**1R41AG078096-01 STAMM, STEFAN**

**RESUME AND SUMMARY OF DISCUSSION:** This Phase I STTR application by CirCure Corporation proposes to develop an intranasally delivered siRNA therapy targeting tau circRNAs for treating Alzheimer's Disease (AD). An stimulating discussion took place for this innovative application and reviewers highlighted many score strengths driving their great enthusiasm. The exciting scientific foundation relies on recent findings from the group showing that backsplicing of a 5' splice site to a upstream 3' splice site generates two human-specific circular RNAs (circRNAs) from microtubule associated protein tau (MAPT) gene (namely 12 ->7 and 12->10 tau circRNAs). Importantly, both tau circRNAs are translated into multimers of tau microtubule-binding domain capable to form neurofibrillary tangles (NFTs), a hallmark AD pathology. Described by reviewers as brilliant, the highly innovative targeting strategy consists of generating siRNAs against the backsplicing junction site for 12 ->7 and 12->10 tau circRNAs. Potentially revolutionary, this approach targets a tau mechanism that is unique in primates, imbuing the committee with hopes regarding its translatability to a human-specific disease such as AD. The commercialization potential is excellent - reviewers mentioned notable strengths such as the strong IP position with patent filled for the siRNAs against tau circRNAs and the general interventions to stop translation of tau circRNAs. The stellar investigative team combine solid expertise in AD, splicing, pre-miRNA processing, and pioneered the initial discoverers of the MAPT circRNAs. Nevertheless, a lack of expertise in therapeutic nucleic acids and drug formulation/delivery was noted. Including a viable alternative approach, the experimental design describes optimization of the siRNAs targeting 12 ->7 and 12->10 tau circRNAs followed by evaluation of nasal brain delivery and efficacy in appropriate rodent model. While recognizing the innovation on determining the efficacy of the siRNAs in human cells grafted in mouse brain, concerns were expressed that the tumor itself could rupture the blood-brain-barrier and defeat the evaluation of the nasal delivery. Overall, the committee expressed tremendous enthusiasm for this highly innovative study with potential to revolutionize the targeting strategies in AD by exploring a "new physical form (circRNA) of an old established target (Tau)".

**DESCRIPTION (provided by applicant):** The formation of neurofibrillary tangles (NFT) formed by aggregation of the microtubule-associated protein tau (MAPT) is a hallmark of Alzheimer's disease (AD) and the likely cause of neuronal death. Currently, there are no rational treatments available that directly target NFT formation and thus address the direct molecular cause for AD. Based on our academic research, we will develop an siRNA-based product that can be delivered nasally and prevents NFT formation in early stages of AD. In prior studies, we identified human-specific circular RNAs from the MAPT gene that are generated through backsplicing of exon 12 to either exon 7 or exon 10. These tau circRNAs are translated after epigenetic modifications that change adenosine residues to inosines (A>I editing). The translated proteins correspond to multimers of the microtubule-binding domain and promote NFT formation. Our company, CircCure, will develop siRNAs that selectively target tau circRNAs and thus prevent NFT formation; we will test this product in two specific aims: Aim #1: Define the optimal siRNA backbone sequence for the 12->7 and 12->10 tau circRNA by performing an oligo-walk to identify the backbone sequence with the highest efficacy toward tau circRNAs and lowest efficacy against linear RNAs. After undergoing standard chemical optimization, in Aim #2, we will test the siRNA efficacy (IC50) using intranasal and injection delivery in an in vivo model of human neuroblastoma cells grafted in mouse brains. This product is highly innovative, as it will directly address NFT formation targeting recently discovered circular RNAs. The final product of these Phase I studies will be an siRNA that can be delivered intranasally that reduces tau circRNAs and NFT formation in human cells embedded in a mouse brain. The product will thus directly target the cause of neuronal death in AD, which will provide supportive evidence for Phase II studies, where we will test pharmacology and safety to prepare for an investigational new drug (IND) application.

**PUBLIC HEALTH RELEVANCE:** Currently, no rational treatment for Alzheimer's disease is available. We will develop small RNAs that specifically target 'pathological' tau circular RNAs that promote neuronal death by causing protein aggregation and test them in human cell models and in human cells grafted in mouse brains.

**CRITIQUE 1**

Significance: 3
Investigator(s): 2
Innovation: 2
Approach: 4
Environment: 2

**Overall Impact:** This phase one SBIR proposes the investigation into a new molecular target for Alzheimer Disease (AD) The target was tau circRNA was discovered by the applicant team and targets neurofibrillary tangles. The development of new therapeutic strategies of AD is of high importance. neurofibrillary tangles (NFT) formed by aggregation of the microtubule-associated protein tau (MAPT) is along with plaques are hallmarks of AD. The applicant proposes to target circRNAS via standard siRNA approaches with typical sequence optimization methods. The siRNA will be delivered after completion with linear PEI via nasal administration. The application has several factors increasing impact including, the identification of a new target, a strong research, team, the ability of easy design and optimization of target sequence, excellent preliminary data. Detractors from the application arises from limited data of the nasal delivery system and some aspects of the approach.

**1. Significance:**

**Strengths**

- Need for new AD therapies and novel targets
- Defined molecular MOA of siRNA approach
- The association of tangles with AD as a target
- Novel model system of evaluation

**Weaknesses**

- Delivery system of nasal delivery could be challenging
- The utility of lPEI is the nucleic acid delivery
- Potential scaling between nasal delivery in rodents and humans

**2. Investigator(s):**

**Strengths**

- The team is strong in terms of understanding AD and the identified target

**Weaknesses**

- The addition of a nucleic acid formulation expert
- The addition of a nasal delivery expert

**3. Innovation:**

**Strengths**

- Identification of a novel target and potential new area of therapeutics
- The use of the grafted model for evaluation

**Weaknesses**

- Nasal delivery could be innovative but not fully developed
- The use of lPEI is as the delivery formulation has been described by others

**4. Approach:**

**Strengths**

- Strong preliminary data demonstrating the role of circRNA
- Good scientific approach for define the optimal siRNA backbone sequence
- Determine the efficacy (IC50) of the siRNAs in human cells grafted in mouse brain
- Interesting model grafted model in vivo system

**Weaknesses**

- Little information provided for nasal delivery system or how it will be optimized, lPEI particle size after formulation, stability of the polyplex with biological fluids
- Impact of the grafted model system would have an impact on nasal delivery
- The n for the animal studies

- The selection of the controls siRNA sequences not fully justified, perhaps a mismatch, in the seed region would be a better control, not clear why a liver targeted siRNA is being proposed.

**5. Environment:**

**Strengths**

- The research environment within the Stamm Lab (University of Kentucky) and CircCure are considered excellent for the proposed research.

**Weaknesses**

- None noted.

**Direct Phase II (Type 1 R44 applications See the SBIR/STTR Info Form):**

Not Applicable

**Fast Track (Type 1 R42 and Type 1 R44 applications See the SBIR/STTR Info Form):**

Not Applicable

**Vertebrate Animals:**

YES, all criteria addressed

**Biohazards:**

Not Applicable (No Biohazards)

**Authentication of Key Biological and/or Chemical Resources:**

Not Applicable (No Relevant Resources)

**Budget and Period of Support:**

Recommend as Requested

Recommended budget modifications or possible overlap identified:

**CRITIQUE 2**

Significance: 1
Investigator(s): 2
Innovation: 1
Approach: 3
Environment: 4

**Overall Impact:** Alzheimers Disease (AD) societal costs are in the $350B range in the US; we need new effective (non-amyloid-directed) drugs to prevent and slow the onset and progression of the disease. As a general rule, backsplicing of a 5'splice site to a upstream 3' splice site cases the generation of CircRNAs. In 2018, the authors discovered that the Microtubule associated protein tau (MAPT) generates two circRNAs through backsplicing of exon 12 to either exon 10 or 7. The resulting tau circRNAs produce Neurofibrillary Tangles (NFTs) which correlates with Braak stages and the propensity of humans to develop AD. The goal here, by employing and easy-to-use nasal spray containing a specific siRNA, would be to remove the tau circRNA seeds from becoming NFTs and blunt AD development. This is a well-written STTR grant proposal that will be carried out at three locations: the host's bioincubator site in the state of Kentucky, the University of Kentucky and a CRO (InVivoTek). Surely, there will be excitement in the field about addressing this new physical form (circRNA) of an old established target (Tau) for creating potential new avenues of Alzheimers therapy.

**1. Significance:**

**Strengths**

- Other existing AD drugs address the symptoms of AD (ex. Donezepil and anti-cholinesterases) or target beta amyloid formation (aducanab). There are antibodies under development which target *linear* tau but that species of protein is essential for cell survival and they do not take into account the A>I RNA editing event that is targeted here.
- Although not required for a Phase I STTR application the authors provide a nice assessment of risk and market size estimation on pp. 45-46. siRNA drugs have been FDA approved in recent years (Givlaari for acute hepatic porphyria and Onpattro for hereditary amyliodogenic transthyretin amyloidosis) and there are publications showing that nucleic acids can be delivered to the brain via the nasal route of administration.

**Weaknesses**

- None noted.

**2. Investigator(s):**

**Strengths**

- De Stefan Stamm is the PI and co-founded the company CirCure in 2018 right after he and Dr Weldon (postdoc) made the discovery and published a paper showing that the human MAPT locus generates circular RNAs, which forms the basis of the company and the provisional IP. He is an expert in splicing and pre-miRNA processing
- Dr Justin Weldon is a very recent Ph.D. (March 2021) who started CircCiure with Dr Stamm. To date he has published five peer reviewed papers, two as first author. His early career strengths, not surprisingly, are circRNAs and Splicing.
- Unlike the other Key Personnel, Dr Peter Nelson does have ongoing and previous funding listed. He is a professor at U. Kentucky Medical Center and is a clinician who has studied tau pathologies for over two decades. He will advise/ consult, as will Dr Dan Rujescu, U. Vienna, who is mentioned on p. 46 and p. 59 letter of support.

**Weaknesses**

- Unfortunately, Drs Stamm and Weldon list *no funding history*, which in the case of Dr Stamm is either an oversight or difficult to imagine across his ~25 year career as professor and postdoc.

**3. Innovation:**

**Strengths**

- The downregulation of the target here, microtubule associated tau (MAPT), via siRNA which will (hopefully) selectively target tau circ RNAs and prevent NFTs is highly innovative. There are no competitors who are planning to target circTau proteins. The authors have submitted a manuscript (Sept 2021) to *Science* which contains data showing that tau circRNAS aid the generation of NFT in a human/primate specific manner. This would be an exciting finding and perhaps partially explain why many of the existing amyloid and tau-based approaches have been unsuccessful to date in the clinic.

**Weaknesses**

- None noted.

**4. Approach:**

**Strengths**

- In Aim 1, the company will synthesize 21 siRNAs designed by performing a oligo walk to determine the backbone which has the best efficacy for the 12>7 and 12>10 tau circ RNA in terms or preventing adenosine to inoside editing. Three cell systems will be used to test the siRNAs: (1) FLAG-tagged HEK293 cells to assess reduction in protein (2) human U87 glioblastoma cells that endogenously express tau circRNA to be assessed via rtPCR and RNase protection analysis and (3) tau CircRNA transfected biosensor cells that form NFTs once transfected.
- In Aim 2, they will test the siRNA efficacy intranasally and by injection to reduce tau circRNAs and NFT. The model uses U87 human glioblastoma cells which naturally express tau circRNAs

**Weaknesses**

- The published design rules for siRNAs do not take into account circRNAs, therefore, this type of target may be resistant or even unapproachable by conventional RNAi methods.
- One wonders whether PROTACS would perform better degradation of the circular RNAs than siRNA? Not known; circRNAs are a very new class of drug target.

**5. Environment:**

**Strengths**

- CircCure resides in the Advance Science and Technology Commercialization Center at the U. Kentucky with 358 sq. ft. lab space which includes a cell culture room with four incubators and three laminar flow hoods, LOS p. 54-55. The U. Kentucky animal facility will be used to test nasal delivery of siRNA *before* Aim2 is attempted and accomplished at a CRO, InVivoTek, which is part of the Genesis group. Price estimated at $67,143 as shown on P. 30 and LOS is included on p. 48.

**Weaknesses**

- Unfortunately, there is no formal price quote included from InVivoTek for the intranasal delivery and injections to test efficacy.
- On p. 29 it is stated that they will be looking for a technician to perform the nasal droplet studies but on p.35 the name Georgi Margvelani is mentioned as the person to fill this role, hence the position seems to be filled but the matter is confusing.

**Phase II (Type 2 R42 and Type 2 R44 applications)**

Not Applicable

**Direct Phase II (Type 1 R44 applications See the SBIR/STTR Info Form):**

Not Applicable

**Fast Track (Type 1 R42 and Type 1 R44 applications See the SBIR/STTR Info Form):**

Not Applicable

**Vertebrate Animals:**

YES, all criteria addressed

**Biohazards:**

Acceptable

**Select Agents:**

Acceptable

**Resource Sharing Plans:**

Acceptable

**Authentication of Key Biological and/or Chemical Resources:**

Acceptable

**Budget and Period of Support:**

Recommend as Requested

Recommended budget modifications or possible overlap identified:

**CRITIQUE 3**

Significance: 1
Investigator(s): 2
Innovation: 1
Approach: 3
Environment: 1

**Overall Impact:** This is a Phase I STTR proposal intended to optimize siRNA to target MAPT circular RNAs and evaluate nasal brain delivery (Aim 1) and determine the IC50 in an in vivo model (Aim 2) to support subsequent Phase II studies and partnerships with pharma. The project is solidly based on exciting recent findings by the investigative team supporting a role for MAPT circular RNA species encoding microtubule-binding domains in promoting Tau aggregation and formation of NFTs in AD. The evidence supporting the scientific premise is strong and the hypothesis that primate-specific RNA editing mechanisms promote translation of these circular RNA species as part of the disease process is compelling. The planned product is protected by two patent filings, and the commercialization potential is very high. The investigative team is ideally suited to conduct these studies, and they have engaged excellent CROs and consultants to support their work. The experimental approach is clearly and thoroughly described with well-articulated rationale, incorporation of state-of-the-art methods, well-designed controls, and consideration of alternative approaches for potential pitfalls. Concerns about the absence of a drug formulation expert on the investigative team as well as some limitations in the approach slightly reduce enthusiasm for this excellent proposal with potential for very high impact.

**1. Significance:**

**Strengths**

- Outstanding scientific premise supporting central role of MAPT and tau aggregates in neurodegeneration.
- Recent discovery of human MAPT circular RNAs encoding microtubule-binding repeat domains of Tau which can influence NFT formation.
- Ability to target potentially pathogenic source of Tau MTBs without impacting normal expression of MAPT from linear RNA.
- Potential applicability for other Tauopathies in addition to AD.
- Very strong IP position and commercialization potential.
- Previous precedents for successful commercialization of siRNA therapeutics.

**Weaknesses**

- None noted.

**2. Investigator(s):**

**Strengths**

- The core investigative team, Drs. Stamm and Weldon, are experts in RNA biology and co-discoverers of human MAPT circular RNAs.
- Subcontract to Genesis Technology Group (Invivotec) to provide appropriate human U87 neuroglioma grafted mice for key *in vivo* testing model.
- Alzheimer's disease expertise provided by Dr. Peter Nelson who was also a co-author on BBA paper identifying human MAPT circular RNAs.

**Weaknesses**

- The investigative team would benefit from a formulation expert to assist in optimization of nasal delivery.

**3. Innovation:**

**Strengths**

- Novel promising target for AD therapeutics.

**Weaknesses**

- None noted.

**4. Approach:**

**Strengths**

- Well-planned application of cutting-edge techniques for optimizing siRNA.
- Focused and rigorous research plan for optimization of siRNAs, evaluation of nasal delivery and dosing, and plans for an appropriate animal model for in vivo assessment of siRNA efficacy.
- Well-controlled experiments with use of well-characterized and FDA-approved siRNAs (ONPATTRO and GIVLAARI).
- Consideration of creative and viable alternative approaches to overcome potential key challenges including insufficient drug delivery via nasal route (coupling siRNA to sertraline) and grafted neuroglioma model for *in vivo* testing (grafting iPS-derived neurons expressing inducible MAPT circular RNA).

**Weaknesses**

- Rationale for number of animals (n=3) is not given, SABV is not explicitly discussed, and statistical considerations were weak.
- There is concern about potential limitation of the U87-grafted rat model as maintenance of normal BBB may be compromised especially with tumor growth. This concern is partially mitigated by viable potential alternative approach through the use of grafted human iPSCs.

**5. Environment:**

**Strengths**

- Strong laboratory resources with all necessary equipment and expertise for proposed work.

- Well-developed planning and support through UK Office of Technology Commercialization and Advanced Science and Technology Commercialization Center.

**Weaknesses**

- None noted.

**Direct Phase II (Type 1 R44 applications See the SBIR/STTR Info Form):**

Not Applicable

**Fast Track (Type 1 R42 and Type 1 R44 applications See the SBIR/STTR Info Form):**

Not Applicable

**Vertebrate Animals:**

YES, all criteria addressed

**Biohazards:**

Not Applicable (No Biohazards)

**Resource Sharing Plans:**

Acceptable

**Authentication of Key Biological and/or Chemical Resources:**

Acceptable

**Budget and Period of Support:**

Recommend as Requested

Recommended budget modifications or possible overlap identified:

**THE FOLLOWING SECTIONS WERE PREPARED BY THE SCIENTIFIC REVIEW OFFICER TO SUMMARIZE THE OUTCOME OF DISCUSSIONS OF THE REVIEW COMMITTEE, OR REVIEWERS' WRITTEN CRITIQUES, ON THE FOLLOWING ISSUES:**

**VERTEBRATE ANIMALS: ACCEPTABLE**

**COMMITTEE BUDGET RECOMMENDATIONS: The budget was recommended as requested.**

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Footnotes for 1 R41 AG078096-01; PI Name: Stamm, Stefan

NIH has modified its policy regarding the receipt of resubmissions (amended applications).See Guide Notice NOT-OD-18-197 at https://grants.nih.gov/grants/guide/notice-files/NOT-OD-18-

197.html. The impact/priority score is calculated after discussion of an application by averaging the overall scores (1-9) given by all voting reviewers on the committee and multiplying by 10. The criterion scores are submitted prior to the meeting by the individual reviewers assigned to an application, and are not discussed specifically at the review meeting or calculated into the overall impact score. Some applications also receive a percentile ranking. For details on the review process, see http://grants.nih.gov/grants/peer_review_process.htm#scoring.

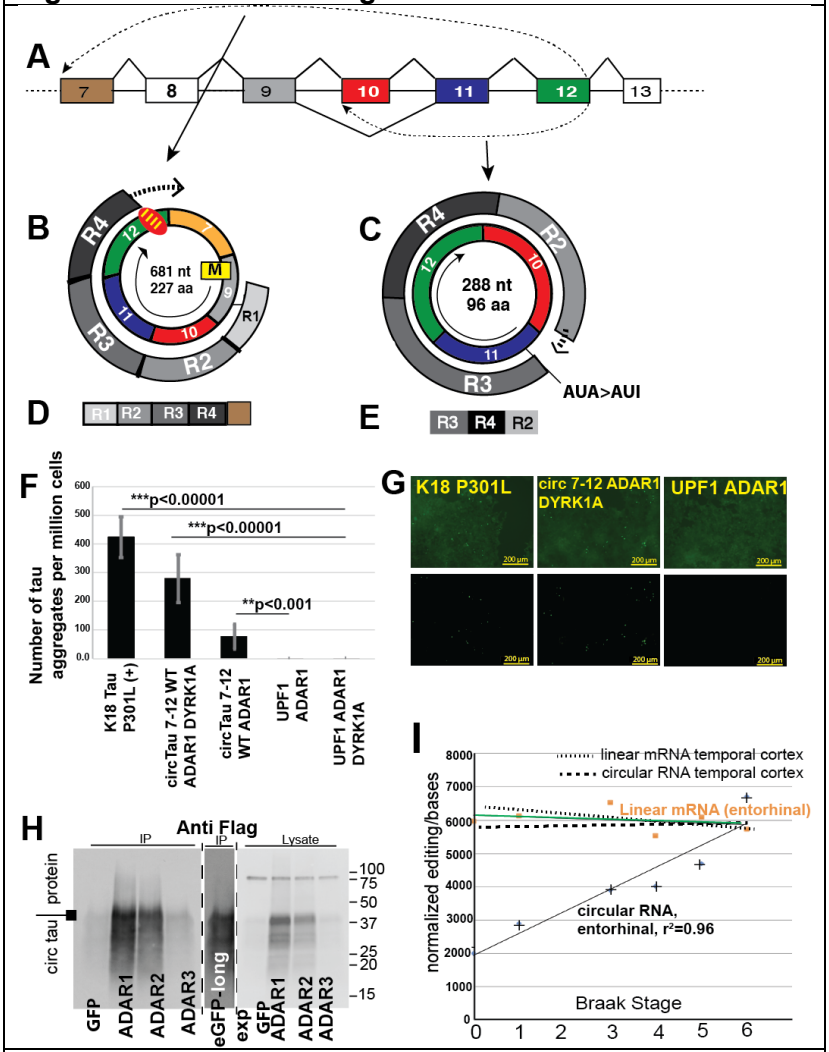
## Background and Significance

### *B1: Significance*

The accumulation of tau protein into neurofibrillary tangles (**NFT**) is the decisive event that causes neuronal death, leading to Alzheimer's disease (**AD**). AD affects approximately 10% of people older than 65, and their number is rising due to the aging population. Currently, there are no drugs available that prevent NFT formation. Aducanumab (Aduhelm) is the only FDA approved drug that directly reverses part of the AD molecular pathology. Aducanumab is a monoclonal antibody against beta-amyloid deposits, the mechanism of which reduces inflammation[2] but has no effect on NFT formation. In addition, cholinesterase inhibitors are used that show limited neuroprotection. Thus, no rationale therapies against the NFT formation that causes neuronal death are available. Given the high socio-economic burden of AD costing $355 billion in 2020[3], rational therapies are needed. The drug market size is estimated to be 18 billion in 2018, growing about 5% annually. *Thus, drugs that target the cause of AD, i.e., NFT-formation, are urgently needed for a large market.*

***Rigor of prior research:*** This work builds on the **scientific premise** that tau pathology has an extremely strong impact on public health[4-8]. The data leading to our product development are based on numerous independent experiments; RNA measurements were done in triplicate using highly specific RNase protection assays, followed by triplicate protein measurement. In addition, expressed proteins were validated using mass-spectrometry and in functional assays showing NFT formation. Nasal delivery of siRNA packaged as nanobodies has been shown in numerous models, including insulin in Alzheimer subjects[9] and siRNAs in rodent models[10, 11].

**Figure 2: Scientific Background**




**A.** Partial *MAPT* pre-mRNA structure. The backsplicing events are indicated by dotted arrows, leading to the **B** 12->7 and **C** 12->10 tau circRNAs (inner circle). Translation of these circRNAs results in peptides that encodes multimers of microtubule binding site repeats (**D, E**). **F:** Adding these tau circ proteins to NFT-indicator cells generates tau aggregates, indicating NFT formation. The statistical analysis of five independent experiments is shown. **G:** Representative experiments showing the tau circ protein induced NFTs (green dots) under experimental conditions indicated. **H.** Cotransfection studies indicate that A>I RNA editing caused by ADAR1 and ADAR2 strongly promote tau circRNA translation. **I.** This correlates with a general increase of A>I editing in human entorhinal cortex that follows Braak stages.

### *B2. Scientific Background*

circRNAs are generated from pre-mRNA through backsplicing of a 5' splice site to an upstream 3' splice site. We discovered that the microtubule-associated protein tau (MAPT) generates two circRNAs through backsplicing of exon 12 to either exon 10 (12->10, most abundant) or

exon 7 (12->7) (**Fig. 2 A-C)**. Both circRNAs lack stop codons and contain a number of nucleotides exactly divisible by 3[12]. The 12->7 circRNA contains a single start codon and translation from this start codon generates a peptide encompassing the microtubule-binding sites 1-4. Although the 12->10 tau circRNA does not contain a start codon, it can be activated through adenosine to inosine RNA editing (A>I). There, an AUA codon is changed to AUI, which serves as a start codon. Translation of the 12->10 circRNA generates multimers of peptides containing the R3-R2 microtubule-binding domains (**Fig. 2B-E**).

Similar to the synthetic K18 peptides that encompass the four microtubule sites[13], protein generated from the tau circRNAs promote the formation of tau aggregates using neurofilament reporter cells (**Fig. 2F, G**). Importantly, the formation of circ tau proteins is induced by A>I editing that increases in progressive Braak stages (**Fig. 2H, I**).

Together, *these data indicate that human tau circRNAs are translated after A>I editing increases during disease progression. The resulting peptides cause NFT formation.* ***We thus will develop siRNAs into a product that selectively removes tau circRNAs as a rational therapy for AD.***

***B3: Innovation and intellectual property***

We recently discovered tau circRNAs and their effect on NFT formation (as of 9/1/2021 under review for publication). Tau circRNA-dependent NFT formation has ***three major new aspects***: (i) As tau circRNA formation depends on primate-specific Alu-elements, circ tau proteins are primate-specific, which might correlate with the propensity of humans to form NFTs and AD[14]; (ii) The translation of tau circRNAs depends on epigenetic modification of the RNA (adenosine to inosine editing), which correlates with Braak stages; and (iii) the A>I editing changes the encoded amino acids, for example, an AUA is changed to an AUG, changing isoleucine to methionine; thus, the protein products of tau circRNAs resemble frontotemporal lobar dementia mutants that cause early onset tauopathies.

The **final product** will be an siRNA that is delivered nasally and reaches the entorhinal and temporal cortex, where it removes tau circRNAs, reducing the NFT load that builds up in early Braak stages[15]. It will be effective in early AD stages to prevent neuronal death due to NFT formation.

Selectively removing circ tau proteins as 'seeds' for NFT formation will be a non-incremental 'game changer' that would establish a new class of drugs. Due to this **high-level innovation**, we have the competitive advantage of being the only product in development that attacks tauopathies at the root cause of NFT formation, i.e., is a *true rational therapy*. This rational aspect is in contrast to all existing AD treatments, which address symptoms (Donepezil, Galantamine, Rivastigmine: Cholinesterase inhibitors, Memantine: Glutamate regulator, Suvorexant: orexin antagonist). Aducanumab targets beta-amyloid plaques, which promote AD, possibly by causing inflammation, and our product will target the decisive NFT formation that cause neuronal death in AD. There are several antibodies in tests that target tau; however, they do not take into account protein sequence changes due to A>I RNA editing and also target linear tau, which is essential for cells[16]. Trials with methylene blue, which inhibits NFT formation *in vitro*, showed no efficacy[17].

Our product is **protected by two patent filings** that protect the use of all possible siRNAs against tau circRNAs (63/137,405, filed on 1/14/2021) and more generally protect interventions to stop translation of circular RNAs after A>I RNA editing (63/224,959, filed on 7/23/2021). The University of Kentucky Office of technology commercialization will help with the ongoing protection of intellectual property rights and will not lease out our technology, *putting us in a secure position to develop the product*.

CircCure will be located in the University of Kentucky Business incubator, by renting space from the Advanced Science and Technology Commercialization Center (see ***letter of support and lab pictures from Tanya Floyd***), where all the work will be done. The only exceptions are radioactive RNase protection analyses which will be done in the Stamm lab, due to specialized equipment and the radiation license and specialized cell grafting in mice that is subcontracted to Invivotek (see equipment and budget).

***B4: Risk assessment***

Several nucleic acids have FDA approval; among them are siRNAs: ONPATTRO (against hereditary amyloidogenic transthyretin amyloidosis)[18] and GIVLAARI (acute hepatic porphyria)[19]. A splicing modifying oligonucleotide (Spinraza) is FDA approved for the treatment of spinal muscular atrophy (SMA) and is delivered to the spinal cord through intrathecal injection[20]. Several oligonucleotides against Duchenne muscular dystrophy are FDA approved. *These approvals indicate that nucleic acid-based therapies are possible, can be delivered to the brain, and can be manufactured in sufficient quality.*

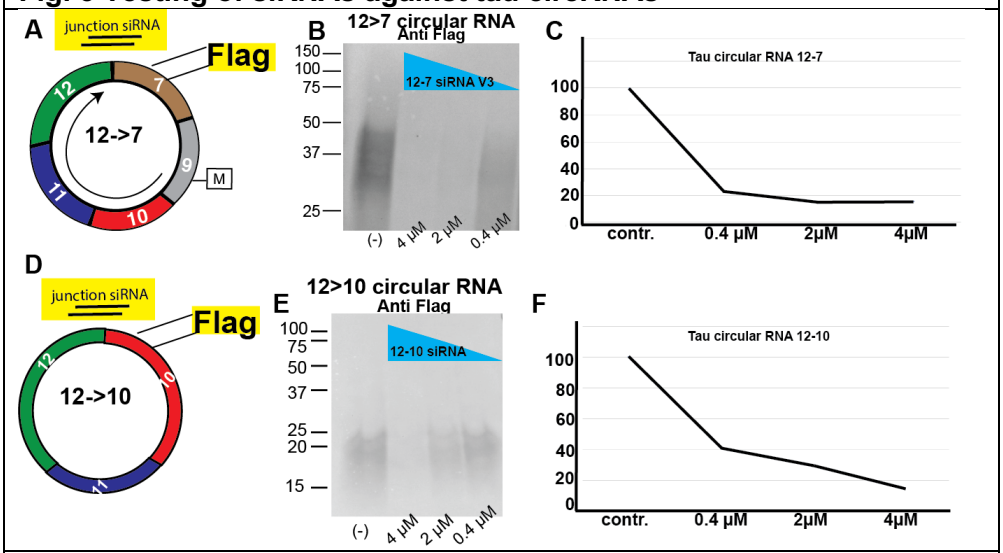
***B5: Market size***

The market size for AD in 2018 is globally $4.36 Billion and estimated to be $8.19 Billion by 2026, growing annually by 8.20% [21]. The societal costs are estimated to be annual $355 billion in the US[3] and will increase with an aging population, affecting 6.2 million patients who are estimated to number 12.2 million in 2050 in the US. These data show the large marked and societal need for treatment that our product will address. Currently, we have no competitors, as nobody is targeting circular tau proteins. Guided by the University of Kentucky small company incubator (see letter), this Phase I application will lay the foundation to partner with pharma companies in Phase II trials. **Current candidates for such a collaboration** are AC Immune (Switzerland, tau vaccination therapeutics), IONIS (RNA therapeutics) and Yumanity/Proteostasis (USA based, misfolding in neurodegenerative diseases).

## Approach

Our team combines technical expertise in RNA biology, clinical expertise in AD, and commercial expertise in incubating start-up companies (University of Kentucky). Stamm is an RNA biologist with 30+ years experience, including tau pre-mRNA processing, and serves as the president of CircCure. Welden earned his Ph.D. working on tau circular RNAs that he discovered in Stamm's laboratory. Together, they discovered the involvement of tau circ proteins in NFT formation. Stamm will oversee the siRNA design and analyze the screening data. Welden will perform and supervise the laboratory work. We will get clinical and regulatory advice from Dr. Nelson, a clinician in AD and expert in tauopathies, and Dr. Rujescu, a gerontopsychiatrist treating age-related dementias (***see their letters***). Governed by an options agreement, the University of Kentucky will provide incubator space, advice with regulatory issues, and depending on the experimental outcomes, will help file an Investigational New Drug application (IND) and connect with industry partners.

**Fig. 3 Testing of siRNAs against tau circRNAs**




**A.** Schematic representation of the 12->7 tau circRNA and the location of the siRNA (sequence in Figure 4). **B**. Representative Western Blot showing the effect on 12->7 circRNA. **C.** Quantification of three experiments. **D.** Schematic representation of the 12->10 tau circRNA and the location of the siRNA (sequence in Figure 4), **E.** representative Western blot showing the effect on protein encoded by circular RNA and **F**. the quantification of three experiments.

***Experimental Design***

***E1: Specific Aim#1: Define the optimal siRNA backbone sequence for the 12->7 and 12->10 tau circRNA***

***E1.1 Preliminary data for Aim #1***

We designed siRNAs against the backsplicing junction site for both the 12->10 tau circRNA and the 12->7 tau circRNA (**Fig. 3A, D**) that showed a reduction starting at 400 nM concentration in HEK293 cells (**Fig. 3C, F**). These siRNAs were generated with standard chemistry, i.e., unmodified ribose, phosphodiester bonds, and nucleotides, with the exception of the last 3' residues, which are deoxyribose and thymidine (**Figure 4**).

We have three cell systems available to test the siRNAs: stably transfected HEK293 cells with FLAG-tag expression constructs, human U87 glioblastoma cells that endogenously express the tau circRNAs and can be tested using RNase protection, and finally, biosensor cells that form NFTs once transfected with tau circRNA expression constructs (**Fig. 2G**).

| Figure 4: Structure of the siRNAs | |
|---|---|
| **A.** 12_7_j_v3<br>5′ AAAAAGGGGGCUGAUGGUGACTdT<br>3′dTTUUUUUCCCCCGACUACCACUG | **B.** 10to12_j<br>5′ AUAAAAAGGUGCAGAUAAUTdT<br>3′dTTUAUUUUUCCACGUCUAUUA |
| **C.** AAAAAGGGGGCUGAUGGUGACTdT | fluoride modification, 2′-O methyl-modification |
| **A, B,** siRNAs against the 12->7 and 12->10 backsplice sites, both sense (top) and antisense strands are shown. Yellow marked nucleotides are in exon 12. **C:** Standard chemical modifications of siRNAs through fluoride and 2′-O-methyl modifications. | |

***E1.2 Determining the optimal siRNA for each splice site***

For an siRNA to work effectively, it must be loaded on argonaut proteins and the passenger strand needs to be removed from the antisense strand. It is optimal when the duplex at the 5′ end of the sense strand has high stability and the middle of the 3′ end has low stability and nucleotide references at positions 3, 10, 13, and 19 are followed, which is reflected in current design algorithms[22]. The splice site junctions of tau circRNAs are suboptimal for siRNAs as they do not adhere to these rules; we will thus test each junction using all possible 20 siRNAs by 'sliding' across the splice site, meaning that in the first siRNA, one nt is in exon 12, and in the last siRNA, 20 nt are in exon 12. After 48 hrs of transfection, the IC50 for each siRNA will be determined by (a) the reduction of protein in stably transfected HEK293 cells using the FLAG-tag construct and (b) by RNase protection of the endogenous RNA in U87 cells. U87 cells are well-characterized glioblastoma cells that endogenously express tau circRNAs (**Fig. 5B**); they are also well-characterized in cell grafting studies (**Fig. 5C**).

Using the U87 system, we will determine the effect of the siRNAs on endogenous linear tau mRNA using qPCR, which will be validated by RNase protection for key siRNAs. We expect that we can identify at least three siRNAs for each junction that have an IC50 of less than 25 nM and show less than 10% reduction of linear mRNA. These siRNAs will be further optimized.

***E1.3 Optimizing siRNAs***

The three best siRNAs showing the highest effect on the backsplice and the least off-site effects on linear RNA will be optimized using standard template chemistry (**STC**). STC increases the stability and efficacy of siRNAs and has been empirically developed by Alnylam Pharmaceuticals[23].

In STC, two phosphorothioates bonds are introduced at the 3′ end of the antisense strand; three consecutive 2′-F ribose derivates at positions 9-11 in the sense strand and three consecutive modifications at positions 11-13 of the antisense strand. In all other positions, we will introduce alternative 2′-O methyl and 2′-F moieties (**Fig. 4C,**). Our unmodified siRNAs have an IC50 from 10-50 nM. The siRNAs will be tested in HEK293 cells expressing FLAG-tag for their ability to reduce circ tau protein production and in U87 cells for their ability to reduce endogenous tau circRNA production. We expect them to drop below 1 nM, as seen for ONPATTRO and GIVLAARI siRNAs[19].

***E1.3 Nasal delivery of siRNAs in mice***

siRNAs have been delivered to the brain intranasally using a cationic linear polyethylenimine (**PEI**) complex, which targets gene expression in the brain[11], demonstrating that siRNA can be functionally and nasally delivered. In addition, large molecules such as insulin have been delivered nasally to AD patients[9].

To test our siRNAs for nasal uptake, the best siRNA designs will be Cy3-fluorescently labeled and complexed with PEI in a nitrogen to phosphate ratio of seven[11]. 5 µl of the siRNA/PEI complex in a 10% glucose solution; these will be administered twice per nostril (40 µl containing a total of 15 µg siRNA). These concentrations have been established for nasal delivery of other siRNAs[11]. Mice will be sacrificed after 24 hrs and the uptake monitored by fluorescence, as described by our lab[24]. First, we will examine the tissue using fluorescence microscopy and then extract the oligos (proteinase K, phenol) and determine the concentration of the oligo through fluorescence.

We will change the amount of siRNA depending on initial outcomes, with the goal of getting about 1 µg siRNA into the brain, as this showed efficacy in previous studies[24].

***E1.4 Statistical analysis, Rigor and reproducibility***

The testing in cell culture will be initially done in triplicate for protein and RNA detection using three concentration points for each siRNA. The five best siRNAs will be tested with six concentration points prior to optimization using STC. For each IC50, a confidence interval will be calculated using ANOVA. The

analysis will be performed by a lab member that is **blinded** for the experimental details. For the nasal delivery, we will use 20 mice (3 mice per concentration) and concentrate on the accumulation in the frontal cortex known to be targeted in siRNA PEI complexes[11]; the accumulation of the siRNA will be determined by microscopy and quantified through fluorescence spectroscopy from phenol-extracted, soluble brain extracts.

***E1.5 Milestones (MS) for Specific Aim#1***

| | |
|---|---|
| **MS 1:** | Determine the backbone of two siRNAs with the lowest IC50 against the 12->7 and 12->10 backsplicing site and lowest off-site effects against linear tau mRNA |
| **MS 2:** | Optimize the two best siRNAs using standard oligo chemistry |
| **MS 3:** | Determine the optimal PEI/siRNA ratio, concentration, and overall amount for nasal delivery |
| **Go/Nogo 1** | If siRNAs cannot be delivered nasally in sufficient quantity, we will follow an injection strategy |

***Expected results and alternatives:*** We expect that we can generate siRNA with an IC50 under 5 nM after STC optimization, similar to ONPATTRO and GIVLAARI. Because siRNA/PEI and drug/PEI complexes have been delivered nasally to various mouse models, we also expect that we can do nasal delivery [25,26]. As an *alternative*, we will couple the siRNA to sertraline for nasal delivery, a compound that binds to serotonin receptors and has been successfully used[27].

***E2: Specific Aim#2: Determine the efficacy of the siRNAs in a grafting model of mouse brain***

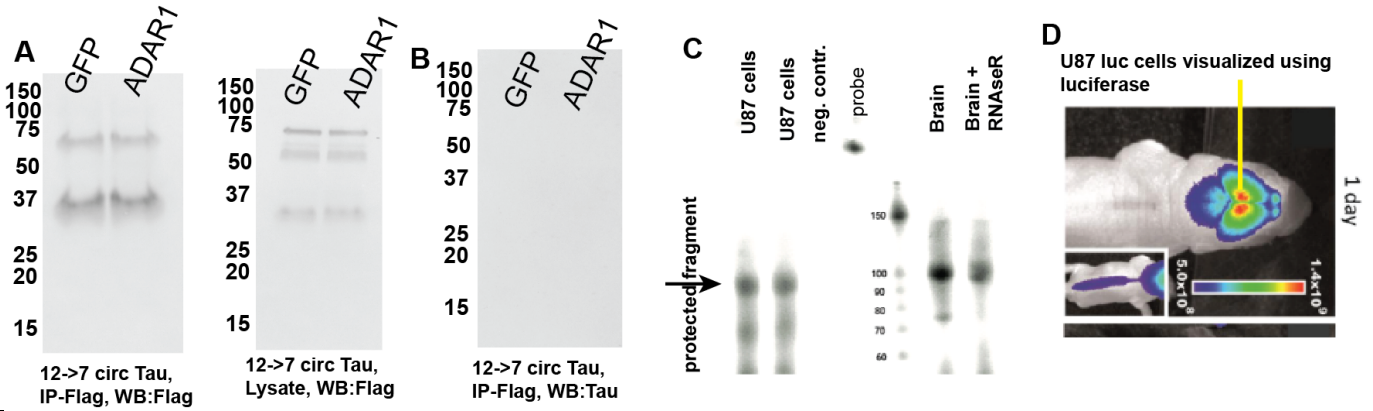
***E2.1 Preliminary data: Why we use a grafting model?***

Tau circRNAs depend on primate-specific Alu elements for their formation. Thus, they are not naturally formed in mice. When transfecting human expression constructs into mouse cells, tau circRNAs are not increased by ADAR1 (**Fig. 5A,B**), and tau immunoreactivity cannot be detected (**Fig. 5B**). In addition, we cannot detect tau circRNAs in mouse brain[12]. These findings suggest that rodents cannot properly process Alu elements in their pre-mRNA, as Alu elements are not part of the rodent genome but make up about 10% of the human genome.

We will therefore use grafts of human U87 neuroglioma cells in mouse brains as a 'humanized' brain model. U87 cells express endogenous tau circRNAs (**Fig. 5C**) and grafted U87 cells can be visualized through luciferase activity (**Fig. 5D**).

For optimal results, we will work together with a specialized US company, Invivotech, New Hamilton, NJ. Invivotech will graft U87-luc glioblastoma cells into mouse brains via injection and follow tumor development for two weeks (**Fig. 5D**), ***see Invivotek letter***.

**Figure 5: Analysis of tau circRNAs in brain grafts**




**A, B**: Tau circRNAs show a different regulation in human vs. mouse cells. The circRNA expression constructs were transfected into mouse NIH3T3 cells. **A:** the 12->7 expression construct has no increase in expression in the presence of ADAR1 and **B:** the protein shows no tau reactivity. The protein structure is different than in human cells, which show ADAR1 dependency and tau immunoreactivity (**Fig. 2H**). **C:** U87 cells express endogenous tau circRNAs, detected by RNAse protection. The probe protects the backsplicing junction site (100 nt). **D:** Visualization of grafted U87-luc cells using luciferase in a mouse brain showing successful grafting.

***E2.2 Test the efficacy of siRNAs on RNA in grafted U87 cells***

In total, 300,000 U87 luc cells will be injected into the striatum of PDX mice as described (**see letter Invivotech**). The growth of these cells can be visualized using luciferase activity (**Fig. 5C**). After 14 days,

when the cells are fully established, 1 µg of siRNA against the 12->10 and 12->7 junctions will be injected, and the grafted cells will be removed after three days. We will use GIVLAARI siRNA as a control. We will compare tau circRNA expression in controls and siRNA-treated cells using RNase protection and determine the effect on tau linear mRNA expression using qPCR (TaqMan). The starting amount of 1µg is based on previous experiments we had with splice site changing oligos[24], and we will adjust the amount of siRNA accordingly.

***E2.3 Test the efficacy of siRNAs on NFT formation in grafted U87 cells***

We will generate U87 NFT-reporter cells by transfecting two linear 2N4R tau cDNA constructs into these cells. These constructs generate cyan and yellow fluorescent protein and aggregates that form can be visualized using green fluorescence, observed as green dots (**Fig. 2G**). These modified cells will be used for further experiments. Prior to injection, we will transfect them with 0.1 µg tau circ expression constructs/300,000 cells to induce NFT formation. These cells will be injected into PDX mice and treated for four days with siRNAs, delivered through injection. We will then determine the number of green dots using automated fluorescence microscopes and image J. The changes in dot numbers will indicate a change in NFT formation as a response to siRNAs. Based on the initial experiments, we will optimize the dosage in three concentrations between 0.5 and 5 µg siRNAs (3 animals per concentration point). The FDA-approved siRNA GIVLAARI will serve as a control, used with matched concentrations.

***E2.4 Test the efficacy of siRNAs on NFT formation in grafted U87 cells through nasal delivery***

Once efficacy through injection has been established, we will deliver siRNAs using nasal delivery. The total amount of nasal delivery will be guided through the effective concentration of the siRNAs through injections. From the pilot experiments in Aim #1, we can estimate the percent of siRNA that accumulate in the brain and will deliver the siRNAs accordingly, possibly through repeated administrations. We will use three time points (2, 5, and 10 days post-delivery, three animals each). The U87 cells will be removed and NFT formation investigated and compared to the GIVLAARI negative control.

***E2.5 Statistical analysis, Rigor and reproducibility***

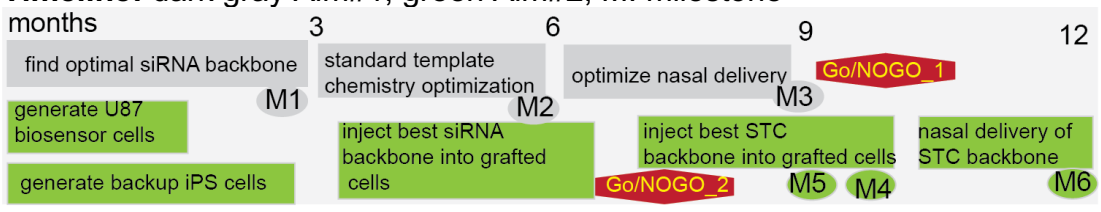
We will use three sets of grafted mice: a) 10 siRNA and 10 control for 1, 2 and 3 µg injection, where 3 animals are one concentration points, b) like (a) using STC-siRNAs and c) 10 siRNA and 10 control for nasal delivery. This allows triplicates for each concentration points, which will be combined using ANOVA.

***E2.6 Milestones (MS) for Specific Aim#2***

| | |
|---|---|
| **MS 4:** | Determine the dosage of siRNAs to reduce 12->7 siRNAs in graft experiments |
| **MS 5:** | Determine the dosage of siRNAs to reduce 12->10 siRNAs in graft experiments |
| **MS 6:** | Test the effect of nasal delivery of NFT formation in grafted cells |
| **Go/Nogo 2** | If U87 model does not generate sufficient NFTs, use human iPS cells that express tau circRNAs and form NFTs; if nasal delivery is not efficient enough, use injection |

***Expected outcomes and alternatives:*** We predict that the siRNAs will show in vivo reduction of the tau circRNAs. It is likely that U87 cells will form NFTs in a biosensor cell setting once linear tau is transfected. However, as an *alternative*, we are constructing human iPS cells that carry a tet-inducible tau circRNA expression cassette that form human neurons with endogenous NFTs upon grafting. These iPS cells are made from CellBio, can be pre-differentiated into neurons prior to injection, and will be used as an alternative to U87 cells.

**Timeline:** dark gray Aim#1, green Aim#2, M: milestone




## Outlook

This project will determine the efficacy of siRNA treatment of tau circRNAs in the prevention of tau circular RNAs and tau aggregate/NFT formation. A successful demonstration of efficacy will provide evidence for Phase II studies, where we will test safety and pharmacology on a larger scale to prepare for an IND and seek investors/a collaboration with pharma with the help of the University of Kentucky incubator.