APOPTOSIS ACTIVITIES OF NICKEL AND COPPER COMPLEXES FROM THYMOQUINONE AND DITHIOCARBAMATE ON ORAL CANCER CELL LINES *IN VITRO*

BY

WASTUTI HIDAYATI SURIYAH

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Kulliyyah of Pharmacy
International Islamic University Malaysia

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ABSTRACT

Oral squamous cell carcinoma (OSCC) has been associated with high morbidity and mortality rate. Metal-based anticancer drugs such as platinum-based agents have been widely used to treat various cancer cells including OSCC. However, their efficiency is limited by the side effects and frequent development of chemoresistant cancer cells. To date, research on metal-based compounds has been extensively continued to develop more promising chemotherapeutic compounds capable to overcome these limitations. Thymoquinone (TQ) has been reported to have numerous biological activities, including anticancer *in vitro* and *in vivo*. Similarly, dithiocarbamate (PEDTC) is also known to have *in vitro* antineoplastic potential against several types of cell lines. Nickel and copper complexes from TQ (NiTQPy, CuTQPy) and PEDTC (NiPEDTC, CuPEDTC) were successfully synthesized and characterized. These metal complexes provide new approach to broaden the spectrum of biological activities their parent ligands. The study is aimed to investigate and determine anticancer potential of the metal complexes derived from TQ and PEDTC. Human OSCC HSC-3 and HSC-4 cell lines were chosen as *in vitro* model. The cells were exposed to various concentrations of the compound substances and examined by MTT assay for cytotoxicity analysis. Normal human oral fibroblast and human keratinocyte (HACAT) cells were also included in the assay. Zebrafish was used as a model for *in vivo* toxicity study. The number of apoptotic cells was quantified by flow cytometry and confirmed by Caspase 3/7 assay. Quantitative RT PCR was performed to analyze the mRNA expression of apoptotic-regulator genes. The protein expression was observed by western blot. The MTT assay demonstrated that the metal complexes induced cytotoxicity on HSC-3 and HSC-4 cells. Exposure of the metal complexes at the concentration similar to cancer cells relatively did not affect the normal cells and zebrafish embryo development, except for those treated with copper complexes. Flow cytometry showed the metal complexes increased the number of sub-G1 population, which represent apoptotic cells. The apoptotic activities were supported by Caspase 3/7 analysis. The various degree of apoptosis induction by metal complexes from TQ and PEDTC are associated with the elevation of BAX/BCL-2 ratio in both transcriptional (mRNA) and translational (protein) levels. NiPEDTC and NiTQPy was shown to be the most effective to induce apoptosis in HSC-3 and HSC-4 cells among the metal complexes. To conclude, these models of study are useful to demonstrate apoptosis activities in OSCC. The metal complexes from TQ and PEDTC are suggested to merit further investigation for its potentiation as anticancer agents.

ملخص البحث

خلايا سرطان الفم الحرشفية (أوسك) مرتبطة بمعدل وفيات عالي. الأدوية المضادة للسرطان القائمة على المعادن مثل معدن البلاتين كانت وما زالت تستخدم على نطاق واسع لعلاج الخلايا السرطانية المختلفة بما في ذلك خلايا سرطان الفم؛ ومع ذلك حتى الآن نتيجة هذا العلاج الكيميائي غير مرضية بسبب الآثار الجانبية والتطور المتكرر للخلايا السرطانية الكيميائية. حتى هذا اليوم الأبحاث المتعلقة بالمركبات القائمة على المعادن مستمرة و على نطاق واسع في تطوير مركبات علاج كيميائي أكثر فعالية. هنالك العديد من الدراسات حول الأنشطة البيولوجية الناتجة من ثيموكينون (تك) وديثيوكاربامات (بيدتك) هذان المركبان يعتبران مضادان للجراثيم و للفطريات و كذلك مضادان للسرطان. تقدم المركبات المعدنية المشتقة من تك و بيدتك نهجا جديدا لتوسيع نطاق أنشطتها البيولوجية. تهدف هذه الدراسة إلى تحديد إمكانيات المركبات المعدنية المستمدة من تك و بيدتك ضد السرطان. تم اختيار خلايا أوسك هسك-3 و هسك-4 البشرية كنموذج في المختبر. تعرضت الخلايا لتراكيز مختلفة من المركبات و تم فحصها باستخدام فحص م ت ت لتحليل السمية الخلوية. وقد أدرجت الخلايا الليفية الفموية البشرية الطبيعية والخلايا الكيراتينية البشرية (هاكات) أيضا في الفحص. تم استخدام سمكة الزرد كنموذج لدراسة السمية في الجسم الحي. و تم قياس عدد الخلايا التي خضعت للموت المبرمج من خلال التدفق الخلوي وأكدت من قبل فحص كاسباس 7/3. كما استخدم فحص ال رت / ب س ر لتحليل ال م-ر ن اي لتحديد الجين المسؤول عن موت الخلايا المبرمج. استخمت لطخة وسترن لملاحظة البروتينان المسؤلة. أظهرت اختبارات السمية الخلوية أن المركبات المعدنية خفضت معدل البقاء لخلايا هسك-3 و هسك-4 ولكن أظهرت تأثير أقل على الخلايا الطبيعية واجنة سمكة الزرد. أظهر اختبار التدفق الخلوي للمركبات المعدنية زيادة في المرحلة ما قبل ال ج-1 و هذا يمثل الخلايا الميتة بطريقة الموت المبرمج. نشاط موت الخلايا المبرمج اكد و دعم بنتائج اختبار الكاسباس 7/3. ارتبطت درجة مختلفة من موت الخلايا المبرمج من قبل المركبات المعدنية المشتقة من تك و بيدتك مع ارتفاع نسبة باكس / بكل-2 في كلا ترانسكريبتيونال (م-ر ن اي) ومستويات متعدية (البروتين). وقد تبين أن نيبيدتك هي الأكثر فعالية لتحريض موت الخلايا المبرمج في خلايا الهسك-3، في حين أن في خلايا هسك-4، نيبيدتك كان الاكثر فعالية، نيتكبي و كوتكبي كانت فعالة نسبيا في إحداث موت الخلايا المبرمج. وختاما، فإن هذه النماذج من الدراسة مفيدة لإثبات أنشطة موت الخلايا المبرمج في خلايا سرطان الفم الحرشفية أوسك.

APPROVAL PAGE

The thesis of Wastuti Hidayati Suriyah has been approved by the following:

_____
Abdul Razak Kasmuri
Supervisor

_____
Fiona How Ni Foong
Co-supervisor

_____
Muhammad Taher
Internal Examiner

_____
Iekhsan Othman
External Examiner

_____
Amin Malik Shah bin Abdul Majid
External Examiner

_____
Kamarul Ariffin Khalid
Chairman

DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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‘Allah is the Light of the heavens and the earth.
The parable of His Light is as a niche within which is a lamp,
the lamp is within glass,
the glass as if it were a pearly star lit from a blessed olive tree,
neither of the east nor of the west,
whose oil would almost glow even if untouched by fire.
Light upon light..
Allah guides to His Light whom He wills. And Allah sets forth parables for mandkind,
and Allah is Knowing of all things’
(Quran, 24:35)

To my parents, my family, and my teachers, who enlighten my world
with love and knowledge
from the light which Allah places in our heart
and to Allah we shall return..

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LIST OF ABBREVIATIONS

A	Adenine
A	Ampere
AAS	Atomic Absorption Spectroscopy
ABL	Abelson murine leukemia viral oncogene
ABC	ATP binding cassette
AIDS	Acquired immunodeficiency syndrome
AIM	Adipogenesis-inducing medium
AJCC	American Joint Committee on Cancer
AKT	Murine thymoma viral oncogene homolog
ANOVA	Analysis of Variance
APAF-1	Apoptotic protease activating factor-1
ATP	Adenosine Tri Phosphate
BAK	BCL-2 homologous antagonist/killer
BAX	BCL-2-associated X
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BCRP	Breast cancer resistance protein
BER	Base excition repair
BH3	BCL-2 homology domain 3
BLAST	Basic Local Alignment Search Tool
BME	β Mercaptoethanol
BQ	Betel quid
BSA	Bovine Serum Albumin
C	Cytosine
Caspases	Cysteine aspartic acid proteoases
CDDP	cis-diamminedichloridoplatinum(II)
CDC	Centers for Disease Control
CDC25A	Cell division cycle 25A
CDK	Cyclin dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDKN2B	Cyclin dependent kinase inhibitors 2B
cDNA	Complementary Deoxyribonucleic Acid
C_3H_8O	Isopropanol
$CHCl_3$	Chloroform
CHNS	Carbon, Hydrogen, Nitrogen, Sulphur
CICD	Caspase-independent cell death
cm	Centimeter
CO_2	Carbondioxide
CT	Computed Tomography
Cu	Copper
CytC	Cytochrome C
DEPC	Diethylpyrocarbonate
DeSigN	Differentially Expressed Gene Signatures-Inhibitors

DEVD	Aspartyl-L-Glutamyl-L-Valyl-L-Aspartic Acid
DISC	Death Inducing Signalling Complex
dH_2O	double Distilled Water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	deoxynucleoside Triphosphate
DTCs	Dithiocarbamates
DTT	Dithiothreitol
ECL	Electro Chemiluminescence
Eds./ed.	Editions/edition
EDTA	Ethylene diamine tetraacetic acid
e.g.	(*exempli gratia*); for example
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(β-aminoethyl ether
ELlSA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-mesenchymal transition
erbB	Erythroblastosis oncogene B
ERK	Extracellular signal
et al	(*et alia*); and others
EtOH	Ethanol
FADD	Fas-associated death domain
FasR	Fas Receptor
FBS	Foetal Bovine Serum
FET	Fish Embryo Toxicity
Fig	Figure
FTIR	Fourier transformed infrared
g	gram
g	gravity
G	Guanine
GC-MS	Gas Chromatography-Mass Spectrometry
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
h	hour/s
HAART	Highly active antiretroviral therapy
HACAT	Cultured Human Keratinocyte Cell
H_2O	Water
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER	Human epidermal growth factor receptor
HHV-8	Human herpesvirus type 8
hNEIL1/2	Human DNA Glycosylase Nei-like 1/2
HNSCC	Head and Neck Squamous Cell Carcinoma
HOF	Human Oral Fibroblast
Hoggi	human 8-oxoguanine DNA glycosylase
hpf	hours post fertilization
HPV	Human papillomavirus
HR	Homologous recombination
HRP	Horseradish Peroxidase
IBD	Inflammatory bowel disease

ICRACU	Integrated Centre for Research Animal Care and Use
IGF	insulin-like growth factor
IR	Infrared
KCl	Potassium Chloride
kBR	Potassium bromide
kDa	kilodalton
LCK	Lymphocyte-specific protein tyrosine kinase
MAPK	Mitogen-activated protein kinase
MeOH	Methanol
min	minute
MIX	Methylisobutylxanthine
mg	miligram
mL	milliliter
MLH1	Mut-L homologin 1
Mm	Millimolar
MMPs	Matrix metalloproteinases
MMR	Mismatch repair
MOS	Moloney murine sarcoma oncogene
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid
MRP	Multidrug-resistance-associated protein
MSH2	Mut-S homologin 2
MT	Metallothionein
mTOR	Mammalian target of rapamycin
MTS2	multiple tumor suppressors 2
MTT	3-(4,5-dimethylthiazol-2-y)2,5-diphenyltetrazolium bromide
Na_3VO_4	Sodium Orthovanadate
NaCl	Sodium Chloride
NaF	Sodium Fluoride
$NaHCO_3$	Sodium Bicarbonate
NaN_3	Sodium Azide
NaOH	Sodium Hydroxide
NER	Nucleotide excision repair
NHEJ	Nonhomologous end-joining
Ni	Nickel
NIST	National Institute of Standards and Technology
nm	nanometer
No.	Number/Numbers
NP40	Nonyl phenoxypolyethoxylethanol
OECD	Organisation for Economic Co-operation and Development
OPC	Oropharingeal cancer
OSCC	Oral Squamous Cell Carcinoma
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor

PDK	PI-3K–dependent kinase
PEDTC	Potassium phenethyl dithiocarbamate
PET	Positron Emission Tomography
pH	Potential of Hydrogen
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PIM1	Proviral integration site 1
PMSF	Phenylmethylsulfonyl fluoride
PRAD1	Parathyroid Adenomatosis 1
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative Real Time-Reverse Transcription PCR
Ras	Rat sarcoma
RAF1	Raf-1 Proto-Oncogene, Serine/Threonine Kinase
Rb	Retinoblastoma
RNA	Ribonucleid Acid
RNA-seq	Sequence-based RNA
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDBS	Spectral Database for Organic Compounds
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec	second
SEM	Standard Error of Means
SH3GL2	SH3 Domain Containing GRB2 Like 2, Endophilin A1
SILAC	Stable Isotope Labeling with Amino Acids in Cell Culture
SOP	Standard Operational Procedure
Src	Sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
SREBP	Sterol Regulatory Element
STAT	Signal transducer and activator transcription
T	Thymine
TBS T	Tris-buffered Saline with Tween 20
TGFs	Transforming growth factors
TNF	Tumor Necrosis Factor
TNM	Tumor, Nodes, Metastases
TRAIL	TNF-related apoptosis-inducing ligand
tRNA	Transfer Ribonucleic Acid
TS	Thymidylate synthase
TSNAs	Tobacco-specific nitrosamines
TSGs	Tumor suppressor genes
TQ	Thymoquinone
UV/VIS	Ultraviolet–visible spectroscopy
V	Volt
WHO	World Health Organization
ZFET	Zebrafish Embryo Toxicity

LIST OF SYMBOLS

α	Alpha
β	Beta
cm^{-1}	Wavenumber
^{o}C	Celcius degree
ΔCt	Delta cycle threshold
µL	Microlitre
µg	Microgram
P	the probability of obtaining the result
*	statistical significance denotation
m/z	mass-to-charge ratio
n	sample sizes
IC_{50}	half maximal inhibitory concentration
LC_{50}	median lethal concentration
®	registered trademark
™	trademark

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Oral squamous cell carcinoma (OSCC) is the most common type of head and neck squamous cell carcinoma (HNSCC), which arises in oral cavity. OSCC causes high morbidity and mortality, with total of 145,300 deaths per year worldwide and estimated of new cancer is 300,400 cases. Overall, the OSCC mortality was common in males than females with ratio of 2:1. There are geographical variations in the incidence of oral cancer across the world, with the highest incidence rate was found in South and Southeast Asia, which is accounted more than 100,000 cases (Vigneswaran & Williams, 2014; Ferlay et al., 2015). Tobacco use, alcohol consumption and human papillomavirus (HPV) as either individually or combination, are some of the risk factors that complicate the increase of the occurrence of the squamous cell carcinoma (Döbróssy, 2005).

The OSCC development is a multistep and progressive in nature. It starts with uncontrolled proliferation of epithelial cells such as basal cell hyperplasia, dysplasia, *carcinoma in situ* and ends up with advanced OSCC (Lehrbach et al., 2003). The early malignant lesion is usually in the form of an erytholeukoplastic lesion that is often asymptomatic (Bagan et al., 2010). OSCC is considered as a cancer with a poor prognosis, for only 50–63% of the five year survival rate is reported. Almost 2/3 of the cases of oral cancers were detected at the late stage of disease (Güneri & Epstein, 2014).

The main principle of the treatments for patients with OSCC is either radiotherapy or radical surgery, which is often combined with adjuvant chemotherapy