PPARgamm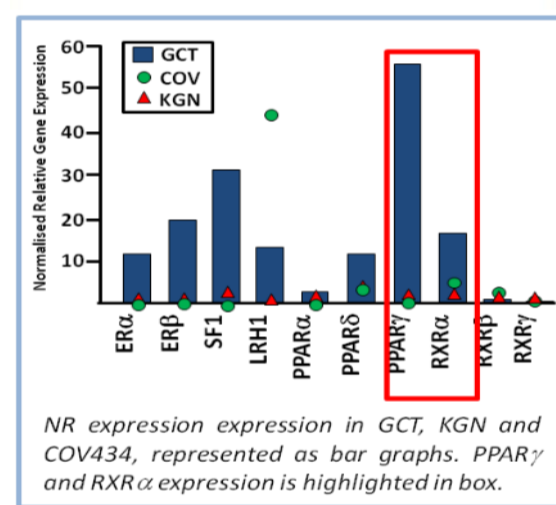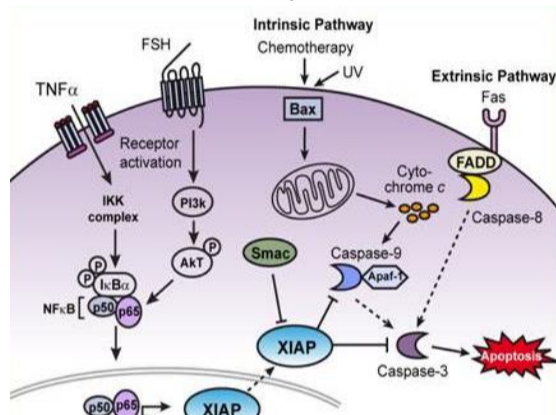a activation and XIAP inhibition mutually augment their respective anticancer effects in Granulosa Cell Tumors of the Ovary

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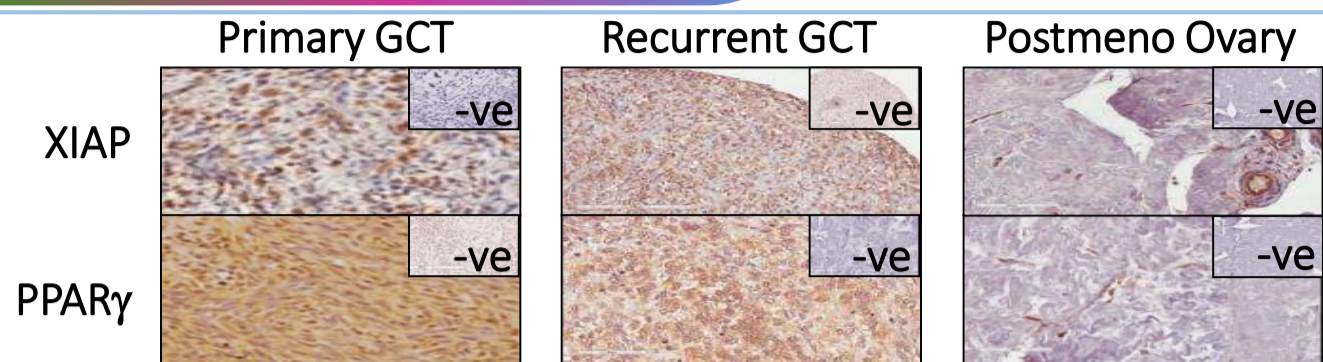
Background

- Granulosa cell tumors (GCT) are the most common type of malignant ovarian sex-cord stromal tumor (1).
- GCT are rare, hormonally active neoplasms characterised by endocrine manifestations, an indolent course, and late relapse.
- Adult GCT are characterized by a missense mutation (C134W) in the FOXL2 gene.
- Chemotherapy and hormonal therapy have proved to be of limited efficacy for GCT.
- We have previously reported that the NF-κB signalling pathway is constitutively activated in two human GCT-derived cell lines, COV434 and KGN (1).
- A key NF-κB regulated protein, the X-linked Inhibitor of Apoptosis Protein (XIAP), is one of eight members of the inhibitors of apoptosis family, and is the most potent caspase inhibitor, blocking both intrinsic and extrinsic apoptotic signals.
- XIAP is regulated by second mitochondria-derived activator of caspases (Smac).
- Given its role in preventing apoptosis, there has been much interest in understanding the role of XIAP in cancer and evaluating XIAP as a therapeutic target.
- Small-molecule inhibitors targeting IAPSs are in various stages of development, from preclinical to phase II clinical trials. These include Smac mimetics which are a new class of small molecule drugs designed to mimic the action of the inhibitory Smac and bind directly to IAPs to neutralize the pro-oncogenic functions of these proteins.
- Nuclear receptors (NR) are well defined targets which have a central pathogenic role in endocrine malignancy, and present as potential targets for therapeutic intervention.
- NR have established roles in granulosa cell biology (2,3); their roles in GCT are largely unexplored.
- Peroxisome proliferator-activated receptor gamma (PPARγ) is highly expressed in GCT (4).
- PPARγ heterodimerizes with retinoid X receptor to bind to peroxisome proliferator responsive element, leading to altered gene expression.
- A number of PPARγ ligands have been identified including synthetic thiazolidinediones, such as rosiglitazone and pioglitazone. Thiazolidinediones are widely used as anti-diabetic agents. Increasing evidence shows a potential application of PPARγ ligands as anti-cancer agents.

NR expression expression in GCT, KGN and COV434, represented as bar graphs. PPARγ and RXRα expression is highlighted in box.

Aims

To investigate whether combined targeting of PPARγ/RXRα and XIAP presents a novel therapeutic strategy for the treatment of GCT.

Methods

- **Cell Lines:** GCT-derived cell lines: KGN and COV434.
- **Reagents:** XIAP inhibitors: *SM* – a bivalent XIAP-specific Smac mimetic (Tetralogic Pharmaceuticals). PPARγ agonists: *Rosiglitazone* (RGZ). PPARγ antagonist: *GW9662*. RXRα agonist: *9-Cis-Retinoic Acid* (RA). NF-κB inhibitor: *BAY11-7082*. High Content Screening Dyes: *Hoechst 33342; YoPro-1; ToPro-3* (Life Technologies).
- **Patients, Tissue Acquisition and Tissue Microarray:** Previously characterized adult GCT (FOXL2 mutations positive) (n=14) collected sequentially at our institution as previously described (1,4, **X**). Normal ovarian tissue was obtained rom premenopausal women who had undergone elective hysterectomy with oophorectomy.
- **Immunohistochemistry:** XIAP and PPARγ protein levels were assessed in fresh-frozen GCT **xCELLigenece Proliferation Assay:** Cell proliferation was assessed in a real-time, non-invasive and label-free manner using the xCELLigence Real-Time Cell Analyzer (RTCA) DP apparatus (Acea Biosciences, San Diego, CA, USA). Briefly, 1x104 KGN cells were seeded in each well of an E-plate 96 in 2% charcoal-stripped serum media with the appropriate treatments. Cell index was assessed in real-time over 120 hours. Relative cell proliferation is presented by the final cell index (difference between the resistance generated by the cells in each time point and the resistance of the medium without cells). Each condition was performed in triplicate.
- **Transient Transfections:** Cells were transfected with a PPRE₄-luciferase reporter construct. Cells were subsequently incubated in serum-free medium with either DMSO, RGZ/RA in the presence or absence of BAY11-7082 or SM for 24 hrs. Luciferase activity was then measured.
- **Apoptosis assays:** Apoptosis was measured using cytofluorometry using a Cellomics Arrayscan VTI High Content Screening Reader.
- **Spheroid Model:** Using KGN cells, spheroids were formed and treated with the appropriate treatment regimen.
- Primary GCT Culture: Primary GCT were collected from oophorectomy of two women, dissected into 1mm³ pieces and plated in 6-well plates in 10% serum-containing DMEM/F12. Following treatment, the explant culture was imaged under light field microscopy.

Results

|	Primary GCT	Recurrent GCT	Postmeno Ovary
XIAP	-ve	-ve	-ve
PPARγ	-ve	-ve	-ve

Using TMA (n=85 cores), high expression of PPARγ and XIAP protein in both primary and recurrent GCT is observed compared to normal postmenopausal ovarian samples. There is low expression of other IAP members in GCT (data not shown).

Marker	GCT Tissue Microarry (n=76: 52 primary tumors only; 4 recurrent tumors only)			
	Low		Medium/High	
	n	(%)	n	(%)
XIAP	24	(32)	52	(68)
PPARγ	27	(36)	49	(64)

TMA revealed that XIAP expression was high in 52/76 (68%) of the primary tumor samples. Consistent with our previous observation of high PPARγ mRNA levels (1), we observed moderate to high expression of PPARγ in 49/76 (64%) of primary GCT
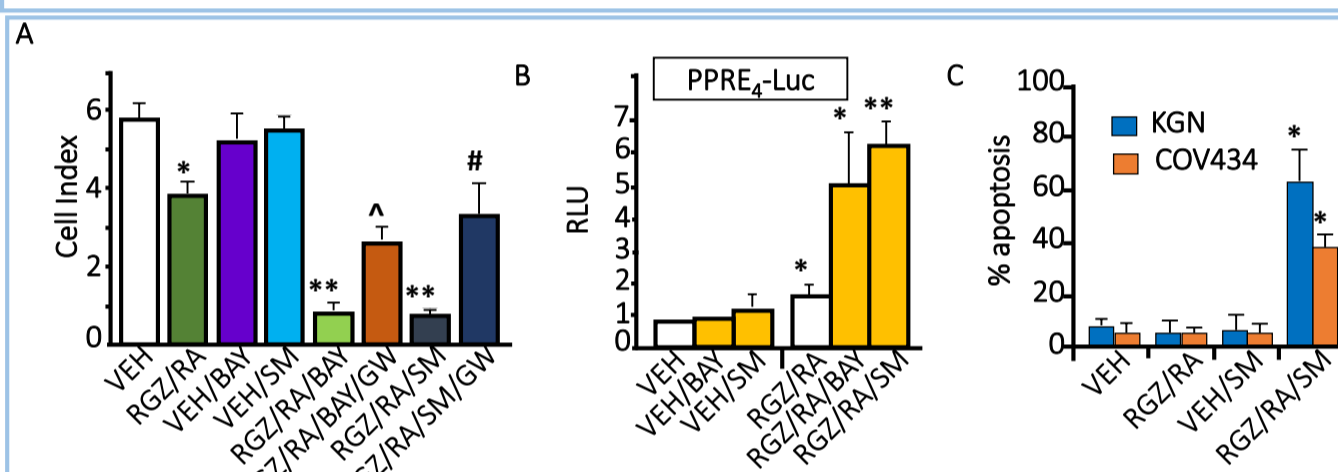
(A) Cell proliferation assay using xCELLigence RTCA. (B) PPARγ-mediated transactivation assay in KGN cells. (C) Effects of XIAP inhibition by SM and PPARγ activation on apoptosis. n>4 experiments; * p<0.05 vs. Veh; ** p<0.01 vs. Veh; ^ p<0.05 vs. RGZ/RA/BAY; # p<0.05 vs. RGZ/RA/SM
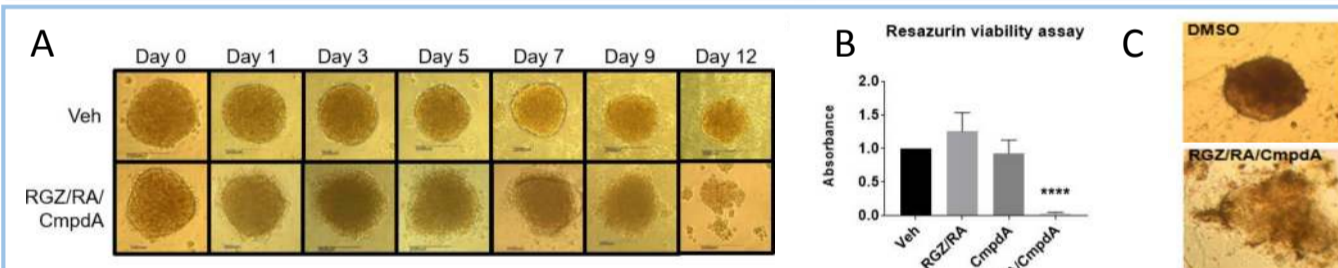
Combination treatment (RGZ/RA/CmpdA) caused disruption of spheroid architecture with gradual but complete spheroid dissociation occurring over 12-days (A). This coincided with a significant decrease in cell viability (B) as assessed using the resazurin viability assay. XIAP inhibition/PPARγ activation disrupts primary GCT explants (C).

Discussion

- The expression of PPARγ and its heterodimeric partner RXRa is elevated in GCT.
- PPARγ plays an antiproliferative role in granulosa cells (2,3), which makes the high levels observed in the tumours unexpected. It suggests that there may be resistance to the actions of PPARγ in GCT.
- PPARγ/RXRα agonists had little effect on cell proliferation or cell viability in two GCT-derived cell lines and minimal transactivation of a PPARγ-response element was observed upon treatment with a PPARγ agonist.
- The non-responsiveness of GCT cells to PPARγ agonists is caused by NF-κB transrepression, when NF-κB is inhibited by BAY11-7082, PPARγ transcriptional activity was restored and cell proliferation decreased.
- Pre-clinical evidence suggests that the NF-κB regulated XIAP protein is an exciting potential target for cancer therapeutics.
- XIAP targeted therapy combined with PPARγ agonism lowers the apoptotic threshold in the GCT cells *in vitro* using monolayer cultures and spheroid cultures. Additionally, combined XIAP inhibition and PPARγ activation disrupts primary patient-derived GCT explants.
- By sensitizing the cells to lower doses of chemotherapy, there is a potential for decreased systemic toxicities normally associated with the higher doses required for a single agent.
- The findings presented in this study may also have broader significance beyond GCT, specifically for malignancies that co-express these proteins such as epithelial ovarian cancer, colorectal cancer and thyroid cancer.

References

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