Journal of Environmental Science and Health, Part B
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597269

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Online publication date: 16 December 2009

To cite this Article Saquib, Quaiser , Al-Khedhairy, Abdulaziz A. , Singh, Braj R. , Arif, Jamal M. andMusarrat, Javed(2010) 'Genotoxic fungicide methyl thiophanate as an oxidative stressor inducing 8-oxo-7,8-dihydro-2' -deoxyguanosine adducts in DNA and mutagenesis', Journal of Environmental Science and Health, Part B, 45: 1, 40 — 45
To link to this Article: DOI: 10.1080/03601230903404325
URL: http://dx.doi.org/10.1080/03601230903404325

Journal of Environmental Science and Health Part B (2010) **45**, 40–45

ISSN: 0360-1234 (Print); 1532-4109 (Online)
DOI: 10.1080/03601230903404325

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Genotoxic fungicide methyl thiophanate as an oxidative stressor inducing 8-oxo-7,8-dihydro-2′-deoxyguanosine adducts in DNA and mutagenesis

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Dimethyl 4,4′-(O-phenylene)bis(3-thioallophanate), commonly known as methyl thiophanate (MT), is a systemic fungicide and suspected carcinogen to humans. In this study, the oxidative potential of this category-III acute toxicant has been ascertained based on its capacity of inducing reactive oxygen species (ROS) and promutagenic 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) adducts in DNA. The discernible MT dose-dependent reduction in fluorescence intensity of a cationic dye rhodamine (Rh-123) in human lymphocytes and increased fluorescence intensity of 2′,7′-Dichlorodihydro fluorescein diacetate (DCFH-DA) treated cells signifies decreased mitochondrial membrane potential ($\Delta\Psi m$) due to intracellular ROS generation. The ^{32}P-post-labeling assay demonstrated the MT-induced 8-oxodG adduct formation in calf thymus DNA. Thus, it is concluded that MT, as a potent oxidative stressor, produces ROS leading to mitochondrial dysfunction, oxidative DNA damage and mutagenesis.

Keywords: Fungicide; methyl thiophanate; 8-oxodG; ROS; mutagenicity; mitochondrial membrane potential.

Introduction

Living organisms are incessantly exposed to reactive oxygen species (ROS) through various environmental contaminants and endogenous metabolic processes. The extraneous sources attributed to ROS-mediated oxidative stress encompass ionizing radiations, xenobiotics and other chemical toxicants including pesticides. Pesticide-induced redox signaling alters cellular redox homeostasis by various mechanisms such as production of ROS, and depletion or impairment of antioxidants enzymes function that ultimately mediate multiple toxic effects.[1,2] Interestingly, the oxidative stress has been reported to play an important role in the toxicity of various pesticides, including organochlorines, and organophosphates,[3] carbamates and pyrethroids.[4] The higher oxidative stress in pesticide sprayers is evidenced by increased concentration of plasma and red blood cells thiobarbituric acid reactive substances (TBARS), changes in antioxidant status, and altered activities of cellular enzymes.[5,6] In general, the primary intracellular location for ROS production is mitochondria, where the electrons, possibly escaped from the electron transport chain, combine with oxygen to form the superoxide anion radical (O_2^-) and other ROS.[7]

Albeit, the presence of cellular antioxidant defense mechanism to counteract ROS, the radical-mediated damage to proteins, lipids, and DNA as well as its gradual accumulation pose a major threat to cellular and genomic integrity. Oxidative damage has a role in the etiology of complex multifactorial diseases such as cancer, arteriosclerosis, neurodegenerative disorders and acquired immunodeficiency syndrome (AIDS).[8,9] Attack of ROS on DNA leads to multitude of oxidative lesions including the most prevalent and stable promutagenic adduct 8-oxodG, implicated in mutagenesis and carcinogenesis.[10,11] Several studies have demonstrated elevated levels of 8-oxodG in human cancer tissues in comparison with the normal[12,13] and 8-oxodG has recently been linked with the Alzheimer's disease and incidence of cancer in humans.[14] Recently, Muniz et al.[2] have reported the pesticide-induced DNA damage in lymphocytes with elevated levels of 8-oxodG in pesticide applicators and farm workers. Our earlier studies have explicitly demonstrated the formation of DNA strand breaks upon treatment of human lymphocytes with a systemic fungicide

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Received June 24, 2009.

methyl thiophanate (MT).[15] Nevertheless, the limited information on MT–induced oxidative stress has prompted us to investigate the extent of the MT-induced (i) ROS production, (ii) 8-oxodG formation, and (iii) reduction in mitochondrial activity/potential in human lymphocytes, as a model. To the best of our knowledge, this is the first report on MT-induced 8-oxodG formation in DNA and the data revealed significant MT engendered ROS production, mutagenesis and oxidative stress in human lymphocytes.

Materials and methods

MT-induced extracellular ROS generation

Superoxide anions were estimated by nitrobluetetrazolium (NBT) reduction assay according to the method of Nakayama et al.[16] In brief, the reaction mixture containing 100 mM Tris-HCl buffer (pH 7.2), 50 μM NBT, 100 μM EDTA, 0.06% Triton X-100 was mixed with varying concentrations (100–600 μM) of MT. The tubes containing reaction mixture were exposed to white light ($20W/m^2$) for 2 h. Parallel reactions with MT in dark and paraquat (in light), a well–known photosensitizer and ROS producer[17] were run as controls. The absorbance of blue color developed was read at 560 nm using Cintra 10e UV-Visible spectrophotometer and plotted as a function of MT concentration.

MT-induced intracellular ROS generation in human lymphocytes

MT-induced ROS generation in human lymphocytes was demonstrated using fluorescent probe DCFH-DA. In brief, the human lymphocytes were treated with increasing concentrations of MT (50, 100 and 200 μM) for 3 h at 37°C in 5% CO_2 atmosphere. The cells were washed adequately and stained with 5 μM of DCFH-DA for 10 minutes at 37°C. Untreated as well as the treated cells with 0.7% Dimethyl sulphoxide (DMSO) (solvent control) and 100 μM H_2O_2 (positive control) were processed in parallel. Cells were visualized under fluorescence microscope (Nikon, Eclipse E600) at the excitation and emission wavelengths of 485 and 530 nm, respectively.

Effect of MT on mitochondrial activity in human lymphocytes

Mitochondrial activity was monitored by observing the changes in the fluorescence intensity of mitochondria specific dye rhodamine (Rh123) in human lymphocytes. Untreated control cells and those treated with MT in concentration range of 50, 100 and 200 μM for 3 h at 37°C were stained with 20 μM Rh123 for 30 minutes at 37°C and visualized through fluorescence microscope (Nikon, Eclipse E600) at the excitation wavelength of 520 nm and emission wavelength of 590 nm. The mitochondrial membrane potential ($\Delta\Psi m$) was determined by flow cytometric analysis. The mean fluorescence intensity (FL1-H 530 nm) was measured using flow cytometer (FACSCalibur, Becton Dickinson, USA). Furthermore, the ultrastructural changes in MT-treated human lymphocytes were studied with transmission electron microscopy (TEM). Ultrathin sections of glutaraldehyde fixed untreated and 200 μM MT-treated cells, embedded in low viscosity araldite resin were prepared, and visualized under high vacuum at 100 KV using JEOI-1011 Electron Microscope (JEOL, Japan).

Enrichment and ^{32}P-postlabeling of calf thymus DNA for 8-oxodg analysis

Calf thymus DNA (1 mg/mL) was treated with 25, 50 and 150 μM MT and photoexposed for 60 min at 37°C. Untreated and methylene blue (MB) (50 and 100 μM) treated DNA were run in parallel as negative and positive controls, under identical conditions. The DNA samples were ethanol precipitated and processed for ^{32}P-postlabeling analysis of 8-oxodG adducts in DNA following the method of Gupta and Arif.[18]

Transformation efficiency and mutagenesis of MT-treated plasmid pUC 19 in E.coli DH5α cells

Transformation of MT-treated plasmid pUC19 in *E.coli* DH5α cells and induced mutagenesis were performed following the methods of Sambrook et al.[19] In brief, plasmid pUC19 DNA (~25 ng) in Tris–HCl buffer (0.1 M, pH 7.5) was treated with increasing concentrations (0.25 -1.0 mM) MT at 37°C for 2 h. The treated plasmid DNA was incubated with 0.1 mL of competent DH5α cells. Cells were then plated on Luria Broth (LB) plates containing 60 μg/mL ampicillin, 40 μL X-gal and 40 μL Iso propyl ß-D-1-thiogalactopyoronoside (IPTG) . The plates were incubated overnight at 37°C followed by 4 h incubation at 4°C, to allow the development of blue color. Colonies were then scored to determine the number of mutants (white) and normal (blue) colonies.

Results and discussion

MT-induced ROS generation and effect on mitochondrial activity

Oxidative stress can be induced by pesticides, either by overproduction of free radicals or alteration in antioxidant defense mechanisms.[9] To determine the oxidative potential of MT, the *in vitro* production of both the extracellular and intracellular ROS have been assessed in MT-treated human lymphocytes. Figure 1 exhibits superoxide anion ($O^{\cdot}{}_2^-$) generation upon photoexcitation of MT in presence of NBT. Most likely, the lone pair of electrons available on the nitrogen and oxygen atoms of the bilaterally symmetrical arms of MT is transferred to NBT and reduces it to form a blue color formazan. The absolute amounts of $O^{\cdot}{}_2^-$anion were estimated to be 24, 42, 82, and 120 μM

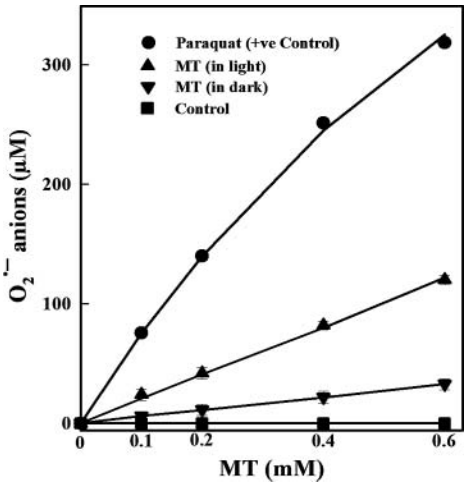


Fig. 1. Assessment of methyl thiophanate (MT)-induced production of $O^{\cdot}{}_2^-$ anions by nitrobluetetrazolium (NBT) reduction assay. The absolute amounts of $O^{\cdot}{}_2^-$ anions produced are plotted as a function of MT concentration upon photosensitization with white light 20 W/m^2 of photosynthetically active radiations (PAR) for 2 h.

at 100, 200, 400 and 600 μM MT, respectively upon photoexcitation with white light vis-à-vis 32.8 μM $O^{\cdot}{}_2^-$ anions generated in dark at the highest concentration. However, the amount of $O^{\cdot}{}_2^-$ anions produced were much lower than paraquat, a well-known photosensitive herbicide and free radical generator,[17] used as a positive control.

The intracellular ROS have been assessed based on detection of peroxide-dependent oxidation of DCFH-DA to fluorescent 2′,7′-dichlorofluorescin (DCF) in MT-treated cells. Qualitative analysis revealed a concentration–dependent increase in the fluorescence intensity of DCF in MT-treated lymphocytes (Fig. 2, panels d-f). The untreated and DMSO (0.7%) treated cells (panels, a and b) as controls have shown much lesser fluorescence intensity. At the highest concentration of 200 μM MT (panel f), the fluorescence intensity of DCF has been found to be almost similar to the positive control (H_2O_2, 100 μM) cells (panel c). These results confirmed the ROS generating capability of MT both outside the cell upon photooxidation and intracellularly possibly due to biochemical oxidation. Furthermore, the role of MT in the induction of oxidative stress in cells was validated with the observed alterations in mitochondrial activity in MT-treated lymphocytes, using a cationic fluorescent probe Rh123. The probe accumulates electrophoretically in the strongly negative charged matrix of mitochondria in untreated cells and undergoes a MT dose-dependent reduction in the mitochondrial Rh123 fluorescent intensity (Fig. 2, panels D-F). The decrease in fluorescence intensity may be in response to dissipation of the mitochondrial membrane potential ($\Delta\Psi$m). The flow cytometric analysis revealed the extent of reduction in $\Delta\Psi$m of MT-treated human lymphocytes as 19.5% and 55.0% at 50 and 100 μM, respectively. The noticeable shifts in the FL1-H peaks (Fig. 3) with reduced fluorescence intensities, suggested perturbation of the inner mitochondrial membrane, and consequent mitochondrial dysfunction as a result of ROS generation in MT-treated lymphocytes. The ultrastructural analysis through TEM (Fig. 4, A-D) demonstrated significant structural changes in the mitochondria and nuclei of treated cells after 24 h treatment with 200 μM MT. The loss of mitochondrial cristae in treated cells suggests sub-cellular alterations typical of ROS-induced mitochondrial damage and possible activation of intrinsic apoptotic pathway, as evident from the disappearance of nucleolus and nuclear condensation in Figure 4 C and D.

Assessment of oxidative stress marker 8-OxodG adducts in calf thymus DNA

DNA damage and oxidative stress have been proposed as mechanisms linking pesticide exposure to health effects such as cancer and neuorological disease.[2] Several

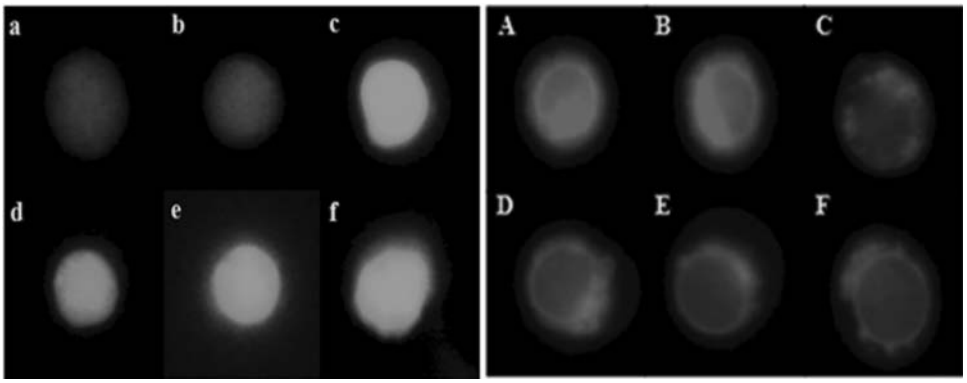


Fig. 2. Methyl thiophanate (MT)-induced reactive oxygen species (ROS) generation and effect on mitochondrial activity in human lymphocytes. Panels (d-f): Increasing concentration of MT (50,100 and 200 μM) enhances the intracellular ROS production in human lymphocytes. Panels (D-F): Concentration dependent reduction in mitochondrial membrane activity in human lymphocytes treated with 50,100 and 200 μM of MT. Panels (a-c and A-C) represents untreated control, dimethyl sulphoxide (DMSO) (0.7% as solvent control) and H_2O_2 (100 μM as positive control), respectively.

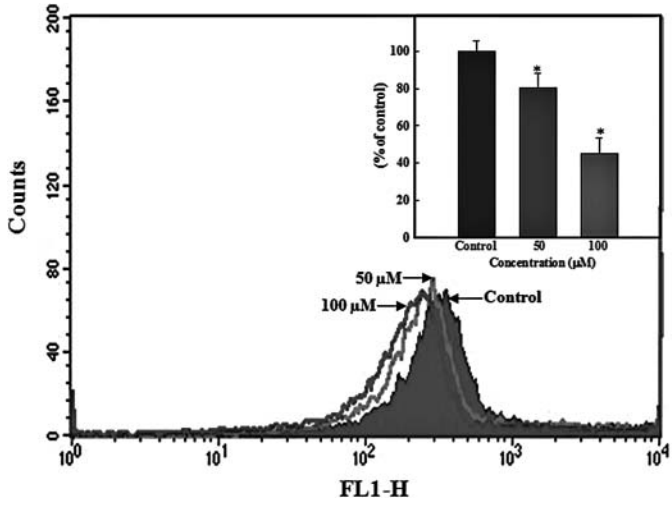


Fig. 3. Flow cytometric analysis of mitochondrial membrane potential ($\Delta\Psi m$) in methyl thiophanate (MT)-treated lymphocytes. Change in the $\Delta\Psi m$ was measured by Rh123, a fluorescent probe, with FACSCalibur at FL1 channel at excitation wavelength of 488 nm. MFI: mean fluorescence intensity. Inset shows the reduction in Rh123 fluorescence intensity of MT-treated human lymphocytes. The results are expressed as the mean ± SD of data obtained in three independent experiments (*p <0.05 relative to control).

fungicides viz. mancozeb, daconil and the metabolites of ortho-Phenylphenol (phenyl-1,4-benzoquinone and phenylhydroquinone) have been reported to produce a concentration dependent increase in the level of 8-oxodG.[20]

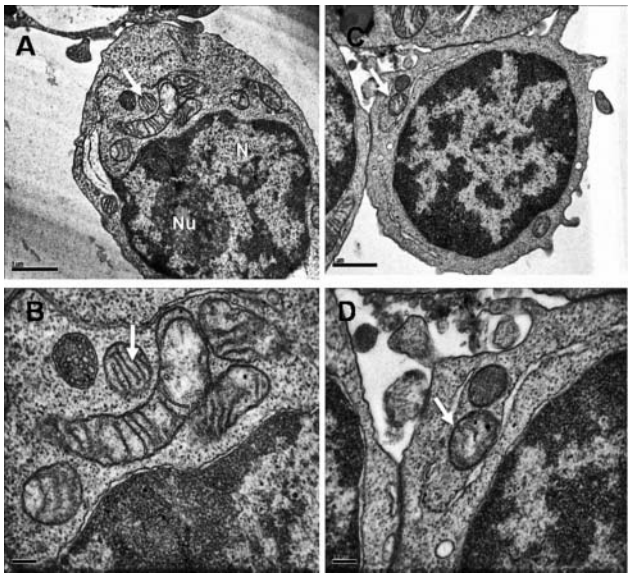
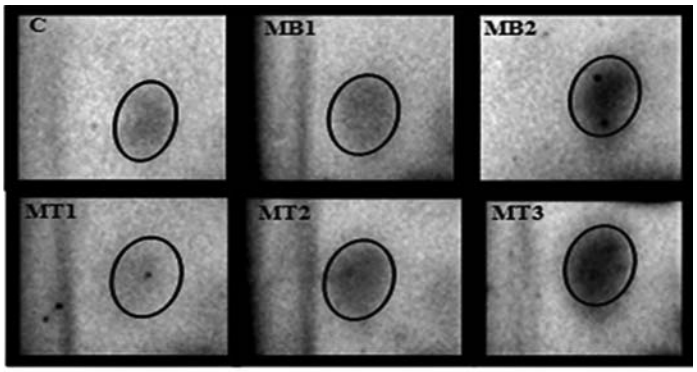


Fig. 4. Transmission electron micrographs (TEM) of untreated and methyl thiophanate (MT)-treated human lymphocytes. Panels A and B: untreated control cells showing typical ultrastructures of mitochondria (white arrow), nucleus (N) and nucleolus (Nu). Panels C and D depict ultra-structural changes in mitochondria and nucleus in cells treated with 200 μM MT for 24 h. Images in panels (A,C) and (B,D) were taken at X 15000 and X 40,000 magnifications, respectively.



Sample	Treatment	8-oxodG /10^8 N
C	Untreated ct-DNA	3.7 ± 0.90
MB1	ct-DNA+MB (50 μM)	4.2 ± 0.50*
MB2	ct-DNA+MB (100 μM)	6.4 ± 0.26*
MT1	ct-DNA+MT (25 μM)	1.6 ± 0.70^{ns}
MT2	ct-DNA+MT (50 μM)	5.3 ± 0.11*
MT3	ct-DNA+MT (150 μM)	6.2 ± 0.01*

MB: methylene blue, (positive control); MT: methyl thiophanate Data are the mean ± SE of two separate analysis. The level of significance was analyzedby performing Tukey test. *= significant at $p<0.05$, ns = non significant.

Fig. 5. Autoradiogram of ^{32}P post-labeled methyl thiophanate (MT)-treated calf thymus DNA depicting 8-oxodG adducts. The spots in the images are represented as C: untreated DNA. MB1and MB2: methylene blue (50 and 100 μM) treated DNA, taken as positive controls. MT1, MT2 and MT3: DNA exposed to 25, 50 and 100 μM MT. Table shows the quantitative analysis of 8-oxodG formation in calf thymus DNA by ^{32}P-postlabeling.

Our results of ^{32}P-postlabelling assay demonstrated significantly higher level of 8-oxodG adducts in MT-treated calf thymus DNA. Figure 5 shows the autoradiograph of typical adduct spots. Quantitative measurements of adduct radioactivity revealed adduct levels as 5.3 ± 0.11 and 6.2 ± 0.01 8-oxodG adduct/10^8 nucleotides ($p<0.05$) with 50 and 150 μM MT, respectively as compared to 3.7 ± 0.9 adducts/10^8 nucleotides in untreated DNA. The level of 8-oxodG was found to be comparable with the MB (positive control), which upon photoexcitation induced 4.2 ± 0.5 and 6.4 ± 0.26 adducts/10^8 nucleotides at 50 and 100 μM. Our 8-oxodG data corroborates with the earlier reports on the potential of other pesticides to form 8-oxodG lesions in DNA.[21] Several other studies have also reported the role of pesticides in the induction of oxidative stress and 8-oxodG DNA adduct formation in pesticide exposed populations.[2,22] However, to the best of our understanding there is no report on MT-induced oxidation stress, although our recent study[15] and some earlier studies[23,24] have demonstrated its genotoxic and clastogenic potential.

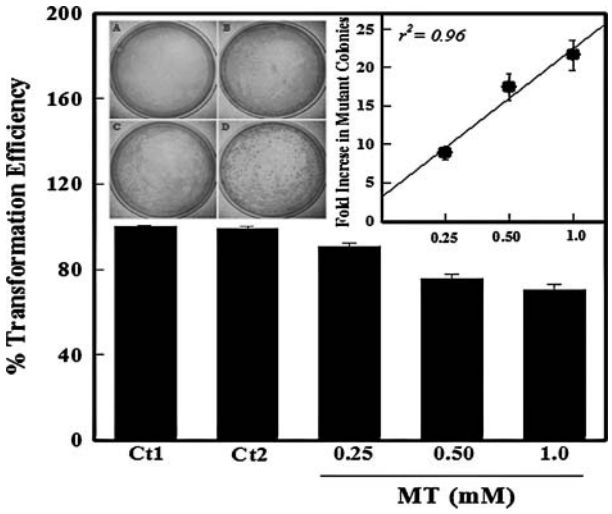


Fig. 6. Effect of methyl thiophanate (MT) on transformation efficiency of plasmid pUC19 in competent *E. coli* DH5α cells. Left inset (A-D) shows the increasing number of mutant (white) colonies with concomitant decrease in the blue colonies upon MT treatment as, A: untreated control, B-D: MT 0.25, 0.50 and 1.0 mM. Fold increase in mutant colonies as an effect of MT concentration is represented in the right inset. The data points are the mean $\pm$ S.D of three independent experiments done in triplicate. Ct1: untreated control, Ct2: DMSO (1%) as solvent control.

MT-induced mutagenesis in lacZ gene

The results shown in Fig. 6 (left inset) exhibited the MT dose-dependent increase in the number of mutants with concomitant reduction in the normal colonies in transformed DH5α cells. Also, the loss of transformation efficiency of the plasmid in DH5α cells occurred upon MT treatment (Fig. 6). Mutants were identified based on the color change from blue to white colonies, which happens due to mutations in the non-essential *lac*Z gene in pUC-19/DH5α system. In fact, pUC19 is a high copy number (2.686 Kb) plasmid carrying a region of *E.coli* Lac operon containing catabolite activator protein (CAP) binding site, promoter P*lac*, Lac repressor binding site and 5′-terminal part of *lac*Z gene encoding the N-terminal fragment of β-galactosidase. This is complemented by a mutant *lac* Z gene in the host DH5α cells, that code for the carboxy terminal portion of the β-galactosidase. The fragment in plasmid, whose synthesis can be induced by IPTG is capable of intra-allelic (α) complementation with a defective (mutation lacZDM15) form of β-galactosidase encoded by the host. However, mutation in the *lac*Z part of the plasmid almost invariably results in production of an amino terminal fragment that is not capable of α-complementation. The results clearly suggest MT-induced mutagenicity, and the approach followed could be used as a suitable alternative for mutagenecity assessment.

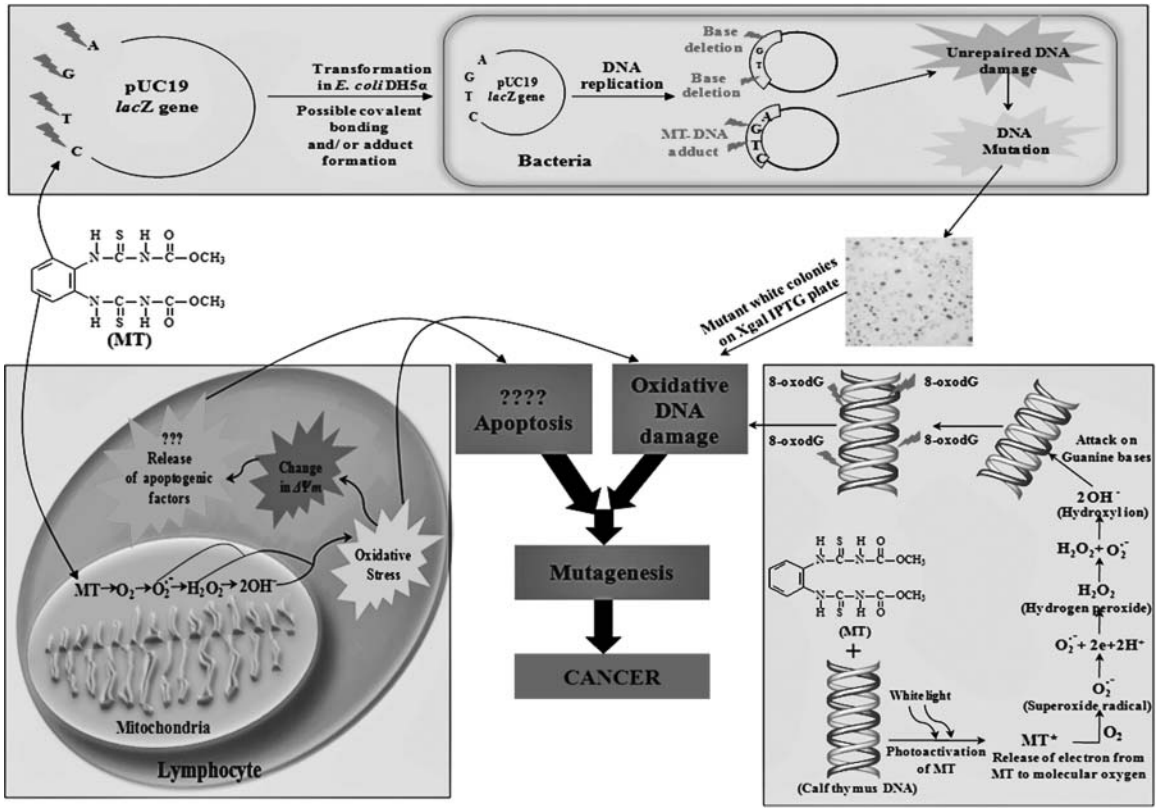


Fig. 7. Integrated modular representation of MT-mediated ROS generation, oxidative DNA damage in human lymphocytes and mutagenesis in *lac*Z gene in *E.coli* DH5α cells.

Conclusion

In conclusion, the MT-mediated ROS generation, oxidative DNA damage in human lymphocytes and mutagenesis in *lac*Z gene elucidated MT as a promutagenic fungicide and potent oxidative stressor. An integrated modular scheme (Figure 7) has been deduced providing a comprehensive view of MT-induced DNA damage and mutagenesis. Owing to its strong ability for inducing oxidative DNA damage and mutagenesis, the chronic exposure to this genotoxic fungicide is envisaged to trigger the initiating events of multi-step carcinogenesis.

Acknowledgments

Financial support through the DNA Research Chair, King Saud University, Riyadh, for completing this work is greatly acknowledged.

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