UNIVERSITI PUTRA MALAYSIA

CYTOTOXICITY, ANTIMUTAGENICITY AND MOLECULAR DOCKING OF BENZIMIDAZOLE DERIVATIVES AS ANTICANCER AGENTS

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By

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Benzimidazole, a fused heterocyclic compound comprising benzene and imidazole rings, is a well-known moiety that has garnered considerable interest in medicinal chemistry due to its wide range of pharmacological activity in a variety of disease models, including cancer. Due to its broad spectrum of therapeutic potential, many modifications and optimizations have been made to its backbone, resulting in the production of many of its derivatives. The purpose of this study was to assess the cytotoxicity, mutagenicity, and antimutagenicity of four benzimidazole derivatives: (*E*)-4-(5,6-dimethyl-1*H*-benzo[d]imidazol-2-yl)-*N*′-(3-hydroxybenzylidene) benzohydrazide **(1)**, (*E*)-*N*′-(2,5-dihydroxybenzylidene)-4-(5,6-dimethyl-1*H*-benzo[d]imidazol-2-yl) benzohydrazide **(2)**, (*E*)-*N*′-(2,4-dihydroxybenzylidene)-4-(5,6-dimethyl-1*H*-benzo[d]imidazol-2-yl) benzohydrazide **(3)**, and (*E*)-4-(5,6-dimethyl-1*H*-benzo[d]imidazol-2-yl)-*N*′-(2-hydroxybenzylidene) benzohydrazide **(4)**. To our knowledge, these benzimidazole derivatives have not been explored for the stated activities. The potential molecular interactions of these compounds with the target proteins Bcl-2, Bcl-xL, and mutant p53-R273H were also investigated. The MTT assay was conducted to determine the cytotoxic effects of benzimidazole derivatives (1 – 4) on HeLa, HT-29, and MDA-MB-231 cancer cell lines. Following that, the Ames test was performed to evaluate the mutagenic and antimutagenic potential of the compounds. Then, the structure-activity relationship (SAR) of benzimidazole derivatives was analyzed based on the results obtained. Further, molecular docking was employed to predict the possible interactions of the selected benzimidazole derivatives with the Bcl-2,

Bcl-xL, and mutant p53-R273H proteins. Except for compound 2, all benzimidazole derivatives demonstrated varying degrees of cytotoxicity against the tested cancer cells. When compared to 5-fluorouracil (5-FU), compound 4 showed the most promising cytotoxicity with lower IC_{50} values against HeLa (48 h: 9.86 ± 1.50 μM), HT-29 (48 h: 4.66 ± 0.16 μM) and MDA-MB-231 (24 h: 7.80 ± 0.19 μM; 48 h: 8.83 ± 0.64 μM) cells. Compound 3 also presented high cytotoxicity compared to 5-FU, with IC_{50} values of 8.89 ± 2.62 μM (48 h) in HT-29 and 10.88 ± 1.39 μM (24 h) and 10.10 ± 2.90 μM (48 h) in MDA-MB-231 cells. Likewise, compound 1 had lower IC_{50} values in HeLa (48 h: 9.09 ± 0.46 μM) and HT-29 (48 h: 13.47 ± 3.50 μM) cells, in comparison to 5-FU. The mutagenicity assessment showed all benzimidazole derivatives were not mutagenic against the TA98 and TA100 strains in the absence of metabolic activation. In the presence of metabolic activation, it was observed that compound 1 induced a frameshift mutation in the TA98 strain, whereas other compounds were not mutagenic in both tester strains. Additionally, all derivatives showed significant antimutagenicity in both tester strains. The SAR analysis proposed that the *ortho*-OH substitution on the phenyl ring has a substantial influence on the cytotoxicity, mutagenicity, and antimutagenicity of benzimidazole derivatives. The molecular docking analysis demonstrated a high-affinity binding of compound 3 to the Bcl-2 protein and compounds 3 and 4 to the mutant p53-R273H protein relative to the reference standards. The results suggested that compound 3 may act as a ligand inhibitor for Bcl-2 and that compounds 3 and 4 may act as ligand activators for the mutant p53-R273H.

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SITOTOKSISITI, ANTIMUTAGENISITI DAN DOK MOLEKUL TERBITAN BENZIMIDAZOL SEBAGAI EJEN ANTIKANSER

Oleh

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Benzimidazol, iaitu sebatian heterosiklik bersatu yang mengandungi cincin benzena dan imidazol, merupakan moieti yang terkenal yang telah mendapat perhatian dalam kimia perubatan disebabkan oleh kepelbagaian aktiviti farmakologinya dalam pelbagai model penyakit, termasuk kanser. Oleh sebab spektrum potensi terapeutik benzimidazol yang luas, pelbagai pengubahsuaian dan pengoptimuman telah dibuat pada tulang belakangnya, lalu menghasilkan pelbagai terbitan benzimidazol. Tujuan kajian ini dijalankan adalah untuk menyelidik sitotoksisiti, mutagenisiti, dan antimutagenisiti empat terbitan benzimidazol: (*E*)-4-(5,6-dimethyl-1*H*-benzo[d]imidazol-2-yl)-*N*′-(3-hydroxybenzylidene) benzohydrazide **(1)**, (*E*)-*N*′-(2,5-dihydroxybenzylidene)-4-(5,6-dimethyl-1*H*-benzo[d]imidazol-2-yl) benzohydrazide **(2)**, (*E*)-*N*′-(2,4-dihydroxybenzylidene)-4-(5,6-dimethyl-1*H*-benzo[d]imidazol-2-yl) benzohydrazide **(3)**, and (*E*)-4-(5,6-dimethyl-1*H*-benzo[d]imidazol-2-yl)-*N*′-(2-hydroxybenzylidene) benzohydrazide **(4)**. Sepanjang pengetahuan kami, terbitan benzimidazol ini belum dikaji untuk aktiviti yang dinyatakan. Potensi interaksi molekul di antara terbitan benzimidazol ini dengan protin sasaran Bcl-2, Bcl-xL, dan mutan p53-R273H juga telah diselidik. Ujian MTT telah dijalankan bagi menilai kesan sitotoksik terbitan benzimidazol (1 – 4) ke atas garisan sel kanser HeLa, HT-29 dan MDA-MB-231. Selepas itu, ujian Ames telah dilakukan untuk menilai potensi mutagenik dan antimutagenik terbitan benzimidazol. Hubungan struktur-aktiviti (SAR) terbitan benzimidazol kemudiannya telah dianalisis berdasarkan hasil kajian yang diperolehi. Seterusnya, dok molekul telah digunakan untuk menjangka interaksi yang mungkin wujud di antara

terbitan benzimidazol yang terpilih dengan protin Bcl-2, Bcl-xL dan mutan p53-R273H. Semua terbitan benzimidazol, kecuali sebatian 2, menunjukkan tahap sitotoksisiti yang berbeza terhadap sel kanser yang diuji. Jika dibandingkan dengan 5-fluorouracil (5-FU), sebatian 4 menunjukkan sitotoksisiti yang sangat memberangsangkan dengan nilai IC_{50} yang lebih rendah terhadap sel HeLa (48 j: 9.86 ± 1.50 μM), HT-29 (48 j: 4.66 ± 0.16 μM) dan MDA-MB-231 (24 j: 7.80 ± 0.19 μM; 48 j: 8.83 ± 0.64 μM). Sebatian 3 juga menunjukkan sitotoksisiti yang tinggi berbanding 5-FU, dengan nilai IC_{50} 8.89 ± 2.62 μM (48 j) dalam sel HT-29 dan 10.88 ± 1.39 μM (24 j) dan 10.10 ± 2.90 μM (48 j) dalam sel MDA-MB-231. Sebatian 1 juga mempunyai nilai IC_{50} yang lebih rendah dalam sel HeLa (48 j: 9.09 ± 0.46 μM) dan HT-29 (48 j: 13.47 ± 3.50 μM), berbanding dengan 5-FU. Penilaian mutagenisiti menunjukkan semua terbitan benzimidazol adalah tidak mutagenik terhadap strain TA98 dan TA100 tanpa kehadiran pengaktifan metabolik. Dengan kehadiran pengaktifan metabolik, sebatian 1 didapati menyebabkan mutasi anjakan bingkai (*frameshift mutation*) dalam strain TA98, manakala sebatian lain tidak mutagenik dalam kedua-dua strain penguji. Selain itu, semua terbitan benzimidazol menunjukkan aktiviti antimutagenik yang signifikan dalam kedua-dua strain penguji. Analisis SAR menyarankan bahawa penggantian orto-OH pada cincin fenil mempunyai pengaruh yang penting ke atas aktiviti sitotoksik, mutagenik, dan antimutagenik terbitan benzimidazol. Analisis dok molekul menunjukkan pengikatan pertalian yang tinggi sebatian 3 kepada protin Bcl-2 dan sebatian 3 dan 4 kepada protin mutan p53-R273H berbanding dengan standard rujukan. Hasil analisis mencadangkan bahawa sebatian 3 mungkin berpotensi untuk bertindak sebagai ligan perencat untuk Bcl-2 dan sebatian 3 dan 4 mungkin boleh bertindak sebagai ligan pengaktif untuk mutan p53-R273H.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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1D	One-dimensional
2D	Two-dimensional
2AA	2-aminoanthracene
2-NF	2-nitrofluorene
3D	Three-dimensional
5-FU	5-fluorouracil
A549	Human lung adenocarcinoma cell line
ADA	*Acanthopanax divaricatus var. albeofructus*
ADME	Absorption, distribution, metabolism, and excretion
ADT	Autodock
ANOVA	Analysis of variance
ALT	Alternative lengthening of telomeres
ATCC	American Type Culture Collection
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BER	Base excision repair
BH3	Bcl-2 homology 3
BHT	Butylated hydroxytoluene

Bid	BH3-interacting domain death agonist
BPB	1-benzyl-2-phenyl benzimidazole
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
DDR	DNA damage response
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ESI	Electrospray ionization
FBS	Fetal bovine serum
FDA	Food and Drug Administration
G_0	Gap 0 phase
G_1	Gap 1 phase
GF	Growth factors
GM	Glucose minimal
GS	Growth signals
HaCaT	Immortalized human keratinocytes
HBV	Hepatitis B virus

HCT 116	Human colorectal carcinoma cell line
HeLa	Human cervical adenocarcinoma cell line
HepG2	Human hepatocellular carcinoma cell line
HER2	Human epidermal growth factor receptor 2
HPLC	High-performance liquid chromatography
HPV	Human papillomavirus
HR	Homologous recombination
HT-29	Human colorectal adenocarcinoma cell line
IARC	International Agency for Research on Cancer
IC_{50}	Half-maximal inhibitory concentration
IR	Infrared
KCl	Potassium chloride
LPS	Lipopolysaccharides
MCF-7	Human breast adenocarcinoma cell line
MDA-MB-231	Human breast adenocarcinoma cell line
MDM2	Mouse double minute 2 homolog
$MgCl_2$	Magnesium chloride
MI	Mutagenic index
MLH1	MutL homolog 1
MMR	Mismatch repair
MQ	Methylene quinuclidinone
MSH2	MutS homolog 2

MSH3	MutS homolog 3
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NA	Not applicable
NADP	Nicotinamide adenine dinucleotide phosphate
NC	Negative control
NCBI	National Center for Biotechnology Information
NCI-H460	Human non-small cell lung carcinoma cell line
n.d	No date
NER	Nucleotide excision repair
NHEJ	Non-homologous end-joining
NIH/3T3	Murine embryonic fibroblast cell line
NMR	Nuclear magnetic resonance
OECD	Organization for Economic Cooperation and Development
OH	Hydroxyl group
p53	p53 tumour suppressor protein
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate-buffered saline
PC	Positive control
PC-3	Human prostate cancer cell line
PDB	Protein Data Bank

PDBQT	Protein Data Bank, Partial Charge (Q), & Atom Type (T)
PI	Percentage of inhibition
PR	Progesterone receptor
pRB	Retinoblastoma protein
Ras	Rat sarcoma
RCSB	Research Collaboratory for Structural Bioinformatics
R-factor	Resistance transfer factor
RMSD	Root-mean-square deviation
ROS	Reactive oxygen species
S9	Supernatant fraction from liver homogenate
SA	Sodium azide
SAR	Structure-activity relationship
SBDD	Structure-based drug design
SDF	Spatial Data File
SEM	Standard error of the mean
SF-268	Human glioblastoma cell line
SHR	Spontaneous hypertensive rats
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TGF-β	Transforming growth factor beta
THP-1	Human monocytic leukemia cell line
TNBC	Triple-negative breast cancer
TP53	Gene encoding the p53 protein

TPA	Tetradecanoyl phorbol acetate
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cancer is defined by the uncontrolled proliferation and spread of abnormal cells resulting from DNA mutations. It is the world's leading cause of death, accounting for 19.3 million new cases and nearly 10 million deaths in 2020 (Sung et al., 2021). When both sexes are considered, female breast cancer has become the most commonly diagnosed type of cancer, followed by lung, colorectal, prostate, and stomach cancers. When it comes to cancer deaths, lung cancer continues to be the leading cause, followed by colorectal, liver, stomach, and breast cancers (Bray et al., 2018; Sung et al., 2021). According to the International Agency for Research on Cancer (IARC), Malaysia reported 48,639 new cancer cases and 29,530 cancer deaths in 2020 (International Agency for Research on Cancer, 2022).

The development of cancer is known to be initiated by mutations, that is, permanent changes in the DNA sequence of an organism, and the agents causing these irreversible changes are referred to as mutagens. Antimutagens, on the contrary, are substances that can either inactivate the mutagen or suppress mutagenicity (Słoczyńska et al., 2014; AbdelHakem & Abdelhafez, 2020). Since the 1970s, the Ames test, which was first introduced by Bruce N. Ames, has been extensively used for assessing mutagenic and antimutagenic profiles of new chemical substances. Not only does this test allow for the early detection of potential cancer-causing agents, but it also allows for the identification of possible antimutagens, which may help reduce the risk of developing cancer (Arriaga-Alba et al., 2012; Słoczyńska et al., 2014).

Benzimidazole, a fused heterocyclic compound bearing benzene and imidazole rings, is a well-known moiety in medicinal chemistry with a wide range of biological activities, including antibacterial, antiviral, anthelmintic, anti-inflammatory, antihypertensive, antidiabetic and anticancer (Pullagura et al., 2016; Akhtar et al., 2017; Purushottamachar et al., 2019). Due to its diverse range of therapeutic potential in various disease models, modifications and optimizations have been carried out around its backbone to improve its biological properties, thus producing many of its derivatives.

For the past few decades, benzimidazole derivatives have been widely explored for their potential anticancer activity. Azam et al. (2015) have reported profound anticancer activity of 2-substituted benzimidazoles in MCF-7, THP-1, PC-3 and A549 cells. A study conducted by Çevik et al. (2018) has demonstrated potent anticancer activity of some benzimidazole derivatives containing hydrazone moiety in A549 and MCF-7 cancer cells. Additionally, Morcoss et al. (2020) have shown moderate to strong antiproliferative activities of new series of benzimidazole-hydrazone derivatives against 60 different cancer cell lines, including leukemia, renal cancer and colon cancer.

1.2 Problem Statement and Justification of Study

Benzimidazole derivatives, as discussed above, exhibit promising anticancer activities. However, some derivatives of benzimidazole have also shown potential mutagenicity. As reported by Seiler (1972), several 2- and 4-substituted benzimidazole derivatives were found to induce a base-pair substitution mutation when tested using the Ames test. Gümüş et al. (2003) have also demonstrated a positive mutagenic response of some newly synthesized Pt(II) complexes of 2-substituted-benzimidazoles. Other than that, Alanyalı et al. (2010) have revealed the presence of mutagenic activity in nitro-bound bisbenzimidazole derivatives. Given that some benzimidazole derivatives have the potential to induce mutations, as reported in earlier research, it is crucial to screen for the mutagenicity of our compounds of interest. Meanwhile, the evaluation of antimutagenicity allows the identification of compounds with potential antimutagenic properties that can inhibit or suppress the effects of mutagenic chemicals (Słoczyńska et al., 2014).

In the current study, we presented a series of benzimidazole derivatives: compounds 1, 2, 3 and 4, which were synthesized by a group of researchers from UiTM, Shah Alam. To the best of our knowledge, the potential cytotoxicity, mutagenicity and antimutagenicity of these compounds have not yet been reported.

1.3 Research Objectives

1.3.1 General Objective

This research was conducted to investigate the cytotoxicity, mutagenicity and antimutagenicity of benzimidazole derivatives, as well as their possible molecular interactions.

1.3.2 Specific Objectives

a. To determine the cytotoxicity of benzimidazole derivatives on HeLa, HT-29 and MDA-MB-231 human cancer cell lines using the MTT assay.
b. To assess the mutagenicity and antimutagenicity of benzimidazole derivatives using the Ames test.
c. To analyze the structure-activity relationship (SAR) based on the results obtained.
d. To predict possible interactions between the most potent benzimidazole derivatives and the proteins Bcl-2, Bcl-xL and mutant p53-R273H using molecular docking.

1.4 Hypothesis

It is hypothesized that benzimidazole derivatives exhibit significant cytotoxic activity against HeLa, HT-29 and MDA-MB-231 cells. Besides, it is expected that benzimidazole derivatives are not mutagenic and display strong antimutagenic activity. It is also predicted that benzimidazole derivatives would interact with Bcl-2, Bcl-xL and mutant p53-R273H proteins.

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