Do novel rho-kinase inhibitor drugs alter the cell cycle duration of HeLa cells, and through what mechanism could they do this?

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Introduction

Rho-kinases (ROCK) are a group of kinases, of the serine-threonine kinase family, that function by phosphorylating their downstream targets, to activate them. ROCKs occur as two natural isoforms in mammals, ROCK1 (found ubiquitously across the body), and ROCK2 (found mostly in heart and brain tissues) **[1]**.

ROCKs are activated themselves by small RhoA GTPases, which function as "molecular switches", and allow the ROCKs to interact with downstream targets to mediate many functions including: promoting cellular contractility (via activating motor proteins), regulation of actin-filament stability, cytokinesis proteins **[2]**, apoptosis, cell-cell adhesion and tumour suppression.

Studies have shown that ROCK inhibitor drugs can effect the progression of the cell cycle, through interaction with cell cycle proteins (i.e. CDKs), cytokinesis motor proteins and apoptotic proteins. These properties of ROCK inhibitors give the drug potential high therapeutic value, with one drug Fasudil, used clinically to treat vasospasms following subarachnoid haemorrhages, with many other drugs in clinical trials.

Preliminary data, prior to starting this summer research project, showed that rho-kinase inhibitor drugs have a marked effect on Drosophila embryonic growth, reducing the rate. Alongside other studies' results stating that ROCK inhibitors reduce rate of cell proliferation, the project would therefore investigate whether these drugs can effectively reduce the rate of human HeLa cell growth, and therefore delay progression of proliferative cell conditions i.e. cancer. The drugs studied in the preliminary data, and so investigated in my project were Y-2, HA-1077 and 962B. Each drug was applied at 25mM.

Methods

Method 1: Using real-time imaging to identify whether ROCK inhibitors have an effect upon cell cycle duration

This part of the project involved using an Andor confocal microscope, to record time-lapse images to form a video showing the cell activity and growth across the time period (time of videos varied from ~10-36hours). Once the videos were produced, actively dividing cells were identified and their cell cycle durations were calculated, which had limited accuracy due to visual identification. The averages of these were taken, along with start-end visual image comparisons, to produce the data below.

Drug	Cell cycle average duration (mins)	Start-end visual comparisons
Control	112.5	Cells appeared healthy and active.
Y-2	200	Cells' attachments remained in a detached form until the end of the recording (~40mins), suggesting a pre-mitotic stage.
HA-1077	110	
962B	N/A	Most cells appear to undergo apoptosis.

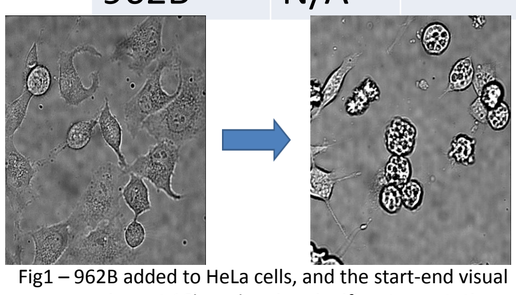


Fig1 – 962B added to HeLa cells, and the start-end visual comparison at 16hrs, showing significant apoptotic activity.

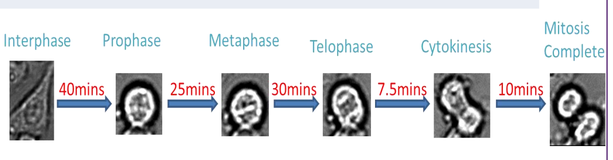


Fig2 – Steps of how the cell cycle duration was identified, in the control experiment.

Evaluating the data from the real-time imaging, we can conclude that HA-1077 had no significant effect, while Y-2 increased the cell cycle duration (potential pre-mitotic arrest occurring), and 962B videos showed no cell division, and potentially induced apoptosis of the HeLa cells, a property of ROCK seen in previous studies **[3]**.

Method 2: Comparison of cell morphology using start-end images

This section involved gathering the dish after real-time imaging, and taking images of the whole dish, which could not be observed during real-time images, to analyse their morphology, to increase reliability of the results.

To produce my data, I took start-end images with each dish used, and manually counted cells that were attached and detached, calculating the average and standard deviations.

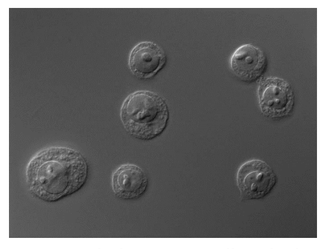


Fig3 – Rounded-up HeLa cells, which occur when the cells are entering mitosis, preparing to divide.

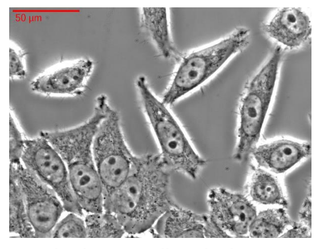
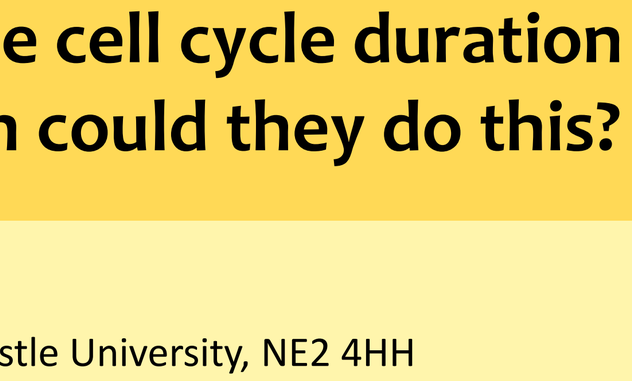


Fig4 – Attached HeLa cells, which are seen when the cells are attached to a surface or to each other, and commonly in interphase.



Evaluating the above bar chart; HA-1077 produced similar results to the control, Y-2 addition led to increased detachment of cells and 962B instigated an increase in detached cells by 33%.

Method 3: Analyse confocal scans to identify whether actin networks have been modified using Olink in situ PLA Staining Technology

This study used in-situ proximity ligation assay (PLA). PLA utilises a fluorescence based analytical approach, using a primary antibody to target a specific protein, and a secondary antibody that binds to the specific primary antibody. The secondary antibody contains a short DNA sequence that can be used for signal amplification by ligation and PCR, to increase the fluorescence signal. The fluorescent signals are detected using confocal microscopy with appropriate excitation wavelengths.

We targeted cyclin B with this technique, a cell cycle protein responsible for stimulating the initiation of mitosis, which can be found within the nucleus when mitosis is occurring. Due to timing of the placement I could only obtain confocal scans for 962B and Y-2. Analysing the scans for Y-2, the cyclin B remained outside the nucleus, consistent for ~50-60% of cells, potentially supporting the results of Method 1, that the cells were arrested at a pre-mitotic phase. 962B scans showed disordered accumulations of cyclin B, also supporting the notion that the cells were apoptosing.

Conclusions

Evaluating each of the drugs:

- ❑ HA-1077 produced no significant effect upon the HeLa cells, suggesting that it may not be an appropriate therapeutic drug.
- ❑ 962B instigated substantial apoptotic effect upon the HeLa cells, also causing them to detach from the dish surfaces.
- ❑ Y-2 significantly increased the cell cycle duration of HeLa cells, and results from the project suggest that this was instigated through a pre-mitotic arrest. Specific biomarkers would be needed in order to establish a more accurate picture of the arrest that is occurring.

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