Toxicology in Vitro 23 (2009) 848–854

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Assessment of methyl thiophanate–Cu (II) induced DNA damage in human lymphocytes

Quaiser Saquib [a], Abdulaziz A. Al-Khedhairy [a], Saud Al-Arifi [a], Alok Dhawan [b], Javed Musarrat [a,*]

[a] Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia
[b] Developmental Toxicology Division, Indian Institute of Toxicology Research, Lucknow, India

ARTICLE INFO

Article history:
Received 19 January 2009
Accepted 29 April 2009
Available online 8 May 2009

Keywords:
Fungicide
Methyl thiophanate
DNA damage
Comet assay
Micronucleus
Reactive oxygen species

ABSTRACT

Dimethyl 4,4′-(*O*-phenylene)bis(3-thioallophanate), commonly known as methyl thiophanate (MT), is a category-III acute toxicant and suspected carcinogen to humans. Hence, the ability of this benzimidazole class of fungicide to engender DNA strand breaks was investigated using alkaline single cell gel electrophoresis (SCGE), alkaline unwinding and cytokinesis-blocked micronucleus (CBMN) assays. The SCGE of human lymphocytes treated with 1 mM MT for 3 h at 37 °C showed much higher Olive tail moment (OTM) value of 40.3 ± 2.6 ($p < 0.001$) vis-à-vis 3.3 ± 0.09 in DMSO control. Treatment of cultured lymphocytes for 24 h resulted in significantly increased number of binucleated micronucleated (BNMN) cells with a dose dependent reduction in the nuclear division index (NDI). Stoichiometric data revealed the intrinsic property of MT to bind with Cu (II) and its reduction to Cu (I), which is known to form reactive oxygen species (ROS). We have detected the intracellular ROS generation in MT treated lymphocytes and observed an elevated level of MT-induced strand breaks per unit of calf thymus DNA in presence of Cu (II). Overall the data suggested that the formation of MT–Cu (II)–DNA ternary complex and consequent ROS generation, owing to Cu (II)/Cu (I) redox cycling in DNA proximity, is responsible for MT-induced DNA damage.

1. Introduction

The use of pesticides including the herbicides and fungicides on crops and weeds has been augmented to a significant extent in the last few decades. Large-scale and indiscriminate application of these agrochemicals pose human health risks, specifically in developing countries, where the pesticide users are often ill-trained and devoid of appropriate protective devices. The associated health hazards are further extended to those exposed occupationally or inadvertently. Excessive use of pesticides resulted in prevalence of a variety of cancerous ailments viz. hematopoietic cancers, non-Hodgkin's lymphoma, leukemia and multiple myeloma (Wiklund and Holm, 1986; Morrison et al., 1992; Zham and Blair, 1992). Several immunological abnormalities as well as the nervous, endocrine, reproductive and developmental disorders have also been related to certain pesticides (Koner et al., 1998; Colosio et al., 2003; Gupta, 2004).

Methyl thiophanate (MT), a broad spectrum fungicides widely used for control of some important fungal diseases of crops (Hassall, 1990; Traina et al., 1998) has been chosen in this study for assessment of the nature and extent of MT-induced DNA damage (as promutagenic and pre-carcinogenic lesions) for carcinogenic risk assessment. MT is a benzimidazole class of compound, classified as "likely to be carcinogenic to humans" as per EPA carcinogen risk assessment guidelines (Proposed Guidelines for Carcinogen Risk Assessment, 1996). Being a category-III acute inhalation toxicant, it has been reported to exhibit a dose-related increase in the incidence of follicular and hepatocellular adenomas in male and female F344 rats. It has also been shown to cause skin papilloma at 75 ppm and pituitary adenoma at 200 ppm in male rats, and mammary gland fibroadenoma in female rats at 1200 ppm (Thiophanate Methyl Revised Report of the Hazard Identification Assessment, 2000). Upon oral administration, MT gets absorbed and metabolized into benzimidazole compounds, mainly carbendazim, which is a reproductive toxicant in male and female rats (Goldman et al., 1989; Cummings et al., 1990). At relatively higher doses, MT acts as a weak endocrine disruptor and adversely affects the endocrine tissue development and thyroid–pituitary homeostasis (Maranghi et al., 2003). It is regarded as a potential spindle poison, impairing the polymerization of microtubule formation in

Abbreviations: BNMN, binucleated micronucleated lymphocytes; BCS, bathocuproine disulfonic acid; CBMN, cytokinesis-blocked micronucleus; Cyto B, cytochalesin B; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DMSO, dimethyl sulfoxide; EMS, ethyl methane sulphonate; LMA, low melting temperature agarose; MT, methyl thiophanate; MMS, methyl methane sulphonate; NMA, normal melting temperature agarose; NDI, nuclear division index; OTM, Olive tail moment; PHA-M, phytohemagglutinin-M; ROS, reactive oxygen species; SCGE, single cell gel electrophoresis; TDNA, tail DNA.

* Corresponding author. Tel.: +966 4677249; fax: +966 4675514.
E-mail address: musarratj1@yahoo.com (J. Musarrat).

0887-2333/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2009.04.017

fungal DNA synthesis (Seiler, 1975; Maranghi et al., 2003). Therefore, it is speculated that the actual risk of genotoxicity from this fungicide might be appreciably higher than that predicted from conventional toxicity tests, as also suggested by Bolognesi (2003) for other pesticides. To the best of our understanding, no systematic study has been carried out which has emphatically demonstrated the MT-induced DNA damage, and role of Cu (II) ions in MT–Cu (II) mediated ROS production in vitro system. We have, therefore, conducted apriori model study to investigate the DNA damaging potential of this broad spectrum fungicide using well established sensitive techniques like single cell gel electrophoresis (SCGE or comet), alkaline unwinding, and cytokinesis-blocked micronucleus (CBMN) assay. The data unequivocally demonstrated the MT-induced DNA strand breaks and predicted the plausible role of intracellular ROS being generated in presence of Cu (II) transition metal ions, in triggering DNA damage.

2. Materials and method

2.1. Chemicals

Methyl thiophanate (dimethyl 4,4′-(*O*-phenylene)bis(3-thioallophanate) CAS No. 23564-05-8, 97% pure (Fig. 1) was obtained from Agrochemical Division, (IARI, New Delhi, India). Deoxyribonucleic acid (DNA), sodium salt, highly polymerized (Type I) from calf thymus, low and normal melting temperature agarose (LMA and NMA), Na_2-EDTA, Tris-buffer, ethidium bromide (EtBr), propiodium iodide, methyl methane sulphonate (MMS), ethyl methane sulphonate (EMS), histopaque 1077, cytochalesin B (Cyto B), phytohemagglutinin-M (PHA-M), 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and DMSO were obtained from Sigma Chemical Company (St. Louis, MO, USA). DMSO (1%) was used as solvent control in experiments where specified, unless otherwise stated. RPMI-1640, foetal bovine serum (FBS) were procured from GIBCO BRL Life Technologies Inc. (Gaithersburg, MD, USA). Phosphate buffered saline (PBS, Ca^{2+} Mg^{2+} free), Triton X-100 and bathocuproine disulfonic acid were obtained from Hi-Media Pvt. Ltd. (India). All other chemicals were of analytical grade. The slides for microgel electrophoresis were purchased from Blue Label Scientifics Pvt. Ltd., (Mumbai, India).

2.2. Alkaline single cell gel electrophoresis (comet assay)

Comet assay was performed with human lymphocytes following methods of Singh et al. (1988) as modified by Bajpayee et al. (2002). Lymphocytes were separated from heparinized whole blood of a healthy male volunteer, aged 26 years, with none of the following habits; smoking, consumption of alcohol, chewing of tobacco, not participating in high physical activities and was not on any type of medication during the period of blood sampling. Freshly isolated cells were treated with varying concentrations (0.25, 0.5, 0.75 and 1 mM) of MT for 3 h at 37 °C. Viability of lymphocytes was checked before and after treatment with MT using 0.4% trypan blue dye. The lymphocytes ($\sim 4 \times 10^4$ cells) both untreated and treated were suspended in 100 μl of Ca^{2+} Mg^{2+} free PBS and mixed with 100 μl of 1% LMA. The cell suspension (80 μl) was then layered on one-third frosted slides, pre-coated with NMA (1% in PBS) and kept at 4 °C for 10 min. After gelling, a layer of 90 μl of LMA (0.5% in PBS) was added. The cells were lysed in a lysing solution for overnight. After washing with Milli Q water, the slides were subjected to DNA denaturation in cold electrophoretic buffer at 4 °C for 20 min. Electrophoresis was performed at 0.7 V/cm for 30 min (300 mA, 24 V) at 4 °C. The slides were then washed three times with neutralization buffer. All preparative steps were conducted in dark to prevent secondary DNA damage. Each slide was stained with 75 μl of 20 μg/ml ethidium bromide solution for 5 min. The slides were analyzed at 40X magnification (excitation wavelength of 515–560 nm and emission wavelength of 590 nm) using fluorescence microscope (Leica, Germany) coupled with charge coupled device (CCD) camera. Images from 50 cells (25 from each replicate slide) were randomly selected and subjected to image analysis using software Komet 3.0 (Kinetic Imaging, Liverpool, UK). The data were subjected to one-way analysis of variance (ANOVA). Mean values of the tail length (μm), OTM and % tail DNA (% TDNA) were separately analyzed for statistical significance. The level of statistical significance chosen was $p \leqslant 0.05$, unless otherwise stated.

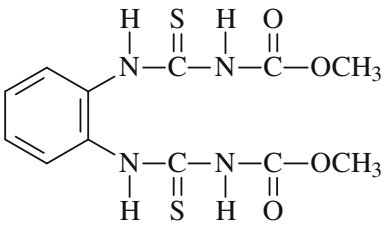

Fig. 1. Structure of methyl thiophanate.

2.3. Alkaline unwinding assay

MT-induced strand breaks in the DNA were quantitated by alkaline unwinding assay using hydroxyapatite batch procedure (Kanter and Schwartz, 1979). In brief, the calf thymus DNA (100 μg) in a volume of 0.5 ml in multiple sterile tubes were treated with MT at 1:2 to 1:10 DNA nucleotide/MT molar ratios in the absence and presence of 100 μM Cu (II). The untreated and EMS treated DNA were taken as negative and positive controls. The treatment was carried out for 30 min at 37 °C. The tubes were immediately placed on ice and subjected to alkaline unwinding by rapid addition of an equal volume of 0.06 N NaOH in 0.01 M Na_2HPO_4, pH 12.5 followed by brief vortexing. Alkaline unwinding was allowed to complete in dark for 30 min. The pH of the reaction mixture was then neutralized to pH 7.0 with the addition of 0.07 N HCl. Subsequently, 20 μM EDTA containing 2% SDS was added and the resultant mixture was transferred to pre-heated stoppered glass tubes containing 0.5 M potassium phosphate buffer, pH 7.0 and 10% formamide. The samples were incubated at 60 °C for 2 h with intermittent vortexing. The relative amount of duplex and single stranded DNA present at the end of alkaline unwinding was quantitated. Single stranded DNA was selectively eluted from the hydroxyapatite matrix with 0.125 M potassium phosphate buffer, pH 7.0 containing 20% formamide. However, duplex DNA was removed with 0.5 M potassium phosphate buffer, pH 7.0 containing 20% formamide. Strand breaks were estimated following the equation $\ln F = -(K/MN)\ t\beta$, where F is the fraction of double stranded DNA remaining after alkali treatment for the time t, MN is the number-average molecular weight between two strand breaks and β is a constant that is less than 1 (Rydberg, 1975). The number of unwinding points (P) per alkaline unwinding unit of DNA were calculated according to the equation, $P = \ln Fx/\ln Fo$ (Kanter and Schwartz, 1979), where, Fx and Fo are the fraction of double stranded DNA remaining after alkaline denaturation of treated and untreated samples, respectively. The number of breaks (n) per unit DNA were then determined using the equation $n = P - 1$.

2.4. Cytokinesis-blocked micronucleus (CBMN) assay

The CBMN assay was performed following the method of Kalantzi et al. (2004). The whole blood (0.5 ml) was cultured in 4.5 ml complete RPMI 1640 medium supplemented with 20% heat-inactivated FBS, L-glutamine (0.02 mM), sodium bicarbonate (2.0 g/L), penicillin