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none

14. ABSTRACT

A member of the ETV/PEA3 subfamily of Ets transcription factors, ETV4 is necessary for anchorage-independent growth of prostate cancer cells and regulates the epithelial-to-mesenchymal transition. Additionally, a recent study demonstrated that activation of ETV4 is occurs alongside activation of Ras and PI3K pathways in metastatic prostate cancer and, importantly, that genetic ablation of ETV4 significant decreases metastasis. Synthetic modulators that perturb ETV4 function have high value as mechanistic probes to dissect the role of this protein in primary tumor growth and the transition to metastatic phenotypes, for which there is conflicting data. The underlying hypothesis for this project is that a small molecule that blocks the ability of ETV4 to function as a transcriptional regulator will correspondingly inhibit key invasion pathways necessary for prostate cancer metastasis. We successfully leveraged our expertise in this area to discover the first inhibitors of ETV4 by targeting a key binding partner, the coactivator Med25.

15. SUBJECT TERMS
ETV4, Med25, prostate cancer, small molecule inhibitor

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1) INTRODUCTION

Dysregulation of the protein-protein interaction (PPI) networks that underpin gene transcription is widespread in many human diseases and is particularly well-characterized in cancer.[1,2] The focus of our proposed work has been dissecting the role of one such network, the complex formed between the transcriptional activator ETV4 and the coactivator Med25. ETV4 becomes highly active in many cancers and turns on invasion and migration pathways. A member of the ETV/PEA3 subfamily of Ets transcription factors, ETV4 is necessary for anchorage-independent growth of prostate cancer cells and regulates the epithelial-to-mesenchymal transition.[3,4] Additionally, a recent study demonstrated that activation of ETV4 occurs alongside activation of Ras and PI3K pathways in metastatic prostate cancer and, importantly, that genetic ablation of ETV4 significant decreases metastasis.[5] Synthetic modulators that perturb the ETV4-Med25 complexes in cells and in vivo have high value as mechanistic probes to dissect the role of this complex in primary tumor growth and the transition to metastatic phenotypes, for which there is conflicting data. Thus, the ETV4•Med25 complex is an outstanding target for inhibitor development. Further supporting this approach are the many innovative and successful screening strategies for targeting PPIs that have recently emerged, including compounds now in late stage clinical trials.[1] The underlying hypothesis for this project is that a small molecule that blocks the ability of ETV4 to function as a transcriptional regulator will correspondingly inhibit key invasion pathways necessary for prostate cancer metastasis. We proposed to use our expertise in small molecule modulators of transcription factors to discover the first inhibitors of ETV4 through targeting its key binding partner, Med25, and test the inhibitors in a battery of in vitro and cell-based experiments.

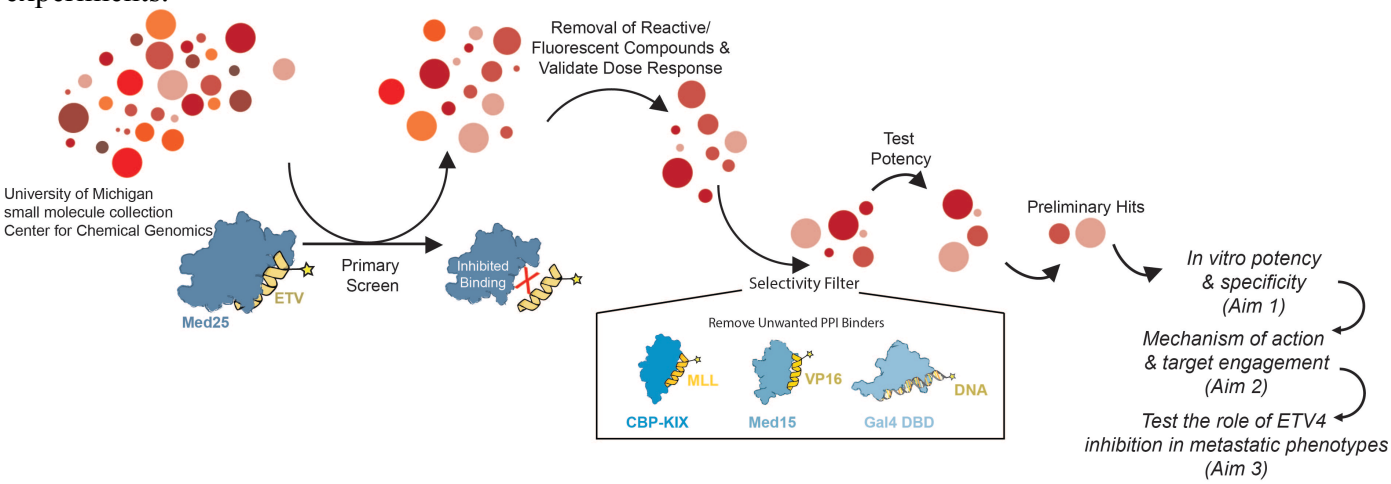


Figure 1 Planned workflow to identify and validate inhibitors of ETV4•Med25 AcID from the UM Center for Chemical Genomics small molecule collection. As noted in the original proposal and SOW, stringent criteria in terms of potency and specificity were planned to carry through the very best quality molecules to more complex studies in Aim 2 and 3.

2) KEYWORDS

ETV4, MED25, AcID, prostate cancer

3) ACCOMPLISHMENTS

This was a productive 12-month period. Highlights include:

- The very first small molecule inhibitors of ETV4 were discovered;
- The mechanism of action and target engagement of the first inhibitors was delineated, and impacts on ETV4-dependent processes (cell migration, for example) were assessed;
- A new allosteric network within Med25 was revealed through the mechanism of action studies, opening a path for the discovery of allosteric modulators of the ETV4-Med25 complex;

• A high-throughput screen of 171,000 compounds was completed to identify next generation molecules with improved potency and druglike properties, with six structurally distinct scaffolds now under investigation.

• One manuscript disclosing the first-in-class inhibitors is ready for submission (Sturlis, S.; Bruno, P.A.; Gray, F.; Cierpicki, T.; Mapp, A.K. *Manuscript in preparation).* A second manuscript based on the full high throughput screen is anticipated to be submitted in mid-2017.

Specific Aim 1: Identification of small molecule inhibitors of the ETV4•Med25 interaction

The **goals** in this Aim were carry out high throughput screens of the small molecule collections at the Center for Chemical Genomics at the University of Michigan to discover the first small molecule inhibitors of the ETV4•Med25 complex, including preliminary assessments of specificity and NMR characterization of the small molecule-Med25 complexes (Proposed timeline months 1-3; actual timeline: months 1-4).

Identification of first generation inhibitors

The first screen was carried out using optimal conditions briefly outlined in the original proposal [384-well plate format, final Med25 concentration 850 nM, tracer (fluorescein-labeled ETV transcriptional activation domain at a concentration of 20 nM, and small molecule concentration 20 μM](12). A screen of 2400 known bioactives (approved drugs, natural products, probe molecules) had a campaign Z' score of 0.87 and a hit rate of 1.6%, with a hit defined as active within three standard deviations above the negative control. Following hit filtering to remove compounds with known chemical reactivity, toxicity, broad activity, aggregation properties and native fluorescence we identified 3 compounds belonging to the depside and depsidone classes of small molecules (Figure 2a); we have previously found depsides and depsidones to be effective modulators of activator-coactivator interactions.[6] Dose-response experiments with re-purchased CCG-38381, CCG-38361, and CCG-40171 confirmed that the molecules were effective inhibitors of ETV4 binding to Med25, with CCG-38381 being the most potent of the three (Figure 2b). The molecules are also specific for the AcID motif, exhibiting no inhibition of either binding site of the related KIX domain, nor the ability of VP16 to bind to the coactivator Med15. A comparison of the structures of these molecules with other depsides and depsidones that did not emerge as hits suggests that key functional groups include the ortho phenolic aldehyde, a carboxylic acid positioned meta or para to the ring junction, and small hydrophobic groups along the rim of the rings. Based upon the modest effectiveness of CCG-40171 (perhaps due to hydrolytic instability) we eliminated it from further consideration, leaving two compounds advanced for further consideration (Aim 2 experiments).

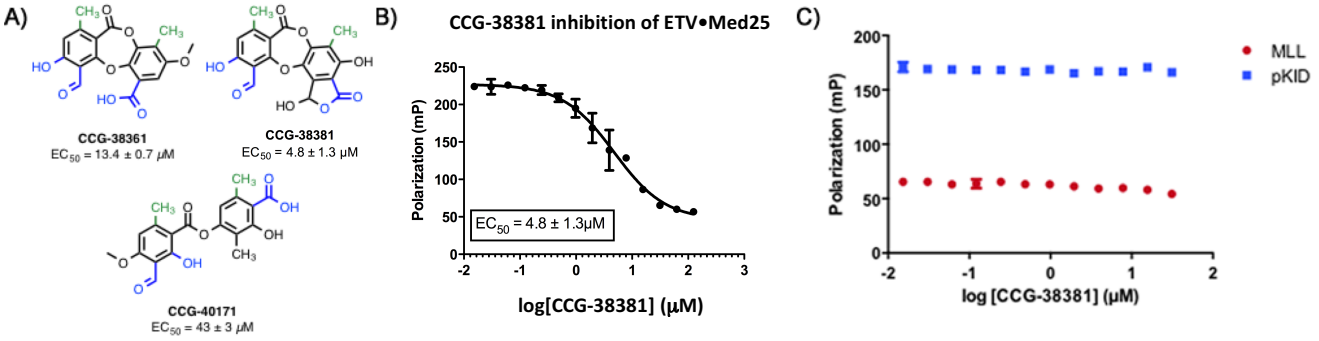


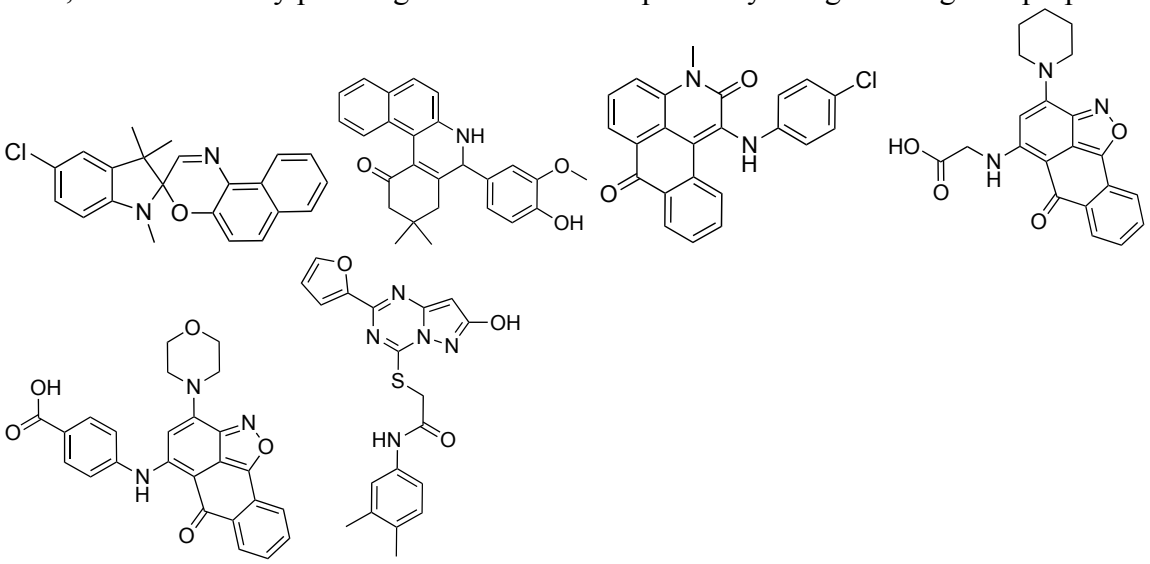
Figure 2. A) Structures of the three first ETV4•Med25 inhibitors along with EC_{50} values from full dose-response experiments. Functional groups colored in blue and green are likely to be key for binding and inhibition, when compared to related depside and depsidones. B) Representative curve of CCG-38381 inhibition against the Med25•ETV transcriptional activation domain complex (average of 3 independent experiments). C) Representative experiment with CCG-38381 showing no inhibition against another coactivator motif, CBP KIX, and two of its binding partners, MLL and the phosphorylated KID domain of CREB (pKID).

As outlined in the original proposal and statement of work, the next task was to carry out HSQC experiments with labeled Med25 to identify the binding sites of the small molecules. This was accomplished, with assistance through a standing collaboration with Prof. Tomasz Cierpicki. As shown in Figure 3, the small molecules induce chemical shift perturbations (red) that overlap with those of ETV activator binding to Med25 (blue). We particularly noted that CCG-38381 altered a flexible, lysine-rich loop that connects the two binding sites, investigated more completely in the Aim 2 studies. Particularly exciting was that, like the native activator, CCG-38381 induces changes in both binding surfaces of Med25 AcID, suggesting that the molecule has a mixed orthosteric/allosteric mechanism of inhibition.

Figure 3 NMR HSQC perturbation data of ETV•Med25 AcID (blue) and CCG-38381 (red). Figure derived from PDB 2XNF.

Identification of second generation inhibitors

As outlined in the statement of work, one goal was to screen a wider array of compounds. The funding provided from Grant #PC141132 was sufficient for 121,000; in order to obtain more complete coverage and leverage the opportunity for finding effective compounds, we expanded this to 171,000 structures (the complete collection of the Center for Chemical Genomics) by using a rainy-day fund not earmarked for any specific project. This expansion required the preparation of additional reagents (protein, tracer), which increased the time needed for completion. Additionally, because we noted allosteric changes induced by the first generation inhibitors, we increased the rigor of our secondary filters for the full screen in order to enrich the hits in allosteric modulators, likely to be more specific and more potent. After dose-response validation, removal of PAINS structures, and toxicity filtration as outlined in the SOW, 64 compounds were verified as hits and approximately 34 compounds with pAC_{50}s below 5 and commercially available were identified. Of these, we are currently pursuing 6 with excellent specificity and good drug-like properties:



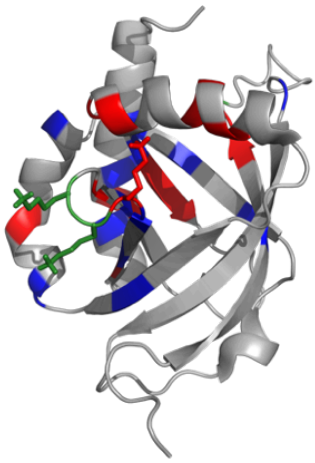
In the original SOW, the goal was to have 10 structures with sub-micromolar IC_{50} values for subsequent studies. The first generation hits emerged above this cutoff but the good specificity profile, the 5 μM activity of CCG-38381, and good cellular permeability properties, CCG-38381 was carried forward as a tool compound. Nonetheless, the second generation hits are significantly improved and represent a variety of chemotypes. Thus, these will be the primary reagents used in final experiments to complete the work in Aims 2 and 3.

Specific Aim 2: Target engagement with Med25 in cells

The **goal** in this Aim was to use a suite of experiments to define the mechanism of target engagement of small molecule inhibitors emerging from Aim 1 with Med25 AcID and to correlate cell-free activities with cellular values (proposed timeline Months 3-6; actual timeline Months 4-11). Somewhat complicating these experimental goals was that analysis of the first generation molecules revealed that they were likely covalent inhibitors of ETV4•Med25 and it was thus vital to identify the site(s) of covalent bond formation in order to establish both mechanism of action and estimate potential toxicity risks, as outlined in the original proposal and SOW.

The phenolic aldehyde present in all three of the first generation inhibitors is the likely point of bond formation and indeed an adduct was observable by mass spectrometry. The HSQC results shown in Figure 3 suggested that the lysine-rich loop was the logical site of modification. Initially proteomics experiments (in collaboration with expert Prof. Brent Martin at the University of Michigan) verified the formation of a 1:1 small molecule•Med25 adduct that was reducible with $NaBH_4$ treatment, suggesting the initial formation of an imine that is reducible. The initial adduct forms on a short time scale (complete in <5 minutes) and is not reversible on a multi-day time scale, as removal of excess small molecule by dialysis followed by long-term incubation in free solution does not lead to re-formation of free protein. Digestion of the small molecule•Med25 adduct with trypsin or chymotrypsin followed by analysis by MS-MS did not lead to identification of the site of modification. Evidently the small molecule adduct is remarkable resistant to proteolysis, although we can obtain excellent sequence coverage of the unmodified protein.

Given the challenges with mass spectrometric-based proteomics approaches, we used standard mutagenesis to identify the site of covalent bond formation for CCG-38381/CCG-38361. We examined more than 25 Med25 mutants and can provide full details if desired. Briefly, these experiments revealed two residues, lysines 518 and 519, that are the key sites of interaction with CCG-38381/CCG-38361 (shown in green in the adjacent structure). Mutation of those resides to arginine abolished covalent bond formation. The molecules do retain affinity for Med25 in the absence of this interaction, however, with an approximately 8-fold shift in IC_{50}. Consistent with this result, reduction of the aldehyde in CCG-3831 does not abolish inhibition, shifting the IC_{50} ~12-fold. Further supporting the specificity of the interaction between CCG-3831 with Med25, reaction of other aldehydes and phenolic aldehydes with Med25 does lead to labeling at lysines 518 and 519 but this does not lead to inhibition of ETV4 binding. The mechanism of action studies were estimated in the original SOW to be accomplished by month 6; given the unforeseen complexities that arose with the first generation molecules, these studies only recently concluded (month 11 of the project).



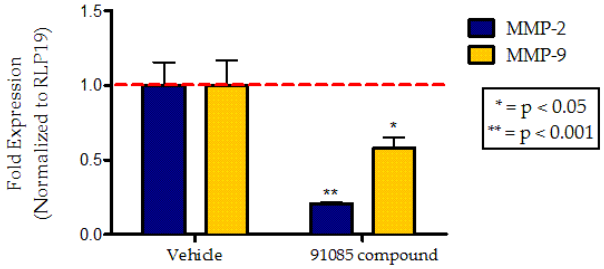


Figure 4. Compound 91085 mediates the knockdown of Med25-ETV dependent genes, MMP-2 and MMP-9, in MDA-MB-231 cells (6 h incubation time).

Cellular activity of candidate inhibitors

As the most potent of the first generation inhibitors, CCG-38381 was tested in a set of primary cellular assays to first assess if inhibition of Med25-dependent transcription tracked with binding experiments and also to test toxicity. As measured by qPCR, CCG-3831 inhibits the Med25-dependent gene HspA5; however, even at 5-fold excess over the in vitro IC_{50} (25 μM) only 20% inhibition is observed. Additionally, toxicity begins to emerge at concentrations above 25 μM, limiting the utility of this structure. Taken together, these

data indicated that CCG-38381 was not an appropriate candidate to move forward beyond these proof of principle experiments and instead, current efforts are focused on the newer chemotypes identified in the large high throughput screen that have much improved properties. At least preliminarily, we do see more significant effects in ETV-dependent genes with these molecules (Figure 4).

Specific Aim 3: *Testing the target: does pharmacological knockdown of ETV4 activity impair metastasis*
The goal in this Aim was to test the best hits that emerged from Aims 1 and 2 in a battery of cellular experiments to determine impact on metastatic phenotypes alone and in combination with PI3K and Ras pathway inhibitors (Months 7-12) Because hits that fulfill the selection criteria outlined in the Statement of Work have not yet emerged, these experiments are not complete. As proof of principle, we tested CCG-38381 in a migration assay (wound healing scratch plate assay) in MDA-MB-231 cells. As shown in Figure 5, CCG-38381 at 100 μM concentration did attenuate cell migration significantly after 24 hours. Nonetheless, the this is a concentration regime at which toxicity has emerged. Thus, we anticipate that the next generation, more potent molecules will have an even greater effect and enable completion of the planned experiments.

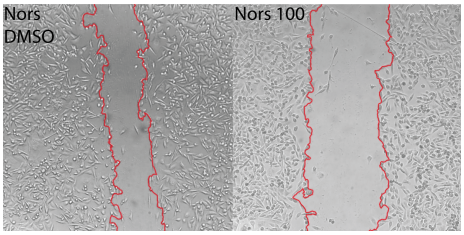


Figure 5. Wound healing assay with CCG-38381. In the left panel, cells were treated with vehicle (DMSO) and in the right panel with 100 μM CCG-3831.

Opportunities for training and professional development
Nothing to report (was not part of the original proposal).

Dissemination of results to communities of interest
As outlined in the introduction to this section, we have a completed manuscript to submit outlining our first generation inhibitors. Additionally, PI Mapp presented this work at ten scientific meetings and universities over the last 12 months (American Chemical Society meeting in San Diego, CA; National Medicinal Chemistry Symposium in Chicago; European Molecule Biology Laboratories in Heidelberg; University of Utah; University of Pennsylvania; University of Massachusetts; Notre Dame; Pacifichem; Tufts; University of Missouri; University of Pittsburgh). PI Mapp also gave a general audience talk to non-science industry leaders at the Life Sciences Institute, with the goal of illustrating the connection between basic research and impacts on human health. Further, PI Mapp ran a tutorial for European graduate students at ISOC (Ischia, Italy) in September 2016 that focused on how chemists can impact biology, with a particular focus on targeting protein-protein interactions such as the complex formed between ETV4 and Med25.

Plan during the next reporting period
This was a 12 month grant and this is the only report.

Selected citations

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5) 'ETV4 promotes metastasis in response to activation of PI3-kinase and Ras signaling in a mouse model of advanced prostate cancer.' Aytesa, A.; Mitrofanava, A.; Kinkade, C.W.; Lefebvre, C.; Lei, M.; Phelana, V.; LeKaye, H.C.; Koutcher, J.A.; Cardiff, R.D.; Califano, A.; Shen, M.M.; Abate-Shen; C. Proc. Natl. Acad. Sci. USA 2013, 110, E3506-E3515. doi: 10.1073/pnas.1303558110
6) Majmudar CY, Hojfeldt JW, Arevang CJ, Pomerantz WC, Gagnon JK, Schultz PJ, Cesa LC, Doss CH, Rowe SP, Vasquez V, Tamayo-Castillo G, Cierpicki T, Brooks CL, 3rd, Sherman DH, Mapp AK. Sekikaic acid and lobaric acid target a dynamic interface of the coactivator CBP/p300. Angewandte Chemie. 2012;51(45):11258-62. doi: 10.1002/anie.201206815. PubMed PMID: 23042634; PMCID: 3711073.

4) IMPACT

Impact on the principle discipline of the project

The metastasis of prostate cancer to is a major contributor to disability and death associated with advanced disease and is thus a fundamentally important area in which to develop innovative therapeutic strategies. Recently the Ets transcription factor ETV4 emerged as a critical player in the metastatic process. Activated alongside Ras and PI3K signaling pathways, ETV4 directly regulates cellular invasion programs and genetic ablation of ETV4 substantially decreases metastasis in vivo. **We have discovered the first small molecule inhibitors of ETV4, and in doing so demonstrated that this is a targetable transcription factor.** Our second generation and more potent small molecule inhibitors have excellent drug-like properties and thus have more tool compound and therapeutic potential.

Impact on other disciplines

Nothing to report

Impact on technology transfer

Nothing current to report, although it is a future goal.

Impact on society beyond science and technology

Nothing to report

5) CHANGES/PROBLEMS

Changes in approach

Nothing to report

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

As outlined in the Accomplishments section, we experienced one unanticipated challenge: the first generation ETV4 inhibitors proved to function through the formation of a covalent complex with Med25 and dissection of the mechanism of action proved to be complex and time consuming. Additionally, the first generation molecules (CCG-38381 in particular) showed dose-limiting toxicity; this is likely due to the redox-active functional groups present in the molecule. Although the official period of the project has ended, we are continuing the work outlined and are using monies from an unrestricted account to finish the project goals. We are happy to provide updates as this is completed.

Changes that had a significant impact on expenditures

None noted

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

None noted

6) **PRODUCTS**

Publications, conference papers or presentations

As outlined in the accomplishments section, we have one manuscript ready to submit describing the first generation inhibitor discovery and characterization.

PI Mapp presented this work at eleven scientific meetings and universities over the last 12 months (American Chemical Society meeting in San Diego, CA; National Medicinal Chemistry Symposium in Chicago; European Molecule Biology Laboratories in Heidelberg; University of Utah; University of Pennsylvania; University of Massachusetts; Notre Dame; Pacifichem; Tufts; University of Missouri; University of Pittsburgh).

Website(s) or other Internet site(s)

None noted

Technologies or techniques

None noted

Inventions, patent applications, and/or licenses

None noted

Other products

None noted

7) PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Personnel

Steve Sturlis, Ph.D.
Postdoctoral fellow
Nearest person month worked: 12
Contribution to the project: Dr. Sturlis performed all aspects of the experimental work.

Change in other support

None noted

Other organizations

None noted

8) SPECIAL REQUIREMENTS

Not applicable

9) APPENDIX

None included