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Title

Inhibition of Cancer Cell Migration by EphA2 Agonistic Agents

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Abstract

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Introduction

Cancer, in general, is an aggressive disease regarding the uncontrolled and rapid proliferation of abnormal cells in various parts of the human body. Normally, cells in the human body grow and divide as necessary for survival and when certain cells are old, damaged, or unneeded, they are either phagocytized by macrophages or undergo apoptosis, also known as programmed cell death. However, in cancer, this pathway is drastically altered. Instead of abnormal cells dying, they strategically elude death by ignoring the signals in the aforementioned mechanisms; and to further compound the condition, additional, unnecessary cells are formed that continuously multiply. These outcomes lead to growths in the body called tumors. Malignant tumors are considered cancerous as they have the ability to spread or invade nearby tissues, sometimes breaking off and traveling to places in the body distant from the source via the bloodstream or lymphatic system; thus, being considered metastatic (National Cancer Institute at the NIH, 2015).

EphA2 is a receptor protein-tyrosine kinase (RTK) that is involved in tumorigenesis, angiogenesis, tumor cell migration, adhesion, and metastatic behaviors of several solid tumors including "prostate cancer, melanoma, urinary bladder, breast, ovarian, pancreatic, brain, esophagus, lung, and stomach cancers, and leukemia" (Gambini and Salem et al., 2018). In various cancer cells, EphA2 has pro-oncogenic activity rendering cancer cells highly aggressive and metastatic; this is caused by the overexpression of the un-ligated EphA2 receptor. Numerous studies have outlined that if the EphA2 receptor is stimulated via binding with its various ephrin-A ligands, the cancerous behavior of the cells is controlled in a method similar to a tumor suppressor. This involves the activation of various tumor suppressive intracellular pathways mediated by EphA2 which lead to the "suppression of cell migration" (Petty et al., 2012).

Evidentially, it seems as if EphA2 can function as both an oncogene and tumor suppressor. Therefore, the utilization of various migration assays in concordance with the testing of small molecule EphA2 agonists from the Pellecchia Laboratory (hence, ephrin-A mimetics) holds great promise for advances in not only cancer research, but in the development of innovative compounds that have the ability to suppress cancer metastases.

Using different cell migration assays, the aim of this research is to test various compounds synthesized by the Pellecchia Laboratory to determine 1) which migration assay is the most robust in testing the rate of cancer cell migration and 2) which of the agonistic agents synthesized by the Pellecchia Laboratory is the most effective at inhibiting cancer cell migration via binding of the EphA2 receptor. With approximately 40% of people predicted to be diagnosed with cancer at some point in their lives, research in these fields is imperative to unlocking the next step in finding the cure for cancer (National Cancer Institute at the NIH, 2018).

Methodology

Over the course of this study, three different types of assays were utilized to determine the effectiveness of the compounds synthesized in the Pellecchia Laboratory. Each of the three assays were created to measure the rate of cancerous cell migration when treated with various agonistic agents.

Cell Preparation

Prior to experimentation, the appropriate cell line was maintained in passage in order to preserve and keep the cells growing for extended periods of time. If an experiment was not yet ready to be performed, the cells were maintained in passage until they were ready to be utilized. Each cell plate was maintained in 10 mL of warm (37 degrees Celsius) "complete media" appropriate for the cell line (e.g. RPMI for BxPC-3 and F-12 for A549) that contained 10% heat-inactivated fetal bovine serum (FBS) and 1% Pen-Strep (mixture of penicillin and streptomycin); these solutions are necessary for the growth and sterility of the cells. In order to further ensure reduced error and optimum sterility and safety, any work involving the cancerous cells was always performed under a laminar flow hood that was sterilized with ethanol and UV light both before and after use. The cells were plated in a 10 cm tissue culture dish with complete media and stored in an incubator at 37 degrees Celsius until they were either in need for another round of passing or ready for use in experimentation.

Every few days, the cells were checked to ensure proper growth and maintenance. The cells would be checked for confluency under a microscope at 10x magnification. If it was estimated that the cells took up more than 90% of the cell plate area, it was ready for a new passage. However, if it was deemed that the cells were not overly confluent, a simple replenish

of fresh complete media was performed. This was done by removing the cell plate from incubation and aspirating the old media with a 10 mL serological pipette. 10 mL of fresh complete media was then added to the plate and re-stored in the incubator. These periodical checks were necessary to ensure the cells were robust and suitable for experimentation.

If the cells were overly confluent (i.e. >90% of the plate area was covered), a cell passage was required in order to maintain optimal growth conditions for the cell line. This method involved the detachment of cells from the old plate to be subsequently diluted and regrown on a new plate. The old media was removed via aspiration with a serological pipette and each plate was washed with 5 mL of room temperature phosphate buffered saline (PBS) free of calcium and magnesium. Calcium and magnesium promote cell adhesion, so its absence is necessary to stimulate detachment of cells from the plate to allow for proper passage. The PBS was then aspirated and 3 mL of warm (37 degrees Celsius) Trypsin-EDTA (0.25%) was added to the plates and gently swished. Trypsin has an optimum activity at 37 degrees Celsius so adding the warm enzyme solution accelerates the detachment process via degradation of extracellular proteins that aid in adhesion of cells to the plate. Additionally, EDTA is added because it acts as a chelating agent; binding and removing calcium and magnesium from the cell surface to weaken adhesion interactions. The Trypsin-cell solution was left in the incubator for approximately four minutes, or until cell detachment was apparent, at which point 7 mL of appropriate media was subsequently added to each plate to dilute the Trypsin and titrated using a serological pipette to ensure complete dispersal of cell clumps. The entirety of the solution was collected and transferred to a Falcon 50mL Conical Centrifuge Tube and centrifuged at 1000 RPM for five minutes at 25 degrees Celsius. The supernatant was removed, the cell pellet was resuspended in 1 mL of warm media and titrated with a P1000 micropipette to remove clumps, and then the

entire solution was completed with 3 mL of media (or as appropriately calculated) and titrated once more. Finally, a new cell plate with 10 mL of fresh media was prepared and 1 mL of the older passage cell suspension was added to the plate. This plate was stored in incubation at 37 degrees Celsius and checked every few days as outlined above. The excess cell suspension was discarded of properly in accordance with EHS Guidelines.

In the case that an experiment was ready to be performed, instead of discarding the excess cell suspension, 20 µl of it was added to a cell counting slide and counted (in cells/mL) using a Nexcelom Bioscience Cellometer Auto T4 Automated Cell Counter. Depending on the desired cell density for the specific experiment, appropriate calculations were done as to how many microliters of cell suspension and media completion was needed. Tubes with appropriate labeling were prepared and the calculated amount of media was added to each tube followed by the calculated amount of cell suspension. After all dilutions were complete, the cells were ready to be plated in accordance with the specific assay.

Manual Scratch Assay

Of the three assays, the first was a scratch assay. Prior to experimentation with the various treatments, the appropriate cell density to use was determined. This was done by plating different densities of BxPC-3 human pancreatic cancer cells (e.g. 5,000 cells/well, 10,000 cells/well, 30,000 cells/well, etc.) on a 24-well plate and leaving them in incubation overnight. The next day, a P20 micropipette tip was used to scratch down the middle of each well using the lid as a ruler and stabilizer. This created a space free of cells on the confluent monolayer with the migration of cells into the empty space being measured at the end. Figure M1 below illustrates the scratching process. The media was aspirated, and 1 mL of PBS was added to each well to

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wash the cells and remove any debris; this was done three times. 1 mL of fresh media was then added to each well. The plate was visualized under a light microscope at either 4x or 10x magnification to determine the best imaging field and it was marked with an Ultra Fine Point Sharpie. The 24-well plate was transferred to an EVOS Imaging System and using the marked field, images of the scratch were manually taken every hour until complete healing was noted. In between images, the plate was stored in an incubator at 37 degrees Celsius. The images were analyzed and the density that had the cleanest scratches were picked for experimentation with our therapeutic compounds. Figure M2 below shows what a proper scratch should look like. When experimenting with our treatments, all the steps above were repeated except the final media that was added to each well after the PBS washes was supplemented with our treatments. The treatments varied in chemical composition, concentration, and included positive and negative controls depending on what was being tested. The images were analyzed using ImageJ to determine the rate of healing using an area method and/or line average method.

Oris™ Cell Migration Assay (Stoppers)

The second assay was the Oris™ Cell Migration Assay which utilized "stoppers" in a 96-well plate to create a center free of cells. Essentially, cells were plated in the presence of a stopper. Once the stopper was removed, the cells would be able to migrate towards the center of the well. Figure M3 below describes this process visually and illustrates what the cells in every well looked like when the stopper was removed. Figure M4 is a picture from our experiment. To conduct this assay, a 96-well plate with stoppers in each well were plated with BxPC-3 and/or BxPC-3 GFP cells at varying densities of 25,000 cells/well, 60,000 cells/well, 100,000 cells/well, and 200,000 cells/well. The plate was incubated overnight to allow for cell attachment in the

outer regions of the stopper. The next day, the stoppers were removed using the Oris™ Stopper

Tool and the old media was removed with a pipette. The cells were gently washed with 100 µl of

cold PBS to remove any debris and 100 µl of fresh media supplemented with our treatments was

subsequently added to each well. The plate was incubated at 37 degrees Celsius and manually

imaged at hourly time intervals to monitor the progress of cell migration. The images were

analyzed using ImageJ to determine the rate of healing using an area method that measured the

empty area of no cells as time progressed. In addition, a Z-factor was calculated to determine the

statistical significance and robustness of our assay (further explanations regarding this will be

outlined in the *Results* portion and *Figure R1* below). Lastly, over subsequent trials of this assay,

it was deemed that 60,000 cells/well for BxPC-3 was the optimum density to test moving

forward.

IncuCyte® Scratch Wound Assay (Semi-Automated)

       The third and most robust assay was the IncuCyte® Scratch Wound Assay. To perform

this assay, the appropriate cell density was first determined using similar cell plating methods

mentioned for the scratch assay. 100 µl of varying cell densities (of either BxPC-3 cells or A549

human lung cancer cells that were either transfected with NucLight Red or simply the parental

line) were plated on a specific IncuCyte® ImageLock 96-well plate using a P300 multichannel

pipette; the lowest densities were plated first. All wells were filled with either media or PBS as

the pins of the scratching tool (IncuCyte WoundMaker) would be damaged otherwise. Once

plating was complete, the cell plate was removed from the hood and sat on the lab bench for 10

minutes in order for the cells to reach equilibrium. The plate was then checked under a light

microscope at 4x and 10x magnification to ensure plating was properly done. The plate was

inserted into the IncuCyte® S3 Live-Cell Analysis System for primary imaging and a schedule was created to automatically image the wells at six-hour time intervals (or as deemed appropriate). The following day, the IncuCyte WoundMaker was cleaned according to manufacturer specifications and the 96-well plate was removed from the IncuCyte machine. Under a laminar flow hood, the old media was removed from the wells and 100 µl of cold PBS was added to every well. The plate was placed into the WoundMaker apparatus and scratched two times. 100 µl of cold PBS was used once again to wash every well in order to remove any cellular debris and then 100 µl of fresh media was added into each well. The plate was placed into the IncuCyte machine and a schedule to image at 10x magnification every 2 hours was created. As new images were being saved, they were analyzed by the IncuCyte software and the density that had scratches that were the cleanest and optimum for this assay and cell line were used for experimentation with our compounds. Figure M5 shows an example picture of the different densities plated and their subsequent scratches in the 96-well plate. Once the appropriate cell density was determined, in order to test our compounds, a 96-well V-bottom plate was pre-prepared with correct treatment types and concentrations in the appropriate wells as they would be in the experimental plate. A P300 multichannel pipette was used to transfer the treatments column by column, making sure all wells were filled. These actions took place right before the imaging steps just mentioned above for the density tests (i.e. treatments instead of fresh media); all other steps were the same. Once the experiment was complete, the WoundMaker was sterilized according to manufacturer specifications and after a few days of image gathering, the data was analyzed using the IncuCyte® Scratch Wound Cell Migration Software Module.

Results

Towards the end of this study, it was apparent that the IncuCyte® Scratch Wound Assay was the most robust migration assay to utilize for our purposes while the scratch assay was the least robust (further explanations regarding this will be outlined in the *Discussion* portion below). Figure R1 shows the definition of the Z-factor and further explains its use in evaluating the robustness of each assay. Performing a Z-factor calculation for each assay revealed that the IncuCyte® Scratch Wound Assay was the strongest assay given that the manual scratch assay and Oris™ Cell Migration Assay only had Z-factors of 0.2 (very poor assay) and 0.7 (very good assay), respectively, while the IncuCyte® exceeded those (Z > 0.7 → an excellent assay). As such, data from the IncuCyte® Scratch Wound Assay was deemed the most relevant in determining the efficacy of the compounds synthesized in the Pellecchia Laboratory. Hence, the soon to be mentioned results will illustrate the data in regard to the IncuCyte® Scratch Wound Assay only.

After treating the lung cancer cell line A549 NucLight Red cells (density of 30,000 cells/well) with either one of the Pellecchia Lab's agonistic agents termed "135H11" or "135H12," it is apparent from Figure R2 that 5 µM of the 135H12 compound was slightly effective at slowing the migration of the cancerous cells. Ephrin-A1 and Fc were positive and negative controls, respectively. This result is drawn from the fact that the cell density in the wound area relative to the cell density outside the wound area for the 135H12 5 µM compound is lower as time goes forward compared to the other compounds.

After treating the pancreatic cancer cell line BxPC-3 NucLight Red cells (density of 30,000 cells/well) with either one of the Pellecchia Lab's agonistic agents termed "135H11" or "135H12," it is apparent from Figure R3 that 10 µM of the 135H12 compound was slightly

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effective at slowing the migration of the cancerous cells. Ephrin-A1 and Fc were positive and negative controls, respectively. Once again, this result is drawn from the fact that the cell density in the wound area relative to the cell density outside the wound area for the 135H12 10 μM compound is lower as time goes forward compared to the other compounds.

One important distinction between ephrin-A1 and 135H11 or 135H12 is that the former is a pan Eph-RTK agent while the synthetic agents discovered in the Pellecchia Laboratory are very selective for the EphA2 subtype only (Gambini and Salem et al., 2018). Hence, the observed results can discriminate on the importance of activating the EphA2 receptor only versus activating other receptor subtypes on the same family (i.e. EphA3, EphA7, EphA10; all of which are also highly expressed in some cancers).

Lastly, the BxPC-3 parental cell line was treated with Fc or Ephrin-A1 either clustered or non-clustered. Clustered means that there is an antibody, anti-Fc, bound to the Fc region of Ephrin-A1 (or just the Fc protein itself in the case of the Fc control) which results in the dimerization and subsequent activation of Ephrin-A1 (or Fc) and other important downstream pathways that play a role in inhibiting cell migration (or inducing migration in the case of Fc). On the other hand, non-clustered essentially is the absence of the bound antibody and this results in less activation due to soluble, non-membrane bound Ephrin-A1 (or Fc) being harder to activate. This treatment was performed to determine whether or not there was a significant difference between the clustered or non-clustered proteins. Data from this test revealed an interesting phenomenon in which cells treated with either Fc or Ephrin-A1 where retracting rather than migrating to the center (further explanations regarding this will be outlined in the *Discussion – Error Analysis* portion below). Figure R4 shows an example of this retraction and

the detailed mechanisms of migration or retraction mediated by the EphA2 receptor are still not completely understood.

Discussion

Conclusion

Based off of the analysis of the experiments depicted in Figures R2 and R3, preliminary findings suggest that the compound 135H12 seems to be the most effective at moderately slowing down the rate of cancer cell migration in BxPC-3 and A549 cells in comparison to other treatments tested. It seems as if the dosage or concentration of the compound depends on which cell line is being tested. Additionally, based off of the calculated Z-factors mentioned in the *Results* section, the IncuCyte® Scratch Wound Assay is the best assay to test for cancer cell migration thus far. Lastly, as ephrin-A1 is a pan Eph-RTK agonist (as mentioned in the *Results* section above), while our agents are selective towards EphA2, these experiments can conclude whether or not activating EphA2 alone is sufficient to inhibit migration. Due to only the slight inhibition of migration as seen in Figures R2 and R3, it is evident that EphA2 alone is not sufficient in completely inhibiting cancer cell migration. Further testing in the lab has revealed that knocked out EphA2 receptors in BxPC-3 cells indeed did not completely stop migration and therefore, our agents cannot fully stop migration either. Nevertheless, further trials, different treatments, and different cell lines are needed in order to validate and confirm these findings and produce more confident results.

Error Analysis

In terms of the primary migration assay performed, the manual scratch assay, multiple issues came up leading to the movement away from this assay. First off, performing the scratches by hand lead to major inconsistencies in the shape and "cleanliness" of the scratch. It was never known if the scratch would be straight, if it would be clean with no cellular debris in the middle,

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if there would be no lifted cells on the edges of the scratch, etc. Furthermore, in order to image the scratch and monitor the migration, sites were "cherry-picked" based on how good they looked under the camera and how proper the scratch itself was. This caused further inconsistency as each well had a different portion that was being photographed. On the topic of photos, each photo for each scratch in each well was hand taken. This resulted in the cell plate being out of incubation for extended periods of time which most certainly affected the growth and migration of the cells as they were not in a constantly stable environment. Secondly, this type of assay required a greater volume of drugs to be utilized which was neither cost nor time effective in the long run. Lastly, due to the largely manual nature of this assay, in that all the scratches, washing steps, treatment additions, media replenishments, and photographs were all done by hand, it was rather unlikely to perform the scratch assay in a high-throughput manner and consistently reproduce it multiple times over. Data analysis was also performed by hand using ImageJ which may have resulted in small inconsistencies when using the straight line or free line drawing tool to calculate the area as time progressed.

In terms of the secondary migration assay performed, the Oris™ Cell Migration Assay, it was deemed that this assay was not robust enough due to a few reasons described below. Primarily, even though the center "hole" void of cells was more consistent in its "cleanliness" around the edges and center in comparison to the previous scratch assay, there were still many times where cells would find their way under the stoppers, the circles would not be perfectly round, or there would be debris around the edges of the circle. This was most likely due to the fact that the stoppers had to be placed and removed by hand and slight variations in the method of taking them out or putting them in caused inconsistent performance. Furthermore, even though all the "holes" were in a consistent field of view, it was still necessary to photograph each

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well manually at every time point. As mentioned above, this seemingly endless cycle of removing the cell plate from the incubator for long periods of time and putting it back resulted in drastic changes of environment which most definitely affected the data. Lastly, the problems plaguing the scratch assay in regard to the volume of treatment and data analysis also came up in experiments that utilized this assay. Cost was also a large issue in this assay as the stoppers were not meant to be re-used; rather, they were supposed to be bought brand-new for each experiment which would have been highly expensive. As such, throughout all our experiments with this assay, the stoppers were sanitized with ethanol and re-used. This may have negatively impacted the data as well. Overall, although the Oris™ Cell Migration Assay fixed a few of the problems troubling the scratch assay, many still persisted.

The IncuCyte® Scratch Wound Assay solved almost all of the problems affecting both of the previous assays and therefore, it was deemed the most robust assay thus far. Unfortunately, due to time constraints, only one experiment for each cell line was able to be performed. This lack of variety in cell lines and treatments, in addition to a deficiency in the amount of trials performed and the reproduction of the trials, led to a lack of robustness in our experiments and greatly decreased the confidence of the data. As for the retraction of cell migration in the experiment mentioned previously and outlined in Figure R3, it may not be due to a problem with the IncuCyte® Scratch Wound Assay but instead, could either be a fluke, error in plating, procedural error, etc. This problem would benefit from a replication experiment to rule out possible causes of error. One theory is that the cells were plated at too high of a density and as such, were utilizing nutrients too quickly which resulted in adverse effects. Overall, any conclusions deduced from this experiment would be deemed premature and further trials and experimentation would be required in order to come to a logical and concrete conclusion.

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<u>Future Directions</u>

Moving forward, different assays to measure migration could be discovered and utilized in order to properly mimic cancer metastasis and cancerous environments in vitro. This experiment opens avenues for different methods that utilize the IncuCyte® Scratch Wound Assay such as measuring the cancer cell migration of a 3-D cancer cell spheroid suspended in a gel matrix or even mimicking environmental conditions by utilizing angiogenesis assays. Not only should it be a hope to expand this experiment, but an emphasis should be placed on refining the current experiment by adding additional cell lines, additional treatments (both types and concentrations), performing more trials, etc. in order to increase confidence in the data. When utilizing these migration assays, if a suitable drug is found that can effectively inhibit cancer cell migration, testing the drug in animal models would be a logical future direction to pursue as well. All in all, steps to strengthen the hypothesis and assays to gain actionable conclusions and opening up solid opportunities for future research are necessary future directions towards finding cures to cancer.

Figures

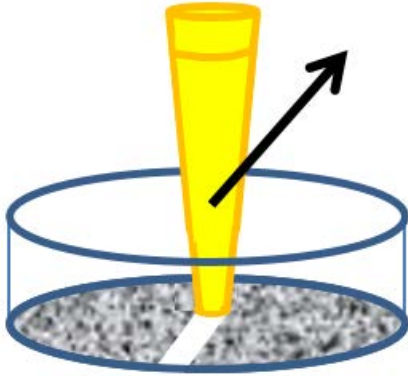Figure M1: Visualization of an individual well when a scratch is being performed with a pipette tip. (Image Source: https://www.yokogawa.com/library/resources/application-notes/lsc-time-lapse-analysis-of-cell-migration-and-proliferation-in-scratch-assay/)
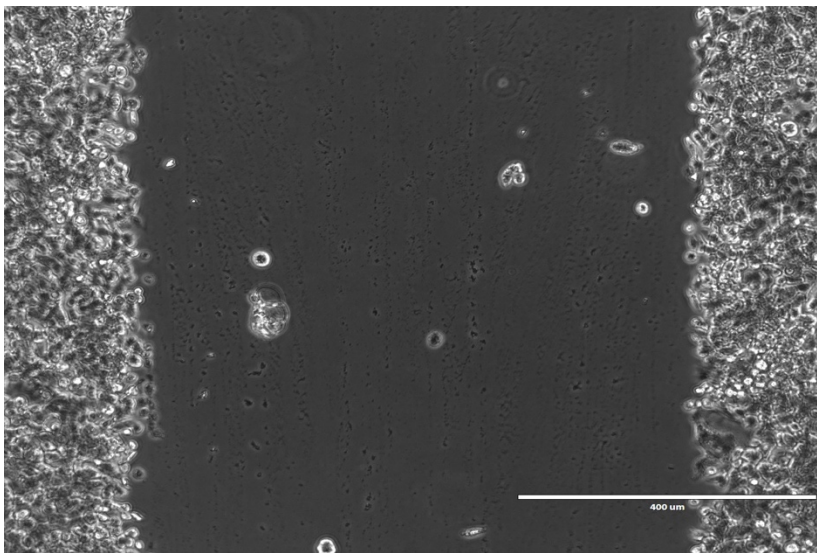

Figure M2: An optimum scratch. Little to no cellular debris and a straight line from top to bottom. The scale indicates a measure of 400 μm.
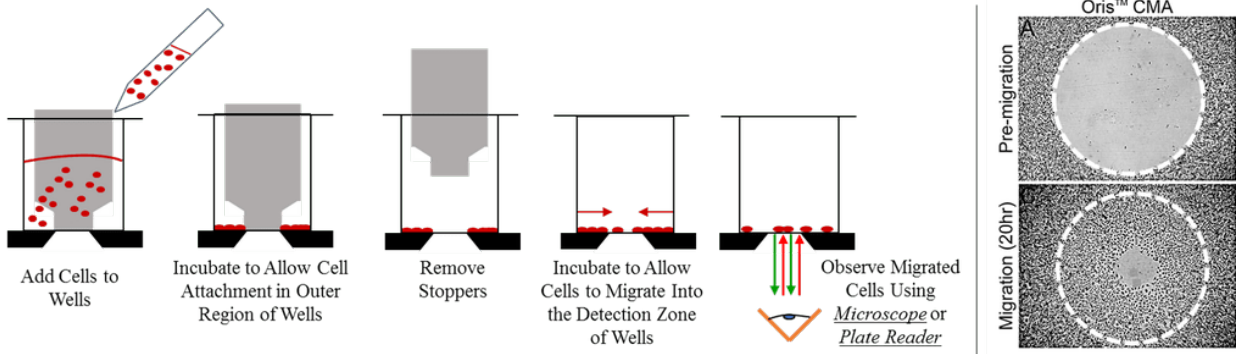
Figure M3: Basic steps of the Oris™ Cell Migration Assay with a picture of what the cell monolayers look like in each well before and after migration. (Image Source: https://www.platypustech.com/cell-based-assays/oris-cell-migration)
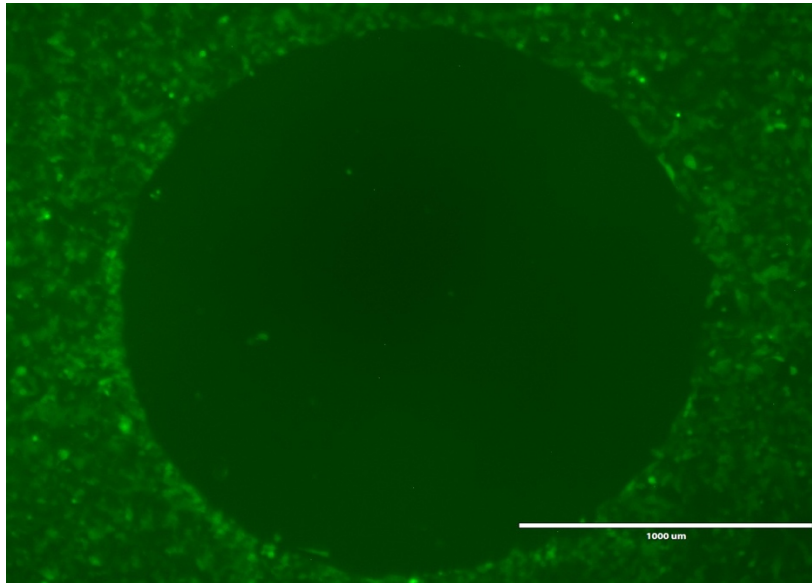
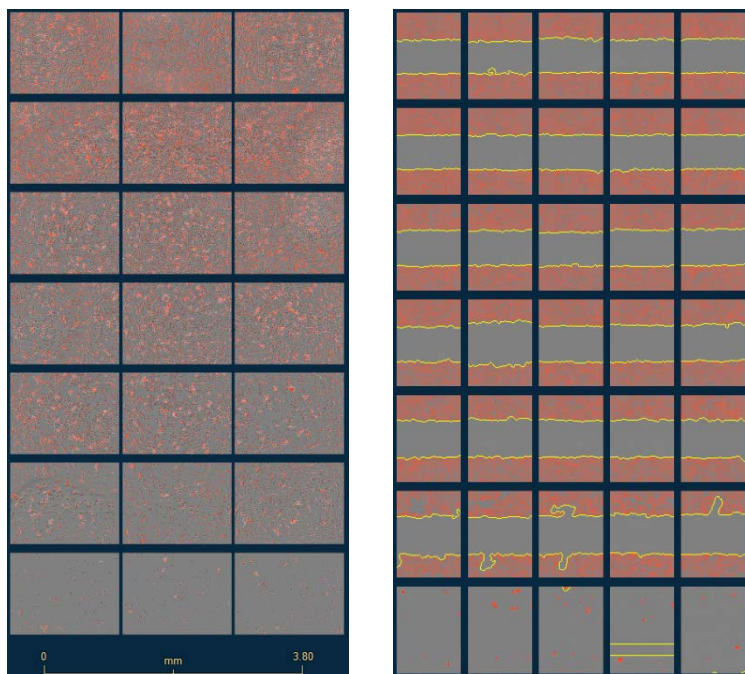Figure M4: Experimental BxPC-3 GFP cells pre-migration in the Oris™ Cell Migration Assay.

Figure M5: The left-most picture shows the varying plated densities of BxPC-3 cells going from 10K (bottom-most row), 30K, 40K, 50K, 60K, 70K, and 80K (top-most row). The right-most picture shows the after-scratch product of the varying densities.

$$Z' = 1 - \left| \frac{3\sigma_+ + 3\sigma_-}{\mu_+ - \mu_-} \right|$$

Figure R1: The formula to calculate Z-factor. A Z-factor is performed in high-throughput assays to determine whether or not that specific assay is robust enough to utilize for further experimentation. The closer the Z-factor is to 1.0, the greater the quality of the assay. Z-factors can never be at or above 1.0. (Image Source: https://pubs.rsc.org/en/content/articlelanding/2015/an/c4an01694g#!divAbstract)
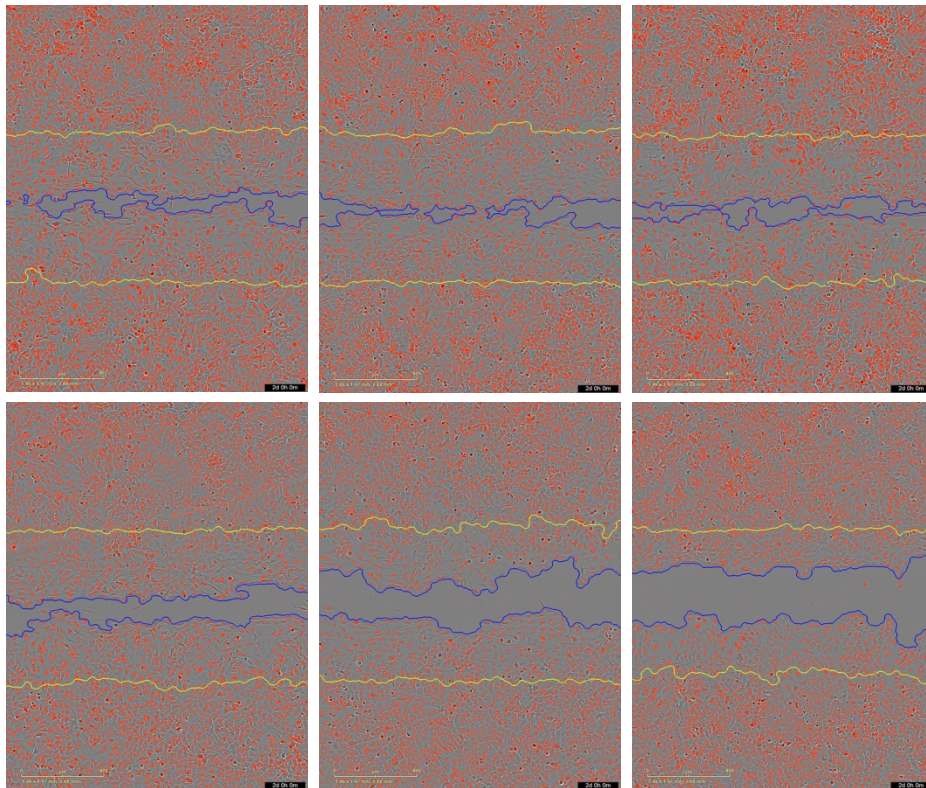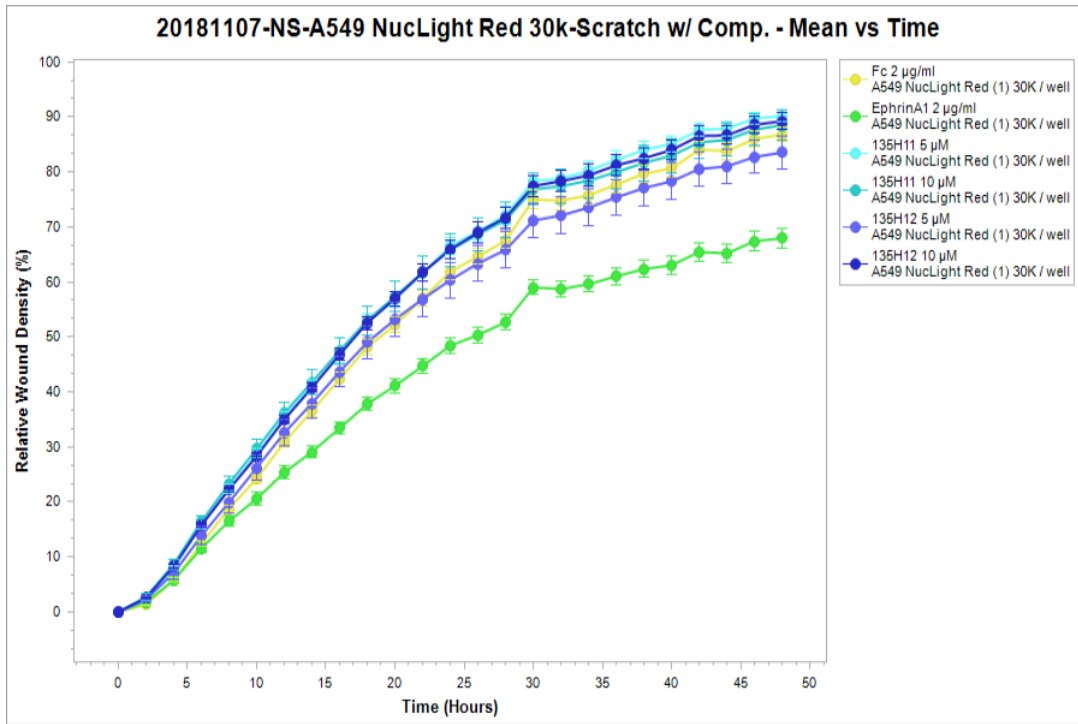
Figure R2: Graph representing the migration of A549 NucLight Red cells treated with various cancer anti-migration compounds till Day 2. "30K" means 30,000 cells/well. The pictures on the bottom of the A549 cells are representative wells for each of the treated groups at Day 2 (blue line). They are displayed in the following order: 135H11 5 μM, 135H12 10 μM, 135H11 10 μM, Fc, 135H12 5 μM, and lastly, Ephrin-A1.
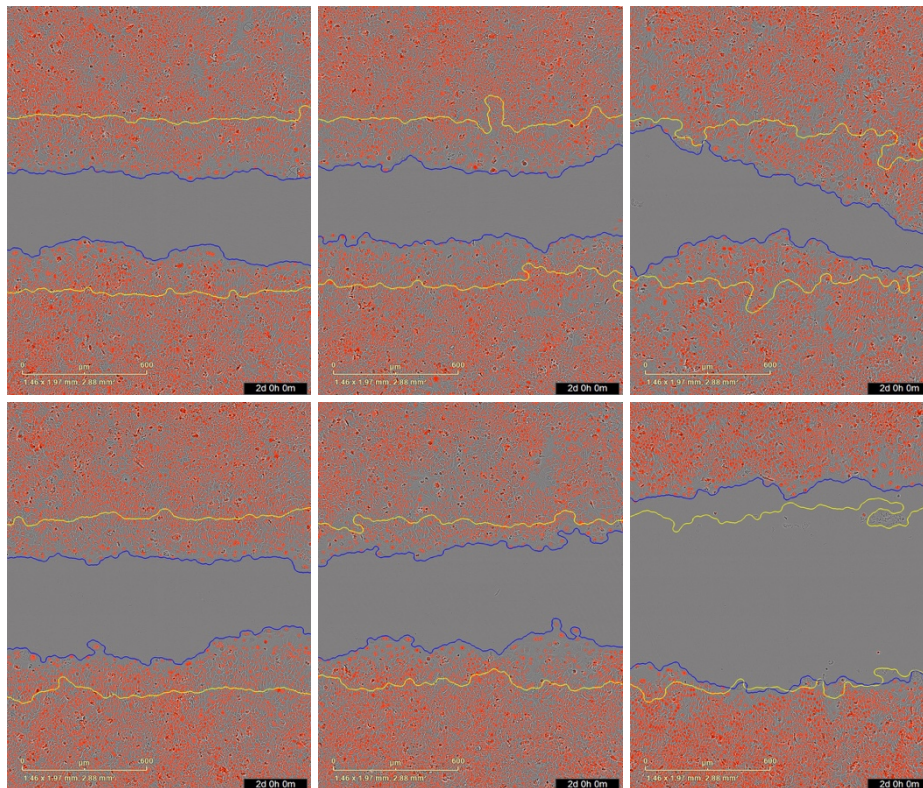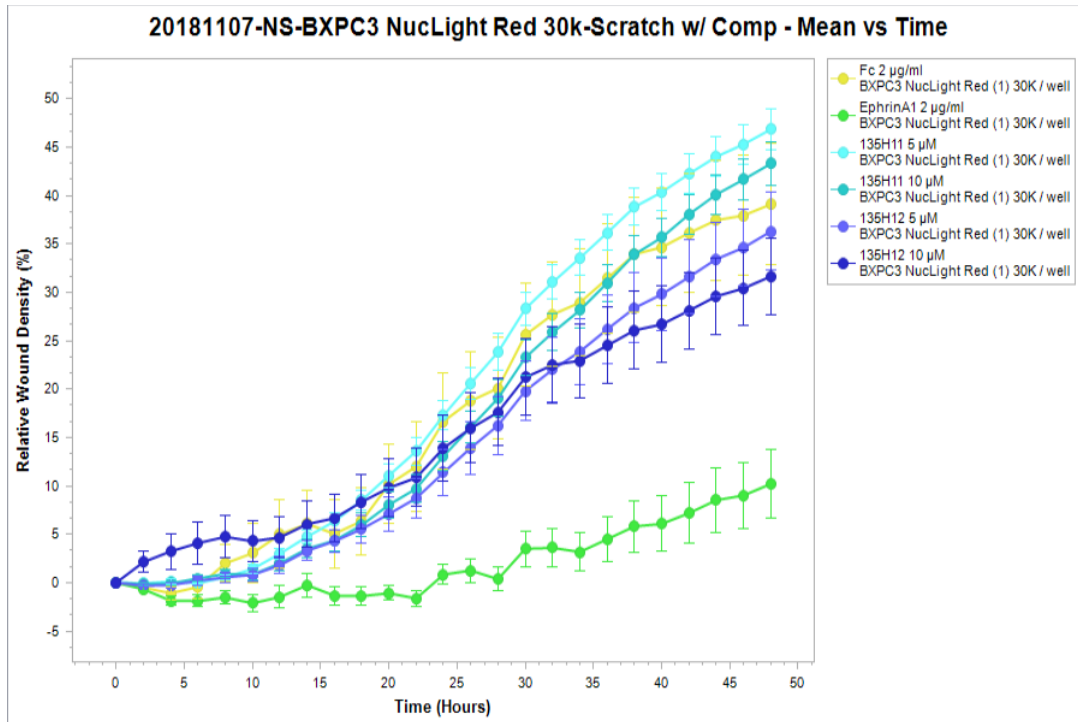
Figure R3: Graph representing the migration of BxPC-3 NucLight Red cells treated with various cancer anti-migration compounds till Day 2. "30K" means 30,000 cells/well. The pictures on the bottom of the BxPC-3 cells are representative wells for each of the treated groups at Day 2 (blue line). They are displayed in the following order: 135H11 5 µM, 135H11 10 µM, Fc, 135H12 5 µM, 135H12 10 µM, and lastly, Ephrin-A1.
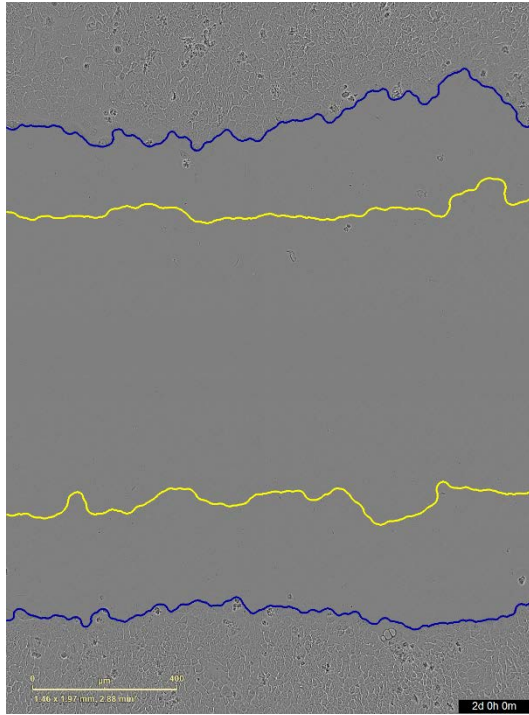
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Figure R4: The yellow line represents the scratch at Day 0 while the blue line represents the scratch at Day 2. Instead of the cells migrating towards the center to heal the wound completely, they are retracting; likely due to the activation of the EphA2 receptor by the agent (Ephrin-A1 Non-Clustered 2 µg/mL) or simply too high a cell density (40,000 cells/well).

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