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February 21, 2008

Dr. Chris Penland
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Cystic Fibrosis Foundation
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Dear Chris:

The following constitutes the final scientific report for our grant number MCCART06P0, which was graciously supported by the Foundation under the CFF/NIH bridge funding program.

We requested support from the Foundation on December 22, 2005, following the failure to receive support from the NIH for our grant entitled "Peptide Inhibitors Probe Structure and Function in Chloride Channels" (1-R01- DK075016-01). The goals of the NIH project were to isolate and achieve initial characterization of two different peptide toxins from scorpion venom: one which inhibits CFTR and another which inhibits ClC-2 voltage-gated chloride channels. At the time of submission to the Foundation in December, we already had isolated the toxin active at ClC-2 (*see* Thompson et al., 2005), but had not yet determined the complete sequence. We had not yet isolated the toxin active at CFTR. The major goal of the CFF-NIH grant submitted for Foundation support was to complete the isolation of the toxin active at CFTR. The Foundation provided one year of support at a budget of $75,000; funding period was 07-01-06 through 06-30-07, with a no-cost extension to 09-30-07.

I. Lay Abstract

The protein defective in Cystic Fibrosis, CFTR, functions as a chloride channel in the outer membranes of cells in the airway. Over the past decade, we have greatly increased our understanding of how this protein works in normal airway cells, with the hope that this information could help us understand how to fix CFTR in the airway cells of CF patients. In order to gain deeper insight into how CFTR works, and how the structure of this protein relates to its function, we need to develop molecular probes that can serve as experimental tools. Similar studies of other ion channel proteins, such as potassium channel proteins and sodium channel proteins, have been aided by the identification of peptide toxins that interact with these proteins with very high specificity. Many such peptide toxins, proteins which are much smaller than the ion channel proteins with which they interact, have been isolated from the venom of animals such as scorpions, snakes, spiders, and other animals that rely on these toxins for either their defense or feeding. However, never before has a peptide toxin been identified that interacts with a specific chloride channel protein such as CFTR.

The goal of this project was to isolate a peptide toxin that might interact with CFTR. We found previously that the venom of the Giant Yellow Israeli Scorpion contained a peptide component which binds to CFTR, and changes its function. Based on this exciting observation, we used a variety of molecular separation techniques to isolate the active peptide from among the hundreds of other peptides that comprise this scorpion's venom. The chemical separation techniques we used included separating the components of venom according to their hydrophobicity (essentially, how sticky they are), along with separating the components according to their molecular size. These steps were guided by testing each fraction of the venom, produced by the separation techniques, to ask how

good it was at changing the function of the CFTR channel when it is studied using high-resolution electro-physiology techniques. This way, we could follow the activity of the active component through the separation process.

The end result of this effort was the isolation of a novel peptide, which has a sequence that has never before been described, but is shaped very much like the peptide toxins that are known to interact with potassium or sodium channel proteins. However, this is the first peptide toxin ever found that interacts with a specific chloride channel protein. We named the toxin GaTx1. This toxin interacts with the CFTR channel with higher strength than almost all other compounds that are known to interact with CFTR, and is absolutely specific -- we have not found any other protein that interacts with GaTx1. We are now using GaTx1 to understand how CFTR functions, and particularly how CFTR changes its shape during the opening and closing of the chloride channel pathway. Furthermore, because GaTx1 appears to interact with the parts of CFTR that are defective in most mutated versions of the protein, it is possible that this small peptide could help hide the fact that CFTR is misfolded, leading to an increase in functional CFTR protein in the airway cells. This would be expected to help minimize the consequences of the defect in the CFTR protein.

II. Detailed Report

A. Specific Aims (for the CFF-NIH proposal):

Specific Aim #1. Isolate the toxin active at CFTR and confirm biological activity. Toxin isolation will be completed by coupling separations chemistry, using reversed-phase HPLC, with electrophysiological bioassays. We will then use the isolated toxin to confirm its mechanism of action, to construct true dose-response curves, and to determine the forward and reverse kinetics of interaction with CFTR channels.

Specific Aim #2. Characterize the peptide toxin using proteomic approaches. We will determine the molecular weight of the toxin, sequence it, identify any post-translational modifications, and then compare the primary sequence to those of known toxins. We will also construct homology models, to guide structure-function studies.

Specific Aim #3. Produce the peptide toxin exogenously. We will isolate or synthesize cDNA encoding the peptide toxin, and use this construct for the preparation of recombinant toxin by bacterial expression. We will also produce the toxin by solid phase chemistry, and will then determine the efficacies of both the recombinant and synthetic toxins in electrophysiological experiments. These steps are necessary to facilitate the production and study of mutant peptide toxins.

Specific Aim #4. Identify the structural elements underlying biological activity. Informed by the results of the modeling studies of Aim#2, we will design, prepare, and test mutant versions of the toxin to determine what structural features confer efficacy and selectivity against its target. These experiments may lead to identification of modifications that can improve efficacy, in order to make this molecular probe even more powerful.

B. Studies and Results

1. Progress toward overall goals Venoms from snakes, scorpions, marine snails, and spiders are rich sources of peptide toxins that have proven to be of great value in the functional exploration of voltage-gated and ligand-gated cation channels. Unfortunately, although several potential chloride channel toxins have been identified, peptide inhibitors of chloride channels of known molecular identity have not been described. In 1992, Strichartz and colleagues described the isolation of chlorotoxin (ClTx), a small basic peptide capable of inhibiting low-conductance Cl^- channels from rat colon or rat brain reconstituted into lipid bilayers. However, ClTx has not been found to inhibit any anion channel of known molecular identity; indeed, its only known target is a matrix metalloprotease.

Prior to our initiation of this project, we showed that venom from the scorpion *Leiurus quinquestriatus hebraeus* inhibits CFTR by binding to the channel from the cytoplasmic side (Fuller et al., 2004); we also showed that ClTx does not inhibit CFTR. We then showed that the component (or components) that inhibits CFTR does so in a strongly state-dependent manner, interacting with the

channel only during closed states (Fuller et al., 2005). Our characterization of this activity of venom served a major purpose of forming the bioassays that we would then use for the isolation of the active component(s).

We made significant progress toward the goals of this project, both before and since the CFF funding began in July 2006. Most importantly, we have completed the isolation of the toxin that inhibits CFTR, completing Aims 1 and 2.

2. Progress toward Aim #1: Sequential purification of components of scorpion venom using size-exclusion chromatography and reversed-phase HPLC (RP-HPLC) resulted in the isolation of a single peptide whose biological activity was sufficient to recapitulate the interburst inhibitory activity of crude venom. This was accomplished by combining these chemical separation approaches with the difficult bioassay of recording of CFTR channel current from membrane macropatches and single-channel patches. Single-channel recordings of WT-CFTR activity in the absence and presence of the active final fraction indicated that the fraction inhibited CFTR by inducing long-lived interburst closings (Fig. 1). The active fraction caused a 58.2 ± 11.3 % decrease in P_o from 0.19 ± 0.06 in control conditions to 0.06 ± 0.004 (n = 3) in presence of the active fraction. From these results we concluded that this fraction contained a peptide toxin which inhibited WT-CFTR; we named this toxin "GaTx1".

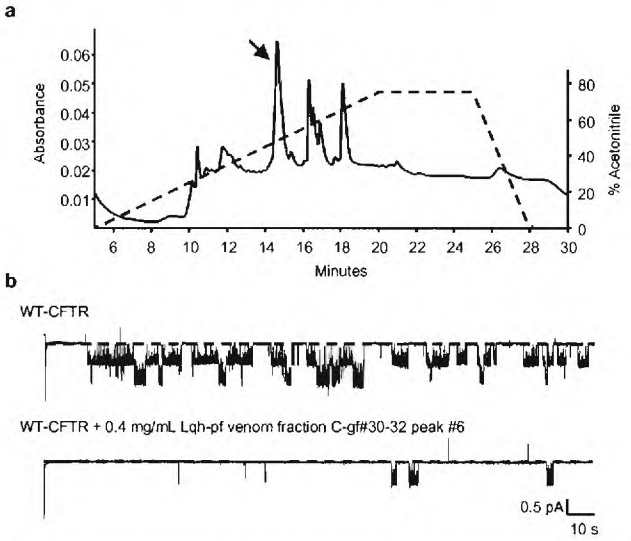

Figure 1: Isolation of the active peak. **(a)** Representative RP-HPLC chromatogram of fraction C-gf#30-32. Individual peaks were collected and tested for activity. The arrow indicates the active peak, peak #6. **(b)** Representative single channel trace in the absence and presence of peak #6. The isolated toxin inhibits CFTR by increasing the interburst closed duration.

Figure 2 shows data from a single-channel recording of WT-CFTR in the absence of toxin (*upper and lower traces*) and in the presence of 60 nM GaTx1 (*middle trace*), indicating reversible inhibition. Exposure of single CFTR channels to GaTx1 in an inside-out patch led to the introduction of very long closed states without affecting the duration of the open states. Toxin-induced closed durations are very long; the mean toxin-induced closed duration in this experiment was >5 seconds. However, evident in the dwell-time distribution (Fig. 2e) are toxin-induced closed states that last nearly a minute. Hence, GaTx1 interacts with CFTR ~1,000-fold longer than most known blockers, and longer than the thiazolidinone $CFTR_{inh}$-172, the best CFTR inhibitor found to date. This intimate interaction means that GaTx1 will be a very informative probe of CFTR structure.

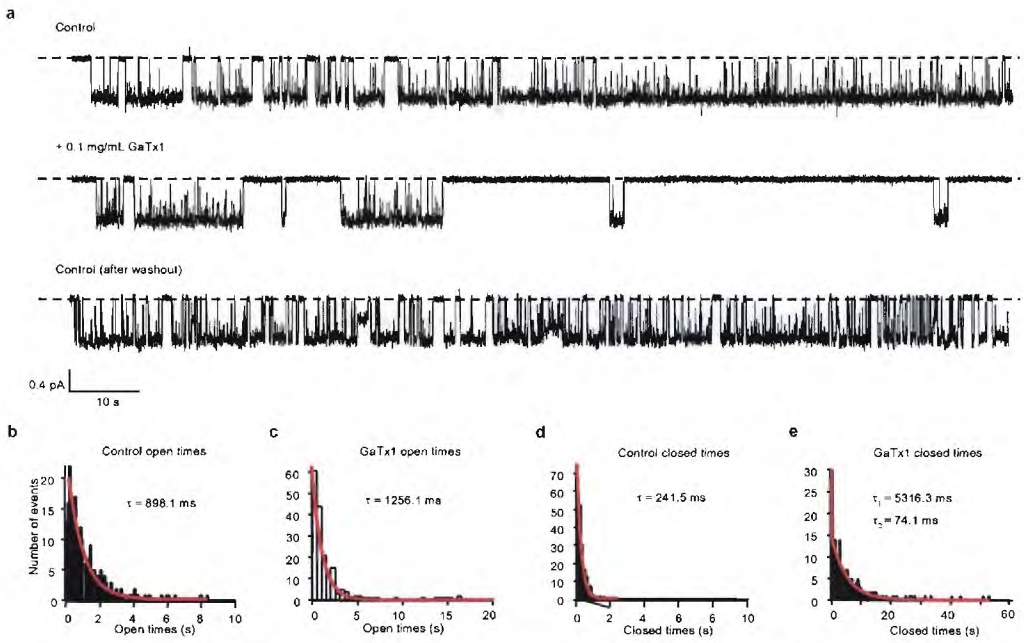

Figure 2: Native GaTx1 inhibits CFTR by increasing the interburst closed duration. **(a)** Representative single channel trace of WT-CFTR in the absence and presence of 0.1 mg/mL equivalent GaTx1. Open probability was significantly reduced in the presence of GaTx1. **(b-e)** Burst analysis of CFTR open durations in the absence **(b)** and presence **(c)** of GaTx1, and CFTR closed durations in the absence **(d)** and presence **(e)** of GaTx1. For this analysis, the record after toxin washout was used as control, to account for any potential loss of channel activity due to rundown. Toxin increased the interburst closed time, but had no effect of CFTR open duration. A concentration of 0.1 mg/mL venom equivalent, as shown in this experiment, was determined by amino acid analysis to provide 60 nM native GaTx1.

3. Progress toward Aim #2: Initial characterization of the active peptide contained in the final chromatographic fraction was accomplished by subjecting a small aliquot of the material to MALDI-TOF analysis. A single peptide was identified with a molecular mass of 3,674.6 Da (Fig. 3a). Additional, less abundant peaks with similar mass were also identified that likely represent different oxidative states of the same toxin; a doubly charged species also was observed (m/z = 1,838.8 Da). The peptide in this fraction then was subjected to automated amino terminal sequencing following destruction of all putative disulfide bridges by reduction with DTT and alkylation of free cysteines by iodoacetamide. The reduction and alkylation of the native toxin resulted in an increase in molecular mass to 4,138.5 Da as determined by MALDI-TOF, which is in agreement with the modification of eight cysteines; expected mass = 4,138.6 Da. The primary sequence was determined to be: ^{1}**CGP-CFT-TDH-QME-QKC-AEC-CGG-IGK-CYG-PQC-LCN-R**^{34}, including C-terminal amidation as the only post-translational modification other than formation of disulfide bridges. The calculated mass of the isolated toxin based upon primary sequence and assuming post-translational C-terminal amidation was in close agreement with the mass determined by MS. The same toxin has been purified from multiple batches of scorpion venom. Because the sequencing yield from the full-length modified peptide dropped below the 1-pmol level toward the very C-terminus, an aliquot of the modified toxin was digested with Lys-C protease which is specific for Lys-Xxx peptide bonds. The digest was separated by RP-HPLC and the resulting three fractions were manually collected and subjected to Edman sequencing. The sequences of the three fragments corresponded to: (1) *N-term.*: CGPCFTTDHQMEQK; (2) *Middle*: CAECCGGIGK; and (3) *C-term.*: CYGPQCLCNRamide. The C-terminal fragment was further analyzed by ESI-MS/MS, yielding a molecular ion of 1,325.5 Da, which agrees with the calculated mass of this fragment based on sequence assuming a C-terminal amide.

The ESI-MS/MS data also confirmed the presence of three carboxymethyl-Cys residues in this fragment. Hence, the sequence listed above has been verified.

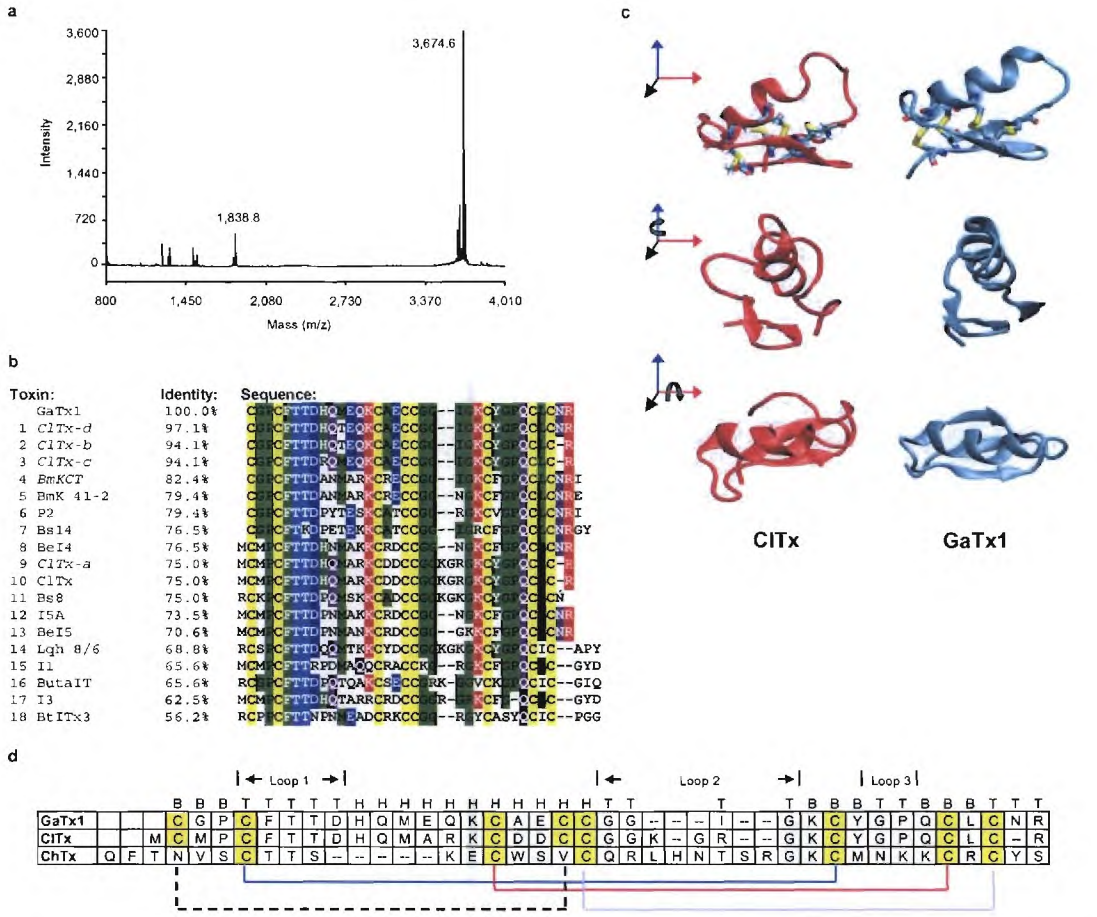

Figure 3: Proteomic characterization of GaTx1. **(a)** MALDI-MS spectrum of peak #6, showing a prominent mass of ~3.7 kDa. **(b)** Alignment of the GaTx1 sequence with other scorpion toxins shows that GaTx1 is a novel peptide. **(c)** Comparison of the ClTx NMR structure (*left*) and the GaTx1 homology model (*right*), in three orientations. Disulfide bridges are shown in the upper panels. **(d)** Sequence alignment of GaTx1 with the sequences of chlorotoxin (ClTx) and charybdotoxin (ChTx). Predicted secondary structure is indicated above the sequences while disulfide bridge linkage between conserved cysteines is indicated below. The dashed line indicates the disulfide bridge absent in ChTx.

Comparisons of the primary sequence with those of other previously characterized known or putative peptide toxins suggested that the isolated toxin, which we named GaTx1, is novel, with ClTx-d recognized as its closest relative (Fig. 3b). ClTx-d is not known to inhibit any ion channel; indeed, the sequences of ClTx-b, -c, and -d are speculative, being predicted from the sequences of cDNAs cloned from scorpion venom gland. The primary sequence of GaTx1, the molecular mass, and the presence of four disulfide bridges between conserved cysteines place this toxin in the family of insectotoxins active at K^+ channels (which includes charybdotoxin) and putative toxins active at Cl^- channels. GaTx1 bears 75% sequence identity to ClTx, but is smaller by ~ 400 Da. The calculated pI values for GaTx1 and ClTx are 6.71 and 8.13, respectively.

A homology model of GaTx1 was created, based on the known NMR structures of CITx and insectotoxin 5A. Figure 3c shows the NMR structure of CITx and the homology model of GaTx1, in three orientations. The two structures are quite similar in shape and secondary structure, although GaTx1 has three regions of anti-parallel β-strand compared to two in CITx; the extra strand is at the immediate N-terminus. The I5A toxin structure (*not shown*) includes three β-strands and a longer α-helix compared to CITx. GaTx1 is also somewhat more compact than CITx, partly due to the insertion of two amino acids C-terminal to the α-helix in CITx, and due to the lack of defined secondary structure in the first loop of CITx, resulting in a bulge of relatively disordered sequence at the N-terminal end of the α-helix in CITx. Figure 3d identifies the borders of regions of predicted secondary structure for GaTx1, and compares the GaTx1 sequence with that of CITx and charybdotoxin (ChTx). While the most striking primary structure differences between short insectotoxins (including all three of these) and the longer toxins that inhibit Na^+ channels and Ca^{2+} channels are the lengths of loops 2 and 3, the major differences between GaTx1 (which inhibits CFTR) and CITx (which does not) are in the α-helix. Hence, GaTx1 is the first peptide toxin ever found that inhibits an anion channel of known molecular identity. It is also the first toxin known to inhibit an ABC Transporter, the superfamily of which CFTR is a member. The GaTx1 sequence has been deposited with UniProt as entry # P85066.

4. Progress toward Aim #3: To confirm that the inhibitory activity in venom could be ascribed to the sequenced toxin, and did not arise from a contaminant, we prepared GaTx1 by solid-phase chemistry. After purification by HPLC, MS analysis indicated that the synthetic linear peptide has a mass in agreement with the theoretical mass for the linear form (*i.e.*, all disulfides reduced to sulfhydryls) of the native toxin. The linear peptide was subjected to oxidative cyclization and refolding was performed over three days under equilibrating conditions, in order to promote the formation of the most stable disulfide bridges. The mass of the folded synthetic peptide was within one a.m.u. both of the theoretical expected value and of the observed mass of the native toxin isolated from venom.

We used multichannel recordings from oocytes expressing Flag-cut-ΔR-CFTR to test for activity of synthetic GaTx1 (Fig. 4), taking advantage of the insensitivity of this mutant CFTR to dephosphorylation-mediated rundown; we showed previously that Flag-cut-ΔR-CFTR is inhibited by venom to a similar degree as WT-CFTR. Because GaTx1 binds channels in the closed state, we used intracellular solution containing only 0.2 mM ATP, which leads to reduced open probability, thus increasing the likelihood of a toxin binding event. In the presence of 250 nM synthetic GaTx1, channel current was reduced by 71.1 ± 2.6% ($p < 0.001$). With 50 nM synthetic GaTx1, channel activity was reduced by 56.2 ± 5.0% ($p < 0.001$), suggesting that the IC_{50} for synthetic GaTx1 under these conditions may

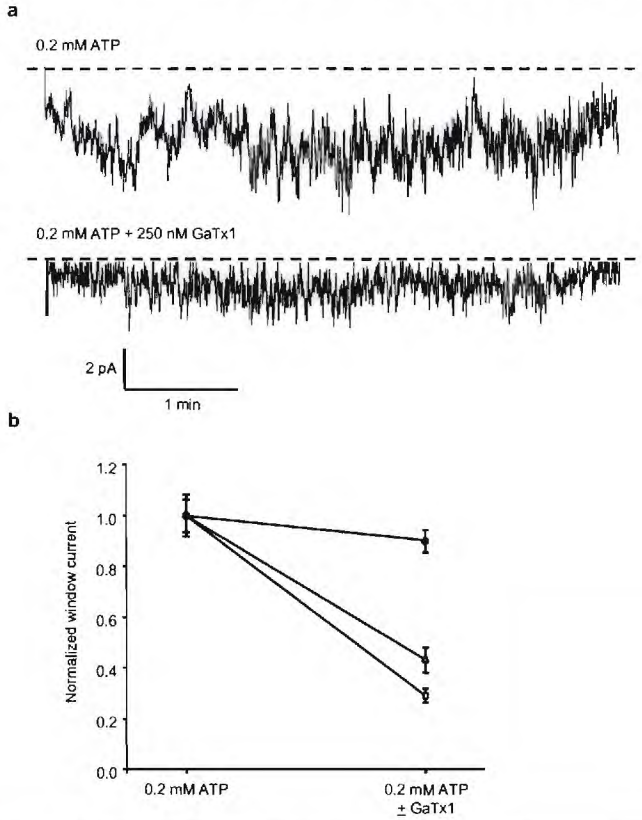

Figure 4: Synthetic GaTx1 inhibits CFTR. **(a)** Representative multi-channel trace of Flag-cut-ΔR-CFTR in the absence and presence of synthetic 250 nM GaTx1. **(b)** Window current over three-minute widows was measured in the presence of 0.2 mM MgATP under control conditions, and in the presence of 0.2 mM ATP (closed circles), 0.2 mM ATP plus 50 nM GaTx1 (open triangle), or 0.2 mM ATP plus 250 nM GaTx1 (open square), and normalized to control conditions. Each point represents a single patch where macroscopic window current was calculated within five windows each under control and experimental conditions.

be ~20 nM. Since the end of the funding period, we have been doing more experiments to construct a true dose-response curve. We point out that the strong state-dependence of action exhibited by GaTx1 means that the apparent IC_{50} for the toxin will be very protocol dependent; under conditions that increase P_o, the apparent IC_{50} will be elevated.

Because the toxin produced exogenously by solid-phase chemistry has worked so well, we chose not to attempt to make GaTx1 using recombinant methods. This decision is also supported by the facts that: (1) we obtain large amounts of toxin from a single synthesis, and (2) the synthetic GaTx1 elutes from a silica RP-HPLC column with the same retention time as the major component of the active fraction of venom, suggesting that they are similar in hydrophobicity.

5. Summary of progress In short, we have completed the most important parts of Aim 1, all of Aim 2, and all of Aim 3.

6. Plans for the near term We will continue experiments with the wildtype synthetic GaTx1 to construct true dose-response curves under a variety of conditions (*i.e.*, ATP concentrations), and then will perform more single-channel experiments to better quantify the kinetics of interaction between toxin and channel. With the help of our colleagues at Calgary, we also will begin to produce mutant toxins for the experiments proposed under Aim 4.

7. References

1) Fuller, M.D., Z.-R. Zhang, G. Cui, J. Kubanek, and **N.A. McCarty** (2004) Inhibition of CFTR channels by a peptide component of scorpion venom. *Am. J. Physiol. (Cell)* **287**:C1328-1341.

2) Thompson, C.H., D.M. Fields, Olivetti, P.R., M.D. Fuller, Z.-R. Zhang, and **N.A. McCarty** (2005) Inhibition of ClC-2 Cl^- channels by a peptide component of scorpion venom. *J. Membr. Biol.* **208**: 65-76.

3) Fuller, M.D., Z.-R. Zhang, G. Cui, and **N.A. McCarty** (2005) The block of CFTR by scorpion venom is state-dependent. *Biophys. J.* **89**: 3960-3975.

C. Grant submission

Consistent with CFF policy, we submitted a revised proposal to the NIH for the next phase of work with GaTx1 within the first month of Foundation funding. Principal Investigator, National Institutes of Health, "Peptide inhibitors probe structure and function in chloride channels", 1-R01-DK075016-01A1. The proposal earned a score of 1.6 at study section, but did not reach funding. A resubmission is planned for this year.

III. Publications resulting from this CFF support

A. Papers

1) Fuller, M.D., C.H. Thompson, Z.-R. Zhang, C. Freeman, B. Sarkadi, G. Szakacs, D. McMaster, R.J. French, J. Pohl, J. Kubanek, and **N.A. McCarty** (2007) State-dependent inhibition of CFTR chloride channels by a novel peptide toxin. *J. Biol. Chem.* **282**:37545-37555.

2) Thompson, C.H., P.R. Olivetti, M.D. Fuller, D. McMaster, R.F. French, J. Pohl, J. Kubanek, and **N.A. McCarty**. Isolation of a peptide toxin inhibitor of ClC-2 voltage-gated chloride channels. *Proc. Natl. Acad. Sci., USA (invited for revision)*

3) Fuller, M.D., C.H. Thompson, and **N.A. McCarty**. Pharmacology of the CFTR chloride channel. (*in preparation*)

B. Abstracts

1) Fuller, M.D., and **N.A. McCarty** (2006) GaTx1: A novel peptide toxin inhibitor of CFTR chloride channels. *Pediatric Pulmonology* Suppl. 29: 222.

2) Thompson, C.H., M.D. Fuller, J. Pohl, J. Kubanek, and **N.A. McCarty** (2007) GaTx2: a novel peptide inhibitor of ClC-2. *Biophys. J.* **92**: 272a.

3) Fuller, M.D., C.H. Thompson, J. Pohl, J. Kubanek, and **N.A. McCarty** (2007) GaTx1: a novel peptide toxin inhibitor of CFTR. *Biophys. J.* **92**: 275a.

C. Presentations

1) "Unique tools for the study of CFTR and ClC-2 airway epithelial chloride channels," Department of Pediatrics, Emory University School of Medicine, September 21, 2006.

2) "Finally: two novel peptide toxins active at chloride channels, CFTR and ClC-2," Membrane Biophysics Subgroup of the Biophysical Society, Baltimore, MD, March 3, 2007.

D. News release (CFF-approved)

http://www.gatech.edu/newsroom/release.html?id=1717
and subsequent news releases, such as:
http://www.eurekalert.org/pub_releases/2008-02/giot-pdi021408.php
http://www.sciencedaily.com/releases/2008/02/080214114521.htm
http://www.scienceblog.com/cms/scorpion-venom-may-hold-key-cystic-fibrosis-fix-15478.html

IV. Intellectual property/Patents

In May of 2006, we submitted a provisional patent application through Georgia Tech's Office of Technology Licensing (GTRC project number 3872, filed May 19, 2006). This was necessary because Matt Fuller, the then-PhD student whose dissertation work resulted in isolation of the toxin, was scheduled to defend his dissertation in late May. We wanted to be sure to protect this piece of technology before making any public dissemination. At that time, we had not yet begun using the support for this project provided by the Foundation (in essence, we completed the isolation before starting to use the CFF's support). However, the subsequent characterization of GaTx1 has been fully supported by our CFF grant. Therefore, we imagined that the Foundation would be interested in helping support the submission of a full patent for this technology. With input from you (Penland) and Melissa Ashlock, Diane Wetmore arranged to help with the costs of filing. A full patent application was filed as follows. We also received support from the CFF for help with filing costs for a patent application on GaTx2, a toxin which inhibits CIC-2 channels with incredibly high affinity.

1) "ABC Transporter Ligand", for a peptide toxin that interacts with the CFTR chloride channel, filed May 18, 2007, International PCT number PCT/US07/69243. Inventors: N.A. McCarty, M.D. Fuller, and J. Kubanek. Patent held by Georgia Tech Research Corporation.

2) "ClC Channel Ligand", for a peptide toxin that interacts with the ClC-2 chloride channel, filed July 12, 2007, International PCT number PCT/US07/73325. Inventors: N.A. McCarty, C.H. Thompson, and J. Kubanek. Patent held by Georgia Tech Research Corporation.

This concludes our final scientific report. Please let me know if you require anything else. We greatly appreciate the Foundation's support of our work.

Best regards,

Nael A. McCarty, Ph.D.
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And Senior CF Scientist
Emory University
Associate Director for Research,
Emory University Cystic Fibrosis Center
Adjunct Professor of Biology, Georgia Tech
Adjunct Professor of Physiology, Emory University