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TITLE: Therapeutic Targeting of p300/CBP in Clear Cell Renal Cell Carcinoma

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14. ABSTRACT

The majority of clear cell renal cell carcinoma (ccRCC) lacks von Hippel-Lindau (VHL) protein. Loss of VHL results in the aberrant accumulation of HIF proteins, and thereby enhanced expression of pro-angiogenic factors, the leading cause of tumor angiogenesis. In the case of VHL-defective ccRCC, HIF-2α acts as an oncogene whereas HIF-1α acts as a tumor suppressor. HIF-2α signaling is regulated via its acetylation by CBP. CBP also target a significant number of non-histone proteins for acetylation, including proteins involved in metabolic processes. Furthermore, lack of VHL function causes a metabolic switch to aerobic glycolysis or "Warburg effect". This is associated with a high glucose influx, a decreased gluconeogenesis and an increased lactate concentration in the tumor microenvironment, which is associated with an impaired immune recognition. Moreover, lactate promotes histone acetylation and gene expression through histone deacetylase (HDAC) inhibition contributing to tumor metastasis and resistance to therapy. P300/CBP serve as transcriptional coactivators of LDH-A. Notably, p300 knockout reduces lactate production. The proposed experiments will evaluate the therapeutic efficacy of novel clinically relevant p300/CBP inhibitor CCS1477 as monotherapy or in combination with TKIs. The experiments will provide rationale for transitioning CCS1477 to a clinical trial addressing the treatment of patients with ccRCC.

15. SUBJECT TERMS

Clear cell Renal cancer; p300; CBP; HIF-1α; HIF2α; LDH-A; p300/CBP inhibitor (CCS1477)

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INTRODUCTION: The vast majority of sporadic and hereditary clear cell renal cell carcinoma (ccRCC) lacks functional von Hippel-Lindau (VHL) protein. Loss of VHL results in the aberrant accumulation of HIF proteins, and thereby enhanced expression of pro-angiogenic factors, the leading cause of tumor angiogenesis. In the case of VHL-defective ccRCC, HIF-2α appears to act as an oncogene whereas HIF-1α has a role as a tumor suppressor. HIF-2α signaling is prominently regulated via its acetylation by CBP. p300 and CBP also target a significant number of non-histone proteins for acetylation, including cytosolic proteins involved in metabolic processes. Furthermore, lack of VHL function causes a metabolic switch to aerobic glycolysis or "Warburg effect". This is associated with a high glucose influx, a decreased gluconeogenesis and an increased lactate concentration in the tumor microenvironment, which is associated with an impaired immune recognition. Moreover, lactate promotes histone acetylation and gene expression through histone deacetylase (HDAC) inhibition mechanism contributing to tumor metastasis and resistance to anticancer therapy. p300/CBP serve as transcriptional coactivators of LDH-A. Notably, p300 knockout reduces lactate production. Although p300 and CBP play distinct roles, they are highly homologous and their functions are largely redundant. The proposed experiments will evaluate the therapeutic efficacy of novel p300/CBP inhibitor CCS1477. CCS1477 is the first drug to be used in patients that specifically targets p300/CBP. It is currently under Phase I/II clinical trials for the treatment of some solid tumors. The proposed studies are focused to examine, for the first time, the therapeutic efficacy of novel p300/CBP inhibitor CCS1477 as monotherapy or in combination with clinically relevant TKIs. The experiments will provide rationale and lay the groundwork for transitioning CCS1477 to a clinical trial addressing the treatment of patients with ccRCC.

KEYWORDS: Clear cell Renal cancer; p300; CBP; HIF-1α; HIF2α; LDH-A; p300/CBP inhibitor (CCS1477)

ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1. To evaluate the therapeutic efficacy of CCS1477 using cell and PDX models of ccRCC.

1. To examine the antitumor activity of p300/CBP inhibitor CCS1477 using a panel of six established long-term cultured and two short-term cultured primary ccRCC cell lines.

2. To examine the therapeutic efficacy of CCS1477 using PDX ccRCC model.

Milestone achieved: These experiments will provide preclinical support and justification for transitioning CCS1477 to a clinical trial for the treatment of ccRCC. 100% completed.

Aim 2. To determine the link between p300/CBP expression, clinical outcomes and response to TKIs in ccRCC patients.

3. To determine the link between p300/CBP expression, clinical outcomes and response to TKIs in ccRCC patients.

Milestone achieved: These studies will determine if p300/CBP might provide useful biomarkers for prognostic and therapeutic purposes in ccRCC. 10% completed

What was accomplished under these goals?

Major activities

During the reporting period we were performing the following studies: (i) evaluation the antitumor activity of p300/CBP inhibitor CCS1477 using three ccRCC cell lines; (ii) examining of the role of p300/CBP inhibition on the acetylation levels of HIF2α and expression of HIF2α dependent genes, VEGFA and LDHA; (iii) solving anti-Acetyl-HIF2α antibody-related issues and optimized conditions of Western Blotting analysis; (iv) testing of anti-tumor activity of combination of CCS1477 plus cabozantinib using xenograft PDX mouse model of ccRCC, PNX0010.

Specific objectives

To establish for the first time a proof of principle of our concept whether clinically relevant p300/CBP inhibitor, CCS1477, can be used as an effective therapeutic approach for ccRCC alone, or in combination with anti-angiogenic TKIs.

Significant results/Major findings

1. p300/CBP inhibitor, CCS1477, potently inhibits viability of ccRCC cells and has synergistic effect in combination with anti-angiogenic TKIs.

To address the proposed concept whether CCS1477 can represent a novel therapeutic approach against ccRCC, we have evaluated the effects of CCS1477 on ccRCC cells viability *in vitro* using Cell Titer Blue (CTB) assay. Three ccRCC cell lines (786-O, 769-P and SK-RC-45) were analyzed. Briefly, cells were plated on 96 well plates (500 cells per well). 24 hours post-plating, CCS1477, sunitinib and cabozantinib were added to the wells at escalating concentrations alone, or in combinations (CCS1477 + Sunitinib, or CCS1477 + cabozantinib). Cells were incubated with drugs for 96 hours. Viability was evaluated using CTB assay (Promega, Madison, WI) according to the manufacturer instructions. To establish the potential synergistic effect between CCS1477 and TKIs, we first, established Effective doses (ED50) values for those drugs. ED50 were calculated using XLfit, a Microsoft Excel add-in (Table 1).

Cell line	ED50 CCS1477 (μM)	ED50 Sunitinib (μM)	ED50 Cabozantinib (μM)
786-O	11.36	2.95	5.09
769-P	9.80	3.56	11.25
SK-RC-45	6.51	2.90	5.80

Table 1. Determination of ED50 values of indicated drugs for ccRCC cell lines.

Next, 786-O, 769-P and SK-RC-45 cells were treated with various dosing regimens of CCS1477 plus sunitinib, or CCS1477 plus cabozantinib to examine a synergistic antitumor effect for the combination of these agents. The data analysis was performed using CalcuSyn 2.0 software. Combination Index (CI) values meanings are following: CI 0.85–0.9: slight synergism; CI 0.7–0.85: moderate synergism; CI 0.3–0.7: synergism; CI 0.1–0.3: strong synergism; CI < 0.1: very strong synergism. Excitingly, as demonstrated in the Table 2, the combination index (CI) values revealed a high levels of synergistic interaction between both pairs of used drugs at all ED dosing regimens (excepting only CCS/Cab combination at ED25 for 786-O cells; CCS/Sut combination at ED50 for SK-RC-45 cells, and CCS/Sut combination at ED90 for 769-P cells).

Cell line	ED90		ED75		ED50		ED25	
	CCS/Sut	CCS/Cab	CCS/Sut	CCS/Cab	CCS/Sut	CCS/Cab	CCS/Sut	CCS/Cab
786-O	0.46002	0.21837	0.50461	0.37672	0.55380	0.65016	0.60798	1.12229

769-P	1.12590	0.63875	0.78086	0.48043	0.54192	0.36156	0.37639	0.27233
SK-RC-45	0.92705	0.57101	0.98329	0.64073	1.04296	0.71897	N/A	N/A

Table 2. CI values for combinations of CCS1477 plus sunitinib (CCS/Sut), and/or CCS1477 plus cabozantinib (CCS/Cab) in three tested ccRCC cell lines treated at indicated ED dosing regimens.

2. CCS1477 affects the expression of HIF2a dependent genes, VEGFA and LDHA, as well as expression of FGF2, potent alternative pro-angiogenic cytokine involved into anti-VEGFR TKI resistance.

To evaluate the effects of CCS1477 on the expression of HIF2a dependent genes, VEGFA and LDHA, we incubated 786-O and 769-P ccRCC cell lines with escalating concentrations (5 and 10μM) of CCS1477 for 24 hours in complete medium. Then, total RNA was isolated and the levels of target genes were evaluated by qRT-PCR. As demonstrated in the figure 1, the levels of VEGFA and LDHA were decreased in dose-dependent manner. Importantly, we have also analyzed expression levels of FGF2, which serves as major alternative pro-angiogenic cytokine involved into anti-VEGFR TKI resistance. qRT-PCR analysis demonstrated decrease in FGF2 expression when cells were treated with 10μM of CCS1477 (Figure 1). This data indicates the potential of

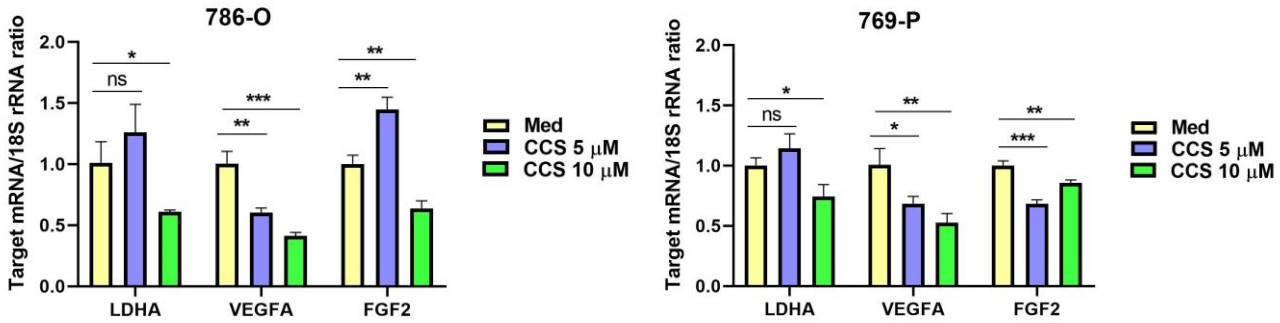

Figure 1. qRT-PCR analysis of LDHA, FGF2 and VEGFA genes expression. 786-O and 769-P cells were treated at indicated concentrations of CCS1477 for 24 hours. The graphs represent the average data from three independent repeats. *, P<0.05; **, P<0.01; ***, P<0.001.

CCS1477 not only to decrease VEGF/VEGFR signaling, but also suppress, or at least significantly delay the development of resistance to anti-VEGFR TKIs.

Taken together, the data obtained during the reporting period provide a clear proof of concept about anti-tumor efficacy of combinatorial use of CCS1477 and TKIs as a novel therapeutic approach for ccRCC management.

3. Combination of CCS1477 plus cabozantinib markedly inhibits ccRCC tumor growth *in vivo*.

Given that cabozantinib has demonstrated the best synergy with CCS1477, this TKI had been chosen for the future *in vivo* studies. 8 weeks old NSG mice (n=5 per group) were subcutaneously injected with $2x10^6$ PNX0010 cells. This cell line was established from ccRCC PDX model. Importantly, PNX0010 TKI-resistant model was developed from ccRCC patient with enormously short relapse periods after TKI therapeutic courses. Animals were fed sterile AIN-93M diet (Harlan Teklad, Madison, WI) and water ad libitum. When tumor volumes reached approximately 150 mm^3, animals were randomly assigned to the control or experimental groups (n=5 mice/group). The mice were treated with (i) 0.15M NaCl with 10% (2-Hydroxypropyl)-β-cyclodextrin (HPCD) solution (vehicle); (ii) CCS1477 (10 mg/kg in 0.15M NaCl with 10% HPCD solution, daily, by oral gavage); (iii) cabozantinib (20 mg/kg in 0.15M NaCl with 10% HPCD solution, daily, by oral gavage); or (iv) combination of drugs. Tumors were measured twice weekly and their volumes were calculated with the formula: [volume = 0.52 x (width)2 x length].

Excitingly, consistent with our *in vitro* observations, the combinatory treatment with CCS1477 and cabozantinib resulted in dramatic reduction of tumor growth and increase in survival (Figure 2). This confirms the strong synergistic effect between these two drugs. Importantly, such combinatorial treatment was well tolerated in mice. We did not observe any adverse clinical side effects (severe diarrhea, cachexia, or 20% weight loss from baseline and/or compared untreated controls).

We also analyzed the levels of proliferation and micro-vessel density in tumor samples using IHC staining for human Ki67 and mouse CD31. Consistently with tumor growth dynamics, CCS1477 alone didn't have any effects on proliferation and angiogenesis, whereas, in tumors from mice treated with cabozantinib only, we observed significant decrease in vascularization accompanied with central necrotic areas, however proliferation of tumor cells was not affected in peripheral and non-necrotic zones. Oppositely, in the tumor samples from mice treated with the combination with two drugs we observed significantly decreased levels of both, proliferation and micro-vessel density (Figure 3).

Taken together, we believe that our successful completion of the studies outlined in the proposal provide rationale and lay the groundwork for transitioning CCS1477 to a clinical trial addressing the treatment of patients with ccRCC.

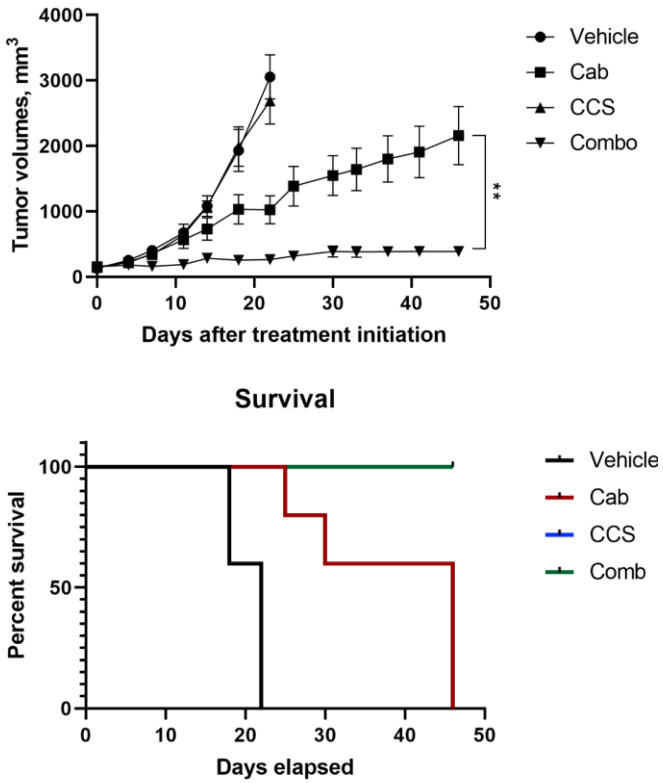

Figure 2. PNX0010 tumor growth dynamics (upper panel) and survival (lower panel) of mice treated with Vehicle, CCS1477 (CCS) and cabozantinib (Cab) alone, or in combination (Combo). Error bars represented by SEM. Statistical analysis was performed using unpaired two tailed t-test. **, p=0.0043. Survival was calculated by the Kaplan-Meier method. Survival curves for Vehicle and CCS1477 are overlapping.

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

Nothing to Report.

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?

Nothing to Report.

IMPACT:

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

Nothing to Report.

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:

Changes in approach and reasons for change

Nothing to Report.

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

We were unable to determine the link between p300/CBP expression, clinical outcomes and response to TKIs in ccRCC patients due to inconsistent IHC staining of TMA.

Changes that had a significant impact on expenditures

Nothing to Report.

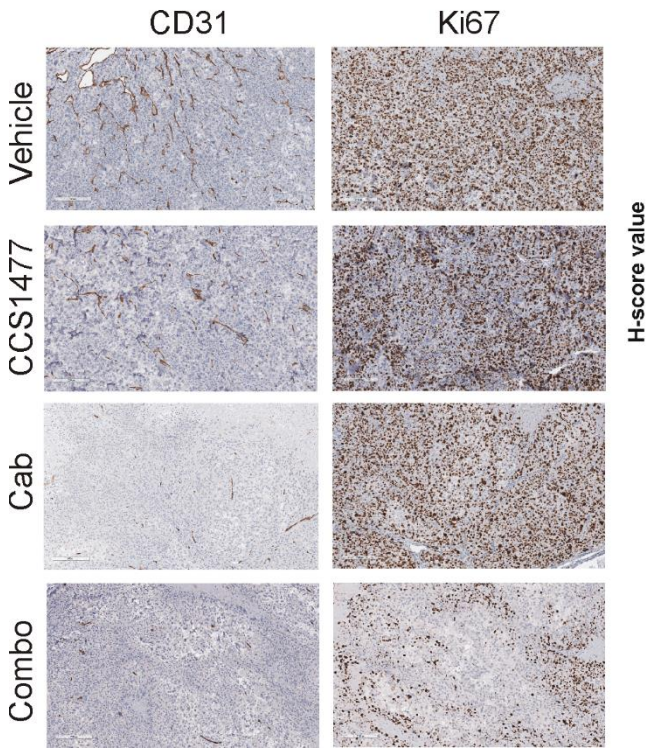

Figure 3. Representative image of IHC analysis of micro-vessel density (anti-mouse CD31 antibody) and proliferation (anti-human Ki67) in mouse xenograft models of ccRCC. Animals were treated with Vehicle, CCS1477 and cabozantinib (Cab) alone, or in combination (Combo). IHC analysis and H-score calculations were performed with the assistance of FCCC Histopathology Facility. Immunostained slides were scanned using an Aperio ScanScope CS 5 slide scanner (Aperio, Vista, CA, USA). Scanned images were then viewed with Aperio's image viewer software (ImageScope, version 11.1.2.760, Aperio). Selected regions of interest were outlined manually by a pathologist. The Ki-67 quantifications were performed with Aperio V9. 10 LOIs (lesion of interest) were selected for each case. The H-score, which ranges from 0 to 300, was calculated using the following formula: [1(% cells 1+) + 2 (% cells 2+) + 3 (% cells 3+)]. Error bars represented by SEM. Statistical analysis was performed using unpaired two tailed t-test. ****, $p<0.0001$.

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

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

PRODUCTS:

Publications, conference papers, and presentations:

Journal publications. Nothing to Report.

Books or other non-periodical, one-time publications. Nothing to Report.

Other publications, conference papers, and presentations. Nothing to Report.

Website(s) or other Internet site(s). Nothing to Report.

Technologies or techniques:

Nothing 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?

Name:	Petr Makhov, M.D., Ph.D.
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.00
Contribution to Project:	Dr. Makhov was responsible for the overall administration and guidance of the research project. He was directly responsible for the training and management of personnel engaged in achieving all specific aims outlined in the research proposal. His major responsibilities included planning experiments, evaluating the results and overseeing the budget. He was also performed animal studies and calculated ED50 and CI values for drugs and their combinations. In addition,

	he was responsible for the preparation of progress reports generated from this project.
Funding Support:	DOD
Name:	Ekaterina Davydova
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3.00
Contribution to Project:	Ekaterina Davydova was responsible for Cell Titer Blue analysis of ccRCC cells proliferation upon drugs treatment, Western Blot analysis of protein expression levels, qRT PCR analysis of LDHA, FGF2 and VEGFA genes expression. In addition, she assisted Dr. Makhov with generation of mouse xenograft ccRCC models, drug treatments of animals and general necropsy.
Funding Support:	DOD

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

Not applicable.

What other organizations were involved as partners?

Nothing to Report.

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Not applicable.

QUAD CHARTS:

Not applicable.

APPENDICES:

None.