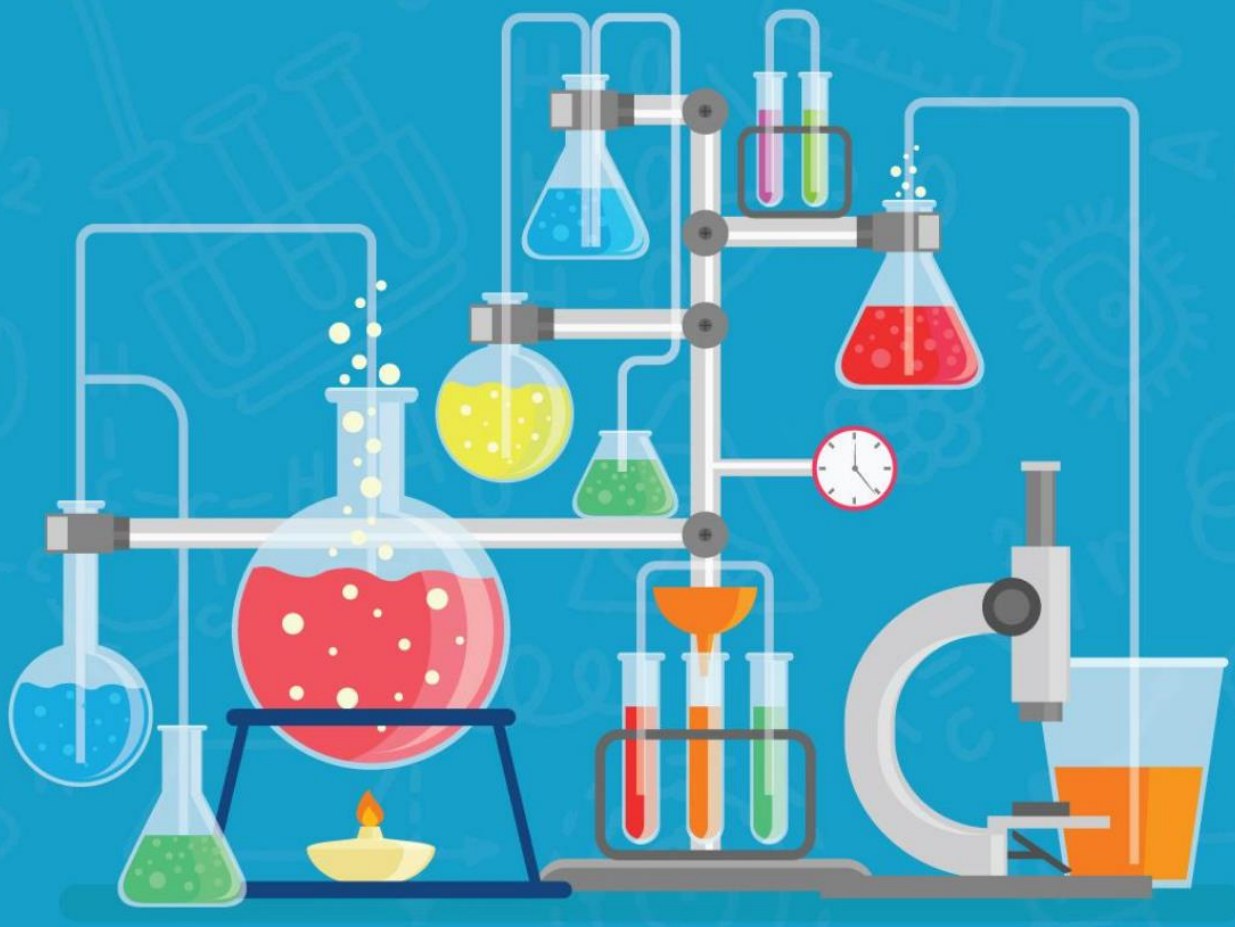
Drug Interaction and Pharmacodynamics Laboratory Module

Leonny Yulita Hartiadi and Agnes Anania Triavika Sahamastuti

Department of Pharmacy
Indonesia International Institute for Life Sciences (i3L)



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i3L PRESS

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ISBN : 978-623-92687-2-5
Page number : 35
Authors : Leonny Yulita Hartiadi
Agnes Anania Triavika Sahamastuti
Editor : Audrey Amira Crystalia
Cover Design : Gilang Dwi Valentino
Published by : © i3L Press

Preface

Drug Interaction and Pharmacodynamics is one of the fundamental courses in Pharmacy that enable the students to predict possible drug interactions, identify and manage the outcomes. Thus, this course is in an essential foundation in clinical decision making. As an inseparable part of the course, the Drug Interaction and Pharmacodynamics Laboratory provide the students with hands-on experience in evaluating drug interactions at pharmaceutical, pharmacokinetics, and pharmacodynamic stages.

In vitro and in vivo, using mice and zebrafish, systems are used to assess the impact of drug interactions. This laboratory also covers the basic concept of pharmacodynamics, such as dose-response curve and ligands. As the drug response is affected by genetic variability, detection of genetic polymorphisms will also be studied using molecular biology techniques. Each laboratory sessions is collected from various original research articles and laboratory testing guidelines.

We hope that this laboratory module represents all aspects in drug interaction and pharmacodynamics evaluations and at the same time through writing report of each session the students can build critical thinking and scientific writing ability. Lastly, we would like to express our appreciation to Indonesia International Institute for life sciences for their continuous support.

Jakarta, July 2018

Authors

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Pharmacodynamics Principle in *in vitro* System ($GABA_ARs$)

Session 1

Overview

Pharmacodynamics studies the mechanism of action of a drug to produce their pharmacologic effects. In most cases, a drug binds to its receptors to exert its actions. As you have learned basics of pharmacodynamics, you will get hands-on experience on how to construct dose response curve from *in vitro* raw data. The data that you will analyze is obtained using electrophysiological experiments of $GABA_A$ receptors ($GABA_ARs$) expressed in *Xenopus* oocytes.

GABA, as the major neurotransmitter systems, mediates its action through $GABA_ARs$. $GABA_ARs$ is a selective chloride ion channel. When GABA binds to $GABA_ARs$, GABA induces a series of conformational change of the receptors that leads to channel opening and allows chloride ions to enter predominantly from the outside to the inside of cells, causing hyperpolarization. The activity of $GABA_ARs$ is measured electrophysiologically using two-electrode voltage-clamp that is recorded as current traces.

Raw data analysis of dose response curve is performed using the following formula:

$$I = I_{max}\ ([A]^{nH}/([A]^{nH} + EC_{50}{}^{nH}))$$

Where [A] is the agonist concentration, n_H is Hill slope, and the EC_{50} value is the concentration of agonist that produces a response that is 50% of the maximum current.

Other substances can also bind to $GABA_ARs$, producing similar effect as GABA. This chemical is called agonist. Muscimol, a psychoactive compound of mushroom *Amanita muscaria*, is an agonist of $GABA_ARs$ that has a sedative-hypnotic activity and psychoactivity. There are also subtances that bind to the agonist site but doesn't activate the receptors. These substances are known as competitive antagonist. The most well-known of competitive antagonist of $GABA_ARs$ is bicuculine. Meanwhile, a non-competitive antagonist, such as picrotoxin, bind to a site, other than the agonist site. Other substances can only produce effect on $GABA_ARs$ while GABA is bound to the receptors. These substances are classified as modulators. A modulator that increases the activity of GABA-induces chloride currents is called positive allosteric modulator while a modulator that reduces the activity of GABA-induces chloride currents known as negative allosteric modulator.

Equipment

Computers in Computer Laboratory room

Procedure

a. Select computer that has been installed with **LabChart Reader** (free version) (https://www.adinstruments.com/products/labchart-reader) and **Graphpad prism** (trial version) (https://www.graphpad.com/scientific-software/prism/).
b. Open data in the following folder: Drug interaction and pharmacodynamic-session 1 → raw traces

c. Raw traces data obtained is from α4β2δ and α4β2γ $GABA_A$Rs. These receptors were exposed to GABA and an agonist known as THIP.
d. Open the data using LabChart Reader and locate the data that you need. You can zoom in the trace for a better view.

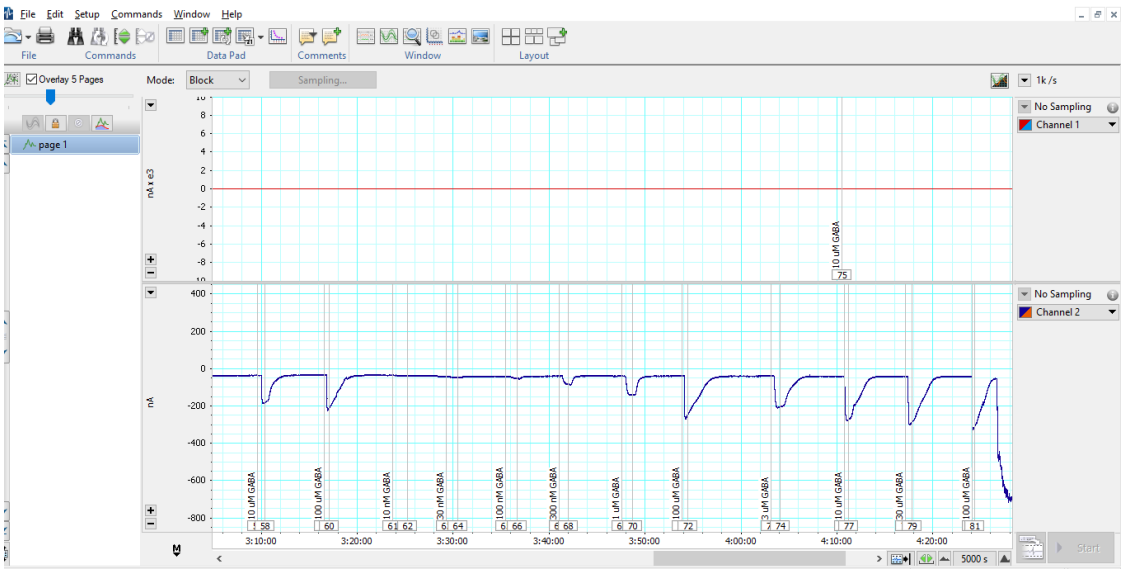


Figure 1.1 Example of traces produced when the receptors are exposed to GABA

e. To measure the trace response, go to commands → set market. Place the marker before the trace as a minimum point and point the cursor to the lowest point of the trace. Record the response showed on the right side.

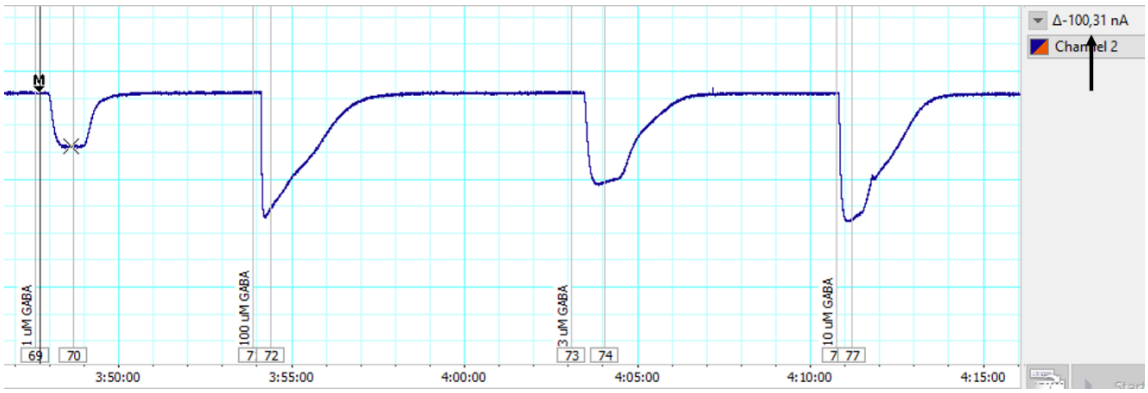


Figure 1.2. Tracer measurement. M is marker which is set as a minimum point. Measurement result is pointed by the arrow on the right side of the figure

f. Measure the rest of the traces produced by increasing concentrations of GABA and THIP.
g. Open Graphpad prism
h. Open a new table & graph under XY selection. Choose Y: enter and plot a single Y value for each point as shown here:

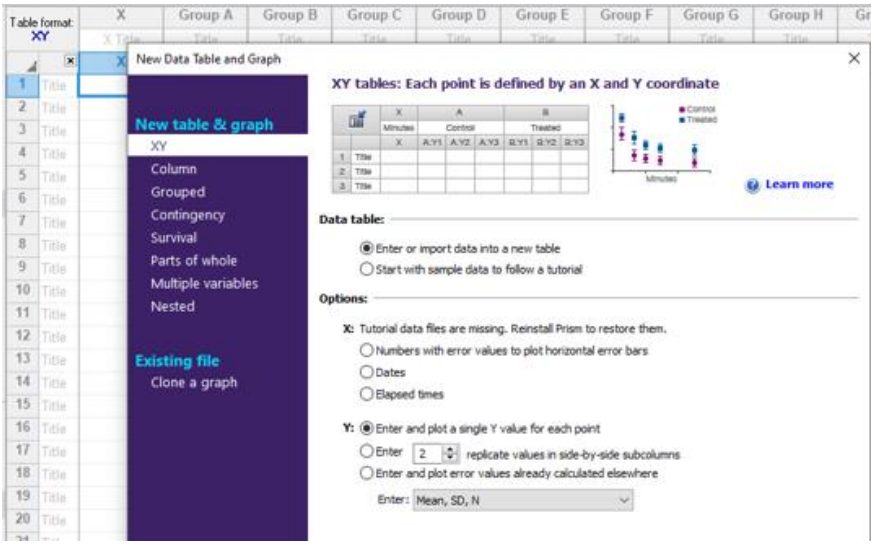


Figure 1.3. Interface of opening a new table & graph

i. Perform individual data analysis by doing the following:
   - Analyze: transform: transform X values using X = log [X]
   - XY analyses: non-linear regression (curve fit)
   - Dose response – stimulation
     - log (agonist) vs response-variable slope (four parameters)
     - constraint bottom = 0
   - Take the top from individual data (non-linear) and paste to the bottom of the raw data
   - XY analyses: non-linear regression (curve fit)
   - Normalize; Y = 0; 100 %: last value

j. Perform group analysis by doing the following:
   - Open new graph & table. Choose Y and enter 10 replicates
   - Input the transformed concentration value
   - Copy paste the data from normalized of transform data
   - Analyze the data using Nonlinear regression
   - Constraint the bottom to zero

Result and Observation

a. Trace measurement

Cell identity & receptor type	GABA concentration (µM)	Response (nA)	THIP concentration (µM)	Response (nA)

b. Write down the EC_{50}, Hill Slope, and Emax for THIP for the α4β2δ and α4β2γ $GABA_A$Rs

Analysis and Discussion

a. Compare the dose response curve of α4β2δ and α4β2γ $GABA_A$Rs Why do these 2 dose response curves are different? What is the consequences of having different subunit composition of $GABA_A$Rs?
b. What is the difference between potency and efficacy? Name 1 compound that can increase GABA potency at $GABA_A$Rs without increases its efficacy
c. Name three class of drugs affecting $GABA_A$Rs that are clinically used!

Antibiotic Interaction

Session 2, 3

Overview

Antibiotic is a substance that may inhibit the growth or kill the bacteria and hence antibiotic can be divided into bacteriostatic and bactericidal. Bacteriostatic inhibit the growth of bacteria until the body's immune system take over the rest of the bacteria. Meanwhile bactericidal directly kill the bacteria, and usually is more desirable. Generally, it is not recommended to combine antibiotics with bactericidal and bacteriostatic, respectively because bactericidal antibiotic is only effective in actively dividing bacteria population.

Based on the mechanism of action, antibiotics are divided into 5 classes, namely anti-metabolites, inhibitor of DNA synthesis, inhibitor of protein synthesis, inhibitor of cell wall synthesis and disruptor of cell membrane. Antibiotics should demonstrate selective toxicity, where the antibiotics should only have toxic effect to bacteria but not to host cells.

In this session, you will learn how combination of antibiotic with different mechanism of action may result in synergistic, additive or antagonistic effect against bacteria colonies *in-vitro*. One of the most popular methods to evaluate the synergistic, additive or antagonistic effects are checkerboard and E-test method. Several antibiotics combinations will be assessed against *E. coli* and *B. subtilis*.

Materials

E. coli	1% $BaCl_2$	Water type III
Mueller-Hinton broth	Tetracycline	1% H_2SO_4
Ciprofloxacin	Penicillin	$MgCl_2$
Chloramphenicol	$CaCl_2$	Agar
B. subtilis		

Equipment

96 well plates	Incubator	Glass tube
Micropipette	Autoclave	Shaker incubator
Erlenmeyer flask	Spectrometer UV-Vis	Whatman filter paper no.1

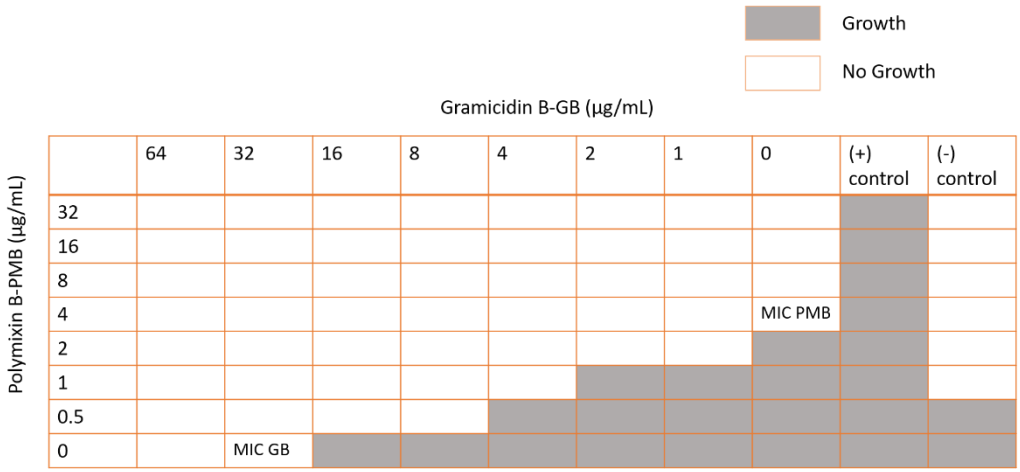


Figure 2.1 An example of checkerboard result

Procedure

1. Preparation of bacteria colonies
   a. The medium used for growing of *E. coli* and *B. subtilis* is Mueller-Hinton broth. This broth is prepared by dissolving 21 grams of Mueller-Hinton powder in 1000 ml distilled water. Heat the solution to dissolve the medium completely, and then add 25 mg/L $CaCl_2$ and 12.5 mg/L $MgCl_2$. Stir it homogeneously and sterilize the medium using autoclave at 121^0C for 15 minutes. Prepare 100 ml of medium for each group. This medium should be used fresh.
   b. Take 10 ml of sterile medium and place it into glass tubes. Inoculate *E. coli* and *B. subtilis* colonies, respectively in the medium. Incubate the medium at 37^0C for 24 hours in the shaker incubator. After 24 hours, check the viability of bacteria colonies. The concentration of bacteria colonies then matched into 0.5 McFarland turbidity standard (equal to bacterial concentration of $1.5x10^8$ CFU/ml. **Please check how to make McFarland standard in www.dalynn.com/dyn/ck_assets/files/tech/TM53.pdf!**) by visual detection or using spectrometer UV-Vis at 625 nm (absorbance 0.08 - 0.1).
   c. Before checkerboard testing, the bacterial colonies should be diluted using Mueller-Hinton broth medium until concentration of $10x10^5$ CFU/ml.

2. Preparation of Mueller-Hinton agar medium
   a. Mueller-Hinton agar medium is prepared by dissolving 21 grams of Mueller-Hinton powder in 1000 ml distilled water. Add 1.7% agar into medium. Heat the solution until it completely dissolved. Sterilize the medium using autoclave at 121^0C for 15 minutes. Prepare 1L for each group. Pour the medium into several petri dishes and let it solidify. At least each group should prepare for 40 petri dishes.

3. Preparation of antibiotic solution

For checkerboard testing:

- Tetracycline solution series are prepared by dissolving tetracycline in sterile Mueller-Hinton broth at concentration of 0.25; 0.5; 1; 2; 4; 8; 16; 32 µg/ml at least 2 ml for each concentration.

- Ciprofloxacin solution series are prepared by dissolving ciprofloxacin in sterile Mueller-Hinton broth at concentration of 0.007813; 0.01563; 0.03125; 0.0625; 0.125; 0.25; 0.5; 1 μg/ml at least 2ml for each concentration.
- Penicillin solution series are prepared by dissolving penicillin G in sterile Mueller-Hinton broth at concentration of 1; 2; 4; 8 ;16 ;32; 64; 128 μg/ml at least 3 ml for each concentration.
- Chloramphenicol solution series are prepared by dissolving chloramphenicol in sterile Mueller-Hinton broth at concentration of 0.5; 1; 2; 4; 8; 16; 32; 64 μg/ml at least 1 ml for each concentration.
- For E-test testing: tetracycline, ciprofloxacin, penicillin G, and chloramphenicol solution is made in sterile water type III at a concentration of 10 μg/ml.

4. Checkerboard testing

Checkerboard testing method is prepared by adding 50 μl antibiotic solution and 50 μl of *E. coli* at concentration of $10x10^5$ CFU/ml in each well of 96-well plates. This means that the final concentration of *E. coli* is $5x10^5$ CFU/ml, while the concentration of antibiotics are half of the stock solution. The final concentration after mixing will follow these pictures:

	Tetracycline concentration (μg/ml)								
Ciprofloxacin concentration (μg/ml)	0	0.125	0.25	0.5	1	2	4	8	16
	0.0039								
	0.00781								
	0.01563								
	0.03125								
	0.0625								
	0.125								
	0.25								
	0.5								

	Tetracycline concentration (μg/ml)								
Penicillin concentration (μg/ml)	0	0.125	0.25	0.5	1	2	4	8	16
	0.5								
	1								
	2								
	4								
	8								
	16								
	32								
	64								

	Penicillin concentration (μg/ml)								
Ciprofloxacin concentration (μg/ml)	0	0.5	1	2	4	8	16	32	64
	0.0039								
	0.00781								
	0.01563								
	0.03125								
	0.0625								
	0.125								
	0.25								
	0.5								

	Penicillin concentration (µg/ml)								
Chloramphenicol concentration (µg/ml)	0	0.5	1	2	4	8	16	32	64
	0.25								
	0.5								
	1								
	2								
	4								
	8								
	16								
	32								

The plates then incubated at 37^0C for 24 hours. The MIC (minimal inhibitory concentration) will be interpreted as the lowest concentration of antibiotic that completely inhibit the bacterial growth as detected by naked eye. The MIC data then will be analyzed in terms of the fractional inhibitory concentration index (FIC). FIC is calculated by comparing the value of the MIC of individual antibiotic with MIC of the combination antibiotics using the following formula:

$$\text{FIC} = \frac{MICA\ combination\ A\ \&\ B}{MIC\ A} + \frac{MICB\ combination\ A\ \&\ B}{MIC\ B}$$

In which MIC A = minimal inhibitory concentration of A antibiotic; MIC B = minimal inhibitory concentration of B antibiotic; MICA combination A & B = minimal inhibitory concentration of combination A & B antibiotic in which the concentration of A is the smallest; MICB combination A & B = minimal inhibitory concentration of combination A & B antibiotic in which the concentration of B is the smallest. FIC from all wells need to be calculated to acquire ΣFIC.

The interpretation of the ΣFIC value is listed below:

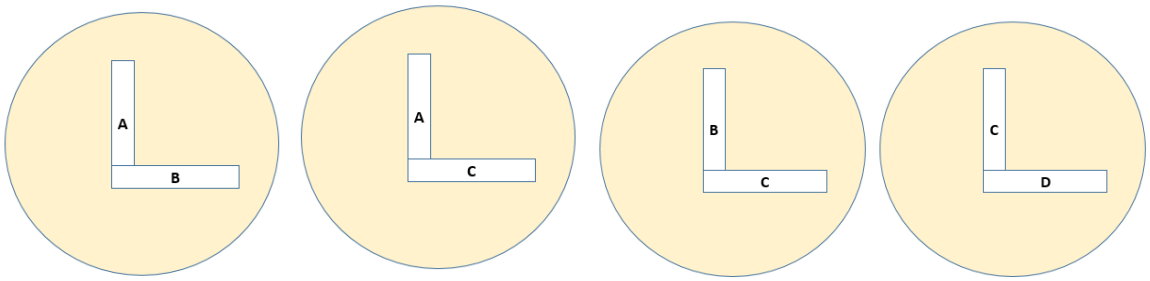
ΣFIC value	**Interpretation**
≤0.5	Synergistic
> 0.5 - 1	Additives
1 - 4	Indifference
>4	Antagonism

5. E-test using conventional antibiotic strips

The antibiotic strips are prepared by cutting Whatman filter paper no 1 with length of 6 cm and width of 1 cm. These strips are dipped and soaked into each antibiotic solution placed on a small petri dish. The concentration of antibiotics as followed (prepared at least 5 ml of each antibiotic solution):

- Tetracycline (A): 10 µg/ml in water type III.
- Ciprofloxacin (B): 10 µg/ml in water type III.
- Penicillin G (C): 10 µg/ml in water type III.
- Chloramphenicol (D): 10 µg/ml in water type III.

The bacterial colonies were inoculated by spreading 10 µl of $1.5x10^8$ CFU/ml *E. coli* in Mueller-Hinton broth medium into Mueller-Hinton agar medium aseptically. *S. aureus* is also used in the experiment. After bacterial inoculation, the antibiotic strips were placed onto agar plates in the following formation:



The petri dishes were incubated at 37^0C for 24 hours. The synergistic or antagonistic interaction is observed by inhibition area in the overlapping between two antibiotic strips.

Result and Observation

a. Determine the MIC of individual antibiotic from Checkerboard test and Etets!
b. Determine the interaction between Tetracycline-Ciprofloxacin, Tetracycline-Penicillin, Penicillin-Ciprofloxacin, Penicillin-Chloramphenicol based on FIC of Checkerboard testing and qualitative data of E-test!

Analysis and Discussion

a. Explain the mechanism of each individual antibiotic!
b. Explain the interaction between tetracycline-ciprofloxacin, tetracycline-penicillin, penicillin-ciprofloxacin, penicillin-chloramphenicol!
c. Explain the effectivity of checkerboard method and E-test for measuring the synergistic interaction!

Effect of Broccoli Juice Towards Ethanol-Induced Liver Damage

Session 4, 5

Overview

Broccoli is a vegetable known for its nutritional value such as vitamin A, vitamin C, and minerals. Broccoli also has antioxidant activity owing to its nutrients and other phytochemical compounds, hence, it is presumed that broccoli have the ability to protect cells from oxidative damage.

Alcohol is widely consumed as drinks. Alcohol is easily absorbed in the upper small intestine due to its relatively small size, has no charge, and is both hydrophilic and lipophilic. Alcohol consumption affects different region of the brain, therefore it can impair brain processes, behavior and emotion. Alcohol is metabolized via oxidation process in the liver by alcohol dehydrogenase into metabolites that damage the liver. Excessive consumption of alcohol in the long-term results in fatty liver disease, which may lead to liver cirrhosis.

In this session, you will evaluate the effect of broccoli juice on a mouse model of ethanol-induced liver damage. Comparison will be made between mice receiving broccoli juice with mice that do not receive any treatment.

Materials

Mice	Xylene	Hemotoxylin & eosin
Ethanol	Pot for liver tissue	Ketamine/xylazine
Drinking water	Paraffin	Broccoli juice
Formalin	Eukitt	

Equipments

Staining jars	Oral gavage needle
Microtome	Syringe
Surgery kits	Juice mixer

Procedure

a. Mice are divided into the following groups:
   - Control group: no treatment
   - Ethanol group: treated with 15% ethanol for day one and two, 20% ethanol for day three and four, 25% ethanol for day five and six, 30% for day seven, eight, and nine with a dose of 0.028 ml/ 20 g bw via oral injection
   - Broccoli group: mice are simultaneously given alcohol such as those received by ethanol group and broccoli juice with 0.2 ml/20 g bw via oral injection.

b. On day 10, mice are sacrificed by injecting ketamine/xylazine intraperitoneally with a dose of Ketamine 100 mg/kg and Xylazine 10mg/kg. Check the toe reflex by pinching their paws using a tweezer. If the mice have no reflex, proceed with surgery

c. Perform a surgery by making a sagittal incision. Harvest the liver and weigh the liver. Collect a small liver section and keep it in a jar containing 10 % formaldehyde.

d. Three days after, dehydrate the liver in increasing concentration of alcohols (70, 80, 90, 96, and 100 %) for 30 minutes each. Transfer the tissue to xylene and paraffin (3x) for histology that has been kept at 60 ºC, respectively. Transfer the tissue in a histology mold and pour liquid paraffin to cover the tissue. Once it is cooled down, section the liver using microtome with a thickness of 5 µM.
e. Placed the sections onto warm water bath and carefully transferred the section into microscopic slide. Let the slides dry.
f. Slides are immersed in xylene and descending concentrations of alcohol for 1 minute. Then, dip the slides into water before placing them into hematoxylin & eosin jar for 2 minutes. Quickly rinse the slides with water and dehydrate the slides with increasing concentration of alcohols for 2 minutes. Transfer the slides into xylene for 1 minutes and close the slides with objective glass with the addition of eukitt.

Result and Observation

- Observe the slides under microscope. Identify the morphological differences between the 3 different groups. Capture representative pictures for each group

Analysis and Discussion

a. Describe how ethanol cause damage to the liver!
b. Explain in detail about the compounds or substances in broccoli juice that have antioxidant activity!
c. Identify what type of interaction occurs between broccoli and ethanol! Describe how broccoli and ethanol interact!

Combination of Acetaminophen-Codeine as Analgesic in Mice

Session 6

Overview

Analgesics are drugs that relieve pain feeling, which used widely from acute and chronic physical injuries until terminal stage of cancer. Analgesics are divided into opioid analgesic and non-opioid analgesic, depends on their site of action. Opioid analgesics act in the brain and spinal cord (central nervous system) and inhibit the neurotransmission of pain. Meanwhile, non-opioid analgesics act in the peripheral nervous system to inhibit the formation of pain-producing substances, such as prostaglandins.

In this session, you will learn how combination of analgesics that have different mechanism can produce synergistic effect in mice. You will also learn how to induce pain in mice and how to analyze the analgesic effect by using writhing method.

Materials

12 male mice	Acetaminophen
Codeine phosphate	Gum arabic
Sterile saline solution	Acetic acid
Warm water (60^0C)	

Equipment

Mice cage	Beaker glass	Plexiglass cylinder
Eppendorf tube	Falcon tube	Oral gavage needle
Syringe		

Procedure

1. Preparation of the pain inducer (acetic acid solution)
   The pain inducer contains acetic acid 0.1% v/v in sterile normal saline. Please prepare 10 ml of acetic acid 0.1% v/v in the falcon tube.

2. Preparation of the drugs solutions
   a. First, prepare 25 ml solution of gum arabic 2% in mortar by dispersing gum arabic powder on hot water. Let it swell for about 5 minutes. Then mix the solution using stamper until all the gum arabic dissolved and a thick solution is obtained.
   b. Acetaminophen solution is prepared by mixing acetaminophen in aqueous solution of gum arabic (2%) to get acetaminophen concentration of 10 mg/ml. Prepare 7.5 ml of acetaminophen solution.
   c. Codeine phosphate solution is prepared by mixing codeine phosphate in aqueous solution of gum arabic (2%) to get the codeine phosphate concentration of 0.6 mg/ml. Prepare 7.5 ml of codeine phosphate stock solution.
   d. Mixture of Acetaminophen and codeine phosphate is mad by mixing 2.5 ml of acetaminophen solution and 2.5 ml codeine phosphate solution that have been prepared previously.

3. Writhing test
    a. Groups of male mice were fasted for 18 hours before the experiment. Access to water is given. Then each mouse should be weighed before treatment to determine the drug dosages.
        - The acetaminophen treated mice were administered orally 0.25 ml/20 g body weight (BW) acetaminophen solution of 125 mg/kg BW. (**Please calculate first how many mg of acetaminophen needed for each mouse and how many ml you need to inject the mice!**) Do it in triplicate!
        - The codeine phosphate treated mice were administered orally 0.25 ml/20 g body weight (BW) codeine phosphate solution of 7.5 mg/kg BW. (**Please calculate first how many mg of codeine phosphate needed for each mouse and how many ml you need to inject the mice!**) Do it in triplicate!
        - The acetaminophen-codeine phosphate treated mice were administered orally 0.5 ml/20 g body weight (BW) mixture of acetaminophen 125 mg/kg BW and codeine phosphate 7.5 mg/kg BW. (**Please calculate first how many mg of codeine phosphate needed for each mouse and how many ml you need to inject the mice!**) Do it in triplicate!
    b. As for control mice, they only received gum arabic solution 0.5 ml/20 g BW. Do it in triplicate!
    c. 1 hour later, give i.p. injection of 0.1% v/v acetic acid solution (10 ml/kg BW) to each of the mouse.
    d. Place the mouse in plexiglass cylinder. Writhing and stretching responses are observed and counted from the fifth to tenth minutes after acetic acid injection (total writhes for 5 minutes). Writhes is defined as contraction of the abdominal muscle together with a stretching of hind limbs or rotation of the trunk. (please watch https://www.youtube.com/watch?v=Ib63O4F856w for detail).

Example of solution calculation that will be given to mouse

For example: the mouse BW is 30 mg.

Acetaminophen need to be delivered = 125 mg/kg BW = 3.75 mg/30 mg BW.

Acetaminophen solution concentration = 10 mg/ml.

Acetaminophen solution taken = (3.75 mg/10mg) x 1 ml = 0.375 ml.

Conclusion: for 30 mg BW mice, we need to give 0.375 ml acetaminophen solution (10 mg/ml)

Result and Observation

a. Insert the data into this table:

Group	Mice no.	BW (g)	Treatment given (name of drug, how many ml of drug, how many ml of gum arabic solution)	Number of writhes for 5 minutes
Control	1			
	2			
	3			
Acetaminophen	1			
	2			
	3			
Codeine phosphate	1			
	2			
	3			
Combination acetaminophen-codeine phosphate	1			
	2			
	3			

b. Observe the number of writhes from acetaminophen treated mice, codeine phosphate treated mice and combination of acetaminophen-codeine phosphate treated mice for total 5 minutes!

Analysis and Discussion

a. Discuss the mechanism of action of individual drug in reducing pain!
b. Discuss the result, is there any synergistic effect?
c. Discuss the interaction mechanism between acetaminophen and codeine-phosphate!

Pharmacokinetic Interaction Between Diazepam and Cimetidine

Session 7

Overview

One of the drug-drug interaction result is pharmacokinetic alteration of one drug by another drug. This pharmacokinetic interaction may cause changes in the blood level of drugs, the metabolism rate of drugs and the excretion rate of drugs in the body. Generally, pharmacokinetic drug-drug interaction could prolong the drug's effect, or inversely, make the treatment not effective. If the first drug inhibits the metabolism of the second drug, most probably this will prolong the action of the second drug due to high level of second drug in blood. However, if the first drug increases the metabolism of the second drug, most probably this will shorten the action of the second drug due to increase of elimination rate of the second drug.

Diazepam is classified as sedative-hypnotic drugs that widely used in the world. The effect also known as anxiolytic effect, in which it able to calm or reduce anxiety, while also induce drowsiness and promote sleep. Diazepam is metabolized by oxidation or reduction prior to glucuronide conjugation. However, this metabolism process can be influenced by administration of cimetidine. Cimetidine is H2 receptor antagonist that mostly used to treat peptic ulcers due to its ability to inhibit stomach acid production. Cimetidine is CYP3A4 inhibitor, which can inhibit the metabolism rate of diazepam.

In this session, you will observe the interaction between diazepam and cimetidine based on its pharmacokinetic process in mice. The interaction will be evaluated from the sedative-hypnotic effect of diazepam in the treated mice.

Materials

9 male mice	Diazepam tablet (5 mg/tab)
Cimetidine tablet (200 mg/tab)	CMC Na
Water type III	

Equipment

Mice cage	Beaker glass
Stopwatch	Hot plate and stirrer
Mortar and pestle	Oral gavage needle

Procedure

1. Preparation of CMC-Na 1%
   Weigh 50 g of CMC-Na and disperse them onto 50 ml of hot water. Wait for 5 minutes until CMC-Na swell and mix the solution until homogenous

2. Preparation of drug solution
   a. Prepare 2 ml of diazepam solution with a concentration of 1 mg/ml:

- First, weigh the diazepam tablet (contain 5 mg diazepam/tablet). Crush the tablet and then take the amount of diazepam as needed based on the following calculation:
  - Diazepam powder taken = $\frac{3\text{ mg}}{5\text{ mg}}$ x weight of 1 tablet
- Add the powder into 3 ml of CMC Na 1% solution, vortex until homogenous.

b. Prepare 2 ml of cimetidine solution with a concentration of 2.6 mg/ml:
   - First, weigh the cimetidine tablet (contain 200 mg cimetidine/tablet). Crush the tablet and then take the amount of cimetidine as needed based on the following calculation:
     - Cimetidine powder taken = $\frac{5.2\text{ mg}}{200\text{ mg}}$ x weight of 1 tablet
   - Add the powder into 2 ml of CMC Na 1% solution, vortex until homogenous.

c. Prepare 2 ml of diazepam and cimetidine solution in 1:1 ratio from previous solutions

3. Delivery of diazepam and cimetidine solution
   Dose of diazepam: 10 mg/kg
   Dose of cimetidine: 26 mg/kg
   a. Divide the mice into 3 groups of mice with 3 mice on each group, i.e. control group, diazepam-treated group, and combination of diazepam-cimetidine-treated group.
   b. Weigh each mouse and determine the amount of drug solution to be injected.
   c. For control group, the mice are given 1% CMC-Na solution orally in 10 ml/kg. Do it in triplicate!
   d. The amount of solution needed for each mouse is given orally to diazepam-treated group and diazepam-cimetidine-treated group, respectively. Do it in triplicate!
   e. Take note at what time the treatment is given and calculate the time when the mouse starts to sleep or lose its gripping ability (the drug's onset) and the time when the mice awake or gain its gripping ability (duration of drug's action).

Result and Observation

a. Insert the data into this table:

Group	Mice no.	BW (g)	Treatment (name of drugs, how many ml of drugs given)	Time of drugs given	Drug onset (time of mice sleep)	Time when mice is wake up	Duration of drugs action
Control	1						
	2						
	3						
Diazepam	1						

	2						
	3						
Cimetidine-Diazepam	1						
	2						
	3						

b. Write the time when drug is given, when the mice starts to sleep and when the mice starts to wake up by evaluating its gripping ability or flipping the mice on supine position
c. Calculate the duration of drugs action!

Analysis and Discussion

a. Compare the duration of drugs action between diazepam treated mice and combination of cimetidine-diazepam treated mice!
b. Discuss the interaction between cimetidine and diazepam related to the sedative-hypnotic/muscle relaxant effect of diazepam!

Combination of Taurine & Caffeine on Platelet Aggregation

Session 8

Overview

Energy drinks are widely consumed throughout the world with annual consumption exceeding 5.8 billion liters. Energy drinks are believed act as energy booster with the main component, caffeine. Caffeine is often combined with taurine, vitamin B, inositol, ginseng, carnitine, and other components.

Caffeine is a naturally occurring alkaloid that can be found in coffee beans, cocoa beans, tea leaves and cola nuts. Caffeine is also known as a non-selective adenosine receptor blocker. The adenosine receptors, particularly A_2-type adenosine receptors, have been shown to be expressed on platelets and the activation of the receptors stimulate cAMP generation, causing platelets aggregation inhibition. Meanwhile, taurine, which is classified as essential amino acids, is known to stabilize platelets against various of pro-aggregating agents which act through Ca^{2+} influx. Additionally, taurine has been shown to inhibit platelet aggregation in gestosis patients with hypertension, proteinuria, and edema.

This session will investigate whether the combination of caffeine and taurine will influence platelet aggregation by looking at bleeding time and coagulation time from mice.

Materials

Taurine	12 male mice
Caffeine	Capillary blood collection tube
Tissue	

Equipment

Oral gavage needle
Syringe
Surgery scissors

Procedure

1. Preparation of taurine, caffeine and taurine-caffeine solution
    - Caffeine: solution of caffeine is made in drinking water in a concentration of 1.04 mg/ml
    - Taurine: solution of taurine is made in drinking water in a concentration of 10.04 mg/ml
    - Taurine-caffeine solution: taurine-caffeine solution is made by mixing the above taurine and caffeine solution in 1:1 ratio.

2. Calculation of dose and the amount of solution needed
    a. Weigh each of the mouse and take note of the weight.
    b. Mark each of the tail.

c. Divide the mice into 4 groups, i.e. vehicle, taurine, caffeine, and taurine-caffeine group (containing 3 mice for each group).
d. Calculate the dose and the solution needed for each mouse with the dose of 13 mg/kg for caffeine and 130 mg/kg for taurine.
e. Give all of the drugs orally and wait for an hour.
f. Following an hour after oral injection, coagulation time is determined from each mouse.

3. Coagulation time measurement
   a. The end of mouse tail is clipped and the blood that comes out is absorbed into capillary tube.
   b. Capillary containing blood is snapped every 15 seconds until there is a formation of fibrin in the snapped capillary.
   c. Coagulation time is the time needed to form the fibrin.

Result and observation

Group	Mice number	BW (g)	Amount of drug (mg) and amount of drug solution (ml)	Bleeding time	Coagulation time
Vehicle group	1				
	2				
	3				
Caffeine group	1				
	2				
	3				
Taurine group	1				
	2				
	3				
Taurine-caffeine group	1				
	2				
	3				

Analysis and discussion

a. Explain about the physiological process of fibrin formation!
b. Compare the bleeding and coagulation time of each group! Is there any significant difference between the group?
c. Please explain about possible mechanism by which caffeine, taurine, and combination of them can affect aggregation of platelet!

Drug Incompatibility

Session 9

Overview

In general, drug interaction comprised of pharmacodynamic and pharmacokinetics interaction. Pharmacodynamic interaction is correlated to the pharmacological activities of the drugs that interact with each other. Pharmacokinetics interaction is more common and it involves either the absorption, distribution, metabolism and excretion of the drugs. However, drug interaction can also happen prior drug administration. In some cases, a drug is mixed with another drug or infusion solution through an infusion line. This process sometimes leads to an undesirable reaction that is known as drug incompatibility.

Most of drugs (> 90 %) are organic, weak electrolytes that lack of solubility in water. Thus, they are predominantly available in liquid (this include injectable dosage form) dosage form as their salts or ionized forms. As a consequence, the major cause of drug incompatibility is acid-base reaction, resulting in precipitation of a non-ionized forms. The pH and drug pKa values play a key role in the incompatibility. For an example, oxytetracycline-HCl injection becomes relatively unstable in solution that has a pH > 6 (especially solution that contains calcium or magnesium), forming an insoluble complex.

Drug incompatibility often results in visible precipitation (turbidity, haziness or crystal formation) and are therefore clinically hazardous. The consequences range from minor effects such as thrombophlebitis to major effects such as organ failure. Preventive measure is essential to avoid these consequences. First, we must perform thorough compatibility checking from databases, literature and information material. Planning and assessment regimes in order to avoid mixing of drugs that have to be administered separately also need to be performed. If two drugs are prescribed, they need to be administered at different time or administered differently.

Materials

Diazepam injection	
Furosemide injection	0.9 % NaCl
Lactated Ringer's injection	5 % dextrose
Calcium gluconate	Midazolam injection

Equipment

Test tubes	Test tubes racks
Pipette	pH indicator paper

Procedure

a. Fill test tube with the following solution: 0.9% NaCl, lactated Ringer's injection, 5 % dextrose, Calcium gluconate for about 2 ml. Take up some drug solutions and add to each of the test tube drop by drop (not more than 0.5 ml).

b. Check the pH of furosemide and midazolam using pH indicator paper. Fill 1 ml furosemide injection into a test tube. Add midazolam injection drop by drop.

Result and Observation

Observe changes that occur when the drugs and or infus solutions are mix together.

Drugs	Drugs or infus solution	Observation
Diazepam injection	0.9 % NaCl Lactated Ringer's injection 5 % dextrose Calsium gluconate	
Oxytetracycline-HCl injection	0.9 % NaCl Lactated Ringer's injection 5 % dextrose Calsium gluconate	
Furosemide injection	0.9 % NaCl Lactated Ringer's injection 5 % dextrose Calsium gluconate	
Furosemide injection	Midazolam Injection	

Analysis and Discussion

a. What are the mechanisms of the incompatibility that occur in the experiments?
b. What are the consequences of drug incompatibility in clinical setting? Please explain.
c. What can be done to prevent drug incompatibility in clinical setting?

Pharmacogenetic Polymorphisms of CYP2C19

Session **10, 11, 12**

Overview

Pharmacogenetics is dedicated to study how human genetic variations affect a person's response to drugs. This field of study has increasingly gained importance in clinical settings because pharmacogenetics allows the development of personalized medicines that maximizes the drug efficacy while at the same time minimizes the drug toxicity. Genetic polymorphisms occur in genes encoding the enzymes responsible for either the absorption, distribution, metabolism or excretion of drugs. Thus, polymorphism results in the changes of kinetics and dynamics of drugs in human, leading to variability in drug responses.

The Cytochrome P450 (CYP) is the main enzyme responsible for the metabolism for most drugs. CYP2C19, one of the major isoforms of CYP, catalyzes many commonly used drugs metabolism such as diazepam, omeprazole, and phenytoin. The CYP2C19 exhibits high genetic polymorphism frequency in Asia where CYP2C19*2 and *3 alleles contribute to poor metabolizer. In this session, you will learn how to detect whether an individual has genetic variations of the CYP2C19.

Materials

CYP2C19*2 primers

sense primer	5'-AATTACAACCAGAGCTTGGC-3'
antisense primer	5'-TATCACTTTCCATAAAAGCAAG-3'

CYP2C19*3 primers

sense primer	5'-AAATTGTTTCCAATCATTTAGCT-3'
antisense primer	5'-ACTTCAGGGCTTGGTCAATA-3'

QIAamp DNA Mini Kit	Agarose
Nuclease Free Water	TAE buffer
Go Taq® Green Master Mix	Gel red DNA stain
SmaI	BamHI
CutSmart® Buffer	DNA ladder 100 bp

Equipment

Cotton swab	Eppendorf tubes	Nanodrop
PCR tubes	Pippette	Tips
PowerPac™ Basic Power Supply	GelDOC™ 1000	
Thermal cycler		

Procedure

Caution

Please do not touch the end of the cotton swab to avoid sample contamination. Remember to avoid eating, drinking and smoking for 30 minutes before sampling.

1. Sample acquisition
   Use cotton swab to obtain sample of your friend's inner mouth mucosa.

2. DNA extraction and purification
   Use **QIAamp DNA Mini Kit** (*make sure that the amount of the reagent is sufficient*)

   a. Place the cotton swab in a microcentrifuge containing 400 µL water. Carefully shake the cotton to release the sample.
   b. Add 20 µL QIAGEN Protease stock solution (or proteinase K) and 400 µL Buffer AL to the sample. Mix immediately by vortexing for 15 s.
   c. Incubate at 56°C for 10 min. Briefly centrifuge to remove drops from inside the lid.
   d. Add 400 µL ethanol (96–100%) to the sample and mix again by vortexing. Briefly centrifuge to remove drops from inside the lid.
   e. Carefully apply 700 µL of the mixture from previous step to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
   f. Repeat the previous step by applying remaining mixture to the QIAamp Mini spin column.
   g. Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min.
   h. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided) and discard the collection tube containing the filtrate.
   i. Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.
   j. Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
   k. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube and discard the collection tube containing the filtrate.
   l. Carefully open the QIAamp Mini spin column and add 150 µL Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min. (*pure DNA is obtained at this stage*)
   m. Check the quality and the purity of the DNA using nanodrop and electrophoresis on 1 % agarose gel containing 1x gel red DNA stain

3. PCR of CYP2C19 polymorphism
   Genotyping of CYP2C19 is done using Polymerase Chain Reaction (PCR)-Restriction Fragment Length Polymorphism (RFLP) technique. (**Please watch tutorial youtube videos about PCR and RFLP**)

a. Dissolve the primers for CYP2C19*2 and CYP2C19*3 to 100 µM using Nuclease Free Water (1 set of primers contain sense and antisense primers respectively). Dilute the primers to 10 µM.
b. Prepare 25 µL of PCR mixture:

Go Taq® Green Master Mix, 2x	12.5 µL
Sense primer	0.5 µL
Antisense primer	0.5 µL
DNA template	< 250 ng
Nuclease Free Water	up to 25 µL

c. Set the PCR cycle for CYP2C19*2 and CYP2C19*3 as follow on a thermal cycler:

Table 1. PCR cycles for CYP2C19*2

Segment	Cycles	Temperature	Time
1	1	94°C	300 secs
2	37	94°C	60 secs
		55°C	30 secs
		72°C	30 secs
3	1	72°C	300 secs
4	1	4°C	∞

Table 2. PCR cycles for CYP2C19*3

Segment	Cycles	Temperature	Time
1	1	94°C	300 secs
2	37	94°C	60 secs
		52°C	45 secs
		72°C	30 secs
3	1	72°C	300 secs
4	1	4°C	∞

4. Enzyme restriction
   Take 10 µL of PCR products and digest the sample of CYP2C19*2 with SmaI at 25°C for 1 hour. While the sample of CYP2C19*3 is digested using BamHI at 37°C for 1 hour. The reaction mixture is prepared according to the following:

PCR product	10 µL
Restriction enzyme	1 µL
CutSmart® Buffer	5 µL
Nuclease Free Water	34 µL

5. Fragmentation patterns visualization
   a. The DNA fragments is electrophoresed on a 3% agarose gel containing 1x gel red DNA stain
   b. Fragmentation patterns of subjects with *1/*1 and *1/*2 or *1/*3 will result in 1 and 3 bands, respectively.

Result and Observation

Record the result from nanodrop and take the pictures of the electrophoresis gel, then interpret the data.

Analysis and Discussion

a. Please explain the concept of Polymerase Chain Reaction (PCR)-Restriction Fragment Length Polymorphism (RFLP) technique and how it is used to detect genetic polymorphisms!
b. Describe the importance of Cytochrome P450 enzymes, specifically the CYP2C19 and further explain the polymorphisms in CYP2C19, including its consequences!

Interaction of Cisapride and Erythromycin Induced Bradycardia in *Danio rerio* larval

Session 13, 14

Overview

Bradycardia is the condition in which the heart rate is less than 60 beats per minutes (BPM) in adults. However, this rate depends on age, physical condition and species. Due to slower heart rate, the heart does not pump enough oxygen-rich blood to whole body, and therefore It can be a serious problem. There are several causes of bradycardia. One of the factors is due to medications, in which some drugs can disrupt the heart rhythm and change the heart beats.

In this session, you will learn how the combination of two drugs which individually has different purpose, may cause unexpected side effect. Here, you will use cisapride, which stimulates the motility of the upper gastrointestinal tract and erythromycin, an antibiotic, and observe their interaction in the zebrafish larva's heart beats.

Materials

Zebrafish	Filtered Type III Water
PTU (1-phenyl 2-thiourea)	Cisapride 5 mg/ tablet
Erythromycin 500 mg/ tablet	6-well plates
DMSO	Ethanol

Equipment

Pasteur Pipette
Stereo Microscope

Procedure

1. Prepare the stock PTU solution (0.4 mM)
   PTU solution is prepared by mixing PTU (0.4 mM) in DW type 3. Prepare 50 ml of PTU for each group. (Mw of PTU = 152.22 g/mol)

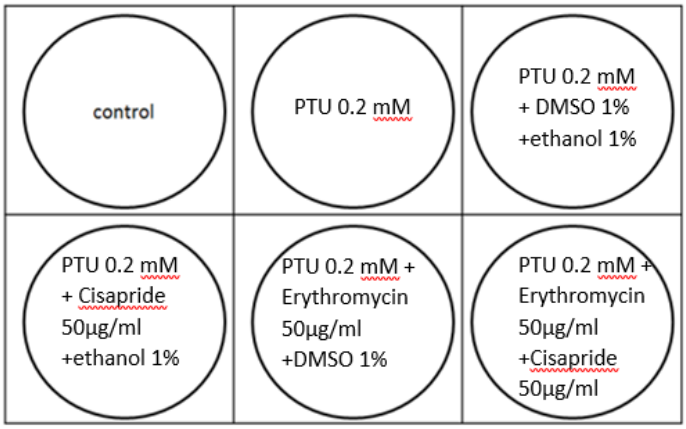
2. Prepare the stock of Cisapride solution
   Cisapride soluble in DMSO. Each tablet contains 5 mg of cisapride. The maximum amount of DMSO per well is 1% (1 well filled with 6 ml medium. Maximum amount of DMSO is 60 µl per well). The stock solution should contain **300 µg cisapride in 60 µl DMSO**.
   a. Prepare 1 ml of cisapride stock solution (5 mg cisapride in 1 ml DMSO). First, weigh 1 tablet of cisapride, record the weigh. Then crush 2 tablets using mortar and pestle. Weigh the powder by using this formula:
   b. Cisapride taken = $\frac{5\ \text{mg}}{5\ \text{mg}}$ x weigh of 1 tablet

c. Dissolve the amount into 1 ml of DMSO in an Eppendorf tube. Vortex it thoroughly for 5 minutes and centrifuge the solution at 10,000 rpm for 1 minute to remove insoluble parts.
d. Take out the supernatant and put into a new Eppendorf tube.

3. Prepare the stock of Erythromycin solution
   Erythromycin is soluble in ethanol. Each tablet contains 500 mg of erythromycin. The maximum amount of ethanol per well is 1% (1 well filled with 6 ml medium. Maximum amount of ethanol is 60 µl per well). The stock solution should contain **300 µg erythromycin in 60 µl ethanol**.
   a. Prepare 1 ml of erythromycin stock solution (5 mg erythromycin in 1 ml ethanol). First, weigh 1 tablet of cisapride, record the weigh. Then crush 1 tablet using mortar and pestle.
   b. Weigh the powder by using this formula:
      $$\text{Erythromycin taken} = \frac{5 \text{ mg}}{500 \text{ mg}} \times \text{weigh of 1 tablet}$$
   c. Dissolve the amount into 1 ml of ethanol in an Eppendorf tube. Vortex it thoroughly for 5 minutes and centrifuge the solution at 10,000 rpm for 1 minute to remove insoluble parts.
   d. Take out the supernatant and put into a new Eppendorf tube.

4. Preparing Zebrafish for Treatment
   a. Breed zebrafish in the afternoon or early evening at day 0 with male: female ratio 1:3.
   b. Harvest the embryo in the morning at day 1, collect living embryos and remove unfertilized and died embryos.
   c. Put 1 healthy <24 hpf embryos on six well plate. Add 6 ml of PTU 0.2 mM to the plate to inhibit melanization that will disturb cardio-observation.
   d. Treat the zebrafish at day 3 (when the zebrafish are 2dpf).

5. Treatment
   a. Distribute 2dpf zebrafish embryos into six-well microplates in 6mL fish water (added PTU 0.2 mM per well) containing the drug following this treatment map. Each mixture should be prepared before added into the wells.



b. Expose embryo with drug for 4 h prior to heart rate assessment.
c. After drug treatment, examine larva zebrafish under a stereo microscope at room temperature

Note:

- The PTU+DMSO+ethanol wells only contain PTU 0.2mM, 1% DMSO and 1% ethanol in total 6 ml of DW type 3. Calculate how many PTU 0.4 mM, DMSO and ethanol should be added in each well!
- Calculate how many cisapride and erythromycin solutions should be taken from cisapride and erythromycin stock to get the concentration of 50 µg/ml of total 6 ml of medium!

Result and Observation

1. Heart Rate Examination
   After incubation, zebrafish were visualized on a stereo microscope, and ventricular contractions for 30 s were counted manually. The number of contractions was multiplied by 2 to calculate heart rate, reported in beat per minute (bpm).

2. Cardiac Morphology & Pericardial Edema Observation
   a. After compound treatment, move the zebrafish larval into petri dish.
   b. Examine the zebrafish larval under stereomicroscope after 72 hours. Use minimum 8x magnification during observation. The zebrafish heart has one atrium and one ventricle. In normal condition, the atrium and ventricle are side by side; blood flows into the atrium from *sinus venosus* and out the ventricle through *bulbus arteriosus*. Morphological abnormalities, including the size of the heart and number position of the heart chamber need to be examined. Use consistent magnification during recording the data.
   c. Pericardium is the membrane enclosing the heart. Pericardial edema or pericardial effusion is an abnormal accumulation of fluid in the pericardial cavity. Examine the presence of pericardial edema in zebrafish larval after treatment.
   d. Abnormalities in heart will lead abnormalities of blood circulation which can lead to other abnormalities including hemorrhage. Hemorrhage is any profuse internal or external bleeding from the blood vessels.
   e. Record the abnormalities of the heart in the following table:

Observation	Control	PTU	PTU+ DMSO + Ethanol	PTU+ Erythromycin + DMSO	PTU+ Cisapride + Ethanol	PTU+ Erythromycin + Cisapride
Mean of heart rate (bpm)						
Size of the heart (cm)						
Presence of Edema						

Analysis and Discussion

Compare the result from your observation from each treatment group. Try to explain the mode of action of each drug and the combination of the drugs on the heart of zebrafish larvae. Collect the data from all groups.

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ISBN 978-623-92687-2-5