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Cell Cycle Effects of Simultaneous Treatment with U0126 MEK Inhibitor and Nocodazole

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Nataliya Pyatka is a senior biochemistry student at Case Western Reserve University. In her free time, she enjoys drawing, dancing, reading, and volunteering. At Case, she is a member of WISER (Women in Science and Engineering Roundtable), Discussions, Global Medical Initiative, and HCSL. After graduation, Nataliya plans to pursue an MD degree.

ABSTRACT

This project evaluated the phosphorylation state of intracellular signaling molecules Histone H3 and ERK in Human Umbilical Vein Endothelial Cells (HUVEC) by using iCyte Laser Imaging Cytometry. The hypothesis is that phosphorylation of state-specific antibodies can be used to classify hematological malignancies and monitor drug effects. This project studied the MAPK/ERK (MEK) signaling pathway that is commonly activated in cancer because of mutations involving growth factor receptors.

The effects of nocodazole and MEK 1/2 inhibition by U0126 were analyzed by studying cell cycle arrest as well as the phosphorylation states of ERK and Histone H3. Nocodazole, which disassembles microtubules and interrupts formation of spindle fibers, caused a decrease in G1 and G2/M cell cycle arrest (Fig. 1). U0126 disrupted MEK growth signals, causing the cells to accumulate in G1, G2, and M. In the double treatment (nocodazole plus U0126), cells arrested in G1, G2, and M, and decreased in S phase. Cells moved through mitosis slower in the presence of U0126 and faster when nocodazole was present.

Since the main drug targets are often signaling molecules themselves, studying their pathways will yield drugs with lesser side effects and greater target specificity. The long-term project will utilize a systems biology approach to integrate high-content and high-throughput bioassay data to create models for predicting the potential efficacy, toxicity, and pharmacokinetics of specific pharmaceutical agents in humans.

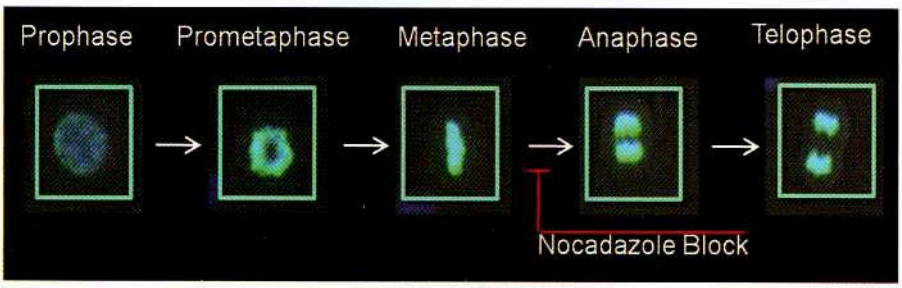

Figure 1: The effects of nocodazole on mitosis. Cells stained with pHistine-H3 (cyan), DAPI (blue), pERK (red).

-Acknowledgments-

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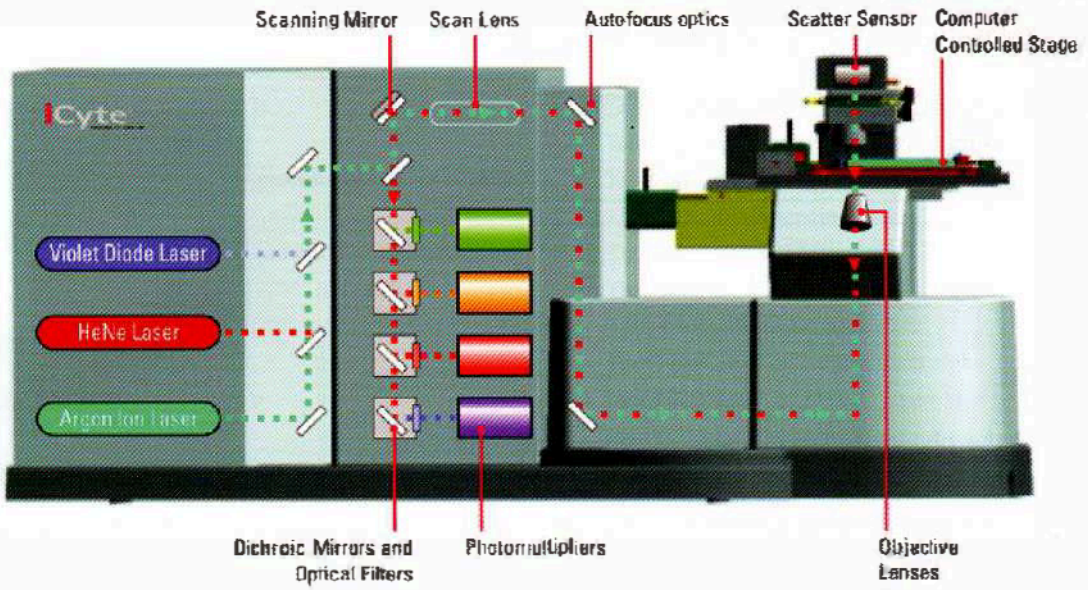

Figure 1: The effects of nocodazole on mitosis. Cells stained with pHistine-H3 (cyan), DAPI (blue), pERK (red).

INTRODUCTION

Since their invention in the 1600's, microscopes have been essential for the advancement of our scientific knowledge. A microscope-based machine called the iCyte Laser Imaging Cytometer is one of the most powerful instruments for cellular analysis. Unlike a simple microscope, the iCyte detects multicolor fluorescence and measures cell properties using lasers and photomultiplier tubes (Fig. 2). Similar to a light microscope, the iCyte displays visual images of the scanned area without destroying the samples. Imaging cytometry provides visual verification and analytical characterization of cellular events (such as apoptosis and cell signaling).

Cytometry measures signals from each cell individually and can correlate two or more parts of data to provide a more complete picture of the cell's behavior. Therefore, cell populations are easily separated based on their statistical differences (such as antibody fluorescence or DNA content). For iCyte analysis, cells can be stained for surface markers because the antibody will form a strong bond to its corresponding antigens. To visualize this bond, a fluorescent tag (such as Alexa Fluor 488-Goat anti-mouse) is used. The presence of proteins like phospho-Histone H3 or phospho-ERK is associated with certain stages of the cell cycle.

The nucleosome, composed of H2A, H2B, H3 and H4 histone proteins, makes up eukaryotic chromatin. Transcription is regulated by chromatin structure modulation. As a result of various stimuli, histones can be phosphorylated, acetylated, methylated, or ubiquitinated. These post-translational modifications have a direct effect on gene expression by making chromatin available (or unavailable) to transcription factors. Chromosome condensation during mitosis is correlated to the phosphorylation of Histone H3 at Ser10. Phosphorylation of Histone H3 begins in late G2, reaches a maximum at metaphase, starts to decrease at metaphase, and is gone at telophase (Hendzel, 1997). Most interphase cells do not show significant levels of phospho-Histone H3. Histone H3's amino-terminus phosphorylation helps to facilitate protein-protein interactions necessary for the binding of trans-acting factors that regulate chromatin condensation and decondensation during mitosis. If the kinase that phophorylates Histone H3 is inhibited, the cell will not be able to enter mitosis (Van Hooser, 1998).

Mitogen-activated protein kinases (MAPKs) are important for cell proliferation, differentiation, and death. The study of MAP kinase cascade is essential for the development of pathway-specific kinase inhibitors and identification of abnormal kinase activity in disease states. Mitogens, growth factors, and cytokines can activate the

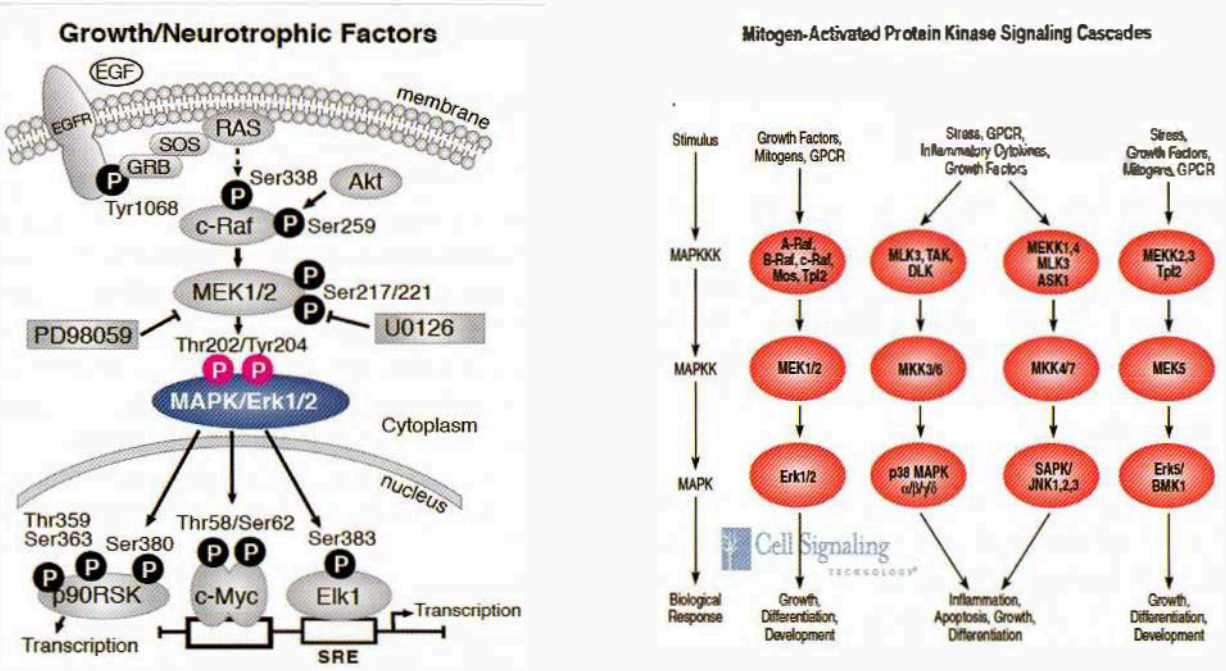

Figure 3: The MAPK/ERK signaling pathway. When stimulated by growth factors, MEK activates cell growth by stimulating transcription. U0126 inhibits MEK phosphorylation and cell proliferation.

p44/42 MAPK (ERK1/2) signaling pathway (Meloche, 2007). Phosphorylation of kinases usually enhances their activities as enzymes and helps to propagate downstream signals. The phosphorylation of ERK1/2 can be used to measure the activation of G protein-coupled receptors. The signaling cascade usually starts the cellular surface and each kinase is activated by being phosphorylated by its upstream member (Fig. 3). To detect the levels of phosphorylated ERK1/2, the mouse antibody phospho-p44/42 MAPK (ERK1/2) is used. It is visible in G1, S, and G2 phases of the cell cycle.

U0126 is a highly selective and potent MEK1&2 inhibitor that causes G1 cell cycle arrest. Decreased MEK activity leads to cessation of cell division but not apoptosis (Blank, 2002). Nocodazole disassembles microtubules, interrupts formation of spindle fibers, and causes fragmentation of the Golgi complex. Thus interphase cells' entry into mitosis is slowed. As an antimitotic agent, nocodazole causes G2/M arrest by binding to tubulin and preventing interchain disulfide links. When nocodazole disassembles microtubules in early prophase, cells return to antephase for 2–4 hours and then ultimately enter mitosis (Rieder, 2002). There is a several hour lag period during which the percentage of mitotic cells does not change due to the disassembled microtubules (Rieder, 2000).

MATERIALS & METHODS

Human Umbilical Vein Endothelial (HUVEC) cell line was cultured in Lonza Endothelial Cell Basal Medium-2 (EBM-2) with 2% fetal bovine serum, FBS, Hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic Acid, hEGF, GA-100, and heparin. Cells were kept in a humidified incubator with 5% CO2 and 95% air at 37°C.

Samples were prepared in 24-well plates or 4-well chamber slides using 43,000 cells per well. Cells were grown for 2-3 days followed by nocodazole and/or U0126 treatment. 1 mg/mL stock nocodazole was diluted to 3 different concentrations: 50, 100, 200 ng/mL/well. Nocodazole was added and cells were incubated for 0, 2, 4, or 6 hours. For the zero time points, nocodazole was added and immediately aspirated out.

In the experiment with U0126 only, there were four different concentrations: 0, 1, 10, and 100 μM and seven time points: 5, 10, 15, 30, 60, 120, and 360 minutes. The total

treatment volume was 500 µL and each point had triplicate samples. 1 mg of stock U0126 was diluted in 26 µL anhydrous DMSO for a final concentration of 100 µM. When cells were treated with the inhibitor, it was diluted in Lonza culture medium.

In the next experiment with the double treatment (nocodazole and U0126), cells were treated for 30 minutes with 100 ng/mL nocodazole and different U0126 concentrations: 0, 0.4, 2, 10, 50, 100, and 200 µM. Some wells had the inhibitor by itself and nocodazole by itself. There were also control samples with nothing added and each point had quadruplicates.

For the nocodazole experiment (where cells were stained with DAPI DNA probe only), 8 µl of 16% formaldehyde was added to 1 mL culture medium for the total concentration of 0.125%. The cells were then incubated at 37°C for 10 minutes followed by addition of -20°C methanol (1mL per sample). For cells that were to be stained with pERK, the fixation procedure was optimized: formaldehyde was used at 1% concentration was (instead of 0.125%) and the formaldehyde incubation took place at room temperature (26°C). Due to the transient nature of intracellular signaling events, cell fixation was done rapidly to ensure that dephosphorylation did not occur. Methanol acted as a stabilizer to prevent polymerization.

After fixation, two PBS washes (phosphate buffered saline) and one PBS/BSA wash (PBS + bovine serum albumin) were done. The antibodies were diluted with PBA/BSA. 10µl of phospho-Histone H3 (Ser10) antibody and 20 µl of phospho-ERK½ (T202/Y204) antibody was added to 245 µl of PBS/BSA for the total antibody staining volume of 275 µL per sample. The cells were incubated in the dark at 4°C for 60 minutes. All reagents, pipettes, and tubes were kept at 4°C in order to ensure stable temperatures during the staining procedure. Two more PBS/BSA washes were done (with 15 minutes in between each wash) to remove any weakly or nonspecifically bound proteins. The DNA probe was added (DAPI: 0.5 µl/mL concentration, 1mL DAPI/tube) and the samples were run on the iCyte laser imaging cytometer.

In the analysis, the percentage of mitotic cells was measured by phosphorylated Histone H3. ERK phosphorylation monitored the activity of U0126. Cell cycle effects were monitored by phase fractions. Mitotics and new cells were gated out from G2 and G1 and analyzed separately.

RESULTS

Nocodazole

Analysis of cell cycle distribution followed nocodazole treatment. Nocodazole demonstrated a dose and time dependent response: G1 decrease, G2 increase, S decrease, and M increase (Figures 4&5). Prolonged nocodazole treatment also led to an increase in endroreduplicated cells, indicating that DNA replication continued but nuclear division had ceased. 50 and 100 ng/mL nocodazole gave similar results. 200 ng/mL dose was too high because nocodazole's effects seemed to be diminished.

U0126

Cell cycle distribution was determined by measuring the DNA content and the relative amounts of phosphorylated Histone H3. 30 minutes and 10 µM U0126 was determined to be optimal. 1 µM U0126 did not provide the maximum possible cell cycle effects. 10 µM concentration showed strongest the inhibition, but only until 30 minutes (Figure 6&7). Due to U0126's inhibition of MEK, the following effects were observed initially: G1 increase, G2 increase, S increase, M increase, and growth (new cells) decrease. After this time point, the inhibition seemed to have been reversed: G1 decreased, G2 increased, S decreased, M de-

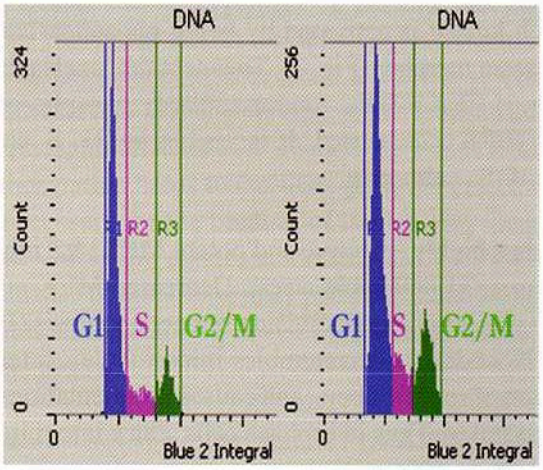

Figure 4: No treatment vs. 4 hr 200 ng/mL nocodazole treatment. The histogram shows the G1 and S area decrease and the G2 increase.

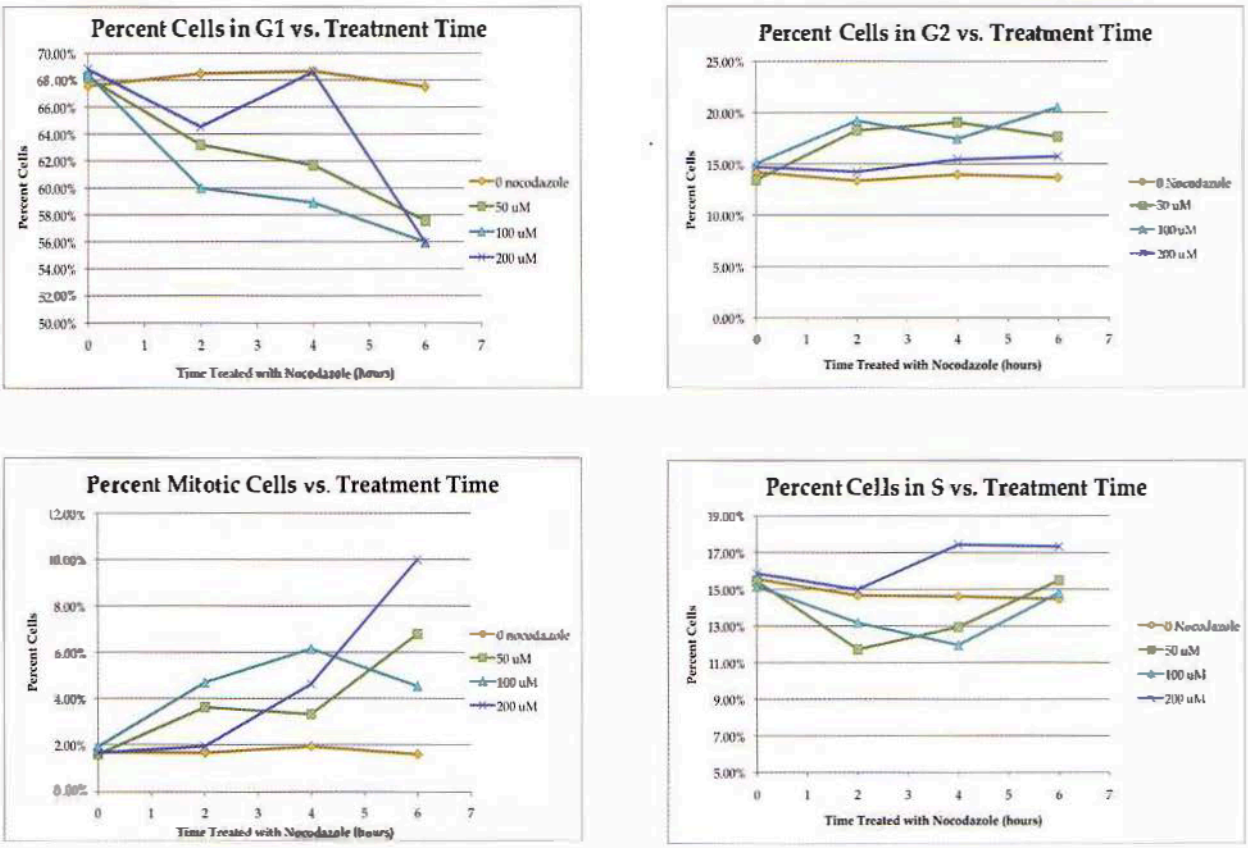

Figure 5: The effects of nocodazole treatment.

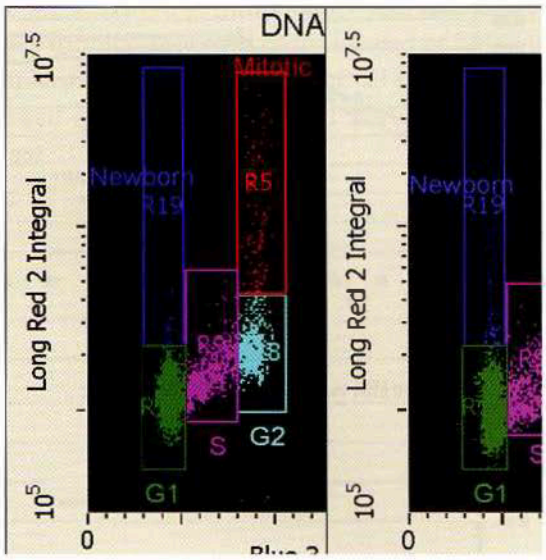

Figure 6: Untreated (left) vs. 10 min, 1μM U0126 treated (right) HUVEC Histone H3 fluorescence. The increase in mitotic, G2 and G1 population due to U0126 is clearly visible.

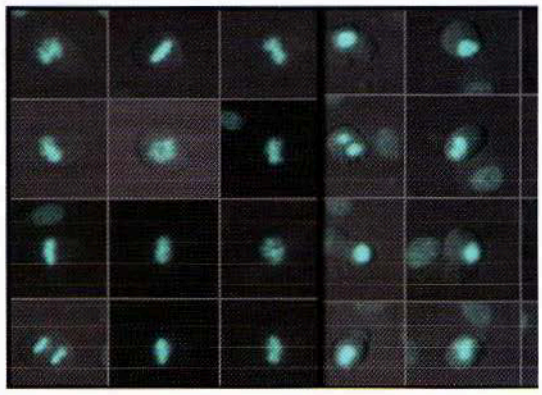

Figure 9: Sample mitotic population of untreated (left) and 4 hour, 100 ng/mL nocodazole treated cells (right). Abnormal morphology can be seen in the treated cells where chromosomes did not line up properly at the metaphase plate during mitosis.

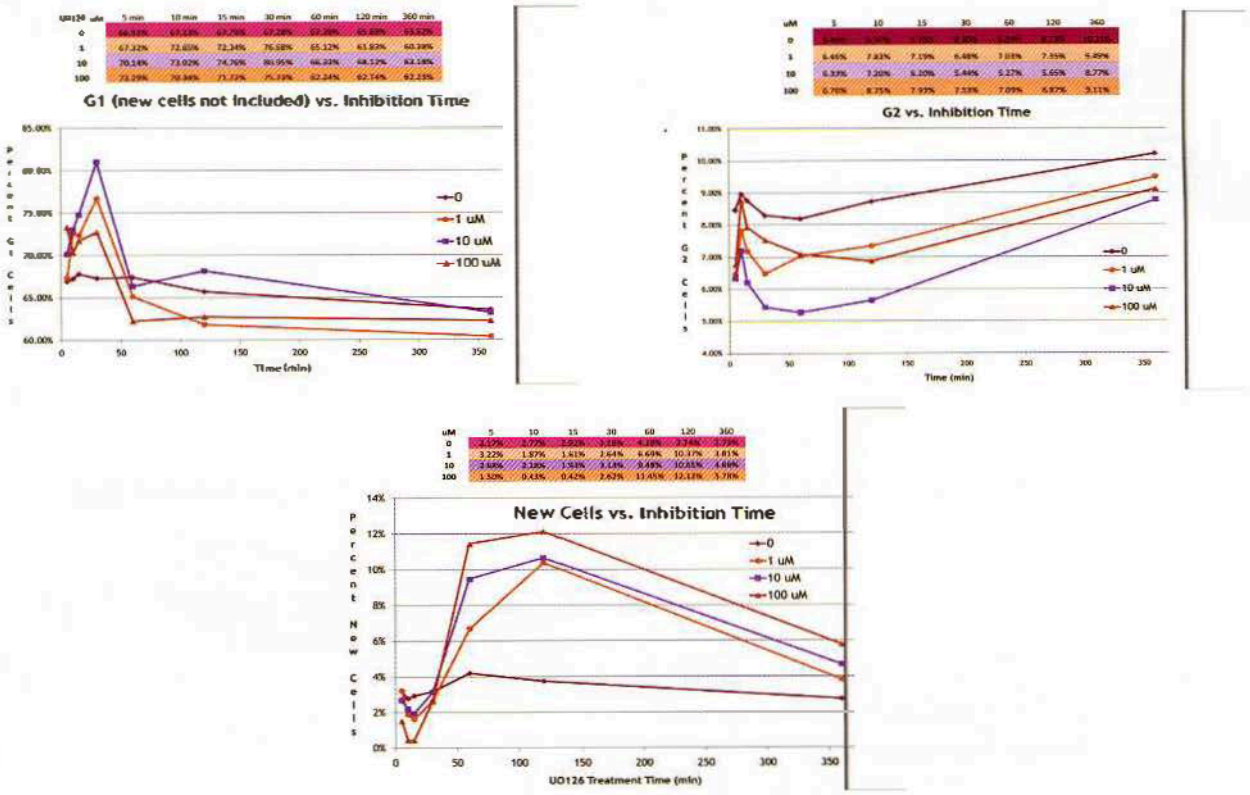

Figure 7: The effects of U0126 treatment.

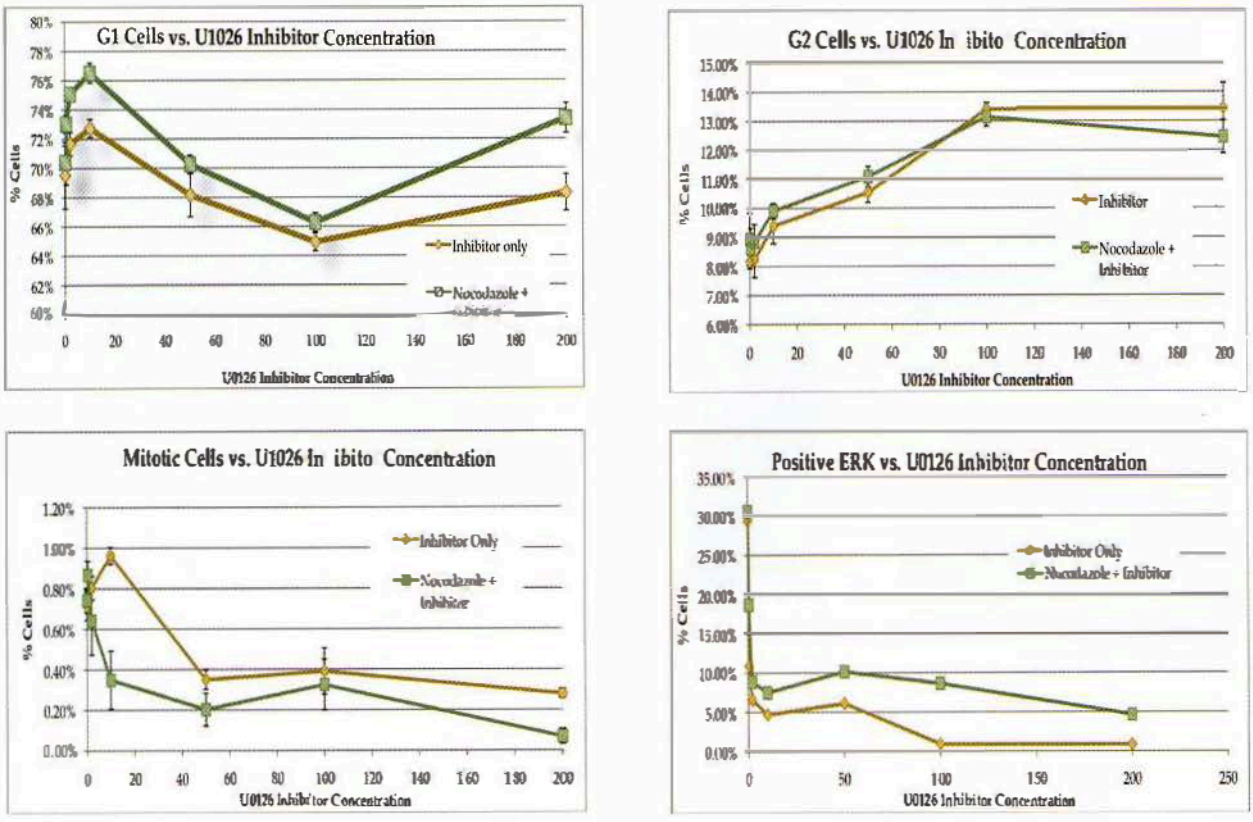

Figure 8: The effects of nocodazole+U0126 treatment.

creased, and growth (new cells) decreased (Figure 7). The ERK signal showed a consistent, dose dependent decrease. These observations demonstrate that cell proliferation was slowed due to decreased growth signals from MEK inhibition.

Nocodazole and U0126

In the double treatment, cells arrested in G1, G2, and M. As a result, S phase decreased (Figure 8). Again, U1026's effects were reversed at concentrations above 10 μM. Nocodazole did not a have dominant effect over U0126 in G2. Cells moved through mitosis slower in the presence of U0126 and faster in the presence of nocodazole. ERK signal was inhibited again, but not as greatly as in the presence of U0126 alone.

DISCUSSIONS & CONCLUSION

From image data it was evident that the ERK signal started at the cellular surface, went to the cytoplasm, and then became localized mostly in the nucleus. This is consistent with ERK's signaling pathway: activating stimuli (such as growth factors) initiate a protein kinase cascade that regulates transcription factors. Based on this information, data analysis focused primarily on nuclear ERK fluorescence.

Nocodazole led to abnormal morphology characterized by chromosomes forming spherical blobs instead of lining up at the metaphase plate (Figure 9, page 21). Cell scatter images showed cells rounding up and starting to detach from the plate.

Up to 50μM U0126 concentrations had potent cell cycle effects. U0126 led to inhibition of transcription because MEK was inhibited and could not stimulate growth. Cells could not get through late G1 phase because MEK growth signals were not present. Thus, S phase decreased because cells were exiting G1 slower and passing through G2 and M at a slower rate.

In the double treatment (nocodazole plus U0126), nocodazole showed an additive, but not synergistic, effect on G1 and S. At higher U0126 concentrations, nocodazole was not strong enough to overcome U0126's effects on G2. G2 showed a consistent increase as a function of U0126. Since the effects of nocodazole are usually not seen until at least 2 hours, the control samples and the samples with nocodazole were not much different.

The study of MAP kinase cascade is essential for the development of pathway-specific kinase inhibitors and the identification of abnormal kinase activity in disease states. Since the main drug targets are often signaling molecules themselves, studying their pathways will yield drugs with lesser side effects and greater target specificity. The long-term project will utilize a systems biology approach to integrate high-content and high-throughput bioassay data to create models for predicting the potential efficacy, toxicity, and pharmacokinetics of specific pharmaceutical agents. Data-driven modeling will also help to identify the mechanism of action of the drugs and improve the utility of early clinical trials.

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