FAT-RELATED PATHWAYS MAY REPRESENT THERAPEUTIC TARGETS FOR MULTIPLE MYELOMA TREATMENT

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Submitted to the

Faculty of the College of Arts and Sciences

of American University

in Partial Fulfillment of

the Requirements for the Degree

of Master of Science

In

Biology

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Dean of the College of Arts and Sciences

December 9, 2011
Date

2011
American University
Washington, D.C. 20016

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ABSTRACT

Multiple myeloma is a blood cancer for which few therapies exist, most of which are limited by toxicity and tumor resistance. Fatty acid synthase, an enzyme which catalyzes the conversion of carbohydrate to fatty acids, is highly expressed in multiple myeloma, among other cancers. This research investigated the potential of orlistat, a fatty acid synthase inhibitor, as a multiple myeloma therapeutic in vitro. In vitro, orlistat reduced proliferation of multiple myeloma cells, induced apoptosis, slowed progression through the cell cycle, and altered expression of survival and mitotic genes.

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CHAPTER 1

INTRODUCTION

Cancer

Cancer is a general term for neoplastic growth, originating from a single cell, characterized by genetic alterations, acquirement of traits unlike those of its normal precursor population, and eventually metastasis (Hanahan & Weinberg, 2000). These events are classically modeled as initiation, promotion, and progression. Initiated cells proliferate during promotion; promoters facilitate additional mutation, proliferation, and the phenotypic alterations referenced above.

Cancers account for between 22.7% and 23.2% of deaths in the United States annually between 1999 and 2007, second only to heart disease as the most common cause of death (J Xu, KD Kochanek, SL Murphy, 2010). There are over 100 different cancers, many of which can be further sub-grouped by different tumor characteristics (Hanahan & Weinberg, 2000). Cancers are neoplastic growths of polyclonal populations (i.e.: cancers are marked by genetic instability and undergo clonal expansion) of cells undergoing up-regulated replication and unrestrained by senescence or apoptosis. Tumorigenesis is a multi-step process beginning with initiation (Hanahan & Weinberg, 2000). The initiating mutations may be due to DNA damage caused by a chemical stressor, radiation, or a virus and effect proto-oncogenes and/or tumor suppressor genes. A proto-oncogene, once mutated is termed simply an oncogene, and increases expression of a neoplastic

trait, such as proliferation. A tumor suppressor gene codes for proteins that protect normal cells from neoplastic hijacking; when mutated, the cell loses a protection against conversion (Hanahan & Weinberg, 2000; Kuehl & Bergsagel, 2002). Promoters, substances supporting the neoplasm, facilitate malignant phenotype innovation through genetic and epigenetic events (Kridel, Axelrod, Rozenkrantz, & Smith, 2004; Pemble, Johnson, Kridel, & Lowther, 2007). The necessary and sufficient phenotypic changes are independence from cell cycle control (growth signaling/suppression); capacity for clonal expansion (inhibited apoptosis and senescence); angiogenic properties (growth of new blood vessels); and metastatic capacity (ability to spread into other tissues). As the clonal neoplasm passes through promotion, the up-regulated cell cycle and error-prone replication creates a polyclonal tumor, where selection favors the cells with favorable mutations (i.e.: contributing an even more pro-neoplastic phenotype). Conversion marks neoplastic 'maturity,' where the cancer has achieved the necessary and sufficient mutations for malignancy; autonomous growth and immune system evasion ensues, with the malignancy invading nearby tissue (basement membrane penetration), with metastatic cells entering the circulatory system, capable of seeding tumors elsewhere in the host.

Inflammation is a response to tissue injury, but can contribute to cancer when dysregulated (Balkwill & Mantovani, 2001; Coussens & Werb, 2002). During an inflammatory response, leukocytes migrate to the damaged tissue, attack pathogens and mediate repair of the damaged cells, extra-cellular matrix, and vasculature. Homeostatic inflammation resolves when the tissue is repaired. Chronic inflammation can exert powerful mutagenic and cancer promoting effects—the presence of inflammatory signals in the tumor microenvironment can contribute to the cancer's metastatic phenotype.

Cytokines and growth factors secreted by inflammatory cells (e.g.: interleukin (IL)-6, tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF) are among those important in multiple myeloma progression) contribute to tumor cell replication, resistance to apoptosis, angiogenesis, and migration (Balkwill & Mantovani, 2001; Coussens & Werb, 2002; Hayashi, Hideshima, & Anderson, 2003; Lauta, 2003).

B-Cells

B-cells are antibody producing cells forming part of the adaptive immune system (Abbas, Lichtman, & Pillai, 2007; Martin & Kearney, 2000; Meffre, Casellas, & Nussenzweig, 2000; K. Schubart et al., 2001). As part of the adaptive immune system, they convey upon the human body the capacity to mount an immune response to a myriad of pathogens, and shortening response time to re-invasion by retaining a 'memory' of previous pathogens. B-cells originate from hematopoietic stem cells in the bone marrow and undergo recombination to become immature B-cells. Immature B-cells localize through the circulatory system to the spleen, where they undergo additional recombination to produce specific antibodies, making them mature, naive B-cells. Naive B-cells are localized to the spleen and lymph nodes; upon activation (i.e.: antigen binding), they can localize to the site of an immune response.

Antibodies are protein structures that are produced by B-cells to mediate an adaptive immune response (Abbas et al., 2007). These receptors exist in two forms: bound to the membrane of the producing B-cell or circulating. Antibodies exhibit astounding variability for recognition of antigens, which may be small chemical molecules or macromolecules, and specificity for primary and higher-level structure.

Antibodies share a basic ‘Y’ shaped structure, comprised of two heavy chains positioned such that the C-terminal globular domains of each are adjacent (the Fc region of each chain) with a ‘hinge’ in each heavy chain (the vertex of the ‘Y’), and two additional globular domains on the N-terminal of the protein. Next to the two N-terminal domains of each heavy chain is a light chain (2 globular domains in length), forming the Fab region (‘arms’ of the ‘Y’). The N-terminal globular domain of each chain is the antigen binding site, the variable (V) region. The C-terminals of the heavy chains form the tailpiece of the complex. Hydrogen and disulfide bonds maintain the polypeptides in their secondary, tertiary, and quaternary structures. The V-region of the complex mediates antigen binding, while signal transduction (i.e.: the effector function, effecting an immune response) is mediated by the tailpiece. While antibodies certainly vary in their V-regions, they also vary in their heavy chain isoforms, and can be classified accordingly; the ‘Y’ structure described above is generally conserved across all isoforms.

Multiple Myeloma

Multiple myeloma (MM) is malignancy of B-cells in which primary tumors localize to the bone marrow (BM), and are characterized by their secretion of the unique antibody called Bence-Jones protein (Sirohi & Powles, 2004). The tumor microenvironment (MM tumor and adjacent BM stromal cells (BMSC), osteoclasts, and BM endothelial cells (BMEC)) is integral in supporting the growing tumor, through bidirectional paracrine signaling, during promotion (Hayashi et al., 2003; Lauta, 2003; Zhang, Klein, & Bataille, 1989). To that end, MM is characterized by an inflammatory microenvironment, with interleukin (IL)-6 secreted by neighboring osteoclasts, BMSCs,

and BMECs; nuclear factor (NF)-κB is a prominent inflammatory pathway up-regulated in MM and in surrounding BMSCs. During progression, the neoplasms become independent of BMSC signals and frequently become aneuploid.

5 year survival for MM patients is 35% (Jemal et al., 2009). Few risk factors have been identified for MM; the established risk factors are: age, male sex, black race, family history, and monoclonal gammopathy of undetermined significance (MGUS; a condition that predisposes the patient to MM) (Alexander et al., 2007). Many conventional and novel therapeutics used to treat MM are limited by the toxicity of their side-effects.

Obesity as a MM Risk Factor

Obesity is emerging in the epidemiological literature as a risk factor for MM (Alexander et al., 2007). Obesity is defined as having body mass index (BMI, the quotient of body mass in kg over the square of height in meters) of 30 or greater for adults; increased adipose tissue volume is generally assumed to be responsible for increased BMI (Calle & Kaaks, 2004). As the volume of adipose tissue increases, incidences of many diseases increase, including cardiovascular disease, diabetes, and many cancers. Aside from the accumulation of lipids within adipocytes, physiological changes occur in obesity that may contribute to these diseases. Research over the past 20 years has identified functions of adipose tissue other than as an insulator against thermal, physical, and caloric shocks; that it has a role as an endocrine organ is now well established, however the breadth of processes it might govern remains unclear (Fantuzzi, 2005; P Trayhurn, 2007; Paul Trayhurn & I. S. Wood, 2004). In obesity macrophages are recruited to adipose tissue, altering the signals secreted in favor of inflammatory

molecules, including IL-6 and TNFα (Tilg & Moschen, 2006). As a result, obese patients have chronic, systemic elevated levels of inflammatory factors (Fantuzzi, 2005). This has been hypothesized as a mechanism by which obesity contributes to many linked pathologies, including MM (MacInnis, English, Hopper, & Giles, 2005).

The link between fat metabolism and cancers is not limited to endocrine mechanisms; fat metabolism within the tumor may contribute to the pathology (Javier a Menendez & Ruth Lupu, 2007; W.-qin Wang, Zhao, H.-yan Wang, & Liang, 2008). Glucose taken up by the tumor may enter the fatty acid synthase pathway, where it is incorporated into fatty acids. Fatty acids are then used for energy, structure, and communication. Interestingly, tumor localized fat metabolism may not be independent of remote fat depots. In a cohort study of colon-cancer patient survival, fatty acid synthase over-expression was associated with increased all-cause and colon-cancer-specific mortality hazard-ratios among patients with BMI at least 27.5, whereas hazard-ratios were slightly lower among patients with lower BMI (Ogino et al., 2008).

Fatty Acid Synthase

Fatty acid synthase (FASN) is an enzyme heterodimer complex which catalyzes the condensation reaction of the substrates acetyl-CoA, malonyl-CoA, and NADPH yielding the fatty acid (FA) palmitate, and is the mechanism of lipogenesis (Chirala & Wakil, 2004; Kusakabe et al., 2000; J. A. Menendez, Luciano Vellon, & Ruth Lupu, 2005). Once synthesized, palmitate is stored as triacylglycerol for later oxidization or is incorporated into cell structures. In non-neoplastic cells, FASN expression is regulated by nutrition and steroid hormones, and its expression is restricted to breast, endometrial,

adipose, and hepatic cells (Kusakabe et al., 2000; Javier a Menendez & Ruth Lupu, 2007). Under nutritional control, FASN is up-regulated by insulin (i.e.: high carbohydrate) and down-regulated in the presence of glucagon and/or leptin (marking the absence of circulating carbohydrate and/or increased dietary FAs, respectively). Growth signal control is mediated by steroid hormones and growth factors.

While FASN is silent in most normal adult tissues, many neoplastic cells exhibit marked over-expression of FASN, earning it attention as a novel therapeutic target (Kusakabe et al., 2000; Javier a Menendez & Ruth Lupu, 2007). In vitro FASN inhibition in breast, colon, melanoma, multiple myeloma, prostate, and stomach cancer cells has resulted in substantial reductions in proliferation (Carvalho et al., 2008; Knowles, Yang, Osterman, & Smith, 2008; Kridel et al., 2004; Kuhajda et al., 1994; Li et al., 2001; J. A. Menendez, Luciano Vellon, et al., 2005; W.-qin Wang et al., 2008). Unlike many other cancer therapeutics, FASN inhibitors demonstrate selectivity. This can be explained by several properties of the FASN pathway: as discussed above, few normal cell phenotypes constitutively express FASN; neoplastic FASN expression is substantially higher than normal levels; dietary fatty acids are favored over lipogenic palmitate as cell structure substrates; and the rapid division of transformed cells increases their sensitivity to substrate deprivation (Kusakabe et al., 2000; Javier a Menendez & Ruth Lupu, 2007).

Several mechanisms have been proposed through which FASN inhibition may in turn inhibit cancer cell proliferation. Because proliferation is the net result of mitosis and cell death, it can be inhibited through slowing the cell cycle and/or increasing apoptosis. In breast cancer cells, FASN inhibition led to a dose-dependent decrease in glycerol

synthesis, and the vast majority of FASN products are synthesized into membrane phospholipids (Kuhajda et al., 1994; Pizer, F. D. Wood, Pasternack, & Kuhajda, 1996). Inhibition of phosphatidylcholine production (e.g.: FASN inhibition leading to a reduction in intracellular palmitate concentration) can induce apoptosis (Cui & Houweling, 2002). Evidence also points to ceremide-mediated apoptosis. Knowles, et al, found FASN inhibition induced caspase-8, inhibited the mTOR pathway, and induced TRAIL sensitivity in tumor cells (Knowles et al., 2008). FASN blockade may induce cell-cycle arrest by disrupting lipid-rafts, blocking mitogenic signals (Javier a Menendez & Ruth Lupu, 2007).

Treatments

Several promising drug therapies—notably bortezomib, dexamethazone, and thalidomide—exist for MM, however, like many cancer treatments, they are limited by intolerable side-effects and/or induced resistance (Schmidmaier, Mörsdorf, Baumann, Emmerich, & Meinhardt, 2006). Genetic instability, rapid replication, and extensive alterations in cell functioning combined with drug treatment yields a tumor microenvironment that is selective for drug resistance (Hayashi et al., 2003). Moreover, the unstable nature of cancers makes drug targeting difficult, and therapies often rely on inhibitors with systemic effects (i.e.: replication inhibitors have non-specific effects).

Orlistat

This project is investigating the anti-neoplastic potential of orlistat (tetrahydrolipstatin), an anti-obesity drug manufactured by Roche and sold in the United States under the names Xenical and Alli. This drug is reportedly an inhibitor of

pancreatic and intestinal lipases (Kridel et al., 2004; Ruth Lupu & Javier a Menendez, 2006). Additionally, in an activity-based assay of serine hydrolases in prostate cancer, orlistat was identified as an irreversible inhibitor of fatty acid synthase and an attractive novel therapeutic. Orlistat induced apoptosis and inhibited proliferation of prostate cancer and murine melanoma cells in vitro and reduced tumor growth/metastases in murine xenograph model (Carvalho et al., 2008; Kridel et al., 2004). Orlistat exhibited a time- and duration-dependent increase in sub-G1 and S-phase cells and decrease in G2-M phase cells, suggesting impairment to membrane synthesis and DNA repair processes leading to apoptosis (J a Menendez, L Vellon, & R Lupu, 2005).

Objectives

Orlistat was hypothesized to inhibit multiple myeloma proliferation by blockade of ectopic endogenous fatty acid synthesis, leading to increased apoptosis and decreased cancer cell proliferation.

The objectives of this study were to:

1. Assess the response of 2 MM cell lines and normal B-cells in terms of proliferation to multiple concentrations of orlistat;
2. Assay the degree to which orlistat effects are FASN-mediated by palmitate rescue study and western blotting of FASN and down-stream proteins;
3. Assay the effects of orlistat treatment on apoptosis and cell cycle by flow cytometry and western blotting of cell cycle and apoptotic proteins ; and
4. Quantify the effects of orlistat treatment on expression of metastatic proteins that have prognostic implication in vivo.

These questions were investigated using the methods described below.

Scope of Impact

Multiple myeloma 5 year survival is 35%, and while deaths-per-100,000-individuals has decreased in the past 20 years, it is decreasing at a much lower rate than cancers generally (Greenberg & Obin, 2006; Jemal et al., 2009). FASN is a promising target of MM treatment, and orlistat is an attractive candidate compound for inclusion in FASN therapy because of its strong effect and the level of FASN dysregulation observed in MM (Kridel et al., 2004; Javier a Menendez & Ruth Lupu, 2007; W.-qin Wang et al., 2008). Moreover, where many MM treatments are limited by the tolerability of their side effects, orlistat, and other FASN inhibitors, promise selectivity because of the differential FASN activity associated with cancers (Kusakabe et al., 2000; W.-qin Wang et al., 2008). The implications of this research extend beyond MM: FASN activity is implicated in many cancers (Kuhajda, 2000).

CHAPTER 2

MATERIALS AND METHODS

Cell culture

To test the effects of orlistat on MM cell proliferation and apoptosis this project use in vitro cell culture models. Because of the aforementioned genetic instability of cancers, and MM in particular, two human MM cell lines procured from ATCC (Manassas, VA)—RPMI 8226, and U266B1 (abbreviated U266)—were used to preclude the possibility that an individual cell line was uniquely sensitive/resistant to a particular treatment. RPMI8226 and U266B1 cultures were grown in RPMI 1640 media (Invitrogen, Carlsbad, CA, catalog # 22400-089) plus 10% fetal bovine serum (Cell generation, Fort Collins, CO, catalog # CGF-0500) plus 1% glutamine (Invitrogen, Carlsbad, CA, catalog # 25030-081) and 1% PenStrep (Invitrogen, Carlsbad, CA, catalog # 15140-122). Primary, normal human cord blood B-cells (Lonza, Walkersville, MD, catalog # 2C-300) were used to test the effect of orlistat on non-cancerous B-cells and to compare gene expression. Normal B-cells were maintained in a modified preparation of supplemented RPMI1640 media described above: 20ml supplemented RPMI1640 plus 0.4mg anti-human IgM (Millipore, Temecula, CA, catalog # AP114) and 1 mg E.coli O111:B4 LPS (Sigma-Aldrich, St. Louis, MO, catalog # L4391-1MG). All cultures were maintained at 5% CO2 and 37°C in a humidified incubator. Cell-lines cultures were

reseeded after approximately 20 passages (i.e.: approximately 60 days); primary cells were cultured 12 days.

Drugs

Orlistat (Sigma, St Louis, MO, catalog # O4139-25MG and Cayman Chemical, Ann Arbor, MI, catalog # 10005426) was dissolved in dimethyl sulfoxide (DMSO) yielding a 40.3 mM solution (initial lot was dissolved to a 38.3 mM solution). Sodium palmitate (Sigma, St Louis, MO, catalog # P9767-5G) was dissolved in ethanol yielding a 18.0 mM solution. Vehicle controls were prepared for experiments. To preclude DMSO-induced effects on proliferation, final DMSO concentration in experiments was limited to 0.2% by volume.

Proliferation Assay

Spectrophotometric proliferation assays were performed using WST-1 (G-Biosciences, St Louis, MO, catalog # 786-212; Roche, Mannheim, Germany, catalog # 11 644 807 001). The assay relies on the reduction of WST-1 tetrazolium salt to formazan, catalyzed by the dehydrogenases of the cells to be assayed, the concentration of which is then measured by absorbance at 450nm and is proportional to cell number (Berridge, Tan, Mccoy, & R. U. I. Wang, 1996; Peskin & Winterbourn, 2000; Tominaga et al., 1999). To perform this assay 10-parts media containing cells are transferred by pipette from culture to a 96-well plate (such that there are between 5x104 and 5x105 cells per well), to which 1-part WST-1 solution is added, followed by incubation at 37°C for 30 min to 4 hours. The plate is shaken and absorbance (A) is measured for each well; proliferation assays are performed in triplicate.

qPCR

Gene expression was measured at the transcriptional level by quantitative polymerase chain reaction (qPCR). qPCR is a technique to compare the relative expression of a target gene, normalized to a housekeeping gene, between two samples (S A Bustin, 2002; Nolan, Hands, & Stephen A Bustin, 2006; VanGuilder, Vrana, & Freeman, 2008). It relies on reverse-transcribing a particular mRNA to a fluorescent labeled cDNA; until the quantity of template/transcript strands exceed the quantity of primers, sample fluorescence will double with each cycle. The cycle at which the quantity of template/transcript strands exceed the quantity of primers (i.e.: point at which the increase in fluorescence will become linear) is inversely related to the quantity of mRNA of the particular gene at the start of the assay, and as such, relative gene expression can be compared (Pfaffl, 2001).

Total RNA was collected from treated cells using a PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA, catalog # 12183018A). Cells were lysed, homogenized, and RNA was collected in a silica membrane, from which it was eluted, treated with Dnase (Invitrogen, Carlsbad, CA, catalog # 12185-010), and stored at -80°C. RNA concentration was measured. RNA template (10 ng/µl) and primers (200 nM) were added to the reaction mix containing SYBR green mix and reverse-transcriptase. The qPCR reactions were prepared using qScript One-Step SYBR Green qRT-PCR Kit (Quanta Biosciences, Gaithersburg, MD, catalog # 95086-200). Primers were procured from realtimeprimers.com for genes of interest.

Western Blotting

Protein expression was measured by western blotting. Proteins were resolved by polyacrylamide gel, transferred to a membrane, blocked with 5% non-fat milk, and then probed with a set of antibodies for visualization.

To collect proteins, cells were pelleted by centrifugation, media removed and washed twice with cold PBS. Cold (4ºC) lysis buffer (preparation of RIPA (Cell Signaling, Danvers, MA, catalog # 9806S) containing Halt protease & phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA, catalog # 1861281)) was added to the pelleted cells, which was then resuspended and incubated 10 minutes at room temperature followed by 35 minutes on ice. Following lysis, the cell-lysis buffer preparations were centrifuged to pellet debris; the supernatants, containing the protein lysates, were removed to a new microfuge tube for storage at -80°C.

Prior to measuring expression of a particular protein, the concentration of total protein in each lysate was established colorimetrically using a bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, catalog # 23225). The BCA assay relies on the reduction of Cu+2 to Cu+1 by peptide bonds in alkaline conditions; a concentration dependent reaction, the quantity of Cu+1 produced is linear in relationship to the concentration of protein between 20 and 2,000µg/ml (Noble, Knight, Reason, Di Matola, & M. J. A. Bailey, 2007; Schoel, Welzel, & Kaufmann, 1995). BCA is chelated by Cu+1, yielding a purple complex, the concentration of which is determined by absorbance at 562 nm. 20µl of 9 bovine serum albumin (BSA) standards and lysis buffer blank are transferred by pipette to a 96-well plate. 1µl lysate plus 19µl lysis buffer were transferred to the 96-well plate, such that each lysate sample were in triplicate. BCA

working reagent was prepared and 200µl was added to each well. The 96-well plate was incubated at 37°C for 30 minutes and absorbance at 562nm was measured. The absorbance of the BSA protein standards establishes the standard curve relating protein concentration and absorbance; the protein concentration of each sample was determined using the standard curve and the observed absorbance.

Upon determination of lysate protein concentration, proteins were diluted in lysis buffer such that 20µg of total protein for each sample are loaded on a NuPAGE 4-12% Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA, catalog # NP0336BOX). 10 µl Novex Sharp Standard (protein standard) was loaded in lane 1 one of each gel (Invitrogen, Carlsbad, CA, catalog # LC5800), with bands at 260, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10, and 3.5 kDa.

Gels were electrophoresed at 200 volts for 35 minutes (BioRad PowerPAC 300). Gels were removed from the apparatus and electrophoretically transferred using PVDF membrane (Invitrogen, Carlsbad, CA, catalog # LC2005) and transfer buffer (Invitrogen, Carlsbad, CA, catalog # NP0006) at 25 volts for 80 minutes. Following transfer, the membranes were blocked in 5% non-fat milk prepared with TBST (Amresco, Solon, OH, catalog # 0788-2PK) overnight at 4°C. Membranes were probed with primary antibody at concentrations ranging from 0.1% to 0.2% for each desired protein, washed, probed with secondary antibody conjugated with horseradish peroxidase (HRP), washed, and then treated with HRP substrate (Thermo Scientific, Rockford, IL, catalog # 34076) for visualization. Membranes were visualized using UVP ChemiDoc-It Imaging System with Vision Works software. Membrane images were processed (i.e.: rotated, inverted,

despeckled) using GNU Image Manipulation Program and densitometry was performed using ImageJ.

Flow Cytometry

Apoptosis and cell cycle were assayed by flow cytometry. Flow cytometry is a protocol to mechanize cell counting and sorting by set of desired phenotypic markers. Cells are suspended in medium and streamed in droplets containing one cell past a detector that directs the stream to a collector corresponding to the phenotype of the cell in the droplet. Markers are fluorescent and may be proteins (i.e.: annexin V conjugated to FITC) or intercalating agents (i.e.: propidium iodide). Annexin V-FITC was used to detect apoptosis by flow cytometry. This assay relies on the apoptosis-specific localization of phosphatidylserine to the extracellular surface of the cell membrane, which annexin V binds (Koopman et al., 1994). Necrotic cells were differentiated from apoptotic cells, both of which bind annexin V, by treatment with propidium iodide (PI), which stains necrotic cells. Similarly, because PI intercalates DNA, the quantity of DNA within a cell—a marker of its position in the cell cycle—is related to the degree of PI staining.

Apoptosis was assayed using annexin V-FITC (FITC Annexin V Apoptosis Detection Kit II; BD Pharmingen, Franklin Lakes, NJ, catalog # 556570). Following treatment, cells were washed with cold (i.e.: 4ºC) PBS twice. Binding buffer was added to each sample to resuspend cells such that final cell concentration was 1 million cells per ml binding buffer; for each sample, 100 µl cell suspension were transferred to a new culture tube, to which 5 µl FITC Annexin V solution and 5 µl PI were added. Samples

were vortexed and incubated at room teperature, shielded from light, for 15 minutes. 400 µl binding buffer was added to each sample. Flow cytometric data were collected by Dr. Kathleen DeCicco-Skinner.

Cell cycle was assayed by PI staining. Following treatment, cells were washed in PBS and suspended in PBS such that final cell density was 10-20 million cells per ml. For each sample, 100 µl of cell suspension were transferred to a new culture tube and incubated in an ice bath for 15 minutes. To fix the cells, 1 ml of 70% ethanol was added to each sample, which was then gently vortexed and stored at 4ºC overnight. Cells were pelleted and ethanol solution was removed. 100 µl RNase A dissolved to 1000 units/ml (Sigma, St. Louis, MO, Catalog # R-4875) was added to each sample and incubated at 37ºC for 20 minutes. 500 µl PI (50 µg/ml) was added to each sample and incubated at 4ºC, shielded from light, for 30 minutes. Flow cytometric data were collected by Dr. Kathleen DeCicco-Skinner.

Experiments

Inhibitory Effects of Orlistat

The effects of orlistat on MM cells were assessed by measuring proliferation (figure 1). MM cells were cultured with orlistat at 5µM,10 µM, 20 µM, and DMSO control. Proliferation was measured after 48h, 72h, and 96h by colorimetry. Retreatment with Orlistat occurred daily. A dose- and time-dependent decrease in proliferation was expected. Primary non-cancerous B-cells were used as a control (described below).

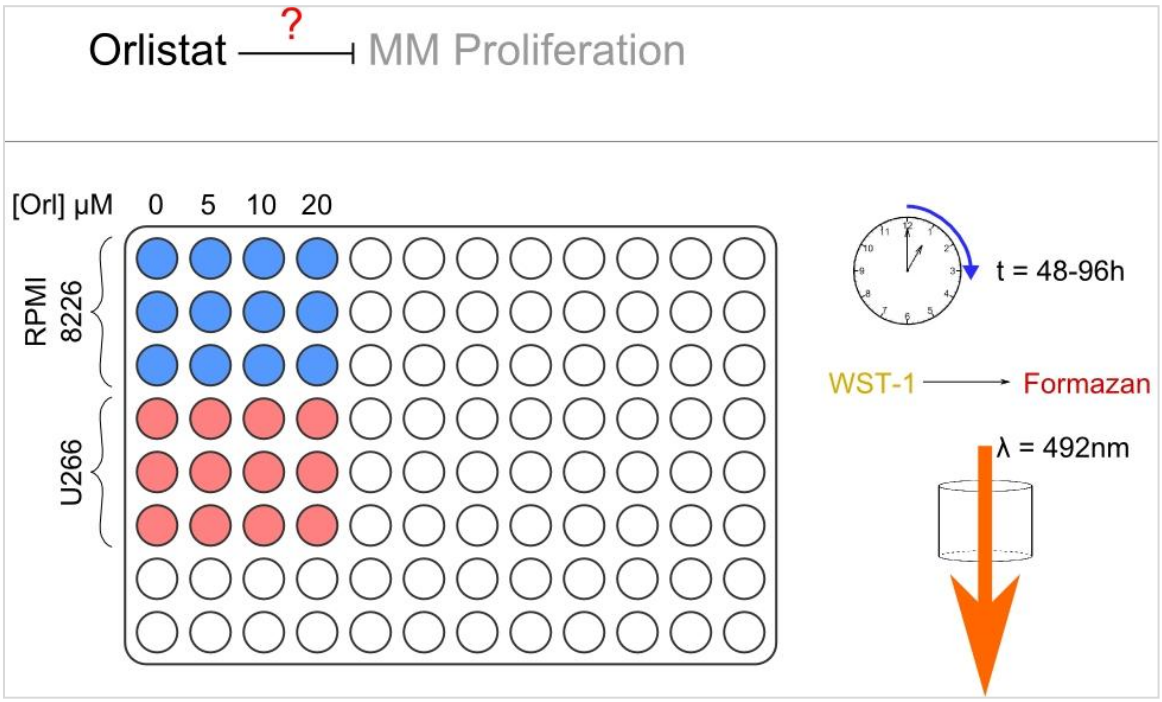

Figure 1. Schematic of Proliferation Assay. Multiple myeloma cell-lines RPMI8226 and U266 were cultured with orlistat at 0, 5, 10, or 20 µM for 48, 72, or 96 hours. Reduction of WST-1 to formazan was measured colorimetrically; absorbance at 492nm was proportional to cell density. This setup was also used in assaying the effect of orlistat on primary B-cells.

Palmitate-Rescue

To test the hypothesis that orlistat's inhibitory effect on MM proliferation is mediated through FASN blockade (thus decreased endogenous palmitate production), a palmitate-rescue study was performed (figure 2). MM cells were cultured with orlistat in 0µM, 10µM, and 20µM concentrations, overlaid with palmitate in 0µM, 7.5µM, and 15µM concentrations. Cultures were incubated for 48h, with retreatment at 24h. All treatment groups were treated such that each contains equal concentrations of DMSO and ethanol vehicles. Proliferation was assayed colorimetrically as discussed above. Palmitate was expected to have a dose-dependent rescue effect on orlistat-induced MM inhibition, adjusted for MM dose.

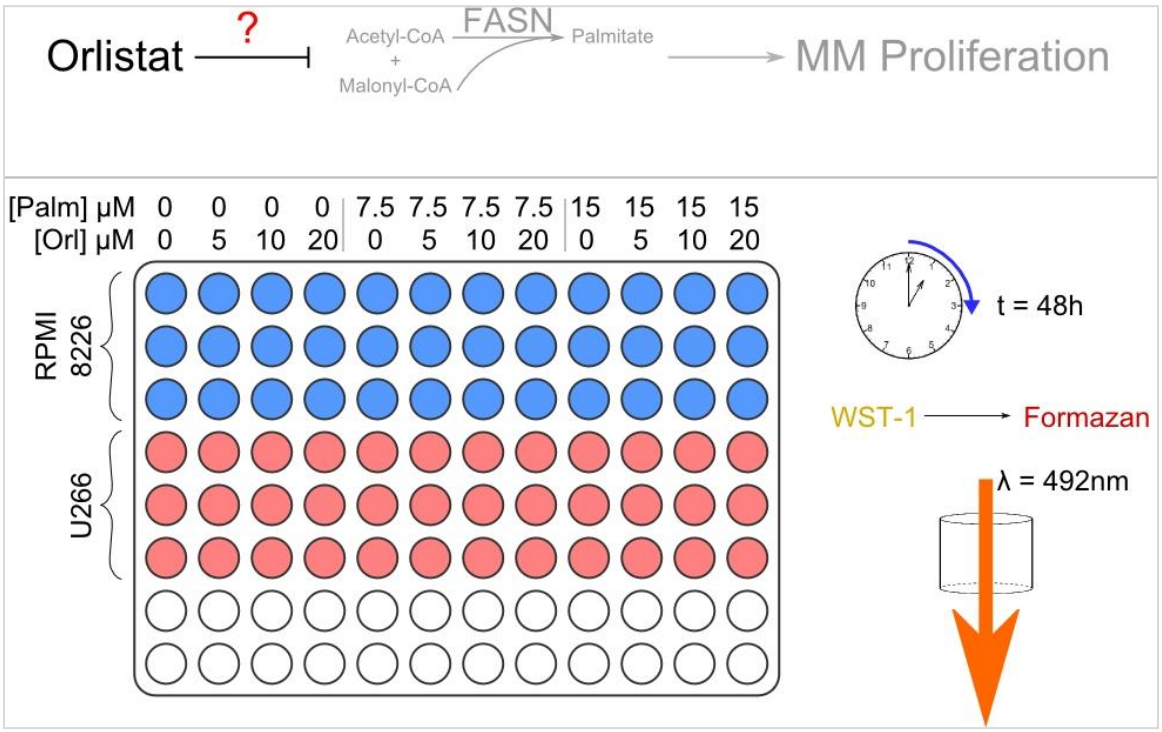

Figure 2. Schematic of Palmitate-Rescue Assay. Multiple myeloma cell-lines RPMI8226 and U266 were cultured with orlistat at 0, 5, 10, or 20 µM and palmitate at 0, 3.75 (not shown in this schematic), 7.5, or 15 µM for 48 hours. Reduction of WST-1 to formazan was measured colorimetrically; absorbance at 492nm was proportional to cell density.

Orlistat Specificity

It was necessary to determine whether the observed orlistat-induced inhibition of MM proliferation was due to the drug acting on a character specific to the neoplastic phenotype rather than broad toxicity to B-cells, including non-transformed B-cells. Normal, primary B-cells were cultured and treated with orlistat at DMSO control, 5µM, 10µM, and 20µM for 48h, 72h, and 96h, following the proliferation assay protocol above. Proliferation of normal B-cells was expected to be equal between the treatment and control groups.

FASN protein expression in normal primary B-cells and MM cells was compared by western blotting. FASN expression was expected to be greater in MM cells.

Cytotoxic and Cytostatic Effects of Orlistat

Orlistat-induced inhibition of proliferation may be due to cytostatic and/or cytotoxic effects. To elucidate the degree of involvement of each, cells were treated with varying concentrations of orlistat, stained with PI, and counted by flow cytometry (figure 3). As PI staining reflects the DNA content of the stained cell, where 2n indicates a cell

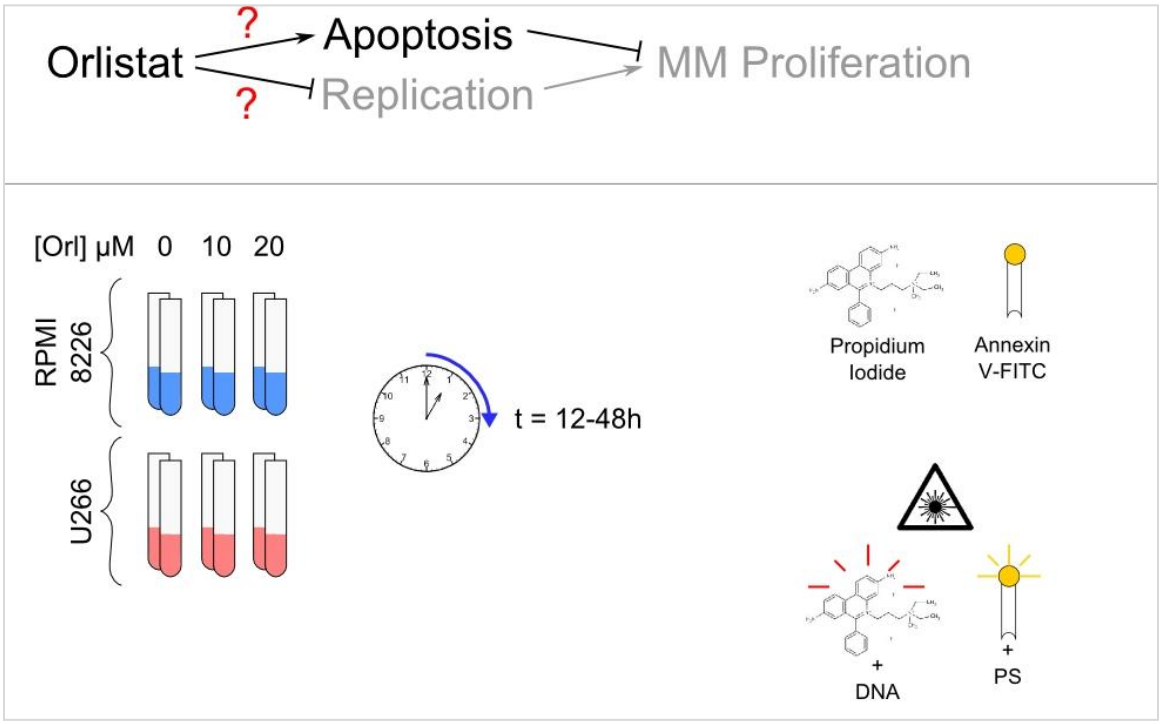

Figure 3. Schematic of Apoptosis and Cell-Cycle Assays. Multiple myeloma cell-lines RPMI8226 and U266 were cultured with orlistat at 0, 10, or 20 μM for 12 (cell-cycle assay only), 24, or 48 hours. Cells were singly stained with propidium iodide (DNA label) in the cell-cycle assay or dual stained with propidium iodide and annexin V-FITC (phosphatidylserine (PS) label) in the apoptosis assay and measured by flow cytometry.

in G_0/G_1, 2n-4n indicates a cell in S, 4n indicates a cell in G_2-M, and less than 2n indicates an apoptotic cell, distributions of stained cells were compared between treatment groups and controls to determine changes in the cell cycle. The S and apoptotic fractions were expected to increase in orlistat treated cells, while the G_2-M fraction was

expected to decrease. An increase in S and decrease in G_2-M fractions are consistent with cell cycle inhibition, while an increase in the apoptotic fraction suggest decreased proliferation due to apoptosis. An annexin V assay further quantified the changes in frequency of apoptotic cells due to orlistat treatment.

Apoptotic and cell cycle gene expression was examined at the protein level by western blot and transcription level by qPCR (figure 4). In orlistat treated cells where

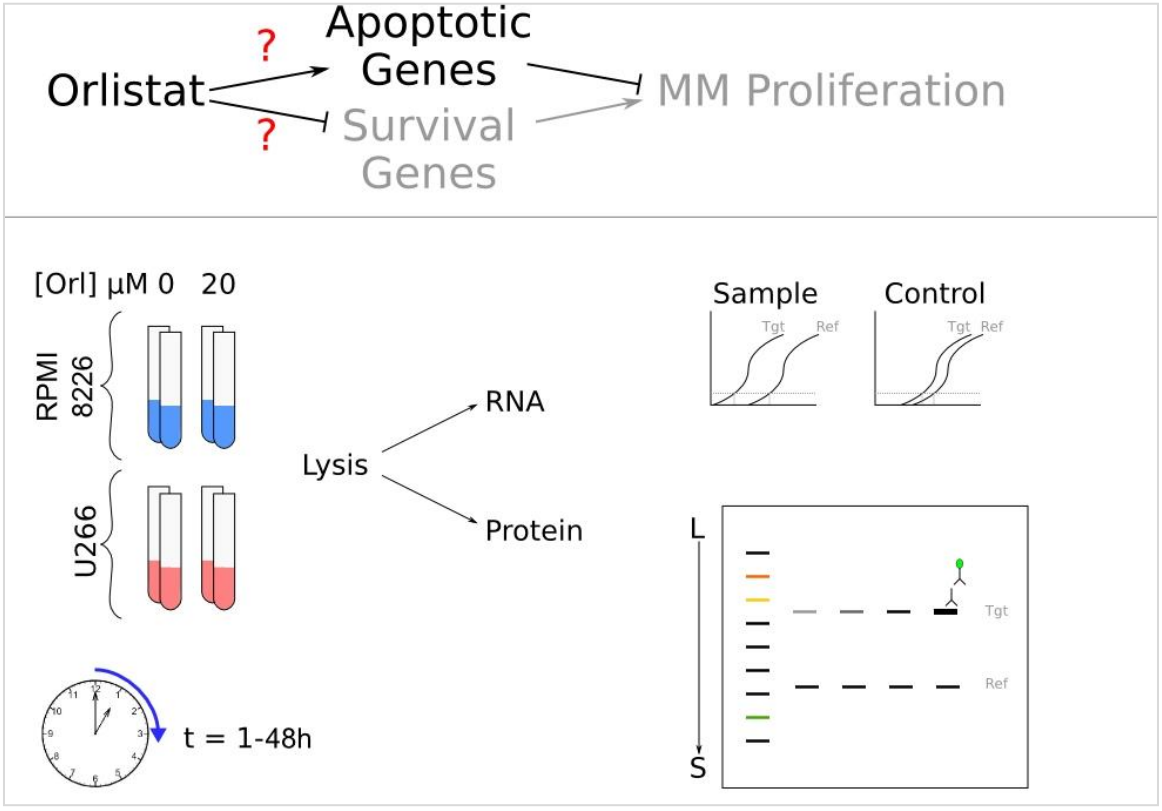

Figure 4. Schematic of Gene Expression Assays. Multiple myeloma cell-lines RPMI8226 and U266 were cultured with orlistat at 0 or 20 µM for time-points between 0 and 48 hours. Cells were lysed and RNA or protein collected. Target (Tgt) and reference (Ref) gene expression was assayed by qPCR (mRNA) and/or Western blot (protein).

proliferation inhibition is mediated by apoptosis, apoptotic gene expression (e.g.: caspase 9) was expected to increase, while survival gene expression (e.g.: Akt, Bcl-2) was

expected to decrease. In orlistat treated cells where proliferation is inhibited by cell cycle interruption, cell cycle gene expression (e.g.: CDK4, E2F1) was expected to be reduced.

Tumor Microenvironment

Several genes have significant clinical implications vis-à-vis tumor aggressiveness. The expression of these genes were assayed by qPCR and/or western blot. Orlistat treatment was expected to decrease expression of genes associated with tumor aggressiveness (i.e.: IL-6, NF-κB, TNFα).

Statistics

Gene Expression Ratio

Relative gene transcription data was analyzed computing the expression ratio as defined by the Pfaffl equation (Equation 1),

$$expressionratio = \frac{\left(E_{target}\right)^{\Delta CP_{target}(control-sample)}}{\left(E_{reference}\right)^{\Delta CP_{reference}(control-sample)}}, \quad (1)$$

The Pfaffl equation relates the difference in cycle number (ΔCP) at which the reaction crosses the critical point (CP) such that PCR reaction product increases linearly as a function of subsequent cycles for a target and reference gene in control and sample groups (Pfaffl, 2001). For this analysis, efficiency (E) was assumed to be 100% (i.e.: one PCR cycle was assumed to double the quantity of target mRNA), the reference gene was beta Actin (ACTB), and the reference sample was the negative control (i.e.: 0 hours).

Linear Models of Colorimetric Data

Colorimetric data were adjusted for the value of the blank (i.e.: background) absorbance, then scaled as a percentage of the negative control. The data were analyzed

using SPSS Statistics (releases 17 and 18, IBM Corporation). The data were split along two variables: trial and time-point. Grouping output by trial prevents conflation of subtle differences between iterations; for example, early trials use cells from an early passage while subsequent trials use cells from later passages, and differences in media, particularly inter-lot variability in the composition of fetal bovine serum (FBS), would violate the independence assumption. Grouping output by time-point further resolves an independence-assumption violation: the absorbance measurements collected at each time-point were from discrete samples.

Models were fit using the analysis of covariance (ANCOVA) procedure. The ANCOVA procedure is conceptually a combination of an analysis of variance (ANOVA) and a linear regression, where the response variable is predicted by one or more nominal variable(s) and one or more scale variable(s). The full model describes the data falling on separate lines (i.e.: the nominal variable convey different intercepts) with distinct slopes (i.e.: the magnitude of the effect of the scale variable differs with the nominal variable). In the general form, the ANCOVA model is written $\hat{y}_i = \beta_0 + \beta_1 x_i + \beta_2 I_i + \beta_3 x_i I_i$, where $\hat{y}_i$ is the predicted value of the response variable for the i^{th} observation, x_i is the value of the scale variable x for the i^{th} observation, and I_i is the value of the indicator variable I for the i^{th} observation. The full model was fit and significance tested where the null hypothesis, H_0, is given: $\beta_0 = \beta_1 = \beta_2 = \beta_3 = 0$ and the research hypothesis, H_A, is given: at least 1 $\beta_n \neq 0$; the null hypothesis is rejected in favor of the alternate hypothesis when $p<0.05$. The significance of the terms of the model were then evaluated: when an interaction term was not significant ($p>0.05$), a reduced model was fit consisting of the nominal and scale terms but not an interaction term; when both an interaction term and variable term were

not significant a reduced model was fit using only the significant variable term. Note that when a variable term was found not to be significant, but the interaction term was significant, the variable term was left in the model to satisfy the hierarchical principle. Reduced models were tested for significance as described above. A reduced model describes data on separate lines but equal slopes (i.e.: the response variable is predicted by the category variable and the scale variable, but the effect of the scale variable is uniform across all categories), and can be further reduced to describe data falling on the same line (i.e.: category variable does not predict the response variable). The full model was then tested against the reduced model using a sum-of-squares F-test, and the reduced model was rejected in favor of the full model when $p<0.05$.

CHAPTER 3

RESULTS

Proliferation

Orlistat has been demonstrated to reduce the number of cancer cells in numerous in vivo and in vitro models and fatty acid synthase (FASN) inhibitors have been demonstrated to reduce the number of multiple myeloma (MM) cells in vivo. To test the hypothesis that orlistat inhibits (i.e.: reduces) MM cell proliferation, an in vitro cell proliferation assay was employed using the human MM cell lines RPMI8226 and U266. These cells were treated with orlistat at doses between 0 and 20 μM and cell proliferation was determined using a colorimetric assay. Colorimetric data were adjusted for the value of the blank (i.e.: background) absorbance, scaled as a percentage of negative control, and fit to a linear model. Proliferation was greatly reduced in orlistat treated groups (figure 5), were the proliferation of the treatment group was lower than that of the control group by 0.7 % to 4.3 % ($p<0.01$) per μM orlistat. The inhibitory effect was greater in RPMI8226 cells compared to U266 cells.

Primary B-cells

The hypothesis that orlistat's inhibitory effect on MM cells is mediated by a mechanism unique to cancer cells was tested in an in vitro cell culture model using primary normal B-cells from human cord blood. In this model, the primary B-cells were

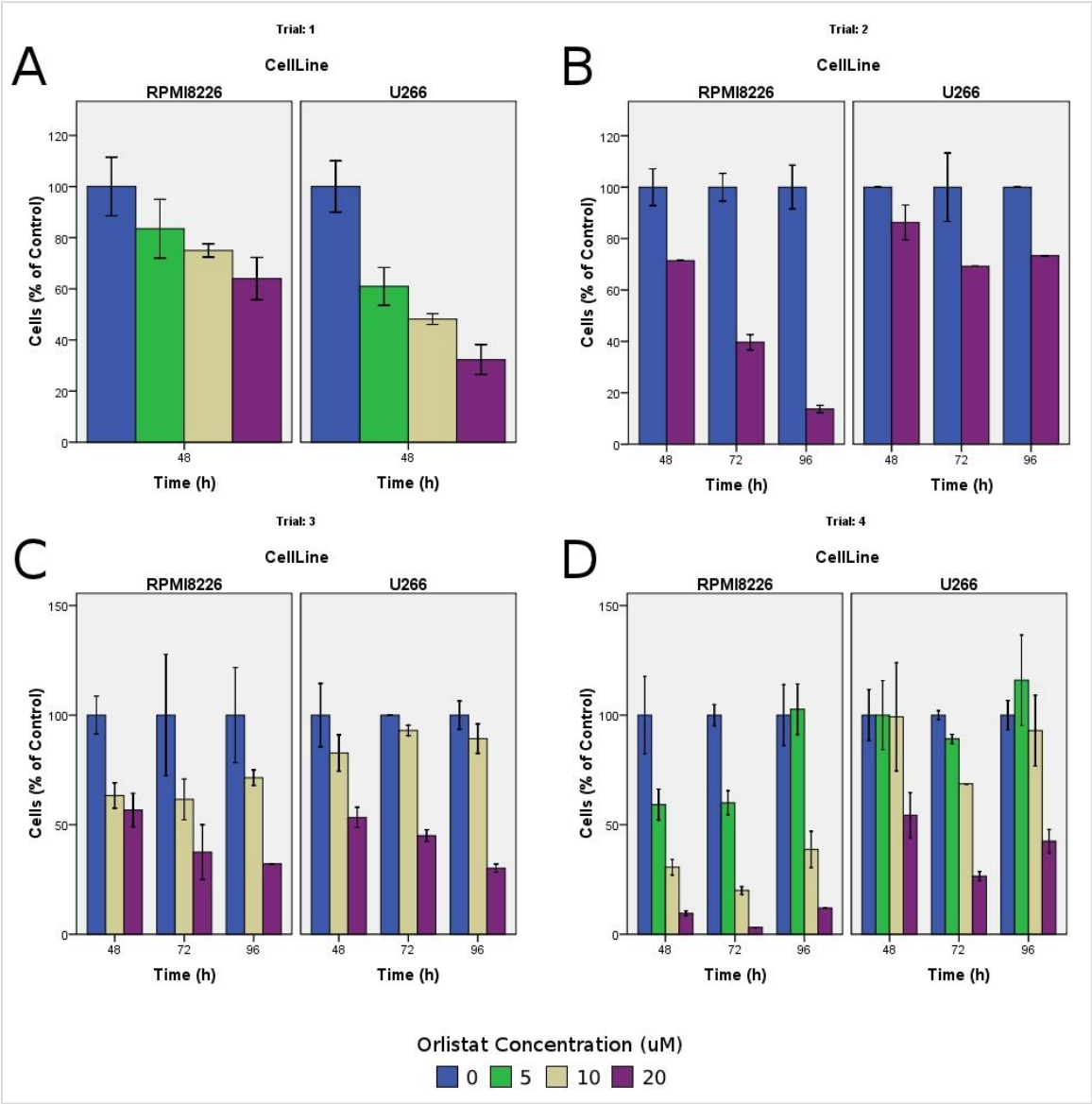

Figure 5. Effect of Orlistat On Multiple Myeloma (MM) Cell Proliferation. MM cell lines RPMI8226 and U266 were cultured for 48, 72, and 96 hours with orlistat at 0, 5, 10, and 20 μM. Proliferation was measured colorimetrically and stated in terms of the negative control. Values are treatment group mean ± SD. N = 3.

treated with orlistat at doses used in the proliferation experiments. It was predicted that orlistat would not affect cell proliferation. Relative cell counts were measured colorimetrically. Colorimetric data were adjusted for the value of the blank (i.e.:

background) absorbance, scaled as a percentage of negative control, and fit to a linear model. At the 48 hour and 72 hour time-points, mean proliferation is higher in the treatment groups than control (figure 6), and at the 96 hour time-point, mean proliferation was lower in the treatment groups than control. However, when these data were fit to a general linear model and tested for equality of means (H0: $y=\mu+\varepsilon$ vs. HA: $y\neq\mu+\varepsilon$), the strength of the model was p=0.833, p=0.094, and p=0.390, for time-points 48 hours, 72 hours, and 96 hours,respectively; the observed differences in mean proliferation were not significant at the 5% level.

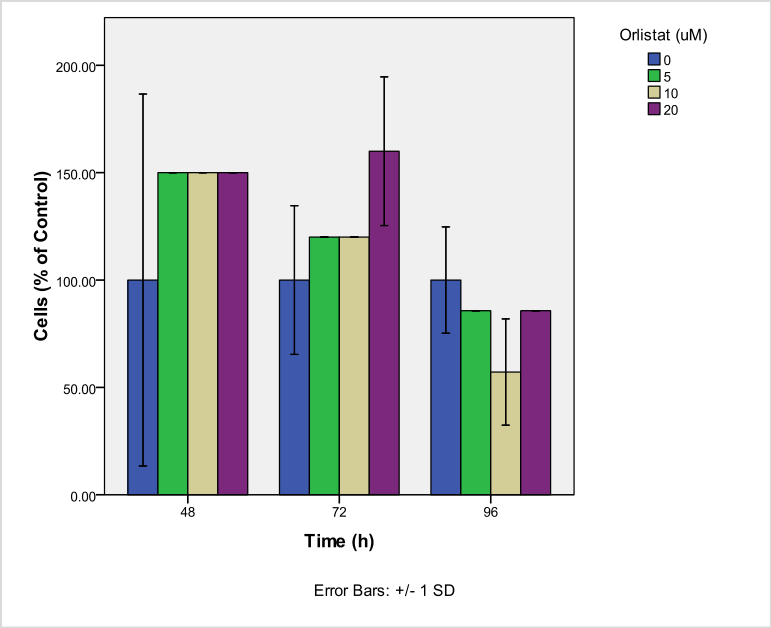

Figure 6. B-cell Proliferation. Primary, normal B-cells were cultured for 48, 72, and 96 hours with orlistat at 0, 5, 10, and 20 μM. Proliferation was measured colorimetrically and stated in terms of the negative control. Values here are treatment group mean ± SD. N = 3.

Palmitate

To test the hypothesis that orlistat's inhibitory effect on MM cells is mediated by FASN inhibition, an in vitro cell proliferation assay was conducted. In this model,

human MM cell lines RPMI8226 and U266 were treated with orlistat at doses used in the proliferation experiments. Palmitate was then added to the cell populations. It was predicted that palmitate would rescue orlistat treated cells (i.e.: reduce the inhibitory effect of orlistat) in a dose-dependent manner. Relative cell counts were measured colorimetrically. Colorimetric data were adjusted for the value of the blank (i.e.: background) absorbance, scaled as a percentage of negative control, and fit to a linear model.

Proliferation is reduced in orlistat treated groups, in a dose-dependent manner, consistent with the results of the proliferation data (figures 7 and 8). Moreover, these data suggest that proliferation is restored with the application of palmitate to some, but not all, orlistat treated groups. The palmitate rescue effect is more robust in RPMI8226 compared to U266.

Apoptosis

To test the hypothesis that orlistat reduces the number of cells in populations by apoptosis (rather than slowing replication), an in vitro cell culture model was used. In this model, human MM cell lines RPMI8226 and U266 were treated with orlistat at doses used in the proliferation experiments for multiple time points. Orlistat was predicted to shift the distribution of cells away from the viable fraction (toward the apoptotic fraction) in a dose-dependent and time-dependent manner. Viable and apoptotic fractions were assayed by Annexin-V- FITC and propidium iodide staining, the intensity of which was measured by flow cytometry.

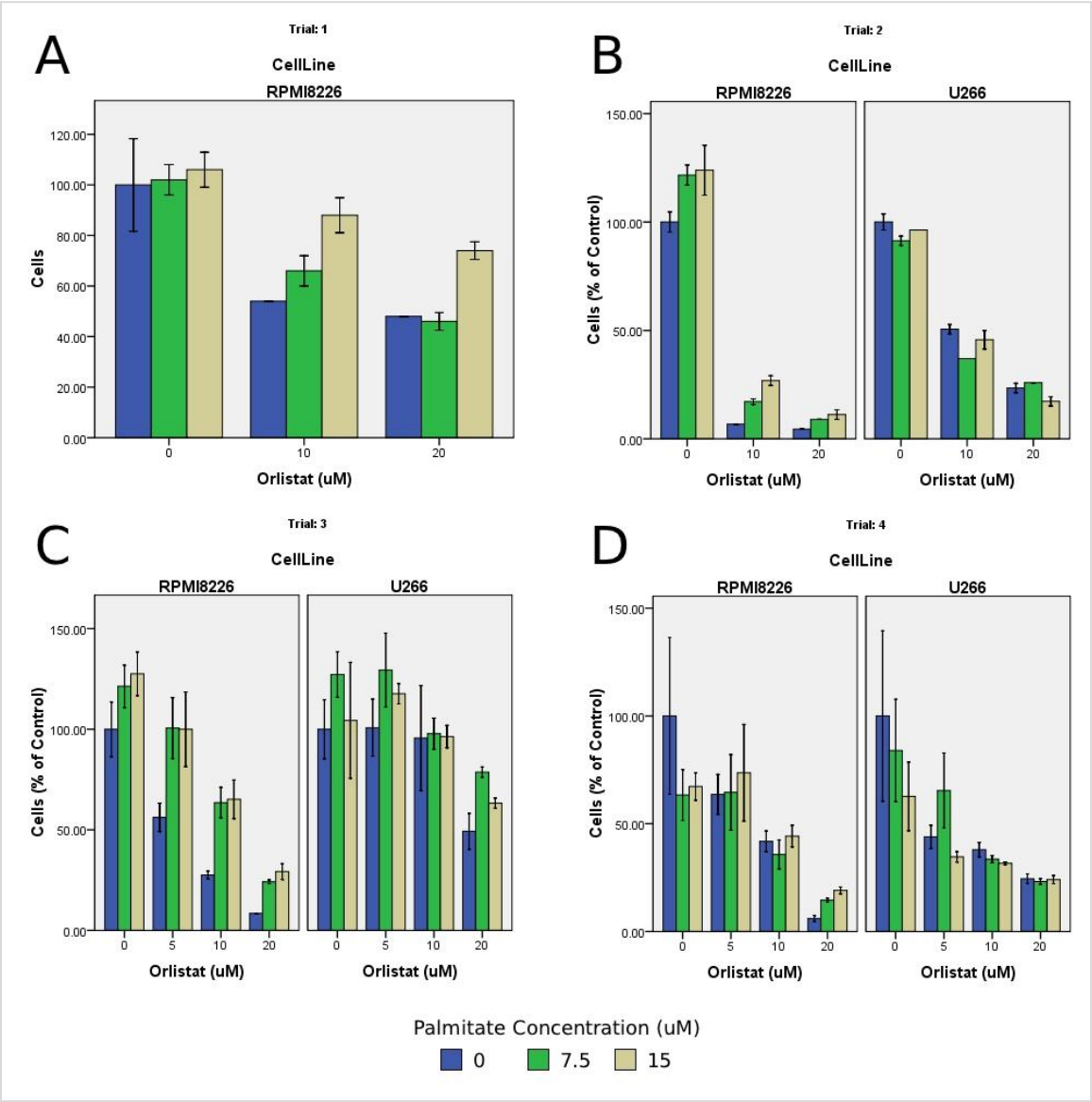

Figure 7. Effect of Palmitate On Proliferation of Multiple Myeloma (MM) Cells Treated With Orlistat, Trials 1 to 4. MM cell lines RPMI8226 and U266 were cultured for 48 hours with orlistat at 0, 5, 10, or 20 μM and with palmitate at 0, 7.5, or 15μM. Proliferation was measured colorimetrically and stated in terms of the negative control. Values here are treatment group mean ± SD. N = 3.

The data suggest that viability is greater in U266 populations compared to RPMI8226 populations across time-points, orlistat concentrations, and trials (figure 9).

Further, U266 population viability was nearly equal across time-points, orlistat

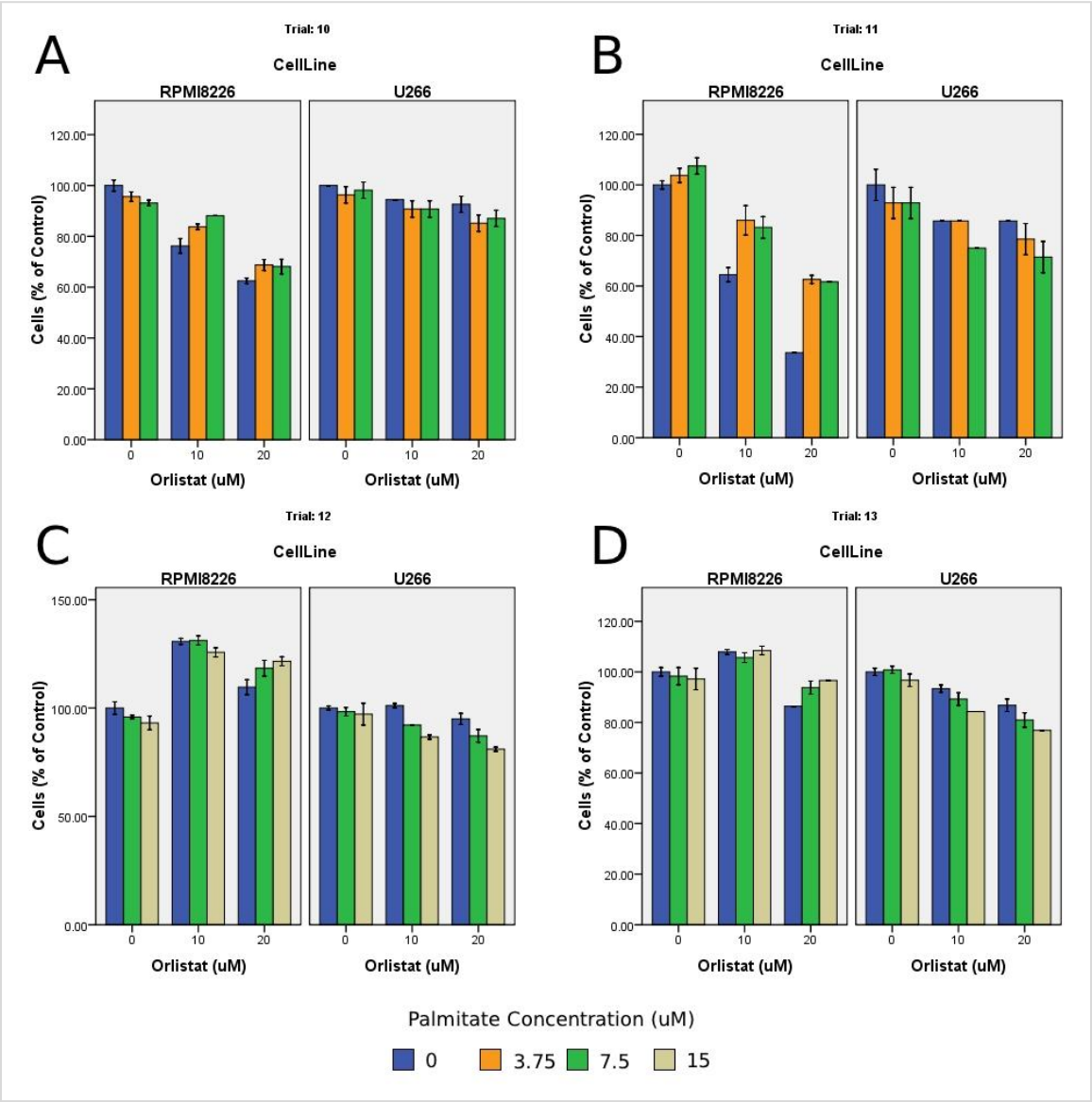

Figure 8. Effect of Palmitate On Proliferation of Multiple Myeloma (MM) Cells Treated With Orlistat, Trials 10 to 13. MM cell lines RPMI8226 and U266 were cultured for 48 hours with orlistat at 0, 10, or 20 µM and with palmitate at 0, 3.75, 7.5, or 15µM. Proliferation was measured colorimetrically and stated in terms of the negative control. Values here are treatment group mean ± SD. N = 3.

concentrations, and trials. In trial 1, RPMI8226 population viability was directly related to orlistat concentration at the 24 hour time-point, however at the same time-point in trial 2, RPMI8226 viability decreased between the negative control and 10µM orlistat

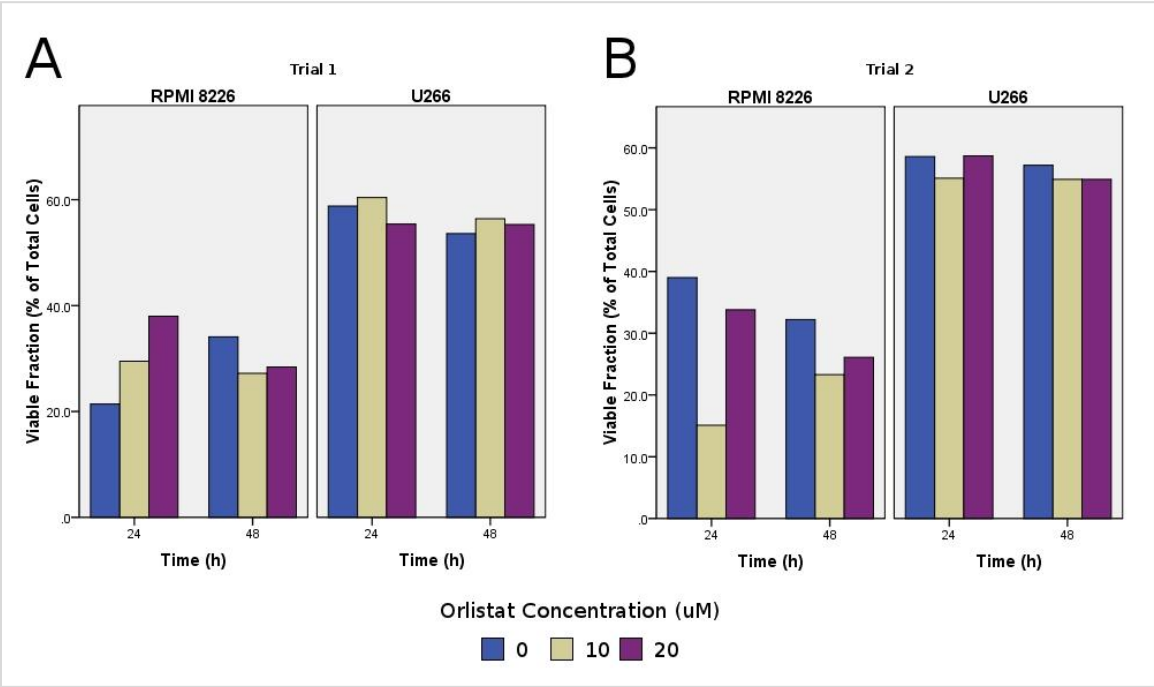

Figure 9. Effect of Orlistat Treatment On Multiple Myeloma (MM) Cell Apoptosis, Trials 1 and 2. MM cell lines RPMI8226 and U266 were cultured for 24 or 48 hours with orlistat at 0, 10, or 20 µM. Cells were stained with annexin V-FITC and propidium iodide and measured by flow cytometry. Values here are percentage of viable (non-apoptotic) cells in each sample.

populations and partially recovered at the 20µM orlistat population. In both trials, at the 48 hour time-point, RPMI8226 population viability fell between the negative control and 10µM orlistat populations and partially recovered at the 20µM orlistat population, but the changes were smaller in magnitude than those exhibited by the trial 2 populations at the 24 hour time-point.

Cell Cycle

To test the hypothesis that orlistat treatment inhibits the progression of MM cells through the cell cycle, and in vitro cell culture model was used. MM cell lines RPMI8226 and U266 were treated with orlistat at 0, 10, and 20µM orlistat for 12, 24, and

48 hours. Distribution in the cell cycle was assayed by propidium iodide staining measured by flow cytometry.

For every sample for each cell line, an aneuploid and a diploid population was identified (figure 10). Among the RPMI8226 populations, there were no differences in

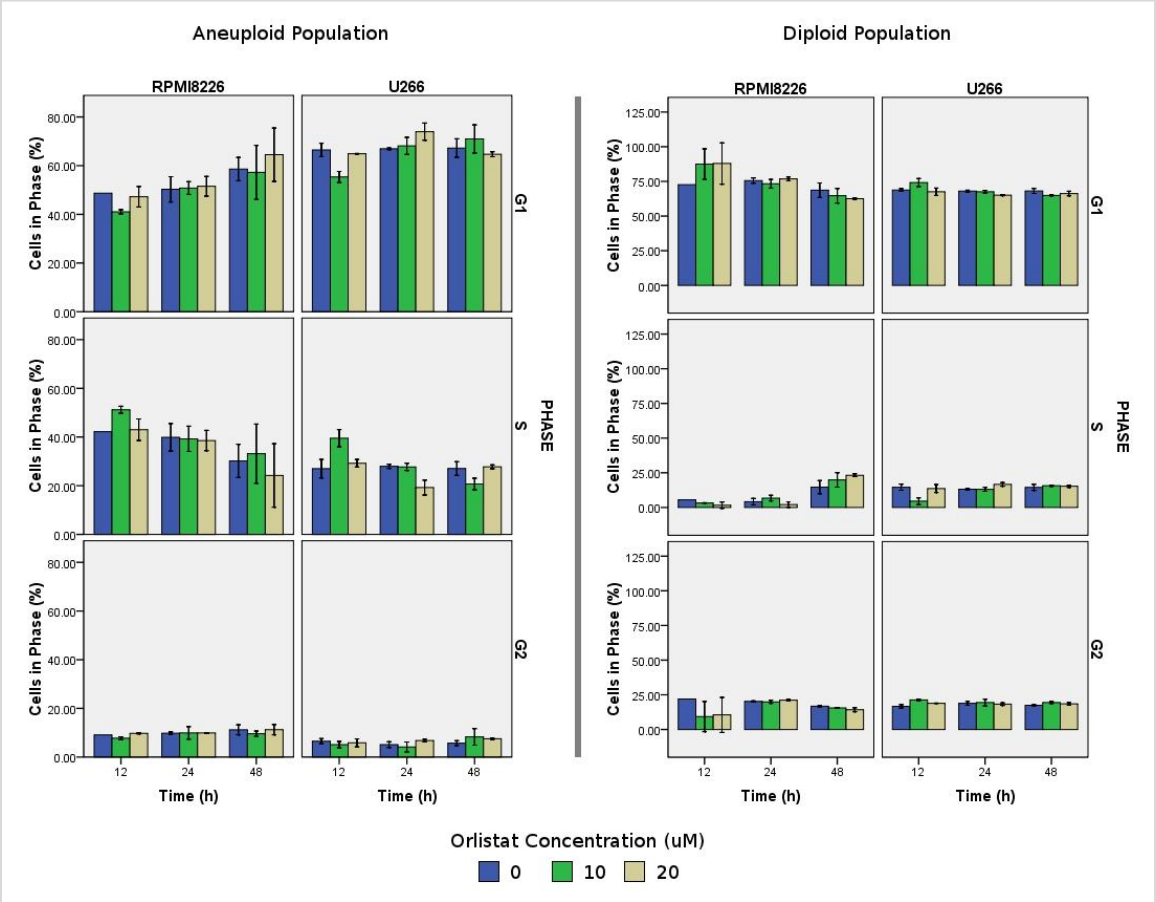

Figure 10. Effect of Orlistat Treatment On Multiple Myeloma (MM) Cell Cycle. MM cell lines RPMI8226 and U266 were cultured for 12, 24, or 48 hours with orlistat at 0, 10, or 20 μM. Cells were stained with propidium iodide and measured by flow cytometry. Aneuploid (left) and diploid (right) populations were observed. Values are treatment group/population mean cell-cycle fraction ± SD. N = 2.

the distribution of populations across the cell cycle at the 5% level. At the 12 hour time-point, the G1 fraction of the aneuploid U266 population treated with 10 μM orlistat was 11.070 points less than control (p=0.025). At the 24 hour time-point, the S fraction of the

aneuploid U266 population treated with 20 µM orlistat was 8.705 points less than control (p=0.045). At the 12 hour time-point, the G2 fraction of the diploid U266 population treated with 10 µM orlistat was 4.660 points greater than control (p=0.018).

Gene Transcription

To test the hypothesis that orlistat alters gene expression, an in vitro cell culture model was used. In this model, human MM cell lines RPMI8226 and U266 were treated with orlistat at 20µM, RNA was collect at 0, 1, and 4 hours of treatment, and quantities of mRNA were compared across samples by qPCR.

Orlistat was predicted to result in a pro-apoptotic, non-mitotic, and less-malignant (measured) transcriptome. Expression ratios of two or greater were interpreted as up-regulated, while those of one-half or less were interpreted as down-regulated (table 1). Whereas fold-change in gene expression is defined as the ratio of gene expression (i.e.: the Pfaffl equation), 'fold-change' and 'expression ratio' are used interchangeably (Pfaffl, 2001; Tusher, Tibshirani, & Chu, 2001).

The apoptotic genes assayed were Akt (survival), Bcl-2 (survival), and Casp9 (apoptotic). Akt expression ratios were 0.73 and 0.35 for RPMI8226 at 1 and 4 hours, respectively, and 1.82 and 1.53 for U266 at 1 and 4 hours, respectively. Bcl2 expression ratios were 0.92 and 0.76 for RPMI8226 at 1 and 4 hours, respectively, and 2.56 and 0.73 for U266 at 1 and 4 hours, respectively. Casp9 expression ratios were 0.96 and 0.84 for RPMI8226 at 1 and 4 hours, respectively, and 2.32 and 0.99 for U266 at 1 and 4 hours, respectively.

The mitotic genes assayed were E2F1 and Akt (both pro-mitotic). E2F1 expression ratios were 0.64 and 0.21 for RPMI8226 at 1 and 4 hours, respectively, and 0.51 and 0.62 for U266 at 1 and 4 hours, respectively. Akt expression ratios were 0.73 and 0.35 for RPMI8226 at 1 and 4 hours, respectively, and 1.82 and 1.53 for U266 at 1 and 4 hours, respectively.

Table 1. Expression Ratio of Select Genes.

	RPMI8226		**U266**	
Gene	**1h**	**4h**	**1h**	**4h**
ACACA	0.76	0.61	2.24	3.08
Akt	0.73	0.35	1.82	1.53
Bcl2	0.92	0.76	2.56	0.73
Casp9	0.96	0.84	2.32	0.99
EGFR	1.03	1.31	2.06	0.98
ERBB2	4.17	2.00	0.10	0.00
E2F1	0.64	0.21	0.51	0.62
IκB	0.14	0.21	0.67	1.14
IL6		(no data)		
NFκB	0.80	0.38	1.10	0.49
TNFα	0.17	0.24	0.55	0.96
β-actin	1.00	1.00	1.00	1.00

Note. Multiple myeloma cell lines RPMI8226 and U266 were cultured with orlistat at 20 μM for 0, 1, or 4 hours. Transcription of select genes was assayed by qPCR. Expression was normalized to β-actin and stated as a ratio of vehicle treated control.

The malignancy genes assayed were ACACA, EGFR, ERBB2, IκB, IL6, NFκB, and TNFα (all but IκB are associated with increased malignancy). ACACA expression ratios were 0.76 and 0.61 for RPMI8226 at 1 and 4 hours, respectively, and 2.24 and 3.08 for U266 at 1 and 4 hours, respectively. EGFR expression ratios were 1.03 and 1.31 for RPMI8226 at 1 and 4 hours, respectively, and 2.06 and 0.98 for U266 at 1 and 4 hours,

respectively. ERBB2 expression ratios were 4.17 and 2.00 for RPMI8226 at 1 and 4 hours, respectively, and 0.10 and 0.00 for U266 at 1 and 4 hours, respectively. IκB expression ratios were 0.14 and 0.21 for RPMI8226 at 1 and 4 hours, respectively, and 0.67 and 1.14 for U266 at 1 and 4 hours, respectively. NFκB expression ratios were 0.80 and 0.38 for RPMI8226 at 1 and 4 hours, respectively, and 1.10 and 0.49 for U266 at 1 and 4 hours, respectively. TNFα expression ratios were 0.17 and 0.24 for RPMI8226 at 1 and 4 hours, respectively, and 0.55 and 0.96 for U266 at 1 and 4 hours, respectively.

IL6 expression ratios were not obtained.

Transcription of each of the three assayed apoptotic genes was decreased at the 1 hour time-point for the RPMI8226 cells when compared to control (but not at the 2-fold threshold), while transcription of each of the three assayed apoptotic genes was increased at the 1 hour time-point for U266 cells. Expression ratios decreased for all apoptotic genes between the 1 hour and 4 hour time-point, for each cell line. The mitotic genes Akt and E2F1 where down-regulated in RPMI8226 at 1 hour and further down-regulated (below the 2-fold threshold) at 4 hours. Akt and E2F1 were slightly up- and down-regulated, respectively, in U266 at 1 hour, with values nearer to control at 4 hours. IκB and TNFα were down-regulated in RPMI8226 at 1 hour, and slightly less so, at the 4 hour time-point (beyond the 2-fold threshold at both time-points). IκB and TNFα were down-regulated slightly (i.e.: less than 2-fold change in expression) in U266 at 1 hour; IκB was slightly up-regulated and TNFα was and slightly below control at the 4 hour time-point. NFκB was slightly down- and up-regulated in RPMI8226 and U266, respectively, at 1 hour; transcription was down-regulated (beyond the 2-fold threshold) at the 4-hour time-point in both cell lines. EGFR transcription was slightly up-regulated at 1 hour and

further up-regulated (but not beyond the 2-fold threshold) at the 4-hour time-point in RPMI8226; conversely, EGFR transcription was up-regulated (greater than 2-fold) at the 1-hour time-point in U266, but had returned to baseline at the 4-hour time-point. ERBB2 transcription was up-regulated at the 1-hour and, to a lesser degree, 4-hour time-point (both greater than 2-fold) in RPMI8226; transcription was down-regulated (greater than 2-fold) in U266 at the 1-hour time-point, and further down-regulated at the 4-hour time-point. ACACA was slightly down-regulated at both the 1-hour and 4-hour time-points in RPMI8226; it was up-regulated (greater than 2-fold) in U266 at the 1-hour time-point and further up-regulated at the 4-hour time-point.

Protein

To test the hypothesis that orlistat alters protein levels in target genes of interest, Western blotting was conducted. In this model, human MM cell lines RPMI8226 and U266 were treated with orlistat at 20μM and protein was collected at 0, 3, 6, 12, 24, and 48 hours of treatment. Protein quantity for target genes was determined by Western blot. Expression was compared within and then across replicates.

Orlistat treatment was predicted to result in a pro-apoptotic, non-mitotic, and less-malignant profile among the assayed proteins.

Akt protein level was reduced with orlistat treatment for 48 hours compared to 12 hours and 24 hours for both RPMI8226 and U266 populations (figure 11). Akt was also reduced with orlistat treatment for 12 hours compared to 6 hours in RPMI8226 populations.

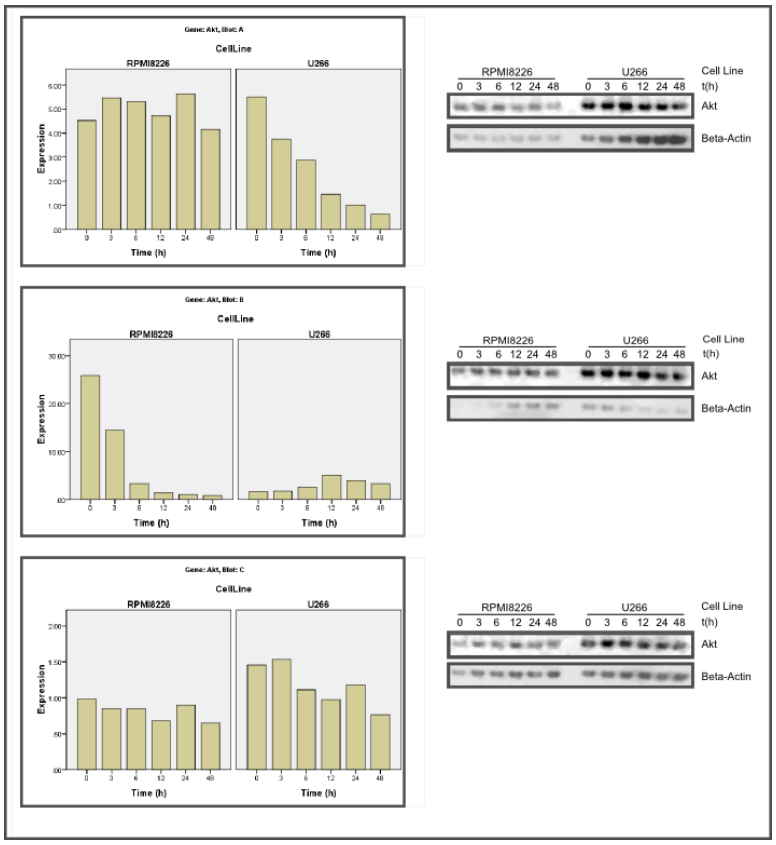

Figure 11. Akt protein expression by Western blot. Multiple myeloma cell lines RPMI8226 and U266 were cultured for 0, 3, 6, 12, 24, or 48 hours with orlistat 20 μM. Protein expression was normalized to β-actin (left) as visualized by Western blots (right) for blot A (top), blot B (middle), and blot C (lower).

Bcl2 was reduced with orlistat treatment for 6 hours compared to 0 hours and 3 hours, and at 3 hours compared to 0 hours among U266 populations (figure 12). Bcl2 was also reduced (or equivalent) with orlistat treatment for 12 hours when compared 0 hours among U266 populations.

CDK4 was reduced with orlistat treatment for 48 hours when compared with treatment for 0 hours and 3 hours among RPMI8226 populations (figure 13). CDK4 was reduced with orlistat treatment for 12 hours when compared with 0 hours, 24 hours, and

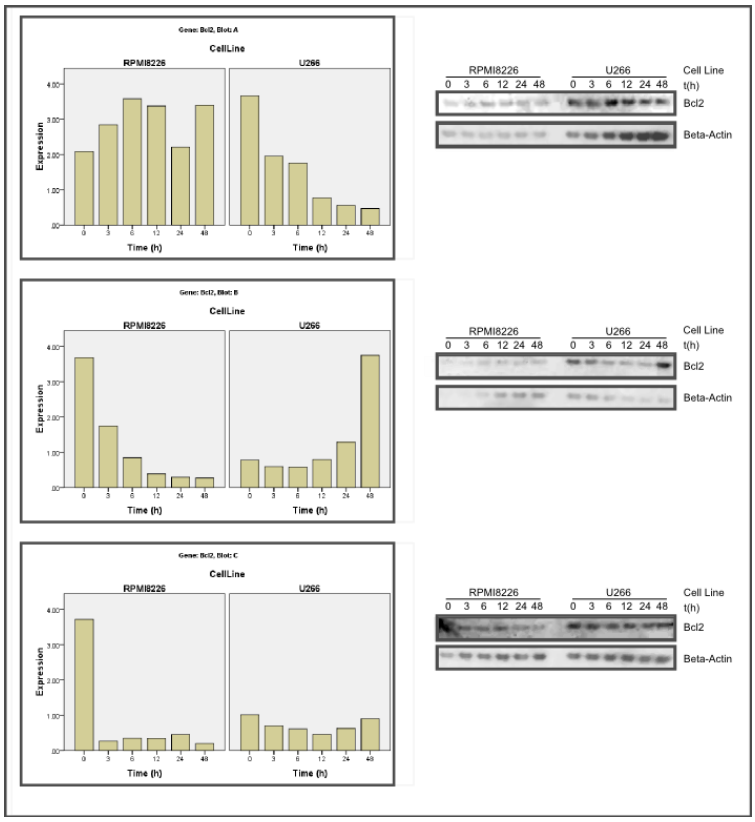

Figure 12. Bcl2 protein expression by Western blot. Multiple myeloma cell lines RPMI8226 and U266 were cultured for 0, 3, 6, 12, 24, or 48 hours with orlistat 20 μM. Protein expression was normalized to β-actin (left) as visualized by Western blots (right) for blot A (top), blot B (middle), and blot C (lower).

48 hours and CDK4 was reduced at 0 hours when compared to 48 hours among U266 populations.

FASN was reduced with orlistat treatment for 48 hours when compared to 24 hours among RPMI8226 populations (figure 14). FASN was reduced with orlistat treatment for 12 hours when compared to 24 hours and 48 hours and was reduced (or equivalent) with treatment for 24 hours when compared to 48 hours among U266 populations. Primary, normal B-cells did not yield sufficient protein to compare FASN expression with MM cells.

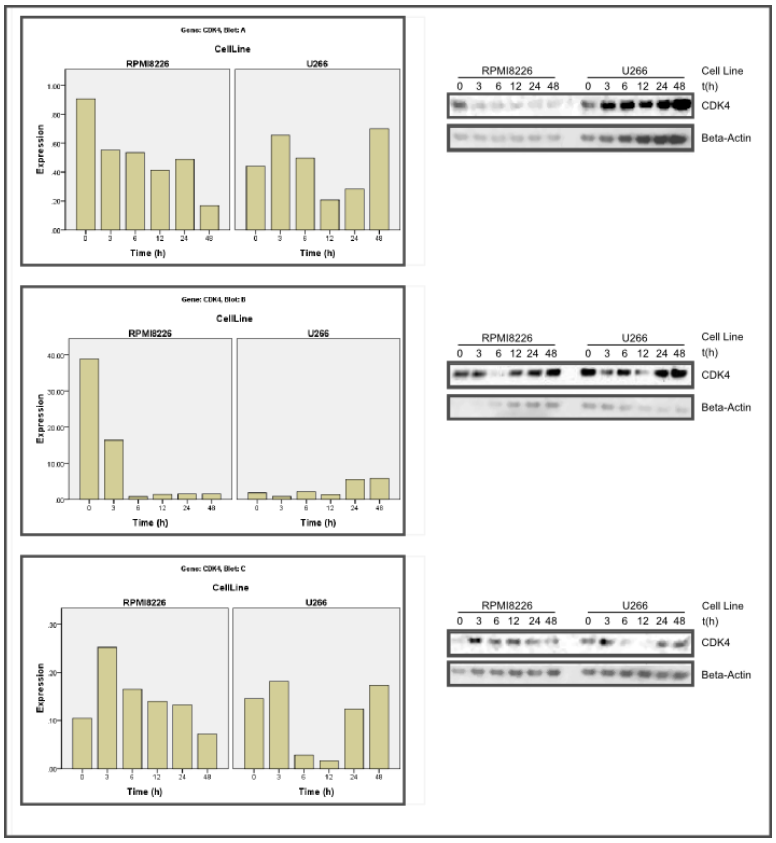

Figure 13. CDK4 protein expression by Western blot. Multiple myeloma cell lines RPMI8226 and U266 were cultured for 0, 3, 6, 12, 24, or 48 hours with orlistat 20 μM. Protein expression was normalized to β-actin (left) as visualized by Western blots (right) for blot A (top), blot B (middle), and blot C (lower).

IκB was reduced with orlistat treatment for 3 hours when compared to 0 hours and reduced (or equivalent) with treatment for 48 hours when compared to 0 hours among U266 populations (figure 15).

NFκB was reduced with orlistat treatment for 6 hours and 24 hours when compared to 12 hours among RPMI8226 populations (figure 16). NFκB was reduced (or equivalent) with orlistat treatment for 24 hours when compared to 0 hours and was reduced with treatment for 48 hours when compared to 0 hours among U266 populations.

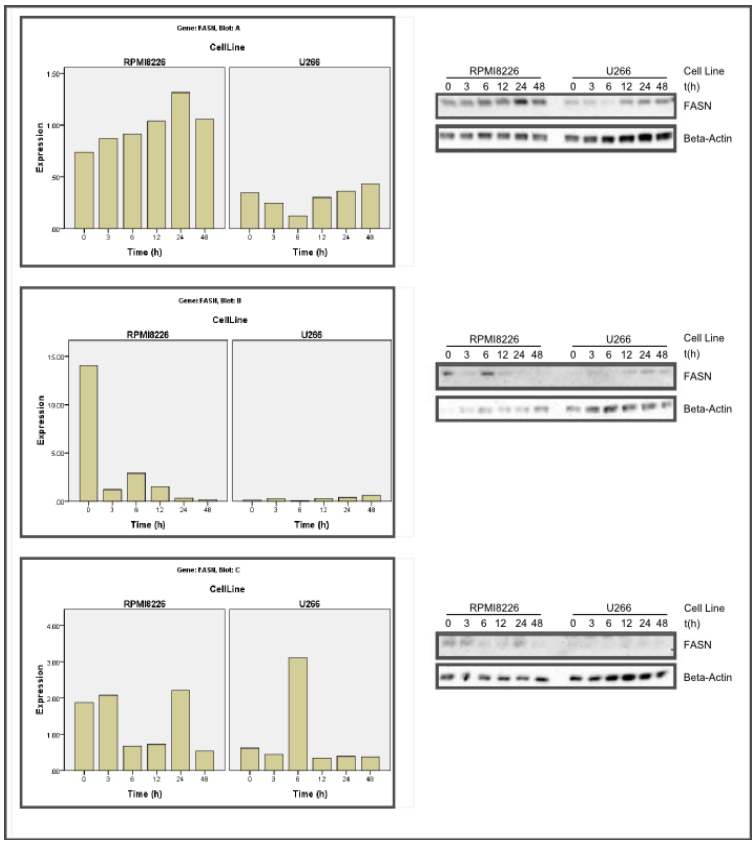

Figure 14. FASN protein expression by Western blot. Multiple myeloma cell lines RPMI8226 and U266 were cultured for 0, 3, 6, 12, 24, or 48 hours with orlistat 20 μM. Protein expression was normalized to β-actin (left) as visualized by Western blots (right) for blot A (top), blot B (middle), and blot C (lower).

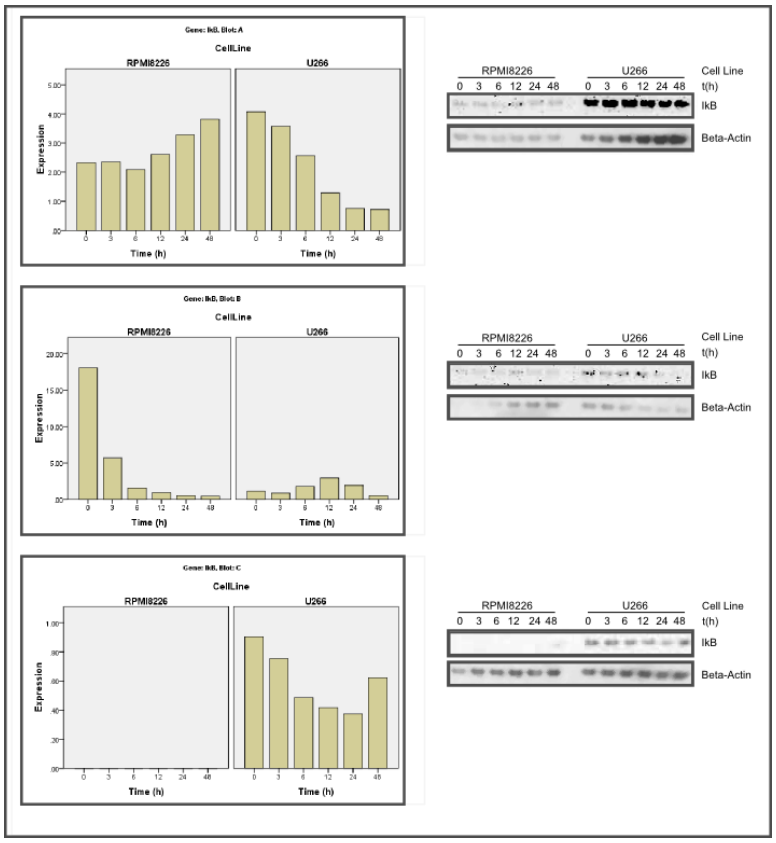

Figure 15. IkB protein expression by Western blot. Multiple myeloma cell lines RPMI8226 and U266 were cultured for 0, 3, 6, 12, 24, or 48 hours with orlistat 20 μM. Protein expression was normalized to β-actin (left) as visualized by Western blots (right) for blot A (top), blot B (middle), and blot C (lower).

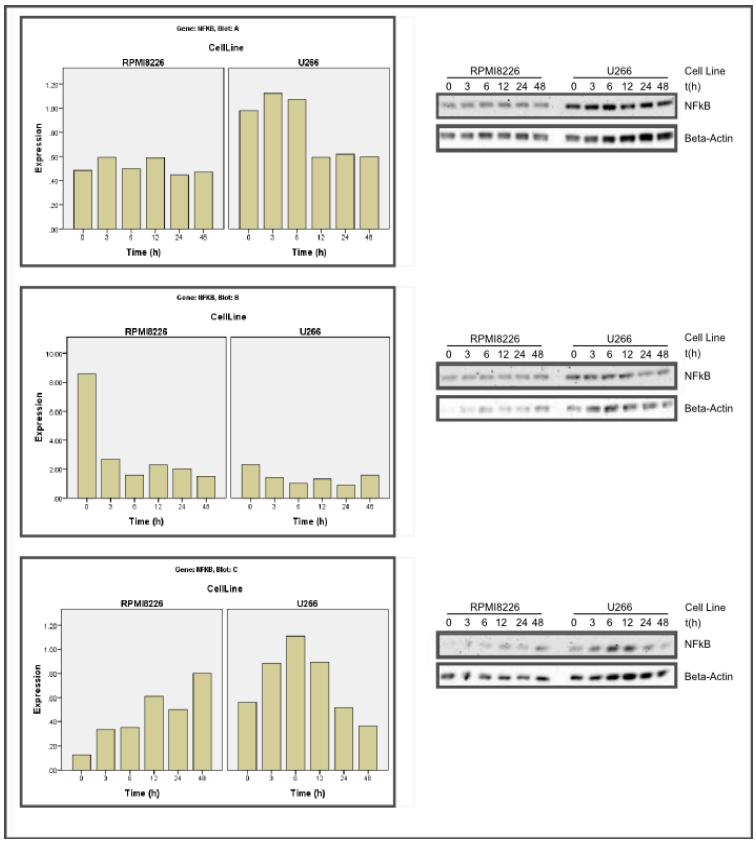

Figure 16. NFkB protein expression by Western blot. Multiple myeloma cell lines RPMI8226 and U266 were cultured for 0, 3, 6, 12, 24, or 48 hours with orlistat 20 μM. Protein expression was normalized to β-actin (left) as visualized by Western blots (right) for blot A (top), blot B (middle), and blot C (lower).

CHAPTER 4

DISCUSSION

Orlistat Treatment Reduces MM Population

Orlistat is a GI lipase inhibitor used in prescription and over-the-counter preparations to block intestinal uptake of dietary fat for the treatment of obesity (Ruth Lupu & Javier a Menendez, 2006; Javier a Menendez & Ruth Lupu, 2007). It has an off-target effect as an irreversible inhibitor of fatty acid synthase, for which it has garnered much attention as a cancer therapeutic. In vivo and in vitro, FASN inhibitors have been demonstrated to reduce proliferation in many cancers, including MM (Bandyopadhyay et al., 2006; Carvalho et al., 2008; Knowles et al., 2008; Li et al., 2001; Javier a Menendez & Ruth Lupu, 2007; Thupari, Pinn, & Kuhajda, 2001). The effect of orlistat on MM proliferation has never been reported; because of the success of other FASN inhibitors in reducing MM proliferation, orlistat was tested on MM (W.-qin Wang et al., 2008). This research demonstrated that orlistat exerts an inhibitory effect on the accumulation of MM cells in vitro. In each of four proliferation trials, proliferation decreased (compared to control) in an orlistat-dose-dependent manner. Further, in the palmitate rescue study, proliferation was reduced in the orlistat-treated, palmitate-untreated groups when compared to control. These results are consistent with the prediction that orlistat treatment reduces proliferation in a dose-dependent manner.

The inhibitory effect of orlistat on MM cell accumulation differs by cell-line. In the first proliferation trial, controlling for orlistat dose, the magnitude of reduction in U266 proliferation was approximately two-fold that of RPMI8226 proliferation. However, in trials 2 and 4 (t=48h), the magnitude of reduction in RPMI8226 proliferation was at least two-fold that of U266 proliferation. The differential decrease in proliferation by orlistat is observed in the palmitate rescue study where, controlling for orlistat dose, the reduction in proliferation is larger in magnitude for RPMI8226 groups compared to U266 groups.

Primary, non-cancerous human B-cells were treated with orlistat at the doses used in the proliferation study to assay the effect of the drug on normal cells. No change in proliferation was observed with orlistat treatment at 5, 10, and 20 μM for 48, 72, and 96 hours, compared to controls, consistent with the prediction that orlistat's effect on MM cells is mediated by a trait (or traits) unique to cancerous phenotypes.

Proliferation (i.e.: the change in the number of cells) is determined by the cumulative effect of replication, death, immigration, and emigration, thus determining the drivers of differential population growth requires the evaluation of each of these factors. Because orlistat's effect on proliferation was evaluated using an in vitro cell culture model, migration was effectively eliminated bidirectionally. Introduction of spores to a culture would amount to immigration (while not the same species, the colorimetric reagent will not differentiate), because the proliferation studies were plated with cells from the same stock, the prospect of spores consistently contaminating and proliferating to form colonies in such a pattern so as to appear as a dose-dependent inhibitory effect of orlistat is dubious. More realistically, loss of media (i.e.: by evaporation) from a culture

would have the same effect as immigration by increasing the number of cells per unit of media. If evaporation was consistently higher among the control groups compared to the treatment groups, then 'immigration' could contribute to the result. However, the study was designed to minimize the effect of evaporation, thus it is a weak explanation. Opportunities for emigration are restricted to removal from culture by clinging to a pipette tip; this is not an appreciable factor considering the numbers of cells plated and that this is expected to be unbiased with respect to treatment group. For these reasons, migration is not further addressed in this analysis.

Decreased replication and increased apoptosis can both yield decreased proliferation between which the colorimetric assay cannot distinguish. While the effect on proliferation will appear identical in the colorimetric assay, the mechanism by which the effect occurs is important. In a reductionist illustration where all other variables are constant, to achieve a particular reduction in population, the fraction of individual cells that must receive the effective dose is lower when the effect is apoptosis-mediated compared to cell cycle arrest mediated. If a hypothetical population-reducing agent is applied to a population such that 50% of the individual cells receive the effective dose, after one cell cycle, the population is expected to decrease by 50% if the drug acts by inducing apoptosis, while the population is expected to decrease by 25% if the drug acts by inducing cell cycle arrest, when compared to the untreated control by the colorimetric assay. This disparity in effect arises because a cell-cycle-arrest-inducing agent prevents the treated cell from contributing to the increase in population, while an apoptosis-inducing agent prevents the treated cell from contributing to the increase in population as well as removes the treated cell from the population.

The effect of FASN inhibition has been associated with p53-status, such that when FASN is inhibited in p53-wildtype cancer cells, they often undergo apoptosis, but p53-mutant cancer cells tend to undergo cell cycle arrest (Bullock & Fersht, 2001; Li et al., 2001). Further, the effects of orlistat have been mediated chiefly by an increase of the G1 fraction in some cancers (including breast and prostate cancers) but by inducing apoptosis in others. When the FASN inhibitor cerulenin was studied in the MM cell-line U266, there was an increase in apoptosis observed, however the effect of the drug on cell-cycle was not studied (W.-qin Wang et al., 2008). To measure the effect of orlistat on apoptosis and cell-cycle, flow cytometry was employed. If orlistat's effect is apoptosis mediated, then the viable fraction of cells will be decreased when measured by an Annexin-V-FITC protocol; if its effect is mitosis-mediated, then the distribution of cells through the cell-cycle will shift. Between 40% and 80% of cells in the control groups where not viable. At the 48 hour time-point, orlistat further decreased average viability by 7.9 points (from 33.2% to 25.3%) and 5.9 points (from 33.2% to 27.3%) among RPMI8226 populations at 10μM and 20μM, respectively, while U266 populations did not differ from control. These observations for RPMI8226 may be a type I error, owing to the small number of samples and large apoptotic fraction observed in the control group. While the results for U266 do not match those previously reported, because of the low number of samples in this study, these results may be a type II error. Interestingly, in the aforementioned study, control group contained a large fraction of apoptotic cells, as observed in this study (Knowles, Axelrod, Browne, & Smith, 2004; Li et al., 2001; J a Menendez, L Vellon, et al., 2005).

FASN blockade has been reported to induce cell cycle arrest in the G1 phase, which is concomitant with the greatest phospholipid synthesis activity (i.e.: a substantial demand for fatty acid) (Li et al., 2001). In this study, distribution of cells through the cell-cycle was assayed by flow-cytometry; diploid and aneuploid (DI approximately 1.2 for both cell lines) populations were identified in each replicate of each treatment and cell line. Among the RPMI8226 groups, there were no differences at the 5% level between treatment and controls. Among the U266 groups, there was a decrease in the fractions of cells in G1 (10 μM orlistat, t=12h) and S (20 μM orlistat, t=24h) phases among the aneuploid populations and an increase in G2-M (20 μM orlistat, t=12h) phase among the diploid populations. These data suggest that orlistat treatment slows the progression of cells in G2-M, causing the fraction of cells in that phase to increase while causing the fraction of cells in the G1 and S phases to decrease. These data are consistent with the larger trend of cell cycle changes induced by FASN-blockade in those cell-lines which predominantly respond through cell-cycle arrest: an early decrease in G1, followed by a decrease in S (Hideshima & Anderson, 2002; Javier a Menendez & Ruth Lupu, 2007). While the data do not indicate that the effect on RPMI8226 is cell-cycle mediated, inspection of the data on a case-by-case basis reveals biologically significant (albeit not statistically significant) changes in distribution that warrant evaluation with a redesigned experiment. The interpretation of these data for both cell lines is complicated by the bifurcation into diploid and aneuploid populations: particularly G1 diploid cells may overlap with sub-G1 and G1 aneuploid cells, and so on with the other ploidy/cell-cycle-phase combinations.

Gene Expression

Whereas orlistat treatment resulted in a dose-dependent reduction in cell number for treatment groups of both cell-lines, a similar change in phenotype at the transcriptional and translational level was induced with orlistat treatment.

Akt has been identified as a mechanism by which FASN inhibition begets reduced proliferation, has been suggested as a target for novel MM therapeutics, and lies in the PI3K pathway—a regulator of FASN expression; for these reasons, Akt expression was of principle interest in this study (Carvalho et al., 2008; Hideshima & Anderson, 2002; Kandel et al., 2002; Javier a Menendez & Ruth Lupu, 2007; W.-qin Wang et al., 2008). Akt plays an important role in cell survival by interacting with proteins so as to inhibit pro-apoptotic factors and induce survival factors; it has been implicated as a mechanism by which fatty acid synthase inhibitors (e.g.: orlistat) inhibit tumors (Javier a Menendez & Ruth Lupu, 2007). By phosphorylating (i.e.: inhibiting) the apoptotic protein Bad, thereby preventing it from binding and sequestering the survival proteins Bcl2 and BclXl, Akt contributes to a survival phenotype (Song, Ouyang, & Bao, 2005). Additionally, Akt can contribute to the survival phenotype by phosphorylating (i.e.: inactivating) caspase 9, inhibiting the caspase cascade, and through FOXO phosphorylation. Akt can also tilt the transcriptome toward survival; Akt phosphorylates IKK-α, which in turn phosphorylates IκB and causes its degradation, allowing NFκB to localize from the cytosol to the nucleus where it transcribes survival genes. The viable fractions were decreased with orlistat treatment for 24 and 48 hours in RPMI8226 (i.e.: apoptotic fractions increased), which is matched by a decrease in the survival protein Akt at 12 and 48 hours. Consistent with the

decrease in Akt protein, Akt transcripts were decreased at the 1 hour (0.73 fold change) and 4 hour (0.35 fold change) time-points.

Because this study is interested in the response of neoplasms to orlistat induced FASN inhibition, and because Bcl2 is modulated by Akt, Bcl2 expression was examined (Cory & Adams, 2002; Song et al., 2005). Bcl2, a protein which prevents apoptosis (i.e.: pro-survival) by blocking the passage of cytochrome c through mitochondrial pores into the cytosol, was reduced (or equivalent) at the 24 hour time-point, consistent with the observed decrease in apoptosis. Bcl2 transcripts were reduced at the 1 hour (0.92 fold change) and the 4 hour (0.76 fold change) time-points, consistent with the observations at the protein and survival levels, providing evidence that orlistat effects in RPMI8226 are mediated through both Akt and Bcl2.

Among the U266 populations, consistent changes in the viable fractions were not observed. Akt protein expression among the U266 populations was decreased at the 48 hour time-point, when compared to the previous time-point. Transcripts, however, are slightly elevated (i.e.: consistent with a survival phenotype) at the 1 hour (1.82 fold change) and 4 hour (1.53 fold change) time-points. Bcl2 protein expression was also decreased at the 6 hour and 12 hour time-points; transcripts where elevated at the 1 hour (2.56 fold change) but decreased at the 4 hour (0.73 fold change) time-points.

Because the replication of tumors is of principle interest in evaluating anti-neoplastic drugs, and because FASN blockade has previously been described as preventing the progression of treated cells past the G1 restriction point, E2F1 and CDK4 were evaluated (Carvalho et al., 2008; Hanahan & Weinberg, 2000; Knowles et al., 2004; Massagué, 2004). Although changes to the distribution of cells through the cell-cycle

among RPMI8226 populations were not statistically significant, transcription and translation level changes were observed consistent with the changes in cell number. Transcription factor E2F1, which is involved in the progression from G1 to S phase, was decreased at the transcriptional level at the 1 hour (0.64 fold change) and 4 hour (0.21 fold change) time-points (Massagué, 2004). Further, CDK4, also involved in the progression from G1 to S phase, was decreased at the protein level (Massagué, 2004). As Akt also has pro-mitotic functions, the decrease in Akt transcripts and protein discussed in the context of apoptosis may also contribute to a non-mitotic phenotype (Gottlieb, Leal, Seger, Taya, & Moshe Oren, 2002; M Oren, 2003). Akt induces cell-cycle progression through activation of MDM2, which in turns inhibits p53.

Three changes in the distribution of U266 cells through the cell-cycle were observed that were significant at the 5% level. At the 12 hour time-point, 10 μM orlistat treatment induced a decrease in the G1 fraction and an increase in the G2-M fraction, and at the 24 hour time-point, 20 μM orlistat treatment induced a decrease in the S fraction. The decrease in G1 and increase in G2-M observed at the 12 hour time-point are consistent with cell-cycle arrest in G2-M: cells continue to enter into G2 and continue to pass from G1 into S, but because cells cannot pass through G2-M, the G2-M fraction increases while the G1 fraction decreases. These data suggest blocking progression of the cell-cycle at the G2-M and/or metaphase checkpoints. The decrease in the S Phase fraction at 24 hours suggests inhibition of the cell-cycle at the restriction point (i.e.: G1 checkpoint). This latter result is in keeping with the reported effects of FASN inhibition on other cancers as detailed above.

For the cell-line U266, cell-cycle observations are consistent with gene expression observations. At the protein level, CDK4 was decreased at the 12 hour time-point; this protein is necessary for the phosphorylation of the Rb protein, to release E2F-family transcription factors, without which the cell-cycle cannot progress into the S-phase (Gottlieb et al., 2002; Massagué, 2004; M Oren, 2003). At the transcriptional level, E2F1 is decreased at the 1 hour (0.51 fold change) and the 4 hour (0.62 fold change) time-points, further suggesting a decrease in cell-cycle progression. Several growth factor receptors have been identified as central in the lipogenic phenotype and associated with cancer aggressiveness. Expression of these growth factor receptor genes was altered by orlistat treatment. EGFR (activation of which can induce mitosis) transcripts were increased at the 1 hour (2.06 fold change) but not at the 4 hour (0.98 fold change) time-points (J a Menendez, L Vellon, et al., 2005; J. A. Menendez, Luciano Vellon, et al., 2005; Javier a Menendez & Ruth Lupu, 2007). ERBB2 transcripts were reduced markedly at the 1 hour (0.10 fold change) and 4 hour (<0.01 fold change) time-points. The virtual transcriptional silencing of ERBB2 is noteworthy for its role in activating the MAPK and PI3K pathways, both of which are involved in mitosis as well as transcription of FASN.

Orlistat Treatment Reduces Cell Count Through the Lipogenic Phenotype

These data provide several pieces of powerful evidence for the inhibition of the lipogenic phenotype at the pre- and post-expression levels. The combined effects of this inhibition lead to decreases in the number of cancer cells.

FASN inhibition leads to inhibited proliferation in a dose-dependent fashion in cancer cells, including MM, however it does not affect non-cancerous cells (Kridel et al., 2004; Javier a Menendez & Ruth Lupu, 2007; W.-qin Wang et al., 2008). Orlistat treatment induces an immediate, dose-dependent decrease in cell number among MM cell-lines RPMI8226 and U266, but not primary, non-cancerous B-cells. This difference in response is indicative of the drug acting through a pathway (or pathways) expressed in the cancerous, but not normal, cells. To the extent that orlistat reduces cancer proliferation through FASN, and FASN is not expressed in most adult tissues, the effect of orlistat treatment observed in cancer cells is not reported in normal cells of the same tissue. The FASN pathway is restricted to a few normal adult tissues (not B-cells), however its expression in cancers in general, and MM in particular, has been documented (Kridel et al., 2004; Kuhajda, 2000; Kuhajda et al., 1994; Kusakabe et al., 2000; W.-qin Wang et al., 2008).

Exogenous palmitate rescues cancer cells, including prostate and breast cancer, from FASN-blockade induced proliferation inhibition, suggesting that deprivation of endogenously synthesized fatty acids mediates FASN-blockade induced effects on proliferation (Li et al., 2001). The inhibitory effect of FASN-blockade has been reported to remain intact even when cells were treated with an ACACA inhibitor (i.e.: precluding the accumulation of malonyl-CoA), further suggesting that the effect of FASN-blockade is, at least in part, mediated by fatty acid deprivation (Li et al., 2001). To the extent that orlistat reduces cell counts among MM cells by depriving the cells of the FASN-catalyzed end-product, supplemental palmitate (i.e.: the primary FASN product) would alleviate the cells of their need for FASN, rendering them unaffected by FASN inhibition.

Groups of MM cells treated with orlistat were rescued from the reduction in population size by the application of exogenous palmitate. The rescue effect of palmitate fell on a curve with respect to orlistat, where for a particular orlistat concentration, the rescue effect was maximized at a particular palmitate concentration, diminishing to either extreme. This observation is consistent with expectations. Where orlistat has inhibited endogenous fatty acid synthesis, leading to a reduction in proliferation, exogenous palmitic acid (the predominant FASN product) would attenuate the reduction in proliferation, as if negating the last n units of the orlistat dose; palmitate has a proliferation-inducing effect. Simultaneously, palmitate has a dose-dependent proliferation-reducing effect on cells. For a particular orlistat dose, when a palmitate dose is such that the proliferation-inducing effect exceeds the proliferation-reducing effect, the dose is a net-proliferation-inducer.

Gene expression changes at both the transcriptional and protein levels induced by orlistat are indicative of FASN inhibition. Inhibition of the lipogenic phenotype in a tumor is reported to result in Akt inhibition, which has been observed in these studies, at both the protein and transcript levels (Javier a Menendez & Ruth Lupu, 2007). This inhibition is noteworthy for the protein's position in the lipogenic, apoptotic, and mitotic pathways. Akt lies downstream of EGFR and ERBB2 in the PI3K pathway, whereby stimulation of these receptors leads to activation/cleavage of the SREBP1c transcription factor, which then localizes to the nucleus to transcribe genes in the FASN pathway (Javier a Menendez & Ruth Lupu, 2007). Additionally, Akt sits at a node in apoptotic and mitotic genes, mediating signals to activate survival proteins, inhibit apoptotic proteins, and induce the transcription of survival and mitotic genes. Indeed, in these

studies, Akt transcription was decreased in the RPMI8226 cell-line with treatment and Akt protein was decreased in both cell-lines with orlistat treatment.

Interestingly, fatty acid synthesis is rate-limited by the upstream process of converting acetyl-CoA to malonyl-CoA, permitting tight regulation of malonyl-CoA concentration, as a single FASN protein clears malonyl-CoA at a higher rate than a single ACACA produces it (Javier a Menendez & Ruth Lupu, 2007). This malonyl-CoA clearance default is critical for a nascent tumor's progression to metastasis as accumulation can induce apoptosis as well as drive the binding of the Erbb2 promoter by the suppressor PEA3 (J a Menendez, L Vellon, et al., 2005; Javier a Menendez & Ruth Lupu, 2007). The level of FASN protein was decreased in both cell lines after orlistat treatment, amplifying the effect of the drug vis-a-vis fatty acid end-products by reducing the supply of newly uninhibited enzymes. Because of malonyl-CoA's effect on apoptosis, the effect due to the decrease in FASN was augmented by the effect of the accumulation of malonyl-CoA. In U266, increased ACACA but decreased Erbb2 transcription was observed, suggesting that in this cell-line orlistat treatment establishes a tumor-suppressing feed-forward mechanism: orlistat inhibits FASN enzymes; malonyl-CoA concentration increases behind inhibited FASN enzymes; new ACACA mRNA are transcribed (and may contribute to increased ACC protein); elevated malonyl-CoA induces an increase in PEA3, which blocks the Erbb2 TATA box; elevated malonyl-CoA inhibits CPT1, leading to an increase in ceramide concentration, inducing apoptosis. That Erbb2 transcription is up-regulated in RPMI8226 following orlistat treatment suggests a difference in the sensitivity to the accumulation of malonyl-CoA. Collectively these data are modeled in figure 17.

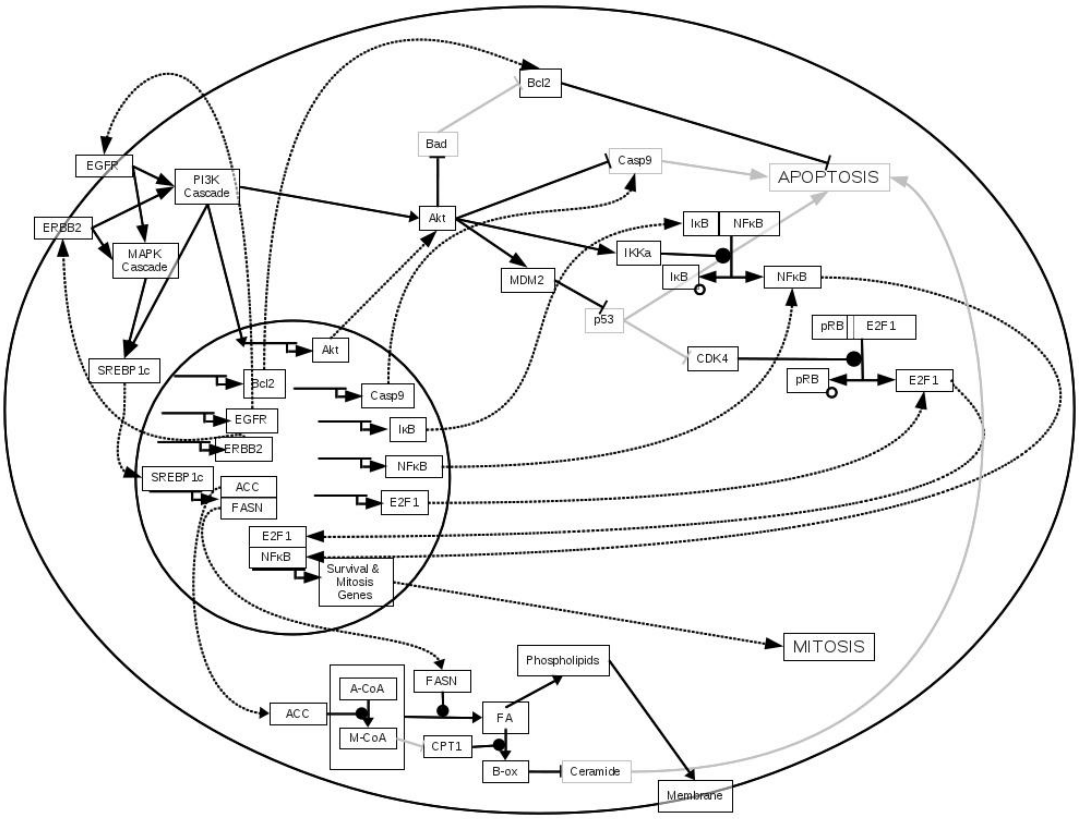

Figure 17. Diagram of The Putative Effects of Orlistat on MM Cells.

Differential Response to Orlistat

Differential Akt and Bcl2 expression provides insight into the mechanism by which orlistat affects RPMI8226. Transcription of NFκB and genes under its control (IκB and TNFα) is decreased in RPMI8226 following orlistat treatment to a much greater degree than in U266; NFκB protein (p105) levels drop in both cell lines, however the effect is visible at 6 hours in RPMI8226 versus 24 in U266. Insofar as these observations are evidence that RPMI8226 is more sensitive to NFκB suppression, orlistat exploits this sensitivity, inducing inhibition of other NFκB-regulated genes, including Akt and Bcl2.

The differential effect of orlistat on proliferation with respect to cell line may be attributable to differential sensitivity to apoptosis and cell-cycle-inhibition. As described, the decrease in population will depend, in part, on the mechanism. That orlistat was observed to effect a larger reduction in population among RPMI8226 groups compared to U266 groups raises the possibility that the drug induces both apoptosis and cell-cycle-arrest in both cell lines, but to different degrees.

In addition to metabolic differences writ large, which may contribute to sensitivity to the drug, the speed with which each cell line completes a cell-cycle may influence response to the drug vis-à-vis cell-cycle arrest. When two otherwise identical populations with different cell cycles, are treated with a replication inhibitor, the decrease in population is expected to be greater among those cells with the shorter cell-cycle. The RPMI8226 cell-cycle is shorter than that of U266 (observed doubling time of 43.7 and 117.9 hours, respectively). If the population-reductive effect of orlistat is solely mitosis-inhibition-mediated, then at the 48-hour time-point the RPMI8226 groups will have been exposed to the drug for an entire cell-cycle, while the U266 groups will have been exposed for less than one-half of a cell-cycle, thus a greater effect is expected among the RPMI8226 group. Because the longest time-point of the proliferation study was 96-hours (approximately 80% of the U266 cell-cycle period), a new study will be be necessary to draw meaningful inferences about the effect of the drug on a cell-cycle-adjusted basis. To be sure, this raises a limitation of the study of the effect of orlistat on primary B-cells: because normal B-cells have a replication rate much slow than that of MM, it is possible for orlistat to exert a cytostatic effect on the primary B-cells but appear to have a constant population size.

Limitations

This analysis is complicated by a low sample number (n=2 for each treatment group/time) and ambiguous ploidy. The matter of the sample size requires little explanation: when an observation is wildly divergent, the reliability of the estimated mean is proportional to the sample size. To that end, the null hypothesis of equal means between treatment groups may not be rejected even when differences between treatment groups apparent on visual inspection, because of insufficient power. The multiple populations observed in the course of measuring cell-cycle may be attributable to several explanations, chiefly: a contaminating organism, polyploidy among the cell lines, or a modeling error. Contamination is a reasonable starting point in studying this problem, particularly in light of this investigator's unconfirmed and confirmed contamination events. However, because the DNA index for the observations was approximately 1.20 (where the population with lower DNA content was arbitrarily deemed the diploid population), the contaminating organism would have a DNA content very similar to that of a human cell, precluding the possibility that the cause of the second curve was due to measuring a microbe (e.g.: mycoplasma or fungus, the aforementioned contaminating organisms). However, reciprocal contamination of the cell lines presents a scenario where the DNA content would be very similar between the cultured cell and the contaminating organism, and that similarity would extend to the DNA indexes between cell-lines. In such a scenario however, RPMI8226 is expected to displace U266 because of its shorter observed doubling time. Moreover, this scenario does not explain the morphological changes observed. Whereas tumors are not clonal, and polyploidy has been documented among cancer cell-lines, including MM, the multiple curves might be a

reflection of the inherent instability among cancer cells (Fonseca et al., 2003; Latreille et al., 1980). The clonal expansion explanation is appealing, however does not address the similarity in the DNA indexes. A third possibility is a modeling error. Under such a scenario, the first spike (i.e.: that which was labeled G1 for the diploid population) would represent a sub-G1 fraction, the second spike (i.e.: labeled G1 for the aneuploid population) would represent the true G1 fraction, the (labeled) diploid G2-M peak would be a type I error, and the true G2-M fraction corresponding to the (labeled) aneuploid G2-M peak.

The cell-lines used in this study, RPMI8226 and U266, are widely used in models. Modeling MM with well characterized cell-lines is advantageous as it facilitates drawing meaningful interpretations of observations through leveraging primary literature. However, as cancers are inherently genetically unstable, restricting candidate cell-lines to those most extensively documented necessarily limits the genetic and phenotypic variation against which the drug can be examined. Whereas this study employed an in vitro model with two cell-lines, interpreting differential response between cell-lines is impeded by correlated characters. A particular example is parsing the importance of NFκB and replication rate in the strong response to orlistat by RPMI8226 relative to U266; RPMI8226, for purposes of this analysis, has a high replication rate and high NFκB expression (the "high/high" phenotype) and U266 has a low replication rate and low NFκB expression (the "low/low" phenotype), meaning that inferences can be drawn about those traits in combination (i.e.: the high/high phenotype has a strong response compared to the low/low phenotype), but inferences about the contribution of each character require the inclusion of high/low and low/high phenotypes in the model.

Implications

This research bolsters the growing body of evidence that fatty acid synthase blockade is a viable target for cancer therapeutics, and this research is the first to demonstrate the proliferation-inducing effect of orlistat in MM. As many of the chemotherapeutics used to treat MM are limited by systemic toxicity (often such that the tolerable dose is below the optimally effective dose) and drug resistance, with the former contributing to the latter, this treatment modality is remarkable in that it addresses both shortcomings of conventional therapy (Hideshima & Anderson, 2002). Because FASN is generally restricted in expression to adult tissues, and because its expression in select normal tissues is substantially lower than in MM, FASN-blockade therapy, such as with orlistat, is promising in that it demonstrates a class of therapeutics that act selectively on tumor cells (Kuhajda, 2000; Kuhajda et al., 1994; Kusakabe et al., 2000; W.-qin Wang et al., 2008). Perhaps more importantly, the efficacy of orlistat can nucleate further innovation and discovery of non-canonical treatment targets. FASN blockade down-regulates many pathways involved in tumor growth and survival, overcoming mechanisms of drug resistance, which makes it an attractive candidate for inclusion in adjuvant therapies, where it may serve to increase a tumor's response to another therapeutic (reducing the required dose) and/or restoring drug-sensitivity in acquired resistance (Hideshima & Anderson, 2002; Hideshima, Mitsiades, Tonon, Richardson, & Anderson, 2007; Javier a Menendez & Ruth Lupu, 2007).

APPENDIX A

DATA TABLES

Table 2. Proliferation Trial 1 Model Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Corrected Model	-	<0.001
Intercept	87.561	<0.001
$CellLine_R$	8.039	0.259
Orlistat	-3.108	<0.001
$(CellLine_R)$*(Orlistat)	1.397	0.032
n = 24		
Adjusted $R^2 = 0.790$		

Table 3. Proliferation Trial 2, T=48 Hours, Model Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Corrected Model	-	<0.001
Intercept	100.000	<0.001
$CellLine_R$	0.000	1.000
Orlistat	-0.686	0.009
$(CellLine_R)$*(Orlistat)	-0.742	0.031
n = 12		
Adjusted $R^2 = 0.857$		

Table 4. Proliferation Trial 2, T=72 Hours, Model Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Corrected Model	-	<0.001
Intercept	100.000	<0.001
$CellLine_R$	0.000	1.000
Orlistat	-1.538	0.001
$(CellLine_R)$*(Orlistat)	-1.479	0.008
n = 12		
Adjusted $R^2 = 0.926$		

Table 5. Proliferation Trial 2, T=96 Hours, Model Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Corrected Model	-	<0.001
Intercept	100.000	<0.001
$CellLine_R$	0.000	1.000
Orlistat	-1.333	<0.001
$(CellLine_R)$*(Orlistat)	-2.981	<0.001
n = 12		
Adjusted $R^2 = 0.986$		

Table 6. Proliferation Trial 3, T=48 Hours, Model Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Corrected Model	-	<0.001
Intercept	98.500	<0.001
Orlistat	-2.250	<0.001
n = 18		
Adjusted $R^2 = 0.784$		

Table 7. Proliferation Trial 3, T=72 Hours, Model Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Corrected Model	-	<0.001
Intercept	102.222	<0.001
Orlistat	-2.938	<0.001
n = 18		
Adjusted $R^2 = 0.715$		

Table 8. Proliferation Trial 3, T=96 Hours, Model Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Corrected Model	-	<0.001
Intercept	104.925	<0.001
Orlistat	-3.444	<0.001
n = 18		
Adjusted R^2 = 0.841		

Table 9. Proliferation Trial 4, T=48 Hours, Model Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Corrected Model	-	<0.001
Intercept	108.976	<0.001
$CellLine_R$	-21.085	0.055
Orlistat	-2.353	0.001
$(CellLine_R)$*(Orlistat)	-1.997	0.039
n = 24		
Adjusted R^2 = 0.798		

Table 10. Proliferation Trial 4, T=72 Hours, Model Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Corrected Model	-	<0.001
Intercept	108.379	<0.001
$CellLine_R$	-25.295	<0.001
Orlistat	-4.262	<0.001
n = 24		
Adjusted R^2 = 0.905		

Table 11. Proliferation Trial 4, T=96 Hours, Model Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Corrected Model	-	<0.001
Intercept	123.679	<0.001
$CellLine_R$	-24.498	0.005
Orlistat	-4.097	<0.001
n = 24		
Adjusted R^2 = 0.746		

Table 12. Palmitate Rescue Trial 1. Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Model	-	<0.001
Intercept	43.000	<0.001
$Orlistat_0$	56.666	<0.001
$Orlistat_{10}$	9.333	0.161
Palmitate	1.733	0.001
$(Orlistat_0)$*(Palmitate)	-1.333	0.057
$(Orlistat_{10})$*(Palmitate)	0.533	0.430
n = 27		
Adjusted R^2 = 0.869		

Table 13. Palmitate Rescue Trial 2. Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Model	-	<0.001
Intercept	24.691	<0.001
$CellLine_R$	-24.940	0.056
$Orlistat_0$	73.663	<0.001
$Orlistat_{10}$	22.222	<0.001
Palmitate	-0.329	<0.001
$(CellLine_R)$*$(Orlistat_0)$	33.303	<0.001
$(CellLine_R)$*$(Orlistat_{10})$	-13.516	<0.001
$(CellLine_R)$*(Palmitate)	1.457	<0.001
n = 54		
Adjusted R^2 = 0.984		

Table 14. Palmitate Rescue Trial 3. Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Model	-	<0.001
Intercept	59.222	<0.001
$CellLine_R$	-54.844	<0.001
$Orlistat_0$	46.814	<0.001
$Orlistat_5$	52.206	<0.001
$Orlistat_{10}$	32.843	<0.001
Palmitate	0.600	0.136
$(CellLine_R)*(Orlistat_0)$	48.879	<0.001
$(CellLine_R)*(Orlistat_5)$	12.775	0.194
$(CellLine_R)*(Orlistat_{10})$	-1.382	0.887
$(CellLine_R)*(Palmitate)$	1.562	0.007
n = 72		
Adjusted R^2 = 0.841		

Table 15. Palmitate Rescue Trial 4. Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Model	-	<0.001
Intercept	15.419	0.011
$Orlistat_0$	81.637	<0.001
$Orlistat_5$	42.018	<0.001
$Orlistat_{10}$	23.054	0.007
Palmitate	0.420	0.490
$(Orlistat_0)*(Palmitate)$	-2.756	0.002
$(Orlistat_5)*(Palmitate)$	-0.396	0.645
$(Orlistat_{10})*(Palmitate)$	-0.550	0.522
n = 72		
Adjusted R^2 = 0.691		

Table 16. Palmitate Rescue Trial 10. Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Model	-	<0.001
Intercept	91.049	<0.001
$CellLine_R$	-27.404	<0.001
$Orlistat_0$	8.025	<0.001
$Orlistat_{10}$	2.778	0.174
Palmitate	-0.741	0.015
$(CellLine_R)*(Orlistat_0)$	28.017	<0.001
$(CellLine_R)*(Orlistat_{10})$	10.347	0.001
$(CellLine_R)*(Palmitate)$	1.491	0.001
$(Orlistat_0)*(Palmitate)$	0.494	0.241
$(Orlistat_{10})*(Palmitate)$	0.247	0.555
$(CellLine_R)*(Orlistat_0)*(Palmitate)$	-2.160	0.001
$(CellLine_R)*(Orlistat_{10})*(Palmitate)$	0.586	0.323
n = 27		
Adjusted $R^2 = 0.943$		

Table 17. Palmitate Rescue Trial 11. Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Model	-	<0.001
Intercept	85.714	<0.001
$CellLine_R$	-47.085	<0.001
$Orlistat_0$	13.095	0.004
$Orlistat_{10}$	1.786	0.678
Palmitate	-1.905	0.004
$(CellLine_R)*(Orlistat_0)$	48.275	<0.001
$(CellLine_R)*(Orlistat_{10})$	28.121	<0.001
$(CellLine_R)*(Palmitate)$	5.643	<0.001
$(CellLine_R)*(Orlistat_0)*(Palmitate)$	-2.741	0.003
$(CellLine_R)*(Orlistat_{10})*(Palmitate)$	-1.246	0.165
$(CellLine_U)*(Orlistat_0)*(Palmitate)$	0.952	0.286
$(CellLine_U)*(Orlistat_{10})*(Palmitate)$	0.476	0.592
n = 54		
Adjusted $R^2 = 0.901$		

Table 18. Palmitate Rescue Trial 12. Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Model	-	<0.001
Intercept	94.693	<0.001
$CellLine_R$	15.858	<0.001
$Orlistat_0$	5.214	0.007
$Orlistat_{10}$	5.866	0.003
Palmitate	-0.931	<0.001
$(CellLine_R)*(Orlistat_0)$	-15.994	<0.001
$(CellLine_R)*(Orlistat_{10})$	15.311	<0.001
$(CellLine_R)*(Palmitate)$	1.726	<0.001
$(Orlistat_0)*(Palmitate)$	0.745	<0.001
$(Orlistat_{10})*(Palmitate)$	-0.037	0.845
$(CellLine_R)*(Orlistat_0)*(Palmitate)$	-1.999	<0.001
$(CellLine_R)*(Orlistat_{10})*(Palmitate)$	-1.094	<0.001
n = 54		
Adjusted $R^2 = 0.974$		

Table 19. Palmitate Rescue Trial 13. Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Model	-	<0.001
Intercept	86.501	<0.001
$CellLine_R$	0.693	0.670
$Orlistat_0$	14.325	<0.001
$Orlistat_{10}$	7.025	<0.001
Palmitate	-0.661	<0.001
$(CellLine_R)*(Orlistat_0)$	-1.613	0.483
$(CellLine_R)*(Orlistat_{10})$	12.843	<0.001
$(CellLine_R)*(Palmitate)$	1.339	<0.001
$(Orlistat_0)*(Palmitate)$	0.441	0.011
$(Orlistat_{10})*(Palmitate)$	0.055	0.743
$(CellLine_R)*(Orlistat_0)*(Palmitate)$	-1.307	<0.001
$(CellLine_R)*(Orlistat_{10})*(Palmitate)$	-0.695	0.005
n = 54		
Adjusted $R^2 = 0.942$		

Table 20. Apoptosis Trail 1. Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Model	-	<0.001
Intercept	65.550	<0.001
$CellLine_R$	-55.633	0.001
Orlistat	-0.425	0.303
Time	-0.235	0.127
$(CellLine_R)$*(Orlistat)	2.370	0.010
$(CellLine_R)$*(Time)	0.711	0.015
(Orlistat)*(Time)	0.011	0.326
$(CellLine_R)$*(Orlistat)*(Time)	-0.057	0.013
n = 12		
Adjusted R^2 = 0.976		

Table 21. Apoptosis Trail 2. Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Model	-	<0.001
Intercept	56.567	<0.001
$CellLine_R$	-28.317	<0.001
n = 12		
Adjusted R^2 = 0.850		

APPENDIX B

PALMITATE TRIALS 5 THROUGH 9

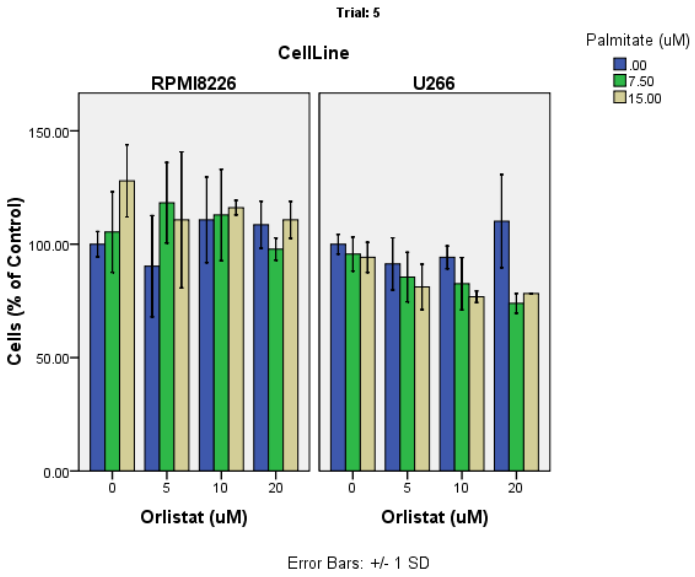

Figure 18. Palmitate Rescue Trial 5. MM Cell Proliferation in Terms of Negative Control.

Table 22. Palmitate Rescue Trial 5. Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Model	-	<0.001
Intercept	101.346	<0.001
$CellLine_R$	5.351	0.298
$Orlistat_0$	-5.696	0.432
$Orlistat_5$	-10.371	0.155
$Orlistat_{10}$	-2.115	0.770
Palmitate	-1.963	0.004
$(CellLine_R)*(Orlistat_0)*(Palmitate)$	3.517	0.001
$(CellLine_R)*(Orlistat_5)*(Palmitate)$	3.318	0.001
$(CellLine_R)*(Orlistat_{10})*(Palmitate)$	2.801	0.005
$(CellLine_R)*(Orlistat_{20})*(Palmitate)$	1.943	0.016
$(CellLine_U)*(Orlistat_0)*(Palmitate)$	1.886	0.036
$(CellLine_U)*(Orlistat_5)*(Palmitate)$	1.293	0.147
$(CellLine_U)*(Orlistat_{10})*(Palmitate)$	0.324	0.714
n = 72		
Adjusted R^2 = 0.432		

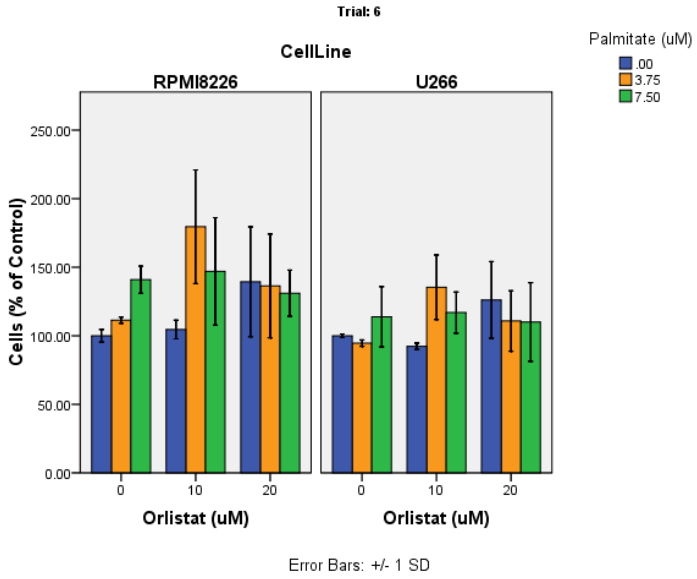

Figure 18. Palmitate Rescue Trial 6. MM Cell Proliferation in Terms of Negative Control.

Table 23. Palmitate Rescue Trial 6. Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Model	-	0.004
Intercept	106.951	<0.001
$CellLine_R$	21.128	0.005
$Orlistat_0$	-15.501	0.081
$Orlistat_{10}$	3.656	0.676
Palmitate	21.128	0.069
n = 54		
Adjusted R^2 = 0.206		

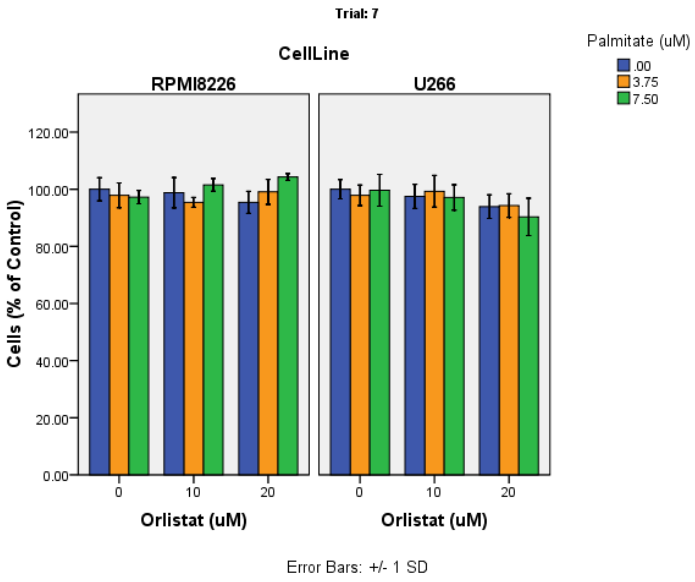

Figure 19. Palmitate Rescue Trial 7. MM Cell Proliferation in Terms of Negative Control.

Table 24. Palmitate Rescue Trial 7. Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Model	-	0.003
Intercept	94.893	<0.001
$CellLine_R$	-0.005	0.997
$Orlistat_0$	4.653	0.015
$Orlistat_{10}$	2.782	0.141
Palmitate	-0.521	0.136
$(CellLine_R)*(Orlistat_0)*(Palmitate)$	0.187	0.719
$(CellLine_R)*(Orlistat_{10})*(Palmitate)$	0.812	0.121
$(CellLine_R)*(Orlistat_{20})*(Palmitate)$	1.747	<0.001
$(CellLine_U)*(Orlistat_0)*(Palmitate)$	0.441	0.338
$(CellLine_U)*(Orlistat_{10})*(Palmitate)$	0.549	0.233
n = 72		
Adjusted R^2 = 0.265		

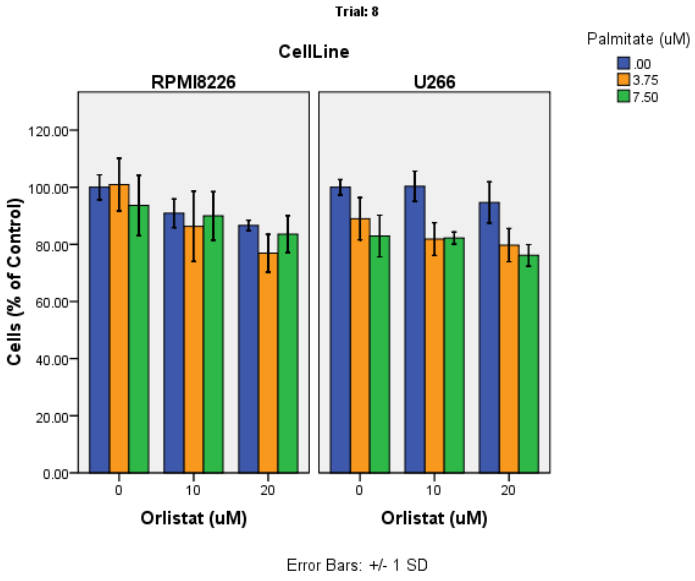

Figure 20. Palmitate Rescue Trial 8. MM Cell Proliferation in Terms of Negative Control.

Table 25. Palmitate Rescue Trial 8. Parameter Estimates and P-Values.

Parameter	**B**	**p-Value**
Model	-	<0.001
Intercept	92.764	<0.001
$CellLine_R$	-8.873	0.061
$Orlistat_0$	6.406	0.173
$Orlistat_{10}$	4.448	0.342
Palmitate	-2.467	0.001
$(CellLine_R)*(Orlistat_0)$	11.072	0.097
$(CellLine_R)*(Orlistat_{10})$	1.175	0.859
$(CellLine_R)*(Orlistat_0)*(Palmitate)$	1.616	0.097
$(CellLine_R)*(Orlistat_{10})*(Palmitate)$	2.346	0.017
$(CellLine_R)*(Orlistat_{20})*(Palmitate)$	2.062	0.036
$(CellLine_U)*(Orlistat_0)*(Palmitate)$	0.190	0.844
$(CellLine_U)*(Orlistat_{10})*(Palmitate)$	0.047	0.961
n = 72		
Adjusted R^2 = 0.480		

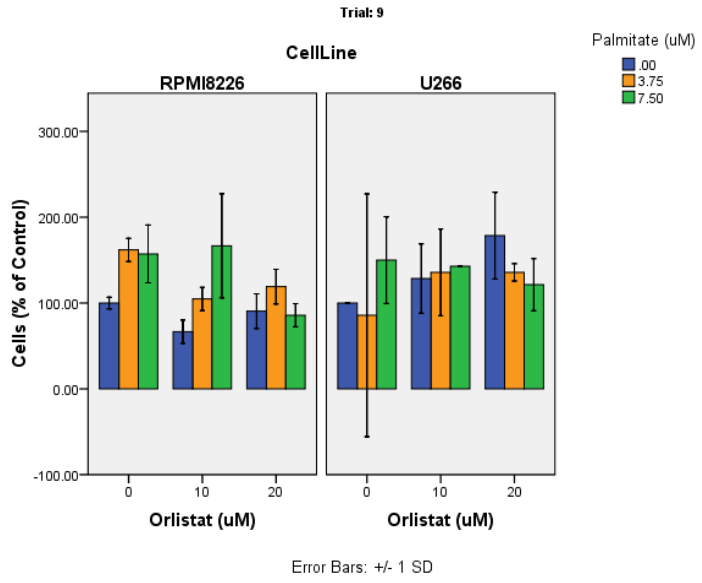

Figure 21. Palmitate Rescue Trial 9. MM Cell Proliferation in Terms of Negative Control.

Table 26. Palmitate rescue trial 9. Parameter estimates and p-values.

Parameter	B	p-Value
Model	-	0.178

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