



Virginia Commonwealth University
VCU Scholars Compass

Theses and Dissertations

Graduate School

2016

Celecoxib enhances sorafenib/sildenafil lethality in cancer cells and reverts platinum chemotherapy resistance

Timothy A. Webb
Virginia Commonwealth University

Follow this and additional works at: <https://scholarscompass.vcu.edu/etd>

 Part of the [Biochemistry Commons](#)

© The Author

Downloaded from

<https://scholarscompass.vcu.edu/etd/4084>

This Thesis is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.

Celecoxib enhances sorafenib/sildenafil lethality in cancer cells and reverts platinum chemotherapy resistance

By
Timothy A. Webb

Major Director: Paul Dent, Ph.D.
Department of Biochemistry

Virginia Commonwealth University
Richmond, Virginia
May 2016

Acknowledgements

I would like to acknowledge my advisor Paul Dent, PhD, for providing me the opportunity to work in his laboratory from my undergraduate career until the completion of my Master's degree. Also, I would like to acknowledge my committee members Sarah Spiegel, PhD, and William McGuire, MD for their efforts in helping me earn my Master's degree.

I want to take this opportunity to thank my lab mates Laurence Booth, PhD, Jane Roberts-Booth, PhD, and Mehrad Tavallai, M.S., for their mentoring at the benchside.

Thanks to my family and friends for providing continual support and encouragement during this project, as they have for every other one of my goals.

Thanks to my sister for being my greatest ally when it is time to dig ditches.

Lastly, thank you to Marla for expanding my mind, despite how uncomfortable it was.

Table of Contents

	Page Number
List of Tables and Figures.....	4
List of Abbreviations.....	6
Abstract.....	10
Introduction.....	10
Ovarian Cancer.....	10
Standard of Care.....	13
Targeted Therapy.....	16
Platinum Drugs.....	17
Platinum Resistance.....	19
Sorafenib.....	21
Sildenafil.....	23
Celecoxib.....	27
Mitogen-Activated Protein Kinase (MAPK) Pathways.....	30
Unfolded Protein Response.....	32
Materials and Methods.....	35
Materials.....	35
Methods.....	36
Tumor Dissociation.....	36
Cell Culture.....	38
Cell Counting and Plating.....	38

Drug Treatment.....	39
Transfections of plasmid and siRNA.....	39
Immunofluorescence.....	40
Live/Dead Assay.....	40
Data Analysis.....	40

Results

The role of cellular efflux pumps ABCB1 and ABCG2 on celecoxib, sorafenib, and sildenafil toxicity.....	51
The unfolded protein response (UPR) plays a role in CSS toxicity.....	58
Assessment of apoptotic role of CSS killing.....	66
Impact of drug combination on signal transduction pathways linked to tumor growth/invasion and chemotherapeutic resistance.....	71
Celecoxib, sorafenib, sildenafil mediated resensitization to platinum standard of care drugs.....	75
Discussion.....	81
Conclusion.....	88
Bibliography.....	89

List of Tables and Figures

Table 1: Ovarian cancer cell lines used in this study along with their location of origin and characteristics.....	13
Figure 1: Chemical structure of cisplatin.....	19
Figure 2: Chemical structure of carboplatin.....	19
Figure 3: Chemical structure of oxaliplatin.....	19
Figure 4: Chemical structure of sorafenib.....	23
Figure 5: Chemical structure of sildenafil.....	25
Figure 6: Diagrammatic representation of sildenafil's mechanism of action, including proposed cancer therapeutic pathway.....	26
Figure 7: Chemical structure of celecoxib.....	28
Figure 8: Diagrammatic representation of sorafenib and celecoxib's mechanisms of action.....	29
Figure 9: Diagrammatic representation of the four major MAPK pathway.....	31
Figure 10: Diagrammatic representation of the basic UPR pathway.....	34
Figure 11: Celecoxib dose response in OVCAR and SK-OV-3 cells.....	44
Figure 12: Assessment of cell viability in multiple ovarian cancer cell lines treated with combinational drug therapy.....	46
Figure 13: Assessment of cell viability in Spiky and PA-1 cell lines treated with combinational drug therapy.....	47
Figure 14: Assessment of cell viability in PDX cell lines treated with combinational drug therapy.....	49

Figure 15: Celecoxib dose response on ABCB1 and ABCG2 in OVCAR and SK-OV-3.....	53
Figure 16: Immunofluorescent HSP27 expression after drug combination.....	55
Figure 17: Assessment of efflux pump modulation/function.....	56
Figure 18: Cell death mechanism determination.....	60
Figure 19: Immunofluorescence of GRP78 and p-eIF2 α	62
Figure 20: Cell viability following transfection with siGRP78 or siEIF2 α	64
Figure 21: Assessment of apoptotic involvement in CSS mediated killing.....	69
Figure 22: Assessment of necrotic and autophagic involvement in CSS mediated killing.....	7
0	
Figure 23: Assessment of drug triplicate on signaling pathways.....	73
Figure 24: Assessment of drug triad effect on sensitization to cisplatin.....	78
Figure 25: Assessment of drug triad effect on sensitization to platinum drugs.....	80
Figure 26: Abbreviated diagrammatic representation of potential resensitization mechanism of CSS.....	87

List of Abbreviations

Full Name	Abbreviation
Activating transcription factor 4	ATF4
Activating transcription factor 6	ATF6
Autophagy protein 5	ATG5
6-diamidino-2-phenylidole	DAPI
B-cell lymphoma 2	Bcl-2
B-cell lymphoma extra-large	BCL-XL
Bcl-2-associated death promoter	Bad
Bcl-2-associated X protein	Bax
BH3 interacting-domain death agonist	BID
Cancer Antigen 125	CA-125
Carbon dioxide	CO ₂
Carboplatin	CARBO
C/EBP homologous protein	CHOP
Cellular FLICE-inhibitory protein-short c-FLIP-s	
Celecoxib	cel
Celecoxib, sorafenib, and sildenafil	CSS
Cisplatin	CIS

Cisplatin, etoposide, and bleomycin	PEB
Celsius	C
Constitutively active	
ca	
Cyclooxygenase-2	COX-2
Cyclic guanosine monophosphate	cGMP
Degrees	°
Dimethyl sulfoxide	DMSO
Dulbecco's Modified Eagle's Medium	DMEM
Endoplasmic reticulum	ER
Endoplasmic-reticulum-associated protein degradation	ERAD
Eukaryotic initiation factor 2 alpha	eIF2 α
pancreatic ER kinase(PKR)-like ER kinase	PERK
Extracellular signal-regulated kinase	ERK
Fetal Bovine Solution	FBS
Fms-like tyrosine kinase 3	FLT3
Food and Drug Administration	FDA
G-force	g
Glucose-regulated protein 78	GRP78(BiP)
Glutathione	GSH
Glioblastoma multiforme	GBM
Harvey rat sarcoma viral oncogene homolog	H-RAS

IκB kinase	
IKK	
Inositol requiring enzyme 1	IRE1
Jun N-terminal kinase	JNK
Mast/stem cell growth factor receptor	SCFR(c-Kit)
Microgram	μg
Milligram	mg
Milliliter	mL
Millimeter	mm
Minimum Essential Media	MEM
Mitogen-activated protein	
MAP	
Mitogen-activated protein kinase	MAPK
Mitogen-activated protein kinase kinase	MAPKK(MKK)(MEK)
Mitogen-activated protein kinase kinase kinase	MAPKKK (MEKK1)
Myeloid cell leukemia 1	
MCL-1	
National Cancer Institute	NCI
Nanogram	ng
Nonsteroidal anti-inflammatory drug	NSAID
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-κB
Oxaliplatin	OX

Phosphodiesterase type 5	PDE5
Phosphoinositide-dependent kinase 1/2	PDK1/2
Platinum (ionic)	Pt ²⁺
Platelet-derived growth factor receptor-beta	PDGFRβ
Prostaglandin E2	PGE2
Protein Kinase B	PKB(AKT)
Protein Kinase G	PKG
Rapidly accelerated fibrosarcoma	Raf
Reactive oxygen species	ROS
Rearranged during transfection	Ret
Receptor-interacting serine/threonine-protein kinase 1	RIP-1
Revolutions per minute	rpm
Roswell Park Memorial Institute Medium	RPMI
Patient-derived xenograft	PDX
Phosphoinositide 3-kinase	PI3K
Phosphate Buffer Solution	PBS
Scramble	SCR
Sildenafil	sil
Small interfering ribonucleic acid	siRNA
Signal transducer and activator of transcription 3/5	STAT3/5
Sorafenib	sor
Tumor Necrosis Factor	TNF

Unfolded Protein Response	UPR
Vascular Endothelial Growth Factor Receptor	VEGFR
Vehicle	veh
Volume	vol

Celecoxib enhances sorafenib/sildenafil lethality in cancer cells and reverts platinum chemotherapy resistance

By Timothy A. Webb

Major Director: Paul Dent, Ph.D.
Department of Biochemistry

Abstract

Ovarian cancer is a dismal disease, affecting thousands of women in the US alone, with a five year survival rate of only 45.6%. Standard of care for ovarian cancer typically includes surgery and chemotherapy, which often only prolongs the struggle against the disease rather than end it. Platinum-based chemotherapeutics are generally effective against ovarian tumors for a time, but the cancer usually relapses with a resistance to platinum. Targeted therapeutics have proven effective in treating other tumor types as part of a multi-drug treatment. Such drugs, celecoxib, sorafenib, and sildenafil, were used in combination to activate or inhibit relevant kinase cascades, cellular efflux pumps, and chaperone proteins which caused established and newly isolated ovarian cancer cells to undergo apoptosis and necrosis. Additionally, the drug

combination was able to resensitize resistant ovarian cancer cells to the platinum drugs through an unknown mechanism.

Introduction

Ovarian Cancer

In the United States alone, approximately 22,000 women will be diagnosed with some form of ovarian cancer in 2015. Of those already diagnosed, 14,180 are expected to die from the disease this year.⁸⁶ While the prevalence of ovarian cancer is relatively rare, accounting for only 1.3% of all new cases, and 2.4% of all cancer deaths, current research in this area is severely underdeveloped.⁸⁶ Total funding for ovarian cancer research from the National Cancer Institute was \$100.6 million, or 2.1% of the total NCI budget, in 2013.⁶⁷ Compared to the \$559.2 million spent on breast cancer research, the current allocation for ovarian cancer seems dismally anemic.⁶⁷ When considering that, by a matter of physiology, only women are at-risk for developing ovarian cancer, the relatively low prevalence does not seem entirely relevant. Considering the five-year survival rate is therefore a better point of reference for the understanding of the ferocity of ovarian cancer.

Five-year survival rates are a standard reference point in explaining the aggressiveness and mortality rate of any given cancer type.^{21,36,19} This convention began within the medical field in the 1930s when surviving past the initial, palpable onset of disease was the major hurdle associated with cancer treatment.³⁶ In the 1970s, the NCI adopted this metric for describing different cancer types, which solidified the

five-year survival rate as a standard within the fields of cancer research and treatment.³⁶ Between the years of 2005 and 2011, the average five-year survival rate of all women diagnosed with any stage of ovarian cancer was 45.6%.^{86,19,80} The individual story, like with any given cancer, changes drastically based on when the disease is diagnosed, and which type of cells within the organ are affected. For instance, women diagnosed with Stage I germ cell tumors of the ovary have a 98% chance of reaching the five year post-treatment mark, whereas women diagnosed with Stage IV invasive epithelial ovarian cancer have only a 17% rate of five year survival.^{21,19} Currently, the best biomarker for diagnosis of epithelial ovarian cancer is Cancer Antigen 125, (CA-125).^{79,18,49,66} Within the ovarian cancer community, CA-125 is known to be a fairly poor biomarker as it lacks in both specificity and sensitivity.^{79,49} Unfortunately, due to a lack of early detection methods, and seemingly ambiguous initial symptoms, only about 20% of all ovarian cancers are found at an early stage, resulting in the overall dismal prognosis.^{20,19,68,79,18} Moreover, the types of ovarian cancers diagnosed early are typically from biological origins which are less virulent than those of the tumor types which are diagnosed at later stages.^{79,18} All told, ovarian cancer is the most lethal type of gynecologic malignancies in developed countries.^{79,18}

Table 1 provides the names and characteristics of the ovarian cancer lines used throughout this study.

Cell Line	Cell Type	Characteristics ^{5,6,19,68,8,35,117}
OVCAR-3	Epithelial	Resistant to cisplatin
SK-OV-3	Ascites	Cisplatin resistant. p53 gene deletion
CAOV-3	Epithelial	Over-expresses mutant p53
CAOV-4	Epithelial	Loss-of-function in p53 gene, sensitive to cisplatin
PA-1	Metastatic site ascites	N-Ras activated
Spiky	PDX	Intrinsic resistance to cisplatin and taxane
MCVH OP1	PDX	Unknown
CTG-1677 #1 and CTG-1677 #2	PDX	Unknown
CTG-1703 #1 and CTG-1703 #2	PDX	Unknown

Table 1. Ovarian cancer cell lines used in this study along with their location of origin and characteristics.

Standard of Care

The care administered to any given woman will vary significantly depending on the aggressiveness and accessibility of the woman's cancer. While the first course of action for most cases will be a debulking surgery in which the goal is to remove as much of the cancerous tissue as possible, some women (with Stage III or Stage IV (2)) are given neoadjuvant chemotherapy prior to debulking.^{106,110,22,64,97} Women considered good candidates for the surgical laparotomy benefit from a physical reduction in the amount of cancerous tissue as well as a more accurate diagnosis and staging, which will educate the medical team on which course of treatment is best indicated.^{106,110,64,97} The relative invasiveness of the surgery depends largely on the perceived progression of the disease. It is possible, though not common, and only for germ cell tumors, to remove only part of one ovary, or simply the reproductive structures on the affected side of the parallel system in order to preserve the fertility of the woman.^{106,22,64,33} In a very limited number of women, this relatively simple surgery suffices for their entire treatment.^{22,33} Often, the cancer is bilateral, and both ovaries must be completely or partially removed.^{22,64,33} The aggressiveness of the surgical plan, and how much tissue will be removed, depends largely on the perceived invasiveness of the cancer, and the woman's desire to bear children.^{106,22,64}

After the initial surgery, women with ovarian cancer will undergo three to six cycles of a combinational drug chemotherapy regimen which, notably, typically includes platinum-based alkylating-like agents like cisplatin (CIS), carboplatin (CARBO), or oxaliplatin (OX).^{23,110,64,22,33} CARBO is the most commonly prescribed medication, with CIS being the second most, and OX used rarely for the treatment of ovarian cancer. In

germ cell ovarian carcinomas, the common combination is cisplatin, etoposide, and bleomycin, known as PEB.^{22,23,67,11} The standard of care for epithelial ovarian cancer, which is the lineage of interest to this study, is CARBO with a taxane.^{62,107} The debulking phase of treatment enables penetration of the drugs into the active tumor cells so as to maximize effectiveness of the chemotherapeutics.^{23,64,22,33} Much of the benefit of a debulking procedure is in the removal of resistant clones.^{62,107} In clinical terms, “optimal debulking” is achieved when the residual tumor has a volume of less than 1 cm, with “suboptimal debulking” encompassing all tumors larger than 1 cm. Delivery mechanisms for the chemotherapeutics vary depending on the stage and precise location of the cancerous cells as well as the debulking status. Both intravenous and intraperitoneal routes can be taken, and are decided upon based on the individual case with which the healthcare team is confronted, with intraperitoneal being the route of choice for the more aggressive and advanced tumors.^{23,64} For instance, intraperitoneal injections are reserved for optimally debulked tumors. Radiation therapy is not currently utilized for ovarian cancer due to the risk of damaging otherwise healthy abdominal organs, and the typical spread of the disease is already outside the compact range of radiotherapy death.^{64,22,33,17,52}

The bleak prognosis for many women with an ovarian cancer diagnosis is due to recurrence of disease rather than the initial onset.^{17,18,22,20,60,23} Maintenance therapy is used to keep the aberrant cell growth at bay, but this does little to eradicate the cancer.^{33,17,22} Many of these tumors display a resistance to the first-line chemotherapeutics, specifically the platinum-based drugs; there is a high mortality rate

associated with recurrence.^{33,17,22,20,18,23,102,60,48} As platinum-based drugs damage DNA, leading to apoptosis, the resistance to such drugs appears to be rooted in the selection of cells with more effective survival pathways against platinum-based therapeutics by virtue of their up-regulating survival and down-regulating apoptotic signals.^{23,47,112,27,38}

Targeted Therapy

Conventional chemotherapy does not distinguish cancer cells from healthy cells. Acting cytotoxically, and distributed systemically, traditional chemotherapeutics kill all cells which are rapidly dividing. Not only does this lack of specificity to cancer cells cause the side effects most closely associated with chemotherapy, but the general cytotoxic effects limit the scope of impact on the tumor.³ Patients typically experience only brief and incomplete tumor reduction from treatment with conventional chemotherapeutics.^{3,49} Targeted therapy is a relatively new approach to chemotherapy which may address these issues inherent in conventional chemotherapy.^{3,10} Instead of interfering with the cellular machinery which is common to all actively dividing cells, targeted therapy is an attempt to exclusively inhibit cancer growth via interaction with markers specific to the cancerous tissue which are not present, or not active, in the healthy tissue.^{3,10} Ideally, targeted chemotherapy kills cancer cells selectively, allowing for specific tumor death without the deleterious effects on the patient's normal

systems.^{3,10} However, cancer cells are categorically adept at developing and utilizing crosstalking survival pathways in an attempt at continued growth and proliferation. Therefore, it is unlikely a single drug therapy will eradicate the disease from a given patient, but will instead lead to developed resistance to the drug. Combinational targeted drug therapy may very well be the answer to the problem of conventional chemotherapy toxicity and targeted drug resistance.

Targeted therapy comes in multiple forms. Some of the more recently used therapeutic interventions are monoclonal antibodies designed to harness the patient's immune system to attack the cancer.⁴⁹ Similarly, immunotoxins are another type of targeted therapy which selectively bind to specific cancer markers using an antibody fragment.^{49,71} Instead of using leukocytes to kill the mutated cells, immunotoxins release a cytotoxic substance into the cell once endocytosed to effectively, yet exclusively, kill cancer.^{49,71} Finally, many targeted therapies are small molecule inhibitors.⁴⁹ The structure of these drugs mimic that of endogenous molecules, allowing them to bind to active sites of proteins such as receptors or enzymes to limit or completely inhibit their function, and so disrupt aberrantly activated intercellular pathways involved in cell survival, potentially leading to cell death..^{3,49} The targeted therapies used in this study are all small molecule inhibitors, and are individually detailed below.

Unfortunately, ovarian cancer does not have any druggable targets which are exceptional in relation to other cancers. Thus, the targeted therapies used in this study were chosen as they interfere with pro-survival pathways generally rather than any specific ovarian cancer survival mechanism.

Platinum Drugs

Almost forty years after receiving FDA approval, the use of platinum-based chemotherapeutics is still standard for treating several tumor types such as testes, head, neck, cervical, non-small-cell lung, and ovarian.^{74,23,111,47} Like many drugs, the discovery of the effects of platinum compounds on cell division was entirely accidental.^{74,83} During an experiment concerning *Eschireichia coli* and electricity, platinum electrodes were used as they were assumed to be inert.^{74,83} After noticing the bacteria were growing to extraordinary lengths, the research team concluded that the platinum electrode had interacted with the solution to produce the compound now known as cisplatin.^{74,47,82} Without being entirely clear on the mechanism of action, the team understood the potential implications of this compound in human diseases such as cancer. Today, despite being commonly used to treat various cancers, exactly how cisplatin and other platinum-based drugs actually inhibit cell growth and cause apoptosis is not entirely explained.^{23,111}

What is known is that platinum-based drugs interact with DNA. Cisplatin is a neutral, inorganic, square planar molecule which must undergo several spontaneous aquation reactions to become primed as a reactive species.^{23,32} Once activated, cisplatin can then interact with purine bases in DNA resulting in DNA-protein and DNA-DNA adducts both interstrand and intrastrand, with the intrastrand being the most detrimental to cell division.^{23,111,103} Unless the cell is able to repair the damage caused by the cisplatin, the cell will eventually enter apoptosis.^{23,111} The three platinum drugs used in

this study are cisplatin, the original, as well as the newer carboplatin and oxaliplatin which are better tolerated by patients. Both of the newer drugs are thought to have mechanisms of action similar to that of cisplatin with perhaps carboplatin also releasing cytotoxic Pt^{2+} *in vitro*.²⁹ Structures of all three drugs are below.

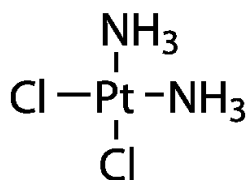


Figure 1. Chemical structure of cisplatin⁸⁸

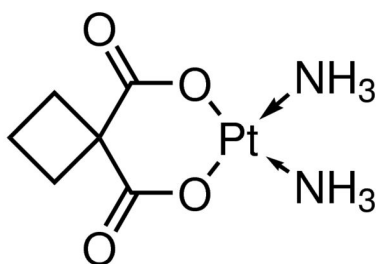


Figure 2. Chemical structure of carboplatin⁹⁴

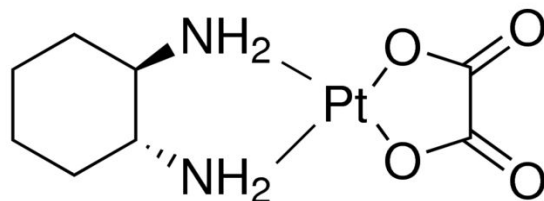


Figure 3. Chemical structure of oxaliplatin⁹⁷

Platinum Resistance

Platinum therapeutics are often effective for a time, but gradually lose their ability to remain lethal to a given tumor after a short period. Even within ovarian cancer, platinum drugs illicit an initial response of tumor reduction in up to 70% of women, but, as the survival rate shows, quickly become fairly ineffective in those same women.^{93,70} Interestingly, tumors which become platinum resistant often develop resistance to completely unrelated antitumor drugs.^{93,70}

Without anyone knowing precisely how platinum work to kill cancer cells, it is difficult to pinpoint why they lose their effectiveness or how resistance develops. The working model for how tumors become platinum resistant involves intracellular drug reduction, accumulation of intracellular thiols, and up-regulation of multiple antiapoptotic pathways.^{93,111,70,46} Intracellular drug levels become reduced in many cell lines after exposure to platinum drugs, and have been diminished by as much as 90% compared to initial concentrations.^{93,46} Both efflux and influx are implicated in this form of resistance, with perhaps influx being the major culprit.^{93,46} With lingering uncertainty as to how exactly the drugs are taken into the cell, or how large a part drug efflux plays in resistance, it is difficult to simply up- or down-regulate cellular pumps as a means of reversing resistance.^{93,46}

Another mechanism of cisplatin, and all platinum, resistance is linked to increased glutathione concentrations in several cisplatin-resistant tumor models.^{93,77} Much in the same way as cisplatin itself forms adducts within DNA, GSH binds with cisplatin leading to the drug's inactivation.^{93,77} GSH-bound cisplatin cannot then go on to

bind to DNA, so the relative increase of GSH as cells are treated with platinum diminishes any response the patient has to further platinum treatment.^{93,77} Ironically, the high reactivity aquated cisplatin utilizes to fight cancer cells enzymatic independently is exactly the same property GSH utilizes to undercut cisplatin. Of course, GSH is a potent endogenous antioxidant.^{93,77,46} As such, the increased levels of GSH in platinum treated tumors probably lead to cell survival by aiding in DNA repair and buffering oxidative stress, both of which actions prevent apoptosis, as well as through direct inhibition of cisplatin.^{93,77,46}

Cancer cells by definition have altered survival and apoptotic protein expression and function. The prosurvival and antiapoptotic pathways are exacerbated even more in platinum resistant tumor lines.^{93,46} With copious crosstalk between pathways, cancer cells ward off platinum-drug induced apoptosis through upregulating phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT) activity, deregulating the mitogen activated protein kinase (MAPK) pathway, increasing the cell's tolerance to DNA damage, suppressing caspase activity, increasing Bcl-2/xL, downregulating Bcl-2-associated X protein (Bax) or Bcl-2-associated death promoter (Bad), and overexpressing Harvey rat sarcoma viral oncogene homolog (H-RAS).⁹³ The culmination of this effort at survival vastly overwhelms the therapeutic effect of the platinum drugs, and the patient's tumor ceases to respond.

The aim of the current study was to restore and retain the cytotoxicity of platinum drugs in ovarian cancer cells. This study utilized combinational targeted drug therapy to directly interfere with these resistance mechanisms.

Sorafenib

Sorafenib (also known as BAY 43-9006 or Nexavar) is a multi-kinase inhibitor which, after only 11 years of development, was FDA approved in 2005 for oral administration in the treatment of renal cell carcinoma.^{115,46,97} Further use has shown the drug has broad inhibitory effects on wild-type and mutant rapidly accelerated fibrosarcoma (Raf) kinase and several tyrosine kinase receptors, to include rearranged during transfection (Ret), Mast/stem cell growth factor receptor (c-Kit), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor-beta (PDGFR β), and Fms-like tyrosine kinase 3 (FLT3) (Figure 8).^{78,1,79,90,92,55,114,115,53,39,96,61} Crystallography has shown that sorafenib works, in part, by binding to the ATP-binding pocket of Raf which inhibits substrate binding and phosphorylation (Figure 8).⁸¹ Despite the number of kinases sorafenib inhibits, the drug remains a form of a targeted therapy as it retains a level of specificity, not binding to protein kinases like mitogen-activated protein kinase kinase (MEK) or extracellular signal-regulated kinase (ERK).⁸¹ By blocking the action of several tyrosine kinase receptors, and Raf, sorafenib has been shown to be anti-proliferative, anti-angiogenic, and anti-survival both *in vitro* and *in vivo*.^{78,1,79,114,115,53,96}

The Dent lab has previously shown sorafenib (sor) also acts to induce endoplasmic reticulum stress (ER stress) in human leukemia cells.^{78,79} Cells treated with sorafenib contained higher than baseline levels of reactive oxygen species, decreased

levels of the chaperone protein GRP78, and reduced levels of the anti-apoptotic protein, myeloid cell leukemia 1 (Mcl-1).^{78,79}

Sorafenib is typically given in 400 mg doses twice daily in the clinic.^{90,92,53,61,46} Clinical trials using sorafenib have had mixed results. Patients who received the clinically relevant doses at times enjoyed only modest results, but often experienced symptoms of rash, diarrhea, edema, and hand-foot syndrome.^{53,39,61} However, other studies have shown positive results from clinical trials with tumors responding well with easily tolerated side effects.^{90,92,115,96} Adverse side effects of sorafenib may be mitigated by temporarily reducing the dosage of the drug, or interrupting treatment briefly.⁴⁶ Interestingly, the more responsive tumors were often later stage, and the sorafenib was given in combination with other drugs.^{90,92,115,96}

Sorafenib was used in this study for its inhibition of multiple tyrosine kinases and Raf, as well as for its demonstrated pro-apoptotic qualities via ER stress. Sorafenib is currently used in the clinic, and is soon to be generic, so accessibility of the drug also is a motivating factor for testing its effectiveness in killing ovarian cancer as well as resensitizing the cancer to platinum therapeutics.

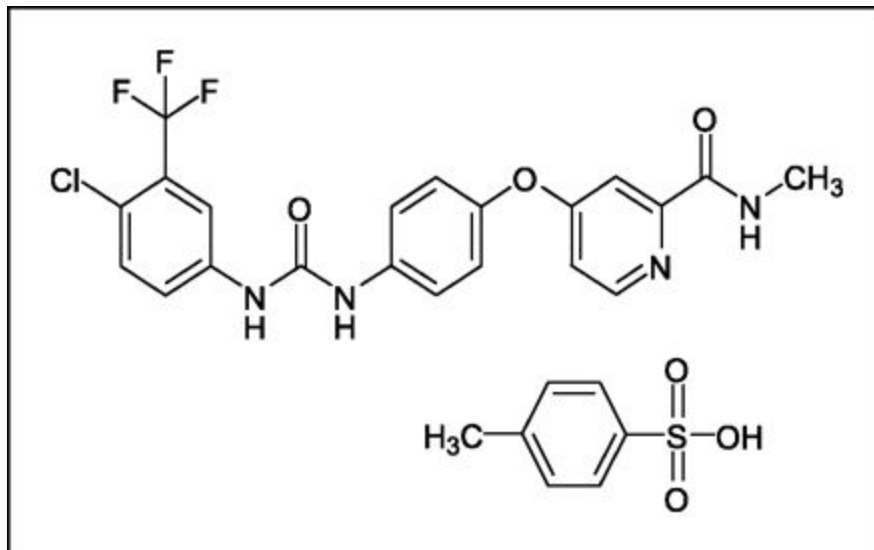


Figure 4. Chemical structure of sorafenib¹¹⁴

Sildenafil

Sildenafil (also known as Revatio or Viagra) is an oral phosphodiesterase type 5 (PDE5) inhibitor, which has proven to have broader applications than originally anticipated. Formulaically intended to aide in the treatment of angina pectoris, sildenafil was found to have limited cardiovascular effects.¹² Instead, the drug was noted to have profound effects as a novel therapeutic for the treatment of erectile dysfunction, eventually receiving approval from the FDA as such in 1998. The drug acts by inhibiting PDE5, the enzyme responsible for breaking down cyclic guanosine monophosphate (cGMP) within cells.^{12,116,43,84} Sildenafil (SIL) is able to selectively inhibit PDE5 due to the drug's structural similarity of the drug to cGMP, thus binding in the active site of PDE5 and blocking the enzyme's degradative activity (Figure 6).^{116,12} With an inhibited PDE5, the cell accumulates nitric oxide (NO) and cGMP, which activates protein kinase G (PKG), and, in turn, decreases the calcium concentration within the cell.^{12,116,43,84} This mechanism leads to smooth muscle relaxation, thus, penile erection.^{12,116,43,84} Sildenafil

is typically well-tolerated, and taken orally at 50mg or 100mg doses for the treatment of erectile dysfunction.⁵⁷

Given that sildenafil was originally formulated for a purpose other than the one for which it received FDA approval, it is ironic that the drug has proven to be an effective therapeutic or preventative agent for a variety of cardiac conditions and other diseases with common comorbidities of erectile dysfunction.^{26,43,84} In the context of this study, sildenafil is used for its demonstrated anti-cancer properties.^{104,14,59,68,16,15}

Many studies, including others produced in the Dent lab, have used sildenafil to complement combinational therapy regimens against various tumor types.^{104,14,59,68,16,15,100} Often, PDE5 is over-expressed in many tumors, so the selective inhibition of such is a relatively safe, direct, and effective mode of therapy.^{104,14} Other studies have noted that abnormally raised cellular concentrations of cGMP activate the mitogen-activated protein kinase kinase kinase 1 (MEKK1)/ mitogen-activated protein kinase kinase (MKK)/ Jun N-terminal kinase (JNK) pathway, resulting in apoptosis (Figure 6).^{105,99,34} PKG itself can trigger apoptosis through caspase activation.¹⁰⁵ In addition to mechanistically increasing cellular cGMP levels, and activating proteins thereby, sildenafil has been credited with aiding to cause ER stress, eventually forcing cancer cells into apoptosis, by directly increasing the amount of ROS, and with reducing resistance to medications by blocking the cellular efflux pumps ABCB1 and ABCG2.^{104,14,59,68,16,15,91,105}

Finally, it is worth noting that low-dose sildenafil in the form of Revatio is currently off-patent, and higher dose sildenafil in the form of Viagra will be generic within the next

five years. While doing nothing mechanistically for the therapy, logistically the therapy proposed in this study is more realistic given an affordable drug combination.

For these reasons, sildenafil was chosen as one of the targeted therapies in the combination.

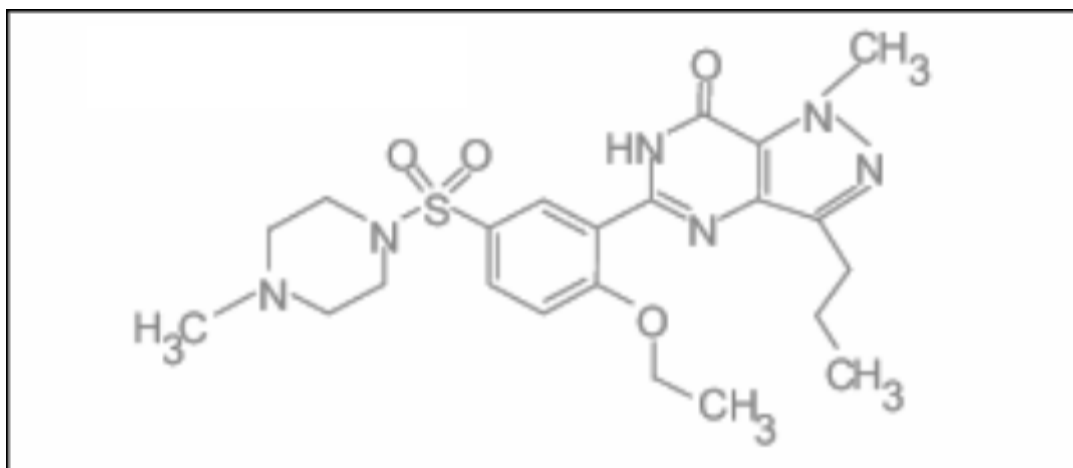


Figure 5. Chemical structure of sildenafil¹¹⁶

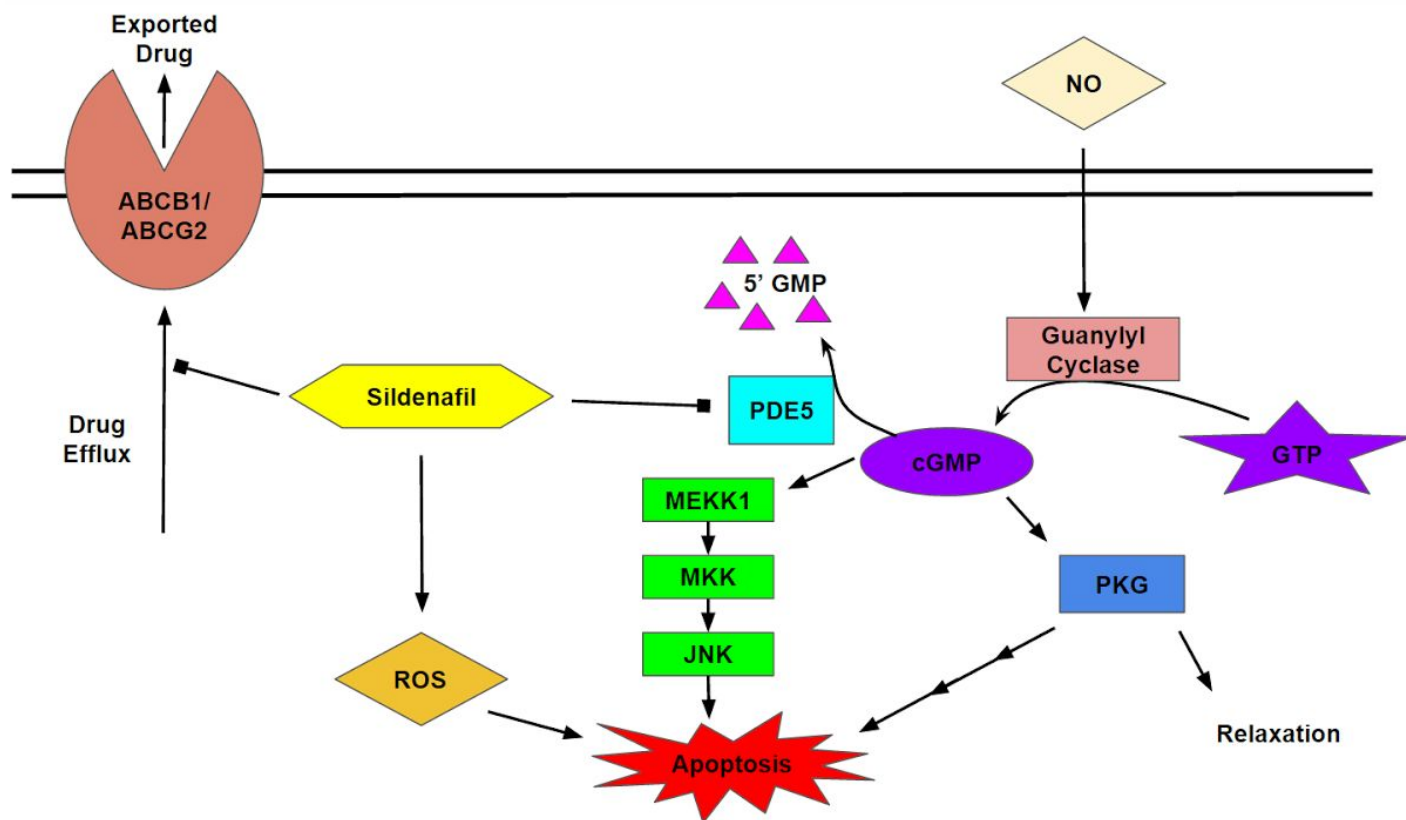


Figure 6. Diagrammatic representation of sildenafil's mechanism of action, including proposed cancer therapeutic pathway.^{105,99,34} Sildenafil potentially acts through three mechanisms as an anti-cancer drug. Sildenafil blocks drug efflux through inhibition of cellular pumps ABCB1 and ABCG2. Sildenafil directly increases the oxidative stress of the cell through generation of reactive oxygen species, which can lead to apoptosis. Sildenafil blocks PDE5 degradative activity of cGMP. This leads to an increased concentration of cGMP which can either activate PKR or the MEKK1/MKK/JNK pathway and trigger apoptosis.

Celecoxib

Celecoxib (also known as Celebrex) is a nonsteroidal anti-inflammatory drug (NSAID) pain reliever which was introduced in the United States in 1999 for the treatment of rheumatoid arthritis and osteoarthritis by selectively blocking cyclooxygenase-2 (COX-2).^{73,28} While other NSAIDs are nonselective, thus interfering with the intestinally protective COX-1, celecoxib reduces pain and inflammation without causing gastrointestinal distress.^{28,109,73} The cyclooxygenase family of enzymes is responsible for converting arachidonic acid into prostaglandins, thus the inhibition of the enzymes results in the reduction of prostaglandins and pain relief (Figure 8). The drug

has comparable efficacy to ibuprofen as an analgesic, and is typically administered orally in 200mg or 400mg doses.²⁸

In the time since FDA approval, celecoxib (cel) has proven to have therapeutic effects beyond pain relief. While the direct mechanism is unclear, COX-2 is implicated in multiple conditions such as Alzheimer's disease, depression, premature labor, and cancer.^{54,98,109,65,41} Celecoxib has been shown to alleviate or slow the progression of these conditions.^{54,98,109,4,65} With regards to this study, cancer is a disease of chronic inflammation. Celecoxib's inhibition of inflammatory prostaglandins may be the source of the drug's anti-cancer properties as prostaglandins stimulate pro-survival pathways in a paracrine and autocrine fashion once secreted from the original cell (Figure 8).^{15,28,54,98,109,65,73,41} Reportedly, the drug acts to inhibit activation of pro-survival protein, AKT, by preventing PI3K from activating phosphoinositide-dependent kinase 1/2 (PDK1/2) (Figure 8).^{110,109} Blocking the actions of AKT would facilitate forcing cells into apoptosis.

An alternative explanation for celecoxib's effectiveness against cancer pertains to the cell-cell adhesion protein, Cadherin-11.⁴ Several tumor types have upregulated cadherin-11 expression, and some seem dependent on the protein for continued progression.⁴ Celecoxib has been shown to bind to cadherin-11, thus disabling the protein.⁴

Regardless of the precise mechanism, celecoxib is regularly demonstrated, by the Dent lab and several others, to be an effective targeted therapy against cancer as both a single agent, and in combination with other targeted therapies.^{15,28,54,98,109,4,73,41} A

recent report has even used celecoxib in combination with sorafenib, as this study does, and showed synergistic effects on hepatic carcinomas *in vitro*.⁶³

Of practical note, celecoxib is now available generically, which reduces the patient's burden of cost for the treatment. For this, and the reasons above, celecoxib was chosen for the combinational therapy in this study.

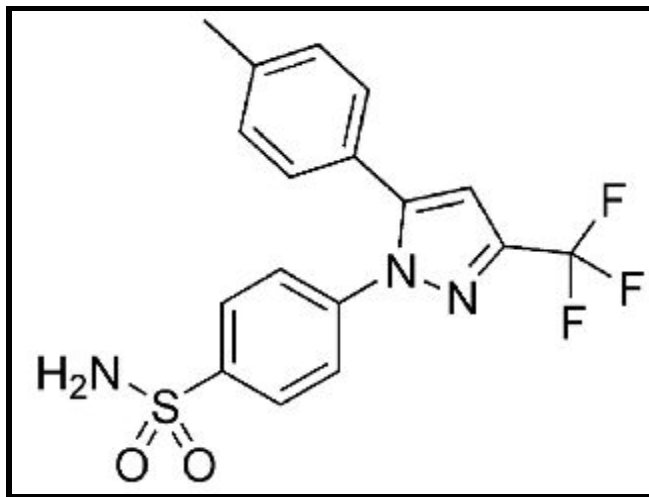


Figure 7. Chemical structure of celecoxib⁴¹

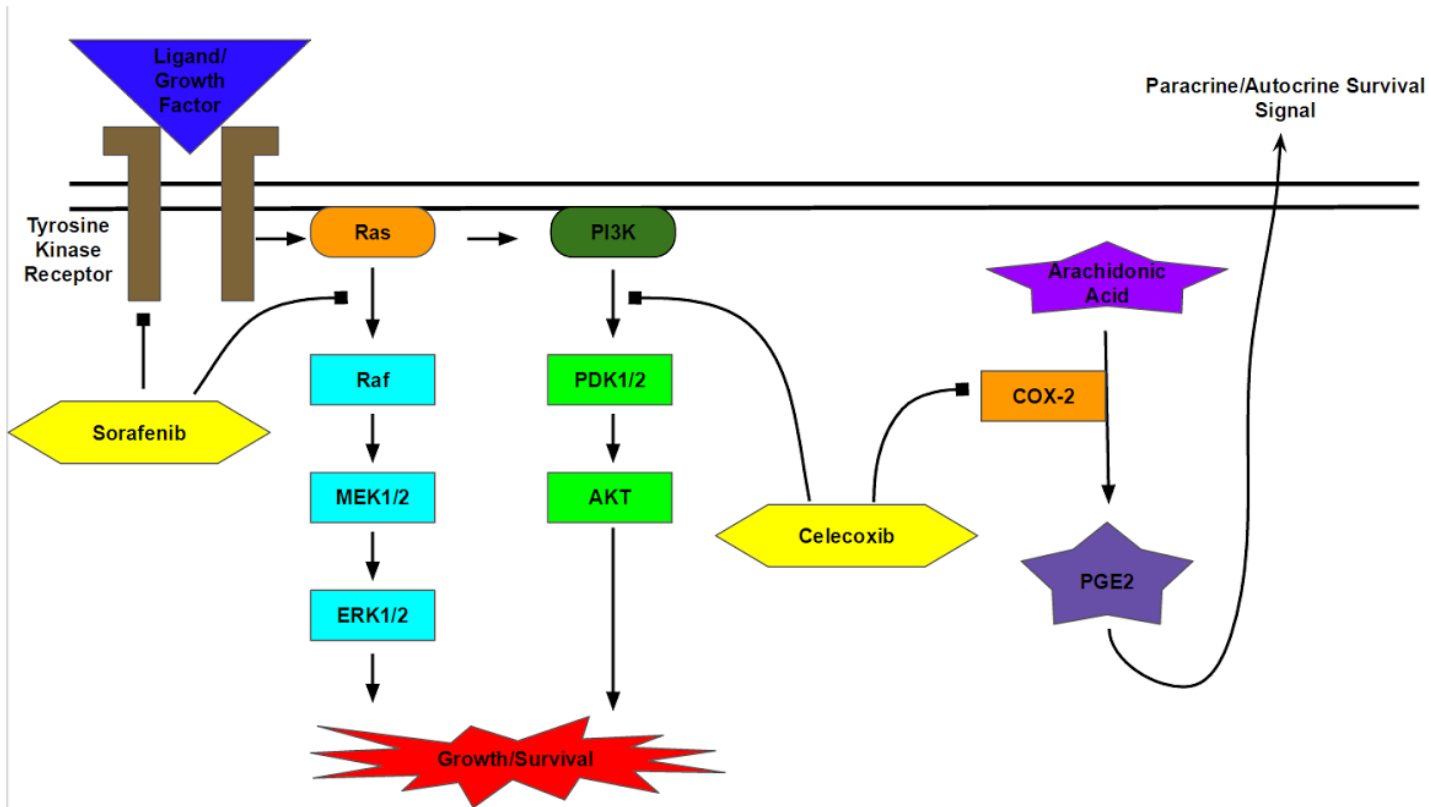


Figure 8. Diagrammatic representation of sorafenib and celecoxib's mechanisms of action.^{97,110,63,85,56,117,58} Sorafenib inhibits tyrosine kinase receptors as well as the Raf/MEK/ERK survival pathway. Celecoxib inhibits the conversion of arachidonic acid conversion to PGE2 by blocking the action of COX-2. Celecoxib also inhibits PI3K-mediated activation of PDK1/2, blocking activation of AKT.

Mitogen-Activated Protein Kinase (MAPK) Pathways

The MAPK pathways are a family of highly conserved, tiered signaling cascades responsible for the transmission and amplification of extracellular stimuli to the nucleus

of a given cell.^{72,87,24,82,75} The hallmark of these cascades is the three-tiered system comprised of a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK).⁷⁵ The three tiers are in series with one another, and each downstream kinase is activated by phosphorylation from its upstream kinase kinase (Figure 9).^{72,24} The cascade is activated when an extracellular signal binds to its appropriate receptor on the plasma membrane.^{72,87,24} The signal is then transduced, transmitted, and amplified as it travels down the phosphorylation cascade to the nucleus of the cell where it activates effector proteins responsible for a diverse set of cellular responses to include differentiation, proliferation, and death (Figure 9).^{75,72,24}

The four typically accepted, major MAPK pathways are the extracellular signal-regulated kinases (ERK1 and ERK2), p38 kinase isozymes (p38 α , p38 β , p38 γ , and p38 δ), Jun N-terminal kinase (JNK1, JNK2, and JNK3), and ERK5 (Figure 9).^{75,72,37} Importantly, each MAPK pathway has the ability for cross-talk with other MAPK pathways within the cellular signalling network for the sake of regulation and resolution of opposing cellular cues.³⁷ They are not totally isolated systems. In normal, differentiated cells, regulation of the MAPK network is well-balanced and regulated.^{72,37} However, cancer cells often have one or several deregulated MAPK pathways, allowing for the cell's propensity for growth and proliferation rather than stasis or apoptosis.^{72,37,30,31,97}

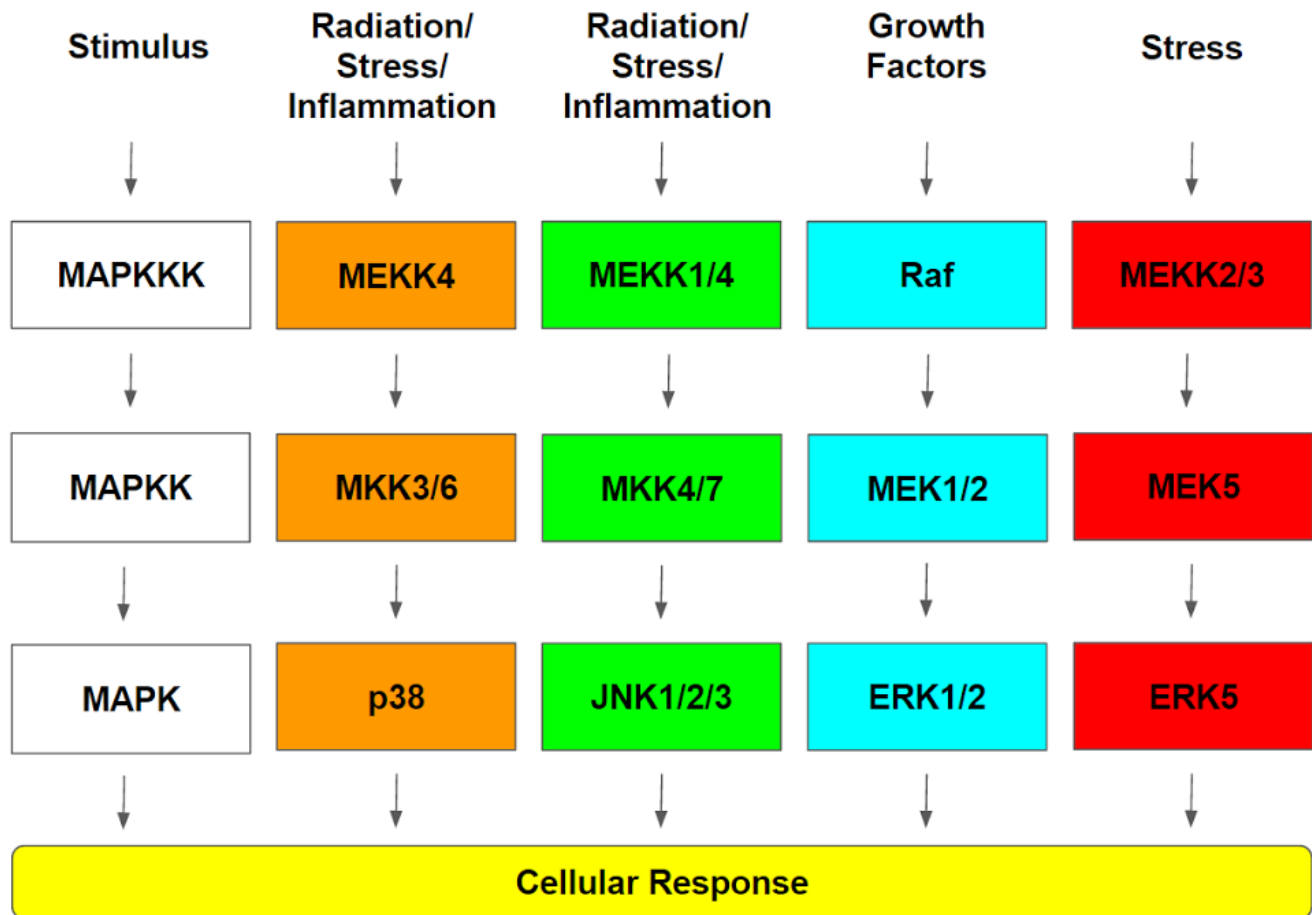


Figure 9. Diagrammatic representation of the four major MAPK pathways^{72,75,37}

MAPK pathways are signalling cascades comprised of three-tiers of kinases in which the upstream MAPKKK phosphorylates the appropriate MAPKK, which phosphorylates the appropriate MAPK, which results in a cellular response specific to the original signal. Both the p38 and JNK1/2/3 pathways can respond to a radiation, stress, and inflammation. The ERK1/2 pathway responds to growth factors. The ERK5 pathway responds to stress.

Unfolded Protein Response

In eukaryotic cells, the ER is responsible for the folding and assembly of proteins to be passed on to other cellular compartments or secreted extracellularly.^{101,42} Proteins which have been folded properly are transported out of the ER, while those which are in some way defective are sequestered for remedial processing.⁴² Such defections can occur through mutation, hypoxia, infection, improper and, like in cancer, high metabolic demand.^{42,101} Due to the inherent inefficiency of cancer cells, the ER often becomes saturated with misfolded and unfolded proteins.¹⁰¹ The accumulation of defective proteins triggers the unfolded protein response (UPR) in an attempt to restore homeostasis and ease the ER stress.^{101,89,51} The innate metabolic weakness of cancer makes exacerbating the UPR, and forcing the cell into apoptosis, an obvious goal of targeted therapies. Initiation of the UPR is characterized by the activation of PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 (IRE1) following their dissociation from glucose-regulated protein 78 (GRP78(BiP)).^{101,42,69,113}

Chaperone proteins, like GRP78, are evolutionarily conserved polypeptides essential to the normal functioning of the ER.^{13,76} These chaperones are tasked with binding to misfolded proteins and altering their structure to a normal, functioning configuration.^{13,76} A relative increase in the abundance of unfolded proteins within the lumen of the ER causes GRP78 to decouple from ER membrane-bound PERK, ATF6, and IRE1 (Figure 10).^{101,42,69,113,13} The active GRP78 then attaches to the misfolded protein and works to correct the deficient structures before their overabundance triggers

apoptosis.¹³ Apoptosis, of course, is an evolutionarily-conserved controlled death mechanism by which the cell enters a programmed form cell death, through DNA fragmentation, cell shrinkage, membrane blebbing.^{101,51,69,42,13}

While GRP78 attempts to resolve the ER stress, PERK, ATF6, and IRE1 each trigger a cascade to cope with the cellular crises.^{51,113,50} Activated PERK phosphorylates eIF2 α which then both halts bulk protein synthesis, so as to prevent additional backlog of unfolded proteins, and activates transcription factor ATF4 (Figure 10).^{51,69,42,113} In turn, ATF4 leads to the translation of additional proteins to alleviate the ER stress, or C/EBP homologous protein (CHOP).^{101,51,44,113} If the ER stress is not alleviated, CHOP downregulates the antiapoptotic BCL-2 and triggers programmed cell death, apoptosis.^{101,51,44,113} Meanwhile, the active ATF6 and IRE1 activate simultaneous cascades to cope with the stress (Figure 10).^{113,101, 89} Both ATF6 and IRE1 activity cause an increase in the folding capacity of the ER, through an increase in chaperones such as GRP78, and in the activity of endoplasmic-reticulum-associated protein degradation (ERAD), a process through which misfolded proteins are degraded outright rather than refolded.^{51,69,42,113,55} Both of the previous are pro-survival adaptive pathways. Additionally, the IRE1 cascade has the ability to promote apoptosis through RNA decay and JNK activation.^{113,69}

Apoptosis and the UPR were examined in this study as they are implicated as mechanisms of action for the drug combination's toxic effects.

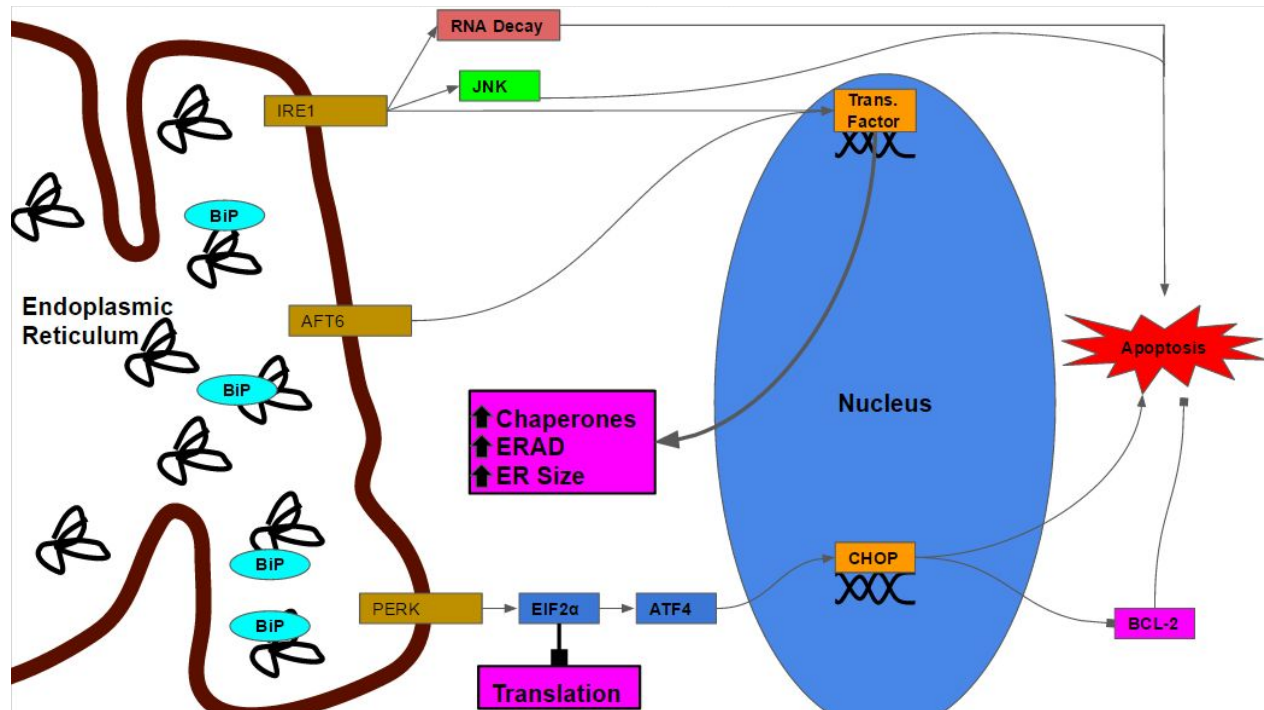


Figure 10. Diagrammatic representation of the basic UPR pathway^{89,44,113} In response to unfolded proteins, BiP (also referred to as GRP78) dissociates from PERK, ATF6, and IRE1, respectively, to refold the protein. The membrane-bound PERK, ATF6, and IRE1 become activated following dissociation from BiP. Active IRE1 triggers signaling which results in RNA decay and JNK activation to promote apoptosis. Concurrently, IRE1, and ATF6, upregulate transcription factors which result in an increase in chaperone proteins, ERAD, and the size of the ER. Concurrently, PERK activation causes phosphorylation of eIF2 α which both halts bulk protein translation, and activates ATF4. Activated ATF4 translocates to the nucleus to activate transcription factor CHOP, which results in suppression of the BCL-2 family, and an overall promotion of apoptosis.

Materials and Methods

Materials

All drugs used in the the course of this study (to include sorafenib tosylate, sildenafil citrate, celecoxib, cisplatin, carboplatin, and oxaliplatin) were purchased from Selleck Chemicals in Houston, TX. Established cell lines of OVCAR-3, SK-OV-3, CAOV-3, and PA-1 were purchased from ATCC. The PDX models, including CTG-1677 #1, CTG-1677 #2, CTG-1703 #1, CTG-1703 #2, MCVH OP1, and the de novo carboplatin/paclitaxel resistant Spiky, were procured from Karen Paz, PhD, the chief scientific officer with Champions Oncology, NJ. The Mayo Clinic repository in Rochester, MN provided the GBM cells. The cellular culture materials DMEM, MEM, RPMI, penicillin-streptomycin, trypsin-EDTA, β -estradiol, insulin, and PBS were purchased from GIBCOBRL (Invitrogen-GIBCOBRL Life Technologies, Grand Island, NY). The FBS used in this study was purchased from HyClone Laboratories, Inc (Thermo Scientific Hyclone, South Logan, UT). The 4% paraformaldehyde in PBS came from Alfa Aesar, a subsidiary of ThermoFisher Scientific. Both the calcein and the ethidium homodimer-1 used in live/dead assays came from Life Technologies, another ThermoFisher Scientific subsidiary. Rat serum, DAPI, and DMSO were all purchased from Sigma Chemical (St. Louis, MO). For transfections, the lipofectamine reagent and Opti-MEM came from ThermoFisher Scientific as well. The plasmid to express GRP78 was obtained by the Dent laboratory from the generous collaboration with A.S. Lee, PhD of the University of Southern California in Los Angeles, CA. Other plasmids used in this study were purchased from Addgene in a process governed by material transfer

agreements. The Dent lab purchased all the validated siRNA from Qiagen (Valencia, CA). Phospho- and total- primary antibodies used for immunofluorescence were obtained from Cell Signaling Technologies (Danvers, MA) and from Santa Cruz Biotech (Santa Cruz, CA). Secondary antibodies (IRDye 680LT GOat anti-rabbit IgG and IRDye 800CW Goat anti-mouse IgG) came from LI-COR Biosciences (Lincoln, NE). The Corning 96-well plates came from Sigma Aldrich. Flasks and graduated tubes were purchased from ThermoFisher Scientific. Core lab equipment such as the centrifuge and tissue culture hood belong to Massey Cancer Center of Virginia Commonwealth University. The tumor dissociator used in the study, the gentleMACS Dissociator, and all enzymes used within that process, came from Miltenyi Biotec. Images shown for live/dead assays as well as immunofluorescence were captured using the Hermes WiScan unit from Accela, which was paid for through the generosity of the Betts family fund.

Methods

Tumor Dissociation

The Spiky, MCVH OP1, CTG-1703 #1, CTG-1703 #2, CTG-1677 #1, and CTG-1677 #2 are PDX cell lines isolated in the Dent lab. Prior to utilization in the course of this research, the tumors were dissociated from the bulk tissue and established as stable cancer cell lines. The protocol used was provided by Miltenyi Biotec, and is detailed here. Enzymes used in this procedure are referred to by the name attributed to them by Miltenyi Biotec.

For the dissociation of ovarian tumors, all of which are deemed “soft” by Miltenyi Biotec, an enzyme mix was prepared by adding 4.7 mLs DMEM, 200 μ L of Enzyme H, 100 μ L of Enzyme R, and 25 μ L of Enzyme A into a gentleMACS C Tube. Concurrently, the patient’s tumor was cut into small pieces (2-4 mm) using a simple scalpel. During the cutting process, an attempt was made to remove any associated adipose, fibrous, or necrotic areas from the tumor itself. The tumor sections were placed into the gentleMACS C Tube containing the enzyme mix already prepared. The C Tube was then closed and placed upside-down into the designated slot in the gentleMACS Dissociator. Once the program was complete, the C Tube was removed from the gentleMACS Dissociator and incubated for 30 minutes at 37⁰C under continuous rotation at 70 rpm. The C Tube was then placed upside-down into the gentleMACS Dissociator once again. At the end of the dissociator program, the tube was again removed from the machine, and incubated for 30 minutes at 37⁰C under continuous rotation at 70 rpm followed by a further treatment in the gentleMACS Dissociator.

The C Tube contents were filtered through a MACS SmartStrainer, 70 μ m, positioned over a standard 50 mL tube. The strainer was washed with 20 mLs of DMEM to ensure the dissociated cells were all collected into the tube below. The 50 mL tube was centrifuged at 300 g for 7 minutes. The supernatant was completely removed, and the pellet resuspended in appropriate media. The dissociated cells were placed in a sterile flask and cultured in the standard procedure detailed below.

Cell Culture

SK-OV-3 cells were cultured in McCoy's medium supplemented with 10% (vol/vol) FBS and 100 µg/mL (1% vol/vol) penicillin-streptomycin. OVCAR cells were cultured in RPMI medium supplemented with 20% (vol/vol) FBS, 100 µg/mL (1% vol/vol) penicillin-streptomycin, and 1 mg/mL insulin. CAOV-3 cells were maintained in DMEM supplemented with 10% (vol/vol) FBS and 100 µg/mL (1% vol/vol) penicillin-streptomycin. PA-1 cells were cultured in MEM supplemented with 10% (vol/vol) FBS and 100 µg/mL (1% vol/vol) penicillin-streptomycin. The PDX lines, Spiky, MCVH OP1, CTG-1677 #1, CTG-1677 #2, CTG-1703 #1, and CTG-1703 #2, cells were maintained in DMEM supplemented with 10% (vol/vol) FBS, 100 µg/mL (1% vol/vol) penicillin-streptomycin, and 500 ng/mL β-estradiol. All cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Cell Counting and Plating

All the cells used in this study were adherent. Prior to use, cells were removed from the adherent surface by trypsinization.

Cells were plated at different densities depending upon the assay. Cells were plated at a density of 5.0×10^5 (per well of a 96-well plate) for live/dead assays and at a density of 3.0×10^3 (per well of a 96-well plate) for immunofluorescence. Cells were allowed to adhere to the well surface under standard incubation conditions for 24-30 hours prior to treatment.

Drug Treatment

Drug concentrations used in this study were selected based upon previous studies published by the Dent laboratory. The concentration of each drug used in this study was as follows: sorafenib, 2 μ M, sildenafil, 2 μ M, celecoxib, 5 μ M, unless otherwise noted. The platinum drugs, cisplatin, carboplatin, and oxaliplatin, 2 μ M. Drug solutions were prepared fresh from powder drug and solubilized in DMSO. Final drug dilution for addition to cells was in the appropriate cell culture media. Solvent concentration did not exceed 0.1% in final drug dilutions.

Transfections of plasmid and siRNA

For the transfection of both plasmids and siRNA plated on the 96-well plates, 2 μ L (per a transfection of 16 wells) of each plasmid was added to 200 μ L of Opti-MEM and incubated in solution for 5 minutes at room temperature. Concurrently, 2 μ L (per a transfection of 16 wells) of lipofectamine was added to 200 μ L of Opti-MEM in a separate graduated tube, and also allowed to sit for 5 minutes. After 5 minutes, the contents of the two tubes were mixed into a single tube, and then incubated for 20 minutes at room temperature.

1.6 mL (per a transfection of 16 wells) of the relevant FBS supplemented media was added to the transfection solution. 100 μ L of the solution was added to each well on the plate. The transfected plates were then incubated under standard incubation conditions for 24 hours prior to drug treatment or data acquisition.

Immunofluorescence

To prepare a plate for image acquisition of fluorescent-tagged proteins, the media was removed from each of the wells on the plate. 100 μ L 4% paraformaldehyde was added to each well to fix the cells, and the plate was incubated under standard cell culture conditions for 20 minutes. The 4% paraformaldehyde was removed, and the cells were washed with 100 μ L PBS twice. A 10% rat serum solution was made with PBS, and added to the cells as a blocking buffer. The fixed cells were placed in a cold room for 24 hours. Blocking buffer was removed and primary antibodies were added to the plate in a 10% rat serum solution. The plates were returned to the cold room overnight. The plates were washed with PBS to remove any unbound primary antibody. The secondary antibody was added in 10% rat serum solution, and left overnight. A final wash with PBS was done, and a rat serum solution with DAPI added.

The plate was then loaded onto the Hermes WiScan machine and viewed for fluorescence. All images recorded were saved to the local hardware and a removable flash drive.

Live/Dead Assay

To determine the effectiveness of a given drug treatment on the killing of cancer cells, a Live/Dead assay was performed using the Hermes WiScan machine. 10 mLs/plate FBS was prepared with 5 μ L calcein (shows live cells) and 5 μ L ethidium bromide (shows dead/dying cells) as a live/dead solution. The live/dead solution was added to the plate in a volume of 100 μ L/well. The plate was centrifuged at a rate of

800 rpm for 3 minutes, and loaded into the Hermes WiScan for image acquisition.

Images taken were a representative sample from all wells.

Percentages of cell death were determined by a simple count of the cells within the collected image.

Data Analysis

Data shown is representative of at least three groups receiving the experimental condition. Representative images were collected from each group, and one was selected for presentation within this thesis. The various treatments were analyzed for statistical significance using a one-way analysis of variance and a two-tailed Student's *t*-test. Results with a *p*-value <0.05 were considered statistically significant.

Results

The three drugs used as the combination targeted therapy in this study were sorafenib (referred to as sor), sildenafil (referred to as sil), and celecoxib (referred to as cel). The three drugs have been utilized individually, and as a part of a combination, in multiple clinical trials and previous studies, as anticancer agents. Sorafenib and sildenafil have shown particular promise as a drug diad at a concentration of 2 μM .¹⁰⁴ As such, and with the intent to use celecoxib as part of the triad, a concentration dose response was performed in OVCAR and SK-OV-3 cells to determine a role for cel within its clinically relevant range. OVCAR and SK-OV-3 are established cell lines which are resistant to the standard of care, cisplatin. Both OVCAR and SK-OV-3 cells responded

to the triad in a greater than additive fashion ($p < 0.05$). In OVCAR cells, the sorafenib/sildenafil lethality was increased at concentrations of celecoxib as low as 1-2 μM (Figure 11A,B). SK-OV-3 cells proved to be less responsive to the triad, but cell death was seen at 3 μM celecoxib (Figure 11A,C). As a result, both sildenafil and sorafenib continued to be used at a concentration of 2 μM , and celecoxib was used at 5 μM unless otherwise stated.

To assess the specific cell killing potential of celecoxib/sildenafil and sorafenib/sildenafil, and to verify that the triad's effects were replicable across multiple cell lines, a live/dead assay was performed. MCVH OP1 (a PDX line), CAO-4, and CAO-3 (both established cell lines with p53 mutations) were treated with vehicle (DMSO), and a combination of either sorafenib and sildenafil, celecoxib and sildenafil, or celecoxib, sorafenib, and sildenafil for 24 hours (Figure 12). Viability was determined by the Hermes WiScan system. The patient derived cells, MCVH OP1, were the most sensitive to the triad while CAO-4 and CAO-3 still had greater than additive differences in death over the control ($p < 0.05$)(Figure 12A,B). The last established cell line to be tested was PA-1 (with an N-Ras mutation). After 24 and 48 hours of drug treatment, cell viability was assessed using the Hermes WiScan. Cell death in the triad group was significantly higher than that of either the diads or the vehicle ($p < 0.05$)(Figure 13A,B).

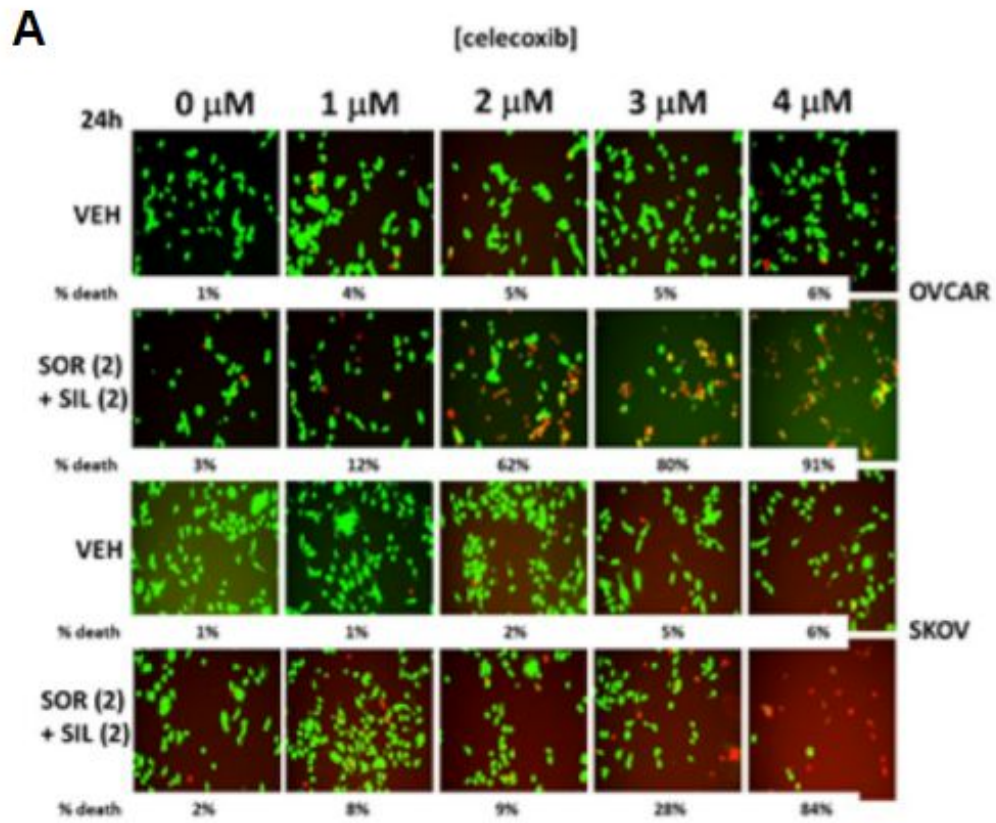
Other PDX cell lines established in the Dent lab were assessed for sensitivity to the drug combination. One such line was called Spiky. Spiky showed a significant response in cell death to each drug diad at both the 24 and 48 hour timepoints ($p < 0.05$),

but did not show a significant increase in response when treated with the triad. (Figure 13A, B).

Four additional PDX lines of unknown genetic characteristics were used in this study. Interestingly, despite originating from the same patient, isolates sent to the Dent lab after having been tumorized in mice often responded differently to treatments. As such, they are referred to as separate lines resulting in CTG-1677 #1, CTG-1677 #2, CTG-1708 #1, and CTG-1708 #2, respectively. The four lines were treated with the standard drug concentrations of veh, sor/sil, cel/sor, and CSS for 24 hours and assessed for cell viability using the Hermes WiScan. Neither CTG-1677 #1 nor #2 showed significant death at 24 hours. CTG-1708 #1 had a less than significant response, while CTG-1708 #2 had a significant response to the drug triad over vehicle ($p < 0.05$) (Figure 14A,C). After 48 hours, CTG-1677 #1, CTG-1677 #2, CTG-1708 #1, and CTG-1708 #2 all exhibited cell death as a result of the triad over vehicle ($p < 0.05$) (Figure 14 B,D).

Considering that all cell lines exhibited a response to the drug combination, the drugs and concentrations described above were used throughout this study. All lines tested had some response to the combination, and were used in this study accordingly. SK-OV-3 served as a model of resistance in terms of cell death to both the combinational therapy and platinum drugs. OVCAR-3 was used as a triad sensitive, cisplatin resistant line. PA-1 is killed by platinum, and also had a profound response to the drug triad ($p < 0.05$). Each of the PDX lines was continuously used with the exception of MCVH OP1.

Fig. 11



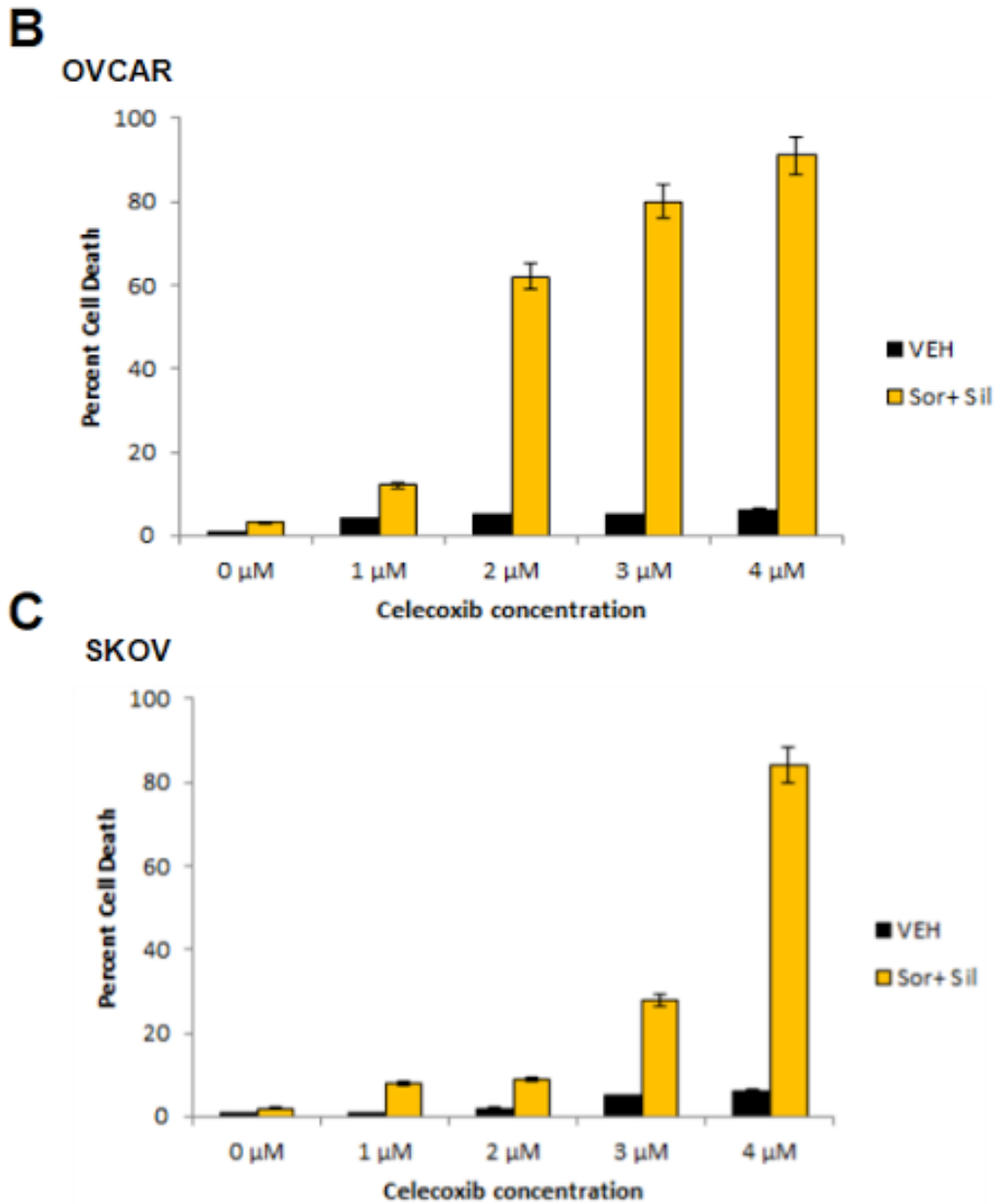
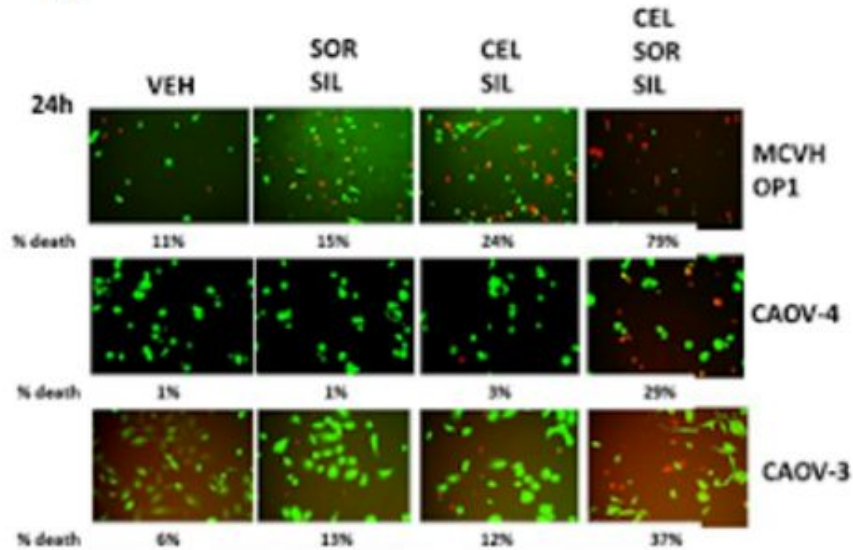


Figure 11. Celecoxib dose response in OVCAR and SK-OV-3 cells. OVCAR and SK-OV-3 cells were treated with 2 μ M sorafenib and sildenafil, and varying concentrations of celecoxib within the clinically relevant range, as indicated above. (A) Cell viability was determined by live/dead assays using the Hermes WiScan system after 24 hour drug exposure. Images shown are representative of each experimental group. This data was quantified for (B) OVCAR and (C) SK-OV-3 and presented graphically

Fig. 12

A



B

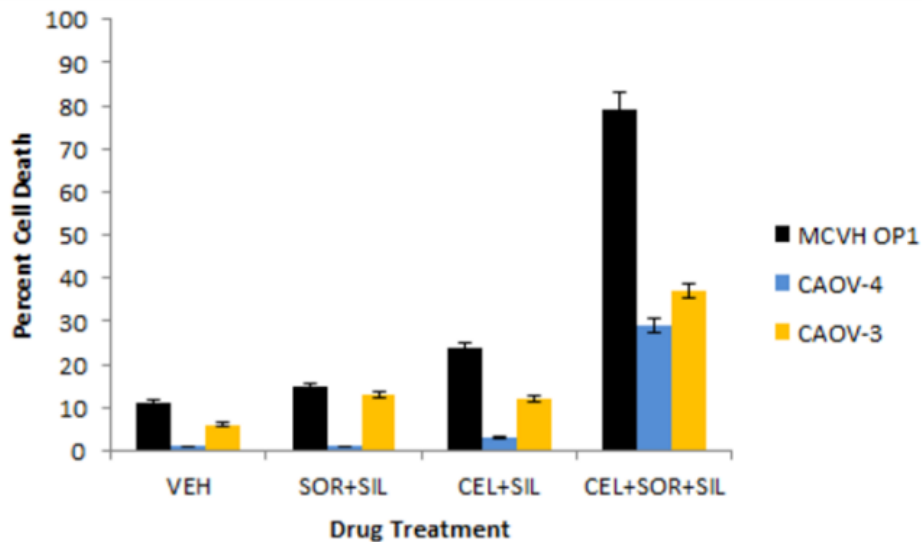
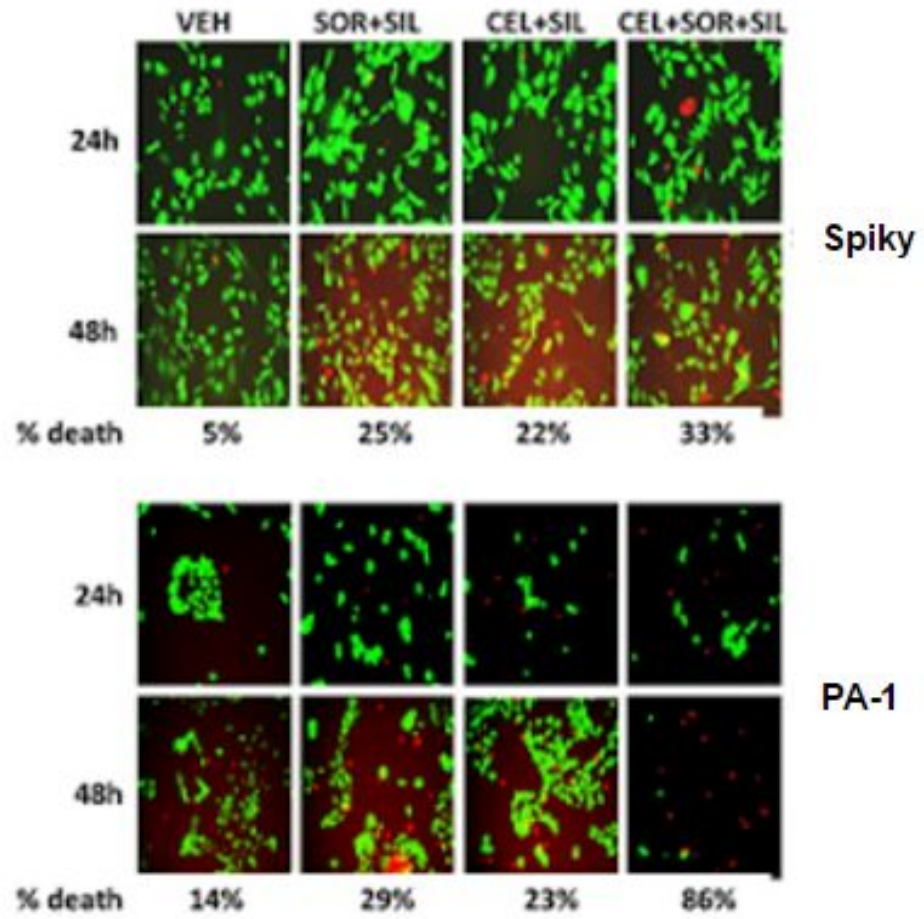


Figure 12. Assessment of cell viability in multiple ovarian cancer cell lines treated with combinational drug therapy. MCVH OP1, CAOV-4, and CAOV-3 were treated with 2 μ M sorafenib, 2 μ M sildenafil, and 5 μ M celecoxib for 24 hours. (A) Cell viability was determined by live/dead assays using the Hermes WiScan system. Images shown are representative of each experimental group. (B) This data was quantified for all three cell lines and is presented graphically

Fig. 13

A



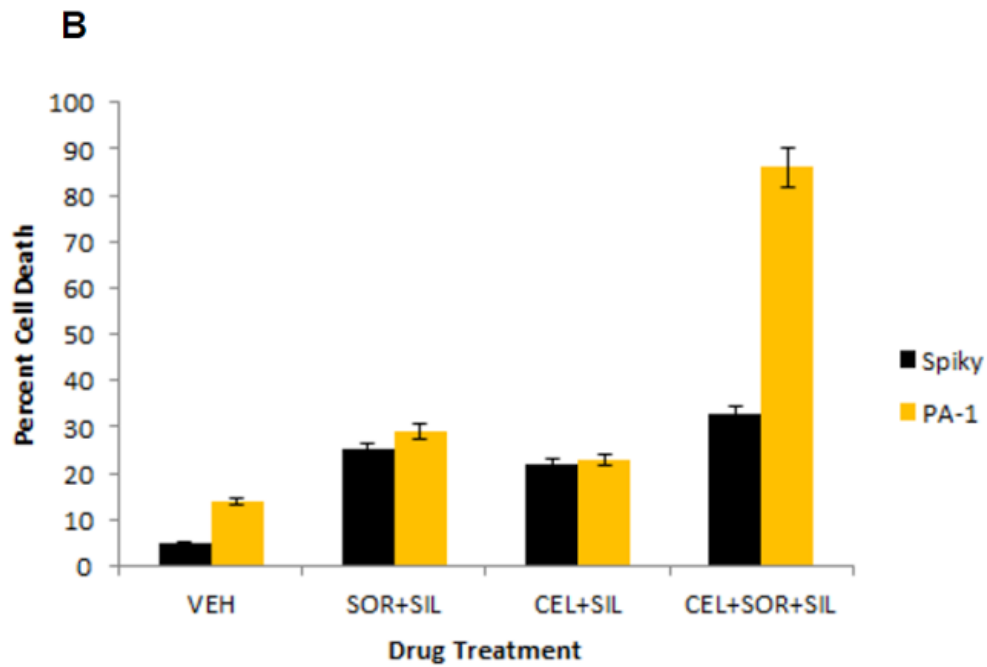
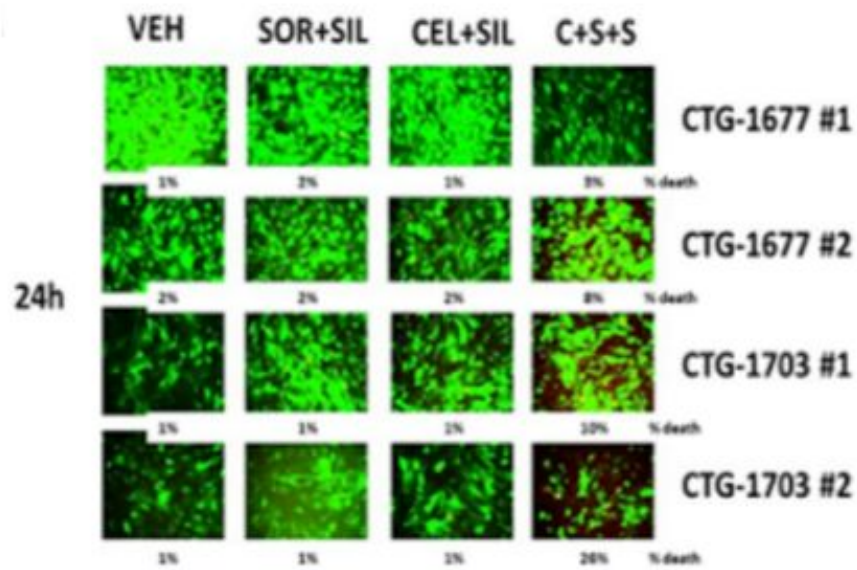


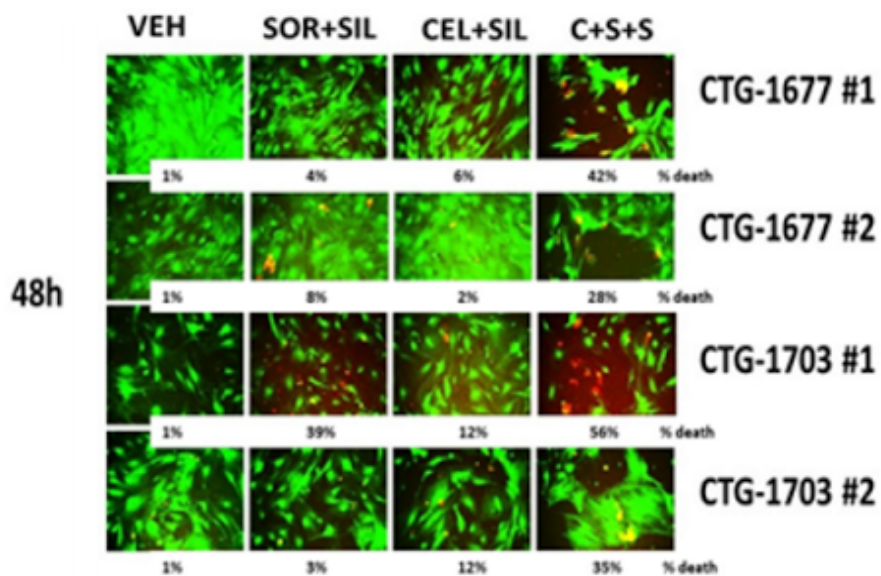
Figure 13. Assessment of cell viability in Spiky and PA-1 cell lines treated with combinational drug therapy. Spiky and PA-1 cells were treated with 2 μ M sorafenib, 2 μ M sildenafil, and 5 μ M celecoxib for 24 and 48 hours. Cell death percentage shown is for 48 hour treatment. (A) Cell viability was determined by live/dead assays using the Hermes WiScan system. Images shown are representative of each experimental group. (B) This data was quantified for both cell lines and is presented graphically

Fig. 14

A



B



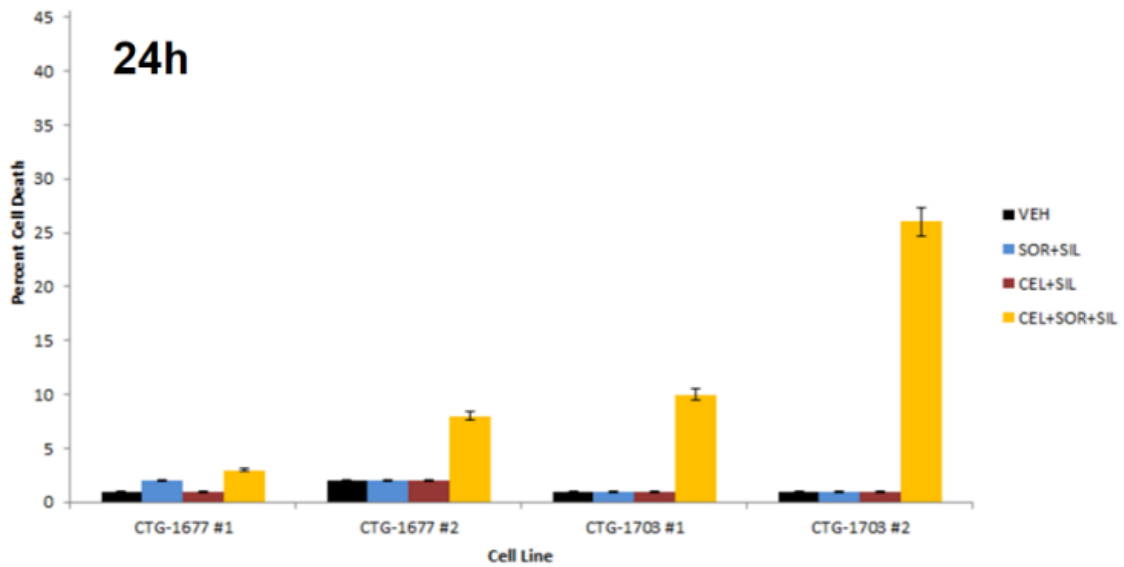
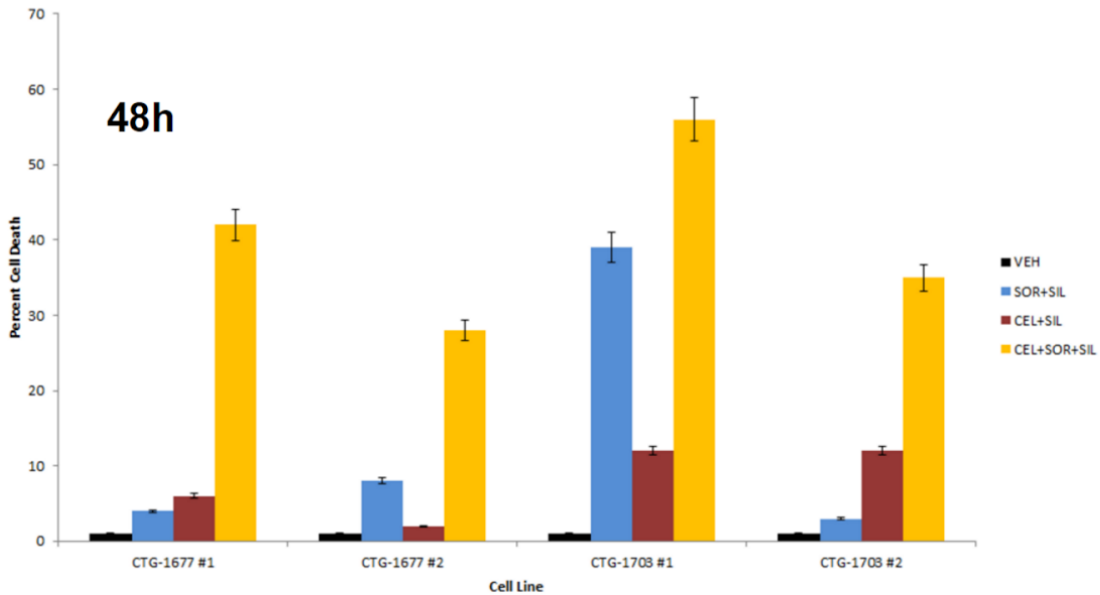
C**D**

Figure 14. Assessment of cell viability in PDX cell lines treated with combinational drug therapy. CTG-1677 #1, CTG-1677 #2, CTG-1703 #1, CTG-1703 #2 were treated with 2 μ M sorafenib, 2 μ M sildenafil, and 5 μ M celecoxib. Cell viability was determined by live/dead assays using the Hermes WiScan system at (A) 24 hours and (B) 48 hours after treatment. Images shown are representative of each experimental group. This data was quantified for both cell lines and is presented graphically for both the (C) 24 and (D) 48 hour timepoints.

The role of cellular efflux pumps ABCB1 and ABCG2 on celecoxib, sorafenib, and sildenafil toxicity.

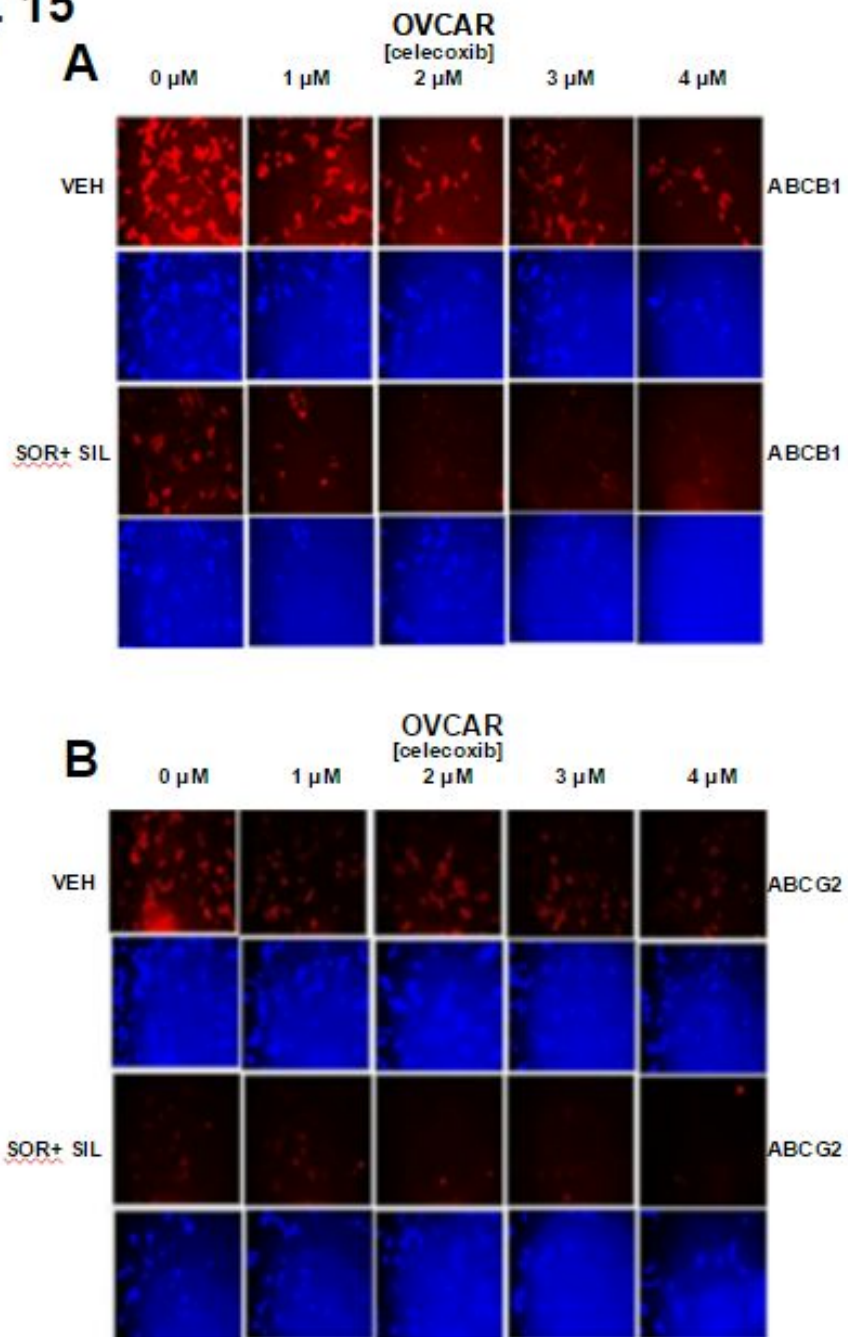
To determine the mechanism by which the drug combination causes cell toxicity, cellular efflux pumps were examined. Increased expression of plasma membrane associated ABCB1 and ABCG2 are a well-established mechanism of chemotherapeutic resistance. ABCB1 and ABCG2 are able to cause sorafenib efflux from the cell. I hypothesized that the drug combination may work, to a degree, by inhibition of these pumps. A dose response experiment was performed with 2 μ M sorafenib and 2 μ M sildenafil and a concentration range of celecoxib. OVCAR and SK-OV-3 cells were used as they are established lines with known resistance to chemotherapy. In OVCAR and SK-OV-3, the sorafenib/sildenafil diad was able to decrease ABCB1 and ABCG2 expression over the vehicle even without celecoxib (Figure 15 A,B,C,D). OVCAR pump levels were further inhibited with increasing concentrations of celecoxib (Figure 15 A,B). SK-OV-3 exhibited a change of ABCB1 expression after the addition of 1 μ M celecoxib, but increased levels of celecoxib did not have an effect past a dose of 2 μ M (Figure 15 C,D).

Chaperone protein HSP27 is, in part, an upstream regulator of cellular efflux pumps. With an interest in chaperones, and efflux pumps, this study utilized immunofluorescence to visualize HSP27 expression following 6 hours exposure to the combinational drug treatment. In Spiky, OVCAR, CAO-3, and PA-1 HSP27 levels were reduced following treatment (Figure 16).

To further explore the link between chaperone proteins and cellular efflux proteins, GRP78 and HSP27 were overexpressed individually and in combination in Spiky and OVCAR. The transfected cells were then subjected with CSS used for the first time or veh. Overexpression of the chaperone proteins prevented the drug combination from reducing the expression of ABCB1 and ABCG2 (Figure 17A).

As the final assessment of the role of cellular efflux pumps in CSS mechanistic killing, the potent pump-inhibitor drug, elacridar (referred to in the figure as “Pi”), was administered to SK-OV-3 and OVCAR cells along with CSS. The addition of Pi did not have a significant effect on the lethality of CSS in either SK-OV-3 or OVCAR cells after 12 hours of treatment (Figure 17B). While the drugs have an effect on cellular pump expression, they do not completely knockout ABCB1 or ABCG2. Since the potent Pi, did not increase cell death in the presence of CSS, the data suggests that alteration of pump expression or function does not fully explain how CSS can kill ovarian cancer cells.

Fig. 15



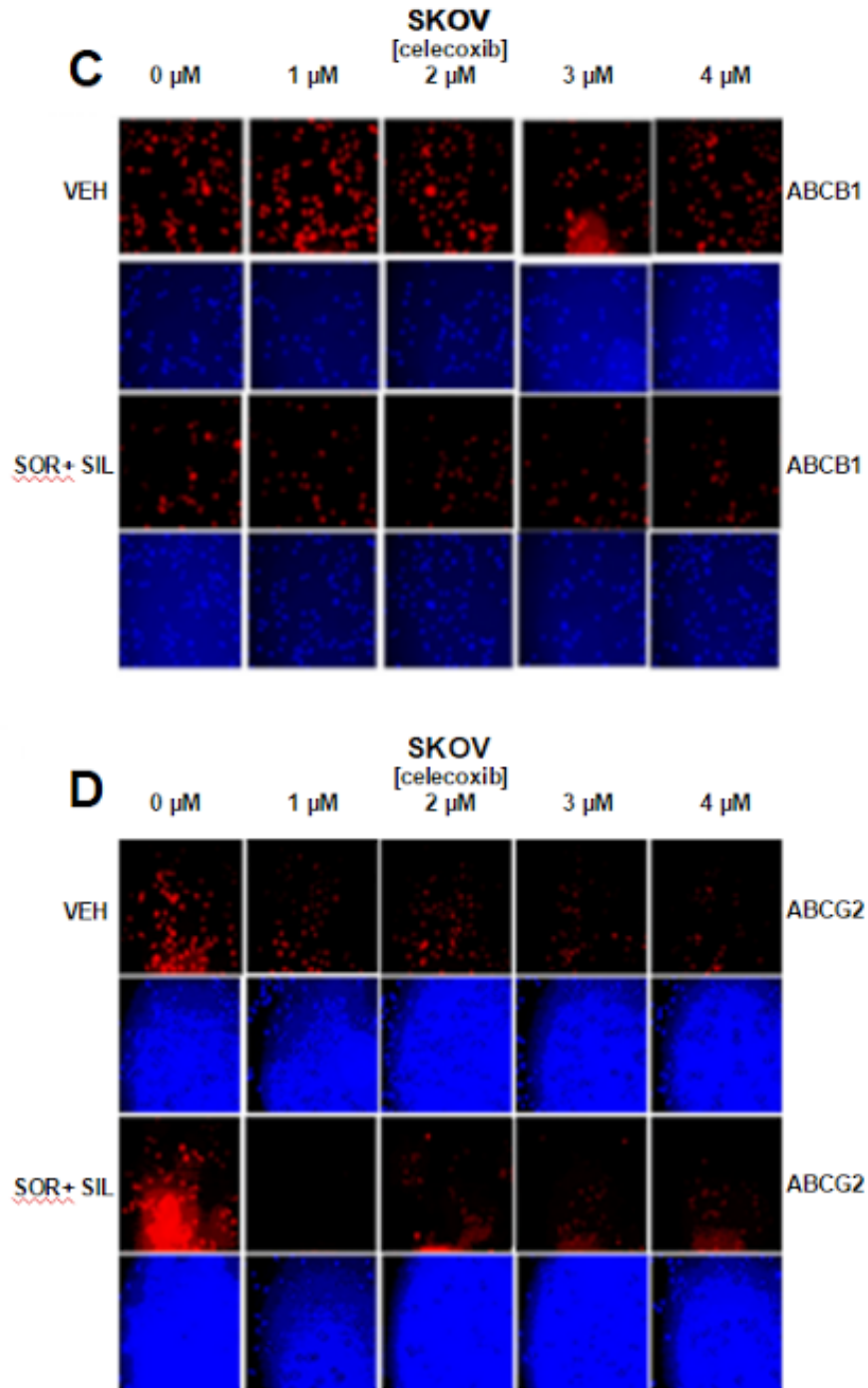


Figure 15. Celecoxib dose response on ABCB1 and ABCG2 in OVCAR and SK-OV-3 OVCAR and SK-OV-3 cells were treated with 2 μ M sorafenib and sildenafil, and varying concentrations of celecoxib within the clinically relevant range for 24 hours. Images shown are representative of each experimental group. Proteins of interest are tagged with red fluorescent antibodies. Cell nuclei are stained blue with DAPI. (A) ABCB1 and (B) ABCG2 expression was assessed in OVCAR cells. (C) ABCB1 and (D) ABCG2 expression was assessed in SK-OV-3 cells

Fig. 16

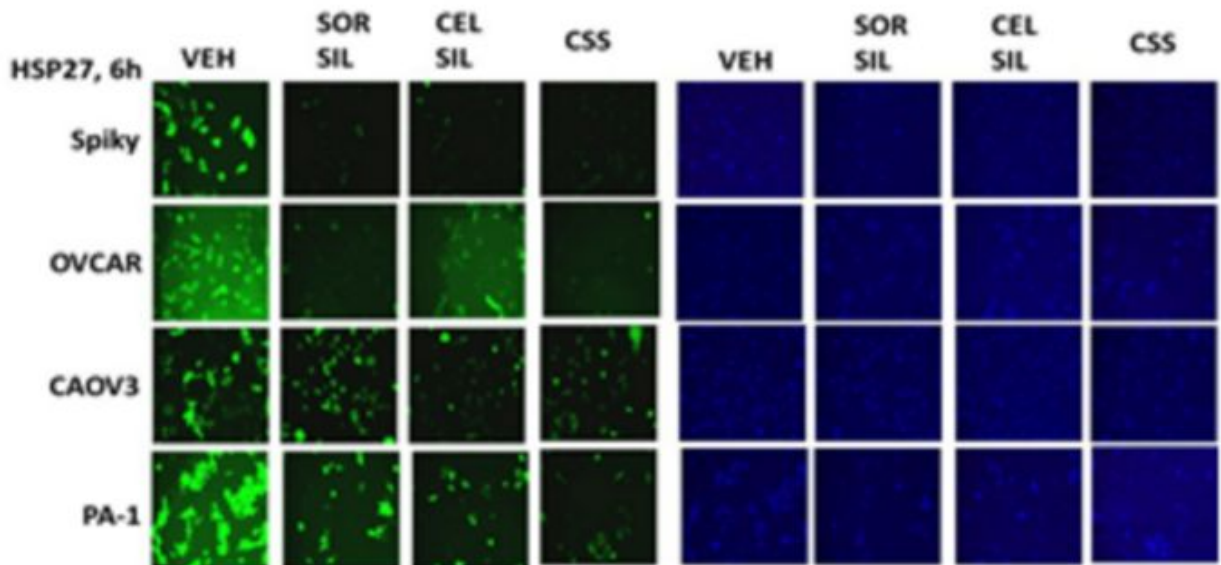
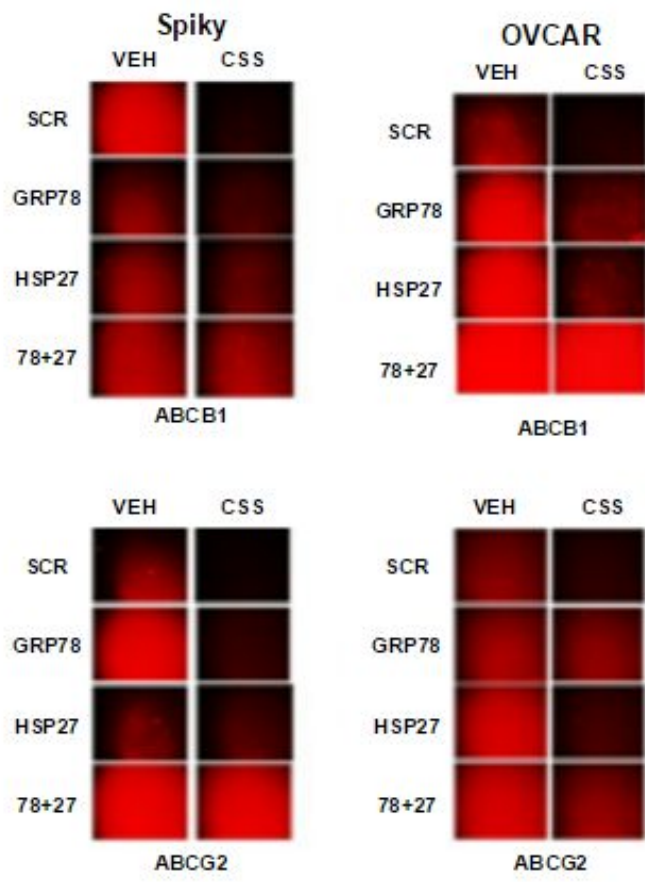


Figure 16. Immunofluorescent HSP27 expression after drug combination Spiky, OVCAR, CAOV-3, and PA-1 cells were treated as indicated for 6 hours. HSP27 expression is shown in green on the left with DAPI staining on the right. Images shown are representative of each experimental group.

Fig. 17
A



B

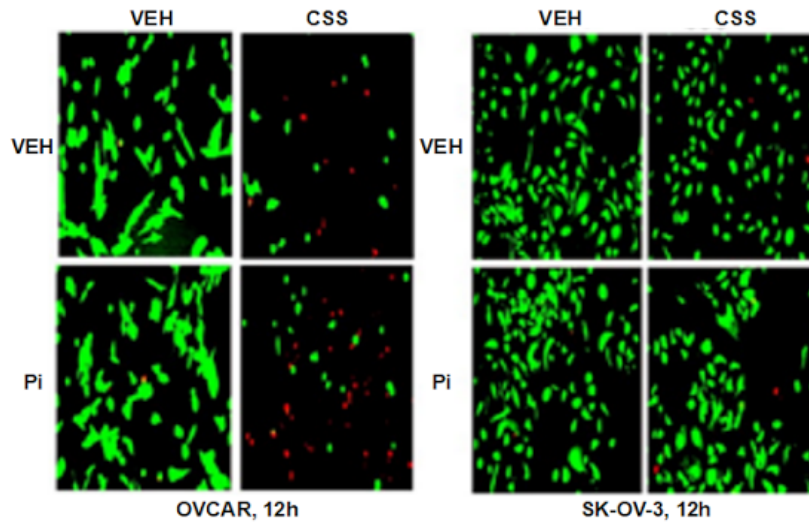


Figure 17. Assessment of efflux pump modulation/function. (A) Spiky and OVCAR cells were transfected to over-express GRP78, HSP27, GRP78+HSP27, or scramble as indicated. Cells were then treated with the drug combination or veh for 24hr and fixed for immunofluorescence. Cells were then probed for ABCB1 and ABCG2 expression. Proteins of interest are tagged with red fluorescent antibodies.(B) OVCAR and SK-OV-3 cells were treated with veh or css and veh or pump inhibitor drug, elacridar, for 12 hr. Live/dead was performed to determine cell viability. Images shown are representative of each experimental group.

The unfolded protein response (UPR) plays a role in CSS toxicity.

OVCAR cells were transfected with siRNAs to inhibit essential proteins of the unfolded protein response. The UPR proteins targeted by siRNAs included PERK-like endoplasmic reticulum kinase (PERK) and its downstream effectors activating transcription factor 4 (ATF4), and C/EBP homologous protein (CHOP). The cells were treated with CSS for 24 hours. Results indicated that knockdown of PERK, ATF4, and CHOP decreased CSS induced cell death (Figure 18 A,B).

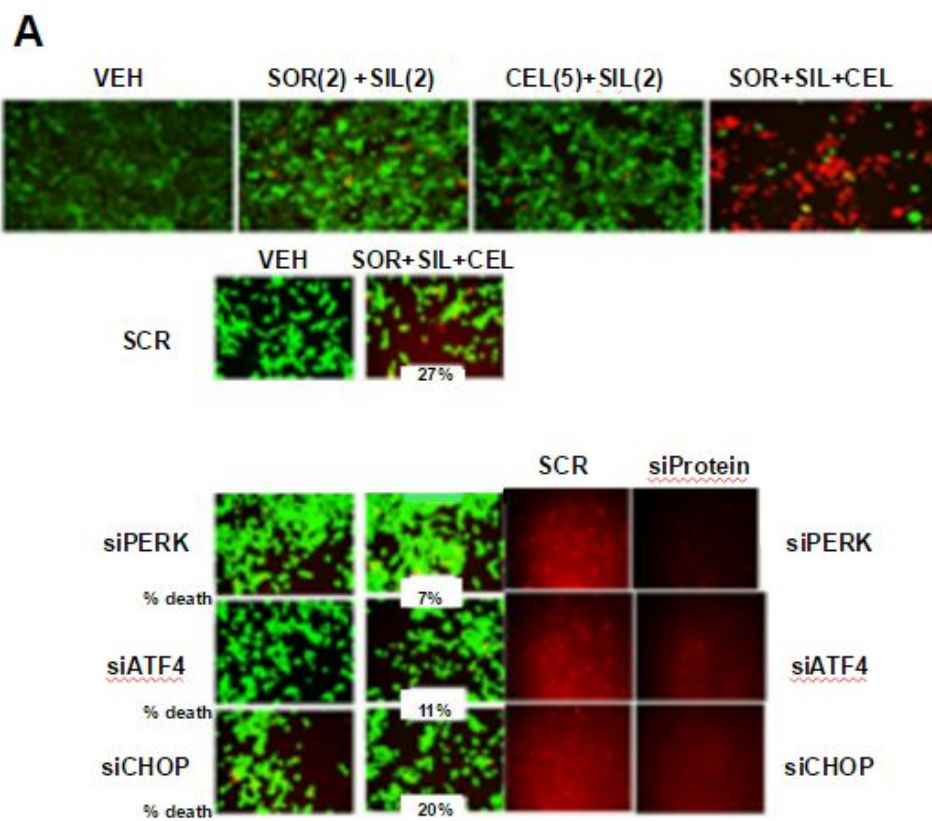
To further assess role of the UPR in the CSS mediated cell death, the study examined GRP78 and p-eIF2 α expression. GRP78 (or BiP) is a chaperone protein whose dissociation from PERK is responsible for the initiation of one branch of the UPR cascade which then phosphorylates eIF2 α to affect transcription factors which can halt translation as well as potentiate the cell for apoptosis. Using immunofluorescence, after just 6 hours of treatment with CSS, Spiky cells showed a reduction in GRP78 expression, and an increase in p-eIF2 α (Figure 19A). To verify these results, Spiky and OVCAR cells were both treated for 12 hours with CSS and probed for GRP78 and p-eIF2 α under immunofluorescence. Spiky cells continued to show CSS mediated inhibition of GRP78 expression (Figure 19B) and increase in p-eIF2 α (Figure 19C). OvcAR showed the same trend of lowered GRP78 expression (Figure 19D) and of increased p-eIF2 α (Figure 18E).

OVCAR and Spiky cell lines were transfected with either siRNA for scramble (SCR), GRP78, or siEIF2 α followed by treatment for 12 hours with the drug regimen. Both cell lines were analyzed using the Hermes WiScan. Results indicated that the SCR

group showed a significant increase in killing in the CSS group over the VEH group ($p < 0.05$)(Figure 20A,B,C). Knockdown of GRP78 increased the amount of CSS mediated killing in both cell lines ($p < 0.05$)(Figure 20A,B,C). Knockdown of eIF2 α significantly reduced the cell death induced by the drug combination in both cell lines compared to SCR ($p < 0.05$)(Figure 20A,B,C).

The above results indicate that the UPR is heavily involved in the celecoxib, sorafenib, sildenafil mediated killing of ovarian cancer cells *in vitro*. Specifically, drug inhibition of GRP78 may increase the phosphorylation of PERK and the subsequent phosphorylation of eIF2 α . Continued stress caused by the drug combination used in this study may induce apoptosis through ATF4 activation of CHOP.

Fig. 18



B

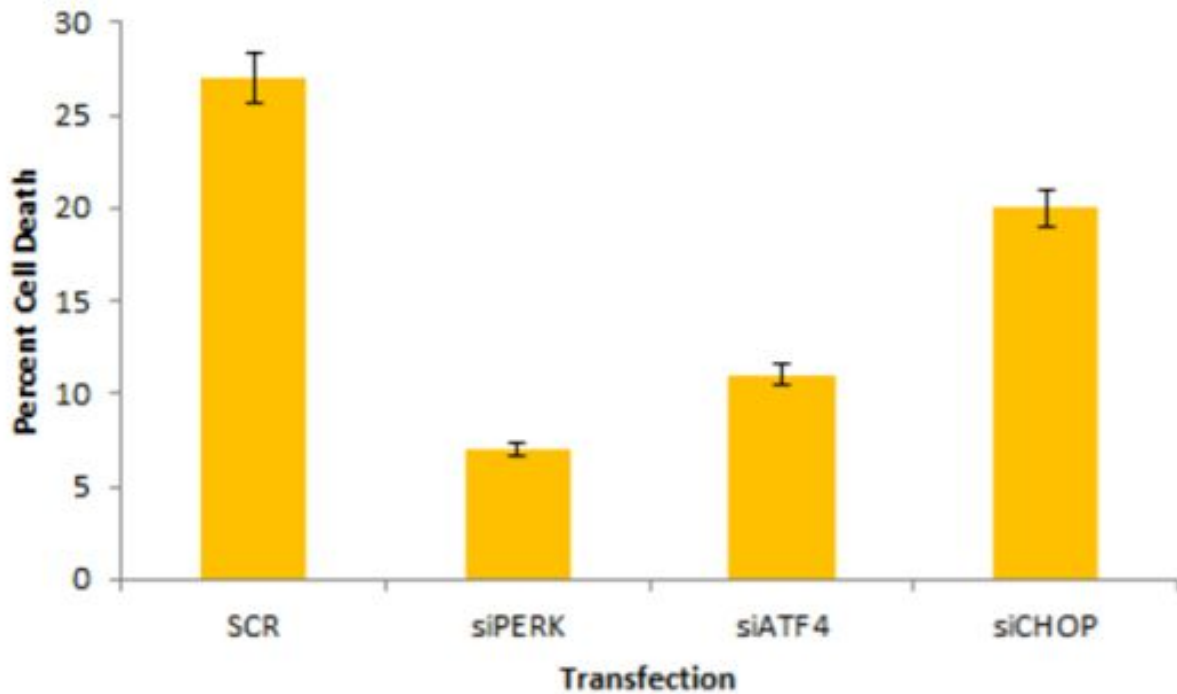
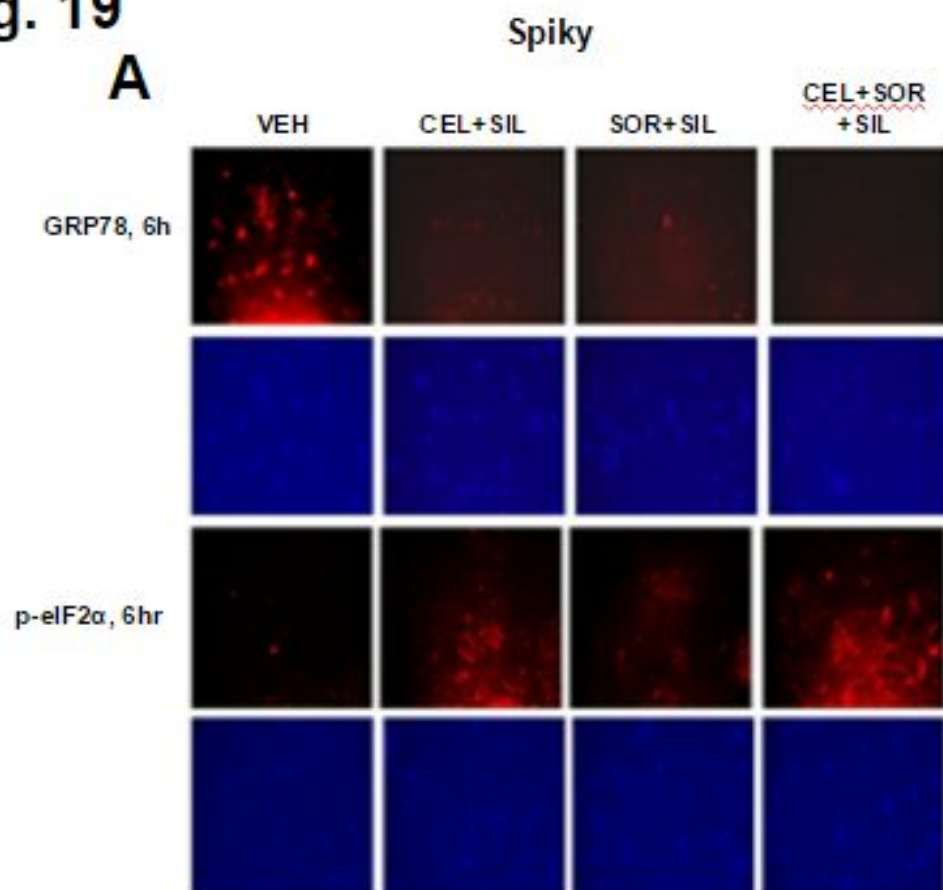


Figure 18. Cell death mechanism determination (A, top) OVCAR cells were treated for 24hr and then assessed for viability. (A, bottom, left) OVCAR cells were transfected with the indicated siRNAs and then treated with the drug combination. Viability was determined after 24 hours. (A,bottom,right) IF of designated proteins as indicator of successful transfection. Images shown are representative of each experimental group. Proteins of interest are tagged with red fluorescent antibodies. (B) Quantification of cell death after transfections and treatment in A.

Fig. 19
A



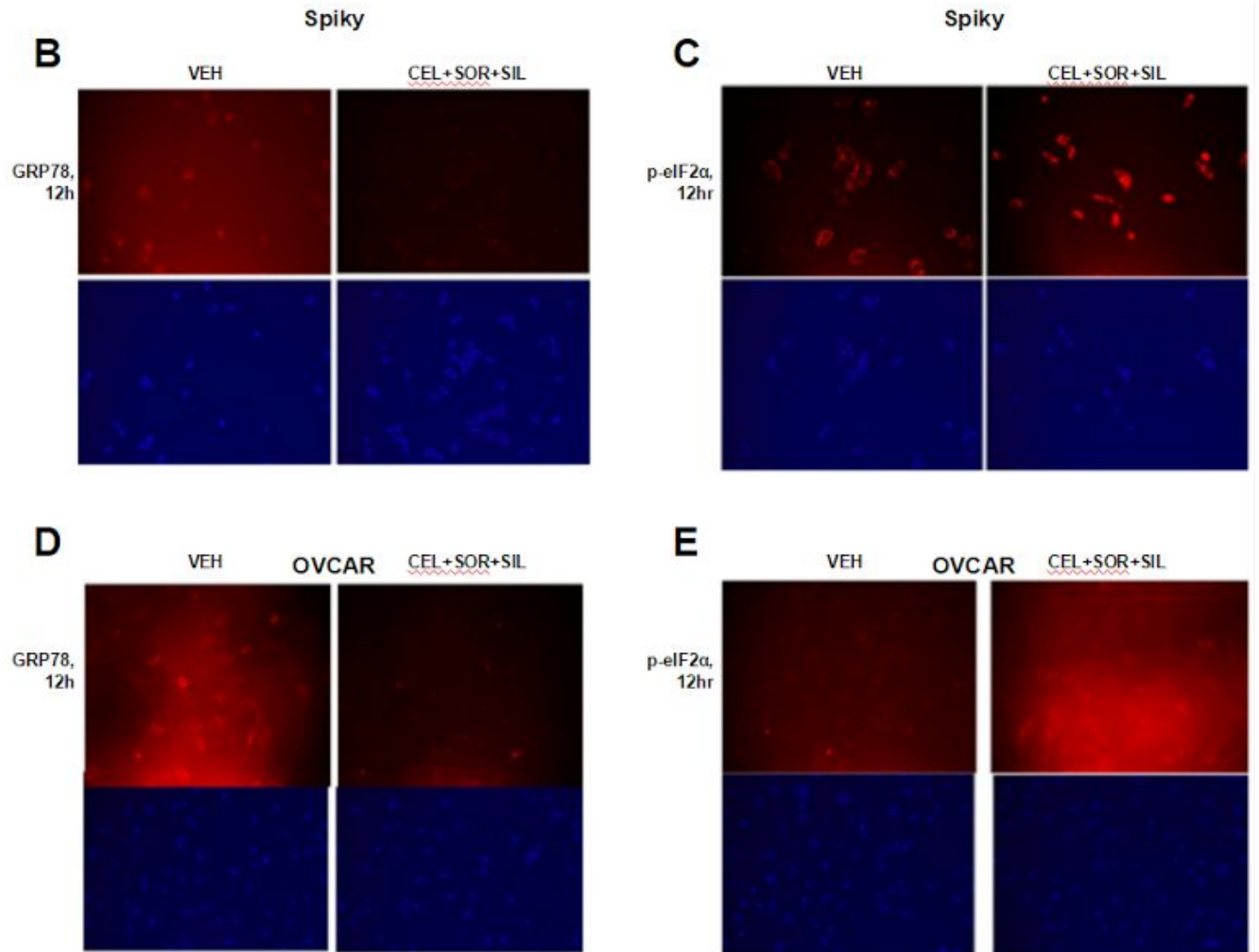
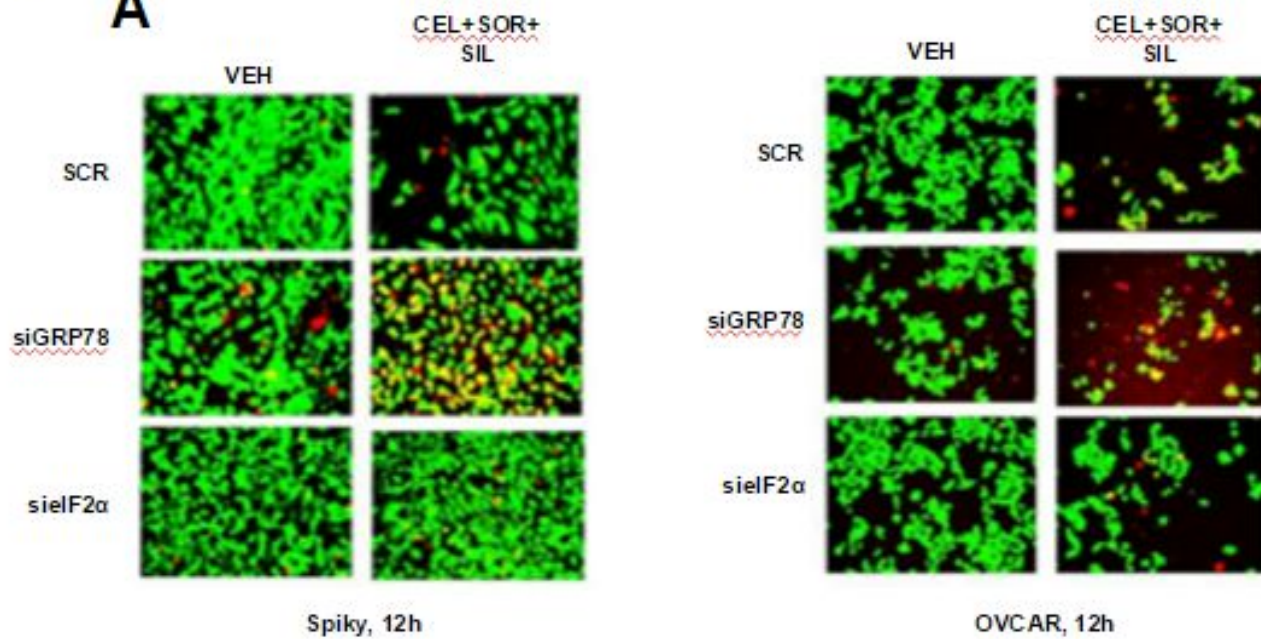


Figure 19. Immunofluorescence of GRP78 and p-eIF2 α Spiky and OVCAR were treated as indicated for 6 hours or 12 hours. Proteins of interest are tagged with red fluorescent antibodies. Cell nuclei are stained blue with DAPI. (A) GRP78 and p-eIF2 α expression after drug treatment in Spiky cells. (B) GRP78 expression in Spiky cells after 12 hour treatment. (C) p-eIF2 α expression in Spiky cells after 12 hour treatment. (D) GRP78 expression in OVCAR cells after 12 hour treatment. (E) p-eIF2 α expression in OVCAR cells after 12 hour treatment. Images shown are representative of each experimental group.

Fig. 20

A



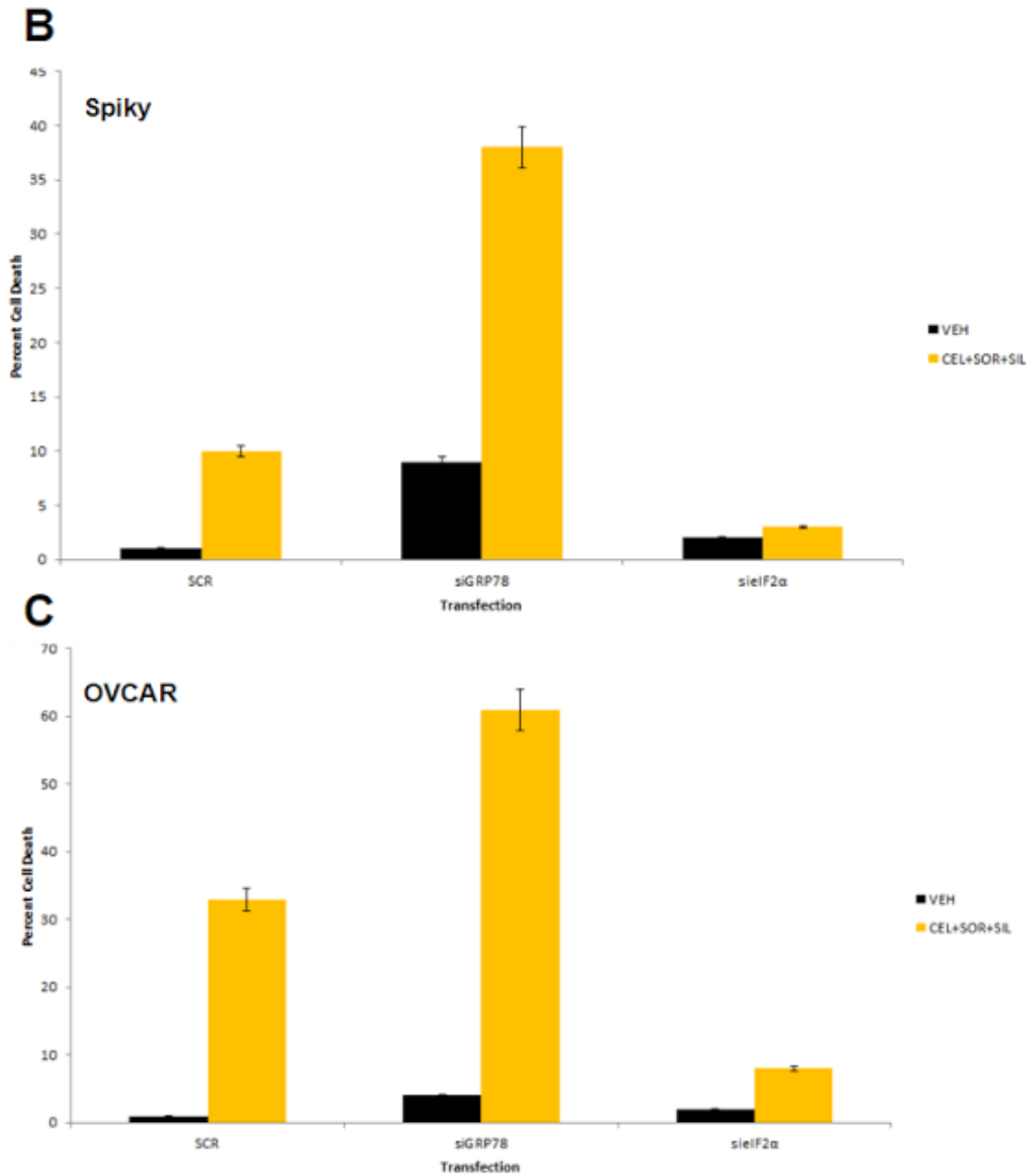


Figure 20. Cell viability following transfection with siGRP78 or siEIF2 α (A) Spiky and OVCAR were transfected with the indicated plasmid, then treated with the drug combination for 12 hours. Images shown are representative of each experimental group. The results were quantified for and represented graphically for (B) Spiky and (C) OVCAR.

Assessment of apoptotic role of CSS killing

Assessment of results concerning chaperone proteins and the UPR, apoptosis was implicated as the effector of CSS mediated killing. Active eIF2 α leads to an activated CHOP which inhibits anti-apoptotic proteins such as B-cell lymphoma extra-large (BCL-XL), myeloid leukemia cell differentiation protein (MCL-1), and cellular FLICE-inhibitory protein short (c-FLIP-s). Knockdown of these three proteins leads to the cleavage of procaspase 3 to yield the pro-apoptotic cleaved caspase 3. To assess if CSS act to kill through apoptosis via an eIF2 α -dependent manner, OVCAR and Spiky were transfected with siRNA for SCR or siEIF2 α , followed by treatment with CSS (Figure 21A). Protein expression was assessed through immunofluorescence. Within the SCR siRNA group of both cell lines, CSS treatment led to a decreased expression of anti-apoptotic proteins c-FLIP-s, BCL-XL, and MCL-1 in both OVCAR and Spiky, with a greater down regulation seen in OVCAR ($p < 0.05$) (Figure 21A). Predictably, the decrease in anti-apoptotic proteins was coupled with an increase in cleaved caspase 3 (Figure 21A). The endogenous expression of the anti-apoptotic proteins in the vehicle group was much higher ($p < 0.05$) in the cells transfected with siRNA for eIF2 α than in the cells transfected with SCR siRNA (Figure 21A). Conversely, the endogenous expression of cleaved caspase 3 was lower in cells transfected with siRNA for eIF2 α when compared to cells transfected with SCR siRNA (Figure 21A). When eIF2 α was knocked down, the expression of anti-apoptotic proteins assessed in this study was not inhibited to the same extent by drug treatment in either cell line when compared to cells

not transfected with siRNA for eIF2 α (Figure 21A). Interestingly, cleaved caspase 3 recruitment was minimally affected in siEIF2 α (Figure 21A).

Transfections with siRNA for EIF2 α was shown to significantly down regulate the endogenous expression of the anti-apoptotic proteins investigated in this study in OVCAR cells. Overexpression of BCL-XL, MCL-1, and c-FLIP-s in OVCAR cells was shown to protect cells from the cell death induced by CSS (Figure 21B). Cells were infected with a dominant negative caspase 9. Following infection, cells were treated 12 hours with CSS. Cell viability was determined using a Live/Dead assay on the Hermes WiScan. (Figure 21B). Cells infected to overexpress the anti-apoptotic proteins were significantly ($p < 0.05$) protected from CSS induced cell death. Similar results were seen for the cells infected with dominant negative caspase 9 (Figure 21B,C). Of note, the overexpression of anti-apoptotic proteins did not affect a decrease in cell numbers seen following drug treatment.

The presence of CSS-mediated cell death following modulation of proteins involved in apoptosis suggests a potential for additional mechanism(s) of killing. A part of the cell death seen in these studies may be caused by necrosis, and was thus examined in this study. Receptor-interacting serine/threonine-protein kinase 1 (RIP-1) is an upstream regulator of necrosis. As such, it was knocked down via siRNA to RIP-1 in order to determine the role for necrosis in the killing mechanism of CSS. Using the same design as above, with 12 hour drug treatment after transfection, knockdown of RIP-1 was shown to significantly reduce drug induced cell death ($p < 0.05$)(Figure 22A). Downstream from RIP-1 in the necrotic pathway are BH3 interacting-domain death

agonist (BID) and caspases 2 and 4. When BID or caspase 2 and caspase 4 were knocked down via siRNA, both Spiky and OVCAR cells were protected from CSS toxicity (Figure 22B). These results implicate that necrosis plays a part in the mechanism of CSS in ovarian cancer cell killing.

To determine a role for autophagy in the cell death induced by CSS, key autophagic proteins Beclin1 and ATG5 were knocked out via siRNA transfection in OVCAR and SK-OV-3 cells prior to CSS treatment. Results suggested that autophagy does not play a significant role in the cell death induced by CSS (Figure 22C).

The above results suggest CSS acts via stressing the ER of the treated cells, thus triggering the UPR, and overwhelming the same to prompt the cell to enter apoptosis. Concurrently, the CSS treated cell is undergoing the more chaotic route of necrosis.

Fig. 21

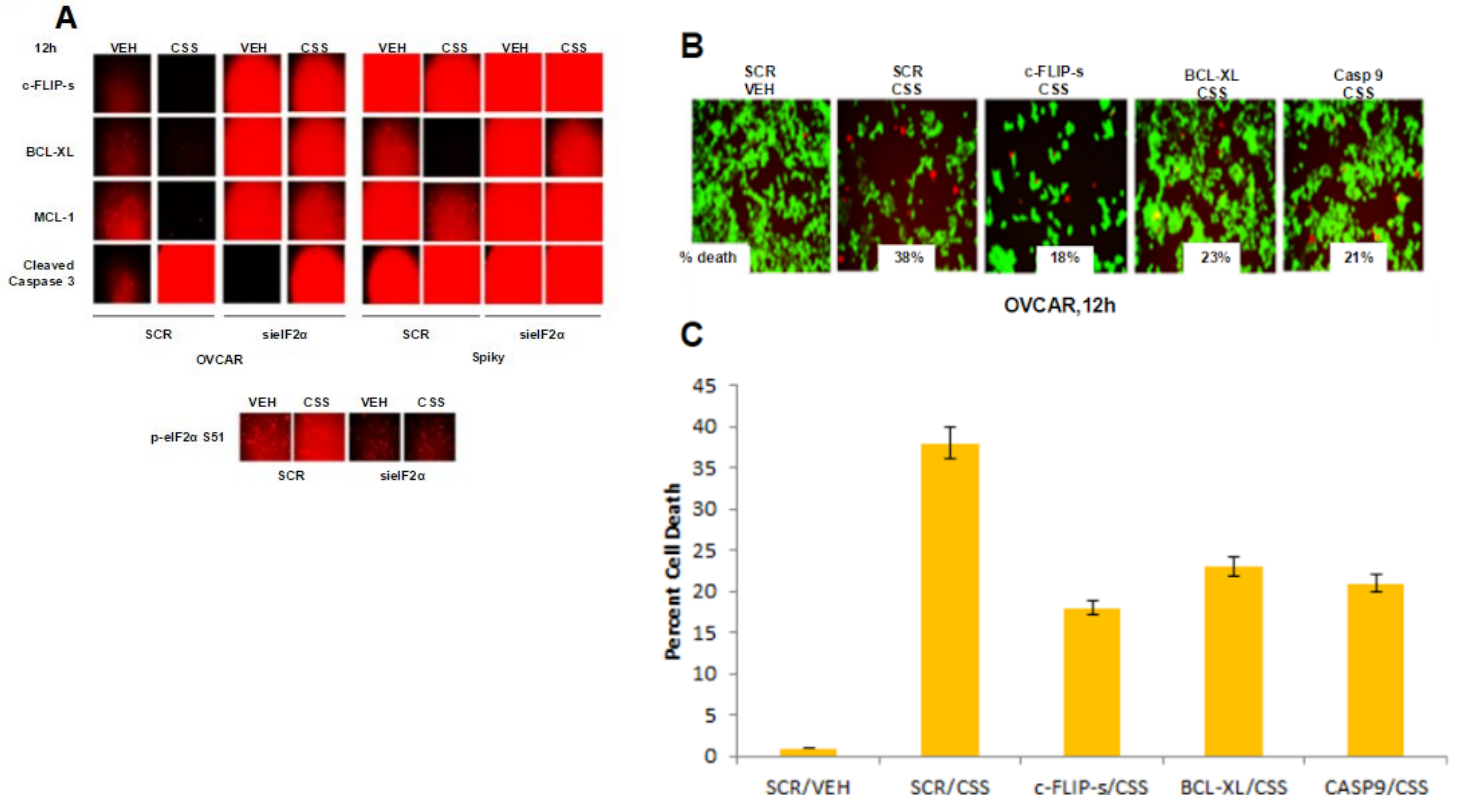


Figure 21. Assessment of apoptotic involvement in CSS mediated killing (A, top) Spiky and OVCAR cells were transfected with either empty plasmid (SCR) or siEIF2 α , and then treated with either vehicle or the drug combination. After 12 hours, the cells were probed for the indicated proteins with red fluorescent antibodies. (A, bottom) Indication of successful inhibition of eIF2 α . (B) Live/Dead assay for cell viability following transfection/treatment as indicated in OVCAR cells. (C) The results were quantified for and represented graphically. Images shown are representative of each experimental group.

Fig. 22

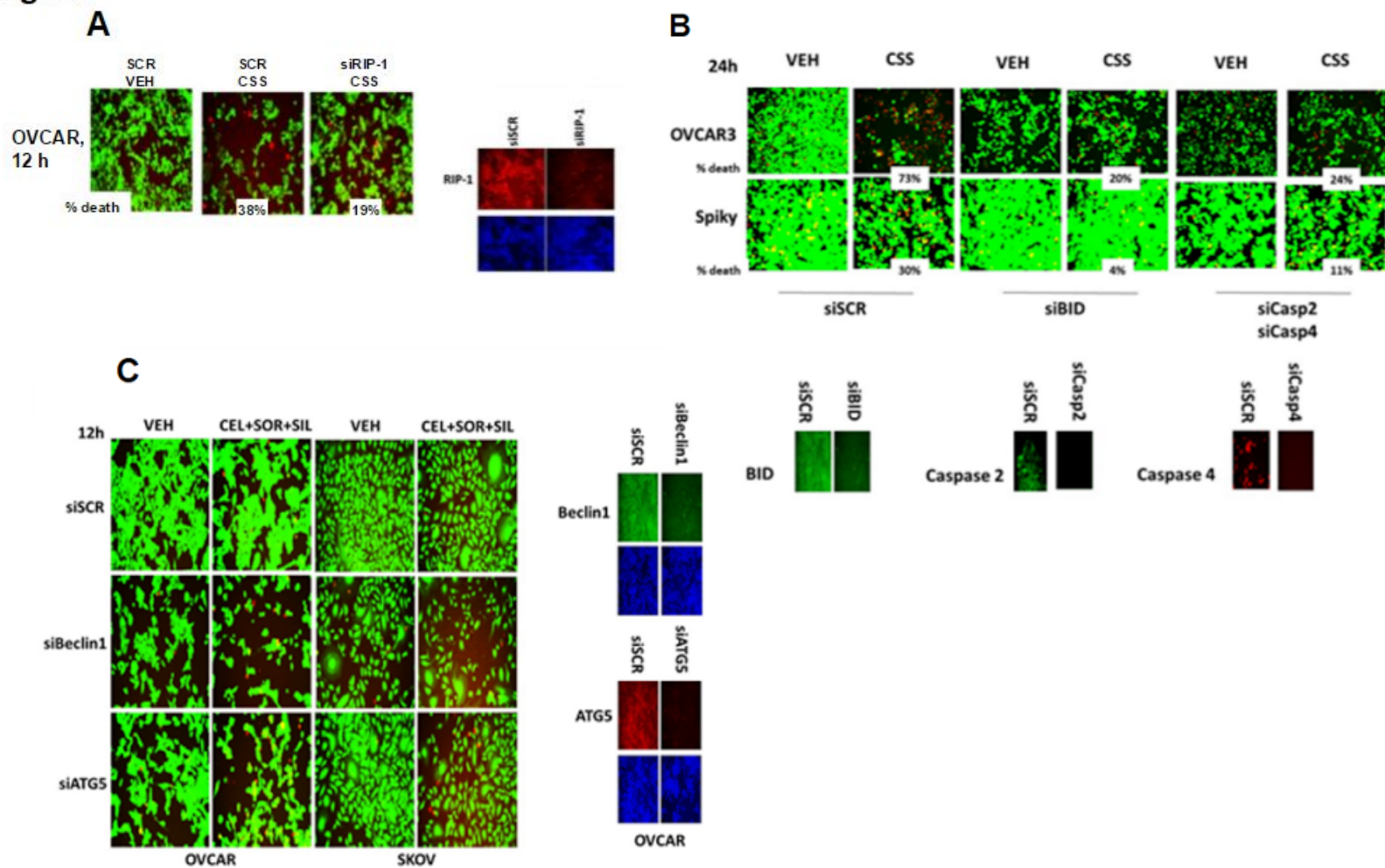


Figure 22. Assessment of necrotic and autophagic involvement in CSS mediated killing (A, left) OVCAR cells were transfected with either empty plasmid (SCR) or siRIP-1, and then treated with either vehicle or the drug combination. After 12 hours, a live/dead assay was performed and quantified. (A, right) Indication of successful inhibition of RIP-1. (B, top) Live/Dead assay for cell viability following transfection/treatment as indicated in OVCAR and Spiky cells after 24hr drug treatment. (B, bottom) Indication of successful inhibition of BID, Casp2, and Casp4. (C, left) Live/Dead assay for cell viability following transfection/treatment as indicated in OVCAR and Spiky cells after 12hr drug treatment. (C, right) Indication of successful inhibition of Beclin1 and ATG5

Impact of drug combination on signal transduction pathways linked to tumor growth/invasion and chemotherapeutic resistance.

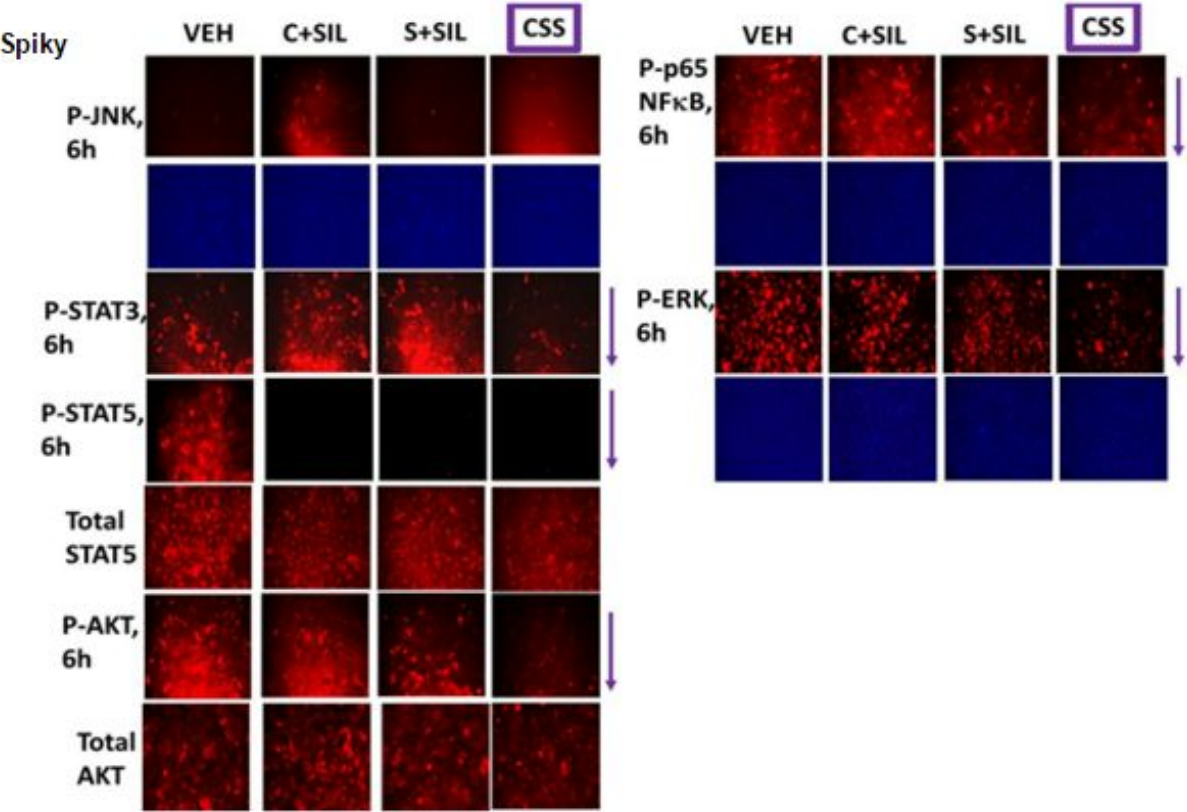
The next goal of this study was to determine the effect of celecoxib, sorafenib, and sildenafil combination therapy on well-known signal transduction pathways linked to cancer growth, invasion, and resistance to chemotherapeutics. Spiky cells were treated for six hours with either DMSO as the vehicle or CSS. The cells were then probed for proteins of interest within the signaling pathways. JNK expression was investigated due to its role in promoting apoptosis and by virtue of JNK expression being indirectly increased through sildenafil.¹⁰⁵ Cel/Sil had a noticeable upregulatory effect on the expression of phosphorylated JNK. This effect was also seen in the triplicate group (Figure 23A). Signal transducer and activator of transcription (STAT) proteins are anti-apoptotic transcription factors essential to the survival of many cancers. AKT is a well-documented anti-apoptotic protein which is indirectly inhibited by celecoxib. Accordingly, STAT3, STAT5, and AKT were probed as part of this assay. While the total STAT5 and total AKT expression remained constant across the treatment groups, the phosphorylated forms of the proteins were suppressed by the drug triad (Figure 23A). Of note, active STAT5, a transcription factor, was blocked by both diads, and the triplicate (Figure 23A). Active STAT3 levels were decreased by treatment with CSS (Figure 23A). However, STAT3 was not decreased by either diad. The final transcription factor probed was phosphorylated p65 NF- κ B, which was significantly inhibited by the sor/sil diad compared to veh, an effect more significant in CSS compared to the veh. (Figure 23A). Lastly, expression of phospho-ERK was examined due to its role as an

anti-apoptotic protein downstream of the sorafenib target, Raf. Following the trend, while P-ERK was slightly inhibited by sor/sil, the most drastic effect was in the triad group where the P-ERK expression was more inhibited (Figure 23A).

With an idea of some of the pathways affected by CSS treatment, we next sought to further determine their involvement by observing their effects on cell viability. As such, OVCAR and Spiky cells were plated and treated inhibitory peptide of JNK, infected with a dominant negative I κ B kinase (IKK), or constitutively active forms of STAT3, MEK1, or AKT. Inhibition of JNK was shown to significantly ($p < 0.05$) protect Spiky and OVCAR cells from cell death ($p < 0.05$) (Figure 23 B,C,D). As expected, constitutively activating STAT3, MEK1, or AKT was protective for both OVCAR and Spiky cells ($p < 0.05$) (Figure 23 B,C,D). IKK is an inhibitor of NF- κ B. Infection of cells with dominant negative IKK was expected to lead to an increase in cell death, and in Spiky it did ($p < 0.05$) (Figure 23 B,D). However, OVCAR cells did not show any decrease in cell viability, and IKK demonstrated a very mild protective effect in OVCAR cells (Figure 23 B,C). It is likely IKK, thus NF- κ B, are insignificantly affected by CSS, and this apparent protection could be attributed to simple variability between treatment groups.

Fig. 23

A



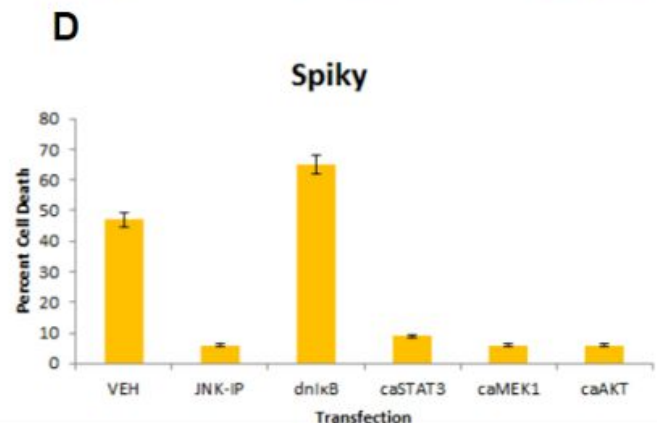
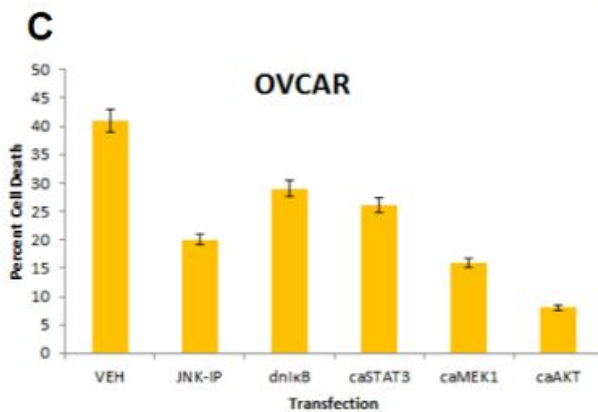
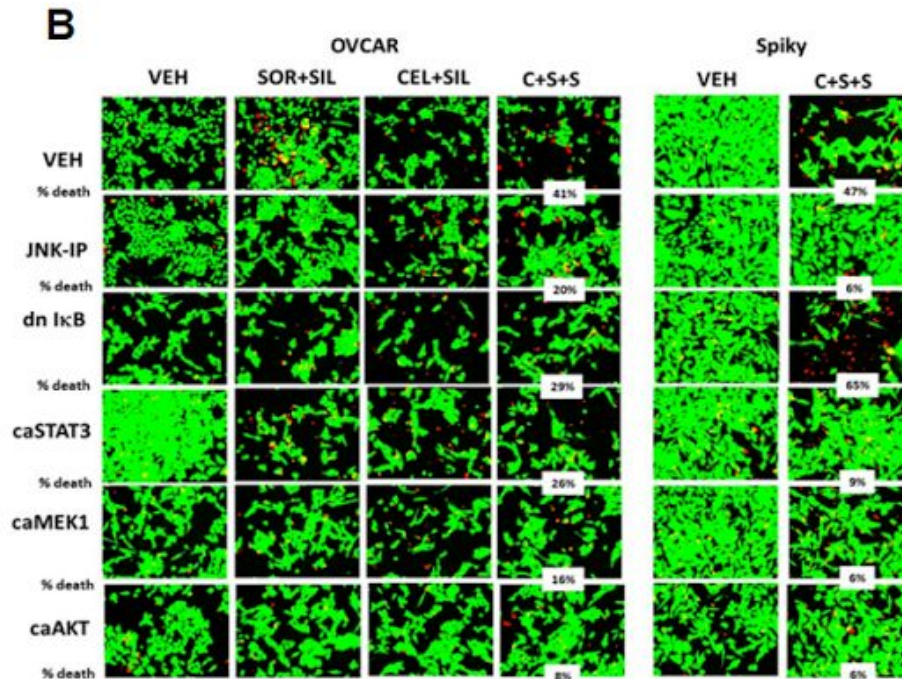


Figure 23. Assessment of drug triplicate on signaling pathways (A) Spiky cells were treated with the indicated drugs for 6 hours, and then probed for proteins of interest with red fluorescent tags. DAPI stained the nuclei blue to serve as point of reference. (B) OVCAR and Spiky cells were transfected and treated as indicated. After 24 hours, cell death was determined using a Live/Dead assay. Images shown are representative of each experimental group. Results of the Live/Dead were quantified and graphed for (C) OVCAR and (D) Spiky

Celecoxib, sorafenib, sildenafil mediated resensitization to platinum standard of care drugs.

The main aim of this study was to determine if combinational drug therapy would prove an effective treatment against platinum resistant ovarian cancer lines, ideally resensitizing those same lines to the standard of care drugs once again. Towards this aim, PA-1,CAOV-3, SK-OV-3, and Spiky were plated. PA-1 and CAOV-3 were chosen due to having different oncogenic mutations. SK-OV-3 was chosen due to being an established cell line with cisplatin resistance. Spiky was chosen as it is a PDX line with *de novo* platinum resistance. All four lines were treated with either DMSO (vehicle) or the CSS triplicate for 12 hours. Half of the vehicle group for each cell line received DMSO (vehicle) while the other half received cisplatin. Similarly, twelve hours after initial treatment half of the CSS group received DMSO while the other half received cisplatin. All four groups in each of the four lines were then incubated for twelve hours, at which point a live/dead assay was performed. As seen previously, Spiky and SK-OV-3 cells exhibited significant ($p<0.05$) increases in cell death compared to control cells (Figure 24 A,B). PA-1 and CAOV-3 cells showed significant levels of cell death with just CSS compared to the control cells ($p<0.05$) (Figure 24 A,B). However, CSS treatment significantly increased cisplatin toxicity in Spiky, PA-1, and CAOV-3 cells ($p<0.05$) (Figure 24 A,B). SK-OV-3 showed no significant response to either the triad alone or the triad mediated cisplatin (Figure 24 A,B).

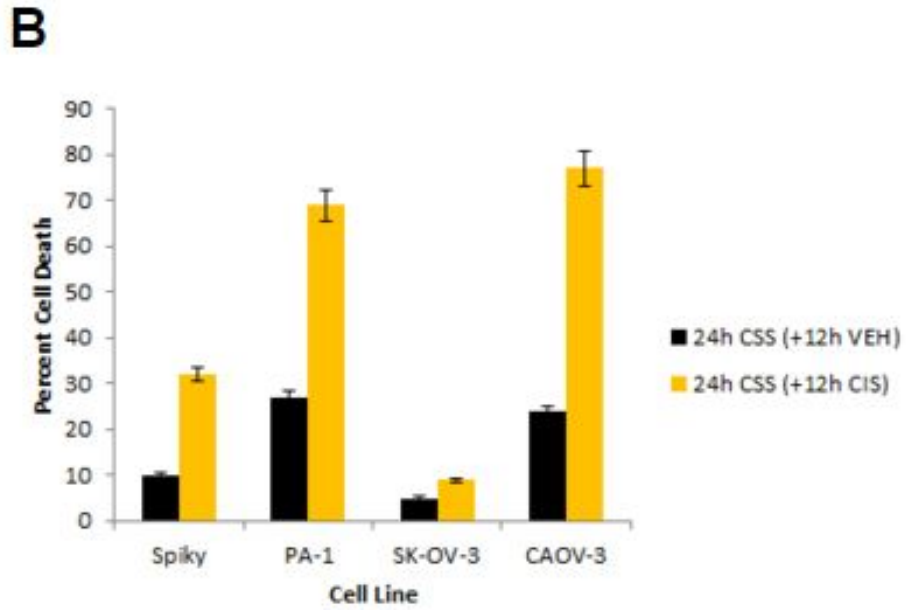
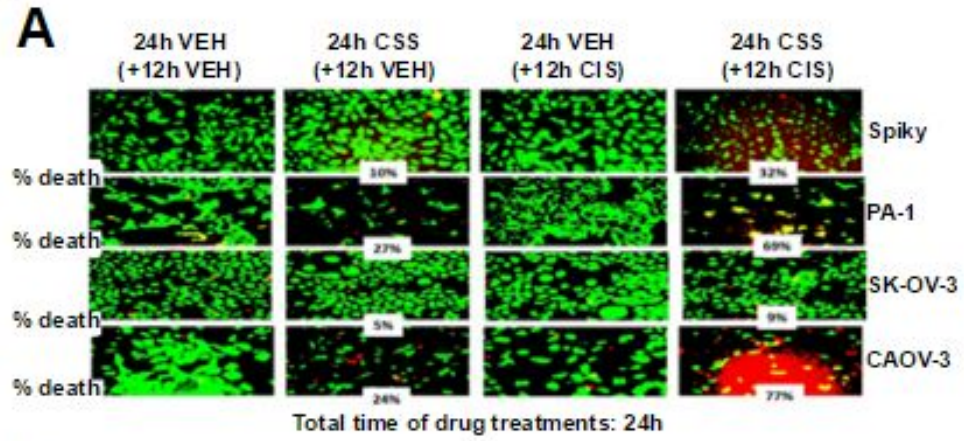
OVCAR cells were chosen for our next assay as SK-OV-3 cells did not respond to the CSS/cisplatin treatment, and OVCAR are also an established cell line with

cisplatin resistance. Our next step was to determine whether CSS works only in combination with cisplatin as a fourth drug, or if CSS was actually resensitizing the cancer cells to cisplatin. The cells were treated for six hours with DMSO (vehicle), cel/sil, sor/sil, or CSS. Drug was removed, and the cells washed with PBS, at which point cisplatin or DMSO (vehicle) was added for 18 hours. By the end of the total 24 hour period, there was no significant difference between the cells which had been treated with CSS for 6 hours and then DMSO to those which had only been treated with DMSO ($p < 0.05$) (Figure 24 C,D). There was significant cell death ($p < 0.05$) in the group which had been exposed to CSS for just six hours, and then cisplatin for a further 18 hours (Figure 24 C,D).

Cisplatin was the first of the platinum based chemotherapeutics, but is not the only one in use today. Carboplatin and oxaliplatin are both also used, and are generally tolerated better by patients than cisplatin. We sought to determine if CSS was able to potentiate platinum-based killing for the other platinum drugs. PA-1 and CAOV-3 were chosen as neither are platinum resistant, so any CSS potentiation should be more distinct. The cells were treated with either a singular platinum drug for twelve hours, or the platinum drug along with CSS. While showing some level of cell death, the platinum drugs alone were not significantly effective (Figure 25 A,C). In both PA-1 and CAOV-3 cells, however, both cisplatin and carboplatin were able to increase the percentage of cell death as compared to the platinum drug alone (Figure 25 A,B,C). Interestingly, cisplatin was more effective than carboplatin against CAOV-3 cells whereas the

opposite was true for PA-1 cells (Figure 25 A,B,C). Oxaliplatin exhibited essentially no effect in either cell line regardless of the addition of CSS (Figure 25 A,D).

Fig. 24



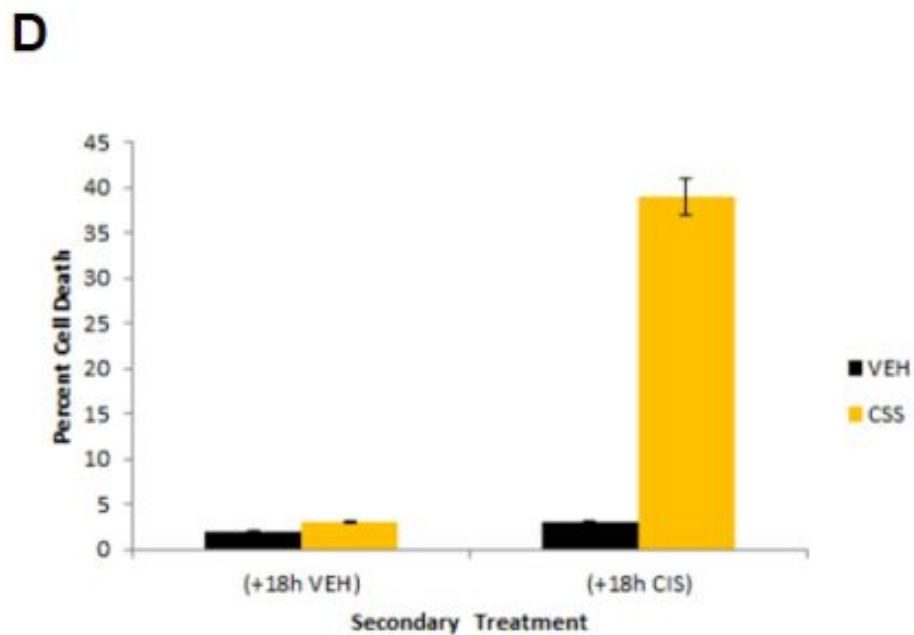
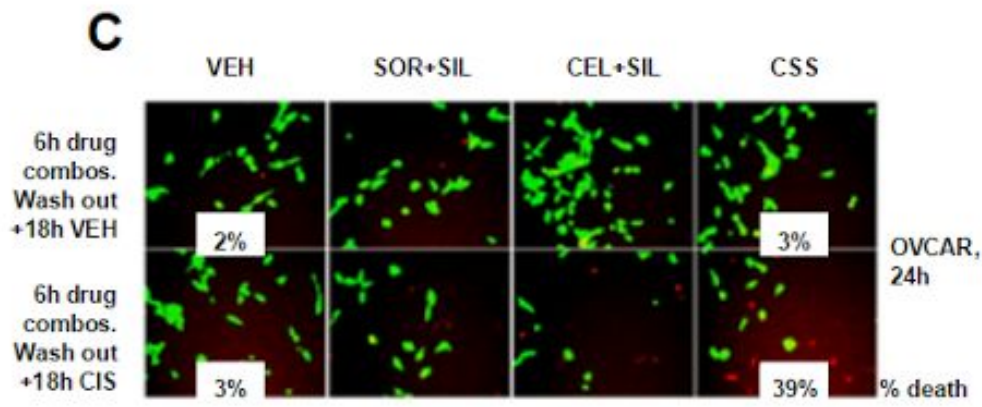


Figure 24. Assessment of drug triad effect on sensitization to cisplatin (A) Spiky, PA-1, SK-OV-3, CAOV-3 were treated with 2 μ M sorafenib, 2 μ M sildenafil, and 5 μ M celecoxib. 12 hrs after the initial treatment, the indicated treatment was added for 12 additional hours. Total drug treatment time was 24 hours. Cell viability was determined by live/dead assays using the Hermes WiScan system. Images shown are representative of each experimental group. (B) This data was quantified for all cell lines and is presented graphically. (C) OVCAR cells were treated with the indicated drug combination for 6 hrs. At that time, the drug was removed, and the cells were washed with PBS. Either DMSO or cisplatin was then added for an additional 18 hours at which time cell viability was determined by live/dead assay using the Hermes WiScan system. Images shown are representative of each experimental group. (D) This data was quantified and is presented graphically.

Fig. 25

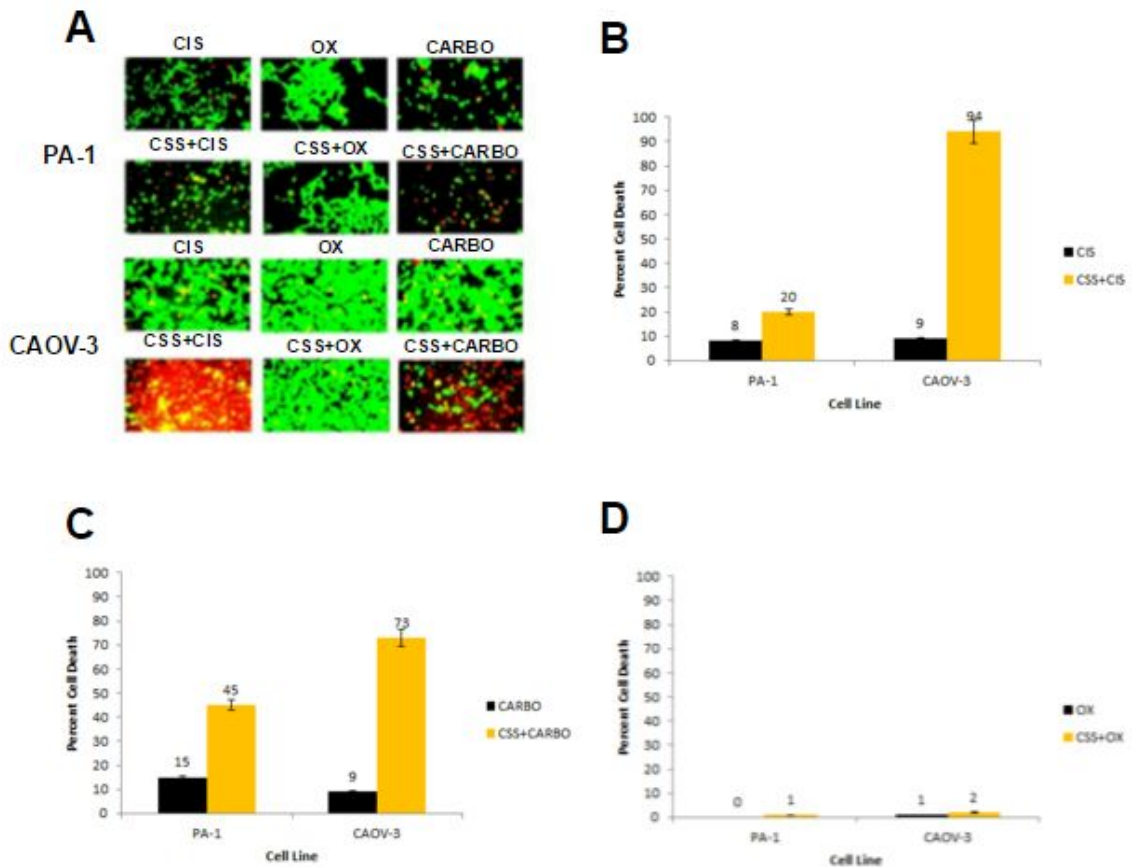


Figure 25. Assessment of drug triad effect on sensitization to platinum drugs
 PA-1 and CAO V-3 were treated with either a platinum drug for 12 hours, or a platinum drug in combination with celecoxib, sorafenib, and sildenafil (CSS). (A) Cell viability was determined by live/dead assays using the Hermes WiScan system. Images shown are representative of each experimental group. (B) This data was quantified for cisplatin (CIS) sensitization (C) This data was quantified for carboplatin (CARBO) sensitization. (D) This data was quantified for oxaliplatin (OX) sensitization

Discussion

Women facing ovarian cancer today must confront bleak realities of common relapses, and chemotherapy desensitized tumors. This is often due to the acquired resistance of the tumor cells to platinum based drugs. While the initial response rate to platinum of 70% of women experiencing tumor reduction sounds promising, the sad reality is that only 45.6% of women survive five years after their diagnosis.^{1,93} The main theories of how ovarian cancer becomes resistant to platinum drugs center around intracellular drug reduction, accumulation of thiols, and up-regulation of pro-survival pathways.^{93,44,46} The Dent lab has published multiple studies detailing multiple drug targeted therapy mediated death on otherwise hardy cell lines. As such, the aim of this study was to potentially develop a novel therapeutic drug treatment for ovarian cancer.

The first step in this process was identifying potential drugs which may prove effective against ovarian cancer. The multi-kinase and Raf inhibitor sorafenib has been demonstrated to be an effective treatment against multiple tumor types as an individual treatment, the results of which are amplified by the PDE5 inhibitor, sildenafil.^{78,104} Additionally, celecoxib has shown to inhibit the otherwise over-expressed AKT pathway in cancer cells.^{110,63} Again, the Dent lab has demonstrated the greater than additive cell death of celecoxib with sildenafil over either drug singularly.¹⁵ The present study also was inclined to use these three drugs as they are generally well-tolerated, and either currently are, or about to be losing patent protection. Thus, the proposed combinational therapy would be more financially accessible for patients as well.

We were able to show early on in the study that our combination of 2 μ M sorafenib and 2 μ M sildenafil was effective in combination with 3 μ M celecoxib for killing platinum resistant ovarian cancer cells (Figure 11). Once the drug combination demonstrated a capacity to increase the cell death against the two initial platinum resistant lines, the combination was tested against most established well-established lines currently available as well as against PDX lines isolated within the Dent lab for the purposes of this study (Figure 12, 13, 14).

Our next step was to elucidate the potential mechanisms by which the drug triad was causing cell death. To do this, we examined the typical pathways by which ovarian cells generally gain resistance to platinum drugs. In a previous study, Shi *et al*⁹¹ outlined how sildenafil reverses the cellular efflux pumps', ABCB1 and ABCG2, ability to contribute to chemotherapeutic resistance in cancer cells. The increased activity of these two types of pumps are part of the system which results in a reduced intracellular drug concentration.⁹¹ We were able to demonstrate that CSS inhibited ABCB1 and ABCG2 expression in both OVCAR and SK-OV-3 cells (Figure 15). Similarly, chaperone proteins associated with increased cell survival through both UPR and efflux pump expression were inhibited by CSS treatment (Figure 16, 17A). Interestingly, however, the highly potent pump inhibitor drug, elacridar, administered in combination with the drug triad had a negligible effect on cell death, so it is unlikely efflux pumps are solely responsible for CSS mediated death (Figure 17B).

Chaperone proteins, already shown to be suppressed by the experimental treatment, play an integral role in the unfolded protein response. Since the efflux pumps

were only partially responsible for the observed death, the UPR was examined next. After transfection to inhibit crucial UPR proteins downstream of the chaperone protein GRP78, the drug combination had a significantly diminished killing capacity as demonstrated in Figure 18. If inhibition of specific proteins involved in the UPR was to be implicated in the CSS mechanism differences in protein expression had to be demonstrated. Figure 19 demonstrates that increased phospho-eIF2 α expression is increased concurrently with reduced expression of GRP78. These results are not surprising as GRP78 dissociation with PERK is what leads to phosphorylation of eIF2 α .¹¹³ To further verify these results, both GRP78 and eIF2 α were knocked down. Expectedly, the presence of the triad drug combination in the absence of GRP78 significantly increased the amount of cell death, whereas removing the pro-death pathway of eIF2 α protected cells against the triad (Figure 20). Based upon these findings, we concluded that the UPR is heavily involved in the drug combination toxicity to ovarian cancer cells, specifically the PERK pathway.

Downstream of active eIF2 α , the cell activates CHOP whose effectors inhibit anti-apoptotic proteins to trigger apoptosis.¹⁵ In the presence of CSS, expression of anti-apoptotic c-FLIP-s, BCL-XL, and MCL-1 were significantly decreased compared to control while the pro-apoptotic cleaved caspase 3 was significantly increased (Figure 21). When eIF2 α was experimentally knocked down by siRNA, the anti-apoptotic proteins MCL-1, BCL-XL, and c-FLIP-s were expressed even in the presence of CSS (Figure 21). When the anti-apoptotic proteins were overexpressed experimentally, the CSS mediated cell death was decreased significantly, but not totally blocked (Figure

21), which suggested the drug combination acts through more than just apoptosis to kill cells.

Necrosis is essentially the chaotic alternative to apoptosis for a dying cell. RIP-1, BID, and Caspases 2 and 4, all necrotic drivers, were experimentally knocked down using specific siRNAs to determine the potential of necrosis as the missing mechanistic source of cell death. Since both cell lines with an inhibited necroptotic pathway were protected from CSS, the drug treatment must kill, in part, through necrosis (Figure 22).

In previous studies from the Dent lab, sorafenib and sildenafil, or celecoxib and sildenafil have been shown to increase the levels of autophagosomes within cells.^{15,14,104} Autophagy is an initially protective cellular response to recycle unnecessary or inoperational cellular components. However, autophagy can lead to apoptosis if the cellular stress causing the autophagy is not eased. As a result, apoptosis activity was examined in this study, but found to play essentially no role in this particular drug mediated killing (Figure 22). Thus, it appears that CSS kills ovarian cancer through both apoptosis and necrosis directly, but has little impact on triggering the adaptive autophagy. It is yet to be determined whether apoptosis or necrosis plays more of a role in CSS mediated cell death.

The final mechanistic goals of this study were to determine where the drugs used were affecting signal transduction pathways within the cell to begin the stress which ultimately leads to apoptosis and necrosis. Sorafenib acts to inhibit multiple tyrosine kinase receptors as well as Raf, both of which lead to the activation of ERK, which serves as a pro-survival kinase.⁷⁸ Celecoxib, as an NSAID, directly inhibits COX-2, an

essential enzyme in the formation of prostaglandin E2 (PGE2).²⁸ However, celecoxib is of interest to this study due to the ability to prevent PI3K from activating PDK1/2, thus preventing AKT activation.²⁸ Sildenafil is a PDE5 inhibitor, which prevents the degradation of cGMP, thus leading to an increase in active PKG, and, in parallel, a triggering of the MEKK1/MKK/JNK cascade.^{116,99} Both of these pathways lead to apoptosis. The anti-apoptotic active forms of AKT and ERK were both significantly decreased in the presence of the CSS treatment (Figure 23A). Also, p-JNK increased with administration of CSS (Figure 23A). The data also showed a protective result when the specific drug targets were constitutively activated (Figure 23 B,C,D). These results verified the precise interactions of the three drugs within signal transduction pathways.

With platinum resistance such a problem in the treatment of ovarian cancer, a main goal of this study was to develop an experimental treatment to complement the standard of care, and potentially resensitize resistant tumors to platinum. Knowing the general mechanism of CSS killing, we then treated lines both with and without resistance with the drug combination and cisplatin. While SK-OV-3 cells showed a negligible difference in cell death between the treated and control groups, the *de novo* platinum resistant PDX cell line, Spiky, showed a significant increase in death in the treated group, as did the other lines tested. (Figure 24 A,B). Not satisfied with simply adding cisplatin to the triad therapy, we decided to see if we could achieve a true resensitization of cells to cisplatin. Even after a brief exposure to CSS, and then total removal of the drug combination prior to cisplatin administration, we were able to demonstrate a significant increase in cell death in the group treated with cisplatin after

CSS over the group which only received CSS (Figure 24 C,D). This data suggests we had truly resensitized these platinum resistant cells to the standard of care therapy. Since cisplatin is not the preferred platinum drug in use today, we tested its derivatives, oxaliplatin and carboplatin. While cells displayed an apparent resensitization to carboplatin post-CSS treatment, those treated with oxaliplatin showed no apparent cell death (Figure 25). Oxaliplatin resistance may then be maintained through a separate mechanism than that of either cisplatin or carboplatin. More research would have to go into the investigation of the specific resistance against this drug in ovarian cancer. The mechanism is not known as to how CSS resensitizes cells to cisplatin or carboplatin. Platinum drugs trigger apoptosis by forming adducts within DNA, something which the drug triad does not affect. It is possible that simply increasing ER stress and pushing the cells toward either apoptosis or necrosis overloads the already stressed cell beyond its capacity to both repair the damaged DNA and cope with the added drug insult (Figure 26). However, more investigation into the precise mechanism is necessary.

Fig. 26

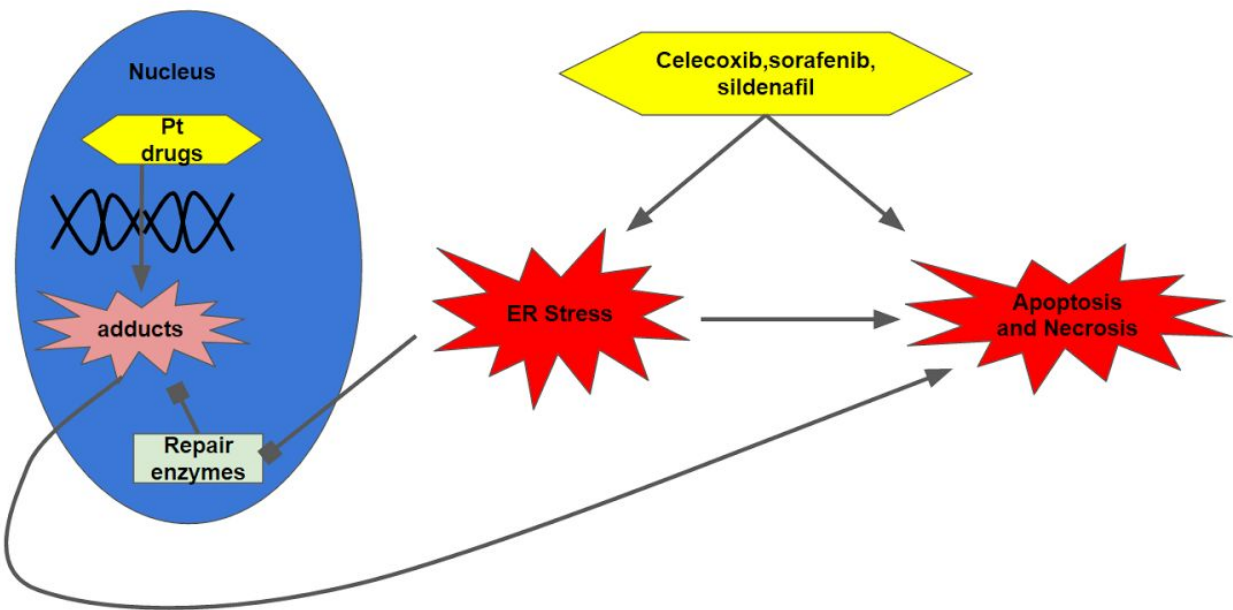


Figure 26. Abbreviated diagrammatic representation of potential resensitization mechanism of CSS

The proposed mechanism of action resulting in the observed responses to drug treatment in this study is represented diagrammatically above. Platinum drugs form adducts within the structure of nuclear DNA, which may be repaired enzymatically in platinum resistant cells. CSS leads to apoptosis and necrosis outright, but also causes ER stress which can lead to apoptosis and necrosis. ER stress also inhibits the action of repair enzymes which may provide an explanation as to how CSS restores platinum sensitivity.

Conclusion

To conclude, this study demonstrated that sorafenib, celecoxib, and sildenafil work in combination as a targeted therapy treatment effective in killing ovarian cancer cells *in vitro* with a broad spectrum of mutations. The process for such killing is linked through ER stress, apoptosis, and necrosis. In addition to the ability to kill tumor cells outright, the drug combination also resensitizes resistant ovarian cancer cells *in vitro* to the platinum-based standard of care chemotherapeutics through an as yet unknown mechanism.

Bibliography

1. Adnane, L., Trail, P., Taylor, I. and Wilhelm, S. (2006). Sorafenib (BAY 43-9006, Nexavar®), a Dual-Action Inhibitor That Targets RAF/MEK/ERK Pathway in Tumor Cells and Tyrosine Kinases VEGFR/PDGFR in Tumor Vasculature. *Regulators and Effectors of Small GTPases: Ras Family*, pp.597-612.
2. Al-Badawi, I., Brasher, P., Ghatage, P., Nation, J., Schepansky, A. and Stuart, G. (2002). Postoperative chemotherapy in advanced ovarian granulosa cell tumors. *International Journal of Gynecological Cancer*, 12(1), pp.119-123.
3. Arora, A. 'Role Of Tyrosine Kinase Inhibitors In Cancer Therapy'. *Journal of Pharmacology and Experimental Therapeutics* 315.3 (2005): 971-979. Web. 11 Oct. 2015.
4. Assefnia, S., Dakshanamurthy, S., Guidry Auvil, J., Hampel, C., Anastasiadis, P., Kallakury, B., Uren, A., Foley, D., Brown, M., Shapiro, L., Brenner, M., Haigh, D. and Byers, S. (2014). Cadherin-11 in poor prognosis malignancies and rheumatoid arthritis: common target, common therapies. *Oncotarget*, 5(6), pp.1458-1474.
5. Atcc.org, (n.d.). *Caov-3 [Caov3] ATCC ® HTB-75™ Homo sapiens ovary adenocarcin.* [online] Available at: <http://www.atcc.org/Products/All/HTB-75.aspx#generalinformation> [Accessed 24 Sep. 2015].
6. Atcc.org, (n.d.). *NIH:OVCAR-3 [OVCAR3] ATCC ® HTB-161™ Homo sapiens ovary aden.* [online] Available at: <http://www.atcc.org/Products/All/HTB-161.aspx#characteristics> [Accessed 24 Sep. 2015].
7. Atcc.org, (n.d.). *PA-1 [PA1] ATCC ® CRL-1572™ Homo sapiens ovary; derived from.* [online] Available at: <http://www.atcc.org/Products/All/CRL-1572.aspx#characteristics> [Accessed 24 Sep. 2015].
8. Atcc.org, (n.d.). *SK-OV-3 [SK-OV-3-3; SK-OV-33] ATCC ® HTB-77™ Homo sapiens ovary: a.* [online] Available at: <http://www.atcc.org/Products/All/HTB-77.aspx#characteristics> [Accessed 24 Sep. 2015].

9. B. Shirode, A. and W. Sylvester, P. (2011). Mechanisms Mediating the Synergistic Anticancer Effects of Combined γ -Tocotrienol and Celecoxib Treatment. *JBABM*, 03(01).
10. Baudino, Troy. 'Targeted Cancer Therapy: The Next Generation Of Cancer Treatment'. *Current Drug Discovery Technologies* 12.1 (2015): 3-20. Web. 11 Oct. 2015.
11. Billmire, D., Cullen, J., Rescorla, F., Davis, M., Schlatter, M., Olson, T., Malogolowkin, M., Pashankar, F., Villaluna, D., Krailo, M., Egler, R., Rodriguez-Galindo, C. and Frazier, A. (2014). Surveillance After Initial Surgery for Pediatric and Adolescent Girls With Stage I Ovarian Germ Cell Tumors: Report From the Children's Oncology Group. *Journal of Clinical Oncology*, 32(5), pp.465-470.
12. Boolell, M., Allen, M., Ballard, S., Gepi-Attee, S., Muirhead, G., Naylor, A., Osterloh, I. and Gingell, C. (1996). Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. *International Journal of Impotence Research*, 8(2), pp.47-52.
13. Booth, L., Roberts, J., Cash, D., Tavallai, S., Jean, S., Fidanza, A., Cruz-Luna, T., Siembiba, P., Cycon, K., Cornelissen, C. and Dent, P. (2015). GRP78/BiP/HSPA5/Dna K is a universal therapeutic target for human disease. *J. Cell. Physiol.*, 230(7), pp.1661-1676.
14. Booth, L., Roberts, J., Cruickshanks, N., Grant, S., Poklepovic, A. and Dent, P. (2014). Regulation of OSU-03012 Toxicity by ER Stress Proteins and ER Stress-Inducing Drugs. *Molecular Cancer Therapeutics*, 13(10), pp.2384-2398.
15. Booth, L., Roberts, J., Cruickshanks, N., Tavallai, S., Webb, T., Samuel, P., Conley, A., Binion, B., Young, H., Poklepovic, A., Spiegel, S. and Dent, P. (2015). PDE5 Inhibitors Enhance Celecoxib Killing in Multiple Tumor Types. *J. Cell. Physiol.*, 230(5), pp.1115-1127.
16. Booth, L., Roberts, J., Tavallai, M., Nourbakhsh, A., Chuckalovcak, J., Carter, J., Poklepovic, A. and Dent, P. (2015). OSU-03012 and Viagra Treatment Inhibits

- the Activity of Multiple Chaperone Proteins and Disrupts the Blood-Brain Barrier: Implications for Anti-Cancer Therapies. *J. Cell. Physiol.*, 230(8), pp.1982-1998.
17. Brown, A., Jhingran, A., Klopp, A., Schmeler, K., Ramirez, P. and Eifel, P. (2013). Involved-field radiation therapy for locoregionally recurrent ovarian cancer. *Gynecologic Oncology*, 130(2), pp.300-305.
 18. Brucks, J. (1992). Ovarian cancer. The most lethal gynecologic malignancy. *Nursing Clinics of North America*, 27(4), pp.835-845.
 19. Cancer Research UK, (2015). *Ovarian cancer survival statistics*. [online] Available at: <http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/ovarian-cancer/survival#heading-Three> [Accessed 24 Sep. 2015].
 20. Cancer.org, (2015). *Can ovarian cancer be found early?*. [online] Available at: <http://www.cancer.org/cancer/ovariancancer/detailedguide/ovarian-cancer-detection> [Accessed 23 Sep. 2015].
 21. Cancer.org, (2015). *Survival rates for ovarian cancer, by stage*. [online] Available at: <http://www.cancer.org/cancer/ovariancancer/detailedguide/ovarian-cancer-survival-rates> [Accessed 23 Sep. 2015].
 22. Cancer.org, (2015). *Treatment for germ cell tumors of the ovary*. [online] Available at: <http://www.cancer.org/cancer/ovariancancer/detailedguide/ovarian-cancer-treating-germ-cell-tumors> [Accessed 25 Sep. 2015].
 23. Cancer.org, (n.d.). *Chemotherapy for ovarian cancer*. [online] Available at: <http://www.cancer.org/cancer/ovariancancer/detailedguide/ovarian-cancer-treating-chemotherapy> [Accessed 8 Oct. 2015].
 24. Cobb, M. (1999). MAP kinase pathways. *Progress in Biophysics and Molecular Biology*, 71(3-4), pp.479-500.
 25. Corte-Rodríguez, M., Espina, M., Sierra, L., Blanco, E., Ames, T., Montes-Bayón, M. and Sanz-Medel, A. (2015). Quantitative evaluation of cellular uptake, DNA incorporation and adduct formation in cisplatin sensitive and resistant cell lines: Comparison of different Pt-containing drugs. *Biochemical Pharmacology*.

26. Das, A., Durrant, D., Salloum, F., Xi, L. and Kukreja, R. (2015). PDE5 inhibitors as therapeutics for heart disease, diabetes and cancer. *Pharmacology & Therapeutics*, 147, pp.12-21.
27. Decollogne, S., Joshi, S., Chung, S., Luk, P., Yeo, R., Nixdorf, S., Fedier, A., Heinzelmann-Schwarz, V., Hogg, P. and Dilda, P. (2015). Alterations in the mitochondrial responses to PENAO as a mechanism of resistance in ovarian cancer cells. *Gynecologic Oncology*, 138(2), pp.363-371.
28. Derry, S. and Moore, R. (1996). Single dose oral celecoxib for acute postoperative pain in adults. *Cochrane Database of Systematic Reviews*.
29. Di Pasqua, A., Wallner, S., Kerwood, D. and Dabrowiak, J. (2009). Adsorption of the Pt II Anticancer Drug Carboplatin by Mesoporous Silica. *C&B*, 6(9), pp.1343- 1349.
30. Downward, J. (2003). Targeting RAS signalling pathways in cancer therapy. *Nature Reviews Cancer*, 3(1), pp.11-22.
31. Dumaz, N. and Marais, R. (2005). Integrating signals between cAMP and the RAS/RAF/MEK/ERK signalling pathways. Based on The Anniversary Prize of the Gesellschaft fur Biochemie und Molekularbiologie Lecture delivered on 5 July 2003 at the Special FEBS Meeting in Brussels. *FEBS Journal*, 272(14), pp.3491- 3504.
32. El-Khateeb, Mahmoud et al. 'Reactions Of Cisplatin Hydrolytes With Methionine, Cysteine, And Plasma Ultrafiltrate Studied By A Combination Of HPLC And NMR Techniques'. *Journal of Inorganic Biochemistry* 77.1-2 (1999): 13-21. Web. 11 Oct. 2015.
33. Emedicine.medscape.com, (2015). *Ovarian Cancer Treatment Protocols: Treatment Protocols*. [online] Available at: <http://emedicine.medscape.com/article/2006723-overview> [Accessed 28 Sep. 2015].
34. Fallahian, F., Karami-Tehrani, F., Salami, S. and Aghaei, M. (2011). Cyclic GMP induced apoptosis via protein kinase G in oestrogen receptor-positive and -negative breast cancer cell lines. *FEBS Journal*, 278(18), pp.3360-3369.
35. Fan, S., Twu, N., Wang, J., Yuan, R., Andres, J., Goldberg, I. and Rosen, E. (1998). Down-regulation of BRCA1 and BRCA2 in human ovarian cancer cells exposed

- to adriamycin and ultraviolet radiation. *International Journal of Cancer*, 77(4), pp.600-609.
36. Feiler, B. (2015). Cancer Survivors Celebrate Their Cancerversary. *The New York Times*, [online] p.ST2. Available at: http://www.nytimes.com/2013/12/08/fashion/Cancer-Survivors-five-year-Celebration-Day-known-as-cancerversary.html?_r=0 [Accessed 23 Sep. 2015].
37. Fey, D., Croucher, D., Kolch, W. and Kholodenko, B. (2012). Crosstalk and Signaling Switches in Mitogen-Activated Protein Kinase Cascades. *Frontiers in Physiology*, 3.
38. Garcia-Cano, J., Ambroise, G., Pascual-Serra, R., Carrion, M., Serrano-Oviedo, L., Ortega-Muelas, M., Cimas, F., Sabater, S., Ruiz-Hidalgo, M., Sanchez Perez, I., Mas, A., Jalon, F., Vazquez, A. and Sanchez-Prieto, R. (2015). Exploiting the potential of autophagy in cisplatin therapy: A new strategy to overcome resistance. *Oncotarget*, 6(17), pp.15551-65.
39. Hainsworth, J., Thompson, D., Bismayer, J., Gian, V., Merritt, W., Whorf, R., Finney, L. and Dudley, B. (2014). Paclitaxel/carboplatin with or without sorafenib in the first-line treatment of patients with stage III/IV epithelial ovarian cancer: a randomized phase II study of the Sarah Cannon Research Institute. *Cancer Med*, 4(5), pp.673-681.
40. Half, E. and Arber, N. (2009). Colon cancer: preventive agents and the present status of chemoprevention. *Expert Opinion on Pharmacotherapy*, 10(2), pp.211-219.
41. Hill, R., Li, Y., Tran, L., Dry, S., Calvopina, J., Garcia, A., Kim, C., Wang, Y., Donahue, T., Herschman, H. and Wu, H. (2012). Cell Intrinsic Role of COX-2 in Pancreatic Cancer Development. *Molecular Cancer Therapeutics*, 11(10), pp.2127-2137.
42. Hiramatsu, N., Chiang, W., Kurt, T., Sigurdson, C. and Lin, J. (2015). Multiple Mechanisms of Unfolded Protein Response–Induced Cell Death. *The American Journal of Pathology*, 185(7), pp.1800-1808.

43. Jackson, G., Gillies, H. and Osterloh, I. (2005). Past, present, and future: a 7 year-update of Viagra (sildenafil citrate). *International journal of clinical practice*, 59(6), pp.580-691.
44. Jiang, H., Wek, S., McGrath, B., Lu, D., Hai, T., Harding, H., Wang, X., Ron, D., Cavener, D. and Wek, R. (2004). Activating Transcription Factor 3 Is Integral to the Eukaryotic Initiation Factor 2 Kinase Stress Response. *Molecular and Cellular Biology*, 24(3), pp.1365-1377.
45. Kane, R., Farrell, A., Saber, H., Tang, S., Williams, G., Jee, J., Liang, C., Booth, B., Chidambaram, N., Morse, D., Sridhara, R., Garvey, P., Justice, R. and Pazdur, R. (2006). Sorafenib for the Treatment of Advanced Renal Cell Carcinoma. *Clinical Cancer Research*, 12(24), pp.7271-7278
46. Kelland, L. (1993). New platinum antitumor complexes. *Critical Reviews in Oncology/Hematology*, 15(3), pp.191-219.
47. Kelland, L. (2007). The resurgence of platinum-based cancer chemotherapy. *Nature Reviews Cancer*, 7(8), pp.573-584.
48. Kohn, E., Sarosy, G., Bicher, A., Link, C., Christian, M., Steinberg, S., Rothenberg, M., Adamo, D., Davis, P., Ognibene, F., Cunnion, R. and Reed, E. (1994). Dose-Intense Taxol: High Response Rate in Patients With Platinum-Resistant Recurrent Ovarian Cancer. *JNCI Journal of the National Cancer Institute*, 86(1), pp.18-24.
49. Ledermann, J. (1997). Tumor Markers in Ovarian Malignancies. *The Oncologist*, 2(5), pp.324-329.
50. Lee, A. (2005). The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. *Methods*, 35(4), pp.373-81.
51. Lee, D., Lee, J. and Sugden, B. (2008). The Unfolded Protein Response and Autophagy: Herpesviruses Rule!. *Journal of Virology*, 83(3), pp.1168-1172.
52. Lee, S., Park, S., Kim, Y., Kim, Y., Choi, E., Kim, D., Kim, J., Nam, J. and Kim, Y. (2011). Radiation therapy is a treatment to be considered for recurrent epithelial ovarian cancer after chemotherapy. *Tumori*, 97(5), pp.590-5.

53. Leone Roberti Maggiore, U., Valenzano Menada, M., Venturini, P. and Ferrero, S. (2013). Sorafenib for ovarian cancer. *Expert Opinion on Investigational Drugs*, 22(8), pp.1049-1062.
54. Li, N., Li, H., Su, F., Li, J., Ma, X. and Gong, P. (2015). Relationship between epidermal growth factor receptor (EGFR) mutation and serum cyclooxygenase-2 level, and the synergistic effect of celecoxib and gefitinib on EGFR expression in non-small cell lung cancer cells. *International Journal of Clinical and Experimental Pathology*, 8(8), pp.9010-20.
55. Lin, W. and Popko, B. (2009). Endoplasmic reticulum stress in disorders of myelinating cells. *Nature Neuroscience*, 12(4), pp.379-385.
56. Liu, M., Li, C., Chen, Z., Ji, R., Guo, Q., Li, Q., Zhang, H. and Zhou, Y. (2014). Celecoxib regulates apoptosis and autophagy via the PI3K/Akt signaling pathway in SGC-7901 gastric cancer cells. *International Journal of Molecular Medicine*.
57. Loran, O., Ströberg, P., Lee, S., Park, N., Kim, S., Tseng, L., Collins, S. and Stecher, V. (2009). Sildenafil Citrate 100 mg Starting Dose in Men with Erectile Dysfunction in an International, Double-Blind, Placebo-Controlled Study: Effect on the Sexual Experience and Reducing Feelings of Anxiety About the Next Intercourse Attempt. *Journal of Sexual Medicine*, 6(10), pp.2826-2835.
58. Maroon, J., Bost, J., Borden, M., Lorenz, K. and Ross, N. (2006). Natural antiinflammatory agents for pain relief in athletes. *Neurosurgical Focus*, 21(4).
59. Marques, J., Gaspar, V., Markl, D., Costa, E., Gallardo, E. and Correia, I. (2014). Co-delivery of Sildenafil (Viagra®) and Crizotinib for Synergistic and Improved Anti-tumoral Therapy. *Pharm Res*, 31(9), pp.2516-2528.
60. Martin, L., Hamilton, T. and Schilder, R. (2008). Platinum Resistance: The Role of DNA Repair Pathways. *Clinical Cancer Research*, 14(5), pp.1291-1295.
61. Matei, D., Sill, M., Lankes, H., DeGeest, K., Bristow, R., Mutch, D., Yamada, S., Cohn, D., Calvert, V., Farley, J., Petricoin, E. and Birrer, M. (2010). Activity of Sorafenib in Recurrent Ovarian Cancer and Primary Peritoneal Carcinomatosis:

- A Gynecologic Oncology Group Trial. *Journal of Clinical Oncology*, 29(1), pp.69-75.
62. McGuire, W. (2003). Current Status of Taxane and Platinum-Based Chemotherapy in Ovarian Cancer. *Journal of Clinical Oncology*, 21(90100), pp.133s-135.
63. Morisaki, T., Umebayashi, M., Kiyota, A., Koya, N., Tanaka, H., Onishi, H. and Katano, M. (2013). Combining celecoxib with sorafenib synergistically inhibits hepatocellular carcinoma cells in vitro. *Anticancer Research*, 33(4), pp.1387- 1395.
64. Mskcc.org, (n.d.). *Ovarian Cancer: Treatment | Memorial Sloan Kettering Cancer Center*. [online] Available at: <https://www.mskcc.org/cancer-care/types/ovarian/diagnosis-treatment-msk/treatment> [Accessed 25 Sep. 2015].
65. Na, K., Lee, K., Lee, J., Cho, Y. and Jung, H. (2014). Efficacy of adjunctive celecoxib treatment for patients with major depressive disorder: A meta-analysis. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 48, pp.79-85.
66. National Cancer Institute, (2011). *Tumor Markers*. [online] Available at: <http://www.cancer.gov/about-cancer/diagnosis-staging/diagnosis/tumor-markers-fact-sheet> [Accessed 24 Sep. 2015].
67. National Cancer Institute: 2013 Fact Book. (2014). 1st ed. [ebook] Bethesda: U.S. Department of Health and Human Services: National Institutes of Health, p.B-8. Available at: <http://www.cancer.gov/about-nci/budget/fact-book-2013/fact-book-2013.pdf> [Accessed 23 Sep. 2015].
68. Ocrf.org, (2015). *Symptoms of Ovarian Cancer | Ovarian Cancer Research Fund*. [online] Available at: http://www.ocrf.org/about-ovarian-cancer/what-is-ovarian-cancer/signs-and-symptoms-of-ovarian-cancer?gclid=CLKgsfyAkMgCFYoUHwodVwYJ_w [Accessed 24 Sep. 2015].
69. Ogata, M., Hino, S., Saito, A., Morikawa, K., Kondo, S., Kanemoto, S., Murakami, T., Taniguchi, M., Tanii, I., Yoshinaga, K., Shiosaka, S., Hammarback, J., Urano, F. and Imaizumi, K. (2006). Autophagy Is Activated for Cell Survival after Endoplasmic Reticulum Stress. *Molecular and Cellular Biology*, 26(24), pp.9220- 9231.
70. Ozols, Robert F. 'Ovarian Cancer: New Clinical Approaches'. *Cancer Treatment Reviews* 18 (1991): 77-83. Web. 11 Oct. 2015.

71. Pastan, Ira et al. 'Immunotoxin Treatment Of Cancer *'. *Annual Review of Medicine* 58.1 (2007): 221-237. Web. 11 Oct. 2015.
72. Pearson, G., Robinson, F., Beers Gibson, T., Xu, B., Karandikar, M., Berman, K. and Cobb, M. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocrine Reviews*, 22(2), pp.153-83.
73. Penning, T., Talley, J., Bertenshaw, S., Carter, J., Collins, P., Docter, S., Graneto, M., Lee, L., Malecha, J., Miyashiro, J., Rogers, R., Rogier, D., Yu, S., Anderson, G., Burton, E., Cogburn, J., Gregory, S., Koboldt, C., Perkins, W., Seibert, K., Veenhuizen, A., Zhang, Y. and Isakson, P. (1997). Synthesis and biological evaluation of the 1,5-diarylpyrazole class of cyclooxygenase-2 inhibitors: identification of 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide (SC-58635, celecoxib). *Journal of Medicinal Chemistry*, 40(9), pp.1347-65.
74. Prestayko, A., D'Aoust, J., Issell, B. and Crooke, S. (1979). Cisplatin (cis-diamminedichloroplatinum II). *Cancer Treatment Reviews*, 6(1), pp.17-39.
75. Qi, M. (2005). MAP kinase pathways. *Journal of Cell Science*, 118(16), pp.3569-3572.
76. Quinones, Q., de Ridder, G. and Pizzo, S. (2008). GRP78: a chaperone with diverse roles beyond the endoplasmic reticulum. *Histology and histopathology*, 23(11), pp.1409-16.
77. Rahman, K. (2007). Studies on free radicals, antioxidants, and co-factors. *Clinical Interventions in Aging*, 2(2), pp.219-236.
78. Rahmani, M., Davis, E., Bauer, C., Dent, P. and Grant, S. (2005). Apoptosis Induced by the Kinase Inhibitor BAY 43-9006 in Human Leukemia Cells Involves Down-regulation of Mcl-1 through Inhibition of Translation. *Journal of Biological Chemistry*, 280(42), pp.35217-35227.
79. Rahmani, M., Davis, E., Crabtree, T., Habibi, J., Nguyen, T., Dent, P. and Grant, S. (2007). The Kinase Inhibitor Sorafenib Induces Cell Death through a Process

- Involving Induction of Endoplasmic Reticulum Stress. *Molecular and Cellular Biology*, 27(15), pp.5499-5513.
80. Rauh-Hain, J., Krivak, T., del Carmen, M. and Olawaiye, A. (2011). Ovarian Cancer Screening and Early Detection in the General Population. *Review in Obstetrics and Gynecology*, 4(1), pp.15-21.
81. Roberts, P. and Der, C. (2007). Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene*, 26(22), pp.3291-3310.
82. Robinson, M. and Cobb, M. (1997). Mitogen-activated protein kinase pathways. *Current Opinion in Cell Biology*, 9(2), pp.180-186.
83. Rosenberg, B., Vancamp, L. and Krigas, T. (1965). Inhibition of cell division in escherichia coli by electrolysis products from a platinum electrode. *Nature*, 13(205), pp.698-9.
84. Sadovsky, R., Miller, T., Moskowitz, M. and Hackett, G. (2001). Three-year update of sildenafil citrate (Viagra) efficacy and safety. *International journal of clinical practice*, 55(2), pp.115-28.
85. Schubbert, S., Shannon, K. and Bollag, G. (2007). Hyperactive Ras in developmental disorders and cancer. *Nature Reviews Cancer*, 7(4), pp.295-308.
86. SEER: Surveillance, Epidemiology, and End Results Program, (2015). *SEER Stat Fact Sheets: Ovary Cancer*. [online] Available at: (1) [Seer.cancer.gov](http://seer.cancer.gov),. Cancer of the Ovary - SEER Stat Fact Sheets
<http://seer.cancer.gov/statfacts/html/ovary.html> (accessed Sep 23, 2015).
[Accessed 23 Sep. 2015].
87. Seger, R. and Krebs, E. (1995). The MAPK signaling cascade. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, 9(9), pp.726-35.
88. Selleckchem.com, (n.d.). *Buy Cisplatin | DNA/RNA Synthesis inhibitor | Price | IC50 | Research only*. [online] Available at:
<http://www.selleckchem.com/products/Cisplatin.html> [Accessed 11 Oct. 2015].

89. Senft, D. and Ronai, Z. (2015). UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. *Trends in Biochemical Sciences*, 40(3), pp.141-148.
90. Shacham-Shmueli, E., Geva, R., Figer, A., Bulocinic, S., Nalbandyan, K., Shpigel, S., Atsmon, J. and Brendel, E. (2012). Phase I Trial of Sorafenib in Combination With 5-Fluorouracil/Leucovorin in Advanced Solid Tumors. *The Journal of Clinical Pharmacology*, 52(5), pp.656-669.
91. Shi, Z., Tiwari, A., Shukla, S., Robey, R., Singh, S., Kim, I., Bates, S., Peng, X., Abraham, I., Ambudkar, S., Talele, T., Fu, L. and Chen, Z. (2011). Sildenafil Reverses ABCB1- and ABCG2-Mediated Chemotherapeutic Drug Resistance. *Cancer Research*, 71(8), pp.3029-3041.
92. Shinsako, K., Mizuno, T., Terada, T., Watanabe, J., Kamba, T., Nakamura, E., Ogawa, O. and Inui, K. (2010). Tolerable sorafenib therapy for a renal cell carcinoma patient with hemodialysis: a case study. *Int J Clin Oncol*, 15(5), pp.512-514.
93. Siddik, Z. (2003). Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene*, 22(47), pp.7265-7279.
94. Sigmaaldrich.com, (n.d.). *Carboplatin | Sigma-Aldrich*. [online] Available at: <http://www.sigmaaldrich.com/catalog/product/sigma/c2538?lang=en®ion=US> [Accessed 11 Oct. 2015].
95. Sigmaaldrich.com, (n.d.). *Oxaliplatin solid | Sigma-Aldrich*. [online] Available at: <http://www.sigmaaldrich.com/catalog/product/sigma/o9512?lang=en®ion=US> [Accessed 11 Oct. 2015].
96. Smolle, E., Taucher, V., Petru, E. and Haybaeck, J. (2014). Targeted treatment of ovarian cancer- the multiple-kinase-inhibitor sorafenib as a potential option. *Anticancer Research*, 34(4), pp.1519-1530.
97. Smolle, E., Taucher, V., Petru, E. and Haybaeck, J. (2014). Targeted treatment of ovarian cancer - the multiple-kinase-inhibitor sorafenib as a potential option. *Anticancer Research*, 34(4), pp.1519-1530.

98. Sobolewski, C., Rhim, J., Legrand, N., Muller, F., Cerella, C., Mack, F., Chateauvieux, S., Kim, J., Yoon, A., Kim, K., Dicato, M. and Diederich, M. (2015). 2,5-Dimethyl-Celecoxib Inhibits Cell Cycle Progression and Induces Apoptosis in Human Leukemia Cells. *Journal of Pharmacology and Experimental Therapeutics*, 355(2), pp.322-342.
99. Sousa, M., Amaral, S., Tavares, R., Paiva, C. and Ramalho-Santos, J. (2014). Concentration-dependent Sildenafil citrate (Viagra) effects on ROS production, energy status, and human sperm function. *Syst Biol Reprod Med*, 60(2), pp.72-79.
100. Sponziello, M., Verrienti, A., Rosignolo, F., De Rose, R., Pecce, V., Maggisano, V., Durante, C., Bulotta, S., Damante, G., Giacomelli, L., Di Gioia, C., Filetti, S., Russo, D. and Celano, M. (2015). PDE5 expression in human thyroid tumors and effects of PDE5 inhibitors on growth and migration of cancer cells. *Endocrine*, [Epub ahead of print].
101. Suh, D., Kim, M., Kim, H., Chung, H. and Song, Y. (2012). Unfolded protein response to autophagy as a promising druggable target for anticancer therapy. *Annals of the New York Academy of Sciences*, 1271(1), pp.20-32.
102. Swisher, E., Sakai, W., Karlan, B., Wurz, K., Urban, N. and Taniguchi, T. (2008). Secondary BRCA1 Mutations in BRCA1-Mutated Ovarian Carcinomas with Platinum Resistance. *Cancer Research*, 68(8), pp.2581-2586.
103. Takahara, Patricia M. et al. 'Crystal Structure Of Double-Stranded DNA Containing The Major Adduct Of The Anticancer Drug Cisplatin'. *Nature* 377.6550 (1995): 649-652. Web. 11 Oct. 2015.
104. Tavallai, M., Hamed, H., Roberts, J., Cruickshanks, N., Chuckalovcak, J., Poklepovic, A., Booth, L. and Dent, P. (2015). Nexavar/Stivarga and Viagra Interact to Kill Tumor Cells. *J. Cell. Physiol.*, 230(9), pp.2281-2298.
105. Tiwari, A. and Chen, Z. (2013). Repurposing phosphodiesterase-5 inhibitors as chemoadjuvants. *Frontiers in Pharmacology*, 4.

106. Trimbos, B., Timmers, P., Pecorelli, S., Coens, C., Ven, K., van der Burg, M. and Casado, A. (2010). Surgical staging and the treatment of early ovarian cancer: long-term analysis from a randomized trial. *Journal of the National Cancer Institute*, 102(13), pp.982-7.
107. Tummala, M., Alagarsamy, S. and McGuire, W. (2008). Intraperitoneal chemotherapy: standard of care for patients with minimal residual stage III ovarian cancer?. *Expert Review of Anticancer Therapy*, 8(7), pp.1135-1147.
108. Vakifahmetoglu, H., Olsson, M., Tamm, C., Heidari, N., Orrenius, S. and Zhivotovsky, B. (2007). DNA damage induces two distinct modes of cell death in ovarian carcinomas. *Cell Death Differ*, 15(3), pp.555-566.
109. Vane, J. and Botting, R. (1998). Anti-inflammatory drugs and their mechanism of action. *Inflammation Research*, 47(2), pp.78-87.
110. Vergote, I., Trope, C., Amant, F., Kristensen, G., Ehlen, T., Johnson, N., Verheijen, R., van der Burg, M., Lacave, A., Panici, P., Kenter, G., Casado, A., Mendiola, C., Coens, C., Verleye, L., Stuart, G., Pecorelli, S. and Reed, N. (2010). Neoadjuvant chemotherapy or primary surgery in stage IIIc or IV ovarian cancer. *New England Journal of Medicine*, 363(10), pp.943-53.
111. Wang, D. and Lippard, S. (2005). Cellular processing of platinum anticancer drugs. *Nature Reviews Drug Discovery*, 4(4), pp.307-320.
112. Wang, G., Wang, X. and Xu, X. (2015). Triptolide potentiates lung cancer cells to cisplatin-induced apoptosis by selectively inhibiting the NER activity. *Biomarker Research*, 3(1).
113. Wang, M. and Kaufman, R. (2014). The impact of the endoplasmic reticulum protein-folding environment on cancer development. *Nature Reviews Cancer*, 14(9), pp.581-597.
114. Wilhelm, S., Adnane, L., Newell, P., Villanueva, A., Llovet, J. and Lynch, M. (2008). Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf

- and VEGF and PDGF receptor tyrosine kinase signaling. *Molecular Cancer Therapeutics*, 7(10), pp.3129-3140.
115. Wilhelm, S., Carter, C., Lynch, M., Lowinger, T., Dumas, J., Smith, R., Schwartz, B., Simantov, R. and Kelley, S. (2006). Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. *Nature Reviews Drug Discovery*, 5(10), pp.835-844.
116. Wright, P. (2006). Comparison of Phosphodiesterase Type 5 (PDE5) Inhibitors. *International Journal of Clinical Practice*, 60(8), pp.967-975.
117. Xia, S., Zhao, Y., Yu, S. and Zhang, M. (2010). Activated PI3K/Akt/COX-2 Pathway Induces Resistance to Radiation in Human Cervical Cancer HeLa Cells. *Cancer Biotherapy & Radiopharmaceuticals*, 25(3), pp.317-323.