In Vitro Genotoxic Effects of Zerumbone and Cisplatin Combination in CHO Cell Lines

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Abstract

Objective: Several natural products are being increasingly used in the treatment of cancer to minimize the adverse side effects of cancer chemotherapy. Zerumbone (ZER), the sesquiterpene derived from Zingiber zerumbet Smith, has been reported to have an in vitro anticancer effects against various human tumour cells as well as in vivo against a number of induced malignancies in mice. Previously we have reported the genotoxic effects of ZER in vitro against CHO cell lines. Material and Method: The aim of this study was to investigate the genotoxic effects of the combination of ZER along with cisplatin in CHO cells. Two cytogenetic endpoints were used, namely Chromosomal Aberrations assay (CA) and Micronucleus test (MN). Both cytogenetic endpoints were performed without any metabolic activation. Result: ZER treated cultures showed the significant increase in the frequency of the chromosome aberrations and MN induction. In CA assay, marked changes have been observed after co-treatment of CHO cell lines with different concentration of ZER along with 5 µM Cisplatin when compared to ZER treatment alone, suggesting a possible synergistic genotoxic effects. Whereas, treatment of CHO cell lines with different concentrations of ZER along with 2.5 µM Cisplatin was found to reduce chromosomal aberrations, suggesting an antagonistic genotoxic effect. On the other hand, in MN induction test, co-treatment of CHO cell lines with both 2.5 µM and 5 µM Cisplatin and different concentration of ZER found to reduce the genotoxic effects compared to the 2.5 µM and 5 µM Cisplatin alone suggesting an antagonistic genotoxic effect. Conclusion: The genotoxic effects of combined low concentrations of Cisplatin with different concentrations of ZER could have an antagonist genotoxic potential in vitro in CHO cell lines.

Keywords: Zerumbone- Cisplatin- CAs- MNi- CHO cell lines- genotoxicity

Introduction

Among the most serious possible side effects of the anticancer chemotherapeutic drugs is their genotoxicity effect which may consequently lead to the development of secondary malignancies. Despite the curative activity in patients with testicular, head and neck or ovarian cancers, cisplatin was shown to have nephrotoxic and neurotoxic side effects [1]. Cancer chemotherapy directly or indirectly affects not only the target tumour cells, but non-tumoural cells DNA structure and function also affected. These effects on the DNA appear primarily in the form of alterations of the DNA structure as a result of alkylations, cross-links, or formation of DNA adducts, which subsequently leads to DNA repair mechanisms. These DNA breaks result as a consequence of DNA repair mechanisms that could be considered as important markers of genotoxicity [2]. Cisplatin or cis-diammine-dichloroplatinum II considered one of the most promising and widely used platinum-based therapeutic anti-cancer drugs in clinical practice [3-4]. Its therapeutic effect is attributed to the formation of adducts with DNA [5], that are not removed and may block DNA replication and transcription. It has also been reported that cisplatin may inhibit the activity of telomerase [6]. Herbal medicine has been practiced since ancient times in traditional medicine, in the form of raw plant material or plant extracts [7]. The considerable attention gained by the herbal medicine is due to the premise that these herbal plants contain natural substances that can promote health...
and eliminate the disease. Previous studies have reported that about half the population of many industrialized countries use complementary and alternative medicine, and the proportion is as high as 80% in many developing countries [8]. According to the World Health Organization, 80% of the earth population use herbal formulations in traditional medicine [9]. Plant-derived therapies represent 25% of the drugs prescribed worldwide, and 121 of these compounds still used in the treatment of diseases [10].

Zingiber zerumbet Smith, the wild ginger, belongs to Zingiberaceae family and is native to South East Asia, and is locally known in Malaysia as ‘lempoyang’. It’s widely cultivated in village gardens throughout the tropical and subtropical area and its rhizomes with the active ingredient, zerumbone (ZER), is used in some South East Asian countries as anti-inflammation while the shoots are used as condiments. The activity of ZER has recently drawn the attention of many researchers due to its activity towards many diseases in vitro and in vivo. Many published researches have reported the anti-tumour effects of zerumbone in different cancer cell lines, including ovarian, colon, breast, myeloid, pancreatic, lung and gastric cancers [11-19]. Our previous work in vivo, we have reported the activity of ZER against DES-induced mice Cervical Intraepithelial Neoplasia (CIN) [20]. In addition, It has been reported to inhibit both azoxymethane-induced rat aberrant crypt foci and phorbol ester-induced papilloma formation in the mouse skin cancer [21].

In chemotherapy, the use of the combination of two anticancer agents often has the advantage of minimizing the toxicity due to lowering the drug dosage as well as reducing the development of drug resistance by the cancer cells, providing the ability of synergism between drugs with different mechanism of action. In combination chemotherapy, non-toxic or less toxic phytochemicals are combined with chemotherapy agents to enhance the efficacy and reduce the toxicity to normal tissues [22], therefore anticancer phytochemicals are being studied as possible candidates for their synergistic efficacy in combination with the anticancer chemotherapy [22-24].

In this study, we attempted to report the results obtained, for the first time, on the genotoxic effects of the combination of both ZER and Cisplatin in Chinese hamster ovary (CHO) cell lines, by using chromosomal aberrations assay (CAs) and micronucleus (MN) formation as cytogenetic endpoints. This paper describes the results of those investigations and comments on the in vitro genotoxic effects of the combination of both ZER and Cisplatin in CHO cell line.

Materials and Methods

Zerumbone extraction

ZER was extracted in the laboratory of cancer research MAKNA-UPM, University Putra Malaysia, from the rhizomes of Zingiber zerumbet plant. The fresh rhizomes obtained from the wet market in Kuala Lumpur, Malaysia. Methanol extraction and column chromatography (CC) method were used to extract, isolate and purify the compound. 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. A stock solution of ZER was prepared immediately before use in absolute ethanol (HmbG Chemicals).

Chemicals

Mitomycin C (MMC), Cytochalasin B (CB [CAS 4930-96-2] and Cisplatin [CAS 15663-27-1] were obtained from Sigma, Giemsa stain [CAS 67-56-1] and Colcemid from (PAA Laboratories).

Chromosomal aberration assay

In genotoxicity testing, the ability of the chemical to induce the formation of chromosomal aberrations and damage as well as gene mutations is investigated. Therefore ZER was evaluated for its potential to produce chromosome aberrations in CHO.

CHO cells line

Chinese hamster ovary (CHO) cells were purchased from ECACC (UK). The atypical cell contains 21 chromosomes. The cells grow as an adherent monolayer in appropriate tissue culture vessels, doubling approximately every 12 hours. Health status of the cultures was monitored throughout each experiment by cell counting and microscopic observation. Cells were maintained in RPMI 1640 medium (PAA Laboratories GmbH, Germany) supplemented with 10% foetal bovine serum (PAA Laboratories GmbH, Germany). Sterile Nunc tissue culture flasks (Nunc, Denmark) were used. The cells were incubated in a humidified tissue culture incubator at 37°C and 5% CO2. When the cells reached approximately 60-80% confluence, they were dislodged with 0.05% trypsin (PAA Laboratories GmbH, Germany), collected by centrifugation and seeded in fresh medium. Each trypsinization was recorded as one passage. A continued culture of CHO cell line (passage15) was used. On the day before the experiment, approximately 5×10⁴ cells from a culture with approximately 60-80% confluence were seeded into each T-25cm² (Nunc, Denmark) flasks. The cells were incubated overnight in a humidified incubator at 37°C and 5% CO2.

Treatment with the test substance

Tested compound was prepared as a 1mg/ml 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. They appeared healthy, well adherent, evenly distributed and 60-80% confluent. 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. The treatment medium was 5 ml of the cell culture medium with 10% foetal bovine serum, with the treatment concentration or a control solute. The final concentrations were 5, 10,
20, 40.0 and 80 μM and the cells were incubated in the treatment medium for 24 hours.

**Cells Harvest and Preparation of chromosome slides**

The cells were harvested at the end of treatment and two hours prior to harvesting, colcemid (PAA Laboratories GmbH, Germany) was added at 0.2 ml to arrest cells in metaphase. After this, the medium with suspended cells was transferred into centrifuge tubes. The remaining cell monolayer was dissociated with 0.05% trypsin and pooled with the collected cell suspension. The cells were centrifuged at 800 rpm and were resuspended with phosphate buffered saline (Sigma) and then collected by centrifugation, swelled in 0.075 M KCl (Sigma) and fixed in a 3:1 mixture of methanol and glacial acetic acid for three times. Next, the tubes were centrifuged 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 in 6% Giemsa stain ([CAS 67-56-1] Gibco, Invitrogen, Germany). For each duplicate culture, at least 1000 cells were examined to score mitotic index. The mitotic index (MI) was calculated as the percentage of cells at the mitotic stage. Relative mitotic index (RMI) was calculated as:

\[
\text{RMI} (%) = \frac{\text{test concentration MI}}{\text{solvent control MI}} \times 100
\]

**Analysis of chromosome aberrations**

All slides were randomly coded. Well-spread metaphase cells with 19-23 chromosomes were analysed for chromosome aberrations as defined. At least 200 metaphase cells from each negative control and ZER-treated culture (100-400 cells per concentration were scored). The number of each type of aberration and the percentage of cells with aberrations was recorded and summarized. The number of chromatid gaps and chromosome gaps was recorded when encountered, but not included in the calculations. This practice concerning chromosome gaps was recorded when encountered, but not included in the calculations. This practice concerning chromosome gaps was used [25], as discussed in literary sources. The percentages of cells with aberrations from each concentration were compared to the solvent control values using \(\chi^2\) analyses.

**Micronucleus test (MN) in CHO**

Chinese hamster ovary (CHO) cells grow as an adherent monolayer in appropriate tissue culture vessels and were maintained in RPMI 1640 medium (PAA Laboratories, Germany) supplemented with 10% foetal bovine serum (PAA Laboratories, Germany). The cells were incubated in a humidified tissue culture incubator at 37°C and 5% CO\(_2\). 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) was used as a positive control. The treatment medium was 5 ml of the cell culture medium with 10% foetal bovine serum, with the treatment concentration or a control solution and the final concentrations were 5, 10, 20, 40 and 80 μM ZER. Cells were cultured in the treatment medium for 24 hours. After treatment, cells were washed twice with 10 mL PBS, trypsinized with 0.05% trypsin and centrifuged for 5 min at 800 rpm. CHO cells were then harvested and scored using the same method of human lymphocytes micronucleus mentioned above.

**Statistical analysis**

Statistical analysis was performed using SPSS 23. Results were analyzed using Chi-square analysis. All statistical tests were performed at the \(p<0.05\) level of significance.

**Results**

**Mitotic index analysis**

A twofold series of six concentrations of ZER was determined based on the IC\(_{50}\) obtained from the MTT cytotoxicity assay [13]. The relative mitotic index (RMI) values (Table 1) revealed the inhibitory effect with all concentrations of ZER. Meanwhile, the mitotic index for all test concentrations was found to be reduced compared to that seen in the untreated control (\(p<0.05\)). On the other hand treatment of CHO cell lines with both compounds in combination, revealed a marked reduction in MI and RMI as compared to the control or cultures treated with ZER alone, particularly co-treatment of cell lines with 5 μM Cisplatin and different concentrations of ZER as observed in (Tables 2,3).

**Chromosome aberrations (CA) assay**

The CA assay, clastogenicity determined was not significantly observed in 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 to induce chromosome aberrations in an increasing dose-response pattern (Table 1). Gaps, breaks as well as endoreduplication, ring chromosomes and dicentrics were the main types of aberration induced by ZER. In treated cultures of CHO cell line, chromosome breaks were found to be the most prominent aberrations observed whether in the ZER treated or in the combinations of ZER and Cisplatin treated cultures (Tables 1, 2, 3). However, ring chromosomes and dicentrics were also observed to be increased in all treated. The number of aberrant cells with structural aberrations in cultures treated with both compounds were observed to be increased significantly p<0.05 (Table 1). Treatment of the cell lines with the lower dose of Cisplatin (2.5 µM) together with different concentrations of ZER seems to reduce the percentage of chromosomal aberrations markedly. In contrast treatment of the cell lines with 5 µM Cisplatin and different concentrations of ZER enhanced the genotoxic effects as shown in (Tables 2, 3).

**MN induction**

Treatment concentrations used in the MN test were 5.0 - 80.0 µM ZER. The potential of ZER and Cisplatin to induce micronuclei is shown in (Tables 4, 5). Treatment of CHO cells with ZER caused dose-dependent MN induction. An increased number of binucleated cells with micronuclei was found at the higher concentrations (> 5.0 µM) of ZER and was statistically significantly different from the control (Table 4). In the same time treatment of CHO cell lines with different concentrations of Cisplatin-induced the formation of Micronuclei significantly in all the used concentrations (Table 5). Treatment of CHO cell lines with ZER for 24 hours for Micronucleus test (MN) * P-value < 0.05 (compared to vehicle control).

| Treatment | Binucleated cells (BN) | MNi | % MN |
|-----------|------------------------|-----|------|
| Ethanol   | 1,000                  | 12  | 1.2  |
| ZII 0 µM  | 2,000                  | 30  | 1.5  |
| ZII 5.0 µM| 3,000                  | 55  | 1.8  |
| ZII 10.0 µM| 3,000                   | 70* | 2.3  |
| ZII 20.0 µM| 2,200                   | 100*| 4.55 |
| ZII 40.0 µM| 2,000                   | 126*| 6.3  |
| ZII 80.0 µM| High toxicity          |     |      |
| MMC       | 1,000                  | 55  | 5.5  |

* P-value < 0.05 (compared to vehicle control).

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Table 2. CAs Assay of CHO Cell Lines after Treatment with Different Concentrations of ZER in Combination with 2.5 µM Cisplatin for 24 hrs

| ZER (µM) | % MI | RMI | % Ab cells | Gaps | Breaks | Acentrics | Translocat | Ring | Di-centric | Endor |
|----------|------|-----|------------|------|--------|-----------|------------|------|------------|-------|
| 5        | 6    | 96  | 9          | 2    | 1      | 2         | 4          |      |            |       |
| 10       | 5    | 80  | 5          | 2    | 2      | 1         |            |      |            |       |
| 20       | 5.5  | 88  | 7          | 1    | 1      | 1         | 2          | 1    |            |       |
| 40       | 5.4  | 86  | 39         | 5    | 16     | 1         |            |      |            |       |
| 80       | 4*   | 64  | 6.8        | 2    |        | 1         |            |      |            |       |

* P-value < 0.05 (compared to corresponding ZER concentration).

Table 3. CAs Assay of CHO Cell Lines after Treatment with Different Concentrations of ZER in Combination with 5 µM Cisplatin for 24 hrs

| ZER (µM) | % MI | RMI | % Ab cells | Gaps | Breaks | Acentrics | Translocat | Ring | Di-centric | Endor |
|----------|------|-----|------------|------|--------|-----------|------------|------|------------|-------|
| 5        | 2.3* | 37  | 81         | 33   | 1      | 38        | 5          | 5    |            |       |
| 10       | 1.8* | 29  | 93         | 49   | 2      | 35        | 3          | 4    |            |       |
| 20       | 2*   | 32  | 82         | 46   | 34     | 2         | 8          | 2    |            |       |
| 40       | 2*   | 32  | 81.8       | 5    | 3      | 3         | 1          |      |            |       |

* P-value < 0.05 (compared to corresponding ZER concentration).

Table 4. MN test, treatment of CHO cell lines with ZER for 24 hours for Micronucleus test (MN) * P-value < 0.05 (compared to vehicle control).

| Treatment | Binucleated cells (BN) | MNi | % MN |
|-----------|------------------------|-----|------|
| Ethanol   | 1,000                  | 12  | 1.2  |
| ZII 0 µM  | 2,000                  | 30  | 1.5  |
| ZII 5.0 µM| 3,000                  | 55  | 1.8  |
| ZII 10.0 µM| 3,000                   | 70* | 2.3  |
| ZII 20.0 µM| 2,200                   | 100*| 4.55 |
| ZII 40.0 µM| 2,000                   | 126*| 6.3  |
| ZII 80.0 µM| High toxicity          |     |      |
| MMC       | 1,000                  | 55  | 5.5  |

* P-value < 0.05 (compared to vehicle control).

Table 5. Micronucleus test (MN) for CHO cell lines after treatment with different concentration of Cisplatin for 24 Hours

| Cisplatin | Binucleated cells (BN) | Micronucleated binucleate (MNi) | % MN |
|-----------|------------------------|--------------------------------|------|
| DMSO      | 2000                   | 30                             | 1.5  |
| 1.25 µM   | 3000                   | 193                            | 6.43*|
| 2.5 µM    | 2800                   | 145                            | 5.2* |
| 5.0 µM    | 3000                   | 393                            | 13.1*|
| 10.0 µM   | 750                    | 147                            | 19.6*|

* P-value < 0.05 (compared to vehicle control).
cell lines with combination of both compounds, ZER and Cisplatin-induced increase in micronucleus formation but only in the 5 μM Cisplatin with different ZER concentrations, while co-treatment of the cell lines with 2.5 μM Cisplatin and different concentrations of ZER produced even lower MNi formation than the 2.5 μM Cisplatin alone (Tables 6, 7).

**Discussion**

The purpose of this study was to evaluate for the first time, the genotoxic effects of the combination of ZER and the classic chemotherapeutic agent, Cisplatin in CHO cell lines in vitro. The investigation was conducted using the chromosome aberration assay and micronucleus test as cytogenetic endpoints. ZER alone induced higher CAs frequencies as compared to control values as well as micronucleus formation as revealed by the CA assay and MN test as reported recently [26]. Thus results found, at least under the experimental conditions used, showed the ability of this compound to induce genotoxicity in vitro in rodent cells. Genotoxic agents can interact with DNA causing damage that result in chromosome fragments, dicentrics, and anaphase or from acentric chromosomal fragments. An increase in the percentage of MN in a population of cells at anaphase or from acentric chromosomal fragments, dicentrics, and anaphase or from acentric chromosomal fragments is commonly used as a test of mutagenicity in order to evaluate cytogenetic responses to chemical exposure. The purpose of this study was to evaluate for the first time, the genotoxic effects of the combination of ZER and Cisplatin-induced increase in micronucleus formation but only in the 5 μM Cisplatin with different ZER concentrations, while co-treatment of the cell lines with 2.5 μM Cisplatin and different concentrations of ZER produced even lower MNi formation than the 2.5 μM Cisplatin alone (Tables 6, 7).

The genotoxic effects of the combination of test compounds, ZER and 2.5 μM and 5 μM Cisplatin were tested. The tested concentrations were chosen according to the cytotoxicity tests [13-28]. The results of the present study demonstrated that the frequency of breaks, gaps, fragments, dicentrics, and endoreduplication was increased significantly in cultures of CHO cell lines treated with ZER alone and in combination with Cisplatin when compared with the control. The percentage of total aberrant cells recorded were significantly increased at P<0.05 when compared with untreated control. The percentage of structurally damaged cells in the MMC (positive control) treatment group was statistically increased compared to the solvent control indicating the responsiveness of the cells in this test system.

The genotoxic effects of the combination of both compounds on CHO cell lines were studied in the present work. Two concentrations of the chemotherapeutic anticancer agent, Cisplatin has been used in this study, 2.5 μM and 5 μM, representing concentrations around the median inhibitory concentration of the drug as we reported previously [13-28]. In CAs assay co-treatment of CHO cell lines with 2.5 μM Cisplatin and different concentrations of ZER was found to reduce the incidence of chromosomal aberrations as compared to cultures treated with ZER alone, suggesting an antagonistic potential of ZER against the genotoxic effects of Cisplatin. In contrast, co-treatment of 5 μM Cisplatin with different concentrations of ZER was shown to increase the percentage of the aberrant cells markedly and hence the genotoxicity of the Cisplatin, suggesting an additive synergistic genotoxic effect of ZER.

MN assay is another widely used cytogenetic endpoint to investigate in vitro chromosomal damage. The occurrence of micronuclei in treated cells provides a comparatively rapid and sensitive indication of both chromosomal damage and chromosome loss that lead to numerical chromosomal anomalies [29-30]. Micronuclei are chromatin masses in the cytoplasm left behind during the cell division; arise from chromosome fragments at anaphase or from acentric chromosomal fragments. An increase in the percentage of MN in a population of cells
indicates chromosomal damage occurred as a result of an exposure to either clastogenic or an aneuploïdogenic effects [31]. The results of the present study showed that exposure of CHO cells to ZER or Cisplatin significantly increased the frequency of MN. These results support our previous observations that ZER causes chromosomal damage in Chinese hamster cell lines, indicating its potential to cause genotoxic effects [26-27].

Treatment of CHO cell lines with the combination of 2.5 μM Cisplatin and different concentrations of ZER was found to reduce the percentage of MN formation when compared to the treatment of the cell lines with Cisplatin alone suggesting an antagonistic genotoxic potential of ZER. In contrast, treatment of cultures with 5 μM Cisplatin and different concentrations of ZER, result in significantly increased percentage of MN, however, this increase in the MN formation is not as much as the effect of the 5 μM Cisplatin alone. The effect of ZER to reduce the genotoxicity of Cisplatin at its IC50 concentration could have a beneficial application in co-treatment of cancer with both compounds.

In conclusion, the present results provide evidence that ZER compound in concentrations around the IC50 could have a potential effect in antagonizing the harmful genotoxic effects of the chemotherapeutic anticancer agent, Cisplatin. This genotoxicity effects on cultured Chinese hamster cell line, bringing into highlight the need for further studies to better understand the molecular mechanisms of action of ZER compound for a better comprehension to be used in combination with other anticancer drugs and in particular Cisplatin. Thus results found, at least under the experimental conditions used in the present work, showed the ability of this compound to reduce the genotoxicity of Cisplatin in vitro in CHO cells.

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