TITLE: Pilot study of the neuroprotective effects of hydrogen and minocycline in acute ischemic stroke (v14.9)

INVESTIGATORS

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A. SPECIFIC AIMS

The aim of this pilot randomized control trial (RCT) is to explore the possible beneficial effect of a novel combination therapy, consisting of molecular hydrogen H_2 plus minocycline ("H2M"), on neurological recovery after acute ischemic stroke.

B. BACKGROUND AND SIGNIFICANCE

The cellular and molecular processes responsible for driving brain cell death after hypoxic-ischemic insults have been progressively delineated over the past 30 years, but the development of an inaugural neuroprotective agent capable of reducing human brain damage after stroke has remained elusive (Ginsberg, 2008; Tymianski, 2014). Many putative therapeutic countermeasures have been identified that show promising neuroprotective effects in rodent models of stroke, but none so far have shown efficacy in human clinical trials. A wave of candidate drugs was brought forward into Phase II and even Phase III clinical stroke trials by both large and small pharmaceutical companies in the 1990s, but with disappointing results. These failures have led to widespread abandonment of the stroke therapeutic area by industry, further accelerated in recent years by a progressive de-investment by pharmaceutical companies in the entire CNS sector (Choi et al, 2014).

Compounding this reduction in investigative bandwidth, there are growing reasons to suspect that the biology of hypoxic-ischemic brain injury might be refractory to single interventions. As more injury mechanisms proceeding in parallel have been identified, the possibility looms that even an intervention powerful enough to halt a major injury mechanism might exhibit limited benefit when applied alone, with brain cells still dying due to parallel pathophysiology. An analogous situation has emerged in other therapeutic areas, including cancer and infections such as by HIV.

There is currently little experience with testing combination treatments (multiple concurrent drugs) for efficacy in reducing stroke-induced brain damage. The number of possible drug combinations is dauntingly high, making a fully systematic approach to testing impractical even in experimental models. Furthermore, predictive limitations of rat stroke models for human stroke have become clear, leading even to a suggestion that baboon models be deployed instead (Kwiecien et al, 2014). The bandwidth of baboon stroke model testing is of course modest, and applicability to man also unproven. A more practical path forward given these issues may be to identify promising combinations based on underlying principles, known mechanisms of action and the demonstration of individual efficacy in rodent stroke models; and then to test these rationally selected combinations directly in small exploratory clinical trials powered only to detect marked therapeutic effects. Only combinations showing high promise in such grass roots exploratory testing would move forward into rigorous, multicenter clinical trials. Ultimately, the demonstration of success with any approach, whether commercializable or not, could provide a major boost for the stroke field, increasing industry engagement and the prospects of developing more effective therapies in the future.

With these ideas in mind, we propose here to perform a clinical pilot study, exploring the ability of a novel combination ("H2M") of a promising antioxidant agent, molecular **hydrogen**, with a widely used antibiotic, **minocycline**, to protect brain tissue from ischemia/reperfusion injury, and hence aid recovery after stroke. Minocycline is known to inhibit the activation of matrix metallo-proteinase-9 (MMP-9) and poly(ADP-ribose) polymerase (PARP), events hypothesized to contribute to the later pathogenesis of ischemic brain tissue damage. Both hydrogen and minocycline have excellent safety profiles, have been previously demonstrated individually to reduce infarction in animal models of stroke, and have potentially synergistic mechanisms of action against ischemic brain damage. The mechanisms of action of both agents would be specifically relevant to patients receiving tissue plasminogen activator (tPA) and achieving some degree of therapeutic reperfusion. Both agents can be easily administered to subjects intravenously or orally. Additional background information relevant to our selection of these agents and doses is presented below in section D, Research Design and Methods.

C. PRELIMINARY STUDIES

The proposed study is a pilot study, so there are no directly applicable preliminary studies utilizing the proposed H2M combination therapy. Relevant prior work with H2 or minocycline is summarized below under D.1, Rationale/overview.

D. RESEARCH DESIGN AND METHODS

D.1. Rational/overview

Hydrogen

While the chemical ability of molecular hydrogen (H_2, a gas at temperatures above minus 252°C) to react with oxygen free radicals has been long known, recent studies have identified a remarkable and biologically favorable selectivity: H_2 efficiently quenches toxic hydroxyl and peroxynitrite free radicals, but has little effect on superoxide, nitric oxide, or hydrogen peroxide. The former radicals are highly pathogenic, whereas the latter radicals play important roles in normal cellular signaling (Ohsawa et al, 2007). H_2 diffuses through tissues and penetrates cell membranes readily, even quenching hydroxyl radicals generated in cell nuclei (Ohsawa et al, 2007).

Furthermore, exposure to H_2 is not associated with any known biological toxicity. Human exposure to molecular hydrogen occurs naturally as a trace gas in air (about 0.5 ppm), and can reach 7,500 ppm (0.75%) in a closed environment such as aboard a nuclear submarine during long underwater periods (Hagar 2003). A 2008 National Academies study group was charged with considering the effects of long term hydrogen inhalation in submariners, and concluded that it was devoid of known toxic activity, beside a risk of asphyxia at concentrations high enough to replace ambient oxygen. That group recommended setting occupational exposure standards based solely on consideration of explosivity, noting that a concentration of 4.1% H_2 in air was level at which hydrogen first becomes flammable (Committee on Emergency and Continuous Exposure Guidance Levels for Selected Submarine Contaminants, 2008). Inhalation of 49% H_2 (with 50% helium and 1% O_2) in a gas mixture called hydreliox is used by human divers in deep diving.

Reactive oxygen species are postulated to be a downstream mediator of several acute neurodegenerative pathways including excitotoxicity (Choi, 1988; Coyle and Puttfarcken, 1993), and both excitotoxicity and free radical-induced cellular damage have been implicated in the pathophysiology of brain tissue damage after ischemic insults (Chan, 2001). Oxidative damage is specifically promoted by reperfusion after ischemia (e.g., after successful mechanical thrombectomy or use of tPA), as well as the availability of

iron after hemorrhage (due to Fenton chemistry). In a rat model of transient focal ischemia (90min MCA occlusion), administration of 2% H_2 gas by inhalation during ischemia-reperfusion, or only during reperfusion, reduced infarction 1d later; behavioral deficit 7d later was also reduced (Ohsawa et al 2007).

A range of other cytoprotective effects have been observed after H_2 administration (by inhalation, or in fluids delivered IV or PO) in rodent models of disease (Ohta, 2011), including against ischemic hepatic injury (Fukuda et al 2007), ischemic myocardial infarction (Hayashida et al 2008), inflammatory injury to cardiac allografts (Noda et a 2012), radiation-induced lung damage (Terasaki et al 2011), and 6-OH-dopamine-induced nigrostriatal degeneration (Fu et al, 2009). Besides direct scavenging effects, indirect mechanisms, e.g. activation of transcription factor Nrf2, have been suggested to contribute to observed protective effects in these animal models of organ injury.

Minocycline

Minocycline is a second generation tetracycline, shown to have neuroprotective and anti-inflammatory effects in a wide range of acute and chronic injury models. These unexpected tissue protective effects may in part stem from minocycline's ability to inhibit matrix metalloproteinases (MMPs) including MMP-9, at standard antimicrobial doses. Brain MMP-9 cellular expression (multiple cell types) and release to the extracellular space is increased after ischemic insults, likely contributing to inflammation, cell death, and breakdown of the blood-brain barrier (Chaturvedi and Kaczmarek, 2014). tPA is known to activate MMP-9, a side effect that has been postulated to promote blood-brain barrier breakdown and limit tPA's therapeutic benefit, so there may be special advantage in inhibiting MMP-9 in conjunction with tPA administration. Minocycline also has other potentially beneficial actions, including reducing apoptosis, inflammation, and the activity of PARP. PARP inhibition occurs at even lower concentrations (submicromolar) of minocycline than needed for MMP-9 inhibition (Alano et al., 2006), and may reduce several neuronal injury cascades, including zinc neurotoxicity (Sheline et al., 2003), a process the co-PI's laboratory identified and implicated in the pathogenesis of late infarction after focal ischemic insults (Lee et al., 2002).

The first reported human trial of minocycline in stroke was a single blind RCT that allocated 152 patients 6-24h after stroke onset to placebo or 200 mg minocycline PO qd x 5d. At day 9, the odds of handicap-free survival (modified Rankin clinical outcome scale, mRS <= 2) was 91% in the minocycline group vs 47% in the placebo group (Lampl et al., 2007). A second small (n=50) open label pilot trial was similarly positive (72% mRS <=2 in minocycline treated vs 37% control) (Srivastava et al., 2012). However a third single blind RCT (n=95) did not see a benefit with 100 mg minocycline every 12h for 5 doses intravenously (2.5d therapy, 66% mRS <=2 in the minocycline-treated group vs 70% in the control group) (Kohler et al., 2013). This last study cooled enthusiasm in the stroke field for use of minocycline as a stroke neuroprotectant treatment, but to our eye, its design deviated importantly from the earlier positive studies. It enrolled all comers including patients with minor strokes (NIHSS > 0) who would be expected to recovery to mRS <=2 naturally, whereas the Lampl and Padma studies excluded such minor strokes, requiring the NIHSS upon entry to be >5 or >4, respectively. Inclusion of minor strokes, together with steady improvement in standard care, could account for the much better placebo outcomes seen in the Kohler study compared to the Lampl and Padma studies. Furthermore, the Kohler study only administered minocycline for 2.5d, instead of the 5d therapy duration used in the Lampl and Padma studies.

ClinicalTrials.gov lists a fourth RCT of minocycline in stroke (NeuMAST) that was conducted in Singapore between 2009-2012 (R. Singh, PI): https://clinicaltrials.gov/show/NCT00930020. This trial enrolled 139 patients and administered 200 mg minocycline or placebo 3-48 hr after stroke onset, and was stopped to futility. However, enrollment of patients again deviated importantly from that utilized in the

earlier positive studies, as beginning treatment up to 48 hr after stroke onset would limit impact on earlier injury processes. Furthermore, this trial used an elevated and perhaps unrealistic primary criterion for efficacy, mRS 0 or 1.

In our view, another look at minocycline as a stroke neuroprotectant is justified. We believe the proposed combination of minocycline with H_2 has a better chance than minocycline alone for producing the dramatic neuroprotective effect needed to gain the large funding required to conduct a definitive stroke neuroprotection trial.

D.2. Research Site

Eligible subjects will be recruited at Stony Brook University Hospital. Follow up assessments will be conducted by telephone or in the SB Neurology outpatient clinic.

SUNY Stony Brook is the only Level 1 Trauma Center in Suffolk County (population 1.5 million) in eastern Long Island, and has a highly active clinical stroke service, recognized for the last few years by the American Heart Association with a "Gold-Plus" award for outstanding adherence to recommended care paths. The Stony Brook stroke team responds within minutes to patients presenting to SBUH with a stroke at any time day or night. In 2015, the SB stroke team evaluated 575 patients presenting to the emergency room (or already on SBUH inpatient services) with acute ischemic stroke; 46 of these received tPA at an outside hospital prior to transfer here, and 36 received tPA here; 39 received mechanical thrombectomy at SB. Of these 575 patients, 196 had a baseline admission NIHSS between 5 and 25.

D.3. Study Sample

Subjects admitted to SBUH with an acute ischemic stroke (or developing an acute ischemic stroke while already in the hospital for other reasons), who meet the inclusion/exclusion criteria in Table 1 below and provide consent (directly or through their legally authorized representative, LAR) will be recruited and randomized sequentially 1:1 to receive either H2M or placebo.

We will seek to enroll a total of 100 patients meeting enrollment criteria and randomized to H2M vs placebo arms. Subjects who receive mechanical thrombectomy (likely <10% of total) will be stratified separately. While they also may benefit from H2M, these patients not uncommonly exhibit a marked and rapid recession of stroke symptoms after successful mechanical thrombectomy, which could make detection of additional treatment benefit difficult (ceiling effect). We project a study duration of 2y with one interim analysis at 1y to look for futility.

D.4. Screening

When a patient presents to SBUH with a new onset stroke from outside, or when hospitalized as an inpatient, code BAT is called and the stroke team responds immediately. A stroke team member will review the patient's medical record to evaluate whether he/she is eligible for the present study (Table 1). If eligible, the subject's capacity to provide informed consent will be assessed by a stroke team clinician (attending, resident, fellow, nurse practitioner, or physician's assistant). This clinician will determine if the subject understands: 1) that the study procedures constitute research, not standard treatment; 2) the risks and benefits of a study; 3) the alternatives that are available if they do not participate; and 4) that if they choose not to participate, that decision will be accepted without penalty, i.e., without jeopardizing clinical care. If the subject is capable of understanding the treatment and is interested in participating in the study, he/she will be asked to sign the consent form. In cases where the subject is unable to provide consent, the subject's legally authorized representative (LAR) will be asked to provide consent. If

possible, the subject will also be asked to indicate their assent on the consent form. A study staff will review the consent form, including the risks and potential benefit of the study medications, with the subject and/or his/her LAR. If no LAR is present in the hospital we will use a verbal consent by a LAR. A LAR/next of kin to verbally consent to research on behalf of the subject will be contacted by telephone. The person conducting the informed consent discussion will discuss the study with the LAR/next of kin and answer any questions they have. An email address will be requested, and the ICF will be sent (via email or mail) to the LAR/next of kin. If the LAR/next of kin agrees to participate they will print the document (either on paper or to PDF) and sign the document with either a pen or a stylus if they are able to do so. The signed document can be scanned, or if no scanner is available, a cell phone photograph can be taken of the document and returned as soon as possible after the consent process. The subject will be enrolled in the study **after** a signed consent form has been received. For Spanish-speaking patients, we will request an in-house Spanish interpreter to assist with oral presentation and serve as an impartial witness. The Spanish consent form will be signed by the subject/LAR. Patients who speak a language other than English or Spanish (<1% of SBUH patients) will not be enrolled in this study, as interpreters for other languages are not in-house at SBUH, and we do have the resources to make a range of other language interpreters available to us in person and on short notice. The subject and his/her LAR will be allowed to ask questions and discuss in private before deciding to sign the consent form. Subjects will be informed during the consenting process that they will bear responsibility for the costs of treating any AEs resulting from study medication.

Table 1: Inclusion/exclusion criteria

Inclusion criteria	1. Aged 18 years old or over
	2. Presenting to/at SBUH with acute ischemic stroke
	3. Baseline (at admission to study) National Institute of Health Stroke Scale (NIHSS) of ≥ 5
	4. Administration of study medication possible within 24 hours of last known well
Exclusion criteria	1.
	2. Pre-existing neurological disability (historical NIHSS >3); unable to live independently
	3. Severe stroke or comorbidities likely to result in patient dying within 3 months
	4. Acute or chronic renal failure with calculated creatinine clearance < 30
	5. Liver disease leading to > 3x elevation in liver transaminases or significant loss of synthetic capacity*
	6. Thrombocytopenia (<$100x10^9$platelets / L blood)
	7. Pre-existing infectious disease requiring antibiotic therapy that have a negative interaction with minocycline (Penicillin, amoxicillin, ampicillin, bacampicillin, carbenicillin, cloxacillin, dicloxacillin, methicillin, mezlocillin, nafcillin, oxacillin, piperacillin, ticarcillin)
	8. Pregnancy or nursing. Females of reproductive age will be required to use barrier contraception or abstain from sexual intercourse while on study medications, as minocycline may render oral contraceptives less effective.
	9. Known allergy to tetracycline group of drugs
	10. Concurrent treatment with retinoids or ergot alkaloids
	11. Inability to safely tolerate the 850 ml fluid load (IV NS or PO water) associated with study medication*
	12. Treatment with another investigational drug within the last 30 days that may interfere with this study's medications*
	13. Inability to tolerate or comply with study procedures*

*These criteria will be judged by the attending physician.

D.5. Procedures

D.5.a Study Procedures

This will be a double blinded, placebo-controlled trial. Subjects will be sequentially randomized in a 1:1 ratio to receive H2M, or corresponding dual placebos, additional to all standard-of-care treatments. Enrollment goal is 100 subjects over 2 years.

For subjects meeting enrollment criteria and providing consent, treatment with H2M or placebo will start as soon as possible after arrival in the emergency department or SBUH, and assessment of vital signs, baseline NIHSS, and baseline modified Rankin Score (mRS). The subject will then be randomly assigned by the study coordinator to either the H2M treatment group or the placebo group. A blocked randomization list will be generated using this website: https://www.sealedenvelope.com/simple-randomiser/v1/lists. The study coordinator will be in charge of using the generated list to assign treatment to subjects as they are enrolled. This study coordinator may have interaction with subjects when obtaining consent or during follow up, but he/she will not be assessing any study outcome. The study staff who will assess study outcomes will remain blinded to treatment assignment. Masking of active and placebo treatments will be preserved by creating IV bags, capsules, and containers that appear identical.

For each subject we will use one of three routes of administration of hydrogen and minocycline depending on the subjects' ability to tolerate oral administration:

1) PO; we use this if subject can tolerate oral administration.
2) Nasogastric intubation (NG); we use this for patients that are unable to swallow but able to have an NG tube placed.
3) IV; we use this in patients that are unable to swallow and NG tube placement is clinically contraindicated.

If the subject passes the dysphagia test, we will switch from NG intubation or IV to PO for the next doses.

Minocycline (200 mg daily) will be given for 5 days, either as 200 mg in 100cc normal saline (NS; IV) over 60 min, or in capsules (per NG and PO). Hydrogen will be given TID x 3d, either as 250 ml of H_2-enriched water (per NG or PO), or as 250 ml of H_2-enriched NS (IV over 20-60 min). The first dose of hydrogen will be given STAT and all other doses per SBUH TID schedule (8 am, 12,pm, 4 pm), but for convenience a dose may be given a few hours earlier or later, as no available information or expected mechanism of action indicates that dose timing would be critical for efficacy) x 3d. Subjects will be monitored for their duration of stay for adverse events. If the subject is discharged before study therapy is completed, the study coordinator will follow up with subjects via phone call to ask about adverse events during study treatment.

To assess neurologic deficits and level of handicap, study staff blind to treatment assignment will assess the mRS (see below) on 1) day 45 ± 7 days via a phone call, and 2) day 90 ± 7 days at the subject's routine follow up clinical visit (preferred) or by phone call. If the 90d follow-up can be performed in person at a clinic visit, the NIHSS will also be assessed at that time. For Spanish speaking subjects, the language line will be used for the assessments.

Subjects can withdraw from the study at any time. Study staff will try to assess the mRS on the subject's last phone call.

Subjects who have undergone one MRI scan within the first 48 hours after stroke will be offered the opportunity to have a second unenhanced MRI scan at 80 to 100 days after stroke to assess the evolution of infarct volume. We will perform additional analysis for research purpose on the patients clinical MRI as comparison. The second MRI will be performed at the SB outpatient MRI facility. The first MRI scan

will be billed to the patient, but the second MRI will be provided to the patient for free unless clinically indicated. The patient will only be responsible for transportation costs to and from the MRI scan appointment. We do not expect to obtain this second MRI scan for all patients, but we would like to be able to obtain this data when circumstances permit.

D.5.b Study Drug

The FDA has approved this study protocol with minocycline and molecular hydrogen (IND#131621).

D.5.b.i Hydrogen

Preparation

The optimal route for administering H_2 in the present study would be via gas inhalation, but the associated environmental risk of gas leakage, buildup, and explosion would have to be managed by hospital engineering. This could be accomplished using a tightly-fitted face mask and room ventilation, but for the purposes of this initial study we propose the logistically easier route of administration of pre-dissolved H_2 in IV or oral fluids. H_2 can be dissolved in water at atmospheric pressure up to a saturating concentration of 0.8 mM (1.6 ppm), and tissue protection has been demonstrated in a variety of rat models utilizing either infusion of H_2 -enhanced IV fluids or ad lib access to H_2 –enhanced drinking water.

We will use one of three methods for hydrogen preparation:

1) H_2 drinking water obtained by dissolving an over-the-counter (dietary supplement) magnesium-containing tablet in tap water.
2) Diffusion of hydrogen into IV bags (containing saline) by placing the IV bag in a chamber filled with water that is bubbled with pure H2 gas, obtained from a gas tank.

The drug administration route flow chart depicted below summarizes this process (Figure 1).

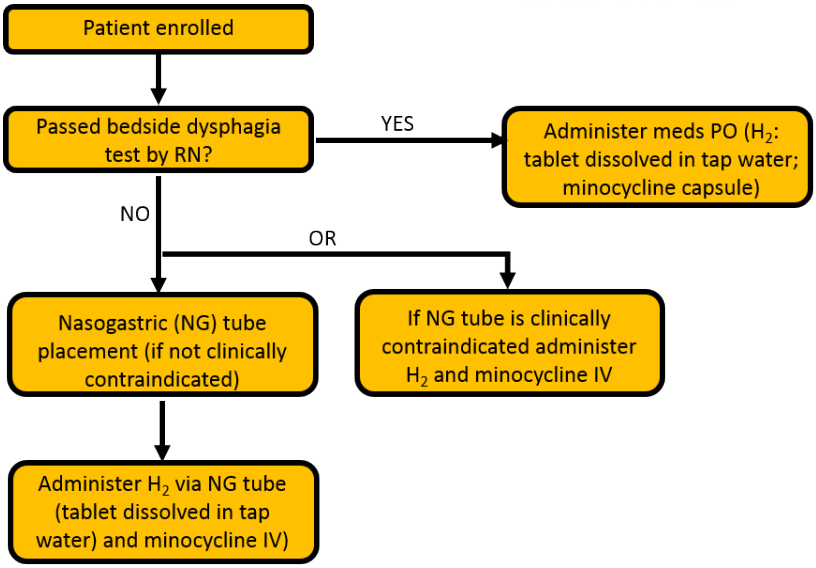


Figure 1: Study drug administration flow chart

Method 1 H_2 drinking water obtained by dissolving an H2 magnesium tablet in tap water for enteral administration (PO or NG): as an alternative method to prepare H_2 drinking water, we would use the hydrogen producing tablets from HRW (https://drinkhrw.com/shopping/sc_cart/sc_CatTy.asp?SectionID=1&treeUID=1040&guid=60614F5C-6904-4E9A-959B-1448EB4BFCC6). These tablets are sold commercially under the general category of dietary supplements, for the purpose of enhancing the hydrogen dissolved in drinking water.

The ingredient in the HRW tablets producing H_2 is magnesium. The tablets contain a safe level of magnesium (80 mg) well below recommended daily allowance (420 mg/d for men; 320 mg/d for women.

Dissolving one tablet in ~250ml of water will achieve a H_2 concentration of approximately 0.9 to 1.6 ppm. The tablet will be added to the water in an open glass/cup at bedside as the hydrogen dissipates quickly. As soon as the tablet is dissolved near-completely and rises to the surface the patient will be instructed to drink the water quickly all at once. For a matching placebo, we will use tablets with same size, same magnesium content and similar dissolve times provided by HRW Natural Health Products Inc. While the nurse may be able to tell the difference between the active study drug and placebo (different color), he/she will not be involved in the assessment at day 45 or 90.

Figure 2: Nutrition label of hydrogen producing tablets from HRW.

The study coordinator will be responsible for preparing H_2 –enhanced drinking water or IV fluid and untreated drinking water or IV fluid as placebo in HSC laboratory room 12-048 and maintaining the master list of treatment assignments. The study co-PI (DWC) has experience operating a cell biology laboratory and will ensure that all preparation surfaces are kept clean with no risk of cross contamination from other laboratory procedures (no concurrent procedures in the same space are currently anticipated). The Stony Brook Pharmacy will be responsible for preparing the H_2 magnesium tablets and placebo. The route of administration and formulation for each dose per patient will be recorded. The study drug will be labeled with the patient's name, MRN, name of drug or matching placebo, preparation time, and expiration.

Method 2 H_2 –enhanced fluids for IV administration: we will first obtain ordinary intact 250 ml IV bags of NS from Stony Brook Hospital supplies. For H_2 to diffuse into an intact IV bag while keeping it dry in the water bath, the vendor-supplied thick plastic VIAFLEX overwrap will be replaced at time of use by a standard plasma overwrap (a thin plastic bag that is currently used by the SB Blood Bank to thaw frozen plasma in a water bath). According to Baxter Healthcare Corporation (see letter on IRBnet), the primary purpose of the VIAFLEX overwrap is to retard water evaporation from the inner IV bag over long storage, which is not an issue here. At room temperature and for 100-1000 ml bags, Baxter recommends that the VIAFLEX overwrap not be removed longer than 30d before use. The inner IV fluid bag is adequate to keep contents sterile and is normally handled with ungloved hands prior to clinical use.

The IV fluid bag will be sealed water-tight inside the plasma overwrap bag using a heat sealer on the open edge; a test strip moisture indicator will also be placed inside the bag to detect any failure of the seal. The overwrapped IV bag will then be placed into a galvanized steel chamber filled with H_2 water (obtained from the chained Pure Hydration system and Iontech IT-380 water treatment systems, see below). The H_2 water in the steel chamber will then be slowly bubbled with pure H_2 gas (Ultra High Purity grade; Airgas) at a pressure of 10-15 psi over atmosphere to further increase dissolved H_2 to saturation. This will be carried out inside of a standard laboratory exhaust hood so that escaping excess H_2 gas will be safety vented to the outside (this procedure has been reviewed with SB EHS). A H_2 gas monitor mounted near the ceiling and hood will assure that the exhaust hood is working properly and that H_2 gas is not accumulating in the laboratory room.

Consistent with data published by Nagatani et al (2013) and others (eg, Ishibashi et al, 2014), we have found that H_2 gas is able to permeate the sealed plasma overwrap and intact IV fluid bag, elevating the level of dissolved H_2 in the bag's saline contents to about 0.9 ppm after 18-24 hours incubation, measured with a dissolved hydrogen meter (Sata Shouji ENH-1000, Kawasaki City, Japan; accuracy +/- 5 ppb). Since the IV bag remains dry and intact, there is no risk of introducing non-gaseous contaminants or infectious agents into its fluid contents by this procedure. We will nonetheless keep the water chamber scrupulously clean, scrubbing it out after each use (at least every week) with a spray of alcohol and then rinsing thoroughly with water. In addition, if the IV bag is not used within 1 week, it will be discarded. The Ultra High Purity H2 gas from Airgas is assessed at >99.999% pure. The CO + CO_2 concentration in this gas is assessed at < 1ppm, so even directly breathing this gas mixture would not carry a risk of CO toxicity (current OHSA permissible exposure limit is 50 ppm). We record cleaning and hydrogen preparation in a cleaning log and H_2 preparation log. We prepare extra quality control IV bags and on every day that we administer an IV bag to a subject, we measure the hydrogen concentration in the quality control IV bag that has been prepared simultaneously with the IV bag for the subject.

The study coordinator will check the moisture indicator before transporting it to the hospital floor for timely administration by the subject's nurse. If moisture is detected inside the overwrap (via the moisture indicator), the IV bag will be discarded. For placebo use, the IV fluid bag and moisture indicator will be sealed in the plasma overwrap bag without exposure to water. At point of administration, H_2–enhanced and ordinary IV fluid (placebo) will be indistinguishable. The placebo IV bags will be discarded if they are not used within 30 days following Baxter's instructions as stated above.

Administration

Subjects will start H_2 treatment (concurrent with minocycline treatment, see below) as soon as possible after arrival at SB (see above). The outside window for enrollment will be 24h after onset of brain ischemia, as ascertained by the stroke team based on last known well. The study coordinator will provide the hydrogen-rich water in a stainless steel water bottle or the hydrogen-rich NS IV bag. In case of IV treatment, the subject's nurse will open the plasma overwrap at bedside and administer IV fluid using the same procedures as a normal IV bag. The subject will receive 250cc H_2–enhanced water (if enteral) or NS (if IV, over 20-60 minutes) TID x 9 doses. If logistical issues preclude preparing a dose of H_2–enhanced IV NS, or the attending physician would prefer to limit fluid delivery, a dose may be skipped, dropping administration for that day from TID to BID. As noted above and immediately below, available evidence and mechanistic arguments suggest that some flexibility around timing and dose of H_2 delivery should not preclude efficacy. For subjects discharged from SBUH before the 3d H_2 treatment ends, they will be instructed to continue drinking 250ml H_2–enhanced water TID. The study coordinator will provide the H_2 magnesium tablets (or placebo) and a stainless steel water bottle for subjects to take with them. Subjects who cannot tolerate thin liquids can use commercial pre-thickened water or commercial thickener to get the water to the consistency they can tolerate (nectar-think or honey-thick).

Dosage

In the study of 6-OH-dopamine-induced nigrostriatal degeneration mentioned above, Fu et al (2009) found that 3ml of 50% saturated (0.8 ppm) H_2 –enhanced water instilled into the rat stomach was roughly equivalent to inhaling 2% gas in raising blood levels to roughly 10 nM; free access to such H_2 -enhanced drinking water was provided beginning 3d after surgery, and neuroprotection was observed at 2, 3, and 4 week post-operative time points. Infusion of H_2 -enhanced IV saline (5 ml/kg) given immediately after and 8h after cerebral hypoxia-ischemia reduced brain infarction in rat pups (Cai et al, 2008), and (at 6 ml/kg x 2 doses) reduced L-arginine-induced acute pancreatitis in rats (Chen et al, 2010). These latter doses would correspond to 300-360 ml IV saline in a 60kg human, scaled by weight/water volume.

Ishibashi et al (2014) performed a RCT of H_2 -enhanced IV saline in 26 patients with active rheumatoid arthritis. They found that 500 ml daily x 5d of IV saline containing approximately 1 ppm of dissolved H_2 was sufficient to reduce the joint disease activity score immediately post first infusion, with benefits increased further by 4 weeks after treatment onset. They noted that circulating blood cells in the vein receiving IV infusion are exposed to H_2 at local concentrations much higher than calculated blood equilibrium concentrations. These circulating immune cells participate in the pathogenesis of rheumatoid arthritis, and are also thought to participate in the pathogenesis of brain injury after ischemic insults.

For these reasons, we have selected a dosage target of 250 ml of H_2 -enhanced IV saline or drinking water TID. Duration of therapy was set at 3d, balancing the desirability of limiting later phases of oxidative tissue damage with practicality, and recognizing the difficulty of maintaining this treatment once subjects are discharged from SBUH.

D.5.b.ii Minocycline

Preparation

The study coordinator will maintain the master list with the treatment assignments and fill out the order form for the Stony Brook Pharmacy. Stony Brook pharmacy will compound the minocycline-infused IV bag using minocycline for injection (MINOCIN IV). For placebo, untreated IV fluid bags will be used. The Stony Brook pharmacy will also compound minocycline and placebo into identical gelatin capsules for subjects who can tolerate oral medication. The route of administration and formulation for each dose per patient will be recorded.

Administration

Minocycline will be given at 200 mg qd x 5d, either via IV solution or in capsule form. NS 100cc will be administered over 60 minutes. For subjects discharged from SBUH before the 5d treatment ends, they will be instructed to continue taking 200 mg capsules PO qd. The capsules have to be swallowed whole. Patients who do not pass the swallow test are not likely to be discharged and will continue treatment via IV.

Dosage

Neuroprotective doses of minocycline in experimental studies are generally similar to antibiotic doses. Dose ranging in stroke patients (Fagan et al., 2010) showed that 3mg/kg was well-tolerated and sufficient to achieve neuroprotective plasma levels, as defined in rats subjected to transient focal ischemia (about 4 mg/L = about 8 micromolar given MW 457; 12 mg/L had a slightly greater protective effect – (Xu et al.,

2004)). For this reason, and because two prior positive clinical studies of minocycline in stroke patients utilized a single daily dose of 200 mg x 5d, we have selected 200 mg qd x 5d as our minocycline dosage level.

D.5.b.iii Compliance

Compliance will be monitored by the study coordinator and recorded in a log. For the subject's inpatient stay, his/her nurse will make note of the administration and completion of each study drug dose in Powerchart. After discharge, the study coordinator will call the subject/caregiver to check compliance and for adverse effects.

D.5.c Outcome measures

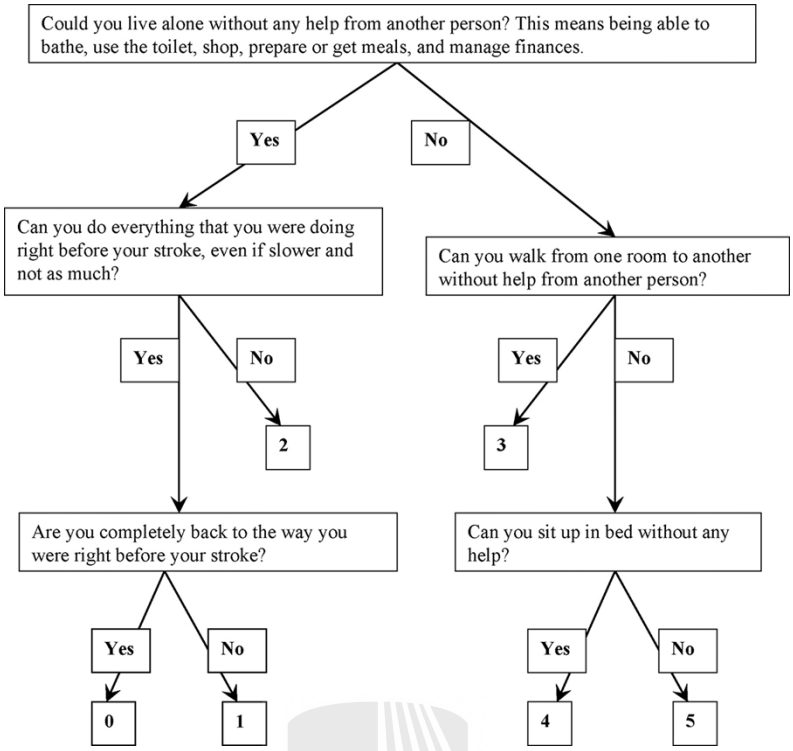
Each subject's neurological function will be evaluated at 45 and 90 days after stroke onset. The primary outcome measure will be the modified Rankin Scale (mRS), a global outcomes rating scale to assess level of functional independence for patients post-stroke with reference to pre-stroke activities, ranging from 0 (no symptoms) to 6 (dead).

Table 2: Modified Rankin Scale

Score	Description
0	No symptoms at all
1	No significant disability despite symptoms; able to carry out all usual duties and activities
2	Slight disability; unable to carry out all previous activities, but able to look after own affairs without assistance
3	Moderate disability; requiring some help, but able to walk without assistance
4	Moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance
5	Severe disability; bedridden, incontinent and requiring constant nursing care and attention
6	Dead

To limit subjectivity in mRS scoring, the simplified mRS questionnaire (sMRSq), a simple and validated questionnaire tool, will be used (Figure 3). It can be successfully administered via telephone, with telephone scores correlating well with in-person rating scores (Bruno et al., 2011). The smRSq will be administered in person at baseline, over the phone at day 45 ± 7 days, and in person or over the phone at day 90 ± 7 days.

Figure 3: Simplified Modified Rankin Scale Questionnaire



A secondary outcome measure will be the NIH Stroke Scale (NIHSS), a 15-item neurologic examination stroke scale used to evaluate the effect of stroke on several domains of neurological function: level of consciousness, language, attention, visual-fields, extraocular movement, motor strength, motor coordination, speech, and sensation. A trained observer rates the patent's ability to answer questions and performs a limited neurological examination. Ratings for each item are scored with 3 to 5 grades, 0 being normal, with allowance for untestable items. The single patient assessment requires less than 10 minutes to complete. Item scores are summed to yield a total score. A total NIHSS of 0 is normal; 1-4 considered a minor stroke; 5-15 moderate; 16-20 moderate to severe; and 21-42 severe. The NIHSS will be administered at baseline and day 90 ± 7 days for subjects (a majority) who are able to be seen in-person in the neurology outpatient clinic for a follow-up visit.

An additional outcome measure for subjects who undergo the second MRI will be the change in volume of the infarct area, measured as the difference between the volume of diffusion restriction in the first 48 hours and the T2 FLAIR infarct volume at 80 - 100 days after the stroke.

D.5.d Enrollment failure

Subject enrollment will be examined continuously. Our original goal was to enroll the targeted 100 subjects within 2y of enrolling the first subject. In the first 15 months we enrolled 9 patients. To increase the number of eligible patients we changed the inclusion criteria to be able to include patients with National Institute of Health Stroke Scale (NIHSS) of 5-25 instead of 5-22. Since last spring we have made the following adjustments to avoid missing any eligible patients: addition of personnel, enrollment during weekends, and addition of the option to administer hydrogen by nasogastric intubation.

We hope that these adjustments will improve enrollment rate to the point that a reasonable extension of time will enable completion of original study goals; or at least, enrollment of enough patients that a preliminary analysis of study data is worthwhile. Such a preliminary analysis may enable us to compete for outside grant support, permitting hiring of additional study coordinator(s) and increasing enrollment duty cycle during the week, or partnership with another medical center. In any case, prior to end of the originally proposed 2y enrollment period, we will either terminate the study, or submit an appropriate amendment request to the IRB.

E. STATISTICS

This is an exploratory study. It will be powered only to detect a large superiority of H2M treatment compared to placebo on the primary outcome measure, which will be a favorable functional outcome as assessed by mRS, 90d after the stroke.

Favorable outcome will be defined using a sliding dichotomy, an approach sometimes designated "responder analysis". mRS scores at 90 days will be classified as favorable or unfavorable based on the baseline NIHSS measured at time of enrollment. Subjects in the lowest baseline severity tertile (NIHSS 5–7) will need to have a 90 day mRS score of 0 to be considered to have a favorable outcome. Subjects with baseline NIHSS 8–14 will need a 90 day mRS score 0–1 to be considered to have a favorable outcome; those with baseline NIHSS 15–25 will need a 90 day mRS score 0–2 to be considered to have a favorable outcome. This approach, in use by the Stroke Hyperglycemia Insulin Network Effort (SHINE) trial (Bruno et al, 2014) requires subjects with milder strokes to achieve a higher level of recovery 90d after treatment than subjects with more severe strokes, to reach the study's favorable outcome endpoint. Many previous stroke therapy trials have used a simpler, straight 90d mRS 0-2 definition of favorable outcome, but this simpler approach runs the risk of a ceiling effect when patients with mild strokes naturally achieve a good functional recovery. This problem may have occurred in the Kohler et al (2013) study which enrolled patients with mild strokes (NIHSS >0), used a mRS 0-2 definition of favorable outcome, and observed a high rate of favorable outcome in both placebo and minocycline arms (66-70%).

The primary study population will be subjects presenting with acute ischemic stroke who are treated medically, with or without tPA. As noted above, this population constitutes the large majority of acute ischemic stroke patients at SBUH. A small number (<10%) of acute stroke patients, with strokes caused by an accessible large artery thrombus, are taken to surgery for mechanical thrombectomy, usually additional to administration of tPA. Mechanical thrombectomy not uncommonly produces marked and rapid alleviation of stroke symptoms, challenging detection of additional benefit due to an experimental therapy. But there is still room for improvement – mechanical thrombectomy typically does not eliminate cerebral infarction entirely, and as noted above, H2M therapy is predicted to specifically reduce the reperfusion injury induced by thrombectomy. We will enroll these surgical patients if they meet inclusion/exclusion criteria, but stratify them separately from the primary target population of subjects receiving medical therapy only.

Statistical analyses will be performed according to intention-to-treat using the Pearson chi-square statistic. Subjects lost to follow up will be considered to have an unfavorable outcome. No correction for additional exploratory analyses will be performed. Final statistical analyses will be performed under the guidance of an expert statistical consultant.

Power

Using the large effect size for minocycline seen in the original Lampl et al study (2007), with 47% favorable outcome in the placebo arm and 91% in the treatment arm, study size estimate yields a total

sample size of 44 (22 in each arm) needed to detect therapeutic effect (reject null hypothesis) in a comparison between two independent proportions at $p < 0.05$ and with a power of 0.90, using a two-tailed test. Using the large effect size seen in the Srivastava et al (2012) study, with 37% favorable outcome in placebo arm and 72% favorable outcome in treatment arm yields total sample size requirement of 82 for $p < 0.05$ / power 0.90, with sample size requirement falling to 62 for power 0.80.

Futility

A single interim analysis will be carried out once 45 primary study subjects have been enrolled (medical therapy only) to see if the study should be stopped for futility or harm, using the same Pearson chi-square statistic and the guidance of an expert statistician. Given pre-specification of this single interim test and enrollment point, it should have limited impact upon the power of the final analysis (O'Brien and Fleming, 1979).

FUNDING STATUS, DETAILS

This is an investigator-initiated pilot study that will be funded at launch from the investigators own uncommitted research funds, possibly with some help from the Department of Neurology. The investigators may seek outside funding once they have established their ability to conduct the study, and certainly will seek outside funding if interim study results are encouraging.

F. HUMAN SUBJECTS RESEARCH PROTECTION FROM RISK

F.1 Risk to Subjects

Based on known biology, we do not anticipate clinically significant adverse effects associated with the proposed brief administration of H_2. Several published studies have administered H_2-enhanced drinking water or IV saline to human subjects without observing any serious treatment-associated AEs. Nakao et al (2010) administered a higher, more sustained dose of H_2-enhanced drinking water (1.5-2L qd x 8 weeks) to 20 subjects with potential metabolic syndrome. They observed a small decrease in high density lipoprotein cholesterol considered therapeutically promising, but no abnormalities in weight, blood pressure, hematological parameters, or routine clinical chemistry values. No serious adverse events were noted. 4 subjects had mild AEs (1 with headache, 1 with heartburn, 3 with loose stools) but no definitive relationship to treatment could be established in such a small study lacking a control arm.

In the study of Ishibashi et al (2014) noted above, H_2 -enhanced IV saline (500 ml qd x 5d, approximately 1 ppm H_2) was given to 20 normal volunteers in a separate safety test. No clinical AEs or changes in routine hematology or blood chemistry were observed. Nagatani et al (2013) specifically carried out an open label exploratory safety study of H_2 –enhanced IV fluid in 38 patients with acute stroke, given together with another anti-oxidant, edaravone. If tPA was administered (11 patients), H_2 was given concurrently. They immersed regular plastic IV fluid bags in a water tank in which water was hydrolyzed to produce H_2; the IV fluid reached about 1 ppm via diffusion through the plastic bags. 200ml of H_2 –enhanced IV fluid was given q12h. 2 patients (5%) had possible adverse effects, consisting of diarrhea in one, and late cardiac failure in another 90 days later, but it would not be unusual to see similar occurrences in stroke patients administered placebo. No acute deterioration in laboratory tests or EKGs were observed, and no patients treated with tPA developed symptomatic intracranial hemorrhage. The investigators concluded that their data did not reveal any safety concerns with administration of IV H_2 to acute stroke patients.

One US company, MitoGene, has recently begun to sell pouches of H_2 –enhanced drinking water on the US market (http://htwo.com/about/). In recent correspondence with the FDA, MitoGene indicated their view that H_2 –enhanced drinking water met criteria to be classified as GRAS. While the FDA has not made a decision on this issue, they indicated in a response letter that they had no questions at this time (see attached).

The proposed administration of 850 ml qd of saline IV or water PO to subjects who have just sustained an acute ischemic stroke should not pose significant risk. While administration of water can transiently increase brain swelling, this volume is less than needed to keep a patient hydrated and much less than typically administered to acute stroke patients at SB (75cc/hr IV NS = 1800 cc qd). Of course the volume of maintenance IV fluids will be adjusted to take study medication fluid load into account. Furthermore, any subject whose attending physician is concerned about study medication-associated fluid load is excluded from study. Patients with acute ischemic stroke at SBUH are closely monitored by a stroke care team expert in managing fluid status, blood pressure, and brain swelling.

Similarly, we do not expect much risk from administration of minocycline in this study. Minocycline administered as an antibacterial agent at the proposed dose has an extensive safety record and is also available for pediatric use. There is no reason to believe that concurrent administration of H_2 will interact negatively with minocycline.

The most important contraindications to minocycline use in adults are allergic hypersensitivity, renal failure (increased azotemia and systemic accumulation of drug leading to liver toxicity), hepatic failure, and pregnancy (teratogenesis). These are all exclusion criteria. Patients with infections requiring antibiotic therapy that minocycline could interfere with are also excluded. Pseudotumor cerebri is a rare complication of longer term use (weeks-months) of tetracycline drugs, typically resolving when drug is discontinued. It is unlikely to develop during 5d administration, but any prior history of this disorder is another exclusion criterion.

Reported minor adverse effects in a small number of individuals that typically resolve when drug is stopped include photosensitivity / exaggerated sunburn reaction, pruritus, arthralgia, light-headedness, dizziness, vertigo and tinnitus. We will make subjects aware of the possibility of these adverse effects and stop study medication if any of these symptoms occur and prove troublesome within the framework of 5d administration.

Subjects who are unable to swallow will undergo nasogastric intubation for administration of study drug, if not clinically contra-indicated. Nasogastric intubation is typically a routine part of care of stroke patients who have difficulty swallowing, but may be performed earlier in the hospital course in patients enrolled into the study. The potential risks of nasogastric intubation include discomfort, and injury of the tissue inside of the nose, sinuses, throat, esophagus, or stomach. Rare risks include internal bleeding, abdominal cramping, abdominal swelling, diarrhea, nausea, vomiting, regurgitation of medicine, and aspiration.

The potential risks of IV drug administration include discomfort, bruising, and pain at the site of injection. Rare risks include inflammation of the vein used for injection, phlebitis, metabolic disturbances, and injury. Very rarely will there be severe reaction, anaphylaxis, cardiac arrest, or death.
MRI is a routine part of the stroke workup, and generally considered safe. Patients who have contraindications for MRI (e.g. patients with pacemakers or metallic implants) would not get an MRI in the acute stroke period, and thus would automatically not qualify for the second MRI. A second MRI scan would involve discomfort similar to the first MRI scan. Some patients experience a claustrophobic

sensation, and the scan can be noisy. Adverse events for MRI scans are very rare. If metallic objects are not removed prior to the scan, second degree burns may occur, and are the most commonly reported patient problem. Other reported problems include injuries from magnetic objects being brought too close and drawn toward the MRI scanner, crushed and pinched fingers from the patient table, patient falls, dizziness, tingling sensation and hearing loss or a ringing in the ear (tinnitus). A clinical MR imaging facility typically has numerous safeguards in place to prevent the above injuries and adverse events.

F.2 Adequacy of Protection Against Risks

The risks of the H2M therapy proposed here are small and thus dwarfed by the significant risks of death or major morbidity associated naturally with acute ischemic stroke. Patients with acute stroke at SBUH are very closely monitored by the stroke team, which includes neurologists, neurosurgeons, and radiologists with subspecialty expertise in the care of stroke patients. The PI and Data Safety Monitoring Committee will monitor safety and risks throughout the study. See section H for more details.

Signed informed consent will be obtained from each subject. A copy of the consent form countersigned by study staff will be given to the subject.

The PI and all key personnel involved in the study will have completed the Collaborative IRB training initiative and HIPAA training. The PI will be responsible to report adverse events experienced by study subjects to the IRB according to IRB guidelines.

Confidentiality will be maintained, as all subject research data will be coded with subject ID number and initials. The paper files will be kept in a secure, locked area. The electronic database used during the trial will be secured with a password and saved on a secured shared drive only accessible to key personnel. Subject results will never be discussed in any form in the presence of other subjects in the study or with non-laboratory personnel. A subject will be referred to by his/her randomization ID number wherever possible. Subjects' names will not be used in manuscripts or presentations about this research.

F.3 Potential Benefits of Proposed Research to the Subject and Others
F.4 Importance of the Knowledge to be Gained

The proposed H2M therapy may protect the subject's brain from injury pathways triggered by brain ischemia and/or reperfusion, thus reducing the brain damage and disability that ultimately results, and facilitating functional recovery. Even if the study is negative, observations made during the study may aid future refinements in H2M therapy or the development of other therapies for ischemic stroke. As stroke is the 2nd leading cause of death or adult disability globally, more effective treatments for acute stroke are much needed.

G. DATA SAFETY MONITORING PLAN

G.1 Adverse events monitoring

The Data Safety Monitoring Committee (DSM) will consist of three experienced physicians who are not study investigators or members of the acute stroke team, and who will be unblinded to treatment. The chair of the DSM will be Dr. Patricia Coyle, Professor and Vice Chair for Clinical Affairs in Neurology. Other members of the DSM will be Dr. Guy Schwartz, Assistant Professor of Neurology, and Dr. Agnieszka Kowalska, Co-Director of the Neuro-Oncology Center. Assisted by study staff, who will ensure that study data are accurate and complete, the DSM will assess protocol compliance by auditing protocol documentation. The DSM will check that inclusion/exclusion criteria are followed, adverse

events are reported, and protocol amendments are approved in advance by the IRB. If in the DSM's judgment a pattern of unexpected and clinically serious adverse events emerges, or if protocol compliance is inadequate, the Committee will terminate the study. The DSM will proactively conduct full reviews of safety data after the first 5 and then 15 patients have been enrolled; proactive reviews will then take place annualy.

Any unfavorable and unintended symptom, sign (including a clinically significant abnormal laboratory finding) or disease that is temporally associated with a study will be considered a study AE. The study staff will carefully monitor each subject throughout the study for possible AEs, and document such in an AE log. Minimum information recorded for each AE includes type of event, duration (start and end dates), severity, seriousness, causality to study drug, action taken, and outcome. The PI or one of the Co-Is will grade the severity of AEs based on the DHHS/NIH Common Terminology Criteria for Adverse Events (CTCAE) v4.0. Baseline stroke symptoms and signs documented prior to initiation of study medication will not be considered AEs, but any adverse change in these stroke symptoms and signs will be recorded as AEs for review by the unblinded DSM; those symptoms and signs judged by the PI or Co-Is to be expected given the usual course of stroke under current management will be noted in the AE log as unlikely to be caused by study drug. Hemorrhage into bland infarction, with associated clinical decline, occurs naturally in a small percentage of ischemic stroke patients, and a higher percentage of patients who receive tPA or mechanical thrombectomy. The DSM will specifically watch rates of hemorrhagic conversion, although we have no *a priori* reason to think that study medication will enhance this rate. The PI will proactively bring to DSM attention any serious AEs judged possibly associated with study medication.

Subjects will be monitored by the stroke team for AEs from the time they sign consent until completion of their participation in the study. We expect most drug AEs to surface during their inpatient stay when they are receiving both H_2 and minocycline, as the likelihood of a troublesome AE surfacing during the last day or two of oral minocycline therapy, or after drug discontinuation, is low. Subjects and any LARs will be made aware of the specific AEs known to be associated with H2M treatment (listed above) and will be instructed to discontinue minocycline and contact study staff immediately if they experience any troublesome AEs. Study staff will also proactively inquire about the occurrence of any AEs after discharge, at the follow up phone call 45d after stroke onset, and at the follow up phone call or clinic visit 90d after stroke onset.

Table 3: Schedule for safety monitoring and assessments

	Assessments	Frequency
H_2 IV fluid	Water chamber will be cleaned with spray of alcohol and rinsed thoroughly with water	After each use or at least once a week
	Moisture indicator in overwrap will be checked	Before each administration
	Hydrogen concentration of a quality control IV bag will be measured	Every day IV bags are used for subjects
H_2 tablet and placebo	Stony Brook pharmacy will compound this following USP 797 guidelines.	At each preparation
Minocycline IV fluid	Stony Brook pharmacy will compound this following USP 797 guidelines.	At each preparation
Minocycline capsule and placebo	Stony Brook pharmacy will compound this following USP 795 guidelines.	At each preparation
Study safety	The Data Safety Monitoring committee will review adverse events and protocol compliance	After the first 5 and then 15 patients enrolled; proactive reviews will then take place annually

G.2 Data safety

All paper study data will be kept in a secured, locked area. All identifiable electronic study data will be password-protected and saved in a secure shared drive on the Stony Brook server only accessible by study staff. The study coordinator will check the data for accuracy. Variable ranges and types will be assessed to ensure they are accurate. Missing data will be identified and obtained (if possible) in a timely fashion. Study staff will take steps to make sure all the information obtained is kept private. The subjects' name will not be used wherever possible. Study staff will use codes instead. The electronic data will be banked on the same secure server after the study ends. The study staff may submit a new proposal to obtain approval from the IRB to use this data.

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