Investigation of Microbial Extracts as a Source of Neurological Agents

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Neurological Disorders

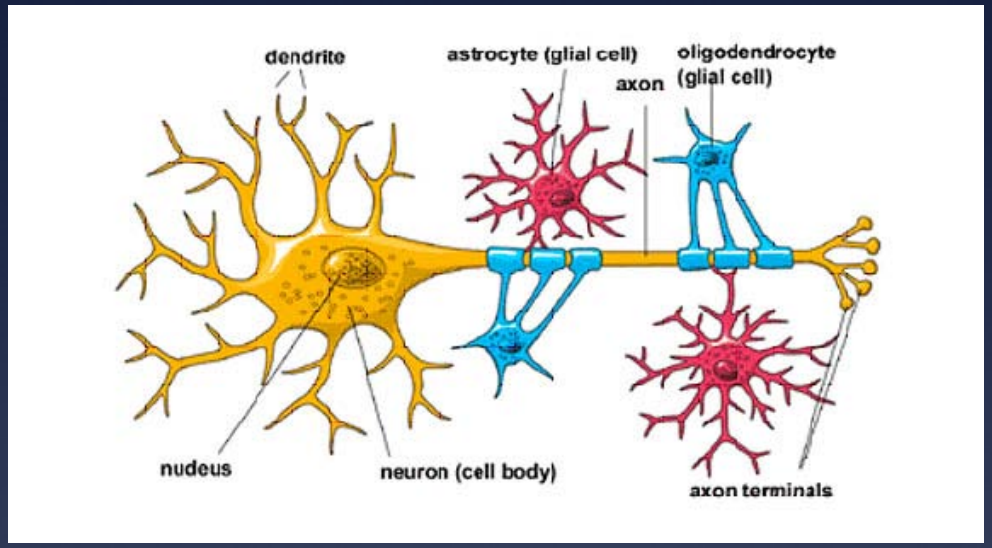
- **Neurological disorder**: irregular conditions of the brain and spinal cord
- Symptoms: memory loss, coordination problems, pain, heart conditions, etc.
- Developed by diseases (Alzheimer's), illnesses (depression) or physical trauma
- Disease prevalence is increasing, few cures, research needed

Estimated Prevalence of Diseases and Illnesses

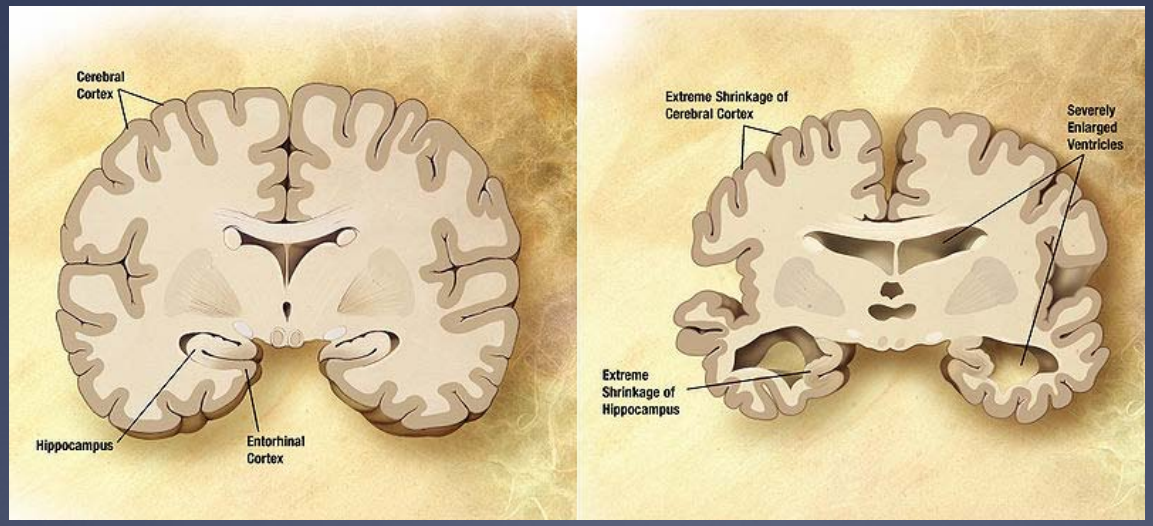
Disease/Illness	U.S. (millions)	Worldwide
Alzheimer's Disease	4.5	15
Mild Cognitive Impairment	12	76
Attention Disorders	15	260
Insomnia	67	238
Depression	19	350
Bipolar Disorder	2.8	30
Anxiety (OCD, GAD, PSTD)	19.1	490
Chronic Pain	86	290
Hearing Loss/Deafness	35	140
Epilepsy and Seizure	2.5	50
Blindness	3.3	45
PD	1.5	4
MS	0.4	2.5
Vision (AMD)	15	35
Schizophrenia and Psychosis	2.2	25
Alcohol Addiction	6	75

Data compiled in 2000 by NIMH, WHO, and UN

Neuron/Gilal Cell Diagram



Loss of Tissue from Neurological Disorder

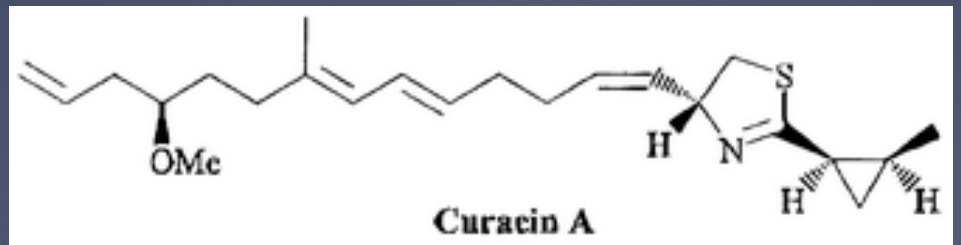


Natural Product Research

- **Natural Products:** any compound produced by living organisms
- Have shown high potential as lead compounds for drug discovery and drug design projects
  - From 1981-2006, 63% of newly developed drugs were derivatives of natural products (Newman & Craig, 2006)
- Despite the high success, a small percentage of natural products have been studied

Marine Organisms as Natural Product Source

- Marine organisms have a wealth of biologically potent products
- Ex: *Lyngbya majuscula*
  - Cyanobacteria off the shores of Curacao
  - Produces curacin A, a potent neurotoxin
  - Curacin A has a unique thiazoline and cyclopropyl ring, has shown anti-tumor activity
- Many products from marine organisms haven't been studied yet



Curacin A

Collection of Marine Organisms

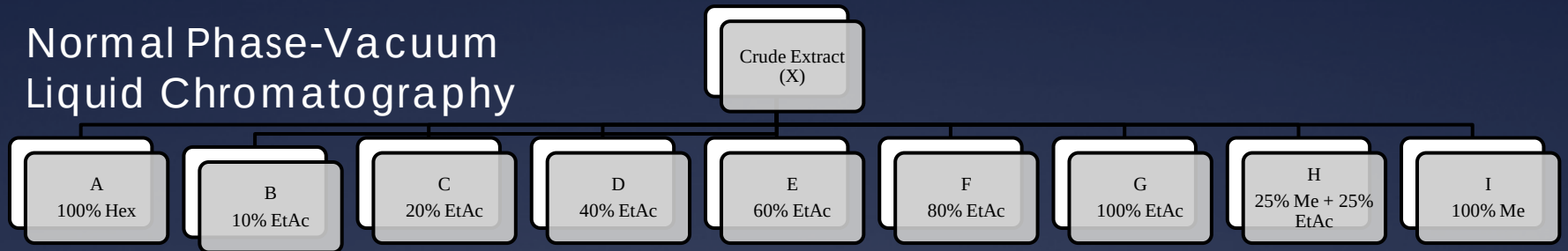
- In 2007, Dr. Kerry McPhail traveled to the Red Sea and South Africa to collect marine organisms that may contain neurotoxins
- Red Sea, collected cyanobacteria
  - Salt water inlet between Africa and Asia
  - High salt concentration, high pH
  - Unique cyanobacteria thrive in these conditions
- Algoa Bay (South Africa), collected tunicates
  - Large coral reef provides diverse ecosystem
  - Contains a high amount of tunicates
  - Tunicates lack research compared to other filter-feeding organisms (sponges, coral) but show promise (Aplidin)

Natural Product Purification

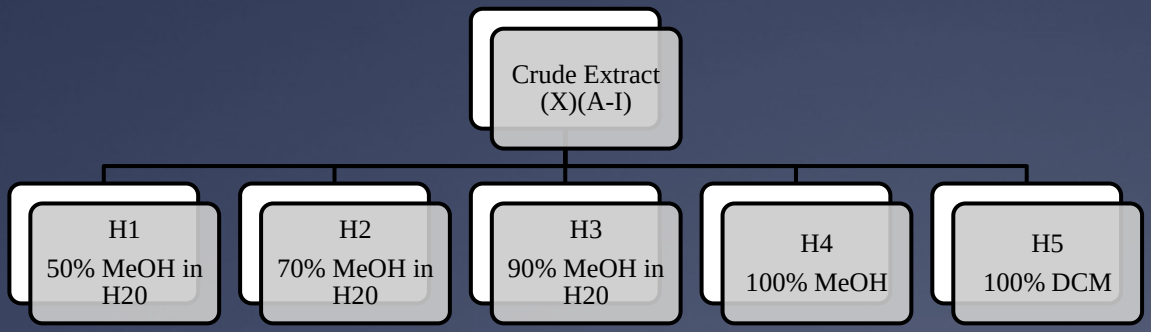
- Products contain multiple compound structures, generally only specific components of the product has activity
- Dr. McPhail separated (fractionated) the crude products in order to identify the most active components
- Her laboratory used a specific chromatography flow chart with different techniques to fractionate the product

Fractionation Flow Chart

Normal Phase-Vacuum Liquid Chromatography



Increasing solvent polarity for Normal Phase VLC



Increasing solvent polarity for Reverse Phase SPE

Reverse Phase Solid Phase Extraction

H=Hexanes,
EtAc=Ethyl Acetate
MeOH=Methanol,
DCM=Dichloromethane

Investigation Overview

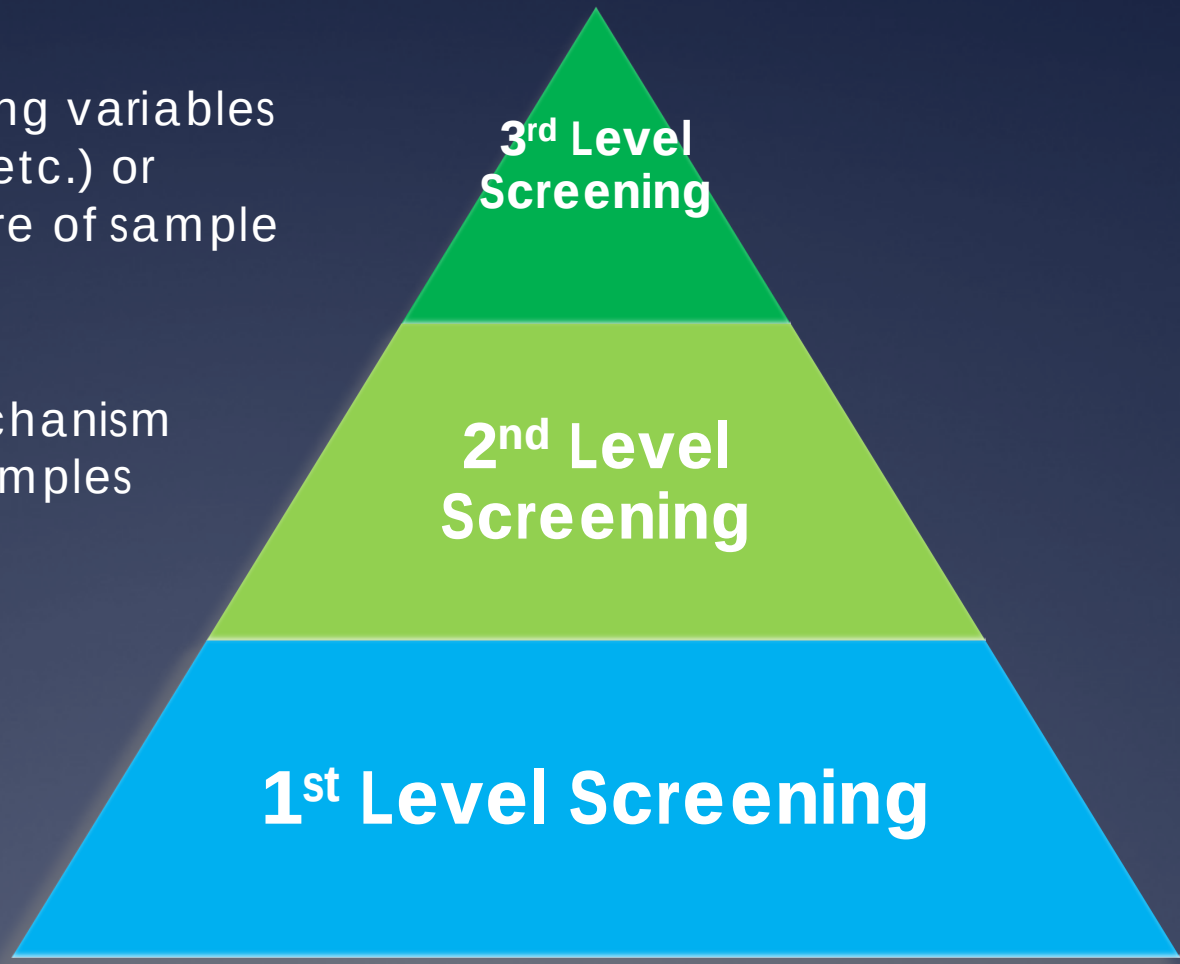
- I received crude and fractionated samples of these products from Dr. McPhail
- **My initial goal was to first screen these crude and fractionated samples for cytotoxicity**
- The highest cytotoxic samples have the most potential for biological activity
- We can do two things with these results:
  - Fractionate the compound to pursue isolating a more active component
  - Run different screenings to understand specific cytotoxic mechanisms

Overview of Drug Discovery Process

Mechanism is understood, testing variables (concentration, time, cell line, etc.) or analyzing the chemical structure of sample

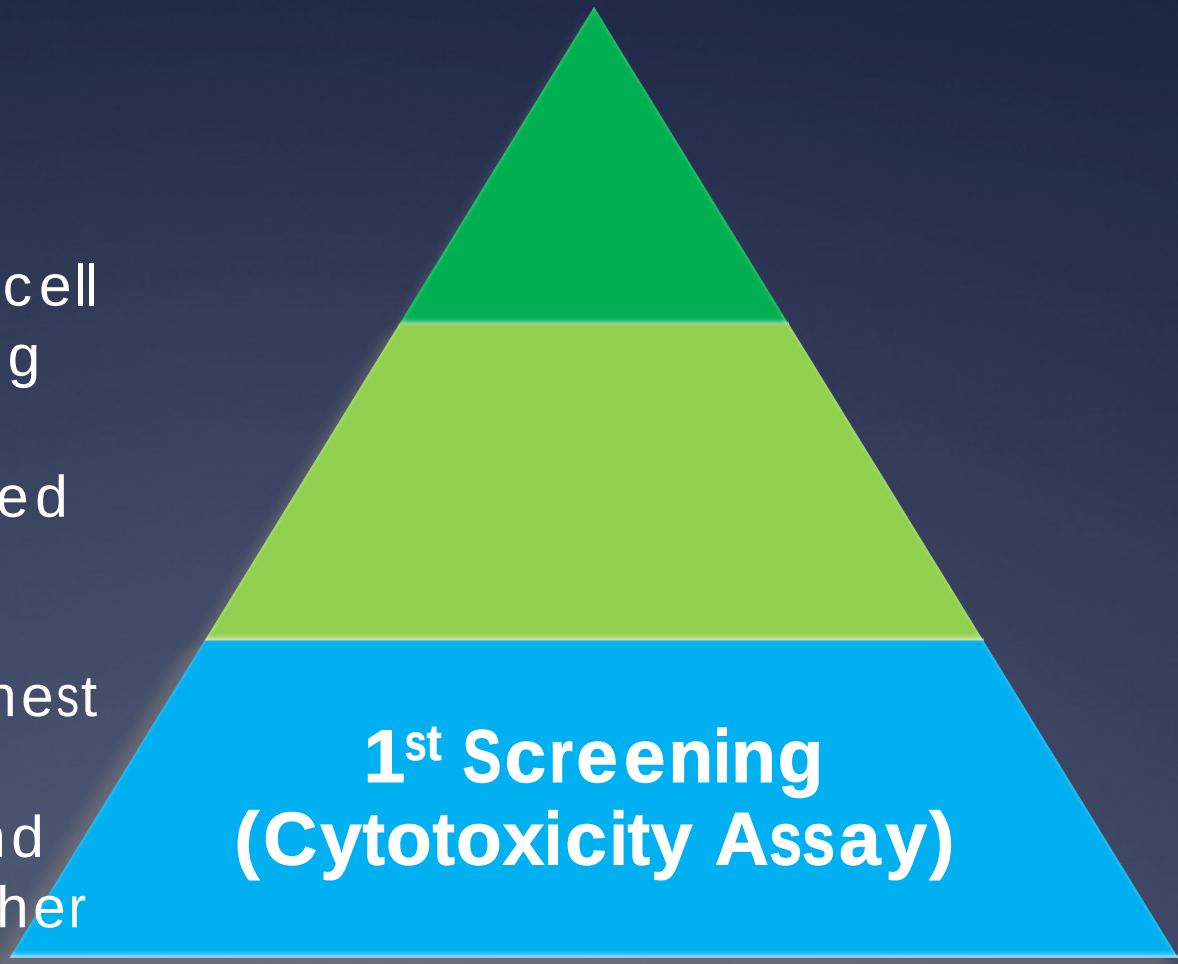
Run tests that help explain mechanism of toxicity in the most active samples

Initial testing for products, find most active samples, disregarding mechanisms



Cytotoxicity Assay

- Identify cytotoxic affects
- Observe changes in cell viability by comparing cells treated with extracts with untreated cells
- Extracts showing highest cytotoxicy suggest biological activity and should be tested further

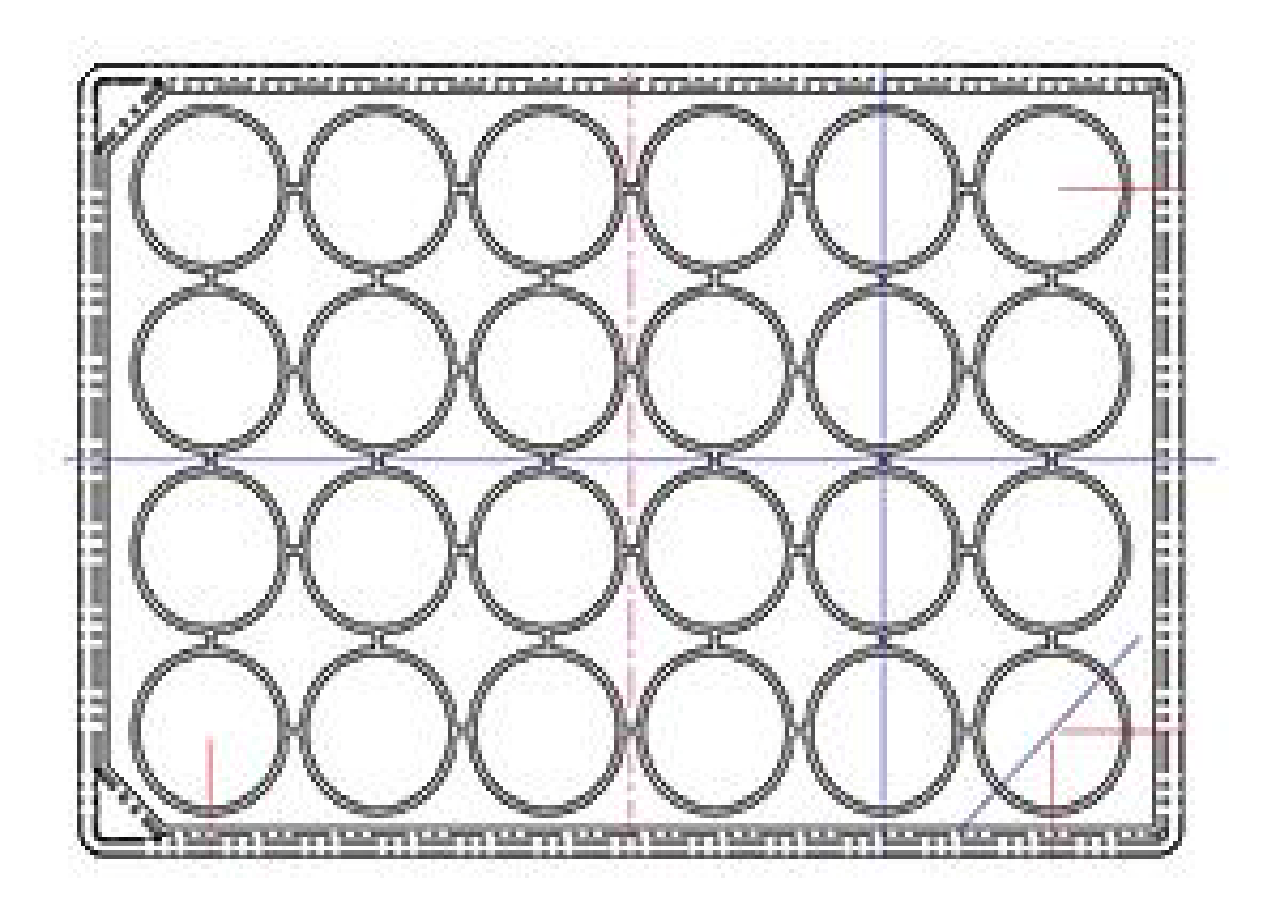


Cytotoxicity Assay Procedure

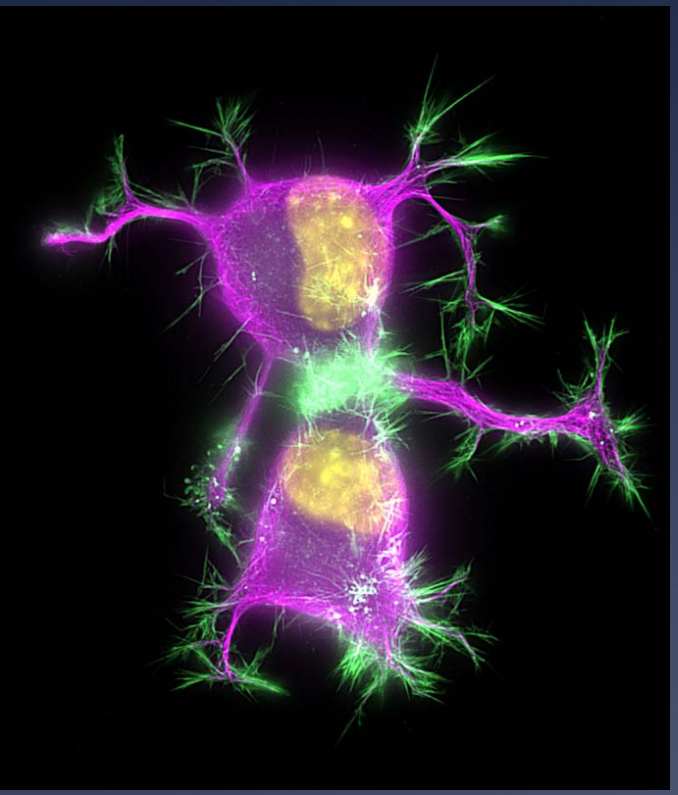
1. Seeded Neuro-2a cells at 450,000 cells per well on 24 well plates and incubated overnight
2. Added samples dissolved in DMSO at 30 ug/mL to wells in quadruplicates, incubate for 24 hour period
3. Quantify cell viability with MTT assay
4. Normalize the data by comparing treated wells to control wells

Cytotoxicity Layout

Control Extract 1 Extract 2 Extract 3 Extract 4 Extract 5



Neuro-2A as a Cell Line Model

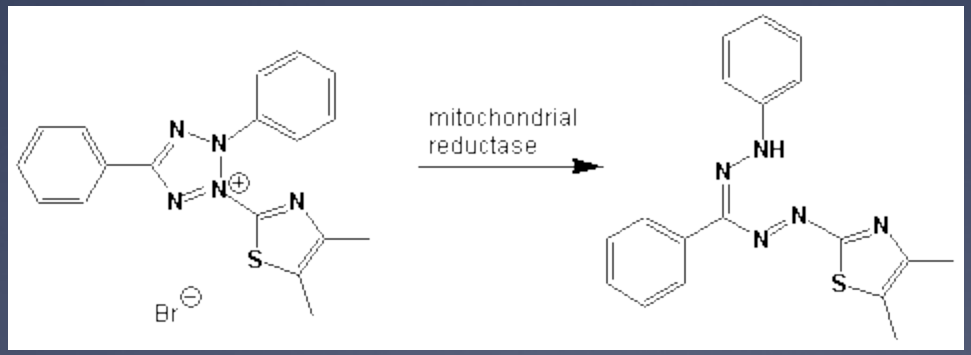


- Neuroblastoma cell line from mice tumors in CNS
- These cells are useful as a model to discover lead compounds for voltage-gated sodium channel activity (2nd Screening)
- High voltage-gated sodium channel density, similar to neurons
- Some medications that treat epilepsy and cardiac arrhythmias act on voltage-gated sodium channels

MTT Assay

- Colorimetric assay to quantify cell viability
- Active mitochondria reduce yellow MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide from a yellow tetrazole to a purple formazan in living cells
- This purple formazan can be quantified by a spectrophotometer and cell viability is determined

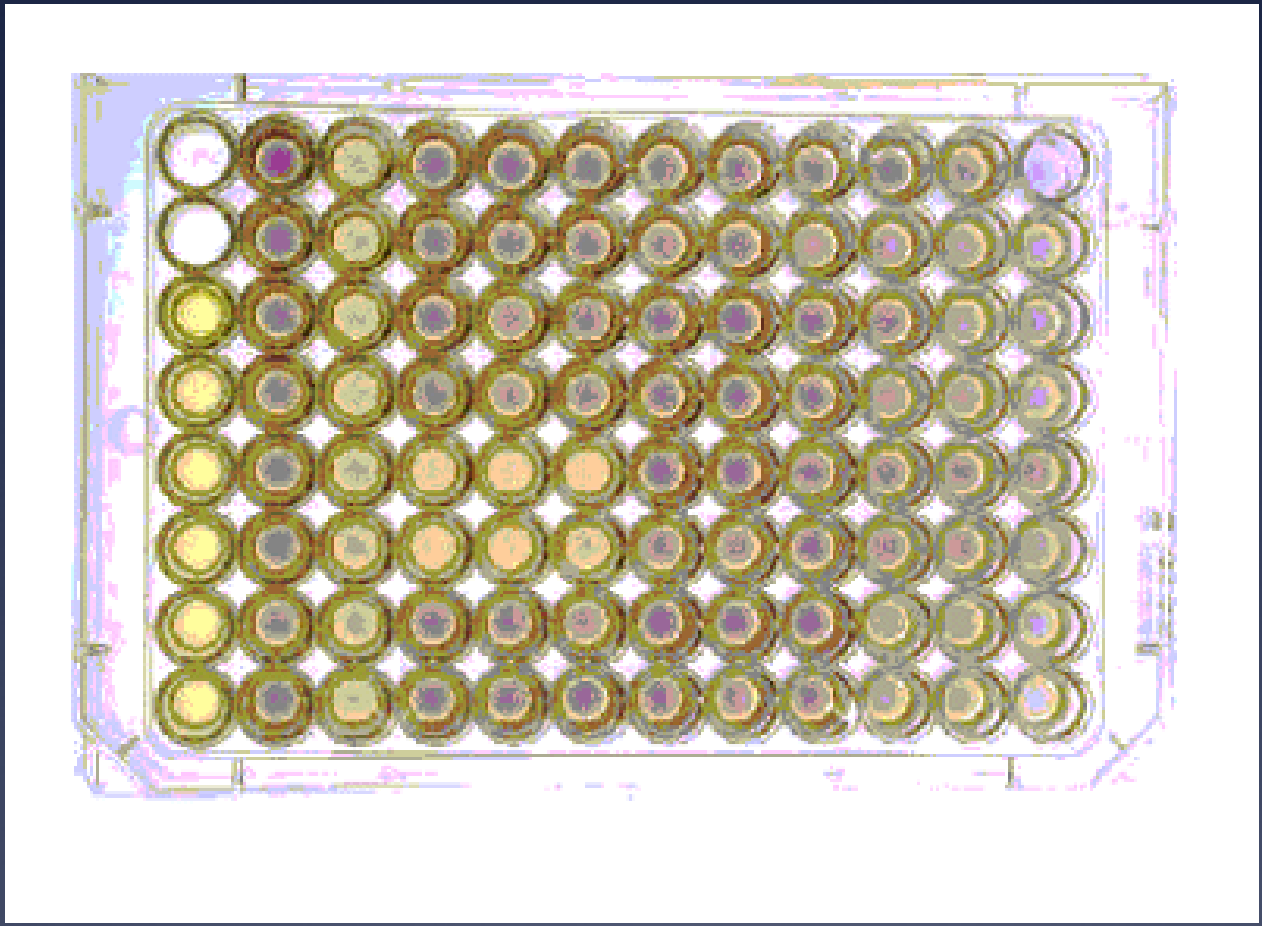
Yellow MTT Purple Formazan



MTT Assay Procedure

- After 24 hour treatment with samples, yellow MTT dissolved in PBS was added to each well at a 1:10 mL ratio
- The wells are left for a 45 min incubation period which living cells reduce yellow MTT
- Formazans are dissolved in acid isopropanol to make the amount of purple formazan quantifiable via spectrophotometer
- Absorbance is measured on spectrophotometer at 570 nm

Plate Exposed to Yellow MTT



96 well plate after 45 minute treatment of yellow MTT

Data Analysis

- After collecting absorbance measurements, the data was normalized and reflected with percentages
  - (avg abs of treated wells/avg abs of untreated wells)*100%
- Dr. Doug Goeger ran a similar cytotoxicity protocol and provided a scale he used to help identify the potency of the samples
- In terms of cell viability normalized by the control wells
  - 100%-86% = no activity
  - 85%-76% = very slight activity
  - 75%-66% = slight activity
  - 65%-56% = moderate activity
  - 55%-0% = strong activity

Cytotoxicity Results Outline

Red Sea

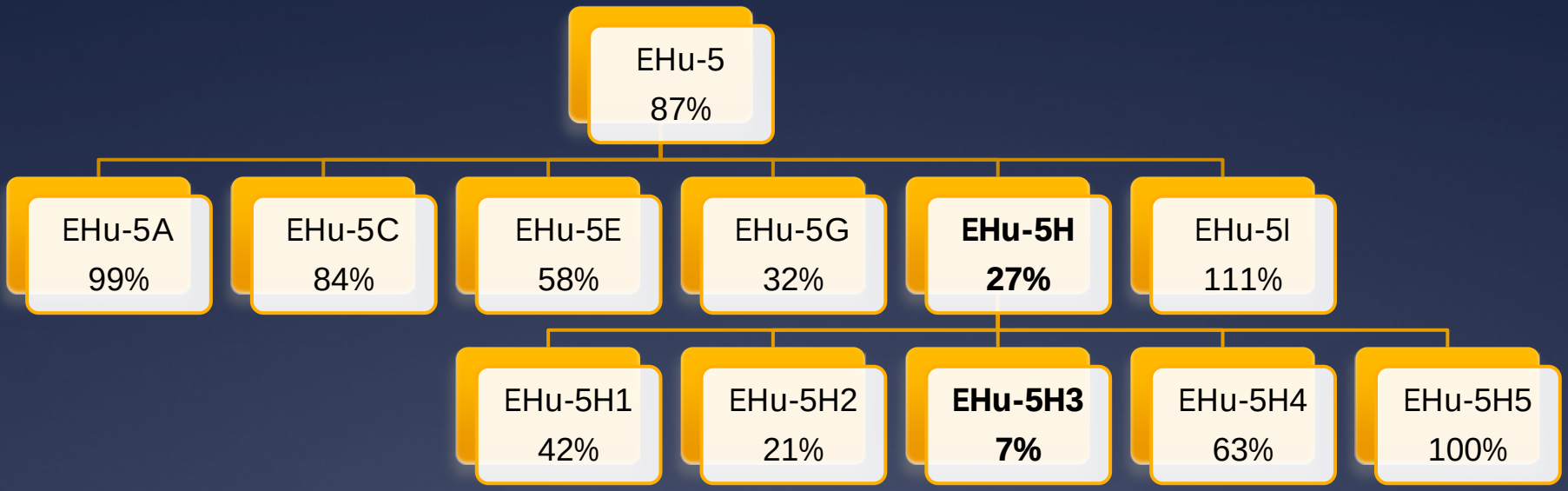
- Ehu-5(A-I)
- Ehu-5H-(1-5)
- Ehu-4H-4(A-G)*
- Ehu-1(A-I)

South Africa

- Priority Samples
- Non-Priority Samples

*A-G were fractionated with NP VLC

Ehu-5 Cell Viability Results



The 5H3 fraction was most active at 7%

Ehu-5H3, Ehu-5H2, Ehu-5H1, Ehu-5G, Ehu-5H all displayed strong cytotoxic activity

The Ehu-5 family was the easiest to track of all the red sea samples

Red Sea Ehu-4H-4(A-G)

Sample Code	Absorbance Average	Standard Deviation	Trials	Standard Error	Control Absorbance	Cell Viability
Ehu-4H-4	0.177	0.0190	4	0.00475	0.224	78.79%
Ehu-4H-4A	0.258	0.0220	4	0.00550	0.224	114.96
Ehu-4H-4B	**0.055**	**0.0750**	**4**	**0.0188**	**0.224**	**24.33%**
Ehu-4H-4C	0.191	0.0180	4	0.00450	0.230	83.04%
Ehu-4H-4D	0.222	0.0220	4	0.00550	0.230	96.41%
Ehu-4H-4E	0.193	0.0270	4	0.00675	0.230	84.02%
Ehu-4H-4F	0.260	0.0250	4	0.00625	0.230	112.93%
Ehu-4H-4G	0.253	0.0370	4	0.00925	0.230	109.89%

Ehu-4H-4, Ehu-4H-4C, and Ehu-4H-4E were all identified as very slightly active
Ehu-4H-4B was identified as strongly active

Red Sea Ehu-1(A-I)

Sample Code	Absorbance Average	Standard Deviation	Trials	Standard Error	Control Absorbance	Cell Viability
Ehu-1A	0.234	0.0150	4	0.00375	0.265	88.2%
Ehu-1C	0.238	0.0230	4	0.00575	0.265	89.5%
EHu-1E	0.210	0.0250	4	0.00625	0.265	79.1%
Ehu-1G	0.231	0.0410	4	0.0103	0.265	86.9%
Ehu-1H	0.192	0.00500	4	0.00125	0.265	72.5%
Ehu-1I	0.235	0.0170	4	0.00425	0.224	105%

Ehu-1E was identified as very slightly active
Ehu-1H was identified as slightly active

South Africa Priority Samples

Sample Code	Absorbance Average	Standard Deviation	Trials	Standard Error	Control Absorbance	Cell Viability
SAF04-18	0.189	0.0460	4	0.0115	0.237	79.6%
SAF04-19	0.148	0.0490	4	0.0123	0.237	62.2%
SAF04-23	0.245	0.0190	4	0.00475	0.243	101%
SAF04-30	0.210	0.0190	4	0.00475	0.214	98.4%
SAF04-53	0.201	0.0210	4	0.00525	0.186	108%
SAF04-55	**0.005**	**0.00100**	**4**	**0.000250**	**0.186**	**2.42%**
SAF04-60	0.237	0.0160	4	0.00400	0.223	106%
SAF04-62	0.134	0.0220	4	0.00550	0.223	60.1%
SAF04-65	0.279	0.0280	4	0.00700	0.198	14.0%

SAF04-55 was the most exciting discovery of the trial, with viability at 2%
SAF04-65 was also surprising by increasing viability by a significant margin (41%)

South Africa Non-Priority

Sample Code	Absorbance Average	Standard Deviation	Total Trials	Standard Error	Control	Cell Viability
SAF04-59	0.188	0.0290	4	0.00725	0.223	84.1%
SAF04-70	0.194	0.0120	4	0.00300	0.236	82.2%
SAF04-71	0.177	0.0230	4	0.00575	0.236	74.9%

There were 23 non-priority SAF samples, only 3 had at least very slight activity

Cytotoxicity Activity Summary

Very Slight	Slight	Moderate	Strong
Ehu-5C (84.0%)	Ehu-4G-4 (74.4%)	Ehu-5E (58.3%)	Ehu-05/27/07 (21.3%)
Ehu-4H-4 (78.8%)	Ehu-1H (72.5%)	Ehu-5H-4 (62.9%)	Ehu-5G (31.7%)
Ehu-4H-4C (83.0%)	SAF04-71 (74.9%)	SAF04-19 (62.2%)	Ehu-5H (26.6%)
Ehu-4H-4E (84.0%)		SAF04-62 (60.1%)	Ehu-5H-1 (41.8%)
Ehu-1E (79.1%)			Ehu-5H-2 (20.8%)
SAF04-18 (79.5%)			Ehu-5H-3 (7.21%)
SAF04-59 (84.1%)			Ehu-4H-4B (24.3%)
SAF04-70 (82.2%)			SAF04-55 (2.42%)

23 total extracts were identified as having some activity

Optimization of Cytotoxicity Assay

- After being comfortable working with 24 well plates, we optimized the protocol with the aid of a multi-channel pipette
- This capability allows trials on smaller 96 well plates
- Benefits:
  - Conserves limited supplies
  - More extracts can be tested on a single plate
  - Improved accuracy in data by having more trials

Most Important Optimization Benefit

- The increase in wells allows us to **include entire families of fractionated samples, including the parent, on a single plate**
- It's difficult to keep all plates uniform with equal amounts of cells
- The accuracy of tracking activity for a family should improve
- Dr. McPhail provided additional samples for the optimization as well as a suggestion of following up on a few samples

SAF04-30(A-I)

Sample Code	Absorbance Average	Standard Deviation	Total Trials	Standard Error	Cell Viability
Control	0.218	0.0300	8	0.00375	
SAF04-30	0.218	0.0300	8	0.00375	107%
SAF04-30A	0.206	0.0340	8	0.00425	101%
SAF04-30C	0.195	0.0220	8	0.00275	95.8%
SAF04-30E	0.149	0.0340	8	0.00425	73.5%
SAF04-30F	0.153	0.0350	8	0.00438	75.1%
SAF04-30G	0.175	0.0270	8	0.00338	85.9%
SAF04-30H	0.190	0.0240	8	0.00300	93.3%
SAF04-30I	0.110	0.0220	8	0.00275	54.0%

Activity was tracked to SAF04-30I
Parent sample was the least active

SAF04-60(A-G)

Sample Code	Absorbance Average	Standard Deviation	Total Trials	Standard Error	Cell Viability
Control	0.171	0.0340	14	0.00243	
SAF04-60	0.121	0.0180	14	0.00129	72.6%
SAF04-60A	0.148	0.0310	14	0.00221	86.8%
SAF04-60B	0.144	0.0260	14	0.00186	94.1%
SAF04-60C	0.157	0.0350	14	0.00250	107%
SAF04-60D	0.179	0.0500	14	0.00357	107%
SAF04-60E	0.185	0.0430	14	0.00307	111%
SAF04-60F	0.172	0.0530	14	0.00379	103%
SAF04-60G	0.176	0.0540	14	0.00386	105%

Fractionated samples were less active than the parent
May suggest some sort of “synergistic affect” that makes the parent more active

SAF04-23(A-I)

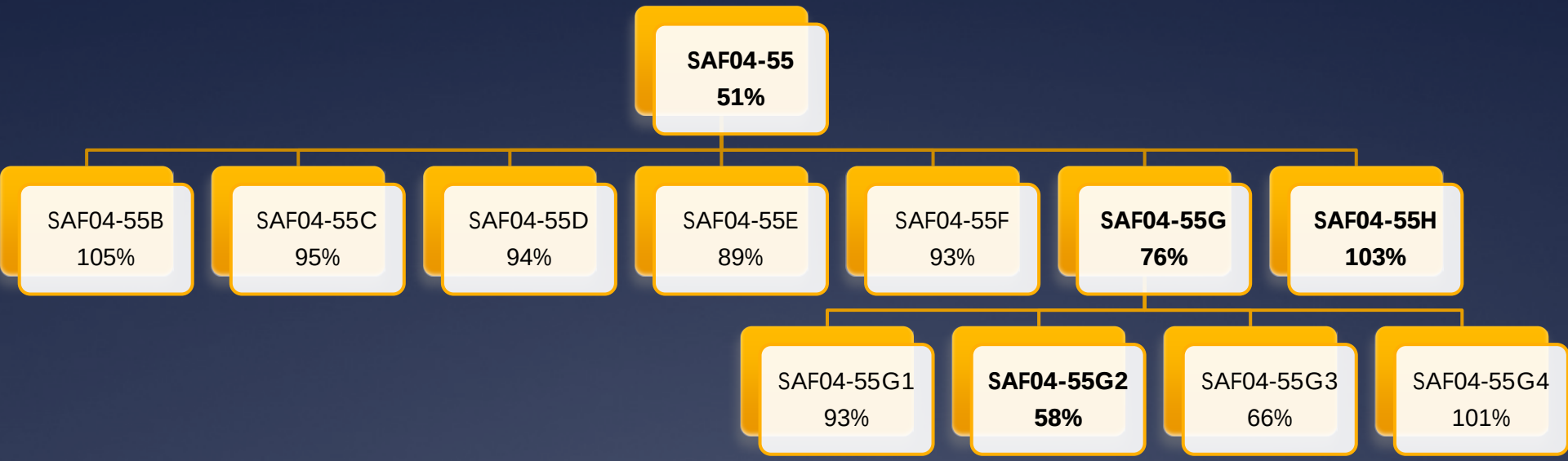
Sample Code	Absorbance Average	Standard Deviation	Total Trials	Standard Error	Cell Viability
Control	0.193	0.0390	6	0.00650	
SAF04-23	0.156	0.0420	6	0.00700	80.5%
SAF04-23A	0.182	0.0240	6	0.00400	94.1%
SAF04-23C	0.171	0.0240	6	0.00400	88.3%
SAF04-23E	0.190	0.0250	6	0.00417	98.4%
SAF04-23F	0.204	0.0330	6	0.00550	106%
SAF04-23H	0.220	0.0320	6	0.00533	114%
SAF04-23I	0.198	0.0200	6	0.00333	102%

Fractionated samples were less active than the parent
May suggest some sort of “synergistic affect” that makes the parent more active

Most Exciting Sample: SAF04-55

- The strong cytotoxic activity of SAF04-55 was a surprise, prompting investigation
- SAF04-55 was not initially identified as a priority sample
- Dr. McPhail's laboratory fractionated it in an attempt to track its activity
- The most active sample from the initial fractionation would be fractionated

SAF04-55



Fractionated samples were less active than the parent

Dramatic increase in viability for SAF04-55 was concerning (2% in the 1st screen)

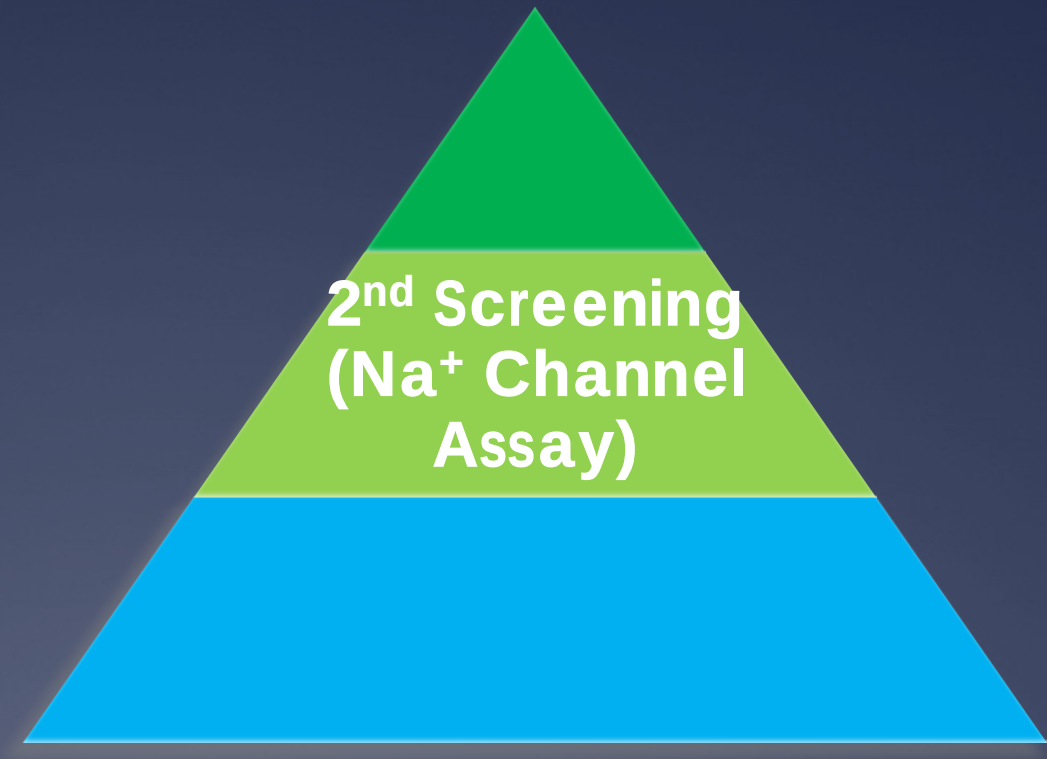
Issues Along the Way...

- DMSO
  - DMSO % higher than recommended 0.5%
  - No DMSO was used in control wells
- Comparisons can still be made

- Accidental change in cytotoxicity protocol during transition to 96 well
  - Wells had a brief period without media, possibly jeopardizing cell survival
- Comparisons can only be made for the specific protocols

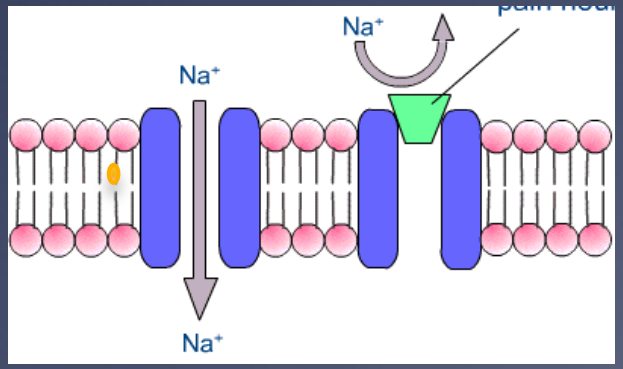
Voltage-Gated Sodium Channel Activity Assay

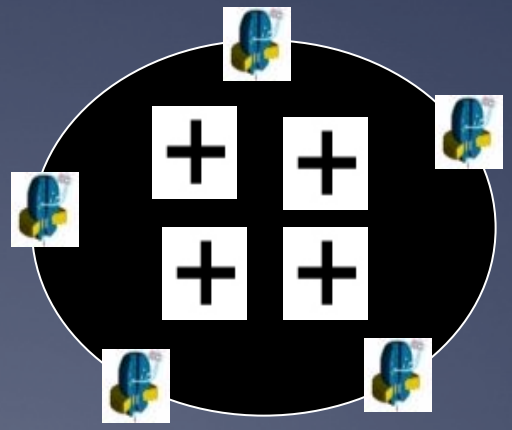
- The most active samples were considered for mechanism screenings
- We chose to investigate voltage-gated sodium channel activity
- We ran two separate protocols to identify either channel activation or channel inhibition



Sodium Channel Assay Explanation

- Similar to the cytotoxicity assay, except adding neurotoxins Ouabain and Veratridine
  - Ouabain/Veratridine causes the cell to increase Na+ ion concentration and block Na+ ions from escaping
  - **Veratridine:** excites sodium voltage gated channels increasing sodium ions in the cell.
  - **Ouabain**: then blocks Na+/K+ ATPase, a pump that regulates sodium concentrations release, thereby not allowing the ions to exit the cell
- Situation is unfavorable for cells due to swelling of the cell





Neurotoxin Setup

- Seed cells at same concentrations overnight
- Add Ouabain/Veratridine to media at given concentration with extracts, 24 hour incubation
- Three Controls: No treatment, O/V, and O/V + positive control

Activation Experiment

O/V at 50/500 mM

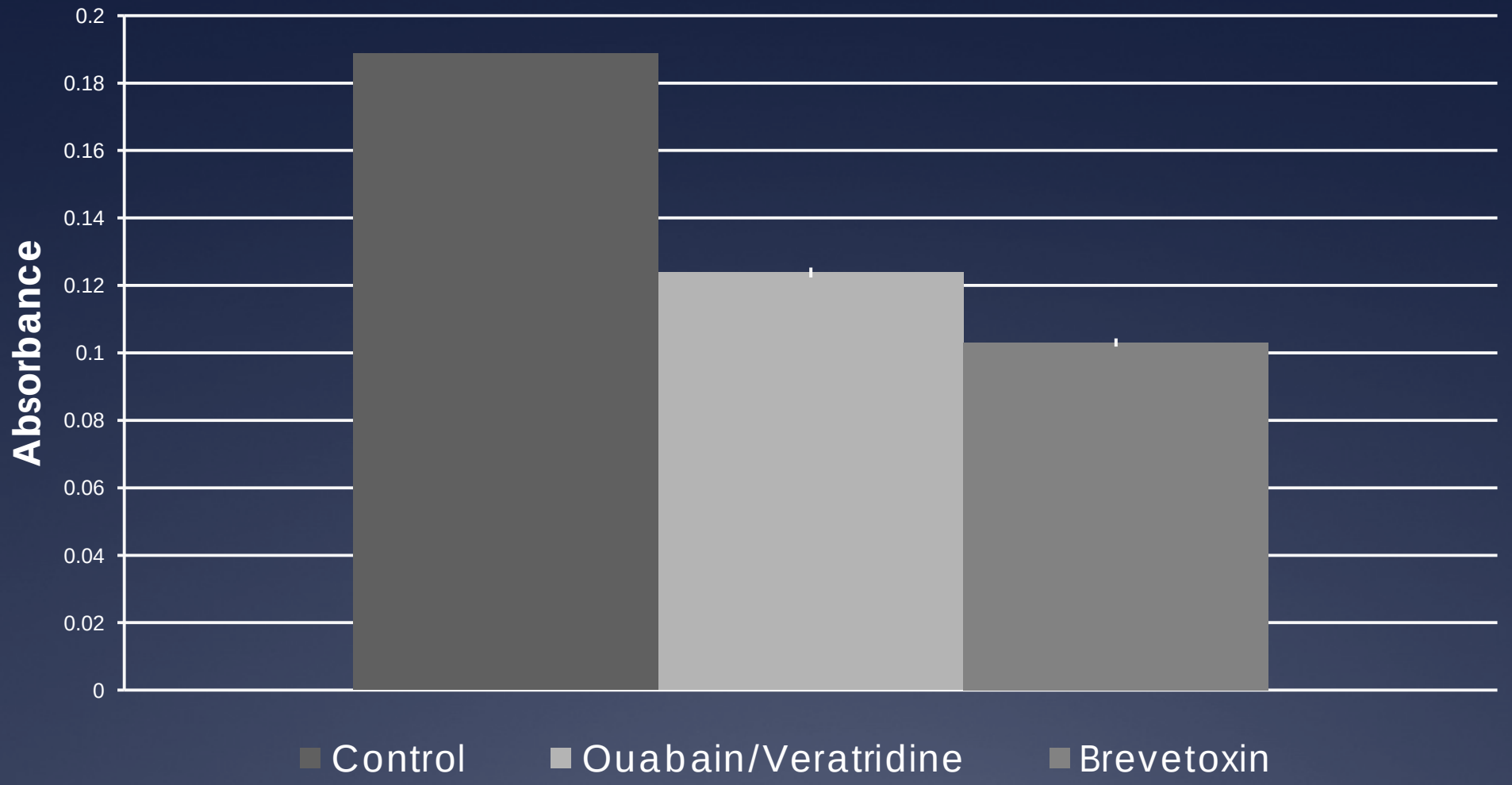
Tetrodotoxin used as positive control

Inhibition Experiment

O/V at 30/300 mM

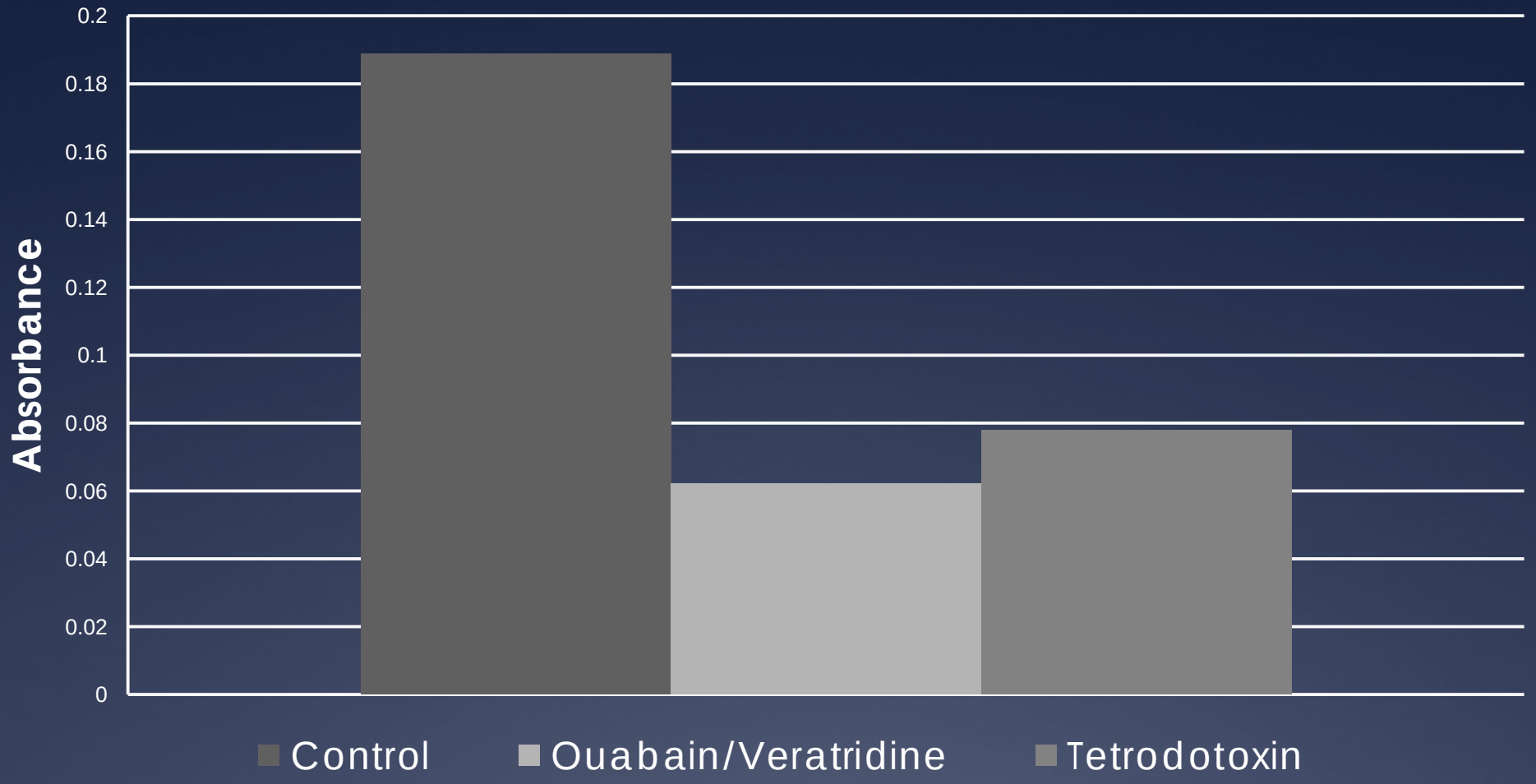
Brevetoxin used as positive control

Channel Inhibition Results



Control Average	+O/V	SD	SE	Cell Viability	O/V +B	SD	SE	Cell Viability
0.18875	0.1238	0.023433	0.001464	65.61%	0.1030	0.019433	0.001214	54.61%

Channel Activation Results



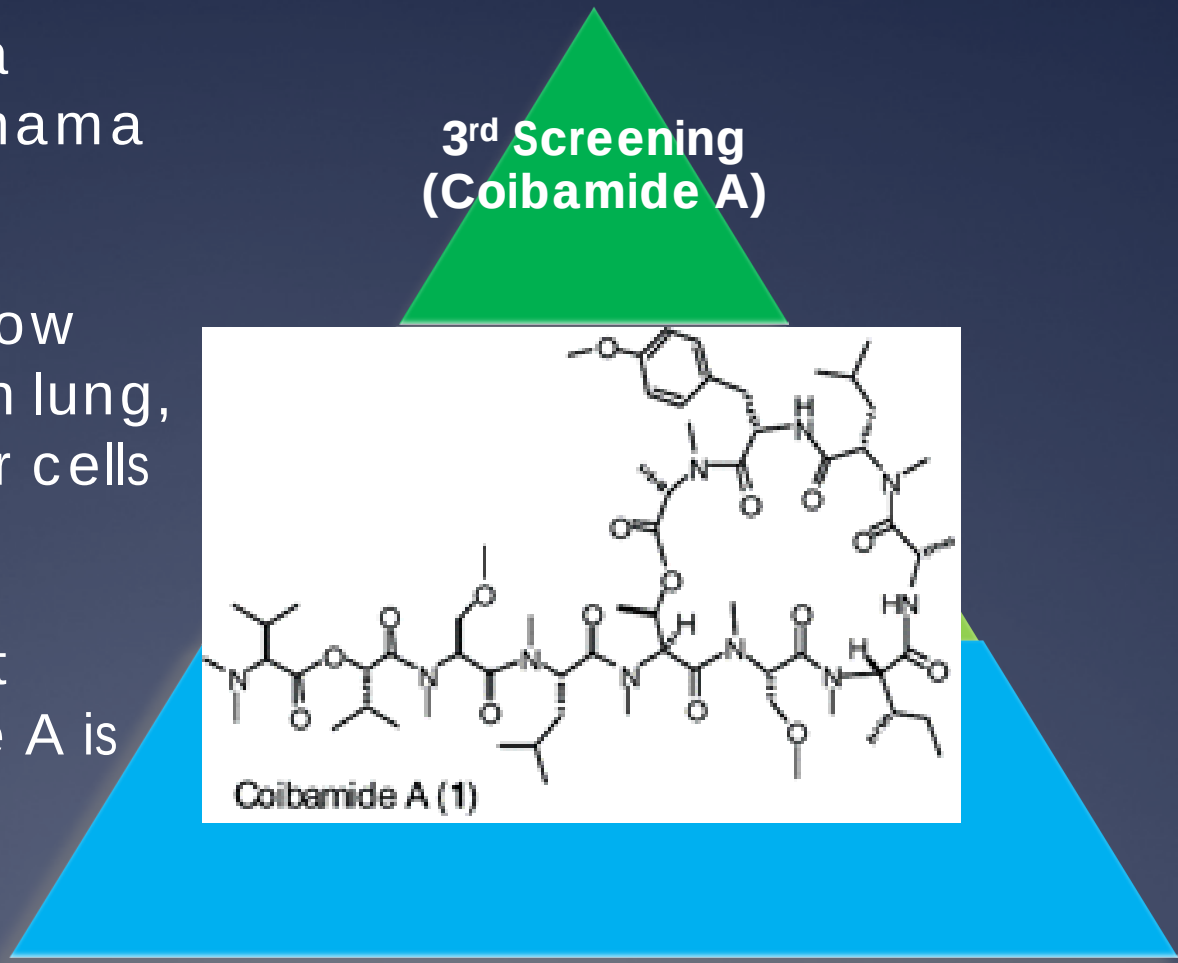
Control Average	+O/V	SD	SE	Cell Viability	+O/V +T	SD	SE	Cell Viability
0.18	0.0621	0.0111	0.000693	34.51%	0.0778	0.020633	0.001289	43.24%

Sodium Channel Assay Summary

- Due to a lack of time, we only attempted establishing the positive controls
- Positive controls worked, but were not as significant as needed to make the assay reliable
  - Expected approximately 35% increase/decrease in viability, only had around 10%
- Until proper controls are established, the assay is unreliable

Coibamide A: A Model of What This Project May Lead

- Dr. McPhail isolated a potent toxin from Panama (Coibamide A)
- Its been proven to show cytotoxicity to human lung, CNS and colon tumor cells
- Culturing of the cyanobacterium that produces Coibamide A is an issue



Possible Future Projects:

- Different cell lines
  - Ex: Lung tumors, colon tumors, etc.
- Different mechanism screenings
  - Ex: AMPA receptors, Steroid receptors
- Time variable cytotoxicity assay
  - Some samples may take longer to show activity?
- Concentration variable cytotoxicity assay
  - See if concentration plays a substantial role in activity?

BRR Project Outcomes

- My BRR project provided me the opportunity to understand the exciting field of drug discovery
- The research has also laid the groundwork for future projects in understanding the medicinal chemistry and pharmacological possibilities of these natural products
- I have also gained valuable skills with running experiments that can not be gained in a classroom setting

Acknowledgements

- Project Advisors: Jane Ishmael and Kerry McPhail
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- BRR Advisor: Wanda Crannell
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Questions?