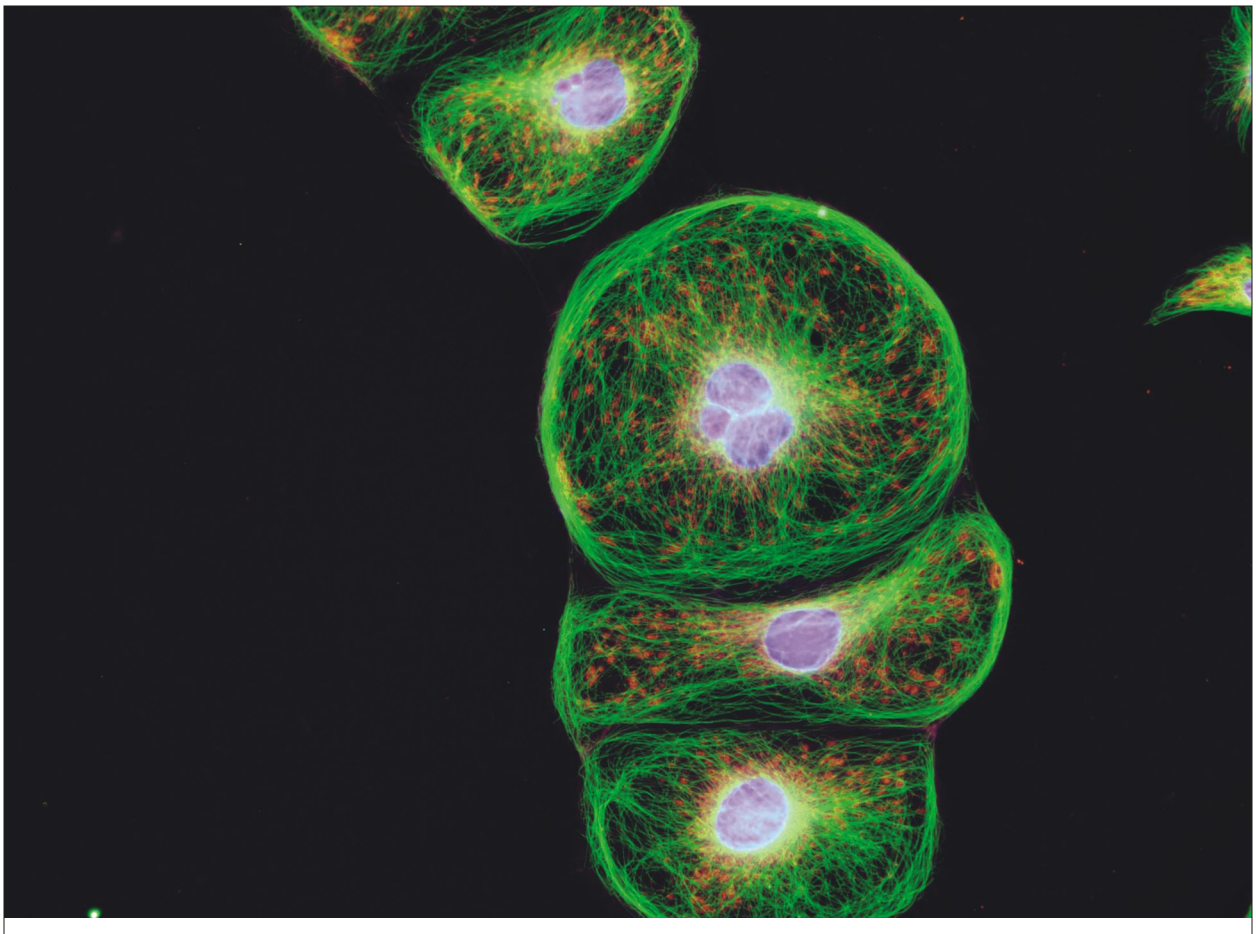

Asian Journal of Cell Biology

ISSN 1814-0068

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS Asian Journal of Cell Biology

ISSN 1814-0068
DOI: 10.3923/ajcb.2016.13.20

Research Article
Evaluation of Cytogenetic and DNA Damage Effects Induced by Zerumbone

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Abstract

Background and Objective: Zerumbone (ZER) is a sesquiterpene isolated from the rhizomes of *Zingiber zerumbet* smith, family Zingiberaceae. Anti-cancer and apoptosis-inducing properties of zerumbone against various human tumour cells have been elucidated earlier. The aim of this study was to further evaluate *in vitro*, the cytogenetic and DNA damage effects induced by ZER in Chinese Hamster Ovary (CHO) cells and in human peripheral blood lymphocytes. **Methodology:** Two cytogenetic end points were used to investigate the clastogenic effects of ZER, namely Chromosomal Aberrations (CA) assay in cultured human peripheral blood lymphocytes and micronucleus test (MN) in CHO cell lines. The alkaline single cell gel electrophoresis assay (comet assay) was used to evaluate the ability of ZER to induce DNA damage in stimulated cultured human Peripheral Blood Lymphocytes (PBL). The chromosome aberrations induction and comet assays were performed on human PBL without any metabolic activation. While S9 liver metabolic activation system was used in micronucleus test. **Results:** The obtained results showed that ZER has no significant effects on human peripheral blood lymphocyte chromosomes in all treatment concentrations, while the comet assay results showed that high concentrations can induce DNA damage, however the DNA damage was less pronounced than that caused by cisplatin. **Conclusion:** In addition, treatment with high concentrations was found to be genotoxic in MN induction in CHO cell cultures.

Key words: ZER, DNA damage, chromosome aberrations, micronucleus, comet assay

Received: November 07, 2015 Accepted: December 01, 2015 Published: December 15, 2015

Citation: Adel Sharaf Al-Zubairi, 2016. Evaluation of cytogenetic and DNA damage effects induced by zerumbone. Asian J. Cell Biol., 11: 13-20.

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

World Health Organization (WHO) estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary health care in which most of this therapy uses plant extracts or their active components[1]. Worldwide considerable attention has been focused on herbal medicine which is based on the premise that these herbal plants may contain natural substances that can promote health and alleviate diseases[1].

Zingiber zerumbet is used in local traditional medicine as a cure for a number of illnesses. Scientific research towards *Zingiber zerumbet* proved that it contained a suppressive effect which was conducted by a bioactive compound, zerumbone (ZER). In some Southeast Asian countries, the rhizomes of the plant are employed as traditional medicines for anti-inflammation, while the young shoots and inflorescence are used as condiments. Zerumbone has gained a great attention due to its activity towards many diseases *in vitro* and *in vivo*. Recently[2], reported ZER as modulator for osteoclastogenesis induced by RANKL and breast cancer. In addition, ZER was reported to effectively suppress mouse colon and lung carcinogenesis through multiple modulatory mechanisms[3] and colonic tumour marker formation in rats and induces apoptosis in human colo-rectal cancer cell lines[4]. The compound was shown to inhibit the proliferation of human colonic adenocarcinoma cell lines in a dose-dependent manner, while the growth of normal human dermal and colon fibroblast was less affected[4,5]. It has also been shown to be active *in vivo* against DES-induced mice Cervical Intraepithelial Neoplasia (CIN)[6] and was previously demonstrated to inhibit both azoxymethane-induced rat aberrant crypt foci and phorbol ester-induced papilloma formation in mouse skin a further indication of its efficacy to prevent colon and skin cancers[7].

Many plant products contain compounds known to cause various diseases or even death in animals and humans as well as synthetic substances present as environmental pollutants and toxicants may cause similar effects[8,9]. In addition, many natural and synthetic compounds have been reported to act as mutagens and/or carcinogens as well[10,11]. A variety of *in vitro* genotoxicity test systems have been developed including cultured mammalian cell systems such as human Peripheral Blood Lymphocytes (PBL) or Chinese Hamster Ovary (CHO) cells, for the screening of potentially mutagenic, carcinogenic and/or teratogenic agents[12].

Taking into account the lack of information about the genotoxic potential of ZER, we continued to provide some data on the cytogenetic activity of this compound. Here we report the results obtained on the genotoxic effects of ZER in Chinese Hamster Ovary (CHO) cell lines by using micronucleus (MN) formation in the presence and absence of metabolic activation system (S9) and in human peripheral blood lymphocytes using chromosomal aberrations assay (CAs) as cytogenetic endpoint in addition to the comet assay as a genotoxicity assessment test. This paper describes the results of those investigations and comments on the *in vitro* genotoxic effects of ZER.

MATERIALS AND METHODS

Zerumbone (ZER): The ZER was extracted in the laboratory of cancer research MAKNA-UPM, University Putra Malaysia from the rhizomes of *Zingiber zerumbet* plant[13]. Zerumbone was extracted, isolated and purified using methanol extraction and Column Chromatography (CC) method. The isolated and purified ZER crystals were subjected to High Performance Liquid Chromatography (HPLC) and Liquid Chromatography Mass Spectrometry (LCMS) to confirm its purity and molecular weight. Further, 13C NMR and 1H NMR analysis were conducted towards the ZER crystals to confirm its molecular structure (Fig. 1). A stock solution of ZER is prepared immediately before use in absolute ethanol.

Chromosomal aberration assay in human peripheral blood lymphocytes: The study was carried out by using blood sample from one healthy, non-smoking male donor, aged 36 years. Approximately, 10 mL of blood was collected, by venepuncture, into tubes containing sodium heparin as anticoagulant. Whole blood was centrifuged at 1000 rpm and 0.5 mL of buffy coat-rich plasma was used for cultures. Test compound was prepared as 1 mg mL^{-1} stock solution in absolute ethanol just prior to use and a dosing volume of stock concentration diluted with medium ensured a final ethanol concentration of <1%. Human lymphocytes were exposed to different concentrations (10, 20, 40 and 80 μM) of ZER. Dose selection was based on the cytotoxicity levels (IC_{50}) of the compound effect on tumour cell lines[13]. Control cultures were handled in a manner identical to the treated ones. Mitomycin-C (Sigma, Germany) was used as a positive control.

The CA assay was carried out using conventional techniques. Lymphocyte cultures were set up by adding 0.5 mL of buffy coat-rich plasma to 4.5 mL of RPMI 1640 medium supplemented with 20% heat inactivated foetal bovine serum, antibiotics (penicillin and streptomycin) and L-glutamine (PAA laboratories). Lymphocytes were stimulated by adding 2% phytohaemagglutinin (PHA) (Gibco). The cultured lymphocytes were incubated for 24 h before treatment with ZER. A control untreated culture and ethanol

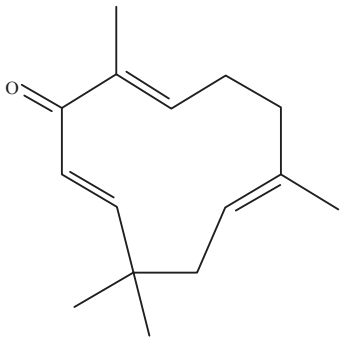

Fig. 1: Chemical structure of zerumbone

(vehicle control) treated cultures were established as well. Cultures were harvested 24 h after the treatments. Colcemid (PAA Laboratories) (0.2 mL) was added to the culture medium, 2 h prior to harvesting. Lymphocytes were stained with 6% Giemsa stain ([CAS 67-56-1] Gibco, Germany) in phosphate buffer, pH 6.8 (Sigma, USA).

Preparation of chromosome slides: Cells were collected by centrifugation, swelled in 0.075 M KCl (Sigma) pre-warmed at 37°C and fixed in a 3:1 mixture of methanol and glacial acetic acid. Next, the tubes were centrifuged at 800 rpm for 5 min, the supernatant discarded and the cell suspension dropped onto pre-chilled slides previously cleaned with non-toxic detergent and soaked in distilled water at 5°C. Chromosome slides were prepared, air dried and stained with 6% Giemsa stain. For each duplicate culture, 1000 cells were examined to score mitotic index. The Mitotic Index (MI) was calculated as the percentage of cells at the mitotic stage.

Analysis of chromosomal aberrations: The end points analyzed were mitotic index, total CAs and percentage of aberrant metaphases. The mitotic index was determined by scoring the number of metaphases in 1000 cells per culture for a total of 1000-4000 cells per treatment and control. A total of 200 well-spread metaphases containing 46 ± 1 chromosomes were scored. All slides were randomly coded and well-spread metaphase cells were analyzed for CAs as defined by Scott *et al.*[14,15] and OECD[16]. The number of each type of aberration and the percentage of cells with aberrations were recorded and summarized. The number of chromatid gaps was recorded when encountered, but not included in the calculations. The percentages of cells with aberrations from each concentration were compared to the solvent control values using χ^2 analyses.

Comet assay: The comet assay was conducted under alkali conditions according to Singh *et al.*[17]. In the comet assay the concentration doses used were 5, 10 and 20 µM and treatment conditions was as in the chromosome aberration test without adding colcemid to the culture flasks before harvest. Lymphocytes cultures were harvested and cells were washed in PBS (Ca^{2+} and Mg^{2+} free) (Amresco) and combined with LMA (low melting agarose) (at 37°C) at a ratio of 1:10 (v/v) and immediately 75 µL pipetted onto pre-coated comet assay slides. Slides were placed flat at 4°C in the dark for 40 min to be solidified. After the solidification, slides were immersed in pre-chilled lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris-HCl, 1% Triton X-100) in DMSO (Amresco), left for 30 min and immersed in freshly prepared alkaline solution (NaOH and EDTA, from Amresco), pH>13 for 40 min to allow for DNA unwinding. Electrophoresis was conducted in a horizontal electrophoresis platform containing pre-chilled alkaline solution (300 mM NaOH, 1 mM EDTA), pH>13. The voltage was set to about 1 V cm^{-1} with approximately 300 mA current for 30 min. Finally slides were removed from the electrophoresis solution and rinsed by dipping several times in dH_2O, then immersed in 70% ethanol for 5 min, air dried and 20 µg mL^{-1} of ethidium bromide placed onto each circle of dried agarose. Slides were then viewed by epifluorescence microscopy. Images of 50 randomly selected cells were analyzed from each slide and examined visually. Comets from the broken ends of the negatively charged DNA molecule become free to migrate toward the anode in the electric field. The assay provides direct determination of the extent of DNA damage in individual cells and the extent of DNA damage can be assessed from the length of DNA migration. Cells were scored visually into five categories according to tail size[18], from no tails-zero (undamaged, no migration) to maximally long tails-four (extensive migration with increasing numbers of breaks, DNA pieces migrate freely into the tail forming comet images), resulting in a single DNA damage score for each treatment studied. Arbitrary units were calculated by multiplying comet class with number of cells in that class and then summing up the total. Thus, the Damage Index (DI) of the group can range from 0 (all cells with no tails: 50 cells 0) to 200 (all cells with maximally long tails: 50 cells 4).

Micronucleus test (MN) in CHO cell lines: Chinese Hamster Ovary (CHO) cells were purchased from ECACC (UK). Atypical cell contains 21 chromosomes. The cells grow as an adherent monolayer in appropriate tissue culture vessels, doubling approximately every 12 h. They were maintained in RPMI 1640 medium (PAA Laboratories GmbH, Germany) supplemented with 10% foetal bovine serum (PAA Laboratories GmbH, Germany). The cell lines were cultured in sterile Nunc tissue

culture asks (Nunc, Denmark) and were incubated in a humidified tissue culture incubator at 37°C and 5% CO_2. When reached approximately 60-80% confluence, cells were dislodged with 0.05% trypsin (PAA Laboratories GmbH, Germany), collected by centrifugation and seeded in fresh medium. On the day before the experiment, approximately 5×10^5 cells were seeded into T-25 cm^2 culture flasks and were incubated overnight in a humidified incubator at 37°C and 5% CO_2.

Test compound was prepared as 1 mg mL^{-1} stock solution in absolute ethanol just prior to use and a dosing volume of stock concentration diluted with medium ensured a final ethanol concentration of <1%. The overnight cell cultures were examined under an inverted microscope. Duplicate cultures were prepared for each test substance concentration and controls. Control cultures were handled in a manner identical to the treated ones. Mitomycin-C (Sigma, Germany) was used as a positive control in the absence of metabolic activator (S9) while ethyl methanesulfonate (EMS) was used as a positive control in the presence of S9. The treatment media was 5 mL of the cell culture medium with 10% foetal bovine serum and the treatment concentrations 0, 12.5, 25.0 and 50 μg mL^{-1} or a control solution. The cell cultures were incubated with the treatment medium for 3 h with or without S9 liver fraction. The media was then replaced by media containing Cytochalacin-B and incubated for further 18 h.

Cells Harvest and slides preparation: In this study, the technique utilized to examine binucleated cells for micronuclei *in vitro* was that described by Fenech[19]. After completing the treatments, the cells were trypsinized and suspended in pre-warmed hypotonic solution (0.075 M KCL) and carefully homogenized with a Pasteur pipette. This cell suspension was centrifuged again and re-suspended in pre-chilled 10 mL of fixative, methanol/acetic acid (3:1 v/v), centrifuged and dropped onto pre-chilled slides. The slides were stained with 6% Giemsa diluted in phosphate buffer (pH 6.8) for 10 min, washed with distilled water, air-dried and examined under the microscope.

Scoring: The induction of MN was determined in at least 1000 binucleated cells with the cytoplasm well preserved and clearly surrounded with nuclear membrane, having an area of less than one third of that of the main nucleus[20]. In a blind test, using a Nikon microscope, cells containing 1 micronucleus were scored. The criterion for the identification of MN was according to Fenech[21]. In each treatment, the numbers of mononucleated, binucleated and polynucleated cells per 1000 cells were counted for cell cycle kinetic analysis and the nuclear division index or Cytochalasin B Proliferation Index (CBPI) as determined in a blind test. Cells with well-preserved cytoplasm, containing 1-4 nuclei, were scored. The CBPI was calculated according to OECD[22] guideline number 487 using the following formula:

$$CBPI = \frac{MI+2MII+3MIII+4MIV}{N}$$

A minimum of 1000 cells/concentration were analysed (N).

Statistical analysis: Data were analyzed for Chi square analysis using SPSS version17.0. All statistical tests were performed at the $p<0.05$ level of significance.

RESULTS

Mitotic index analysis: A two fold series of four concentrations of ZER was determined based on the IC_{50} obtained from the cytotoxicity assay. The Mitotic Index (MI) values (Table 1) revealed an increasing inhibitory effect with increasing concentrations of ZER, however this effect was non-significant except after treatment with the highest dose (80 μM) in which it was significantly higher than the vehicle control ($p<0.05$). On the other hand, mitotic index of the lower test concentration was found to be non-significantly different from that seen in the vehicle control ($p>0.05$).

Chromosome Aberrations (CA) assay: The CA assay, clastogenicity determined was not significantly observed in all ZER treated cultures of human PBL when compared to the untreated control or the solvent treated control. Positive control (MMC) was significantly shown to induce chromosome aberrations. On the other hand, all the clastogenic indices of ZER were found not to induce chromosome aberrations significantly (Table 1). The Gaps, breaks as well as interchanges, ring chromosomes and dicentrics were the main types of aberration induced by ZER.

Comet assay: Figure 2 shows damaged and normal cells in single cell gel electrophoresis after 24 h incubation with or without treatment. Comet cells were scored visually and the extent of DNA damage measured in terms of arbitrary units. The results of the treatment concentration 20 μM ZER and 2.5 μM cisplatin were shown to be significantly different ($p<0.05$) when compared to the untreated control cultured cells, while the lower concentrations treatments were found not to be significantly different from the control untreated cultured cells.

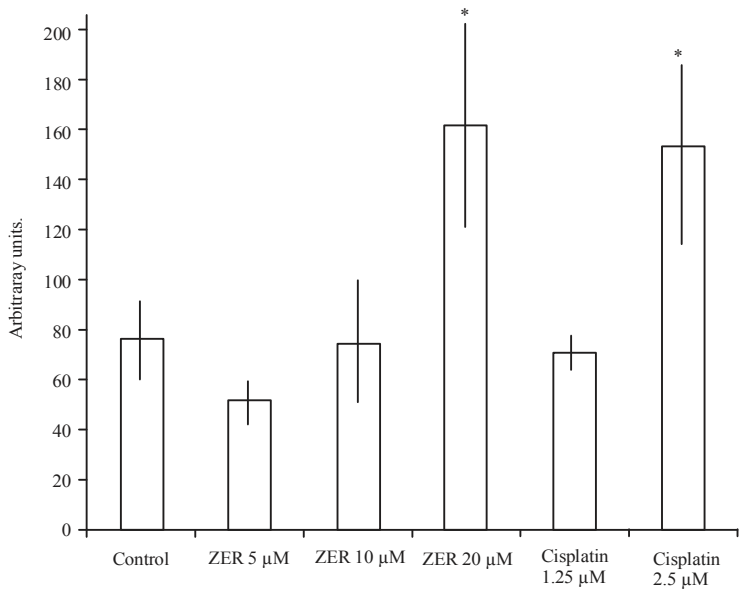

Fig. 2: Damage index of comet assay in zerumbone and cisplatin treated PHA stimulated peripheral blood lymphocytes, compared to untreated negative control, *p<0.05

Table 1: Frequencies of Chromosome Aberrations (CAs) induced in Human PBL cultures treated for 24 h with different concentrations of ZER and the positive and negative control

					Chromosome Aberrations					
Treatment	Conc	MI	Metaphase scored	Aberrant metaphase	G	B	A	E	R	Percentage of aberrant metaphases (%)
Solvent		2.4	200	6	2	2	0	0	2	3.0
MMC (µg)	1.0	0.7	200	24*	0	12	8	4	0	12.0*
ZII (µM)	0	2.3	200	4	2	2	0	0	0	2.0
	10.0	2.0	200	6	0	4	0	2	0	3.0
	20.0	1.8	200	8	2	4	0	0	2	4.0
	40.0	1.7	200	6	0	4	0	0	2	3.0
	80.0	1.0*	200	8	2	4	0	2	0	4.0

G: gap, B: breaks, A: acentrics, E: chromatid exchange, R: chromosome ring, *p<0.05, significantly different from control

MN induction: Treatment concentrations used in MN test were 12.5-50 µg mL^{-1} ZER. The potential of ZER to induce micronucleus is shown in Table 2. Treatment of CHO cells with ZER in the presence of metabolic activator caused a significant increase in MN induction at 25 and 50 µg mL^{-1} concentrations, while treatment without metabolic activation caused a significant MN induction at treatment concentration 50 µg mL^{-1}. An increased number of binucleated cells with micronuclei was found at the higher concentrations of ZER and was statistically significantly different from the control. In the cell cycle kinetics analysis or CBPI, treatment with ZER in the concentration of 50.0 µg mL^{-1} with metabolic activator was found to exert an inhibition of cell proliferation in CHO cells. Meanwhile, treatment of cells with ZER in the absence of metabolic activator was found to significantly reduce the CBPI in all the concentrations compared to the control.

DISCUSSION

The aim of this study was to evaluate, the cytogentic effects and DNA damage induced by ZER in CHO cells and normal human peripheral blood lymphocytes. The study was carried out using the chromosome aberration assay and single cell gel electrophoresis assay (comet assay) on stimulated cultured human peripheral blood lymphocytes. While cytochalacin B blocked micronucleus induction test was performed on CHO cell lines with and without S9 rat liver microsomal fraction as metabolic activation system. The ZER induced higher MN frequencies in CHO cell lines with and without the metabolic activation system as compared to control.

Genotoxic agents have the ability to interact with DNA and can cause DNA damage. Chromosomal aberrations assay

Table 2: Frequencies of micronucleus (MN) formation and cell cycle kinetics on CHO cultures treated for 3 h with and without S9

					Cell cycle kinetics[a]			
Treatment	Conc.	BN	MNi	Mni (%)	M1	M2	M3	CBPI
Treatment with S9								
ZER (μg mL^{-1})	0	1000	18	1.80	87	96	17	1.65
	12.5	1000	19	1.90	88	94	18	1.65
	25	1000	68**	6.80	120	50	30	1.55
	50	1000	60**	6.00	160	26	14	1.27*
EMS (μg mL^{-1})	125	1000	101**	10.10	143	52	5	1.31*
Treatment without S9								
ZER (μg mL^{-1})	0	1000	20	2.0	41	140	10	1.755
	12.5	1000	38*	3.8	150	44	6	1.280**
	25	1000	34*	3.4	116	76	8	1.460*
	50	1000	57**	5.7	154	40	6	1.260**
MMC	1.0	1000	68*	6.8	74	107	19	1.725

a: The numbers of mononucleated (M1), binucleated (M2) and polynucleated (M3) cell per 1000 cells were quantitated for cell cycle kinetic analysis, *p<0.05, **p<0.001

formation in proliferating cells is considered as a manifestation of damage to the genome. Chromosomal aberrations assay has been widely used as a mutagenicity assay to test for cytogenetic responses to chemical exposure. In the present study, ZER non-significantly decreased the mitotic index in cultures of stimulated peripheral blood lymphocytes, except the highest concentration (80 μM), in which the mitotic index reduced significantly.

The concentrations used for the genotoxic effects of the test compound started from 10-80 μM for a 24 h in CA assay and comet assay, while the concentrations used for MN test was from 56-220 μM (12.5-50 μg mL^{-1}). These tested concentrations were chosen according to the cytotoxicity test. The number of total aberrant cells recorded at all concentrations was non-significantly different when compared with the untreated control. The cells with structural damage in the positive control (MMC) treatment group were statistically increased compared to the solvent control indicating the responsiveness of the cells in this test system. The results of CA assay were found to be in the same line with our previous finding, since we previously treated the cells[23] with ZER for 48 h.

The MN assay is another commonly used cytogenetic method to assess *in vitro* chromosomal damage. Analysis of the frequency of micronuclei formation in treated cells provides a comparatively rapid and sensitive indication of both chromosomal aberrations and chromosome loss that lead to numerical chromosomal anomalies[24,25]. Micronuclei are cytoplasmic chromatin masses appear as small nuclei that arise from chromosome lagging at anaphase stage or from acentric chromosomal fragments. They provide a quantifiable measure of recent DNA injury that result when acentric fragments or whole chromosomes are left behind the main nucleus at telophase stage of the cell mitosis[26]. An increase in the occurrence of MN in the cells indicates that chromosome damage has occurred as a result of an exposure that caused either clastogenic or an aneuploidogenic effect[27]. In the present study, ZER showed no significant effects on CBPI index in cultures of Chinese Hamster Ovary (CHO) cell lines when treated for 3 h with low concentrations in the presence of metabolic activation system, while in the highest concentration treatment 50 μg mL^{-1}, the effect was significant. Meanwhile, the concentration range of 12.5-50.0 μg mL^{-1} was observed to decrease the proliferation index at significant level p<0.05 in the absence of the metabolic activation system. This observation is in agreement with our previous report on the effect of ZER on CHO treatment for 48 h[28,29].

Exposure of CHO cells to ZER, in the presence and absence of metabolic activation system, significantly increased the MN frequency. These results of micronucleus formation support our previous observations[29] that showed ZER can cause chromosomal damage in CHO cell lines, indicating its potential to cause genotoxic effects in these cells. These results were in contrast to our previous finding of the Ames mutagenicity assay, in which a negative results were obtained that indicated ZER to have no potential to cause point mutations in the *Salmonella typhimurium* strain[28] TA100. In addition, data on the effects of ZER on normal human peripheral blood lymphocyte chromosomes showed to have less impact when compared to the commercial anticancer drug cisplatin, as well as the higher concentrations that were found to exert less significant effects on the chromosomes. This later observation was in the same line with our results of the comet assay in which treatment of stimulated cultured human peripheral blood lymphocytes resulted in

significant difference compared to the control, but only when treated with the highest dose (20 μM). However these effects were less pronounced when compared to that produced by the anti-cancer drug, cisplatin.

This unexplained genotoxicity is much more common in the *in vitro* cytogenetics[30,31]. Some of the positive cytogenetic results may result due to true covalent adducts formation whereas, others are most likely cytotoxicity artefacts[32] or non-covalent drug/DNA interactions, i.e. DNA intercalation or groove-binding[31,33]. Structurally, ZER carried no obvious structural alerts and no obvious mechanism-based genotoxicity had been identified such as nucleoside analogues that can cause a genotoxic effects. The present results provide additional evidence that ZER compound have a genotoxic and cytotoxic effects at high concentrations on cultured CHO cell line, making it necessary for further studies to better understand the molecular mechanisms of action of ZER compound for a better comprehension. Under the experimental conditions used in the present work, ZER showed the ability to induce genotoxicity and cytotoxicity *in vitro* in CHO cells, while less effects were observed on human peripheral blood lymphocytes.

ACKNOWLEDGMENTS

The author would like to extent his utmost gratitude and appreciation to MOSTI (Ministry of Science, Technology and Innovation) and The National Cancer Council Malaysia (MAKNA) for providing the research grants IRPA No: 06-02-04-0720-EA001. The author also would like to convey his thanks to Dr. Ahmad Bustamam, for his supervision and Dr. Syam Mohan, Dr. Siddig I Abdelwahab for their help in completing this work.

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