AWARD NUMBER: W81XWH-18-9-0009

EGS NUMBER: MT180009

TITLE: Novel cell-based Therapy to Treat Muscle Atrophy Associated with Peripheral Nerve Injury

PRINCIPAL INVESTIGATOR: Dr. Mitchell Zakin

PERFORMING ORGANIZATION: Clear Scientific (previously Nano Terra)

REPORT DATE: 1/10/2022 for Fiscal Year 3 (Oct 2020 – Sep 2021) Activities

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

MTEC
Medical Technology Enterprise Consortium

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Annual Report 3

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Dr. Mitchell Zakin; Dr. Piercen Oliver; Dr. Madeline Vara; Mr. Philip Graf

E-Mail: mzakin@clearsci.com; poliver@clearsci.com; mvara@clearsci.com; pgraf@clearsci.com

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Clear Scientific, LLC

737 Concord Ave
Cambridge, MA 02138

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2018-680

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U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

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14. ABSTRACT

During this performance period, we found no difference between control and cell-therapy arms in mitigation of muscle atrophy in the Lewis rat surgical model (gastrocnemius muscle whose innervation, the sciatic nerve, was severed). After extensive literature review, we identified acetylcholine modulation as a therapeutic target for mitigation of muscle atrophy, with early promising studies using atropine as the therapeutic dating to the 1940s. We confirmed and further demonstrated that local modulation of acetylcholine (ACh) is a promising therapeutic target for mitigation of muscle atrophy caused by damage to the innervating nerves. Nerve damage degrades and/or interrupts signaling between the CNS and muscle, de-modulating ACh release at muscle ACh receptors and in the neuromuscular junction (NMJ), which causes the classic spastic contractile muscle activity that results in muscle atrophy. We showed, in the Lewis rat surgical model, that intramuscular injection of atropine (which reduces ACh receptor activity), **essentially eliminated atrophy** in a 7-day study, confirming our hypothesis, **regardless of the co-injection of cell therapy.** Thus we decided to discontinue our cell therapy approach and focus solely on ACh modulation. Since the toxicity profile of atropine is too severe for practical use in this application, we selected an alternative mechanism of reducing local ACh activity, namely, direct binding and removal of ACh via small-molecule ACh chelators. A previous *ex-vivo* study by one of our collaborators in chick muscle, which demonstrated that ACh chelation modulates muscle contraction, supports this approach. We have constructed an 3-stage experimental plan to test the efficacy of our library of small-molecule ACh chelators towards mitigation of muscle atrophy: (1) *in-vitro* measurement of the strength of chelator binding to ACh (K_a, the association constant), via isothermal microcalorimetry to select the strongest chelators; (2) *ex-vivo* measurement of the effectiveness of local ACh chelation to reverse ACh-induced spasms in invertebrate muscle tissue to select the most effective candidates; and 3) *in-vivo* studies of the most effective candidate chelators towards mitigation of muscle atrophy in an animal model, most likely an invertebrate model due to time constraints.

15. SUBJECT TERMS

Peripheral nerve injury; muscle atrophy; muscle wasting; cell therapy; muscle atrophy treatment

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					Clear Scientific, LLC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Unclassified	17	**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified			(617) 621-8500

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Annual Technical Status Report for

Novel cell-based Therapy to Treat Muscle Atrophy Associated with Peripheral Nerve Injury

Research Project No. 2018-680-001

EGS# MT180009

Reporting Period: 01 Oct '20 – 30 Sep '21

MTEC Research Project Awardee

Clear Scientific

Massachusetts General Hospital

Research Project Technical POC

Dr. Mitchell Zakin

737 Concord Ave

Cambridge, MA 02138-1002

617-621-8500

mzakin@clearsci.com

Submitted: 10 Jan '22

1. Project Status

a. Accomplishments

During this performance period, we found no difference between control and cell-therapy arms in mitigation of muscle atrophy in the Lewis rat surgical model (gastrocnemius muscle whose innervation, the sciatic nerve, was severed. After extensive literature review, we identified acetylcholine modulation as a therapeutic target for mitigation of muscle atrophy, with early promising studies using atropine as the therapeutic dating to the 1940s. We confirmed and further demonstrated that local modulation of acetylcholine (ACh) is a promising therapeutic target for mitigation of muscle atrophy caused by damage to the innervating nerves. Nerve damage degrades and/or interrupts signaling between the CNS and muscle, de-modulating ACh release at muscle ACh receptors and in the neuromuscular junction (NMJ), which causes the classic spastic contractile muscle activity that results in muscle atrophy. We showed, in the Lewis rat surgical model, that intramuscular injection of atropine (which reduces ACh receptor activity), **essentially eliminated atrophy** in a 7-day study, confirming our hypothesis, **regardless of the co-injection of cell therapy**. Thus we decided to discontinue our cell therapy approach and focus solely on ACh modulation. Since the toxicity profile of atropine is too severe for practical use in this application, we selected an alternative mechanism of reducing local ACh activity, namely, direct binding and removal of ACh via small-molecule ACh chelators. A previous *ex-vivo* study by one of our collaborators in chick muscle, which demonstrated that ACh chelation modulates muscle contraction, supports this approach. We have constructed an 3-stage experimental plan to test the efficacy of our library of small-molecule ACh chelators towards mitigation of muscle atrophy: (1) *in-vitro* measurement of the strength of chelator binding to ACh (K_a, the association constant), via isothermal microcalorimetry to select the strongest chelators; (2) *ex-vivo* measurement of the effectiveness of local ACh chelation to reverse ACh-induced spasms in invertebrate muscle tissue to select the most effective candidates; and 3) *in-vivo* studies of the most effective candidate chelators towards mitigation of muscle atrophy in an animal model, most likely an invertebrate model due to time constraints.

b. Reportable Outcomes

See 1a above.

c. Progress Detail

REFERENCED TO NEW OR REVISED SOW?
Cell work.
Updated IACUC and ACURO for atropine studies.
Atropine work.
Latest Monthly Report(s).
New work.

Milestone 19: Deliverable 3 (Task 1.3), Year 2020 Initial rat dosing studies using Task 1.3 formulation(s)

i. CSInc and MGH completed the initial rat dosing studies (conducted at MGH). The study encompassed 48 rats with 4 different study arms, as follows:
    1. 12 rats – sciatic nerve cut, no repair; daily tacrolimus treatment
    2. 12 rats – sciatic nerve cut, repaired; daily tacrolimus treatment
    3. 12 rats – sciatic nerve cut, no repair; CSInc cell therapeutic and daily tacrolimus treatment
    4. 12 rats – sciatic nerve cut, repaired; CSInc cell therapeutic and daily tacrolimus treatment

Table 1.

Rat Study Group	Atrophy in Gastroc	Standard Deviation
Sciatic Cut, No Repair	-69.3%	±1.6%
Sciatic Cut, Repair	-63.3%	±7.8%
Sciatic Cut, No Repair + Cells	-74.1%	±3.3%
Sciatic Cut, Repair + Cells	-62.5%	±5.7%

ii. Initial results indicated that no statistically significant difference when the CSInc cell therapeutic is applied (over the 28-day study). This may be due to the long length of the study compared to previous literature, immunorejection of the human motoneuron cells (despite the tacrolimus treatment), premature death of the muscle before the cells can take, or ineffective treatment. Notably, the repair of the sciatic nerve appears to have the most statistically significant impact on the amount of atrophy observed in the muscle.

iii. CSInc and MGH discussed amendments to the study design to better isolate critical variables impacting the atrophication process and recovery. After examining literature and understanding of muscle atrophy, it was decided that muscle damage can occur after peripheral nerve injury due to dysregulation of acetylcholine, resulting in excessive muscle twitching and accelerating the atrophy process. To this end, given precedence in the literature, a new arm of the study was developed, aimed at monitoring the effect of adding atropine sulfate (FDA-approved anti-muscarinic) as part of the daily treatment for the rats. Only rats with sciatic nerve cut and no repair will be used for this study. The proposed amended rat study is as follows:
    1. Rat group 1 (6 rats): sciatic nerve cut no repair, injection of 10 mg atropine sulfate per 100 g into the gastrocnemius muscle, followed by daily treatment of Tacrolimus (as already set) as well as 15 mg atropine per 100 g.
    2. Rat group 2 (6 rats): sciatic nerve cut no repair, injection of 10 mg atropine sulfate per 100 g PLUS cell therapeutic formulation into the gastrocnemius muscle, followed by daily treatment of Tacrolimus (as already set) as well as 15 mg atropine per 100 g.

iv. The IACUC and ACURO approval were both received for the above amended study.

v. The amended two-armed study in rat was performed at MGH to investigate the effect of addition of atropine sulfate to the therapeutic regimen to better modulate the localized muscle environment (control arm: daily atropine and tacrolimus injection; treatment arm: single CSInc cell therapeutic injection followed by daily atropine and

tacrolimus injection). **The control and treatment groups showed no statistically significant difference in loss of muscle mass over the 7-day study: 0-8% (average: 5%, n=11) reduction relative to the healthy leg.** This is in comparison to the 30% loss after 7 days reported in the 2007 Craff study, which utilized neuron injection only. These results confirm the long-standing hypothesis that reducing muscle twitching lessens denervation-driven muscle atrophy. Some adverse effects (e.g., weight loss, ocular porphyrin expression) were observed, but were not unexpected given the known systemic toxicity profile of atropine; accordingly, the study was ended early at 7 days. The lack of differential between the control and therapeutic arms brings into question the value of injecting neurons as part of a therapeutic regimen. Thus, **next studies will build upon our mechanism-validating results using atropine and focus on small-molecule agents to calm muscle activity**, i.e., by delivery of less toxic FDA-approved drugs (e.g., relaxants, anti-seizure medications) and/or sustained local delivery of atropine to the muscle. This mechanism-driven approach has the advantage of simpler implementation and faster approval pathway through the FDA, thus accelerating the availability of the therapy to warfighters.

vi. Based on our atropine studies and an extensive review of the literature and discussion with our clinical collaborators, **we have hypothesized that neuromuscular junction support via restoration of modulation of the neurotransmitter acetylcholine, in particular downregulation of the effect of ACh until the damaged nerve(s) are restored, will mitigate muscle atrophy. This will be the focus of our effort moving forward**. There are three basic strategies for ACh downregulation: (1) use of ACh receptor antagonists/blockers (e.g., neuromuscular blocking agents, Botox, -caine-based anesthetics, etc.); (2) reduction of ACh release; and (3) rapid removal and clearance of ACh upon release using chelators. Given the toxic and/or clinically-impractical nature of ACh receptor antagonists, and the packaging of ACh in intracellular vesicles (thus, restricting access to the ACh), we have chosen to follow strategy (3).

    1. The study by Cisterna et. al., "Active acetylcholine receptors prevent the atrophy of skeletal muscles and favor reinnervation," NATURE COMMUNICATIONS | (2020) 11:1073 | https://doi.org/10.1038/s41467-019-14063-8, provides strong confirmation of our hypothesis that ACh modulation, in particular downregulation of the effect of ACh, mitigates muscle atrophy after denervation.

vii. Thus, we will focus on exploration of our library of small molecule drugs that bind and remove ACh as injectable and oral therapeutics for muscle atrophy, in both *ex-vivo* and *in-vivo* models. The *ex-vivo* study will generally follow the protocols outlined in Cisterna et. al. using muscle contractility. The *in-vivo* proof-of-concept studies will generally follow the current ACURO-approved protocol used in our rodent studies with atropine as the therapeutic.

viii. We selected and procured a commercial system for *measuring ex-vivo* muscle contractility and associated electrical activity (iWorx AK-TA Animal Physiology Teaching Kit, Figure 1), and an experimental protocol for use with this system that will be utilized with our ACh chelators. We specified binding assay protocols for measurement of the

association constant K_a between ACh and our small molecule chelators. A meeting was held with Dr. Inna Williams, MTEC representatives, and MRMC sponsor representatives to discuss our proposed path forward and the granting of a 6-month no-cost extension to complete the effort. All parties were in agreement with the plan details, and we submitted a modified SOW to MTEC for Contracting Officer approval. We conducted discussions with our Mass General Hospital collaborators regarding ACURO modifications required for our planned in-vivo studies.

Milestone 23: Tasks 1-4 (Deliverables 1-5): Execution of all prior tasks & development of revised approach

i. We requested and were granted the following scope change for the project: 1) deletion of NHP studies; 2) deletion of IND submission to the FDA; 3) addition of a detailed ex-vivo study (see below) in muscle tissue to screen small-molecule acetylcholine (ACh) modulators as a therapeutic for mitigation of muscle atrophy post-neural injury. The ex-vivo study will be used to generate the data required for initiation of nonclinical studies in rodent; and 4) in-vivo studies of a selected small molecule agent, and motoneuron/small molecule combination, in rat, in the laboratory of our partner, Dr. Curtis Cetrulo at Mass General Hospital. A minor amendment to the current ACURO will be required for this study.

ii. Details of the associated rationale, as discussed with Dr. Williams, MTEC representatives, and MRMC sponsor representatives, are as follows:

    1. In the process of investigating the use of injected motoneurons as a therapeutic for mitigating muscle atrophy post-neural injury, we co-administered an FDA-approved small molecule anticholinergic agent (muscarinic receptor antagonist) to block the action of acetylcholine and minimize muscle spasticity, in ACURO-approved studies. This was to best achieve survivability and performance of the local neural network formed by the motoneurons, for acute injury treatment. We found that, for the acute phase of injury, cell therapy alone appears not to be sufficient. However, with inclusion of the anticholinergic agent **atropine**, the results are very promising, **with only ~5% atrophy over 7 days vs ~50% for control.**

    2. These results teach us that anticholinergic agents are a viable treatment option for the short-term acute phase, but the regenerative ability of cell-based treatments is more ideally suited for the long-term phase (i.e., until the damaged innervating nerve is sufficiently healed). This suggests that a combination treatment using small molecules in conjunction with cells could be ideal and merits further study. However, atropine, while effective, has a toxicity profile unsuitable for human use in this application. The challenge is to identify an anticholinergic that has the potency of atropine without the toxicity.

    3. Our studies are designed to identify safe and effective anticholinergic agents. Follow-on studies will explore combination therapy for long-term muscle preservation.

4. Rationale for the *ex-vivo* study is as follows: There is strong evidence, both in the *in-vivo* studies with atropine described above, and in the published literature (Nature Communications 2020 11:1073) tying the modulation of ACh (in particular the reduction of ACh activity at receptors in the neuromuscular junction and those sprouted at the muscle surface post-neural injury) to mitigation of atrophy following neural injury, both *ex-vivo* and *in-vivo*. In these studies reduction of ACh activity was achieved using small molecule antagonists; however, none of the drugs used (atropine, neuromuscular blocking agents, etc.) are safe enough for use in humans in our intended purpose. A second, more tenable, means to reduce local ACh activity is the administration of ACh chelators that bind, inactivate, and eliminate ACh from the local site. We will investigate the activity of several of our small-molecule chelators that exhibit significant ACh binding affinity ($K_a = 10^5 M^{-1}$ to $10^7 M^{-1}$), which have been shown to be well tolerated in animal models. Preliminary studies indicate the potential viability of this approach (*Toxicol Res (Camb)* 2014; 3(6): 447–455). Contractions in fresh excised muscle (sourced in a manner not subject to IACUC and ACURO approval) will be stimulated via administration of ACh, and quantified via contractility (force, electromyography) measurements. Candidate chelators will subsequently be administered, and their ability to dampen muscle contractions, via binding to ACh, will be measured. These studies will provide key data, including chelator type, dose, dose rate, timing, and site of administration, required for initiation of nonclinical studies in rodent. Based on our findings and the extant literature, it is strongly anticipated that chelators with potent anti-contractile activity will exhibit strong anti-atrophy activity *in-vivo*.

Figure 1. iWorx system.

iii. The experimental protocol for use of iWorx in *ex-vivo* muscle/ACh chelator studies is presented here:

iv. http://web.as.uky.edu/biology/faculty/cooper/Bio450-AS300/Earth%20worm%20experiment/earthworm%20saline%202.pdf

2. Future Plans
   a. Over the next quarter, we plan to confirm ACh binding constants to selected small-molecule chelators from our library, using Isothermal Microcalorimetry. The execution of *ex vivo* studies will be initiated.

3. Problems / Issues
   a. Current Problems / Issues

      None

   b. Anticipated Problems / Issues

      None

4. Financial Health

Comment on the financial health of the study. Was the study financially on track during this annual reporting period and cumulatively for completion as proposed within the period of performance? If not, describe the cause(s), whether this will have a short-term or long- term impact, the likelihood this can be overcome, and provide remediation strategy. Provide amount expended this year and cumulatively. State if there was any major equipment procured, sub-award implemented, and/or travel conducted.

   a. This is a Cost Reimbursable, Cost Sharing Milestone effort. The spending for the year of October 2020 - September 2021 was $627,041.23. The cumulative program total is $1,785,960.28 of the $2,327,616 total government funded award. There has been $0 in cost share out of a total budgeted amount of $23,774. We have $541,655.72 (23.3%) of the government funded base period remaining in the award.

5. Personnel Effort

Provide names of current staff along with their roles and percent effort of each on this project. Add additional rows if necessary to list the complete team. If there is more than one project on this award, breakdown according to each project (one table per project).

Personnel	Role	Percent Effort
Philip Graf	Product Manager	4%
Piercen Oliver	Director R&D	11%
Xinhua Li	EVP Chemistry	13%
Shekar Shetty	CEO	16%
Mitchell Zakin	CSO	26%
Madeline Vara	Senior Scientist	9%
Amanda Code	Scientist	6%
Patrick Reust	Senior Scientist	1%

6. Protocol and Activity Status

For awards involving the use of human subjects, use of human cadavers, and/or use of animal subjects, prepare a summary in accordance with the following subsections. For all other awards, including those involving the use of human anatomical substances (such as tissue or cells or identifiable private information), mark as directed below.

a. Human Use Regulatory Protocols

TOTAL PROTOCOLS:

"No human subjects research will be performed to complete the Statement of Work."

b. Use of Human Cadavers for RDT&E, Education or Training

TOTAL ACTIVITIES:

"No RDT&E, education or training activities involving human cadavers will be performed to complete the Statement of Work (SOW)."

c. Animal Use Regulatory Protocols

TOTAL PROTOCOLS: The total number of animal use protocols required to complete the Statement of Work is still being determined. It is anticipated there will be 3 animal use research protocols required.

PROTOCOLS:

Rat in-vivo determined and approved

Non-human primate in-vitro to-be-determined

Protocol [ACURO Assigned Number]: Log MT180009.e001

Title: Rat in-vivo (to-be-determined)

Target required for statistical significance: In-progress

Target approved for statistical significance: In-progress

Submitted to and Approved by:

- Both IACUC and ACURO for rat studies have been approved.
- IACUC Assigned Number: 2020N000005

Annual Business Status Report for

Novel cell-based Therapy to Treat Muscle Atrophy Associated with Peripheral Nerve Injury
Research Project No. 2018-680-001
EGS# MT180009
Reporting Period: 01 Oct '20 – 30 Sep '21

MTEC Research Project Awardee
Clear Scientific
Massachusetts General Hospital

Research Project Technical POC
Dr. Mitchell Zakin
737 Concord Ave
Cambridge, MA 02138-1002
617-621-8500
mzakin@clearsci.com

Submitted: 10 Jan '22

1. CURRENT STAFF

Personnel	*% of Effort on project*
Product Manager	4%
Director R&D	11%
EVP Chemistry	13%
CEO	16%
CSO	26%
Senior Scientist	9%
Scientist	6%
Senior Scientist	1%

2. CURRENT EXPENDITURES

A. Cost Reimbursable Contracts: Complete only if your contract is Cost Reimbursable or Cost Plus Fixed Fee.

Expenditures should be reflective of cost incurred to date, not exceeding awarded project ceiling. Expenditures should coincide with the latest invoice for the reporting period. For cost reimbursable contracts please use the table below.

Contract Expenditures	Current Year Expenditures	*Cumulative To Date Expenditures*
Labor (Personnel and Fringe)	$228,700.13	$641,083.38
Supplies/Materials	$25,807.02	$80,462.78
Travel	$0	$26.10
Equipment	$0	$0
Subcontractors and Consultants	$68,362.90	$217,194.11
Other Direct Costs	$0	$0
Indirect Costs	$304,171.18	$847,193.91
Total	**$627,041.23**	**$1,785,960.28**

B. Cost Share Contributions: Complete only if you're reporting Cost Share:

Funding Source (Cash)	This Period	Cumulative to Date
Cash	$0.00	$0.00
Labor Dollars	$0.00	$0.00
Indirect Labor Rates (Overhead/Fringe Benefits)	$0.00	$0.00
Travel	$0.00	$0.00

General & Administrative Services	$0.00	$0.00
Equipment (New)	$0.00	$0.00
Material	$0.00	$0.00
Other Direct Costs	$0.00	$0.00
Other *	$0.00	$0.00
Sub-Total	$0.00	$0.00
Funding Source (In-Kind)	**This Period**	**Cumulative to Date**
Use of Existing Equipment (Estimated fair market value)	$0.00	$0.00
Use of Existing Software (Estimated fair market value)	$0.00	$0.00
Intellectual Property (Estimated fair market Value)	$0.00	$0.00
Space (Land or buildings)	$0.00	$0.00
Sub-Total	$0.00	$0.00
Cost Share Total	$0.00	$0.00

3. STATUS OF MILESTONES – FILL OUT FOR ALL CONTRACT TYPES (all project milestones are to be included)

All project milestones from the Milestone Payment Schedule, in the project award, should be accounted for below.

Milestones reported below are from the contract modification M03 signed on November 8, 2021.

MTEC Milestone Number	Milestone Description	Due Date	% Completed this Reporting Period	Cumulative % Complete
1	**Project Kick Off**	**9/30/2018**	0%	100%
2	Quarterly Reports 1 (October-December, Technical and Business Reports)	1/25/2019	0%	100%
3	Quarterly Reports 2 (January - March, Technical and Business Reports)	4/25/2019	0%	100%
4	Quarterly Report 3 (April - June, Technical and Business Reports)	7/25/2019	0%	100%
5	Annual Report 1	10/25/2019	0%	100%
6	Quarterly Report 4 (October-December, Technical and Business Reports)	1/25/2020	0%	100%
7	Determination of commercially available human motoneuron source	2/29/2020	0%	100%
8	Development of initial formulation(s) using human motoneurons in PBS	2/29/2020	0%	100%

9	HRPO approval	4/25/2020	0%	100%
10	Quarterly Report 5 (January - March, Technical and Business Reports)	4/25/2020	0%	100%
11	Rat IACUC approved for in vivo studies	6/30/2020	0%	100%
12	Rat ACURO submitted for in vivo studies	6/30/2020	0%	100%
13	Quarterly Report 6 (April-June, Technical and Business Reports)	7/25/2020	0%	100%
14	Rat ACURO approved for in vivo studies	9/30/2020	0%	100%
15	Annual Report 2	10/25/2020	0%	100%
16	Initial rat dosing studies using Task 1.3 formulation(s)	11/30/2020	14%	100%
17	Quarterly Report 7 (October-December, Technical and Business Reports)	1/25/2021	100%	100%
18	Development of adapted human motoneuron formulation(s) (on-going)	3/31/2021	2%	100%
19	Determination of final human motoneuron formulation(s)	12/31/2021	35%	50%
20	Quarterly Report 8 (January-March, Technical and Business Reports)	4/25/2021	100%	100%
21	Additional rat dosing studies	3/31/2022	40%	40%
22	Quarterly Report 9 (April-June, Technical and Business Reports)	7/25/2021	100%	100%
23	Execution of all prior tasks & development of revised approach	9/29/21	100%	100%
24	Annual Report # 3	10/25/2021	100%	100%
25	Screening – *in-vitro*	12/31/2021	0%	0%
26	Screening –*ex-vivo*	12/31/2021	0%	0%
27	Quarterly Report 10 (Oct-Dec, Technical and Business Reports)	1/25/2022	0%	0%
28	Rat dosing study	3/31/2022	0%	0%
29	Final Reports (Business and Technical)	3/31/2022	0%	0%

4. DEVIATION FROM PROJECT PLAN

Any major deviations from the agreed to project plan shall be explained with a discussion of proposed actions to address the deviations.