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<b>14. ABSTRACT</b> Pheochromocytomas (PCC) are catecholamine-producing neuroendocrine tumors. Up to 30% give rise to metastases, for which there is no effective treatment. There are also no human PCC cell lines for pre-clinical drug testing. In this research period we used cultures of mouse pheochromocytoma(MPC) cell lines developed in our laboratory as the principal model for pre-clinical testing of strategies to improve the efficacy of existing chemotherapeutic drugs and for testing potential new drugs that might be used to treat patients with metastatic PCC. Findings with the mouse model were validated against primary cultures of human PCC. Cytotoxicity assays showing cooperative effects of topoisomerase 1 inhibitors with 5-azacytidine, an inhibitor of DNA methylation, were completed and results were submitted for publication. Cytotoxicity testing of a new type of drug, Gamitrinib, was also published as part of a collaborative study relating the mechanism of action of the drug to the <i>SDHB</i> gene, which when mutated is a major cause of metastatic PCC. Gamitrinib is highly toxic to both MPC cells and primary human PCC cells, and might prove to be both the most effective drug and the one most specifically targeting the defect in the majority of malignant PCC. Despite these positive results there are differences between MPC and human PCC cells in responsiveness to some drugs including 5-azacytidine, and a pressing need for human cell lines remains. However, human PCC cells immediately and completely cease proliferating when placed into conventional cell cultures. We extensively tested potential ways to propagate human PCC cells from primary tumors in mouse xenografts but these efforts were unsuccessful, even with cells that grew rapidly in a patient with widely metastatic tumor. A major focus in the next funding period will therefore be on testing novel approaches to developing human cell lines from primary cultures.					
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## **1. Introduction**

### **Purpose and scope of the research effort.**

Pheochromocytomas (PCC) are catecholamine producing neuroendocrine tumors that arise from chromaffin cells in the adrenal medulla or other parts of the peripheral sympathetic nervous system. Extra-adrenal PCCs are arbitrarily classified as paragangliomas (PGLs) (1). For convenience, the two are often referred to collectively as PCC/PGL. These tumors occur sporadically or in association with at least 10 hereditary diseases. Between 10% and 30% of give rise to metastases, for which there is currently no effective treatment. Metastasis is particularly likely when tumors harbor mutations of the *SDHB* gene.. A major deficiency in current treatment strategies is that they do not account for the fact that, in contrast to many other types of malignant tumor, metastatic PCC/PGLs usually grow very slowly and most of the cells are quiescent at any given time. Mitotic counts and expression of cell cycle markers both in primary tumors and in their metastases are often very low. Treatments that target replicating tumor cells or tumor angiogenesis have therefore met with only limited success. Patients with metastases often die from complications of catecholamine hypersecretion, or from invasive and expansile tumor growth that occur over many years. The need to improve treatment of metastatic PCC/PGLs requires new strategies and a valid experimental model for pre-clinical testing of those strategies. Development of a model has itself been hampered by failure to establish any human cell lines from the tumors for cell culture or xenograft studies, despite many efforts to establish them and several initially promising reports.

This project aims both to develop treatment paradigms targeting quiescent and replicating tumor cells in PCC/PGL metastases using existing models generated in the PI's laboratory, and to develop new models. Specific Aim 1 uses a mouse pheochromocytoma cell line (MPC) *in vitro* as a model to test new approaches to enhance the effectiveness of chemotherapeutic agents by optimizing the combination and timing of their use. Aim 2 uses primary cultures of human PCC/PGL cells derived from individual patient's tumors with diverse genetic backgrounds to validate the findings in Aim 1, and Aim 3 tests the ability of drug-administration paradigms developed in specific aims 1 and 2 to kill disseminated PCC *in vivo* using MPC cells as a model in nude mice. Aim 4 tests novel approaches to develop cell lines of human PCC/PGL for cell culture and xenografts, thereby providing a foundation for future studies. Because the tumors are rare, an important aspect of the project is collaboration with other investigators in order to maximize access to human tumors and to new drugs. Progress in this second funding period builds on results from the first year and the goals stated in the in the first year progress report

## **2. Key Words**

Pheochromocytoma, paraganglioma, cell culture, cell line, xenograft, nude mouse, chemotherapy, pre-clinical testing, camptothecin, 5-azacytidine, irinotecan, topoisomerase-1, bioluminescence, imaging, Gamitrinib

### 3. Overall Project Summary During the Second Year (15 August 2012- 14 September 2013).

Progress in this second funding period builds on results from the first year and reflects the goals for the second year as stated in Section III of the first year progress report. Those stated goals from the first year progress report are repeated below in italics. The actual tasks performed in the second year are enumerated below each goal.

*Goal 1. Marshal 3 papers thus far written, but not published, through the review process to publication.*

Task 1. Responded to critiques, performed additional experiments as required, resubmitted papers.

Results:

- One paper is submitted for publication reporting cell culture cytotoxicity studies of topoisomerase 1 (TOP1) inhibitors and 5-azacytidine against human and mouse pheochromocytoma (specific aims 1 and 2). These drugs were the major emphasis of the first funding period, as summarized in 2011-2012 Progress Report.

Powers JF, Fliedner S, Giubellino A, Pacak P, Tischler AS. Cooperative Cytocidal Activity of Camptothecin and 5-Azacytidine Against Pheochromocytoma Cells in Primary Cultures and Cell Lines Submitted to PLOS1

The paper shows that enhanced cytotoxic efficacy is achieved by combining 5-azacytidine, which increases transcriptional activity, with TOP1 inhibitors, which block enzymes that protect against DNA damage during transcription. For optimal timing, the two drugs are initially administered together and 5-azacytidine is then removed.

- Two papers are published reporting cell culture cytotoxicity studies of novel/alternative drugs against human and mouse pheochromocytoma (specific aims 1 and 2):

1. Chae YC, Angelin A, Lisanti S, Kossenkov AV, Speicher KD, Wang H, Powers JF, Tischler AS et al. Landscape of the mitochondrial Hsp90 metabolome in tumours. Nature Communications. 2013;4:2139

This paper in a high impact journal is based on toxicity of the new drug Gamitrinib toward human and mouse pheochromocytoma. Gamitrinib specifically targets the mitochondrial complex containing succinate dehydrogenase B (SDHB), the protein encoded by the *SDHB* gene. *SDHB* mutations are

responsible for many cases of metastatic PCC/PGL. The paper is therefore a major contribution to mechanistic understanding of the relationships of SDHB to mitochondrial organization and mechanisms of Gamitrinib as an anti-cancer drug.

2. Giubellino A, Bullova P, Nolting S, Turkova H, Powers JF, Liu Q, Guichard S, Tischler AS et al. Combined inhibition of mTORC1 and mTORC2 signaling pathways is a promising therapeutic option in inhibiting pheochromocytoma tumor growth: in vitro and in vivo studies in female athymic nude mice. *Endocrinology*. 2013;154:646-55.

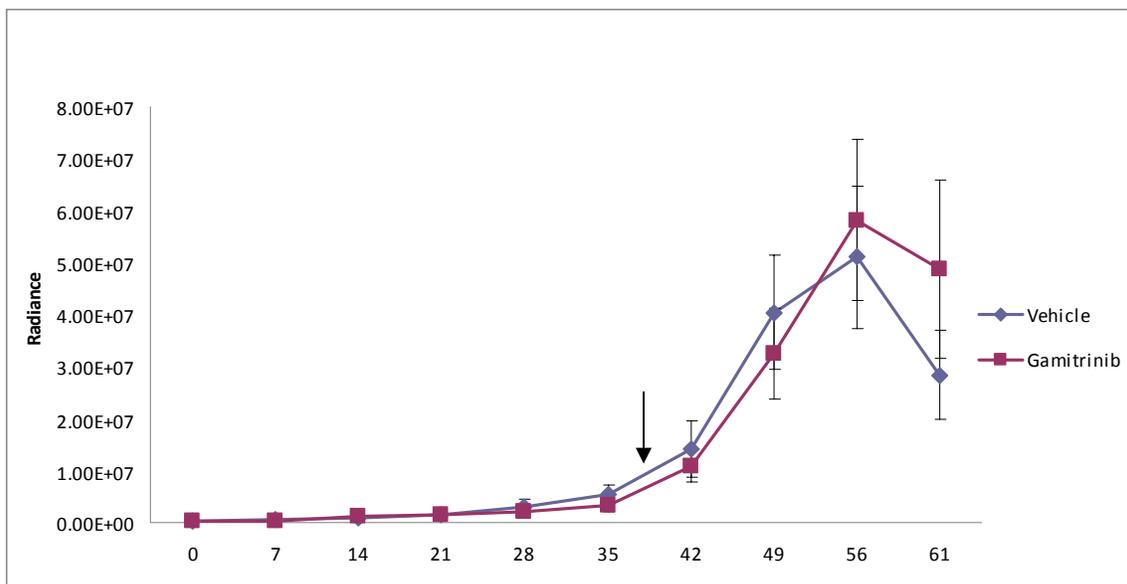
This paper highlights the possible use of new rapamycin-related drugs for treatment of metastatic pheochromocytoma

*Goal 2. Follow up promising results thus far obtained in specific aims 1 and 2*

Task 1. We tested the toxicity of Gamitrinib against disseminated MPC GL-9 cells in nude mice (specific aim 3). We first propagated sufficient cells to inject 20 mice,  $2 \times 10^6$  cells/mouse (~3 weeks), then waited for discrete tumor masses to form. (~4 weeks) and performed treatment, with interval luminescence imaging

Results:

- There was no detectable effect of Gamitrinib in vivo using the protocol tested (Fig 1). This study was based on one of two published protocols by our collaborators who developed Gamitrinib. It entailed a cycle of 3 days on followed by 2 days off treatment for 21 days ((2)). It has subsequently become clear that a continuous administration described in the second protocol is likely to be more effective, while still causing minimal toxicity (3). We intend to repeat this study with the second protocol in the next funding period.



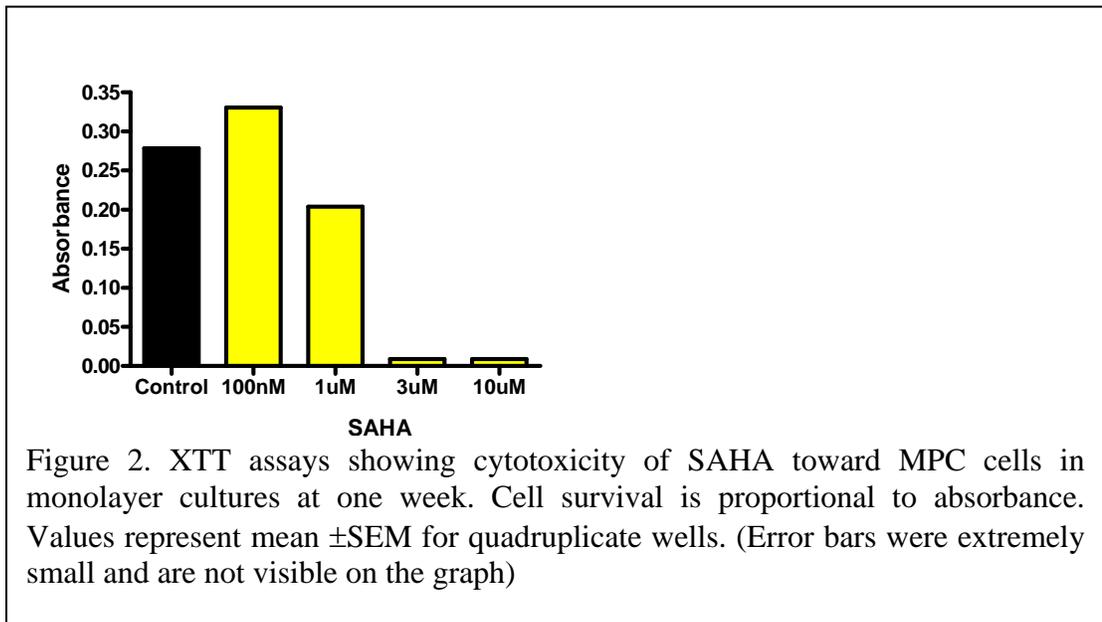
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Figure 1. Sequential luminescence imaging of subcutaneously injected MPC GL-9 cells in nude mice before and after start of cyclic Gamitrinib treatment (arrow). There is no detectable effect of the drug using this treatment protocol. The dip in luminescence at day 61 is an artefact resulting from a new lot of luciferin.

Task 2. The HDAC inhibitor, SAHA (vorinostat, Zolinza) was tested against MPC cells in monolayer culture (specific aim 1).

Results:

- SAHA is highly effective against MPC cells as a single agent (Figure 2). This finding might suggest a distinctly important role for HDAC inhibitors in treating pheochromocytoma because these drugs have serious limitations for treating other types of solid tumors, including ineffectively low concentrations and cardiac toxicity (4). This provides a rationale for subsequent testing to minimize cardiac and other systemic toxicity by using lower concentrations of the drugs in combination with low concentrations of TOP1 inhibitors (see Task 4).



Task 3. The HDAC inhibitor, trichostatin was tested against MPC cells in monolayer culture (specific aim 1). (A third HDAC inhibitor mentioned in our original grant proposal, valproic acid, was not further tested because preliminary studies shortly after approval of the grant showed a paradoxical protective effect most likely unrelated to HDAC inhibition. We therefore tested trichostatin in addition to SAHA as a further confirmation of specificity).

Result

- Like SAHA, trichostatin was highly effective against MPC cells as a single agent (Figure 3).

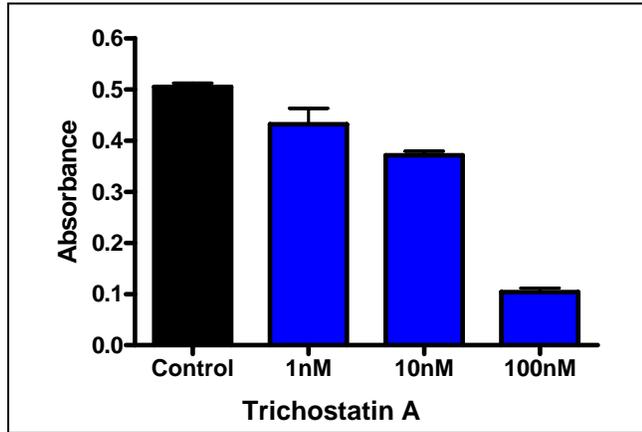


Figure 3. XTT assays showing cytotoxicity of trichostatin A toward MPC cells in monolayer cultures at one week. Cell survival is proportional to absorbance. Values represent mean ±SEM for quadruplicate wells.

Task 4. SAHA was tested for cytotoxicity against two primary human tumors in primary cultures (specific aim 2). We focused on SAHA for this purpose because SAHA is one of only two HDAC inhibitors currently approved for clinical use (4).

Results

- Both pheochromocytoma (tumor 1) and extra-adrenal paraganglioma (tumor 2) were susceptible to SAHA as a single agent within the concentration range effective against MPC cells. Variation between tumors was evident, as for drugs tested during the first funding period (Table 1).

Table 1. Cytotoxicity of SAHA Against Primary Human Pheochromocytoma/Paraganglioma

Tumor	Genotype	Surviving Cells/Dish (% of Control)*	
		1.0 uM SAHA	10 uM SAHA
1	<i>SDHB</i>	67.1	60.0
2	Sporadic-	122.6	22.4

\*Representative primary human PCC/PGL cells were treated with the indicated concentrations of SAHA or maintained in control medium for one week in monolayer cultures. At the end of the treatment period, cultures were fixed and stained for tyrosine hydroxylase TH to discriminate the tumor cells from fibroblasts and other cell types.

Task 5. SAHA was tested in conjunction with two TOP1 inhibitors, camptothecin and SN38, for cytotoxicity against MPC cells. These experiments were performed to determine whether cooperative interactions between the two classes of drugs (HDAC inhibitors and TOP1 inhibitors) would lower the effective concentrations and thereby potentially reduce systemic toxicity.

Results

- Both TOP1 inhibitors decreased cell survival alone, as shown in the first funding period. There was no apparent cooperativity between these agents and SAHA. However, SAHA alone was effective as a single agent at high concentrations (Figure 3)

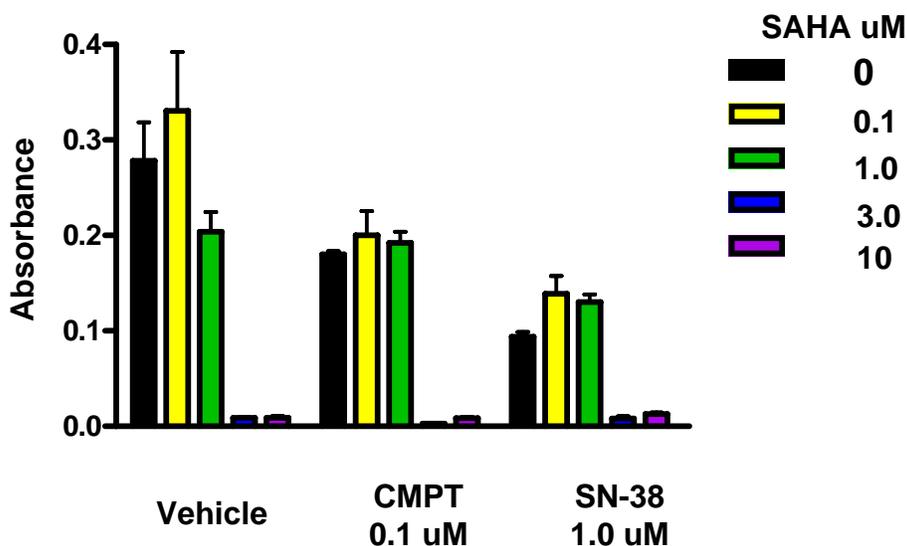


Figure 4. Cytotoxic effects of SAHA and TOP1 inhibitors are not cooperative. MPC cells were maintained in monolayer cultures for one week in control medium or with the indicated concentrations of camptothecin (CMPT), SAHA and SN-38. Cell survival was then measured by XTT assay. Values represent mean  $\pm$ SEM of quadruplicate cultures. (Note: For increased clarity, the data for SAHA alone versus control from this same experiment are shown as a separate figure in Fig 2.)

Task 6 We tested cytotoxicity toward cultured MPC cells using TOP1 inhibitors in conjunction with lithium, one of the alternate drugs to 5-azacytidine described in the original project narrative and approved SOW. Lithium was tested for this purpose because it can dramatically increase gene transcription in the rat pheochromocytoma cell line PC12 (5). This concentration is somewhat above the toxic threshold for serum concentration in patients treated with Li for bipolar disorders but is not toxic to PC12 cells.

Results:

- At the concentration previously tested with PC12 cells, lithium alone markedly decreased MPC cell number measurable by XTT assay after 1 week. There was also a trend toward cooperativity between Li and 0.5  $\mu\text{M}$  camptothecin or SN-38 during the same time period (Fig 5).

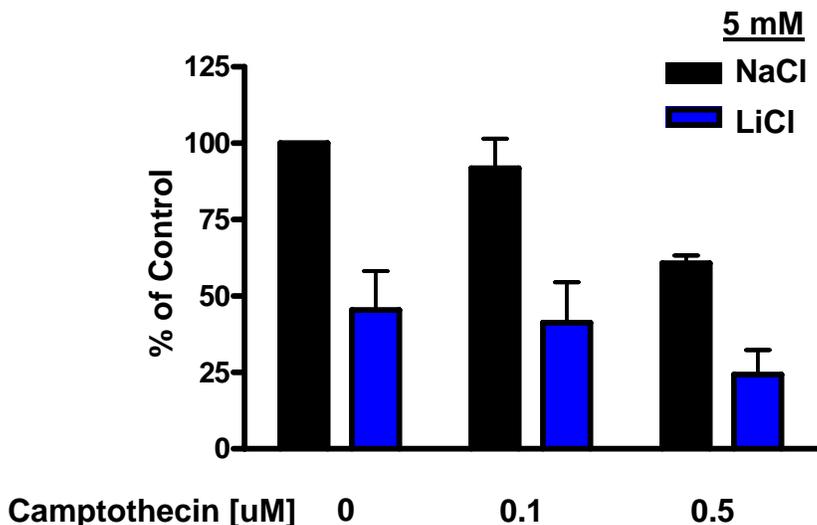


Figure 5. MPC cells were maintained in monolayer cultures for one week in control medium or with the indicated drug concentrations. Cell survival was measured by XTT assay. Values represent mean  $\pm$ SEM of 3 independent experiments, each with quadruplicate cultures. Similar results were obtained with SN-38 as the TOP1 inhibitor in place of camptothecin (not shown)

Task 7 To determine whether the dramatically reduced cell number seen with lithium in task 6 was caused by cell death or cytostasis, MPC cultures were cultured with 5mM LiCl or equimolar NaCl as a control for 3 days in the presence of bromodeoxyuridine (BrdU) to label replicating cells. The cultures were then fixed and stained immunocytochemically for BrdU. Cells were counted and labeled cells were expressed as percentage of total.

#### Result

- BrdU labeling was present in 47% of MPC cells after 3 days, versus 8.9% with LiCl. This indicates that the short term effect on cell number is caused by cytostasis rather than increased cell death as seen with TOP1 inhibitors. This effect could itself be useful in treatment of metastatic pheochromocytoma and will be further studied in the next funding period.

Task 8 We tested cytotoxicity toward cultured MPC cells using TOP1 inhibitors in conjunction with caffeine, another alternate drug to 5-azacytidine described in the original project narrative and approved SOW. Caffeine was tested for this purpose because it can dramatically increase gene transcription by releasing  $\text{Ca}^{2+}$  from

intracellular stores (6). We performed a two-week experiment with caffeine added during the first and/or second week in the presence or absence of camptothecin.

Results

- Caffeine did not detectably increase cytotoxicity when added to camptothecin (Fig. 6). However, an interesting observation was that caffeine alone decreased cell survival compared to control medium. This finding is consistent with preliminary data in our original grant proposal showing that caffeine causes a burst of apoptosis in MPC cultures. The effect is variable and small compared to that of the TOP1 inhibitors. However the concentration of caffeine in these experiments was very low (equivalent to the serum caffeine concentration after consuming two cups of instant coffee (7)). Further studies of caffeine will be performed in the next funding period.

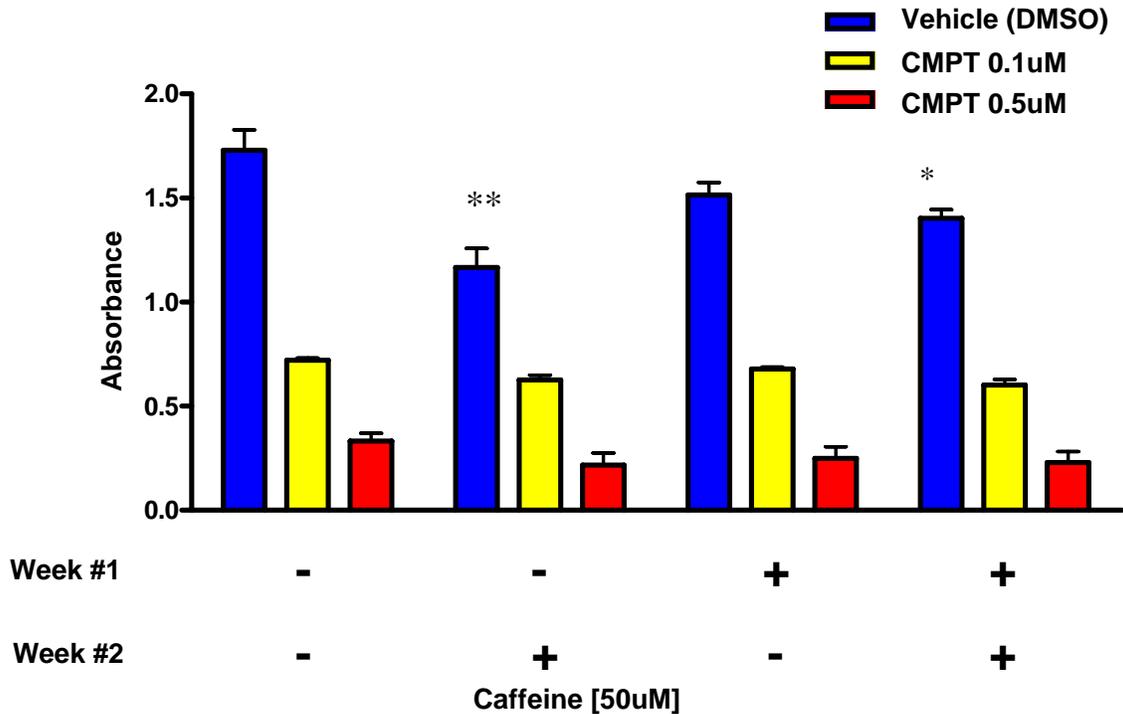


Figure. 6. Caffeine does not enhance toxicity of camptothecin but exerts a small apoptotic effect alone. The effect is most apparent when caffeine is added during week 2, probably indicating that the effect is transient and becomes masked by continued cell replication when caffeine is present continuously from week 1. MPC cells were maintained in monolayer cultures for 2 weeks in control medium or with the indicated drug combinations. Cell survival was measured by XTT assay. Values represent mean  $\pm$ SEM of quadruplicate cultures. \*\*  $p < .01$ ; \*  $p < .05$

Goal 3. Attempt to develop high throughput bioluminescence-based cytotoxicity assay for human PCC/PGL in cell cultures (Problem Area 1 in previous funding period).

Task 1. We performed a dilution curve with luminescent MPC GL-9 cells to determine the minimal number of luminescent cells detectable in monolayer culture in our luciferin-luciferase bioluminescence assay. These cells, which we generated in the first funding period, stably express the firefly luciferase gene integrated into their genome.

Task 2. Before progressing to human cells we next used an adenoviral luciferase vector (AD-CMV-luciferase, Vector Biolabs) to transiently introduce firefly luciferase into non-luminescent MPC cells. For this purpose we used AD-CMV –luciferase at a MOI of 10, which we had found to infect 100% of PCC/PGL cells in the previous funding period. The luminescence of these MPC cells would be comparable to that of primary human PCC/PGL cells, which can be made luminescent only by infection with the adenovirus construct. We then performed a dilution curve and luminescence assay directly comparing the luminescent signal intensity of of the AD-CMV-luciferase MPC cells to luminescent MPC GL-9 cells

Results (Fig. 7).

- Although 100% of PCC/PGL cells are infected with AD-CMV-luciferase, the signal intensity is far lower than with MPC GL-9. Based on Fig. 6, we estimate that a starting number of at least several thousand luminescent human cells per well would be required in order to obtain reliable results from a cytotoxicity assay using this approach. The combined obstacles of limited tumor cell yield and variable tumor cell enrichment within a dissociated cell population continued to make these attempts unsuccessful. We therefore continued to use our immunohistochemical method for testing primary human cultures.

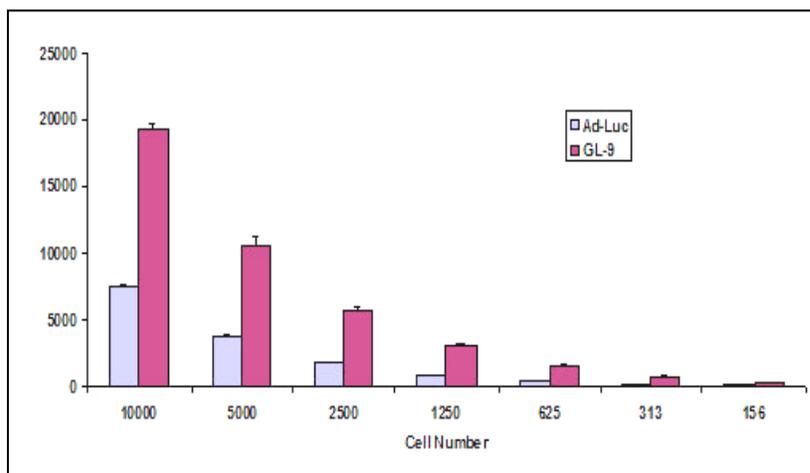


Figure 7. Comparative luminescence intensities of equal numbers of AD-CMV-luciferase MPC cells and luminescent MPC GL-9 cells. Values represent mean  $\pm$  SEM for quadruplicate wells.

*Goal 4. Finalize choice for route of administration of human PCC cells to mice and choice of mouse strain (Specific Aim 4).*

Task 1. We first generated luminescent human PCC/PGLs using an adenovirus luciferase construct as described in *Goal 3*.

Task 2. Continuing studies begun and preliminarily reported in the first funding period, we compared survival of luminescent human cells introduced by tail vein vs intrahepatic injection and subcutaneous injection in 3 mouse strains (nude, SCID, NOD-SCID). Because the adenovirus construct is not integrated into the genome, we simultaneously maintained cells from representative tumors in cell culture to determine how long luminescence persists before spontaneously being extinguished.

## Results

In total, 56 mice were grafted by the end of the second funding period (Table 2). Regardless of the type of graft (dissociated cells or tumor tissue fragments), route of tumor administration, site of grafting or mouse strain, there were no tumor takes from any tumors during periods so far ranging from 21 to 294 days. In the tumors that were tagged with luciferase, luminescence of cell or tissue grafts invariably extinguished before luminescence of tumor cells in culture (Figure 8). Mice that died or were euthanized for reasons unrelated to tumor were dissected at the end of the observation periods and confirmed that there were no tumor takes *in vivo* that had ceased to be luminescent.

<b>Table 2. Summary of Mouse Xenografts of Pheochromocytoma (PCC) and Extra-adrenal Paraganglioma (PGL) through August 2013</b>								
<b>Age</b>	<b>Sex</b>	<b>Tumor Type</b>	<b>Gene</b>	<b>Mouse</b>	<b>#</b>	<b>Route</b>	<b>Result</b>	<b>Tag</b>
47	F	PGL	Sporadic-Neg	Nude	4	Liver implant	No Tumor take	Ad-Luc
				Nude	2	Liver implant	No Tumor take	Ad-Luc
55	F	PCC	Not Tested	Nude	4	Liver implant (coated with matrigel)	No Tumor take	Ad-Luc
54	M	PGL-CB	SDHB	Nude	2	Liver injection	No Tumor take	Ad-Luc
51	M	PCC	Sporadic-Neg	Nude	1	Liver injection	No Tumor take	Ad-Luc

11	F	PGL	SDHB	Nude	1	Liver injection	No Tumor take	Ad-Luc
	F	PCC	MEN2A	SCID-Balb	3	Liver injection	No Tumor take	Ad-Luc
66	F	PCC	Not Tested	SCID-Balb	3	Liver injection	No Tumor take	Ad-Luc
33	M	PGL	Sporadic-Neg	SCID-Balb	2	Liver injection, SC-neck	No Tumor take	Ad-Luc
				NOD-SCID	2	Liver injection, SC-neck	No Tumor take	Ad-Luc
27	M	PGL	SDHB+	NOD-SCID	3	Liver injection	No Tumor take	Ad-Luc
50	F	PGL	Sporadic-Neg	NOD-SCID	3	Liver injection	No Tumor take	Ad-Luc
39	F	PGL	SDHB+	NOD-SCID	1	IV-Tail vein	No Tumor take	Ad-Luc
61	M	PCC	Not Tested	Nude	2	SC-flank, SC-tail	Pending	Ad-Luc
25	F	PCC	Pending	Nude	2	SC-flank	Pending	Ad-Luc vs. GreenFire
30	M	PGL	SDHB	Nude	1	SC-flank	Pending	Ad-Luc
9	F	PGL	SDHB	Nude	2	SC-flank	Pending	Ad-Luc
48	M	PGL	SDHB	Nude	1	SC-flank	Pending	Ad-Luc
45	M	PGL	SDHB	Nude	1	SC-flank	No Tumor take	Ad-Luc
27	M	PGL	SDHB+	Nude	1	SC-flank	Pending	Ad-Luc
46	F	PCC	SDHB	Nude	1	SC-flank	Pending	Ad-Luc
39	M	PGL	SDHB	Nude	1	SC-flank	Pending	Ad-Luc
54	M	PGL	SDHB	Nude	1	SC-flank	Pending	Ad-Luc
28	F	PCC	SDHB	Nude	1	SC-flank	Pending	Ad-Luc
55*	M	PGL-skull metastasis	SDHB	Nude	2	SC-flank (tissue chunks)	1- negative 1- pending	
		PGL-liver metastasis	SDHB	Nude	2	Liver injection through skin	No Tumor Take	
				Nude	3	Abdomen	1-	

						through incision (tissue chunks)	negative 2- pending	
				Nude	4	Liver injection through skin	Pending	Ad-Luc vs. GreenFire

\* Highlighted case is discussed in Task 2

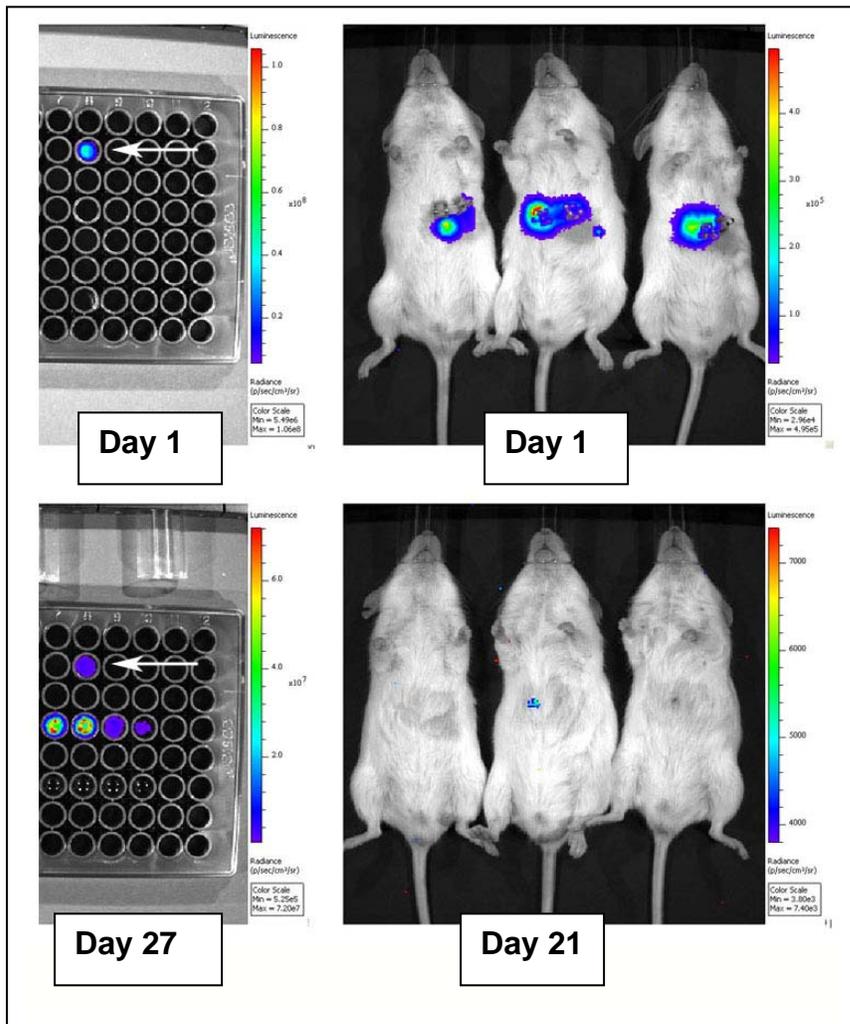


Figure 8. Right: Bioluminescence imaging of SCID mice with intrahepatic grafted human PGL cells tagged with adenovirus-luciferase. Left, arrows: Concurrent cell cultures of the tagged cells. Signal is essentially gone in the mice by day 21 while still persistent, though diminished, in the cultures at day 27. Luminescent wells in the row below the arrow at day 27 contain dilutions of mouse MPC GL-9 cells.

Task 2. To rule out the possibility that the absence of tumor takes *in vivo* might have merely reflected the slow growth typical of most PCC/PGLs, we used rapidly growing metastatic tumor cells from a single tumor derived from a patient with hepatic and peritoneal metastases. This unusual tumor also enabled us to test and compare multiple routes of administration from a single tumor, which was not possible in most other cases because of limited cell yields. We tested subcutaneous and intrahepatic tumor cell injection and intraperitoneal implantation of tumor fragments from this tumor in a total of 11 nude mice (highlighted case in Table 2).

#### Result

- All but 2 of the mice with cells or tissue from this tumor are alive with no evidence of tumor after 7 months, despite the fact that these aggressive tumor cells were returned to the same anatomic sites from which they were derived. The two dead mice were euthanized for unrelated lesions and necropsies showed no evidence of tumor. Tumor cells in culture have survived for the same period with no cell proliferation.

Task 3 We attempted to develop a protocol to use lentivirus for transduction and stable luciferase expression in human PCC/PGL. Using the Greenfire 2 lentivirus luciferase construct that we previously employed to generate luminescent MPC GL-9 cells, we tested transduction efficiency comparing our previously optimized protocol vs 2 new commercial reagents purported to increase transduction rate (Transdux reagent, from System Biosciences and MACSDuctin, from Miltenyi Biotec). Two tumors were tested using the manufacturers' suggested protocols.

#### Results

- Regardless of the protocol employed, only extremely rare primary human cells (estimated < 1/1000 cells) express the lentivirus construct. Our existing protocol was subjectively superior to the others tested.

*Goal 5.* Attempt to address lack of human PCC/PGL cell proliferative response to hypoxic culture conditions (Problem Area 2, Specific Aim 4A in previous Progress Report)

Although we framed this goal in terms of hypoxic cultures, it soon became clear that this was not the appropriate perspective because there is no proliferation under any culture conditions. This is the case even for tumors with *SDHB* or *VHL* mutations, which are characterized by intrinsically activated hypoxic signaling pathways (8). We therefore reframed the goal in order to address the broader problem and reformulated the tasks in order to begin testing new concepts.

Task 1. We tested all tumors in Table 2 for proliferation under routine culture conditions as described in the first funding period. We focused on tumors with *SDHB* mutations for two reasons. The first is their intrinsic hypoxic signaling profile, which makes a hypoxic

culture system redundant. The second is that *SDHB*-mutated tumors are most likely to be malignant and therefore pose the most urgent need for cell lines to be used for pre-clinical drug testing. For each tumor, dissociated cells were plated in RPMI 1640 medium with 15% fetal bovine serum. Proliferation was assessed by labeling with bromodeoxyuridine (BrdU) as a marker for DNA replication as we have previously reported (9). BrdU was added immediately upon plating and cultures were maintained one week prior to fixation. Double immunohistochemical staining was performed for BrdU and tyrosine hydroxylase to discriminate tumor cells from proliferating fibroblasts and other cell types in primary cultures. Some cultures were studied at two or more time points.

## Results

- There was no BrdU labeling of tumor cells under any conditions, while labeling of fibroblasts and other irrelevant cell types was readily detected. It was especially informative that the tumor highlighted in the Table, which grew rapidly *in vivo*, was the same as all of the other tumors in showing absolutely no labeled tumor cells, even though unlabeled cells have survived for > 7 months. This suggests that culture conditions either lack a required mitogenic signal (a missing “on” switch) or provide signals that suppress mitogenesis (an “off” switch).

Task 2 We reviewed > 20 years of our laboratory notes describing testing of human PCC/PGL for responses to a variety of small molecules that we had previously found to be mitogenic for rodent chromaffin cells, including several activators of adenylate cyclase/protein kinase A (principally cholera toxin and forskolin), and phorbol esters that activate protein kinase C (10).

## Results

- We confirmed that we have already tested tumors representing a variety of genotypes including *SDHx* and *VHL* mutations and found no response to activators of PKA or PKC. We concluded that these agents are therefore unlikely to be effective as single agents under either normoxic or hypoxic conditions and would be more appropriately incorporated into combinatorial protocols to provide the missing “on” switch (Task 3).

Task 3 It is now apparent that expression of markers associated with embryonic stem cells often drives neoplastic progression, and that these markers can in some tumors be acquired in response to external signals (11). We therefore began to systematically test protocols that reprogram “stemness”. To begin this new approach we tested a protocol based on the use of a Rho kinase (ROCK) inhibitor, Y-27632, that increases proliferation of keratinocytes and a variety of normal stem cells (1, 12) This method employs the ROCK inhibitor, a feeder cell layer of irradiated mouse fibroblasts, and a medium containing several small molecule growth factors and a low concentration of serum(1). We extensively tested the separate and combined components of this protocol using cells from 2 human tumors as diagrammed in Tables 3A-C. Both tumors were also routinely cultured in RPMI 1640 medium with 15% fetal bovine serum and tested according to our standard protocol described in Task 1, where they showed no tumor cell proliferation.

Results

- There was no significant proliferation using this protocol as reported or with any modifications tested

Table 3A  
 Human PGL Cells seeded on  $2.5 \times 10^4$  J2 3T3 feeder cells in 2 different media  
 \* indicates complete protocol as reported (1) except for cholera toxin, which was toxic to this tumor under these conditions

Medium	Y27632 (uM)	Hydrocortisone (ug/mL)	Insulin (ug/mL)	rhEGF (ng/mL)	Adenine (ug/mL)	Result (BrdU+)
F3(F12:DME M 3:1, FBS 5%)	10	0.4	5	10	24	
+		+	+	+	+	0
+ *	+	+	+	+	+	0
+	+	+	+	+		0
+	+	+	+			0
+	+	+				0
+		+				0
+						0

Medium	Y27632 (uM)	Hydrocortisone (ug/mL)	Insulin (ug/mL)	rhEGF (ng/mL)	Adenine (ug/mL)	Result (BrdU+)
McCoy's FBS (15%)	10	0.4	5	10	24	
+		+	+	+	+	0
+	+	+	+	+	+	0
+	+	+	+	+		0
+	+	+	+			0
+	+	+				0
+		+				0
+						0

Table 3B: Human PGL Cells plated immediately in 2 different media, no feeder cells

Medium	Y27632 (uM)	Hydrocortisone (ug/mL)	Insulin (ug/mL)	rhEGF (ng/mL)	Adenine (ug/mL)	Cholera Toxin (ng/mL)	Result (BrdU+)
F3(F12:DMEM 3:1, FBS 5%)	10	0.4	5	10	24	8.4	
+	+	+	+	+	+	+	0
+	+	+	+	+	+		0
+	+	+	+	+			0
+	+	+	+				1 cell
+	+	+					1 cell
+	+						0

Medium	Y27632 (uM)	Hydrocortisone (ug/mL)	Insulin (ug/mL)	rhEGF (ng/mL)	Adenine (ug/mL)	Cholera Toxin (ng/mL)	Result (BrdU+)
McCoy's FBS (15%)	10	0.4	5	10	24	8.4	
+	+	+	+	+	+	+	0
+	+	+	+	+	+		0
+	+	+	+	+			0
+	+	+	+				0
+	+	+					0
+	+						0

Table 3C Cultures started in high serum medium (Mc Coy's 15%FBS) then switched to indicated conditions at 48 hours, no feeder layers

Medium	Y27632 (uM)	Hydrocortisone (ug/mL)	rhEGF (ng/mL)	Insulin (ug/mL)	Adenine (ug/mL)	PMA (nM)	Cholera Toxin (ng/mL)	Result (BrdU +)
F3(F12:DMEM 3:1, FBS 5%)	10	0.4	10	5	24	50	20	
+	+	+	+	+	+			0
+	+	+	+	+	+	+	(48 hrs)	0

## **Section II - Problem Areas**

1. We have not been able to develop a high throughput method for cytotoxicity testing of primary human PCC/PGL cultures. We attempted to develop a bioluminescence-based assay as discussed in the previous progress report but limitations of cell number and purity made this approach unworkable. We do not plan to pursue this objective and will continue to use our current immunocytochemistry-based assay as needed.
2. We have not been able to devise any means to make human PCC/PGL cells proliferate in culture. Even tumor cells that proliferated rapidly in a patient *in vivo* immediately ceased proliferating in culture. Our next step will be to test new approaches based on the possibilities of restoring tumor stem cell properties or preventing the differentiation of tumor stem cells as discussed in Goal 5 section I.
3. Xenografts of human PCC/PGL apparently do not take in any strain of mouse tested, regardless of the type of graft (tissue fragments or dissociated cells) or the anatomic site of the graft. Even tumor cells that proliferated rapidly in a patient's hepatic and peritoneal metastases have failed to graft when introduced to the corresponding sites in nude mice. Because most PCC/PGL grow slowly, we will maintain the mice already grafted for their full lifespans and we will continue grafting newly acquired tumors. However, we now believe the ultimate solution to the problem will be finding a way to obtain human cell lines directly from primary cultures.
4. Although Lentivirus is known to be able to transduce non-proliferating cells, in our experience and that of other investigators it is very inefficient with PCC/PGL cells. We tested methods to improve the ability of Lentivirus to transduce human cells with luciferase in order to track xenografts that might ultimately proliferate, but we were unsuccessful. Because human PCC/PGL cells do not replicate, adenovirus-luciferase will continue to serve as at least a short term indicator of graft location and survival. If we are able to trigger proliferation in cell culture, the proliferating cells will be more susceptible to transduction by Lentivirus.

## **Section III - Description of work to be performed during the next reporting period.**

*Goal 1.* Marshall 1 paper submitted but not accepted as of September 2013, through the review process to publication.

Task 1. Respond to critiques, perform additional experiments as required, resubmit paper

*Goal 2.* Follow up promising results thus far obtained in specific aims 1 and 2

Task 1. Repeat testing toxicity of Gamitrinib against MPC GL-9 cells in nude mice (~ 2 months).

- 1a. Propagate sufficient cells to inject 20 mice,  $10^6$  cells/mouse (3 weeks)
- 1b. Wait for discrete tumor masses to form. (1 month)
- 1c. One-month treatment protocol with luminescence imaging (4 weeks).
- 1d. Harvest tumor tissue from control and treated animals at termination of experiment for histologic sections and RNA extraction.

Task 2. Using MPC cells, follow up and continue cell culture testing of non-toxic or minimally toxic drugs including lithium and caffeine as single agents or adjuncts to conventional chemotherapy drugs including TOP1 inhibitors.

Task 3. Test protocols developed with MPC cells in Task 2 against human cells in primary cultures

Task 4. Proceed with in vivo testing if promising regimens are developed in tasks 1 & 2 above, using MPC GL-9 cells and bioluminescence imaging as in Goal 1.

Task 5. Prepare papers for publication if warranted by the results of tasks 1-4

*Goal 3.* Continue attempts to develop xenografts of human PCC/PGL.

Task 1. Procure new tumors as available. Dissociate the tumors and graft up to 3 mice depending on cell yield. Because no mouse strain offered advantages over nude mice in tests during the previous funding period, we will routinely use nude mice, which are least costly and most easily imaged. Because no anatomic site offered any apparent advantage, subcutaneous grafts will be performed to facilitate long-term monitoring and intrahepatic grafts will be performed because the liver is a favored metastatic site.

*Goal 4.* Continue and expand attempts to establish human PCC/PGL cell lines from primary cultures.

Task 1. For each newly acquired tumor, plate dissociated cells routinely in RPMI 1640 medium with 15% fetal bovine serum which we have previously found optimal for survival of almost all human PCC/PGL. Test for proliferation under routine culture conditions immunocytochemical staining for BrdU incorporation according to our standard protocol described in Section I Goal 5 Task 1.

Task 2 Test a novel approach to maintaining stemness of PCC/PGL cells using protocols that were developed for normal neural stem cells and is based on the presence of recombinant leukemia inhibitory (hrLIF) plus several other growth factors. We will test 2 protocols as described by Zhang et al (13). The studies will be performed initially using frozen freshly dissociated viable cells from the highlighted tumor in Table 2. Because that tumor contained an unusually high proportion of proliferating cells *in vivo*, it is particularly likely to respond to this approach. Proliferation will be assessed as in Task 1

Task 3. Test a novel approach to maintaining stemness of PCC/PGL cells using a protocol developed for normal neural stem cells and based on the use of a low concentration of the HDAC inhibitor trichostatin A (14). This at first seems paradoxical because in the present progress report we describe a cytostatic/cytotoxic effect of trichostatin A on MPC cells. However, in preliminary studies we have observed a biphasic effect of the drug, with low concentrations increasing MPC cell proliferation. These experiments will be performed as for Task 2, starting with cells from the same tumor.

Task 4. Test a novel approach to forcing growth-arrested PCC/PGL cells to resume proliferation by introducing cyclinD1 + constitutively active CDK4 as described by Kendall et al (15). This will be accomplished using a cyclinD1 + CDK4R24C construct that is currently being cloned into a lentivirus vector by our collaborator at Tufts University School of Medicine, Prof. Brent Cochran. This approach has been successful with normal somatic human cells but has never been attempted with growth-arrested tumor cells. These experiments will be performed with cells from the same tumor as for Tasks 1 and 2.

## **2. Key Research Accomplishments During the Second Year (15 August 2012- 14 September 2013).**

- Completed the major experiments proposed in our original SOW showing cooperative interactions between topoisomerase 1 inhibitors and 5-azacytidine and submitted a paper for publication.
- Completed a large collaborative mechanistic study of Gamitrinib, a new type of drug that we found to be highly effective against PCC/PGL cells, resulting in a published a paper on Gamitrinib in a high impact journal.
- Demonstrated effects of 2 drugs with little or no systemic toxicity, lithium and caffeine, that could potentially supplement conventional chemotherapeutic agents in targeting malignant PCC/PGL. These will be further tested in the next funding period.
- Tested the toxicity of histone deacetylase inhibitors against PCC/PGL cells as described in the original SOW and identified potentially useful effects of these drugs as single agents.
- Extensively tested different approaches to grafting human PCC/PGL cells in immunodeficient mice and concluded that primary xenografts are unlikely to be effective in establishing cell lines from these tumors.
- Began to test novel approaches for directly deriving cell lines from primary cultures using protocols designed to maintain or induce stem cell properties in the cultured tumor cells.

**4. Appendices** Not applicable

**5. Conclusion**

We have used cultures of mouse pheochromocytoma cell lines developed in our laboratory as the principal model for pre-clinical testing of strategies to improve the efficacy of existing chemotherapeutic drugs and for testing potential new drugs that might be used to treat patients with malignant pheochromocytoma. Findings made with the mouse model are secondarily tested against primary cultures of human tumors and then tested *in vivo* with grafts to mice of the mouse cell lines. This approach is necessary because there are no human pheochromocytoma cell lines. Using this approach, we have completed most of the cell culture studies for Specific Aims 1 and 2 in the approved SOW and Project Narrative for this grant. Two papers reporting work funded by the grant were published in this funding period and one has been submitted for publication. Additional *in vivo* studies will be completed in the next funding period, and we anticipate additional papers to follow. However, there are important differences between the mouse pheochromocytoma cells and their human counterparts, caused in part by the fact that the human cells immediately and completely cease proliferating when placed into cell cultures. There remains a pressing need for human cell lines to perform more valid pre-clinical drug testing. In the past two funding periods we have extensively tested potential ways to propagate human pheochromocytoma cells from primary tumors in conventional and hypoxic cultures and in mouse xenografts. These efforts have been completely unsuccessful, even with cells that grew rapidly in a patient with widely metastatic tumor. A major focus in the next funding period will therefore be on testing novel approaches to developing human cell lines from primary cultures. These will be based on protocols developed for maintenance of normal embryonic stem cells and will be directed at maintaining or inducing “stemness” characteristics that drive tumor progression.

**6. Publications, Abstracts and Presentations**

1. Chae YC, Angelin A, Lisanti S, Kossenkov AV, Speicher KD, Wang H, Powers JF, Tischler AS et al. Landscape of the mitochondrial Hsp90 metabolome in tumours. *Nature Communications*. 2013;4:2139
2. Giubellino A, Bullova P, Nolting S, Turkova H, Powers JF, Liu Q, Guichard S, Tischler AS et al. Combined inhibition of mTORC1 and mTORC2 signaling pathways is a promising therapeutic option in inhibiting pheochromocytoma tumor growth: in vitro and in vivo studies in female athymic nude mice. *Endocrinology*. 2013;154:646-55.

**7. Inventions, Patents and Licenses** None

**8. Reportable Outcomes** None

**9. Other Achievements** Not applicable

## **10. References**

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