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Synthesis of a-Cyanostilbenes And Testing of Their Anticancer Properties

Gabrielle Cook
Andrews University, gabriellec@andrews.edu

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Andrews University

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Gabrielle Cook

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Advisor: Denise Smith PhD

Primary Advisor Signature: Denise L Smith

Department: Biology

Abstract

This research project involves synthesizing cyanostilbenes and testing their anticancer properties in breast cancer cells. Breast cancer is a global issue, one that great improvement has been found in the last few decades, but also one that remains a large killer. The components of the cyanostilbene hybrids have all been shown to have anticancer properties on their own, and we would like to see if we can increase these properties by combining multiple into a single hybrid. Then these cyanostilbene compounds are tested on Her2+ human breast cancer cells. The goal is to assess whether or not the cyanostilbenes have any effect on breast cancer cells. This project encountered many difficulties and as a result did not have significant results, but did produce many solutions for the problems encountered which will aid future research.

Introduction

Cancer is undoubtedly of the utmost importance in medical research. According to the CDC in 2012 8.2 million people died from cancer and 14.1 million new cancer cases were diagnosed worldwide (CDC). Clearly cancer has a huge effect on healthcare systems internationally and on people internationally. Research into cancer treatment is important since it finds new cures/treatments, better options for treatments, and also because it can reduce costs with new, less expensive treatments. Breast cancer is among the most common cancers at second place with 12% of all cancers diagnosed (just behind lung cancer at 13%). This is a rather astounding figure since it was calculated using both sexes and women have a much higher incidence of breast cancer. Breast cancer also accounts for 6% of the cancer deaths each year (5th internationally). These statistics indicate that breast cancer is a large international heath issue that needs improved treatment.

Today breast cancer is treated in a variated of ways that vary depending on the type of cancer, the stage of progression, the overall health of the patient/comorbidities, patient choice, and many other factors. The National Cancer Institute of the NIH gives five types of standard treatment, surgery, radiation therapy, chemotherapy, hormone therapy, and targeted therapy. In many cases multiple types are used on the same patient. In this research the focus is on drug research for breast cancer treatment, which typically falls under the chemotherapy type. The cancer used in testing is Her2+ breast cancer which is a type of breast cancer where the cells overexpress the human epidermal growth factor 2. This type of cancer spreads faster and has a decreased response to treatment as compared to Her2- breast cancer. Therefore, historically Her2+ has meant a decreased rate of survival for patients, as it is more aggressive. More recently targeted therapies have been developed that have greatly improved survival rates, but there is still more progress to be made.

All of the compounds synthesized in this project are α-cyanostilbenes, see Figure 1, which are part of the broader class called stilbenes (same structure just no nitrile group). Stilbenes have been researched as cancer treatment for decades, the most notable being resveratrol. Resveratrol, a phytoestrogen, is found in grapes and has been thought to be effective in cancer treatment. These anticancer properties are often the subject of research, with the aim of improving them. This is done by taking the backbone of resveratrol and making synthetic changes to the compound by adding and removing functional groups. Chalal and colleagues synthesized and tested hydroxylated stilbenes and ferrocenyl-stilbenes and compared their anticancer properties to resveratrol. They found that five of their compounds were more potent than resveratrol (Chalal et al 2014). Penthala and colleagues also synthesized different analogs of resveratrol and tested them against 60 different cancer cell lines. They then chose a few promising compounds and analyzed their binding activity. They found that the compounds were showing anticancer properties by binding to tubulin, which prevents cell growth. These research papers show the promise of editing the stilbene backbone.

Cyanostilbenes can be seen as an example of these modifications as they are a single functional group different from stilbenes. Cyanostilbenes are notable in their structure in that they have significant resonance with allows for conjugation abilities. This gives some of these compounds fluorescence and they are used in lasers and other optics that use this characteristic. In addition to optics research they have also been researched for their anticancer properties. Penthala and colleagues created novel cyanostilbenes and tested them against 60 different human cancer cell lines. 2 compounds showed promise against all lines and many worked well for specific cancer types. Overall these compounds worked best on solid tumor cancers, which is promising since breast cancer is a solid tumor cancer (Penthala et al 2014).

In many cases the functional groups chosen to add to the cyanostilbenes are just guesses at what researchers think many have an increased effect, but sometimes the groups are chosen

specifically because they themselves have shown anticancer properties. An example of this is boronic acid which has been shown to have broad application in medicinal chemistry as discussed in a review paper by Trippier and Mcguigan (2010). The idea is that if you can combine 2 compounds that are anticancer on their own (cyanostilbenes and boronic acid in this case) maybe you can actually increase the potency of the compound. Another way that functional groups are chosen is to attempt to manipulate certain properties of the compound. An example would be adding certain polar groups in order to increase solubility.

In conclusion the goals of this research project are to synthesize novel α-cyanostilbenes and to test them for anticancer properties. This project hopes to find compounds that show significant anticancer properties, but it would still be a success if certain compounds were eliminated from further testing. In addition this project, as one of the first of many, would like to deal with many of the issues encountered and propose solutions for future projects. This project outline has the potential to be continued for many students in the future who are testing new compounds and the more optimized the process can become the better results we will hopefully see.

Methods

The synthesis of the cyanostilbenes is the first step of my research. Overall this process involves combining an aldehyde compound with a nitrile compound in a nucleophilic addition and then elimination reaction. This is conducted in the following steps:

1) 16ml of water and 4 ml of ethanol were put into a clean 50 ml round bottom flask
2) In the following order each compound was added:
    a. 2.5 mmol aldehyde compound
    b. 2.5 mmol nitrile compound
    c. 10 mmol of calcium reagent
3) The mixture was then stirred and refluxed for 3 hours.
4) Mixture was poured into ice with 30 ml of saturated NH4Cl to neutralize.
5) Reaction mixture was then dried and NMR taken.

After synthesis the compounds must then be assessed to see if they can be used in cancer testing. First a percent yield is taken. Very low percent yields indicate that a reaction was not very successful and most likely very little product was collected. Percent yields over 100% indicate that the compound is holding on to water, which does not automatically disqualify it from my research. Next an NMR or IR is taken of the compound. Since this reaction is very specific, there is essentially only one major product, therefore if we can determine from the NMR that a reaction did in fact take place it is reasonable to assume that it was the correct reaction. How we can assess this from the NMR is by analyzing the reaction itself. While one of our reactants is an aldehyde, the cyanostilbenes created do not have an aldehyde group. This means that the disappearance of an aldehyde peak in the NMR spectra (around 9-10 ppm) indicates that the reaction happened. Therefore if a compound is still displaying an aldehyde peak, it will not be further used in the research process. Also further examination of the NMR and comparison to predicted NMR for products could lead to further

elimination of products. The IR can also show the presence of an aldehyde. The carbon double bonded to oxygen has a strong stretch around 1740-1720 intensity and the aldehyde hydrogen has a medium strength double peak at 2820-2850 and 2720-2750 intensity.

Next the cyanostilbenes need to be tested on the Her2+ cancer cells. For this the following timeline is used:

Day 1 – Setup plates with cancer cells in wells

Day 2 – Treat cells with drug

Day 3 – Nothing (Allow cells to grow)

Day 4 – Add CellTiter-Blue Dye

Day 5 – Analyze via fluorometer

On day one the cancer cells need to be pipetted into the wells. Cells need to be taken from the 100mm culture dishes where they are grown. Cells are treated with 2 mililiters of trypsin to remove them from the culture dish. Next the cancer cells are counted with the hemocytometer using 90 microliters of trypan blue and 10 microliters of cells. The trypan blue gives the ability to distinguish between living and dead cells. Then the cells are diluted to have around 10,000 cells per well. Then 100 microliters is pipetted into each well (we are using 96 well plates).

On day 2 the compounds are dissolved in 100% DMSO and then diluted using media to 4 different concentrations, 0.2 mg/ml, 0.1 mg/ml, 0.02 mg/ml, and 0.01 mg/ml. While water would be a more ideal solvent due to its low toxicity our compounds are not soluble in water. Previous studies done in Dr. Smith's lab have shown that concentrations of DMSO less than 1% are completely nontoxic and therefore DMSO will not affect cell viability. Then 100 microliters is pipetted into each well (we are using 96 well plates).

On day 4 CellTiter-Blue is added to the wells. This is an assay used to assess cell death. It works by harnessing a simple reduction reaction that living cells are able to conduct. A chemical

called Resazurin is added to the wells. Resazurin is dark blue in color and has very little fluorescence. When the cells are living they convert Resazurin into Resorufin. Resorufin is pink in color and is highly fluorescent (584 nm emission). Therefore if the drug has killed a lot of the cells, the dye should remain somewhat blue and the intensity of fluorescence will be low, but if the drug has not killed the cells, the dye should turn pink and the intensity of the fluorescence will be high. 40 microliters is pipetted into each well (protocol is 20 microliters per 100 microliters of volume in the wells and the wells have 200 microliters).

On day 5 the cells are analyzed via fluorometer. The raw data collected is the intensity of the fluorescence at the wavelength 590. The values can then be converted into cell concentration as a percent of the control value (no drug). Therefore a percent below 100% shows greater cell death than the control and a percent above 100% shows an increase in survival compared to the control. The t-test requires the use of the raw data.

In addition to the methods above the compounds were also tested for autofluorescence to ensure that the fluorometer results can be fully attributed to the Resorufin and not to the compounds themselves. This is done using the same methods as above but no cells are added to the wells. If there is autofluorescence this value will be subtracted from the raw data.

The last step is to conduct statistical analysis of the data; a t-test was used.

Results

First the products are described and a percent yield calculated and these results are given in Table 1. Using the percent yields it was determined that GC10 and GC29 were too low to be of any use in further research. Next an NMR was taken of compounds GC1, GC4, GC7, GC10, GC13, GC16, GC19, and GC21 which is shown in Figure 2 and an IR taken of compounds GC23, GC25, GC27 which is shown in Figure 3. In the analysis of the NMR compounds GC7 and GC10 showed retention of the aldehyde peak and were therefore eliminated from future testing and compounds GC13 and GC19 had poor, inconclusive NMRs and were also eliminated. Based on the NMR and IR data 7 compounds were chosen to begin testing, GC1, GC4, GC16, GC21, GC23, GC25, GC27. The raw data collected from the fluorometer is given in Figure 4. Then these values are used to calculate to calculate the corrected data, given in Figure 5. This data is shown graphically in Figure 6 with the structures of the compounds synthesized. The p values of the t-tests were all significant and are given in Figure 5 and the caption of Figure 6. Figure 7 shows the compounds that we attempted to synthesize, but were not chosen for testing.

Discussion

While it may seem a success that all the p values are significant, this data should be taken lightly. The sample size for this project was not large enough to correct for the large standard deviations between the raw data and this data did not have any significant differences. Once the autofluorescence was factored in everything became significant since so many values went to zero. During the course of this project many roadblocks were encountered as this was one of the first projects completed using these methods. Many of the issues were able to be fixed during the course of the research, many were not, and all of them will be discussed here.

Differences between Plates

In order to have data that is free from error the drugs must be tested in different runs on different plates. This creates a problem though when the amount of cells per wells varies between plates. Even though the procedure is designed to average about 10,000 cells per well, this doesn't always happen. Variations in this number are caused by many factors include experimenter error, number of cells available, and the researcher who sets the plate up. Then when the raw data is compared using a t-test there is rarely significance because the standard deviations are so high. One solution was to convert the raw data into a percent of control, therefore correcting for the differences between plates (since the controls were also different). But this fix made statistical comparisons to the control very difficult (since the control data all turns to 1 or 100%). Future improvements in this area could be to research less common statistics that could work for percent from control data, improvements to the procedure to get close to the 10,000 cells/well, and make sure that the same person sets up all the plates in a single project.

Differences between Wells

In addition to differences between plates there was also differences between different wells on the same plate. There was two main issues with the wells, the first being an edge effect. On the

edge of the plate the liquid in the wells tended to evaporate faster than in the wells that were more centrally located. When water evaporates the concentration of the drug changes, as does the composition of the media and this can obviously skew the data. The first attempted solution was to vary the placement of the drugs in the wells to try and average out the edge effect, but this only works if you can have a very large sample size (since to edge effect creates a very noticeable difference). Another solution is to use a special kind of material that seals the plate more tightly (similar to parafilm), thereby reducing the chance of evaporation. There is also a second fact is well to well variation, the absorption of water. DMSO is hygroscopic, meaning that it has a tendency to absorb water from the air and increase the volume of liquid in the wells. This also changes the concentration of the media and drugs and therefore can skew data as well. We believe that when this does occur it's because the wells are absorption water from the humid air in the incubator and therefore the solution from above, to seal the plate better, should also work to fix this issue as well. Future work could also look at the best, most random way to distribute the samples onto the plate, ensure that with new method there are no significant differences between wells, and if there still is a difference research new methods of eliminating this factor.

Cell Growth

Human cells are more difficult to grow in culture than bacteria or yeast cells, although human breast cancer seems to grow fairly well. That being said there are still some issues that were encountered with the growing of the cancer cells. First the largest concern was contamination as it spreads rapidly in the incubator. Whenever this occurs all the cells must be thrown out, new cells taken from cold storage, and these cells must be given time to fully being dividing again. Therefore whenever this occurs it sets everyone's research back a week or so. During the time of this project this occurred once and the solution was to better sterilize the source of the contamination, which happened to be the warm water bath used to heat up media. Another issue with growing the cells is

that over time their growth seems to slow, which can slow down research as well. It is hypothesized in the lab that this decrease in growth is due to the cells picking up new mutations that caused them to die off, leaving only those without those mutations left. This changes the cell line over time and therefore they should be discarded and new cells taken from the freezer.

Autofluorescence of Compounds

All of the compounds used in this research had fluorescent properties of their own and this can interfere with the collection of data, since the raw data collected is intensity of fluorescence. The current solution to this is to test the compounds for autofluorescence and then subtract that value from all other values of intensity. This seems to be working okay, but can lead to issues such as negative values. In the future a new methodology could be used to properly evaluate these compounds without the use of fluorescence in data collection.

In addition to these problem in the research, there were also significant successes. Of the 12 synthesis reactions, 7 were successful and at large percent yields. These compounds will be available for future testing for many years. This project also documented the autofluorescence of these cyanostilbenes. Knowing beforehand that your compounds fluoresce could be very helpful to future researches. In addition to this the data collected is still valuable and can be strengthened by many more repetitions of this research. Future projects could create more cyanostilbene compounds, test the drugs created here on normal breast cells or other cancer cell types, optimization of the methods specifically for fluorescent compounds, and many more.

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Tables and Figures

#	Aldehyde Compound	Nitrile Compound	% Yield	Product Description
GC1	4-methoxybenzaldehyde	chlorophenylacetonitrile	68.0%	Yellow/white, powder, forms clumps
GC4	4-nitrobenzaldehyde	chlorophenylacetonitrile	63.5%	Dark, burnt orange, metallic
GC7	4-dimethylaminobenzaldehyde	chlorophenylacetonitrile	85.5%	Yellow, sticky
GC10	fluorene-2-carboxyaldehyde	chlorophenylacetonitrile	21.0%	Brown and yellow mix
GC13	3-formylphenylboronic acid	chlorophenylacetonitrile	120.5%	Milky white, powder, very fine
GC16	4-formylphenylboronic acid	chlorophenylacetonitrile	134.9%	Off white powder, granules
GC19	2-formylphenylboronic acid	chlorophenylacetonitrile	146.2%	White, large granule size, forms clumps
GC21	5-formyl-2-thienylboronic acid	chlorophenylacetonitrile	66.7%	Tan, powder, sand-like, forms clumps
GC23	4-methoxybenzaldehyde	2-thiopheneacetonitrile	68.5%	Tan, powder, fine, forms clumps
GC25	4-methoxybenzaldehyde	2-pyridineacetonitrile	84.4%	Reddish brown, fine, powder, forms flakes
GC27	4-methoxybenzaldehyde	4-nitrophenylacetonitrile	95.3%	Yellow/green, powder, fine, forms clumps
GC29	4-methoxybenzaldehyde	indole-3-acetonitrile	11.1%	Tan, forms flakes

Table 1. **Reagents, Percent Yields, and Product Descriptions.**

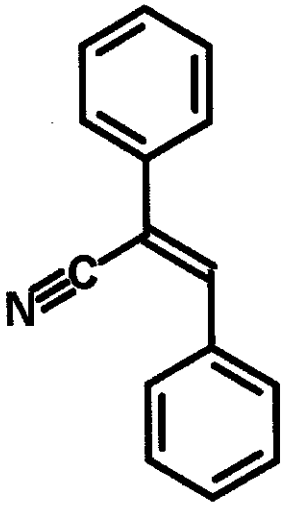


Figure 1. **α-Cyanostilbene Structure.** Two benzene rings connected by a double bond and on the double bond is a nitrile group. This structure allows for resonance and conjugation between both rings and the nitrile group. It's high resonance abilities give cyanostilbenes their fluorescent abilities and usefulness in optics.

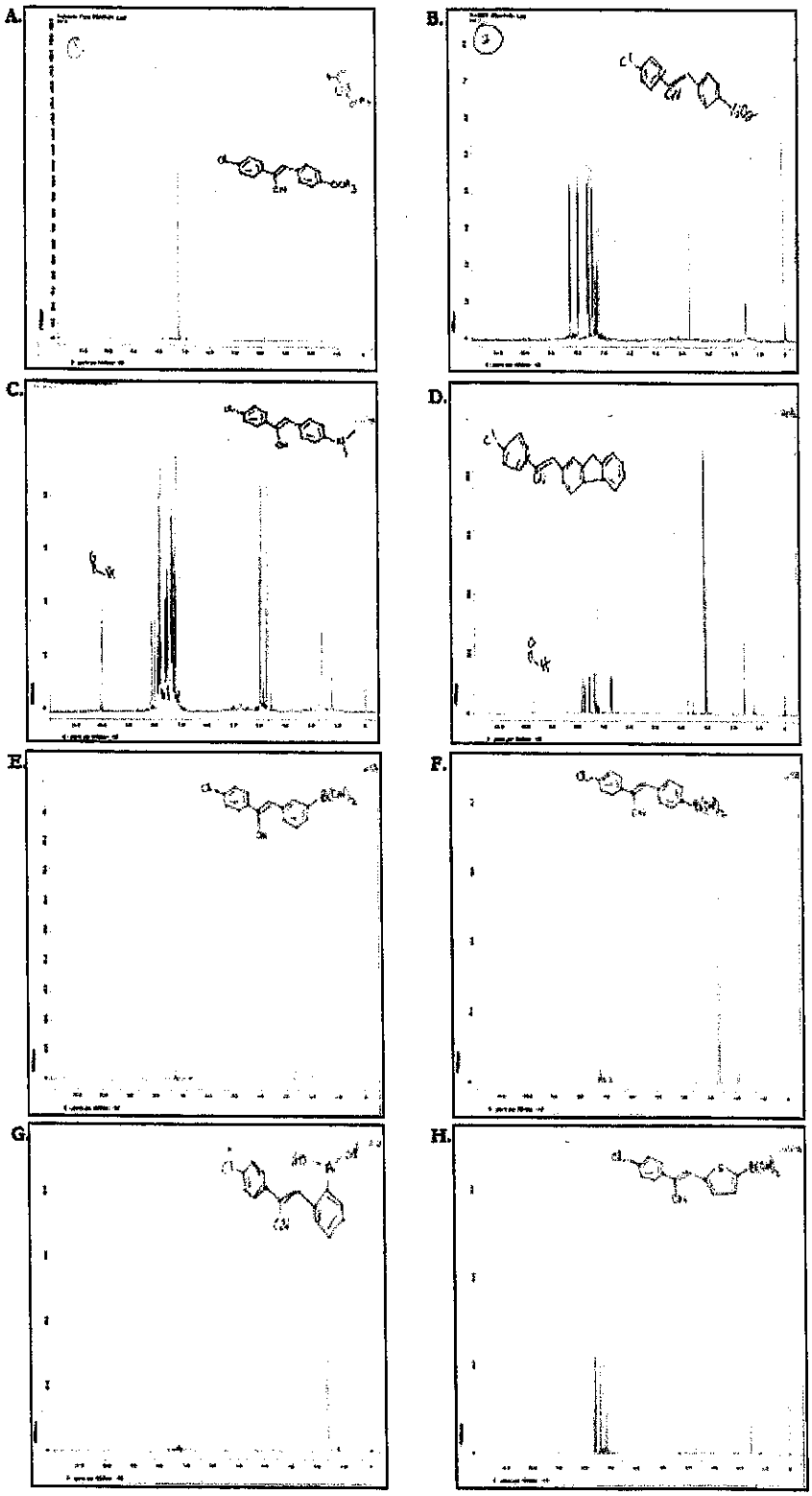


Figure 2. **NMR Spectra.** In NMR aldehydes peak around 9-10ppm. A) GC1. No aldehyde peak, reasonable other peaks, successful product formation, okay for testing. B) GC4. No aldehyde peak, reasonable other peaks, successful product formation, okay for testing. C) GC7. Aldehyde peak between 9-10, product not formed, eliminated from testing. D) GC10. Aldehyde peak between 9-10,

product not formed, eliminated from testing. E) GC13. No aldehyde peak, other peaks not what was expected, inconclusive, eliminated from testing. F) GC16. No aldehyde peak, reasonable other peaks, successful product formation, okay for testing. G) GC19. No aldehyde peak, other peaks not what was expected, inconclusive, eliminated from testing. H) GC21. No aldehyde peak, reasonable other peaks, successful product formation, okay for testing.

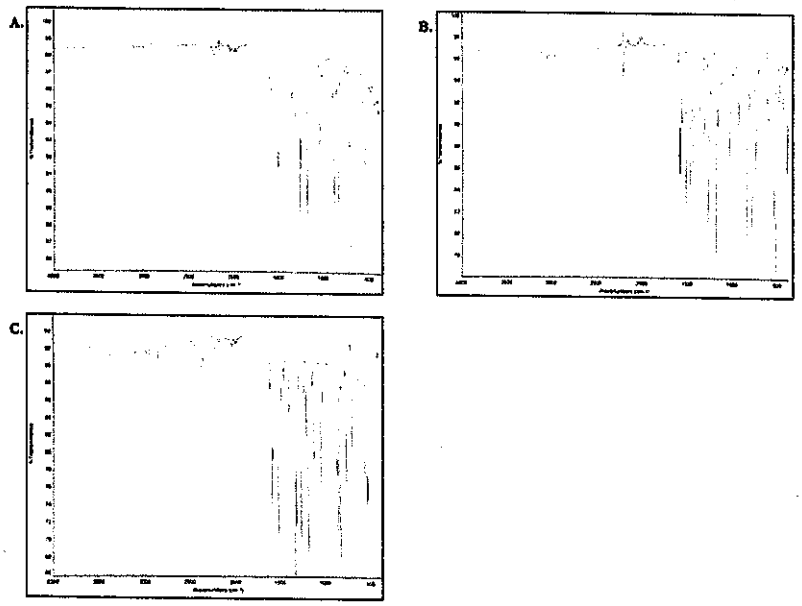


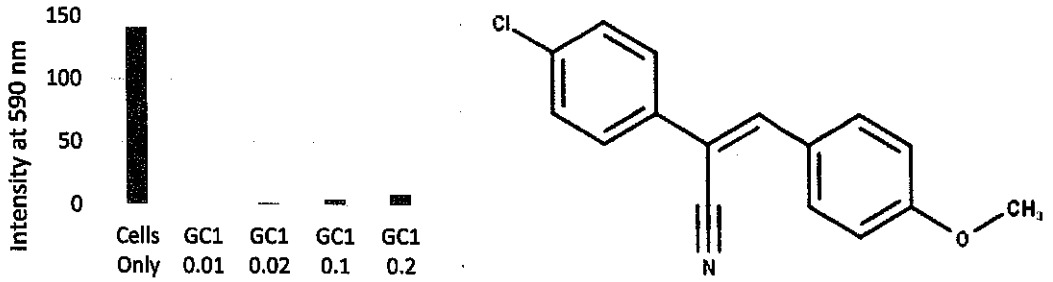
Figure 3. **IR Spectra.** In IR aldehydes the c double bond to oxygen peaks around 1740-1720 intensity. A) GC23. No aldehyde peak, reasonable other peaks, successful product formation, okay for testing. B) GC25. No aldehyde peak, reasonable other peaks, successful product formation, okay for testing. C) GC27. No aldehyde peak, reasonable other peaks, successful product formation, okay for testing.

	Plate 1 - 3/2/2017			Plate 2 - 3/17/2017			Average	STDV
Cells Only	229.289	214.965	219.227	85.127	94.289	90.950	155.641	71.983
GC1 0.01	95.777	102.984	88.649	11.119	13.673	15.482	54.614	45.369
GC1 0.02	92.203	100.450	96.213	4.897	4.878	4.614	50.542	50.180
GC1 0.1	6.786	6.670	6.416	74.677	73.181	72.553	40.047	36.620
GC1 0.2	32.864	29.573	30.734	85.464	79.471	76.677	55.797	27.270
GC4 0.01	150.841	160.273	159.252	8.264	7.092	9.297	82.503	81.445
GC4 0.02	131.160	133.340	155.662	5.250	5.167	4.907	72.581	74.409
GC4 0.1	8.104	8.078	7.999	63.802	74.943	62.492	37.570	32.614
GC4 0.2	50.360	53.076	57.114	76.490	77.610	70.199	64.142	12.103
GC16 0.01	122.355	129.797	124.588	21.701	24.773	26.062	74.879	55.610
GC16 0.02	107.824	115.561	107.198	7.302	8.967	5.761	58.769	56.420
GC16 0.1	6.743	7.072	6.137	78.383	77.159	54.670	38.361	35.749
GC16 0.2	19.932	22.561	21.717	81.355	91.664	90.204	54.572	36.515
GC21 0.01	109.368	116.049	100.035	16.374	17.566	20.293	63.281	49.794
GC21 0.02	86.650	88.267	84.614	7.009	5.310	5.862	46.285	44.083
GC21 0.1	11.029	9.706	8.359	75.961	77.366	77.902	43.387	36.920
GC21 0.2	47.624	46.720	55.759	89.510	83.856	86.763	68.372	20.412
	Plate 3 - 3/30/2017 - 1			Plate 4 - 3/30/2017 - 2			Average	STDV
Cells Only	48.215	44.793	35.756	41.620	44.685	42.655	42.954	4.188804
GC23 0.01	49.714	51.105	52.027	6.822	7.672	6.981	29.053	23.99808
GC23 0.02	53.152	51.987	52.861	41.544	41.294	41.754	47.099	6.113187
GC23 0.1	45.898	48.028	46.194	54.214	54.495	53.949	50.463	4.182469
GC23 0.2	6.840	7.002	7.082	52.864	53.392	52.461	29.940	25.1593
GC25 0.01	55.522	52.797	51.791	7.104	7.384	7.157	30.292	25.30962
GC25 0.02	49.440	49.904	48.433	40.412	42.904	41.044	45.356	4.378883
GC25 0.1	47.058	45.814	44.325	56.489	49.775	50.308	48.962	4.339715
GC25 0.2	7.262	6.602	6.628	52.181	48.927	52.109	28.951	24.2616
GC27 0.01	55.381	58.341	56.585	7.579	7.996	9.970	32.642	26.45899
GC27 0.02	50.966	50.162	47.088	32.269	38.921	33.806	42.202	8.294163
GC27 0.1	42.024	48.730	44.686	47.536	50.807	40.623	45.734	3.97353
GC27 0.2	6.432	6.311	6.578	42.066	55.471	51.651	28.085	24.10955

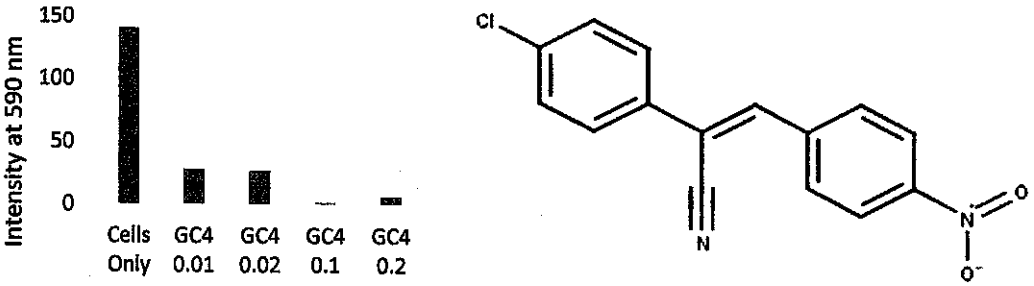
Figure 4. **Raw Data for Compounds.** This is the data that the fluorometer directly provides and the units are intensity at a wavelength of 590. The averages of the 3 wells are given as well as the percent of control. The highlighted values indict a different use of data. The control for that plate was placed on the edge wells, which led to a severe edge effect and essentially no control values. The values given were on the same plate but of cells treated with 1% DMSO. Previous studies in our lab have shown that 1% DMSO has no effect on cell viability and is non-toxic. Therefore using these values is reasonable and more accurate than using the actual control values.

	Plate 1 - 3/2/2017			Plate 2 - 3/17/2017			Average	STDV	T-Test
Cells Only	215.096	200.771	205.034	70.934	80.096	76.757	141.448	71.983	
GC1 0.01	0.000	4.110	0.000	0.000	0.000	0.000	0.685	1.678	0.004738
GC1 0.02	0.000	5.962	1.725	0.000	0.000	0.000	1.281	2.395	0.004655
GC1 0.1	0.000	0.000	0.000	10.516	9.020	8.392	4.655	5.145	0.007384
GC1 0.2	0.000	0.000	0.000	21.859	15.866	13.072	8.466	9.699	0.010287
GC4 0.01	50.095	59.528	58.507	0.000	0.000	0.000	28.022	30.870	0.001179
GC4 0.02	45.072	47.252	69.574	0.000	0.000	0.000	26.983	30.778	0.001366
GC4 0.1	0.000	0.000	0.000	0.320	11.461	0.000	1.964	4.655	0.005789
GC4 0.2	0.000	0.000	0.000	15.068	16.188	8.777	6.672	7.733	0.008804
GC16 0.01	29.555	36.998	31.789	0.000	0.000	0.000	16.390	18.116	0.002436
GC16 0.02	27.713	35.449	27.086	0.000	0.000	0.000	15.041	16.738	0.002614
GC16 0.1	0.000	0.000	0.000	1.630	0.407	0.000	0.339	0.653	0.004989
GC16 0.2	0.000	0.000	0.000	20.438	30.747	29.287	13.412	15.110	0.015192
GC21 0.01	36.532	43.213	27.199	0.000	0.000	0.000	17.824	20.177	0.002271
GC21 0.02	11.491	13.108	9.454	0.000	0.000	0.000	5.675	6.324	0.003923
GC21 0.1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004827
GC21 0.2	0.000	0.000	0.000	33.567	27.912	30.820	15.383	16.946	0.017761
	Plate 3 - 3/30/2017 - 1			Plate 4 - 3/30/2017 - 2			Average	STDV	T-Test
Cells Only	34.022	30.599	21.563	27.427	30.492	28.462	28.761	4.188804	
GC23 0.01	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.36E-05
GC23 0.02	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.36E-05
GC23 0.1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.36E-05
GC23 0.2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.36E-05
GC25 0.01	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.36E-05
GC25 0.02	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.36E-05
GC25 0.1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.36E-05
GC25 0.2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.36E-05
GC27 0.01	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.36E-05
GC27 0.02	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.36E-05
GC27 0.1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.36E-05
GC27 0.2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.36E-05

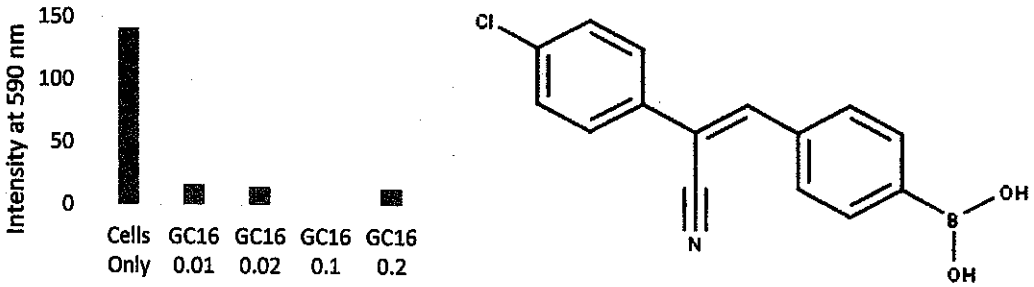
Figure 5. **Data Corrected for Autofluorescence.** The compounds had significant fluorescence on their own which can interfere with the data. The intensity of the autofluorescence was subtracted from the values in Figure 4. A T-Test was also performed comparing each drug concentration to the control values. While the p values are all significant, this should be considered carefully since the n values were rather low. Also in most cases the values were negative, but 0 was reported since negative intensity is not possible.



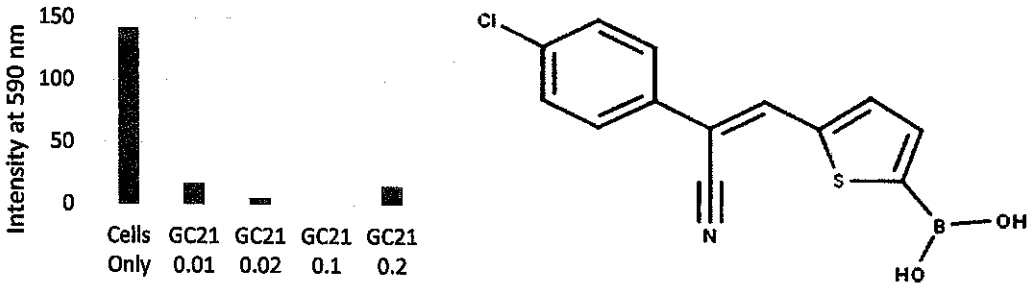
A)


Intensity at 590 nm
150
100
50
0
Cells Only
GC4 0.01
GC4 0.02
GC4 0.1
GC4 0.2
Cl
N
N
O
O⁻

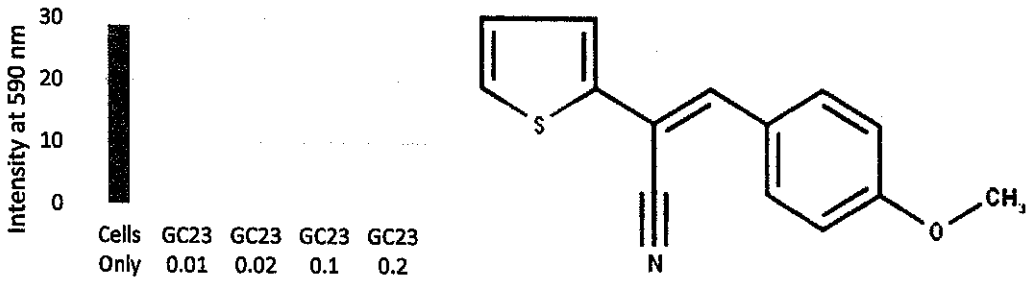
B)


Intensity at 590 nm
150
100
50
0
Cells Only
GC16 0.01
GC16 0.02
GC16 0.1
GC16 0.2
Cl
N
B
OH
OH

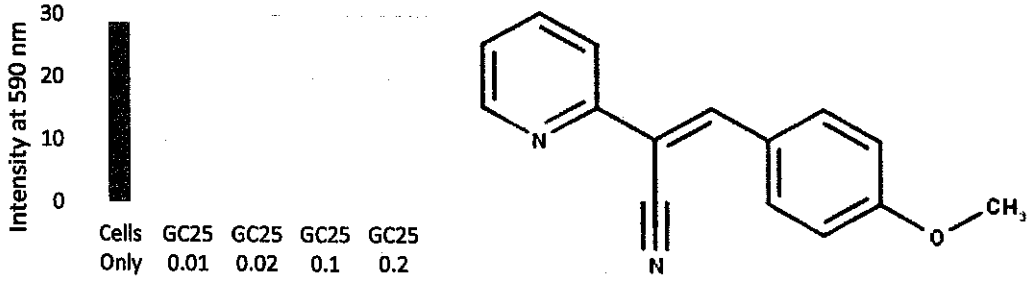
C)


Intensity at 590 nm
150
100
50
0
Cells Only
GC21 0.01
GC21 0.02
GC21 0.1
GC21 0.2
Cl
N
S
B
OH
HO

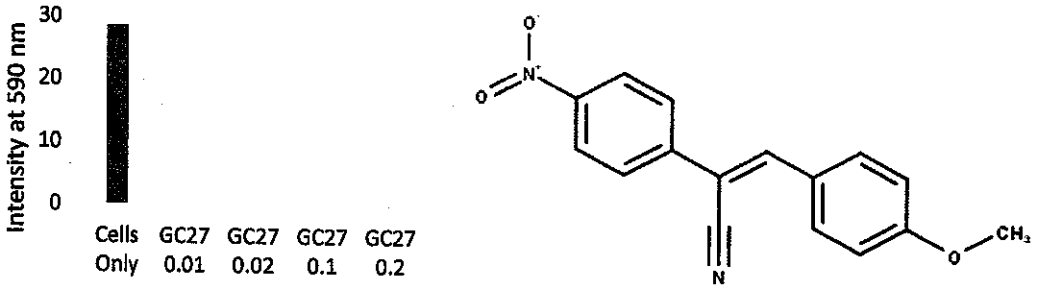
D)


Intensity at 590 nm
30
20
10
0
Cells Only
GC23 0.01
GC23 0.02
GC23 0.1
GC23 0.2
S
N
O
CH₃

E)


Intensity at 590 nm
30
20
10
0
Cells Only
GC25 0.01
GC25 0.02
GC25 0.1
GC25 0.2
N
N
O
CH₃

F)



G)

Figure 6. **Graphs and Structures of each Compound Tested.** The values used to make the graphs are the corrected values from Figure 5. Since the autofluorescence creates large differences between the control and the drug concentrations, the p values that will follow are significant even though the data gives us little confidence in those numbers. Until the n numbers can be increased or the autofluorescence dealt with the information given in these graphs should be taken lightly. A) GC1 0.01 p= 0.004738, GC1 0.02 p= 0.004655, GC1 0.1 p= 0.007384, GC1 0.2 p= 0.010287. B) GC4 0.01 p=0.00117881, GC4 0.02 p=0.001365713, GC4 0.1 p=0.005788996, GC4 0.2 p=0.008804332. C) GC16 0.01 p=0.002435571, GC16 0.02 p=0.002614341, GC16 0.1 p=0.004989021, GC16 0.2 p=0.015192314. D) GC21 0.01 p=0.002270646, GC21 0.02 p=0.003922644, GC21 0.1 p=0.00482686, GC21 0.2 p=0.017760543. E) GC23 0.01 p=1.35851E-05, GC23 0.02 p=1.35851E-05, GC23 0.1 p=1.35851E-05, GC23 0.2 p=1.35851E-05. F) GC25 0.01 p=1.35851E-05, GC25 0.02 p=1.35851E-05, GC25 0.1 p=1.35851E-05, GC25 0.2 p=1.35851E-05. G) GC27 0.01 p=1.35851E-05, GC27 0.02 p=1.35851E-05, GC27 0.1 p=1.35851E-05, GC27 0.2 p=1.35851E-05.

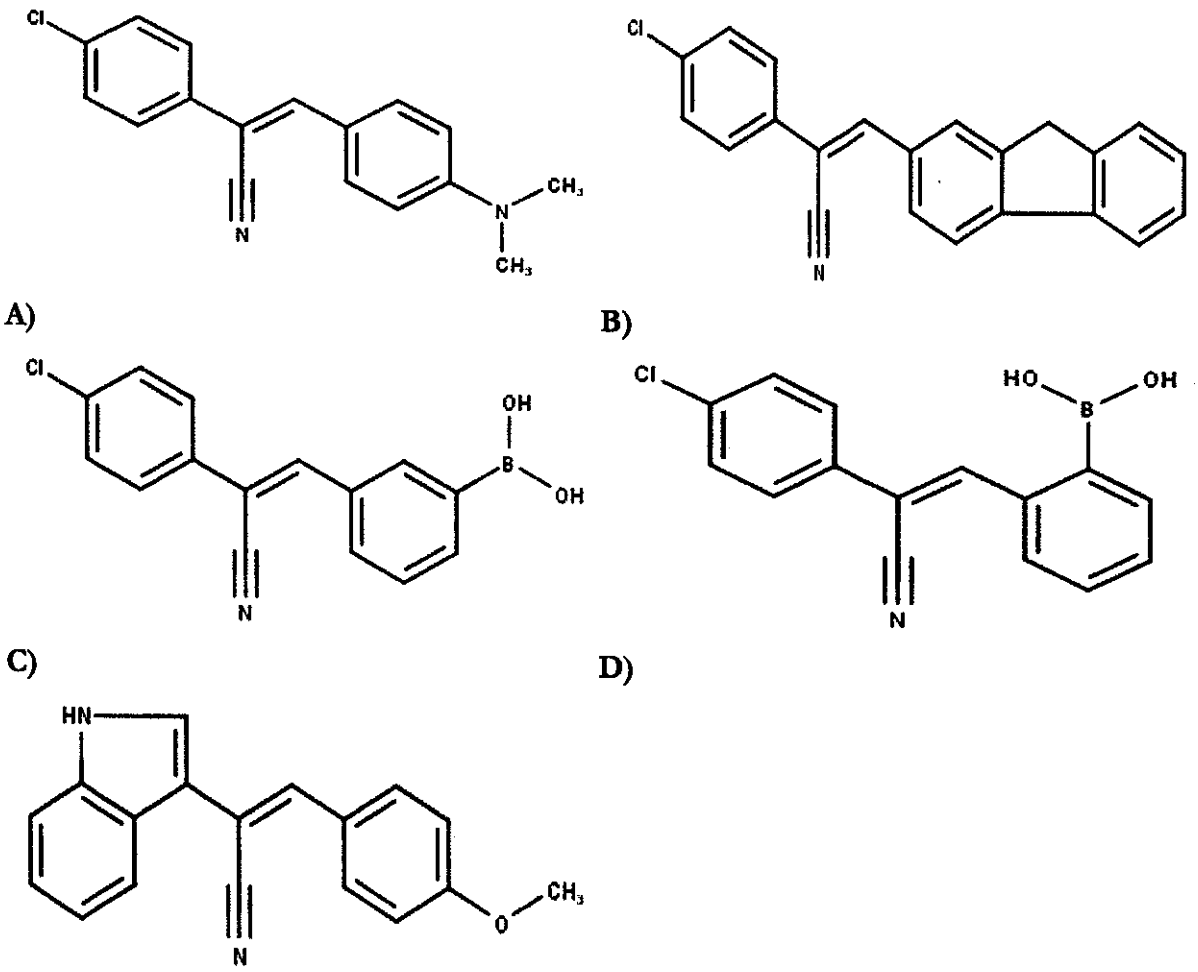


Figure 7. **Structures of Compounds not Successfully Created.** These are the structures of the compounds that were attempted to be created but were eliminated from testing. A) GC7 B) GC10 C) GC13 D) GC19 E) GC29