OP449 inhibits breast cancer growth without adverse metabolic effects

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Abstract

Hyperinsulinemia is associated with a decrease in breast cancer recurrence-free survival and overall survival. Inhibition of insulin receptor signaling is associated with glycemic dysregulation. SET is a direct modulator of PP2A, which negatively regulates the PI3K/AKT/mTOR pathway. OP449, a SET inhibitor, decreases AKT/mTOR activation. The effects of OP449 treatment on breast cancer growth in the setting of pre-diabetes, and its metabolic implications are currently unknown. We found that the volumes and weights of human MDA-MB-231 breast cancer xenografts were greater in hyperinsulinemic mice compared with controls (P<0.05), and IR phosphorylation was 4.5-fold higher in these mice (P<0.05). Human and murine breast cancer tumors treated with OP449 were 47% and 39% smaller than controls (P<0.05, for both, respectively). AKT and S6RP phosphorylation were 82% and 34% lower in OP449-treated tumors compared with controls (P<0.05, P=0.06, respectively). AKT and S6RP phosphorylation in response to insulin was 30% and 12% lower in cells, pre-treated with OP449, compared with control cells (P<0.01, P<0.05, respectively). However, even with decreased AKT/mTOR activation, body weights and composition, blood glucose and plasma insulin, glucose tolerance, serum triglyceride and cholesterol levels were similar between OP449-treated mice and controls. Xenografts and liver tissue from OP449-treated mice showed a 64% and 70% reduction in STAT5 activation, compared with controls (P<0.01 and P=0.06, respectively). Our data support an anti-neoplastic effect of OP449 on human breast cancer cells in vitro and in xenografts in the setting of hyperinsulinemia. OP449 led to the inhibition of AKT/mTOR signaling, albeit, not leading to metabolic derangements.

Introduction

Over the past several years, there has been a progressively growing body of evidence suggesting that obesity, which pre-disposes to the metabolic syndrome (MetS) and type 2 diabetes mellitus (T2DM), infers an increased risk of breast cancer incidence, metastases and recurrence (Calle et al. 2003, Stattin et al. 2007, Giovannucci et al. 2010,

While anti-neoplastic pharmacology has progressed immensely during the past decade, many anti-cancer drugs that target the insulin signaling pathway, including phosphoinositide-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) inhibitors and the insulin-like growth factor 1 receptor (IGF1R)-targeted therapies, have been associated in clinical trials with high rates of glycemic dysregulation and frank T2DM development (Shlomai et al. 2016). Therefore, there is an increasing need for effective, targeted anti-neoplastic agents that will maintain or even improve the patients’ metabolic profiles.

Protein phosphatase 2 (PP2A) is a ubiquitously expressed serine/threonine phosphatase, that plays a pivotal role in regulating cell cycle, cell growth, proliferation and survival (Janssens et al. 2005). PP2A is considered an important tumor suppressor gene, as it is a negative regulator of several oncogenic signaling pathways, including the PI3K/AKT/mTOR pathway, MYC, RAS-RAF-mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) family and the WNT/β-catenin pathway (Silverstein et al. 2002, Sablina et al. 2010, Zhang & Claret 2012). Decreased activity of PP2A has previously been observed in colorectal cancer and breast cancer (Baldacchino et al. 2014, Cristobal et al. 2014). In addition, the expression and function of PP2A may be impaired during prostate cancer progression (Bhardwaj et al. 2011, Pandey et al. 2013). SET (also known as PP2A inhibitory protein, I2PP2A) is an oncogenic protein that has been identified as an endogenous inhibitor of PP2A (von Lindern et al. 1992, Adachi et al. 1994). SET is a direct regulator of PP2A, binding to its catalytic subunit and forming an inhibitory protein complex that impairs PP2A activity (Li et al. 1995, 1996). SET expression is upregulated in many types of cancers including malignant brain tumors, testicular tumors, tumors of the head and neck regions, pancreatic cancer, several hematological malignancies and breast cancer (Westermarck & Hahn 2008, Christensen et al. 2011a, Agarwal et al. 2014, Janghorban et al. 2014).

COG112, a novel peptide based on a short fragment of apolipoprotein-E, has been shown to inhibit the ability of SET to associate with the catalytic unit of PP2A, thus increasing PP2A activity in immune cells (Christensen et al. 2011b) and in MDA-MB-231 hormone receptor-negative human breast cancer cells (Switzer et al. 2011). OP449, (formerly COG449), a dimerized derivative of COG112 (Li et al. 2006, Singh et al. 2008), is a cell-penetrating physiologically stable peptide, that specifically binds to SET and antagonizes its inhibition of PP2A (Perrotti & Neviani 2008, Christensen et al. 2011a,b). Treatment of several breast cancer lines, including MDA-MB-231 cells, with OP449 has been shown to decrease tumor growth in vivo and induce cell apoptosis in vitro (Janghorban et al. 2014).

The effects of OP449 on breast cancer growth and metastases in the setting of pre-diabetes are currently unknown, and data regarding the metabolic implications of OP449 treatment are lacking. Here, we present findings supporting an anti-neoplastic effect of OP449 on human MDA-MB-231 breast cancer xenografts and murine MVT-1 tumors in the Rag/MKR immunodeficient mouse model of hyperinsulinemia (Zelenko et al. 2016). Moreover, while breast tumor growth is inhibited, OP449 treatment was not associated with metabolic derangements.

Materials and methods

Animal models

All mice used in these studies were females, on an FVB/n background. The hyperinsulinemic MKR mouse model has been previously described (Fernandez et al. 2001, Novosyadlyy et al. 2010, Ferguson et al. 2012). Briefly, the male mouse phenotype includes insulin resistance with hyperinsulinemia, hyperglycemia and hyperlipidemia, while female mice have isolated insulin resistance and hyperinsulinemia, but are normoglycemic and normolipidemic. For injection of human breast cancer cells, an immunodeficient MKR –/– mouse was utilized. The Rag1+/MKR–/ (Rag/MKR) mouse was generated by crossing the hyperinsulinemic MKR–/– mouse with the Rag1-knockout (Rag1–/–) mouse, which lacks mature T and B lymphocytes. Rag/MKR mice have a similar metabolic phenotype compared with MKR mice and have been previously described (Zelenko et al. 2016).

Animal studies

All mice procedures were in compliance with the current standards specified in the Guide of the Care and Use of
Laboratory Animals provided by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and approved by Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee (IACUC). The mice were housed 4–5 per cage, kept on a 12-h light:12-h darkness cycle and fed a regular chow diet (PicoLab 5053, Brentwood, MO, USA).

**Tumor studies** MDA-MB-231 human breast cancer cells were received from ATCC, and cell line authentication was performed by IDEXX BioResearch (Columbia, MO, USA). After thawing, cells were passaged 4–5 times before injection. 5 × 10^6 MDA-MB-231 cells, resuspended in 100 μL of sterile phosphate buffered saline (PBS) were injected into the 4th mammary fat pad of Rag/MKR mice and Rag1−/− (Rag/WT) mice. In addition, 1 × 10^6 MVT-1 cells were injected to Rag/MKR or Rag/WT mice as described above. The murine mammary MVT-1 cell line was derived from an explant culture of an MMTV c-Myc/Vegf transgenic female mouse as described elsewhere (Pei et al. 2004).

Body weight and body condition were monitored weekly, and tumor size was measured with calipers twice weekly. The formula used for calculating tumor volume was: volume = 4/3 × π × (length/2 × width/2 × depth/2). All mice were killed and dissected, once tumor volumes reached 15 mm in diameter in greatest dimension, or if there was deterioration in the body condition of the mice reached 15 mm in diameter in greatest dimension, or if there was deterioration in the body condition of the mice (humane endpoints in the IACUC protocol). In order to analyze the pulmonary macrometastases, postmortem lungs were inflated with 10% formalin and visually inspected.

For evaluation of OP449 effects on tumor growth, mice were allocated to two groups with equal tumor size, 6–10 per group, and treated intraperitoneally with 5 mg/kg of OP449 (Oncotide Pharmaceuticals Inc., Research Triangle Park, NC, USA) or vehicle (PBS), five times per week, consistent with previously published studies (Janghorban et al. 2014).

**Metabolic studies** Rag/MKR female mice, age 8–10 years were allocated, 3–4 mice per cage and treated intraperitoneally with 5 mg/kg OP449 or vehicle, five times per week for three consecutive weeks. Body weight was measured weekly. Non-fasting glucose was measured in blood samples from the capillary tail vein once weekly, using a Bayer Contour Next Glucometer (Bayer). Glucose tolerance test was performed on mice fasted for 10 h. Glucose (1.5 g/kg) was injected intraperitoneally. Capillary tail vein blood glucose levels were measured immediately before glucose injection at time 0 and at 15, 30, 60 and 120 min using Bayer Contour Next Glucometer (Bayer). Body composition was determined in non-anesthetized mice using the EchoMRI 3-in-1 NMR system (Echo Medical Systems, Houston, TX, USA). At the end of the treatment period, fasting plasma and serum were collected for subsequent analysis.

Insulin levels were measured using the Meredia Mouse Insulin ELISA (Mercodia AB,Upsala, Sweden). Serum triglyceride and cholesterol levels were determined using the Pointe Scientific Liquid Triglyceride and Liquid Cholesterol kits, respectively, per the manufacturer’s instructions (Pointe Scientific, Canton, MI, USA).

**Cell culture and in vitro studies**

All cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies), 100 U/mL penicillin and 100 μg/mL streptomycin (Mediatech, Manassas, VA, USA). All cells were grown at 37°C in 5% CO₂ atmosphere.

For the viability assay, 1 × 10⁴ MDA-MB-231 cells were plated on a 96-well plate in full medium (DMEM, supplemented with FBS and penicillin/streptomycin) and allowed to adhere overnight. Cells were then grown in serum-free medium supplemented with 0.1% bovine serum albumin (BSA) (Sigma-Aldrich) and allocated in triplicates for incubation with OP449 at 0, 0.5, 1, 1.5 and 2 μM for 24 h. Cell viability was assessed, using the Cell Counting Kit-8 (CCK-8), per the manufacturer’s instructions (Dojindo Molecular Technologies, Inc. Rockville, MD, USA).

For assessment of OP449 effects on breast cancer cells signaling pathways, MDA-MB-231 cells were plated on 60 mm dishes in full medium until reaching 60–80% confluence. Cells were then serum-starved with 0.1% BSA and allocated in triplicates for incubation with 2 μM of OP449 for 2, 3, 4, 5, 6 and 8 h in the presence or absence of 10 nM regular insulin (Humulin R, Lilly) for 30 min.

**Western blotting**

MDA-MB-231 cells tumor tissue and Rag/MKR liver tissue were lysed in ice-cold lysis buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1.25% CHAPS, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 8 mM β-glycerophosphate and...
Complete Protease Inhibitor Cocktail tablet (Roche). The protein concentration of the cell and tumor lysates was measured using the BCA protein assay kit (Thermo Scientific). Twenty-five micrograms of each protein sample was resuspended in 3x sodium dodecyl sulfate (SDS) loading buffer supplemented with dithiothreitol (DTT) (Cell Signaling Technologies). The samples were denatured at 96°C for 5 min and loaded on an SDS-PAGE 8–16% Tris–glycine gel (Invitrogen Life Technologies) and transferred onto nitrocellulose membrane. After overnight incubation at 4°C with primary antibodies, the membrane was incubated with secondary antibodies (Li-Cor Biosciences, Lincoln, NE, USA) and scanned using the Li-Cor infrared imaging system. The protein bands were quantified using Image Studio Lite software (Li-Cor Biosciences).

Antibodies

In Western blot analyses, the nitrocellulose membranes were probed with the following primary antibodies: anti-phospho-IGF1 receptor β(Tyr1150/1151)/IR β(Tyr1135/1136) (#3024), anti-phospho-Akt(Ser473) (#9271), anti-total Akt (#2920), anti-phospho p44/42 mitogen-activated protein kinase (ERK1/2)(Thr202/Tyr204) (#9101), anti-total p44/42 mitogen-activated protein kinase (ERK1/2) (#9107), anti-phospho S6 Ribosomal Protein (Ser235/236) (#2211), anti-total S6 Ribosomal Protein (#2317), anti-phospho-STAT5(Tyr694) (#9359) (Cell Signaling Technology), anti-total STAT5 (#SC-835) anti-IR (#SC-711) anti-IGF-1R (#SC-711) (Santa Cruz) and β-actin (#A5441) (Sigma-Aldrich).

Statistical analysis

All in vitro studies were performed at least in duplicates. All data are expressed as mean ± s.e.m. Statistical analysis to determine significance of intergroup differences in biomarkers of interest was performed with two-sample Student’s t tests (two-sided) for studies with 2 groups with equal variance or one-way ANOVA for studies comparing more than 2 groups (IBM SPSS, version 22.0).

Results

Mice with endogenous hyperinsulinemia developed larger MDA-MB-231 xenografts than control mice

To determine the effects of hyperinsulinemia on growth and metastasis of human breast cancer cells, we injected MDA-MB-231 breast cancer cells into the mammary fat pad of immunodeficient female Rag/MKR hyperinsulinemic mice and Rag/WT controls. By the end of the study, tumor volumes and tumor weights were significantly greater in Rag/MKR compared with Rag/WT mice (P < 0.05) (Fig. 1A and B). We also observed more lung metastasis in Rag/MKR mice compared with Rag/WT mice (P < 0.05) (data not shown). To examine the effects of endogenous hyperinsulinemia on insulin receptor (IR) signaling in the immunodeficient hyperinsulinemic mouse model, we analyzed protein lysates from the MDA-MB-231-derived tumors for IR signaling. We found that in tumors from Rag/MKR mice, IR phosphorylation was 4.5-fold higher than Rag/WT tumors (P < 0.05) (Fig. 1D). These studies show that hyperinsulinemia enhanced the growth of human MDA-MB-231 breast cancer xenografts with activation of IR/IGF-1R signaling.
OP449 treatment reduced tumor growth in mice with endogenous hyperinsulinemia

To examine the anti-neoplastic effects of OP449 in the setting of endogenous hyperinsulinemia, we injected MDA-MB-231 breast cancer cells into the mammary fat pad of female Rag/MKR mice and treated the mice with OP449 or vehicle for 5 weeks. At the end of the study, in OP449-treated Rag/MKR mice, tumors were 47% smaller than tumors in vehicle-treated Rag/MKR mice ($P < 0.05$) (Fig. 2A). Tumor weights were $0.82 \pm 0.11$ in OP449-treated mice and $1.33 \pm 0.24$ in vehicle-treated mice ($P = 0.08$) (Fig. 2B). Four of nine of the Rag/MKR vehicle-treated mice had pulmonary macrometastases, while two of 9 of the OP449-treated mice had macrometastases (data not shown). Similar to its effects on MDA-MB-231 xenografts, we found that OP449 reduced MVT-1 tumor volumes in Rag/MKR and Rag/WT by 39% and 40%, respectively, compared with PBS ($P < 0.05$ for both) (Fig. 2C). Tumor weights were also reduced in OP449-treated mice compared with PBS controls (60% and 58% in Rag/MKR and Rag WT, respectively, $P < 0.01$ for both) (Fig. 2D).

We also found less lung metastasis in Rag/MKR mice treated with OP449 compared with Rag/MKR mice treated with PBS (4.6 vs 6.6, respectively) and in Rag/WT mice treated with OP449 compared with PBS controls (2.1 vs 4.1, respectively); however, the differences did not reach statistical significance (Fig. 2E).

Therefore, OP449 reduced the growth of human MDA-MB-231 and murine MVT-1 xenografts in a hyperinsulinemic mouse model. The data show that OP449 has a significant inhibitory effect in hyperinsulinemic Rag/MKR mice as well as on WT mice and that the relative reduction in tumor growth is similar for Rag/MKR mice and Rag/WT mice.

OP449 treatment decreased AKT/mTOR signaling in MDA-MB-231 xenografts from hyperinsulinemic mice

To examine the mechanisms by which OP449 treatment inhibited tumor growth in the hyperinsulinemic Rag/MKR mice, we analyzed the protein lysates from the MDA-MB-231-derived tumors. In the tumors from mice treated with OP449, AKT phosphorylation was 82% lower compared with tumors from vehicle-treated mice ($P < 0.05$) (Fig. 3A). Expression levels of both phospho-S6RP and total S6RP levels were found to be lower in tumors derived from OP449-treated mice compared with vehicle-treated mice ($P = 0.06$, $P < 0.01$, respectively) (Fig. 3B). We did not observe a statistically significant difference in the ratio of ERK1/2 phosphorylation to total Erk1/2 between tumors from OP449-treated and vehicle-treated mice; however, total ERK1/2 expression was decreased in the tumors from OP449-treated mice ($P < 0.01$) (Fig. 3C). These data show that OP449 decreased Akt/mTOR signaling in MDA-MB-231 xenografts of hyperinsulinemic Rag/MKR mice and led to decreased total protein levels of ERK1/2 and S6RP.
OP449 inhibited insulin-stimulated activation of AKT and S6RP

To examine if the observed in vivo effects of OP449 were due to direct effects on the tumor cells, we treated MDA-MB-231 cells with 2 μM of OP449, in the presence and absence of insulin, and analyzed protein lysates for AKT, S6RP and ERK1/2 phosphorylation. We found that OP449 blunted the phosphorylation of AKT and S6RP in response to insulin (Fig. 4A and B). AKT phosphorylation in response to insulin was 30% lower in cells pre-treated with OP449 compared with cells pre-treated with vehicle (P < 0.01) (Fig. 4A). We also observed a 20% decrease in total Akt in cells pre-treated with OP449 compared with the absence of OP449 (P < 0.05) (Fig. 4A). S6RP phosphorylation was 12% lower in cells pre-treated with OP449 and stimulated with insulin, compared with cells treated with insulin alone (P < 0.05) (Fig. 4B). The decrease in total S6RP levels in the OP449-treated cells, compared with vehicle-treated cells, did not reach statistical significance (P = 0.06) (Fig. 4B). We did not observe differences in phospho-ERK1/2 or total ERK1/2 expression between treatment groups (Fig. 4C). Longer incubation times (up to 8 h) with OP449 further decreased AKT phosphorylation in response to insulin, but complete...
inhibition of AKT phosphorylation was not achieved (Supplementary Fig. 2A, see section on supplementary data given at the end of this article). Incubated for 24h with OP449 at increasing concentrations up to 2 µM led to a 90% reduction in the viability of MDA-MB-231 cells (Supplementary Fig. 2B).

Similar to our results from the tumor xenograft studies in the hyperinsulinemic mice, our in vitro studies demonstrate that OP449 partially inhibits AKT/mTOR signaling in the presence of insulin.

**OP449 did not worsen the metabolic phenotype of the pre-diabetic Rag/MKR mice**

Previous animal and human studies have found that inhibiting AKT and mTOR signaling worsens insulin resistance and induces hyperglycemia and hypertriglyceridemia (Fierz et al. 2010, Gallagher et al. 2012). To determine the effects of OP449 on the metabolic phenotype of the pre-diabetic Rag/MKR mice, female Rag/MKR mice were treated for 3 weeks with either OP449 or vehicle. Body weights, and composition, as determined by EchoMRI 3-in-1 NMR, were not different between OP449 and vehicle-treated groups after 3 weeks of treatment (Fig. 5A and B, respectively). Fasting blood glucose did not differ significantly between groups (Fig. 5C), and a glucose tolerance test did not show any significant difference between the OP449 and vehicle-treated groups (Fig. 5D). Furthermore, there were no significant differences in fasting serum triglyceride, serum cholesterol levels or plasma insulin levels between the groups (Fig. 5E, F and G). We also evaluated the metabolic characteristics of Rag/MKR mice at the end of the tumor study, i.e. 35 days post OP449 treatment. Similar to the results of the metabolic study, we found no significant difference in non-fasting serum cholesterol and triglyceride levels as well as in non-fasting plasma insulin levels and non-fasting blood glucose level between Rag/MKR treated with OP449 and those treated with PBS control (Supplementary Fig. 1A, B, C, D and E).

To understand why no metabolic detrimental effects of OP449 were observed in the Rag/MKR mice, despite inhibition of AKT and S6RP signaling, we analyzed STATS phosphorylation, which has been shown to play a role in the development of insulin resistance (Gurzov et al. 2014, Kaltenecker et al. 2017). We observed a 64% and 70% reduction in phospho-STAT5 levels in xenografts and hepatic tissue from OP449-treated mice, compared with vehicle-treated mice (P<0.01, P=0.06, respectively) (Fig. 6A and B, respectively). Overall, these results showed that treatment of mice with OP449 produced no adverse effects on body weight, body composition, glucose tolerance or lipids.

**Discussion**

Obesity and T2DM are becoming increasingly prevalent worldwide. Cohort studies have clearly shown an association between obesity, T2D and a 20–60% increased risk of breast cancer metastases, recurrence and mortality.
A number of causal factors have been proposed for the increased risk of breast cancer metastases, recurrence and mortality in the setting of obesity and T2D, one of which is endogenous hyperinsulinemia (Goodwin et al. 2002, LeRoith & Roberts 2003, Renehan et al. 2006, Dankner et al. 2012). Studies in women with breast cancer have demonstrated that hyperinsulinemia and higher levels of C-peptide are associated with decreased recurrence-free survival and overall survival (Irwin et al. 2011, Goodwin et al. 2012).

The MKR mouse is a hyperinsulinemic mouse model of T2DM. These mice overexpress dominant-negative human IGF1Rs in the skeletal muscle, which form hybrid receptors with endogenous IRs and IGF1Rs, causing skeletal muscle insulin resistance (Fernandez et al. 2001, Novosyadlyy et al. 2010). MKR male mice are hyperinsulinemic, hyperglycemic and hyperlipidemic, but not obese. The female MKR mice develop insulin resistance and hyperinsulinemia, but are not hyperglycemic (Fernandez et al. 2001, Novosyadlyy et al. 2010). When injected with mouse breast cancer cells, the MKR mice develop larger tumors and more numerous pulmonary metastases than the control mice (Ferguson et al. 2012). The PI3K/AKT/mTOR pathway is the main signaling pathway that shows increased activation in tumors from MKR mice (Novosyadlyy et al. 2010).

We have previously generated an immunodeficient MKR mouse model, the Rag/MKR mouse, which has a similar metabolic phenotype as MKR mice (Zelenko et al. 2016). In the current study, we found that endogenous hyperinsulinemia enhanced the growth of human MDA-MB-231 breast cancer xenografts in an immunodeficient Rag/MKR mouse model and that there was increased IR/IGF-1R phosphorylation in these tumors.

Targeted inhibitors for components of the IR signaling cascade have been the focus of many pre-clinical and clinical studies (Shlomai et al. 2016). However, several of these agents, in particular, inhibitors of the PI3K/AKT/mTOR pathway and IGF-1R-targeted therapies, have been associated with high rates of metabolic abnormalities in clinical trials (Shlomai et al. 2016). We have previously shown that mTOR inhibition with rapamycin, significantly suppressed tumor growth in the presence of hyperinsulinemia; however, it also markedly impaired glucose and lipid homeostasis and altered body composition (Fierz et al. 2010). Furthermore, we have demonstrated that inhibiting PI3K hampered tumor growth in the setting of hyperinsulinemia; yet, it was also associated with hyperglycemia and hypertriglyceridemia in the pre-diabetic female MKR mice (Gallagher et al. 2012). In addition to the well-established role of insulin resistance, hyperglycemia and hyperlipidemia in the pathogenesis of atherosclerosis and cardiovascular disease,
these metabolic derangements may also carry potential tumor-promoting activity (Vona-Davis et al. 2007, Lann & LeRoith 2008, Gallagher & LeRoith 2015). Thus, there is an increasing need for anti-neoplastic agents that would target the IR signaling cascade, without worsening of the metabolic phenotype.

SET is an oncoprotein that plays a role in cancer progression, by inhibiting the tumor suppressor PP2A (Schonthal 2001, Eichhorn et al. 2009, Switzer et al. 2011). OP449 binds to SET causing its release from PP2A and increasing PP2A activity (Christensen et al. 2011a, Switzer et al. 2011). In this study, we have demonstrated that treatment of pre-diabetic female mice with OP449 reduced the growth of human MDA-MB-231 xenografts and decreased AKT/mTOR signaling. We also found that, in vitro, OP449 partially inhibited AKT/mTOR signaling in the presence of insulin. Previous studies have found that treating mice with OP449 significantly inhibits human tumor xenograft growth in vivo (Janghorban et al. 2014). Previous studies also found that treatment of human breast cancer cells with OP449 in vitro resulted in decreased activation of both AKT and its downstream effector mTOR (Switzer et al. 2011). However, until now, the effect of OP449 on tumor growth or signaling has not been previously investigated in the setting of pre-diabetes.

Notably, even in the setting of decreased AKT/mTOR signaling, treatment with OP449 in the pre-diabetic Rag/MKR mice did not result in adverse metabolic effects, as evaluated by body weight, body composition, glucose tolerance and lipids. To the best of our knowledge, this is the first study that evaluated the effects of OP449 on metabolic characteristics in mice. The observation of an anti-neoplastic drug capable of inhibiting AKT/mTOR signaling without causing glucose and lipid dysregulation is novel and intriguing. STAT5 is a member of the STAT family of transcription factors that are phosphorylated by the receptor-associated kinases (Emanuelli et al. 2000), and then translocate to the cell nucleus where they act as transcription activators for various genes (Chia et al. 2006). Enhanced STAT5 activation has been shown to exacerbate the development of obesity and T2DM (Gurzov et al. 2014). Elevated phosphorylated STAT5 levels have been found in hepatic tissue of high fat-fed mice compared with controls, in association with lower AKT phosphorylation consistent with development of insulin resistance (Gurzov et al. 2014). In addition, it has been recently shown that compared with control mice, mice with an adipocyte-specific knockout of STAT5 had lower insulin levels and lower HOMA-IR (homeostatic model assessment of insulin resistance) (Kaltenecker et al. 2017). Inhibition of SET protein with OP449 results in increased PP2A levels (Christensen et al. 2011b, Switzer et al. 2011, Janghorban et al. 2014). Given the broad substrate specificity of PP2A phosphatase activity, it is plausible that it may reduce the levels of multiple phosphorylated proteins, including STAT5. Indeed, our results show that in mice treated with OP449, there is a reduction in STAT5 activation in liver tissue. The decreased activation of STAT5 may explain the lack of metabolic deterioration that we observed in OP449-treated pre-diabetic mice, even in the presence of decreased AKT/mTOR signaling.

Conclusions
We have shown that SET inhibition with OP449 inhibits human triple-negative MDA-MB-231 breast cancer xenograft and murine MVT-1 tumor growth in a mouse model of hyperinsulinemia through decreased activation of the AKT/mTOR signaling. Furthermore, OP449 did not alter the metabolic phenotype of the pre-diabetic mice even in the presence of decreased AKT/mTOR pathway activation, potentially due to decreased activation of STAT5 in metabolic tissues. The comparable relative reduction in tumor growth in Rag/MKR mice and Rag/WT mice treated with OP449 compared with control mice suggests that OP449 does not entirely overcome the relative increase in tumor growth observed in the setting of hyperinsulinemia. Therefore, SET inhibition may be a useful therapeutic strategy in triple-negative breast cancer while not causing adverse metabolic side effects.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-17-0077.

Declaration of interest
Dr Vitek is a Founder and Shareholder of Cognosci, Inc. Matters of conflict of interest are managed by the Duke Conflict of Interest Committee. All other authors declare that they do not have competing interests.

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