Regorafenib Enhances Lethality of Sildenafil and Curcumin in Colorectal Cancer Cells

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Regorafenib Enhances Lethality of Sildenafil and Curcumin in Colorectal Cancer Cells

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Richmond, Virginia
May 2019
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<table>
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<th>Full Name</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>AMP-activated protein kinase</td>
<td>AMPK</td>
</tr>
<tr>
<td>Apoptosis-inducing factor</td>
<td>AIF</td>
</tr>
<tr>
<td>Ataxia-telangiectasia mutated kinase</td>
<td>ATM</td>
</tr>
<tr>
<td>Autophagy protein 5</td>
<td>ATG5</td>
</tr>
<tr>
<td>Binding immunoglobulin protein (BiP)</td>
<td>(BiP)GRP78</td>
</tr>
<tr>
<td>B-cell lymphoma 2</td>
<td>Bcl2</td>
</tr>
<tr>
<td>B-cell lymphoma extra large</td>
<td>BCLXL</td>
</tr>
<tr>
<td>Bcl2 associated death promoter</td>
<td>Bad</td>
</tr>
<tr>
<td>Bcl2 associated X protein</td>
<td>Bax</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>Cath B</td>
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<td>Cluster of differentiation 95</td>
<td>CD95</td>
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<tr>
<td>Colorectal cancer</td>
<td>CRC</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>DMSO</td>
</tr>
<tr>
<td>Dominant-negative caspase-9 protein</td>
<td>DN9</td>
</tr>
<tr>
<td>Endothelial NOS</td>
<td>eNOS</td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>ER</td>
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<tr>
<td>Epidermal growth factors</td>
<td>EGF</td>
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<tr>
<td>Epidermal growth factor receptor 1,2,3,4</td>
<td>ERBB1,2,3,4(EGFR)</td>
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<tr>
<td>Eukaryotic initiation factor 2 alpha</td>
<td>eIF2α</td>
</tr>
<tr>
<td>Extracellular Signal-Regulated Kinase</td>
<td>Erk</td>
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<tr>
<td>Fas-associated death domain protein</td>
<td>FADD</td>
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<td>Fibroblast growth factors</td>
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<tr>
<td>FLICE-inhibitory protein</td>
<td>FLIP</td>
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<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>GAPDH</td>
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<td>Heat shock protein 70</td>
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Hand foot skin reaction  HFSR
Heat shock protein 90  HSP90
High motility group protein B1  HMGB1
Histone Acetyltransferases  HATs
Histone Deacetylases  HDACs
Inducible NOS  iNOS
Indoleamine 2,3-dioxygenase 1  IDO1
Kirsten rat sarcoma viral oncogene  K-ras
Major Histocompatibility Complex  MHCA1
Mammalian target of rapamycin  mToR
Microgram  μg
Milliliter  mL
Millimeter  mm
Mitogen activated protein kinase  MAPK
Myeloid cell leukemia 1  MCL1
Nitric Oxide Synthase  NOS
Non-small cell lung cancer  NSCLC
Nuclear factor kappalightchainenhancer of activated B cells  NFκB
Ornithine decarboxylase  ODC
Phosphodiesterase type 5  PDE5
Platelet derived growth factor receptor beta  PDGFRβ
Programmed Cell Death Ligand-1  PDL1
Programmed Cell Death Ligand-2  PDL2
Protein kinase B  Akt
Protein kinase C  PKC
Protein kinase G PKG
Protein kinase R (PKR)-like endoplasmic reticulum kinase  Perk
Rapidly accelerated fibrosarcoma-1  Raf-1
Reactive oxygen species  
ROS
Reactive nitrogen species  
RNS
Revolutions per minute  
rpm
Ribosomai protein S6 kinase beta-1  
p70
Phosphoinositide 3kinase  
PI3K
Phorbol-12-myristate-13-acetate-induced protein 1  
Noxa
Phosphate Buffer Solution  
PBS
Proto-oncogene c-RAF  
Raf-1
p53 upregulated modulator of apoptosis  
Puma
Scramble  
Scr
Scrambled siRNA  
siSCR
Small interfering ribonucleic acid  
siRNA
Signal transducer and activator of transcription 3  
STAT3
Standard of care  
SOC
Standard error of the mean  
SEM
Two times a day  
BID
Unc-51 like Autophagy Activating Kinase 1  
Ulk-1
Vasodilator-stimulated phosphoprotein  
VASP
Vehicle  
veh
Volume  
vol
V-raf murine sarcoma viral oncogene homolog B  
B-raf
Vascular endothelial growth factor receptors  
VEGFR
Regorafenib Enhances Lethality of Sildenafil and Curcumin in Colorectal Cancer Cells

By Kervin B Owusu

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

Abstract

In the United States, more than 130,000 people will be diagnosed with colorectal cancer (CRC) each year and an estimated 50,000 people will die from the disease. Standard of care (SOC) therapies for CRC combine multiple cytotoxic chemotherapeutic drugs. These combinations have varying degrees of effectiveness and can often result in significant patient morbidity. For second recurrence patients, the multi-kinase inhibitor, regorafenib, is an approved agent, but is often poorly tolerated at current doses. In the current study, we propose to develop therapeutic regime of combining agents with modest toxicity profiles: curcumin and sildenafil with regorafenib. Using clinically achievable enterohepatic drug concentrations (~2.0 μM), our laboratory has shown that both sildenafil and curcumin interact to synergistically down regulate the expression of multiple cyto-protective molecular chaperones and kill CRC cells in a greater than additive manner in vitro and suppress the growth of colon cancer tumors in vivo. In this study, the expression of PDGFRβ and PDGF in the plasma was increased in colon tumor bearing
mice previously exposed to curcumin and sildenafil. Further, the *in vitro* killing potential of curcumin and sildenafil was shown to be reduced in evolved tumor cells from these mice.

The purpose of this study was to determine whether down regulation of PDGFRβ using regorafenib would increase the lethality of curcumin and sildenafil in colorectal cancer cell lines *in vitro* and *in vivo*. In the current study, we have shown that with the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination reduces of cyto-protective proteins, enhances cytotoxicity and creates a carnage of CRC cells in a greater than additive *in vitro*. The combination also suppressed growth of colon cancer tumors *in vivo*, when compared to curcumin and sildenafil alone. In addition we have shown that the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination can modulate immune checkpoint proteins *in vitro*. These results suggest that this drug combination may enhanced the anti-tumor efficacy of anti-PD-1 and anti-CTLA4 antibodies.
Introduction

Colorectal Cancer

Colon cancer is cancer of the large intestine, which is the final part of the digestive tract. Colorectal cancer (CRC) is the third most common cancer in the world, with about 1.4 million cases diagnosed worldwide in 2012. The prognosis for CRC patients is largely dependent on the stage of the tumor at diagnosis. In the United States, the 5-year survival rates following the surgical removal of tumors for localized (stage I), regional (stages II and III) and distant (stage IV) cases are 91.1, 71.7 and 13.3%, respectively. Thus, the mortality rates for each stage, 8–13% (stage I/II), 11–47% (stage III) and approximately 89% (IV), represent the limitations of initial diagnoses and current treatments, indicating that more precise diagnostic measures and effective treatments are required. In most cases, colon cancer begin as small, benign clumps of cells called adenomatous polyps. On common occasions, polyps may produce few, if any, symptoms but can develop into malignant tumors. For this reason, doctors recommend regular screening tests to help prevent colon cancer by identifying and removing polyps before they develop into cancer.

The American Cancer Society’s estimates for the number of colorectal cancer cases in the United States for 2018 are 97,220 new cases of colon cancer 33,030 new cases of rectal cancer. The risk of developing colorectal cancer is about 1 in 22 (3.39%) for men and 1 in 23 (3.15%) for women. A number of factors such as old age, race, family history, heavy alcohol consumption, smoking and high fat diets can increase the risk for developing colorectal cancer. Colorectal cancer is expected to cause about 50,630 deaths during 2018. The death rate (the number of deaths per 50,000 people per year) from colorectal cancer has been dropping in both
men and women for several decades. There are a number of likely reasons for this. One, is that colorectal polyps are now being found more often by screening and removed before they can develop into cancers. The total funding for colorectal cancer research from the National Cancer Institute was 212.2 million dollars, or 3.1% of the total NCI budget in 2016 and is steadily increasing every year.

*Treatments for Colorectal Cancer*

Current options for standard treatment of CRC include surgical removal for stage I and for most of stage II CRCs and adjuvant 5-fluorouracil (5-FU)-based chemotherapy for high-risk stage II and stage III CRCs. For metastatic stage IV disease, surgical removal of the primary CRC and/or metastatic lesions is followed by therapy using a variety of chemotherapy and targeted treatments. In addition, treatment for CRC has improved over the last few decades. As a result, there are now more than 1 million survivors of CRC in the United States. Overall, cancer death rates have declined 20% from their peak in 1991 (215.1 per 50,000 population) to 2009 (173.1 per 50,000 population).

Surgery is the most common treatment for CRC that has not spread. A permanent colostomy, which is the creation of an abdominal opening for elimination of body waste, is a last resort when dealing for colon cancer and not usually required for rectal cancer. In combination with radiation for rectal cancer, patients are given chemotherapy before or after surgery to reduce tumors that have penetrated the bowel wall before there is metastasis. For CRC that has spread to other parts of the body (metastatic colorectal cancer) a palliative care approach is another option.
Chemotherapy is one of the foremost therapeutic interventions in cancer. Chemotherapy is a cancer treatment that uses drugs to stop the growth of cancer cells, either by killing the cells or by stopping them from dividing. When chemotherapy is taken by mouth or injected into a vein or muscle, the drugs enter the bloodstream and can reach cancer cells throughout the body. This is known as systemic chemotherapy. When chemotherapy is placed directly into a body cavity such as the abdomen, the drugs mainly affect cancer cells in those areas. This type of treatment is called regional chemotherapy. The way the chemotherapy is given depends on the type and stage of the cancer being treated. Chemotherapy is an important part of treatment for many people with CRC and doctors are constantly trying to make it more effective and safer. The use of chemotherapy drugs have been a relevant element in medicine. Moreover, there have been innovative ways in the approach to treat CRC. However, the use of plant alkaloids have normally been an afterthought in colon cancer treatment. The Dent lab has been implementing the drug combination technique with known drugs to work against CRC. For example, the use of curcumin along with sildenafil (Viagra) have shown optimistic results. However, advances in drug discovery and treatment protocols are still a work in progress as researchers are finding innovative ways to treat cancer. Nonetheless, efforts to increase the effectiveness of chemotherapy is still the number one priority.

Targeted therapy drugs work differently from standard chemotherapy drugs. Targeted therapy is a type of treatment that uses drugs or other components to identify and attack specific cancer cells without harming normal cells. Types of targeted therapies used in the treatment of CRC used in this study include monoclonal antibodies. Monoclonal antibodies can identify target receptors on the cell surface of cancer cells. The antibodies attach to specific receptors resulting in cancer cell death, reduction in cancer cell growth, or a reduction in metastasis. Monoclonal
antibodies are given by infusion. They may be used alone or to carry drugs, toxins, or radioactive material directly to cancer cells.\(^8\)

In the current study we use a specific multi-kinase inhibitor to treat CRC called regorafenib. This drug used to treat CRC that has metastasized and proved resistant to previous therapy.\(^{12}\) Regorafenib blocks the action of certain proteins, including vascular endothelial growth factor (VEGF). This may help keep cancer cells from growing and potentially lead to programmed cell death. It may also prevent the growth of new blood vessels that tumors need to grow.\(^{13,14,15}\) In this lab, we have determined that sorafenib or its derivate regorafenib interacts with sildenafil to kill tumor cell.\(^{10}\) Studies have shown that this type of therapy remains extremely challenging because of the chance of a resurgence of cancer due to survival enhancing mutational events. For example, this lab conducted experimental treatments of gastrointestinal cancers with curcumin and sildenafil. After running a multiplex analyses of mouse plasma and tumor samples taken after drug exposure, \textit{in vivo} surviving tumors were shown to express platelet derived growth factor beta (PDGFR\(\beta\)), which may represent a survival signal for these evolved cells. Regorafenib was used in the current study as a means to down regulate the PDGFR\(\beta\) and so enhance the tumor cell killing potential of curcumin and sildenafil.\(^{11}\)

Previous studies in our laboratory have shown that curcumin and sildenafil can modulate the cell surface expression of a number of check point proteins and can be exploited to enhance immunotherapeutic tumor cell death. Immunotherapy is a treatment that uses the patient's immune system to fight cancer. Substances made by the body or made in a laboratory are used to boost, direct, or restore the body's natural defenses against cancer. This type of cancer treatment is also called biotherapy or biologic therapy.\(^7\) Past studies in the Dent lab have shown that experimenting on immunogenicity can prove to treat colorectal cancer.\(^{16,17}\) In this study, the
focus is on a number of immune markers such as PDL-1 and MHCA1. PDL-1 is a protein on the surface of cancer cells. When PD-1 on the cell surface of T cells attaches to PDL-1 on the cell surface of a cancer cell, the potential of the T cell to recognize and initiate an immune response, potentially leading to cancer cell death, is neutralized. PD-1 inhibitors such as PD-1 antibodies attach to the PD-1 receptor on the cell surface of T cells, preventing the PD-1 from binding to the PDL-1 on the surface of cancer cells. This in turn prevents T cell neutralization and allowing the T cells to recognize the cancer cell without being inactivated and so can potentially kill cancer cells.8,16,17,18 MHCA1 complexes are presented on nucleated cells and are recognized by cytotoxic CD8+ T cells. Once CD8+ T cells recognize the cell as either foreign or ill, they induce cell death.19,20
Curcumin

This study involves the use of curcumin to induce cell death in colon cancer cells. Curcumin is a yellow pigment from *Curcuma longa*, is a major component of turmeric and is commonly used as a spice and food-coloring agent.\(^{11,21,23,24,25,26}\) Curcumin is a low-molecular-weight polyphenol, first chemically characterized in 1915, with the molecular formula of \(\text{C}_{21}\text{H}_{20}\text{O}_6\) (Figure 1). It is generally regarded as a very active constituent and is comprises 2–8% of most turmeric preparations.\(^{27}\) It has long been incorporated as the yellow spice in the India subcontinent diet for centuries and reviews have claimed colorectal cancer rates are much lower in India compared to other places in the world.\(^{13,28}\) Curcumin has been used as a treatment of inflammatory diseases for hundreds of years.\(^{11,15,23}\) Moreover, it is also used as a cosmetic and in some medical preparations.\(^{11,21,23,24,25,26,26,54}\) It is worthwhile to outline its role in the creation of free radicals and its potent antioxidant abilities in health and disease.\(^{11,21,23,24,30,47}\) Because of the free radical-mediated peroxidation of membrane lipids and oxidative damage of DNA and proteins, curcumin is believed to play a therapeutic role in a variety of chronic pathological complications such as cancer, atherosclerosis, neurodegenerative diseases.\(^{11,21,23,25,31}\) Hence, the past few decades have witnessed intense research devoted to the antioxidant activity of curcumin.\(^{11,21,23,24,30,31,33,34,44}\) It also exhibits antitumor properties.\(^{11,21,23,25,30,33}\) Curcumin has been known to modulate many signal transduction events including those involving MAPK, PDGF FGF, EGF, VEGFR, c-KIT pathways as well as up-regulation and down-regulation of specific genes.\(^{11,22,26,31,35,36,37,38,39}\) For example; curcumin inhibits the activity of histone deacetylases (HDACs), suppresses the activation of transcription factor NF-\(\kappa\)B/AP-1 signaling and increases reactive oxygen species (ROS), which in turn causes endoplasmic reticular stress.\(^{11,36,37,38,39,46}\) In Paul Dent’s laboratory, it has been shown that
Curcumin has potent and reactive effects on autophagy, apoptosis, endoplasmic reticulum proteins, and histone deacetylase inhibitors.\textsuperscript{11,30,31} Curcumin usually does not cause significant side effects but research has shown that some people have experienced upset stomachs, nausea, dizziness, diarrhea, increased bleeding at very high doses surpassing 1500 milligrams more than once a daily. High doses can also lead to abnormal heartbeat.\textsuperscript{40,41,42} Studies have shown that the uptake of curcumin isn’t as high but is very achievable in the body at 2μM.\textsuperscript{11} In this study, the main focus was whether regorafenib is able to enhance the cell killing potential of curcumin and sildenafil. Curcumin’s cost and availability also makes it a forerunner to use in treatment. For this reason, curcumin is used in this combinational drug study.\textsuperscript{109}
Figure 1. Chemical Structure of Curcumin\textsuperscript{106}
Sildenafil Citrate

In this study, we combined curcumin with sildenafil. In our laboratory, we have shown that sildenafil can enhance the cell killing potential of a number of FDA approved chemotherapeutics. Sildenafil Citrate (also known as Revatio or Viagra), (Figure 2), was released to the public in 1998 after FDA approval as an oral treatment for heart related illnesses but it was later realized that it aids in erectile dysfunction. More specifically, Viagra is used for its cardio-protectiveness in many cardiovascular conditions such as heart failure, coronary artery disease, hypertension and heart transplantation. Sildenafil also acts by inhibiting cGMP-specific phosphodiesterase type 5 (PDE5), an enzyme that promotes degradation of cGMP. PDE5 is overexpressed in several types of human carcinoma including colorectal, genitourinary and liver cancer. In Dent’s laboratory, studies have shown the effectiveness of sildenafil on tumor cells. The PDE5 inhibitor is also known to increase reactive nitrogen species (RNS) at a very significant level. PDE5 inhibitors elevate cGMP levels which in turns elevates expression of nitric oxide synthase (NOS) enzymes, particularly endothelial NOS (eNOS) and inducible NOS (iNOS), activating protein kinase C (PKC) isoforms and protein kinase G (PKG). This ultimately leads to the inactivity of DNA, proteins, and lipids and activates apoptosis and the activity of caspases via forming the toxic oxidant peroxy-nitrite (ONOO-). Clinical side effects of Viagra have been more severe with increased dosage. The side effects include flushing, headaches, nasal congestion, painful erections, heart burn, and lightheadedness. Other studies, including ones out of the Dent laboratory have noted the effect on intracellular pathways such as the Mitogen Active Protein Kinase (MAPK) pathway and the c-Jun N-terminal kinases (JNK) pathway. In the context of this study, sildenafil is used for its anti-cancer properties as well as its readily availability and cost.
Figure 2. Chemical Structure of Sildenafil$^{107}$
Regorafenib

In the study previous to this, the evolved tumor cells from curcumin and sildenafil treated mice were shown to over express PDGFR. The over expression of PDGFR may act as a survival signal to these tumor cells and may represent a mechanism for them to survive curcumin and sildenafil treatment. We added regorafenib to the curcumin and sildenafil regimen in an attempt to circumvent this survival signal by knocking down PDGFR. Regorafenib (as known as BAY73-3506 or Stivarga) is a potent multi-kinase inhibitor that was approved for treatment in April 27th, 2017.\textsuperscript{14-16} Studies have shown that regorafenib is involved in the suppression of tumor angiogenesis, oncogenesis, and tumor niche formation.\textsuperscript{62,63,65} More specifically, regorafenib has been shown to inhibit tyrosine kinase receptors such as vascular endothelial growth factor receptors one, two and three (VEGFR1, VEGFR2, VEGFR3), stromal receptor tyrosine kinases such as platelet derived growth factor receptor beta (PDGFRβ), and oncogenic receptor tyrosine kinases KIT, RET, RAF, fibroblast growth factor receptor (FGFR) in liver and renal cancer cells.\textsuperscript{62,63,77} Recent studies have shown that regorafenib is effective in treating metastasis in CRC.\textsuperscript{62,63} Regorafenib is also known for increasing reactive oxygen species (ROS) and reactive nitrogen species (RNS).\textsuperscript{110} Regorafenib side effects include; hypomagnesemia, hypopotassemia, hypertension, proteinuria, pain, hand-foot skin reaction (HFSR), asthenia/fatigue, diarrhea, decreased appetite, infection, dysphonia, elevated bilirubin, fever, mucositis, weight loss, rash, high, blood pressure and nausea.\textsuperscript{59,64-68} Regorafenib has a very similar chemical structure to that of sorafenib the only difference is there is a fluorine substitution to the middle benzene ring.\textsuperscript{12,14-16} (Figure 3)
Figure 3. Chemical Structures of Sorafenib and Regorafenib\textsuperscript{108}
The Focus

In this study, advanced tumor cells from mice that were previously treated with curcumin and sildenafil were shown to over express PDGFR. Over expression of PDGFR indicates there is an activating survival signal for these tumor cells. Regorafenib was added to the curcumin and sildenafil regimen to circumvent this survival signal by reducing the expression of PDGFR, which could potentially lead to more tumor cell death. Potential signal transduction mechanisms involved in apoptosis, autophagy and cell survival were investigated.
Materials and Methods

Materials

All drugs used in this study (Curcumin, Sildenafil Citrate, and Regorafenib) were purchased from Selleck Chemicals in Houston, Texas. The cellular culture materials DMEM, RPMI, and McCoy’s penicillin-streptomycin, trypsin-EDTA, and PSB were purchased from GIBCOBRL (Invitrogen-GIBCOBRL Life Technologies, Grand Island, New York) The Fetal Bovine Serum (FBS) was bought from HyClone Laboratories, Inc. (Thermo Scientific Hyclone, South Logan, Utah. The 4% paraformaldehyde in PBS can from Alfa Aesar, a subsidiary of ThermoFisher Scientific. Both the calcein and the ethidium homodimer-1 used in the 24 hour live dead assay came from Life Technologies, another ThermoFisher Scientific subsidiary. Rat serum, and DMSO were all purchased from Sigma Chemical (St. Louis, Missouri). For transfections, the lipofectamine reagent and Opti-MEM came from Thermo Fisher Scientific. The Phospho- and total- primary antibodies used from immunofluorescence were obtained from Cell Signaling Technologies (Danvers, Massachusetts) and from Santa Cruz Biotech (Santa Cruz, California). Secondary antibodies IRDye 680LT goat anti-rabbit IgG an IRDye 800CW goat anti-mouse IgG came from LI-COR Biosciences (Lincoln, Nebraska). 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 the Massey Cancer Center of Virginia Commonwealth University.
Methods

Cell Culture (In Vitro)

CT-26 cells were cultured in RPMI Medium 1680 1X supplemented with 2.5 milliliters of sterile glucose (250mg/ml⁻¹), 5 milliliters of 1M HEPES, 25 milliliters of 5% fetal bovine serum and 5 milliliters of penicillin-streptomycin. HCT-116 cells were cultured in McCoy’s medium with 25 milliliters of 5% fetal bovine serum and 5 milliliters of penicillin-streptomycin. HT-29 cells were cultured in DMEM supplemented with 25 milliliters of 5% fetal bovine serum and 5 milliliters of penicillin-streptomycin. All cells lines were incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Cell Counting and Cell Plating

All the cells used in the study were adherent to the flasks provided. For proper use, cells were removed from the adherent surface of the flasks via trypsinization with 0.25 Trypsin-EDTA. Cells were plated at different densities depending on a specific assay. Cells were plated at a density of 3.0 x 10^3 (per well of a 96-well plate) for immunofluorescence. Cells were plated at a density anywhere from 2.5 x 10^4 to 3.5 x 10^4 (per 12 well plate) for Trypan Blue exclusion assays. Cell lines were then allowed to adhere to the well surface under standard incubation conditions for 24-36 hours prior to treatment.

Drug Treatment

Drug concentrations were chosen based on prior studies performed in the Dent laboratory. The concentrations of each drug used in this study were curcumin, sildenafil citrate and Regorafenib. Drug solutions were prepared fresh via the combination of the powder drug
and solubilized DMSO. Final drug dilution for addition did not exceed 0.1% in final drug dilutions.

**Transfections of Cells with Plasmids or with siRNA**

**For Plasmids:** Cells were transfected 24h after plating. Plasmids expressing a specific mRNA (or siRNA) or appropriate vector control plasmid DNA was diluted in 50μl serum-free and antibiotic-free medium (1 portion for each sample). Concurrently, 2μl Lipofectamine 2000 (Invitrogen), was diluted into 50μl of serum-free and antibiotic-free medium (1 portion for each sample). Diluted DNA was added to the diluted Lipofectamine 2000 for each sample and incubated at room temperature for 30 min. This mixture was added to each well / dish of cells containing 200μl serum-free and antibiotic-free medium for a total volume of 300 μl, and the cells were incubated for 4 h at 37 °C. An equal volume of 2x medium was then added to each well. Cells were incubated for 24h, then treated with drugs.

**Transfection for siRNA:** Cells from a fresh culture growing in log phase as described above, were transfected 24h after plating. Prior to transfection, the medium was aspirated and serum-free medium was added to each plate. For transfection, 10 nM of the annealed siRNA, the positive sense control doubled stranded siRNA targeting GAPDH or the negative control (a “scrambled” sequence with no significant homology to any known gene sequences from mouse, rat or human cell lines) were used. Ten nM siRNA (scrambled or experimental) was diluted in serum-free media. Four μl Hiperfect (Qiagen) was added to this mixture and the solution was mixed by pipetting up and down several times. This solution was incubated at room temp for 10 min, then added drop-wise to each dish. The medium in each dish was swirled gently to mix, then incubated at 37 °C for 2h. Serum-containing medium was added to each plate, and cells
were incubated at 37°C for 24h before then treated with drugs (0-24h). Additional immuno-fluorescence / live-dead analyses were performed at the indicated time points.

**Detection of Cell Death by Trypan Blue**

Trypan Blue Exclusion was used to assess cell viability at each experimental time point. Floating cells were isolated along with attached cells that were harvested by trypsinization with Trypsin/EDTA for approximately 3 to 5 minutes at 37°C. Following isolation, the total cell population for each experimental point was assessed for cell viability.

**Detection of Protein Expression and Protein Phosphorylation by Immuno-Fluorescence using a Hermes WiScan Machine**

Cells (4 x 10^3) were plated into each well of a 96 well plate and allowed to grow over night. Depending upon the specific experiment, cells were then either genetically manipulated, or treated with drugs. For genetic manipulation, cells were transfected with plasmids or siRNA molecules and incubated for an additional 24 hours. Cells were treated with vehicle control or with drugs at the indicated final concentrations, alone or in combination. Cells were isolated for processing at various times following drug exposure. For immuno-fluorescence studies, after centrifugation, cell growth media was removed and cells were fixed in place in 4% paraformaldehyde for 10 minutes at room temperature. The cells were then permeabilized using ice cold PBS containing 0.5% Triton X-100. After 30 min the cells were washed three times with ice cold PBS and pre-blocked with rat serum for 3 hours. Following pre-blocking, cells were incubated with a primary antibody for the detection and expression / phosphorylation of a given protein (usually at 1:100 dilution from a commercial vendor) overnight at 37°C. Following
overnight incubation, cells were washed three times with PBS followed by incubation with a secondary antibody containing an associated fluorescent red or green chemical tag, for 3 hours. Following this incubation, the cells were washed three times in PBS and 100 μl of PBS was added to each well for assessment of protein expression. The cells were visualized at either 10x or 60x in the Hermes machine. All immunofluorescent images for each individual protein / phosphoprotein were recorded using the standardized settings to ensure that signal level for each image was directly comparable to signal level in the control and drug treated cells. Similarly, for presentation, the enhancement of image brightness/contrast using PhotoShop CS6 was simultaneously performed for each individual set of protein/ phospho-protein to permit direct comparison of the image intensity between treatments. All immune-fluorescent images were initially visualized at 75 dpi using an Odyssey infrared imager (Li-Cor, Lincoln, NE), then processed at 9999 dpi using Adobe Photoshop CS6. For presentation, immunoblots were digitally assessed using the provided Odyssey imager software. Images have their color removed and labeled figures generated in Microsoft PowerPoint.

**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. 10mls/plate FBS was prepared with 5μl of calcein (shows live cells) and 5μl of ethidium bromide (shows dying/dead cells) as a live dead solution. The live/dead solutions was added to the plate in a volume of 100 μL/well. The plate was centrifuged at a rate of 200 rpm for 3 minutes and loaded into the Hermes WiScan for imaging. Images taken were a representative sample from all wells. The Percentages of cell death were determined by a simple count of the cells within the collected image.
**Data Analysis**

Comparison of the effects of various treatments was performed using one-way analysis of variance and two tailed Student’s *t*-test. Differences with a p-value of < 0.05 were considered statistically significant. Experiments shown are the means of multiple experiments (± SEM).

**Cell Lines:**

The cell lines used in this study as well as their major attributes are found in the table below.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Major Attributes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29</td>
<td>Mutant p53, CD95, Wild Type K-Ras, Mutant B-Raf</td>
</tr>
<tr>
<td>HCT-116</td>
<td>Wild Type p53, CD95, Mutant K-Ras</td>
</tr>
<tr>
<td>CT-26</td>
<td>Mutant K-Ras</td>
</tr>
</tbody>
</table>

**HCT-116:**

A human colon cancer cell line used in therapeutic research and drug screenings. HCT116 cells have a mutation in codon 13 of the K-RAS proto-oncogene, and are suitable transfection targets for gene therapy research. This human colorectal carcinoma cell line initiated from an adult male. The cells are adherent with an epithelial morphology. Following implantation into immunocompromised mice, the cells form primary tumors and distant metastases. In vitro, HCT116 cells grow with a doubling time of approximately 18 hours. They are suitable for in vitro and in vivo experimentation. Immunocompromised mice should be used for in life studies, and will form tumors and metastases following implantation of the cells.
HT-29:

A human colorectal adenocarcinoma (a type of cancer that starts in the glands that line the inside of one of your organs. Adenocarcinoma can happen in many places, like your colon, breasts, esophagus, lungs, pancreas, or prostate) cell line with epithelial morphology. HT-29 cells overproduce the p53 tumor antigen, but have a mutation in the p53 gene at position 273, resulting in a histidine replacing an arginine. The cells proliferate rapidly in media containing suramin, with corresponding high expression of the c-myc oncogene. However, c-myc is deregulated, but may have a relation with the growth factor requirements of HT-29 cells.

These cells are sensitive to the chemotherapeutic drugs 5-fluorouracil and oxaliplatin, which are standard treatment options for colorectal cancer. In addition to being a xenograft tumor model for colorectal cancer, the HT-29 cell line is also used as an in-vitro model to study absorption, transport, and secretion by intestinal cells. Under standard culture conditions, these cells grow as a nonpolarized, undifferentiated multilayer. Altering culture conditions or treating the cells with various inducers, however, results in a differentiated and polarized morphology, characterized by the redistribution of membrane antigens and development of an apical brush-border membrane. This cell line was established in 1964 from the primary tumor of a 44-year-old Caucasian female with colorectal adenocarcinoma.

CT-26:

Murine CT26 (Colon Tumor #26) cells were developed in 1975 by exposing BALB/c mice to N-nitroso-N-methylurethane (NMU), resulting in a rapid-growing grade IV carcinoma that is easily implanted and readily metastasizes. K-ras, is mutated in CT26. Used in over 500 published studies, the CT26 colon carcinoma is one of the most commonly used cell lines in drug
development. Numerous cytotoxic agents as well as therapeutics targeting specific signaling pathways have been studied with these cells. Moreover, as the CT26 model in BALB/c mice provides a syngeneic in vivo test system, it is frequently used for developing and testing immunotherapeutic concepts. In sharp contrast to its frequent use in drug development, there have been no comprehensive studies of the genome and transcriptome of CT26. Cdkn2a is homozygously deleted. Proliferation and stem-cell markers, including Top2a, Birc5 (Survivin), Cldn6 and Mki67, are highly expressed while differentiation and top-crypt markers Muc2, Ms4a8a (MS4A8B) and Epcam are not. Myc, Trp53 (tp53), Mdm2, Hif1a, and N-ras are highly expressed while EGFR and FLT1 are not. MHC class I but not MHC class II is expressed.

**Antibodies and SiRNAs**

Antibodies to BAX, BAK, BCL-XL, CHOP, c-FLIP, FADD, Cathepsin B, mTOR, phospho-mTOR S2448 and S2481, AIF, NOXA, PUMA, ATG5, phospho-ATG13 S318, Beclin-1, AKT, phospho-AKT T308, eiF2α, phospho-eiF2α S51, ATM, phospho-ATM S1981, AMPKα, phospho-AMPK T172, phospho-ULK1 S757, S317, STAT3, p70 S6K, phospho-ERK1/2, GRP78, HSP70 and HSP90, phospho-γH2AX, were purchased from Cell Signaling Technology, (Danvers, MA). PERK, CD95 and caspase 9 antibodies, were purchased from Santa Cruz Biotechnology, (Dallas, TX).
Results:

Regorafenib interacts with sildenafil and with curcumin to induce apoptosis in GI tumor cells.

Previously, tests on HCT-116 and HT-29 cells were only performed with sildenafil (also known as Viagra) and curcumin. The results show that there was significant cell death on these colorectal cancer cells. However, cells isolated from in vivo treated [curcumin + sildenafil] tumors were resistant to in vitro [curcumin + sildenafil] exposure. The surviving cells had an upregulation of PDGFRβ. However, we hypothesized that the late stage colorectal cancer therapeutic and PDGFRβ inhibitor regorafenib would restore the curcumin and sildenafil toxicity to a level found in naïve cells. The use of regorafenib in the presence of curcumin and sildenafil may represent an effective second line therapy for colorectal cancer patients. Regorafenib (as known as BAY73-4506 or Stivarga) is a potent multi-kinase inhibitor that is involved in the suppression of tumor angiogenesis, oncogenesis, and tumor niche formation. The lab decided to use the [curcumin + sildenafil + regorafenib] drug combination to overcome the proliferation signaling provided by the upregulation of PDGFRβ. 6, 12, and 24 hour treatments to ascertain the lethality of the drug combination was measured via trypan blue exclusion assay. There was a significant increase in cell death in the CT-26, HCT-116, and HT-29 cell lines due to drug combination exposure. (Figures 4-6)
Trypan Blue Exclusion Assay

CT-26 cell line were plated into 12 well plates at a density of 5 x 10^3/ml and allowed to adhere to the plate surface for 12 hours. Cells were then treated a vehicle DMSO, [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6, 12 and 24 hour period on the 12 well plates. Cell death was determined by the trypan blue exclusion assay. The graph depicts a quantification of the percentage of cell death. (n=3+/−SEM) # = p <0.05 greater killing compared to the vehicle *= p < 0.05 greater killing compared to the [curcumin + sildenafil] treatment.

Figure 4. Regorafenib interacts with sildenafil and with curcumin to induce apoptosis in CT-26 cell line.
Figure 5. Regorafenib interacts with sildenafil and with curcumin to induce apoptosis in HCT-116 cell line.

HCT-116 cell lines were plated into 12 well plates at a density of 5 x 10³/ml and allowed to adhere to the plate surface for 12 hours. Cells were then treated a vehicle DMSO, [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6, 12 and 24 hour period on the 12 well plates. Cell death was determined by the trypan blue exclusion assay. The graph depicts a quantification of the percentage of cell death. (n=3+/-SEM) # = p <0.05 greater killing compared to the vehicle *= p < 0.05 greater killing compared to the [curcumin + sildenafil] treatment.
Figure 6. Regorafenib interacts with sildenafil and with curcumin to induce apoptosis in HT-29 cell line.

HT-29 cell lines were plated into 12 well plates at a density of 5 x 10^3/ml and allowed to adhere to the plate surface for 12 hours. Cells were then treated a vehicle DMSO, [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6, 12 and 24 hour period on the 12 well plates. Cell death was determined by the trypan blue exclusion assay. The graph depicts a quantification of the percentage of cell death. (n=3+/−SEM) # = p <0.05 greater killing compared to the vehicle *= p < 0.05 greater killing compared to the [curcumin + sildenafil] treatment.
Drug combination activates DNA-damage-ATM-AMPK pathway signal transduction pathway and inactivates signal transduction pathways that lead to cell proliferation and survival in colorectal cancer cells.

Initially, it was determined that the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination causes cell death over time. Logically the next step of inquiry would be to observe the activity of signal transduction pathways. Previous studies in the Dent lab have focused on the PI3K and MAPK pathways and their roles in autophagy and apoptosis. Frequent activation of these pathways occurs when intracellular proteins are phosphorylated by active kinases at serine, tyrosine and/or threonine sites. The goal was to investigate the level of autophagy/apoptosis by looking at the activity of intracellular proteins in the colorectal tumor cells. Treatment of colorectal tumors with [2μM curcumin + 2μM sildenafil + 2μM regorafenib] increased the phosphorylation of ATM, AMPK, ULK1 at serine reside 317, ATG-13 at serine 318, eIF2α, Beclin1 and ATG 5. Concurrently, there was a decrease in phosphorylation with proteins normally involved in cell proliferation and cell survival. Proteins such as ULK1 at serine reside 757, mToR at serine reside 2448, mToR at serine residue 2481, Bcl-XL and MCL1 all had reduced expression. (Figures 7-12)
CT-26 cell line was plated into a 96 well plate at a density of $5 \times 10^3$ cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/−SEM) * = (p < 0.05 less fluorescence than the vehicle) # = (p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment) ** = (p < 0.05 more fluorescence than the vehicle) ## = (p < 0.05 more fluorescence compared to the [curcumin + sildenafil] treatment)
CT-26 cell lines was plated into a 96 well plate at a density of $5 \times 10^3$ cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/SEM) *= (p < 0.05 less fluorescence than the vehicle) # = (p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment) **= (p < 0.05 more fluorescence than the vehicle) ## = (p < 0.05 more fluorescence compared to the [curcumin + sildenafil] treatment)
Figure 9. Drug combination treatment activates cascade that leads to cell death and deactivates cascade that lead to cell proliferation and survival in HCT-116 cancer cells.

HCT-116 cell lines was plated into a 96 well plate at a density of $5 \times 10^3$ cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. ($n=3+/−$ SEM) *= (p < 0.05 less fluorescence than the vehicle) # = (p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment) **= (p < 0.05 more fluorescence than the vehicle) ## = (p < 0.05 more fluorescence compared to the [curcumin + sildenafil] treatment
HCT-116 cell lines was plated into a 96 well plate at a density of $5 \times 10^3$ cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/-SEM) *= (p < 0.05 less fluorescence than the vehicle) # = (p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment) **= (p < 0.05 more fluorescence than the vehicle) ## = (p < 0.05 more fluorescence compared to the [curcumin + sildenafil] treatment)

**Figure 10. Drug combination treatment activates cascade that leads to cell death and deactivates cascade that lead to cell proliferation and survival in HCT-116 cancer cells.**
HT-29 cell lines was plated into a 96 well plate at a density of $5 \times 10^3$ cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/SEM) *= (p < 0.05 less fluorescence than the vehicle) # = (p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment) **= (p < 0.05 more fluorescence than the vehicle) ### = (p < 0.05 more fluorescence compared to the [curcumin + sildenafil] treatment)
Figure 12. Drug combination treatment activates cascade that leads to cell death and deactivates cascade that lead to cell proliferation and survival in HT-29 cancer cells.

HT-29 cell lines was plated into a 96 well plate at a density of $5 \times 10^3$ cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/−SEM) *=(p < 0.05 less fluorescence than the vehicle) #=(p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment) **=(p < 0.05 more fluorescence than the vehicle) ##=(p < 0.05 more fluorescence compared to the [curcumin + sildenafil] treatment)
Drug treatment induces downregulation of ER stress chaperone proteins and upregulates production of apoptotic proteins in CT-26, HCT-116 and HT-29 cells.

In previous studies done in this lab, we have described how curcumin and sildenafil induces reactive oxygen species (ROS) to facilitate ER stress on colorectal cancer cells. The next step was to test if the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination had any effects on heavily studied ER, autophagic and chaperone proteins. There is an increased level of expression on eukaryotic initiation factor 2α (eIF2α). When the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination was used on the CT-26, HCT-116 and HT-29 there was a significant upregulation of ATG5 and Beclin1. The drug combination, however, decreased levels of expression on chaperone proteins heat shock protein 70 and heat shock protein 90 (HSP70 and HSP90) and binding immunoglobulin protein (GRP78 as known as BIP). These cyto-protective proteins aid in the folding and assembly of proteins inside and outside of the ER. The activity of these proteins attach to other misfolded proteins and works to correct the deficient structures before their accumulation triggers apoptosis. In cancer, there is an influx misfolded proteins due to mutations but HSP 70, HSP 90 and GRP 78 are all upregulated which decreases misfolded proteins and ultimately decreases the likelihood of apoptosis. Reducing the expression of HSP 70, HSP 90 and GRP78 will mean that drug-induced cell killing will significantly increase. The hypothesis is synonymous with the results (Figures 13-15)
Figure 13. Drug treatment induces downregulation of ER stress chaperone proteins and upregulates production of apoptotic proteins in CT-26

CT-26 cell lines were plated into a 96 well plate at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO 2μM), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] drug combination for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with immune-marker antibodies to determine the expression of respective proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/SEM) *= (p < 0.05 less fluorescence than the vehicle) # = (p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment) **= (p < 0.05 more fluorescence than the vehicle) ### = p < 0.05 more fluorescence compared to the [curcumin + sildenafil] treatment.
Figure 14. Drug combination treatment induces downregulation of immune receptors, enzymes and DNA protective proteins in HCT-116 cells.

HCT-116 cell lines were plated into a 96 well plate at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO 2μM), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] drug combination for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with immune-marker antibodies to determine the expression of respective proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/SEM) *= (p < 0.05 less fluorescence than the vehicle) # = (p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment) **= (p < 0.05 more fluorescence than the vehicle) ## = (p < 0.05 more fluorescence compared to the [curcumin + sildenafil] treatment).
Figure 15. Drug combination treatment induces downregulation of immune receptors, enzymes and DNA protective proteins in HT-29 cells.

HT-29 cell lines were plated into a 96 well plate at a density of $5 \times 10^3$ cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO 2μM), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] drug combination for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with immune-marker antibodies to determine the expression of respective proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/SEM) *= (p < 0.05 less fluorescence than the vehicle) # = (p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment) **= (p < 0.05 more fluorescence than the vehicle) ## = (p < 0.05 more fluorescence compared to the [curcumin + sildenafil] treatment)
Modulation of autophagy and intracellular proteins can suppress [2μM curcumin + 2μM sildenafil + 2μM regorafenib] lethality.

Small interfering RNAs (siRNAs) were used to knock down specific proteins. Initial studies have indicated that knockdowns of certain key proteins linked to autophagy and apoptosis suppress [curcumin + sildenafil] drug lethality. The same step was taking in this study with the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination. The following siRNAs were used to evaluate the effectiveness of the drug combination on the HCT-116 and HT-29 cell lines: ATG5, Beclin-1, Bax, Bak, Noxa, Puma, ATM, AMPK, ULK1, CD95, FADD, eIF2α, Perk, AIF and Cath B. Beclin and ATG5 siRNAs were used because both are essential for autophagy. Expression of all proteins that were previously mentioned were suppressed as a result of the utilization of siRNAs across both HCT-116 and HT-29 cell lines. With a cytomegalovirus (CMV) used as the control, there is a significant reduction in cell death when proteins FLIP, BCL-XL, DN caspase9, GRP78, HSP70 were upregulated across both the HT-29 and HCT-116 cell lines. (Figures 13-20)
**Transfections (12 Hour Treatment)**

**HCT-116**

![Graph showing knockdown of key autophagy/apoptosis proteins suppressed [2μM curcumin + 2μM sildenafil + 2μM regorafenib] lethality.]

**Figure 16. Knockdown of key autophagy/apoptosis proteins suppressed [2μM curcumin + 2μM sildenafil + 2μM regorafenib] lethality.**

HCT-116 cell lines were plated into 12 well plates at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HCT-116 cell lines were transfected in 12 well plates using Opti-MEM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 12 hour period. The number of dead cells were counted via a trypan blue exclusion assay (n=3+/SEM) * = p < 0.05 less killing compared to the values in scrambled siRNA (siSCR) # = p < 0.05 more killing than compared to the [curcumin + sildenafil] knockdown treatments.
Figure 17. Knockdown of key autophagy/apoptosis proteins suppressed [2μM curcumin + 2μM sildenafil + 2μM regorafenib] lethality.

HT-29 cell lines were plated into 12 well plates at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HT-29 cell lines were transfected in 12 well plates using Opti-MUM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 12 hour period. The number of dead cells were counted via a trypan blue exclusion assay (n=3+/SEM) * = p < 0.05 less killing compared to the values in scrambled siRNA combination treatment (siSCR) # = p < 0.05 more killing than compared to the [curcumin + sildenafil] knockdown treatments.
Figure 18. Knockdown of key autophagy/apoptosis proteins suppressed [2μM curcumin + 2μM sildenafil + 2μM regorafenib] lethality.

HCT-116 cell lines were plated into 12 well plates at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HCT-116 cell lines were transfected in 12 well plates using Opti-MEM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 12 hour period. The number of dead cells were counted via a trypan blue exclusion assay (n=3+/−SEM) * = p < 0.05 less killing than compared to the scrambled siRNA combination treatment (siSCR) # = p < 0.05 more killing than compared to the [curcumin + sildenafil] knockdown treatments.
Figure 19. Knockdown of key autophagy/apoptosis proteins suppressed [2μM curcumin + 2μM sildenafil + 2μM regorafenib] lethality.

HT-29 cell lines were plated into 12 well plates at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HT-29 cell lines were transfected in 12 well plates using Opti-MUM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 12 hour period. The number of dead cells were counted via a trypan blue exclusion assay (n=3+/−SEM) * = p < 0.05 less killing compared to the values in scrambled siRNA combination treatment (siSCR) # = p < 0.05 more killing compared to the [curcumin + sildenafil] treatments.
Figure 20. Knockdown of key autophagy/apoptosis proteins suppressed [2μM curcumin + 2μM sildenafil + 2μM regorafenib] lethality.

HCT-116 cell lines were plated into 12 well plates at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HCT-116 cell lines were transfected in 12 well plates using Opti-MUM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 12 hour period. The number of dead cells were counted via a trypan blue exclusion assay (n=3+/−SEM) * = p < 0.05 less killing compared to the scrambled siRNA (siSCR) # = p < 0.05 more killing compared to the [curcumin + sildenafil] treatments.

HT-29 cell lines were plated into 12 well plates at a density of 5 x 10³ cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HT-29 cell lines were transfected in 12 well plates using Opti-MUM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 12 hour period. The number of dead cells were counted via a trypan blue exclusion assay (n=3+/−SEM) * = p < 0.05 less killing compared to the scrambled siRNA (siSCR) # = p < 0.05 more killing compared to the [curcumin + sildenafil] treatments.

Figure 21. Knockdown of key autophagy/apoptosis proteins suppressed [2μM curcumin + 2μM sildenafil + 2μM regorafenib] lethality.
Figure 22. Upregulation of pro-survival proteins suppressed [2μM curcumin + 2μM sildenafil + 2μM regorafenib] lethality.

HCT-116 cell lines were plated into a 12 well plates at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HCT-116 cell lines were infected with CMV plasmids in 12 well plates using Opti-MUM. The respective plasmids were deposited into these wells and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 12 hour period. The number of dead cells were counted via a trypan blue exclusion assay (n=3+/−SEM) * = p < 0.05 less killing compared to cytomegalovirus control (CMV) # = p < 0.05 more killing compared to the [curcumin + sildenafil] treatments.
Figure 23. Upregulation of pro-survival proteins suppressed [2μM curcumin + 2μM sildenafil + 2μM regorafenib] lethality.

HT-29 cell lines were plated into a 12 well plates at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HT-29 cell lines were infected with CMV plasmids in 12 well plates using Opti-MUM. The respective plasmids were deposited into these wells and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 12 hour period. The number of dead cells were counted via a trypan blue exclusion assay. (n=3+/SEM) * = p < 0.05 less killing than corresponding values in cytomegalovirus (CMV)
[2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination induces an overall inhibition of HDACs in CT-26, HCT-116, and HT-29 cell line

In the CT-26, HCT-116 and HT-29 cell line there was an overall decrease in expression of HDACs proteins when the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination was used. The Dent lab has shown that autophagy can facilitate the rapid degradation of a wide variety of signal transduction regulatory proteins. Moreover, it was demonstrated that autophagy can contribute to the degradation of HDAC proteins. Treatment of CT-26 cells with the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination had decreased fluorescent intensity for HDACs 1-7, 9-11 compared to the vehicle. The [2μM curcumin + 2μM sildenafil] drug combination had decreased fluorescent intensity for HDACs 1-11 compared to the vehicle. With regorafenib, there were decreases in fluorescent intensity for HDACs 1-3, 6, 8-11 compared to the vehicle however the decrease wasn’t as significant as the curcumin/sildenafil and [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combinations. HDAC expression was decrease across the HCT-116 and HT-29 cell lines as well except HDAC 10 in the HCT-116 (Figure 21-23).
CT-26 cell lines was plated into a 96 well plate at a density of $5 \times 10^3$ cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 12 hour period on 96 well plates. After treatment, the cells were fixed and probed with HDAC antibodies to determine the expression of HDACs. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3 +/-SEM) * = p < 0.05 less HDAC expression compared to the vehicle. # = p < 0.05 less HDAC expression compared to the [curcumin + sildenafil] treatment. ## = p< 0.05 more HDAC expression compared the vehicle.

**Figure 24. Histone Deacylases (HDACs) 1-11 are repressed in CT-26 tissues.**
Figure 25. Histone Deacylases (HDACs) 1-11 are repressed in HCT-116 tissues.

HCT-116 cell lines was plated into a 96 well plate at a density of $5 \times 10^3$ cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 12 hour period on 96 well plates. After treatment, the cells were fixed and probed with HDAC antibodies to determine the expression of HDACs. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3 +/- SEM) * = p < 0.05 less HDAC expression compared to the vehicle. # = p< 0.05 less HDAC expression compared to the [curcumin + sildenafil] treatment. ## = p< 0.05 more HDAC expression compared the vehicle.
Figure 26. Histone Deacetylases (HDACs) 1-11 are repressed in HT-29 tissues.

HT-29 cell lines was plated into a 96 well plate at a density of $5 \times 10^3$ cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 12 hour period on 96 well plates. After treatment, the cells were fixed and probed with HDAC antibodies to determine the expression of HDACs. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3 +/- SEM) *= p < 0.05 less HDAC expression compared to the vehicle. # = p < 0.05 less HDAC expression compared to the [curcumin + sildenafil] treatment.
Drug combination treatment induces downregulation of immune checkpoint proteins and enzymes in CT-26 HT-29 and HCT-116 cells.

The next step in this study is to determine if there is a correlation between reduced HDAC expression and proteins levels that alter immunogenicity of colorectal tumor cells to checkpoint inhibitory immunotherapies. The immune checkpoint protein and enzyme levels of PDL1, PDL2, MHCA1, ODC and IDO were observed. There is an apparent downregulation of Programmed Cell Death Ligand-1 (PDL1), Programmed Cell Death Ligand-2 (PDL2), Indoleamine 2,3-dioxygenase 1 (IDO1) Ornithine decarboxylase (ODC) across the CT-26, HCT-116 and HT-29 cell lines when the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination is used. Major Histocompatibility Complex (MHCA1) had a significant increase in expression across all three colorectal tumor cell lines (Figures 24-26).
CT-26 cell lines was plated into a 96 well plate at a density of $5 \times 10^3$ cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with immune-marker antibodies. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3 +/-SEM) *= p < 0.05 less expression compared to the vehicle. # = p < 0.05 less expression compared to the [curcumin + sildenafil] treatment. **= p < 0.05 more expression compared to the vehicle. ###= p < 0.05 more expression to the [curcumin + sildenafil] treatment.

Figure 27. Drug combination treatment induces downregulation of immune checkpoint proteins and enzymes in CT-26 cells.
Figure 28. Drug combination treatment induces downregulation of immune checkpoint proteins and enzymes in HCT-116 cells.

HCT-116 cell lines was plated into a 96 well plate at a density of $5 \times 10^3$ cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with immune-marker antibodies. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3 +/-SEM) * = p < 0.05 less expression compared to the vehicle. # = p < 0.05 less expression compared to the [curcumin + sildenafil] treatment. **= p < 0.05 more expression compared to the vehicle. ##= p < 0.05 more expression to the [curcumin + sildenafil] treatment.
HT-29 cell lines was plated into a 96 well plate at a density of $5 \times 10^3$ cells/ml and allowed to adhere to the well surface for 12 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with immune-marker antibodies. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3 +/-SEM) *= p < 0.05 less expression compared to the vehicle. # = p < 0.05 less expression compared to the [curcumin + sildenafil] treatment. **= p < 0.05 more expression compared to the vehicle. ###= p < 0.05 more expression to the [curcumin + sildenafil] treatment.
Activations of PDGFR, Raf-1, ERBB1, ERBB2, ERBB3, and B-raf were halted with [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination in colorectal tumor cells.

Previous attempts to treat colorectal cancer cells in the lab proved to be noteworthy. At the time, the [curcumin + sildenafil] drug combination were significantly increasing ROS. This in turn increased lethality and caused cell death. However, when cells isolated from in vivo treated [curcumin + sildenafil] tumors were resistant to in vitro [curcumin + sildenafil] exposure, the lab decided to utilize the efficacy of regorafenib. It is known that regorafenib is shown to be effective in treating colorectal cancers, more specifically it can reduce the activity levels of PDGFRβ, B-raf, and Raf-1. When using [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination, there was a decrease in PDGFRβ, Raf-1, ERBB1, ERBB2, ERBB3, and B-raf in the HCT-116 and HT-29 cell lines. However, ERBB3 in HT-29, however showed an increase in activity (Figures 30-33)
Figure 30. Activations of PDGFR, Raf-1, ERBB1, ERBB2, ERBB3, and B-raf were halted with [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination in colorectal tumor cells.

HCT-116 cell lines were treated with a vehicle (DMSO 2μM), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] drug combination for a 4 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine receptor activity. The images were acquired via a Hermes WiScan machine. The graph depict a quantification of the imaging based off of fluorescent intensity. (n=3+/− SEM) * = p < 0.05 more receptor expression compared to the vehicle. ** = p < 0.05 less receptor expression compared to the vehicle. # = p < 0.05 more receptor expression compared to the [curcumin + sildenafil] treatment.
Figure 31. Activations of PDGFR, Raf-1, ERBB1, ERBB2, ERBB3, and B-raf were halted with [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination in colorectal tumor cells.

HCT-116 cell lines were treated with a vehicle (DMSO 2μM), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] drug combination for a 4 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine receptor activity. The images were acquired via a Hermes WiScan machine. The graph depict a quantification of the imaging based off of fluorescent intensity. (n=3+/- SEM) * = p < 0.05 less receptor expression compared to the vehicle. **= p < 0.05 more receptor expression compared to the vehicle. # = p < 0.05 less receptor expression compared to the [curcumin + sildenafil] treatment.
Figure 32. Activations of PDGFR, Raf-1, ERBB1, ERBB2, ERBB3, and B-raf were halted with [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination in colorectal tumor cells.

HT-29 cell lines were treated with a vehicle (DMSO 2μM), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] drug combination for a 4 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine receptor activity. The images were acquired via a Hermes WiScan machine. The graph depict a quantification of the imaging based off of fluorescent intensity. (n=3+/- SEM) ** = p < 0.05 less receptor expression compared to the vehicle. * = p < 0.05 more receptor expression compared to the vehicle. # = p < 0.05 less receptor expression compared to the [curcumin + sildenafil] treatment. ## = p < 0.05 more receptor expression compared to the [curcumin + sildenafil] treatment.
Figure 33. Activations of PDGFR, Raf-1, ERBB1, ERBB2, ERBB3, and B-raf were halted with [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination in colorectal tumor cells.

HT-29 cell lines were treated with a vehicle (DMSO 2μM), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] drug combination for a 4 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine receptor activity. The images were acquired via a Hermes WiScan machine. The graph depict a quantification of the imaging based off of fluorescent intensity. (n=3+/- SEM) ** = p < 0.05 more receptor expression compared to the vehicle. * = p < 0.05 less receptor expression compared to the vehicle. # = p < 0.05 less receptor expression compared to the [curcumin + sildenafil] treatment. ## = p < 0.05 less receptor expression compared to the [curcumin + sildenafil] treatment.
Immunofluorescence reveals knockdown of essential downstream regulators increases cytoprotective and anti-apoptotic protein productivity even when [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination is used on colorectal tumor cells.

The next step in this study was to utilize siRNAs to detect the activity of cytoprotective proteins. Initial studies have indicated that knockdowns for certain key proteins that are linked to autophagy and apoptosis will suppress drug lethality. The effort was refocused on three specific proteins BCL-XL, MCL1, and mToR due to the fact that they have high activity levels in tumor cells. Moreover, ATG13 was looked at as well to see its activity as a result of the knockdowns. The siRNAs knockdown PERK, eIF2α, Beclin, CD95, ATM and AMPK. Expression of BCL-XL and MCL1 proteins were increased across the HCT-116 and HT-29 cell lines as a result of the utilization of siRNAs, even with the use of [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination. There was a decrease in the expression of ATG13 and mToR when [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination was used. (Figures 34-41)
**Figure 34.** Knockdown of essential downstream regulators increases cyto-protective and anti-apoptotic protein productivity even when [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination is used on colorectal tumor cells

HCT-116 cell lines were plated into a 96 well plate at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HCT-116 cell lines were transfected in 12 well plates using Opti-MUM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/−SEM) *= p < 0.05 less fluorescence compared to the scrambled vehicle **= p <0.05 more fluorescence compared to the scrambled vehicle # = p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment.
Figure 35. Knockdown of essential downstream regulators increases cyto-protective and anti-apoptotic protein productivity even when [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination is used on colorectal tumor cells

HCT-116 cell lines were plated into a 96 well plate at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HCT-116 cell lines were transfected in 12 well plates using Opti-MUM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/−SEM) * = p < 0.05 less fluorescence compared to the scrambled vehicle ** = p <0.05 more fluorescence compared to the scrambled vehicle # = p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment. ## = p < 0.05 more fluorescence compared to the [curcumin + sildenafil] treatment.
HCT-116 cell lines were plated into a 96 well plate at a density of $5 \times 10^3$ cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HCT-116 cell lines were transfected in 12 well plates using Opti-MUM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. ($n=3+/−$SEM) * = $p < 0.05$ less fluorescence compared to the scrambled vehicle # = $p < 0.05$ less fluorescence compared to the [curcumin + sildenafil] treatment.
Figure 37. Knockdown of essential downstream regulators affect mToR productivity even when [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination is used on colorectal tumor cells

HCT-116 cell lines were plated into a 96 well plate at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HCT-116 cell lines were transfected in 12 well plates using Opti-MUM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/−SEM) * = p < 0.05 less fluorescence compared to the scrambled vehicle # = p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment.
Figure 38. Knockdown of essential downstream regulators increases cyto-protective and anti-apoptotic protein productivity even when [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination is used on colorectal tumor cells

HT-29 cell lines were plated into a 96 well plate at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HCT-116 cell lines were transfected in 12 well plates using Opti-MUM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/−SEM) * = p < 0.05 less fluorescence compared to the scrambled vehicle # = p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment.
HT-29 cell lines were plated into a 96 well plate at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HCT-116 cell lines were transfected in 12 well plates using Opti-MEM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/−SEM) *= p < 0.05 less fluorescence compared to the scrambled vehicle # = p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment.

Figure 39. Knockdown of essential downstream regulators increases cyto-protective and anti-apoptotic protein productivity even when [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination is used on colorectal tumor cells
HT-29 siKnockdown (ATG13 S318 Probe)

Figure 40. Knockdown of essential downstream regulators decreases pro-apoptotic protein productivity even when [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination is used on colorectal tumor cells

HT-29 cell lines were plated into a 96 well plate at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HCT-116 cell lines were transfected in 12 well plates using Opti-MUM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/-SEM) *= p < 0.05 less fluorescence compared to the scrambled vehicle # = p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment.
Figure 41. Knockdown of essential downstream regulators affect mToR productivity even when [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination is used on colorectal tumor cells

HT-29 cell lines were plated into a 96 well plate at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HCT-116 cell lines were transfected in 12 well plates using Opti-MEM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these wells and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/−SEM) *= p < 0.05 less fluorescence compared to the scrambled vehicle # = p < 0.05 less fluorescence compared to the [curcumin + sildenafil] treatment.
Immunofluorescence reveals knockdown of essential downstream regulators decreases eIF2α activity levels when [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination is used on colorectal tumor cells.

96 well plates were re-probed with P-eIF2α to view the levels of activity. Across the HCT-116 and HT-29 cell line, there is decreased activity in protein synthesis when Perk, eIF2α, Beclin, CD95, ATM and AMPK were knockdown. There is a significant downregulation of eIF2a across both the HCT-116 and HT-29 cell lines. (Figure 42-43)
Figure 42. Knockdown of essential downstream regulators decreases pro-apoptotic protein productivity even when [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination is used on colorectal tumor cells

HCT-116 cell lines were plated into a 96 well plate at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HCT-116 cell lines were transfected in 12 well plates using Opti-MUM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/−SEM) * = p < 0.05 less fluorescence compared to the scrambled vehicle ## = p < 0.05 more fluorescence compared to the [curcumin + sildenafil] treatment.
Figure 43. Knockdown of essential downstream regulators decreases pro-apoptotic protein productivity even when [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination is used on colorectal tumor cells.

HT-29 cell lines were plated into a 96 well plate at a density of 5 x 10^3 cells/ml and allowed to adhere to the well surface for 12 hours. After that, the HCT-116 cell lines were transfected in 12 well plates using Opti-MUM and Lipofectamine 2000. The respective small interfering RNAs (siRNAs) were deposited into these well and were left to enter the cells for a span not lasting more than 30 hours. Cells were then treated with a vehicle (DMSO), [curcumin (2μM) + sildenafil (2μM)], [regorafenib] (2μM) and [curcumin (2μM) + sildenafil (2μM) + regorafenib (2μM)] for a 6 hour period on 96 well plates. After treatment, the cells were fixed and probed with antibodies to determine the expression and/or phosphorylation of the indicated proteins. The images were acquired via a Hermes WiScan machine. The graph depicts a quantification of the imaging based off of fluorescent intensity. (n=3+/−SEM) *= p < 0.05 less fluorescence compared to the scrambled vehicle ### = p < 0.05 more fluorescence compared to the [curcumin + sildenafil] treatment.
Animal studies reveal the efficacy of [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination over a course of 30 days.

Immuno-competent BALB/c mice were ejected with CT-26 colorectal tumor cells and were allowed to grow for 7 days. The results show that, compared to the vehicle, drug combination significantly reduced tumor size at the end of the study. (Figures 44)
Figure 44. Animal Studies reveal the efficacy of [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination over the course of a 30 day period

Immuno-competent BALB/c mice (~20 g) were injected with 1x10^6 CT-26 cells into their rear flank. Tumors were permitted to form for 7 days with tumors at the time exhibiting a volume of ~35 to ~45mm³. Mice were treated by oral gavage once every BID for 30 days with vehicle, curcumin (50mg/kg), sildenafil (10mg/kg), regorafenib (10mg/kg) on days 1-5 or in combination as indicated. Tumors were callipered and tumor volumes were determined before and following drug exposure. (n=18 +/- SEM) * = p < 0.05 less tumor growth compared to vehicle.
Discussion

The aforementioned study was designed to determine whether very low clinically achievable concentrations of curcumin and sildenafil could have their anti-cancer properties enhanced by the multi-tyrosine kinase inhibitor, regorafenib. The rationale behind this study is derived from previous findings in our laboratory that demonstrated that tumors that were previously exposed to curcumin and sildenafil, displayed an increase in activated PDGFRβ, which was correlated with elevated levels of PDGF in mouse plasma.11 These tumor cells were resistant to subsequent re-exposure to curcumin and sildenafil. Thus, increased activation of PDGFRβ signaling represents a key evolutionary survival mechanism of gastrointestinal tumor cells exposed to curcumin and sildenafil. The precise mechanisms downstream of PDGFRβ that mediate resistance to curcumin and sildenafil are presently unknown. The hypothesis of this study is that a late stage colorectal cancer therapeutic and PDGFRβ inhibitor regorafenib would restore curcumin and sildenafil lethality to a level found in naïve cells. The use of regorafenib in the presence of curcumin and sildenafil may represent an effective second line therapy for colorectal cancer patients. Regorafenib (as known as BAY73-4506 or Stivarga) is a potent multi-kinase inhibitor that is involved in the suppression of tumor angiogenesis, oncogenesis, and tumor niche formation.62,64,65 More specifically, Regorafenib has been shown to inhibit tyrosine kinase receptors such as vascular endothelial grow factor receptors one, two and three (VEGFR1, VEGFR2, VEGFR3), platelet derived growth factor receptor beta (PDGFRβ), Kit, B-Raf, RET, Raf-1, fibroblast growth factor receptor (FGFR), halt metastasis in liver and colorectal cancer cells and increase the levels of ROS and RNS.11,16,60,62. Isoforms and receptors of PDGF have important functions in the regulation of growth and survival of certain cell types during embryonal development and e.g. tissue repair in the adult. Upregulation of PDGF receptor
signaling, by over expression of receptor / ligand or receptor mutational events, may drive tumor cell growth.\textsuperscript{92} Studies in colorectal cancer patients suggest PDGF over-expression correlates with vascular invasion and invasion was significantly greater in patients expressing PDGFB\textbeta at a high level.\textsuperscript{93} Further, patients with high PDGFB\textbeta expression also had a significantly poorer survival rate.\textsuperscript{94}

Firstly, the goal was to see if cell death could be exacerbated with the \([2\mu M \text{ curcumin} + 2\mu M \text{ sildenafil} + 2\mu M \text{ regorafenib}]\) drug combination. Results indicate that although \([\text{curcumin} + \text{sildenafil}]\) drug combination does attain cell death, the \([2\mu M \text{ curcumin} + 2\mu M \text{ sildenafil} + 2\mu M \text{ regorafenib}]\) drug combination achieves significantly greater cell death over a 6, 12, and 24 hour period. (Figures 4-6) Cell treatment with \([2\mu M \text{ curcumin} + 2\mu M \text{ sildenafil} + 2\mu M \text{ regorafenib}]\) was shown to affect endoplasmic reticulum stress (ER Stress) signaling. ER stress signaling is mediated through three self-regulatory pathways: eIF2\(\alpha\), IRE1, and ATF6. Signaling by eIF2\(\alpha\) reduces transcription from many cyto-protective gene that code for proteins like MCL-1, Bcl-XL, Erk, AKT and K-Ras resulting in the rapid reduction of protein expression for those with short half-lives. The reduced expression of multiple chaperones/cyto-protective proteins as a result of using the \([2\mu M \text{ curcumin} + 2\mu M \text{ sildenafil} + 2\mu M \text{ regorafenib}]\) drug combination significantly enhanced eIF2\(\alpha\) S51 phosphorylation in HT-29, HCT-116 and CT-26 cells. This led to an eIF2\(\alpha\) dependent dephosphorylation of mTOR and the mTOR substrate ULK1 S757 and the phosphorylation of ULK1 S317 which ultimately led to ULK1 activation. ULK1 activation resulted in a significant increase in ATG13 S318 phosphorylation and a significant increase in Beclin 1 expression. This implies that the \([2\mu M \text{ curcumin} + 2\mu M \text{ sildenafil} + 2\mu M \text{ regorafenib}]\) drug combination mediates ER stress, apoptosis and autophagy in the cell. Moreover, the \([2\mu M \text{ curcumin} + 2\mu M \text{ sildenafil} + 2\mu M \text{ regorafenib}]\) was not only shown to mediate reduction of
mTORC1 and mTORC2 phosphorylation but increase the activity of autophagy proteins e.g. Beclin1 and ATG5, indicating a role for eIF2α dependent reduction in expression of many cytoprotective genes e.g. HSP70, HSP90 and GRP78. (Figures 7-15) Past studies in this lab have accurately illustrated autophagy signaling and mitochondrial control of apoptosis.11

These data indicate that the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination could have a significant effect on killing colorectal cancer cells in vitro. To further validate this, transfection of small interfering RNA (siRNA) experiments were completed in order to create a holistic idea that will further verify the potency of this drug combination. Prior studies done in the Dent laboratory have indicated knockdown of pro-apoptotic and autophagic proteins suppress cell death.11 Knockdown of autophagic proteins e.g. (ATG5 and Beclin1) and pro-apoptotic proteins (e.g. Noxa, Puma, Bax and Bak) in junction with the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug significantly reduced cell death in the HCT-116 and HT-29 colorectal cell lines. After observing the significance of pro-apoptotic proteins, the next steps included assessing molecular pathways that deal with ER stress, apoptosis and autophagy signaling because, prior to experimentation, there was a significant increase in activity in proteins such as ATM, AMPK and eIF2α with the use of the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug. The next step was to see if stable knock down of ATM, AMPK, ULK1, CD95, FADD, eIF2α, Perk, AIF, and Cath B to further understand the underlying mechanisms effected by the drug combination. After intensive experimentation, knockdown of these proteins significantly reduced cell death across the HT-29 and HCT-116 cell lines. This indicates these proteins must be active along with the drug in order to achieve lethality. An infection experiment was performed with a cytomegalovirus (CMV) to test the effect of [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug treatment on cyto-protective proteins e.g. FLIP, BCL-XL,
DN caspase 9, GRP78, and HSP70 when they are upregulated. The reduction of cell death in these proteins suggests that they play a significant role in protecting the CRC. (Figures 16-23)

In addition to reducing the expression of a number of molecular chaperone proteins, previous studies in our laboratory have indicated that the induction of autophagy can lead to reduced expression of histone deacetylase enzymes (HDACs). In this study HT-29, HCT-116 and CT-26 cells, curcumin and sildenafil plus regorafenib mediated a down regulation of HDACs. Histone acetylation and deacetylation is a dynamic process balanced by histone acetyltransferase enzymes (HAT) and histone deacetylase enzymes (HDAC). In general, addition of acetyl groups to histones via HATs promote gene expression by creating an “open” chromatin conformation. Removal of acetyl groups by HDACs results in a “closed” conformation and represses transcription. The aim of the study was inhibit HDAC expression so that the tumor cells could produce pro-apoptotic proteins via transcription and translation that could activate and facilitate cell death. The [2μM curcumin + 2μM sildenafil + 2μM regorafenib] combination drug decreased the expression of all HDACs across all three cell lines. This suggests that [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination is acting as potent HDAC inhibitor, and concurrently inducing increased DNA damage and ultimately inducing apoptosis and autophagy. (Figures 24-26)

One aspect of HDAC down-regulation shown from other studies was that reduced expression of HDACs was correlated with altered expression of multiple cellular proteins; including alterations in the levels of proteins that could potentially modify the immunogenicity of the tumor cells to checkpoint inhibitory immunotherapies. Recent studies have linked altered HDAC expression in cancer cells to an altered expression of a number of immunogenic biomarkers predicted to enhance tumor cell immunogenicity and to decrease tumor cell
viability. In the Dent laboratory several model systems, including melanoma and NSCLC were used in vitro were confirmed in vivo. In the present studies, in HCT-116, HT-29 and CT-26 cells, [curcumin+ sildenafil+ regorafenib] drug combination rapidly decreased the expression of PD-L1, PD-L2, ODC and IDO. The [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination used increased the levels of MHCA. This indicates that the cancer cells are now being presented as foreign material instead of self-antigens and can be eliminated by a person’s own immune system. (Figures 27-29)

The next step in this study was to see if the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination had any effect on receptor activity. The main objective was to decrease the activity of PDGFRβ, however we also observed the activity of other protein kinases as well. They include: ERBB1, ERBB2, ERBB3, Raf-1, and B-Raf. Whenever a receptor tyrosine kinase is activated it is phosphorylated on certain residues that lead to a cascade of events. In this study, we assessed levels of phosphorylated receptors verses the total amount of receptor activity and concluded that the drug combination does have significant effects on receptor expression. These findings suggest that receptor tyrosine kinases that aid in cell growth and cell proliferation in the HCT-116 and HT-29 colorectal tumor cell lines are halted by the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination and are subsequently activating autophagy and apoptosis. (Figures 30-33)

Since the study had revealed that apoptotic and autophagic proteins were essential to achieve cell death, the next objective was to see the level of activity in MCL1, BCL-XL, mToR phosphorylation at serine residue 2448 and ATG-13 phosphorylation at serine reside 318 when Perk, eIF2α, Beclin, CD95, ATM and AMPK were knocked down via siRNA. The results reveal that the cyto-protective proteins MCL1 and BCL-XL were significantly upregulated compared to
the scramble vehicle, even in the presence of [2μM curcumin + 2μM sildenafil + 2μM regorafenib]. This indicates that in order to achieve cell toxicity, the proteins that were knockdown have to be viable and active. There was an apparent decrease in ATG-13 phosphorylation at protein reside 318 compared to the scramble vehicle which indicative of knockdown proteins aiding in the phosphorylation. The data shown for the activity of mToR across the HCT-116 and HT-29 cell lines indicates the following: since the AMPK proteins are knocked down, there is no sense of energy deprivation or autophagy signaling. As a result, the CRC continue to proliferate as a result via activation of mToR. Knockdown of ATM indicates there is a loss of the ability to sense DNA breaks which will then lead to the avoidance cell cycle checkpoints and the continuation of mutations as well as proliferation. With the CD95 knockdown, CRC can no longer activate extrinsic apoptosis and CRC cells proliferate. The knockdown of Beclin1 suggest the likelihood of autophagy is close to nonexistent and knockdown of eIF2α and PERK means that there’s no protein synthesis. Thus, mToR cannot cause cells to proliferate because the lack of proteins. (Figures 34-41) When the HT-29 and HCT-116 colorectal tumor cells were reprobed with eIF2α antibody to investigate its activity with the respective knockdowns, it was apparent that activity was suppressed compared to the scramble vehicle. This is indicative to the idea that the activity of eIF2α has be high in order to generate pro-apoptotic and autophagic structures to commence cell killing. (Figures 42-43)

Lastly, an animal study was performed using CT-26 colorectal cancer cells growing in their syngeneic C57/BL6 mice. Five days of treatment with the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination significantly reduced tumor growth rate and continued to do so over 25 days compared to the vehicle treated mice. Two administrations of an anti-PD-1 antibody at days two and four caused a further significant reduction in the rate of tumor growth
when the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination was used, whereas administration of the antibody combined with vehicle control had no appreciable effect on tumor growth (Figure 44). PDL1 plays a critical role in the maintenance of autoimmunity. It has also been shown to suppress the adaptive immune system during particular events e.g. tumor growth. The binding of PD-L1 to the inhibitory checkpoint molecule PD-1 transmits inhibitory signals via immuno-receptor tyrosine-based switch motif (ITSM). This reduces the proliferation of antigen-specific T-cells in lymph nodes, while simultaneously reducing T cells’ ability to activate apoptosis in tumor cells. The findings presented in this manuscript suggest that [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination not only has having an effect on PD-1/PDL-1 binding affinity but also has some influence on MHCA levels as well.

During this study, there was a significant upregulation in ERBB3 tyrosine receptor kinase in the HT-29 cell line even in the presences of the [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination. ERBB3 is closely associated with mutant B-raf. This protein plays a role in regulating the MAPK/ERK signaling pathway, which affects cell division and differentiation. Mutations in this gene, most commonly occur when valine is substituted with glutamic acid at amino acid position 600 in a protein (V600E mutation). This mutation is most frequently identified in melanoma and other cancers, including non-Hodgkin lymphoma, colorectal cancer, thyroid carcinoma, non-small cell lung carcinoma, hairy cell leukemia and adenocarcinoma of lungs. Drugs that treat cancers driven by B-raf mutations have been developed. Two of these drugs, vemurafenib and dabrafenib, are approved by the FDA for treatment of late-stage melanoma. Vemurafenib was the first drug to come out of fragment-based drug discovery. The next logical step in treating upregulated ERBB3 in the HT-29 colorectal
tumor cell line is to use dabrafenib with sildenafil and curcumin to see if there would be any significant effect.

Studies performed outside of the Dent lab suggest that curcumin in the perfect candidate for treatment of colorectal cancers. Specifically, curcumin has been previously reported to alter AMPK, Akt, and Ulk-1 levels significantly by adding cytotoxicity to cells.\(^9^9\) There have also been reports of treating colorectal cancer stem cells with curcumin by heightening their chemosensitivity to chemotherapeutic treatment, thus markedly increasing positive therapeutic outcome, especially on cell lines used in this study (e.g. HCT-116 tumor cells).\(^1^0^0\) Many studies have reported curcumin interacting with other drugs in an additive fashion to kill colorectal cancer cells. Drugs such as dasatinib, 5-fluorouracil, irinotecan which are used for chronic myelogenous leukemia (CML), breast and pancreatic cancers and a topoisomerase-1 inhibition respectively along with curcumin have enhanced lethality.\(^7^4,9^9,1^0^1,1^0^2\) The approach for this study in particular has taken similar steps with the use of curcumin and its ability to halt proliferation and increase drug interactions. The key difference is that, in the current study, sildenafil and regorafenib were utilized. Moreover, the data presented in this study suggest that the [sildenafil + curcumin + regorafenib] drug combination is a very good for targeted therapy. It is understood that resistance to targeted drug combination therapy in colorectal cancer cells can and does ultimately happen. For example, the pervious study performed in the Dent lab determined that cells that were isolated from in vivo treated [curcumin + sildenafil] tumors became resistant in vitro.\(^1^1\) To combat the resistance, regorafenib were added to induce cell death.

Previous reports state the clinical relevance of regorafenib. For example, treatment with regorafenib resulted in a significant improvement of overall survival.\(^1^0^3\) A clinical trial showed the efficacy of regorafenib during phase III of colorectal cancer after failure of standard
therapy.\textsuperscript{104} There are also findings that include regorafenib working in tandem with other target therapy drugs such as cetuximab.\textsuperscript{105} Cetuximab is an epidermal growth factor receptor (EGFR) inhibitor used for the treatment of metastatic colorectal cancer. The efficacy of cetuximab against EGFR was lowered due to tumor cell resistance. However, the combined treatment with cetuximab and regorafenib induced synergistic anti-proliferative and apoptotic effects which caused significant tumor growth inhibition.\textsuperscript{105} The cetuximab/regorafenib study not only provides a rationale for the use of drug combination therapy but it gives rise to the idea that preventing and/or overcoming colorectal cancer drug resistance in patients has practical reasoning. Hence the reason why regorafenib was added to the study: to lower tumor cell resistance. Overall, the data in this report suggest that the \([2\mu\text{M curcumin} + 2\mu\text{M sildenafil} + 2\mu\text{M regorafenib}]\) drug combination is a great choice for a mechanistic cell killing.

In terms of the future direction of this study, the Dent laboratory will have to look into the proto-oncogene tyrosine-protein kinase Src. Src is short for sarcoma and is responsible for phosphorylating certain tyrosine residues on other proteins. More specifically, there are two major phosphorylation sites on Src: one is at tyrosine 416 (or Y416), the other at tyrosine 527 (Y527).\textsuperscript{95} The Dent laboratory should turn their attention to the activity of Src by look at these phosphorylation sites. The animal studies have successful results and the next step is to promote clinical trials on human so far as the \([2\mu\text{M curcumin} + 2\mu\text{M sildenafil} + 2\mu\text{M regorafenib}]\) drug combination are given at clinically suitable levels. More experimentation on different types of colorectal cancer cell lines is also need to see how far the \([2\mu\text{M curcumin} + 2\mu\text{M sildenafil} + 2\mu\text{M regorafenib}]\) drug combination can go in treatment.
Figure 45. Curcumin, sildenafil and regorafenib drug combination interact synergistically to kill colorectal tumor cells^{11}

Exposure to [2μM curcumin + 2μM sildenafil + 2μM regorafenib] increases reactive oxygen species (ROS) and reactive nitrogen species (RNS) in colorectal tumor cells that induces DNA damage and inactivates chaperone proteins. This, in turn, inactivates cyto-protective proteins and increases apoptotic. Concurrently, DNA damage increases the activity of AMPK, deactivates mToR, and activates ATG13 which then stimulates autophagosome production. The [2μM curcumin + 2μM sildenafil + 2μM regorafenib] drug combination increases cytotoxicity which in turn activates CD95 and ultimately leads to cell death.
Conclusion

To conclude, this study demonstrated that curcumin and sildenafil work alongside the multi-kinase inhibitor regorafenib as a potent targeted therapy treatment. This drug combination effectively killed colorectal cancer cells in vitro as well as in vivo in mice via stimulating endoplasmic reticulum stress and increasing reactive oxygen/nitrogen species. These processes ultimately led to autophagosome and autophagolysosome formation and apoptosis.
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