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Metabolic Regulation of Ovarian Cancer Cell Death

PRINCIPAL INVESTIGATOR:

Sally Kornbluth, Ph.D.

CONTRACTING ORGANIZATION:

Duke University School Of Medicine
Durham, NC 27710

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14. ABSTRACT Following treatment with chemotherapeutic agents, responsive ovarian cancer cells undergo apoptotic cell death. Several groups have shown that the apoptotic protease, caspase 2 (C2), is an essential activator of cell death in ovarian cancer cells treated with cisplatin and we have found, by knock-down of C2 in ovarian cancer cells, that C2 is also required for responsiveness to microtubule-perturbing agents such as paclitaxel. Work from our laboratory has demonstrated that C2 is normally controlled by the metabolic status of the cell in that high levels of flux through the pentose phosphate pathway (PPP) prevents activation of C2. Because ovarian cancers exhibit increased glucose uptake and increased fatty acid synthesis, we hypothesized that susceptibility of ovarian cancers to front-line chemotherapeutic agents, reflect, at least in part, the metabolic status of the cells and, consequently, the phosphorylation state of caspase 2. In this past year, we have found that fatty acid synthesis inhibition kills ovarian cancer cells through the induction of a protein known as REDD1. REDD1 induction leads to (and is required for) caspase 2 activation, leading to death of the cells.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusion.....	7
References.....	7
Appendices.....	N/A

Narrative:

This proposal stemmed from our observations in model systems demonstrating that the apoptotic protease, caspase 2, is critical for cell death in response to various chemotherapeutic agents [1, 2]. In addition, we had found that caspase 2 activity could be modulated by altering the metabolic status of cells. We proposed to determine whether ovarian cancer cells were, indeed, dependent on caspase 2 for cell death and, in addition, whether metabolic manipulation could enhance ovarian cancer cell death. If so, we wished to determine whether this enhanced cell death could be correlated with changes in caspase 2 phosphorylation or oligomerization status, which are indicative of caspase 2 activation. Finally, we proposed to determine whether the phosphorylation status of caspase 2 or the metabolomic profile of ovarian cancer cells could predict responsiveness of ovarian tumors to chemotherapy.

Body of report:

In this second year of work, we have focused our attention on Task 2 as this has yielded highly promising results. We are currently finishing a manuscript for submission. Task 2 from the original statement of Work:

Task 2: To assess the role of metabolic intermediates in controlling ovarian cancer cell death (months 10-24):

- Determine the responsiveness of ovarian cancer cell lines to fatty acid synthesis inhibitors C75 and C93 (months 10-13)
- Determine the phosphorylation and oligomerization status of caspase 2 in cell lines treated with agents that alter metabolism (months 13-19)
- Perform proof-of-concept experiments to determine if CaMKII inhibitors would be viable chemotherapeutic agents in ovarian cancer and assess their effects on caspase 2 in ovarian cancer cells (months 19-24).

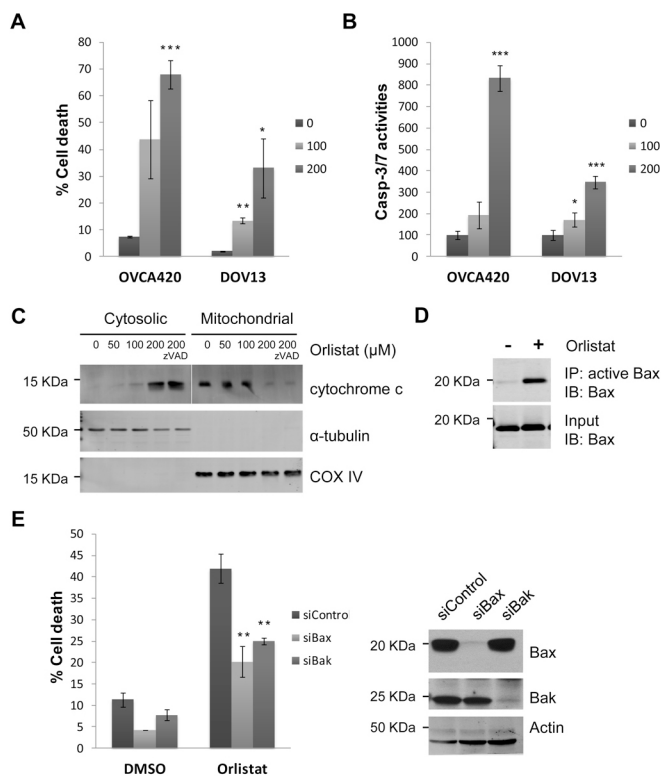


Figure 1. Orlistat induces apoptosis in ovarian cancer cells through the intrinsic pathway. **A**, Ovarian cancer cells were treated with indicated dose of orlistat (0, 100, or 200 μM). The PI-positive population was monitored 24h post treatment by flow cytometry. **B**, Orlistat induced dose-dependent activation of effector caspases. Cells were treated as shown in A and then lysed for a caspase assay. **C**, Inhibition of FASN by orlistat triggered cytochrome c release from mitochondria in OVCA420 cells. COX IV and α-tubulin were used as loading controls and markers for mitochondrial and cytosolic fractions. **D**, Orlistat-induced Bax activation in OVCA420 cells was examined using a monoclonal antibody (6A7) raised against the active form of Bax. **E**, Downregulation of either Bax or Bak, the primary MOMP regulators, by RNAi significantly rescued orlistat-induced cell death in OVCA420 cells. Lysate from transfected cells was resolved by immunoblotting for the efficacy of different siRNAs. (*P<0.05, **P<0.01, ***P<0.001)

Inhibition of de novo lipogenesis activates the intrinsic apoptotic pathway

We have observed robust effects of fatty acid synthesis (FASN) inhibitors on ovarian cancer cells using a variety of inhibitors (though orlistat has proven the most reproducible). We show here data from orlistat-treated cells. As shown in Fig. 1A, cells were treated with various doses of orlistat and monitored for cell death by PI staining and flow cytometry. In this figure and in 1B, where similarly treated ovarian cancer cells were assayed for caspase 3 activity, it is clear that orlistat very effectively induced cell death. This was mediated by the intrinsic pathway of apoptosis in that orlistat treatment clearly triggered the release of mitochondrial cytochrome c (Fig. 1C). Moreover, orlistat induced activation of Bax, a key mediator of cytochrome c release, as indicated by immunoprecipitation using an antibody specific to the Bax active conformation (Fig 1D). In addition, when we silenced either Bax or Bak using siRNA, we had a dampening effect on orlistat-induced apoptosis of the ovarian cancer cells. Collectively, these data show that FASN inhibition leads to activation of apoptosis in ovarian cancer cells.

Caspase-2 is required for apoptosis induced by inhibition of fatty acid synthase.

We monitored caspase 2 activation in ovarian cancer cells treated with orlistat using cleavage of the caspase 2 substrate VDVAD-pNA as an indicator. As shown in Fig. 2A, orlistat induced caspase 2 activation in both OVCA420 and DOV13 cells. This conclusion was bolstered by the detection of cleaved caspase 2 (Fig. 2B) and the appearance of cleaved Bid (a known substrate of caspase 2), which was blocked using the caspase 2 inhibitor VDVAD (Fig. 2C). In addition, Bax activation by orlistat was blocked by treating ovarian cancer cells with siRNA directed against caspase 2. Similarly, cell death induced by orlistat treatment, as measured by PI staining and flow cytometry, was inhibited by siRNA-mediated knock down of caspase 2 (Fig. 2D).

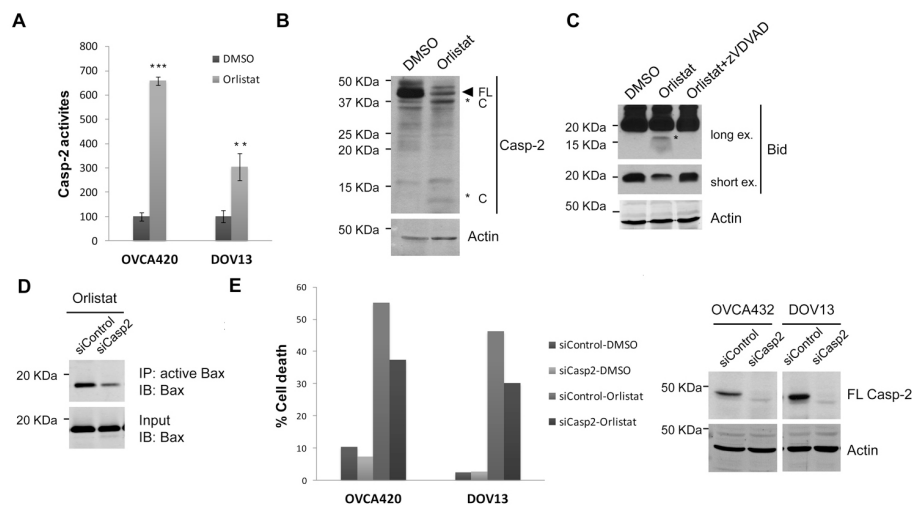


Figure 2. Orlistat activates caspase-2 to transmit death signal through Bid and Bax/Bak. **A**, Caspase-2 activity was measured by caspase assay using lysates collected 24 h post orlistat treatment. **B**, Caspase-2 cleavage was used as an indicator for its activation in OVCA420 cells. (Arrow, full-length caspase-2; asterisks, cleaved caspase-2) **C**, Caspase-2 selective inhibitor, zVDVAD, blocked tBid generation upon orlistat treatment in OVCA 420 cells (Asterisk, truncated Bid, tBid). **D**, Knockdown of caspase-2 in OVCA420 cells dampened orlistat-induced Bax activation. Active Bax was analyzed as shown in Fig.1D. **E**, Caspase-2 is involved in the ovarian cancer cell death induced by orlistat. PI-positive cells were analyzed 24 h post treatment by flow cytometry. The efficacy of siCasp2 was examined by immunoblotting. (**P<0.01, ***P<0.001)

REDD1 accumulation mediates caspase 2 activation upon FASN inhibition

We had reported previously that caspase 2 is inhibited by phosphorylation and binding to the small acidic protein 14-3-3 ζ . Moreover, apoptotic stimuli appeared to result in the dissociation of 14-3-3 and dephosphorylation of caspase 2. In the course of our work, we became interested in a protein known as REDD1, that appeared to be pro-apoptotic in some contexts and anti-apoptotic in others. Our interest was driven by the fact that REDD1 appeared to be able to participate in the dissociation of 14-3-3 from other proteins following stress stimuli. This is, in part, because REDD1 acts as a 14-3-3 “sink” binding up released caspase 2. Thus, we speculated that REDD1 might be involved in 14-3-3 release from caspase 2 and apoptosis in the presence of FASN inhibitors.

Indeed, orlistat induced the accumulation of REDD1 protein in ovarian cancer cell lines (Fig. 3A) and REDD1 levels were elevated prior to caspase -2 activation (Fig. 3B). Importantly, siRNA-mediated down-regulation of REDD1 blocked orlistat-induced caspase 2 cleavage (Fig. 3C) and Bax activation (Fig. 3D). Linking this to the effects of REDD1 via 14-3-3 protein, REDD1 overexpression in OVCA432 cells promoted ectopic activation and cleavage of caspase 2 in the absence of exogenous stimuli, but a REDD1 mutant unable to bind 14-3-3 did not do so (Fig. 3E).

REDD1 knock-down protects ovarian cancer cells from orlistat and tunicamycin-induced death

To determine whether REDD1 was, indeed, critical in the death of ovarian cancer cells induced following orlistat treatment, we knocked down REDD 1 using siRNA and monitored cell death following orlistat treatment. As shown in Fig. 4, ovarian cancer cells were largely protected from orlistat-induced death following REDD1 treatment. If REDD1 is the mechanism by which orlistat enhances cell death, it might suggest that REDD1 elevation would render tumors more susceptible to death induced by other agents. This will be evaluated by examining patient samples for REDD1 expression and correlating this with (blinded) treatment outcome data. In addition, it suggests that in the future treating

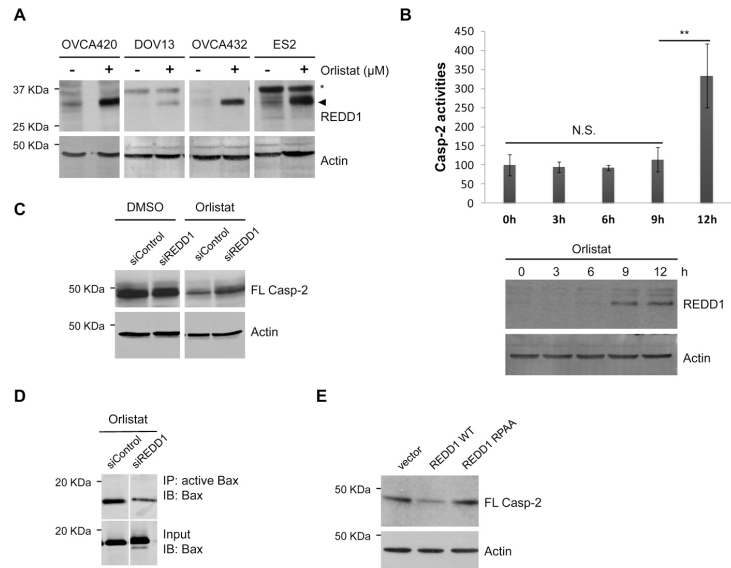


Figure 3. REDD1 modulates caspase-2 activation through its 14-3-3 binding ability. **A**, Orlistat induced REDD1 expression in ovarian cancer cells. Lysates were collected 24 h post 200 μ M orlistat treatment in the presence of 50 μ M zVAD. (Arrow, REDD1; asterisk, nonspecific band) **B**, REDD1 levels escalated prior to caspase-2 activation. OVCA420 cells were treated with 200 μ M orlistat and collected for caspase assay and immunoblotting. (N.S., not significant; ** $P < 0.01$) **C**, Downregulation of REDD1 blocked orlistat-induced caspase-2 activation in OVCA420 cells, which was monitored by the loss of full-length caspase-2. **D**, Removal of REDD1 decreased orlistat-induced Bax activation. Lysates from treated OVCA420 cells were assayed as shown in Fig 1D. **E**, Overexpression of wild-type REDD1, but not a mutant lacking of 14-3-3 binding ability (RPAA), activated caspase-2 in OVCA432 cells.

patients with low levels of FASN inhibitors might synergize with conventional chemotherapeutics if REDD1 is induced.

We are currently undertaking experiments described in Task 3.

Key research accomplishments:

- Identification of the intrinsic pathway as mediator of orlistat-induced death of ovarian cancers
- Demonstration that caspase-2 is activated by orlistat leading to activation of Bak/Bax
- Demonstration that the 14-3-3 binding ability of REDD1 is required for its ability to mediate caspase 2 activation and cell death in response to orlistat
- Demonstration that REDD1 is required for orlistat-induced death in ovarian cancer cells

Reportable outcomes:

Manuscript in preparation describing role of REDD1 in ovarian cancer death after inhibition of fatty acid synthesis inhibition.

Conclusion:

We have demonstrated that inhibition of fatty acid synthesis in ovarian cancer cells engages caspase 2 to promote cell death. This activation of caspase 2 depends upon the ability of fatty acid synthesis inhibition to promote activation of REDD1. We will be following up on this, examining REDD1 expression in ovarian tumor samples and also, if time allows, examining ovarian cancer xenografts for REDD1 expression following treatment of mice with fatty acid synthesis inhibitors.

References:

1. Nutt, L.K., M.R. Buchakjian, E. Gan, R. Darbandi, S.Y. Yoon, J.Q. Wu, Y.J. Miyamoto, J.A. Gibbons, J.L. Andersen, C.D. Freel, W. Tang, C. He, M. Kurokawa, Y. Wang, S.S. Margolis, R.A. Fissore and S. Kornbluth, *Metabolic control of oocyte apoptosis mediated by 14-3-3zeta-regulated dephosphorylation of caspase-2*. Developmental cell, 2009. **16**(6): p. 856-66.
2. Nutt, L.K., S.S. Margolis, M. Jensen, C.E. Herman, W.G. Dunphy, J.C. Rathmell and S. Kornbluth, *Metabolic regulation of oocyte cell death through the CaMKII-mediated phosphorylation of caspase-2*. Cell, 2005. **123**(1): p. 89-103.

Appendices: N/A

Supporting data are included in the text of this report.

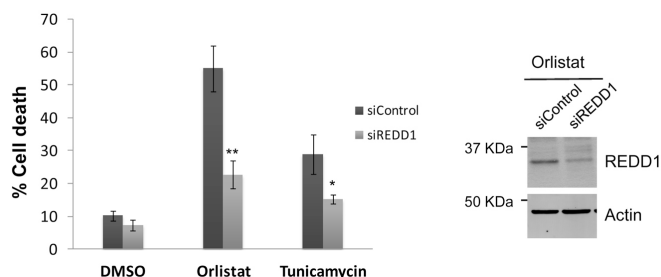


Figure 4. REDD1 RNAi protects cells from orlistat- and tunicamycin-induced death. Transfected OVCA420 cells were treated with DMSO, 200 μ M orlistat or 10 μ g/ml tunicamycin for 24 h. PI-positive cells were analyzed by flow cytometry. The efficacy of REDD1 siRNA was examined by immunoblotting. (* P <0.05, ** P <0.01)