e.g.*, current use of warfarin) for which we established exclusion criteria or modification plans to minimize associated risks. We could find no reports of Fisetin toxicity in the literature. Some flavonols have been associated with DNA damage *in vitro*, but Fisetin exerts little or no genotoxic effects[60]. To the contrary, Fisetin has been noted to be anticarcinogenic[48,61,62]. Indeed, we found that chronic Fisetin administration delays cancer in mice and increases remaining lifespan in old mice as well as DNA damage/ genotoxicity highly-prone *Ercc1*$^{-/\Delta}$ mice by over 17%[63]. Since up to 50% of most mouse strains die from cancers, this shows that the beneficial effects of Fisetin on such pro-carcinogenic factors as senescent cell burden and tissue chronic inflammation appear to more than counteract any theoretical effects of flavonol-associated genotoxicity. **Fisetin actually reduces cancer risk**, as opposed to increasing it, as would be expected if Fisetin-induced DNA damage were a risk for the subjects in the clinical study proposed here.

Overall, we do not anticipate substantial risk from the natural product, Fisetin, which is present in many foods, including cucumbers, strawberries, and other fruits and vegetables, albeit at levels lower than those that will be used here. Thus, the risks of using this drug are likely minimal compared to the anticipated benefits for patients with CoV infection severe enough to require hospitalization and the knowledge that may be gained from these clinical investigations.

2 STUDY OBJECTIVES

The primary objective of this study is to determine whether short-term treatment with Fisetin reduces

the rate of complications related to COVID-19 (CoV) disease using a 7 point score adapted from the WHO Ordinal Scale for Clinical Improvement of CoV (Appendix A). The secondary objective is to determine safety and tolerability of treatment with Fisetin in this patient population. Exploratory objectives are to evaluate links between soluble and genetic markers of senescent cell abundance, SASP factors, inflammation, viral load, and treatment effects.

3 STUDY DESIGN

This will be an enterprise wide, randomized, double-blind, placebo-controlled study. Following signature of the informed consent form, approximately 150 subjects meeting all inclusion and no exclusion criteria will be randomized to receive either Fisetin or placebo (1:1 allocation ratio) for days 0 and 1 AND days 8 and 9. Follow-up phone assessments will occur at 15, 30, and 60 days following randomization for evaluation of the occurrence of any trial endpoints or other adverse events. Exploratory objectives are to evaluate links between soluble and genetic markers of senescent cell abundance, SASP factors, inflammation, viral load, and treatment effects and if Fisetin treatment during acute CoV infection decreases Long-hauler syndrome at Days 60 .

3.1 Study Schedule

The schedule of visits for this study is outlined in Table 1. However, a patient may be evaluated at any time for safety concerns.

3.2 Enrollment

Informed consent will be obtained from patients who volunteer to participate in the study prior to the conduct of any study-specific procedures. The patient will be considered “enrolled” into the study at the time an informed consent is provided.

Biomarker samples collection including senescent biomarker analysis will be optional for participants. The informed consent form will reflect this option and patients will specifically be asked to consent to this biomarker evaluation.

3.3 Screening Evaluations and Randomization

Screening evaluations will include a review of the patient’s medical history and assessment of concomitant medications to determine if the patient qualifies for the study. Patients who meet all inclusion criteria and no exclusion criteria will be randomized to receive the study drug or placebo. All patients must be randomized within 10 days of the diagnosis of COVID-19 infection.

Women of childbearing potential must have a negative urine pregnancy test result after the time of randomization, but before IP administration in order to qualify. Qualifying patients will be randomized to receive placebo or fisetin administered in a blinded manner. Blinded randomization will be performed through an automated Interactive Web Response System (IWRS). 75 patients will be randomized to active treatment and 75 patients will be randomized to placebo for a total of 150 randomized patients.

3.4 Active Treatment Period

All patients will receive study medication dosing (either Fisetin 100 mg or matching placebo) (PO) ON day 0 and 1 And Day 8 and 9. If a dose is missed, it should not be replaced. Throughout the study, patients will undergo phone visits (at 15, 30, and 60 days) to assess for potential study endpoints and other adverse events (AEs). Patients will be dispensed the entire supply of study medication on Day 0 to use for the duration of the study.

At each phone contact, patients will be:

1) questioned in a non-specific manner for the occurrence of AEs and any change in concomitant medications
2) 7 point score adapted from the WHO Ordinal Scale for Clinical Improvement of CoV.

Fecal, urine and buccal swab samples for biomarker are optional for the participants. The informed consent form will reflect this option and patients will specifically be asked to consent to this biomarker evaluation.

Please refer to Table 1 and Appendix A for Timetable of Visits and Requirements.

Table 1 Timetable of Visits and Requirements

Visits	**1 Screening/Randomization Phone call**	**2[a] Randomization/ Baseline Phone call**		**3[b] Phone contact**		**4[b] Phone contact**	**5[b] Phone contact**	**6[b] Phone contact**
Days		**0**	**1**	**8 (-/+ 2 days[d])**	**9**	**15 (-/+ 2 days)**	**30 (-2/+7 days)**	**60 (-/+ 14 days)**
Informed consent	X							
Medical/Surgical history	X							
Review Concomitant Medications	X	X		X		X	X	X
Phone contact	X	X		X		X	X	X
Review Inclusion/Exclusion criteria	X	X						
Urine pregnancy test (only women of childbearing potential)		X		X				
Randomization		X						
SF-36 Health Survey		X		X		X	X	X
Buccal, fecal and urine sample for biomarkers (optional)		X				X	X	X
Record potential study endpoints (ordinal scale)		X		X		X	X	X
Review Adverse Events		X		X		X	X	X
Study medication dispensing		X						
Study medication dosing		X	X	X	X			

a) Screening Visit 1 and Visit 2 may be performed on the same day; under these circumstances, requirements at both Screening Visit 1and Visit 2 will be performed once.
b) Phone contact
c) Saliva sample for pharmacogenomics evaluation will be collected using saliva collection kit.
d) Phone contact can occur + or – 2 days; however study drug administration should still occur on days 8 and 9.

4 STUDY POPULATION

4.1 Source and Number of Patients

A total of 150 patients will be randomized.

4.2 Patient Selection Criteria

4.2.1 Inclusion Criteria:

All of these criteria must be met:

- Males and females, at least 18 years of age, capable and willing to provide informed consent;
- Patient must have received a diagnosis of COVID-19 infection within the last 10 days;
- Outpatient setting (not currently hospitalized or under immediate consideration for hospitalization);
- Patient must possess at least one of the following high-risk criteria: 70 years or more of age, obesity (BMI ≥30 kg/m^2), diabetes mellitus, immune deficient state (primary or secondary immunodeficiency), history of hypertension (systolic blood pressure ≥150 mm Hg), known respiratory disease (including asthma or chronic obstructive pulmonary disease, current or previous smoking), known heart failure, known coronary disease, fever of ≥38.4°C within the last 72 hours, dyspnea at the time of presentation, bicytopenia, pancytopenia, or the combination of high neutrophil count and low lymphocyte count;
- Female patient is either not of childbearing potential, defined as postmenopausal for at least 1 year or surgically sterile, or is of childbearing potential and practicing at least one method of contraception and preferably two complementary forms of contraception including a barrier method (e.g. male or female condoms, spermicides, sponges, foams, jellies, diaphragm, intrauterine device (IUD)) throughout the study and for 30 days after study completion;
- Patient must be able and willing to comply with the requirements of this study protocol.

4.2.2 Exclusion Criteria:

None of these exclusion criteria should be met:

- Patient currently hospitalized or under immediate consideration for hospitalization;
- Patient currently in shock or with hemodynamic instability;
- Patient with severe hepatic disease (as *per* clinical judgement) or liver enzymes >2x the upper limit of normal;
- Female patient who is pregnant, breast-feeding, or is considering becoming pregnant during the study or for 1 day after the last dose of study medication;
- Patient currently taking Sirolimus, Tacrolimus, or other mTOR inhibitors for other indications (mainly chronic indications represented by organ transplantation or autoimmune diseases);

- On Warfarin therapy; Subjects taking any of the medications listed in Appendix C may participate if they are otherwise eligible AND the medication can be safely held during the following times:
    - Immediately before the 1st IP administration (Day 0) until at least 10 hours after the 2nd IP administration (Day 1)
    - Immediately before the 3rd IP administration (Day 8) until at least 10 hours after the 4th IP administration (Day 9)
- Patient with a history of an allergic reaction or significant sensitivity to Fisetin;
- Patient undergoing chemotherapy for cancer;
- Patient is considered by the investigator, for any reason, to be an unsuitable study candidate.
- History of diverticulitis or diverticulosis with GI bleeding, as per clinical judegement.

4.3 Prohibited, Allowable, and Concurrent Medications

The use of concomitant medications at the time of randomization will be recorded in the eCRF (with the exceptions of concomitant medication taken "when necessary" (PRN)).

5 STUDY METHODOLOGY

5.1 Efficacy Outcomes

The primary endpoint will be the composite of death or the need for hospitalization due to COVID-19 infection in the first 30 days after randomization. The secondary endpoints will consist of the components of the composite primary endpoint; and the need for mechanical ventilation in the first 30 days after randomization. Exploratory endpoints will include associations between senescent biomarkers and long hauler syndrome effects.

Biomarkers endpoints:
Samples received from participants will be kept frozen until evaluation of biomarkers.
Samples will be stored and processed at Mayo Clinic Rochester.

5.2 Safety Monitoring

Drug safety will be assessed by an evaluation of types, frequencies, severities and duration of any reported AEs. Patients will be monitored for signs and symptoms of drug toxicity.

For all toxicities that require the study therapy to be temporarily or permanently discontinued, relevant clinical and laboratory tests will be repeated as clinically needed until there is final resolution, stabilization of the toxicity, or another cause for the abnormality is determined.

5.2.1 Adverse Event Reporting

Information regarding AEs will be collected from the first dose of study medication (at Visit 2) through and including the last visit. Any AEs prior to randomization will be recorded in the medical history and kept in the patients' chart.

In addition to standard reporting of Serious Adverse Events (SAEs) as defined and outlined in Section 5.2.1.1 below, information regarding SAEs that occur within 30 days following the last study visit, and reported to the investigational site, will be collected. Each patient will be observed and queried in a non-specific fashion at each visit during the study for any new or continuing symptoms since the previous visit.

All SAEs will be recorded on the appropriate eCRF section. The only other AEs to be recorded on the eCRF are those that are either related to the gastrointestinal system, that are judged related to the study medication by the investigator or laboratory abnormalities judged clinically significant by the investigator. Information collected will include the onset, duration, severity, relationship to study drug, and the management as outlined in Appendix C.

5.2.1.1 Serious Adverse Events

Serious Adverse Events (SAE) are those that meet any of the following International Council for Harmonisation (ICH) criteria:

- Is fatal or immediately life-threatening;
- Results in persistent or significant disability/incapacity;
- Requires or prolongs inpatient hospitalization;
- Is a congenital anomaly/birth defect in the offspring of the patient;
- Is a cancer;
- Is an overdose (intentional or accidental);
- Is judged to be medically important.

Medically important events may not be immediately life-threatening or result in death or hospitalization but may jeopardize the patient or may require intervention to prevent one of the outcomes listed in the definition above. Serious Adverse Events are to be reported if they are known to occur within 30 days following the last study visit.

Medical and scientific judgment should be exercised in deciding whether other AEs meet these criteria and are immediately reportable to the sponsor or designee.

In the event of a serious or life-threatening adverse event, or in the event of death, immediately report the event on the appropriate SAE form in the eCRF.

If any SAE occurs, the study treatment may be interrupted or discontinued at the Investigator's discretion. If an acute medical emergency occurs, the investigator should make every effort to reach the contact person listed above, before breaking the randomization code, via the automated Interactive Web Response System (IWRS). However, the investigator may break the randomization code (via IWRS) at any time if this is required for proper treatment of the patient and even if the contact person could not be reached.

Reports to the Health Authorities must be made within 7 calendar days, (for death and life-threatening events) and within 15 calendar days (for other serious events) after being informed of

an SAE by the investigator.

5.2.1.2 Lack of Efficacy of study medication

Any signs or symptoms defined in the protocol as lack of efficacy or collected as efficacy parameters (endpoints) will be captured as AE in the database.

5.2.1.3 Adverse Event Follow-Up

Record all reportable serious and non-serious AEs on the appropriate eCRF including the onset, duration, severity, relationship to the study drug, and ultimate management. All AEs reported during the treatment phase should be recorded and followed until the AE has subsided, or stabilized or until the end of the study, whichever occurs first. The study will be stopped at any time if new knowledge is gained and the risk-benefit ratio is no longer favorable for the participating patients

5.2.2 Other Safety Monitoring

5.2.2.1 Laboratory Evaluations

Optional biomarker with buccal, urine and stool samples will be collected for the biomarker analysis. At day 0/1, day 15, day 30 and day 60. The samples will be kept in a laboratory at Rochester campus. These samples may be used to assess (but not limited to) markers of inflammation and senescence.

5.3 Withdrawal of Patients from the Study

Patients have the right to withdraw from the study at any time during the course of the study. However, every effort should be made, within the bounds of safety, patient choice and the provisions of informed consent, to have each patient complete the study up to and including the last protocol-specified study visit. If the study medication jeopardizes the patient's health or if the patient wishes to discontinue for any reason, study medication can be discontinued but the patient should be encouraged to remain in the study for the follow-up visit up to the End of Study visit. If the patient does not wish to pursue the protocol follow-up visit, the option for a last contact at the end of the study to obtain their vital status should be proposed. Patients who are not compliant during the active treatment period should be counseled on the importance of complying with study requirements and be allowed to remain in the study. No patient who has withdrawn their consent from the study during the active treatment period should be replaced.

In the case of an adverse event or safety concern, the study medication may be withheld temporarily, or the dose reduced, as per investigator judgment.

Patients withdrawn at any time from the study during the active treatment period should complete all last protocol-specified visit requirements (End of Study Visit, the reason for withdrawal from the study, the date of the last visit and the date of the last dose of double-blind medication will be clearly documented in the eCRF).

5.4 Study Completion

The study will end when the last randomized patient will have completed his (or her) 60 day follow-up phone contact. Completion of the study by a patient should be clearly indicated in the eCRF, along with the date of the last visit and the date of the last dose of double- blind medication.

Patients will be remunerated for participation in the study; they will receive $25 after completing Day 15, Day 30 and Day 60.

6 STUDY MEDICATION

Mayo Research pharmacy will prepare and dispense study medication (fisetin and placebo) with appropriate labeling to include a statement that these products are for investigational use only.

6.1 Medication Dispensing

Study medication may be dispensed by the designated pharmacist or a qualified investigative site representative, according to a detailed set of dispensing instructions. Study medication may also be shipped directly to the patient's home depending on evolving circumstances related to COVID-19.

6.2 Dosage Regimen

At randomization, patients will be dispensed Fisetin capsules or placebo to match Fisetin capsules. Patients will be instructed to take 20mg/kg on days 0 and 1 and another 20mg/kg on days 8 and 9 according to the detailed set of dispensing instructions outlined on the label.

7 DATA COLLECTION

Electronic Case Report Forms (eCRF) for all patients will be supplied. These are to be completed as instructed. Original source documents and other study documentation will be maintained at the study site. Risk-based monitoring will be performed by the study team.

8 DATA ANALYSIS AND STATISTICAL CONSIDERATIONS

8.1 Statistical Power and Sample Size Considerations

All main analyses in COVFIS-HOME will be conducted on an intention-to-treat basis. Assuming 75 subjects/ group with a significance level of 0.05, the study will have 80% power to detect an odds ratio of 2.24 comparing the placebo- to Fisetin-treated group, using the 7-point severity score at Day 30 as the endpoint in an ordinal logistic regression model.

Analysis population

8.1.2 Intent to Treat (ITT) population

All patients randomized will be included in the ITT population. Patients will be assigned to treatment groups as randomized for analysis purposes.

8.1.3 Safety population

All patients who received at least one dose of study medication will be included in the safety analysis population. Patients will be assigned according to the true treatment received for analysis purposes.

8.2 Data analysis

8.2.1 Analysis of efficacy outcomes

The primary analysis of efficacy will be based on the intent-to-treat principle. The primary endpoint will be compared between the two treatment groups using a chi-square test. Logistic regression models might also be used to compare the primary endpoint between the two treatment groups while accounting for important baseline characteristics. Secondary endpoints will be analyzed similarly. Exploratory analysis (biomarkers) will include logistic regression models with the primary/secondary endpoints as dependent variables and with treatment group, biomarker and treatment group x biomarker interaction as independent variables.

All statistical tests will be two-sided and conducted at the 0.05 significance level, with the exception of the primary analysis that will be conducted at a slightly lower level to account for the interim analysis. Statistical analyses will be done using SAS version 9.4 or higher.

8.2.2 Analysis of safety outcomes

Safety of Fisetin will be evaluated by presenting descriptive statistics for various safety endpoints broken down by group. This will be done for the population of patients who received at least one dose of study medication (safety population).

8.2.3 Interim analysis

A fully independent 5-member Data and Safety Monitoring Board (DSMB) will be established and will review unblinded safety data as detailed in the DSMB charter. An interim analysis is planned after approximately 50% of randomized patients have completed 30 days of follow-up. The DSMB charter will pre-specify the methods of interim efficacy analyses and the rules for early study termination, approved by all board members. The stopping rules for efficacy will be based on the O'Brien-Fleming alpha-spending function or on a similar conservative approach so that the impact on the final alpha level will be negligible. Futility will be assessed by computing conditional power. The DSMB will have the option of recommending early study termination because of overwhelming efficacy, early termination for futility, or continuation of the trial as planned.

The final analysis of the primary endpoint will be conducted at a significance level slightly below

the 0.05 level to account for the interim analysis. However, since this will have a negligible impact on power, the sample size calculation was calculated using a significance level of 0.05.

9 STUDY COORDINATION

Mayo Clinic Rochester will be responsible for processing and quality control of the data. The handling of data, including data quality control, will comply with all applicable regulatory guidelines, and the study Data Management Plan.

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LIST OF APPENDICES

Appendix A Detailed Study Safety Parameters

1. Demographics and Medical/Surgical History

The following elements of demographics, medical/surgical history will be recorded at the Screening Visit:

- age
- ethnic origin
- sex
- smoking history
- history of diabetes
- history of hypertension
- history of dyslipidemia
- prior cardiac events/heart failure
- prior obstructive pulmonary disease
- prior other respiratory disease

2. Physical Appearance

1. weight
2. height
3. BMI ≥ 30 kg/m^2

4. Detailed Visit Summary

Screening Visit (Days -2 to 0)
Patients will be pre-screened and inclusion and exclusion criteria will be reviewed prior to being recruited for the study.

- Screening Labs (within 6 months prior to randomization): CBC with diff and comprehensive metabolic panel with assessment of liver and kidney function tests
- Pregnancy test (women of childbearing potential)

Subjects who qualify should be immediately randomized. Randomization and dosing should occur on the same day if possible.

Baseline (Day 0 & Day 1) – IP Administration

- Mail in kit for buccal swab, urine and stool sample prior to first dose of IP administration, if applicable
- SF 36 Health Survey
- Determination of Ordinal Scale Score.
- The 1st IP administration will occur on Day 0.
- The 2nd IP administration will occur on Day 1 (12 -36 hours after the 1st IP administration).

- **Day 8 & Day 9 – IP Administration** Phone call visit with the study team as a reminder for IP administration. This call can occur within a 2 day window (+ or -) of day 8; study drug administration will still occur on day 8 and day 9.
- Determination of Ordinal Scale score.
- Review of adverse events.
- SF-36 Health Survey

- The 3rd IP administration will occur on Day 8.
- The 4th IP administration will occur on Day 9 (12- 36 hours after the 3rd IP administration).

Day 15 – (±2 days)
- Determination of Ordinal Scale score.
- Review of adverse events.
- SF-36 Health Survey
- Mail in kit for buccal swab, urine and stool sample, if feasible

Day 30 (-2 to +7 days)
- Determination of Ordinal Scale score.
- Review of adverse events.
- SF-36 Health Survey
- Mail in kit for buccal swab, urine and stool sample, if feasible

Day 60 (±14 days)
- Determination of Ordinal Scale score.
- Review of adverse events.
- SF-36 Health Survey
- Mail in kit for buccal swab, urine and stool sample, if feasible

Appendix B Administrative Procedures for the Reporting of Adverse Events

The following administrative procedures for reporting AEs are to be followed during the conduct of this clinical trial.

1 ADVERSE EVENTS DURING THE TRIAL

Each patient will be observed and queried in a non-specific manner by the investigator or study coordinator at each visit for any new or continuing AE since the previous visit. Any AEs prior to the first dose of study medication will be recorded in the medical history and kept in the patients' chart. All SAEs will be recorded in the appropriate eCRF section. In addition to SAEs, the only AEs to be recorded in the eCRF are those that are either related to the gastrointestinal system, that are judged related to the study medication by the investigator or that are laboratory abnormalities judged clinically significant by the investigator. Information collected will include the onset, duration, severity, relationship to study drug, and the management. SAEs are to be reported if they are known to occur within 30 days following the last study visit.

The investigator will review the clinical laboratory test results in a timely fashion when received from the laboratory. Those results qualifying as AEs as defined in this appendix will be recorded on the AE eCRF section and will be handled according to these AE reporting procedures.

The investigator will review concomitant medications being taken by the patient.

Definitions

1.1 Pre-existing condition

A pre-existing condition is one that is present prior to randomization. A worsening of a pre-existing condition after taking the first dose of investigational product should be reported as an AE.

1.2 Adverse Event (AE)

An AE is defined as any unfavorable and unintended sign (including a clinically meaningful abnormal laboratory finding), symptom, or disease temporally associated with the use of an investigational product, whether or not related to the investigational product.

A medical procedure is not considered and should not be reported as an AE. However, the medical condition which led to the procedure should be considered as an AE and be reported as such.

1.3 Related Adverse Event

A related AE is one where, according to the Investigator, there is a reasonable possibility that the event may have been caused by the study drug.

1.4 Serious Adverse Event (SAE)

Serious Adverse Events (SAE) are those that meet any of the following International Council for Harmonisation (ICH) criteria:

- Is fatal or immediately life-threatening (NOTE: the term "Life-Threatening" refers to an event in which the patient was at immediate risk of death at the time of the event; it does not refer to an event which could hypothetically have caused death had it been more severe);
- Results in persistent or significant disability/incapacity;
- Requires or prolongs patient hospitalization;
- Is a congenital anomaly/birth defect in the offspring of the patient;
- Is a cancer;
- Is an overdose (intentional or accidental);
- Is judged to be medically important.

Medically important events may not be immediately life-threatening, result in death or hospitalization, but may jeopardize the patient or may require intervention to prevent one of the outcomes listed in the definition above. Serious adverse events are to be reported if they are known to occur within 30 days following the last study visit.

Medical and scientific judgment should be exercised in deciding whether other AEs meet these criteria and are immediately reportable to the sponsor or its designee.

Hospitalization is defined as a patient admission to a hospital for medical treatment or observation; a visit to the emergency room for an outpatient consultation is not considered a hospitalization. Moreover, the following hospitalizations are not considered SAEs:

- Hospitalizations for diagnostic or elective surgical procedures for a pre-existing condition.
- Hospitalization for therapy of the target disease(s) of the study if the protocol explicitly anticipated and defined the symptoms or episodes.
- Hospitalization for study efficacy measurement, as defined in the protocol.

1.5 Life-Threatening Adverse Event

A life-threatening AE is an AE that, in the opinion of the investigator, places the patient at immediate risk of death from the reaction as it occurred.

1.6 Unexpected Adverse Event

An unexpected AE is any AE that is not consistent with findings previously observed.

1.7 Clinical Laboratory Adverse Event

A clinical laboratory abnormality is regarded as an AE if it has been confirmed by at least 1 repeat test and suggests a disease and/or organ toxicity severe enough to require active management.

1.8 Treatment-Emergent Signs and Symptoms (TESS)

A TESS event is any AE that was not present prior to randomization or that worsens in character, intensity or frequency while the patient is in an active treatment period.

1.9 Post-treatment Adverse Event

A post-treatment AE is any AE that occurs after treatment is discontinued.

2 HANDLING OF ADVERSE EVENTS

2.1 Treatment-Emergent Signs and Symptoms

Any condition/diagnosis that meets the definition of a TESS event is captured in the appropriate eCRF AE form and kept in the patient's chart.

2.2 Serious Adverse Events

All SAEs are to be immediately reported. Serious Adverse Events, within 24 hours of the Investigator's first knowledge of the event.

2.3 Intensity

The following criteria are used to assess the intensity of each AE:

- Mild: The patient is aware of the sign or symptom, but finds it easily tolerated.
- Moderate: The patient has enough discomfort to cause interference with or change in usual activities.
- Severe: The patient is incapacitated and unable to work or participate in many or all usual activities.

2.4 Relationship to Study Drug – Physician's Assessment

There are 3 categories for the physician's assessment of the causal relationship between study drug and an AE as follows: not related, possibly and probably.

2.5 Clinical Outcome

The following categories are used to assess the clinical outcome of each AE:

- Death related to the adverse event
- Recovered – The patient has fully recovered from the AE without observable residual effects.
- Recovered with sequelae – The patient has recovered from the AE with observable residual effects.
- Not Recovered – The patient is still being treated for the residual effects of the original AE. This does not include treatment for pre-existing conditions including the indication for the study drug.
- Recovering
- Unknown

3 CAPTURING AVDERSE EVENTS

3.1 Pre-Existing Condition

A pre-existing condition should be captured in the medical history and kept in the patient's chart. If the frequency, intensity, or character of the condition worsens during study treatment, and is either related to the gastrointestinal system, is judged related to the study medication by the investigator or is a laboratory abnormality judged clinically significant by the investigator, it must be documented in the appropriate eCRF AE form.

3.2 Lack of Efficacy of Study Medication

Signs or symptoms defined in the protocol as lack of efficacy or collected as efficacy parameters (endpoints) will be captured as AEs.

3.3 Clinical Laboratory Adverse Event

A clinical laboratory abnormality should be reported as an AE only if it is considered to be clinically significant by the investigator and confirmed by repeat testing.

3.4 Hospitalization or Surgery/Procedure

Any AE reported as study endpoint should not be reported as an SAE. Any condition/diagnosis responsible for surgery/procedure should be reported as an AE if it meets the criteria for an AE. A medical procedure is not considered and should not be reported as an AE. The surgery/procedure itself will be reported as a Clinical Outcome of the underlying event. Events that prolong any hospitalization are reported as SAEs.

3.5 Death

The cause of death should be reported as an AE.

3.6 Other Adverse Events

Study endpoints are to be reported in the eCRF within 24 hours of awareness of the event.

In addition to SAEs and study endpoints, the only AEs to be recorded on the eCRF are those that are either related to the gastrointestinal system, that are judged related to the study medication by the investigator or that are laboratory abnormalities judged clinically significant by the investigator. Information collected will include the onset, duration, severity, relationship to study drug, and the management.

3.7 Follow-Up Period

For SAEs, the patient must remain under observation until the SAE has subsided or stabilized and all serious findings have returned to normal or stabilized. Any follow-up information to an initial SAE report must be updated in the eCRF. Serious Adverse Events are to be reported if they are known to occur within 30 days following the last study visit.

Appendix C. Excluded Medications and Dosing Modifications

Subjects taking any of the medications listed in Appendix C may participate if they are otherwise eligible AND the medication can be safely held during the following times:

- **Immediately before the 1st IP administration (Day 0) until at least 10 hours after the 2nd IP administration (Day 1)**
- **Immediately before the 3rd IP administration (Day 8) until at least 10 hours after the 4th IP administration (Day 9)**

Cardiac:
- Digoxin
- Flecainide
- Amiodarone

Psychiatric:
- Duloxetine (Cymbalta)
- Lithium
- Clozapine (Clozaril)
- Thioridazine (Mellaril or Melleril)

Neurologic:
- Carbamazepine (Tegretol)
- Phenobarbital (Luminal and Solfoton)
- Phenytoin (Dilantin, Phenytoin Sodium, Phenytek)
- Fosphenytoin (Cerebyx)
- Riluzole (Rilutek; Used to treat amyotrophic lateral sclerosis ALS)

Antimicrobial/fungal:
- Aminoglycosides (Eg. amikacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin, tobramycin)
- Azole antifungals (fluconazole, miconazole, voriconazole) – (if absolutely necessary, levels must be subtherapeutic or therapeutic)
- Macrolides (clarithromycin)
- Antivirals (nelfinavir, indinavir, saquinavir)

Oral hypoglycemic/Anti-Diabetes drugs:
- Glimepiride (Amaryl)
- Glyburide (DiaBeta, Glynase PresTab, Micronase)

Anticoagulants/ Antiplatelet:
- Warfarin
- Full dose aspirin

Others:
- Cyclosporine
- Methotrexate
- Nitroglycerin
- St. John's Wort
- Theophylline
- Tyrosine Kinase Inhibitors (list included further down in this appendix)
- Tizanidine (Zanaflex)
- Tacrine

- Diclofenac

Continuation of Excluded Medications

Sensitive Substrates CYP2C9, CYP2C19, CYP1A2, Other (OATP1B1)

	Sensitive substrate	**Drugs with a narrow therapeutic range**
CYP2C9	**Glyburide** **Losartan**	Fosphenytoin Phenytoin Warfarin Glimepiride Diclofenac Bosentan
CYP2C19		Phenytoin
CYP 1A2	Clozapine Olanzapine	Theophylline Tizanidine Warfarin Ramelteon Tacrine Duloxetine Mexiletine Riluzole
Other (OATP1B1)	Corticosteroids Thyroid hormones Eluxadoline Eltrombopag *Pioglitazone (Actos)* - monitor blood glucose levels closely or ½ dose QD Enzalutamide Colchicine (½ dose QD until 10 hours after the last (2nd) IP administration Venlafaxine (skip dose on study drug Day 2; then resume as usual) **Atorvastatin** (QOD or ½ dose QD until 10 hours after the last (2nd) IP administration; unless willing to modify as directed.) **Rosuvastatin** (QOD or ½ dose QD until 10 hours after the last (2nd) IP administration unless willing to modify as directed.) Desipramine (QOD or hold until 10 hours after the last (2nd) IP administration) Atomoxetine (hold) *Repaglidine*- monitor blood glucose level closely (CYP2C8)	

Tyrosine Kinase Inhibitor List (CYP2C8)

afatinib axitinib Bosulif bosutinib cabozantinib Caprelsa Cometriq	crizotinib dasatinib erlotinib Gilotrif Gleevec ibrutinib Iclusig	imatinib Imbruvica Inlyta lapatinib Nexavar nilotinib pazopanib	ponatinib regorafenib sorafenib Sprycel Stivarga sunitinib Sutent	Tarceva Tasigna Tivopath tivozanib Tykerb vandetanib Votrient	Xalkori Zaltrap ziv-aflibercept