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TITLE: Translation of Novel Serotonin 5-HT7 Agonist Drug Candidates in Rodent Models of Fragile X Syndrome

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14. ABSTRACT

The objective of this grant is to synthesize 5-PAT-type 5HT7 receptor agonists and assess their effectiveness to correct FXS phenotypes in Fmr1-KO mice and other mouse models of FXS symptoms. We completed several objectives as described in the Statement of Work. We successfully synthesized 35 novel 5-PAT analogs, and determined their affinities at the human 5HT7 receptor. Seven compounds (including two racemates) met 5HT7 affinity potency criterion ($K_i \leq 25$ nM) for further pharmacological assessment. Five of seven were tested in functional assays, and each was a 5HT7 agonist, as determined by 5HT7-Gs-cAMP signaling in HEK293 cells stably expressing the human 5HT7 receptor; the racemates were not tested. All seven compounds were screened for off-target affinity. One compound met criteria for ≥ 10-fold 5HT7 selectivity, and scale up synthesis is currently underway to advance this compound for *in vivo* assessment.

15. SUBJECT TERMS
5HT7 agonist; off-target activity; 5HT1A

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INTRODUCTION:

The subject of this research is preclinical drug discovery and development for Fragile X syndrome. The purpose is to design, synthesize, and test novel 5-phenylaminotetralin compounds targeting serotonin type 7 (5HT7) receptors as medication candidates for Fragile X syndrome. The scope of this research includes medicinal chemical syntheses, *in vitro* molecular pharmacology, and *in vivo* behavioral pharmacology in mouse models of core symptoms of Fragile X syndrome.

KEYWORDS:

Serotonin, 5HT7 receptor, Fragile X syndrome, agonist, drug discovery and development, preclinical, molecular pharmacology, cAMP signaling, radioligand binding, phenylaminotetralin

ACCOMPLISHMENTS:

What were the major goals of the project?

Major Goal 1: Design and synthesize 46, 5-PAT-type ligands (23 racemates separated into 46 single enantiomers). Target date ≤ 12 months: 100% completed*.
Major Goal 2: Assess molecular pharmacology of 5-PATs. Target date ≤ 13 months. Part 1 of Statement of Work (SOW): 100% completed. Part two of SOW: 83% completed. Part 3 and Part 4 of SOW: 64% completed.
Major Goal 3: Translate 5-PATs in mouse models of Fragile X syndrome. Target date ≤ 18 months: 8% completed.
*12 racemates have not been separated into single enantiomers, as they showed no 5HT7 selectivity, or their affinities were >10x the cut-off for 5HT7 potency criterion; thus, single enantiomers would not meet criterion for further testing. Separation of these 12 racemates would yield 47 compounds.

What was accomplished under these goals?

1) Major Activities: Chemical synthesis and *in vitro* molecular pharmacology screening of 35 novel 5-PATs.

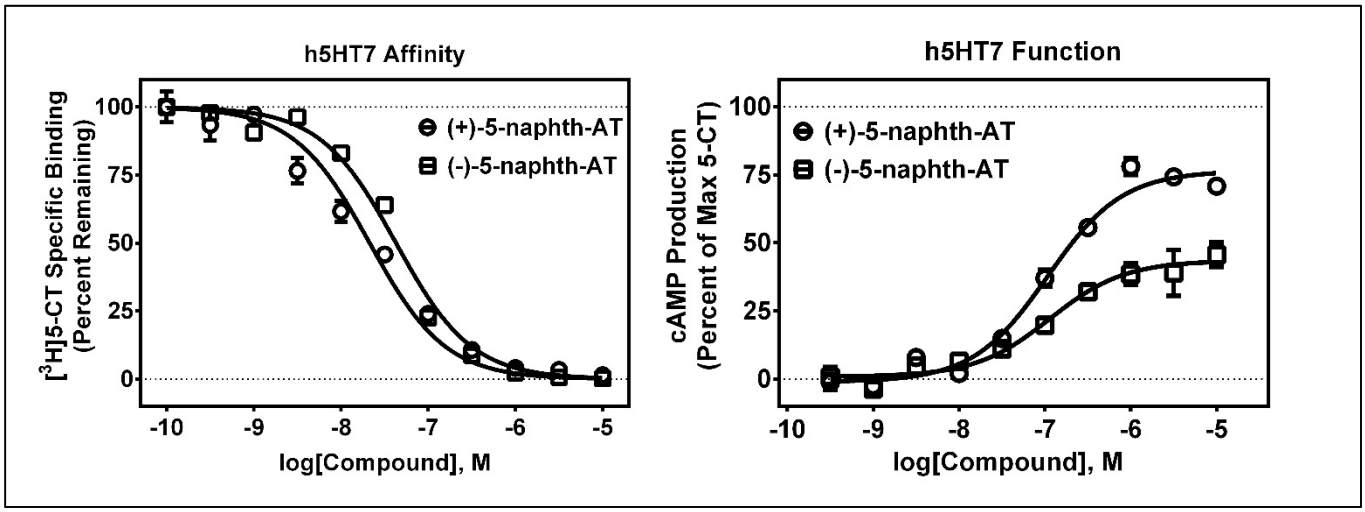
2) Specific Objectives: We synthesized 35 unique 5-PAT compounds. We assessed their affinity (K_i) at the human 5HT7 receptor. Based on criteria for lead identification, we discovered two new 5HT7 receptor agonists.

3) Significant Outcomes: Seven of the compounds we synthesized met affinity criterion ($K_i \leq 25$ nM; emboldened in table above), and five were tested in 5HT7 functional assays, measuring 5HT7-Gαs-cAMP; two compounds that met criterion were racemic, and therefore were not tested in functional assays. [Racemates are two stereoisomers, and one isomer may affect the function of the other.] All five compounds were agonists. Based on our objective criterion, 5HT7 agonists with $K_i \leq 25$ nM were tested for affinity at off-targets. All seven compounds were screened for affinity at at least one relevant off-target, the 5HT1A receptor. Three compounds were screened at all off-targets, i.e. 5HT1A, 5HT2A, 5HT2B, 5HT2C, H1, D2.

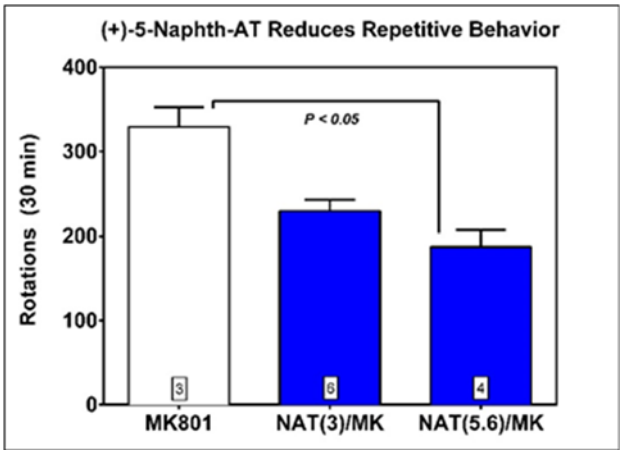
5-PAT	Median Affinity (K_i in nM)						
	5HT7	**5HT1A**	**5HT2A**	**5HT2B**	**5HT2C**	**H1**	**D2**
(+)-5-PAT	>1000	959	752	27	202	>1000	n.d.
(—)-5-PAT	**10**	6	535	66	547	>1000	>1000
(+)-o-Cl-5-PAT	**6**	36	569	11	100	630	>1000
(—)-o-Cl-5-PAT	45	235	>1000	n.d.	>1000	>1000	>1000
(*S*)-o-F-5-PAT	**6**	24	830	67	268	>1000	720
(—)-o-F-5-PAT	463	905	130	674	>1000	>1000	>1000
(±)-m-F-5-PAT*	28	20	759	96	196	608	n.d.
(±)-di-m-F-5-PAT*	175	234	>1000	110	433	648	n.d.
(±)-m-Cl-5-PAT*	**17**	101	121	17	30	641	n.d.
(±)-di-m-Cl-5-PAT*	433	>1000	>1000	96	272	>1000	n.d.
(±)-m-CF_3-5-PAT*	718	>1000	299	38	284	948	n.d.
(+)-di-m-CF_3-5-PAT	>1000	>1000	>1000	n.d.	604	>1000	n.d.
(—)-di-m-CF_3-5-PAT	853	>1000	>1000	n.d.	>1000	>1000	n.d.
(±)-m-methoxy-5-PAT*	185	n.d.	410	310	894	710	n.d.
(±)-p-F-5-PAT*	140	25	888	313	786	>1000	n.d.
(±)-5-anthracene-AT*	395	>1000	776	53	126	623	n.d.
(±)-o-benzyl-5-PAT*	>1000	>1000	460	90	629	103	n.d.
(+)-7,8-methoxy-5-PAT	>1000	n.d.	129	231	>1000	n.d.	989
(—)-7,8-methoxy-5-PAT	>1000	n.d.	158	52	870	n.d.	>1000
(±)-5-cyclopentyl-AT*	249	100	692	n.d.	188	n.d.	n.d.
(+)-5-naphthalene-AT	**15**	180	906	n.d.	188	n.d.	n.d.
(—)-5-naphthalene-AT	**15**	69	>1000	n.d.	375	n.d.	n.d.
(±)-5-isoquinoline-AT*	103	648	n.d.	n.d.	>1000	n.d.	n.d.
(+)-5-(2'-furanyl)-AT	>1000	997	n.d.	n.d.	n.d.	n.d.	n.d.
(—)-5-(2'-furanyl)-AT	31	14	n.d.	n.d.	n.d.	n.d.	n.d.
(±)-o-NH_3-5-PAT*	94	601	n.d.	n.d.	n.d.	n.d.	n.d.
(+)-p-Cl-5-PAT	83	14	n.d.	n.d.	n.d.	n.d.	n.d.
(—)-p-Cl-5-PAT	152	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
(±)-p-F-o-Cl-5-PAT	**16**	33	n.d.	n.d.	n.d.	n.d.	n.d.
(+)-N,N-dipropyl-5-PAT	138	20	n.d.	n.d.	n.d.	n.d.	n.d.
(—)-N,N-dipropyl-5-PAT	89	12	n.d.	n.d.	n.d.	n.d.	n.d.
(+)-N,N-dipropyl-o-F-5-PAT	31	12	n.d.	n.d.	n.d.	n.d.	n.d.
(—)-N,N-dipropyl-o-F-5-PAT	66	52	n.d.	n.d.	n.d.	n.d.	n.d.
(+)-N,N-dipropyl-p-Cl-5-PAT	373	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
(—)-N,N-dipropyl-p-Cl-5-PAT	>1000	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

*No 5HT7 selectivity or affinity >10x cut-off for 5HT7 potency criterion, thus, not resolved into single enantiomers.
n.d. = not determined

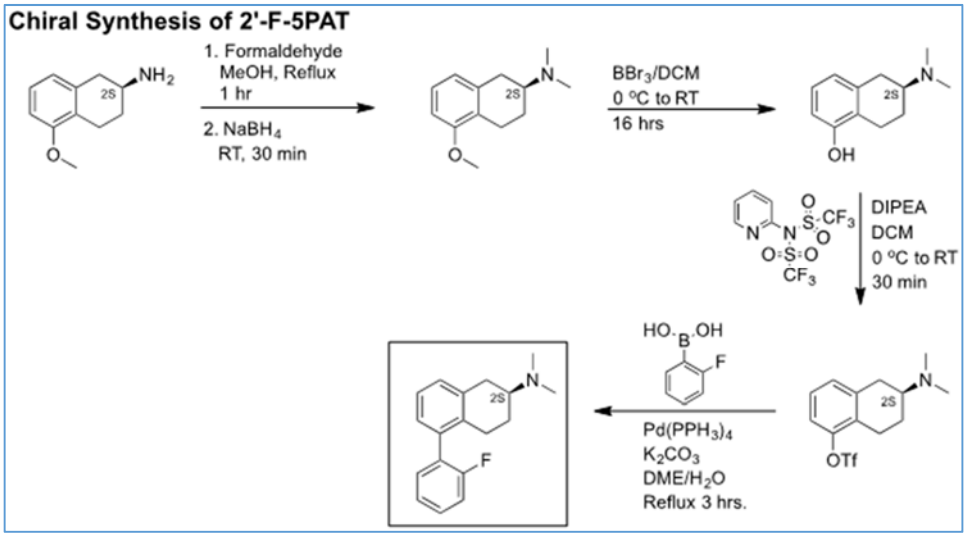
4) Major findings include the discovery of two new, selective 5HT7 agonists, one with ≥ 10-fold 5HT7 selectivity (+)-5-naphthalene-AT (NAT or (+)-5-Naphth-AT), and the other with 5-fold 5HT7 selectivity, (—)-5-naphthalene-AT (Figure below). (+)-5-NAT met criteria defined in the SOW for advancement to *in vivo* studies—with the exception that determinations of affinity values at 5HT2B, H1, and D2 receptors are still in progress. Scale up synthesis is currently underway to advance (+)-5-NAT for *in vivo* assessment in all assays. (+)-5-NAT was assessed in the MK801 model of repetitive behavior.



5) Preliminary results show (+)-5-Naphth-AT is active at reducing repetitive behavior caused by MK801-mediated glutamate NMDA receptor inactivation (See Figure on right; numbers in squares equal number of animals per group).



6) A chiral (asymmetric) synthetic route was developed to avoid the need to separate racemic mixtures of 5-PATs.



Succinct Methodology for Molecular Pharmacology: Assays measuring ligand affinity were performed with membrane preparations of GPCRs expressed in HEK293 or CHO-K1 clonal cells, labeled with tritiated radioligands. For measurements of 5HT7-Gαs-cAMP signaling in HEK293 cells stably expressing high density (~5 pmol/mg protein) 5HT7 receptors, the Lance Ultra cAMP kit (Perkin-Elmer, MA, USA) was used.

Succinct Methodology for MK801 Repetitive Behavior: Adult, male C57BL/6J mice were injected subcutaneously with vehicle or (+)-5-Naphth-AT (3 or 5.6 mg/kg). 10 min later, mice were injected with MK801 (0.3 mg/kg), and placed in a 43 x 43 cm open field. Video monitoring and calculation of number of rotations (360 degree, circling behavior) in 30 min was performed by Ethovision XT software. Data were analyzed using One-Way ANOVA with GraphPad Prism 6.0 software.

We have currently not met the goal of testing lead medication candidates in the FMR1 or the 5HT7 knockout mouse models (Target date ≤ 18 months).

Succinct Methodology for Chemical Syntheses:

Synthesis of the lead (+)- and (–)-5-naphthyl-2-dimethylaminotetralin (5-naphth-AT). 5-bromo-1-tetralone was reduced with sodium borohydride to give the corresponding alcohol that underwent an acid catalyzed dehydration to obtain the C(1)–C(2) olefin compound. *m*-Chloroperbenzoic acid was reacted with the olefin to obtain the C(1)–C(2) epoxide that underwent an acid catalyzed epoxide opening to yield 5-bromo-2-tetralone[59]. Suzuki-Miyaura cross coupling with naphthylboronic acid, followed by reductive amination with dimethylamine, gave racemic 5-naphth-AT, that was converted to the hydrochloride (HCl) salt for characterization ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.55-1.63 (m, 1H), 1.98-2.07 (m, 1H), 2.32-2.41 (m, 1H), 2.49-2.52 (m, 1H), 2.58 (s, 6H), 2.97-3.06 (m, 2H), 3.17-3.23 (m, 1H), 7.12 (dd, *J*= 7.5, 6.5 Hz, 1H), 7.21-7.23 (m, 1H), 7.25-7.28 (m, 2H), 7.31 (dd *J*= 6.0, 1.0 Hz, 1H), 7.34-7.4 (m, 1H), 7.44-7.54 (m, 2H), 7.89 (dd, *J*= 18.0, 8.5 Hz, 2H); 12.82 [bs, 1H] The free base racemate was resolved to (+)- and (–)-5-napth-AT by semi-preparative polysaccharide-based chiral stationary phase (CSP)-HPLC[60] (EtOH:Hexane [15:85] + 0.1% of diethylamine modifier + 0.1% trifluoroacetic acid

modifier; flow rate = 2.0 mL/min), and the HCl salt form of each enantiomer was characterized for optical (stereochemical) purity: (+)-5-FPT: CSP-HPLC t =16.15 min, $[\alpha]^{22}_{D}$ (Perkin Elmer 343 series polarimeter) = (+) 2.42º (c 0.1, CH_2Cl_2); (–)-5-FPAT: CSP-HPLC t = 21.05 min, $[\alpha]_{25}^{D}$ = (–) 2.94º (c 0.1, CH_2Cl_2).

Chiral (asymmetric) Synthesis of (+)-S-5-(2'fluorophenyl)-2-dimethylaminotetralin. (*S*)-5-methoxy-1,2,3,4-tetrahydronaphthalen-2-amine under went reductive alkylation with formaldehyde to give (*S*)-5-methoxy-*N*,*N*-dimethyl-1,2,3,4-tetrahydronaphthalen-2-amine, which was then treated with BBr_3 to convert the 5-methoxy group to the corresponding alcohol. The alcohol was treated with N-(2-Pyridyl)bis(trifluoromethanesulfonimide) to convert the alcohol to (*S*)-6-(dimethylamino)-5,6,7,8-tetrahydronaphthalen-1-yl trifluoromethanesulfonate. The sulfonate underwent Suzuki-Miyaura cross coupling, along with 2'-F-benzylboronic acid, to give (+)-S-5-(2'fluorophenyl)-2-dimethylaminotetralin, which was converted to the hydrochloride (HCl) salt for characterization. ^{1}H NMR (500 MHz, $CDCl_3$): 1.78-1.92 (m, 1H), 2.38 (d, J= 11.5 Hz, 1H), 2.58-2.74 (m, 1H), 2.76-2.96 (bs, 7H), 3.16-3.21 (m, 1H), 3.32-3.42 (m, 1H), 7.11-7.13 (m, 1H), 7.18-7.27 (m, 4H), 7.35-7.39 (m, 1H).

What opportunities for training and professional development has the project provided?

This project provided training to several students, including:

Postdoctoral trainees—Yue Liu (now scientist at Pfizer)
Ph.D. candidates—Charles Perry and Daniel Felsing (earned Ph.D. in Medicinal Chemistry during project; now post-doc at University of Texas, Galveston)
Masters candidates—Yiming Chen, Hima Patel, Kirin Gada
Undergraduates—Jessica Mecklosky (earned B.S. in Neuroscience during the project), Jiaxing Guo, Christopher Chang

Dr. Booth provided training for Charles Perry and Daniel Felsing; Dr. Canal provided training for the remaining students and Dr. Liu.

As part of his professional development, Dr. Canal participated in the Training Seminar by Terrence Kenakin, Ph.D. "Applying Pharmacology to New Drug Discovery" (06/15/16-06/16/16, Westin Boston Waterfront Hotel, Boston, MA).

How were the results disseminated to communities of interest?

Results were presented to the founders of FRAXA, Fragile X research foundation (http://www.fraxa.org/), Katie Clapp and Michael Tranfaglia, as well as to the (now former) President of DELSIA (Delivering Science Innovation for Autism) and Vice President, Innovative Technologies at Autism Speaks, Daniel Smith, who expressed interest in the technology. Both Dr. Smith and Dr. Tranfaglia have since given their support, as consultants for our newly established LLC, Seropeutics, a biotechnology start-up company aimed to deliver novel medications for Fragile X syndrome.

What do you plan to do during the next reporting period to accomplish the goals?

Lead candidates, meeting *in vitro* molecular pharmacology criteria will be tested in preclinical models of core symptoms of Fragile X syndrome.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We are the first to show that compounds that activate the serotonin 5HT7 receptor can reduce repetitive behavior and enhance social interactions, behaviors impacted in autism and Fragile X syndrome. Notably, there are no

pharmacotherapies approved for core symptoms of autism or Fragile X syndrome, thus, our technology offers a potential, therapeutic breakthrough.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

The project has made a direct impact on commercial technology. The project led to the discovery and preclinical development of novel serotonin 5HT7 receptor agonists for Fragile X syndrome. Discussions are underway with Seropeutics LLC, a Boston-based start-up biotechnology company (co-founded by the MPI Raymond G Booth), to commercialize the drug candidates.

What was the impact on society beyond science and technology?

The project will impact society—improving the lives of persons with Fragile X syndrome—if we obtain additional resources to advance our compounds to clinical trials for Fragile X syndrome, and if the lead candidate medication is efficacious in clinical trials.

CHANGES/PROBLEMS:

Changes in approach and reasons for change.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them.

Nothing to Report

Changes that had a significant impact on expenditures.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Nothing to Report.

Significant changes in use or care of human subjects.

Nothing to Report.

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

PRODUCTS:

Presentations.

Canal, C.E., Felsing, D.E., Perry, C.K., Liu, Y., Booth, R.G. Preclinical development of a serotonin (5-HT)7 and 5-HT1A partial agonist for autism. 6th Cisbio HTRF symposium (Brewster, MA), September 14-17, 2015. *Acknowledged DOD funding*.

Teaching Lectures.

Canal, C.E. Research on Autism: Neurobiology to Treatments. Health 1555, Northeastern University (Boston, MA), Pharmacy program, November 17, 2015.

Journal publications.

Canal, C.E., Felsing, D., Liu, Y., Zhu, W., Wood, J., Perry, C.K., Vemula, R., Booth, R.G. (2015). An orally-active phenylaminotetralin-chemotype serotonin 5-HT7 and 5-HT1A receptor partial agonist that corrects motor stereotypy in mouse models. *ACS Chemical Neuroscience*, *6*, 1259-1270 PMID: 26011730. *Acknowledged NIH support*; this paper was published a few months before receiving DOD funding.

Books or other non-periodical, one-time publications.

Daniel Felsing PhD Thesis (May, 2016): Drug discovery targeting serotonin G protein-coupled receptors for neuropsychiatric disorders.

Other publications, conference papers, and presentations.

Nothing to Report.

Website(s) or other Internet site(s)

A website for Seropeutics LLC (see above under Technology Transfer) is under development.

Technologies or techniques

Newly discovered 5HT1A and 5HT7 receptor agonists, as summarized in the table above. These products are patent-protected (see below).

Inventions, patent applications, and/or licenses Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

U.S. Pat. No. 9,422,229 issued on August 23, 2016.

Other Products

Nothing to Report beyond descriptions noted above.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project? Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Clinton Canal, no change
Raymond Booth, no change
Charles Perry, no change
Daniel Felsing, PhD graduate student worked approximately 1 person month

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Clinton Canal, PI, received a new award on 07/01/2016. His percent effort on the DOD project is unchanged.

1R21DA040907-01 (Canal, Murnane), 07/01/2016-06/30/2018, 7 calendar months (effort), NIH/NIDA. “Receptor Pharmacology and Toxicology of Second-Generation Pyrrolidine "Bath Salt" Cathinones”

There is no overlap of the above project with the DOD project.

What other organizations were involved as partners? If there is nothing significant to report during this reporting period, state "Nothing to Report." Describe partner organizations - academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) - that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed. Provide the following information for each partnership: Organization Name: Location of Organization: (if foreign location list country) Partner's contribution to the project (identify one or more). Financial support; In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff); facilities (e.g., project staff use the partner's facilities for project activities); Collaboration (e.g., partner's staff work with project staff on the project); Personnel exchanges (e.g., project staff and/or partner's staff use each other's special reporting requirements facilities, work at each other's site); and Other.

Nothing to report.

SPECIAL REPORTING REQUIREMENTS

Nothing to Report.

APPENDICES:

None.