Diphenyl ditelluride (DPDT) is a potential prototype for the development of novel biologically active molecules. Thus, it is important to evaluate the toxic effects of this compound. In the present study, we evaluated the cytotoxic, genotoxic and mutagenic properties of DPDT in Chinese hamster fibroblast (V79) cells, in strains of the yeast Saccharomyces cerevisiae both proficient and deficient in several DNA repair pathways and in Salmonella typhimurium. DPDT induced frameshift mutations in both S. typhimurium and a haploid wild-type strain of S. cerevisiae. Mutants of S. cerevisiae defective in base excision repair and recombinational repair were more sensitive to DPDT. The results of a lactate dehydrogenase leakage assay suggest that DPDT is cytotoxic to V79 cells. At cytotoxic concentrations, this compound increased thiorbituric reactive species levels and decreased the glutathione:GSSH ratio in yeast and V79 cells. DPDT generated single- and double-strand DNA breaks in V79 cells, both with and without metabolic activation, as revealed by alkaline and neutral comet assays. Moreover, an induction of oxidative DNA base damage was indicated by a modified comet assay using formamidopyrimidine DNA glycosylase and endonuclease III. Treatment with DPDT also induced micronucleus formation in V79 cells. Pre-incubation with N-acetylcysteine reduced DPDT’s oxidative, genotoxic and mutagenic effects in yeast and V79 cells. Our results suggest that the toxic and mutagenic properties of DPDT may stem from its ability to disturb the redox balance of the cell, which leads to oxidative stress and the induction of DNA damage.

Introduction

Tellurium (Te), which is a chemical element with a name derived from the Latin ‘tellus’ (Earth), rarely occurs in a free state in nature. Moreover, this metalloid is known to be present in plants, particularly in members of the Allium family. A number of studies have shown that trace amounts of Te are present in body fluids such as blood and urine (1). Moreover, Te has been shown to be present as tellurocysteine and tellurumethionine in several proteins in bacteria, yeast and fungi. However, until now, no telluroproteins have been identified in animal cells (2).

Currently, inorganic Te is used in the vulcanization of rubber, in metal oxidizing solutions used to blacken or tarnish metals and in the nanoparticulate semiconductor industry. Moreover, the use of organic Te compounds will increase due to their importance as catalysts in inorganic and organic synthesis, as stabilizers for polymers, as components of insecticides and phase-change optical magnetic disks and as compounds used in the photography industry (1). Risks from occupational and environmental human exposure to this element may be implied due to this increased use. The main focus on the biological effects of this element has been its toxicity since Te is a non-essential and harmful metalloid. Inorganic and organic Te compounds are highly toxic to the central nervous system of rodents, causing demyelination, probably by disrupting cholesterol synthesis in Schwann cells through the inhibition of squalene monooxygenase (3). This inhibition sequentially affects the expression of myelin proteins themselves at the transcriptional level (4). In addition, it causes persistent neuromotor impairment and deficits in learning several tasks of investigation and memory (5).

Synthetic organotellurium (OT) compounds have found limited use in the past, but they have become a promising and advantageous alternative for numerous applications, as seen in the increase of reports on OT chemistry appearing in the literature. OT compounds have been synthesized since 1840; however, in the last few decades, evidence has been accumulating that OT molecules are promising pharmacological agents. Several reports have been published showing immunomodulatory, antioxidant, anticancer and anti-inflammatory properties of OT compounds (6–8). Diphenyl ditelluride (DPDT) (Figure 1) is a solid, non-volatile, hydrophobic, simple and stable OT compound used as an important and versatile intermediate in organic synthesis. It is extremely toxic to rodents, causing marked neurotoxic effects in mice after acute or prolonged exposure (5,9–11). DPDT can also be teratogenic, causing various morphologic abnormalities in mice foetuses during development (12). In addition, it produces renal and hepatic toxicity in rodents as well as haematological disorders during development (12). In contrast to these toxic effects, studies have demonstrated that DPDT has glutathione (GSH) peroxidase-like activity and is able to prevent oxidative stress induced by several oxidizing agents (15).

The aim of the present study was to investigate the cytotoxic effects of DPDT, particularly its effects on cellular redox status, genotoxicity and mutagenicity. To this end, we employed three different test systems: the Salmonella/microsome assay, repair proficient and deficient Saccharomyces cerevisiae strains and a permanent lung fibroblast cell line.
Fig. 1. Chemical structure of DPDT.

derived from Chinese hamster (V79 cells). In S. cerevisiae and V79 cells, we measured DPDT’s cytotoxicity as well as its effect on GSH levels and lipid peroxidation. DPDT-induced DNA damage was estimated by the comet assay and assessment of micronucleus formation in mammalian cells. The mutagenic potential of DPDT was also evaluated in bacteria and in the simple eukaryote S. cerevisiae, where haploid strains with different DNA repair defects were tested. This study is relevant to human health since it can be expected that exposure of humans to OT will increase in the near future through both occupational and environmental avenues. Moreover, it is important to evaluate DPDT’s safety for possible future pharmacological applications and to explore the biological properties of OT and its derivatives.

Materials and methods

Chemicals

DPDT (CAS registry number 32294-60-3) was provided by Dr Antônio Braga, Federal University of Santa Catarina, Florianópolis, Brazil. The chemical purity of DPDT (99.9%) was determined by gas chromatography/high-performance liquid chromatography (16). Yeast extract, bacto-pezanto and bacto-agar were obtained from Difco Laboratories (Detro, MI, USA). Dulbecco’s modified Eagle’s Medium (DMEM), foetal bovine serum (FBS), trypsin–ethylenediaminetetraacetic acid (EDTA), L-glutamine and antibiotics were purchased from Gibco BRL (Grand Island, NY, USA). Aflatoxin B1, 4-nitroquinoleine (4-NQO), sodium azide, L-histidine, L-threonine, L-methionine, L-tryptophan, L-leucine, L-lysine, nitroguanine bases (adenine and uracil), reduced glutathione (GSH), oxidized GSH prepared according to Maron and Ames (18). DPDT was dissolved in dimethyl formamide (99.9%) was determined by gas chromatography/high-performance liquid chromatography (16). Yeast extract, bacto-peptone and bacto-agar were obtained from Difco laboratories (Detroit, MI, USA). Formamidopyrimidine DNA glycosylase (FPG), also known as MutM and endonuclease III (Endo III, also known as Nth) were obtained from New England BioLabs. The overnight cultures contained 1–2 × 10⁸ cells/ml with 2–3% budding cells. Cells were harvested and washed twice with saline solution. The number of cells with or without buds was determined by counting in a Neubauer chamber.

Cytotoxicity in yeast strains proficient and deficient in DNA repair. Sensitivity to DPDT was assayed by incubation of stationary cultures (2 × 10⁶ cells/ml) in phosphate-buffered saline (PBS, 0.067 M, pH 7.0) with different concentrations (10, 50, 100, 500 and 1000 μM) of the compound in a rotary shaker at 30°C for 2 h. After treatment, cells were harvested by centrifugation at 12 000 × g for 1 min, washed twice with PBS, counted, diluted and plated on solid YPD. Plates were incubated at 30°C for 3–5 days before counting.

Detection of DPDT-induced reverse and frameshift mutation in S. cerevisiae. Mutagenesis was measured in the S. cerevisiae strain XV185-14c. The highest DPDT concentration used, providing ~60% survival, was determined in the previous cytotoxicity test. A suspension of 2 × 10⁶ cells/ml in stationary phase was incubated for 2 h at 30°C with different concentrations of DPDT in PBS. Cells were pretreated with NaN₃ for 18 h before DPDT treatment. Cell survival was assessed in SC medium at 30°C, and mutation induction in LYS, HIS or HOM locus was assessed in the appropriate omission media (7–10 days, 30°C). Whereas his3-7 is a non-suppressible missense allele, and reversions result from mutation at the locus itself, lys1-1 is a suppressible ochre non-sense mutant allele, which can be reverted either by locus-specific mutation or forward mutation in a suppressor gene. True reversions and forward (suppressor) mutations at the lys1-1 locus were differentiated according to Von Borstel et al. (20). It is believed that hom3-10 contains a frameshift mutation due to its response to a range of diagnostic mutagens (20). Assays were repeated at least three times, and plating was done in triplicate for each dose.

Preparation of yeast cell-free extracts. Crude yeast extracts were prepared by glass bead lysis as follows. Cells were suspended in lysis buffer (50 mM Tris, 150 mM NaCl, 50 mM EDTA, pH 7.2) with an equal volume of acid-washed glass beads and PMSF; vortexed for 10–15 cycles (30 s each) and cooled for 30 s. The mixture was then centrifuged for 5 min at 6000 × g to remove cellular debris and the glass beads (19). The supernatant was kept on ice for immediate use. Protein concentration was determined by the Bradford assay (21).

Measurement of lipid peroxidation in yeast. The extent of DPDT-induced lipid peroxidation was determined by the reaction of TBA with malondialdehyde (MDA), a product formed by lipid peroxidation. The assays were performed according to Salgo and Pryor (22), with minor modifications. To the lysate, 2 ml of 0.4 mg/ml TCA in 0.25 M HCl was added. The mixture was then incubated with 6.7 mg/ml TBA for 15 min at 100°C then centrifuged at 750 × g for 10 min. As TBA reacts with other products of lipid peroxidation in addition to MDA,
Mutagenicity of DPDT

<table>
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<td>—</td>
<td>Von Borst®</td>
</tr>
</tbody>
</table>

Table I. Yeast strains used in this study

Notes:

1 Obtained from EUROSCARF, Frankfurt, Germany.
2 Kindly provided by Dr. Rebecca L. Swanson, Departments of Biochemistry and Biology, Emory University School of Medicine, Atlanta, GA.
3 Described in Von Borstel et al. (20).

The results are expressed in terms of thio-barbituric reactive species (TBARS), which were determined by absorbance at 532 nm. Hydrolysed TMP was used as the standard. The results were normalized to protein content (21).

Determination of total GSH, GSH and GSSG in yeast. Total GSH (GSH plus GSSG) levels were determined by photometric determination of 5-thio-2-nitrobenzoate, which was produced from DTNB in a kinetic assay, according to Akerboom and Sies (23) with minor modifications. An equal volume of 2 M HClO₄ with 4 mM EDTA was added to the cell extract and the precipitated proteins were sedimented by centrifugation at 8000 × g for 10 min at 4°C. The supernatant was neutralized with 2 M KOH and 0.3 M 3-(N-morpholino)propanesulfonic acid, and the insoluble residue was removed by centrifugation under the same conditions. For spectrophotometric determination, 910 l of the cell extract was mixed with 50 μl of 4 mg/ml NADPH in 0.5% (w/v) NaHCO₃, 20 μl of 6 U/ml GSH reductase in phosphate–EDTA buffer and 20 μl of 1.5 mg/ml DTNB in 0.5% NaHCO₃. The increase in absorbance was measured at 412 nm. Total GSH content was calculated as nanomole per 10⁶ viable cells. For GSSG determination, 4-vinylpyridine is able to react with all GSH without interfering with GSSG determination. GSH was determined for 1 h at room temperature. At this concentration, 4-vinylpyridine is able to react with all GSH without interfering with GSSG determination. GSH was determined based on the total GSH and GSSG concentration results. The results were expressed as GSH/GSSG ratios.

Assays in V79 cells

V79 cell culture and treatments. V79 cells were cultured under standard conditions in DMEM supplemented with 10% heat-inactivated FBS, 0.2 mg/ml l-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were kept in tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO₂ in air and were harvested by treatment with 0.15% trypsin and 0.08% EDTA in PBS. Cells were seeded (3 × 10⁶ cells) in 5 ml of complete medium in a 25-cm² flask and grown for 2 days up to 60–70% confluence before treatment with the test substance. DPDT was dissolved in DMSO and added to FBS-free medium to reach the different desired concentrations. The final DMSO concentration in the medium never exceeded 0.2%, and the control group was exposed to an equivalent concentration of solvent. In some experiments, cells were pre-treated with 5 mM Na-acetate for 24 h in complete medium.

Cytotoxicity evaluation in V79 cells using a lactate dehydrogenase leakage assay. After treatment, the fraction of total lactate dehydrogenase (LDH) activity in the supernatant was taken as an indicator of membrane leakage or cell death (24). CytoTox assay kit (Promega, Madison, WI, USA) was used for enzymatic assessment of LDH release, following the manufacturer’s instructions. This method involves assessing the rate of conversion of reduced nicotinamide adenine dinucleotide (1.5 mmol/l) to oxidized nicotinamide adenine dinucleotide in the presence of l- (+)-lactic acid (50 mmol/l) in culture supernatants, and in the remaining cells (C) after lysis with serum-free medium containing 1% Triton X-100. The percentage of LDH leakage was calculated as follows: % leakage = S/(S + C) × 100.

Preparation of V79 cell-free extracts. Briefly, 3 × 10⁶ cells were incubated with various concentrations of DPDT for 2 h in FBS-free medium and after they were lysed with Tris–HCl (15 mM for 1 h).

Measurement of lipid peroxidation in V79 cells. Lipid peroxidation in V79 cells was measured as described in Measurement of lipid peroxidation in yeast.

Determination of total GSH, GSH and GSSG in V79 cells. The total GSH, GSH and GSSG levels in V79 cells were measured as described in Determination of total GSH, GSH and GSSG in yeast.

Comet assay. The alkaline comet assay was performed as described by Singh et al. (25), with minor modifications. V79 cells were incubated with various concentrations of DPDT for 2 h in FBS-free medium. After treatment, cells were washed with ice-cold FBS, trypsinized and resuspended in complete medium. Then, 20 μl of cell suspension (3 × 10⁶ cells/ml) was dissolved in 0.75% low-melting point agarose and immediately spread on to a glass microscope slide pre-coated with a layer of 1% normal melting point agarose. The agarose was allowed to set at 4°C for 5 min. The slides were then incubated in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH 10.0) at 4°C for at least 1 h in order to remove cellular proteins and membranes, leaving the DNA as “nucleoids”. The neutral assay was performed at pH 8.5, essentially according to the same procedure as the alkaline version, except at lower pH. In the neutral version, electrophoresis was carried out in a buffer consisting of 100 mM Tris and 0.1% Triton X-100. The percentage of LDH leakage was calculated as follows: % leakage = S/(S + C) × 100.

Measurement of lipid peroxidation in V79 cells. Lipid peroxidation in V79 cells was measured as described in Measurement of lipid peroxidation in yeast.

Determination of total GSH, GSH and GSSG in V79 cells. The total GSH, GSH and GSSG levels in V79 cells were measured as described in Determination of total GSH, GSH and GSSG in yeast.
concentration of 2 various DPDT concentrations for 2 h in FBS-free medium. After treatment, cells were separated from the flask by trypsinization, and the cell suspension

activation, S9 mix was prepared according to Ku

mutagenic index (MI) when: (i) the number of revertants was at least double the spontaneous yield

Salmonel software. A compound was considered positive for mutagenicity only in the sample over the number of spontaneous mutants in the negative control], (ii) MI/C20

The micronucleus assay was performed according to

was carefully added. This fixation step was repeated twice, and finally, cells were centrifuged, and methanol:acetic acid (3:1) solution was added. Cells were

7.5), washed in di-distilled water and stained using a silver staining protocol as described previously (26). After the staining step, gels were left to dry at room temperature overnight and analysed. One hundred cells (50 cells from each of two replicate slides) per concentration of each test substance were selected and analysed visually using an optical microscope for tail length and amount of DNA present in the tail. When selecting cells, cells around air bubbles or at the edge of the gel were discarded according to tail length into five classes: (1) class 0: undamaged, without a tail; (2) class 1: with a tail shorter than the diameter of the head (nucleus); (3) class 2: with a tail length one to two times the diameter of the head; (4) class 3: with a tail longer than twice the diameter of the head and (5) class 4: comets with no heads.

A value [damage index (DI)] was assigned to each comet according to its class. The genotoxic effect of DPDT in V79 cells was estimated by two different parameters, DI and damage frequency (DF). The DI ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4) (26).

DF (%) was calculated based on the number of cells with tails as compared with those with no tail. International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method (26). The vehicle was used as a negative control, MMS treatment at 4 × 10^{-5} M for 1 h was used as a positive control in the absence of metabolization and 1.5 × 10^{-5} M CP was used as a positive control in the presence of metabolization. In the modified comet assay, the vehicle was used as a negative control, and treatment with 150 μM H_{2}O_{2} for 5 min at 4°C was used as a positive control. In the experiment involving metabolization activation, S9 mix was prepared according to Ku et al. (27). Cells were incubated with S9 mix for 30 min and then treated with several concentrations of DPDT at 37°C for 20 min.

Micronucleus test. The micronucleus assay was performed according to Bonacker et al. (28), with minor modifications. V79 cells were incubated with various DPDT concentrations for 2 h in FBS-free medium. After treatment, cultures were washed twice with medium, and Cyt-B was added at final concentration of 2 μg/ml. Cultures were harvested 21 h after Cyt-B addition. Cells were separated from the flask by trypsinization, and the cell suspension was centrifuged at 1000 × g for 5 min. Cells were then resuspended in 0.075 M KCl solution and maintained at 4°C for 3 min (mild hypotonic treatment). Subsequently, cells were centrifuged, and methanol:acetic acid (3:1) solution was carefully added. This fixation step was repeated twice, and finally, cells were resuspended in a small volume of methanol:acetic acid and dropped onto clean slides. Slides were stained with 10% Giemsa (pH 6.8) for 3–4 min. Slides were mounted and coded prior to analysis. MMS, at 50 μg/ml concentration, was used as a positive control, and the vehicle was used as a negative control. Micronuclei were counted in 2000 binucleated cells (BNCs) with well-preserved cytoplasm. The identification of micronuclei was carried out according to Fenech (29).

Statistical analysis

Mutagenicity data in the Salmonella/microsome assay were analysed with Salmonom software. A compound was considered positive for mutagenicity only when: (i) the number of revertants was at least double the spontaneous yield [mutagenic index (MI) ≥ 2]. MI is defined as the number of induced colonies in the sample over the number of spontaneous mutants in the negative control; (ii) a significant response for analysis of variance (P ≤ 0.05) was found and (iii) a reproducible positive dose response (P ≤ 0.01) was present as evaluated by the Salmonom software (30). A cytotoxic effect was considered when MI ≤ 0.6.

All experiments with yeast and V79 cells were independently repeated at least three times, with triplicate samples for each treatment. Results are expressed as means ± standard deviations and were analysed by one-way analysis of variance followed by Tukey’s test. P < 0.05 was considered statistically significant.

Results

Salmonella/microsome assay

The DPDT dose range was determined in the TA100 strain, with and without metabolization, and cytotoxicity was observed at concentrations >20 μg/ml (data not shown). Metabolization was used in order to verify possible generation of reactive intermediates during DPDT detoxification. The effect of DPDT on the frameshift mutation-detecting strains TA97a (detects frameshift mutations in –C–C–C–C–C–C–; +1 cytosine) and TA98 (detects frameshifts in the DNA target –C–G–C–G–C–G–C–G) indicates a clear mutagenic induction response in the absence or presence of metabolic activation, as shown in Table II. No mutagenicity of DPDT was seen in either strain detecting base pair substitutions in the absence of metabolic activation: TA1535 [detects base pair substitutions of a leucine (GAG) by proline (GGA)] or the corresponding isogenic strain TA100 (harbouring the plasmid pKM101, which enhances induced mutagenesis via an increase in the error-prone recombinational DNA repair pathway). However, in TA1535, a weak mutagenic response was detected at the highest dose of DPDT in the presence of metabolic activation. Negative results for mutagenicity were also observed in TA102 (detects transversions or transitions in a TAA DNA sequence), which is sensitive to oxidative and alkylating mutagens (Table II).

DPDT cytotoxicity and mutagenicity in strains of the yeast S.cerevisiae

DPDT induced moderate dose-dependent cytotoxicity in stationary phase cultures of the wild-type (WT) S.cerevisiae XV185-14c haploid strain (Table III). With respect to mutagenicity, Table III indicates that the mutation frequencies at the his1 and lys1 loci were significant at higher concentrations of DPDT, whereas the mutation frequency at the hom3 locus was significant at almost all doses. Moreover, this DPDT-induced mutagenesis was abolished when cells were pre-treated with N-ac (Table III).

In order to evaluate the interaction of DNA repair pathways in response to DPDT-induced DNA damage, we also performed survival assays to compare the sensitivities of single, double, triple and quadruple mutants defective in base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR), non-homologous end joining (NHEJ) and translesion synthesis (TLS). The single mutants rad1, rad6 and rev3 alone (Figure 2B) exhibited the same sensitivity as that observed for WT cells. In addition, the mutant strains rad50, xrs2 and mre11A (subunits of the MRX complex involved in both HR and NHEJ) showed elevated DPDT sensitivity in relation to the isogenic BY4741 WT strain (Figure 2C). The recombination-deficient rad52A mutant showed the highest sensitivity to DPDT (Figure 2C). Finally, the single BER mutants ntg1, ntg2 and ngs1 had significantly higher sensitivity to DPDT than WT (Figure 2A). It is important to note that the double mutant ntg1Δntg2Δ was more sensitive than the single mutants. Figure 2D shows that the triple mutant ntg1Δntg2Δapn1Δ was more sensitive than the single and double mutants. The quadruple mutants ntg1Δntg2Δapn1Δrad52Δ is more sensitive than the triple mutant ntg1Δntg2Δapn1Δ. ngs1Δrad52Δ shows the same sensitivity to DPDT as the triple mutant. However, the mutant ntg1Δntg2Δapn1Δrad52Δ shows the same sensitivity as the single mutant rad52Δ.

Oxidative stress biomarkers in yeast

In order to determine the oxidative damage triggered by DPDT, we examined two well-established oxidative stress biomarkers: the concentration-dependent degree of lipid peroxidation, measured by TBARS levels, and the GSH:GSSG ratio. As can be seen in Figure 3A, the treatment of cells with DPDT resulted in a dose-dependent increase in TBARS production in WT yeast. This increase was statistically significant after incubation with DPDT doses >10.0 μM for 2 h. Pre-incubation with N-ac reduced DPDT-generated oxidative damage at all doses, reducing the levels of TBARS in yeast. With respect to intracellular GSH content, treatment with 50, 100, 500 or 1000 μM DPDT for 2 h

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significantly reduced the GSH:GSSG ratio in *S. cerevisiae* (Figure 3B).

**Cytotoxic effects in V79 cells**

Dose- and time-dependent changes in the viability of DPDT-treated cells were assessed by monitoring LDH leakage, which is an indicator of plasma membrane damage. When compared to the negative control, significant cytotoxic effects of DPDT were detectable starting at concentrations of 0.1 μM for 6-h treatments and 1 μM for 2-h treatments (Table IV). Moreover, at all tested DPDT concentrations, the viability of the cells decreased further with an increase in the concentration or the incubation time. This finding suggests that DPDT is cytotoxic at a concentration range of 0.5–50 μM. Since our interest is focused on the genetic toxicity of cells exposed to DPDT, we chose to perform all subsequent experiments using a treatment...
of 2 h in light of the low cytotoxicity observed at this time point (<30%).

Oxidative stress biomarkers in V79 cells

As can be seen in Figure 4A, treatment of cells with DPDT resulted in a dose-dependent increase in TBARS production in V79 cells at concentrations starting at 0.5 \text{ \mu M}. Pre-incubation with N-ac reduced DPDT-generated oxidative damage at all doses, thereby reducing the levels of TBARS. In addition, 2-h treatments within a concentration range of 0.5–50 \text{ \mu M} significantly reduced the GSH:GSSG ratio in V79 cells (Figure 4B).

DNA damage in V79 cells

Table V shows the effects of DPDT on DI and DF in V79 cells according to the comet assay with and without metabolic activation. DPDT clearly induced a dose-dependent increase in both DI and DF both in the alkaline and neutral comet assays at concentrations ranging from 1.0 to 50.0 \text{ \mu M}. The comet assay under alkaline conditions simultaneously detects single- and double-strand DNA breaks and alkali-labile sites, while the neutral conditions allow only the detection of DNA double-strand breaks (DSBs) (25). We can therefore infer that DPDT induces DNA single-strand breaks (SSBs) and DSBs in the presence or absence of metabolic activation (Table V). The extent of DNA damage in cells exposed to these compounds was concentration dependent.

In order to determine the nature of the DPDT-induced DNA damage in V79 cells, we carried out a modified comet assay. While the alkaline version of the comet assay detects DNA SSBs and DSBs, crosslinks and alkali-labile sites, the modified comet assay is more specific than the standard method. In this version, there is an incubation step combining lysed cells with lesion-specific enzymes that recognize certain damaged bases, and the resultant abasic sites are converted to SSBs. The enzyme FPG is specific for oxidized purines, including 8-oxo-7,8-dihydroguanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 4,6-diamino-5-formamidopyrimidine, as well as other ring-opened purines. Endo III recognizes oxidized pyrimidines, including thymine glycol and uracil glycol (31). In this manner, the DI increases in a dose-related fashion after incubation with the lesion-specific enzymes, and this increment represents the induction of oxidative DNA damage. The amounts of Endo III- and FPG-sensitive sites were calculated from the scores obtained with enzyme minus the scores obtained without enzyme (i.e. incubation with buffer alone), and these were considered the oxidative damage scores. It can be seen in Figure 5B and C that H$_2$O$_2$ treatment increases the extent of oxidative DNA damage recognized by Endo III and...
FPG in V79 cells. These results indicate the presence of oxidized pyrimidines and oxidized purines, respectively, validating the experiment. The oxidative damage scores in DPDT-treated V79 cells without (Figure 5B and C) and with (Figure 6A and B) metabolic activation indicated the occurrence of oxidative DNA damage. CP is an alkylating agent widely used as a positive control in tests using metabolic activation.

Our results demonstrate that \( N \)-ac pre-treatment was able to decrease DPDT-induced DNA damage in V79 cells (Figure 5A). Moreover, the induction of oxidative damage recognized by FPG (Figure 5B) and Endo III (Figure 5C) decreased significantly, indicating that pre-treatment with \( N \)-ac reduces DPDT-induced oxidative DNA damage. Treatment with DPDT decreased the percentage of binucleated V79 cells in a dose-dependent manner, reinforcing the cytotoxic effect of this molecule on cell proliferation (Table VI). Furthermore, exposure at concentrations ranging from 0.5 to 50 \( \mu \)M significantly increased the frequency of micronucleus formation relative to the untreated control (Figure 7).

**Discussion**

In this study, we evaluated the toxic and mutagenic properties of DPDT in bacteria, yeast and cultured mammalian cells. As expected, the cytotoxic threshold of DPDT was different in...
Table IV. Effects of DPDT on LDH leakage (% of total) in V79 cells

<table>
<thead>
<tr>
<th></th>
<th>Control*</th>
<th>0.1 µM DPDT</th>
<th>0.5 µM DPDT</th>
<th>1.0 µM DPDT</th>
<th>5.0 µM DPDT</th>
<th>10.0 µM DPDT</th>
<th>50.0 µM DPDT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>11.8 ± 0.6</td>
<td>12.3 ± 2.2</td>
<td>12.1 ± 1.0</td>
<td>11.6 ± 2.3</td>
<td>12.4 ± 1.7</td>
<td>14.2 ± 6.2</td>
<td>18.6 ± 2.3</td>
</tr>
<tr>
<td>2 h</td>
<td>15.1 ± 1.3</td>
<td>16.2 ± 1.9</td>
<td>17.3 ± 2.1</td>
<td>19.4 ± 0.9*</td>
<td>20.5 ± 1.5*</td>
<td>22.3 ± 1.4*</td>
<td>28.1 ± 2.0**</td>
</tr>
<tr>
<td>4 h</td>
<td>14.5 ± 0.5</td>
<td>18.0 ± 2.0</td>
<td>20.2 ± 1.7*</td>
<td>22.7 ± 0.6*</td>
<td>26.4 ± 1.0**</td>
<td>30.5 ± 1.9**</td>
<td>41.3 ± 1.7***</td>
</tr>
<tr>
<td>6 h</td>
<td>14.9 ± 0.7</td>
<td>19.8 ± 0.3*</td>
<td>21.9 ± 1.3*</td>
<td>25.9 ± 1.7**</td>
<td>29.9 ± 3.0**</td>
<td>36.8 ± 2.1**</td>
<td>52.1 ± 3.2***</td>
</tr>
<tr>
<td>8 h</td>
<td>15.4 ± 1.6</td>
<td>22.0 ± 2.4*</td>
<td>27.0 ± 3.2**</td>
<td>35.5 ± 1.8***</td>
<td>42.0 ± 2.6***</td>
<td>50.4 ± 1.5***</td>
<td>64.3 ± 5.1***</td>
</tr>
<tr>
<td>12 h</td>
<td>17.6 ± 2.6</td>
<td>30.2 ± 4.4**</td>
<td>37.7 ± 4.3***</td>
<td>48.2 ± 2.5***</td>
<td>56.5 ± 5.8***</td>
<td>69.5 ± 2.7***</td>
<td>83.3 ± 2.3***</td>
</tr>
<tr>
<td>24 h</td>
<td>21.4 ± 2.4</td>
<td>44.6 ± 6.7***</td>
<td>58.6 ± 4.9***</td>
<td>68.8 ± 4.2***</td>
<td>84.7 ± 5.6***</td>
<td>87.6 ± 6.2***</td>
<td>96.7 ± 3.2***</td>
</tr>
</tbody>
</table>

Data significant in relation to the control group *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA Tukey’s Multiple Comparison Test.

*Negative control (solvent). Data are expressed as mean ± SD, n = 12.

Fig. 4. Determination of TBARS (A) and GSH:GSSG ratios (B) in V79 cells treated with DPDT at the indicated concentrations for 2 h. The concentration of 5 mM N-ac was used for the pre-treatment. Solvent was used as a negative control. Data are expressed as means ± SDs, n = 4. Significantly different in relation to the negative control or in relation to the treatment at the same DPDT concentration without N-ac pre-treatment, *P < 0.05, **P < 0.01 and ***P < 0.001 by one-way ANOVA with Tukey’s Multiple Comparison Test.
Table V. Genotoxicity induced by 2 h DPDT treatment in V79 cells evaluated by neutral and alkaline comet assay without and with metabolic activation

<table>
<thead>
<tr>
<th>Agent</th>
<th>Treatment (µM)</th>
<th>DF* Without S9 mix</th>
<th>With S9 mix</th>
<th>DF (%) Without S9 mix</th>
<th>With S9 mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline condition (pH 13.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCb</td>
<td>35.3 ± 21.9</td>
<td>35.7 ± 20.3</td>
<td>30.0 ± 21.7</td>
<td>34.5 ± 23.4</td>
<td></td>
</tr>
<tr>
<td>PCc</td>
<td>117.5 ± 6.8**</td>
<td>217.0 ± 8.3**</td>
<td>75.0 ± 1.2**</td>
<td>88.3 ± 2.3**</td>
<td></td>
</tr>
<tr>
<td>DPDT</td>
<td>0.1</td>
<td>34.5 ± 10.9</td>
<td>48.3 ± 14.7</td>
<td>28.8 ± 8.4</td>
<td>38.5 ± 5.6</td>
</tr>
<tr>
<td>0.5</td>
<td>46.3 ± 12.1</td>
<td>51.7 ± 19.8</td>
<td>39.2 ± 10.8</td>
<td>44.9 ± 17.0</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>99.3 ± 7.3**</td>
<td>129.0 ± 14.1**</td>
<td>55.3 ± 3.3**</td>
<td>68.4 ± 2.5**</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>127.3 ± 9.5**</td>
<td>169.5 ± 14.6**</td>
<td>66.8 ± 2.7**</td>
<td>78.4 ± 1.9**</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>170.5 ± 10.5***</td>
<td>217.5 ± 8.6***</td>
<td>75.7 ± 3.5**</td>
<td>86.3 ± 2.7**</td>
<td></td>
</tr>
<tr>
<td>50.0</td>
<td>248.3 ± 8.0**</td>
<td>292.5 ± 14.0***</td>
<td>93.2 ± 2.9**</td>
<td>96.2 ± 1.6**</td>
<td></td>
</tr>
<tr>
<td>Neutral conditions (pH 8.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCb</td>
<td>19.8 ± 4.8</td>
<td>24.3 ± 5.9</td>
<td>18.5 ± 5.1</td>
<td>27.8 ± 14.5</td>
<td></td>
</tr>
<tr>
<td>PCc</td>
<td>102.3 ± 5.9**</td>
<td>224.5 ± 22.2**</td>
<td>50.8 ± 3.9**</td>
<td>82.1 ± 2.8**</td>
<td></td>
</tr>
<tr>
<td>DPDT</td>
<td>0.1</td>
<td>24.5 ± 7.2</td>
<td>31.8 ± 6.3</td>
<td>15.0 ± 8.3</td>
<td>27.9 ± 3.6</td>
</tr>
<tr>
<td>0.5</td>
<td>27.5 ± 6.8</td>
<td>36.3 ± 6.9</td>
<td>20.5 ± 1.7</td>
<td>34.2 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>52.3 ± 11.6**</td>
<td>79.3 ± 9.3**</td>
<td>46.8 ± 5.9**</td>
<td>52.1 ± 3.9**</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>66.5 ± 16.4**</td>
<td>87.3 ± 11.6**</td>
<td>50.7 ± 3.3**</td>
<td>59.2 ± 3.5**</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>79.0 ± 14.5**</td>
<td>105.3 ± 7.3**</td>
<td>56.0 ± 2.4**</td>
<td>63.6 ± 1.3**</td>
<td></td>
</tr>
<tr>
<td>50.0</td>
<td>108.8 ± 15.5**</td>
<td>149.5 ± 13.4***</td>
<td>64.3 ± 2.5**</td>
<td>73.9 ± 2.4**</td>
<td></td>
</tr>
</tbody>
</table>

Data significant in relation to DPDT treatments as compared to the negative control *P < 0.05, **P < 0.01 by one-way ANOVA Tukey’s Multiple Comparison Test. 

*Mean values and SD obtained from four experiments in triplicate per dose for each substance.

†Negative control (solvent).

‡Positive control: MMS treatment at 4 × 10⁻⁵ M in absence of metabolization and 1.5 × 10⁻⁵ M CP in presence of metabolization.

measured the extent of lipid peroxidation in DPDT-treated yeast and mammalian cells. The occurrence of an increase in TBARS levels, shown in Figures 3A and 4A, suggests that ROS play an important role in DPDT cytotoxicity in S.cerevisiae and V79 cells. In accordance with this, Borges et al. (38) observed that the repeated administration of DPDT induced lipid peroxidation in rat liver and kidney. Moreover, DPDT treatment of female rats during lactation caused elevated levels of lipid peroxidation in several cerebral structures in their progeny through passage in maternal milk (39).

The present study also provides evidence that in the presence of DPDT, oxidation of GSH markedly increases in S.cerevisiae and V79 cells in a dose-dependent manner. This result suggests that DPDT can react with biological thiols. GSH depletion represents a decrease in non-enzymatic antioxidant defenses, which can lead to oxidative stress and consequently to induction of oxidative lesions, cell injury and loss of viability (40). If GSH is involved in the effects of DPDT, pre-treatment with compounds that stimulate an increase in cellular GSH should lower or even abolish this effect. N-ac is an antioxidant molecule that acts primarily by two mechanisms: (i) by promoting GSH synthesis, as it is readily deacetylated in cells to yield L-cysteine, that acts primarily by two mechanisms: (i) by promoting GSH synthesis, and (ii) as an ROS scavenger and reacting (GSH-like) with various types of ROS, including hydroxyl radicals (41). In agreement with this, our results show that DPDT genotoxicity in V79 cells (Figure 5A) and mutagenicity in yeast (Table III) can be reduced by N-ac pre-treatment, thereby reducing the decline of GSH levels in the cell. This finding is consistent with the several reports about the interactions between selenium and tellurium and biological thiols, including our previous results about the toxic effects of diphenyl diselenide in yeast, V79 cells and mouse organs (42–44). In this context, DPDT inhibits δ-aminolevulinate dehydratase in several tissues and organs of rats and in human erythrocytes, as well as voltage-dependent calcium channels and Na⁺-K⁺-ATPase in mouse brain (45). The enzyme’s activity can be restored and protected by treatment
with GSH and dithiothreitol, indicating that this organochalcogen interacts with the sulphydryl groups essential for enzyme activity. DPDT also disturbs calcium influx in hippocampal brain slices in vitro and glutamatergic neurotransmission in several models (11,46). These effects were attributed, at least in part, to modulation of cellular redox status affecting proteins-containing thiol moieties.

In order to increase knowledge of the toxicity of DPDT, an investigation of its genotoxic profile was performed. The observed induction of frameshift mutations in the Salmonella/microsome assay in strains TA97a and TA98 (Table II) cannot be related to direct oxidative damage as DPDT does not induce mutagenesis in the strains testing base pair substitution used. Alternatively, the frameshift mutation can be a result of DPDT intercalating ability considering the planar structure of this compound. Interestingly, negative results for mutagenesis were obtained in S. typhimurium TA102, a strain that has a proven ability to detect mutagens that induce base pair substitutions in a TAA DNA sequence (17), indicating that oxidative lesions of this kind are not induced by DPDT. Similarly, diphenyl diselenide showed the same behaviour as DPDT in the Ames test (44). These results suggest that the lesion induced by these compounds could be rather specific and not detectable in the test system used or that only limited direct oxidative damage was induced by DPDT at the experimental conditions of our study. Although not mutagenic by itself, diphenyl diselenide enhances the hydrogen peroxide-induced mutagenesis in TA102, supporting an idea of indirect effect via GSH depletion (44). In this manner, the more accentuated oxidative damage on macromolecules observed in the eukaryotic yeast and V79 cells could be attributed to effects of DPDT on mitochondria and/or effects on the mitochondrial and cytosolic GSH pools.

In the present work, we also showed that DPDT treatment clearly induces frameshift mutations in yeast (Table III). In order to gain insight into these mechanisms, we studied the response of S. cerevisiae mutants defective in DNA repair to treatment with this OT. The results of survival assays demonstrated that DPDT leads to pronounced sensitivity in apn1Δ, ntg1Δ and ntg2Δ single mutants (Figure 2A), suggesting that the frameshift mutations observed in our experiment may be caused, at least partially, by base damage and/or apurinic/apyrimidinic (AP) sites induction in yeast test system. Apn1p, Ntg1p and Ntg2p are important proteins of the BER pathway: Apn1p is the major AP endonuclease in S. cerevisiae, and Ntg1p and Ntg2p are DNA glycosylases and AP lyases that recognize and remove oxidized purines and pyrimidines. The AP sites generated by glycosylase activity can be removed by the lyase activity of the enzyme or by the endonuclease Apn1p (47). In the absence of these proteins, there is an accumulation of AP sites generated after removal of damaged bases, and these are mutagenic and potentially lethal lesions that can block DNA replication and transcription (47). Restart of stalled forks can occur through a recombination-associated pathway or mutagenic TLS. The fact that ntg1Δntg2Δ mutants were sensitive to DPDT (Figure 2D) may indicate that both Ntg1p and Ntg2p are important in the repair of DPDT-induced lesions. In agreement with this, the ogg1Δ single mutant was sensitive to DPDT. The OGG1 gene encodes a DNA glycosylase with associated lyase activity that excises 8-hydroxyguanine and oxidized formamidopyrimidines (47).

The rad1Δ (deficient in the DNA endonuclease subunit of nucleotide excision repair factor 1, which cleaves single-stranded DNA during NER and DSB repair), rad6Δ (ubiquitin-conjugating enzyme (E2), involved in TLS) (48) and rev3Δ (deficient in the catalytic subunit of DNA polymerase zeta, which is involved in DNA repair and TLS) (49) strains did not present sensitivity for DPDT (Figure 2B). These data suggest that NER and TLS pathways alone are not important in repairing DPDT-induced DNA damage. Reinforcing this
point, our data showed that quadruple mutants involving BER and NER (ntg1Δntg2Δapn1Δrad1Δ) or BER and TLS (ntg1Δntg2Δapn1Δrev3Δ) show sensitivity similar to the ntg1Δntg2Δapn1Δ triple mutant after treatment with DPDT (Figure 2D). The hypersensitivity of the rad52Δ mutant strain to DPDT (Figure 2C) suggests that HR is critical for the processing of potentially lethal genetic lesions and/or the rescue of collapsed replication forks resulting from DPDT-induced DNA damage. In addition, after treatment with DPDT, the quadruple mutant ntg1Δntg2Δapn1Δrad52Δ showed the highest sensitivity (Figure 2D), indicating that both the BER and HR pathways are important for the repair of DPDT-induced lesions. Thus, the BER pathway could initiate the repair by recognizing and removing the damaged DNA base through the activity of Apn1p, Ntg1p and Ntg2p, leading to strand break formation. These repair intermediates could stall the replication forks, resulting in DSBs that are substrates for the HR repair pathway. If not repaired, the DSBs lead to chromosomal breakage and rearrangements, which in mammalian cells lead to chromosomal aberrations and micronucleus formation (as observed in our experiment; Figure 7), and that can trigger cell death (50,51). Moreover, we observed enhanced sensitivity in strains deficient in subunits of the MRX complex (Figure 2C), which, in addition to its role in the HR, is also involved in DSBs processing by the NHEJ pathway in an error-prone manner and may participate in the mutagenesis observed after DPDT treatment (51).

In V79 cells, DPDT treatment induced DNA damage detected by neutral and alkaline comet assays. We found that DNA DSBs, as detected by the neutral comet assay, were significantly higher in number in V79 cells following exposure to DPDT (Table V). The DNA damage observed in V79 cells after DPDT exposure may be the result of the attack of free radicals on DNA as a consequence of their ability to impose oxidative stress since ROS may cause base damage, SSBs, DSBs and mutations (37,52). In fact, the results of the modified comet assay employing Endo III and FPG reinforce the oxidative nature of the DNA damage in DPDT-exposed cells. In addition, as N-ac pre-treatment was able to prevent DPDT-induced oxidative DNA damage (Figure 5B and C), DNA strand breaks can be attributed indirectly to decreasing GSH levels, which propitiate a pro-oxidant cellular status.

**Table VI. Effect of DPDT treatment on the cell proliferation measured as percentage of BNCs in micronucleus assay**

<table>
<thead>
<tr>
<th>DPDT (μM)</th>
<th>% BNC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>89.0 ± 3.2</td>
</tr>
<tr>
<td>0.1</td>
<td>91.8 ± 4.1</td>
</tr>
<tr>
<td>0.5</td>
<td>85.7 ± 2.2</td>
</tr>
<tr>
<td>1.0</td>
<td>63.2 ± 1.4*</td>
</tr>
<tr>
<td>5.0</td>
<td>54.4 ± 1.6**</td>
</tr>
<tr>
<td>10.0</td>
<td>21.5 ± 0.2**</td>
</tr>
<tr>
<td>50.0</td>
<td>9.2 ± 0.5**</td>
</tr>
<tr>
<td>Positive control</td>
<td>59.1 ± 0.8**</td>
</tr>
</tbody>
</table>

MMS was used as positive control. Data are expressed as mean ± SD, n = 4; *P < 0.05, **P < 0.01 (ANOVA, Tukey’s test): untreated cells versus DPDT exposure and positive control versus negative control.

**V79 cells were exposed to DPDT for 2 h and the BNC per 1000 cells were counted.**

**Fig. 6.** Oxidative damage induced by 2-h DPDT treatment with metabolic activation in V79 cells as evaluated by the modified comet assay using FPG (A) and Endo III (B) enzymes. The oxidative damage score was calculated as the difference between the score obtained after incubation with the respective enzyme or with the enzyme buffer. Data are expressed as means ± SDs, n = 4. Significantly different in relation to the untreated control, *P < 0.05 and **P < 0.001 by one-way ANOVA with Tukey’s Multiple Comparison Test.

**Fig. 7.** Micronuclei induction in V79 cells by 2-h DPDT treatment. The micronucleus (MN) frequency is expressed per 2000 BNC. MMS was used as positive control. Data are expressed as mean ± SD, n = 4. Significantly different in relation to the untreated control, *P < 0.05, **P < 0.01 and ***P < 0.001 by one-way ANOVA Tukey’s Multiple Comparison Test.
addition, the DSBs observed after DPDT treatment could indicate the ability of this compound to intercalate into DNA and/or affect topoisomerase activity. In agreement, DPDT treatment induced a time-dependent increase in the number of apoptotic cells from the S and G2 + M portions of the cell cycle in HL-60 cells, whereas no cell cycle specificity of apoptosis induction was noted for two other OT compounds, 3,3'-diaminophenyl ditelluride and 4,4'-disopropylidiphenyl ditelluride (8). This suggests that DPDT interferes either with DNA replication or with cell division processes. The proposed ability of DPDT to intercalate into DNA disturbing the topoisomerase function is also consistent with our unpublished data about sensitivity of yeast topoisomerase mutants to DPDT.

Although the cytotoxicity and systemic toxicity of tellurium and OT in OT in mammals have been reported, the genotoxic properties of these compounds are still unclear. In agreement with our data about the genotoxicity of DPDT, diaminophenyl telluride and dihydroxyphenyl telluride at 30 μM are able to induce DNA breakage in trout erythrocytes as evaluated by the comet assay (53). Sodium tellurite induces oxidative stress, which leads to DNA breakage and increases the frequency of micronucleus formation in human lymphocytes (54). It is important to mention that the mutagenic potential of DPDT may explain the reproductivity described for this OT in rodents (12,13).

Several observations suggest that organotellurides are more reactive than the structurally related organoselenium compounds due to their higher electronegativity in relation to carbon, which is associated with a larger atomic volume (36). This enhanced reactivity is evidenced in our study, where DPDT was cytotoxic, genotoxic and mutagenic in V79 cells at a concentration range lower than that of diphenyl diselenide evaluated under the same experimental conditions (42).

In summary, the cellular effects of DPDT appear to be very complex and linked to its ability to disturb cellular redox homeostasis, which leads to DNA base damage, DSBs formation and cell death. In addition, the DPDT induced frameshift mutation in S. typhimurium and S. cerevisiae cells, point mutation in yeast cells, as well as micronucleus formation in V79 cells. Moreover, N-ac pre-treatment was able to decrease the cytotoxic, genotoxic and mutagenic effects of this OT compound counteracting intracellular GSH depletion.

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References