Cytotechnology (2015) 67:939–946
DOI 10.1007/s10616-014-9729-9

BRIEF REPORT

Cytotoxicity and genotoxicity of zingiberene on different neuron cell lines in vitro

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Received: 19 February 2013 / Accepted: 7 April 2014 / Published online: 7 May 2014
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Abstract The main objective of this study is to investigate the cytotoxic, genotoxic and antioxidant properties of zingiberene (ZBN) in an in vitro rat brain cell culture study. The cytotoxic effect was determined against the rat neuron and N2a neuroblastoma (N2a-NB) cell lines using the 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, while the antioxidant activity was assessed using the total antioxidant capacity (TAC) and total oxidative stress (TOS) assays. The effects on DNA damage were also evaluated in this study by the single cell gel electrophoresis assay. The results indicated that ZBN has an anti-proliferative activity suppressing the proliferation of N2a-NB cells at concentrations over 50 mg L^{-1} and neuron cells at concentrations over 150 mg L^{-1}. In addition, ZBN treatments at higher doses ($\leq$50 mg L^{-1}) led to increases of TOS levels in N2a-NB cell cultures. However 25 mg L^{-1} of ZBN treatment caused increases of TAC levels in cultured neuron and N2a-NB cell cultures while ZBN at doses of 10–400 mg L^{-1} did not increase the number of total damage score in both cell lines. This study clearly indicates that ZBN has a significant potential to be used as a natural anticancer agent in cultured N2a-NBs.

Keywords Zingiberene · N2a neuroblastoma · Total oxidative stress · Total antioxidant capacity · MTT assay · Single cell gel electrophoresis

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Introduction

Neuroblastoma (NB) stands as the most common, frequently fatal, extracranial solid tumor of early childhood, with a median age of onset of 17 months. The incidence is 10.5 per million children under 15 years of age per year, with little variation between Europe and North America, and there are 700 new cases diagnosed in the United States each year (Stiller and Parkin 1992; Brodeur 2003). NBs grow rapidly; often giving rise to metastasis (Wassberg et al. 1999). Therapy resistance to anticancer drugs represents the major limitation to the effectiveness of clinical treatment. This is a major reason for the high frequency of fatal outcome of the disease (Svensson and Larsson 2003). The incidence of cancer is a global health problem and cancer treatments do not have effective drugs as the currently available drugs are causing

side effects in some instances. Therefore, the natural products derived from medicinal plants have gained significance in the treatment of cancer (Boopathy and Kathiresan 2010). Recently, there is a growing interest in the use of natural products for anticancer activity against cancer development (Ben Ammar et al. 2009; Kilani-Jaziri et al. 2009; Ben Sghaier et al. 2011). These include fatty acids, vitamins, minerals, lichens, flavonoids and terpenoids (Chaudhary et al. 2009; Iranshahi et al. 2009; Ben Sghaier et al. 2011; Ranković et al. 2011). Terpenoids are one of the most important substances of this which are naturally occurring in plants that are thought to have positive effects on human and animal health (Hamułka et al. 2012; Styrczewska et al. 2012). Sesquiterpenes, which are one of the most common terpenes, are a class of natural products with a diverse range of attractive industrial properties (Scalcinati et al. 2012). They are compounds containing three isoprene units, which is fifteen carbons and twenty-four hydrogens per molecule ($C_{15}H_{24}$). There are more than 10,000 kinds of sesquiterpenes (Davıs and Croteau 2000). They have long been investigated for biological activities; anticarcinogenic (Afoulous et al. 2013), antimicrobial (Wang et al. 2013), antifungal (Kundu et al. 2013), anti-inflammatory (Wang et al. 2013), and, more recently, antioxidant (Abolaji et al. 2013) activities.

Zingiberene (ZBN) is a sesquiterpene hydrocarbon present as the main active component of the essential oils in ginger rhizomes (Jeena et al. 2011; Antonious and Kochhar 2003). ZBN has been used in the odors and cosmetics industry for a long time (Denyer et al. 1994). Furthermore, previous reports indicated that ZBN has antifertility, antiviral, antiulcer and anticancer effects (Denyer et al. 1994; Millar 1998; Bou et al. 2013).

To our best knowledge, there has been no report of cytotoxic and genotoxic investigation for ZBN in rat neuron and N2a-NB cell cultures so far. Therefore, we aimed to assess the cytotoxic (by MTT assay), oxidative (by TAC and TOS levels) and genotoxic (by SCGE assay) effects of ZBN in cultured rat neuron and N2a-NB cells for the first time.

Materials and methods

Test compound and chemicals

Zingiberene (Cas: 495-60-3, $C_{15}H_{24}$, (5R)-2-methyl-5-[(2S)-6-methylhept-5-en-2yl] cyclohexa-1,3-diene

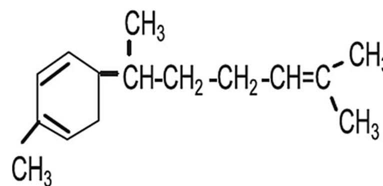

Fig. 1 Chemical structures of zingiberene

(Fig. 1; Antonious and Kochhar 2003) was purchased by Guide Chem® (Beijing, China). Dulbecco modified Eagles medium (DMEM), Hank's balanced salt solution (HBSS), neurobasal medium (NBM), sodium phosphate (NaH_2PO_4), monobasic potassium phosphate (KH_2PO_4), ethylenediaminetetraacetic acid (EDTA), phosphate buffer solution (PBS), dimethylsulfoxide (DMSO), Triton-X-100, Tris, low melting point agarose, normal melting point agarose were purchased from Sigma® Co. (St. Louis, MO, USA). Hydrogen peroxide was purchased from Merck® (Darmstadt, Germany). Fetal calf serum (FCS) and trypsin–EDTA were purchased from Biological Industries® (Beit-Haemek, Israel). All other chemicals were of analytical grade.

N2a neuroblastoma cell cultures

We employed the N2a-NB cell line widely used as a model for brain cancer. The rat brain NB cell line N2a was obtained from the Turkey FMD Institute (Ankara, Turkey). Prior to the experiments, the cells were thawed and grown in tissue culture flasks as a monolayer in DMEM supplemented with 1 % glutamine, 0.5 % penicillin/streptomycin (Pan Biotech®, Aidenbach, Germany) and 10 % fetal bovine serum at 37 °C in a humidified (95 %) incubator with CO_2 (5 %). The cultured cells were trypsinised with trypsin/EDTA for a maximum of 5 min and seeded with a subcultivation ratio of 1:3–1:8.

Healthy neuron cell cultures

This study was conducted at the Medical Experimental Research Centres at the Ataturk University (Erzurum, Turkey). The Ethical Committee of Ataturk University approved the study protocol (B.30.2.ATA.0.23.85-73). Primary rat cerebral cortex neuron cultures were prepared using rat foetuses as described previously (Ban et al. 2006). Briefly, a total of nine new-born Sprague–Dawley rats were used in the study. The rats

were decapitated by making a cervical fracture in the cervical midline and the cerebral cortex was dissected and removed. The cerebral cortex was placed into 5 mL of HBSS, which had already been placed in a sterile petri dish and macromerotomy was performed with two lancets. The cerebral cortices were dissociated with HBSS, were pulled into a syringe and treated at 37 °C for 25–30 min in 5 mL HBSS plus 2 mL Trypsin–EDTA (0.25 % trypsin–0.02 % EDTA) and chemical decomposition was achieved. Eight µL of DNase type 1 (St. Louis, MO, USA) was added to this solution and treated for 1–2 min, and centrifuged at 800 rpm for 3 min. After having thrown away the supernatant, 31.5 mL of NBM and 3.5 mL FCS were added to the residue. The single cells which were obtained after physical and chemical decomposition were divided into 3.5 mL samples in each of 10 flasks coated with poly-D-lysine formerly dissolved in PBS. The flasks were left in the incubator including 5 % CO_2 at 37 °C. The single cell which was obtained after physical and chemical decomposition was divided into 3.5 mL samples in each of 10 flasks coated with poly-D-lysine formerly dissolved in PBS. The flasks were left in the incubator including 5 % CO_2 at 37 °C. The neuron cell cultures were used in experiments after 8 days in vitro.

Treatments

ZBN was dissolved in hexane and hexane was evaporated to dryness at ambient temperature (final concentration of hexan <0.2 %). The concentrations were selected according to the works of Wang et al. (2009). In this study, ZBN was used in eight distinct doses at concentrations of 10, 25, 50, 75, 100, 150, 200 and 400 mg L^{-1}.

Cytotoxicity assay

Viability of cells was assessed by measuring the formation of formazan from MTT spectrophotometrically via a commercial kit (Cayman Chemical®, Ann Arbor, MI, USA). Rat neuron and N2a-NB cells were seeded in each 48-well plates. After incubation with compounds for 24 h, MTT solution (final amount 10 µL) was added to each well and re-incubated for 4 h at 37 °C. After washing, the blue formazan was extracted from the cells with isopropanol/formic acid (95:5) and was photometrically determined at 560 nm (Lewerenz et al. 2003; Turkez et al. 2012b; Aydın et al. 2014).

Biochemical assay

The automated total antioxidant capacity (TAC) and total oxidant status (TOS) assays were carried out in the culture medium by a commercially available kit (Rel Assay Diagnostics®, Gaziantep, Turkey) on rat neurons and N2a-NB cell cultures for 24 h (Erel 2004, 2005; Turkez et al. 2012a, c).

Genotoxicity assay

In this study, the DNA damage evaluation was performed by Comet (SCGE) assay (Singh et al. 1988). After the application of coverslips, the slides were allowed to gel at 4 °C for 30–60 min. The slides were immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10 in which 10 % DMSO and 1 % Triton X-100 were added) and refrigerated overnight followed by alkali treatment (300 mM NaOH, 1 mM EDTA pH >13), electrophoresis (25 V, 300 mA) and neutralization (0.4 M Tris, pH 7.5). The dried slides were then stained using Ethidium bromide (50 µL^{-1} of 20 µg mL^{-1}), (Sigma-Aldrich®, St Louis, MO, USA) after appropriate fixing. The whole procedure was carried out in dim light to minimize artefacts. DNA damage analysis was performed at a magnification of 100× using a fluorescence microscope (Nikon Eclips E6600, Japan) after coding the slides by one observer (Togar B). A total of 100 cells were screened per slide. A total damage score for each slide was derived by multiplying the number of cells assigned to each grade of damage by the numeric value of the grade and summing over all grades (giving a maximum possible score of 400, corresponding to 100 cells at grade 4; Ozkan et al. 2009).

Results

The MTT assay was used to quantify cell viability in response to ZBN. The results of MTT analysis showed that ZBN significantly suppressed the proliferation of N2a-NB cells, at higher concentrations than 50 mg L^{-1} (100, 150, 200 and 400 mg L^{-1}) compared to control value. However, ZBN treatments showed anti-proliferative activity on healthy neuron cells at concentrations over 150 mg L^{-1} (200 and 400 mg L^{-1}; Fig. 2).

Table 1 presents the comparison of oxidant–antioxidant profile of ZBN on cultured primary rat neurons and

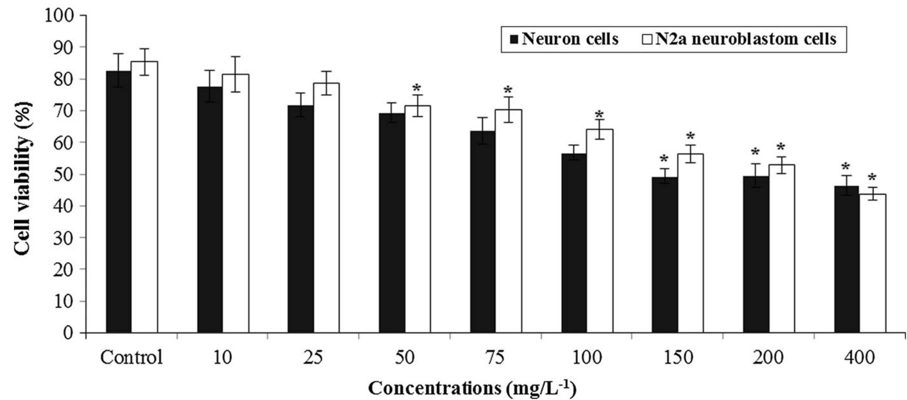

Fig. 2 Cytotoxic effect of zingiberene (ZBN) on cultured rat neurons and N2a-NB cells. The results are given as the mean ± SD from six independent experiments. Compared with control, *$p < 0.05$

Table 1 In vitro TAC and TOS levels in cultured neuron and N2a-NB cells maintained 24 h in the presence of zingiberene (ZBN)

Cells	Healthy neuron		N2a Neuroblastoma	
Concentrations (mg L^{-1})	TAC (mmol Trolox Equiv. L^{-1})	TOS (mmol H_2O_2 Equiv. L^{-1})	TAC (mmol Trolox Equiv. L^{-1})	TOS (mmol H_2O_2 Equiv. L^{-1})
Control	28.6 ± 3.0	1.7 ± 0.1	6.1 ± 0.5	2.3 ± 0.2
10	28.9 ± 2.9	1.6 ± 0.2	6.8 ± 0.5	2.3 ± 0.1
25	31.6 ± 2.3*	1.7 ± 0.1	7.3 ± 0.6*	2.4 ± 0.3
50	29.0 ± 4.0	1.8 ± 0.2	6.8 ± 0.8	2.6 ± 0.2*
75	28.7 ± 2.7	1.8 ± 0.3	6.4 ± 0.7	2.8 ± 0.3*
100	28.3 ± 3.1	2.0 ± 0.2	5.3 ± 0.7*	2.7 ± 0.2*
150	23.5 ± 2.8*	2.4 ± 0.1*	4.2 ± 0.6*	2.9 ± 0.1*
200	22.3 ± 2.6*	2.5 ± 0.1*	3.7 ± 0.4*	2.9 ± 0.1*
400	22.1 ± 2.3*	2.5 ± 0.1*	3.9 ± 0.5*	2.9 ± 0.1*

TAC total antioxidant capacity; *TOS* total oxidant status

The results are given as the means ± SD from six independent experiments. Compared with control, *$p < 0.05$

N2a-NB cell cultures. As seen from the Table 1, 150, 200 and 400 mg L^{-1} concentrations of ZBN did not lead to any alterations in TAC levels, while 25 mg L^{-1} of ZBN treatments caused significant increases of TAC levels in cultured primary rat neuron cells as compared to control value. Also, ZBN caused statistically important ($p < 0.05$) increases in TOS levels at concentrations higher than 100 mg L^{-1} in comparison with control values on rat neuron cell line. Likewise, ZBN (at 25 mg L^{-1}) did not cause any alterations in TAC levels on rat N2a-NB cell cultures. However, 100, 150, 200 and 400 mg L^{-1} of ZBN applications caused significant decreases of TAC levels when compared to controls. On the other hand, the TOS levels increased at 50, 100, 150, 200, and 400 mg L^{-1} concentrations of ZBN in cultured N2a-NB cells, respectively.

Comet assay was performed on healthy neuron and N2a-NB cell lines to measure the genotoxicity of ZBN. As shown in Fig. 3, the mean values of the total scores of cells showing DNA damage were not found to be significantly different from the control values in both cells. Besides, the neuron and N2a-NB cultures were found to be sterile at higher concentration of ZBN (400 mg L^{-1}).

Discussion

In the present study, we evaluated the cytotoxic effect of ZBN on rat neuron and N2a-NB cells via 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT) assay, for the first time. In fact, the MTT

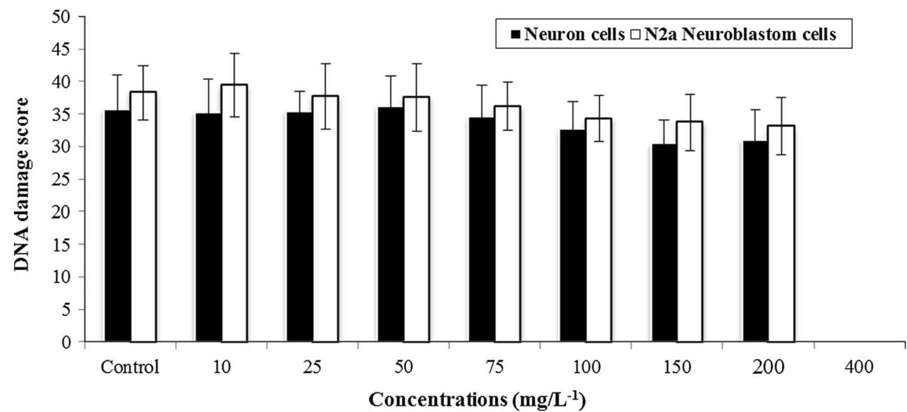

Fig. 3 Effect of varying concentrations of zingiberene (ZBN) on induction of DNA damage in vitro for 24 h

colorimetric assay is an established method for determining viable cell number in proliferation and cytotoxicity studies. Since the MTT assay is rapid, convenient, and economical, it has become a very popular technique for quantification of viable cells in culture (Sylvester 2011). We found that ZBN at 150 mg L^{-1} significantly reduced the cell viability of cultured primary rat neurons, while ZBN at concentrations above than 50 mg L^{-1} significantly reduced the cell viability of N2a-NB cells. Our findings were in line with previous reports. Similar to our findings, it was reported that the purified α-ZBN from the essential oil from leaves of *Casearia sylvestris* showed a cytotoxic activity against HeLa, U-87, Siha and HL60 cell lines (Bou et al. 2013). Our results are also in agreement with previous studies which have reported that parthenolide demonstrated strong cytotoxicity towards human lung carcinoma (A549), human medulloblastoma (TE671), human colon adenocarcinoma (HT-29) cells in the MTT in vitro assay (Parada-Turska et al. 2007). Also, Wang et al. (2001) revealed that artesunate showed evident cytotoxicity to human hepatocarcinoma SMMC-7721 cells. In addition, Taha et al. (2010) demonstrated that zerumbone (a monosesquiterpene found in the subtropical ginger), showed a strong cytotoxic effect in rat liver (DEN/AAF-induced) cancer cell lines. These results suggest a dose-dependent cytotoxic and antiproliferative effect of ZBN on neuron and N2a-NB cell cultures.

Many studies reported that while the in vitro or in vivo antioxidant capacity of several sesquiterpenes exists, antioxidant capacity of ZBN did not see in different cell types. For assessing the antioxidant/oxidant effects of ZBN, total antioxidant capacity (TAC) and total oxidant status (TOS) assays were performed. Actually, rapid and reliable automated colorimetric assay for TAC and TOS are frequently used to determine the impact of increasing increased oxidative damage with altered antioxidative status (Kusano and Ferrari 2008). Our results reported that a concentration of ZBN of 25 mg L^{-1} caused an increase of TAC level in neuron and N2a-NB cell cultures. On the other hand our results showed that ZBN (higher than 100 mg L^{-1}) caused increases in TOS levels in healthy neurons. And high concentrations of ZBN (higher than 50 mg L^{-1}) caused significant increases in TOS levels in N2a-NB cells. The exact mechanisms of the cytotoxic action of ZBN are not known, but oxidative stress is thought to be the main responsible mechanism in its cellular toxicity. Previous studies reported that different mechanisms have been linked to cytotoxicity of plant products, (I) including proteasome inhibition, (II) topoisomerase inhibition, (III) inhibition of fatty acid synthesis, (IV) accumulation of p53, (V) induction of cell cycle arrest, (VI) inhibition of phosphatidyl-inositol 3-kinase or (VII) enhanced expression of c-fos and c-myc including oxidative stress (Constantinou et al. 1995; Lepley et al. 1996; Plaumann et al. 1996; Agullo et al. 1997; Chen et al. 1998, 2005; Kazi et al. 2004; Brusselmans et al. 2005).

In this study, we investigated the genotoxic potential of ZBN by using the comet assay. The comet assay is a rapid, sensitive and relatively simple assay for measurements of DNA damage (Singh et al. 1988). As far as we know, the genotoxicity of ZBN has not been investigated on cultured rat neurons and N2a-NB cells. Also, there are no studies on the genotoxic effect of zingiberene on any cell line. Our findings also indicate that ZBN is neither genotoxic nor mutagenic on neurons and N2a-NB cells using the comet assay since the observed mean values of the total scores of

cells showing DNA damage was not found significantly different from the control values. To the best of our knowledge, there is no information on the genotoxic effect of ZBN on cell culture systems. Therefore, we have discussed its genotoxicity potential as compared with other sesquiterpenes. Similar to our finding it was suggested that beta-caryophyllene by itself did not produce any cytotoxic and genotoxic effect, as shown by the value of the nuclear division index (NDI) and the frequency of micronuclei (MN) in human lymphocytes cultures (Di Sotto et al. 2010). Likewise, Anter et al. (2011) reported that apigenin, bisabolol, and protocatechuic acid did not exhibit any genotoxic effect in DNA fragmentation assay. Additionally, it was found that gossypol significantly depressed the mitotic index but did not alter chromosome numbers or increase the frequency of chromosomal structural abnormalities. Moreover, Al-Zubairi et al. (2010) demonstrated that zerumbone is a cytotoxic but not a clastogenic substance in human peripheral blood lymphocytes. On the other hand, Mishima et al. (2005) reported that baccharin and drupanin induced a significant genotoxic effect on the tumor cells sarcoma S-180 in comparison with normal splenocytes using comet assay. In addition, it was demonstrated that deoxynivalenol an epoxy-sesquiterpenoid, was able to induce lymphocyte DNA damage in chickens (Awad et al. 2012). These divergent results suggest the relevance of the chemical structure in the biological effect of sesquiterpenes and indicate as well the importance of using various test models to reach a valid conclusion.

In conclusion our in vitro cytotoxic studies suggest that high concentrations of ZBN used in the present investigation may be cytotoxic. This study concludes that ZBN has weak anticancer activity in vitro. However, further studies are needed to further elucidate the mechanisms of ZBN to improve our understanding of their anticancer effect using different in vivo and in vitro cancer models.

Acknowledgments This work was supported by the Scientific & Technological Research Council of Turkey (TÜBİTAK, Project Number: 210T142).

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