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Research Paper

In-vitro free radical scavenging, antiproliferative and anti-zearalenone cytotoxic effects of 4-(methylthio)-3-butenyl isothiocyanate from Tunisian *Raphanus sativus*

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Abstract

Objectives The aim of this study was to investigate the antiradical and antioxidant properties of 4-(methylthio)-3-butenyl isothiocyanate (MTBITC) extracted from *Raphanus sativus* and to assess the effects of MTBITC on tumour cell growth, cytotoxicity induced by zearalenone, an oestrogenic mycotoxin, and modulation of the expression of the genes involved in these aspects of cell behaviour.

Methods A murine leukaemia cell line (L1210) was grown *in vitro* and supplemented with MTBITC (2, 4, 8, 16 and 32 μM) for 48 h. Cell growth was evaluated by the MTT assay. The chemopreventive role of MTBITC on the cytotoxic effect of zearalenone in a Balb/c mice keratinocyte cell line (C5-O) was also evaluated. Apoptosis and lipid peroxidation were assessed, as well as the expression of genes involved following zearalenone treatment alone or in combination with MTBITC.

Key findings MTBITC showed a significant ability to inhibit nitroblue tetrazolium reduction by superoxide radicals in a non-enzymatic superoxide generating system, to scavenge free radicals and to cause a decrease in L1210 cell growth. The C5-O cells treated with zearalenone alone showed a high frequency of apoptotic cells and lipid peroxidation, typical of oxidative stress generated by zearalenone. The cotreatment with MTBITC reduced the cytotoxicity of zearalenone and the subsequent gene expression analysis demonstrated that MTBITC decreased the expression of caspase 8, implicated in the physiological mechanism to eliminate injured or abnormal cells.

Conclusions The results suggest that MTBITC was able to inhibit L1210 cell growth and counteract the zearalenone oxidative stress to C5-O cells through caspase 8 inhibition of apoptosis.

Keywords antioxidant; apoptosis; MTBITC; caspase 8; *Raphanus sativus*; zearalenone

Introduction

Mycotoxins represent a class of dietary mutagens and carcinogens to which humans are exposed. Zearalenone (ZEN) is genotoxic and responsible for potent reproductive toxicity in humans and animals.[1,2] Humans are exposed to ZEN through consumption of food that has been directly contaminated by the growth of fungi, and by food products derived from exposed animals.[3,4] ZEN has been shown to be immunotoxic,[5,6] hepatonephrotoxic,[7] apoptotic[8] and an enhancer of lipid peroxidation.[7,9,10] Epidemiological studies show that ZEN intake is associated with the aetiology of human and animal cancers.[1,11] In addition, ZEN has been reported to cause an earlier onset of puberty in children,[3] endometrial adenocarcinomas, hyperplasia and breast cancer in women.[1] It induces DNA fragmentation, micronuclei production, chromosome aberrations[2,11] and DNA adduct formation.[12]

The carcinogenic risk imposed by this probable human carcinogen can be reduced by other dietary factors that influence its uptake and biotransformation. There is sufficient scientific evidence indicating that populations consuming diets rich in fruits and vegetables have a reduced risk of developing several types of cancers.[13,14] In Asian and African medicine, the juice of radish is used for the treatment of a variety of ailments, including whooping cough, cancers, coughs, gastric discomfort, liver problems, constipation, dyspepsia, gallbladder problems, arthritis, gallstones, kidney stones and intestinal parasites.

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However, its effectiveness has not been scientifically confirmed. Moreover, radish roots are generally relished as a raw vegetable and as a component of salads. Hence, they may be more beneficial than other cruciferous vegetables which are consumed after cooking because heat inactivates the enzyme myrosinase, which is essential for liberating active isothiocyanates from their glucosinolate precursors.[15]

Recently, several natural compounds with chemoprotective properties have been identified from radish (*Raphanus sativus*). Some of these compounds, particularly isothiocyanates, have been shown to inhibit various human cancers.[16–18] Among these, 4-(methylthio)-3-butenyl isothiocyanate (MTBITC), which is found in radish,[19–21] is one of the most characterised isothiocyanates and is currently under active investigation for its chemopreventive properties against various cancers. It has been reported to induce apoptosis, cytotoxicity and MAPK activation in head and neck squamous cells carcinoma.[22] Also, it induces cell cycle arrest and reduction of α- and β-tubulin isotypes in human prostate cancer cells.[23,24] Mechanistic studies have shown that the chemopreventive activity of MTBITC is due to a favourable modification in c-Jun N-terminal kinase mediated apoptosis by carcinogen metabolism, resulting in increased carcinogen excretion or detoxification and decreased carcinogen–DNA interaction.[25] In-vitro inhibitory studies on various human cytochrome P450 enzymes have shown isothiocyanates to be effective inhibitors of a range of cytochrome enzymes that are essential for metabolic activation of many proximate carcinogens.[26]

The present study was designed to examine the antiradical and the antioxidant activity of MTBITC extract from *R. sativus*. We also wanted to evaluate the effects of MTBITC on the growth of the murine leukaemic cell line L1210 and the chemopreventive role of MTBITC on ZEN-induced cytotoxicity in C5-O cells and modulation of the expression of genes involved in these aspects of cell behaviour in order to elucidate the molecular mechanisms underlying any changes.

Materials and Methods

Chemicals

ZEN and methyl isothiocyanate were purchased as pure crystals from Sigma-Aldrich Chimie, Lyon, France. MTBITC was extracted and purified from the root of *R. sativus* according to the method previously described by Esaki and Onozaki,[27] with a slight modification. The purity of the MTBITC was 96.7% as estimated by high-performance liquid chromatography. Fetal calf serum, RPMI 1640, HEPES and L-glutamine were purchased from Gibco, Life Technologies, Rockville, MD, USA. 40,6-Diamidino-2-phenylindole (DAPI), 3-[4,5-dimethylthiazol]-2,5-diphenyl tetrazolium bromide (MTT), 2-mercaptoethanol, sodium pyruvate and antibiotics (penicillin, streptomycin sulfate, concanavalin A and amphotericin) were obtained from Sigma-Aldrich Chimie. Trizol reagent, sperm DNA and the Fluor S phosphorimager were obtained from Bio-Rad, Hemel Hempstead, Hertfordshire, UK. CPD Star chemiluminescent detection kit and RNeasy Mini Kit were obtained from Super Array Inc, Bethesda, MD, USA. All other chemicals were of the highest purity commercially available.

Plant materials

R. sativus was collected from the Eletha region of Tunisia in October 2005. Identification was carried out by Professor El Ouni (Department of Botany, Faculty of Sciences, Bizerte, Tunisia).[28] A voucher specimen has been kept for future reference (R.S.19-89).

Extraction, measurement and isolation of isothiocyanate from *R. sativus*

Whole roots of *R. sativus* growing in Tunisia were washed with distilled water, peeled, and then cut into 2-cm cubes. After 30 min of incubation, in order to increase the isothiocyanate level,[29] a sample was homogenized in methanol and then extracted. The methanol extracts were combined and evaporated to 100 ml of crude aqueous extract. The extract was combined, and 3 ml of 1 M HCl was added to avoid degradation of isothiocyanates in alkaline pH. The mixture was partitioned three times with *n*-hexane, chloroform and ethyl acetate, in that order, with 100 ml of solvent each time. The *n*-hexane, chloroform and ethyl acetate layers were evaporated to dryness at 35°C and the aqueous layer was lyophilized.

The level of total isothiocyanate was measured using the method of Zhang *et al.*,[30] with a slight modification. In brief, 50 μl of the *n*-hexane extract from *R. sativus* was diluted with a mixture of 0.45 ml of methanol and 0.45 ml of 50 mM $Na_2B_4O_7$-HCl buffer (pH 8.5), and then 50 μl of 8 mM 1,2-benzenedithiol was added and mixed well in a 1.5-ml plastic tube. The tube was heated at 65°C for 1 h, and then the isothiocyanate content was calculated by reading the sample absorption at 365 nm from a linear standard equation derived from the absorption readings of a serial dilution of known methyl isothiocyanate concentrations.

The MTBITC in the *n*-hexane extract was measured by gas chromatography on a Shimadzu (Kyoto, Japan) model GC-12A instrument with flame ionization detector. The operating conditions for gas chromatography were as follows: injection volume, 1.0 μl; injector and detector temperature, 250°C; DB-5 (25 m $\times$ 0.2 mm, 0.33-μm film thickness; J&W Scientific, Folsom, CA, USA); column temperature, 70°C for 70 s increased to 170°C at 3°C/min; carrier gas, 180 kPa He; injection, splitless (closed for 70 s).The obtained MTBITC was characterized by gas chromatography-mass spectroscopy and nuclear magnetic resonance spectroscopy.[31] It was then freeze-dried and the desired dose was prepared in distilled water.

Free radical scavenging activity

Free radical scavenging activity of MTBITC, was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method.[32] A sample (0.5 ml) of ethanol solution containing different amounts of MTBITC was added to 3 ml of daily prepared ethanol DPPH solution (0.1 mM); the maximum concentration of MTBITC used was 100 μg/ml. The optical density change at 517 nm was measured after 30 min using a spectrophotometer. The antioxidant assay was carried out in triplicate and the readings were averaged. The scavenging

activity was measured as the decrease in absorbance of the samples versus DPPH standard solution. Results were expressed as percentage inhibition of DPPH (IC50) by using the following formula:

$$\% \text{ inhibition} = [(OD_{control} - OD_{sample})/OD_{control}] \times 100$$

where $OD_{control}$ is the initial absorbance and OD_{sample} is the value of the added sample concentration.[33] The IC50 parameter is defined as the concentration (μg/ml) of substrate that causes 50% loss of DPPH activity and was calculated by using the method of Litchfield and Wilcoxon.[34]

Evaluation of the inhibition of xanthine oxidase activity and superoxide radical scavenging effect

Xanthine oxidase inhibition and superoxide anion scavenging activity were assessed in an in-vitro assay. Inhibition of xanthine oxidase activity was measured according to the increase in UV absorbance of uric acid at 290 nm as proposed by Cimanga *et al.*,[35] while superoxide anion scavenging activity was detected spectrophotometrically according to the nitrite method described by Oyangagui,[36] with some modifications introduced by Hu *et al.*[37] and Russo *et al.*[38] Briefly, the assay mixture consisted of 100 μl of MTBITC (50, 100, 200 and 300 μg/ml), 200 μl of the substrate xanthine (50 μM), hydroxylamine (0.2 mM), 200 μl EDTA (0.1 mM) and 300 μl distilled water. The reaction was initiated by adding 200 μl xanthine oxidase (5.5 mU/ml) dissolved in phosphate-buffered saline (PBS) (0.2 M, pH 7.5). The assay mixture was incubated at 37°C for 30 min. Before measurement of the uric acid production at 290 nm, the reaction was stopped by adding 0.1 ml of HCl (0.5 M). The absorbance was measured spectrophotometrically against a blank solution prepared as described above but replacing xanthine oxidase with PBS. Another control solution without the tested compound was prepared in the same manner as the assay mixture to measure the total uric acid production. The positive control solution was prepared using allopurinol (50, 100, 200 and 300 μg/ml). The modulation of decrease of uric acid production was calculated from the differential absorbance. To detect the superoxide scavenging activity, 2 ml of the colouring reagent consisting of sulfanilic acid solution (300 μg/ml), *N*-(1-naphthyl) ethylenediamine dihydrochloride (5 μg/ml) and acetic acid (16.7%, v/v) were added. This mixture was allowed to stand for 30 min at room temperature and the absorbance was measured at 550 nm on a Helios Alpha spectrophotometer. The dose–effect curve for MTBITC was linearized by regression analysis and used to derive the IC50 values.

Cell culture

Murine leukaemia cell lines (L1210) and Balb/c mice keratinocyte cell lines (C5-O) were obtained from the European Collection of Animal Cell Culture. L1210 cell lines were cultured in RPMI 1640 culture medium supplemented with 10% (v/v) fetal calf serum, 0.118% (w/v) sodium bicarbonate, 0.029% (w/v) glutamine, 100 000 U/l penicillin and 100 mg/l streptomycin at 37°C in a humidified atmosphere containing 5% (v/v) carbon dioxide. The C5-O cell line was cultured in DMEM culture medium. Media were supplemented with 10% (v/v) fetal calf serum, 0.118% (w/v) sodium bicarbonate, 100 000 U/l penicillin and 100 mg/l streptomycin, and cells cultured at 37°C in a humidified atmosphere containing 5% (v/v) carbon dioxide.

Cell proliferation assay

The effect of MTBITC on the growth of L1210 cells was determined using the MTT dye reduction assay.[39] Cells were plated in 96-well plates with a seeding density of 1.2×10^4 cells per well in RPMI 1640 culture medium for 24 h before the cells were treated with varying concentrations of MTBITC (2, 4, 8, 16 and 32 μM) for 24, 48 and 72 h. At the end of each time period, cells were treated with 50 ml (5 mg/ml) MTT dye and incubated at 37°C for 4 h. Following this, MTT dye was removed and 200 ml dimethylsulfoxide was added in order to solubilize the cells and dissolve the MTT-formazan crystals. The number of viable cells was determined by measuring the absorbance at 570 nm and 630 nm for each well, using a microplate spectrophotometer (DynaTech Laboratories Inc., Chantilly, VA, USA). All experiments were repeated eight times with six replicates per experiment.

Assessment of apoptosis

Viable cells (C5-O) were incubated in 96-well culture plates with a seeding density of 10^5 cells for 24, 48 and 72 h in the presence of medium containing ethanol as vehicle, MTBITC (32 μM), ZEN alone (40 μM) and ZEN (40 μM) plus MTBITC (32 μM). The nuclear chromatin of cells was stained with the fluorogenic compound DAPI in order to assess the morphological changes associated with apoptosis. C5-O cells were fixed in 0.4% (v/v) paraformaldehyde before attaching the cells onto glass microscope slides by cytocentrifugation at 50*g* for 5 min. The slides were then stained with DAPI solution (5 μg/ml) for 10 min. Cells were visualized by fluorescent microscopy, with a blue filter (330–380 nm), to observe morphological features of apoptosis such as cell shrinkage, chromatin condensation and formation of apoptotic bodies. A minimum of 500 cells were counted per slide and the percentage of cells displaying apoptotic characteristics was expressed as an apoptotic index. This was defined as the proportion of apoptotic cells compared with the total number of cells, expressed as a percentage. All the experiments were repeated three times.

Lipid peroxidation inhibitory activity

Lipid peroxidation was assayed by the measurement of malondialdehyde (MDA) according to the method of Ohkawa *et al.*[40] The C5-O cells were incubated in 96-well culture plates with a seeding density of 10^5 cells for 24, 48 and 72 h in the presence of medium containing ethanol as vehicle, MTBITC (32 μM), ZEN alone (40 μM) and ZEN (40 μM) plus MTBITC (32 μM). Cells were then washed with cold PBS and homogenized in ice-cold 1.15% KCl. Samples containing 100 μl of cell lysates were combined with 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid adjusted to pH 3.5 and 1.5 ml of 0.8% thiobarbituric acid. The mixture was brought to a final volume of 4 ml with distilled water and heated to 95°C for 120 min. After cooling to room temperature, 5 ml of a mixture of *n*-butanol and pyridine (15 : 1, v/v) was added to each sample and the mixture was shaken vigorously. After centrifugation at 600*g* for 10 min, the

peroxide content of the supernatant obtained was assayed using a thiobarbituric acid reaction with the molar extinction coefficient (OD532) of MDA. The absorbance was measured at 546 nm[33] and the inhibitory activity towards lipid peroxidation was expressed as the IC50.

cDNA microarray analysis

Total RNA was isolated from C5-O cells, treated as described previously, using Trizol reagent, according to the manufacturer's instructions, and purified using the RNeasy Mini Kit. The resulting RNA was electrophoresed to check its integrity using a 1% (w/v) agarose gel. In addition, the purity and quantity of the RNA was confirmed by measuring the absorbance at 260 nm and 280 nm using a spectrophotometer. Gene expression of cancer pathway genes was determined using the Cancer Pathway Finder gene expression arrays (Super Array Inc.) according to the manufacturer's instructions. Gene-specific biotin-labelled cDNA probes were synthesized using 2.5 mg total RNA with gene-specific primers for reverse transcription using 200 U MMLV reverse transcriptase and 40 U RNase inhibitor (Promega, Southampton, UK). Prior to hybridization, the cDNA membrane array was prehybridized at 60°C for 2 h in the supplied hybridization buffer supplemented with denatured salmon sperm DNA. The cDNA probe was then denatured by heating at 94°C for 5 min and quickly cooled on ice before hybridization with the cDNA membrane array at 60°C overnight. The following morning, the membrane was washed and the hybridization signals were detected using the CPD Star chemiluminescent detection kit. The Fluor-S phosphorimager was used to scan the cDNA membrane array and the resulting image was converted into digital data and analysed by GEArray Analyzer version 1.3 software (Super Array Inc.). All signals were background subtracted using the PUC18 negative control and normalised to the housekeeping gene PPIA (positive control). Changes in gene expression were illustrated as a fold increase or decrease. The cut-off fold induction determining expression was $\leq$ 1.5- or $\geq$ 0.667-fold changes. Genes that met the above criteria were considered to be either increased or decreased when C5-O cells were treated with MTBITC. The experiments were repeated in duplicate with RNA isolated from two independent extractions.

Reverse-transcription polymerase chain reaction analysis

Total RNA (2 μg) was reverse-transcribed as described previously. The following oligonucleotide primers were used: APAF1 forward primer sequence 5′-GCTGC-CATTTCACCAACAGT-3′ and reverse primer sequence 5′CTCTCATTTGCTGATGTCGC-3′, CASP8 forward primer sequence 5′-TTATTCAGGCTTGTCAGGGGG-3′ and reverse primer sequence 5′-GCACCATCAATCAGAAGGGAAG-3′. QuantumRNA 18S internal control standards (Ambion, Huntingdon, UK), which produced a polymerase chain reaction (PCR) product of 489 bp, were used in a ratio of 9 : 1 and 13 : 1 competitors to primers for CASP8 and APAF1, respectively, according to the manufacturer's instructions, to act as an internal loading control to normalize between samples during densitometry. Following an initial denaturation step at 94°C for 2 min, PCR was carried out using 1 × PCR buffer (10 mmol/l Tris, pH 8.3, 50 mmol/l potassium chloride, 0.1 mg/ml gelatin, (Roche Diagnostics, Lewes, East Sussex, UK), 200 μmol/l dNTP, 2 mmol/l magnesium chloride, 0.5 U Taq DNA polymerase (Roche Diagnostics), 10 pmol of each oligonucleotide primer for 25 cycles using the following cycling conditions: 94°C for 30 s, an annealing temperature of 60°C for 60 s (APAF1) and 58°C for 30 s (CASP8), 72°C for 60 s, followed by an extra 10-min extension step at 72°C. PCR products (APAF1 223 bp and CASP8 380 bp) were electrophoresed through a 1% (w/v) agarose gel. Gel images were captured by Gene snap software (Syngene, Cambridge, UK) and band densities calculated using a Fluor S phosphoimager (Bio-Rad). The experiments were repeated in triplicate with RNA isolated from two independent extractions.

Statistical analysis

The Kruskal–Wallis test was used to compare the effect of concentrations on the percentage inhibition of the tested parameters for MTBITC compounds. Statistical analyses were performed using SPSS 12.0 software for Windows (SPSS, Chicago, IL, USA).

Results

Phytochemical study

The phytochemical results showed the presence of an important quantity of total MTBITC in *R. sativus* extract. The amount of MTBITC reached 238.98 ± 4.2 μmol/100 mg.

DPPH free radical scavenging activity

The free radical scavenging activity of MTBITC is reported in Figure 1. MTBITC was a very potent radical scavenger with a percentage decrease versus the absorbance of DPPH standard solution of 96, 90, 85, 77 and 56%, respectively, at 100, 30, 10, 3 and 1 μg/ml and an IC50 value of 0.96 μg/ml. Vitamin E, the positive control, was a very potent free radical scavenger, with a percentage decrease versus the absorbance of DPPH standard

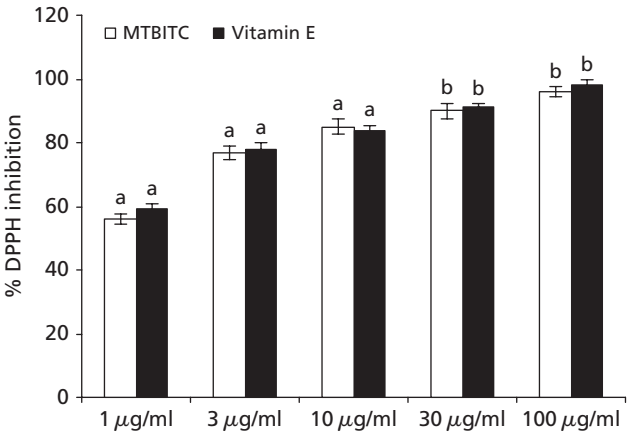

Figure 1 Free radical scavenging effects of 4-(methylthio)-3-butenyl isothiocyanate (MTBITC) against the stable radical DPPH compared with vitamin E. Results are the means ± SD of three experiments. The Kruskal–Wallis test was used to compare the effect of concentration on the % DPPH inhibition for both MTBITC and vitamin E. Means with different letters are significantly different ($P < 0.05$).

solution of 98% at 100 μg/ml and an IC50 value of 1.8 μg/ml. These results indicate that MTBITC compound showed comparable antioxidant activity to vitamin E.

Inhibition of xanthine oxidase activity

Results are expressed as a percentage of inhibition of xanthine oxidase activity with respect to control. At a concentration of 100 μg/ml, MTBITC showed the most potent inhibitory effect of xanthine oxidase activity (77%; Figure 2). Moreover, an increase of xanthine oxidase activity was observed with 300 μg/ml MTBITC, translating to a pro-oxidant effect at this dose. Assessment of the antioxidant or pro-oxidant properties of MTBITC was imperative to understand some of the mechanisms involved in the eventual therapeutic or toxic effects.[41]

The antioxidant effect of MTBITC on superoxide anions (O_2^-) enzymatically generated by the xanthine/xanthine oxidase assay enzymatic system was evaluated *in vitro*. The percentage superoxide anion (O_2^-) scavenging effect of MTBITC is given in Figure 3. MTBITC showed a potent superoxide scavenging effect at all tested doses. At a concentration of 50, 100, 200 and 300 μg/ml, the percentage superoxide anion (O_2^-) scavenging effect was 70, 90.3, 94 and 97.8%, respectively.

Effect of MTBITC on L1210 leukaemia cell proliferation

The effect of MTBITC on L1210 cell growth was assessed using the MTT cell proliferation assay (Figure 4). A decrease in L1210 cell growth at 24, 48 and 72 h was noted with increasing concentration of MTBITC. The lowest concentration of MTBITC, 2 μM, resulted in a statistically significant decrease of cell growth after 48 and 72 h (16% and 18%, respectively).

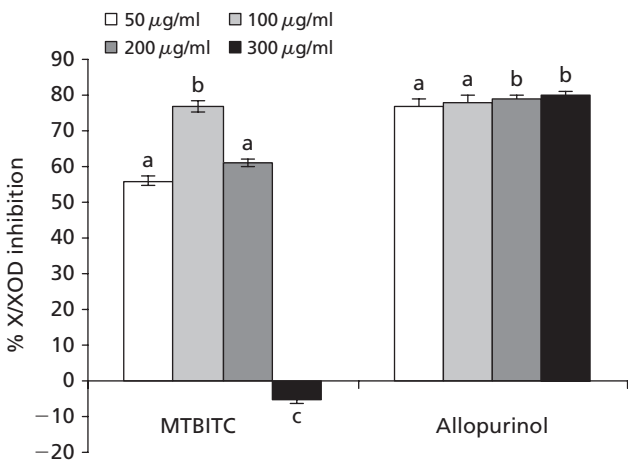

Figure 2 Percentage inhibition of uric acid production in the presence of different concentrations of 4-(methylthio)-3-butenyl isothiocyanate (MTBITC). Allopurinol was used as a positive control for protection against superoxide radicals. Results are the means ± SD, $n = 3$. The Kruskal–Wallis test was used to compare the effect of concentration on the percentage of xanthine/xanthine oxidase (X/XOD) inhibition activity for both MTBITC and allopurinol. Means with different letters are significantly different ($P < 0.05$).

After 24, 48 and 72 h of culture, maximum inhibition of cell growth occurred using 32 μM MTBITC (50, 58 and 66% inhibition, respectively). The IC50 value was 16 μM after 24 h.

Apoptosis assessment in C5-O cells treated with zearalenone and MTBITC alone or in combination

DAPI staining and subsequent morphological assessments of cells were used to determine if ZEN and MTBITC alone or in combination induced apoptosis in C5-O cells. Increased apoptosis in C5-O cells was noticed when cells were cultured with ZEN at 24, 48 and 72 h (Figure 5). After 24, 48 and 72 h, a

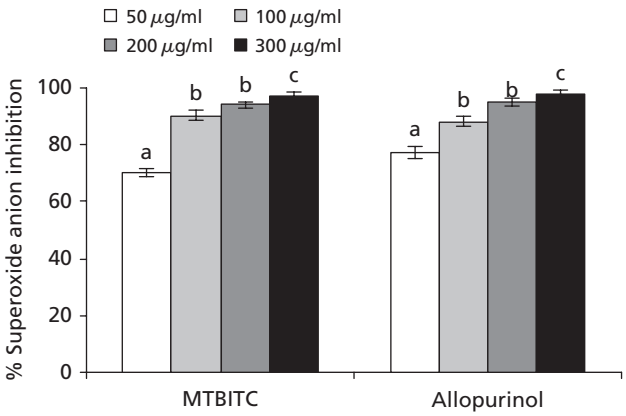

Figure 3 Percentage superoxide anion inhibition in the presence of different concentrations of 4-(methylthio)-3-butenyl isothiocyanate (MTBITC). Allopurinol was used as a positive control for protection against superoxide radicals. Results are the means ± SD, $n = 3$. The Kruskal–Wallis test was used to compare the effect of concentration on the percentage superoxide anion inhibition for both MTBITC and allopurinol. Means with different letters are significantly different ($P < 0.05$).

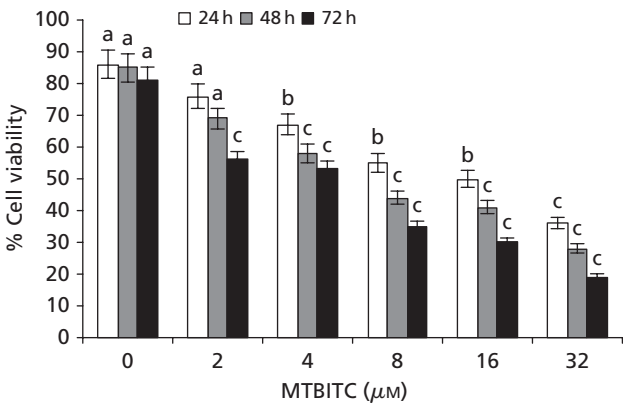

Figure 4 Effects of 4-(methylthio)-3-butenyl isothiocyanate (MTBITC) on cell viability. The percentage of cell viability was measured by MTT assay. Data are means ± SEM of eight experiments with six replicates per experiment. The Kruskal–Wallis test was used to compare the effect of concentration on the percentage of cell viability for MTBITC. Means with different letters are significantly different ($P < 0.05$).

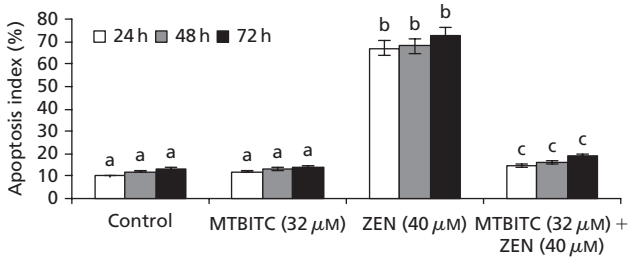

Figure 5 Effects of 4-(methylthio)-3-butenyl isothiocyanate (MTBITC) and zearalenone (ZEN) alone or in combination on apoptosis in C5-O cells. Data are means ± SEM of three experiments. The Kruskal–Wallis test was used to compare the effects of MTBITC on the apoptosis index. Means with different letters are significantly different ($P < 0.05$).

statistically significant increase in apoptotic index (57, 58 and 61%, respectively) was observed with a ZEN concentration of 40 μM when compared with C5-O cells with vehicle supplementation (apoptotic index 12, 13 and 14% at 24, 48, and 72 h, respectively). No significant difference was observed in C5-O cells treated with MTBITC alone compared with control after 24, 48 and 72 h at a concentration of 32 μM (apoptotic index 12, 13 and 14%, respectively). After 24, 48 and 72 h, a statistically significant decrease in apoptotic index (15, 16 and 19%) was noted at 32 μM MTBITC in combination with ZEN (40 μM) when compared with C5-O cells treated with ZEN alone. The percentage inhibition of apoptosis was 50, 52 and 55% after 24, 48 and 72 h, respectively.

Anti-lipid peroxidation activity of MTBITC against zearalenone

The inhibitory effect of MTBITC on MDA production in the C5-O cell line induced by ZEN is shown in Figure 6, expressed as percentage levels of thiobarbituric acid reactive substance (TBARS). In cells treated with 40 μM ZEN, the TBARS level increased and reached 75%. A potential antilipid peroxidation activity was obtained with the co-treatment of ZEN–MTBITC and the percentage inhibition of TBARS levels was 65%, confirming a good antioxidant effect of the tested compound. C5-O cells treated with MTBITC alone at a concentration of 32 μM showed percentage TBARS levels comparable with untreated cells.

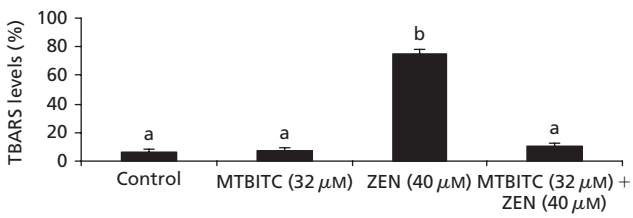

Figure 6 Thiobarbituric acid reactive substance (TBARS) levels in C5-O cells treated with zearalenone (ZEN) and 4-(methylthio)-3-butenyl isothiocyanate (MTBITC) alone or in combination. Data are means ± SEM of six experiments with three replicates per experiment. The Kruskal–Wallis test was used to compare the effects of MTBITC on the percentage TBARS levels. Means with different letters are significantly different ($P < 0.05$).

Effect of MTBITC on gene expression caused by ZEN cytotoxicity

Potential molecular mechanisms associated with inhibition of ZEN toxicity to C5-O cells treated with MTBITC were investigated. The apoptosis noted in C5-O cells treated with 40 μM ZEN and MTBITC alone or in combination for 24 h was evaluated using cDNA microarray technology. Changes in gene expression as a result of ZEN, MTBITC and co-treatment are given in Table 1. Only gene expression changes that were either increased or decreased consistently in each microarray replicate were reported. The increase in gene expression of APAF1 (apoptotic protease activating factor), caspase 8 (apoptosis-related cysteine protease), casper (CASP8 and FADD-like apoptosis regulator) and TNFα (tumour necrosis factor superfamily, member 2) involved in the regulation of cell apoptosis was investigated. Further, by way of validation at the mRNA level, semi-quantitative RT-PCR was carried out. This confirmed a 2.5-, 3-, 2.6- and 3.2-fold increase in APAF1, caspase 8, casper and TNFα mRNA expression following ZEN treatment of C5-O cells, respectively. The gene expression level of the cells cotreated with ZEN and MTBITC were tending towards normal control levels (Table 1). The treatment of C5-O cells with MTBITC alone showed no significant difference in gene expression level compared with untreated cells after 24 h.

Discussion

Epidemiological studies offer evidence that cruciferous vegetables protect humans against cancer, and results from animal experiments show that they reduce mycotoxins, induced toxicity and tumour formation. ZEN is an oestrogenic mycotoxin widely present in foods and environmental water. The ZEN content in food and water is >10-fold the recommended level[42–44] and this toxin can induce several deleterious effects in humans and animal livestock.[3,6,45,46] The liver is the main target organ for ZEN toxicity and chronic exposure to even low levels of ZEN in foodstuffs causes liver fibrosis, endometrial adenocarcinomas and stimulates the growth of human breast cancer cells through its effects on the oestrogen receptor response.[1,47] ZEN is mainly metabolized by oxidase enzyme systems and cytosolic enzymes into many metabolites. Some of these metabolites are non-toxic, others are active and toxic.[48,49] The toxic metabolites can produce genetic alterations *in vitro* and *in vivo*[2,50] as a result of the interaction between these metabolites and DNA; this represents the primary pathological condition for cancer development.[12] It is clear that ZEN is a long-standing and inextricable problem. Concerns about ZEN originate from the strong implications of its involvement in disease and death in humans and animals, and thus scientists and clinicians are still seeking ways to effectively deal with this dangerous and elusive chemical. Chemopreventive strategies against ZEN toxicity have been well documented.[5–7,45,51,52]

The present study has demonstrated that MTBITC isolated from *R. sativus* exhibited radical scavenger and antioxidant activities. Our data show that MTBITC was a very effective scavenger against DPPH radicals. However, the chemical assay using DPPH is far from biological conditions.[53]

Table 1 Gene expression in C5-O cells treated with zearalenone and 4-(methylthio)-3-butenyl isothiocyanate (MTBITC) alone or in combination

Gene	Treatment			
	Control	MTBITC (μM)	Zearalenone (40 μM)	MTBITC + zearalenone
Apaf-1 (apoptotic protease activating factor)	1.2	1.1[a]	3.2[b]	1.6[a]
Caspase 8 (apoptosis-related cysteine protease)	0.8	0.9[a]	2.3[b]	1.2[a]
Casper (CASP8 and FADD-like apoptosis regulator)	1	1.1[a]	2.6[b]	1.1[a]
TNFα (tumour necrosis factor; TNF superfamily, member 2)	0.9	1.1[a]	3.1[b]	1.7[a]

The Kruskal–Wallis test was used to compare the effects of MTBITC on zearalenone-induced gene expression in C5-O cells. Within each row, means with the same superscript letter are not significantly different ($P \leq 0.05$).

Regarding the antioxidant effect of MTBITC, the assay (xanthine/xanthine oxidase system) based on the influence of MTBITC on xanthine oxidase activity in an enzymatic oxidation system and the effect on the superoxide anions (O_2^-) enzymatically generated by this system were evaluated *in vitro*. The enzymatic assay is closer to physiological conditions and MTBITC was a very potent radical scavenger and more active than the positive control, allopurinol, in the assay at dose 100 μg/ml. The enzyme xanthine oxidase plays a crucial role in the production of uric acid, catalysing the oxidation of hypoxanthine and xanthine. During the reoxidation of xanthine oxidase, molecular oxygen acts as an electron acceptor, producing superoxide radical and hydrogen peroxide. Consequently, xanthine oxidase is considered to be an important biological source of superoxide radicals. The results indicated that MTBITC inhibits xanthine oxidase activity, as determined by uric acid production. It seems that this activity is mostly related to the sulfur atom and double bond in the side chain of the MTBITC compound. On the other hand, the antioxidant activity observed in this study might be the result of quenching of singlet oxygen by the isothiocyanates as suggested by Hall and Cuppett.[54] Therefore, isothiocyanates remove protonated species (i.e. OH) and thus interrupt the oxidative chain reaction, as demonstrated by Manesh and Kuttan.[55] Delaquis *et al.*[56] have shown that isothicyanates are able to retard oxidation of meats. Moreover, in Japanese cuisine, radish is used primarily as a condiment for sushi and seafood dishes.

Typical isothiocyanates extracted from cruciferous vegetables are allyl, benzyl and phenethyl isothiocyanates, and they are all well investigated for cancer chemopreventive and chemotherapeutic effects.[57,58] The chemoprotective effect of MTBITC against L1210 cell proliferation seems to be important (Figure 4). A decrease in L1210 cell growth at 24, 48 and 72 h was noted with increasing concentration of purified MTBITC. This behaviour was easily detectable considering the IC50 values (16 μM) of the tested compound. We can make some preliminary considerations on the basis of the structure–activity relationship of this compound. In a previous study we reported that among alkenyl-isothiocyanates, a longer side chain negatively affects the antiproliferative activity of K562 and the stereochemistry of the C5 atom of the two hydroxylalkenyl isothiocyanate epimers, derived from PRO and *e*-PRO, does not affect the biological activity of K562 cells. Moreover, we found that antiproliferative activity appears to be correlated to the lipophilicity of the compounds rather than their nucleophilicity. The most active isothiocyanates are, in fact, characterized by low partition coefficients independently from the group of glucosinolates to which they belong.[29,59]

Concerning the evaluation of ZEN toxicity, our study demonstrated that ZEN inhibited C5-O cell viability and this was associated with increased apoptosis. Our results are in accordance with those of Abid-Essafi *et al.*,[8] who reported that the apoptosis induced by ZEN may be due to three linked processes: sustained DNA injury, DNA lesions or DNA fragmentations, and cell cycle arrest in the G2/M phase. Moreover, ZEN damaged DNA repair capability and initiated the apoptosis pathway.[60] In addition to these effects, an increase of MDA formation was induced by ZEN, confirming oxidation of cell membrane lipids. ZEN is active in inducing oxygen reactive species, which in turn induce several cytokines such as TNF-α, interleukins and nuclear factors with mitogenic activity that may underlie tumour promoter activity.[5,6,61]

The cotreatment of ZEN–MTBITC succeeded to strongly inhibit apoptosis and showed substantial protection against lipid peroxidation initiated by ZEN in C5-O cells. Indeed, membrane lipids are rich in unsaturated fatty acids, which are most susceptible to oxidative processes. It is generally thought that the inhibition of lipid peroxidation by antioxidants may be due to their free radical scavenging activity. The data showed good inhibition of MDA formation, indicating that MTBITC is polar and soluble in a micellar water–lipid system. According to Burda and Oleszek,[62] the solubility of an antioxidant compound in a micellar water–lipid system by making two phases can influence oxidation results. Our results demonstrated that MTBITC is soluble in the cell and thus is an effective scavenger of oxidizing molecules generated by ZEN stress in the treated cells. A previous study showed that isothiocyanate and allyl isothiocyanate inhibit lipid oxidation and food poisoning bacterial activity.[55] These compounds also inhibit the growth of mould and yeast[63] and have been tested for use as food preservation agents. The role of antioxidants has attracted much interest with respect to their protective effects against free radical damage that may be the cause of many diseases including cancer.[64] These studies identified a change in expression of a number of genes involved in key stages of apoptosis and cancer pathways. In particular, there was an increase in expression of caspase 8. This is an important protein which is involved in apoptosis triggered by the extrinsic pathway. Binding of FasL and TNF to their corresponding receptor domains at the surface of the cells

leads to the activation of caspase 8. Subsequently, this initiates a cascade of caspase activation that ultimately results in apoptosis.[65] In this study, the evaluation of the anti-ZEN toxicity role of MTBITC has firmly supported the role of caspase 8 as an apoptotic activator in an in-vitro model. These studies demonstrated apoptosis of cells by ZEN following activation of caspase 8 and inhibition of apoptosis following MTBITC treatment as a caspase inhibitor. There is, therefore, a potential link with regard to MTBITC supplementation in cultured cells. The mechanism by which MTBITC may inhibit caspase 8 remains unclear. However, the underlying mechanisms may be better understood by considering the cellular metabolic enzyme pathways activated by MTBITC. While recognizing the limitations of an in-vitro model for investigating the effects of MTBITC on tumour cell behaviour and ZEN toxicity, the results of our study provide, for the first time, a further insight into how these effects might be exerted at the molecular level. Decreased expression of caspase 8 and inhibition of apoptosis may be a potential mechanism underlying the ability of MTBITC to induce inhibition of ZEN toxicity in cells. MTBITC did not adversely affect the C5-O cells.

Conclusions

The MTBITC extract from *R. sativus* has antioxidant, antitumour cell growth and anti-zearalenone cytotoxic effects and may have potential as a chemotherapeutic and cytostatic agent. MTBITC is phytopharmaceutical molecule of interest and has a range of prospective applications in human healthcare.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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