Targeting Hyperactivated RhoA Signaling for Cancer Therapy by Cysteine-reactive Compounds

Laidlaw Undergraduate Research and Leadership Scholarship Programme

Research Report

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Introduction

This project aims to synthesize cysteine-reactive compounds to target hyperactive RhoA signaling for cancer therapy and investigate the compound's anticancer properties as well as the mechanism of action.

The recognition and identification of cancer-related proteins like RhoA acts as a catalyst for drug discovery and is a breakthrough for the development of targeted and personalized cancer treatments. However, due to the protein's structure-function considerations, like their shallow binding sites, these proteins were once considered to be pharmacologically "undruggable". To mitigate this limitation, cysteine-reactive compounds could be used. They provide the possibility to turn RhoA into a "druggable" protein since covalent binding of the compounds onto cysteine residue on RhoA, which cannot be found in non-covalent drug modalities, can strengthen the interactions and enhance the binding specificity.

RhoA is a member of the Rho family of GTPase and is overexpressed and associated with the influence of cancer signaling cascades in many cancer cell lines like colorectal and prostate cancer and metastatic cancer. However, the exact role of the protein is still relatively unknown. RhoA exists in a GDP-bound and GTP-bound form, which corresponds to their inactive and active forms respectively. This allows the protein to regulate transcription, cell migration and cytoskeleton reorganization when activated by cell surface receptors (Jeong et al., 2015). Moreover, the mutation RHOA Y42C is frequently found in colorectal cancer and is suggested to be associated with gain-of-function of the protein and its overactivation (Benton & Chernoff, 2020). To target RhoA and alter its activities in cancers, its cysteine residues can be one of the most promising targets for drug discovery and development.

Cysteine poses great importance in regulating our biological functions. Despite the low abundance of cysteine in the human body of only 3.3% frequency in protein sequences, it is highly conserved in functionally important sites with more than 50% of cysteine associated with critical biochemical processes. The amino acid is extremely nucleophilic and reactive to oxidative modification, and certain oxidized forms of cysteine are being recognised as important players in cellular signaling. Moreover, the formation of a disulfide bond between two cysteine residues is associated with the shape of the tertiary structure of the protein (Hallenbeck et al., 2017). Therefore, all these factors make cysteine a favourable target for protein modification and biochemical process regulation, and utilizing a covalent approach to target cysteine is being recognised as a viable mechanism (Singh et al., 2011).

I hypothesize that the indole compound synthesized in this project will be able to target cysteine residues on RhoA and hence supress RhoA activity *in vitro.*

Methodology

Chemical synthesis

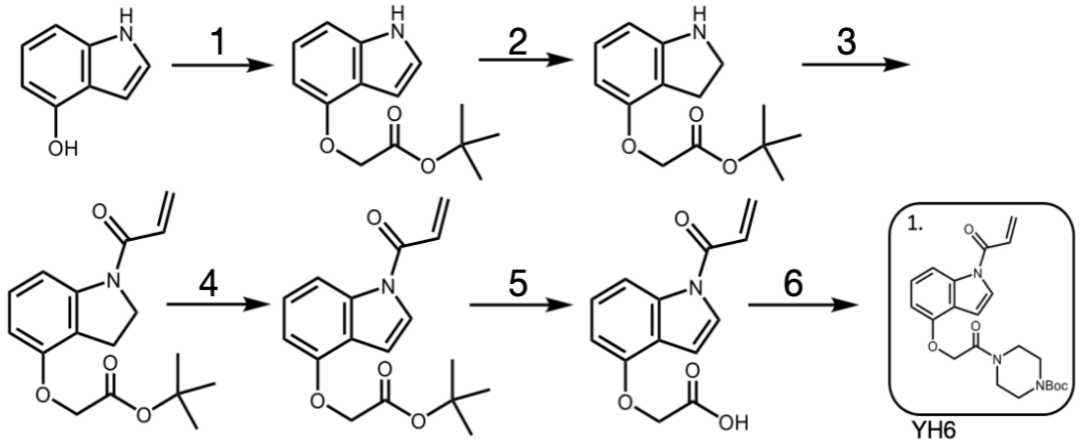

Figure 1. *Process of the chemical synthesis of YH6*

Chemical and reagents were obtained from major chemical suppliers and were used without further purification. The progress of each step was monitored by thin layer chromatography using silica gel.

1) Substitution: 4-hydroxyindole and bromoacetate are dissolved in acetone to form a dark green-brown solution before adding potassium carbonate (K_2CO_3) for overnight reflux. A dark brown product is formed. The product is filtered and then purified with silica column chromatography.

2) Reduction: Product from 1) is dissolved in a minimum amount of acetic acid, then sodium cyanoborohydride is added slowly to the mixture. The solution is mixed at room temperature for 2 hours. The reaction is quenched with sodium hydroxide and undergoes extraction with ethyl acetate (EA) and washed with brine. Product was purified with silica column chromatography and was light yellow oil after vacuum drying.

3) Acylation: In an ice bath, product from 2) was dissolved in minimal volume of tetrahydrofuran (THF) and K_2CO_3 and acryloyl chloride was slowly added to the solution. It was stirred at 4 ^oC for 30 minutes and quenched with brine. Product was extracted using EA and washed with brine. It was a greyish power when dry.

4) Oxidation: Product from 3) was dissolved in toluene before adding 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and refluxed overnight. Reaction was quenched with water, washed with brine and underwent EA extraction, before being purified with column chromatography.

5) Deprotection: Product from 4) was dissolved in acetonitrile (ACN) before adding equal volume of phosphoric acid (H_3PO_4) to the solution, which was stirred overnight at room

temperature. The reaction was quenched with water, washed with brine and extracted with EA.

6) Amide coupling: Product from 5) was dissolved in dimethylformamide (DMF) before adding HATU, and the solution was stirred vigorously for 30 minutes at room temperature. 1-Boc-piperazine was dissolved in triethylamine (TEA) and added to the reaction mixture. The solution was stirred overnight at room temperature. The reaction was quenched and washed with sodium bicarbonate, and additionally washed with brine. The product was extracted with EA and purified with column chromatography.

To confirm the purity of YH6 before biological experiments, ^{1}H NMR conformation was done. Deuterated chloroform was used as the solvent.

Cell culture

SW620 is a human colorectal adenocarcinoma cell line with hyperactive RhoA activity. It was grown in RPMI medium with 10% foetal bovine serum (FBS) and 1% penicillin and streptomycin (PS) in an incubator at 37.0 °C and 5.0% CO_2. After 2 to 3 days, the cells were passaged.

HT29 is a human colorectal adenocarcinoma cell line with hyperactive RhoA activity and lacks the SMAD4 gene, making it insensitive to 5-fluorouracil and oxaliplatin, the standard colorectal cancer treatment (Cohen et al., 1999). It was grown in DMEM medium with 10% FBS and 1% PS or McCoy 5A medium with 10% FBS and 1% in an incubator at 37.0 °C and 5.0% CO_2. After 2 to 3 days, the cells were passaged.

CCK81is a human colorectal adenocarcinoma cell line with RhoA Y42C mutation. It was grown in MEM medium with 10% FBS and 1% PS in an incubator at 37.0 °C and 5.0% CO_2 °C. After 2 to 3 days, the cells were passaged.

CCD-18Co is a normal human fibroblast that is typically found in connective tissue. It was grown in MEM medium with 10% FBS and 1% PS in an incubator at 37.0 °C and 5.0% CO_2 °C. Medium was changed after 2 to 3 days, and the cells were passaged after 6 to 7 days.

Gel-based Activity-based Protein Profiling (ABPP)

ABPP is used to understand the interaction between the compounds and the RhoA protein with Y42C mutation. 2μg of protein in 1mL of PBS was aliquoted into PCR tubes in 50μL increment along with compound (HY8, HY9, HY106B, HY106C, HY106D, HY106E, HY106F, JA03B, and YH6) at final concentration of 20 μM. Sample mixtures were vortexed and incubated at room temperature for 1 hour, with vortexing at 15 min increments. 1μL of 0.05mM of iodoacetamide-rhodamine (IA-Rh) was added to each sample mixture and vortexed. It was then incubated in the dark at room temperature for 1 hour, with vortexing at 15 min increments. 16.7μL of 4X sampling buffer was then added to the sample mixture and thoroughly mixed and spun down before heating at 95°C for 5 minutes. A 26-well midi gel was run with 1X SDS running buffer, 3.5μL of PageMarker as protein ladder, and 10uL of sample mixture. PAGE ran at 110V until dye front passing the well bottom, and the voltage was then increased to 240V for 30 minutes.

The gel was imaged in the Chemidoc™ MP Imaging System using the Rhodamine protocol. Silver staining was done to visualize protein migration using the Pierce™ Silver Stain Kit by Thermo Scientific following manufacturer's protocol.

MTT

MTT assay is a colorimetric assay to assess cell viability after being treated with the compounds. In a 96-well plate, SW620 (16,000 cells per well), HT29 with DMEM medium (8,000 cells per well), CCK81 (18,000 cells per well), and CCD-18Co (6500 cells per well) were cultured for 24 hours in their respective culture media. The cells were then incubated with different concentrations of HY8, HY106f, JA03b, and YH6 (0-40μM by serial dilution) for 24 hours. Afterward, 5 mg/mL of MTT was added and the cells were incubated at 37 ^{o}C in incubator for 4 hours to allow purple formazan crystals to form before lysing the cells with a cell lysis buffer containing 10% SDS in 0.01M HCl. The absorbance of the contents was measured using PerkinElmer VICTOR 3™ at 570 nm. To calculate percentage cell viability, the following equation was used: $\frac{(Absorbance_{compound} - Absorbance_{background})}{(Absorbance_{DMSO} - Absorbance_{background})} \times 100\%$. IC_{50} and the plots for the corresponding compounds were calculated and created using Prism 9.

Wound healing assay

Wound healing assays is an in vitro assay to assess cell migration after being treated with the compounds. HT29 with McCoy 5A medium were seeded in a 24 well plate (280,000 cells per well) and cultured for 24 hours. A P20 sterile pipette tip was used to draw a linear wound and PBS was used to wash the wound twice before incubation. The cells were then incubated in 20μM of HY8, HY106f, JA03b and YH6 for compound dependent analysis or 1μM, 5μM, 10μM and 20μM of YH6 for dose-dependent analysis. 0.4% DMSO (10μL in 2500μL McCoy 5A medium) was used as DMSO control. Images of the wound were taken at 0h, 24h, and 48h. The area of the wound was analysed using ImageJ and the rate of cell migration was calculated with the following equation: $\frac{(Area_{0h} - Area_{24h\ or\ 48h})}{Area_{0h}} \times 100\%$.

Results and Discussion

Investigating *in vitro* binding on RhoA

Here, we have used gel-based activity-based protein profiling to better understand the interaction mechanism between the cysteine-reactive compounds and protein. This technology utilises a probe which consists of a reactive group to label proteins containing reactive amino acids, such as cysteine in this study. The probe also contains a linker site and a reporter group which allows further functionalization for detection and analysis. In the gel-based ABPP experiment, the reporter group is an alkyne which can undergo click chemistry with fluorophore-azide. Therefore, proteins labelled with the probe will show strong fluorescence. On the other hand, if our cysteine-reactive compounds can bind onto the protein, this will compete with the probe binding, resulting in a significant diminishment of fluorescence from the protein as illustrated in figure 2A.

Here, a variety of pre-synthesized indole and indoline compounds, along with YH6 were selected for gel-based ABPP using RhoA protein with Y42C mutation and IA-Rhodamine as the probe. Through this analysis (figure 2B), we identified HY8, HY106F, JA03b, and YH6 as potential candidates due to the significant decrease in fluorescence intensity compared to DMSO control and no significant change in protein loading as found in the silver staining experiment. These compounds were used for further biological experiments. As observed in the silver stain, protein precipitation was not consistent and could be attributed to the compound binding to various cysteines in the protein or the concentration of compound was too high, causing protein denaturation.

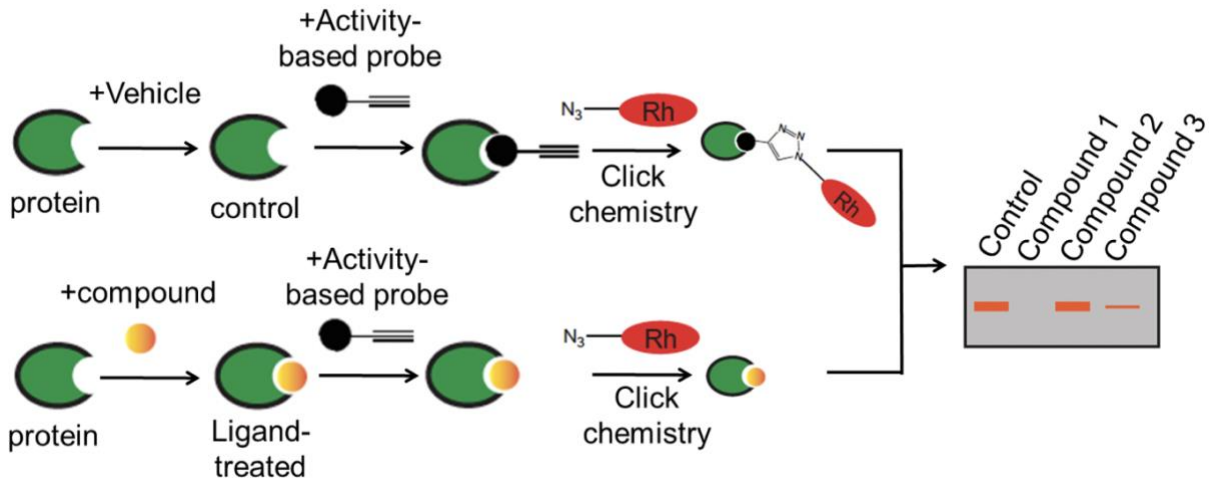

Figure 2A. *Method of gel-based ABPP. ABPP is used to understand the in vitro interaction between the compounds and the RhoA protein with Y42C mutation.*

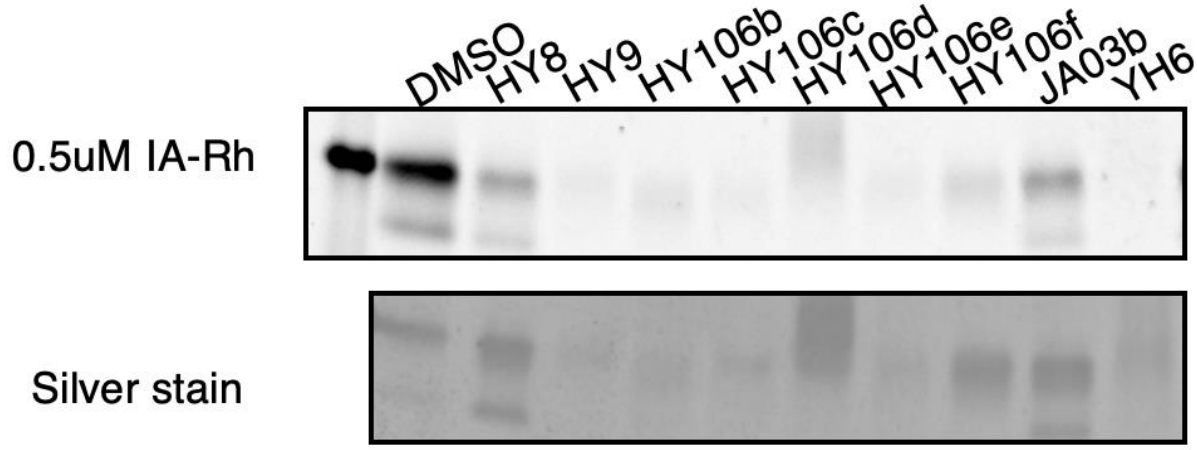

Figure 2B. *Gel-based ABPP analysis of the compounds to target RhoA with Y42C mutation. Shown above is the fluorescence detection of IA-Rh labelling and the silver staining to check protein precipitation.*

Anti-cancer and anti-metastatic effects of lead compound

To investigate the anti-cancer and anti-metastatic effects of HY8, HY106F, JA03b, and YH6, cell viability MTT assay and wound healing assay were completed. The most promising compound was YH6 in both experiments.

MTT assay is colorimetric assay to assess cell viability after being treated with the compounds. MTT assays were performed for HY8, HY106F, JA03b (figure S1 A-C), and YH6 (figure 3A), in some cell lines from SW620, HT29, CCK81, and CCD-18Co. Not all compounds were tested in all these cell lines due to time and resource limitations. Nonetheless, initial results showed YH6 to have the highest potency and better selectivity compared to the other three compounds. Observed in figure 2A, the potency in cell line CCK81 with RhoA Y42C mutation is higher compared to CCD-18Co (normal cells) and HT29 (hyperactive RhoA activity). However, it is most potent in SW620 (hyperactive RhoA activity) and this is a bit unexpected. This could be ascribed to the binding to other functional cysteines or that the SW620 cell line undergoes cell death easily.

Continuing, compound-dependent (figure S1D) and dose-dependent (figure 3B) wound healing assay was completed. Wound healing assays is an in vitro assay to assess cell migration after being treated with the compounds. Due to the large standard deviation for compound-dependent wound healing assay with 20μM, the results were not statistically significant but based on images taken (figure S1E), YH6 showed promising results, hence it was used for dose-dependent experiment.

For dose-dependent (figure 3B) wound healing assay, concentrations of 1μM, 5μM, 10μM, and 20μM were used. Student's t-test has been conducted to compare the change in area of the wound between 0-24 hour and 0-48 hour against DMSO control. It can be concluded that after treatment for 24 hours, 10μM and 20μM of YH6 yielded statistically significant decreases in cell

migration with p-values less than 0.05 of 0.024 and 0.028 respectively. Also, 48 h treatment with 20μM of YH6 had a p-value of 0.005 showing significant difference from the DMSO control sample, but 10μM of YH6 did not result in statistically significant changes.

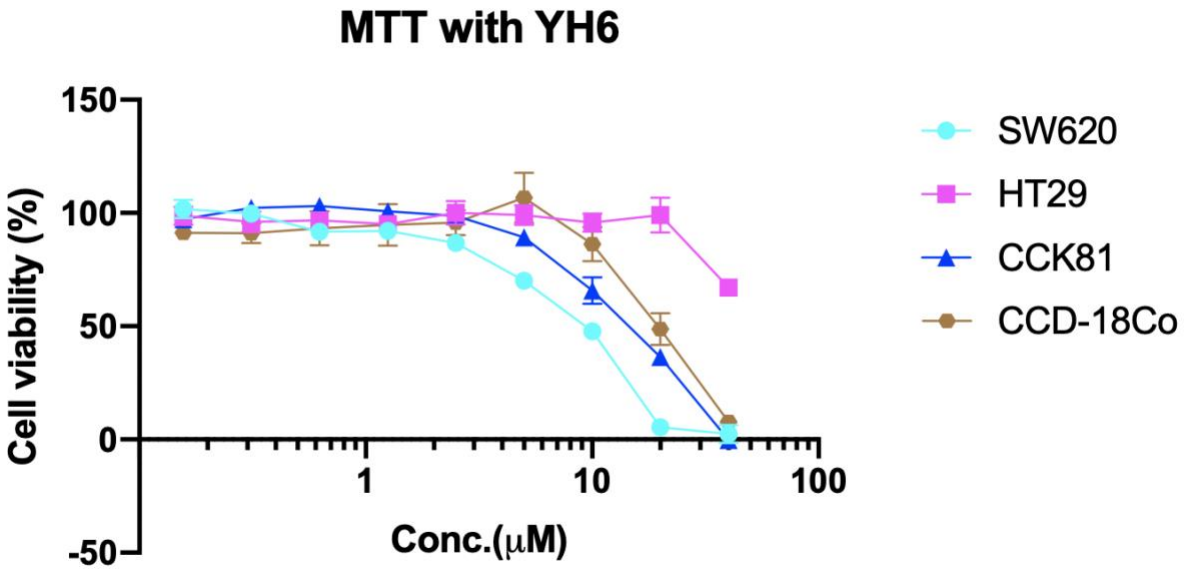

Figure 3A. *MTT assays with YH6 in cell lines: SW620, HT29, CCK81, and CCD-18Co in 0-40μM by serial dilution after being incubated for 24 hours (n=3). It was then incubated in 100μL of 5 mg/mL of MTT for 4 hours before lysing the cells. Absorbance was measured at 570 nm.*

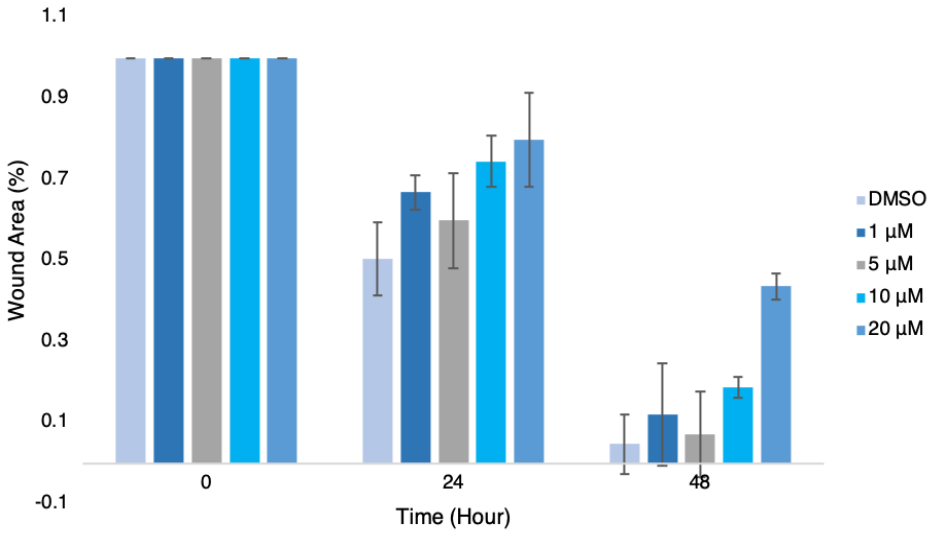

Figure 3B. *Wound Healing assay data of YH6 at concentrations between 1-20μM with DMSO control at 0, 24, and 48 hours with HT29 cells (n=3). Images were taken by the microscope and analysed with ImageJ.*

Conclusion and Reflection

In this project, we synthesized YH6, an indole compound and showed that it has the possibility for its counterpart to be used in targeted cancer therapy through identifying its anti-cancer and anti-metastatic activities. Results from gel-based ABPP shows its interaction between the compound and the protein, however, MTT assay and wound healing assay did not show significantly promising results but showed YH6's potential.

Suggestions and extensions for further study

To further validate and improve the effects of YH6, other experiments can be done. Firstly, it is important to identify the mechanism of action of lead compound on inhibiting RhoA signaling, hence GDP-GTP exchange assay should be completed. As mentioned, RhoA exists in either GDP-bound or GTP-bound form, and by doing this experiment, the effectiveness of YH6 in inhibiting the exchange of the two forms can be determined.

Furthermore, since the scaffold of YH6 is promising, the compound should be optimised to enhance its selectivity through deprotecting the tert-butyloxycarbonyl (Boc) group and incorporating different chemical moieties. This would require all the previous experiments to be conducted to identify a significant effect. Additionally, mass-spectrometry (MS)-based ABPP can be done to investigate the selectivity profile of the compounds toward the cancer-associated proteins. This is critical because humans are made of a complex system consisting of a myriad of proteins and my lead compound may show binding to other proteins that can lead to undesirable side effects. By the MS-based ABPP experiment, these off-target proteins, if any, can be identified and this can help to provide insights into future medicinal chemistry and lead optimization to improve efficacy and reduce toxic side effects.

Impacts of the research beyond the classroom

This project expands the library of the potential compounds to treat cancer in humans and further understand the biological functions of the targeted protein. By doing so, other forms of research and drug discovery can be expedited when done in a collaborative manner. As the scaffolding of YH6 is tested to be promising, various counterparts can be synthesized and tested to identify which structure has the highest selectivity and efficacy.

Difficulties encountered and how they were resolved

Like all scientific investigations, there were a multitude of hurdles with regard to the success of the experiments. Some experiments conducted did not yield conclusive, significant, or the hypothesized results; hence troubleshooting was necessary. To do so, I had to identify the inconsistency within the data to propose potential explanations; this was done by checking the controls, storage and conditions of the reagents, and the procedures. Afterward, relevant action was taken to eliminate the inconsistency. Some of the undesired experimental results were a result of inexperience in using the instruments. Therefore, there were fewer systematic errors and more significant results with practice and repetition of the experiments. Additionally, there were issues with some of the starting reagents, which caused problematic results. To combat this, faulty reagents were remade before repeating the experiment again.

However, it was more difficult to control cell growth to obtain enough cells for biological experiments due to its unpredictable growth at times. Hence, some experiments had to be postponed as a result.

Improvements that could be made if the project were to be repeated

As the cell line SW620 undergoes cell death relatively easily, it is not the most effective control cell line since we cannot confidently attribute the results to the toxicity or the potency of the compound tested. To improve, cell line HCT116 could be used instead.

Moreover, as I was not familiar with wound healing assay, it was difficult to draw a thin linear line onto a 24-well plate with the cells. As the inconsistency of the width of the wound played a part in the large standard deviation and thus the large p-values, this resulted in statistically insignificant results. Therefore, more practice is needed to keep the lines at a consistent and desirable width. Also, different techniques to draw the wound can be experimented with to find the one that is most suitable for me.

Acknowledgement

I would like to thank Dr Clive Chung for his supervision and allowing me to join his lab. I also want to express my unwavering gratitude to Yoyo Lai and Jason Li for mentoring and supporting me throughout my whole internship. Lastly, I would like to thank the rest of the lab members for their wonderful collaboration.

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Supplementary

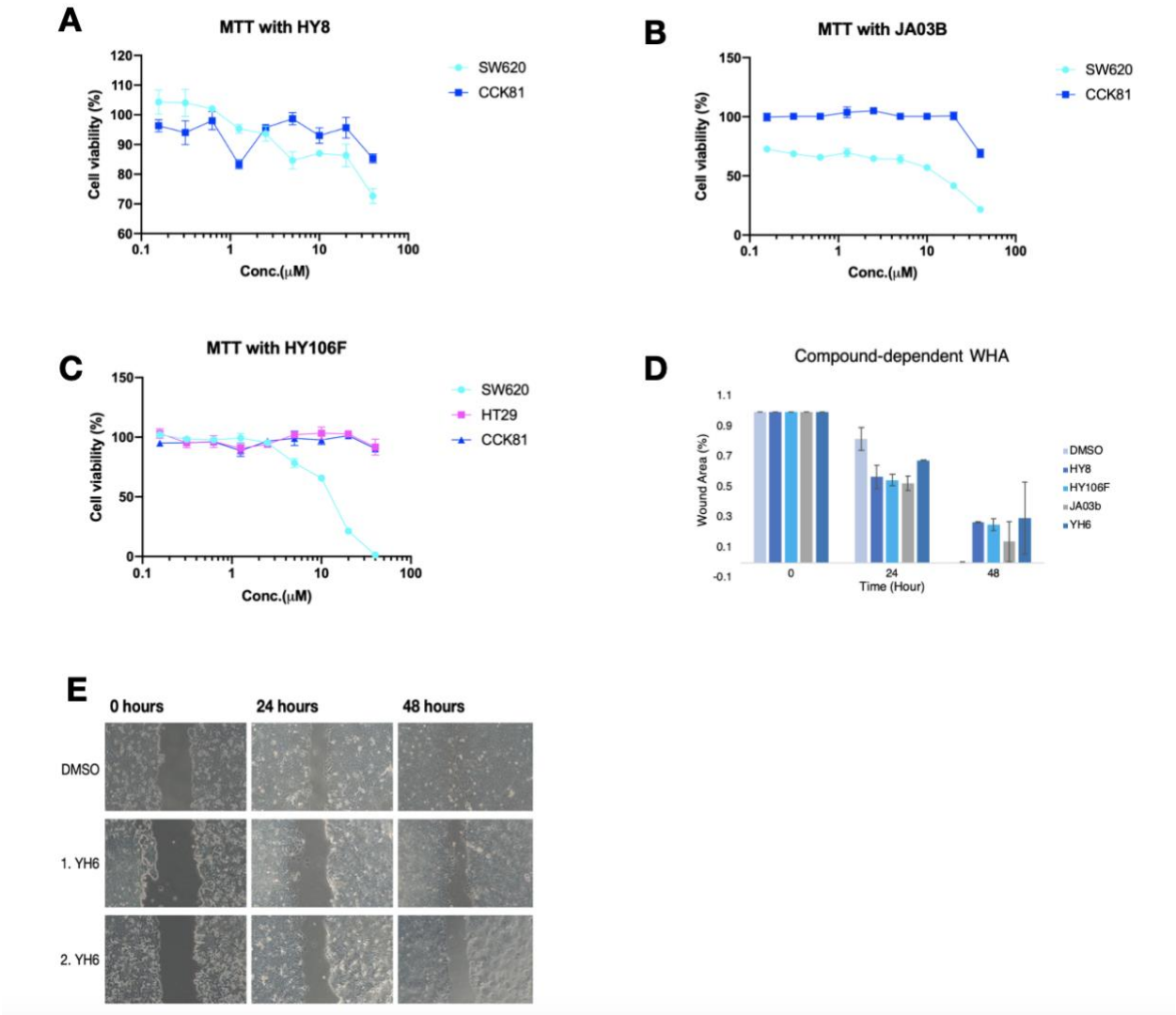

Figure S1. *Cell viability experiments for compounds: HY8, JA03b, HY106F, and YH6*
(A) MTT assays with HY8 in cell lines: SW620, HT29, and CCK81 in 0-40μM by serial dilution after being incubated for 24 hours (n=3). It was then incubated in 100μL of 5 mg/mL of MTT for 4 hours before lysing the cells. Absorbance was measured at 570 nm.
(B) MTT assays with JA03b in cell lines: SW620, and CCK81 in 0-40μM by serial dilution following the method described (n=3).
(C) MTT assays with HY106F in cell lines: SW620, HT29, and CCK81 in 0-40μM by serial dilution following the method described (n=3).
(D) Wound Healing assay data of HY8, HY106F, JA03b and YH6 at the concentration of 20μM with DMSO control at 0, 24, and 48 hours with HT29 cells (n=2). Images were taken by the microscope and analysed with ImageJ.
(E) Wound Healing Assay images of DMSO and 20μM of YH6 (n=2) at 0, 24 and 48 hours.