UNIVERSITI PUTRA MALAYSIA

ANTI-ANGIOGENIC AND ANTI-HEPATOCELLULAR CARCINOMA PROPERTIES OF ZERUMBONE EXTRACTED FROM ZINGIBER ZERUMBET (L.) SMITH

NOZLENA BINTI ABDUL SAMAD

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By

NOZLENA BINTI ABDUL SAMAD

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By

NOZLENA BINTI ABDUL SAMAD

April 2015

Chairman: Ahmad Bustamam Abdul, PhD
Faculty: Institute of Bioscience

Zerumbone (ZER) extracted from Zingiber zerumbet is known to have anti-cancer properties; however, its mechanism in curbing liver cancer growth and spread is still not clear. Thus the objective of this study is determine the in vitro anti-cancer effect of ZER towards HepG2 cell line and the in vivo effect on induced rat hepatocellular carcinoma (HCC). The anti-cancer mechanisms investigated were apoptosis, anti-proliferation and anti-angiogenesis. Zerumbone was shown to be toxic towards HepG2 cells with IC\textsubscript{50} of 6.20±0.70 µg/mL and less toxic towards normal liver cells (WRL68) with IC\textsubscript{50} of 61.00±0.40 µg/mL. The study showed that ZER caused cell cycle arrest at the G2/M phase and apoptosis, demonstrated by chromatin condensation, cell shrinkage and formation of apoptotic bodies in the HepG2 cells in a time-dependent manner. Zerumbone also stimulated caspase-3 and -9 activities in the HepG2 cells, suggesting that the induction of apoptosis was via the mitochondrial pathway. The study employed the diethylnitrosamine-induced rat HCC model and the rat aortic ring to determine the effect of ZER treatment. The study showed that ZER significantly (p<0.05) inhibited microvessel outgrowth in the aortic ring model. Zerumbone at 12.5 µg/mL caused the most significant (p<0.05) 98±1.28% blood vessels inhibition compared with the control and inhibited endothelial tube formation at 96.00±0.72%. This study showed that ZER treatment decreases expression of VEGF, MMP-9 and Ki-67 in the rat HCC tissue as well as and inhibits neovascularization in the chick embryo. The treatment had also induced apoptosis in HCC. The ZER-treated liver tissues with HCC showed normal hepatocyte orientation, unlike the untreated livers, which showed pleomorphic hepatocytes and anaplastic appearance typical of HCC. It can be concluded from the study that the anti-cancer effect of ZER on the HepG2 cell line and HCC is multifaceted involving induction of cell cycle arrest, apoptosis, and suppression of VEGFR, VEGF, MMP-9 and Ki-67 proteins, leading to inhibition of angiogenesis. Since ZER was less toxic to the normal liver cells, this compound is a potentially effective anti-HCC agent, without significant side-effects and can be developed as a therapeutic regime either alone or in combination with other chemotherapeutic agents.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

KANDUNGAN ANTI-ANGIOGENESIS DAN ANTI-KARSINOMA HEPATOSEL BAGI ZERUMBON YANG DI EKSTRAK DARI ZINGIBER ZERUMBET (L.) SMITH

Oleh

NOZLENA BINTI ABDUL SAMAD

April 2015

Pengerusi: Ahmad Bustamam Abdul, PhD
Fakulti: Institut Biosains

Zerumbon (ZER) yang diekstrak daripada Zingiber zerumbet diketahui mempunyai sifat anti-kanser; bagaimanapun mekanisme dalam perencatan pertumbuhan dan perebakkan kanser hati masih belum jelas. Objektif kajian ini ialah untuk menentukan kesan anti-kanser ZER in vitro terhadap titisan sel HepG2 dan kesan in vivo pada karsinoma hepatosel (HCC) tikus. Mekanisme anti-kanser yang diselidik ialah apoptosis, anti-pemproliferatan dan anti-angiogenesis. Perubahan morfologi ditentukan melalui mikroskopi elektron imbasan. Zerumbon didapati toksik terhadap sel HepG2 dengan IC_{50} 6.20±0.40 µg/mL dan kurang toksik kepada sel hati normal (WRL68) dengan IC_{50} 61.00±0.04 µg/mL. Kajian ini menunjukkan ZER menyebabkan sekatan kitaran sel pada fasa G2/M dan apoptosis, yang ternyata sebagai pengenapan kromatin, pengecutan sel dan pembentukan jasad apoptosis pada sel HepG2 yang berlaku secara bersandarkan masa. Zerumbon juga merangsang aktiviti kaspase-3 dan -9 dalam sel HepG2, dimana ini menunjukkan bahawa pengaruh apoptosis adalah melalui arah laluan mitokondrion. Kajian ini telah menunjukkan bahawa ZER pada kepekatan 12.5µg/ml merancat pertumbuhan mikrovesel secara paling ketara (p<0.05) dalam model gegelang aorta serta merancat pembentukan tiub endotelium pada kadar 96.00±0.72%. Apoptosis dalam tisu hati terperlaku ZER ditentukan melalui assai TUNEL. Kajian ini menunjukkan bahawa perencatan ZER telah mengurangkan penyataan protein VEGF, MMP-9 dan Ki-67 pada tisu HCC tikus dan juga merancat neopengvaskulararan pada embrio anak ayam. Perlakuan ini juga telah menunjukkan apoptosis dalam HCC. Tisu hati dengan HCC yang diperlakukan ZER menunjukkan orientasi hepatosit yang normal, bukan seperti pada hati yang tidak terperlaku, yang menunjukkan hepatosit pleomorfik and tampilan anaplasia yang tipikal untuk HCC. Kesimpulan daripada kajian ini ialah, kesan anti-kanser ZER terhadap titisan sel HepG2 dan HCC adalah berperan mengurangkan seketan titisan dan apoptosis serta penindasan protein VEGFR, VEGF, MMP-9 dan Ki-67, yang membawa kepada perencatan angiogenesis. Oleh kerana ZER kurang toksik terhadap titisan sel hati normal, maka sebatian ini adalah berpotensi berkesan sebagai agen anti-HCC, tanpa kesan sampingan yang ketara dan boleh dikembangkan sebagai regim terapeutik sama ada secara bersendirian atau gabungan dengan agen kemoterapi lain.
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I certify that a Thesis Examination Committee has met on 27 April 2015 to conduct the final examination of Nozlena binti Abdul Samad on his thesis entitled “Anti-Angiogenic and Anti-Hepatocellular Carcinoma Properties of Zerumbone Extracted from *Zingiber zerumbet* (L.) Smith” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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<tbody>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>h</td>
<td>Hour/s</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>ML</td>
<td>Milliliter</td>
</tr>
<tr>
<td>Mg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μL</td>
<td>Microliter</td>
</tr>
<tr>
<td>mM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>Pg/ml</td>
<td>Pikogram/milliliter</td>
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<tr>
<td>rpm</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AO</td>
<td>Acridine orange</td>
</tr>
<tr>
<td>ATCC</td>
<td>American tissue culture collection</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaCo₂</td>
<td>Calcium carbonate</td>
</tr>
<tr>
<td>CAM</td>
<td>Chick chorioallantoic membrane</td>
</tr>
<tr>
<td>CDK-2</td>
<td>Cyclin-dependent kinase 2</td>
</tr>
<tr>
<td>CDK-4</td>
<td>Cyclin-dependent kinase 4</td>
</tr>
<tr>
<td>DEN</td>
<td>Diethylnitrosamine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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EGF     Epidermal growth factor
eNOS    Endothelial nitric oxide synthase
FBS     Fetal bovine serum
FITC    Fluorescein isothiocyanate
FFPE    Formalin-fixed paraffin-embedded
G₀      Gap 0 at cell cycle
G₁      Gap 1 at cell cycle
G₂/M    Gap 2/mitosis at cell cycle
H3      Histone 3
H4      Histone 4
HCC     Human hepatocellular carcinoma
HepG2   Human hepatocellular carcinoma cells
HIFCS   Heat inactivated fetal calf serum
HRP     Horseradish peroxidase
HT29    Human colorectal adenocarcinoma cell
HUVEC   Human umbilical vein endothelial cells
IC₅₀    Half maximal (50%) inhibitory concentration
IL2     Interleukin 2
IL6     Interleukin 6
IL8     Interleukin 8
iNOS    Inducible nitric oxide synthase
IUPAC   International Union of Pure and Applied Chemistry
KI67    Protein associated with cell proliferation
LC₅₀    Lethal concentration, 50%
MCF-7   Human breast cancer cell
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<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinase 9</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>Nacl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>pH</td>
<td>A scale that measures how acidic or basic a substance</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonylfluoride</td>
</tr>
<tr>
<td>PO₂</td>
<td>Oxygen partial pressure</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphotidylserine</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute medium</td>
</tr>
<tr>
<td>Rtdt</td>
<td>Terminal deoxynucleotidyl transferase recombinant</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline tween</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>WAF1</td>
<td>Cyclin dependant kinase interacting protein 1</td>
</tr>
<tr>
<td>WRL68</td>
<td>Human normal hepatic cells</td>
</tr>
<tr>
<td>ZER</td>
<td>Zerumbone</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Introduction

Natural products refer to compounds that are derived from animals, plants or microorganisms. Since early civilization, natural products have played important roles in health care and prevention of diseases in humans and animals. Before the 19th century, natural products were the sole mean in the treatment diseases and injuries. Our ancestors chewed herbs to relieve pain and wrapped the leaves around the wound to facilitate healing. By the 19th century, the natural products in its original form began playing a secondary role in therapy when active therapeutic elements were isolated from medicinal plants. In 1806, morphine was isolated from *Papaver somniferum* (Wetzel *et al.*, 2010), atropine from *Atropa belladonna*, ziconotide from a cone snail and Taxol from the bark of the Pacific yew tree (Cragg and Newman, 2013). This was the beginning of evolution of natural products in modern medicine. Based on recent data (WHO), 80% of the world’s population depend on traditional medicine (Koehn and Carter, 2005; Newman *et al.*, 2003). Approximately, 25% of the drugs prescribed today are derived from natural products (Zhang *et al.*, 2013). Natural products have also significantly contributed to the development of vaccines and anticancer drugs. Between 1981 to 2006 more than 100 anticancer drugs were developed and 47% of these were derived from natural products (Newman *et al.*, 2003).

Cancer is a complex disease that develops from single damaged cell, subsequent to the accumulation of errors to its genes (Loeb, 2000). Manifestation of these genetic errors may possibly be the result of exposure of chemicals, viruses and physical assault to the cell (Karpinets and Foy, 2004). These noxious factors can influence or damage cellular pathways. Signaling pathways leading to cancer are numerous; therefore the biological profile of this disease would differ from one cancer patient to another depending on which pathway is affected by the cancer-causing agents (Chin and Gray, 2008).

Liver is a complex organ in the human body, performing approximately 500 functions daily for the maintenance of the organism (Maton *et al.*, 1993). The liver is quite often affected by cancers and these diseases can originate in the liver itself or the result of metastasis. Liver cancer is the fifth most common type of cancers and the third leading cause of cancer-related death (Davis *et al.*, 2008). The majority (80%) of reported liver cancer cases occur in developing countries. Among the countries with highest rates of liver cancers include central and Western Africa, Southeast Asia, China and Mongolia (Mokdad *et al.*, 2014). Liver cancers are typically hypervascular tumours or carcinomas. Treatment of this type of carcinoma is difficult because most patients, especially those in less-developed countries, are diagnosed when the disease is already at an advanced stage (El-Serag and Rudolph, 2007). Furthermore, there is high incidence of recurrence, possibly metastasis after hepatic resection, with the disease becoming non-amenable towards therapy (Davis *et al.*, 2008).
The failure and various side-effects of conventional medicine in treating cancers have led to the growing interest in the search for drugs from natural resources. Among advantages of drugs from natural products are that they are affordable and accessible to the majority of the world population that does not have access to modern conventional pharmacological treatments. Natural products are also claimed to be harmless and have minimal or no side-effect in comparison to synthetic drugs (Rates, 2001).

Angiogenesis is a process of new blood vessel formation. Inhibition of angiogenesis is considered one of the most promising strategies in treating a variety of illnesses including cancers (Adair, 2010). The inhibition of angiogenesis may potentially be a very effective way to treat and inhibit progression and spread of cancers. Angiogenesis is controlled through the balance between pro-angiogenesis and anti-angiogenesis factors, which are vital to the triggering of angiogenesis switch (Keshet and Ben-Sasson, 1999). Several signals that can trigger this switch include low partial oxygen pressure, pH and glucose levels (Kizaka et al., 2003). Anti-angiogenesis drugs are proven to boost anti-tumor activities of several conventional cytotoxic chemotherapeutic drugs (Folkman, 2002). However, different organs and tissues may express different angiogenesis receptors, which pose a great challenge in the development of effective anti-angiogenesis therapy, particularly with receptor-specific compounds, such as monoclonal antibodies. Moreover, the microenvironment of tumor site, for example the endothelium that is phenotypically distinctive for the organ, may influence the efficacy anti-angiogenesis. This phenomenon makes an agent that is therapeutically effective in one organ may not be effective in another (Kerbel, 2000).

Zerumbone (ZER) is a sesquiterpene phytochemical from a type of edible ginger known as *Zingiber zerumbet* (L.) Smith found abundantly in Southeast Asia (Murakami et al., 2002). Zerumbone is currently being explored for its effects on cancers to include leukemia, cervical, colon and breast cancers. To date, there has been no report on the effect of ZER on anti-angiogenesis in liver cancers.

The current study was undertaken to determine the anti-angiogenesis properties as well as the anti-cancer effect of ZER in hepatocellular carcinoma. Previous studies in our laboratory showed that ZER retards cervical intraepithelial neoplasia (CIN) in cervical tissues of female BALB/C, induced prenatally with diethylstilbestrol to develop the cancer (Abdelwahab et al., 2010). The anti-cancer properties of ZER were found to be equivalent to that of cisplatin, a commercial anticancer drug preferentially used in treating cervical cancer in humans (Abdelwahab et al., 2010). Zerumbone also possesses anti-inflammatory activities (Sulaiman et al., 2009), which is beneficial in the inhibition of angiogenesis. Zerumbone was also chosen for this study because of its traditional use in the treatment of several illnesses while possessing high anti-oxidant activities (Yob et al., 2011). This study was conducted *in vitro* on HepG2 cells, *ex vivo* on isolated liver tissue and *in vivo* in a rat hepatocellular carcinoma model.
1.2 **Aims and objective**

General Objectives
To ascertain the anti-angiogenic and anti-cancer effects of ZER in rat hepatocellular carcinoma.

Specific Objectives
To determine the

- anti-proliferative and apoptotic activity of ZER on a liver cancer (HepG2) cell line.
- anti-angiogenesis mechanism of ZER using *in vitro, ex vivo* and *in vivo* assays
- anti-angiogenesis and anti-proliferative effects of ZER in the rat hepatocellular carcinoma model.

1.3 **Hypothesis of the Study**

Zerumbone has anti-cancer effect through inhibition of angiogenesis.
References


