Department of Pharmacology

Qualifying Examination (Part I)

August 8-11, 2017

ALL EXAMS TAKE PLACE IN THE BASS CONFERENCE ROOM, 436 RRB

Date	Exam # and Time	
Tuesday, 08.08.2017	Exam #1 – 10:00 am – 12:00 pm	
	Exam #2 – 1:00 pm – 3:00 pm	
	Exam #3 – 3:15 pm – 5:15 pm	
Wednesday, 08.09.2017	Exam #4 – 10:00 am – 12:00 pm	
	Exam #5 – 1:00 pm – 3:00 pm	
Thursday, 08.10.2017	Exam #6 – 10:00 am – 12:00 pm	
	Exam #7 – 1:00 pm – 3:00 pm	
	Exam #8 – 3:15 pm – 5:15 pm	
Friday, 08.11.2017	Exam #9 – 1:00 pm – 3:00 pm	
	3:00 pm – 4:00 pm	**Committee Meets**
	5:00 pm	**Results sent to students (via secure email, unless requested otherwise)**

The exam consists of seven problem sets. **This is a closed-book examination, so students must not consult with others about the exam in any manner or use resources including your previously prepared notes. Students must answer the DMPK Question (Question #1) and 3 of the 6 remaining questions.**

BEST WISHES FOR YOUR SUCCESSFUL COMPLETION OF THE EXAMINATION!

Warfarin remains one of the most important, orally available anticoagulation medications for the treatment of blood clots associated with pulmonary embolism and deep vein thrombosis and in the prevention of stroke in clinical populations with atrial fibrillation, artificial heart valves and/or valvular heart disease. It has a narrow therapeutic window and dosing has to be tailored to the individual and closely monitored (fitting prothrombin clotting time to the international normalized ratio, INR). If the INR is outside the target range, a high INR indicates a higher risk of bleeding, while a low INR suggests a higher risk of developing a clot.

Under normal conditions within the blood coagulation pathway, the serine protease thrombin converts factor XI to XIa, VIII to VIIIa, V to Va, fibrinogen to fibrin, and XIII to XIIIa. Thrombin is synthesized through proteolytical cleavage of the precursor prothrombin (coagulation factor II) in this normal clotting process.

The primary mechanism of action for Warfarin (Figure) involves inhibition of the vitamin K-dependent synthesis of biologically active forms of the calcium-dependent clotting and regulatory factors, including the anticoagulant regulatory factors protein C and protein S and the procoagulant clotting factors II, VII, IX and X. Importantly, temporary enhancement of clot formation occurs within the first 24 hours of initiation of warfarin doses of greater than 5 mg per day. The only common adverse side effect of warfarin is bleeding.

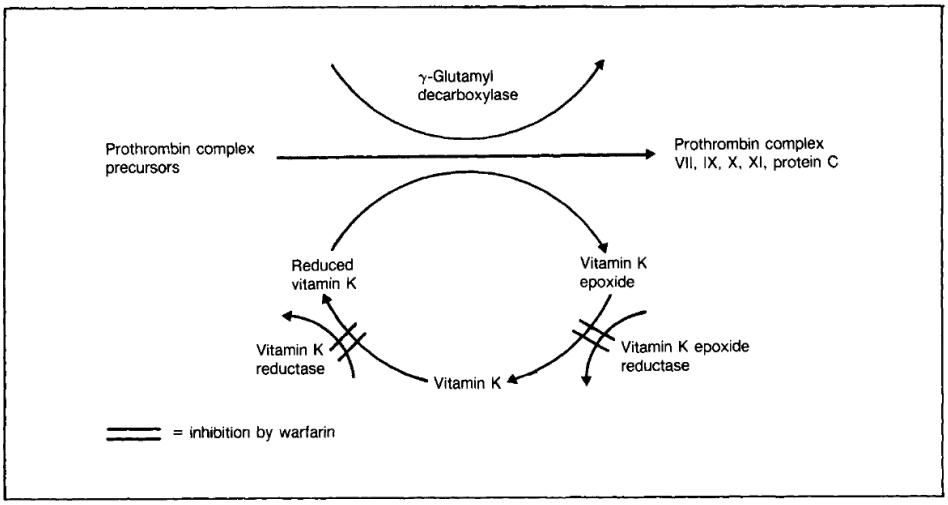


Fig. 3. The vitamin K cycle. Warfarin reduces the synthesis of prothrombin complex by inhibiting vitamin K epoxide reductase and vitamin K reductase. There is some suggestion that in humans the vitamin K reductase may be more important for the anticoagulant action of warfarin.

1) **Using your knowledge of the relationships between fundamental pharmacokinetic parameters, provide (on the graph attached, together with your calculations) a detailed PK profile representing the absorption, distribution, and elimination half-life of a single oral dose of warfarin over a 48 hour period of time. You should consider that warfarin is essentially completely absorbed, reaches a maximum plasma concentration between 2 and 6 hours, distributes into a small volume of distribution (10 L/70kg) and is eliminated by hepatic metabolism with a very low clearance (0.2 L/h/70kg).**

2) **Explain whether the following variables would change the DMPK properties of warfarin over 48 hours and identify the potential clinical impact:**

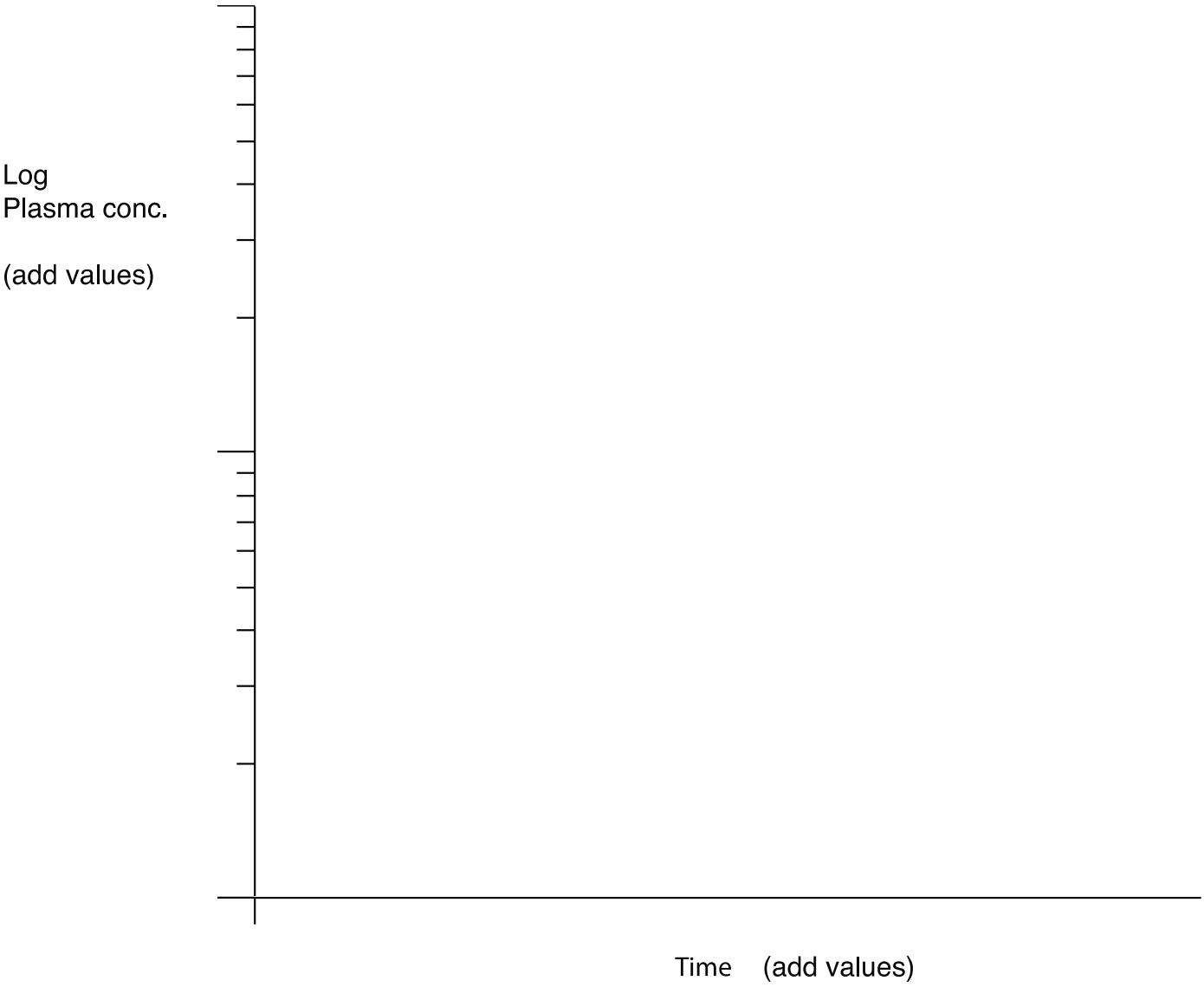
i. **Co-dosing with the antibiotic metronidazole, which reduces the metabolism of warfarin.**

ii. **Co-dosing with an NSAID with anti-platelet activity.**

iii. **Co-ingestion of a diet of cooked Kale and Asparagus with chili powder that are all rich in Vitamin-K.**

iv. **Individuals with severe liver disease, gastrointestinal ulcers, or uncontrolled hypertension.**

3) **It is well recognized that initial dosing with warfarin can result in a temporary enhanced prothrombotic state, and that co-dosing with the anticoagulant heparin IV can be critical for decreasing the risk of thrombosis. Provide a logical explanation for this phenomenon of an initial prothrombotic effect of warfarin.**


Log
Plasma conc.
(add values)
Time (add values)

The heart initially compensates for hypertension-mediated pressure overload (↑afterload) by enhancing its contractile force and developing hypertrophy without dilation. Adaptation mechanisms to reduce cardiac damage during the compensatory phase remain largely unknown. Such mechanisms protect the heart from inappropriate hypertrophy, remodeling, and depressed cardiac function which leads to heart failure. Regulator of G-protein signaling 2 (RGS2) is abundantly expressed in cardiac myocytes. RGS2 null mice were generated to identify any role of RGS2 in basal cardiac function and in the response to pressure overload. Thoracic Aortic Constriction (TAC) was used as a model of increased afterload. The results are depicted below.

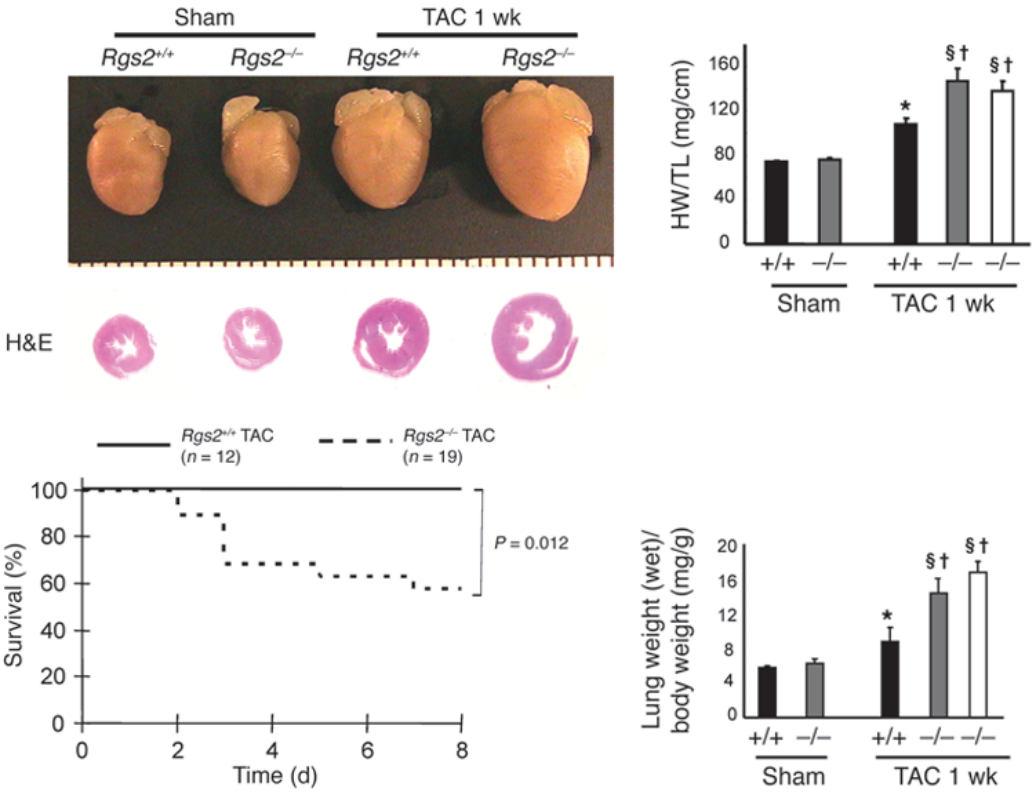


Figure 1. Cardiac phenotype of *Rgs2–/–* mice. Myocyte RGS2 protein expression was detected in *Rgs2*+/+ cells, but not *Rgs2*–/– cells (data not shown). Representative whole hearts, H&E-stained cross sections, and summary results for heart weight normalized to tibia length (HW/TL) in *Rgs2*+/+ and *Rgs2*–/– hearts subjected to 1 week TAC. White bar shows data from deceased mice (top panels). Kaplan-Meier survival curves of *Rgs2*–/– mice subjected to TAC compared with littermate controls. Wet lung weight normalized to body weight (n = 8–13 per group). White bar shows data from deceased mice (bottom panels). *P < 0.05 versus *Rgs2*+/+ sham; §P < 0.05 versus *Rgs2*–/– sham; †P < 0.05 versus *Rgs2*+/+ TAC.

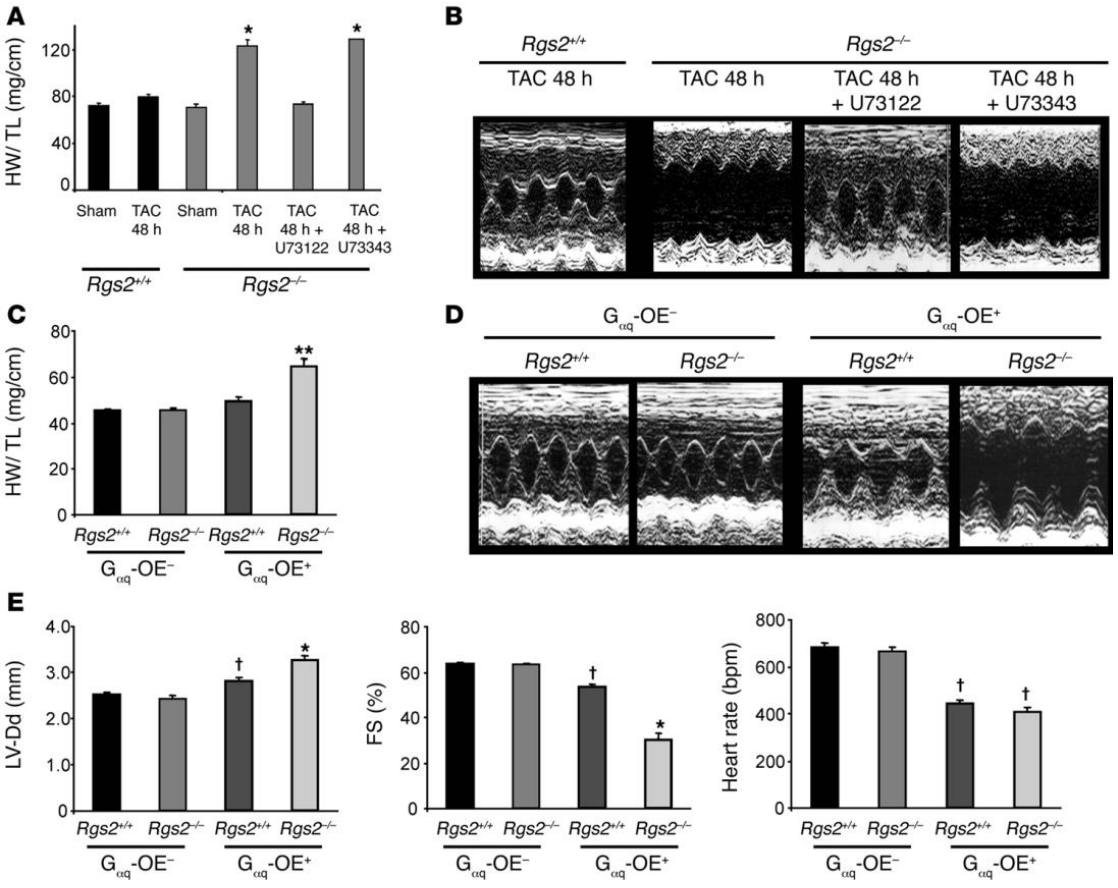


Figure 2. Effects of PLCβ inhibition and Gαq overexpression on cardiac function. (**A, B**) Effect of inhibition of PLCβ with U73122 on hypertrophy (heart weight/tibia length), chamber dilation, and dysfunction (n = 7–14 per group). *P < 0.001 versus other groups. Mice subjected to 48 h TAC. U73343 is an inactive analogue. (**C, D, E**) Effect of Gαq overexpression using a transgenic overexpression model (Gαq-OE+) on cardiac hypertrophy (n = 7–9 per group). **P < 0.01 versus other groups. Summary data for Left Ventricle diastolic dimension (LV●Dd), Fractional Shortening (FS), and heart rate (n = 7–9 per group). *P < 0.05 versus other groups; †P < 0.05 versus all Gαq-OE– groups. (B & D are representative echocardiograms).

In addition to the *in vivo* data shown above, a recent study performed in isolated mouse cardiac myocytes reported that stimulation of α1-adrenergic receptors (normally found on the surface of cardiac myocytes) results in the activation of PLCβ. This observation should be considered in answering the questions below.

1) **Describe succinctly the data and your interpretation.**

2) **Present a hypothesis, that incorporates the data provided above, to describe the role of RGS2 in the regulation of cardiac hypertrophy. Please include a diagram of pathway with your hypothesis.**

3) **Outline both *in vivo* and *in vitro* experiments to test your hypothesis.**

Mutations in **w**ith **n**o **l**ysine (WNK) kinases cause familial hyperkalemic hypertension (FHHt). Thiazide diuretics seem to specifically treat the disease, fostering the view that hyperactivation of the thiazide-sensitive sodium-chloride cotransporter (NCC) in the distal convoluted tubule (DCT) is solely responsible. However, aberrant signaling in the aldosterone-sensitive distal nephron and inhibition of the potassium excretory renal outer medullary potassium (ROMK) channel have also been implicated. To test these ideas, you introduced kinase-activating mutations after Lox-P sites in the mouse *Stk39* gene, which encodes the terminal kinase in the WNK signaling pathway, Ste20-related proline-alanine–rich kinase (SPAK). Renal expression of the constitutively active (CA)-SPAK mutant was specifically targeted to the early DCT using a DCT-driven Cre recombinase.

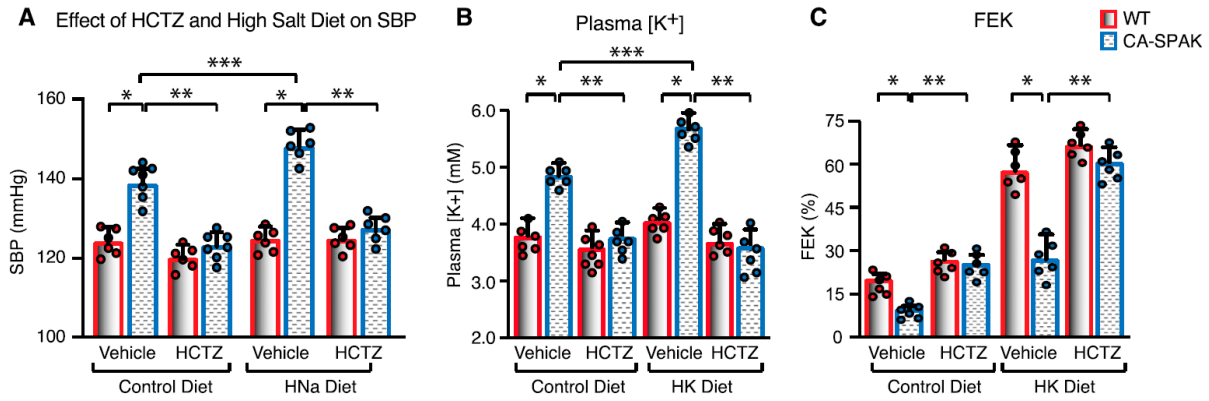


Figure: CA-SPAK mice develop hydrochlorothiazide (HCTZ)-remediated salt-sensitive hypertension and hyperkalemia. (A) Average systolic BP (SBP) during the awake period in the presence of vehicle or HCTZ (25 mg/kg body wt) after 3 days of treatment on control diet or high-sodium diet (HNa). (B) Plasma potassium concentration under basal conditions or after dietary potassium loading (HK) in the presence of vehicle or HCTZ (3 days treatment). (C) Urinary potassium excretion relative to amount filtered (fractional excretion of potassium [FEK]) in the presence of vehicle or HCTZ (3 days treatment). WT (red) and CA-SPAK (blue) bars and wickers are means±SEM, and circles are individual data points from each mouse (n=6 per genotype per treatment). *P,0.05, WT versus CA-SPAK undergoing same treatment; **P,0.05, vehicle versus HCTZ in same genotype; ***P,0.05, control diet versus experimental diet in the same genotype.

Questions:

1) **Explain the results in figure A. Why would SBP be higher in CA-SPAK mice? And, why would it be blocked by HCTZ?**

2) **The mice exhibit hyperkalemia, as is typical of FHHt, figure B. How is this hyperkalemia explained in figure C?**

3) **How is potassium excretion controlled in the distal nephron?**

Hemolysis increases susceptibility to bacterial infections and increases the likelihood of death from sepsis. The mechanisms underlying this increased susceptibility are poorly understood. Hemolysis leads to release of free heme from red blood cells and plasma free heme levels correlate with extent of bacterial infection. Two hypotheses are considered of how high plasma heme levels that result from hemolysis might lead to increased bacterial infection: 1) since growth of bacteria requires free iron that is normally unavailable in human plasma and tissue, the release of heme during hemolysis makes iron available for rapid bacterial growth or 2) excess heme interferes with the normal responses of phagocytes to bacteria. To test these hypotheses, the experiments described on the next page were carried out.

In your response to this question, please do the following:

1) **Interpret the results of the described experiments in regards to the two initial hypotheses.**

2) **Based on these results, choose the most likely of the two hypotheses above and propose a molecular mechanism by which hemolysis leads to increased susceptibility to bacterial infections and a series of experiments to test this hypothesis.**

3) **Briefly describe the normal response of the innate immune system to bacterial infection.**

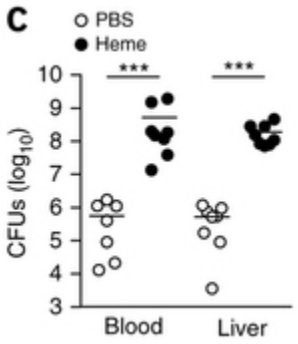


Figure 1. Wild-type mice (n=7 per group) were injected intraperitoneally (i.p.) with either PBS or 25 μmol / kg body weight of free heme. 30 min later both groups were injected i.p. with 10^4 colony forming units (CFU) of *E. coli.* 16 h after infection blood and liver *E. coli* CFU were determined. ***p<0.0001

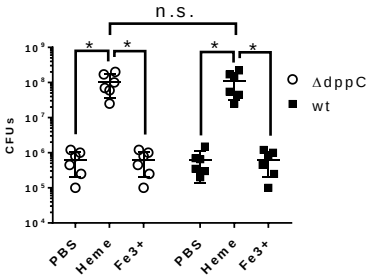


Figure 2. (Note: Previous studies showed *E. coli* requires the heme transporter dppC to acquire iron from extracellular heme.) Wild-type mice (n=5-6 per group) were injected i.p. with PBS, 25 μmol / kg body weight of free heme (heme), or 25 μmol / kg body weight of ammonium iron (III) citrate (Fe^{3+}). 30 min later mice were injected i.p. with 10^4 CFU of either wild-type *E. coli* (black squares) or *E. coli* where dppC has been deleted (white circles) After 16 h, bacterial levels were measured in blood. *p<0.05.

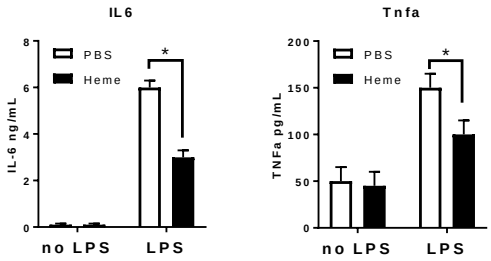


Figure 3. (Left) Mice were pre-treated with PBS or heme as in Figure 1, and then injected i.p. with 40 mg/kg of lipopolysaccharide (LPS), and the levels of plasma IL-6 and TNFα measured at 16 post-LPS injection. *p<0.05 PBS vs heme.

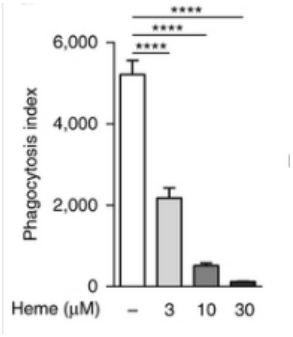


Figure 4 (Right). RAW264.7 macrophages were pretreated with the indicated concentration of heme for 15 minutes, and then heat-killed FITC-labeled *E. coli* was added. Phagocytosis was measured by flow cytometry. **** p<0.0001

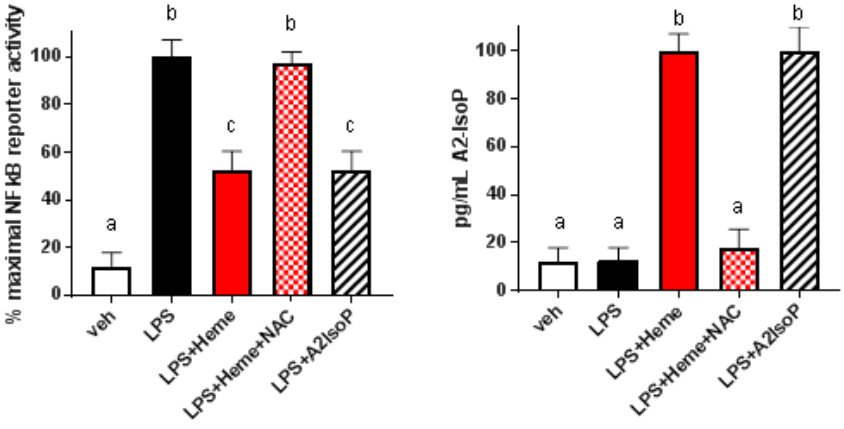


Figure 5 (left): 15-A2 isoprostane (A2-IsoP) is a prostaglandin like lipid formed by iron mediated oxidation of arachidonic acid. A2-IsoP can stably react with thiols such as cysteine to form covalent adducts. RAW264.7 macrophages were transfected with an NFκB luciferase reporter plasmid. Transfected cells were treated with either vehicle (veh); LPS; LPS + heme; LPS + heme + the antioxidant thiol N-acetyl-cysteine (NAC); or LPS + A2-IsoP for 18 hours. **Left panel:** Relative NFkB activity normalized to the 100 ng/mL LPS group. **Right panel:** Total amount of A2-IsoP present in cell supernatants. Groups with same letter significantly differ from groups with other letters.

A recently formed pharmaceutical company, Thinspiration®, initially focused its research efforts upon the growing problem of human obesity by attempting to identify new compounds that suppress food consumption for the 37.9% of Americans who are considered obese with a body mass index (BMI) of 30 or higher. Unfortunately, daily oral administration of one of their newest test compounds caused hyperphagia in rodents, characterized by a significant increase in both food consumption and body mass. Not deterred by this minor setback, Thinspiration® pivoted its business model to focus upon the nearly 2% of American adults (ages 20-74) and 3.5% of children and teens who are chronically underweight and have difficulty in maintaining their body mass and BMI values.

Additional studies revealed that chronic administration of the new drug increased food uptake, yet experimental animals also began to demonstrate weakness, sluggishness and eventually died within about 6 weeks. A post-mortem necropsy of treated animals did not identify an overt pathology for any organ system, yet hyperpigmentation of the mucous membranes of the mouth was noted. A more detailed analysis of these mice after three weeks of daily treatment revealed the fasted, mean blood values indicated below:

Laboratory test	Treated animal value	Normal range
Na^+	102 mmol/L	137-145 mmol/L
Cl^-	71 mmol/L	98-107 mmol/L
K^+	6.4 mmol/L	3.5-5.0 mmol/L
Glucose	80 mg/dL	150-200 mg/dL
Corticosterone	<0.2 μg/dL	AM: 6.2-29.1 μg/dL PM: 3.0-17.3 μg/dL
ACTH	1745 pg/mL	9-52 pg/mL

ACTH, adrenocorticotropic hormone; Cl-, chloride; K^+, potassium; Na^+, sodium

1) **Based upon the blood values, behavioral phenotype, hyperphagia, hyperpigmentation and death, develop a hypothesis to account for the physiological changes in response to your compound and propose a mechanism for its action.**

2) **Describe a series of experiments by which to confirm your proposed mechanism of action.**

Membrane type-4 matrix metalloproteinase (MT4-MMP) and epidermal growth factor receptor (EGFR) are co-expressed in over 70% of triple negative breast cancers (TNBC). The MT4-MMP level and patients' progression-free survival (PFS) in the presence or absence of chemotherapy was calculated (Fig 1). To explore how MT4-MMP modifies the effect of chemotherapeutic agents, MDA-MB-231 cells were used. The effect of anti-cancer agents on cell growth was compared with or without the expression of MT4-MMP (Fig 2).

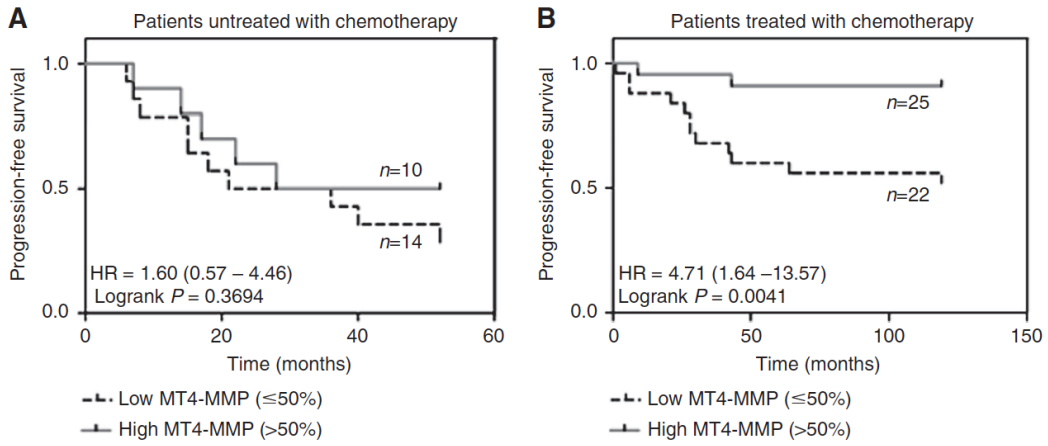


Fig 1: Kaplan–Meier progression-free survival (PFS) curves from patients untreated by chemotherapy (**n**=24) and TNBC patients treated with chemotherapy (**n**=47) according the MT4-MMP expression. Note: 95% confidence interval is represented between the parentheses. HR, hazard ratio. Dash line (low MT4-MMP, ≤50% cells expressing MT-MMP), straight line (high MT4-MMP, over 50%)

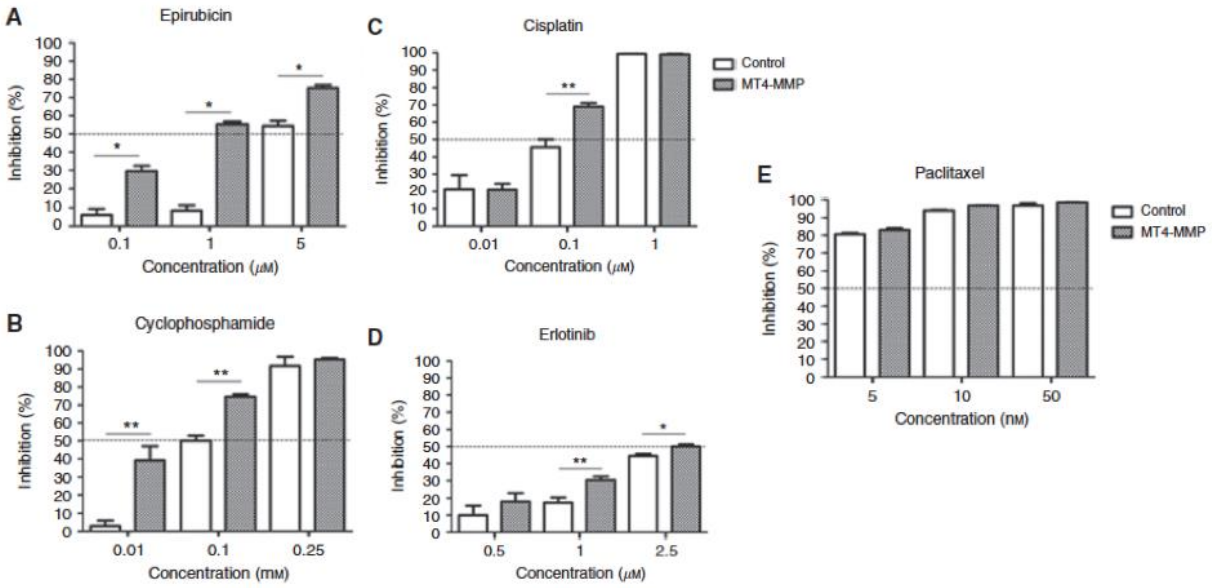


Fig 2: The effect of MT-MMP4 on cell proliferation. MDA-MB-231 cells overexpressing MT4-MMP or not were incubated in a 3D matrix (Matrigel). Cell proliferation was assessed by DNA measurement after 7 days of culture in the presence of different concentrations of epirubicin (**A**), cyclophosphamide (**B**),cisplatin (**C**), erlotinib (**D**) and paclitaxel (**E**) as indicated. Results are expressed as percentage of inhibition.

1) **What conclusion can be drawn from Figure 1?**
2) **What conclusion can be drawn from Figure 2?**
3) **Based on the results, propose a hypothesis including a series of experiments to investigate how MT4-MMP enhances the effectiveness of chemotherapeutic agents.**

Heroin acts through its active metabolite, morphine. Once it enters the organism, heroin is rapidly metabolized by carboxylesterase to 6-monoacetylmorphine and morphine. Morphine, which is commonly used to treat pain, causes addiction in a subset of patients by activating Gi/o receptors. The following experiments were carried out in rodents to further understand the mechanism of action of heroin and morphine.

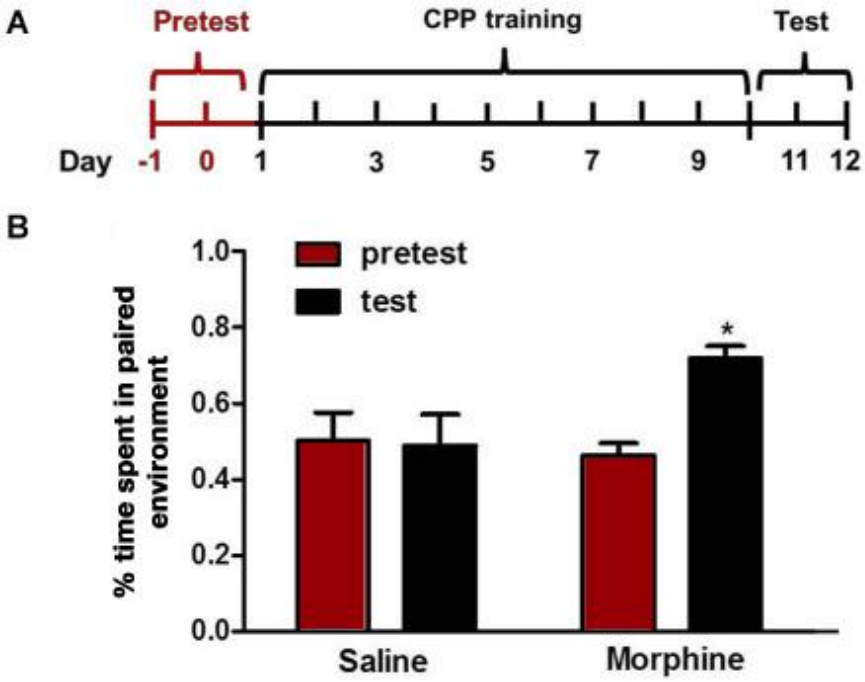


Figure 1: Rats prefer an environment that is associated with morphine administration. A. Experimental setup: during pre-test (red bars), naïve rats are tested in the place conditioning setup with access to two distinct environments to rule out innate preferences for a particular environment. During CPP (conditioned place preference) training, one environment is paired with either morphine or saline in two independent groups of rats. On the test day, rats are allowed to choose between the morphine – or saline – paired environments and a neutral environment, to test for preference or aversion. B. Percent time spent in the paired environment of saline-treated ("Saline") or morphine-treated ("Morphine") rats.

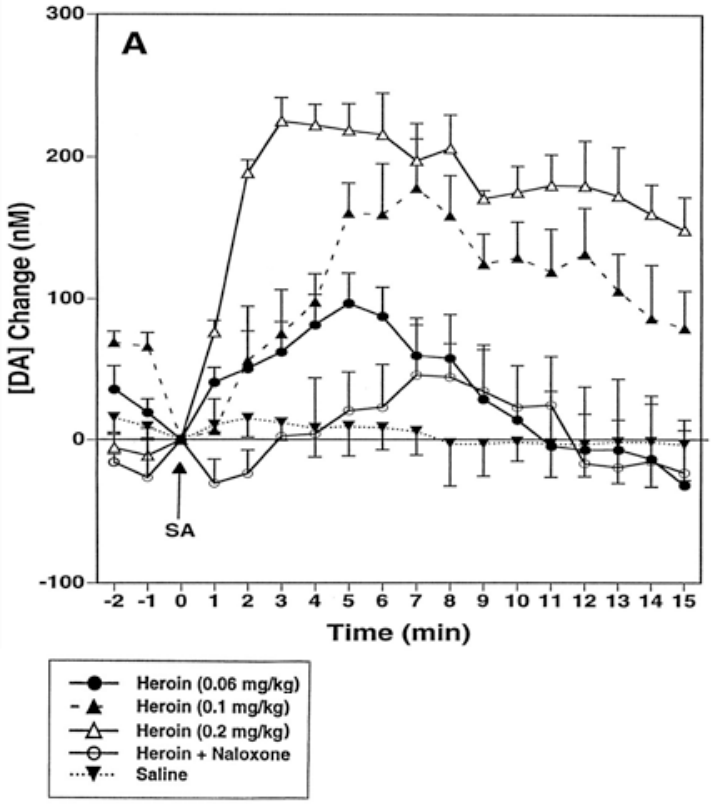


Figure 2: Heroin-mediated dopamine release in the nucleus accumbens. Groups of rats were trained to self-administer heroin at concentrations of either 0.06, 0.1, or 0.2 mg/kg. Dopamine levels were measured in the nucleus accumbens in vivo during the self-administration session. Pretreatment with the inverse μ (mu) receptor agonist naloxone (10 mg/kg i.p.), administered 10 min before heroin self-administration, significantly decreased heroin– mediated (0.06 mg/kg/injection) dopamine release. SA = start of self-administration session

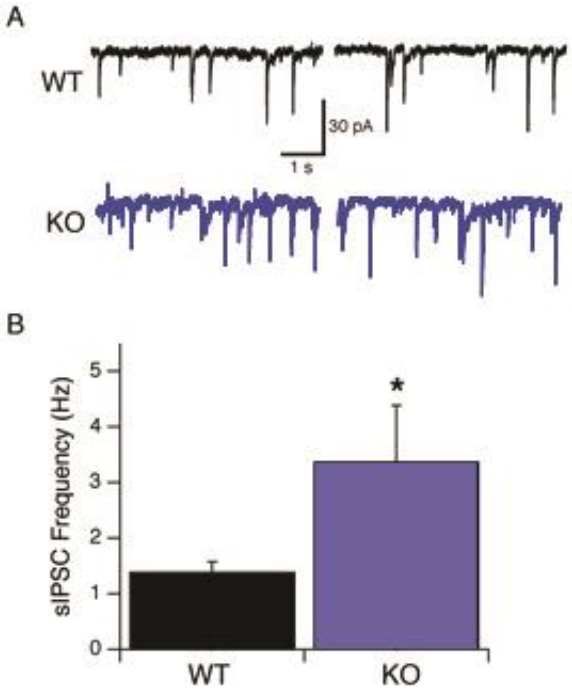


Fig. 3: The frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) in dopaminergic neurons of the VTA is enhanced in μ (mu) opioid receptor (MOR) knockout mice (KO). sIPSCs were recorded from dopaminergic cells in the VTA from MOR knockout mice (KO) and wild-type (WT) controls; * indicates P 0.001; significantly different from WT. (A) Two representative voltage-clamp recordings (5 s) from WT and MOR KO mice, respectively. (B) The average frequency of sIPSCs in WT and MOR KO mice.

1) **Explain the data in figure 1.**

2) **Explain the data in figure 2.**

3) **Explain the data in figure 3**

4) **Which neurotransmitter system drives spontaneous inhibitory postsynaptic currents (sIPSCs) observed in the dopaminergic neurons in figure 3?**

5) **How would an increase in spontaneous inhibitory postsynaptic currents (sIPSCs) of dopaminergic neurons affect transmitter release of dopamine, and how would a decrease in sIPSCs affect transmitter release of dopamine?**

6) **Draw the most direct anatomical pathway from μ opioid receptors to dopamine release that could explain the behavioral, dopamine-release, and electrophysiological data.**

7) **Propose *two* experiments to test the model.**