FINAL SCRIPT: APPROVED FOR FILMING

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Title: Drug-Induced Senescence in Liver Cells Promotes M2 Macrophage Polarization: Implications for Tyrosine Kinase Inhibitor-Associated Hepatotoxicity

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. **Filming location:** Will the filming need to take place in multiple locations? **yes**

   If **Yes**, how far apart are the locations? **Within the university campus only**.

Current Protocol Length
Number of Steps: 22
Number of Shots: 49 (5 SC)

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Anindita Chakrabarty:** The research examines how TKI lapatinib and neratinib induce senescence in liver cells, triggering secretome production to influence macrophage polarization. Such phenomenon may be key to the TKI-induced hepatotoxicity.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.1*

What technologies are currently used to advance research in your field?

1.2. **Abhishek Mitra:** Current technologies include co-culturing liver and immune cells, cellular assays with conditioned media from senescent liver cells, confocal microscopy, western blotting, cytokine profiling, proteomics, examining ROS production, and senescence biomarkers.

1.2.1 INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.1*

What are the current experimental challenges?

1.3. **Vrinda Joshi:** Current challenges include variability in senescence induction, residual drug contamination, limited relevance of immortalized cell lines, differences between murine and human macrophages, neglecting cell-cell interactions, and optimizing conditioned media collection.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.1*

What advantage does your protocol offer compared to other techniques?

1.4. **Gargi Mukherjee:** We link SASP produced by senescent liver cells to macrophage polarization and provide insights into TKI-induced immune modulation. Traditional hepatotoxicity assays or animal models often miss such paracrine interactions.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.2.1*

What research questions will your laboratory focus on in the future?

1.5. **Gargi Mukherjee:** Our lab aims to identify key SASP components and senotherapeutics to mitigate TKI-induced hepatotoxicity, investigate human macrophage responses, and explore the long-term effects of TKI-induced senescence on liver disease and tumorigenesis.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.3.1*

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. MTT Assay for Measuring Cell Viability

Demonstrator: Abhishek Mitra

2.1. To begin, grow HepG2 cells in a 60-millimeter culture dish containing complete growth media composed of Minimum Essential Medium and 10% FBS **[1]**, and place the dish in a cell culture incubator **[2]**.

2.1.1. WIDE: Talent adding the cells and media to the 60-millimeter culture dish.

2.1.2. Talent placing the dish into the incubator.

2.2. Once the cells reach approximately 80 percent confluency, remove the growth media using a pipette **[1]**. Rinse the dish quickly with 1 milliliter of PBS **[2]**, then add 600 microliters of 0.5 times trypsin to the dish **[3]**. Place the dish back into the incubator for 5 to 6 minutes **[4]**.

2.2.1. Talent aspirating and discarding the growth media from a culture dish.

2.2.2. Talent pipetting 1 milliliter of PBS into the dish and swirling briefly.

2.2.3. Talent adding 600 microliters of trypsin to the dish.

2.2.4. Talent placing the culture dish back into the incubator.

2.3. Inside a biosafety level 2 cabinet, neutralize the trypsin by adding 1.2 milliliters of complete growth media to the dish **[1]**.

2.3.1. Talent pipetting 1.2 milliliters of complete growth media into the dish inside a biosafety cabinet.

2.4. Using a pipette, transfer the cell suspension into a 5-milliliter microcentrifuge tube **[1]**. Centrifuge the tube at 180 *g* for 3 minutes at room temperature **[2]**.

2.4.1. Talent transferring the cell suspension into a labelled 5-milliliter microcentrifuge tube.

2.4.2. Talent placing the tube into a centrifuge and starting the spin.

2.5. Discard the supernatant using a pipette **[1]**. Resuspend the cell pellet in 1 milliliter of complete growth media to create a single cell suspension **[2]**, and count the cells using a hemocytometer under a microscope **[3]**.

2.5.1. Talent discarding the supernatant from the centrifuged tube.

2.5.2. Talent pipetting up and down after adding media to ensure a single-cell suspension.

2.5.3. Talent placing a hemocytometer under a microscope.

2.6. Plate HepG2 cells in a 96-well plate at 5,000 to 7,000 cells per well in triplicate for each drug dose, using complete growth media **[1]**. Place the plate in the incubator for 12 to 16 hours to allow the cells to adhere **[2]**.

2.6.1. Talent pipetting cell suspension into the wells of a 96-well plate while working inside the biosafety cabinet.

2.6.2. Talent placing the 96-well plate into the incubator.

2.7. Inside the biosafety cabinet, prepare serially increasing working concentrations of lapatinib and neratinib in DMSO from their stock concentrations of 30 millimolar and 3.5 millimolar, respectively **[1]**. Dilute the drugs in growth media containing Minimum Essential Medium and 5 percent FBS **[2-TXT]**. Prepare one untreated control for each drug **[3]**.

2.7.1. Talent pipetting DMSO into microcentrifuge tubes containing lapatinib and neratinib stocks.

2.7.2. Talent performing serial dilutions using a multichannel pipette and adding to media. **TXT: Lapatinib: 1, 2.5, 5, 10, 15, 25, 50 millimolar; Neratinib: 1, 2, 3, 4, 5, 6, 10 millimolar**

2.7.3. Talent labeling a tube as control and adding only media without the drug.

2.8. Aspirate the old media from the 96-well plate containing the cells **[1]** and add 100 microliters of drug-containing media to each well **[2]**. Incubate the plate for 48 hours inside the incubator **[3]**.

2.8.1. Talent aspirating media from each well of the 96-well plate.

2.8.2. Talent pipetting 100 microliters of drug-containing media into each well.

2.8.3. Talent placing the 96-well plate back into the incubator.

2.9. After 48 hours, add 10 microliters of MTT dye at 5 milligrams per milliliter, dissolved in PBS, directly into the media in each well **[1-TXT]**. Incubate the plate for at least 2 hours until purple crystals appear **[2]**.

2.9.1. Talent pipetting 10 microliters of MTT dye solution into each well. **TXT: MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide**

2.9.2. Talent placing the plate into the incubator and setting a timer.

2.10. Remove the media containing the dye from each well **[1]**. Then, add 100 microliters of DMSO to dissolve the purple crystals **[2]**. After 15 minutes, record the optical density at 595 nanometers **[3]**.

2.10.1. Talent aspirating media from each well.

2.10.2. Talent adding 100 microliters of DMSO to each well.

2.10.3. Talent placing the sample in the plate reader.

3. SA-β Galactosidase Assay

Demonstrator: Vrinda Joshi

3.1. Plate HepG2 cells in 35-millimeter dishes at a density of 4 lakh cells per dish, creating three sets: one for lapatinib, one for neratinib, and one for untreated control **[1]**.

3.1.1. Talent pipetting cell suspension into labeled 35-millimetre dishes.

3.2. Add one microliter of lapatinib from a 5 millimolar working stock and 1.43 microliters of neratinib from a 3.5 millimolar main stock to 2 milliliters of media to prepare drug-containing media **[1-TXT]**.

3.2.1. Talent pipetting lapatinib and neratinib into separate 2-milliliter media tubes using a micropipette. **TXT: Final drug concentration: 2.5 µM**

3.3. Inside the biosafety cabinet, remove the old media from each dish and wash once with 500 microliters of PBS **[1]**. After adding the drug-containing media, place the dishes into the incubator for 48 hours **[2]**.

3.3.1. Talent aspiring the old media and adding PBS to each dish.

3.3.2. Talent placing dishes inside the incubator.

3.4. Next, remove the drug-containing media and rinse the dishes once with 500 microliters of PBS **[1]**. Incubate the cells with 1.5 milliliters of a fixative solution containing 2 percent formaldehyde and 0.2 percent glutaraldehyde in PBS at room temperature for 10 minutes **[2]**.

3.4.1. Talent aspirating media and rinsing dishes with PBS.

3.4.2. Talent placing the dishes on the benchtop for incubation.

3.5. Wash each dish twice with 1 milliliter of PBS to remove the fixative solution **[1]**. Then, incubate the dishes with the staining solution at 37 degrees Celsius for 8 hours without carbon dioxide **[2-TXT]**.

3.5.1. Talent adding the dishes twice with PBS.

3.5.2. Talent placing the dishes into a non-CO_2 incubator set at 37 degrees Celsius. **TXT: Staining solution: 40 mM citric acid/ Na-phosphate buffer; 5 mM $K_4[Fe(CN)_6]$; 5 mM $K_3[Fe(CN)_6]$; 150 mM NaCl, 2 mM $MgCl_2$; 1 mg/mL X-gal in autoclaved water.**

3.6. After staining, wash each plate twice with 1 milliliter of PBS **[1]**. Capture brightfield images at 20 and 40x magnification using an epi-fluorescence microscope, covering a minimum of four to five fields per treatment group **[2]**.

3.6.1. Talent rinsing dishes twice with PBS.

3.6.2. Talent placing the dish under the microscope.

3.7. Now, select one captured field to count the total number of cells along with the number of cells exhibiting a blue signal **[1]**. Manually calculate the percentage of blue-stained cells for at least four images, average the results, and plot them in a spreadsheet graph **[2-TXT]**.

3.7.1. SCREEN: 68725_3.7.1.mp4 00:20-00:30.

3.7.2. SCREEN: 68725_3.7.2.mp4 04:00-04:10. **TXT: Perform a t-test**

4. ROS Detection using Confocal Microscopy

Demonstrator: Gargi Mukherjee

4.1. Plate HepG2 cells in 35-millimeter glass-bottom dishes at a density of 4 lakh cells per dish, setting up three experimental sets: one for lapatinib, one for neratinib, and one for untreated control **[1]**.

4.1.1. Talent pipetting HepG2 cells into glass-bottom dishes and arranging them into three clearly labeled sets.

4.2. Treat the plated cells using the same procedure as previously described for lapatinib and neratinib, including drug preparation, media replacement, and 48-hour incubation **[1]**.

4.2.1. Talent placing the plates in the incubator.

4.3. After 48 hours of treatment, add two micromolar CellROX *(cell-rocks)* Deep Red reagent, one micromolar MitoTracker Green, and 500 nanomolar Hoechst dye to each dish **[1]**. Incubate the cells for 30 minutes **[2]**.

4.3.1. Talent pipetting a dye into the dish.

4.3.2. Talent placing the stained dish inside the incubator.

4.4. After 30 minutes, aspirate the staining solution and wash the cells with PBS to remove excess dye **[1]**. Acquire images using a confocal microscope at 100x oil immersion magnification with appropriate excitation wavelengths **[2]**.

4.4.1. Talent removing dye from the dish and adding PBS.

4.4.2. Talent operating the confocal microscope.

4.5. Use Fiji ImageJ software to quantify the red signal from CellROX Deep Red and compare it with the signal from the untreated control **[1]**. Use the green MitoTracker signal to confirm mitochondrial localization **[2]**. Plot the average signal intensities as bar graphs and perform a t-test for statistical comparison **[3]**.

4.5.1. SCREEN: 68725_4.5.1.mp4 00:36-00:48.

4.5.2. SCREEN: 68725_4.5.2.mp4 00:57-01:04.

4.5.3. SCREEN: 68725_4.5.3.mp4 05:15-05:25.

Results

5. Results

5.1. HepG2 cell viability decreased in a dose-dependent manner after treatment with lapatinib **[1]** and neratinib **[2]**.

5.1.1. LAB MEDIA: Figure 1A. *Video editor: Highlight the bars from left to right sequentially*.

5.1.2. LAB MEDIA: Figure 1B. *Video editor: Highlight the bars from left to right sequentially*.

5.2. Treatment with 2.5 micromolar lapatinib or neratinib caused a strong increase in mitochondrial reactive oxygen species, as shown by MitoSOX *(mito-sox)* Red staining **[1]**, and colocalization with MitoTracker Green confirmed mitochondrial origin **[2]**.

5.2.1. LAB MEDIA: Figure 2. *Video editor: Highlight the mito-SOX cells with red-stained regions in lapatinib- and neratinib panels*.

5.2.2. LAB MEDIA: Figure 2. *Video editor: Highlight the mito-Tracker cells in lapatinib- and neratinib panels.*

5.3. SA-beta-galactosidase staining revealed a marked increase in senescent cells after treatment with lapatinib and neratinib **[1]** compared to the control **[2]**.

5.3.1. LAB MEDIA: Figure 3. *Video editor: Highlight the images for lapatinib and neratinib in A and bars in B for lapatinib and neratinib*.

5.3.2. LAB MEDIA: Figure 3. *Video editor: Highlight the control image and bar*.

5.4. RAW264.7 *(raw-2-6-4-point 7)* macrophages exposed to conditioned media from drug-treated HepG2 cells exhibited a larger, elongated morphology consistent with polarization **[1]**.

5.4.1. LAB MEDIA: Figure 4C. *Video editor: Zoom in on the elongated macrophages in the lapatinib- and neratinib-treated groups compared to the rounder control cells*.

5.5. Western blotting showed increased expression of arginase 1 in RAW264.7 macrophages treated with conditioned media from the lapatinib and neratinib **[1]** groups, while inducible nitric oxide synthase levels were decreased **[2]**.

5.5.1. LAB MEDIA: Figure 4D. *Video editor: Highlight the thicker ARG1 bands in the lapatinib and neratinib lanes and highlight the lapatinib and neratinib boxes in row ARG1*.

5.5.2. LAB MEDIA: Figure 4D. *Video editor: Highlight the NOS2 bands in the lapatinib and neratinib lanes and highlight the lapatinib and neratinib boxes in row NOS2*.

1. HepG2

- **Pronunciation link:** No confirmed link found (it's a cell line name, not a standard dictionary entry)
- **IPA:** /ˈhɛp dʒiˈtuː/
- **Phonetic spelling:** hep-jee-two

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2. confluency

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/confluency
- **IPA:** /ˈkɑnfluənsi/
- **Phonetic spelling:** kon-floo-en-see

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3. trypsin

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/trypsin
- **IPA:** /ˈtraɪpsɪn/
- **Phonetic spelling:** tryp-sin

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4. hemocytometer

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/hemocytometer
- **IPA:** /ˌhiːmoʊˈsaɪˌtɑːmɪtər/
- **Phonetic spelling:** hee-mo-sigh-toh-meh-ter

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5. senescence (in "senescent cells")

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/senescence
- **IPA:** /səˈnɛsəns/
- **Phonetic spelling:** suh-ness-ens