UNIVERSITI PUTRA MALAYSIA

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ABSTRACT

COMPARISON OF ANTIMUTAGENIC ACTIVITY OF TWO BENZIMIDAZOLE DERIVATIVES WITH (+S9) AND WITHOUT (-S9) METABOLIC ACTIVATION

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Introduction: Antimutagenic activity refers to inhibition of mutation that leads to severe disease such as cancer. Benzimidazole is a heterocyclic aromatic compound which consists of benzene and imidazole that plays various important roles as therapeutic agents such as anti-ulcer, anthelmintic, anti-microbial, anti-viral, anti-inflammatory and analgesic drugs. However, the study of benzimidazole derivatives on cancer is still limited. **Objectives:** This study aims to evaluate the potential of antimutagenic activity of two benzimidazole derivatives (NN-1-5 and NN-1-7) using Ames test, followed by the comparison of structure-activity relationship (SAR). **Methodology:** Ames test was carried out by using standard mutagens (sodium azide, 2-nitro-fluorene and 2-aminoanthracene) as mutagenic inducer on two strains of *Salmonella typhimurium* (TA98 and TA100). The bacteria strains, TA98 and TA100 were used to assess the frameshift mutation and base-pair substitution mutation respectively. Five different concentrations (0.31, 0.63, 1.25, 2.5 and 5.0 mg/plate) of benzimidazole derivatives were tested on both strains in the absence (-S9) and presence (+S9) of metabolic activation. Group treated with PBS only was used as the negative control. Then, data of three independent experiments were analysed using two-way ANOVA and followed by post hoc Tukey's test, $p<0.05$ indicate a significant value. It is considered absent/weak, moderate and strong antimutagenic compound if the percentage of mutagenic inhibition are <25%, (25-40%) and >40% respectively. The SAR was analysed based on the antimutagenic activity. **Results:** The data revealed that the compounds without metabolic activation had a significant ($p<0.05$) antimutagenic effect with strong antimutagenic activity in TA98 and less to moderate antimutagenic activity in TA100. In contrast, metabolic activation of tested compounds had produced no significant ($p>0.05$) antimutagenic effect. **Conclusion:** Both benzimidazoles derivatives had potential as antimutagenic agent specifically on frameshift mutations. However, the compound (NN-1-5) with one hydroxyl groups has a stronger antimutagenic effect as compared to (NN-1-7) with two hydroxyl groups.

Keywords: antimutagenic, benzimidazole derivatives, Ames test, structure-activity relationship (SAR)

ABSTRAK

PERBANDINGAN AKTIVITI ANTIMUTAGENIK ANTARA DUA BENZIMIDAZOLE TERBITAN TANPA PENGAKTIFAN METABOLIK (-S9) DAN PENGAKTIFAN METABOLIK (+S9)

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Pengenalan: Aktiviti antimutagenik merujuk kepada perencatan mutasi yang membawa kepada penyakit merbahaya seperti kanser. Benzimidazole adalah sebatian aromatik heterosiklik yang terdiri daripada benzene dan imidazole yang memainkan pelbagai peranan penting sebagai agen terapi seperti anti-ulser, anti-helmintik, anti-mikrob, anti-virus, anti-inflamasi dan ubat analgesik. Walau bagaimanapun, kajian terhadap benzimidazole terbitan terhadap kanser masih lagi terhad. **Objektif:** Kajian ini bertujuan untuk menilai keupayaan aktiviti antimutagenik antara dua benzimidazole terbitan (NN-1-5 and NN-1-7) dengan mengunakan ujian Ames dan diikuti dengan perbandigan hubungan aktiviti-struktur (SAR). **Metodologi:** Ujian Ames dijalankan menggunakan standard mutagen (sodium azide, 2-nitro-fluorene dan 2-aminoanthracene) sebagai perangsang mutagenik dalam dua strain *Salmonella typhimurium* (TA98 and TA100). Bakteria strain, TA98 dan TA100 digunakan untuk menilai mutasi frameshift dan mutasi penggantian pengembalian asas. Lima siri kepekatan (0.31, 0.63, 1.25, 2.5 and 5.0 mg/plate) benzimidazole terbitan dikaji pada kedua-dua strain tanpa (-S9) pengaktifan metabolik dan dengan (+S9) pengaktifan metabolik. Kumpulan yang dirawat dengan PBS sahaja, digunakan sebagai kawalan negatif. Kemudian, data daripada tiga ujikaji dianalisa dengan ANOVA dua-arah dan diikuti ujian lanjut Tukey, $p<0.05$ menunjukkan nilai yang signifikasi. Kompaun dianggap sebagai tiada/lemah, sederhana dan tinggi aktiviti antimutagenik sekiranya peratusan perencatan mutasi adalah <25%, (25-40%) dan >40% masing masing. SAR dianalisa berdasarkan aktiviti antimutagenik. **Keputusan:** Data menunjukkan sebatian tanpa pengaktifan metabolik mempunyai kesan antimutagenik yang signifikasi ($p<0.05$) dengan aktiviti mutagenik yang tinggi dalam TA98 dan aktiviti mutagenik yang kurang dan sederhana dalam TA100. Berbeza dengan kompaun yang diuji dengan pengaktifan metabolik yang menghasilkan kesan antimutagenik yang tidak signifikan ($p>0.05$). **Kesimpulan:** Kedua-dua benzimidazole terbitan mempunyai potensi sebagai agen antimutagenik khusus pada mutasi frameshift. Walau bagaimanapun, kompaun (NN-1-5) dengan satu kumpulan hidroksil mempunyai kesan antimutagenik yang tinggi berbanding dengan kompaun (NN-1-7) yang mempunyai dua kumpulan hidroksil.

Kata kunci: antimutagenik, benzimidazole terbitan, ujian Ames, Hubungan Aktiviti-Struktur (SAR)

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LIST OF ABBREVIATION

2-AA	2-Aminoanthracene
2-NF	2-Nitrofluorene
ATCC	American Tissue Cell Culture Company
ANOVA	Analysis of Variance
DNA	Deoxyribonucleic acid
PI	Percentage Inhibition
PBS	Phosphate Buffer Saline
SA	Sodium Azide
SAR	Structure-Activity Relationship
SEM	Standard Error of Mean
UITM	Universiti Teknologi Mara

CHAPTER 1

INTRODUCTION

1.1 Introduction

Cancer is an uncontrolled growth of cells that arises from a single cell where it metastasizes to other cells or tissues, disrupting the normal function of the cellular activity in the body. According to the Global Cancer Observatory (GLOBOCAN, 2019), the number of new cancer cases arise in Malaysia in 2018 is about 43837 cases while the number of cancer deaths is 26395 people among the population. It estimated that the risk of developing cancer before the age of 75 years is about 14.5% and the number of prevalent cases in 5 years is about 106262 cases. Cancer can occur due to genetic factors and environmental agents such as chemicals, radiation and viruses. Mutagen or carcinogen can cause a mutation that leads to cancer progression.

Mutation is a permanent changes in the structure or nucleotide sequence of DNA that result in cancer development. Mutation act as the initiators in the first stage of carcinogenesis (initiation) causing the irreversible DNA damage and increase the susceptible of mutated cells to transform into neoplastic cells in promotion and progression stages. There are several types of mutation that can occur in the body included point mutation. Point mutation is a mutation which there is a single change on nucleotide sequence. The mutation can affect at the DNA level and protein level. Examples of point mutation are base-pair substitution mutation and frameshift mutation. Base pair substitution is mutation involve single changes in base pair of the nucleotide being replaced with another, affecting the DNA level. Frameshift mutation is a mutation that involves insertion and deletion in the nucleotide sequence, causing the changes in the reading frame of a codon that produce different amino acid different than original, affecting the protein level (Griffiths et al., 1999).

Antimutagens is agents that have ability to inhibit mutagen effects. There are two mechanisms of antimutagens which are desmutagens and bioantimutagens. Desmutagen is an agent that can inactivate the mutagen before they reach the DNA

while bioantimutagen is an agent that can suppress the mutation within the cell after the DNA damage. Several tests can be conducted to evaluate the antimutagenic activity of the compound. The antimutagenic testing can be done in vitro and in vivo. The commonly used for the antimutagenicity test is reverse mutation assay (Ames test) whether using *Salmonella thyphimurium* or *Escherichia coli* WP2 which applied in vitro. Other in vitro test include SOS chromotest that using *Escherichia coli* PQ37 and antimutagenic assay on yeast using *Saccharomyces cerevisiae* strains can be used to determine the antimutagenic activity. For in vivo tests such as micronucleus test and Allium cepa test (Malini et al., 2010; Leme & Marin-Morales, 2009).

Benzimidazole derivatives were studied as potential anticancer drug candidates for treating various disease as it were reported to possess various therapeutic activities included anti-ulcer, anti-proliferative and anti-inflammatory. Currently, there is a limited study that evaluate the potential antimutagenic activity of benzimidazole derivatives.

1.2 Problem Statement and Justification

Antimutagenic study is important to find drug that can inhibit the mutagens from impairing or mutated the DNA structure. Inhibition of the carcinogen or mutagen that act as initiators in cancer progression may benefit the researcher to search the 'magic bullet' for treating cancer with high therapeutic activity and less side effect. Our research group have found that the same benzimidazole derivatives have anti-inflammatory, antioxidant and cytotoxic effect on breast cancer which these findings have been submitted for publication. The same benzimidazole derivatives also reported to produce no mutagenic activity in presence and absence of metabolic activation (Azahar et al., 2019). Therefore, study on antimutagenic activity is crucial to determine whether the same benzimidazole derivatives have potential antimutagenic activity that may add a value for the compound to act as potential anticancer drug.

1.3 Objectives

1.3.1 General Objectives

1. To evaluate the potential of antimutagenic activity of two benzimidazole derivatives.

1.3.2 Specific Objectives

1. To measure the percentage of mutation inhibition of two benzimidazole derivatives.
2. To compare the structure-activity relationship (SAR) of two benzimidazole derivatives.

1.4 Hypothesis

1. The benzimidazole drivatives significantly inhibit the mutation on *Salmonella typhimurium* strains that induced with mutagens.
2. The differences position of hydroxyl group of benzimidazole derivatives attached to benzene ring will affect their antimutagenicity activities.

CHAPTER 2

LITERATURE REVIEW

2.1 Mutation

Mutation defined as a process of inducing the permanent heritable changes in the DNA nucleotide sequences or structure of an organism by insertion or elimination of one or more bases in the gene which altered the gene product. Mutation occurs spontaneously or can be induced by environment, industrial, dietary and natural chemicals result in interruption of the DNA repair, replication and recombination which can lead to cancer and other degenerative diseases. The agents that are capable to induce mutation known as mutagens or mutagenic agents, causing the lesions in DNA such as strand break, base damage and dimerization of bases (Sloczynska et al., 2014). Mutation at certain genes such as suppressor genes, oncogenes and genes involved in DNA repair which can leads to uncontrollable of cell proliferation, resulting in genetic instability. Mutation not only involved in cancer development but can also cause genetic disorders in somatic cells. The effect of the mutagenic effects of genotoxic chemicals are additive, cumulative and sometimes irreversible (Mohammed, 2016).

Point mutation involves single changes on nucleotide sequences either by insertion, deletion or substitution that affected at the DNA level. The substitution mutation is a mutation that replaces a single base pair of nucleotide with another. There are two subtypes of base substitution, transition and transversion. Transition involves a base being replaced by the other same chemical base group. For instance, pyrimidine replaced by pyrimidine (T or C) or purine replaced by purine (A or G). In contrast, transversion is the replacement of a base with other different chemical base groups such as pyrimidine replaced by purine or vice versa. Apart from that, simultaneous insertion and deletion mutation can lead to multiple changes in base pairs, resulting in a frameshift mutation. Frameshift mutation causing the change in the reading frame of codon to shift forward or backwards affected the protein level by producing different

amino acid than original. The base-pair substitutions can also affect the protein level such as silent, missense and nonsense mutations. The silent mutation is a mutation that change single codon for a particular amino acid replaced with another codon that codes for the same amino acid. The missense mutation involves a codon for one amino acid change to a codon for another amino acid that has similar chemical properties (conservatives) or different chemical properties (non-conservatives). The nonsense mutation occurs when a codon for one amino acid replaced with the stop codon, forming inactive protein products (Griffiths et al., 1999).

2.2 Carcinogenesis

Cancer or malignant tumour is an uncontrolled growth of cells that arises from a single cell. It can metastasize to other tissues in the body by disrupt the tissue structure and cause blood vessel growth or angiogenesis. The development of cancer or carcinogenesis can be initiated by genetic factors and environmental agent such as chemicals, radiation or viruses. Factors that cause cancer or carcinogen can damage the DNA of cells and tissues after being exposed to it. There are three process in carcinogenesis which are initiation, promotion and progression.

Initiation is the first steps where the initiators activates via metabolism enzymes, resulting in permanent changed in DNA or mutations. Most of the initiators are mutagenic or genotoxic which form the mutated cells that will produce the daughter cells which also carrying the mutation. Thus, the effects of initiators are irreversible and the cells are susceptible to undergo second stage of cancer development, promotion. However, the conversion of an initiated cells to a fully malignant neoplasm usually prolong process which take years to develop in human (Sutandyo, 2010; Cooper & Hausman., 2000).

Promotion is the process where the initiated cell transformed to neoplastic due to disruption of cellular balance where the cells undergo further proliferation. There are two types of promoters which are specific promoters and nonspecific promoters. Specific promoters interact with receptors on the target cells while nonspecific do not interact with receptor where both promoters can result in alteration of gene expression.

Progression is the process which the neoplasm form into malignant form. The process can be increase by repeated exposures to carcinogens (Malarkey et al., 2013).

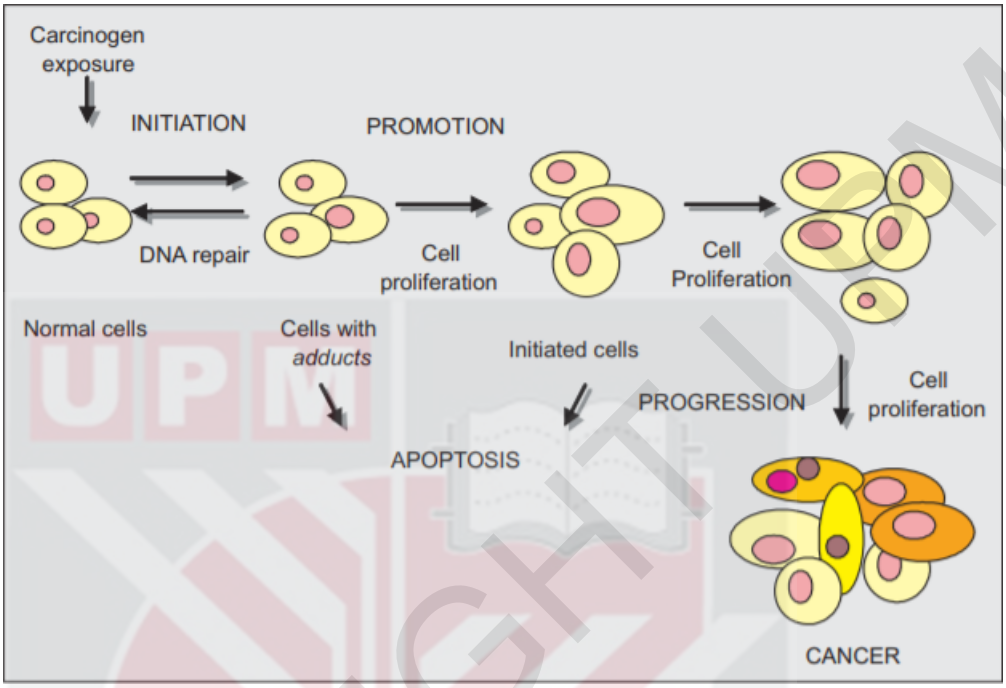


Figure 2.1: The stage of carcinogenesis (Sutandyo, 2010).

2.3 Antimutagen

The agent that able to inhibit or reduce the mutagen effects is called antimutagenic agent or antimutagen. Antimutagens can be natural and synthetic. Example of natural antimutagenic agents are cinnamaldehyde, lycopene, punicalagin and ellagic acid. Mestranol (steroidal hormonal molecules), gallic acid, tannic acid, synthesis β- aminoketones and xanthones are synthetic antimutagens that were reported to show antimutagenic activity (AbdelHakem & Abdelhafez, 2020).

2.3.1 Mechanism of Antimutagen

Antimutagen can also directly or indirectly activate or inactivate the enzymes used in the DNA repair, recombination and replication pathways. Antimutagens can be classified into two categories based on the initiation site of antimutagenicity which

are desmutagen and biomutagen. Desmutagen is agent that act as blocking agent by fully or partially inactivate the mutagens by the enzymatic or chemical interaction of mutagen before it damages the gene. Bio-antimutagen can inhibit the mutation process of genes which damaged by the mutagens. It can act as suppression of neoplastic process, inhibition of error prone re-pair or interruption of cellular DNA fixation process. There are several mechanism of antimutagens that can be classified broadly into antimutagens with antioxidant activity, chemical or enzymatic inactivation, direct interaction with mutagen,

Antimutagenic agents can act as an antioxidant and free radical scavenger which can prevent the Reactive Oxygen Species (ROS) from inactivating the tumour suppressor genes, causing cancer. Several antitumor compounds were reported to act through the antimutagenic mechanism. Antimutagens can also inhibit the activation of mutagens by acting on DNA, protein and enzymes. Antimutagens can suppress the promutagens bioactivation in metabolic activation which involves phase 1 metabolic enzymes such as the cytochrome P450. For example, indole-3-carbinol reported to act as cytochrome P450 enzymes inhibition (Mohammed et al., 2016; Sloczynska et al., 2014).

2.4 Benzimidazole derivatives

Benzimidazole is an organic compound with heterocyclic aromatic ring formed by fusion of benzene and imidazole ring shown in the figure 1 (Azahar et al., 2019).

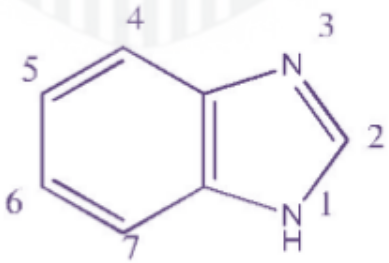


Figure 2.2: The structure of benzimidazole nucleus (Azahar et al., 2019)

Previous studies shown that benzimidazole and its derivatives structure have pharmacological activity and have been utilized in development of drug and materials.

Benzimidazole nucleus was developed as plant fungicides before it discovered and approved as antihelmintics. For example, fenbendazole, oxfendazole, parbendazole and cambendazole. Based on the structure-activity relationship studies, the substitution of C-2 position in the heterocyclic system can highly affected the biological activities. Benzimidazole can act as anthelmintic by kill or expel the infested helminth. For example, mebendazole and albendazole. Omeprazole was used as anti-ulcer drug that inhibit gastric acid secretion in the body (Bansal & Silakari, 2012).

2.4.1 Application of Benzimidazole derivatives

2.4.1.1 Anticancer

Heterocyclic benzimidazole derivatives were synthesized by condensation of succinic acid, homophthalic acid and 2,3-pyrazinedicarboxlic via microwave irradiation along with various substituted diamines. All derivatives exerted good anticancer activity against ovary (IGR-OV-1), breast (MCF-7) and central nervous system (SF-295) human cancer cell lines at 50 mg/kg (Sondhi et al., 2010). Series of benzimidazole such as 2-[(4-oxothiazolidin-2-ylidene)-methyl, (4-amino-2-thioxothiazol-5-yl) benzimidazoles, 2-[(4-fluorobenzylidene and cycloalkylidene)-cyanomethyl] benzimidazoles were evaluated against three cell lines; human hepatocellular carcinoma cell line (HEPG2), human breast adenocarcinoma cell line (MCF7) and colon carcinoma cell line (HCT 116) (Refaat, 2010).

2.4.1.2 Antiproliferative

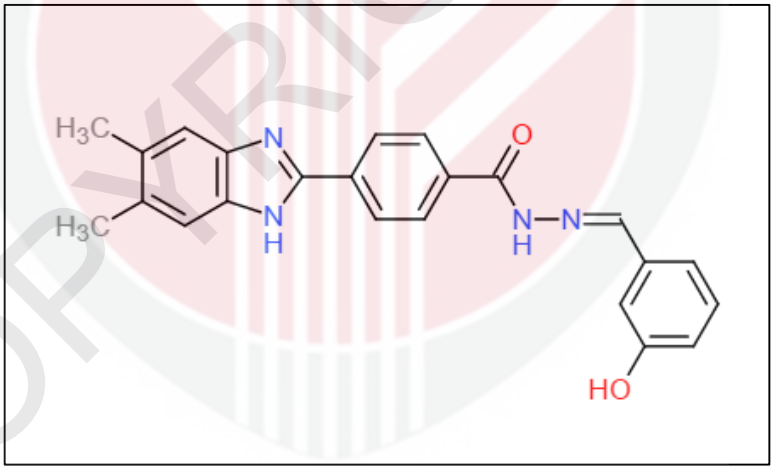
The novel series of benzimidazole substituted Schiff bases were synthesized by aromatic aldehydes reaction with 2-aminobenzimidazoles that exert non-specific antiproliferative activity in vitro. The synthesized Shiff bases were reported to have strong antiproliferative activity on HeLa and MCF-7 cell lines at the highest tested concentration (Hranjec et al., 2011). The series of 2-(5-phenylindol-3yl)benzimidazole derivatives were reported to show antiproliferative activity on MDA-MB-231 (human breast cancer) cells. The tested compounds change the levels of reactive oxygen

species (ROS) by entering the lysosome of breast cancer cells and causing the mitochondrial damage (Wang et al., 2019).

2.4.1.3 Mutagenic and antimutagenic

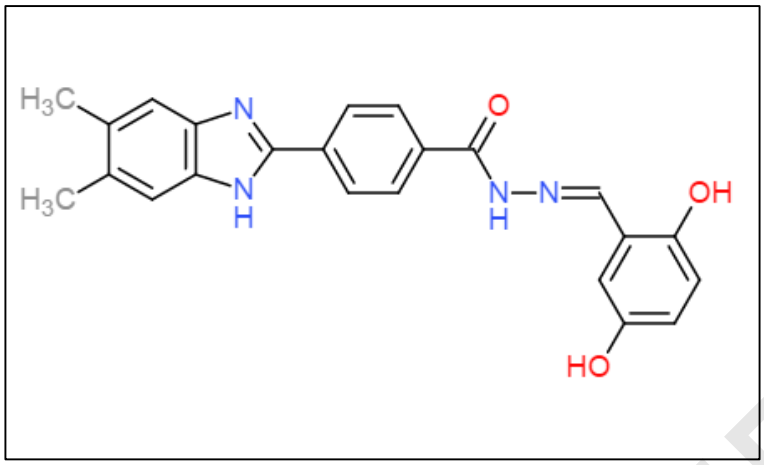
Previous study shown that all novel benzimidazole derivatives showed no mutagenic activity in the absence and presence of metabolic activation in TA98 and TA100 strains (Azahar et al., 2019). 2-(2-Furyl)benzimidazole (FBD) and its derivatives show antimutagenic by inhibit the aflatoxin B_1 biosynthesis. Aflatoxin produced as secondary metabolites in saprophytic fungi and it is mutagenic. The tested derivatives down regulated *aflR* gene that involved in regulation and *aflB* structural gene in aflatoxin B_1 biosynthesis (Dhanamjayulu et al., 2019).

2.4.2 Chemical structure of benzimidazole derivatives



a) NN-1-5 (MW: 384.43 g/mol)

(*E*)-4-(5,6-dimethyl-1H-benzo[*d*]imidazol-2-yl-*N'*-(3-hydroxybenzylidene)benzohydrazide



b) NN-1-7 (MW: 400.43 g/mol)

(*E*)-*N'*-(2,5-dihydroxybenzylidene)-4-(5,6-dimethyl-1H-benzo[*d*]imidazol-2-yl)benzohydrazide

Figure 2.3: The structure of newly-synthesized benzimidazole derivatives.

The benzimidazole derivatives have different number and position of the hydroxyl groups (-OH) attached to phenyl ring. The structure for compound (a) have one hydroxyl group attached at the meta-position of phenyl ring while compound (b) have two hydroxyl groups were attached at the ortho-meta-position of phenyl ring (Azahar et al., 2019).

2.5 Antimutagenic test

There are several tests that can be conducted to evaluate the antimutagenic and genotoxicity activity of the tested compounds. The antimutagenicity testing can be conducted in vitro and in vivo. For in vitro test, researchers commonly used bacteria to evaluate the inhibition of mutation such as Ames test either using *Salmonella thyphimurium* or *Escherichia coli* WP2 and SOS chromotest using *Escherichia coli* PQ37. The antimutagenic assay also can be applied on yeast using *Saccharomyces cerevisiae* strains. For in vivo test, the micronucleus test in mice was used for antimutagenic evaluation. Micronucleus test used cyclophosphamide for treatment and the bone marrow sample were collected to evaluated the frequency of Polychromatic

Erthrocytes (PCE's), Normochromatic Erythrocytes (NCE's), Micronuclesated Normochromatic Erthtocytes (MNNCE's) and P/N ratio were calculated after 24, 48 and 72 hours of cyclophosphamide administration. The antimutagenic activity was evaluated by its ability to inhibit the effect of cyclophosphamide that induce mutation (Golwala et al., 2020). Micronucleus assay with CHO-K1 (blockage of cytokinesis in ovary cells) and Allium cepa test were reported to be applied in studies of antimutagenic activity (Malini et al., 2010; Leme & Marin-Morales, 2009).

2.6 Ames test

Ames test is a bacterial assay that employs the used of bacteria strain such as *Salmonella typhimurium* to identify the mutagenic of tested substances through the induction of reverse mutations in the *his* operon of genetically modified *S. typhimurium* strains (Mortelmans & Zeiger, 2000). The Ames test can be used to detect antimutagenic activity by adding test compound and standard mutagen (Sloczynska et al., 2014).

2.6.1 Bacterial strains

The Salmonella strains that commonly used contain a mutation in the histidine operon include TA100 and TA1535 (*his*G46) and TA98 and TA1538 (*his*D3052). These mutated bacterial strains unable to produce histidine to survive but they gain their original state which they have ability to produce histidine in the presence of mutagenic chemicals. The test used different genotypes of tester strains to evaluate different mutation mechanism. For instance, *his*G46 (TA100) in the strains can detect mutagen that induces the base-pair substitution while *his*D3052 (TA98) in the strains can identify the mutagen that causes the frameshift mutation. The test using *S. typhimurium* can also be applied to *Escherichia coli* WP2 reverse mutation assay with additional amounts of tryptophan instead of histidine to the top agar for detection of AT base pair damage (Mortelmans & Zeiger, 2000).

Table 2.1: The genotypes of *Salmonella thyphimurium* strains.

Histidine operon	Mutation (strain)	uvrB deletion	Lipolysaccharide (LPS) defect	Plasmid
*his*G46	TA1535	Presence	*rfa* mutation	No plasmid
	TA100	Presence	*rfa* mutation	pKM101
*his*D3052	TA1538	Presence	*rfa* mutation	No plasmid
	TA98	Presence	*rfa* mutation	pKM101

Source: Mortelmans & Zeiger, (2000)

2.6.2 Metabolic Activation

The bacteria required the presence of exogenous mammalian metabolic activation for the mutation to occur since the bacteria do not have the ability to metabolize chemicals via cytochrome P450. Some mutagens also needed metabolic activation to become active for reacting with the DNA (Mohammed et al., 2016). There are several standard mutagens were used in Ames test included 2 aminoanthracene (2-AA) and sodium azide (NaN_3). 2-AA involved indirect-acting that can form DNA adducts and sodium azide involved direct-acting that can form point mutation. 2-nitrofluorene (2NF) is used on TA98 while sodium azide was used on TA100 in the absence of metabolic activation. 2-AA is used on both TA98 and TA100 in the presence of metabolic activation (Sloczynska et al., 2014; Di Sotto et al., 2008). Thus, a rodent metabolic activation system (S9 fraction) was included in the test system. S9 fraction contains liver homogenate from rats that consist of phase I and phase II drug metabolite enzymes (Snijman et al., 2007). The animal was pre-treated with mixed-function oxidase inducer Aroclor 1254 to increase the level of metabolizing enzymes. The cytochrome P450 mainly present in the liver is capable to metabolize a large number of carcinogenic chemical to DNA-reactive, electrophilic forms. The rat liver homogenate delivered to the test system in the presence of NADP and cofactors for NADPH-supported oxidation (Mortelmans & Zeiger, 2000).

2.6.3 Positive control and Negative control

The positive controls can be used in the experiment without metabolic activation were 2-nitrofluorene (TA98) and sodium azide (TA100). For presence of metabolic activation, 2- aminoanthracene was used for both strains.

Table 2.2: The range of spontaneous colonies for negative control in absence and presence of metabolic activation for validation.

Spontaneous Revertant Control Values		
Strain	Number of Revertants	
	Without S9	With S9
TA98	20-50	20-50
TA100	75-200	75-200
TA1535	5-20	5-20
TA1538	5-20	5-20

Source: Mortelmans & Zeiger, (2000)

2.6.4 Bacteria counting and percentage of inhibition

The percentage of inhibition (PI) was calculated based on formula:

$$\text{PI} = 100 - \frac{\text{number of revertant colonies in test plates}}{\text{number of revertant colonies in positive control plates}} X\ 100\%$$

The percentage of antimutagenic effect which is less than 25% are consider less or no antimutagenic, 25% to 40% are consider moderate and more than 40% are consider strong antimutagenic (Di Sotto et al., 2008).

2.6.5 Structure Activity Relationship (SAR)

The SAR is conducted to study the relationship between the chemical or 3D structure of a compound to its biological activity. The SAR allows the determination of the chemical groups responsible for producing the desire biological effect in the organism by changing the chemical structures to modify the effect of bioactive compound (Shankar et al., 2014).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Material and Apparatus

Benzimidazole derivatives were obtained and synthesized by researchers from UITM, Shah Alam, Malaysia. Purity of the compound is between 95-99%.

3.1.1 Bacterial Strains

Two strains of *Salmonella typhimurium* bacteria, TA98 and TA100 were purchased from ATCC and were used in the antimutagenicity assay. Both strains possessed histidine dependent, *uvr* genes deletion, *rfa* gene mutation and carry plasmid pKM-101.

3.1.2 Chemicals Reagents and Media

Bacto agar (No. 1), nutrient broth (No. 2), nutrient agar and dextrose (D(+)-glucose) powders were purchased from Oxoid Ltd, France; sodium azide, 2-nitrofluorene, 2-aminoanthracene, magnesium sulphate ($MgSO_4.H_2O$), citric acid monohydrate, potassium phosphate dibasic anhydrous (K_2HPO_4), sodium ammonium phosphate ($Na_2NH_2PO_4.4H_2O$), D-biotin, L-histidine, sodium chloride (NaCl), sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$), disodium hydrogen phosphate ($Na_2HPO_4.H_2O$), Potassium chloride (KCl), magnesium chloride ($MgCl_2.6H_2O$), dimethyl sulphoxide (DMSO), rat liver S9 (Aroclor 1254-induced), NADP and glucose-6-phosphate powders.

3.1.3 Apparatus and Equipment

The apparatus and equipment used for antimutagenicity testing were disposable Petri plates (90×15-mm), Schott Duran and Pyrex test tubes, conical flasks (100mL and 1L volume), test tube racks, Schott bottles (100mL, 250mL, 500mL and 1L volume), micropipettes (1-10µL, 10-100µL, and 100-1000µL), pipette tips in the rack (10µL, 200µL, and 1000µL), sterilized pipettes tips (10µL, 200µL, and 1000µL), pipette gun, sterilized serological pipettes (2ml, 5ml, 10ml and 25ml), cryogenic vial (2ml), measuring cylinders, beakers, autoclave tape, gloves, biohazard bags, autoclave machine, shaking waterbath, incubator, bunsen burner, microwave oven, magnetic stirrers, weighing balances, water purification system, spectrophotometer (660nm wavelength), -80°C and 4°C freezer, biological safety cabinet and fume hood.

3.2 Methodology

3.2.1 Long Term Storage of Bacteria

12mL of nutrient broth followed by 12µL of bacteria from frozen stock which is preserved in -80°C was inoculated into 100ml conical flask. Then, the bacterial culture was incubated for 7 hours at 4°C before transferring into shaking waterbath and incubated for 12 hours at 37°C and 100rpm. After that, 1mL of the overnight bacterial culture was transferred into 250mL nutrient broth in a 1L conical flask and incubated for another 12 hours in shaking waterbath at 37°C and 100rpm. After 12 hours incubation, 800µL of bacteria culture was aliquoted into a cryovial and 80µL of 100% DMSO was added to the same cryovial. The mixture was gently mixed and quickly preserved in -80°C.

3.2.2 Thawing Bacteria

12mL of nutrient broth was aliquoted into a 100 ml sterile conical flask. The frozen stock of bacteria preserved in the cryovial was taken from -80°C and it was allowed to melt. After that, 12µL of bacteria was inoculated into 100 ml conical flask containing the aliquoted nutrient broth.

3.2.3 Growing Overnight Bacteria Culture

The bacteria culture was incubated at 4°C for 7 hours in the chiller after the bacteria were thawed. Then, the bacteria culture was transferred to the shaking waterbath to incubate for another 12 hours at 37°C and 100rpm. After 12 hours, 3ml of bacteria suspension was transferred into a cuvette to determine the concentration of the bacteria by measuring the absorbance using a spectrophotometer at 660nm wavelength. The optical density (OD) value after 12 hours incubation should be between 1.0 to 2.0 and the OD of bacterial suspension must be within the same range before proceeding to the Ames test.

3.2.4 Antimutagenic Assay (Ames test)

3.2.4.1 Stock and Concentration of Positive and Negative Control

25mg/ml (2.5µg/plate) of 2-nitrofluourene for TA98 and 50mg/ml (5.0µg/plate) of sodium azide for TA100 strain were prepared as positive control without the presence of metabolic activation. 50mg/ml (5.0µg/plate) of 2-aminoanthracene for both TA98 and TA100 strains were prepared as a positive control with the presence of metabolic activation. PBS was used as a negative control for both strains with and without metabolic activation. The experiment was done in duplicate for three independent experiments.

3.2.4.2 Stock and Concentration of Benzimidazole Derivatives

Benzimidazole compounds were weighed and dissolved into 100% DMSO to prepare the stock solution. Five concentrations of the compound were used in the experiment which are 0.31µg, 0.63µg, 1.25µg, 2.5µg and 5.0µg per plate. The highest concentration of compound was prepared by diluting with a correct amount of stock solution of PBS to obtain 50µg/ml (5.0µg/plate). Then, serial dilution was conducted to obtain the lower concentrations of compounds which are 25µg, 12.5µg, 6.3µg and 3.1µg per millilitre (2.5µg, 1.25µg, 0.63µg and 0.31µg per plate). Stock and working solutions were prepared before the antimutagenicity assay. The dilution of compounds was calculated by using the formula below:

$$\mathbf{M_1V_1 = M_2V_2}$$

Where,

M_1: Concentration of compound or stock solution of benzimidazole derivatives

V_1: Volume needed to take from the stock

M_2: Highest concentration used

V_2: Total volume needed for plating

3.2.4.3 Treatment with and without metabolic activation

The antimutagenic activity of compounds with five different concentrations (0.31µg, 0.63µg, 1.25µg, 2.5µg and 5.0µg per plate) were tested on two different strains of Salmonella thyphimurium (TA98 and TA100). Firstly, 500µL of 0.1M sodium phosphate buffer was added for without metabolic activation and 500µL of S9 mix was added for the presence of metabolic activation to each sterile tubes. 50µL of mutagen (2-nitrofluorene, sodium azide and 2-aminoathracene) was added to the tubes containing the buffer. Then, 50µL of the test compound was added to the same tube. After that, 100µL of overnight bacteria culture was added to the tubes. The tubes were gently swirled to ensure the content mix properly. Then, the tubes containing the mixture were incubated for 20 minutes. After 20 minutes, 2mL melted top agar

supplemented with 0.5mM histidine and biotin at a temperature between 43°C to 48°C was added to each tube, mixed and quickly poured on top of GM agar plates. After the top agar hardened on GM agar, the plates were inverted and incubated at 37°C for 48 hours.

3.2.4.4 Bacterial Counting and Percentage of Inhibition (PI)

The number of revertant colonies on GM plates were calculated manually. The revertant colonies were compared with negative and positive control plates. The percentage of inhibition was calculated for each treatment group using the formula below. The compound is considered strong antimutagenic if the PI more than 40%, moderate antimutagenic if the PI between 25% to 40% and no antimutagenic if the PI less than 25% (Di Sotto et al., 2008).

$$\text{PI} = 100 - \frac{\text{number of revertant colonies in test plates}}{\text{number of revertant colonies in positive control plates}} X\ 100\%$$

3.2.5 Structure-Activity Relationship (SAR)

Structure-activity relationship analysis was conducted to determine the effect of different structures of benzimidazole derivatives to their antimutagenic activities. In this experiment, the effect of different locations of the hydroxyl group attached to the benzene ring of benzimidazole derivatives was compared.

3.2.6 Statistical Test

All data from 3 independent experiments were presented as mean ± SEM and analysed by using Graphpad Prism 7.0 software. Two-Way ANOVA was conducted followed by post-hoc Tukey test where $p<0.05$ indicate the results obtained were significant.

CHAPTER 4

RESULTS

4.1 Genetic analysis of *Salmonella typhimurium* Strains

The result of genetic analysis for TA98 and TA100 strains was presented in Table 4.1. The genetic analysis was conducted by our research group before the Ames test was conducted.

Table 4.1: The results of genetic analysis for TA98 and TA100 strains of *Salmonella thyphimurium*.

Genetic properties	Presence of bacteria growth on plate
Histidine dependence	Absent
Biotin dependence	Present
Presence of *rfa* mutation	Absent
Presence of *uvrB* deletions	Absent
Presence of plasmid pKM101	Present

4.2 Result of Ames test without Metabolic Activation

4.2.1 The Number of Revertant Colonies of TA98 Bacteria Strain

The effects of the benzimidazole derivatives on the TA98 strain are illustrated in Figure 4.1. The positive groups are significantly different from negative groups at $p<0.05$. All treatments groups show significant different with negative groups and positive groups at $p<0.05$.

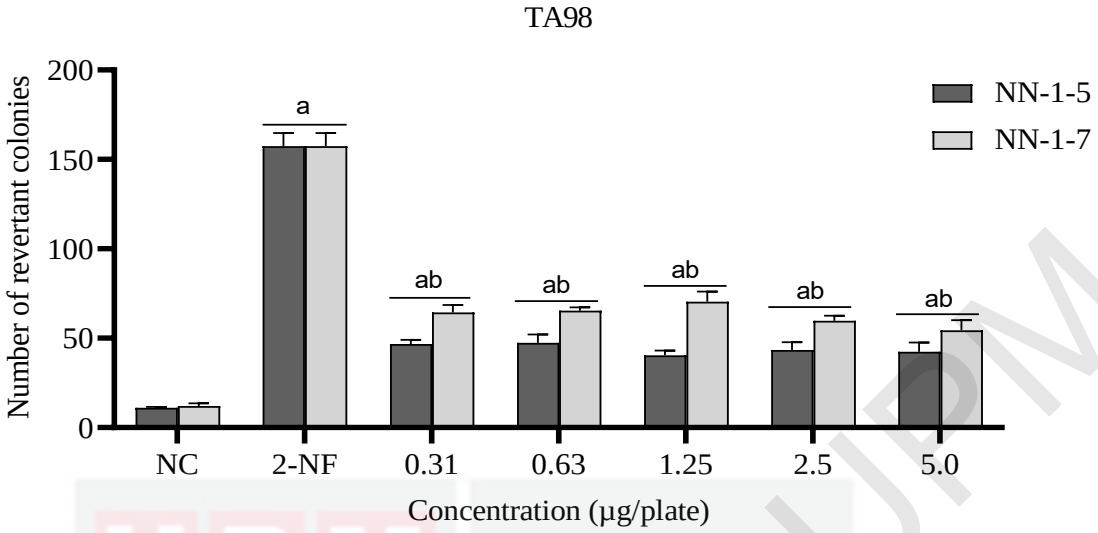


Figure 4.1: The number of revertant colonies in different concentrations of benzimidazole derivatives (NN-1-5 and NN-1-7) in TA98 without metabolic activation (-S9). PBS is the negative control (NC) and 2-nitrofluorene (2-NF) is the positive control. Value were expressed as mean ± SEM of three independent experiments, *a*, significant different ($p<0.05$) from NC group, *b*, significantly different ($p<0.05$) from positive group (2-NF).

4.2.2 The Number of Revertant Colonies of TA100 Bacteria Strain

The effects of the derivatives on the TA98 strain are illustrated in Figure 4.2. The positive groups are significantly different from negative groups at $p<0.05$. All treatments groups show significant difference with negative groups and positive groups at $p<0.05$.

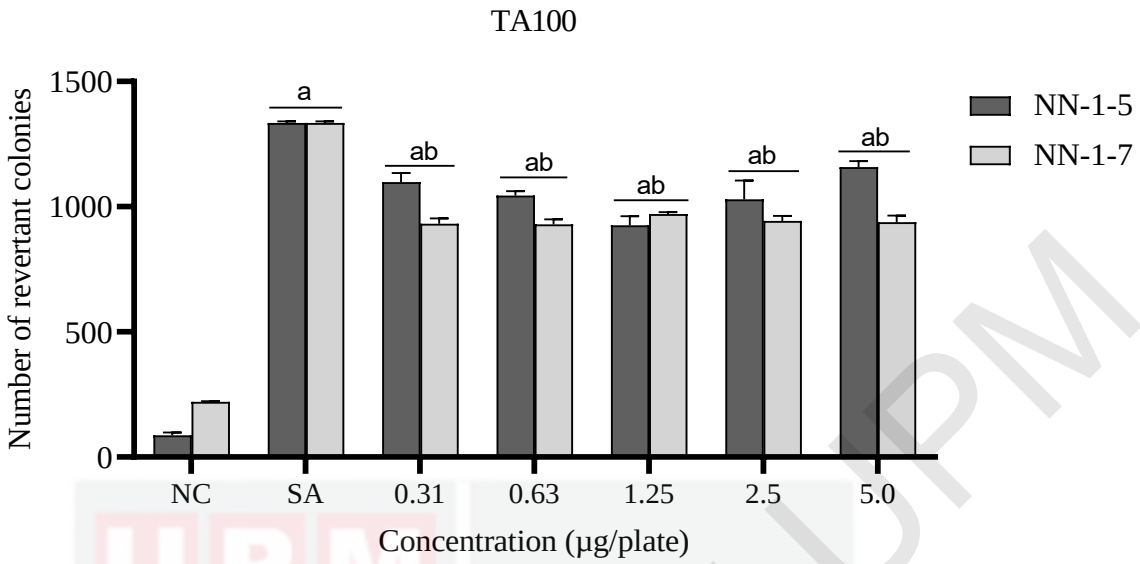


Figure 4.2: The number of revertant colonies in different concentrations of benzimidazole derivatives (NN-1-5 and NN-1-7) in TA100 without metabolic activation (-S9). PBS is the negative control (NC) and sodium azide (SA) is the positive control. Value were expressed as mean ± SEM of three independent experiments, *a*, significant different ($p<0.05$) from NC group, *b*, significantly different ($p<0.05$) from positive group (SA).

4.3 Result of Ames test with Metabolic Activation

4.3.1 The Number of Revertant Colonies of TA98 Bacteria Strain

The effects of the derivatives on the TA98 strain are illustrated in Figure 4.3. The positive groups are no significantly different from negative groups at $p>0.05$. All treatments groups show no significant difference with negative group and positive group at $p>0.05$.

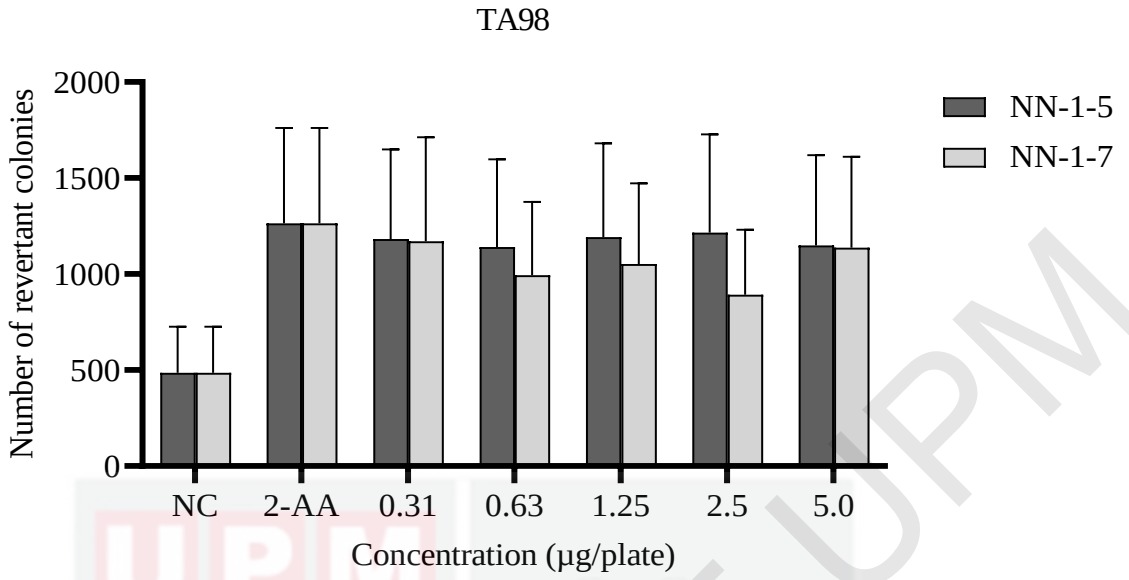


Figure 4.3: The number of revertant colonies in different concentrations of benzimidazole derivatives in TA98 with metabolic activation (+S9). PBS is the negative control (NC) and 2-aminoanthracene (2-AA) is the positive control. Value were expressed as mean ± SEM of three independent experiments.

4.3.2 The Number of Revertant Colonies of TA100 Bacteria Strain

The effects of the derivatives on the TA100 strain are illustrated in Figure 4.4. The positive groups are no significantly different from negative groups at $p>0.05$. All treatments groups show no significant difference with negative group and positive group at $p>0.05$.

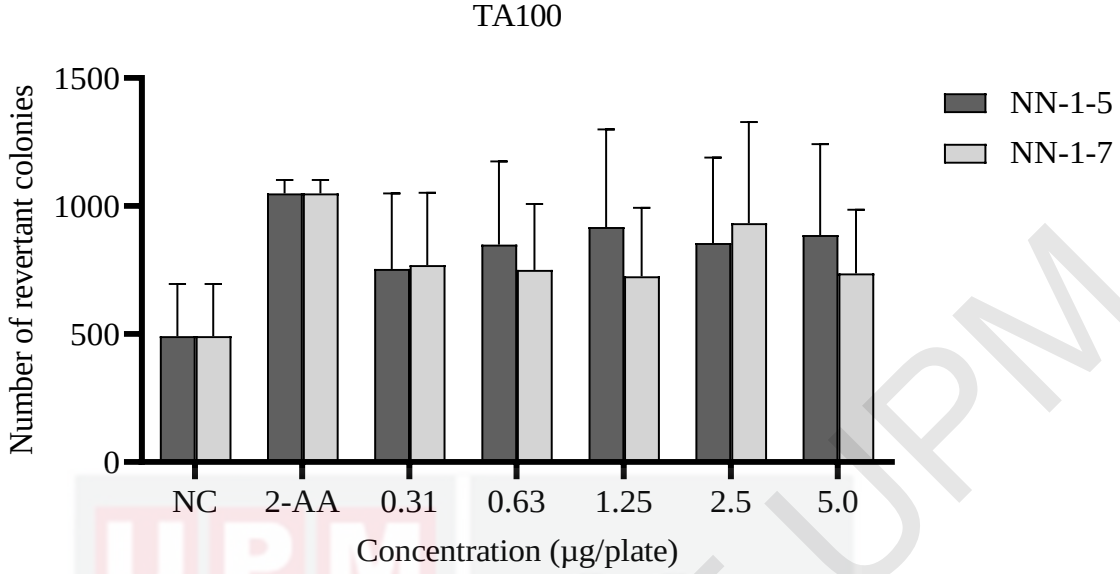


Figure 4.4: The number of revertant colonies in different concentrations of benzimidazole derivatives in TA100 with metabolic activation (+S9). PBS is the negative control (NC) and 2-aminoanthracene (2-AA) is the positive control. Value were expressed as mean ± SEM of three independent experiments.

4.4 Percentage of inhibition

The percentage of inhibition (PI) was calculated based on the formula shown below:

$$\text{PI} = 100 - \frac{\text{number of revertant colonies in test plates}}{\text{number of revertant colonies in positive control plates}} \text{ X } 100\%$$

The percentage of inhibition were calculated to determine the antimutagenic activity of benzimidazole derivatives (NN-1-5 and NN-1-7). According to Di Sotto et al, 2008, if the percentage inhibition is less than 25%, 25% to 40% and more than 40%, the compound considered has weak or absent, moderate and strong antimutagenic activity.

Table 4.2: Percentage of inhibition (PI) of benzimidazole derivatives in TA98 and TA100 strains without metabolic activation.

Type of compound	Treatment	Number of Revertant Colonies/plate			
		TA98		TA100	
		Mean ± SEM	Percentage inhibition	Mean ± SEM	Percentage inhibition
NN-1-5	NC	11±1 [a]	-	87±12 [a]	-
	PC	157±7 [b]	-	1335±6 [c]	-
	0.31	47±2	70.34	1099±35	17.68
	0.63	47±5	69.92	1044±17	21.79
	1.25	40±3	74.36	925±37	30.67
	2.50	43±4	72.46	995±41	25.47
	5.00	42±5	73.09	1158±23	13.19
NN-1-7	NC	12±2 [a]	-	220±2 [a]	-
	PC	157±7 [b]	-	1335±6 [c]	-
	0.31	64±4	59.11	932±21	30.17
	0.63	65±2	58.47	929±20	30.37
	1.25	70±6	55.30	971±7	27.27
	2.50	60±3	62.08	943±21	29.37
	5.00	54±6	65.47	939±26	29.67

TA98: a = PBS (negative control), b = 2.5µg/plate of 2-nitrofluorene (positive control). TA100: c = 5.0µg/plate of sodium azide in the absence of metabolic activation. Percentage inhibition <25% indicate absent/weak, 25% to 40% indicate moderate and >40% indicate strong antimutagenic activity.

Table 4.3: Percentage of inhibition (PI) of benzimidazole derivatives in TA98 and TA100 strains with metabolic activation.

Type of compound	Treatment	Number of Revertant Colonies/plate			
		TA98		TA100	
		Mean ± SEM	Percentage inhibition	Mean ± SEM	Percentage inhibition
NN-1-5	NC	486±241 [a]	-	491±204 [a]	-
	PC	1264±497 [b]	-	1049±53 [b]	-
	0.31	1183±467	6.47	754±295	28.14
	0.63	1142±456	9.72	849±326	19.08
	1.25	1192±489	5.71	918±382	12.55
	2.50	1216±510	3.80	855±333	18.49
	5.00	1149±470	9.14	887±355	15.49
NN-1-7	NC	486±241 [a]	-	491±204 [a]	-
	PC	1264±497 [b]	-	1049±53 [b]	-
	0.31	1171±541	0.13	770±282	26.64
	0.63	994±382	15.25	750±257	28.49
	1.25	1052±420	10.26	726±266	30.80
	2.50	892±339	23.92	934±394	11.02
	5.00	1138±473	2.928	736±250	29.83

TA98 and TA100: a = PBS (negative control), b = 5.0 µg/plate of 2-aminoanthracene (positive control) in the presence of metabolic activation. Percentage inhibition <25% indicate absent/weak, 25% to 40% indicate moderate and >40% indicate strong antimutagenic activity.

CHAPTER 5

DISCUSSION

Ames test can be used to screen the mutagenic and antimutagenic effects of benzimidazole derivatives (NN-1-5 and NN-1-7). The difference between mutagenic and antimutagenic test in the Ames test is the addition of the mutagen. In the mutagenic test, the tested compound was added in the bacterial strain of Salmonella thyphimurium and the increasing in the number of revertant than positive control can be observed if the compound is mutagenic. For the antimutagenic test, the mutagen was added to observe if the tested compound can inhibit the mutagen effect in the bacterial strains by reducing the number of revertant colonies compare to the positive control. The positive control used were standard mutagen that can induce mutation in specific bacterial strains and conditions. For example, the 2-nitrofluorene (2-NF) in TA98 and sodium azide (SA) in TA100 without metabolic activation while 2-aminoanhtracene (2-AA) for both bacterial strain TA98 and TA100 with metabolic activation. The S9 fraction was added together with the test compound and the bacteria in the presence of metabolic activation. The S9 fraction contains liver homogenate from rats treated with the Aroclor-1254 to increase the level of hepatic metabolizing enzymes. Some mutagen can display mutagenicity only after undergoing metabolic activation by microsomal enzymes such as cytochrome P450. Thus, the S9-based metabolic activation was introduced in the test system since the bacteria do not have the metabolic ability.

In this test, two types of Salmonella thyphimurium strain were used which are TA98 and TA100. Both bacterial strains have been mutated in their histidine operon, causing them to lose the ability to produce histidine to grow or survive. The presence of mutagen, an agent that cause mutation can allow the bacterial strains to revert to their original state, allowing them to produce histidine. Thus, the bacterial colonies can be observed on a minimal agar plate with a small amount of histidine. These bacterial strains can be used to determine the frameshift mutation in TA98 and base-pair substitution mutation in TA100 respectively. Genetic analysis is a preliminary test

that was conducted to ensure the correct genotypes of both Salmonella thyphimurium TA98 and TA100 strains. The analysis was carried out by our research group before the Ames test was conducted. Based on Table 4.1, both bacterial strains (TA98 and TA100) shown to have a dependency on histidine with the presence of *rfa* mutation, *uvrB* deletion and plasmid pKM101.

Based on the data without metabolic activation in Figure 4.1 and Figure 4.2, all different concentration of benzimidazole derivatives (NN-1-5 and NN-1-7) significantly lower the number of revertant colonies than the positive control (2-NF and SA) in both bacterial strains (TA98 and TA100). These indicate the compounds can inhibit mutagenic activity in the absence of metabolic activation specifically on frameshift mutation (TA98) and base-pair substitution mutation (TA100). The percentage of inhibition shown in Table 4.2, the data have shown all benzimidazole derivatives (NN-1-5 and NN-1-7) have strong antimutagenic activity in TA98. However, the compound NN-1-5 has stronger antimutagenic activity compare to the compound NN-1-7 in TA98 without metabolic activation.

Compound NN-1-5 may have better ability to inhibit the effect of 2-nitrofluorene which is a direct-acting mutagen that does not require metabolic activation by the microsomal enzyme. 2-nitrofluorene belongs to nitroarenes class that induces frameshift mutation. The nitroarenes required O-transferases or nitroreductases and the presence of plasmid pKM101 of bacteria strain TA98 that increase its mutagenicity (Di Sotto et al., 2011). Compound NN-1-5 deactivating the direct mutagen 2-nitrofluorene, thus protecting from DNA damage effect. It also may inhibit the mutagenic expression of O-tansferases or nitroreductases in 2-nitrofluorene. In the TA100, the percentage of inhibition for the compound NN-1-7 have moderate antimutagenic activity than compound NN-1-5 which have weak to moderate. This might indicate that compounds NN-1-7 are capable to inhibit the base-pair substitution mutation in TA100 better than NN-1-5 but in moderate level. The structural differences between two compounds NN-1-5 and NN-1-7 are the number and the position of hydroxyl groups. Compound NN-1-5 has one hydroxyl group at meta position attached to phenyl ring while compound NN-1-7 have two hydroxyl groups at ortho-meta position attached to the phenyl ring. The absence or presence of ortho and para position of the hydroxyl group or the number of hydroxyl groups could affect the antimutagenic activity of the compounds.

For the data with metabolic activation in Figure 4.3 and 4.4, all different concentration of benzimidazole derivatives (NN-1-5 and NN-1-7) shown no significant number of revertant colonies than negative and positive controls in both TA98 and TA100 strains. Even though the data in for the treatment group seem to be comparable than negative and positive control but the standard error mean for the number of revertant colonies are too large so the data are not valid or accurate. The negative control in both tested strains can be observed to be near 500 revertant colonies. According to Mortelmans and Zeiger in 2000, the negative control should be 20 to 50 in TA98 and 75 to 200 in TA100 to be considered valid.

Antimutagenicity study of the active compound is important in the discovery of new effective anticancer treatments. If the compound shown to has antimutagenic activity, it is an indication that the compound might be the possible anticancer drug candidates (Ghazali et al., 2011). Since the antimutagenic test was conducted using the bacteria in vitro (Ames test), it is not a good model to study antimutagenic in human. But, the test can provide the preliminary data on the antimutagenic potential of tested compounds. Thus, in vivo test should be performed in mice or rats of both sexes such as micronucleus test using mice and genotoxic testing should be done for confirmation to indicate the safe use as a drug (Ntuli et al., 2018).

CHAPTER 6

CONCLUSION

6.1 Conclusion

In conclusion, the compound NN-1-5 may has a stronger antimutagenic effect compared to the compound NN-1-7 specifically on frameshift mutation (TA98) by direct carcinogen (without metabolic activation).

6.2 Further Recommendation

To understand the antimutagenic activity of benzimidazole derivatives with the presence of metabolic activation the Ames test should be repeated to obtain significant data. For better SAR analysis, more benzimidazole derivatives should be tested so that variety of structure and effects can be compared.

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APPENDIX

PREPARATION OF MEDIA AND CHEMICAL REAGENTS

Preparation of Nutrient Broth

Nutrient broth was used to grow the bacteria strain overnight by adding 3.25g of nutrient broth (Oxoid nutrient broth No.2) to 250ml distilled water. The mixture was autoclaved for 15 minutes at 121°C and allowed to cool before stored at 4°C.

Preparation of Glucose Minimal (GM) Agar Plates

Glucose solution (10% w/v)

Glucose was used as carbon source for GM agar plates. 50g of dextrose powder (Oxoid) was added to 500ml distilled water and the mixture was autoclave for 15 minutes at 121°C. Then, the solution allowed to cool and stored at 4°C.

Vogel-Bonner (VB) salts solution (50x)

Ingredients	Per 500 ml
1. Warm distilled water (50°C)	250ml
2. Magnesium sulphate ($MgSO_4.H_2O$)	5g
3. Citric acid monohydrate	50g
4. Potassium phosphate dibasic anhydrous (K_2HPO_4)	250g
5. Sodium ammonium phosphate ($Na_2NH_2PO_4.4H_2O$)	87.5g

The above ingredients were added in the order to warm water in 1L flask. Each salts were mixed until it dissolved thoroughly by stirring on magnetic stirrer before adding the next salts. Then, the volume of VB salts was adjust to 500mL and autoclave for 15 minutes at 121°C. VB salts was stored at room temperature in the dark. VB salts was used as salts for the GM agar plates.

GM Agar Plates (1.5% w/v)

7.5g of bacto agar (Oxoid) was added to 465ml distilled water and autoclave for 15 minutes at 121°C. When the agar reach 60°C, 10ml of VB salts were added and mixed thoroughly to avoid precipitate formed. Then, 25ml of sterile glucose solution was added and also mixed. The final concentration of glucose solution will be 5% in GM agar. The GM agar was maintained at 50°C and the agar was dispensed into the petri plates which approximately 25ml in each per plate. The GM agar plates were allowed to harden and stored upside down in sealed plastic bag at 4°C.

Preparation of Top Agar

Histidine/Biotin Solution (0.5mM)

Ingredients	Per 500ml
1. Distilled water	500ml
2. D-Biotin	62mg
3. L-Histidine	48mg

The distilled water was heated before adding the D-biotin and L-histidine and mixed until completely dissolved. The solution was autoclave for 15 minutes at 121°C and stored at 4°C. The solution was used as supplement for top agar for bacteria to growth.

Top Agar (0.6% w/v) with Histidine/Biotin Solution

Ingredients	Per 200ml
1. Distilled water	180ml
2. Bacto agar	1.2g
3. Sodium chloride (NaCl)	1.2g
4. Histidine/biotin solution (0.5mM)	20ml

Bacto agar (Oxoid) and sodium chloride was added to 180ml distilled water and the mixture was autoclave at 121°C for 15 minutes. The solution was allowed to cool about 60°C before 20ml of sterile histidine/biotin solution was added and mixed properly. The top agar was allowed to harden and stored in room temperature in the dark. It was melted using microwave and maintained at 43°C to 48°C prior to Ames test.

Preparation of Sodium Phosphate Buffer (0.1M) pH7.4

Ingredients	Per 500ml
0.1M Sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$) • 6.9g of sodium dihydrogen phosphate was dissolved in 500ml of distilled water (monobasic).	60ml
0.1M Disodium hydrogen phosphate ($Na_2HPO_4.H_2O$) • 7.1g of disodium hydrogen phosphate was dissolved in 500ml of distilled water (dibasic).	440ml

The above ingredient of monobasic and dibasic solution were mixed and the pH was adjust to 7.4 by adding 0.1M disodium hydrogen phosphate solution. The buffer was autoclaved at 121°C for 15 minutes and stored at room temperature in the dark.

Preparation of S9-Mix

Salt solution (1.65M KCl and 0.4M $MgCl_4$)

Ingredients	Per 100ml
1. Potassium chloride (KCl)	12.3g
2. Magnesium chloride ($MgCl_2.6H_2O$)	8.14g
3. Distilled water	100ml

The above ingredients was dissolved in distilled water and the solution was autoclave for 15 minutes at 121°C before stored at 4°C.

Sodium Phosphate Buffer (0.2M) pH7.4

Ingredients	Per 500ml
0.2M Sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$) • 13.8g of sodium dihydrogen phosphate was dissolved in 500ml of distilled water (monobasic).	60ml
0.2M Disodium hydrogen phosphate ($Na_2HPO_4.H_2O$) • 14.2g of disodium hydrogen phosphate was dissolved in 500ml of distilled water (dibasic).	440ml

The above ingredient of monobasic and dibasic solution were mixed and the pH was adjust to 7.4 by adding 0.1M disodium hydrogen phosphate solution. The buffer was autoclaved at 121°C for 15 minutes and stored at room temperature in the dark.

S9-Mix (Rat liver microsomal enzymes and cofactor)

Ingredients	Per 25ml
1. Sterile distilled water	9.87ml
2. 0.2M phosphate buffer, pH7.4	12.5ml
3. 0.1M NADP	1.0ml
4. 1.0M glucose-6-phosphate	0.125ml
5. Salts ($MgCl_2$-KCl) solution	0.5ml
6. Rat liver S9 (Aroclor 1254-induced)	1.0ml

The above ingredients and must be chilled and were added in the order. The solution was prepared fresh and maintained on the temperature on ice. The solution was mixed thoroughly prior to the Ames test.