Abstract

Ovarian granulosa cell tumors (GCT) are characterized by indolent growth and late relapse. No therapeutic modalities aside from surgery have proven effective. We previously reported overexpression of the nuclear receptor, peroxisome proliferator-activated receptor-gamma (PPARγ), and constitutive activity of the NFκB and AP1 signaling pathways in GCT. PPARγ presents as a potential therapeutic target as it impedes proliferation and promotes terminal differentiation of granulosa cells. However, resistance to the actions of PPARγ is caused by NFκB transrepression in GCT-derived cell lines, KGN and COV434. We showed that abrogation of NFκB signaling in GCT cells enables PPARγ agonists to initiate apoptosis. In addition, we observed overexpression of an NFκB-induced gene, X-linked inhibitor of apoptosis protein (XIAP), in GCT and GCT-derived cells. XIAP is an attractive therapeutic target due to its role in inhibiting the apoptotic pathway. We investigated the antitumor effects of combined XIAP inhibition using Smac mimetics and PPARγ activation using thiazolidinediones (TZD) in the GCT-derived cells. Transactivation assays revealed that NFκB transrepression of PPARγ can be relieved by NFκB or XIAP inhibition. Combined Smac-mimetic and TZD significantly induced apoptosis, reduced cell viability and proliferation in KGN cells in monolayer and 3D spheroid culture, and in GCT explant models. The Smac-mimetic and TZD cotreatment also delayed cell invasion, upregulated proapoptotic genes, and compromised cell metabolism in KGN cells. This study provides evidence that PPARγ and XIAP cotreatment has antineoplastic effects in GCT. As therapeutics that target these proteins are already in clinical or preclinical use, expedient translation to the clinic is possible.

Introduction

Granulosa cell tumors (GCT), which comprise the majority of ovarian stromal tumors, arise from proliferating granulosa cells (GC) of the ovarian follicle, and represent a specific subset of malignant ovarian tumors (1). They frequently present with endocrine manifestations such as estrogenization in prepubertal girls and postmenopausal women. GCTs exhibit many features of normal ovarian GCs (1), including expression of the follicle stimulating hormone (FSH) receptor gene, estrogen synthesis, estrogen receptor (ER) expression, and in subunit expression with synthesis of biologically active inhibin, and anti-Müllerian hormone (AMH) expression. The adult form of GCT (95%) are defined by the presence of the FOXL2 C134W mutation, which is absent in the juvenile subtype (5%). GCTs exhibit an indolent course and have an unexplained propensity for late recurrence; approximately 80% of patients with aggressive or recurrent tumors die from their disease (1). Currently, there are no reliable methods for predicting relapse and, aside from surgery, therapeutic modalities are limited (1).

Nuclear receptors (NR) play a central pathogenic role in several endocrine malignancies and as such represent established therapeutic targets. We have previously shown that peroxisome proliferator-activated receptor-gamma (PPARγ) is highly expressed in GCT (2). PPARγ is implicated in the pathology of numerous diseases including obesity and diabetes. PPARγ binds to DNA at specific sites as an obligate functional heterodimer with retinoid X receptor-alpha (RXRα), which is also expressed in GCT and in two GCT-derived cell lines (2). The potential of PPARγ agonists as anticancer agents has attracted considerable interest, including the treatment of endocrine malignancies such as thyroid, prostate, and pancreatic cancer (NCT00098852; NCT00182052; NCT02475499; https://clinicaltrials.gov/).

The role of the NFκB family of proteins in immune, inflammatory, and antiapoptotic responses is well documented (3, 4). NFκB and the signaling pathways that are involved in its activation are also important for tumor development; activated NFκB increases the expression of genes involved in cell proliferation, metastasis, and antiapoptosis (5). In GCT, we have previously demonstrated that constitutive NFκB signaling transrepresses several NR (6). Reciprocal transrepression of NFκB and NR is well described (7), usually as a consequence of protein–protein interaction without direct binding to DNA. The best characterized
interactions are those of NFκB and the glucocorticoid receptor (GR), while the androgen and progesterone receptors also physically interact with NFκB (7).

Apoptosis is induced by activated caspases. The inhibitors of apoptosis proteins (IAP) family are antiapoptotic regulators, several of which have been shown to be upregulated in various cancers. These proteins are characterized by the presence of baculovirus IAP repeat (BIR) domains, which are responsible for inhibiting caspases. The best characterized IAP is the X-linked inhibitor of apoptosis protein (XIAP) and is the most potent caspase inhibitor (8). XIAP has three BIR domains, and a RING finger domain conferring E3-ubiquitin ligase activity (8). XIAP is predominantly regulated by the mitochondrial protein, Second mitochondria-derived activator of caspases/direct IAP-binding protein with low pi (Smac/DIABLO; refs. 9, 10). When mitochondria become compromised and apoptosis is triggered, Smac is released into the cytoplasm where it binds to the caspase-binding domains of XIAP. This subsequently displaces activated caspases to promote apoptosis (8). Thus, XIAP is an attractive therapeutic target for novel anticancer treatment (11, 12). Clinical trials for small-molecule inhibitors of XIAP tested alone or in combination with other chemotherapy have been outlined in Fulda (2015) (12). This includes trials in solid tumors including head and neck carcinomas, breast and ovarian cancers, as well as hematologic malignancies such as lymphoma and leukemia. In this study, we investigated the effects of PPARγ agonists on GCT-derived cells in vitro, and showed that when combined with Smac-mimetics, they release trans-repression of PPARγ by NFκB, resulting in an induction of apoptosis.

Materials and Methods

Patients, tissue acquisition, and tissue microarray

We used previously characterized ovarian adult GCT samples (all FOXL2 mutation positive; n = 14) collected sequentially at our institution (2, 13–15). Normal ovarian tissue was obtained from 8 premenopausal women who had undergone elective hysterectomy with oophorectomy for conditions not associated with ovarian malignancy. Tissue microarrays (TMA) were provided by Professor Colin Stewart (University of Western Australia, Perth, Australia). The study protocol was approved by the Research and Ethics Committee of Monash Medical Centre (Clayton, Australia) and all women gave written informed consent for the studies.

Cell lines

Cell lines used in these studies are the human GCT-derived cells, COV434 and KGN (16, 17), and the human nonluteinized granulosa cell line, hGrC1 (18). The KGN cells are heterozygous for the FOXL2 mutation and hence derived from an adult GCT. The COV434 cells are wild type for FOXL2, representing a juvenile GCT model (1). The KGN and COV434 cell lines were purchased from and authenticated by ATCC (www.atcc.org). These cell lines have been authenticated by molecular profiling of the short tandem repeats using the PowerPlex 16 HS System PCR Amplification Kit, and data matched through searching cell line data generated by the cells in each time point and the resistance of the medium without cells.

The xCELLigence RTCA system was also utilized to assess invasion of the KGN cells. Electrical current passes through the electrodes on the bottom of the upper chamber of the cell culture plate (CEM-16). As cells seeded in the upper chamber invaded across a layer of Matrigel into the lower chamber, which contains 10% FCS as chemoattractant, they attach onto the electrodes and electrode resistances increase. The electrode resistance is indicative of cell invasion and is represented by cell index. The maximum cell indices across a 72-hour period, which is indicative of number of cells invaded, were compared between the control and treatment groups.

High content screening apoptosis assays

Cells were grown in 24-well plates and treated for 24 hours. Media containing Hoechst 33342 dye, YoPro-1 dye (to detect early apoptosis), and ToPro-3 dye (to detect late apoptosis or necrosis) were added to the cells before imaging and analyzed on a Celsisomics ArrayScan VTI High Content Screening Reader (Thermo Fisher Scientific).

Reverse transcription and real time RT-PCR

The comparative C (ΔΔC) method was used to validate XIAP expression. Fluorescein amidite (FAM) labeled TaqMan Gene Expression assays for X-linked inhibitor of apoptosis protein (XIAP), TNFR superfamily 1B (TNFRSF1B), baculovirus IAP...

www.aacrjournals.org
Mol Cancer Ther; 18(2) February 2019 365

Published OnlineFirst December 7, 2018; DOI: 10.1158/1535-7163.MCT-18-0078

Downloaded from mct.aacrjournals.org on September 2, 2021. © 2019 American Association for Cancer Research.
repeat-containing protein 3 (BIRC3), and transglutaminase 2 (TGM2) were purchased along with an endogenous control probe, FAM labeled 60S acidic ribosomal protein P0 (RPLP0) probe. A 10 µL reaction was prepared with 1 × TaqMan universal PCR Master Mix (Applied Biosystems) and diluted cDNA. All PCR reactions were carried out in triplicate in MicroAmp optical 384-well reaction plates (Applied Biosystems). The cycling parameters were initiated by 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60°C for 1 minute using the 7900HT fast real-time PCR system (Applied Biosystems).

**TaqMan low-density array**
A TaqMan low-density array (Applied Biosystems #4378701) containing gene expression assays for 93 apoptosis genes and 3 internal controls [eukaryotic 18S rRNA (18S), GAPDH and β-actin (ACTB)] was used to profile gene expression. Total RNA (1.5 µg) was reverse-transcribed using random hexamers with SuperScript III reverse transcriptase (Invitrogen, Thermo Fisher Scientific). The arrays were processed and analyzed in accordance with the manufacturer's protocols as described previously (2). Each GCT sample was run once while the cell lines were run as three biological replicates.

**IHC**
Four-micron sections of OCT (optimal cutting temperature)-embedded frozen GCT were prepared. All incubation and washes were performed at room temperature unless stated otherwise. The frozen GCT sections were fixed in 4% paraformaldehyde and endogenous peroxidase quenched using 0.3% hydrogen peroxide/PBS. Triton X-100/PBS (0.1%) was used for membrane permeation. Nonspecific binding was blocked by 10% goat serum in 3% BSA for 1 hour. Incubation with primary antibody, rabbit polyclonal PPARγ (Abcam ab27649; 1:350), XIAP (Sigma-Aldrich PRS3331; 1:200), or goat serum (negative control) was performed at 4°C overnight. After PBS washes, slides were incubated with biotinylated goat anti-rabbit secondary antibody (Dako, Agilent Technologies; 1:200) for an hour. Avidin/biotinylated enzyme complex (Vector Laboratories) was added to sections and incubated for an hour. Staining was visualized by incubation of DAB solution (Dako) for 3 minutes. Sections were counterstained with hematoxylin and dehydrated (70% and 100% ethanol). Staining was analyzed using “positive pixel count v9” algorithm in the Aperio ImageScope version 12.3.0.5056 (Leica Biosystems). Percentage of positive (brown) staining indicating protein levels was determined as low (0%–30%), medium (31%–79%), or high (>80%).

**Transactivation assays**
The KGN cells were transfected using SuperFect reagent (Qiagen) as described previously (6). The reporter constructs, NFκB-luc or AP1-luc, were transfected in the KGN cells and then treated for 24 hours with DMSO (0.1%) as vehicle control, CmpdA (500 nmol/L), and inhibitors of NFκB or AP1.

**Extracellular flux assay**
Cellular bioenergetic profiling of the KGN cells was assessed using the Seahorse XF Cell Mito Stress Test Kit. Briefly, the sensor cartridge was hydrated with calibrate overnight prior to cell seeding. KGN cells (1 × 10⁴) were plated in 6 wells, with 2 further wells containing media only for background correction. Following 24-hour treatments, cells were washed twice with the XFp base medium (supplemented with 1 mmol/L pyruvate, 2 mmol/L glutamine, and 10 mmol/L glucose). Stessors were prepared in XFp base medium with oligomycin (1 µmol/L), FCCP (2 µmol/L), or antimycin A/rotenone (0.5 µmol/L). Cellular bioenergetics was measured on a Seahorse Extracellular Flux XFp Analyzer according to the manufacturer’s protocol. Pierce BCA protein assay (Thermo Fisher Scientific) was performed for normalization. Data analysis was performed with the analyzer’s Wave software version 2.3.0.20.

**Spheroid formation assay**
To generate spheroids, 3,000 KGN cells were seeded in DMEM/F12 with 20% methylcellulose and without additives or serum. Spheroids formed within 24 to 72 hours postseeding. Spheroids were treated in quadruplicate in 2% charcoal-stripped serum-containing DMEM/F12, and replenished every 3 days. Cell viability was assessed using 0.005% resazurin (Sigma-Aldrich) in 2% charcoal-stripped FCS-containing DMEM/F12. Fluorescence signal was measured using excitation at 560 nm and emission at 595 nm (25).

**Primary GCT culture**
Primary GCTs collected from oophorectomy of two women were each dissected into three 1-mm³ pieces, plated in a 6-well plate, and treated in 10% serum-containing DMEM/F12 for 24 hours. Following treatment, the explant culture was pooled to extract RNA for further analysis.

**Statistical analysis**
Drug treatments for all experiments were performed in duplicates and repeated at least three times. Data are presented as mean ± SD. Student’s t test, one-way ANOVA, or the nonparametric equivalent, Kruskal–Wallis test was used for statistical analyses where P < 0.05 is considered statistically significant.

**Results**
Expression of XIAP and PPARγ in GCT and GCT-derived cell lines
We have previously described PPARγ mRNA overexpression in GCT (n = 12) and the GCT-derived cell lines, KGN and COV434 (2). We performed expression profiles for 12 tumors, and also for the KGN and COV434 cell lines. The GCT samples for RT-PCR analysis have been described previously (2, 13–15).

Using quantitative RT-PCR, we observed that XIAP was abundantly expressed in the cell lines and was significantly upregulated in GCT compared with whole normal ovary samples (Fig. 1A). XIAP mRNA expression did not differ between stage I and advanced stage GCT (Supplementary Fig. S1). The expression of XIAP and PPARγ was also examined at the protein level using IHC, which demonstrated strong staining for both proteins (Fig. 1B). TMA analysis revealed that XIAP expression was high in 52 of 76 (68%) of the primary tumor samples (Fig. 1C; Table 1). For those tumor samples with both primary and recurrent cores, we observed that XIAP expression did not differ between tumor samples (Table 1). The TMA included a normal preovulatory ovarian sample with moderate level of XIAP expression, where
staining was observed in the granulosa cells of both small follicles and a prevulatory follicle, as well as in the surrounding theca (Fig. 1C).

We observed very low expression of cIAP1 in all tissues examined, whereas no expression of cIAP2 was observed (Fig. 1C).

Consistent with our previous observation of high PPARγ mRNA levels (2), we observed high levels with IHC for PPARγ in the majority of the GCT cores in the TMA. Moderate to high expression of PPARγ was observed for 49 of 76 (64%) of primary GCTs (Fig. 1C; Table 1). Normal preovulatory ovarian samples showed moderate expression of PPARγ; nuclear staining was observed in the granulosa cells of the small and prevulatory follicle, whereas both nuclear and cytoplasmic staining were observed in the cells of the surrounding stroma (Fig. 1C).

mRNA and protein expression in GCT, GCT-derived cell lines, and premenopausal ovaries. A, XIAP mRNA level was assessed using RT-PCR. The panel includes 8 premenopausal ovary (NO), 8 GCT, and the two human GCT-derived cell lines, KGN and COV434. Mean ± SD. Kruskal–Wallis; Dunn’s post hoc analysis: *P < 0.05; **P < 0.01; ***P < 0.001 when compared with NO. B, PPARγ and XIAP protein expression in GCT was determined using IHC. C, High to low expression of XIAP and PPARγ was observed in the GCT cores in the TMA. XIAP and PPARγ was also detected in the granulosa cells and theca cells in a normal premenopausal ovarian sample. Very low levels of cIAP1 and no expression of cIAP2 was observed in GCT samples.

Figure 1.

Inhibiting XIAP decreases NFkB and AP1 transactivation

We previously reported that the NFkB and AP1 signaling pathways are constitutively activated in KGN and COV434 cells (6). NFkB transcriptionally activates XIAP expression, while XIAP modulates NFkB activation as a consequence of a positive feed-forward loop. XIAP has been reported to regulate NFkB through the ubiquitin ligase activity of its RING domain (26). XIAP also activates NFkB and AP1 signaling pathways via the activation of TGFβ-activated kinase 1 (TAK1; ref. 27). In addition to the removal of PPARγ transrepression, we thus sought to determine if inhibiting XIAP could also decrease the constitutive activity of NFkB and AP1 in the GCT-derived cell lines. When cells were transfected with either a NFkB or AP1 reporter, there was an approximate 6-fold induction of luciferase activity under basal conditions compared with cells transfected with an enhancer-less reporter (pTAL-Luc; Fig. 3A and B). Treating KGN cells with 5 μmol/L PD-98059 (PD; ERK/AP1 inhibitor) suppressed the respective constitutive activity as reported previously (6). When we treated KGN cells with a Smac-mimetic, CmpdA (500 nmol/L), there was an approximate 6-fold induction of luciferase activity (Fig. 3A). When cells were transfected with a PPARγ reporter construct (PPRE-luciferase) and treated with RGZ/RA, there was a small activation of the reporter compared with vehicle (Fig. 2B). However, after 24 hours of RGZ/RA treatment, no significant increase in apoptosis was observed for either KGN or COV434 cells (Fig. 2A). A sublethal dose of the NFkB inhibitor, BAY11-7082 (BAY; 2 μmol/L) alone, did not affect cell proliferation (Fig. 2A) or activate the PPARγ reporter (Fig. 2B). When RGZ/RA was combined with BAY, proliferation was blocked (Fig. 2A) with a significant increase in apoptosis (Fig. 2C and D). This response was likely mediated by PPARγ as the effect was reversed by the PPARγ antagonist, GW9662 (GW; 20 μmol/L; Fig. 2A). The robust activation of the reporter by RGZ/RA in combination with BAY (Fig. 2B) indicated that NFkB transpressed PPARγ signaling. Similar results were observed when another PPARγ agonist, TGZ, was used in these assays (Supplementary Fig. S2A–S2D).

Table 1. XIAP and PPARγ protein expression on GCT tissue microarray

<table>
<thead>
<tr>
<th>Marker</th>
<th>GCT tissue microarray (n = 76: 52 primary tumors only; 4 recurrent tumors only)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low n (%)</td>
</tr>
<tr>
<td>XIAP</td>
<td>24 (32)</td>
</tr>
<tr>
<td>PPARγ</td>
<td>27 (36)</td>
</tr>
</tbody>
</table>

*Staining was analyzed using “positive pixel count v9” algorithm in the Aperio ImageScope version 12.3.0.5056 (Leica Biosystems). Percentage of positive (brown) staining indicating protein levels was determined as low (0–30%), medium (31%–79%), or high (>80%).

Each sample represents a GCT collected from an individual patient. Of the 52 primary tumors, 20 have multiple samples and 4 patients have developed recurrent diseases, which were included in the cohort. The tumor samples with both primary and recurrent cores were scored medium/high for XIAP and PPARγ expression.
activity was abrogated for both pathways, indicating that XIAP is regulating both pathways, potentially through the TAK1–TAB1/2/3 complex (Fig. 3A and B). A similar result was observed for the COV434 cell line (Supplementary Fig. S3A and S3B).

XIAP inhibition prevents NFκB transrepression and potently sensitizes KGN and COV434 cells to PPARγ-mediated apoptosis

As inhibition of XIAP abrogates the constitutive activity of both the NFκB and AP1 signaling pathways, we hypothesized that inhibition of XIAP would remove NFκB-mediated transrepression of PPARγ. Using the xCELLigence RTCA system to assess cell proliferation in real time, we assessed the effect of CmpdA (500 nmol/L) either as a single treatment, or in combination with the PPARγ agonists KGN and COV434 cells, and in a nonluteinized granulosa cell line, hGrC1. When cells were treated with CmpdA alone, there was no effect on cell proliferation for either the GCT-derived cell lines (Fig. 3C and D) or the hGrC1 cells (Fig. 3E) over 72 hours. In addition, CmpdA alone also had no effect on PPARγ transactivation (Fig. 3F) or apoptosis (Fig. 3G and H). PPARγ activation alone with RGZ/RA or TGZ/RA caused a small decrease in proliferation (Fig. 3C–E) with no effect on apoptosis (Fig. 3G and H). Combining CmpdA and with RGZ/RA (20 μmol/L/5 μmol/L) resulted in cessation of cell proliferation in both KGN (Fig. 3C) and COV434 (Fig. 3D) cells over 72 hours. This was accompanied by robust PPARγ-mediated transactivation of the reporter construct (Fig. 3F) and significant increase in apoptosis (Fig. 3G and H), similar to that seen with combined NFκB inhibition and PPARγ activation (Fig. 2B–D). Consistent results were observed when a monomeric Smac-mimetic AZD5582 was used in combination with RGZ/RA or with another PPARγ agonist, TGZ (Supplementary Fig. S3C–S3F).

Combined XIAP inhibition and PPARγ activation disrupts KGN 3D spheroid formation

To determine whether the drug combination would be successful in a more physiologic setting, we tested combined XIAP inhibition and PPARγ activation in a 3D cell culture model (25).
for KGN cells. We observed that the combination treatment (RGZ/RA/CmpdA) at the same concentrations used for the 2D monolayer experiments, caused disruption of spheroid architecture from 24-hour treatment, with gradual but complete spheroid dissociation occurring over 12-day treatment (Fig. 4A). Singular treatments, RGZ/RA and CmpdA, have no effects on the spheroids (Supplementary Fig. S4). This coincided with a significant decrease in cell viability (Fig. 4B) as assessed using the resazurin viability assay.

Combined XIAP inhibition and PPARγ activation disrupts primary patient-derived GCT explants

Combination treatment also disrupted established GCT explants after 7-day treatment, with concomitant loss of cell viability (Fig. 4D). This coincided with a significant decrease in cell viability (Fig. 4B) as assessed using the resazurin viability assay.
viability (Fig. 4C). We also measured the gene expression levels of stearoyl-CoA desaturase-1 (SCD), a PPARγ-induced gene that has been reported to be stimulated following RGZ treatment (28). SCD encodes the SCD protein that was highly induced after the combination treatment in KGN cells (D.T.H. Leung; unpublished observations). We observed that there was robust induction of SCD gene expression after the combination treatment in the explant samples (Fig. 4D), indicating that PPARγ is being activated by the combined treatment.

Combined XIAP inhibition and PPARγ activation delayed invasion

In advanced stage ovarian cancer, aggregates of cancer cells attach to and invade the peritoneal lining to promote metastasis. Utilizing the xCELLigence RTCA system, we investigate whether combined inhibition of XIAP and PPARγ activation can reduce the invasiveness of the KGN cells (D.T.H. Leung; unpublished observations). We observed that RGZ/RA/CmpdA-treated KGN cells have a delayed onset of invasion by approximately 8 hours and were less invasive than vehicle-treated cells (Fig. 5A and B).

Combined XIAP inhibition and PPARγ activation decreases mitochondrial respiration and reduces spare respiratory capacity

We next explored the underlying mechanisms of the cytotoxic effect by the combination treatment. PPARγ plays a pivotal role in lipid and glucose metabolism, and there is upregulation of proteins associated with metabolic processes in combined treated GCT-derived cell lines, consistent with the restoration of PPARγ activity (D.T.H. Leung; unpublished observations). Given the pivotal role of mitochondria for energy metabolism in determining cell fate and the association of oxidative stress with the XIAP/Smac signaling pathway, we investigated whether the combined treatment would affect mitochondrial oxygen consumption in the KGN cells. We performed mitochondrial respiratory analysis using Extracellular Flux Analyzer (XFp) to determine

---

Figure 4.
Effects of combined PPARγ activation and XIAP inhibition in KGN spheroids (A and B) and GCT explant (C and D). Combination treatment (RGZ/RA/CmpdA) at the same concentrations used for the 2D monolayer experiments was used to treat KGN spheroids and GCT explant over 12- and 7-day period, respectively. Cell viability of the KGN spheroids was assessed using the resazurin viability assay (B). n = 3 in quadruplicate wells; one-way ANOVA; Tukey post hoc analysis; ∗, P < 0.05 when compared with vehicle (Veh; 0.1% DMSO). Representative of one GCT sample showing concomitant loss of cell viability after RGZ/RA/CmpdA treatment (C). Expression of a PPARγ-induced gene, stearoyl-CoA desaturase 1 (SCD), was investigated after the combination treatment in the GCT explants (D). n = 2. Mean ± SD.
Figure 5.
Effects of combined PPAR activation and XIAP inhibition in KGN. RGZ/RA (20 μmol/L/5 μmol/L) and CmpdA (500 nmol/L) was used alone or in combination to activate PPAR and inhibit XIAP, respectively. A and B. Invasion assay. Onset of invasion (A, indicated by arrows, representative of 3 separate experiments) and overall invasiveness (B) in the treated cells compared with the vehicle (Veh; 0.1% DMSO) treatment. Mean ± SD. n = 3 in duplicate wells; one-way ANOVA; Tukey post hoc analysis; *, P < 0.05 when compared with vehicle. C–E. Seahorse extracellular flux assay in KGN. A schematic representation of the design and interpretation of the assay (C). Mitochondrial respiration (D), represented by the OCR, was measured as the control and treated KGN cells were subjected to metabolic stressors, oligomycin, FCCP, and antimycin A/rotenone. Spare respiratory capacity (E) represents the ability of the cells to respond to an increased energy demand. Mean ± SD. n = 3 in duplicate wells; one-way ANOVA; Tukey post hoc analysis; *, P < 0.05 when compared with vehicle. F–H. Gene expression analysis. mRNA levels of the proapoptotic genes, TNFRSF1B (F) and TGM2 (G), were investigated using digital PCR following the combined PPAR activation and XIAP inhibition (10 μmol/L Emb or 500 nmol/L CmpdA). H, Quantitative PCR showed changes of an IAP member, cIAP2 or BIRC3, followed by the two Smac-mimetics, CmpdA and AZD (both at 500 nmol/L), alone or in combination with RGZ/RA. Mean ± SD. n = 3–7 in duplicate wells; Kruskal-Wallis; Dunn’s post hoc analysis; *, P < 0.05; **, P < 0.001; ***, P < 0.0001 when compared with vehicle (Veh; 0.1% DMSO).
and rosiglitazone, have been developed to treat patients with polycystic ovarian syndrome (30). PPAR activation significantly decreased OCR (Fig. 5D). By measuring the spare respiratory capacity, which is an estimate of the potential bioenergetic reserve the cell can call upon in times of stress, we observed a significant reduction in the combined treated cells compared with vehicle- or RGZ/RA-treated cells (Fig. 5E). This indicates that the combined treatment affected mitochondrial function, inhibiting the ability of the mitochondria to function at their full potential, causing the reserve capacity to be significantly reduced.

TLDA analysis of XIAP inhibition and PPARγ activation in KGN cells

The expression of 93 apoptosis-related genes was analyzed using TaqMan Low Density Arrays (Supplementary Table S1). Relative gene expression was normalized against the median of geNorm software selected controls. Genes that showed the highest induction after the combined treatment compared with vehicle control were TNFRSF1B (TNF Receptor Superfamily Member 1B, 2.3-fold) and BIRC3 (baculovirus IAP repeat-containing 3 or cIAP2, 139.8-fold).

The induction of these genes, together with TGM2, was validated using qRT-PCR. TGM2 encodes the proapoptotic protein transglutaminase-2 (29), and we previously identified using a proteomic approach called the stable isotope labeling with amino acid in cell culture (SILAC) coupled with mass spectrometry, that the protein levels of TGM2 were upregulated by 2.3-fold after the RGZ/RA/CmpdA treatment in the KGN cells (D.T.H. Leung; unpublished observations). Cells were treated with DMSO (vehicle; 0.1%), PPARγ/RXRa agonists (RGZ/RA 20 μmol/L/5 μmol/L), Smac-mimetics (Cmpd A and AZD at 500 nmol/L, for BIRC3) alone and a combination of Smac-mimetics and PPARγ/RXRa agonists. Gene expression is presented as a change in fold induction, relative to RPLP0 control and normalized to vehicle control. TNFRSF1B expression following RGZ/RA treatment alone was not significantly increased. However, TNFRSF1B was significantly upregulated following RGZ/RA/CmpdA treatment (Fig. 5F). We observed an 8-fold increase of TGM2 mRNA levels after the combined treatment (Fig. 5C). For BIRC3 mRNA levels, CmpdA and AZD treatments resulted in significantly increased induction when used alone or in combination with RGZ/RA. RGZ/RA treatment did not affect BIRC3 mRNA levels (Fig. 5H).

Discussion

The molecular mechanisms of PPARγ action in ovarian function are poorly understood. PPARγ is antiproliferative, promoting terminal differentiation in GC and alterations in steroidogenesis (30). It is involved in GC atresia as activation of PPARγ induces apoptosis in primary rat GCs (30). PPARγ is implicated in the pathology of several diseases including obesity, diabetes, and polycystic ovarian syndrome (30). PPARγ agonist ligands, the synthetic thiazolidinediones (TZD), for example, troglitazone and rosiglitazone, have been developed to treat patients with type II diabetes (31). The potential of PPARγ agonists as anticancer agents has attracted considerable interest due to exerting antineoplastic effects in various solid tumors including endocrine malignancies (32–35). The prodifferentiating role of PPARγ is associated with inhibition of tumor development and progression (36). The overexpression of PPARγ in cancers such as the colon and thyroid allows these cancer cells to be targeted with PPARγ agonists and has led to in vitro studies with PPARγ agonists, which show a reduction in cell viability and inhibition of cell proliferation (37, 38). These antitumor effects have also been observed in xenograft models of solid tumors (39).

High levels of PPARγ in the GCT at the mRNA level (2) and subsequently in this study at the protein level were unexpected given the antiproliferative properties of PPARγ, suggesting that, although potentially targetable, there is resistance to its actions in GCT. Our findings that the TZDs alone have only a small effect on GCT cell proliferation and transactivation activity is consistent with this conclusion (Fig. 2A and B).

We have previously reported that another NR, the antiproliferative ERβ is also upregulated in the GCT-derived cell lines (6). However, ligand-associated ERβ signaling is inhibited by constitutive activation of NFκB signaling. GCT-derived cells transfected with estrogen-responsive reporter constructs showed no response when treated with estradiol (6). Similar, glucocorticoid receptor (GR)–mediated transactivation is also repressed by NFκB signaling in these cell lines (6). Inhibition of the NFκB pathway using the IκBα-specific inhibitor, BAY11-7082, restored both ER- and GR-mediated transactivation (6). These data demonstrated that the functional consequence of constitutive NFκB activity is the transrepression of ERβ-mediated transcription in the COV3434 and KGN cell lines (6). Consistent with these published data, in this study, we demonstrated that the observed PPARγ resistance in the GCT-derived cells was also mediated by constitutive NFκB activity causing transrepression of PPARγ. With inactivation of NFκB signaling, PPARγ agonists led to a profound effect with decreased proliferation (Fig. 2A), increased apoptosis (Fig. 2C and D), and PPARγ-mediated transactivation at the PPRE resulting in a 5-fold induction in response to ligand (Fig. 2B).

Given their respective physiologic roles, mutual transrepression between NFκB and GR results in a cooperative modulation of inflammation and immunosuppression. However, the significance of constitutive activation of NFκB with consequent transrepression of PPARγ in the pathogenesis of GCT remains speculative. In addition to repressing the antiproliferative function of PPARγ in granulosa cells (30, 40), activation of NFκB signaling in GCT may also provide a survival advantage through increasing the expression of genes involved in cell proliferation, metastasis, angiogenesis, and antiapoptosis (3).

The IAPs are critical modulators of apoptosis as they inhibit activation (cIAP1/2) or activity (XIAP) caspases, a process that initiates apoptosis. Among the 8-member IAP family, the best characterized and most potent caspase inhibitor, XIAP, is transcriptionally activated by NFκB. In addition, XIAP has a reciprocal modulatory role in NFκB activation as a consequence of a positive feed-forward loop. XIAP can activate the NFκB and AP1 signaling pathways involving the TAK–TAB protein complex via a physical interaction between XIAP BIR1 domain and the TGFβ-activated kinase-binding (TAB) protein 1 (26). We have demonstrated that NFκB and TGFβ coordinately regulate cell survival in GCT (41). It

Leung et al.
remains to be determined whether the constitutive activity of both NFκB and AP1 (6, 42) in the GCT-derived cell lines is regulated through TAK1–TAB complex.

XIAP is a critical regulator of follicular atresia (43). FSH induces XIAP expression, which in turn suppresses GC apoptosis to facilitate FSH-induced follicular cell expansion and development (43). XIAP is highly expressed in proliferating GC of gonadotropin-primed follicles and downregulated in apoptotic cells from atretic follicles after gonadotropin withdrawal (43). FSH induces XIAP expression in GC in vitro via the NFκB pathway (44). In this study, we showed that XIAP is overexpressed in our cohort of primary GCT and in KGN and COV434 cells, likely due to constitutive activation of the NFκB pathway.

Because of its elevated expression and prominent ability to control cell death in many human cancers, XIAP has become an attractive therapeutic target for novel anticancer treatment (11, 12). Smac-mimetics are small-molecule inhibitors designed to mimic the natural IAP antagonist Smac and bind directly to XIAP with high affinity to neutralize the pro-oncogenic functions of this protein, by preventing caspase binding (11, 12). Mono- or bivalent Smac-mimetics have been designed to target the BIR2 and/or BIR3 functional domains. Preclinical studies have reported high anti-neoplastic activity by both monovalent and bivalent compounds. Several of these compounds have also demonstrated favorable safety profiles and evidence of antitumor activity in early clinical trials (11, 12, 45). Although originally designed to inactivate XIAP, Smac-mimetics are also effective in producing rapid ubiquitylation and proteasomal degradation of other members of the IAP family, in particular cIAP1 and cIAP2 (46, 47).

We have shown that XIAP is the predominant IAP expressed in GCT and the levels of other members are low. In our studies, we utilized a bivalent Smac-mimetic, CmpdA (22), and a commercial monovalent compound, AZD5582. We demonstrated that these compounds have no effect on cell proliferation, PPARγ transactivation or apoptosis when used as a single treatment. However, we observed in cells transfected with either an NFκB or AP1 reporter, that inhibition of XIAP abrogated constitutive activity for both pathways (Fig. 3A and B), indicating that XIAP is regulating both pathways, potentially through the TAK1/TAB1/2/3 complex.

Inhibiting XIAP using single agents has shown some signs of antitumor activity; however, the majority of studies indicate a better efficacy can be achieved with combining drugs (11, 12). In this study, we presented strong evidence that PPARγ agonism in combination with inhibition of NFκB signaling (by XIAP inhibition) is a potential efficacious, molecular-targeted therapy for GCT treatment. XIAP is an attractive therapeutic target due to: (i) its inhibition of intrinsic antiapoptotic properties; (ii) its ability to block NFκB activation; and (iii) its relative tissue specificity. Our data show that this is the case for GCT-derived cell lines, in monolayer and 3D spheroids, which mimic the tumor microenvironment, and is also likely relevant for primary GCT given the similar effects on two in vivo GCT explants from patient tumor samples.

The role of PPARγ in adipogenesis and metabolic homeostasis led us to investigate the effect of the combined treatment on metabolic function by measuring mitochondrial respiration. Generation of ATP by mitochondrial respiration is an indispensable source of energy. Many cells and tissues operate at a basal level, only requiring a part of their total bioenergetic capability thus allowing a reserve respiratory capacity for sudden surges in energy requirement. We observed that the combined treatment reduced the available reserve respiratory capacity, indicating that the combined treatment sensitzes the GCT-derived cells to a sudden surge in ATP demand. However, as the reserve is depleted, it causes the cells to undergo cell death (Fig. 5D–E).

The evidence that XIAP and PPARγ cotreatment induces apoptosis and alters cellular bioenergetics is supported by the gene expression analysis where proapoptotic genes such as TNFRSF1B, TGM2, and BIRC3 are found to be upregulated. In addition, we also identified from our proteomic study (D.T.H. Leung; unpublished observations), the downregulation of fascin, a protein associated with cell motility. Cell motility is another major challenge for GCT treatment as it contributes to the metastatic process of cancer cells by allowing cells to attach to and invade the peritoneal lining to form secondary tumors. In this study, we observed that the RGZ/RA/CmpdA-treated KGN cells when compared to vehicle, significantly delayed the onset and level of invasion. Proliferation of the RGZ/RA/CmpdA-treated cells ceased postinvagination, as represented by the plateau of the cell index (Fig. 5A). This may indicate the cells are undergoing differentiation due to PPARγ activity being restored. It also suggests that the combined treatment strategy may also be advantageous in preventing the metastatic process.

This study provides a proof of concept for combination therapeutic targeting involving XIAP inhibition and PPARγ activation for GCT. We anticipate this targeted therapy will contribute to reducing the use of broad nonspecific standard chemotherapy. As these drugs are already in clinical or preclinical use, expedient translation to the clinic is possible. The findings presented in this study may also have broader significance beyond GCT, specifically for malignancies that coexpress these proteins such as epithelial ovarian cancer (48, 49), colorectal cancer (50), and thyroid cancer (51, 52).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Chu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.T.H. Leung, T. Nguyen, E.M. Oliver, J. Matti, T.W. Jobling, S. Chu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.T.H. Leung, E.M. Oliver, J. Matti, P.J. Fuller, S. Chu
Writing, review, and/or revision of the manuscript: D.T.H. Leung, T. Nguyen, M. Alexiadis, P.J. Fuller, S. Chu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.T.H. Leung, T. Nguyen, J. Silke, T.W. Jobling, S. Chu
Study supervision: P.J. Fuller, S. Chu

Acknowledgments
This work was supported by grants-in-aid from the Cancer Council Victoria (to P.J. Fuller and S. Chu), the Ovarian Cancer Research Foundation (to S. Chu), the Granulosa Cell Tumour of the Ovary Foundation (to S. Chu and P.J. Fuller), the Marshia Bykin Center for Ovarian Cancer Research (to S. Chu and P.J. Fuller), the National Health & Medical Research Council of Australia through a Project Grant (#1058334, to P.J. Fuller and S. Chu), Ian Potter Foundation for the Seahorse Extracellular Flux XfP Analyzer (to S. Chu), and Endocrine Society of Australia through a Research Higher Degree Scholarship (to D.T.H. Leung). The Hudson Institute is supported by the Victorian Government’s Operational Infrastructure Scheme. The authors thank Calvin Chee (Hudson Institute of Medical Research), Nicholas Chee (Hudson Institute of Medical Research), and the Marsha Rivkin Center for Ovarian Cancer Research (to S. Chu and P.J. Fuller), the National Health & Medical Research Council of Australia through a Project Grant (#1058334, to P.J. Fuller and S. Chu), Ian Potter Foundation for the Seahorse Extracellular Flux XfP Analyzer (to S. Chu), and Endocrine Society of Australia through a Research Higher Degree Scholarship (to D.T.H. Leung).
Leung et al.

Medical Research., Daniel Heathcoote (Hudson Institute of Medical Research), and the MHTP Research Platforms for technical support. We would also like to thank Professor Akira Iwase (Nagoya University Graduate School of Medicine) for kindly providing the hGcR cell line (18). We would like to acknowledge MHTP medical Genomics Facility, Melbourne, Australia, as the service provider for quantitative PCR and high content screening.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 22, 2018; revised June 25, 2018; accepted December 4, 2018; published first December 7, 2018.

References
Molecular Cancer Therapeutics

Combined PPARγ Activation and XIAP Inhibition as a Potential Therapeutic Strategy for Ovarian Granulosa Cell Tumors

Dilys T.H. Leung, Trang Nguyen, Edwina May Oliver, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-18-0078

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2018/12/07/1535-7163.MCT-18-0078.DC1

Cited articles
This article cites 51 articles, 7 of which you can access for free at:
http://mct.aacrjournals.org/content/18/2/364.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mct.aacrjournals.org/content/18/2/364.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.