Investigating the Mechanism of Action Behind Novel Anti-Cancer Drugs

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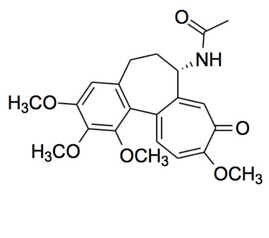
Abstract

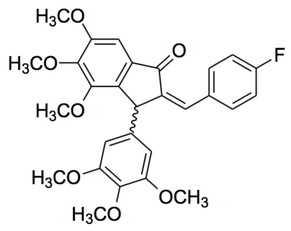
Microtubules serve as a fundamental component of the cytoskeleton. Due to the nature of their role in cell division, microtubules are important targets for potential chemotherapy drugs. We are in the process of attempting to determine the mechanism of action of fluorinated benzylidene indanone 1 (Ind-V). Ind-V was derived from chemical modification of the plant-derived compound gallic acid. Ind-V contains the trimethoxy ring structure that is key to the microtubule-depolymerizing activity of the drug colchicine, which is currently used to treat gout. We found that, like colchicine, Ind-V inhibits the division of cells by arresting them in mitosis. I used three cell-based assays to test the effects of Ind-V on HeLa cells, a cervical cancer cell line. Unlike colchicine, Ind-V did not promote microtubule depolymerization and instead generated mitotic spindle abnormalities very similar to those generated by the microtubule-stabilizing anticancer drug, Taxol. The drug likewise failed to inhibit microtubule polymerization in an *in vitro* assay involving purified bovine brain tubulin. An unusual light scattering pattern was recorded in the *in vitro* assay, suggesting that Ind-V may have a unique effect on microtubules.

Introduction

Microtubules are polymers of tubulin that make up the cytoskeleton and play a vital role in maintaining cellular structure, cell division, and cell migration. These protein polymers exhibit a dynamic equilibrium in which free tubulin subunits attach and detach from the ends at equal rates. An interference in this dynamic equilibrium will result in cell cycle arrest, that is, the cell will no longer divide. These characteristics make microtubules an exceptional target for cancer therapeutics. A recently designed compound isolated from a plant-based acid, fluorinated benzylidene indanone 1 (hereon referred to as Ind-V), is hypothesized to act as a microtubule destabilizer as it shares structural similarities with colchicine, a known microtubule destabilizer. So far, we have carried out viacount assays, in vitro microtubule polymerization assays, and immunofluorescence microscopy to explore if Ind-V can be eventually used as an effective cancer therapeutic. In continuing to study Ind-V, we will perform repeats of experiments to strengthen our initial findings as well as study Ind-V more extensively by performing other experiments, including apoptosis assays.



Colchicine



Ind-V

Methods and Materials

Immunofluorescence microscopy was used in order to determine the mitotic index of HeLa cells drugged with varying concentrations of Ind-V. Mitotic index is a comparison of how many cells are in cell cycle arrest to the total number of cells viewed, thus measuring the effectiveness of Ind-V at various concentrations. To do this, we started with HeLa cell suspensions and added varying concentrations (0µM up to 70 µM) of Ind-V diluted in fresh cell growth media. After 24 hours, the Ind-V solutions are removed and the cells are fixed, which kills the cells but keeps membranes and proteins intact. A fluorescent DAPI stain is added that binds to the DNA, therefore allowing one to determine which cells are in a cellular arrest and which are in interphase. Additionally, fluorescent anti-tubulin antibodies were added to allow visualization of microtubules using confocal microscopy.

The Viacount assay helps determine the EC50 of Ind-V, that is, the concentration of the drug in which 50% of its maximal effect is produced. This experiment measures the inhibition of HeLa cell proliferation. Like the immunofluorescence experiment, the cell suspensions are drugged with varying concentrations of Ind-V. However, the cells are not fixed and treated with fluorescent stains or antibodies, but are instead counted via flow cytometer to determine the concentration (cells/mL) of cells in each well.

In order to test whether Ind-V will directly target microtubules or if they do so via a cellular pathway, we carried out microtubule polymerization assays using PC tubulin purified from fetal calf brains. In this experiment, microtubule seeds were first prepared and then added to different polymerization mixes, the experimental containing 50 µM Ind-V, the negative control containing DMSO (the solvent of the Ind-V solution), and the positive control containing 1 µM vinblastine (a known microtubule depolymerizer). The samples were measured by spectrophotometer every minute for 30 minutes, the time in which microtubules are expected to polymerize during.

Charts and Figures

Fig. 1

Mitotic Index of Cells Treated With Various Concentrations of Ind-V

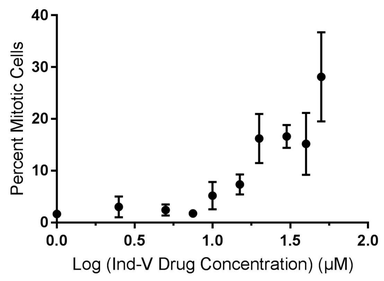


Fig. 2

Inhibition of HeLa Cell Proliferation by Ind-V

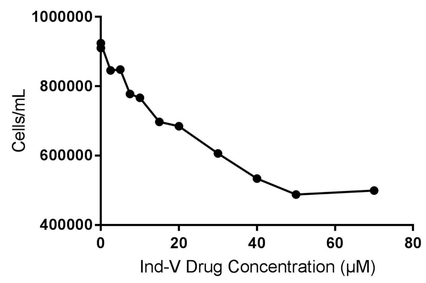


Figure 1: Mitotic Index versus Log [Ind-V]| This graph shows the mitotic indices for increasing concentrations of Ind-V. A direct relationship between increasing Ind-V concentration and mitotic index is observed. **Figure 2: Inhibition of Proliferation Due to Ind-V|** This chart shows the decrease in proliferation due to an increasing number of cells being trapped in mitotic arrest as the concentration of Ind-V is increased. Cell concentration was measured using the Viacount Flow Cytometry assay. Half-maximal inhibition occurred when Ind-V concentration was 15 uM.

Fig. 3

In Vitro Polymerization Levels After Treatment with Experimental Drugs

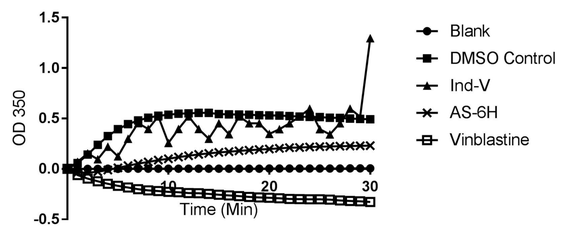


Figure 3: In Vitro Polymerization| Microtubules were polymerized in vitro using PC Tubulin derived from fetal calf brains. These microtubules were then treated with vinblastine (positive control), DMSO (negative control), Ind-V, and AS-6H (second experimental drug). The OD at 350 nm was then measured in real time.

Fig. 4

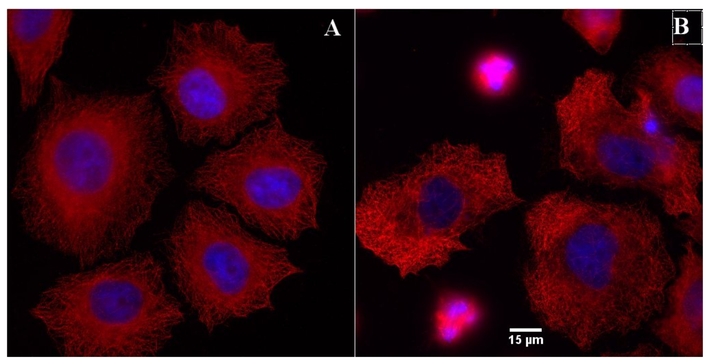


Figure 4: Immunofluorescence Images | Fluorescent microscopy images of control interphase cells (A) and 70 uM Ind-V-treated cells (B). Tubulin is fluorescently labeled with red dye and cell nuclei are labeled with blue dye using conjugated antibodies. The convoluted microtubules found in Image B are a result of Ind-V treatment. Additionally, several mitotic cells are visible in the background of Image B, indicating that the drug induced mitotic block at the 70 µM concentration.

Results and Discussion

Through the use of immunofluorescence microscopy, we found that an increased proportion of cells were in mitotic arrest as the Ind-V concentration rose and abnormal mitotic spindles were observed. This is evidence that Ind-V is inducing cell cycle arrest of HeLa cells in a concentration-dependent manner, consistent with the findings of our partner lab. In the future, we will repeat this experiment, potentially with different cancer cell lines.

The viacount assay illustrates that inhibition of cell proliferation by Ind-V is concentration-dependent, further evidence that the effects of Ind-V are concentration-dependent. The data shows that half of the maximum inhibition of cell proliferation occurs in cells treated with 15 µM, hence the EC50 concentration of Ind-V is 15 µM. In the future, we will repeat this experiment to strengthen our findings.

The microtubule polymerization assay measures tubulin polymerization until a steady state is reached in which the microtubules exhibit dynamic equilibrium. The DMSO negative control shows expected microtubule polymerization and the vinblastine-treated positive control completely inhibits the formation of microtubules. As you can see in the figure, the Ind-V mix resulted in unpredictable polymerization and depolymerization when compared to the controls and the 6H drug, which is another drug our lab studies as well. The exact implications of this result are unclear, but could be caused by spontaneous length increases and decreases that are sometimes observed in microtubules polymerized from PC tubulin. We plan to test this hypothesis by first making sure these results are reproducible by repeating this experiment, and if so, doing electron microscopy of samples taken periodically from the cuvette.

References and Acknowledgments

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