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ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANTIANGIOGENIC ACTIVITY AND ROS-MEDIATED LUNG CANCER CELL LINE INJURY OF ZERUMBONE

ZERUMBONE'UN ANTİANJİYOJENİK AKTİVİTESİ VE ROS ARACILI AKCİĞER KANSERİ HÜCRE HATTI HASARI

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ABSTRACT

Objective: *Zerumbone (ZER) is a well-known natural compound that has been reported to have anti-cancer effect. Thus, this study investigated the ZER potential to inhibit Thymidine Phosphorylase (TP) and the ability to trigger Reactive oxygen species (ROS)-mediated cytotoxicity in non-small cell lung cancer, NCI-H460, cell line.*
Material and Method: *The antiangiogenic activity for ZER was evaluated by using the thymidine phosphorylase inhibitory test. Reactive oxygen species (ROS) production was determined via DCFDA dye by using flow cytometry.*
Result and Discussion: *ZER was found to be potent TP inhibitory with the IC_{50} value of 50.3±0.31 µg/ml or 230±1.42 µM. NCI-H460 cells upon treatment with ZER produced significant ROS by 55.7%. Consequently, ZER exerts anti-angiogenic properties and modulates ROS production in lung cancer cells, serving as leads for better therapeutic index in cancer drug.*
Keywords: *Flow cytometry, NCI-H460, thymidine phosphorylase*

ÖZ

Amaç: *Zerumbone (ZER), kanser önleyici etkisi olduğu bildirilen, iyi bilinen bir doğal bileşiktir. Bu nedenle, bu çalışma, ZER'in Timidin Fosforilaz'ı (TP) inhibe etme potansiyelini ve küçük hücreli dışı akciğer kanser, NCI-H460, hücre hattında Reaktif oksijen türleri (ROS) aracılı sitotoksisiteyi tetikleme yeteneğini araştırmıştır.*
Gereç ve Yöntem: *ZER'in antianjiyogenik aktivitesi, timidin fosforilaz inhibitör testi kullanılarak değerlendirilmiiştir. Akış sitometrisi kullanılarak DCFDA boyası ile reaktif oksijen türleri (ROS) üretimi belirlenmiştir.*
Sonuç ve Tartışma: *ZER'in 50.3±0.31 µg/ml veya 230±1.42 µM IC_{50} değeri ile güçlü bir TP inhibitörü olduğu bulunmuştur. ZER ile işleme tabi tutulduktan sonra NCI-H460 hücrelerinin, %55.7 gibi yüksek bir oranda ROS ürettiği bulunmuştur. Sonuç olarak, ZER'in, anti-anjiyojenik*

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özellikler sergilediği ve akciğer kanseri hücrelerinde ROS üretimini modüle ederek kanser tedavisinde daha iyi terapötik indeks sunduğu tespit edilmiştir.
Anahtar Kelimeler: *Akış sitometrisi, NCI-H460, timidin fosforilaz*

INTRODUCTION

Angiogenesis is a process of the formation of new blood vessels from pre-existing vasculature. This process is very crucial for tumor growth and progression of cancer [1]. To enhance the process of angiogenesis, thymidine phosphorylase (TP) enzyme plays a vital role as growth factor by promoting endothelial cell migration and the release of different angiogenic factors from malignant and stromal cells in the tumor microenvironment site which play critical roles in the evasion of apoptosis and immune cells [2]. In various cancers, up regulation of angiogenesis plays vital role in the progression of glioblastoma [3], melanoma [4], therefore many compounds are under pre-clinical trial to block angiogenesis pathway. Among different triggering factors, thymidine phosphorylase enzyme has received special attention due to its outstanding ability to trigger vascular growth; therefore this enzyme has been used as a validated target to find antiangiogenic compounds [5].

Reactive oxygen species (ROS) are known potential carcinogens and act as a key player in the progression of mutagenesis by converting normal cells into malignant cells, but its increasing might exploit selective killing leading to ROS-mediated cancer cell injury. The anticancer effect of some chemotherapeutic drugs includes elevation of intracellular levels of ROS which raise the level of oxidative stress in cancer cells and causes ROS mediated cell injury leading to cell death. Most chemotherapeutics often failed because of cytotoxicity and drug resistance [6]. Thus, it is necessary to have safe natural products which can modulate intracellular ROS such that it can prevent cell signaling and cellular functions, thereby preventing the progression and metastasis of cancers [7].

Since ancient time, mankind at different geographical locations had been using different plants, animals, fungal, minerals for treating different diseases [8-11]. Recently, many researches are investigating on natural compounds from different plants for their anti-cancer potential and this approach has led to the discovery of many new therapeutic agents [12,13]. Among various anticancer mechanisms proposed, antiangiogenic and modulating intracellular ROS mechanism has also important role, in which immune systems are given opportunity to identify and respond to the tumor cells [14]. ZER, a natural sesquiterpene from edible ginger *Zingiber zerumbet* Smith, has anti-inflammatory, immunomodulatory, antibacterial [15-17], antioxidant, antinociceptive, and anti-proliferation activities against several cancer cells [18-20]. Even though, the pharmacological studies had shown the anti-cancer effects of ZER through the induction of apoptosis on human lung cancer cells [21], the anti-angiogenic activity of ZER and its possible anticancer capacity by modulating intracellular ROS have not been clearly evidenced yet. In the current study, new mechanism of ZER was explored by targeting against thymidine phosphorylase to inhibit angiogenesis. Together, generation of ROS in NCI-H460 cell was also evaluated after ZER treatment.

MATERIAL AND METHOD

ZER crystals were gifted by Professor Dr. Rasedee Abdullah from Universiti Putra Malaysia (UPM), Malaysia. The crystals were diluted with 0.1% dimethyl sulfoxide (DMSO) to obtain the stock solution.

In vitro Thymidine Phosphorylase Inhibition

The protocol of Iftikhar et al [22] was followed. All the chemicals used in this assay were taken from Molecular Bank of International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan.Potassium phosphate buffer with 50 mM and pH 7.0 was prepared and 150 µl of this buffer was kept in tube. In this buffer, 20 µl of TP enzyme (0.058 unit/well) was added. To this solution, 10 µl of test compound (1 mg/ml) was added and whole reaction mixture was incubated at 30^{o}C for 10 min. After incubation, 20 µl thymidine substrate (1.5 mM) was added and continuously monitored at 290 nm to detect any change in the absorbance. This observation was continued for 15

min. Absorbance was taken in 96-well ELISA plate reader (MultiSkan go, ThermoFisher Scientific). For positive control, 7-Deazaxanthine was used as standard drug. Every experiment was run in triplicate.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay of ZER on NCI-H460 Cell Line

The human lung cancer, NCI-H460, cell line was obtained from cell culture bio bank of Dr. Panjwani Center for Molecular Medicine and Drug Research, ICCBS, University of Karachi. The protocol of Albaayit et al [23] was followed in which cells were cultured and passaged using DMEM medium. MTT assay was used to find the cytotoxic effect of the ZER on the NCI-H460 cell lines. After trypsinization, 5×10^5 NCI-H460 cells/well were counted and dispensed in each well in a 96-well plate and incubated at 37°C, 5% CO_2. Next day, cells were properly attached at the bottom of the wells. Cells were then treated with ZER at different concentrations (3.125 to 25 µg/ml), while the solvent control was treated with 0.5% DMSO and incubated for 48 h. On this cell suspension, 20 µl of MTT (0.5 mg/ml) reagent was added and the plate incubated for 4 h at 37°C. MTT dye upon reduction formed the purple formazan crystals at the bottom of the wells. These crystalswere dissolved with 100 µl DMSO and the absorbance measured at 540 nm using an ELISA plate (Multiskan GO, Thermo Scientific). Standard drug doxorubicin at 0.501 µg/ml was used as a positive drug control. The percentage of cytotoxicity of NCI-H460 was determined by using below formula.

$$\%\ \text{cytotoxicity/inhibition} = 100 - \frac{\text{O.D of treated well} - \text{O.D of media control}}{\text{O.D of untreated control} - \text{O.D of media control}}\ \text{X } 100.$$

Reactive Oxygen Species (ROS)

The protocol of Rasul et al [24] was followed to find ROS production by the NCI-H460 cells. Fully grown NCI-H460 cells were trypsinized from culture flask and counted. Approximately 1×10^6 NCI-H460 cells in 500 µl were seeded into each well of 24-well plate. Next day, these cells were treated with 3.05 µg/ml or 14.3 µM of ZER and the plate incubated for 48 h. Cells treated with hydrogen peroxide (50 µM) was considered as positive control. After treatment, the cells were then detached by trypsinization, washed with PBS, and 500 µl of 10 µM 2,7-dichlorofluorescein DCFH-DA dye was added and incubated for 30 min at 37°C in the dark. These cell suspensions were transferred to FACS tube and analyzed the ROS generation by using flow cytometry (FACSCaliburTM Cell Quest Pro Software version 2.0).

RESULT AND DISCUSSION

TP is an important angiogenic enzyme in the malignant progression, which induces migration and angiogenesis in endothelial and tumor cells. It is well known that TP provide pentose for tumor cells from the catalyzing thymidine, augment the expression of several angiogenic growth factors and trigger tumor angiogenesis [25]. There are many drugs that are being used to block angiogenesis and among these are tipiracil, trifluridine. However, these drugs are often compromised with serious side effects including weakness, vomiting, diarrhea, stomatitis, and dysgeusia [26].

Drugs to be used for the treatment for the condition should not only be efficacious but also not to cause significant adverse effects [27-30]. Recently, cancer researchers are focused on the natural sources, especially on traditional medicinal and edible types for the treatment of tumor and for controlling of angiogenesis and metastasis due to their safety issue [31]. In the present study, ZER exhibited TP inhibition with IC_{50} values of 50.3 ± 0.31 µg/ml or 230 ± 1.42 µM. The standard drug (7-deazaxanthine) showed IC_{50} value of 41.0 ± 1.63 µM [23]. Based on the outcome of TP enzyme inhibition study, the potential of ZER to have antiangiogenic effect was observed. This study gave supporting evidence to the previous study by Shamoto et al., 2014 [32] in which zerumbone was reported to inhibit pancreatic cancer through inhibiting angiogenesis process by blocking NF-κB activity.

ZER was reported to have inhibitory effect on several enzymes like pancreatic lipase [33], carbonic anhydrase [33], murA [34], trypsin protease [35], TNF-alpha [36]. From the findings of these similar previous studies, the present study propose that ZER is potential inhibitors of Thymidine

phosphorylase enzyme by interacting to the amino acid residues in the active site of enzyme, and prevent the interaction between enzyme and corresponding substrate and growth of cancer.

Various evidences have shown the role of ROS concentration in the tumor microenvironment and its progression towards malignant state. Numbers of evidences have shown the dual nature of ROS i.e tumor-suppression [37,38] and tumor-promotion effects [39], depending on the level of ROS concentration in the state of tumor cells and its surrounding microenvironment [40]. Overexposure of ROS is toxic to cells and can initiate apoptotic and necrotic pathway [41,42]. The behavior of tumors in response to ROS depends on the threshold concept, in which increase in ROS causes cancer cells to undergo in an adaptive state and later cells will perish upon exceeding the threshold limit [43,44]. The current study showed that ZER was active against NCI-H460 cells and IC_{50} value was found to be 3.05±0.01 µg/ml (Figure 1). NCI-H460 cells, after treatment with 3.05 µg/ml or 16 µM ZER for 24 h, significantly increased ROS by 55.7%. Negative treated cells didn't show increase in ROS whereas, H_2O_2 treated cells showed significant rise in fluorescence due to high ROS production (Figure 2).

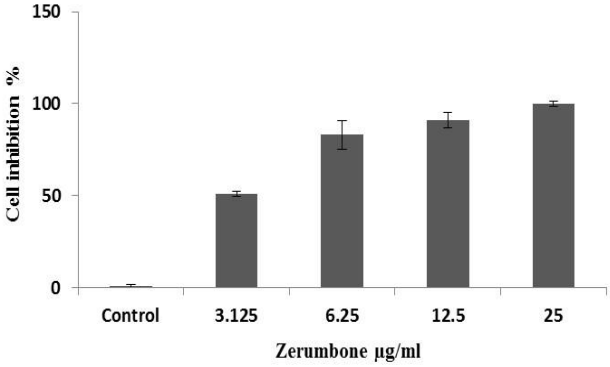

Figure 1. Determination of the cell viability of lung cancer (NCI-H460) cells after treatment with ZER for 48 h exposure

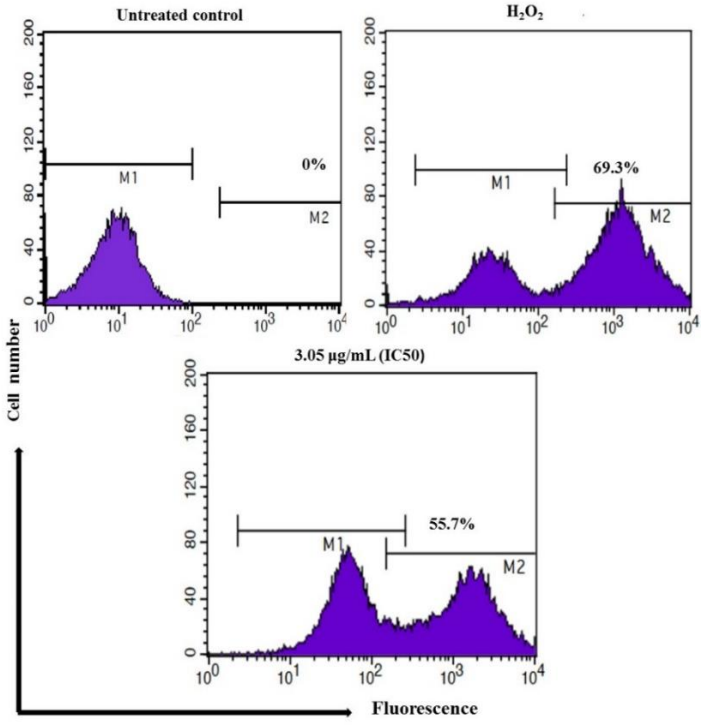

Figure 2. ROS production by NCI-H460 cells after treatment with 3.05 µg/ml of ZER. Cells treated with Hydrogen peroxide (H_2O_2) were the positive control

The present ROS induced cancer cell death result was similar to the previous manuscript in which ZER was reported to produce significant ROS from non-small cell lung cancer line beyond the protective effect of cellular anti-oxidant defense mechanism, resulting in damage to cell membrane and finally cell death [21,45]. ROS kill cancer cells by triggering both intrinsic and extrinsic apoptotic pathways [46]. In addition, zerumbone was reported to have ROS induced cytotoxicity against Glioblastoma multiforme [47], breast cancer [48], colorectal cancer [49], and chronic myelogenous leukemia cells [50,51].

In conclusion, the present study provides supporting evidence for zerumbone to have antiproliferative activity against non-small cell lung cancer through thymidine phosphorylase inhibition and ROS induced cytotoxicity. This support antiangiogenic mechanism of action of zerumbone against many cancers cell lines through suppressing the vascular growth around the tumor.

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AUTHOR CONTRIBUTIONS

Concept: S.F.A.A.; Design: S.F.A.A.; Control: S.F.A.A.; M.K.M.; Sources: S.F.A.A.; Materials: S.F.A.A.; Data Collection and/or Processing: S.F.A.A.; Analysis and/or Interpretation: S.F.A.A.; Literature Review: S.F.A.A.; Manuscript Writing: S.F.A.A.; Critical Review: S.F.A.A., M.K.M.; Other: M.K.M.

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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