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MECHANISM OF ANTI-CANCER ACTIVITY OF (Z)-3-HYDROXY-1-(2-HYDROXYPHENYL)-3-PHENYLPROP–2–EN-1-ONE (DK1) IN HUMAN OSTEOSARCOMA CELLS AND SUBCHRONIC TOXICITY EVALUATION IN BALB/C MICE

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By

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Osteosarcoma (OS) is an aggressive bone cancer that arises from malignant transformation of mesenchymal cells and predominantly diagnosed among children. Poor prognosis of OS patient is due to the capability of this cancer to proliferate uncontrollably and metastasize, leading to reduction of the 5 years survival rate of patients. Even with intensive treatment, the patients' survival rate will drop significantly to 30% if diagnosed with malignant OS. Commonly prescribed chemotherapy drug like doxorubicin exhibits adverse effects including cardiotoxicity, mucositis and myelosuppression. Therefore, discovery of potential anti-cancer agent that derived from natural products and pharmacologically safe for human consumption is imperative. Curcumin is a natural polyphenolic component that is isolated from turmeric and possess wide spectrum of pharmacological benefits including anti-proliferative, anti-metastasis, anti-angiogenesis as well as has an excellent safety profile. However, natural curcumin reportedly has poor cellular uptake, leading to the development of synthetically synthesized curcuminoid analog, namely (Z)-3-hydroxy-1-(2-hydroxyphenyl)-3phenylprop–2–en-1-one (DK1). The aims for this study are to assess the safety of DK1 in BALB/c mice model and to determine the anti-cancer potential of DK1 by evaluating the cytotoxicity effects, apoptosis induction as well as metastasis inhibition in both U-2 OS and MG-63 human osteosarcoma cell lines. Additionally, this study also focused on the anti-cancer mechanism induced by DK1 and to assess the expression of regulatory long non-coding RNA in human osteosarcoma cell lines. Subchronic toxicity study revealed that DK1 did not cause any toxicity effects on BALB/c mice, proven by no significant ($p<0.05$) alteration in the biochemical analysis, organ to body weight ratio as well as no

apparent physical signs of toxicity on the organs histology. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay manifested that DK1 has successfully inhibited the proliferation of U-2 OS and MG-63 osteosarcoma cell lines with the half maximal inhibitory concentration (IC_{50}) at 19.6 ± 0.3 μM and 23.8 ± 0.8 μM respectively without interfering the proliferation of the normal cells. To examine the morphological signs of cell death induced by the DK1, AO/PI double staining assay was performed and subsequently followed by Annexin V/FITC and cell cycle flow cytometry analysis in order to further confirm that DK1 induced cell death via apoptosis. Cell cycle analysis verified that DK1 induced apoptosis as depicted by the accumulation at Sub G0/G1 phase that was substantially ($p<0.05$) increased in both osteosarcoma cell lines. In this study, DK1 also exhibited an anti-metastatic potential by impeding the cell motility of U-2 OS and MG-63 osteosarcoma cell lines, as shown by the finding that the percentage of cell migration were significantly ($p<0.05$) decreased to 37.2 ± 7.4% and 3.5 ± 0.2 % respectively. A substantial ($p<0.05$) reduction in the percentage of migrated and invaded cells also can be observed in both cell lines where reduction from 100% to 44.4 ± 3.6% and 61.6 ± 1.4% was observed respectively. Additionally, DK1 also possessed an anti-angiogenic potential that was proven by the finding that the percentage of tube formation and microvessels sprouting were significantly ($p<0.05$) reduced. From the microarray analysis several cancer pathways such as PI3K/Akt, MAPK, NF-κB, P53 and cell cycle were successfully modulated by the DK1. In addition, DK1 also significantly ($p<0.05$) regulated the expression of several characterized long non-coding RNA namely HOTAIR, TUG1 and GAS5 as well as uncharacterized long non-coding RNA such as lnc-CETP-1, lnc-LOXL1-1, lnc-UBLCP1-1, and lncC17orf62-3 in both human osteosarcoma cell lines. In conclusion, DK1 could be considered as a potential candidate for anti-cancer therapy in near future since it was able to induce apoptosis, inhibit metastasis and regulate the lncRNA expression in osteosarcoma cell lines as well as did not exert any toxicity effects on the tested mice model in similar to natural curcumin.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

MEKANISME SERTA AKTIVITI ANTI-KANSER (Z)-3-HYDROXY-1-(2-HYDROXYPHENYL)-3-PHENYLPROP-2-EN-1-ONE (DK1) DALAM SEL OSTEOSARKOMA DAN PENILAIAN KETOKSIKAN SUB-KRONIK TERHADAP TIKUS BALB/C

Oleh

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Osteosarkoma (OS) merupakan kanser tulang yang agresif yang bermula dengan transformasi sel mesenkima dan biasanya didiagnosis dalam kalangan kanak-kanak. Prognosis bagi pesakit OS menjadi lemah berpunca daripada proliferasi tidak terkawal dan metastasis oleh kanser ini, lantas menyebabkan penurunan dalam kadar kemandirian 5 tahun. Walaupun dengan rawatan intensif, kadar kemandirian pesakit akan menurun dengan ketara sehingga 30% jika didiagnosis dengan OS malignan. Doxorubicin, ubat kemoterapi yang selalu digunakan, memberi kesan sampingan seperti ketoksikan kardio, mukositis dan pengurangan aktiviti sumsum. Oleh itu, penemuan produk semulajadi yang mempunyai potensi dalam melawan osteosarkoma dan selamat digunakan oleh manusia sangat diperlukan. Kurkumin adalah komponen polifenolik semula jadi daripada kunyit dan mempunyai banyak manfaat dari segi farmakologi seperti anti-proliferatif, anti-metastasis, anti-angiogenesis serta mempunyai profil keselamatan yang sangat baik. Namun, kurkumin dilaporkan mempunyai nilai pengambilan sel yang rendah, dan membawa kepada pembangunan terhadap pensintesisan analog kurkumin secara sintetik, (Z)-3-hydroxy-1-(2-hydroxyphenyl)-3phenylprop–2–en-1-one (DK1). Kajian ini bertujuan untuk menaksir profil keselamatan DK1 terhadap model tikus BALB/c dan untuk menentukan potensi anti-kanser oleh DK1 melalui penilaian kesan sitotoksik, dorongan apoptosis termasuk penghalangan metastasis dalam sel osteosarkoma manusia, U-2 OS dan MG-63. Tambahan lagi, kajian ini turut memfokus kepada mekanisme anti-kanser yang dipengaruhi oleh DK1 dan menaksir ekspresi RNA bukan pengekodan panjang pengawal atur dalam sel osteosarkoma manusia. Kajian ketoksikan subkronik menunjukkan bahawa DK1 tidak memberi kesan toksik terhadap tikus BALB/c, tiada nilai yang signifikan ($p<0.05$) dibuktikan dalam analisis biokimia dan nisbah berat badan ke organ serta tiada juga tanda-tanda ketoksikan fizikal

dilihat pada histologi organ. Melalui asai MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, menjelaskan bahawa DK1 berjaya merencatkan proliferasi sel osteosarkoma manusia, U-2 OS dan MG-63 dengan masing-masing menunjukkan nilai kepekatan rintangan (IC_{50}) sebanyak 19.6 ± 0.3 μM dan 23.8 ± 0.8 μM dan tanpa mengganggu proses proliferasi sel normal. Bagi memeriksa tanda morfologi kematian sel yang disebabkan oleh DK1, ujian AO/PI telah dilakukan diikuti dengan Annexin V/FITC dan seterusnya diikuti dengan analisis kitaran sel untuk pengesahan lanjut mengenai keupayaan DK1 dalam menyebabkan kematian sel melalui proses apoptosis. Analisis kitaran sel menunjukkan bahawa DK1 menyebabkan apoptosis seperti yang ditunjukkan oleh pengumpulan pada fasa Sub G0 / G1 yang meningkat secara besar ($p < 0.05$) pada kedua-dua sel osteosarkoma. Kajian ini juga menunjukkan bahawa DK1 mempunyai potensi anti-metastasis dengan menghalang motiliti sel bagi sel U-2 OS dan MG-63 dengan masing-masing menunjukkan penurunan dalam hasil peratusan sel yang bermigrasi sebanyak 37.2 ± 7.4% dan 3.5 ± 0.2 % secara signifikan ($p<0.05$). Pengurangan besar ($p < 0.05$) dalam peratusan sel yang bermigrasi dan invasif juga dapat dilihat pada kedua-dua sel di mana pengurangan masing-masing dari 100% menjadi 44.4 ± 3.6% dan 61.6 ± 1.4%. Tambahan lagi, DK1 turut mempamerkan potensi sebagai anti-angiogenik melalui hasil penurunan peratusan pembentukkan tiub dan pertumbuhan microvesel secara signifikan ($p<0.05$). Melalui analisis jujukan mikro, beberapa laluan kanser seperti PI3K / Akt, MAPK, NF-κB, P53 dan kitaran sel berjaya dimodulasi oleh DK1. Di samping itu, DK1 juga secara signifikan ($p < 0.05$) mengatur ekspresi beberapa RNA bukan pengekod panjang yang dicirikan iaitu HOTAIR, TUG1 dan GAS5 serta RNA bukan pengekodan panjang yang tidak dicirikan seperti lnc-CETP-1, lnc-LOXL1-1, lnc-UBLCP1-1, dan lncC17orf62-3 untuk kedua-dua sel. Kesimpulannya, DK1 boleh dianggap sebagai salah satu calon yang berpotensi sebagai terapi bagi anti-kanser untuk masa depan kerana kemampuannya untuk menggalakkan apoptosis, menghalang metastasis dan mengaturkan ekspresi lncRNA dalam sel osteosarkoma, malah, tidak menunjukan sebarang kesan ketoksikan terhadap tikus yang diuji seperti kurkumin semulajadi.

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3T3	Mouse fibroblast cell line
ACTB	Homo sapiens Actin beta
AJCC	American Joint Committee on Cancer
AKT2	Homo sapiens AKT serine/threonine kinase 2
ALP	alkaline phosphatase
ALT	Alanine transaminase
AO/PI	Acridine orange/propidium iodide
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
BCL2	Homo sapiens BCL2 apoptosis regulator
BCRT	Bone Cancer Research Trust UK
BH3	Interacting domain death agonist
BW	Body weight
CDK1	Homo sapiens cyclin-dependent kinase 1
CDK2	Homo sapiens cyclin-dependent kinase 2
CDKN1A	Homo sapiens cyclin dependent kinase inhibitor 1A
CO_2	Carbon dioxide
COL1A1	Homo sapiens collagen type I alpha 1 chain
CRP	C-reactive protein
CYCS	Homo sapiens cytochrome c
DK1	(Z)-3-hydroxy-1-(2-hydroxyphenyl)-3-phenylprop–2–en-1-one
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylene-diamine-tetraacetic acid
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF1	Homo sapiens fibroblast growth factor 1

FITC	Fluorescein isothiocyanate
FOX-P4	Homo sapiens FOXP4 antisense RNA 1
FRAP	Ferric reducing antioxidant power
G0	Gap 0 phase or resting phase
G1	Gap 1 phase
G2	Gap 2 phase
GAS5	Homo sapiens growth arrest-specific 5
GFR	Glomerular filtration rate
GUSB	Homo sapiens glucuronidase beta
HCl	Hydrochloric acid
H&E	hematoxylin and eosin staining
HOTAIR	Homo sapiens HOX transcript antisense RNA
HTRA2/OMI	Serine peptidase 2
HUVEC	Human umbilical vein endothelial cell line
IC	Inhibitory concentration
IKBKB	Homo sapiens inhibitor of nuclear factor kappa B kinase subunit beta
IKBKE	Homo sapiens inhibitor of nuclear factor kappa B kinase subunit epsilon
JC-1	Tetraethylbenzimidazolylcarbocyanine iodide
LDH	Lactate dehydrogenases
LEF1-AS1	Homo sapiens LEF1 antisense RNA 1
LINC-PINT	Homo sapiens long intergenic non-protein coding RNA, p53 induced transcript
LncRNA	Long non-coding RNA
M	Mitosis phase
MAP	Multi-agent chemotherapy
McCoy's 5A	McCoy's 5A Modified Media
MDA	Malondialdehyde
MET	Mesenchymal to epithelial transition
MG-63	Human osteosarcoma cell line
MMP3	Homo sapiens matrix metallopeptidase 3
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide
NFKBIA	Homo sapiens NFKB inhibitor alpha

NFKBIE	Homo sapiens NFKB inhibitor epsilon
NO	Nitric oxide
OS	Osteosarcoma
PBS	Phosphate-buffered saline
PI	Propidium iodide
PTEN	Homo sapiens phosphatase and tensin homolog
PVT1	PVT1 oncogene
RIN	RNA integrity number
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RT-PCR	Real time polymerase chain reaction
S	Synthesis phase
SDS	Sodium dodecyl sulphate
S.E.M	Standard error of the mean
SMAC/DIABLO	Homo sapiens Diablo IAP-binding mitochondrial protein
SOD	Superoxide radical scavenging activity
TP73	Homo sapiens tumor protein p73
TUG1	Homo sapiens taurine up-regulated 1
U-2 OS	Human osteosarcoma cell line
UCA1	Homo sapiens urothelial cancer associated 1
VEGFA	Homo sapiens vascular endothelial growth factor A
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Cancer is one of the health issues that caused major concerns in medical sector with approximately 9.6 million deaths worldwide (Bray et al., 2018; Cancer Research UK, 2018). This disease has become the fourth leading cause of death in Malaysia with nearly 43, 837 new reported incidences in 2018 and the probability of lifetime risk for Malaysian to diagnose with cancer is 1 in 9 for female and 1 in 10 for male (Malaysian National Cancer Registry Report, 2019). World Health Organization (WHO) described cancer as an uncontrollable proliferation of neoplasm cells with aberrant growth rate and have the ability to spread at the secondary loci.

Osteosarcoma (OS) or bone cancer is one of the most prevalent paediatric cancers, that is frequently diagnosed among younger patients in Malaysia (Synopsis of Childhood Cancer Incidence in Malaysia, 2018; The Star, 2017). Patients who are diagnosed with this particular cancer will manifest several notable symptoms such as swollen, erythema, enlarged palpable mass, brittle bone at the tumor site and acute pain that consistently intensifies for months (Taran et al., 2017). This condition will worsen once the OS patients are further diagnosed with a distant pulmonary metastasis, which consequently reduces the 5 years survival rate substantially to 30% even upon being treated with an intensive course of chemotherapy (Osborne and Khanna, 2012).

Currently, chemotherapy and surgical resection are the standard treatment strategies used to treat the osteosarcoma patients (Ando et al., 2013; Luetke et al., 2014). Doxorubicin is a chemotherapy drug that is frequently prescribed to the osteosarcoma patients and occasionally this drug will be administered in combinatorial format with another chemotherapy agents like cisplatin and methotrexate (Ando et al., 2013; Luetke et al., 2014). Even though, current administration of these chemotherapy drugs has considerably improved the 5 year survival rate of the patients, yet utilization of these drugs especially doxorubicin has been reported to be associated with several complications such as myelosuppression, mucositis, and cardiotoxicity (Janeway and Grier, 2010; Jia et al., 2012; Luetke et al., 2014). Consequently, these adverse side effects will immensely cause health complications to younger patients whose lifespan is left vulnerable due to this treatment efficacy (Harake et al., 2012). Apart from that, OS patients diagnosed in 2017 have been reportedly still receiving similar standard medical therapies that remain indispensably unchanged since late 1970s when the use of MAP (methotrexate, doxorubicin, and cisplatin) was first introduced in OS treatment (Saraf et al., 2018). For the past few decades, this slow advancement has caused stagnancy in the 5 years overall survival rate especially in patients diagnosed with advance and recurrent osteosarcoma, who continue to face poor prognosis and dismal outcomes including onset chemoresistance as well as pulmonary metastasis

(Saraf et al., 2018; Zhang et al., 2018). Therefore, continuous discovery of potent anti-cancer agents with fewer side effects from naturally derived substances is imperative in order to tackle this issue.

Natural products have been widely used in various sectors including agriculture, cosmetics, food production, and most importantly in medical (Sorokina and Steinbeck, 2020). Curcumin is a yellow polyphenol bioactive molecule derived from turmeric, a spice that originates from the roots of Curcuma longa plant (Anand et al., 2007; Stohs et al., 2020). Reportedly, curcumin possesses broad spectrum of pharmacological benefits including anti-inflammatory, anti-oxidant, anti-angiogenesis, and anti-metastasis (Stohs et al., 2020; Paulraj et al., 2019; Hewlings and Douglas, 2017). However, curcumin purportedly has poor cellular uptake which subsequently contributes to poor absorption and bioavailability; this condition will ultimately limit the curcumin efficacy as an anti-cancer agent (Anand et al., 2007; Nagahama et al., 2016; Stohs et al., 2020).

Owing to the excellent bioactivities manifested by curcumin, further improvements were adopted to ameliorate any physiological limitation such as excessive hydrogen bonding motifs in the curcumin structure that causes considerable deleterious effects on the membrane permeability (Nagahama et al., 2016; Alex et al., 2011). Therefore, in this study curcumin analog (Z)-3-hydroxy-1-(2-hydroxyphenyl)-3-phenylprop-2-en-1-one (DK1) with simplified, shorter chain structure and optimal hydrogen bonding motifs was utilized as a potential anti-cancer agent against osteosarcoma. Moreover, this particular analog was found to inhibit proliferation and induce apoptosis in several cancer cell lines such as SW620 and HT29 colon cancer cell lines as well as MCF-7 breast cancer cell line (Hussin et al., 2018; Ali et al., 2017). In fact, just like natural curcumin, DK1 was also reported not to induce any cytotoxicity effects on several normal cells including 3T3, MCF10A and mice's splenocytes (Hussin et al., 2018; Ali et al., 2017).

For this particular study, the effectiveness of curcumin analog DK1 was further evaluated in human osteosarcoma cell lines with the general objective to comprehensively elucidate the DK1 anti-cancer effects as well as its mechanism, in terms of apoptosis, metastasis and long non-coding RNA. Additionally, the DK1 safety was also assessed in order to examine any possible toxicity signs on the BALB/c mice. This study will provide an uttermost evaluation of DK1 potential as an anti-cancer agent to date.

Therefore, this study was performed in accordance to the objectives listed below:

1. To evaluate the cytotoxicity effects and anti-cancer potential of DK1 in term of apoptotic induction in both U-2 OS and MG-63 human osteosarcoma cell lines *in vitro*.

2. To determine the anti-metastatic and anti-angiogenic potential of DK1 in both U-2 OS and MG-63 human osteosarcoma cell lines *in vitro*.

3. To investigate the regulation of anti-cancer mechanism and pathway associated to apoptosis and metastasis activities in both human osteosarcoma cells by DK1.

4. To assess the expression of characterized and uncharacterized long non-coding RNA (lncRNA) regulator in both U-2 OS and MG-63 human osteosarcoma cell lines treated with DK1.

5. To assess the subchronic toxicity of DK1 in BALB/c mice model.

Hypotheses

Curcumin analog DK1 is safe and will exert cytotoxicity and anti-metastatic effects in both human osteosarcoma cell lines. Furthermore the mechanisms and pathways involved in its anti-cancer effects will be elucidated.

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