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Full Length Research Paper

Effects of zerumbone on cisplatin-induced clastogenesis in Sprague-Dawley rats bone marrow cells

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Zerumbone (ZER) is derived from *Zingiber zerumbet* smith from the Zingiberaceae family. It has been shown to have anti-cancer and apoptosis-inducing properties against various human tumour cells. The aim of our study was to assess the effect of ZER on cisplatin-induced clastogenesis in Sprague-Dawley rat bone marrow polychromatic erythrocytes (PCEs) using micronucleus test (MN). Animals treated with two ZER doses for 4 consecutive days plus a single dose of cisplatin following treatment, presented a non-significant effects of ZER on cisplatin-induced clastogenesis. The results also indicated that ZER has no clastogenic effects on rat bone marrow polychromatic erythrocytes after 4 days treatment with 250 and 500 mg/kg body weight when compared to one dose cisplatin of 45 mg/kg body weight. On the other hand, significant decrease in the number of PCE was observed in all treatment groups, indicating cytotoxicity of ZER and cisplatin. Under the present experimental conditions, ZER could not prevent cisplatin-induced clastogenesis in rat.

Key words: Clastogenicity, micronucleus, micronuclei in polychromatic erythrocytes (MNPCEs), zerumbone.

INTRODUCTION

Zingiber zerumbet smith is used in local traditional medicine to cure a number of illnesses. It is locally known as *'lempoyang'* wild ginger which belongs to Zingiberaceae family. It is native to South East Asia but has been widely cultivated in village gardens throughout the tropical and subtropical area around the world and has been naturalized in some areas for its medicinal properties. In some Southeast Asian countries, the rhizomes of the plant are employed as traditional medicines for various ailments, while the young shoots and inflorescence are used as condiments. The biologically active compound in *Z. zerumbet* called zerumbone (ZER) is one of the most promising chemopreventive agents against colon and skin cancer (Murakami et al., 2004). In addition, it was reported to suppress colonic tumour marker formation in rats, induce apoptosis in colon cancer cell lines (Murakami et al., 2002) and 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 (Murakami et al., 2002, 2004). Zerumbone was further demonstrated to inhibit both azoxymethane-induced rat aberrant crypt foci and phorbol ester-induced papilloma formation in mouse skin which is another indication of its efficacy to prevent colon and

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Abbreviation: MNPCEs, Micronuclei in polychromatic erythrocytes; **ZER,** zerumbone; **CC,** column chromatography; **HPLC,** high performance liquid chromatography; **LCMS,** liquid chromatography mass spectrometry; **MTD,** maximum tolerable dose; **DMSO,** dimethyl sulfoxide; **NCE,** normochromatic erythrocytes; **PCE,** polychromatic erythrocytes; **MN,** micronuclei.

Table 1. Experimental groups and treatment protocol.

Treatment	Group	Dose
Control	1	No treatment
ZER	2	250 mg/kg b.w.
ZER	3	500 mg/kg b.w.
DDP	4	45 mg/kg b.w.
ZER + DDP	5	as in (2) and (4)
ZER + DDP	6	as in (3) and (4)
Vehicle only	7	DMSO

skin cancers (Murakami et al., 2004; Tanaka et al., 2001).

Genotoxicity studies of both naturally occurring and synthetic substances are of great interest because of the widespread and often chronic use of herbal remedies, modern medicinal products, food ingredients, as well as other household and environmental chemicals. Many plant products contain compounds known to cause various diseases or even death in animals and humans (Dearfield et al., 2002; Ames and Gold, 1997; Raskin et al., 2002; Rates, 2001). Cisplatin (*cis*-diamminedichloroplatinum II; DDP) is an effective synthetic antitumor agent with a wide spectrum of activity against various solid tumors, but it has serious side effects on non-tumor cells, including free radical generation (Masuda et al., 1994). Cisplatin is highly mutagenic, inducing sister-chromatid exchanges and chromosomal aberrations in cultured mammalian cells, mouse bone marrow cells and peripheral blood lymphocytes in patients (Ohe et al., 1990).

Taking into account the lack of information on *in vivo* cytogenetic effects of zerumbone, we decided to provide some data on the cytogenetic activity of this compound. Here we reported the results obtained on the cisplatin-induced anticlastogenic effects of zerumbone *in vivo* using rat bone marrow erythrocyte micronucleus (MNPCE).

MATERIALS AND METHODS

Zerumbone

Zerumbone was extracted in the laboratory of cancer research MAKNA-UPM, University Putra Malaysia, from the rhizomes of *Z. zerumbet* plant. The rhizomes were obtained from the wet market in Kuala Lumpur, Malaysia. Zerumbone was extracted, isolated and purified using methanol as an extracting solvent and column chromatography (CC) method. The isolated and purified zerumbone crystals were subjected to high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LCMS) to confirm its purity and molecular weight. Further, ^{13}C NMR and ^{1}H NMR analysis were conducted on the zerumbone crystals to confirm its molecular structure (Figure 1). A stock solution of zerumbone is prepared immediately before use in absolute ethanol (HmbG Chemicals).

Animals and their treatment for measurement of micronuclei in polychromatic erythrocytes (MNPCE)

Male Sprague-Dawley rats (6 to 8 weeks old) weighing 170 to 200 g were obtained from the Institute of Medical Research, Kuala Lumpur. The rats were maintained in group per cage at room temperature (25 ± 1°C) and 12-h light: 12-h dark cycle, and were given food and water *ad libitum*. The animals were acclimatised for at least 5 days prior to dosing and were divided into seven groups containing 3 to 5 rats each as described in Table 1. Two dose levels of zerumbone (250 and 500 mg/kg) were given intraperitoneal for 4 consecutive days. Dose selection was based on preliminary experiments in which the maximum tolerable dose (MTD) was identified to be 1000 mg/kg body weight. An untreated control and a positive control (cisplatin 45 mg/kg) were also used to test the validity of the assay. The experiment complied with the guide for Animal Care and Use Committee (ACUC), Faculty of Medicine and Health Sciences, University Putra Malaysia.

After the last ZER dose, 45 mg/kg cisplatin (Sigma) was given to all the rat groups except the vehicle control in which rats were given dimethyl sulfoxide (DMSO). Twenty four hours later, the animals were anesthetized with chloroform and sacrificed. For bone-marrow preparations, both hind femora were isolated and the adherent muscle was removed. The epiphyses were cut off and bone marrow cells were flushed out with foetal bovine serum (PAA Laboratories). The suspension of bone marrow cells and foetal bovine serum was centrifuged for 10 min at 1000 rpm. The resulting sediment was re-suspended in foetal bovine serum. Bone marrow smears were prepared from the resulting cell suspension. After air-drying and fixing for 10 min in absolute methanol, slides were stained with Giemsa-staining method. The slides were analyzed in a blinded fashion using a Nikon light microscope. Both normochromatic erythrocytes (NCE) and polychromatic erythrocytes (PCE) were scored for bone marrow activity and PCEs were scored for micro-nuclei (MN). A total of 2000 polychromatic erythrocytes were scored per animal for MNPCE and 200 erythrocytes were counted for the PCE : NCE ratio according to the Organisation for Economic Co-operation and Development (OECD) guideline for testing of chemicals (mammalian erythrocyte micronucleus test), guideline No. 474 (OECD 1997). For every group of animals, the following parameters were reported: number of MN-containing cells/2000 PCE/animal, the number of PCE, the number of NCE and the NCE: PCE ratio.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Science (SPSS) 15. Rat bone marrow erythrocytes MN results were expressed as mean ± SE and were analyzed with one way analysis of variance (ANOVA), while the results from *in vitro* work were analyzed using Chi square analysis. All statistical tests were performed at the $p < 0.05$ level of significance.

RESULTS AND DISCUSSION

Micronucleus frequencies observed in polychromatic erythrocytes as well as the ratio of polychromatic to normochromatic erythrocytes of male rats are shown in Table 2. The exposure to the given doses of ZER resulted in non-significant increase in the number of micronuclei in polychromatic erythrocytes (MNPCEs) of both the exposed groups when compared to the vehicle control (DMSO) and negative control groups. Rats exposed to cisplatin showed a significant increase in the MNPCEs formation when compared to the negative untreated control and vehicle control (DMSO) groups. In addition, there was suppressive effect observed on pri-administration of ZER on the cisplatin induced MNPCEs

Table 2. Frequencies of MNPCE and PCE in bone marrow of rats after 4 days intraperitoneal treatment with the indicated doses of zerumbone and the positive control, cisplatin.

Treatment	Rats (no)	MNPCE (Mean ± SE)	Total MNPCE/total PCE analyzed (MNPCE/2000 PCE, individual data)	Percentage of PCE
Control (no treatment)	4	3.0±0.40	12/8000 (2,4,3,3)	56.5±1.1
Cisplatin 45 mg/kg b.w.	4	7.0±0.71[c]	28/8000 (8,5,8,7)	24.0±3.10[b]
ZER 250 mg/kg b.w.	4	1.25±0.48	5/8000 (2,1,2,0)	27.6±3.6 [b]
ZER 500 mg/kg b.w.	4	2.25±0.63	9/8000 (4,2,2,1)	31.7±5.4[b]
ZER 250 mg/kg + DDP 45 mg/kg b.w.	3	11.00±2.1[a]	33/6000 (8,15,10)	12.8±1.8 [bc*]
ZER 500 mg/kg + DDP 45 mg/kg b.w.	5	11.60±2.7[a]	58/10000 (7,9,20,16,6)	25.2±5.5[b]
Vehicle control (DMSO)	4	2.75±0.63	11/8000 (1,3,4,3)	43.62±1.8

MNPCE, Micronucleated polychromatic erythrocytes; % PCE, number of PCE that were counted in microscopic fields containing 2000 NCE; the percentage of PCE was calculated as % PCE = [PCE / (PCE + NCE)] × 100. [a]$P < 0.01$, significantly different from control; [b]$P < 0.001$, significantly different from control; [c]$P < 0.05$, significantly different from the control; *significantly different from the same dose without DDP.

formation.

Antitumor agents are known to interact with specific biological molecules, and evidence has been obtained that treatment with antitumor drugs from different categories leads to generation of free radicals in nontumor cells both *in vivo* and *in vitro* (Weijl et al., 1997). Zerumbone has gained a great attention due to its activity towards many diseases *in vitro* and *in vivo*. Very recently, Sung et al. (2009) reported zerumbone 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 (Kim et al., 2009). In cancer chemotherapy, genotoxicity effects should be determined before or during the phase I clinical trials (Pratt et al., 1994). Since the cytogenetic effects of zerumbone *in vivo* and *in vitro* have not been attempted and due to the lack of information available on the cytogenotoxicity of ZER, this study aims to investigate the effects of zerumbone that could be exerted on chromosomes *in vivo* using rat bone marrow MNPCEs test.

As part of its preclinical safety assessment program, ZER was assessed for genotoxic potential by means of a micronucleus test in cultured human lymphocytes and CHO cells as well as *in vivo* rat bone marrow polychromatic erythrocyte micronucleus (PCEMN) test (Al-Zubairi et al., 2010a, 2010b). Besides the possible use of ZER as a therapeutic agent, knowledge about its genotoxic potential is also of interest from the point of view of human ZER-containing food consumption. The use of *in vivo* and *in vitro* assays was decisive in order to obtain a picture of the genotoxic or anti-genotoxic potential of this plant product. When a bone marrow erythroblast develops into a PCE, the main nucleus is extruded and any MN that has been formed may remain behind in the cytoplasm. Visualisation of MN is facilitated in these cells because they lack a main nucleus. An increase in the frequency of micronucleated PCEs in treated animals is an indication of induced chromosomal damage. Genotoxic activity is normally indicated by a statistically significant dose-related increase in the incidence of micronucleated immature erythrocytes and/or chromosome aberrations for the treatment groups when compared with the concurrent control group.

The observed inhibition of cell proliferation in the bone marrow illustrates the cytotoxicity of ZER. Bone marrow cell toxicity (or depression) is normally indicated by a substantial and statistically significant dose related decrease in the proportion of immature erythrocytes (PCEs); a very large decrease in the proportion would be indicative of a cytostatic or cytotoxic effect. However, the present study showed a significant decrease in the number of PCE (or depression in bone marrow) in all treatment groups of animals receiving ZER alone or ZER with cisplatin. This indicates that ZER and cisplatin are very potent inhibitors of mitosis. In the present study, the observed inhibition of cell proliferation in rat bone marrow illustrates the cytotoxicity of ZER.

Genotoxic agents have the potential to interact with DNA and may cause DNA damage. Chromosomal aberration that occurs in proliferating cells is regarded as a manifestation of damage to the genome. It has been commonly used as a test of mutagencity in order to evaluate cytogenetic responses to chemical exposure. Analysis of the frequency of occurrence of MNPCEs in bone marrow of treated animals provides a comparatively rapid and sensitive indication of both chromosomal aberrations and chromosome loss that lead to numerical chromosomal anomalies. An increase in the prevalence of MN in a population of cells indicates that chromosome damage has occurred as a result of an exposure that caused either clastogenic or an aneuploidogenic effect (Gonsebatt et al., 1997; Mahata et al., 2003). MN assay is a widely used cytogenetic method to assess *in vivo* and *in vitro* chromosomal damage, because this method is the most reproducible to show positive effects (Robbiano et al., 1998).

The clastogenic action of cisplatin in somatic cells *in vivo* has already been described (Edelweiss et al., 1995). Chromosomal aberrations as well as abnormal meta-

phases were observed to be highest at 18 and 24 h after cisplatin administration when compared to 72 h. It has been reported that antitumor agents produce a high frequency of aberrations in rodents 24 h after a single dose (Rosselli et al., 1990). The present finding shows that exposure to cisplatin significantly increased the frequency of MNPCEs in rat bone marrow when administered in a single dose intraperitoneally. The pretreatment with ZER showed no effects on cisplatin-induced formation of MNPCEs in the rat bone marrow.

In conclusion, our *in vivo* clastogenesis studies found out that zerumbone could be cytotoxic without any anticlastogenic potential against cisplatin induced clastogenesis. However, it is important to carry out more investigations using various cytogenetic tests under different experimental conditions to get more information on the genotoxic effects of zerumbone.

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