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TITLE: **Targeting Cancer Stem Cells with a Novel Glycolytic Inhibitor to Overcome Metastasis and Chemoresistance in Small Cell Lung Cancer**

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**14. ABSTRACT**

Based preliminary data and additional data generated by this project, we have determined that the glycolytic inhibitor PFK158 reduced cell viability, attenuated glucose uptake, lactate production and ATP levels in several SCLC cell lines (H1408, H1882 and H1876. Consistent with this, KD of PFKFB3, the target of PFK158 also inhibited glycolysis in these cell lines. While PFK 158 treatment induced apoptotic mediated cell death, it was independent of autophagy. Overall, our results show for the first time that synergy with QC and carboplatin involves a complex interplay between AV and apoptosis in OVCa cells and is associated with upregulation of INP2, downregulation of p62, and simultaneous upregulation of CTSL only in resistant cells. While PFK158 treatment resulted in the downregulation of several cancer stem markers such as CD!33, SOX2 and STAT3, it was independent of PFKFB3. More importantly, our FACS analysis of cancer stem cell enriched spheroid cultures positive for ALDH and CD133 of H1048 and 1882 was effectively targeted by PFK158. Additional results also showed that PFK158 sensitized SCLC cell lines to CBPt mediated cytotoxicity. Collectively, these results show that PFK158 treatment in addition to inhibiting the glycolytic rate also significantly reduced the cancer stem cell population to induce apoptotic cell death of SCLC cells.

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Table of Contents


| | Page |
|---|---|
| 1. Introduction…… ……. | 4 |
| 2. Keywords……. | 4 |
| 3. Accomplishments……..……... | 4-10 |
| 4. Impact……...…… | 10 |
| 5. Changes/Problems...…… | 10 |
| 6. Products, Inventions, Patent Applications, and/or Licenses..……. | 11 |
| 7. Participants & Other Collaborating Organizations…….. | 11 |
| 8. Special Reporting Requirements…… | 11 |

**Final Report**
This progress report is a final report covering the time period 15 Aug 2018 - 14 Aug 2020

## INTRODUCTION:

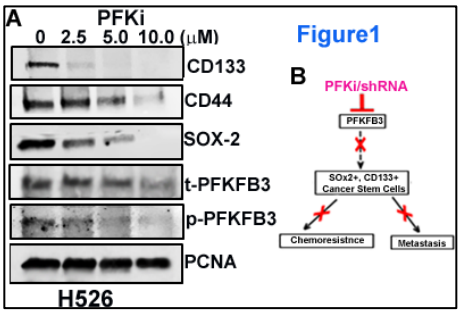
There are very few targeted therapies being tested in small cell lung cancer (SCLC) clinical trials. Given the importance of cancer stem cells (CSC) in chemoresistance and metastasis, new CSC-targeting compounds appear to be a promising strategy to prevent cancer recurrence and metastasis[1-2]. We hypothesize that treatment of SCLC with PFKi158, a novel glycolytic inhibitor that specifically targets 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), a critical control point in the glycolytic pathway alone as well as in combination with CBPt will result in better response rates via targeting the CSC population [3-4]. Our preliminary data shows that increased PFKFB3 expression that is reported to be higher in cancer stem cell (CSC) compared to non-stem cancer cells or induced pluripotent stem cells can be efficiently targeted by PFKi in SCLC cell lines leading to the downregulation of several cancer stem cell markers such as CD133, CD44 and SOX2. The central hypothesis of this proposal is that targeting PFKFB3 with PFKi will result in reversal of chemoresistance and inhibition of metastasis in SCLC both in vitro and in vivo. The work proposed in this grant will provide critical information on whether targeting CSCs with the novel glycolytic inhibitor PFKi, currently in Phase 1 clinical trial, will lead to increased sensitivity to chemotherapy, decreased incidence of metastasis, and an improved clinical outcome in patients with SCLC in military personnel, veterans and their families who have been exposed to cigarette smoking, exposure to radon and environmental pollution. Specific Aims: To therapeutically target the CSC niche with PFKi we propose the following two specific aims. In **Aim 1**, we will elucidate the role of PFKFB3 in glycolytic flux and CSCs targeting and survival in SCLC. Using both pharmacological (PFKi) and genetic (shRNA mediated knock down (KD) approaches we will determine the effect of PFKi and the role of PFKFB3 in targeting CSC in SCLC cell lines in vitro. In **Aim 2:**.We propose to determine the efficacy of PFKi alone and in combination with carboplatin (CBPt) in CSC-enriched population in vivo (H1048 spheroids grown in stem cell media on ultra-low attachment plates) aiming to reverse chemoresistance and decrease metastatic potential.

**KEYWORDS:** 2-NBDG 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose, ALDH-Aldehyde dehydrogenase, CSC- Cancer stem cells, EMT- Epithelial-to-mesenchymal transition, MMP- Matrix metalloproteinase, NTC- Non targeted control, p-FAK- phospho-Focal adhesion kinase, PFKFB3-6-phosphofructo-2-kinase/fructose-2,6-biphosphatase3, PI3K- Phosphoinositide 3-kinase, p-STAT3, SCLS- Small cell lung cancer, shRNA- Short hairpin Ribonucleic acid, SOX2- SRY (sex determining region Y)-box 2 and PFKi-PFK158,

## ACCOMPLISHMENTS:

***Aim1z:*** **To *Elucidate the role of PFKFB3 in CSCs targeting their glycolytic flux and survival in SCLC***

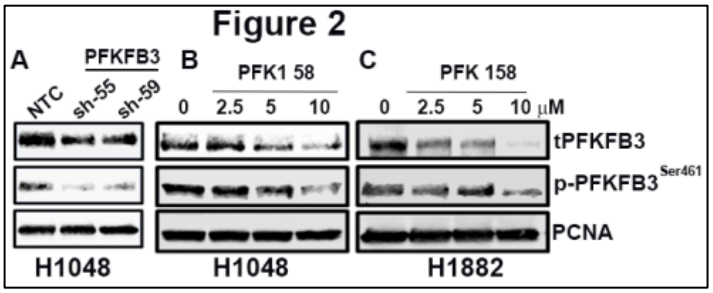
**Rationale:** Cancer is now considered as a stem cell disease. Determining the role of CSC metabolism in carcinogenesis has become a leading focus in cancer research, and substantial efforts are directed to explore new clinical targets. To date, there are no reports on the effects of targeting the glycolytic pathway on CSC in SCLC. It has been shown that CSCs are largely responsible for tumor initiation, relapse, metastasis, and chemoresistance. Conventional chemotherapy alone has little effect in eradicating the CSC population. Thus, a better understanding of the properties and mechanisms underlying CSC resistance is imperative. Identification of novel drugs that target CSC markers and/or associated signaling pathways in order to eradicate SCLC and enhance patient survival is a promising approach. The objective of this study is to explore the use of PFKi in SCLC treatment. While the role of PFKi in inhibiting the glycolytic pathway has been extensively documented in other cancers, there are no reports in SCLC. Increased PFKFB3 expression is reported in CSC compared to non-stem cancer cells or induced pluripotent stem cells[4]. PFKFB3 blockade can reduce cancer cell



intravasation and metastasis. We have generated preliminary data that targeting H526 SCLC cell line with PFKi leads to downregulation of several CSC markers including SOX2, CD133 CD44 and SOX-2 (Figure 1A). SOX-2, a stem cell transcription factor, is able to reprogram human somatic cells to pluripotent stem cells and is frequently amplified in SCLC[5]. SOX2-dependent up-regulation of ATP-binding cassette subfamily C member 1 (ABCC1) is often associated with MCAM (Muc18) that mediates chemoresistance in SCLC via the PI3K/AKT/SOX-2 signaling pathway. The amplification of SOX2 in SCLC has an important role as a "lineage specific oncogene" suggesting that CSC expressing SOX-2 can be targeted with this novel glycolytic inhibitor in SCLC.

*In Aim 1, we had proposed to elucidate the role of PFKFB3 in CSCs targeting their glycolytic flux and survival in SCLC*. Our primary goal is to determine if PFKFB3 inhibition specifically restrains cancer stem cell population.

As we had proposed in **Tasks 1** of this Aim, we genetically knocked down PFKFB3 expression in two different SCLC cell lines H1048 cell line using two different shRNAs to PFKFB3 with nontargeted controls shRNA NTC. The efficiency of knockdown was confirmed by Western blot analysis. In parallel, H1048 and H1882 SCLC cell lines were treated with PFKi in dose dependent manner and the down-regulation of PFKFB3 activity was determined using p-PFKFB3 ($S^{461}$) levels (Figure 2). Downregulation of PFKFB3 led to a significant downregulation of p-PFKFB3 in H1048 cell line (Fig. 2A). PFK158 treatment downregulated the expression of both total PFKFB3 (tPFKFB3) and p-PFKFB3 levels in both cell lines (Figs. 2B and C)




To better understand the effect of PFK158 on SCLC cell, we first performed cell viability assays to establish the IC50 values for PFK158 and carboplatin, as proposed in Task 2. The IC50 for PFK158 was between 3.5 and 5.0μM, for the 4 SCLC cell lines tested while the IC50 for carboplatin was 150μM for H1048, H1882 and H1876 cell line with DMS-53 with 100μM (Figs. 3A-C).

2. Next, to determine if the membrane integrity was lost upon PFK158 treatment, we used using annexin V and PI labeling by FACS analysis (fluorescence-activated cell sorting) (Figs.3D and E) which showed that PFK 158 treatment of H1048 resulted in the loss of cell viability by 25% on an average. The # of Annexin V/PI positive cells increased from 11.9% to 19.1% and 24.1% at 5.0 and 10μM PFK158 treatment compared to control untreated cells respectively.

3. Since the above data indicated that PFK158 inhibited cell proliferation and induced loss of membrane integrity which would lead to apoptosis, we determined the level of apoptosis checking Cl.PARP and Cyt-c) levels and also if the cells underwent autophagy by checking LC3B and p62 levels by western blot analysis which revealed that PFK158 treatment increased the expression of cleaved PARP in a dose dependent manner. However, there was no change in the expression levels of autophagy markers LC3BII and p62

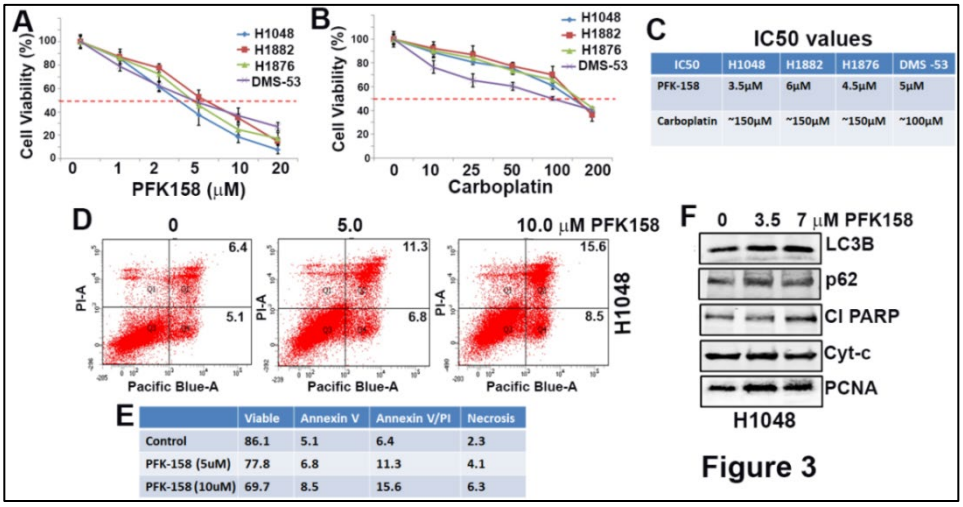
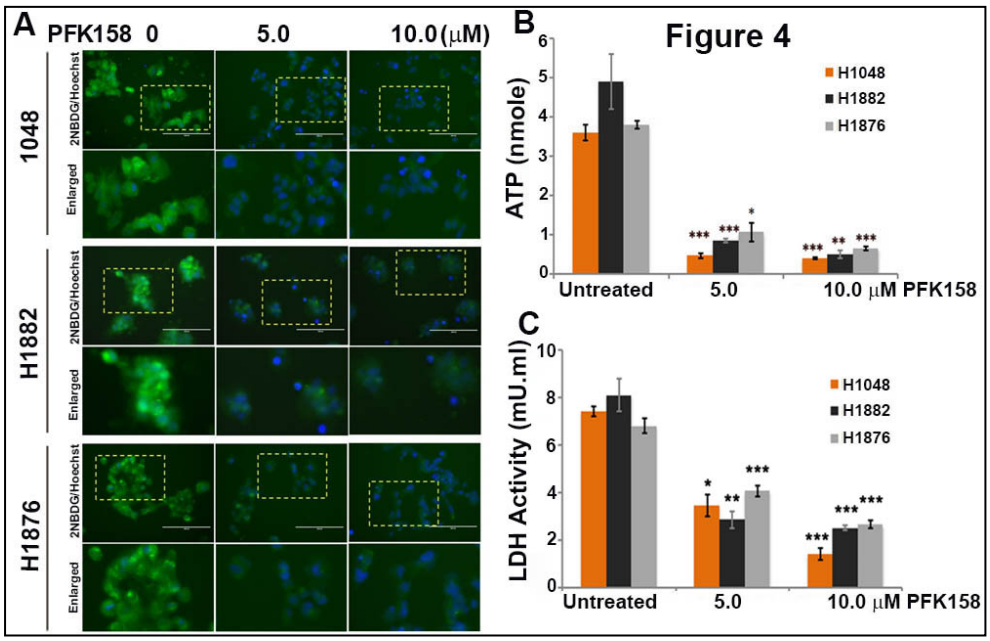


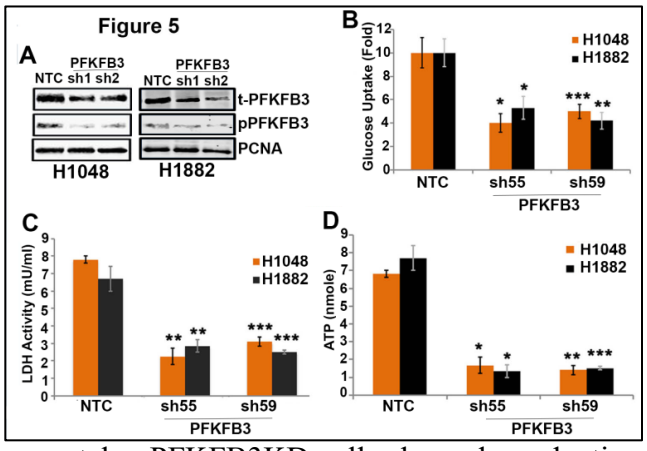
Figure 3

suggesting that unlike what we reported in ovarian cancer, PFK158 treatment does not induce autophagy in SCLC cells (Figures 3F).

Collectively, these results indicate that PFK158 treatment induces apoptosis independent of autophagy.
To evaluate if PFK158 inhibits glycolytic rate, we determined glucose uptake, lactate dehydrogenase activity and intracellular ATP level in SCLC cell lines in the presence and absence of of PFK158 as previously described {Mondal, 2019 #1} (Task 3). Results showed that a concentration-dependent decrease in glucose uptake in H1048, H1882 and H1876 as determined using 2NBDG, a fluorescent glucose analog (Fig. 4A) respectively. Consistent with a decrease in glucose uptake, PFK158 showed a reduction in lactate dehydrogenase activity (Fig. 4C) and intracellular ATP levels (Fig. 4B). This is consistent with the decrease in p-PFKFB3 ($S^{461}$) by immunoblot analysis (Figs. 2C and D) suggesting PFK158 inhibits the glycolytic rate in SCLC cells.




To confirm whether the expression level of PFKFB3 has any impact on the higher glycolytic rate of these cells, we further investigated glucose uptake after genetic manipulation using two different shRNA-against PFKFB3 in H1408 and H1882 cells with nontargeted control (NTC) shRNA as control. Efficient downregulation of PFKFB3 was verified by western blot analysis as shown in Figure 5A in both H1408 and H1882 cells. Consistent with PFKFB3 downregulation, p-PFKFB3 levels were also significantly downregulated. To evaluate if PFKFB3 knockdown inhibits glycolytic rate, we determined glucose uptake, lactate dehydrogenase activity and intracellular ATP level in in PFKFB3 knockdown clones compared to NTCshRNA cells. As shown in figure 5B, KD of PFKFB3 resulted in decreased glucose uptake. Consistent with a decrease in glucose uptake, PFKFB3KD cells showed a reduction in lactate dehydrogenase activity (Fig. 5C) and intracellular ATP levels (Fig. 5D) compared to NTCshRNA cells. Collectively, these results confirm the role of PFKFB3 in modulating the glycolytic rate in SCLC cells.




One of the main objectives of this proposal is that by inhibiting PFKFB3 activity either pharmacologically or genetically will result in the downregulation of cancer stem cell markers thus providing a therapeutic target to treat SCLC patients with PFK158. Our hypothesis is based on our preliminary data using H526 cell line as shown in figure 1A where treating H526 SCLC cell line with PFKi led to downregulation of several CSC markers including SOX2, CD133 CD44 and SOX-2.

To determine if PFK158 treatment or genetic downregulation of PFKFB3 (Task 4) will attenuate cancer stem cell expression in additional cell lines, we initially treated H1048 and H1882 cells with increasing concentration of PFK158 for 24 hrs and analyzed the expression levels of several of the cancer stem cell markers as shown in

Figure 5A. In general, the expression levels of CD44, CD133, SOX2 and p-STA3 were significantly downregulated in H1882 cell line in a dose dependent manner. All the CSC markers were also downregulated in H1048 cells albeit to a lesser extent compared to H1882 cell line (Fig.6A).

To determine if PFKFB3 played a role in maintaining the CSC population in SCLSC cell line, we downregulated PFKFB3 expression using two different shRNAs to PFKFB3 with nontargeted control NTC-shRNA as controls in H1048 cell line. As shown in figure 6B, PFKFB3 expression was ~50% down (compare NTC cells to sh55 and sh59 cells). Consistent with this downregulation, we saw significant downregulation of p-PFKFB3 (Panel 2 in figure 6B). However, the cancer stem cell markers CD44 and CD133 were partially downregulated in H1048 shPFKFB3 cells and remarkably downregulated in in H1882 cells but no changes in SOX2 expression in both cells.

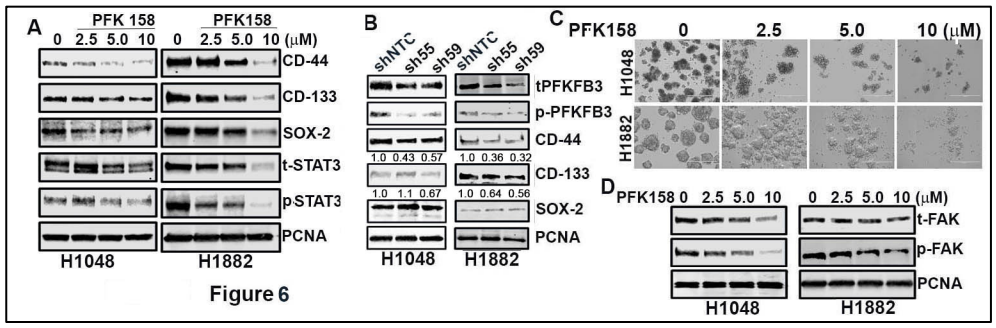


Figure 6

To further characterize the stemness of H1048 and H1882 cells, we grew them as spheroids on low attachment plates. Both the cell lines formed spheroids potentially indicating that they are rich in CSC population which was effectively targeted by PFK158 treatment. PFK 158 targeted the spheroids in a dose dependent manner (Fig. 6C). Consistent with the results shown in figure 6C, the levels of t-FAK and p-FAK ($Y^{397}$) were attenuated. These results imply that by targeting p-FAK, PFK158 may inhibit migration of these cells (Fig.6D).

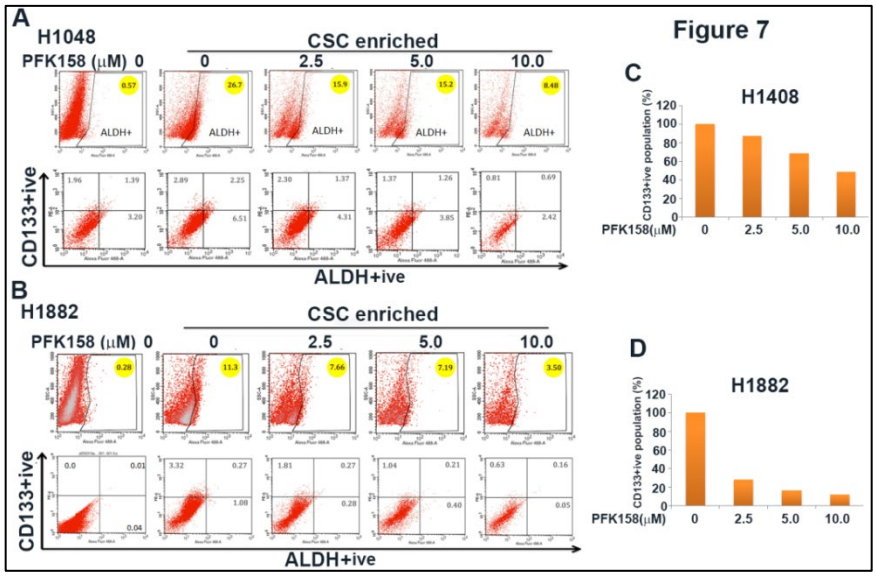


Figure 7

To better understand the role of PFK158 in modulating CSC population, H1048 and H1882 cells were cultured in stem cell media for a week to enrich the CSC population and exposed to increasing doses of PFK158 (0, 2.5, 5, 10μM) for 24h and CD-133 and ALDH level was analyzed by FACS analysis. As shown in figures 7A and B the number of both ALDH and CD133 positive cells were substantially reduced by PFK 158 treatment in both H1048 and H1882 cells respectively. Figures 7C and D shows the % population of CD133 positive cells following PFK158 treatment in H1048 and H1882 cells.

***Aim 2:*** Examine the therapeutic efficacy of PFKi alone and in combination with carboplatin (CBPt) *in vitro and vivo in reversing chemoresistance and metastasis.*

In Task 1of this Aim, we had proposed to determine if PFK158 treatment will sensitize SCLC cells to CBPt-mediated cytotoxicity *in vitro*. To do this, initially, we determined the IC50 values for

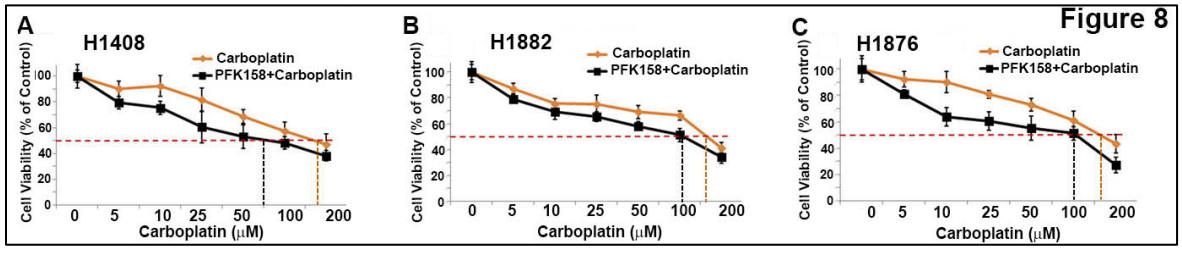
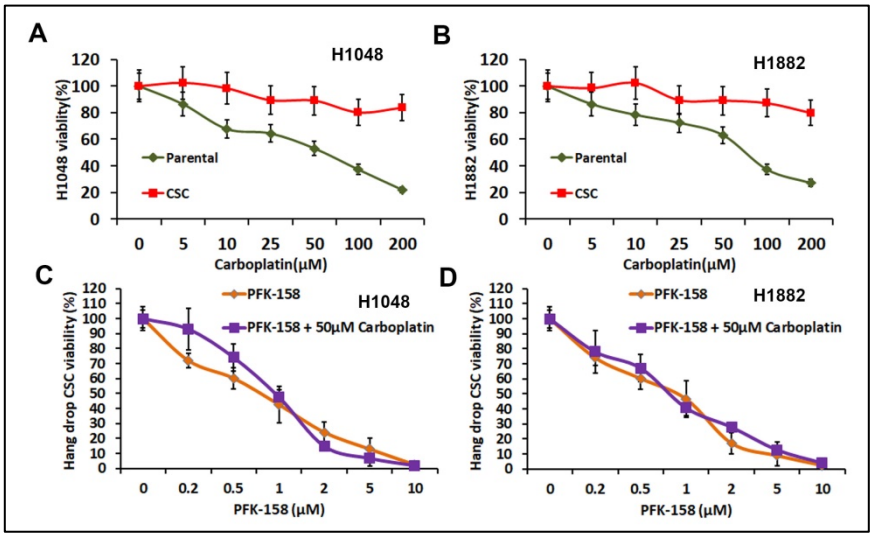


Figure 8

PFK158 and carboplatin (CBPt) in several SCLC cell lines (Figs.3A-C). We treated H1408, H1882 and H1876 cells with 1×$IC_{50}$ (~5.0µM) of PFK158 in combination with increasing concentrations of CBPt. As shown in figures 8A-C, PFK158 sensitized SCLC cell lines to CBPt mediated cytotoxicity. On an average the IC50 of CBPt (150-175µM) was reduced to 75µM in H1048 and to 100µM in H1882 and H1876 cell lines.




Anti-proliferative investigations of CBPt and PFK158 on enriched CSC of H1048 and H1882 cells were extended by MTT and Hangdrop CSC viability assays, due to its promising antiproliferative effect in parental cells (Figures 8A-C). Carboplatin showed negligible cytotoxicity against enriched cancer stemcells of both H1048 and H1882 cells compared to unenriched parental cells (Figures 9A &B) which highlights that cancer stem like cells are resistant to carboplatin treatment. The primary goal of this investigation was to determine whether combining PFK158 with carboplatin would be a better preventive/therapeutic outcome. we examined the effects of incremental concentrations of PFK158 and with 50µM of CBPt in combination on enriched CSC by hangdrop method. As shown in Figures 9C and D, enriched CSC of H1048 and H1882 cells affected by only PFK158 treatement in concentration dependant manner and combination of both was found to be not greater than PFK158 monotherapy, which indicates enriched populations are only sensitive to PFK158 treatment not to carboplatin.

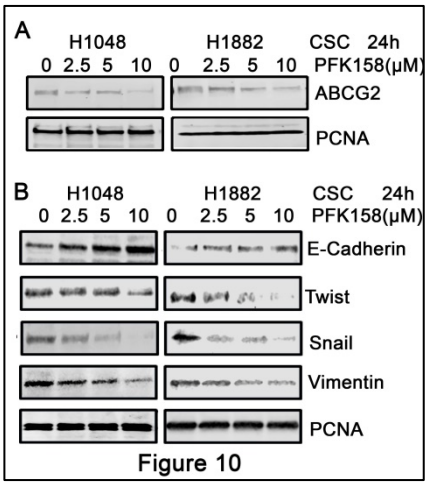


Figure 10

**In Task 2,** we had proposed to determine the effect of PFK158 treatment in cancer stemcell resistance. ABCG2 is a member of the ATP binding cassette (ABC) transporters, which can pump a wide variety of endogenous and exogenous compounds out of cells and also plays an important role in promoting stem cell proliferation and the maintenance of the stem cell phenotype. Following inhibition of enriched cancer stemcells by PFK158, role of PFK158 in chemoresistance was addressed on H1048 and H1882 cells via measuring chemoresistance marker ABCG2 by westenblot. As shown figure 10A, PFK158 exhibited down regulation of ABCG2 in both H1048 and H1882 cells in concentration dependant manner. Further we had proposed to determine if PFK158 treatment will inhibit the migration and invasion of SCLC cell lines and determine the expression levels of Epithelial Mesenchymal Transition (EMT) markers by western blot analysis. EMT has emerged as a key regulator of cell invasion and metastasis in cancers. Besides the acquisition of migratory/invasive abilities, the EMT process is tightly connected with the generation of cancer stem cells (CSCs), thus contributing to chemoresistance.

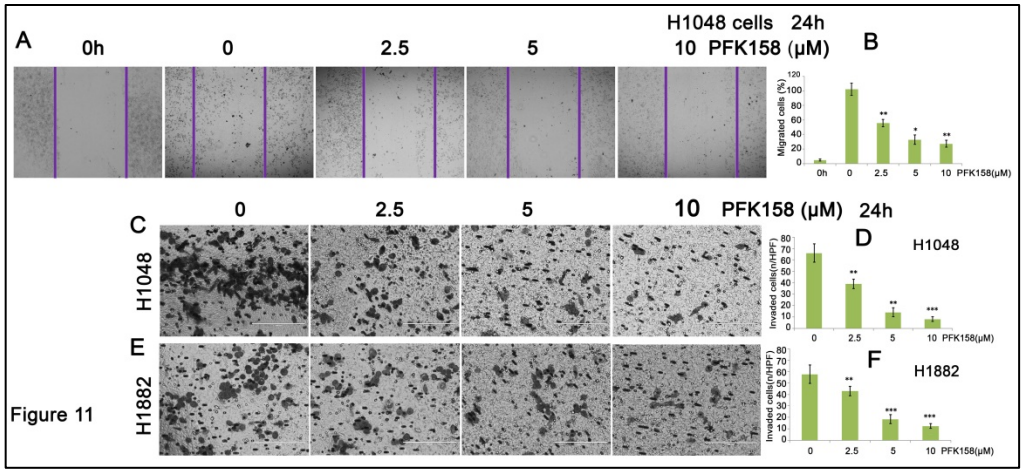


Figure 11

Following promising counteraction of cancer stemness and ABCG2 level, an impact of PFK158 on migration and invasion was examined on H1048 and H1882 cells by validation of EMT markers and migration and invasion assays. PFK158 treatment restrained the critical EMT markers including Twist, Snail and vimentin with gradual upregulation of E-cadherin expression in enriched CSC of both H1048 and H1882 cells as verified by westernblot (Figure 10 B). Furthermore, migration and invasion investigations demonstrated that PFK158 impaired the migration with 47.6%, 77.8% & 71.2% (Figures 11A & B) in H1048 cells at various concentrations and also arrested the invasion with 39±5 to 9±3 cells/HPF in H1048 when untreated showed 66±8 cells/HPF and with 43±6 to 13±4 cells/HPF in H1882 when compared to that of untreated (57±8 cells/HPF) group.

In **Tasks 3 we proposed to d**etermine if PFKi alone and in combination with CBPt will suppress CSC growth in H1048 *in vivo* .The pathophysiological response of PFK158 was investigated to determine whether PFKFB3 perturbation affects cancer stem cells induced tumor growth in mice. As invitro experimental evidences suggest that PFK158 alone inhibits remarkably cancer stem like cells and no improved synergy was exhibited when combined with CBPt (Figure 9A-D),we have studied the impact of PFK158 and PFKFB3 knockdown in CSC tumor growth in vivo. Mice were injected subcutaneously with enriched H1048 cells (2500 cells/mice)(n=20) and enriched PFKFB3 KD (n=10) cells into right flank of scid nod mice. After onset of tumor (100mm$^3$ size) development and mice divided into 3 groups(Group 1-Control, Group 2- PFK158 and Group 3-PFKFB3 KD) adminitstered with PFK158 (25mg/kg bw intraperitoneally) for 6 doses twice per week (Figure 12A) and tumor growth inhibition was recorded by vernier caliper. Mice injected with H1048 parental cells were not developed tumors till end of the study. The treatment of PFK158 on CSC tumors at 25 mg/kg bw concentration or CSC with knockdown of PFKFB3, noticeably reduced tumor establishment as measured by vernier caliper and gradually inhibited tumor growth in dose dependant manner(Figure 12B). Anatomical appearance of flank containing tumor shows that inhibition of PFKFB3 by PFK158 and genetic knockdown with shPFKFB3 regressed CSC tumor size visibly with 0.341±0.046g and 0.136±0.026g of tumour respectively, when corresponded to that of untreated (2.51±0.24g) (Figure 12C). Mice exhibited no secondary tumor formation in any organs like brain, liver and lungs.

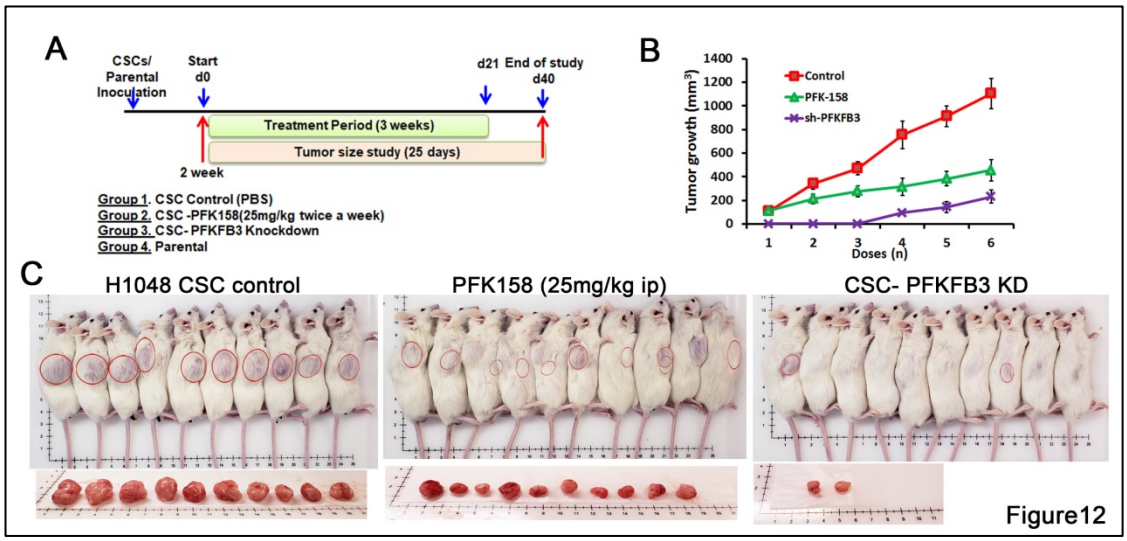


Figure12

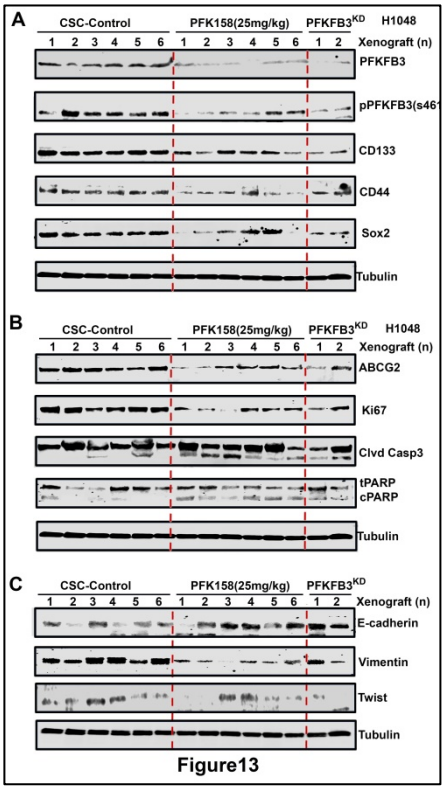


Figure13

The molecular events of PFK158/PFKFB3 knockdown exhibiting anti-pluripotency in CSC tumor were elucidated by performing westernblot in randomly selected 6 tumors from each groups. Results demonstrated that inhibition of PFKFB3 with PFK158 or gentic knockdown repressed the expression of pPFKFB3 and total PFKFB3 which resulted in abolition of expressions of cancer stemcell markers including CD133, CD44 and Sox2

(Figure 13A). Since inhibition of PFKFB3 negatively regulates cancer stemcell marker expressions, role of PFK158 in chemoresistance and apoptotic markers were assessed. ABCG2 overexpression can render the cancer cells resistant to the ABCG2 substrate chemotherapy agents. To the best of our knowledge, no published clinical trial has ever succeeded in reversing the ABCG2-mediated MDR. But our results shows that PFK158 or PFKFB3 KD potentially inhibited level of ABCG2 and proliferative marker Ki67 and as a consequence, inhibition of PFKFB3 activate the apoptotic cell death as verified by cleaved caspase3 and cleaved PARP (Figure 13B). The in-vivo effect of PFK158 on EMT markers evaluated by westernblot, as shown in figure 13C, inhibition of PFKFB3 by PFK158 or shRNA resulted in decrease of vimentin and twist with significant upregulation of E-cadherin, which is parellal to in-vitro results(Figure 10 B).

Taken together, both *in-vitro* and *in-vivo* results highlights that PFK158 acts as potential inhibitor of cancer stemcell proliferation targeting PFKFB3 mediated modulation of CD133,CD44, Sox2 and EMT markers.

**What opportunities for training and professional development has the project provided?** – It has provided training to the postdoc to work on a cancer stem cell project.

**How were the results disseminated to communities of interest?** -Nothing to Report

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

This is the final report and the grant is in closeout

**IMPACT: What was the impact on the development of the principal discipline(s) of the project?** Based on the preliminary data presented in this report, we have shown for the first time that targeting SCLC cells with PFK158 resulted in loss of cell viability, attenuated glycolytic rate and more importantly, significantly reduced the cancer stem cell (CSC) population to induce cell death. Given importance of CSC in chemoresistance and metastasis and the lack of targeted therapies being tested in SCLC clinical trials, new CSC-targeting compounds appear to be a promising strategy to prevent cancer recurrence and metastasis.

**What was the impact on other disciplines?** *Nothing to Report.*

**What was the impact on technology transfer?** *Nothing to Report.*

**What was the impact on society beyond science and technology?** *Nothing to Report.*

**CHANGES/PROBLEMS:** *One of the concerns is the increase in stromal infiltration in treated groups*

**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- Not applicable**

**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:** *"Nothing to Report."*

**Website(s) or other Internet site(s)-** Nothing to report

**Technologies or techniques-** Noting to report

**Inventions, patent applications, and/or licenses-** Nothing to report

**Other Products-** Nothing to report

## PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

### What individuals have worked on the project?

*Provide the following information for: (1) PDs/PIs- No change*

| | |
|---|---|
| Name: | Dr. Prabhu Thirusangu |
| Project Role: | *Post-Doctoral Fellow* |
| Nearest person month worked: | *7* |
| Contribution to Project: | *Worked on Aim1* |

What other organizations were involved as partners? – None

SPECIAL REPORTING REQUIREMENTS: "Nothing to Report"

## REFERENCES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