PART B
STUDY DESCRIPTION

TITLE OF PROTOCOL	**Pharmacokinetic and pharmacodynamic study of oral quercetin and isoquercetin in healthy adults and patients with hypercoagulable states.**
Principal Investigator	**Jeffrey Zwicker, MD**

B1. PURPOSE OF PROTOCOL

The primary goal of this trial is to perform a pharmacokinetic analysis in 60 healthy individuals of quercetin or isoquercetin and determine whether the addition of ascorbic acid and niacin increases absorption. The secondary goal is to evaluate the pharmacodynamic activity of quercetin or isoquercetin metabolites to inhibit protein disulfide isomerase activity. The first 30 patients in this group will receive isoquercetin delivered in the form of a chew. An additional 10 patients will receive isoquercetin delivered in capsule form. Pharmacodynamic studies will also be performed in an additional cohort of 20 patients with evidence of antiphospholipid antibodies or myeloproliferative disorder.

B2. SIGNIFICANCE AND BACKGROUND FOR THE STUDY

Extracellular disulfide isomerization has been explored as a regulatory pathway in platelet activation and fibrin generation. Protein disulfide isomerase (PDI) is a thiol isomerase that is secreted by platelets and endothelial cells following activation and localizes to the membrane surface. The hemostatic targets of extracellular PDI continue to be elucidated and may include tissue factor and platelet integrins such as □IIb□IIIa. The Furie Laboratory recently demonstrated the critical role of extracellular PDI in regulating platelet thrombus formation and fibrin deposition in vivo. Employing intravital fluorescence microscopy to monitor a laser-induced vascular injury in mice, we observed that inhibition of PDI with either bacitracin, a non-specific thiol isomerase inhibitor, or a specific and inhibitory monoclonal anti-PDI antibody completely blocked both platelet thrombus formation and fibrin generation in vivo. The role of PDI in thrombus formation is not unique to the laser injury model as similar observations have been made using carotid artery ligation and ferric chloride injury in mice (Furie, Furie and Bellido-Martin, unpublished results).

Identification of quercetin as a potent inhibitor of PDI

Following the observation that inhibitory antibodies against PDI block platelet thrombus and fibrin generation in vivo, we became interested in investigating PDI as a novel antithrombotic target. Absent a known potent and selective small molecule inhibitor, we performed a high throughput screen of 4900 bioactive compounds using a turbidometric insulin assay. The reduction of insulin by PDI can be monitored spectrophotometrically at OD650 . Notably, several related flavonoids (quercetin-3-rutinoside and isoquercetin) were identified as potent inhibitors of PDI. The Kd of quercetin-3-rutinoside for PDI is 2.8 □M as determined by surface plasmon resonance (SPR). PDI inhibition appears to be specific without evidence of significant inhibition of other thiol isomerases such as ERp57, ERp5, ERp72, or thioredoxin. Flavonoids belong to a larger group of plant polyphenols that are plant pigments characterized by an aromatic ring with at least one hydroxyl group and are subclassified into flavonols, flavonones,

flavones, flavan-3-ols, and isoflavones based on structural variation and degree of oxidation. In Western diets the daily consumption of flavonoids is approximately 20 to 30 mg, the majority of which is composed by the flavanol quercetin and its derivatives present in onions, tea, berries, and apples. As the most common flavonoid present in dietary sources, the quercetins are among the best studied. Purified forms of quercetins are available as nutritional supplements and touted for anti-oxidant properties. The structures of quercetin differ based on type and location of sugar moieties. The parent molecule quercetin as an aglycone is present in only small quantities in food in contrast to quercetin-3-rutinoside (rutin) which has a glycoside group at the 3 position of the pyrone ring. The enzymatic cleavage of the glucose-rhamnose bond in quercetin-3-rutinoside yields isoquercetin (quercetin-3-O-□-D-glucoside).

Quercetin inhibits thrombus formation in animal models
Following the identification of quercetin-3-rutinoside and related flavonoids as potent inhibitors of PDI, we performed laser- induced vascular injury experiments in mice and observed inhibition of platelet thrombus formation and fibrin generation following intravenous and oral administration. A modest dose of oral quercetin (5 mg/kg) yielded a ~50% reduction in platelet accumulation and fibrin generation in mice. The PDI-specific nature of this interaction is confirmed by addition of recombinant human PDI to neutralize quercetin activity.

Quercetin as an anticoagulant in humans
In order to evaluate the potential antithrombotic activity of quercetin in humans, d-dimer levels were measured in plasma samples following administration of oral quercetin. In this previously published clinical trial conducted at University of Utah, study subjects received oral quercetin (365 mg twice daily) for 28 days. Eligible patients were hypertensive and excluded for a history of cardiovascular events, diabetes, renal disease, obesity, or active smoking. In four patients with baseline elevations in d-dimer (>500 ng/ml) there was a significant decrease in mean plasma d-dimer levels compared with baseline (1198 vs. 462 ng/ml, P=0.056). No change in d-dimer levels was observed in patients (N=20) with normal baseline concentrations (174 vs. 184 ng/ml, P=0.97).

The antithrombotic potential of flavonoids first garnered attention in the 1990's following the publication of several large epidemiologic studies. In the Zupthen Elderly Study, the group of individuals who consumed over 30 mg of flavonoids daily experienced a nearly 70% reduction in mortality from myocardial infarction (adjusted RR 0.32, 95% CI 0.15-0.71) compared with those individuals who consumed less than 20 mg daily. A prospective study of nearly 35,000 postmenopausal women demonstrated that flavonoid consumption reduced both cardiovascular and overall mortality which was also confirmed in a meta-analysis of 7 cohort studies. Flavonoid consumption has also been linked with a decreased incidence of nonfatal and fatal stroke. Attempts have been made to identify which thrombotic risk factors are modulated with dietary flavonoid consumption but endpoints such as blood pressure, high and low-density lipoprotein concentrations, and flow-mediated dilatation have yielded inconsistent results. Flavonoids such as quercetin are known to inhibit plate let function in vitro. The ingestion of either onion soup or quercetin -4'-O-□-D-glucoside (the predominant quercetin found in onions) inhibited ex vivo platelet aggregation. However there has never been a prospective clinical study on the use of quercetin to specifically prevent thrombotic events in humans.

Quercetin has been used in numerous clinical studies with a long established history of safety. The compound recently received the designation of Generally Recognized as Safe (GRAS) by

the FDA. Quercetin (>99.5% purity) will be provided by Quercegen Pharma (Sudbury, MA) which adheres to Good Manufacturing Practice guidelines and previously provided study drug for randomized clinical trials conducted in the United States. Based on our preliminary data, the IC50 for PDI inhibition with quercetin analogues is 5-7uM. While we describe a reduction in d-dimer levels following an oral dose
of quercetin at 375mg daily twice daily, the IC50 achieved was only 1.5uM. Recent data provided by Quercegen Pharma suggests that quercetin (500mg) in combination with ascorbic acid and niacin improves both stability and absorption, yielding serum concentrations close to 5uM. Quercegen Pharma formulates quercetin in chews containing ascorbic acid but formal PK studies are lacking. Also, the presence of a glucoside moiety at the 3 position of isoquercetin appears to increase absorption possibly through a sodium-dependent glucose transporter on enterocytes. Quercetin glucosides such as quercetin-3-rutinoside or isoquercetin undergo deglycosylation locally at the enterocyte to yield the aglycone. Although the absorption of the related quercetin compounds may differ, all forms of quercetin are metabolized in the enterocyte and liver and circulate as similar glucuronated, sulfated, and methylated conjugates. These conjugates have demonstrated biologic activity and in preliminary experiments using an insulin turbidometric assay we observed that purified quercetin-glucoronide inhibited PDI activity with greater potency than quercetin or quercetin-3-rutinoside.

In summary, we previously observed that inhibition of PDI in vivo inhibits platelet thrombus formation and fibrin generation in a mouse thrombosis model. Following the identification of quercetin and related flavonoids as potent PDI inhibitors in vitro, we demonstrated antithrombotic efficacy in animal models as well as a reduction in d-dimer in humans. Based on these preliminary findings and unique antithrombotic mechanism of PDI inhibition, we propose to evaluate quercetin as an antithrombotic in specific patient populations. Prior to initiating these trials, we plan to perform a pharmacokinetic and pharmacodynamic study to evaluate the influence of ascorbic acid and niacin on quercetin/isoquercetin absorption. As planned trials include patients at risk of thrombosis such as those with elevated antiphospholipid antibodies, will explore pharmacodynamics specifically in this population.

B3. DESCRIPTION OF RESEARCH PROTOCOL

A. Study Design – Overview, Methods, Procedures

Overall Design

To compare the absorption and activity of quercetin or isoquercetin with or without ascorbic acid, we will perform pharmacokinetic and pharmacodynamic analysis in 4 cohorts. Cohorts A, B, and C will consist of 10 healthy adults and cohort D will consist of 20 participants with positive anti-phospholipid anitbodies or myeloproliferative disorder. Group A will include 10 healthy individual receiving oral chews containing quercetin (500 mg total). Subjects may return and given chews containing quercetin (500 mg) in combination with ascorbic acid (500 mg) and niacin (20mg). Group B will include 10 healthy adults with isoquercetin (500 mg) alone and isoquercetin (500mg) with ascorbic acid (500 mg) with niacin (20mg). Group C will be comprised of 10 healthy adults receiving isoquercetin (1000 mg) alone and isoquercetin (1000mg) with ascorbic acid (1000 mg) with niacin (40mg). Group D will include 20 individuals with antiphospholipid antibodies or myeloproliferative disorder receiving isoquercetin (1000 mg) with ascorbic acid and niacin. Group E will be comprised of 10 healthy individuals who will receive isoquercetin 1000 mg with ascorbic acid 248 mg and niacin 20 mg in a capsule formulation. For group A, B, C and E blood will be drawn prior to the administration of study drug and at different time points over a 24-hour period (0 min, 30 min and 1, 2, 4, 6, 8, and 24 hours) in order to assess pharmacokinetics and pharmacodynamic inhibition of PDI activity. Group D will have blood drawn prior to the administration of study drug and 4 hours following the administration of the study drug in order to assess pharmacodynamic inhibition of PDI activity.

Group A will receive 2 chews, each containing:
1) quercetin 250mg
2) quercetin 250mg, vitamin c 250mg, niacin 10mg

Group B will receive 2 chews, each containing
3) isoquercetin 250mg,
4) isoquercetin 250mg, vitamin C 250g, niacin 10mg

Group C will receive 4 chews, each containing
5) isoquercetin 250mg,
6) isoquercetin 250mg, vitamin C 250g, niacin 10 mg

Group D will receive 2 chews, each containing
7) isoquercetin 500 mg, vitamin C 500g, niacin 20mg

Group E will receive 4 capsules, each containing
8) isoquercetin 250mg, vitamin C 62mg, niacin 5mg

All study drugs will be provided by Quercegen Pharma.

Study Procedures: Healthy participants (Group A, B, C and E)

1. Register patient at the Clinical Research Center (CRC) facility at BIDMC.
2. Urinary pregnancy test (if applicable)
3. Record height and weight of subject and vitals
4. Place an 20-guage (or larger) IV in the upper extremity (patient may elect serial venipuncture).
5. A 15 ml sample of venous blood will be collected into 3 blue top test tubes containing sodium citrate tubes 3.8% (wt/vol)
6. Study medication (e.g. quercetin) will be given to chew along with a cup of water
7. Additional blood samples will be obtained at 30 min, 1, 2, 4, 6, 8 and 24 hours into citrated tubes. Study subjects will be permitted to eat at flavonoid-free lunch following the 4 hour dose.
8. An additional 5ml will be drawn at the 0 and 4 hour time points into a citrated tube.
9. Study subjects will be permitted to go home after the 8 hour lab draw and will return for the 24 draw. (IV must be removed after the 8 hour lab draw)

Study Procedures: Antiphospholipid antibody or myeloproliferative disorder participants (Group D)

1. Register patient at the Clinical Research Center (CRC) facility at BIDMC.
2. Urinary pregnancy test (if applicable)
3. Record height and weight of subject and vitals
4. Place an 20-guage (or larger) IV in the upper extremity (patient may elect serial venipuncture).
5. A 20 ml sample of venous blood will be collected into 4 blue top test tubes containing sodium citrate tubes 3.8% (wt/vol)
6. Study medication (e.g. quercetin) will be given to chew along with a cup of water
7. An additional 20ml will be drawn at the 4 hour time point into 4 blue top test tubes containing sodium citrate 3.8% (wt/vol).
8. Study subjects will be permitted to go home after the 4 hour lab draw.

Platelet studies

Do not process (centrifuge) one of the 5ml citrated tubes at time 0 and 4 hours. Please page Anita Rodrigues or designee as soon as the time 0 and 4 hour blood draws are obtained. These unprocessed tubes will be picked up immediately for platelet aggregation studies to be performed in the Flaumenhaft Laboratory.

Plasma sample preparation and storage

1. Platelet free plasma (PFP) will be generated by differential centrifugation 2,100g x 20 minutes x 2. Initial centrifugation will occur within 60 min of laboratory draw.
2. PFP aliquots (1ml) will be placed into cryotubes (labeled with study ID and time point)
3. Samples will be stored at -80oC until PK/PD analysis

Plasma samples will be used for quantitation of quercetin by liquid chromatography and mass spectrometry.

Microparticles will be isolated from PFP and PDI activity on the surface of plasma microparticles will be measured with a fluorescent substrate (dieosin glutathione disulfide or di-E-GSSG) per previously described methods (Raturi et al Biochimica et Biophysica Acta 2008;1778:2790-2796.

Additional hemostasis-related assays such as microparticle and tissue factor activity will be performed using PFP.

B. Statistical Considerations

a. **Sample Size Justification:** A total of 40 healthy individuals will be enrolled in the trial. Data collected from the pharmacokinetic analysis will be used to identify the formulation of study drug, either isoquercetin or quercetin with or without ascorbic acid. Ten patients per group is the minimum number necessary to establish a difference in values of pharmacologic variables (e.g. Cmax or AUC) of at least one standard deviation of magnitude with a 0.05 level of significance using a paired two-tailed test of log-transformed values. Although this study may be technically underpowered to detect small differences, a clinically significant difference between the relative bioavailability among the four formulations should be readily apparent. If a subject chooses not to participate in the second part of the study (i.e. quercetin in combination with ascorbic acid and niacin) then alternative subjects will be recruited to complete the second PK/PD analysis.

For the cohort with antiphospholipid antibodies or myeloproliferative disorder, we plan to enroll 20 patients (approximately 10 in each arm). The null hypothesis is there is no difference in thrombin-induced thrombin generation measured at 0 and 4 hours. The sample size calculation is based on preliminary data demonstrating that the mean decrease in thrombin generation before and after isoquercetin was -54% with a
standard deviation of mean % difference of .41 in healthy individuals. Accordingly, using a paired t-test analysis for time 0 and 4 hours with a 2-sided alpha of 0.05, the power of the study is 95.8

b. **Data Analysis:** The concentration of quercetin in plasma will be determined by liquid chromatography and mass spectrometry in collaboration. Analysis will include elimination half-life, peak concentration, and area under the curve. We will also evaluate an experiment assay to monitor PDI inhibition in plasma using a dieosin glutathione disulfide (di-E-GSSG) to liberate a self-quenching N-terminal GSSG residue following cleavage of a disulfide bond. There is a planned comparison of peak PDI inhibition for the different groups and correlation with serum quercetin levels. Statistical analysis will be performed in collaboration with Donna Neuberg, PhD and Federico Campigotto at DFCI.

C. Subject Selection

Inclusion Criteria

1. Subject is willing to participate and provide informed consent
2. Subject is considered reliable and capable of adhering to the protocol per the judgment of the Investigator
3. Subjects in group D must exhibit good organ reserves (within prior 4 weeks) defined as:
a. Estimated GFR >35 (formula),
b. Platelet count >65 K/uL,
c. Hemoglobin >10.5 grams/dL
d. Total bilirubin <2.0 mg/dL

4. Minimum age 18 years old
5. Body mass index (BMI) between 18 and 35 kg/m2
6. For cohort D (antiphospholipid antibodies or myeloproliferative disorder)
a. Subjects in group D with antiphospholipid antibodies must have at least one positive antiphospholipid antibody within the last 8 weeks and/or previous confirmed antibodies (2 or more occasions at least 12 weeks apart) :
i. Positive lupus anticoagulant
ii. anticardiolipin antibody IgM or IgG (>40U GPL)
iii. anti-β2 Glycoprotein1 antibody titer (>35 units)
b. Subjects in cohort D with myeloproliferative disorder must have confirmed diagnosis of essential thrombocythemia or polycythemia vera through bone marrow biopsy or molecular testing.

Exclusion Criteria

1. Pregnant. If female of child-bearing age, negative urinary pregnancy test prior to dosing of quercetin or isoquercetin
2. No history of malabsorptive gastrointenstinal disorder
3. Currently taking, warfarin, low-molecular weight heparin or other anticoagulants (such as direct thrombin inhibitors or factor X inhibitors)

4. Prescribed niacin for hyperlipidemia
5. Known HIV
6. History of sensitivity or intolerance to flavonoids, niacin or ascorbic acid
7. May not have uncontrolled intercurrent illness including, but not limited to ongoing or active infection, hepatitis, symptomatic congestive heart failure, unstable angina pectoris or cardiac arrhythmia

Foods containing quercetin that study subjects will be asked to avoid for 72 hours before the first dose of study drug (and between the 8 and 24 hour blood samples) include:
Vegetables: beans (green, yellow, snap), broccoli, capers, kale, lettuce, onion, peppers (hot, sweet, green, jalapeno), red cabbage, spinach, tomato
Fruit: apples, apricot, blackberries, blueberries, cherry, cranberry, grapes, lemon, oranges, plums, raspberries, strawberry.

Other: chocolate/cocoa, honey, tea, wine

B4. POSSIBLE BENEFITS

This study will not directly benefit the study subjects. Pharmacokinetic and pharmacodynamics studies of quercetin will facilitate the conduct of clinical trials of quercetin to treat prothrombotic conditions.

B5. POSSIBLE RISKS AND ANALYSIS OF RISK/BENEFIT RATIO

Quercetin is considered safe without significant toxicities and Quercegen Pharma received GRAS designation from the FDA for their quercetin formulation (Inventory Number GRN 341).Quercetin has been used in numerous clinical studies with a long established history of safety. Quercetin or isoquercetin (>99.5% purity) alone or in combination with ascorbic acid and niacin will be provided by Quercegen Pharma (Sudbury, MA) which adheres to Good Manufacturing Practice guidelines and previously provided study drug for randomized clinical trials conducted in the United States.
As a common dietary flavonoid, quercetin has been evaluated in a number of clinical trials with different endpoints. Quercetin may lower blood pressure slightly as was demonstrated in a randomized trial of 43 individuals treated with quercetin 365 mg twice daily for 28 days. Both systolic and diastolic blood pressure were modestly lower at 28 days in the quercetin treated patients (7 mm Hg and 5 mm Hg, respectively) although this effect was not observed in a other studies. No significant change was observed with lipid profiles or serum glucose. In a randomized trial of 1002 outpatients treated with oral quercetin 500mg or 1000 mg daily for 3 months to prevent upper respiratory infections, there were no significant adverse events reported. In a phase I study where quercetin was administered intravenously at doses greater than 2000 mg, there was evidence of nephrotoxicity in several patients.

Healthy subjects will undergo a venipuncture and placement of an IV with subsequent draw of 80cc over the course of 24 hours. Antiphospholipid subjects will undergo a venipuncture and

placement of an IV with subsequent draw of 40cc over the course of 4 hours. Risks associated with IV placement and lab draws include pain, ecchymosis, and rarely superficial thrombophlebitis. Some individuals can experience a vaso-vagal reaction to venipuncture.

B6. RECRUITMENT AND CONSENT PROCEDURES

Recruitment

Subjects will be recruited at the medical center. Flyers will be posted at different locations at BIDMC and Center for Life Sciences Building (CLS) requesting healthy subjects interested in participating in a the study. Investigators will discuss with the protocol with potential subjects and provide a consent form describing this study for the subjects to review. Recruitment for Cohort D will include discussion with rheumatology and hematology staff of potentially eligible study subjects. We will also access BIDMC CQ2 database to identify eligible participants who have been diagnosed with an antiphospholipid antibodies. Following identification of potentially eligible study subject, physician or nurse will contact subject and discuss whether they may be interested in participating. Based on interest, will be evaluated in clinic and will review consent and eligible criteria.

Following consent, inclusion criteria will be reviewed to confirm eligibility for study participation using a checklist that will be signed and dated by the Investigator confirming eligibility. The subjects will be given list of flavonoid-rich foods to avoid 72 hours prior to scheduled participation.

Consent

The formal consent of a participant, using the IRB approved consent form, will be obtained before the participant is involved in any study-related procedure. The participant will be given a copy of the signed and dated consent document. The original signed copy of the consent document will be retained in a research file and documentation of consent will be placed in the medical record.

Subject Protection

All participants receiving study agents will be evaluated for safety. The safety parameters include spontaneous reports of adverse events reported to the investigator by participants. All toxicities encountered during the study will be evaluated according to the NCI criteria (CTCAE 4.0). Life-threatening toxicities will be reported immediately to Dr. Zwicker and IRB.

B7. STUDY LOCATION

Privacy

Interested participants will be scheduled to meet with a study Investigator at the Harvard Catalyst Clinical Research Center in the Feldberg building at BIDMC or on Shapiro 7 in the hematology suite. Screening questions, consent, and eligibility confirmation will be performed in private exams.

Physical Setting

The clinical study will be conducted at the Harvard Catalyst Clinical Research Center at BIDMC.

B8. DATA SECURITY

All documents, investigative reports, or information relating to the participant are strictly confidential. Study forms will be completed by clinical research coordinator or personnel at CTSA and kept in study binders in a locked office. Pertinent medical history will be obtained to ensure trial eligibility. Demographic data collected for each patient will include age, sex, and race. Only the primary investigators, research coordinator, statistician, and research nurse will have access to protected information of study subjects. Any electronic information containing PHI will remain on a secure server behind the BIDMC firewall.

B9 Multi-Site Studies

Is the BIDMC the coordinating site? ☒ Yes ☐ No

Is the BIDMC PI the lead investigator of the multi-site study? ☐ Yes ☒ No

B10 Dissemination of Research Results

Please explain whether you will be able to thank subjects and provide research results and, if so, how this will be accomplished. If you do not think this is feasible, appropriate or applicable to this research, please specify why.

This is a non-therapeutic trial thus data generated from this study will not directly impact the health of those involved. As such, there are no plans to disseminate the results.