The Viability of Cancerous vs. Non-cancerous Cells

Lung cancer is a disease that affects a number of human beings in the world each and every day. It is the number one cancer-related cause of death in men, and the number two cancer-related cause of death in women. It also has a high mortality rate when compared to other cancers. These factors, combined with the high-profile nature of cancer make it imperative that we study this disease and find ways to defeat it. My research with the University of Kansas' High Throughput Screening laboratory is the beginning of finding ways to defeat this disease. Every drug on the market has some mode of action. A compound will bind to a receptor, or block the synthesis of another compound, or act in some way that affects the cell. Here, we are looking for the differences that cancerous and non-cancerous cells have. With this research, we can find compounds that inhibit the growth of cancerous cells, and do not inhibit the growth of non-cancerous cells, and then find common modes of action for those compounds. If there is a common mode throughout many different compounds, then that mode of action can be isolated and further researched for drug delivery.

In my specific work, we used two different lung cell types: the MRC-5 cell line, which is a normal lung fibroblast, and the A-549, which is a cancerous lung cell. These were not identical cell lines, as they did not originate from the same human being, but they were close matches. The way we decided to approach the problem is to use a cell-based assay to inject the compounds into the cells, and then measure the cells' growth. To find the effects the compounds had on the cells, we needed some way to visualize the viability of the cell lines. To do this, we used the reagent Cell Titer-Glo. This reagent reacts with the amount of ATP present, and luminesces at different intensities corresponding to the amount of ATP. Therefore, if many cells were to survive, the luminescence value would be very high and, contrastingly, if the cells were to die, then the values would be lower. However, before this reagent could be used, preliminary experiments had to be performed.

The first experiment to be performed was to obtain growth curves

LOGAN NICKELS is a senior in Biochemistry at the University of Kansas.

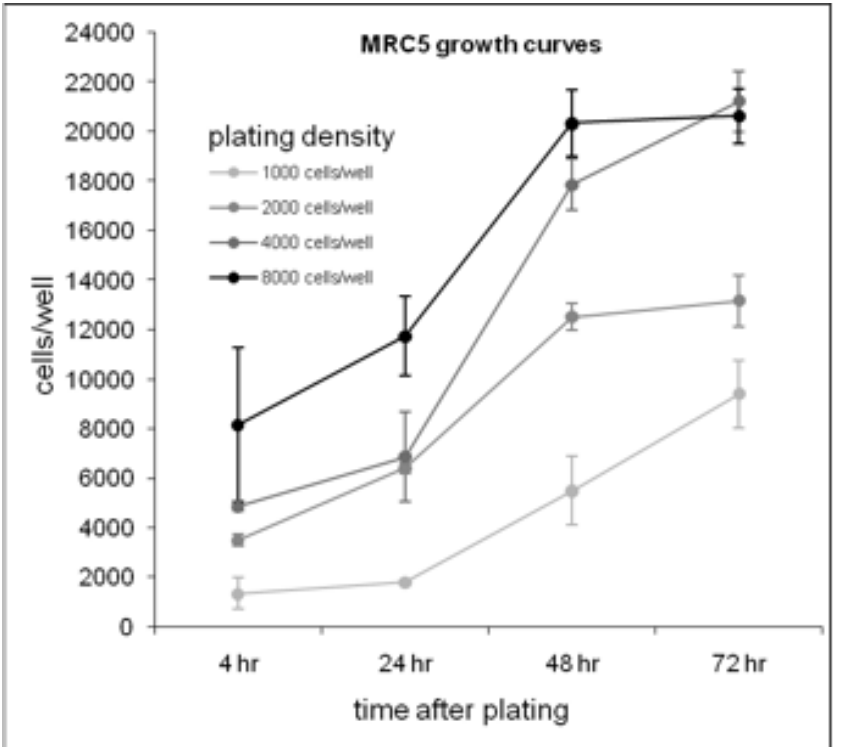

Figure 1. This is the growth curve of the non-cancerous MRC-5 cell line, with an ideal plating density of 1,500 cells / well of a 384-well plate.

for both cell lines. This would allow us to not only find the optimal seeding density for both lines, but to also associate specific luminescence values with specific numbers of cells. This was done by using a hemocytometer to place a specific number of cells within a well of a standard 384-well plate, and using the Cell Titer-Glo reagent combined with the Tecan Safire, 2 a device that measures luminescence values, a luminescence value for that number of cells could be obtained. When that was done, the cells were then plated at specific densities, and allowed to grow. The plates were read with the Cell Titer-Glo reagent every 24 hours, and the luminescence values noted. Since the luminescence values could only be obtained using black plates, a separate set of cells were plated in clear plates, and using a photographic microscope, a good visual representation of the different densities could be obtained as well. The plates with the higher seeding densities began to show cell death after 48 hours, and the lower seeding densities continued to grow. After the full 4 days of growth, the results showed that the optimal seeding density for both the MRC-5 cell line and the A-549 cell line was 1,500 cells per well. This would allow the cells to grow to full confluency over the four days of the project, but not experience cell death due to overconfluency. If the cells are overly confluent, then they

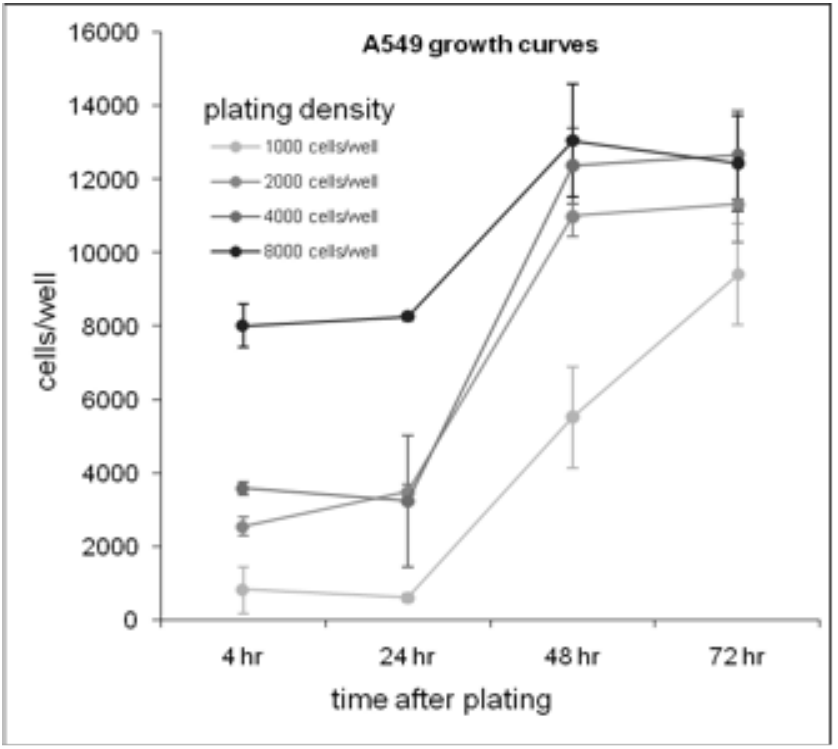

Figure 2. This graph shows the growth curve of the cancerous A-549 cells, which have an ideal plating density of 1,500 cells / well of a 384-well plate.

begin to stack on top of each other, use all of their nutrients, and die in their own waste. This would skew the results of the final project, so it was imperative that we find the proper seeding density.

The second experiment to be performed before the actual assay was a toxicity screen. The compounds used in the screen were stored in 384-well polypropylene plates. The plates contained 20 µl of compound

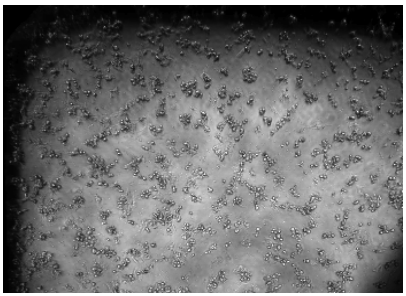

FIgure 3. Day 1 of the highest seeding density, 16,000 cells / well of the MRC-5 line

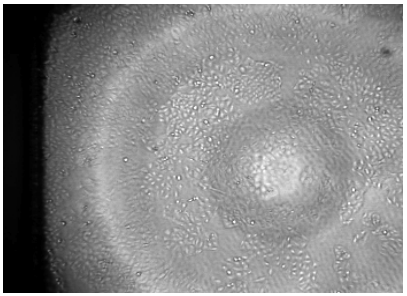

FIgure 4. Day 4 of the A-549 1,000 cells / well density, nearing confluency

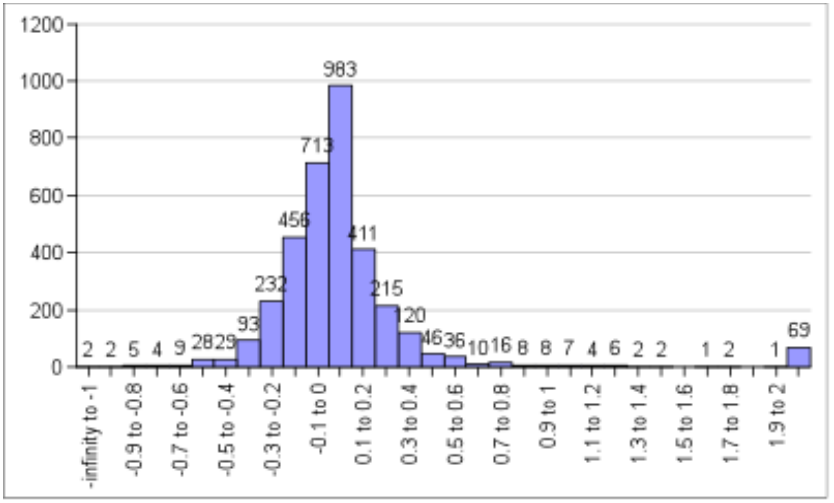

Figure 5. This graph plots the number of compounds vs. their percentages away from normalized values. Overall, there were 79 compounds that were at least 50% below the normalized value, and 103 compounds at least 50% above the normalized value, for a total of 182 compounds classified as "active." Of the 103 active compounds above 50% of the normalized value, 69 of them were of suspiciously high values, and in a concentrated area of one plate, possibly indicating a liquid handling error.

in each well, frozen in 2.25% DMSO in water. Since DMSO is toxic to cells, this test was required to ensure that addition of compound would only induce toxicity due to the effects of compound, not DMSO. By adding different concentrations of DMSO to cell lines, the cell viability was observed by using Cell Titer-Glo and the Tecan Safire2, as in the growth curve experiment. By comparing the luminescence values obtained with the DMSO lines to the growth curve, cell viability was easily established. Since after compound addition, the cells would be in approximately 0.65% DMSO, as long as the cell lines were viable in that concentration of DMSO, then the screen could proceed as planned.

To accomplish this, two black plates were seeded with 1,500 cells per well, one plate of each cell type. This seeding density was found previously in the growth curve experiment. These plates were allowed to grow for two days, and then, as per the protocol for the final assay, a mock compound was added, using the BioMek FX robotic system. This mock compound was simply a salt solution with different concentrations across the board. After addition, the concentrations of DMSO ranged from 0.0%, all the way to 4.0%. This would allow for reliability of results, and a great range of DMSO toxicity results. After the addition of the mock compound, the cells were grown another two days, and then read using Cell Titer-Glo and the Tecan Safire2. The results showed that for both cell lines, the DMSO concentration used in the final assay was well within safe levels, and that the non-cancerous line did not show signs of toxicity until a concentration of 2%. The cancerous line, however, seemed to be more hardy than the non-cancerous line, not showing signs of toxicity until a concentration of nearly 4%.

After these experiments were performed, the final cell assay could continue. The optimal cell densities had been set, luminescence values could be traced back to actual cell numbers, and the DMSO concentration being used in the final assay was not toxic. The cells were plated at their optimal densities in black 384-well plates, and then grown for two days before compound addition. The compounds used came from different libraries, compiled by different laboratories. The libraries used in the screen were the Prestwick (Prestwick Chemical, Illkirch, France) and the Spectrum Collection (Microsource Discovery Systems, Inc., Gaylordsville, CT) libraries, which contain a very small fraction of the total compounds available to the KU HTS lab. The reason for the small selection of compounds was, again, a time limitation associated with the constraints of an independent study. These libraries consist of small molecules with known biological activity. The purpose of these libraries is to see how compounds with a variety of biological activities behave in an assay. Thus, their purpose is more for assay development than for finding lead molecules.

After compound addition using the BioMek FX system, the cells were then grown for another two days, to reach full confluency within their wells. They were then read using the same method of Cell Titer-Glo and the Tecan Safire2 seen in the preliminary experiments. After reading the plates, the data was collected, and normalized so that the difference in growth could be easily seen. Again, the point was to find compounds that adversely affected the growth of the cancerous cell line, but caused no harm to the non-cancerous cell line. For a compound to be classified as "active", there needed to be a difference of at least 50% in the growth of the two lines. The data was then analyzed to find the compounds that differed by at least 50% in growth, and the specific compounds noted.

The screen indicated 182 compounds of interest that should be investigated further, as their effects differed by more than 50%.

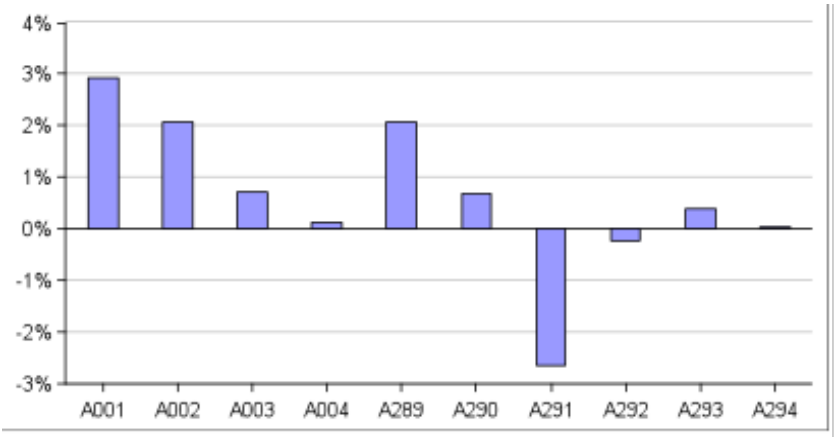

Figure 6. This graph shows the overall effect of certain plates on the cell lines. A positive number means that the plate had an effect that caused toxicity in the cancerous cell line more than the normal cell line, and a negative number means that the normal cell line was more affected than the cancerous line. Plate A001 had a number of abnormalities that could have caused the plate to look as if was more toxic to the cancerous cell line than it actually was.

Unfortunately, due to the time constraints of a student independent study, this work will have to be continued at a later date. The next step with these compounds is to confirm their activity, and ensure that they are not false positives / negatives. Unfortunately, many false positives / negatives are expected during the checks, as there were pipetting errors with the robotic addition system used in the experiment. The next step includes performing the experiment again in the areas that were deemed problematic, and comparing those results against the original results to find false positives and negatives. Compounds that are confirmed as active should be used in dose-response experiments, to show the potency of the compounds. After dose-response curves have been calculated, the compounds should be used in other assays to determine the mechanism of action for the drugs. There are a number of assays to perform with the drugs, such as apoptosis assays, cell-cycle arrest assays, and cytoskeletal assays. Once the mechanism of action is determined, the compounds can be used with toxicity mechanisms to develop new drug targets. Despite this being only the first, small step in fighting cancer, hopefully we can soon find a way to make these compounds deliverable to human beings, and eventually help those in need.