Title:

Randomized, double-blind, placebo-controlled, pharmacokinetic, pharmacodynamic study of Trans Sodium Crocetinate utilizing Transcutaneous Oximetry Measurement in healthy volunteers

NCT Number:

NCT04808622

Document Type:

Protocol

Document Date:

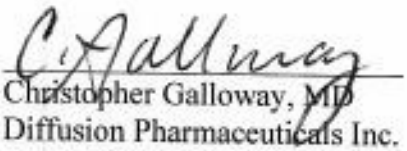
09 March 2021

DOCUMENT TYPE:	Clinical protocol
DOCUMENT NUMBER:	200-301
COMPOUND:	Trans Sodium Crocetinate
STUDY TITLE:	Randomized, double-blind, placebo-controlled, pharmacokinetic, pharmacodynamic study of Trans Sodium Crocetinate utilizing Transcutaneous Oximetry Measurement in healthy volunteers
CLINICAL PHASE:	1
INDICATION:	Healthy volunteers
IND NUMBER:	68579
SPONSOR:	Diffusion Pharmaceuticals Inc. 1317 Carlton Avenue, Suite 200 Charlottesville, VA 22902
DOCUMENT VERSION/STATUS:	Final
DOCUMENT RELEASE/AMENDMENT DATE:	Amendment 2: March 9, 2021 Amendment 1: February 12, 2021 January 14, 2021

SIGNATURES

The following Sponsor's signature confirms acceptance and approval of Protocol 200-301.

CHIEF MEDICAL OFFICER:



Christopher Galloway, MD
Diffusion Pharmaceuticals Inc.
1317 Carlton Avenue, Suite 200
Charlottesville, VA 22902
Tel: 434-220-0718
Email: cgalloway@diffusionpharma.com

CHIEF EXECUTIVE OFFICER:

Robert Cobuzzi, PhD
Diffusion Pharmaceuticals Inc.
1317 Carlton Avenue, Suite 200
Charlottesville, VA 22902
Tel: 434-220-0718
Email: rcobuzzi@diffusionpharma.com

CLINICAL OPERATIONS:

Harry N. Cook, RPh/MBA
Diffusion Pharmaceuticals Inc.
1317 Carlton Avenue, Suite 200
Charlottesville, VA 22902
Tel: 434-220-0718 x107
Mobile: 612-812-3363
Email: hcook@diffusionpharma.com

PRINCIPAL INVESTIGATOR:

Martin K. Kankam, MD, PhD, MPH, FAPCR
Co-Medical Director
Altasciences Clinical Kansas Inc.
10103 Metcalf Avenue
Overland Park, KS 66212
Mobile: 913-696-1601
Email: mkankam@altasciences.com

Table of Contents

PROTOCOL SYNOPSIS 8
1 INTRODUCTION 14
1.1 Study Rationale 14
1.1.1 TSC Background 16
1.2 TSC as a Therapeutic 16
1.3 Preclinical Experience 17
1.3.1 Animal Pharmacokinetics 17
1.3.2 Safety Pharmacology 18
1.3.3 Animal Toxicology 18
1.3.4 Hemocompatibility 19
1.3.5 Intravenous Irritation 19
1.3.6 Genotoxicity 19
1.3.7 Reproductive Toxicology 20
1.3.8 Absorption, Distribution, Metabolism, and Excretion 20
1.4 TSC in Model of Hemorrhagic Shock 23
1.5 Experience in Humans 26
2 STUDY METHODS 30
2.1 Study Overview 30
2.2 Study Objectives 30
2.2.1 Primary Endpoint 30
2.2.2 Safety Endpoints 30
2.3 Study Population 30
2.3.1 Inclusion Criteria 30
2.3.2 Exclusion Criteria 31
2.4 Enrollment and Consent 31
2.5 Withdrawal of Subjects 31
2.5.1 Criteria for Early Withdrawal 31
2.5.2 Screening Failures 32
2.5.3 Subjects Lost to Follow Up 32
2.5.4 Replacement of Subjects 32
2.5.5 Study Stopping Rules 32
2.6 Study Costs 32
2.7 Study Drug 32

2.7.1 TSC Packaging 32
2.7.2 Reconstitution of TSC 33
2.7.3 Placebo 33
2.7.4 Randomization 33
2.7.5 Blinding Protocol 33
2.7.6 Dosing Regimen 34
2.7.7 Drug Accountability 34
2.8 Concomitant Medications 34
2.9 Schedule of Events 35
2.9.1 Screening Evaluation (Day -21 to Day 0) 35
2.9.2 Treatment Visit (Day 0) 35
2.9.3 48 Hour Follow Up Telephone Visit 37
2.10 Laboratory and PK Assessment Parameters 37
2.10.1 Laboratory 37
2.10.2 PK Procedures 37
3 ADVERSE EVENT REPORTING 38
3.1 Definitions 38
3.1.1 Adverse Event 38
3.1.2 Suspected Adverse Reaction 39
3.1.3 Serious Adverse Event 39
3.1.4 Unexpected 39
3.1.5 Unanticipated Problem (UP) 39
3.2 Adverse Events Previously Reported with TSC 40
3.3 Adverse Event Reporting Requirements 40
3.3.1 Expedited Reporting Requirements for Unanticipated Problem SAEs 40
3.4 Research Related Injuries 41
3.5 Contraception and Pregnancy 41
4 DATAMANAGEMENT AND QUALITY ASSURANCE 42
4.1.1 Electronic Case Report Form 42
4.1.2 Clinical Monitoring 42
4.1.3 Source Documentation 43
5 STATISTICAL METHODS 43
5.1 General Considerations 43
5.2 Analysis Populations 43

5.3 Statistical Hypotheses 43
5.4 Sample Size 44
5.5 Missing Data 44
5.6 Primary and Secondary Efficacy Analyses 44
5.7 Safety Analyses 45
6 ADMINISTRATIVE AND SPECIAL PROCEDURES 45
6.1 Protocol Amendments 45
6.2 Investigator Responsibilities 45
6.2.1 Compliance with Protocol 45
6.2.2 Good Clinical Practice (GCP) 46
6.2.3 Institutional Review Board Review and Approval 46
6.2.4 Informed Consent 46
6.3 Protocol Deviations 46
6.4 Monitoring Activities 46
6.5 Quality Assurance Audits and Regulatory Inspections 47
6.6 Disclosure and Confidentiality 47
6.7 Changes in Study Personnel 47
6.8 Study Record Retention 47
6.9 Publication and Data Sharing Policy 47
7 REFERENCES 48
8 APPENDICES 50
8.1 Laboratory Parameters 50
8.2 Schedule of Events 51
8.3 Investigator Signature Page 52

Listing of Tables

Table 1. Rat and Dog PK Half-Life and Clearance of TSC Dosed Once Daily for 14 Days and 90 Days 17
Table 2. Mean Pharmacokinetic Parameters Correlated with Sample Radioactivity in Whole Blood and Plasma..... 21
Table 3. Recovered Radioactivity through 168-hour Post-dose 22
Table 4. Blood Parameters of Systemic Hypoxia Following Hemorrhagic Shock – Baseline, Post-Hemorrhage, and Following TSC vs. Saline Injection..... 24

Listing of Figures

Figure 1. Trans Sodium Crocetinate 16
Figure 2. Effect of TSC On Blood Pressure After Hemorrhage When Therapy Is Delayed 20 Minutes..... 24
Figure 3. Effect of TSC on Heart Rate After Hemorrhage When Therapy Is Delayed 20 Minutes 24
Figure 4. Liver Transaminase Activity At Baseline and 24-Hours Post-Hemorrhagic Shock in TSC vs. Saline Treated Groups [A – aspartate aminotransferase (AST), B – alanine aminotransferase (ALT)]..... 25
Figure 5. Survival with TSC vs. Saline Following Second Hemorrhage 26

LIST OF ABBREVIATIONS AND DEFINITIONS

Abbreviation	Definition
ADME	Absorption, distribution, metabolism and excretion
AE	Adverse event
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase (AST)
AUC	Area under the curve
BMI	Body mass index
BMP	Basic metabolic panel
BZK	Benzalkonium Chloride
CBC	Complete blood count
CMP	Clinical monitoring plan
COT	Claudication onset time
CRA	Clinical Research Associates
CRF	Case report form
CTCAE	Common Terminology Criteria for Adverse Events
DLT	Dose Limiting Toxicities
DSN	Data Safety Navigator
DVT	Deep vein thrombosis
EDC	Electronic data capture
EGC	Electrocardiogram
FDA	Food and Drug Administration

Abbreviation	**Definition**
GCP	Good Clinical Practice
GBM	Glioblastoma multiforme
GOT	Glutamate-oxaloacetic transaminase
GPT	Glutamate-pyruvate transaminase
HBOT	Hyperbaric oxygen therapy
ICF	Informed Consent Form
ICH	International Conference on Harmonisation
IEC	Independent Ethics Committees
IRB	Institutional Review Boards
ITT	Intention-to-Treat
KPS	Karnofsky Performance Score
LAR	Legally Authorized Representative
LOQ	Limit of quantitation
mRNA	Messenger ribonucleic acid
NOAEL	No observable adverse effect level
NOEL	No observable effect level
O_2	Oxygen
ONR	Office of Naval Research
OS	Overall survival
OTC	Over the counter
PAD	Peripheral Artery Disease
PFS	Progression-free survival
PH	Proportional hazards
PHI	Protected Health Information
PI	Principal investigators
PK	Pharmacokinetic
PCO_2	Partial pressure carbon dioxide
PO_2	Partial pressure oxygen
POC	Point of care
PWT	Peak walking time
QOL	Quality of life
RA	Room air
RT	Radiation therapy
SAE	Serious adverse events
SMC	Safety Monitoring Committee
SOC	Standard of care
SpO_2	Oxygen saturation
TCOM	Transcutaneous oximetry
$tcpO_2$	Transcutaneous tissue oxygen tension
TEAE	Treatment emergent adverse event
TSC	Trans Sodium Crocetinate

PROTOCOL SYNOPSIS

Protocol Number	200-301
Title of Study	Randomized, double-blind, placebo-controlled, pharmacokinetic, pharmacodynamic study of Trans Sodium Crocetinate utilizing Transcutaneous Oximetry Measurement in healthy volunteers
Study Phase	1
Study Objective	Determine the dose response of Trans Sodium Crocetinate (TSC) on Transcutaneous Oximetry Measurements ($tcpO_2$) following a single administration of TSC in subjects breathing oxygen (O_2)
Study Population	Healthy volunteers aged 18-50
Number of Subjects	Maximum of 30
Participant Duration	Maximum of 26 days, inclusive of screening, treatment and follow-up
Study Centers	1
Eligibility	**Inclusion Criteria** 1. Healthy male or female, age 18-50 2. Able and willing to lie quietly supine or semi-recumbent for up to 2.5 hours 3. Abstinence from exercise, caffeine, alcohol, nicotine, and a heavy meal prior to testing on the day of the treatment visit 4. Subject is able to communicate effectively with the Investigator and to comply with all study requirements, restrictions, and directions from the study staff 5. Females of childbearing potential must have a negative blood pregnancy test at screening and agree to use one of the accepted contraceptive regimens, or a double method of birth control (e.g. condom and spermicide), during the study and at least 30 days after the last dose of study drug. Females of non-childbearing potential should be surgically sterile or at least one year post-menopausal. 6. Males who engage in sexual activity that has the risk of pregnancy must agree to use a double barrier method (e.g. condom and spermicide) and agree not to donate sperm during the study and for at least 90 days after the last dose of study drug

	Exclusion Criteria 1. Allergy to study medication 2. Pregnant or breastfeeding 3. Current smoker and/or any nicotine use within 4 hours of the start of tcpO2 procedures, to include e-cigarette vaping, snuff, chew, nicotine gum and nicotine patches 4. Body Mass Index (BMI) > 30 5. Positive test results for HIV-1/HIV-2 Antibodies (HIV), Hepatitis B surface Antigen (HBsAg), or Hepatitis C Antibody (HCVAb) 6. Blood donation (excluding plasma donation) of approximately 500 mL within 56 days prior to screening 7. Plasma donation within 7 days prior to screening 8. Treatment with an investigational drug within 30 days or 5 times the half-life (whichever is longer) prior to screening 9. Any skin condition on limbs to be tested that could impair testing (rash, wound, prior radiation therapy, other skin conditions, per Principal Investigator (PI) discretion) 10. Known cardiovascular disease, including treated or untreated hypertension 11. Significant respiratory disease and/or any other significant medical condition 12. Subject has an acute illness (gastrointestinal, influenza, or known inflammatory process) at the Treatment Visit 13. Urine screen positive for drugs or positive breathalyzer for alcohol (at screening and enrollment) 14. Concomitant medications used to treat a diagnosed medical condition 15. Subject who, for any reason, is deemed by the Investigator to be unsuitable for the study; or has any condition that would interfere with the evaluation of tissue oxygen measurements or PK of the investigational drug; or is otherwise unable to comply with the protocol
Safety	Assessment of adverse events, new medications, laboratory (complete blood count [CBC], basic metabolic panel [BMP] HIV, HBsAg, HCVAb), vital signs (blood pressure, heart rate, respiratory rate, temperature), and oxygen saturation (SpO_2)
Study Design	Randomized, double-blind, placebo-controlled, pharmacokinetic, pharmacodynamic study

	Subjects will be randomized to a single IV bolus dose of TSC (0.5, 1.0, 1.5, 2.0, or 2.5 mg/kg) or placebo (normal saline).
Study Overview	A maximum of 30 healthy volunteers will be recruited to participate in this study. Following written informed consent, screening assessments will be obtained, including physical exam, laboratory (CBC, BMP, HIV, HBsAg, HCVAb), urine drug screen, alcohol screen, blood pregnancy test for all females, and vital signs. Following the screening visit, the subject will return to the clinic for study treatment procedures. If the site has Point of Care (POC) laboratory testing available, the screening and treatment procedures may occur on the same day. Subjects will refrain from exercise, caffeine, alcohol, nicotine, and a heavy meal prior to testing on the day of the treatment visit. Subjects will be randomized into one of 6 groups in a 1:1:1:1:1:1 schema, to include the 5 TSC doses and one placebo arm. Each TSC dose will be calculated based on the subject's body weight in kg at the dosing level they are randomized to. Each individual dose of placebo will be normal saline 7 mL. To maintain the double-blind, study drug administration will be performed by unblinded medical staff who will not be involved in other study procedures, including subject assessment. Subjects, investigators and study coordinators will not see the injection or injection site or be aware of randomization. On the day of treatment, subjects will be maintained in a temperature-controlled room (between 22.0 and 25.0˚C), and in a supine position with the head slightly raised on one pillow, or semi-recumbent. One blanket will be provided for comfort. One or two IV catheters will be placed for study drug administration and PK measurement. Direct venipuncture will also be allowed for PK draws, if needed. $TcpO_2$ sensor electrodes will be applied to the left or right lower extremity, per PI discretion. Sensor electrode temperature will be preset to 45˚C, to allow maximum vasodilation. Risk of sensor site superficial burn is minimal given the relatively brief testing period. Four (4) sensors will be applied to the following locations: • Sensor 1: Mid-dorsum of the foot • Sensor 2: 10 cm distal to the lateral femoral epicondyle • Sensor 3: 5 cm proximal to the anterior aspect of the lateral malleolus

- Sensor 4: 5 cm proximal from the center of the medial malleolus

After the $tcpO_2$ sensors have been applied and tested, subjects will be placed on O_2 via simple face mask at 6 L/minute, and will remain on O_2 for 70 minutes prior to study drug administration. The first 10 minutes will allow for equilibration of O_2 levels, and the subsequent 60 minutes will serve as the baseline period. $TcpO_2$ values and SpO_2 will be recorded every 5 minutes during the above periods. At the end of the 70-minute equilibration/baseline period, subjects will receive a single IV bolus injection of TSC at a dose of 0.5, 1.0, 1.5, 2.0 or 2.5 mg/kg, or placebo.

After study drug has been administered, subjects will continue on O_2 and be evaluated for an additional 60 minutes , with $tcpO_2$ values and SpO_2 recorded at 1, 2, and 5 minutes post-dose, and every 5 minutes thereafter.

In addition to assessment of $tcpO_2$ as described, continuous $tcpO_2$ measurements will be provided by the TCOM machine in graphical format.

Vital signs will be assessed prior to study drug dosing, and at 10, 30, and 60 minutes post-dosing. Adverse events will be assessed from the start of the equilibration period.

Prior to and following study drug administration, PK samples will be obtained at the below intervals.

Pre-dose (within 10 minutes prior to injection)
1 minute post end of injection (+1 min)
10 minutes post end of injection (± 1 min)
30 minutes post end of injection (± 1 min)
1.5 hours post end of injection (± 2 min)

After the 60 minute post-treatment evaluation period, oxygen will be discontinued and the $tcpO_2$ sensor electrodes removed. Subjects will remain in the procedure room for an additional 30 minutes to allow for collection of the 1.5-hour PK blood draw and repeat vital signs. Subjects will remain in the clinic overnight for observation.

Subjects will be contacted by telephone at 48 hours (+ 2 days) for a safety follow up to assess adverse events and new medications.

Statistical Analysis Plan	Subjects will be randomized using a non-stratified permuted block randomization scheme. Subjects who fail to complete the study will not be replaced. There will be a single analysis conducted at the end of the study; no interim assessments or analyses are planned for this fixed design. All analyses will be based on the observed data; no data will be imputed for the statistical analyses. The trapezoidal rule will be used for the construct of the area under the curve (AUC) analyses. All probability values will be 2-sided and accepted at face value for this feasibility study; there will not be an adjustment for multiplicity. For interpretation, probability values <0.05 will be considered *significant*; probability values <0.1 but ≥0.05 will be considered *highly suggestive of a significant difference*. The sample size selected for this clinical investigation with 6 treatment groups is based on clinical judgement. While hypotheses will be evaluated at the end of the study, there is no pre-specified statistical power that was used to establish the sample size. The primary analysis will be based on the change over time in the $tcpO_2$ measurements following a single administration of TSC in subjects breathing O_2. The time-matched changes from Period 1 (60 minute run-in on O_2) to Period 2 (60 minute post-drug administration on O_2) will serve as the dependent variable in the repeated measures analysis. The 60 minute run-in on O_2 is intended to account for the intra-subject variability over time and the time-match differences represent the least biased estimate of the effect of the study drug. With 5 subjects per randomized treatment arm, the change over time should provide sufficient clinical information to determine if there is a drug effect, and if the effect in an active drug treatment arm is different than placebo. To compare the time-match changes in $tcpO_2$, a 2-factor (treatment and time) repeated measures (time) analysis of variance model will be used. Contrast statements within the model will facilitate comparisons between individual treatment arms over individual time points, providing guidance relative to the initial time and duration of separation. To compare the AUC based on the time-matched differences, a 1-factor (treatment) analysis of variance model will be used, followed by a Dunnett's test to compare the least square mean AUC values from the 5 active drug groups relative to the placebo group. If the data within a treatment arm is skewed, suggesting that the least square mean value is not the best measure of central tendancy, the data may be ranked and the analyses will be conducted using the ranked scores. Additional information regarding the examination of the distribution of the data will be presented in the Statistical Analysis Plan. To compare the time to the

	maximum increase in $tcpO_2$, the event time distribution functions will be compared using a log rank test and presented using Kaplan-Meier curves. Summaries will be prepared for all parameters using descriptive statistics. For variables recored on a continous scale, results will be summarized by randomized treatment assignment and time. For categorical variables recorded on a multimonial or binomial scale, results will be presented using counts and percentages. All recorded data will be presented in the data listings. Adverse events will be coded using MedDRA and summarized by system organ class and preferred term and presented by randomized treatment assignment, Period (1 or 2), and overall, independent of Period.

1 INTRODUCTION

1.1 Study Rationale

Hypoxia is a condition in which local, regional, or generalized oxygen delivery to tissues is either inadequate or disrupted. If not reversed or corrected, tissue hypoxia can rapidly lead to tissue damage and cell death, which is irreversible. The global delivery of oxygen to tissues is dependent on both convection and diffusion. Convective delivery of oxygen is primarily driven by cardiac output and arterial oxygen content, while the diffusive component at the microcirculatory level (arterioles, capillaries, venules) is dependent on Fick's Law of Diffusion. Stated simply, diffusing particles move from an area of high concentration to an area of low concentration when a gradient is present. This rate of diffusion is dependent not only on how large the concentration differences are across a membrane (the gradient), but also physical and chemical characteristics that can impede movement of particles.

Transcutaneous Oximetry ($tcpO_2$) is a well characterized and a non-invasive method of measuring the partial pressure of oxygen (oxygen tension) under the skin. This is very different from pulse oximetry which uses infrared technology to measure hemoglobin saturation in the blood (not in the tissues). TSC's unique mechanism of action is to enhance diffusion of oxygen, and $tcpO_2$ methodology uniquely measures the amount of oxygen in the tissues that has diffused from the microcirculation. $tcpO_2$ is used extensively in cardiovascular patients being evaluated for revascularization for limb salvage or mapping for amputation. $tcpO_2$ also is used to determine if Hyperbaric Oxygen Therapy (HBOT) may help a patient's particular condition.

This clinical trial is designed to further clarify and optimize the dosing of TSC by directly measuring $tcpO_2$ in multiple locations on the lower extremity of a healthy volunteer after a single dose of TSC, while breathing O2.

The rationale for enrolling healthy volunteers versus patients in the phase 1 trial is the components of oxygen delivery will be highly variable both intra- and inter-subject in patients to a much higher degree than healthy volunteers. Patients already with some evidence of end organ disease (such as PAD mentioned in response) will have varying cardiac outputs, hemoglobin content, baseline oxygen saturation, and probable pulmonary function variability. All of these physiologic variables will confound the ability to detect a specific signal we hope to confidently attribute to TSC in a small cohort of subjects that is appropriately powered for the intra- and inter-subject variances. Additionally, the technicalities of TCOM measurements necessitates a person be quite motionless for accurate readings and any exertional test is not feasible utilizing this modality. We feel a healthy volunteer population is a best model to reduce the confounders in oxygen delivery as in this early phase trial.

The rationale to investigate TSC while on oxygen versus room air is based on two pre-clinical studies:

- "Trans sodium crocetinate increases oxygen delivery to brain parenchyma in rats on oxygen supplementation." Okonkwo et al, Neuroscience Letters 352 (2003) 97-100.

    - Licox brain tissue probes placed within rats demonstrated enhanced oxygen delivery within 30 minutes after TSC was given versus saline placebo while ventilated with varying oxygen concentrations (21%, 60%, 100%). 100% oxygen demonstrated the greatest change in brain parenchyma oxygenation versus the lower concentrations.
- "Effect of trans sodium crocetinate on brain tumor oxygenation." Journal of Neurosurgery 111:226-229, 2009.
    - C6 glioma model rats with Licox probes inserted into the tumor and contralateral healthy cerebral tissue demonstrated improved oxygenation in the tumor tissue within 30 minutes after TSC administration versus saline placebo while the rats were sedated and breathing room air.

Experience with TSC demonstrates there is a necessary diffusion gradient to demonstrate TSC's efficacy and pre-clinical models also demonstrate that the maximal effect is likely when breathing 100% oxygen versus room air, but effects also potentially can be seen at lower concentrations.

Oxygen is vital to sustain life and can be highly beneficial in certain illnesses. Too much oxygen can have strong unintended effects as well, and even cause oxygen toxicity.

Oxygen toxicity is most likely to occur in high risk groups:

- High concentrations (100% oxygen) for long duration (>24hrs)
- Mechanical ventilation (ventilator) for long duration and high oxygen concentrations (>50%)
- Hyperbaric oxygen therapy (patients in high pressure chambers and placed on supplemental oxygen)
- Underwater divers

Symptoms of Oxygen toxicity

- Pulmonary – airway irritation, coughing, congestion, inflammation, shortness of breath
- CNS (central nervous system) – headache, irritability, anxiety, dizziness, facial muscle twitching, ringing in ears, tingling in extremities, seizure

In this study, volunteers will be place on low flow oxygen at 6 L/min via simple face mask for approximately 2 hours in total time and have clinical and research personnel at the bedside all times during oxygenation and monitoring phase.

Each volunteer will be actively monitored by multiple clinical and research personnel at all times while in the clinical research unit, particularly with multiple clinical and research personnel present at all times at the bedside during the oxygenation, dosing, and TCOM monitoring period. After the oxygenation, dosing, and monitoring period, the volunteers will stay in the clinical research unit overnight for further safety and oversight monitoring before being discharged the next day per the principal investigator's discretion.

1.1.1 TSC Background

Diffusion Pharmaceuticals Inc. has developed a novel compound, trans sodium crocetinate (TSC), indicated as a drug to treat diffusion-limited hypoxia by selectively enhancing the re-oxygenation of hypoxic tissues. The ability of TSC to increase the diffusion (movement) of oxygen is the underlying mechanism by which TSC exerts its pharmacodynamic effects. In vitro studies demonstrate that TSC increases the diffusion of oxygen by altering the molecular arrangement of the water molecules which constitute the bulk of blood plasma. This effect creates a more ordered water structure which is less dense, reducing resistance to oxygen diffusion and allowing a more rapid movement of oxygen through the plasma (Laidig et al., 1998; Stennett et al., 2006). TSC exerts its pharmacodynamic effects via the ability to increase the diffusion of oxygen through blood plasma to hypoxic tissues. Thus, it is based on physical-chemical principles, unlike most drugs which are based on biochemistry based mechanisms.

TSC was first made for a project between the University of Virginia and the Division of Battlefield Casualties of the United States Office of Naval Research (ONR). Blood loss is responsible for a significant proportion of battlefield casualties, and ONR supported the development of TSC to the limit of their capabilities, which included considerable preclinical studies plus a Phase 1 clinical trial for safety and dosing in TSC-treated normal subjects. At this point, Diffusion Pharmaceuticals took over the manufacture of TSC as well as the oversight of Phase 2 clinical trials, including the treatment of Peripheral Artery Disease (PAD) and as an adjunct to radiation therapy for glioblastoma multiforme (GBM).

1.2 TSC as a Therapeutic

TSC (Disodium 2,6,11,15 Tetramethyl-hexadeca 2E,4E,6E,8E,10E,12E,14E-heptaene-1,16-diotate) is a bipolar synthetic carotenoid having the chemical formula $Na_2C_{20}H_{24}O_4$, and a molecular weight of 372. The Chemical Abstracts registry number is 64603-92-5. The chemical structure is shown in Figure 1. Carotenoids are a diverse group of highly colored nonpolar, monopolar, and bipolar polyenes (conjugated double-bonded carbon chain backbone). Crystals and concentrated solutions of TSC are orange-red, but in dilute solution, TSC is yellow.

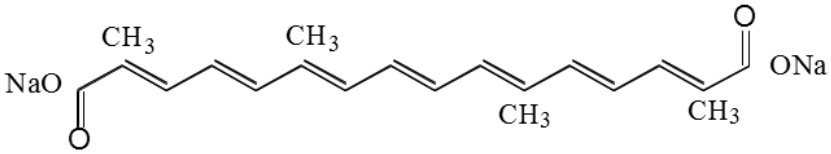


Figure 1. Trans Sodium Crocetinate

The carbon chain structure of TSC is identical to the naturally occurring dicarboxylic acid carotenoid, crocetin. However, crocetin and TSC differ in that crocetin is comprised of a mixture of isomers. TSC is a synthetically produced sodium salt of the single trans isomer. TSC has been the subject of investigation in 5 clinical trials to date (Diffusion Protocols 100-001, 100-201, 100-202, 100-301, 100-206).

Based on in vitro and computer modeling studies, it has been proposed that TSC works by altering the molecular arrangement of the water molecules which constitute the bulk of blood plasma, thereby creating a more ordered water structure (Laidig et al., 1998; Stennett et al., 2006). This altered water structure is less dense, reducing resistance to oxygen diffusion and

allowing a more rapid movement of oxygen through the plasma. More oxygen can thereby reach oxygen-deprived tissue (Giassi et al., 2003). In vitro studies have shown that TSC also increases the diffusion of glucose in a manner similar to oxygen (Stennett et al., 2006).

TSC administered intravenously does not appear to significantly affect oxygen levels in normal animals, but TSC increases oxygen levels in hypoxic animal models. Healthy animals breathing normal ventilated air show little effect on brain PO2 after TSC administration, suggesting that TSC does not significantly affect oxygen tissue levels in normal animals (Okonkwo et al., 2003).

When TSC is administered to animals ventilated with 100% oxygen, the animals show a significant increase in brain PO2 levels over a saline control. It is hypothesized that a sufficient oxygen concentration gradient must be established in order to significantly increase the diffusion of oxygen with TSC. This gradient appears to be present if the tissue is hypoxic, however, it can also be produced with hyper-oxygenation (such as 100% O2). Such a gradient is not present under normal conditions.

1.3 Preclinical Experience

1.3.1 Animal Pharmacokinetics

Pharmacokinetic (PK) studies of TSC have been conducted in rats and dogs. TSC has a relatively short half-life after a single intravenous injection. The half-life of TSC in the rat after a single IV bolus injection of 5, 15, 45, or 100 mg/kg varied from 20 minutes to 2 hours. Clearance varied from 50 to 300 mL/hr/kg. These data are shown below in Table 1 for both the low and high doses used in both 14- and 90-day studies. In addition, the volume of distribution varied from 60 to 140 mL/kg.

Table 1. Rat and Dog PK Half-Life and Clearance of TSC Dosed Once Daily for 14 Days and 90 Days

	Half-Life		**Clearance**	
Species	**Low Dose**	**High Dose**	**Low Dose**	**High Dose**
Rat 14 Day	5 mg/kg/day ~0.4 hr	100 mg/kg/day ~2 hr	5 mg/kg/day 300 ml/hr/kg	100 mg/kg/day 70 ml/hr/kg
Rat 90 Day	5 mg/kg/day	45 mg/kg/day	5 mg/kg/day	45 mg/kg/day
	~0.4	~1.4	233 ml/hr/kg	59 ml/hr/kg
Dog 14 Day	2.6 mg/kg/day ~2 hr	50 mg/kg/day 4 hr	2.6 mg/kg/day 70 ml/hr/kg	50 mg/kg/day 40 ml/hr/kg
Dog 90 Day	2.6 mg/kg/day	50 mg/kg/day	2.6 mg/kg/day	50 mg/kg/day
	8 hr*	6 hr	115 ml/hr/kg	52 ml/hr/kg

*Half-lives for male dogs were 4, 1.91, 0.924 and 48.4 hours, and 4, 1.65, 0.0389 and 2.35 hours for female dogs.

The half-life of TSC in dogs after a single IV bolus injection of 2.6, 5, 25, or 50 mg/kg varied from 2 to 6 hours. The half-life of 8 hr for the low-dose in the 90-day study is an average of values (listed below the table) that included an apparent erroneous value of 48.4 hours. Clearance varied from 40 to 115 mL/hr/kg. Again, these data are included in Table 1. In addition, the volume of distribution varied from 160 to 270 mL/kg.

The PK parameters in rats and dogs in the 14-day and 90-day repeat-dose toxicology studies did not vary significantly after daily injections of TSC compared to a single dose of TSC in rats and dogs. The pharmacokinetics in rats and dogs appears to be non-linear with greater than dose proportional increase for both Cmax and AUC. The half-life increases with dose in rats and dogs. The volume of distribution remained reasonably similar from low to high dose in rats and dogs, and did not exceed the total body weight for either species, indicating that TSC was not highly distributed into the tissues of rats and dogs after intravenous dosing.

1.3.2 Safety Pharmacology

Cardiovascular safety was tested in dogs. Central nervous system and pulmonary safety studies were conducted in rats. Single doses of 0.1, 1, or 10 mg/kg were tested in all safety pharmacology studies. No effects were observed in the cardiovascular studies when assessed by body temperature, blood pressure, heart rate, and QRS, RR, PR, and QT intervals. The no observable effect level (NOEL) was at least 10 mg/kg. TSC did not have any adverse effects on pulmonary function as measured by respiratory rate, tidal volume, or minute volume. Neurobehavioral studies did not show any behavioral changes suggesting neurotoxicity. The no observable adverse effect level (NOAEL) was at least 10 mg/kg in the pulmonary studies.

1.3.3 Animal Toxicology

Toxicology studies up to 14 days in one study and up to 90 days in another study were conducted in rats and dogs by the IV route of administration at maximum technically feasible doses based on limits of solubility and a clinically relevant dosing regimen. In both the 14-day and 90-day rat studies, animals received a daily IV dose of TSC at levels of 5, 15, 45, or 100 mg/kg/day. In the 14-day and 90-day dog studies, animals received a daily IV dose of TSC at levels of 2.6, 25, or 50 mg/kg/day.

In the 14-day study, TSC did not result in any mortality. In both species, TSC produced dose-dependent yellow discoloration of urine, hair, skin, and eyes. The discoloration generally disappeared after a 7-day recovery period indicating that the effects were reversible. The discoloration was not considered adverse to the health of the animals and was considered related to the color of TSC, which is yellow at dilute concentrations. Tissue discoloration appears to be a class effect of carotenoids because of their color in solution. When given in sufficient doses to humans, carotenoids are known to color tissues and particularly contribute to a yellow cast of the skin (Alaluf et al., 2002; Micozzi et al., 1988).

In the 90-day rat study, animals received a daily intravenous dose of TSC at 5, 15, or 45 mg/kg/day. There were no adverse effects noted following daily intravenous dosing of TSC for 90 days in Sprague Dawley rats, so the NOAEL for this study is considered to be 45 mg/kg/day. The observations of yellow discolored hair and skin continued through the recovery period for both males and females at 45 mg/kg/day, with the incidence remaining constant for discolored

hair while the incidence of discolored skin reduced in incidence with time. The observations of discolored skin/hair are, again, attributed to the color of the test article, and since neither the discoloration nor sparse hair were associated with any impact on the health of the animals they were not considered adverse.

In the 90-day dog study, animals received a daily intravenous dose of TSC of 2.6, 25 or 50 mg/kg/day. The NOAEL in dog was considered to be 25 mg/kg/day for the females due to the effects on body weight and euthanasia of 2 females at this level. The NOAEL is considered to be 50 mg/kg/day for the males as there were no adverse effects noted for the males during the course of the study. Yellow discoloration of the body (minimal to severe) that occurred for males and females at ≥ 25 mg/kg/day was generally dose-related in incidence and severity and was attributed to the color of the test article. The yellow discoloration was not considered adverse, due to a general lack of microscopic correlates in most animals and an absence of clear toxicity associated with the color change.

1.3.4 Hemocompatibility

In vitro rat, dog, and human blood plasma and serum compatibility studies indicated that TSC was compatible with blood from each of these species since there was no evidence of coagulation or precipitation. Hemocompatibility studies showed evidence of hemolysis in rat and dog blood, but not in human blood. In animals, a small reduction (5 to 10%) in erythrocytes, hematocrit, and hemoglobin were found in the rat and dog 2-week studies in the highest dose groups tested; 100 mg/kg/day and 50 mg/kg/day, respectively in the rat and dog. In addition, a 46 to 56% reduction in reticulocytes was noted at 50 mg/kg/day in the dog study. The increased bilirubin, when combined with an increase in urea nitrogen and the changes in the values of red cell variables described, may suggest a decreased red blood cell lifespan with impaired regeneration at the dose of 50 mg/kg/day in dogs. The magnitude of effect on hematological variables was small and the findings were reversible.

Consistent with the in vitro hemocompatibility results in human blood, there was no evidence of hemolysis or hemocompatibility issues in a Phase 1 study in 30 healthy human volunteers given a single IV bolus dose of TSC at a rate of 15 ml/min up to a maximum dose tested of 5 mg/kg. There were no laboratory reports of hemolysis or hemocompatibility issues in the Phase 1/2 study in 40 PAD subjects who received multiple doses of 0.25 mg/kg to 2.0 mg/kg TSC with samples collected at multiple time points.

1.3.5 Intravenous Irritation

An IV irritation study was conducted in rabbits. Using the marginal ear vein of rabbits, treatment with TSC resulted in very slight erythema in only 2 of 9 animals. When administered perivascularly, TSC was considered to induce slight- to well-defined erythema and slight edema, with effects persisting for several days.

1.3.6 Genotoxicity

TSC was negative in the in vitro Ames assay and the L5178Y mouse lymphoma cells, in both studies in the presence and absence of metabolic activation.

1.3.7 Reproductive Toxicology

The International Conference on Harmonisation (ICH) Stages C-D (Segment II) nonclinical reproductive toxicology studies in rats and rabbits using TSC as the formulated drug product administered intravenously to both species have been completed. For the rat Segment II study, doses of 0 (0.9% saline), 25, 50, or 100 mg/kg/day TSC were administered intravenously in the tail vein. The data demonstrated that the maternal NOAEL of TSC was 25 mg/kg/day. The 50 and 100 mg/kg/day TSC dosages caused reductions in body weight gain, and the 100 mg/kg/day TSC dosage also reduced food consumption. Injection site reactions occurred at all dosages; the severity of these reactions at 50 and 100 mg/kg/day resulted in the early sacrifice of 2 and 1 rats at these dosages, respectively. The developmental NOAEL was 50 mg/kg/day. The 100 mg/kg/day TSC dosage reduced fetal weight and increased the number of skeletal variations. Based on these data from the rat ICH Stages C-D (Segment II) reproductive toxicology study, TSC should not be identified as a developmental toxicant.

In the rabbit Segment II study, doses of 0 (0.9% saline), 25, 50, or 75 mg/kg/day TSC using the formulated drug product were administered intravenously. The maternal NOAEL of TSC was 50 mg/kg/day. The 75 mg/kg/day TSC dosage caused reductions in body weight gain and feed consumption. The developmental NOAEL was greater than 75 mg/kg/day since no effects were observed at the highest dosage tested. There were no adverse effects on embryo-fetal development as evaluated in this study. Based on these data from the ICH Stages C-D (Segment II) study in rabbits, TSC should not be identified as a developmental toxicant.

1.3.8 Absorption, Distribution, Metabolism, and Excretion

The results from in vitro assessment of reaction phenotyping (enzyme identification) of human CYP450 enzymes by TSC indicate that TSC is not metabolized in large amounts by liver microsomal CYP450 enzymes. Four in vitro metabolism studies within the full profile of absorption, distribution, metabolism and excretion (ADME) studies have been completed. TSC has been evaluated in the following metabolism studies: Cytochrome (CYP) P450 enzyme inhibition and induction studies, reaction phenotyping for CYP P450 enzyme identification, and metabolic stability of TSC in hepatocytes from multiple species. The in vitro studies testing the potential impact of TSC on liver direct enzyme inhibition in human microsomes showed no marked concentration-related increases at concentrations up to 40 μM in all tests done. TSC did not have an effect on the time-dependent inhibition of the standard battery of agents tested and is not likely a mechanism-based inhibitor of these isoforms. No significant induction responses (≥ 40% of adjusted positive control) of CYP1A2, CYP2B6, and CYP3A4 enzyme activity were observed with any of the concentrations of TSC examined in any of the human donor hepatocyte preparations. In addition, TSC treatment resulted in no marked CYP1A2, CYP2B6, and CYP3A4 mRNA induction. The results from these studies suggest a low potential for drug-drug interactions with TSC due to enzyme inhibition or enzyme induction of CYP1A2, CYP2B6, and CYP3A4 at the concentrations examined. A study was done to determine the in vitro metabolic stability of TSC in rat, dog, rabbit, and human cryopreserved hepatocytes. The metabolic stability was evaluated based on analysis of the disappearance of TSC as a function of time. The results from this multi-species metabolic stability study in cryopreserved hepatocytes suggest that TSC is not significantly (> 35%) metabolized via the particular CYP-mediated pathways present in the hepatocytes tested. Ultimately, drug interactions will be dependent on multiple metabolism and PK factors encountered in vivo.

The in vivo ADME of TSC was also investigated using [^{14}C]-labelled Trans Sodium Crocetinate ([^{14}C]-TSC) in the rat. Pharmacokinetic, excretion/balance and tissue distribution experiments were conducted and evaluated from samples collected following single oral and intravenous administration of [^{14}C]-TSC combined with non-radioactive TSC for a total of 50 mg/kg doses to rats.

Maximum mean plasma total radioactivity concentrations (C_{max}) were observed at 0.5 hours (T_{max}) post oral dose administration. Radioactivity/concentrations declined slowly thereafter with an apparent half-life of approximately 18 hours. Systemic exposure to the total radioactivity (AUC_{0-48}) was calculated to be 1700 µg equivalents·h/g (µg eq./g). Mean plasma concentrations (radioactivity) were 8 µg eq./g at 48 hours post dose.

Maximum mean whole blood total radioactivity concentrations were measured at 0.5 hours post oral dose administration. Concentrations declined slowly thereafter with an apparent half-life of 25 hours. Systemic exposure to the total radioactivity (AUC_{0-48}) was 1230 µg eq·h/g. Mean whole blood concentrations/radioactivity equated to 7 µg eq./g at 48 hours post dose.

Pharmacokinetics
The mean pharmacokinetic parameters correlated with sample radioactivity obtained in whole blood and plasma following a single oral gavage dose of [^{14}C]-TSC to male rats at 50 mg/kg, is displayed in Table 2.

Table 2. Mean Pharmacokinetic Parameters Correlated with Sample Radioactivity in Whole Blood and Plasma

Parameter	Male Rats (50 mg/kg)	
	Whole Blood	Plasma
$C_{max\ (measured)}$ (µg eq./g)	196	254
$C_{max\ (extrapolated\ to\ zero\ time)}$ (µg eq./g)	223	279
$T_{max\ (measured)}$ (hours)	0.5	0.5
$T_{½}$ (hours)	25.10	17.78
$AUC_{(0-48)}$ (µg eq·h/g)	1228.53	1675.27
$AUC_{(0-inf)}$ (µg eq·h/g)	1508.4	1861.25

Excretion balance
Excretion of radioactivity was followed through 168 hours after a single intravenous administration of [^{14}C]-TSC. The main route of elimination was through feces as metabolites with a mean recovery of approximately 50% of the dose administered. Expired air accounted for another 23% for each animal representing the next largest route of recovery. Urine accounted for a smaller proportion of radioactivity (approximately 6 % per each animal) with remaining quantitated concentrations of radioactivity measured in the carcass and cage washings. The mean total recovery of administered radioactivity was 83%. A summary of the relative amounts of recovered radioactivity through 168 hour post dose period following single intravenous administration of [^{14}C]-TSC at 50 mg/kg, is shown in Table 3.

Table 3. Recovered Radioactivity through 168-hour Post-dose

Sample	% Recovery Male (50 mg/kg) (n = 3)
Urine	5.90
Faeces	49.47
Expired Air Trap 1	10.43
Expired Air Trap 2	12.87
Cage Wash	0.05
Carcass	4.29
Total	83.01

Normally, the minimum target for radioactive recovery is 90%. The incomplete recovery may be explained by the fact that a large proportion of the radioactivity was eliminated in the first 24 hours via expired air. This could potentially be attributed to volatile components (i.e. CO_2 based metabolites) not being trapped in the expired air trapping solutions.

Tissue distribution
Following a single intravenous dose of [^{14}C]-TSC at 50 mg/kg to male Sprague Dawley rats, distribution of radioactivity was quick and widespread. The highest concentrations of radioactivity at early time points were observed in the gastro-intestinal tract contents, implicating biliary excretion as a route of elimination. Peak concentrations in remaining tissues generally occurred at 1 hour post dose, though some tissues (brain, spinal cord and some secretory glands) achieved peak concentrations at later times.

Elimination of radioactivity from tissues was generally slow; at 168 hours post dose, radioactivity remained quantifiable in most of the tissues analyzed, with greatest concentrations associated with the liver, skin, glandular tissue and brown fat.

Tissue: blood ratios indicate that up to 2 hours post dose, only the small intestine contents contained consistently greater concentrations of radioactivity than levels observed in blood. Six (6) hours post dosing and beyond indicated secretory glands had equal to or greater than the corresponding blood concentrations. Distribution of radioactivity in pigmented animals followed a similar pattern as non-pigmented animals, with no evidence of affinity for melanin-containing tissues observed.

1.4 TSC in Model of Hemorrhagic Shock

The objective of this study was to determine if TSC could enhance survival in rats with severe hemorrhage in a time-dependent fashion, mirroring a battlefield scenario where there is often a treatment delay prior to resuscitation (Giassi et al. Shock 2002). A constant-volume hemorrhage protocol was used in Male Sprague-Dawley rats. In the first set of experiments, the protocol involved removing 60% of the estimated blood volume, and 20 minutes elapsed before TSC (or saline) was first injected. TSC injections were repeated four times, every 10 minutes. After an additional 30 minutes, fluid resuscitation with saline was initiated. Blood pH, base excess, and lactate levels were monitored for 90 minutes after hemorrhage, prior to the point of initiation of fluid resuscitation. Possible liver damage was assessed 24 hours later by measurement of enzymes.

Blood pressure decreased by 40-45% of its baseline value following hemorrhage, with a commensurate increase in heart rate. Following the 20 min delay and initial TSC administration, blood pressure rose immediately by around 10-15 mmHg, but there was no change in response to saline injections. This improvement declined over 10 min and TSC was injected again with a similar increase in blood pressure, with no change in the saline group. This discrepancy in blood pressure improvement between TSC and saline was reproduced at each of the serial five injections (Figure 2).

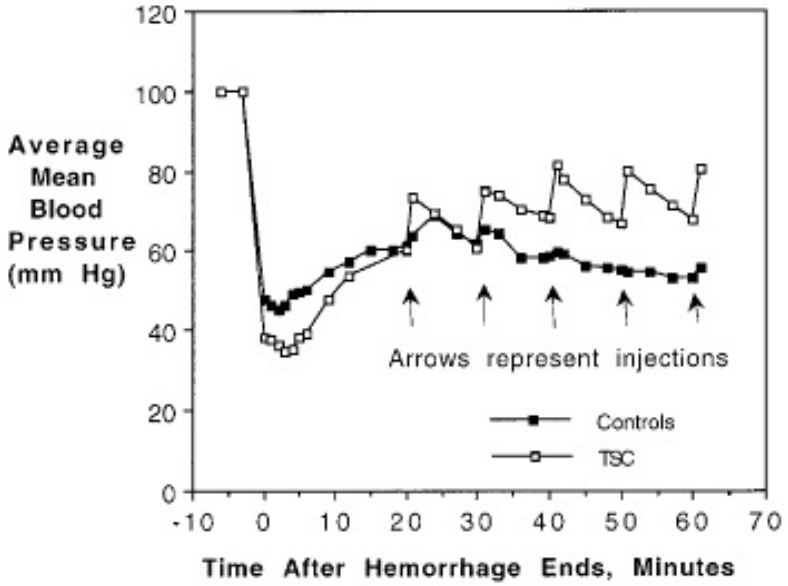


Figure 2. Effect of TSC On Blood Pressure After Hemorrhage When Therapy Is Delayed 20 Minutes

Similarly, a significant decrease in heart rate was noted following TSC injection compared to saline, and with each serial injection following the 20 min delay from hemorrhage (Figure 3).

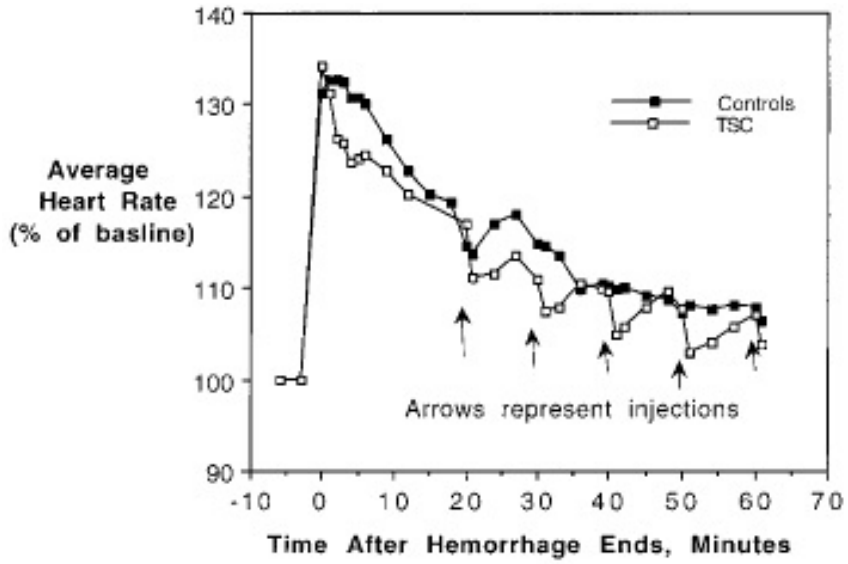


Figure 3. Effect of TSC on Heart Rate After Hemorrhage When Therapy Is Delayed 20 Minutes

Blood parameters of systemic hypoxia were taken at baseline, 15 min post-hemorrhage prior to injection, and 90 min post-hemorrhage following TSC (vs. saline) injection. TSC resulted in significant improvement in blood pH and base deficit compared saline, and demonstrated non-significant improvements in PO2, PCo2, lactate, and bicarbonate (Table 4).

Table 4. Blood Parameters of Systemic Hypoxia Following Hemorrhagic Shock – Baseline, Post-Hemorrhage, and Following TSC vs. Saline Injection

	Baseline (before hemorrhage)		15 minutes post-hem. (before injection)		90 minutes post-hem. (before infusion)		
Measurement	Control	TSC	Control	TSC	Control	TSC	p
PO_2 (mmHg)	98 ± 5	100 ± 5	129 ± 6	126 ± 11	130 ± 12	135 ± 7	NS
PCO_2 (mmHg)	37.2 ± 5.5	36.2 ± 4.3	24.3 ± 4.7	27.5 ± 3.3	30.2 ± 6.3	29.2 ± 5.6	NS
pH	7.42 ± 0.03	7.42 ± 0.03	7.23 ± 0.02	7.24 ± 0.04	7.33 ± 0.03	7.41 ± 0.07	0.04
Base Deficit (mmoles/liter)	–0.9 ± 1.8	–1.4 ± 1.4	14.6 ± 2.2	13.1 ± 2.9	8.1 ± 2.1	4.3 ± 1.5	0.01
Lactate (mmoles/liter)	0.98 ± 0.08	0.87 ± 0.10	5.6 ± 1.0	5.1 ± 1.4	3.5 ± 2.1	2.6 ± 1.1	NS
Bicarbonate (mmoles/liter)	24.5 ± 2.5	25.1 ± 1.3	10.4 ± 2.4	12.0 ± 2.5	15.9 ± 2.9	18.4 ± 1.5	NS

Liver injury was assessed by serum levels of glutamate-oxalacetic transaminase (GOT) and glutamate-pyruvate transaminase (GPT) (i.e. aspartate aminotransferase (AST) and alanine

aminotransferase (ALT)) before hemorrhage and 24 hours later. At the 24-hour mark post-hemorrhage, liver enzymes doubled in the saline group but remained stable in the TSC group ($p<0.05$).

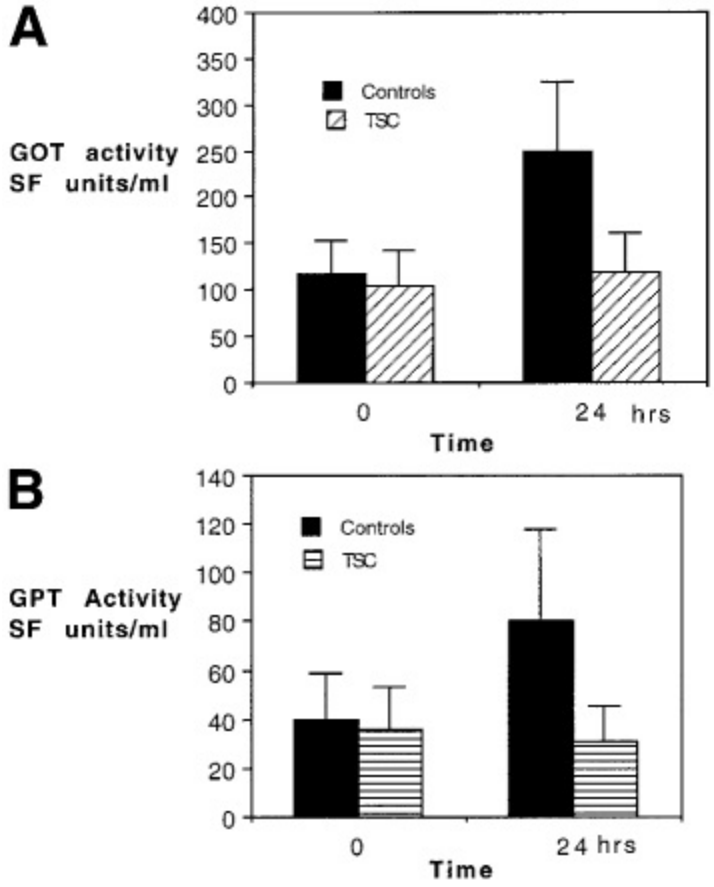


Figure 4. Liver Transaminase Activity At Baseline and 24-Hours Post-Hemorrhagic Shock in TSC vs. Saline Treated Groups [A – aspartate aminotransferase (AST), B – alanine aminotransferase (ALT)]

In a second experiment, a smaller hemorrhage (10% blood volume) was incurred ten minutes after the initial severe hemorrhage (60% blood volume). TSC (vs. saline) was given immediately following the second hemorrhage and every ten minutes for a 60-minute period. No subsequent fluid resuscitation was provided. In the saline group, almost all of the animals died within 50 minutes, whereas the majority treated with TSC survived.

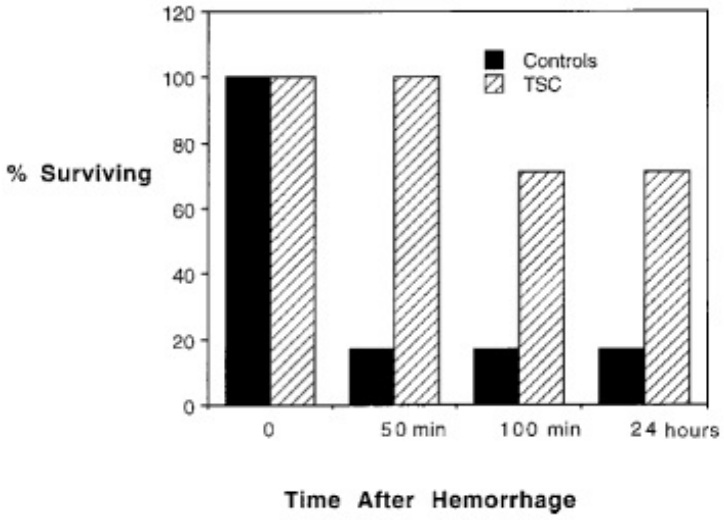


Figure 5. Survival with TSC vs. Saline Following Second Hemorrhage

These sets of experiments in rats with hemorrhagic shock suggest that TSC has a measurable effect on markers of systemic hypoxia, including normalization of acidosis and acute liver injury. TSC's effect on correcting hypotension and tachycardia following severe hemorrhage also supports the hypothesis that TSC enhances oxygenation and systemic oxygen transport.

1.5 Experience in Humans

Phase 1 - Safety

The safety and tolerability of the lyophilized injectable formulation of TSC in humans has been evaluated in a randomized, double-blinded, placebo-controlled Phase 1 trial in 40 normal healthy subjects (Diffusion Pharmaceuticals Clinical Study Report 100-001, 2008). Tolerability and PK were assessed after a single intravenous dose of 0.1, 0.5, 1, 2.5, and 5 mg/kg (6 subjects per TSC dose) through a 1 month follow-up visit after a single IV bolus injection of 20 mg/ml TSC. A total of 30 subjects received TSC and 10 received placebo. A single IV dose of TSC was very well tolerated and the maximum tolerated dose was 2.5 mg/kg based on mild, transient, yellow visual field disturbances seen in 2 subjects at a dose of 5 mg/kg. There were no clinically significant dose-dependent changes in laboratory parameters, vitals, or ECG up to the maximum dose evaluated of 5 mg/kg. There were no serious adverse events (SAEs) or deaths and no subject withdrew from the study.

Phase 2

Peripheral Artery Disease

A randomized, double-blinded, placebo-controlled Phase 1/2 study in 48 subjects has been conducted to evaluate the safety, PK, and dose response of TSC in subjects with symptomatic peripheral artery disease (PAD) and claudication (Diffusion Pharmaceuticals Clinical Study Report 100-301, 2010; Mohler et al., 2011). A total of 40 subjects received TSC and 8 subjects received placebo. Overall, multiple dosing by IV injection of TSC from 0.25 mg/kg to 2.00 mg/kg in elderly subjects with PAD was well tolerated when compared to placebo. The distribution of adverse events (AEs) observed in the study did not suggest predominance in any dosing arm compared to placebo. No treatment-emergent or dose limiting toxicity was

discovered in this study and no subject withdrew due to an AE. The 3 AEs reported by the principal investigators (PIs) as related to TSC were single incidents during dosing of mild to moderate burning or irritation at the injection site, and a yellow pigmented streaking at the injection site at the time of dosing; all resolved without treatment. These were likely associated with the IV line catheter placement or dosing technique, since reconstituted TSC has a yellow color. One subject receiving TSC at 2.00 mg/kg reported a single episode of a mild visual disturbance of "spots" that completely resolved without treatment. The PI evaluated this event as not related to TSC. The Sponsor determined this event was not a suspected adverse reaction, since it was not yellow in nature as was observed in the Phase 1 study and did not re-occur with repeated exposure to TSC. There were no AEs considered by the PI or Sponsor to be associated to the highest TSC dose (2.00 mg/kg).

In the 48 PAD subjects enrolled in Protocol 100-301, there were a total of 4 SAEs in TSC-treated subjects, including 1 death which occurred after the protocol-defined reporting period. There were 2 SAEs, including 1 death, reported in the placebo group. All of the SAEs were unexpected and considered unrelated to blinded study medication by the PI and Sponsor with the exception of the SAE of deep vein thrombosis (DVT). The DVT occurred in a subject who was randomized to 0.50 mg/kg TSC, and it was considered possibly related by the PI to blinded study medication. Although the DVT was an unexpected AE, the Sponsor determined this event was not a suspected adverse reaction since there was no evidence to suggest a causal relationship. While a causal attribution to study medication cannot be excluded, neither the PI nor the Sponsor can offer a probable pathophysiological mechanism by which TSC could result in a DVT. Study blind was not broken for any of these AEs.

The efficacy and dose response of TSC was evaluated in Protocol 100-301 using objective measurements from exercise treadmill tests in subjects with PAD and intermittent claudication symptoms. The change in claudication onset time (COT) showed a significant increase in the 0.25 mg/kg TSC dosing arm after Dose 1 and Dose 5 as well as lesser signals of improvement at TSC doses 1.25 mg/kg to 1.75 mg/kg. Notable signs of clinical benefit were observed after Dose 1 and Dose 5 of TSC above 1.0 mg/kg for both increased peak walking time (PWT) and COT on the exercise treadmill test, as well as subject-perceived increases in walking distance from the Modified Walking Improvement Questionnaire survey.

The PK profile of TSC from Protocol 100-301 in the elderly PAD subject population after multiple doses was similar to the PK results observed in the Phase 1 study in normal healthy subjects. TSC was eliminated quickly and the PK profile of TSC appears to be nonlinear after IV doses of 0.25 to 2.0 mg/kg in PAD subjects. The mean elimination half-life (t½) increased with dose and at doses greater than 1 mg/kg, t½ appeared to fluctuate in the 1.5 hour range. For the higher doses, 0.75 to 2.00 mg/kg, plasma concentrations appeared to decay at the same rate. Due to the short half-life of TSC relative to the 24-hour dosing interval, essentially 12.5 times the longest mean t½, and since only 4 subjects had a pre-dose plasma TSC concentration that was ≥ 10 ng/ml limit of quantitation (LOQ) and < 0.15% of corresponding maximal plasma concentration (C_{max}), the Dose 3 PK data may be viewed as if a single dose.

Glioblastoma Multiforme (GBM)
Diffusion Pharmaceuticals completed a phase 2 trial with a phase 1 safety lead-in (Diffusion Pharmaceuticals, Clinical Study Report 100-202, 2015) that evaluated the safety, tolerability, PK profile, efficacy, progression-free survival (PFS), quality of life (QOL), and overall survival (OS) in adults with GBM who are treated with TSC in addition to the standard of care (SOC) consisting of radiation therapy (RT) and temozolomide. The Phase 1 safety lead-in portion of the protocol incorporated a TSC dose escalation approach. The Phase 2 study portion enrolled 56 subjects at the recommended dose based on the safety lead-in study results. A previously conducted study by Stupp et al (2005) was used as a historical control.

During the conduct of Phase 1, monitoring for dose-limiting toxicity (DLT) events was performed. A Safety Monitoring Committee (SMC) evaluated safety data from the Phase 1 cohort and recommended a dosage regimen for Phase 2.

Safety and efficacy variables were evaluated at scheduled time points over 2 years after administration of study treatment. Investigators and independent reviewers assessed tumors and reported on tumor status. Tumor scans were subsequently analyzed by a single, central group, Biomedical Medical Systems.

The SOC treatment regimen, administered concomitantly with TSC treatment in both phases of the study, included RT and temozolomide, which were administered in a manner similar to that utilized in the Stupp et al (2005), which was used as a historical control study. Treatment with RT began within 5 weeks after tumor resection surgery or definitive biopsy. The RT treatment regimen included 5 sessions per week for 6 weeks (30 sessions total). Temozolomide 75 mg/m^2 was taken orally (capsule formulation) daily for 6 weeks (ie, 42 days) concomitantly with RT.

- Phase 1: 3 weeks of TSC .25mg/kg with concomitant RT and temozolomide; TSC administered 45 to 60 minutes before RT (9 doses total).
- Phase 2: 6 weeks of TSC .25 mg/kg with concomitant RT and temozolomide, TSC administered 45 to 60 minutes before RT (18 doses total).

After completion of administration of the study treatment, efficacy and safety evaluations were continued for a total of 2 years (24 months).

Overall survival (modified Intent-to-Treat [mITT] population; TSC 18 doses group)
- Two years after initiation of treatment with TSC, 35 subjects (62.5%) had died while 21 subjects (37.5%) remained alive. The probability (Kaplan-Meier analysis) of OS was 71.2% after 1 year and 36.3% after 2 years.
- The mean standard deviation (SD) OS duration was 16.31 (7.313) months, and the median (95% confidence interval [CI]) OS duration (using a Kaplan-Meier analysis) was 16.3 months (13.27, 23.66).

Progression-free survival (mITT population; TSC 18 doses group)
- The median (95% CI) PFS duration (using a Kaplan-Meier analysis) was 3.3 (3.15, 5.16) months. This may be a misleading value as many tumors that initially increased in size later decreased in size (see below).

Tumor size (mITT population; TSC 18 doses group)

- A mean change from Baseline of 28.7% in tumor size (sum of tumor area) was seen at Week 10. However, at the next time point (Week 18) mean tumor size was smaller, and subsequently mean tumor size remained smaller than at Baseline and continued to progressively decrease throughout the remainder of the 2-year treatment evaluation period. At the 1-year time point, mean tumor size in surviving subjects was approximately half of the mean tumor size at Baseline, and by the 2-year time point, 11 subjects had a 100% reduction in tumor size, indicating essentially complete disappearance of the GBM tumor.
- Of 14 subjects who had a complete resection before Baseline, 6 were alive at 2 years and showed no tumor present.

Quality of life and performance measures

- Mean and median scores for the European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30 and Karnofsky Performance Score (KPS) remained fairly constant over time, with subscale mean scores suggesting continued relatively good function and global health and generally low levels of symptoms.

Safety

A total of 56 subjects (94.9%) had a treatment emergent adverse event (TEAE), most of whom had more than one TEAE. The most frequently occurring TEAEs included fatigue (40.7%), alopecia (35.6%), nausea (27.1%), and constipation (27.1%). Most TEAEs were categorized as either common terminology for adverse events (CTCAE) Grade 1 (mild) or 2 (moderate); 10 subjects (16.9%) had a Grade 3 (severe) TEAE and 4 subjects (6.8%) had a total of 6 Grade 4 (life-threatening) TEAEs (ie, brain oedema, neutropenic sepsis, tooth abscess, pulmonary embolism, febrile neutropenia, drug hypersensitivity). There were no CTCAE Grade 5 (death related to AE) TEAEs.

A total of 24 TEAEs in 12 subjects (20.3%) were assessed as related to TSC. The most frequently reported TSC-related AEs were fatigue (5 events in 4 subjects [6.8%]) and headache (3 events in 2 subjects [3.4%]).

Eleven subjects (18.6%) had a total of 19 SAEs. The most frequently reported SAEs were hydrocephalus, pulmonary embolism, and muscular weakness, each of which occurred in 2 subjects (3.4%). All other SAEs occurred in 1 subject each. None of the SAEs were considered related to TSC.

No subject had an AE that resulted in death, and no subject had an AE that resulted in discontinuation of TSC.

Overall, diastolic and systolic blood pressure and pulse rate mean values over time showed variability, but no concerning patterns of clinically meaningful TSC-related changes from Baseline were discerned for evaluated vital signs, ECGs, or clinical laboratory tests.

In summary, TSC 0.25 mg/kg administered as an IV bolus 3 times per week for 6 consecutive weeks (18 doses total) as concomitant treatment along with SOC treatments of RT and

temozolomide was well tolerated in the subjects in this study with newly diagnosed GBM, and no safety findings were identified that would preclude further clinical development for the indication of treatment of GBM.

2 STUDY METHODS

2.1 Study Overview

This is a randomized, double-blind, placebo-controlled, pharmacokinetic, pharmacodynamic study of Trans Sodium Crocetinate (TSC) utilizing Transcutaneous Oximetry Measurement ($tcpO_2$) in healthy volunteers breathing O_2. Study assessments include $tcpO_2$ levels, SpO_2, and PK.

2.2 Study Objectives

2.2.1 Primary Endpoint

Determine the dose-response of TSC on $tcpO_2$ following a single administration of TSC in subjects breathing O_2.

2.2.2 Safety Endpoints

Assessment of adverse events, new medications, laboratory (CBC, BMP), vital signs, and SpO_2.

2.3 Study Population

Subjects will be healthy adult volunteers meeting all inclusion and exclusion criteria. Up to 30 subjects will be randomized to a single IV bolus injection of TSC at a dose of 0.5, 1.0. 1.5, 2.0, or 2.5 mg/kg or placebo normal saline with concomitant O_2 administration.

2.3.1 Inclusion Criteria

1. Healthy male or female, age 18-50
2. Able and willing to lie quietly supine or semi-recumbent for up to 2.5 hours
3. Abstinence from exercise, caffeine, alcohol, nicotine, and a heavy meal prior to testing on the day of the Treatment Visit
4. Subject is able to communicate effectively with the Investigator and to comply with all study requirements, restrictions, and directions from the study staff
5. Females of childbearing potential must have a negative blood pregnancy test at screening and agree to use one of the accepted contraceptive regimens, or a double method of birth control (e.g. condom and spermicide), during the study and at least 30 days after the last dose of study drug. Females of non-childbearing potential should be surgically sterile or at least one year post-menopausal.
6. Males who engage in sexual activity that has the risk of pregnancy must agree to use a double barrier method (e.g. condom and spermicide) and agree not to donate sperm during the study and for at least 90 days after the last dose of study drug

2.3.2 Exclusion Criteria

1. Allergy to study medication
2. Pregnant or breastfeeding
3. Current smoker and/or any nicotine use within 4 hours of the start of tcpO2 procedures, to include e-cigarette vaping, snuff, chew, nicotine gum and nicotine patches
4. Body Mass Index (BMI) > 30
5. Positive test results for HIV-1/HIV-2 Antibodies, Hepatitis B surface Antigen (HBsAg), or Hepatitis C Antibody (HCVAb)
6. Blood donation (excluding plasma donation) of approximately 500 mL within 56 days prior to screening
7. Plasma donation within 7 days prior to screening
8. Treatment with an investigational drug within 30 days or 5 times the half-life (whichever is longer) prior to screening
9. Any skin condition on limbs to be tested that could impair testing (rash, wound, prior radiation therapy, other skin conditions, per Principal Investigator (PI) discretion)
10. Known cardiovascular disease, including treated or untreated hypertension
11. Significant respiratory disease and/or any other significant medical condition
12. Subject has an acute illness (gastrointestinal infection, influenza, or known inflammatory process) at the Treatment Visit
13. Urine screen positive for drugs or positive breathalyzer for alcohol (at screening and enrollment)
14. Concomitant medications used to treat a diagnosed medical condition
15. Subject who, for any reason, is deemed by the Investigator to be unsuitable for the study; or has any condition that would interfere with the evaluation of tissue oxygen measurements or PK of the investigational drug; or is otherwise unable to comply with the protocol

2.4 Enrollment and Consent

An investigator or delegated site staff will interview and examine the volunteer in the clinic, and will discuss inclusion in the clinical investigation, the details of the investigation, and other information contained in the informed consent document; and that the subject's participation in the study may be discontinued at any time without penalty or loss of benefits to which the subject is otherwise entitled.

2.5 Withdrawal of Subjects

2.5.1 Criteria for Early Withdrawal

The following events are considered sufficient reason to discontinue a subject from the study:

- Subjects are free to withdraw from the study at any time, for any reason, and without prejudice
- The subject experiences an adverse event (AE) that in the investigator's opinion precludes continued participation

- The subject incurs a significant protocol violation that constitutes a safety hazard or significantly confounds the interpretation of the data from that subject
- At the investigator's request
- The study is terminated

2.5.2 Screening Failures

After the screening evaluations have been completed, the investigator or designee will review the inclusion and exclusion criteria and determine the subject's eligibility for the study. Only the reason for ineligibility will be collected on screening failures. Subjects who are found to be ineligible will be told the reason for ineligibility.

2.5.3 Subjects Lost to Follow Up

The investigator will repeatedly attempt to contact any subject that is unreachable by telephone for the 48 Hour visit to obtain follow-up safety information. All attempts to contact the subject should be documented, including the use of registered mail with return receipt.

2.5.4 Replacement of Subjects

Subjects who have received study drug will not be replaced. The analysis will be conducted on an intention-to-treat basis.

2.5.5 Study Stopping Rules

The Sponsor reserves the right to terminate the study at any time for reasonable medical or administrative reasons. Any premature discontinuation will be appropriately documented in compliance with applicable requirements and/or regulations (e.g., IRB/EC, regulatory authorities, etc.).

2.6 Study Costs

There is no cost to subjects for the research tests, procedures, evaluations, and study drug while taking part in this study.

2.7 Study Drug

2.7.1 TSC Packaging

Study drug will be supplied as a sterile lyophilized powder in 10 mL vials and will be in bulk cardboard boxes stored at room temperature (15 to 30° Celsius or 59 to 86° Fahrenheit) under secure conditions with limited access. Each vial will contain:

	10 mL Vial
Trans Sodium Crocetinate	100 mg
Gamma cyclodextrin	400 mg
Glycine USP	18.75 mg

Mannitol USP	115 mg

The label will contain appropriate labeling in accordance with all relevant labeling regulations.

2.7.2 Reconstitution of TSC

The 10 mL vial will be reconstituted with 5 mL of Sterile Water for Injection (USP). TSC should **not** be reconstituted with any other diluent other than Sterile Water for Injection. The lyophilized powder should rapidly reconstitute to a clear deep orange-red solution. Each reconstituted vial will contain: 20 mg/mL TSC, 8% gamma cyclodextrin, 50 mM glycine, 2.3% mannitol at an approximate pH of 8.0 - 8.2.

TSC is dosed based on the subject's baseline weight, obtained on the day of screening, on a milligram per kilogram basis. Several vials may be needed depending on the subject's weight. Reconstitution of multiple vials will be carried out per institutional Standard Operating Procedures relative to preparation of IV injectable drugs.

The reconstituted study drug will be prepared separately for each subject at room temperature (15 to 30°C or 59 to 86°F). TSC must be administered within 4 hours of reconstitution and any remaining TSC in the vial should not be used for further dosing of the same subject or another subject. Reconstituted TSC should **not** be diluted prior to administration.

2.7.3 Placebo

Placebo normal saline will be supplied from the bulk stock of the Investigational Pharmacy. Placebo is dosed at 7 mL per administration.

2.7.4 Randomization

Volunteers will be randomized to one of 5 TSC doses or placebo in a 1:1:1:1:1:1 schema, with 5 subjects randomized to each of the 6 cohorts.

2.7.5 Blinding Protocol

Because TSC is highly colorized, this study will utilize unblinded and blinded personnel as follows:

- Unblinded study team: Pharmacist(s) and administering personnel
- Blinded study team: Investigator(s) and study coordinators

The unblinded study team will take specific steps to ensure that the subjects and blinded study personnel are not made aware of treatment assignment.

- Prior to release from the pharmacy, the pharmacist will package the prepared syringe(s) in foil or acceptable masking material that masks the contents (i.e. cannot see through the material). The study drug should not be removed from the masking material at the time of administration to the subject.
- The medical staff assigned to administer the masked study drug will do so using a shroud to insure that the patient is blinded to treatment. The shroud may be made of any material and be of any size as long as it sufficiently blocks the patient's view of the

injection site and injection syringe at the time of administration. The shroud need not be in place at times other than during the time of study drug injection.

- A cloth or paper drape will be added underneath the injection site in the event that a droplet falls from the syringe as part of the injection process. The drape will be removed and folded up following the injection and before the shroud is removed so that it is not visible to the study team or subject.
- If in the treatment room at the time of injection, the blinded study team must position themselves so that the injection site and syringe(s) are not visible to them during study drug injection. The subject will also be asked to look away from the injection site.

2.7.6 Dosing Regimen

Study drug will be administered as a one-time IV bolus injection to 5 unique subjects per dose cohort, to include 5 TSC dose levels and placebo. Placebo will consist of 7 mL of normal saline. Subjects will be randomized to the following dose cohorts:

Dosing Cohort	# of Subjects in Cohort	TSC Dose (mg/kg)	Placebo (mL)
1	5	0.5	N/A
2	5	1.0	N/A
3	5	1.5	N/A
4	5	2.0	N/A
5	5	2.5	N/A
6	5	N/A	7 mL

2.7.7 Drug Accountability

It is the responsibility of the Principal Investigator to ensure that study drug at the site is inventoried upon receipt, accounted for throughout the study, and the results of drug accountability documented. Return of drug to the sponsor or sponsor designee, or the destruction of study medication, will also be documented.

Drug accountability tasks may be delegated to a pharmacist or other appropriately trained party at the site.

2.8 Concomitant Medications

Subjects may only be enrolled in this trial if they are healthy and not taking any medication prescribed for a diagnosed medical condition. Over the Counter (OTC) medications and supplements will require a 7-day washout period. Concomitant medications, such as Tylenol, may be given prior to study drug administration at the PI's discretion.

2.9 Schedule of Events

The charted schedule of events is included in Appendix 8.2

2.9.1 Screening Evaluation (Day -21 to Day 0)

Following completion of the informed consent process and signing of the Informed Consent Form (ICF), site staff will obtain the following.

- Demography
- Medical History
- Concomitant medications
- Physical exam
- Vital signs (heart rate, blood pressure, respiratory rate, temperature)
- Body Mass Index (BMI = kg/m^2)
- Oxygen saturation by pulse oximetry (SpO_2)
- 12-lead ECG
- Complete blood count (CBC)
- Basic metabolic panel (BMP)
- HIV, HBsAg, and HCVAb
- Urine drug screen
- Alcohol screen
- Serum pregnancy test for all females

Subjects meeting all inclusion/exclusion criteria and who have completed the informed consent process may be enrolled in the study.

2.9.2 Treatment Visit (Day 0)

Following the screening visit, the subject will return to the clinic for study treatment procedures. If the site has Point of Care (POC) laboratory testing available, the screening and treatment procedures may occur on the same day. Subjects will refrain from exercise, caffeine, alcohol, nicotine, and a heavy meal prior to testing on the day of the treatment visit.

Subjects will be randomized into one of 6 groups in a 1:1:1:1:1:1 schema, to include the 5 TSC doses and one placebo arm. Each TSC dose will be calculated based on the subject's body weight in kg at the dosing level they are randomized to. Each individual dose of placebo will be normal saline 7 mL. To maintain the double-blind, study drug administration will be performed by unblinded medical staff who will not be involved in other study procedures, including subject assessment. Subjects, investigators, and study coordinators will not see the injection or injection site or be aware of randomization. Please refer to Section 2.7.5 for specific blinding protocols.

2.9.2.1 Subject Arrival and $TcpO_2$ Sensor Electrode Placement

When subjects arrive to clinic for the treatment visit, medical history, physical exam, body weight, vital signs, concomitant medications, urine pregnancy screen, urine drug screen, and breathalyzer alcohol screen will be assessed (unless the Screening and Treatment visits are done on the same day).

Subjects will be maintained in a temperature-controlled room (between 22.0 and 25.0˚C), and in a supine position with the head slightly raised on one pillow, or semi-recumbent. One blanket will be provided for comfort. One or two IV catheters will be placed for study drug administration and PK measurement. Direct venipuncture will also be allowed for PK draws, if needed.

$TcpO_2$ sensor electrodes will be applied to the left or right lower extremity, per PI discretion. Sensor electrode temperature will be preset to 45˚C, to allow maximum vasodilation. Risk of sensor site superficial burn is minimal given the relatively brief testing period. Four (4) sensors will be applied to the following locations:

- Sensor 1: Mid-dorsum of the foot
- Sensor 2: 10 cm distal to the lateral femoral epicondyle
- Sensor 3: 5 cm proximal to the anterior aspect of the lateral malleolus
- Sensor 4: 5 cm proximal from the center of the medial malleolus

2.9.2.2 O_2 Equilibration and Baseline Period

After the $tcpO_2$ sensors have been applied and tested, subjects will be placed on O_2 via simple face mask at 6 L/minute, and will remain on O_2 for 70 minutes prior to study drug administration. The first 10 minutes will allow for equilibration of O_2 levels, and the subsequent 60 minutes will serve as the baseline period. $TcpO_2$ values and SpO_2 will be recorded every 5 minutes during the above periods. Baseline vital signs will be measured within 10 minutes prior to study drug dosing.

Once the equilibration phase begins, it is important that subjects remain lying quietly and minimize bodily movement through the entire baseline and treatment periods.

2.9.2.3 Treatment Evaluation Period

At the end of the 70-minute equilibration/baseline period, subjects will continue on O_2 and receive a single IV bolus injection of TSC at a dose of 0.5, 1.0, 1.5, 2.0 or 2.5 mg/kg, or placebo. After study drug administration, subjects will be evaluated for 60 minutes, with $tcpO_2$ values and SpO_2 recorded at 1, 2, and 5 minutes post-dose and then every 5 minutes thereafter. In addition to assessment of $tcpO_2$ as described, continuous $tcpO_2$ measurements will be provided by the TCOM machine in graphical format.

Vital signs during the treatment and evaluation period will be assessed at 10, 30, and 60 minutes post-study drug dosing. Adverse events will be assessed from the beginning of the equilibration period.

2.9.2.4 PK Measurements

Prior to and following study drug administration, PK samples will be obtained at the below intervals.

Pre-dose (within 10 minutes prior to injection)
1 minute post end of injection (+1 min)
10 minutes post end of injection (± 1 min)
30 minutes post end of injection (± 1 min)
1.5 hours post end of injection (± 2 min)

2.9.2.5 Post-Treatment Evaluation Period

After the 60-minute treatment evaluation period, the $tcpO_2$ sensor electrodes will be removed. Subjects will remain in the procedure room for an additional 30 minutes to allow for collection of the 1.5-hour PK blood draw and repeat vital signs.

2.9.2.6 Overnight Stay in Clinic

Each subject will remain in clinic overnight for observation.

2.9.3 48 Hour Follow Up Telephone Visit

Subjects will be contacted by telephone at 48 hours (+ 2 days) for a safety follow up to assess for adverse events and new medications.

2.10 Laboratory and PK Assessment Parameters

2.10.1 Laboratory

- Clinical laboratory evaluations:
    - Fasting is not required before collection of laboratory samples
    - Blood will be collected at the time points indicated in the protocol

- Venipuncture volumes:

Day +/- Window	**Day -21 to Day 0**
CBC, BMP, HIV, HBsAg, HCVAb	18 mL
PK samples (5 draws X 6 mL)	30 mL
Total all study days	48 mL

2.10.2 PK Procedures

Blood samples of approximately 6 mL will be collected at each timepoint for measurement of plasma concentrations of TSC.

The actual date and time will be recorded by 24-hour clock time noting the hour and minute of the end of the TSC infusion.

The same digital clock will be consistently used to record the actual hour and minute of the start of each blood specimen collection.

Each sodium heparinized vacutainer blood collection tube will be clearly labeled with the patient identifier number, date and time point of the specimen collection.

1. About 6 mL samples of blood will be collected in a sodium heparinized vacutainer tube and the blood will be centrifuged to separate plasma.
2. The plasma will be separated into two approximately equal volumes in separate tubes. The plasma should be approximately 1.5 mL in each tube.
3. Protocol-specific instructions will be provided to the clinical site in a lab manual.
4. The plasma samples will be frozen at -70°C. One aliquot will be shipped to the bioanalytical lab identified in the PK Laboratory Manual, and the other stored at -70°C as a back-up.
5. PK samples that are remaining after all PK analyses have been completed, may be used for additional analysis. Participant confidentiality will be maintained.

3 ADVERSE EVENT REPORTING

3.1 Definitions

3.1.1 Adverse Event

An adverse event (AE) is any untoward medical occurrence associated with the use of the drug in humans, whether considered drug related or not. An AE is any unfavorable and unintended sign (e.g., an abnormal laboratory finding), symptom, or disease temporally associated with the use of a drug, without any judgment about causality. This includes changes in anatomical, physiological, or metabolic functions as indicated by physical examination signs, symptoms, and/or laboratory changes or medical occurrence which develops or worsens while enrolled in this study regardless of whether the event is considered related to the investigational drug.

Signs and symptoms should be grouped into a single medical diagnosis and reported as a single AE when appropriate.

Abnormal laboratory values or test results constitute AEs if they are deemed clinically significant by the PI and induce clinical signs or symptoms or require therapy.

Adverse events will be collected from the start of the equilibration period through the 48 hour follow up phone visit.

3.1.2 Suspected Adverse Reaction

A suspected adverse reaction means any AE for which there is a reasonable possibility that the drug caused the AE. For the purposes of IND safety reporting, "reasonable possibility" means there is evidence to suggest a causal relationship between the drug and the AE. The sponsor will evaluate the available evidence and make a judgment about the likelihood that the drug caused the AE.

3.1.3 Serious Adverse Event

Serious adverse events (SAEs) are AEs that pose a threat to the subject's life or functioning based on the following outcome/actions regardless of whether the event is considered related to the investigational drug:

- Results in death
- Is life-threatening
- Requires in-patient hospitalization or prolongation of existing hospitalization
- Results in persistent or significant disability/incapacity
- Is a congenital anomaly/birth defect
- Other events, based on medical judgment, which jeopardize the subject and require medical/surgical intervention to prevent one of the outcomes above

Events **not** considered to be SAEs are:

- Treatment on an emergency outpatient basis for an event not fulfilling any of the definitions of serious and not resulting in hospital admission
- A hospital admission of less than 24 hours in duration

After the initial SAE report, the investigator is required to proactively follow each participant at subsequent visits/contacts. All SAEs will be followed until resolution, stabilization, until the event is otherwise explained, or the participant is lost to follow-up.

3.1.4 Unexpected

An unexpected adverse event is any adverse event where the nature, specificity or frequency of the event is not consistent with either: 1) the known or foreseeable risk associated with the procedures involved in the research that are described in the protocol or Investigator's Brochure; or 2) the expected natural progression of any underlying disease, disorder, or condition of the subject(s) experiencing the adverse event and the subject's predisposing risk factor profile for the adverse event.

3.1.5 Unanticipated Problem (UP)

An unanticipated problem is an event or outcome that meets all of the following criteria: 1) unexpected; 2) related or possibly related to participation in the research; and 3) places subjects or others at a greater risk of harm than was previously known or recognized.

3.2 Adverse Events Previously Reported with TSC

The Investigator's Brochure for TSC should be used as a reference for up-to-date information regarding non-clinical and clinical data that are available. As outlined in the subject informed consent form, the following risks and side effects may include the following:

- TSC is a yellow-orange color and may cause irritation or burning and/or yellow streaking of the skin at the site of injection and may be due to the IV-line placement or dosing technique.
- At the doses being tested in this study, 1 subject in another study reported a hot feeling in their leg which went away without treatment.
- In another study, 2 subjects reported a brief and mild, reversible yellow visual effect at 5.0 mg/kg, a dose that is higher than the doses used in this study, which resolved without treatment.

In animals, the following have been reported at TSC doses 50 times the highest dose utilized in this study:

- Mild, yellow skin discoloration
- Mild, yellow to orange discoloration of urine
- Mild changes in blood tests (liver enzymes, blood proteins, bilirubin, blood urea nitrogen)

No subjects have withdrawn from any study as a result of an AE related to TSC.

3.3 Adverse Event Reporting Requirements

Each subject will be evaluated for the development of AEs by the site study team. It is the responsibility of the investigator to document all AEs occurring during this investigation by entering them into the Case Report Form (CRF). All AEs occurring from the start of the equilibration period must be recorded, regardless of whether they are considered related to study drug. The nature of each event, date and time of onset, outcome, frequency, intensity, action taken with respect to dosage, whether it was serious or non-serious, expected or unexpected, and relationship to treatment should be documented. Signs and symptoms should be grouped into a single diagnosis and reported as a single AE when appropriate.

SAEs require expedited review and must be reported by the site to the Sponsor or designee via the CRF and/or electronically **within 24 hours** of discovery.

The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 will be used for AE reporting. Adverse events will be coded using the MedDRA coding dictionary.

3.3.1 Expedited Reporting Requirements for Unanticipated Problem SAEs

SAEs will be reported to the appropriate regulatory authorities per the Sponsor Regulatory Reporting Requirements Matrix maintained by Drug Safety Navigator (DSN). For expedited cases, DSN will finalize the required documents on or before Day 7 or Day 15, as applicable, per FDA requirements as outlined in the Reporting Matrix.

3.4 Research Related Injuries

For any potential research related injury, the site PI or designee will assess the subject. Study personnel will try to reduce, control, and treat any complications from this study. Immediate medical treatment may be provided by the participating study site, such as giving emergency medications to stop immediate allergic reactions.

As needed, referrals to appropriate health care facilities will be provided to the subject. The site PI should then determine if an injury occurred as a direct result of the tests or treatments that are done for this trial.

3.5 Contraception and Pregnancy

A female study subject must meet one of the following criteria:

- If of childbearing potential – agrees to use one of the accepted contraceptive regimens during the study, and for at least 30 days after the last dose of the study medication. An acceptable method of contraception includes one of the following:
    - Abstinence from heterosexual intercourse
    - Hormonal contraceptives (birth control pills, injectable/implant/insertable hormonal birth control products, transdermal patch)
    - Intrauterine device (with or without hormones)

      OR

    - Agrees to use a double barrier method (e.g. condom and spermicide) during the study and for at least 30 days after the last dose of the study medication
- If of non-childbearing potential – should be surgically sterile (i.e. has undergone complete hysterectomy, bilateral oophorectomy, or tubal ligation/occlusion) or in a menopausal state (at least 1 year without menses), as confirmed by FSH levels (≥ 40 mIU/mL).

A male study subject that engages in sexual activity that has the risk of pregnancy must agree to use a double barrier method (e.g. condom and spermicide) and agree to not donate sperm during the study and for at least 90 days after the last dose of the study medication.

A pregnancy of a female subject occurring within 28 days of the subject's dose of study drug is considered a reportable event. If a pregnancy is reported or discovered, the investigator should inform the Sponsor or designee within 24 hours of learning of the pregnancy. Pregnancy itself is not considered an AE or SAE, however abnormal pregnancy outcomes (e.g., spontaneous abortion, fetal death, stillbirth, congenital anomalies, ectopic pregnancy) are considered SAEs and should be reported to the Sponsor. If a pregnancy is reported, the Sponsor or designee will follow up on pregnancy outcome 4 weeks after the projected due date.

4 DATA MANAGEMENT AND QUALITY ASSURANCE

4.1.1 Electronic Case Report Form

Study data will be collected on a standardized case report form (CRF) and managed using an electronic data capture tool selected by the sponsor. The EDC tool will be a secure, web-based software platform designed to support data capture for research studies, providing 1) an intuitive interface for validated data capture; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for data integration.

Clinical data will include subject demographics, medical history, vital signs, laboratory assessments, tissue oxygen measurements, and pulse oximetry. Additional clinical information including concomitant medications and adverse events will be included.

4.1.2 Clinical Monitoring

Clinical site monitoring is conducted to ensure that the rights and well-being of trial subjects are protected, and that the reported trial data are accurate, complete, and verifiable. Clinical Monitoring also ensures conduct of the trial is in compliance with the currently approved protocol/amendment(s), ICH, GCP, applicable regulatory requirement(s) and sponsor requirements. Clinical monitoring will also verify that any critical study procedures are completed following specific instructions in the protocol.

Monitoring for this study will be performed by qualified and trained Clinical Research Associates (CRAs) so contracted by the sponsor. Details of clinical site monitoring are documented in a clinical monitoring plan (CMP). The CMP describes in detail who will conduct the monitoring, at what frequency monitoring will be done, at what level of detail monitoring will be performed, and the distribution of monitoring reports. Monitoring visits will include, but are not limited to, review of regulatory files, drug accountability records, CRFs, ICFs, medical and laboratory reports, record storage, training records, and protocol and GCP compliance. Site monitors will have access to each participating site, study personnel, and all study documentation according to the sponsor approved clinical monitoring plan. Study monitors will meet with site PI, sub-Investigators and Study Coordinators to discuss any problems and outstanding issues and will document site visit findings and discussions.

Study Coordinators will fill out a screening log for all study sites that documents all volunteers screened for the trial, and whether they screen-failed or were enrolled. For screen fails, the reason for exclusion will be recorded.

Monitoring will be performed via remote or in-person monitoring. CRFs will be reviewed and compared with the data entered to the subject's medical record (source documents). If remote monitoring is necessary the Study Coordinator will collect the appropriate source documents, copy, and redact all PHI information and then scan and send them to the Study Monitor for review. Any inadequacies or errors will be reviewed with the PI at the site. Subsequently, on-site case report form monitoring visits (if in-person visits are cleared by the clinic) will be performed at regular intervals as appropriate. Otherwise remote monitoring procedures will continue.

Site investigators or Study Coordinators will inform the sponsor of every subject enrollment on the same or next business day, by entering the subject into the eCRF.

4.1.3 Source Documentation

Source data are all information in original records (and certified copies of original records) of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data should be attributable, legible, contemporaneous, original, accurate, and complete. Each participating site will maintain appropriate medical and research records for this trial, in compliance with ICH, GCP, regulatory, and institutional requirements. Data recorded in the CRF derived from source documents should be consistent with the data recorded on the source documents.

Interview of subjects is sufficient for obtaining medical history. Solicitation of medical records from the subject's primary care provider is not required.

5 STATISTICAL METHODS

5.1 General Considerations

Subjects will be randomized using a non-stratified permuted block randomization scheme. Subjects who fail to complete the study will not be replaced. There will be a single analysis conducted at the end of the study; no interim assessments or analyses are planned for this fixed design. The trapezoidal rule will be used for the construct of the area under the curve (AUC) analyses. All probability values will be 2-sided and accepted at face value for this feasibility study; there will not be an adjustment for multiplicity. For interpretation, probability values <0.05 will be considered significant; probability values <0.1 but ≥ 0.05 will be considered highly suggestive of a significant difference. Summaries will be prepared for all parameters using descriptive statistics. For variables recored on a continous scale, results will be summarized by randomized treatment assignment and time. For categorical variables recorded on a multimonial or binomial scale, results will be presented using counts and percentages. All recorded data will be presented in the data listings.

5.2 Analysis Populations

Two analysis populations will be used to summarize the results from this clinical investigation.

- Intent-to-Treat Population: The intent-to-treat (ITT) population includes all randomized subjects. Subjects in the ITT population will be analyzed as randomized.
- Per Protocol Population: The per protocol (PP) population includes subjects in the ITT population who do not have significant protocol deviations and who complete the study. Protocol deviations will be assessed prior to database lock and unmasking. The PP population will be analyzed using observed data only for efficacy variables. Subjects in the PP population will be analyzed as treated.

5.3 Statistical Hypotheses

The primary endpoints will be tested in a hierarchical fixed sequence in the following order:

Based on the 2-factor (treatment and time) repeated measures (time) analysis of variance model using the time-match changes in $tcpO_2$:

H_0: μ (active treatment) = μ (placebo treatment)
H_1: μ (active treatment) ≠ μ (placebo treatment)

Separate examinations of each active dose will be compared to placebo.

Based on the 1-factor (treatment) analysis of variance model using the AUC based on the time-match changes in $tcpO_2$:

H_0: μ (active treatment) = μ (placebo treatment)
H_1: μ (active treatment) ≠ μ (placebo treatment)

Separate examinations of each active dose will be compared to placebo.

Based on the comparison of the time to the maximum time-match increase in $tcpO_2$:

H_0: $exp(\lambda)$ (active treatment) = $exp(\lambda)$ (placebo treatment)
H_1: $exp(\lambda)$ (active treatment) ≠ $exp(\lambda)$ (placebo treatment)

5.4 Sample Size

The sample size selected for this clinical investigation with 6 treatment groups is based on clinical judgement. While hypotheses will be evaluated at the end of the study, there is no pre-specified statistical power that was used to establish the sample size.

5.5 Missing Data

All analyses will be based on the observed data; no data will be imputed for the statistical analyses.

5.6 Primary and Secondary Efficacy Analyses

The primary analysis will be based on the change over time in the $tcpO_2$ measurements following a single administration of TSC in subjects breathing O2. The time-matched changes from Period 1 (60 minute run-in on O2) to Period 2 (60 minute post-drug administration on O2) will serve as the dependent variable in the repeated measures analysis. The 60 minute run-in on O2 is intended to account for the intra-subject variability over time and the time-match differences represent the least biased estimate of the effect of the study drug. With 5 subjects per randomized treatment arm, the change over time should provide sufficient clinical information to determine if there is a drug effect, and if the effect in an active drug treatment arm is different than placebo.

To compare the time-match changes in tcpO2, a 2-factor (treatment and time) repeated measures (time) analysis of variance model will be used. Contrast statements within the model will facilitate comparisons between individual treatment arms over individual time points, providing guidance relative to the initial time and duration of separation.

To compare the AUC based on the time-matched differences, a 1-factor (treatment) analysis of variance model will be used, followed by a Dunnett's test to compare the least square mean AUC values from the 5 active drug groups relative to the placebo group. If the data within a treatment arm is skewed, suggesting that the least square mean value is not the best measure of central tendancy, the data may be ranked and the analyses will be conducted using the ranked scores. Additional information regarding the examination of the distribution of the data will be presented in the Statistical Analysis Plan. To compare the time to the maximum increase in tcpO2, the event time distribution functions will be compared using a log rank test and presented using Kaplan-Meier curves.

5.7 Safety Analyses

Adverse events will be coded using the MedDRA dictionary. Frequencies and percentages of subjects with treatment-emergent adverse events (TEAEs), serious TEAEs, and TEAEs causing premature discontinuation will be summarized by treatment group. An adverse event is treatment emergent if it 1) occurs after the first dose of randomized study treatment or 2) if it is present prior to receipt of randomized study treatment but worsens in severity or increases in frequency after the dose of randomized study treatment. Frequencies will be presented for subjects with TEAEs by system organ class and preferred term; by system organ class, preferred term and maximal severity; by system organ class, preferred term for treatment-related AEs; by system organ class and preferred term for SAEs; and by system organ class, preferred term, day of onset and Period (1 or 2).

6 ADMINISTRATIVE AND SPECIAL PROCEDURES

6.1 Protocol Amendments

Any changes to the protocol will be made in the form of an amendment. Unless the changes are designed to eliminate an apparent immediate hazard to subjects, both the Sponsor and the governing IRB must grant approval of the amendment before any changes may be implemented in study conduct.

6.2 Investigator Responsibilities

The investigator has overall responsibility for the conduct of the study at his/her site. The investigator is responsible for ensuring that study staff has suitable qualifications, training, and authorization to perform any delegated study tasks. The investigator is responsible for the care of the subjects throughout this study. The investigator/authorized person will monitor the subjects for the occurrence of AEs throughout their participation in this study.

6.2.1 Compliance with Protocol

The investigator is responsible for the conduct of the study. No alterations or changes in this protocol will be permitted without written approval from the Sponsor and the IRB unless the amendment is necessary to reduce immediate risk to trial participants. The investigator should document and report to the Sponsor all deviations from the protocol.

6.2.2 Good Clinical Practice (GCP)

The investigator agrees to conduct and monitor the clinical trial in accordance with GCP standards.

6.2.3 Institutional Review Board Review and Approval

It is the responsibility of the investigator to communicate with the IRB. The IRB will be functioning in accordance with current regulations. A copy of the IRB's unconditional written approval of the protocol and the informed consent will be obtained and provided prior to the initial supply of test article and start of the trial. All protocol amendments will be submitted to the IRB and approval sought prior to implementation, unless the amendment is necessary to reduce immediate risk to trial participants. The IRB will be informed of any new safety information as it becomes available. The investigator will provide reports to the IRB as requested, but at least annually, and after the trial is complete.

6.2.4 Informed Consent

The investigator is responsible for ensuring that a current informed consent is obtained from each subject or the subject's LAR. Subjects will be informed in simple terms and all questions answered about the objectives, procedures, and risks of study participation both verbally and in writing in accordance with current regulations. A signed, dated, IRB-approved written informed consent form will be obtained prior to any study-specific procedure.

6.3 Protocol Deviations

A protocol deviation is any noncompliance with the clinical trial protocol, any process that is noted in the protocol or GCP requirements or any critical study procedures with specific instructions in ancillary documents referenced in the protocol.

The noncompliance may be either on the part of the subject, the investigator, or the study site staff. Following a deviation(s), corrective actions should be developed by the site and implemented promptly. All individual protocol deviations will be addressed in subject study records.

It is the responsibility of the site PI and personnel to use continuous vigilance to identify and report deviations within five working days of identification of the protocol deviation, or within five (5) working days of the scheduled protocol-required activity. All deviations must be promptly reported per the protocol deviation reporting procedures. Protocol deviations must be sent to the local IRB/IEC per their guidelines. The site PI and personnel are responsible for knowing and adhering to their IRB requirements. A completed copy of the Protocol Deviation Form must be maintained in the Regulatory File, as well as in the subject's chart if the deviation is subject specific.

6.4 Monitoring Activities

The Sponsor and/or its designee will monitor the clinical trial. At regular intervals, the monitor will visit the investigator's site to assess the progress and conduct of the study. CRFs, as well as source documents, must be available for review. The monitor must have access to all trial-related documents.

6.5 Quality Assurance Audits and Regulatory Inspections

The Sponsor, its designee, or a regulatory authority may audit the study. Study-related documents must be made available for auditing purposes.

The investigator is advised to contact the Sponsor immediately if a regulator contacts the site inquiring or requesting to inspect and/or audit the investigative study site and/or this clinical trial.

6.6 Disclosure and Confidentiality

By signing the protocol, the investigator agrees to keep all information provided by the Sponsor or its designee in strict confidence and to request similar confidentiality from his/her staff and the IRB. Study documents provided by the Sponsor (protocols, investigators' brochure, CRFs/eCRFs, and other material) will be stored appropriately to ensure their confidentiality. Data generated by this study will be considered confidential by the investigator except to the extent that is included in a publication as provided in the investigator's respective contractual agreements. A description of this clinical trial will be available on http://www.ClinicalTrials.gov.

6.7 Changes in Study Personnel

If there is a change of any personnel listed on the FDA form 1572, a new form reflecting the change must be completed and forwarded to the Sponsor or its designee including, when applicable, any new staff member's signed and dated curriculum vitae, current medical license (as appropriate), and signed financial disclosure statement.

6.8 Study Record Retention

Study related records, including the regulatory file, study drug accountability records, consent forms, subject source documents and electronic records should be maintained for a period of 2 years following the date a marketing application is approved for the investigational product for the indication for which it is being investigated; or, if no application is to be filed or if the application is not approved for such indication, until 2 years after the investigation is discontinued and the regulatory authority is notified. These documents should be retained for a longer period, however, if required by local policies or regulations. No records will be destroyed without the written consent of the sponsor.

6.9 Publication and Data Sharing Policy

Reporting and publication processes will follow applicable laws and guidelines and ensure that the design and results of the trial are reported in an accurate and complete manner. Author lists and contributorship statements will accurately reflect all substantial intellectual contributions to the research and be in accordance with the policies associated with the journals where published.

7 REFERENCES

Blake DF, Crowe M, Lindsay D, Brouff A, Mitchell SJ, Pollock N. Comparison of tissue oxygenation achieved breathing oxygen from a demand valve with four different mask configurations. Diving and Hyperbaric Medicine. 2018; 48 (4): 209-217.

Blake DF, Crowe M, Lindsay D, Brouff A, Mitchell SJ, Leggat PA, Pollock N. Comparison of tissue oxygenation achieved breathing oxygen using different delivery devices and flow rates. Diving and Hyperbaric Medicine. 2020; 50 (1): 34-42.

Diffusion Pharmaceuticals, Inc. Investigators Brochure for Trans Sodium Crocetinate, Edition 13, July 2017.

Diffusion Pharmaceuticals LLC. A randomized, double-blinded, placebo-controlled phase 1/2 dose-range-finding study to evaluate the safety, efficacy and pharmacokinetics of multiple once daily intravenous doses of trans sodium crocetinate (TSC) in patients with intermittent claudication (IC). Protocol 100-301. Clinical Study Unpublished Report. September, 2010.

Diffusion Pharmaceuticals LLC. Open-label Phase 1/2 (Safety Lead-in) Study of Trans Sodium Crocetinate (TSC) with Concomitant Treatment of Fractionated Radiation Therapy and Temozolomide in Newly Diagnosed Glioblastoma (GBM) Patients to Evaluate Safety and Efficacy. Protocol 100-202. Clinical Study Unpublished Report. November, 2015.

Diffusion Pharmaceuticals LLC. A double-blind, placebo-controlled phase 1 study to evaluate the tolerability and pharmacokinetics of a single intravenous injection of trans sodium crocetinate in healthy volunteers. Protocol 100-001. Clinical Study Unpublished Report. March, 2008.

Diffusion Pharmaceuticals LLC. The effects of trans sodium crocetinate on infarct volume in a permanent model of ischemic brain injury in the rat. Unpublished Report 1000-P018. January, 2007.

Ercengiz A, Mutluoglu M. Hyperbaric transcutaneous oximetry. StatPearls Publishing Jan 2020.

Fife CE, Smart DR, Sheffield PJ, Hopf HW, Hawkins G, Clarke D. Transcutaneous Oximetry in Clinical Practice: Consensus statements from an expert panel based on evidence. Undersea and Hyperbaric Medicine. 2009; 36 (1): 43-53.

Gainer JL. Trans-sodium crocetinate for treating hypoxia/ischemia. Expert Opin Investig Drugs. 2008; 17(6):917-24.

Greenwood TW, Gilchrist AG. Hyperbaric oxygen and wound healing in post-irration head and neck surgery. Brit J Surg. 1973; 60 (5): 394-397.

Hiatt W, Barz K, Gainer J, Mohler E. Evaluation of Trans Sodium Crocetinate on Safety and Exercise Performance in Patients with Peripheral Artery Disease and Intermittent Claudication. Circulation. 2010; 122:A12341.

Huxley VH, Kutchai H. The effect of the red cell membrane and a diffusion boundary layer on the rate of oxygen uptake by human erythrocytes. J Physiol. 1981; 316:75-83.

Laidig KE, Gainer JL, Daggett V. Altering diffusivity in biological solutions through modification of solution structure and dynamics. Journal of the American Chemical Society. 1998; 120(36):9394-95

Manabe H, Okonkwo D, Gainer JL, Clarke R, Lee KS. Protection against focal ischemic injury to the brain by trans-sodium crocetinate. J Neurosurg. 2010; 113: 802-809.

Mohler ER, Gainer JL, Whitten KM, Eraso LH, Tanaporn PK, Bauer T. Evaluation of trans sodium crocetinate on safety and exercise performance in patients with peripheral artery disease and intermittent claudication. Vascular Medicine. 2011; 16(5):346-353.

Stennett AK, Dempsey GL, Gainer JL. Trans sodium crocetinate and diffusion enhancement. Journal of Physical Chemistry. 2006; 110(37):18078-80.

Trinks TP, Blake DF, Young DA, Thistlewaite K, Vangaveti VN. Transcutaneous oximetry measurements of the leg: comparing different measuring equipment and establishing values in healthy young adults. Diving and Hyperbaric Medicine. 2017; 47 (2): 82-87.

Weaver LK. Transcutaneous Oxygen and Carbon Dioxide Tensions Compared to Arterial Blood Gases in Normals. Respiratory Care Vol. 52 No 11, Nov 2007.

Young DA, Blake DF, Brown LH. Transcutaneous oximetry measurement: normal values for the upper limb. Diving and Hyperbaric Medicine. 2012; 42 (4): 208-213.

8 APPENDICES

8.1 Laboratory Parameters

CBC	White blood cells (WBC), red blood cells (RBC), Hemoglobin, Hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), Platelet Count, mean platelet volume (MPV) and Differential (Absolute and Percent - Neutrophils, Lymphocytes, Monocytes, Eosinophils, and Basophils)
BMP	BUN/Creatinine Ratio (calculated), Calcium, Carbon Dioxide, Chloride, Creatinine with GFR Estimated, Glucose, Potassium, Sodium, Urea Nitrogen (BUN)
Serology	HIV-1/HIV-2 Antibodies (HIV), Hepatitis B surface Antigen (HBsAg), Hepatitis C Antibody (HCVAb)
Pregnancy	Serum pregnancy test at screening, urine pregnancy test at clinic arrival
Urine Drug Screen	Amphetamines, barbiturates, benzodiazapenes, cannabinoids, cocaine, opiates, phencyclidine
Alcohol Screen	Breathalyzer

8.2 Schedule of Events

	Screening	**Treatment (Day 0)**					**Follow Up**
	Day -21 to Day 0	**Clinic Arrival**	**Equilibration/ Baseline: 70 minutes on O_2**	**Treatment/ Evaluation: 60 minutes on O_2**	**Post-Treatment: 30 minutes on Room Air**	**Clinic Observa-tion**	**48 Hours (+ 2 Days)**
Informed Consent	X						
Inclusion/Exclusion criteria	X	X					
Demographics	X						
Medical history	X	X					
Concomitant medications	X	X	X	X	X	X	X
BMI (kg/m^2)	X						
Body weight (kg)		X					
Physical Exam	X	X					
Randomization		X					
Vital signs (HR, BP, RR, Temp)	X	X	X	X	X	X	
SpO_2 [b]	X		X	X			
12-lead ECG	X						
Laboratory (CBC, BMP, HIV, Hep B, Hep C)	X						
Pregnancy test[a]	X	X					
Urine drug screen	X	X					
Alcohol breathalyzer screen	X	X					
Subject resting quietly supine with one pillow, or semi-recumbent			X	X			
$TcpO_2$ sensors in place		X	X	X			
PK measurements			X	X	X		
Oxygen:simple face mask at 6 L/min			X	X			
$TcpO_2$[b]			X	X			
Administer Study Drug				X			
$TcpO_2$ sensors removed					X		
Overnight stay at clinic						X	
Adverse Events			X	X	X	X	X

[a] For all females, with serum pregnancy test at screening and urine pregnancy test at clinic arrival

[b] SpO2 and TcpO2 will be assessed every 5 minutes during the baseline period. After study drug administration they will be assessed at 1, 2, and 5 minutes, and then every 5 minutes thereafter.

8.3 Investigator Signature Page

DOCUMENT TYPE:	Clinical protocol
DOCUMENT NUMBER:	200-301
COMPOUND:	Trans Sodium Crocetinate
STUDY TITLE:	Randomized, double-blind, placebo-controlled, pharmacokinetic, pharmacodynamic study of Trans Sodium Crocetinate utilizing Transcutaneous Oximetry Measurement in healthy volunteers
CLINICAL PHASE:	1
INDICATION:	Healthy volunteers
IND NUMBER:	68579
SPONSOR:	Diffusion Pharmaceuticals Inc. 1317 Carlton Avenue, Suite 200 Charlottesville, VA 22902
DOCUMENT VERSION/STATUS:	Final
DOCUMENT RELEASE/AMENDMENT DATE:	Amendment 2: March 9, 2021 Amendment 1: February 12, 2021 January 14, 2021

This acknowledges receipt of the above protocol.

Investigator Name: _____ (Print)

Investigator Signature: _____ Signature Date: _____