Determining the Effect of a CBC-based Oxime on Cell Viability and Apoptosis in Sea Urchin Embryos.

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Abstract

Natural products are an important source of existing and potential new anti-cancer drugs. We have previously shown that cannabichromene (CBC) oxime and several CBC oxime esters have potent anti-mitotic activity when used in bioassays with sea urchin embryos. In this study we explored the effect of the CBC oxime on cell viability and apoptosis in sea urchin embryos. Newly fertilized or gastrula stage embryos were combined with 10µM or 50uM CBC oxime as well as a DMSO only control. At various timepoints, samples of the embryos were removed from the cultures and stained with three different fluorescent dyes. NucBlue (ThermoFisher) was used to determine cell number, apoptotic effects were observed with CellEvent caspace 3/7 (ThermoFisher), and cell viability was determined with Live-or-Dye (Biotium). As expected, there was a clear effect of the CBC oxime on the rate of cell division. The embryos exposed to both 10uM and 50uM CBC oxime showed a reduction in cell division compared to the controls, with an almost total inhibition in the higher concentration. Cell viability assays using the vital stain Live-or-Dye showed a concentration dependent effect of the CBC oxime on cell viability, with levels of cell death reaching almost 80% in the 50uM concentration by 3 hours after drug addition. Finally, staining with CellEvent caspace 3/7, which indicates the presence of active caspace activity, demonstrated concentration dependent induction of apoptosis following exposure to the CBC oxime drug. Therefore the loss of cell viability observed was due to apoptosis rather than necrosis. There are clear toxic effects seen when this drug is exposed to developing sea urchin embryos, suggesting that it might have potential use as a chemotherapy agent capable of inducing apoptosis in cancer cells. We plan to test this hypothesis on human cancer cell lines in the near future.

Introduction

- Cancer is a disease that causes cells to grow uncontrollably and is a leading cause of death (American Cancer Society 2019).
- A few of the most common treatments that are used to fight cancer are surgery, chemotherapy, radiation, and immunotherapy, but many come with unwanted side effects (National Cancer Institute, 2015).
- One of the biggest problems in cancer treatments is chemotherapy drug resistance, meaning there is a continual need for new effective anticancer drugs (Wang et. al, 2019).
- Natural products that come from plants and microbes are an excellent source of these drugs. They can be synthetically altered to become more potent, and can relieve some of the toxicity and unwanted side effects of traditional cancer treatments (Demain & Vaishnav, 2010).
- Taxol (plant based), Etoposide (plant based), and Statins (microbe based) are examples of drugs that come from natural products that are approved for the treatment of cancer or have anti-tumor activity (Demain & Vaishnav, 2010).
- Natural products can act on cancer cells via several mechanisms including induced apoptosis (Demain & Vaishnav, 2010), which is a useful tool in combating the problem of chemotherapy resistance (Amaral et. al, 2019).
- CBC (cannabichromene) is a compound that comes from the plant *Cannabis sativa*. Its alkane chain was synthetically altered in Dr. Henry's lab (Henry & Parks) with an oxime group to form the product, CBC oxime (cannabichromene oxime).

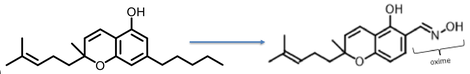

Figure 1. CBC (cannabichromene)

Figure 2. CBC oxime (cannabichromene oxime)

- Apoptosis is programmed cell death and its pathways occur both intrinsically (intracellular stimulus) and extrinsically (external stimulus) (Robertson et. al, 2006).
- The impact of induced apoptosis on cells is an important mechanism in anti-tumor processes.

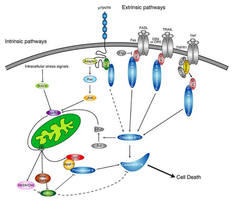

Figure 3. Apoptosis pathways. Both of these pathways activate multiple proteins within the cell, that eventually activate the 3/7 effector caspases that are essential in the promotion of cell death (Robertson et. al, 2006).

- Sea urchins are a great model system to test for the effectiveness of antitumor drugs because they are easy to observe over a short period of time, they go through rapid cell division, and they are a comparable system to humans because they are deuterostomes (Nishioka et. al, 2003; Ettensohn, 2017; McClay, 2011).

Materials and Methods

Sea urchin experiments:

- Adult sea urchins (*Lytechinus pictus*) were spawned by intracoelomic injection of 0.5M KCl.
- Eggs were washed in artificial sea water (Instant Ocean), fertilized with dilute sperm, and the fertilized embryos were cultured at 17 °C.
- Embryos of the selected stage of development were transferred to a non-treated 6 well plate.
- The CBC oxime was added to the cultures from a 25mM stock culture in DMSO to final concentrations of 10-50uM, with an equal volume of DMSO added to the controls.
- Embryos were exposed to the drugs for 150 minutes then concentrated by gentle centrifugation to a volume of 1mL.
- Nuclear staining was done by adding 1-2 drops of NucBlue LiveReady Probes Reagent (ThermoFisher). Caspace activation was detected by adding 1 drop of CellEvent caspace 3/7 Green (ThermoFisher). Cell viability was determined by the addition of 1uL Live or Dye (Biotium).
- Embryos were incubated with the 3 stains in the dark for 30 minutes. Samples were placed on glass slides and imaged on an EVOS Cell Imaging System. One hundred embryos were assessed for each treatment.

Cancer cell experiments:

- A melanoma cell line (A375; ATCC) was cultured in DMEM supplemented with 10% FBS and grown at 37 degrees C at 5% CO_2. Cells were grown to confluency in 24 well or 96 well plates before drug addition.
- CBC oxime was added to final concentrations of 10-50uM with DMSO added to the controls. Etoposide (400ug/ml) was used as a positive control for induction of apoptosis.
- To determine the impact on cell viability and induction of apoptosis, caspace activation, cells were stained with NucBlue, Live or Dye and CellEvent caspace 3/7 Green.
- An additional test of cell viability was utilized, the XTT assay (Sigma). Cells were grown to confluency in 96 well plates, CBC oxime was added for 24 hours, then the XTT solution was added for 4 hours. At 4 hours, the level of staining was determined using an Elisa plate reader. Cell viability was calculated as (absorbance of drug treated cells/absorbance of control cells)X100.

Results and Discussion

- NucBlue shows us the number of nuclei present in the embryos, Live-or-Dye is a red fluorescent dye that permeates the membrane upon cell death, CellEvent caspace 3/7 turns cells with activated 3/7 caspase a fluorescent green to showed induced apoptosis.

CBC Oxime Blocks Cell Division and Induces Apoptosis During Early Development

- As shown in Figure 4, CBC oxime blocks cell division when added at fertilization.
- Control embryos showed normal cell division, with no signs of cell death or induced apoptosis.
- The 10µM of oxime showed a reduced number of nuclei and some cell death and apoptosis in embryos compared to the control.
- The 50µM of oxime had the most significant effects on cell viability. Cell division has slowed and or completely stopped, significantly more dead and apoptotic embryos compared to the control and 10µM of oxime.

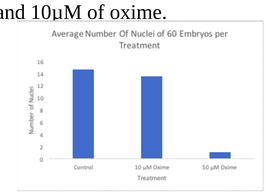

Figure 4. The mean number of nuclei in 60 embryos per treatment 3hrs post fertilization

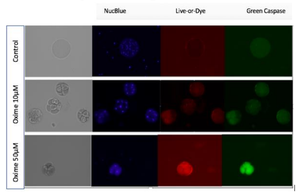

Figure 5. Shows the number of nuclei, cell death, and apoptosis in each treatment of embryos.

- As shown in Figures 5-9, CBC oxime caused cell death primarily by inducing apoptosis.
- Observed 100 embryos per treatment every 30 minutes for 2hrs.
- Strong concentration dependence between the treatments, with 50µM having the most effects on cell death and apoptosis over the 2hr time period.
- Strong correlation between cell death and apoptosis, makes it likely cell death is occurring due to induced apoptosis from the CBC oxime.

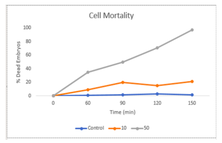

Figure 6. Cell death trends in each treatment of embryos over 2hrs

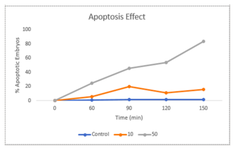

Figure 7. Apoptosis trends in each treatment of embryos over 2hrs

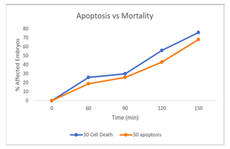

Figure 8. Comparing cell death and apoptosis trends in the 50µM embryos over 2hrs

CBC Oxime Induces Apoptosis at the Gastrulation Stage (24hrs post fertilization)

- Again, concentration dependent results, but 50µM oxime treatment produced significantly stronger effects. As shown in Figure 9, there were high levels of cell death and apoptosis.

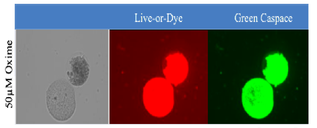

Figure 9. Shows cell death and apoptosis in embryos after 24hrs post fertilization

- Observed 100 embryos per treatment every 30 minutes for 2hrs.
- As shown in Figures 10-12, we again saw high levels of cell death and apoptosis in the treated embryos.

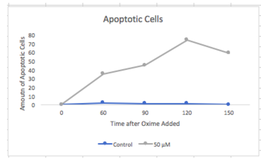

Figure 10. Cell death trends in each treatment of embryos over 2hrs

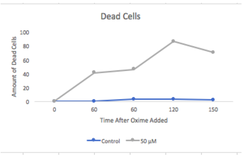

Figure 11. Apoptosis trends in each treatment of embryos over 2hrs

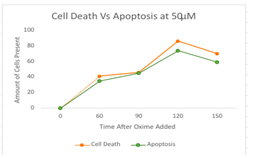

Figure 12. Comparing cell death and apoptosis trends in the 50µM embryos over 2hrs

CBC Oxime Induces Apoptosis in Melanoma A375 Human Cancer Cell Lines

- We observed a concentration dependent increase in the level of cell death and apoptosis following a 24 hour exposure to the CBC oxime (Figure 13).
- An XTT assay to determine cell viability also demonstrated that the CBC oxime reduced cell viability in the A375 cells (Figure 14). Etoposide was used a positive control in both experiments.

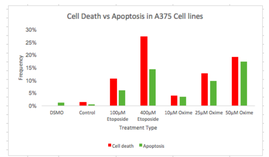

Figure 13. Cell death and apoptosis frequency in A375 cell lines after 24hrs of exposure.

Figure 14. (XXT assay) Cell viability percentages in A375 cell lines after 24hrs exposure.

Conclusion

After testing cannabichromene oxime for anticancer activity using sea urchin embryos and a human cancer cell line, the results were encouraging. We found a concentration effect between the different treatments of the CBC oxime, with the highest concentrations of oxime showing the most significant results. Sea urchin embryos treated with 50µM CBC oxime either immediately following fertilization or at the gastrula stage exhibited high levels of cell death which was strongly correlated with expression of markers for apoptosis. In the A375 human cancer cell lines, we also saw the most significant effects on cell viability with the 50µM CBC oxime treatment. With these compelling results for cannabichromene oxime's anti-tumor properties, it is important that further research should continue on naturally derived compounds like cannabichromene oxime, as they could be the solution to cancer drug resistance, as well as relieving some of the unwanted and toxic side effects that traditional cancer treatments generate.

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