Targeting dopamine receptor D2 as a novel therapeutic strategy in endometrial cancer

Stuart R. Pierce
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Ziwei Fang
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Yajie Yin
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Lindsay West
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Majdouline Asher
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Tianran Hao
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Xin Zhang
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Katherine Tucker
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Allison Staley
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Yali Fan
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Wenchuan Sun
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Dominic T. Moore
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Chang Xu
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Yi-Hsuan Tsai
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Joel Parker
  UNC-Chapel Hill: University of North Carolina at Chapel Hill

Varun Vijay Prabhu
  Oncoceutics, philadelphia

Joshua E Allen

Oncoceutics Philadelphia

Douglas Lee
Omic insight, NC, USA

Chunxiao Zhou (✉ czhou@med.unc.edu)
University of North Carolina at Chapel Hill

Victoria Bae-Jump
UNC-Chapel Hill: University of North Carolina at Chapel Hill

Research

Abstract

Background

ONC201 is a dopamine receptor D2 (DRD2) antagonist that inhibits tumor growth in preclinical models through ClpP activation to induce integrated stress response pathway and mitochondrial events related to inhibition of cell growth, which is being explored in clinical trials for solid tumors and hematological malignancies. In this study, we investigated the anti-tumorigenic effect of ONC201 in endometrial cancer cell lines and a genetically engineered mouse model of endometrial cancer.

Methods

Cell proliferation was assessed by MTT and colony formation assays. Cell cycle and apoptosis were evaluated by Cellometer. Invasion capacity was tested using adhesion, transwell and wound healing assays. LKB1$^{fl/fl}$p53$^{fl/fl}$ mouse model of endometrial cancer were fed a control low fat diet versus a high fat diet to mimic diet-induced obesity. Following tumor onset, mice were treated with placebo or ONC201. Metabolomics and lipidomics were used to identify the obesity-dependent effects of ONC201 in the mouse endometrial tumors. DRD2 expression was analyzed by immunohistochemistry in human endometriod and serous EC specimens. DRD2 mRNA expression from the Cancer Genome Atlas (TCGA) database was compared between the four molecular subtypes of endometrial cancer.

Results

Increasing DRD2 expression in endometrial cancer was significantly associated with grade, serous histology and stage, as well as worse progression free survival and overall survival. Higher expression of DRD2 mRNA was found for the Copy Number High (CNH) subtype when compared to the other subtypes.. ONC201 inhibited cell proliferation, induced cell cycle G1 arrest, caused cellular stress and apoptosis and reduced invasion in endometrial cancer cells. Diet-induced obesity promoted endometrial tumor growth while ONC201 exhibited anti-tumorigenic efficacy in the obese and lean LKB1$^{fl/fl}$/p53$^{fl/fl}$ mice. Metabolomic analysis demonstrated that ONC201 reversed the obesity-driven upregulation of lipid biosynthesis and reduced protein biosynthesis in obese and lean mice.

Conclusion

ONC201 has anti-proliferative and anti-tumorigenic effects in endometrial cancer cells and mouse model, and DRD2 expression was documented in both human serous and endometrioid endometrial cancer. These studies support DRD2 antagonism via ONC201 as a promising therapeutic strategy for endometrial cancer that has already demonstrated pharmacodynamic activity and clinical benefit in both serous and endometrioid endometrial cancer patients.

Introduction

Endometrial cancer (EC) is the most common gynecologic malignancy, and alarmingly, the frequency and mortality from EC continues to rise, in part due to the obesity epidemic (1). In 2020, 65,620 new cases will be diagnosed, and 12,590 women will succumb to this disease (2). Patients with advanced/recurrent EC are unlikely to be cured by surgery, conventional chemotherapy (paclitaxel + carboplatin is the standard 1st line treatment), radiation or a combination of these. In patients with prior exposure to platinum chemotherapy, further chemotherapy, endocrine therapy, targeted therapy, and/or immunotherapy have low response rates between 10–38% with long-term disease-free survival being rare (3–7). Thus, novel treatments are desperately needed.

Interest in personalized medicine and targeted therapies continues to rise. The dopamine receptor pathway has emerged as a novel oncologic target due to its documented role in cellular proliferation pathways (8, 9). Historically, modulation of the dopaminergic pathway has primarily focused on neurologic diseases or anti-psychotic therapy. However, a growing body of literature reveals increased mRNA and protein expression of the G protein-coupled DRD2 (dopamine receptor D2) in many tumors including breast, colon, pancreas, prostate, glioblastoma, lymphoma, and endometrial cancer (10–14). Knockdown of DRD2 or inhibition with pharmacologic antagonists in glioblastoma, pituitary tumor, pancreatic and breast cancer cells leads to inhibition of proliferation and migration as well as anti-tumorigenic effects in number of different xenograft mouse models (11, 13–16).

Imipridones are a new class of anti-cancer compounds that target G protein-coupled receptors. ONC201, the first-in-class clinical imipridone, selectively and competitively antagonizes DRD2, induces the integrated stress response through activation of ClpP and results in activation of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway (17–20). *In vitro* studies in lymphoma, leukemia, glioblastoma, colorectal cancer have shown ONC201 increases apoptosis and inhibits Akt/mTOR signaling, leading to a corresponding decrease in tumor growth in mouse models (17, 19). This is significant particularly since PI3K/AKT signaling is often deregulated in both Type I (endometrioid histology) and II (serous histology) ECs (21). We recently found that initial *in vitro* testing of serous EC cell lines with ONC201 resulted in significant inhibition of cellular proliferation, induction of apoptosis, as well as anti-metastatic effects (22). The results from several phase I and II clinical trials showed that ONC201 is clinically active and exceptionally well-tolerated with favorable pharmacokinetics, pharmacodynamics and clinical benefit in advanced cancers including lymphoma, glioblastoma and ECs (17, 23–26). ONC201 has demonstrated durable objective responses in patients with H3 K27M mutant gliomas and FDA has granted Fast track designation to this investigational agent (26, 27). The objective of this study was to investigate the expression of DRD2 in human EC specimens as well as to evaluate the anti-proliferative and anti-tumorigenic effects of DRD2 antagonist ONC201 in EC cell lines and a genetically engineered mouse model of EC.

Methods

Cell culture and reagents

Four EC cell lines, Ishikawa, ECC-1, HEC1A and KLE, were used for all experiments. The ECC-1 cells were maintained in RPMI 1640 medium with 5% fetal bovine serum (FBS). The Ishikawa and ECC-1 cells were maintained in DMEM/F12 with 10% FBS. HEC1A cells were cultured in McCoy's 5A with 10 % FBS. All medium was supplemented with 100 U/ml of penicillin and 100 ug/ml of streptomycin. The cells were cultured in humidified 5% CO_2 at 37°C. ONC201 was obtained from Oncoceutics, Inc. All antibodies were purchased from Cell Signaling (Beverly, MA).

MTT assay

The four cell lines were plated and grown in 96-well plates at a concentration of 4000 to 6000 cells/well for 24 hours. The cells were subsequently treated with varying doses of ONC201 for 72 hours. MTT (5 mg/ml, Sigma) was added to the 96-well plates at 5 μl/well, followed by an additional hour of incubation. The MTT reaction was terminated through the addition of 100 μl of DMSO. The results were read by measuring absorption at 570 nm with a microplate reader (Tecan, Morrisville, NC). Quest Graph™ IC50 Calculator (AAT Bioquest) was used to calculate IC50 value. The effect of ONC201 was calculated as a percentage of control cell growth obtained from DMSO treated cells grown in the same 96-well plates.

Colony formation assay

ECC-1 and KLE cells were seeded (1000 cells/well in a 6-cm dishes) in regular growth medium overnight, and medium was replaced with fresh complete regular growth medium containing the indicated concentrations of ONC201 for 36 hours. Cells were cultured at 37°C for 14 days, with medium changes every third or fourth day. Cells were stained with 0.5% crystal violet, and colonies were counted under microscope.

Analysis of apoptosis and cell cycle by Cellometer imaging

The ECC-1 and KLE cells were treated with ONC201 for 24 to 36 hours in six well plates. For apoptosis analysis, the cells were collected and resuspended in 100 μl binding buffer containing Annexin V-FITC and 0.5 μl of propidium iodide for 15 minutes. For cycle analysis, the cells were harvested and fixed in a 90% methanol solution for 1 hour. The cells were resuspended in RNase A solution for 30 minutes, followed by incubation with propidium iodide (PI) staining solution for 10 minutes. All samples were immediately measured by Cellometer (Nexcelom, Lawrence, MA) to identify apoptotic cells and assess cell cycle progression (22, 28). The results were analyzed by FCS4 express software (Molecular Devices, Sunnyvale, CA).

Adhesion and invasion assay

For the adhesion assay, 2.5×10^3 cells were added in laminin-1 coated 96-well plates with varying concentrations of ONC201 at 37°C for 1.5 hours. After this time period, the cells were fixed by 5% glutaraldehyde. Adhered cells were stained with crystal violet, and 10% acetic acid was used to solubilize the dye. The absorbance was measured at 570 nm using a microplate reader. For the invasion assay, the

cells were starved for 12 hours and then seeded in the upper chambers coated with BME. The lower chambers were filled with regular medium plus ONC201 for 6 hours at 37°C. After Calcein AM solution was added into lower chamber for 30 minutes, the lower chamber plate was measured using a plate reader for reading fluorescence at EX/EM 485/520 nm.

Wound healing assay

The ECC-1 and KLE cells were plated at 3×10^5 cells per well in a 6-well plate for 24 hours and then replaced with media with 0.5 % charcoal stripped FBS for 12 hours. A uniform wound was created through the cell monolayer using a 200 µl pipette tip. Cells were treated with ONC201 immediately after scratching. Photographs were taken at 0, 24 and 48 hours after scratching, and the area of the scratch was analyzed with ImageJ software (NCI, Bethesda, MD). Percent closure was measured compared to 0 hours, and fold change was determined from percent closure of treated compared to untreated.

ROS assay

The cells were seeded onto a 96-well black culture plate overnight and then treated with ONC201 at the indicated doses for 24 hours. 10µl DCF-DA (10mM) was added into the media for 30 minutes. The fluorescence intensity was measured at EX/EM 485/530 nm using a fluorescence micro-plate reader

Western immunoblotting

The ECC-1 and KLE cells treated with ONC201 for 24-36 hours. Cell lysates were prepared in RIPA buffer. Protein concentration was measured by BCA assay. Equal amounts of protein were separated by 10-12% gel electrophoresis and transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat dry milk and then incubated with a 1:1500 dilution of primary antibody overnight at 4°C. The membrane was then washed and incubated with a secondary peroxidase-conjugated antibody for 1 hour after washing. Antibody binding was detected using SuperSignal™ West Pico on the ChemiDoc™ Image System (Bio-Rad). After developing, the membrane was stripped and re-probed using antibodies against β-actin or α-tubulin to confirm equal loading. Intensity for each band was measured and normalized to β-actin or α-tubulin as an internal control.

LKB1$^{fl/fl}$ p53$^{fl/fl}$ transgenic mouse model of EC

The LKB1$^{fl/fl}$ p53$^{fl/fl}$ mouse genetically engineered mouse model of EC was used in this study as described previously in detail (28). All mice were handled according to protocols approved by UNC-CH Institutional Animal Care and Use Committee (IACUC). To mimic diet-induced obesity (DIO), half of the mice were subjected to a high fat diet (HFD), while the other half were subjected to a low fat diet (LFD) (Research Diets, New Brunswick, USA) at 3 weeks age. Intrauterine Ad-Cre injections of LKB1$^{fl/fl}$ p53$^{fl/fl}$ mice were performed on the left side of uterus at 6-8 weeks of age to induce EC. The LFD and HFD mice were further divided into the vehicle or ONC201 (130mg/kg, weekly, Oral gavage, 4 weeks) treatment groups, The animals were weighed weekly throughout the study. All mice were euthanized after 4 weeks

of ONC201 or vehicle treatment. At sacrifice, endometrial tumors were weighed and blood samples were taken. Half of the endometrial tumor was snap-frozen and stored at −80°C, and the other half was fixed in 10% neutral-buffered formalin and paraffin embedded. The paraffin sections from the endometrial tumors were subjected to immunohistochemistry at the Animal Histopathology Core Facility at UNC. The following primary antibodies were used: Ki-67, vascular endothelial growth factor, phosporylated-p42/44 and phosphorylated-S6. Further processing was carried out using ABC-Staining Kits (Vector Labs, Burlingame, CA) and hematoxylin. Immunohistochemistry slides were scanned by Motic and scored by ImagePro software (Vista, CA).

Immunohistochemistry of human endometrial tumor specimens

Triplicate cores were made of 118 endometrial tumors at our institution from hysterectomy specimens, and tissue microarrays were created. Immunohistochemical analysis DRD2 was performed on 4-µmol/L sections of formalin-fixed, paraffin-embedded tissues using standard methodologies in UNC-CH Translational Pathology Laboratory Core. Individual slides were scanned using the Aperio™ ScanScope (Aperio Technologies, Vista, CA), and digital images were analyzed using Aperio™ ImageScope. Non-parametric tests and Cox regression analysis were used to correlate DRD2 expression with clinical outcomes.

TCGA RNA-Seq Analysis

Using the TCGA data portal, we collected DRD2 RNA-Seq expression for differential gene expression analysis among the 371 EC samples. Information regarding bio-specimen procurement, data processing, quality control and normalization has been described by the Cancer Genome Atlas Research Network and can be reviewed on the TCGA open-access data portal (http://cancergenome.nih.gov). The RNA-seq gene expression level 3 data utilized in this study were TCGA normalized gene read counts, pre-corrected such that the 75th percentile of each patient's set of gene expression measurements were normalized to a value of 1000. Using a multiple linear regression model for DRD2, we tested the association between DRD2 expression and EC genomic subtype, POLE ultramutated (POLE), microsatellite instability hypermutated (MSI), copy-number low (CNL) and copy-number high (CNH)(29).

Metabolomics measurements

Endometrial tumors were analyzed from the four groups (N = 5/group) by Metabolon (Durham, NC) according to their standard protocols(30, 31). Briefly, unbiased global metabolomic profiling was achieved using methanol extracts of tumor tissues normalized to tissue weight. Analysis of extracts consisted of either ultrahigh performance liquid chromatography (Waters Corporation, Milford, MA) coupled with tandem mass spectrometry (UHPLC/MS/MS; Thermo-Finnigan, San Jose CA) in positive and negative ionization modes, or via gas chromatography/MS analysis (Thermo-Finnigan). Metabolites in tumor tissues were positively identified by matching chromatographic retention time, mass and MS/MS fragmentation patterns to a reference library of over 2500 purified, authenticated biochemicals. Identification of known chemical entities was based on comparison to metabolomic library entries of

purified standards based on chromatographic properties and mass spectra. Data are presented as relative measures of "scaled intensity" and median scaling to 1. Missing values were imputed with the minimum.

Statistical Analysis

Descriptive statistics were used to summarize data, particularly medians and inter-quartile ranges and means and standard deviations. Both parametric (Student's t, ANOVA, and linear regression) and nonparametric (Wilcoxon, Kruskal-Wallis) methods were used. The Kaplan-Meier method was used to estimate the time-to event functions of progression-free survival (PFS) and overall survival (OS). The log-rank test was used for comparisons of these functions. Both SAS (version 9.4, Cary, NC) and GraphPad Prism (version 6, La Jolla, CA) statistical software packages were used. All tests were two-sided with p<.05 considered significant.

Results

DRD2 expression was associated with prognosis in EC patients

A cohort of 118 human EC patients was identified that included 98 endometrioid and 20 serous histologies. Median patient age was 60.5 years with a median BMI of 31. Immunohistochemistry staining was performed for DRD2 in these tumors. Using median composite H-scores, increasing DRD2 protein expression was significantly associated with tumor grade (p<0.0001), stage (p<0.0001), and serous histology (p<0.0001) (Figure 1A and B). In addition, expression of DRD2 protein was significantly associated with progression free survival (PFS) (p=0.049) and overall survival (OS) (p=0.02, Figure 1C). Using the TCGA database (N=371 endometrial tumors), DRD2 mRNA expression was higher in serous type compared with endometrioid type EC (p=0.02) (Figure 1D). We further analyzed the expression of DRD2 distribution in molecular subtypes of EC based on molecular classification of endometrioid and serous carcinomas, and found high expression of DRD2 in serous-like copy-number high subtype when compared to the other subtypes (p=0.005) as seen in Figure 6E. These results confirm that DRD2 is expressed in both endometrioid and serous histology ECs; however, increased expression of DRD2 was associated with more aggressive histology's (serous versus endometroid) and molecular subtypes (CNH) as well as higher grade and stage.

Effect of ONC201 on cell proliferation in EC cell lines

Four EC cell lines, Ishikawa, HEC1A, ECC-1 and KLE, were treated with ONC201 at different concentrations for 72 hours. Cell proliferation was assessed by MTT assay. The results showed that ONC201 demonstrated significant reduction in cell proliferation in a dose dependent manner in all cell lines. The IC50 dose was between 1.39 and 4.01 µM in all cell lines (Figure 2A). Colony formation assays were performed to investigate the long-term effect of ONC201 on cell growth in the ECC-1 and KLE cell lines. As shown in Figure 2B, the colony-forming ability of ECC-1 and KLE was reduced by 64% and 57% (p<0.01), respectively, after exposure to 5 uM of ONC201 for 36 hours and subsequent culture of cells for 14 days.

The effects of ONC201 on cellular morphology in both cell lines are shown in Figure 2C. Following ONC201 treatment for 48 hours, both cell lines shrunk and displayed an elongated shape while control cells were round or oval shaped with large nuclei.

In order to determine if the level of DRD2 protein expression was related to the sensitivity to ONC201 in each cell line, we detected expression of DRD2 and DRD5 using western blotting in all four cell lines. The results revealed varying levels of DRD2 and DRD5 expression in the four cell lines. The expression levels of DRD2 and DRD5 were not associated with sensitivity to ONC201 in the cell lines. ECC-1 cells displayed the highest level of DRD2, but was not more sensitive to ONC201 than the other cell lines when compared with the IC50 for each cell line (Figure 2D). Treatment of ECC-1 and KLE cells with ONC201 for 24 hours significantly increased the expression of DRD2 and decreased the expression of DRD5 (Figure 2E).

We next investigated whether the AKT/mTOR pathway was involved in the anti-proliferative effect of ONC201 in the ECC-1 and KLE cell lines. Western blotting showed that phosphorylation of S6 was repressed by ONC201 in a dose dependent manner after 24 hours of treatment in both cell lines. ONC201 increased phosphorylation of AKT expression in the ECC-1 and KLE cells (Figure 2F). Together, these results suggest that ONC201 inhibits cell proliferation in part via the AKT/mTOR/S6 pathway.

Effect of ONC201 on apoptosis and cell cycle in EC cells.

To evaluate the underlying mechanism of growth inhibition by ONC201, the cell cycle profile was analyzed after treating the ECC-1 and KLE cell lines with varying doses (1-50uM) of ONC201 for 36 hours. As illustrated in Figure 3A, ONC201 induced G1 cell cycle arrest and reduced G2 phase in the ECC-1 and KLE cell in a dose-dependent manner. In the ECC-1 cells, G1 phase arrest increased from 36.26% in the control to 46.44% in cells treated with ONC201 at 50 µM (p<0.05). In KLE cells, treatment with ONC201 increased G1 phase arrest from 56.36% in the control to 70.59% at a dose of 50 uM (p<0.01). Western immunoblotting showed that ONC201 down-regulated the cell cycle related proteins, cyclin D and CDK4, and increased p21 expression (Figure 3B). Thus, ONC201 treatment resulted in arresting cells in G1 phase in EC cells.

To further confirm whether the growth inhibition by ONC201 was related to apoptosis, we evaluated its apoptotic effect on ECC-1 and KLE cells by Annexin-V FITC stain analysis, which detects the phospholipid phosphatidylserine (PS) translocation from the inner (cytoplasmic) leaflet of the cell membrane to the external surface in very early apoptotic cells. As shown in Figure 3C, after treatment of the cells with ONC201 at the indicated concentrations for 24 hours, there was an increase in early apoptotic cell populations from untreated to treated cells (4.8%-25.8% in ECC-1 cells and 7.25%-13.7 % KLE cells, respectively). Western blotting results showed that ONC201 significantly reduced MCL-1 and BCL-2 expression in both cells after 24 hours of treatment (Figure 3D). To further explore the effect of ONC201 on the apoptotic pathways, an ELISA assay was used to detect the activity of cleaved caspase-3 in the ECC-1 and KLE cell lines treated with ONC201. We found that ONC201 increased cleaved caspase-3 activity in a dose-dependent manner after 24 hours of treatment. Exposure of cells to 50 µM ONC201 resulted in 1.8 fold increase in cleaved caspase-3 activity in KLE and 2.7 fold increase in ECC-1 cells

(Figure 3E). These data suggest that ONC201 reduces proliferation through induction of apoptosis and G1 cell cycle arrest in EC cells.

Effect of ONC201 on cellular stress in EC cells

Reactive oxygen species (ROS) have been implicated in the cellular response to stress and are involved in mediation of apoptosis via mitochondrial DNA damage. To investigate the involvement of oxidative stress in the anti-proliferative effect of ONC201, intracellular ROS levels were examined by using the ROS fluorescence indicator DCFH-DA. As seen in Figure 4A, treatment with ONC201 for 24 hours significantly increased cellular ROS production in a dose-dependent manner in the ECC-1 and KLE cells. We next examined the changes of markers for endoplasmic reticulum (ER) stress after 24 hours of ONC201 treatment in both cell lines. Western blotting showed ONC201 induced PERK, Bip, PD-1, Erol-1 and IRE1-a protein expression in a dose dependent manner, which is further evidence of ER stress induction by ONC201 (Figure 4B). These results indicate that an increase in ROS might also be involved in the anti-tumorigenic effects of ONC201 in EC cells.

Effect of ONC201 on adhesion and invasion in EC cells

Adhesion and invasion of tumor cells are important steps leading to metastasis. In order to determine the effect of ONC201 on the invasive ability of EC cells, an *in vitro* laminin adhesion assay, transwell invasion system and wound healing assay were employed. Treatment of the ECC-1 and KLE cells with ONC201 for 90 min showed a significant reduction in adhesion in both cells (Figure 5A). Exposure to ONC201 at concentrations ranging from 1-50 uM for 6 hours significantly suppressed invasive ability of the cells compared to vehicle-treated control cells as determined by the transwell invasion assay (Figure 5B). To examine the effect of ONC201 on motility in EC cells, we used a scratch wound healing assay to measure the extent of cell migration into the scratched area. ONC201 significantly slowed down cell migration into the "wounded" area in both cells after 72 hours of treatment (Figure 5C). To further analyze the effect of ONC201 on epithelial-mesenchymal transition (EMT) and vascular endothelial growth factor (VEGF) of EC cells, the levels of expression of E−cadherin and VEGF were analyzed by Western blotting. After 24 hours of treatment, ONC201 increased expression of E-cadherin and decreased expression of snail and VEGF (Figure 5D). Collectively, these results demonstrate that ONC201 inhibits the adhesion and invasion of EC cells.

Anti-tumorigenic effect of ONC201 on LKB1$^{fl/fl}$ p53$^{fl/fl}$ mouse model of EC

Given that ONC201 significantly inhibited EC cell proliferation and induced apoptosis *in vitro*, we sought to evaluate the effect of ONC201 on tumor growth in the LKB1$^{fl/fl}$p53$^{fl/fl}$ mouse model of EC. The mice were divided into four groups including LFD and HFD groups treated with either ONC201 or placebo. The mice were treated weekly by oral gavage with either ONC201 (130mg/kg) or placebo for 4 weeks after tumor induction. This dose schedule mimics the administration of ONC201 in the clinic. The initial body weights of the obese mice at the starting treatment with ONC201 were 36.8 gm, while that of the lean mice was only 25.6 gm (p<0.01, data not shown). Tumor weights were significantly increased in the

control obese mice (HFD) compared to lean mice (LFD) at the completion of treatment, consistent with our previous work that found obesity promotes tumor growth in the LKB1$^{fl/fl}$p53$^{fl/fl}$ mouse model ([28]). In the obese mice, tumor weight decreased by 79.1% (p<0.01) with ONC201 treatment when compared with the obese control group. Among the lean mice, tumor weight decreased by 63.2% after treatment with ONC201 (p<0.01) when compared with control-treated animals. Thus, ONC201 appeared to have a more pronounced impact on the tumor growth in obese mice (Figure 6A). ELISA assay showed that the production of VEGF in serum was significantly reduced following 4 weeks of ONC201 treatment in both obese and lean mice compared to the controls (Figure 6B).

Immunohistochemical analysis was performed on the endometrial tumors to assess effects of ONC201 on proliferation, angiogenesis, and downstream targets of the mTOR and MAPK pathways. We found higher expression levels of Ki67 and phosphorylated-S6 in the tumor of obese mice than in that of lean mice, indicating that obesity promotes tumor proliferation and activates the mTOR pathway in endometrial tumors. The Ki-67 staining was significantly reduced by 51.8% and 34.5% in the obese and lean groups treated with ONC201 compared with the control treated mice, respectively (p<0.01). Treatment with ONC201 significantly resulted in a decrease in the expression of phosphorylated-S6 and BCL-2 in both obese and lean mice, suggesting that ONC201 may result in downregulation of the mTOR pathway and induction of apoptosis *in vivo*. Importantly, we also found a significant decrease in the number of VEGF positive cells in the tumors of ONC201-treated obese and lean mice (Figure 6C).

Effect of ONC201 on metabolomic profiling in LKB1$^{fl/fl}$ p53$^{fl/fl}$ mouse model of EC

Metabolomic analyses of endometrial tumors from LKB1$^{fl/fl}$p53$^{fl/fl}$ mice revealed clear metabolic differences between obese and lean mice, also consistent with our previous work in this model ([28]). To gain insight into the effect of ONC201 on metabolism in obese and lean LKB1$^{fl/fl}$ p53$^{fl/fl}$ mice, metabolic pathways of the significantly altered metabolites in endometrial tumors after treatment of ONC201 were determined using metabolomics and lipidomics. Metabolomic profiling revealed significant differences between obese and lean mice treated with ONC201 or placebo. ONC201 decreased availability of amino acids for protein biosynthesis in both obese and lean ECs. Lipid and protein biosynthesis were dramatically upregulated in ECs from obese control as compared to lean control mice. Treatment with ONC201 reversed the obesity-driven upregulation of lipid biosynthesis in the endometrial tumors, resulting in lipid degradation/oxidation as evidenced by decreased cholesterol esters and diacylglycerols/triacylglycerols and increased lysolipids in obese vs lean mice (Figure 7A and B, Supplemental Figure 1). ONC201 reduced glycolysis in the lean mice as evidenced in the significant decrease in dihydroxyacetone phosphate in lean tumors treated with ONC201 versus lean treated with placebo (p<0.05). Glycolytic metabolites were not significantly altered in obese tumors receiving the same treatment (Figure 7C). In addition, eicosanoids were increased in obese but not lean mice with treated ONC201, with over a 4-fold increase in 12-HHTRE and 6-keto prostaglandin F1, suggesting enhanced immune response in the setting of obesity (Supplemental Figure 1 and 2).

Discussion

Recent epidemiological studies found that patients with schizophrenia in which the dopaminergic pathway is activated have higher cancer risk, while the patients who received DRD2 antagonists treatment (e.g. haloperidol) have a reduced cancer risk in different solid tumors, including those of the rectum, colon, and prostate, suggesting that dopaminergic signaling pathways may be associated with the carcinogenesis of cancer (14, 32–34). Multiple researchers have shown increased expression of dopamine receptors in malignancies (14, 17, 35). Meredith *et. al.* identified frequent expression of DRD2 in neoplastic B cell populations, although treatment response with dopamine or dopamine-like compounds seemed independent of DRD2 expression (10). Similarly, expression of DRD2 mRNA and protein was notably elevated in glioblastoma specimens relative to non-malignant cerebrum (11), and the pro-proliferative influence of DRD2 was mediated in part through the Ras/ERK signaling axis. Blockade of this pathway through DRD2 antagonism or gene silencing has *in vitro* and *in vivo* anti-proliferative effects in glioblastoma (11). Recently, Jandaghi *et. al.* showed significant DRD2 mRNA and protein expression with corresponding activation of the DRD2 cell signaling pathway in human pancreatic tumors (14). In addition, inhibition of DRD2 by siRNA reduced cell proliferation and migration, and slowed tumor growth in xenograft pancreatic cancer mouse models by inducing apoptosis and increasing cellular stress (14).

Targeting DRD2 with ONC201 has shown promising results *in vitro* and *in vivo* preclinical models in some types of cancers. Recent clinical trials showed that ONC201 has demonstrated well tolerated and durable objective responses in patients with H3 K27M mutant gliomas (26, 36). Our recent study showed that ONC201 significantly inhibited cell proliferation, reduced ability of invasion and increased the sensitivity to paclitaxel in serous EC cell lines (22). In this study, we assessed the anti-proliferative, anti-metastatic and anti-tumorigenic effects of ONC201 in EC cell lines and mouse models. Treating EC cell lines with ONC201 inhibited cellular proliferation, induced ROS production and apoptosis, reduced cellular adhesion and invasion. Additionally, ONC201 significantly reduced tumor growth in a genetically engineered mouse model of endometrioid EC (LKB1$^{fl/fl}$ p53$^{fl/fl}$) under both obese and lean conditions, with a suggestion of greater effects seen in the setting of obesity. Metabolomics and lipidomics results confirmed that ONC201 reversed the obesity-driven upregulation of lipid biosynthesis in the endometrial tumors, resulting in lipid degradation/oxidation as evidenced by decreased cholesterol esters and diacylglycerols/triacylglycerols and increased lysolipids in obese vs lean mice. Lastly, this is the first study to demonstrate the correlation between the expression levels of DRD2 and the clinical and histopathological endpoints in human EC tumors (37). Increased DRD2 expression in human EC tumors was significantly associated with PFS and OS, likely related to its correlation with grade, stage, and histology.

Given these data, intuitively one could expect higher DRD2 expression to lead to more sensitivity to ONC201 treatment; however, the high-DRD2-expressing cell line ECC-1 did not demonstrate increased sensitivity to ONC201 as compared to the other cell lines tested. We noted that treatment with ONC201 did increase the expression of DRD2 in this cell line, which potentially provided some proliferative advantage. Additionally, the complicated downstream effects of dopamine receptor pathways, the

multiple potentially undescribed mechanisms of action of ONC201 and activation of pathways independent of DRD2 could explain this surprising result (38). Prabhu *et. al.* recently found a DRD2 + DRD5- biomarker signature that significantly predicts the sensitivity to ONC201 in pre-clinical models and is associated with improved outcomes in patients treated with ONC201 in a Phase II clinical trial for recurrent glioblastoma (37). Whether the level of DRD2 expression or DRD2 + DRD5- signature can be used as a predictive biomarker for response to ONC201 is an intriguing possibility and will be assessed in the ongoing clinical trials of ONC201 in both type 1 and 2 EC.

Using the TCGA database, we found expression of the DRD2 receptor was significantly increased in serous type or in the copy-number high subgroup. While the copy-number high subtype is comprised of primarily serous histology, it should be noted that 25% of high-grade endometrioid tumors fall into this aggressive copy-number high genomic subtype (39, 40). Our immunohistochemistry results for DRD2 from our 118 EC patients further confirmed that higher DRD2 expression is associated with serous histology tumors. These findings suggest that the clinically aggressive nature of this subgroup may in part be related to activation of the dopaminergic pathway; and thus worthy of further exploration in future studies. We acknowledge that interpretation of this association may be limited by the retrospective nature of the data, small sample size and subjectivity of automated immunohistochemical scoring. However, this association makes antagonism of DRD2 a compelling treatment hypothesis for both type 1 and 2 endometrial tumors.

Targeting cancer metabolism seems to hold great promise to uncover novel targets for treatment, particularly in an obesity-driven cancer such as EC. Long-term antagonism of DRD2 with antipsychotic drugs significantly increases metabolic side effects including weight gain and disturbed lipid metabolism in patients with schizophrenia (41). Recent studies have found that DRD2 signaling functionally alters glucose and lipid metabolisms in glioblastoma cells, and the effect of ONC201 on oxidative phosphorylation and glycolytic activity was dependent on genetic background of tumor cells (42, 43). ONC201 treatment decreased oxidative phosphorylation through activation of ClpP to reduce basal oxygen consumption rate and enzymatic activity of respiratory chain complexes I, II, and IV in cancer cells (18, 20, 44). We have previously found significant metabolic pathway differences between endometrial tumors from obese versus lean LKB1$^{fl/fl}$ p53$^{fl/fl}$ mice (28). Thus, we postulated that the anti-tumorigenic effects of ONC201 may be accompanied by changes in glucose and lipid metabolism that are beneficial to cancer cell growth inhibition. Our metabolomics data showed that ONC201 significantly reduced glycolytic activity in endometrial tumors in the lean but not obese mice. Treatment of ONC201 resulted in a switch from obesity-driven upregulation of lipid biosynthesis to lipid degradation and oxidation; and thus, specific to only the obese mice. ONC201 treatment led to a reduction of protein biosynthesis, and this effect was shared between obese and lean mice. These results clearly indicate that inhibition of lipid and protein biosynthesis could contribute to ONC201-mediated tumor suppression, and anti-tumorigenic effects of ONC201 may align with obesity status *in vivo*. Meanwhile, these results may also explain why ONC201 has an improved tumor suppressing effect in obese mice.

DRD2 has been shown to regulate many aspects of tumor behavior, including invasion and migration in cancer cells. Activation of DRD2 by the DRD2-specific agonist BIM53097 inhibits tumoral pituitary cells migration and invasion by activating the cofilin pathway in vitro (45, 46). However, downregulation of DRD2 by trifluoperazine and haloperidol also results in decreased invasion and migration in prostate cancer cells, indicating the functional diversity of DRD2 and its downstream targets in different types of cancer (47). TRAIL signaling, the integrated stress response, and the AKT/mTOR/S6 pathway are all known downstream targets of ONC201, and all have regulatory effects on cancer cell adhesion, migration and invasion(17, 22, 48–50). Wagner et. al. found that ONC201 exerts a potent anti-metastasis effect via inhibition of cell migration and invasion in colorectal cancer cells and colon cancer mouse models (51, 52). We also recently confirmed that ONC201 reduces adhesion and invasion in serous EC cell lines through induction of the epithelial-mesenchymal transition and inhibition of VEGF expression (22). Similarly, in this current study, ONC201 exhibited an inhibitory effect on adhesion and invasion in EC cells and reduced VEGF production in both the serum and tumor tissues of the LKB1$^{fl/fl}$ p53$^{fl/fl}$ mouse model of EC. Thus, future studies are planned to evaluate whether the anti-invasion effects of ONC201 are dependent on DRD2 signaling pathways versus other signaling effects.

Given that ONC201 has anti-tumor efficacy in preclinical models, the safety/tolerability and efficacy of ONC201 has been and is being tested in phase I and phase II clinical trials, respectively. Oral weekly ONC201 is well tolerated with very few adverse events and results in clinical activity in advanced solid tumors, including ECs (23, 53–55). The unique features of ONC201 to occupy DRD2 and DRD3 at physiological concentrations strengthens the excellent safety profile of ONC201 in clinical trials (53). Importantly, multiple ongoing phase I/II trials of ONC201 as a signal agent or in combination with other agents in solid tumors and hematologic malignancies has been initiated in the US. Needless to say, the results of clinical evaluation of ONC201 are highly anticipated in EC, where effective treatment options are limited.

Conclusion

We found that ONC201 had efficacy in the inhibition of EC cell proliferation, tumor growth and metastatic potential in vitro and in vivo. Increased DRD2 expression was more common in human serous versus endometrioid ECs. These preclinical studies provide a fundamental rationale for investigating DRD2 antagonism with ONC201 in EC, with phase 2 clinical trials in type 1 and 2 endometrial cancer ongoing and which will hopefully mature in the upcoming year.

Abbreviations

DRD2: Dopamine receptor D2; EC:Endometrial cancer; TCGA:The Cancer Genome Atlas; CNL:Copy Number Low; CNH:Copy Number High; EMT:Epithelial-mesenchymal transition; VEGF:Vascular endothelial growth factor; TRAIL:tumor necrosis factor-related apoptosis-inducing ligand; FBS:fetal bovine serum; HFD:high fat diet; LFD:Low fat diet; MSI:microsatellite instability hypermutated; ROS:Reactive oxygen species; OS:overall survival; PFS:progression free survival;

Declarations

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Supplementary Information

Supplemental Figures 1 and 2 not available with this version