Arch Toxicol (2003) 77: 30–36
DOI 10.1007/s00204-002-0407-x

MOLECULAR TOXICOLOGY

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Cytometric determination of novel organotellurium compound toxicity in a promyelocytic (HL-60) cell line

Received: 29 April 2002 / Accepted: 9 September 2002 / Published online: 22 October 2002
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Abstract The increasing use of tellurium compounds in organic synthesis, industrial applications, and as a possible component in pesticides means that its introduction into the environment will increase in the future. Therefore, knowledge of the relative toxicity and mode of toxic action of tellurium-containing compounds is important. The studies detailed here used three model compounds: diphenyl ditelluride, 3,3′-diaminodiphenyl ditelluride, and 4,4′-diisopropyldiphenyl ditelluride. Experiments with human promyelocytic (line HL-60) cells indicate that all of the organotellurium compounds induce an apoptotic form of cell death. The induction of apoptosis occurs in a time- and dose-dependent manner as assayed by three different analytical methods: fluorescence microscopy, gel electrophoresis, and flow cytometry. Apoptotic cells were evident as early as 2 h following treatment with 1×10^{-6} M concentrations of the compounds. Based on these results, future care should be afforded these compounds in laboratory as well as industrial settings.

Keywords Organotellurium compounds · Apoptosis · Cytotoxicity · Flow cytometry · Fluorescence microscopy

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Introduction

Synthetic organic tellurium compounds have found limited use in the past, but recently they have become a promising and advantageous alternative for numerous synthetic operations, with a corresponding increase in organotellurium chemistry appearing in the literature (Petragnani and Comasseto 1991a, b; Petragnani 1994). Little or no organotellurium compounds have been detected in the environment (Thayer 1995; Chau and Wong 1986; Hirner et al. 1998; Feldmann et al. 1994), but ingestion of inorganic tellurium appears to interfere with the normal conversion of squalene to cholesterol in rats (Anthony and Graham 1991), and chronic exposure to high amounts of TeO_2 results in liver necrosis in rats (Cerwenka and Cooper 1961). Although the compounds described and tested in this manuscript are not naturally occurring, analogous compounds are currently being used in photography (Eastman Kodak 1985, 1989), as stabilizers for polymers (Engman et al. 1996), as a component of insecticides (Sumitomo Chemical 1993), and for inorganic synthesis reactions (Petragnani and Comasseto 1991a, b; Petragnani 1994). Although inorganic forms of tellurium appear to be similar to selenium in relative toxicity, little toxicity data are available for organotellurium compounds. Because of their current use in industry, a general knowledge of their relative toxicity is important.

Prior studies have indicated that organoselenium compounds and inorganic selenium induce a form of cell death called apoptosis in eukaryotic cell systems (El-Bayoumy et al. 1995; Harrison et al. 1997; Lu et al. 1994; Ronai et al. 1995; Stewart et al. 1997; Zhu et al. 1996). Researchers have hypothesized that both inorganic selenium and organoselenium compounds can function as a redox switch in numerous biochemical pathways, leading to apoptotic cell death (Harrison et al. 1997; Stewart et al. 1997; Zhu et al. 1996). Another proposed hypothesis is that selenium compounds interact with reduced intracellular glutathione, leading to the

production of reactive oxygen species (Spallholz 1994; Stewart et al. 1997), inducing various forms of intracellular damage and apoptotic cell death. This form of cell death may also be triggered by some forms of organotellurium compounds (Sailer et al. 1999).

Apoptosis is a mode of cell death triggered by a diverse range of environmental stimuli (for reviews, see Arends et al. 1990; Kerr et al. 1972; Wyllie et al. 1990). A cell triggered to undergo apoptosis actively participates in its own demise. This is why apoptosis is also referred to as programmed cell death or "cellular suicide." During apoptosis, a cascade of specific biochemical events, the most prominent of which is the activation of endogenous endonucleases that have affinity for internucleosomal DNA regions, is triggered, leading to specific morphological changes in both the cell nucleus and the cytoplasm (Wyllie et al. 1984, 1990). Due to their ability to induce apoptosis in transformed cell lines, selenium compounds have been recommended as dietary supplements for chemoprevention (El-Bayoumy et al. 1995; Harrison et al. 1997). Similar results with organotellurium compounds (Sailer et al. 1999) led us to the current study.

The research was performed to determine the incidence of cellular toxicity associated with exposure to organotellurium compounds, and to determine the actual mode of cell death induced. The research was performed on transformed human promyelocytic (line HL-60) cells, which are commonly used for these studies (Darzynkiewicz et al. 1992, 1997; Gorczyca et al. 1993). Data indicate that diphenyl ditelluride, 3,3′-diaminodiphenyl ditelluride, and 4,4′-diisopropyldiphenyl ditelluride are cytotoxic, and capable of inducing apoptotic cell death, similar to organoselenium compounds. The promising results of this study will form the basis for future examination into this class of compounds as potential chemopreventive agents, similar to organoselenium compounds already in clinical use.

Materials and methods

Chemical synthesis

The organotellurium compounds used in this study (Fig. 1) were generously donated by Thomas Junk, and were synthesized according to the methods described in Junk and Irgolic (1988).

HL-60 cell culture

Human promyelocytic (HL-60) cells from a genetically stable, leukemic cell line were used, as they are the popular model for the analysis of apoptosis-related events (Gorczyca et al. 1993). Cells were maintained in suspension culture at 37 °C in T-25 flasks containing RPMI 1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco BRL), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco BRL), and 1.25 mM l-glutamine (Gibco BRL). Experimental flasks were seeded at 0.02 to 0.05×10^6 cells/ml and allowed to grow for 48 to 72 h before treatment at a cell density of 0.4 to 0.5×10^6 cells/ml. Cell densities were determined by hemocytometer counts following trypan blue (Gibco BRL) staining. The population doubling time ranged between 15 and 18 h.

Treatment of HL-60 cultures

Stock solutions of diphenyl ditelluride, 3,3′-diaminodiphenyl ditelluride, and 4,4′-diisopropyldiphenyl ditelluride were prepared at 1×10^{-3} M in dimethyl sulfoxide. The stock solutions were then added to HL-60 cell cultures to achieve final concentrations ranging from 1×10^{-6} to 5×10^{-5} M. The times of sample harvest and specific dosage information are presented in the results section and figure legends. For each experiment, one flask of cells was treated with 0.15 μM camptothecin (Sigma Chemical, St. Louis, MO, USA) and were harvested at 2.5 h as a positive control (Darzynkiewicz et al. 1992, 1997; Gorczyca et al. 1993). At appropriate time points, cells were harvested from suspension cultures by centrifugation, at room temperature, for 8 min at 600*g* and were thoroughly re-suspended in one volume of cold phosphate buffered saline (PBS). Three volumes of cold 95% ethanol were then added to yield a final cell concentration of 1.0×10^6/ml in 70% ethanol.

Gel electrophoresis

Following at least a 24 h fixation in 70% ethanol as described above, untreated and treated cells were centrifuged at 600*g* for 8 min, re-suspended in 50 μl phosphate–citrate buffer (PC buffer; 192 parts 0.2 M Na_2HPO_4, 8 parts 0.1 M citric acid, pH 7.8) (Gong et al. 1994), and incubated at room temperature for at least 45 min. After centrifugation for 8 min at 800*g*, the supernatant was transferred to a new tube and concentrated by vacuum to a volume of 3 μl. A 3-μl aliquot of 0.25% Igepal CA 630 (Sigma) in distilled water was added to remove any extraneous lipid material, followed by 3 μl of a solution of RNase (1 mg/ml in H_2O, Sigma). After 30 min of incubation at 37 °C, 3 μl of a solution of proteinase K (1 mg/ml in H_2O, Sigma) was added, and the extract was incubated for an additional 30 min at 37 °C. After the incubation, 12 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol) was added, and the entire content of the tube was transferred to the gel. Horizontal 0.8% agarose gel electrophoresis was performed at 100 V/cm for 1 h in a standard Tris, boric acid, and EDTA (TBE) buffer. The DNA in the gels was visualized under ultraviolet light after staining with 5 μg/ml of ethidium bromide (Sigma) (Gong et al. 1994).

Fluorescence microscopy

To quantitate and visually verify the presence of apoptotic cells, fluorescence microscopy was performed on slides prepared from the treated samples (Darzynkiewicz et al. 1992, 1997). A stock

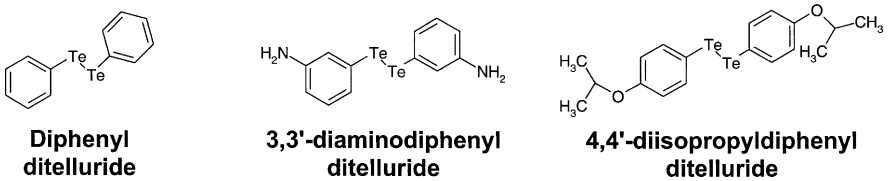

Fig. 1 Structural formulas of the three organotellurium compounds utilized in this study

Diphenyl ditelluride | **3,3'-diaminodiphenyl ditelluride** | **4,4'-diisopropyldiphenyl ditelluride**

solution of ethidium bromide (Sigma) was prepared at 1 mg/ml in PBS. For fluorescence microscopy, cell smears were stained with 5 μg/ml ethidium bromide in the presence of 50 μg/ml RNase (Sigma) in PBS. Direct visualization and counting of apoptotic nuclei was performed on a Nikon Fluophot microscope equipped with a mercury arc excitation source, Model HB-201AN power supply, and a ×100 oil-immersion (1.25 numerical aperture) objective for a total magnification of ×1000 (Nikon, Melville, NY, USA). Slides prepared from camptothecin-treated samples were used as a visual reference for correct identification of apoptotic cells (data not shown) (Darzynkiewicz et al. 1992, 1997).

Flow cytometry

After ethanol fixation for at least 24 h, cells were centrifuged at room temperature for 8 min at 600*g* and the fixative aspirated. Cell pellets were re-suspended in 50 μl of PC buffer and incubated at room temperature for 30 min to extract the fragmented DNA produced during apoptosis (Gong et al. 1994). For cell staining, cells were centrifuged at room temperature for 8 min at 800*g*, and the PC buffer was removed. Staining for DNA content measurements was performed in solutions containing 50 μg/ml propidium iodide (Sigma) and 50 μg/ml RNase in PBS at a final cell concentration of 1×10^6/ml.

DNA content measurements were performed on a BD FACSAnalyzer flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). A mercury arc source provided 488-nm excitation light. Stained, camptothecin-treated cells were used for instrument alignment and verification of the apoptotic subpopulations (data not shown) (Darzynkiewicz et al. 1992, 1997; Gorczyca et al. 1993). A minimum of 10,000 cells were analyzed per sample, and data were analyzed using WinMDI software (J. Trotter, Salk Institute).

Statistical analysis

Statistical analyses were performed using the Student's *t* test function within Microsoft Excel 98 (Microsoft Corporation, Redmond, OR, USA).

Results

Fluorescence microscopy

Figure 2 illustrates the percentage of apoptotic cells present in HL-60 cell populations following treatment with varying concentrations of the three compounds, as measured by direct counting of apoptotic nuclei. At diphenyl ditelluride concentrations of 1×10^{-6} M or higher, there was a significant increase ($P<0.05$) in the number of apoptotic cells at all time points, compared to the control population. The increase in apoptotic cell concentration was both time- and dose-dependent following treatment with diphenyl ditelluride (Fig. 1).

The percentage of apoptotic cells following treatment with either 3,3′-diaminodiphenyl ditelluride or 4,4′-diisopropyldiphenyl ditelluride followed the same pattern as with diphenyl ditelluride. At concentrations of 1×10^{-6} M or higher, there was a significant increase ($P<0.05$) in the number of apoptotic cells at all time points, compared to the control population.

Gel electrophoresis

The PC buffer extracts, containing the fragmented DNA released from the apoptotic cells, were analyzed by gel electrophoresis. Treatment with all three compounds induced the ladder pattern typical of apoptosis (data not shown) similar to that reported in Sailer et al. (1999). The fragments were produced in a time- and dose-dependent manner, similar to the microscopy data. The detection of apoptotic fragments followed the same pattern described below for the flow cytometry results.

Flow cytometry

Flow cytometric DNA content measurements were performed on HL-60 cell samples at 2, 4, 6, and 8 h following treatment with diphenyl ditelluride, 3,3′-diaminodiphenyl ditelluride, or 4,4′-diisopropyldiphenyl ditelluride. The DNA content distributions in Figs. 3, 4, and 5 were obtained following PC buffer treatment of fixed cell populations for removal of the small

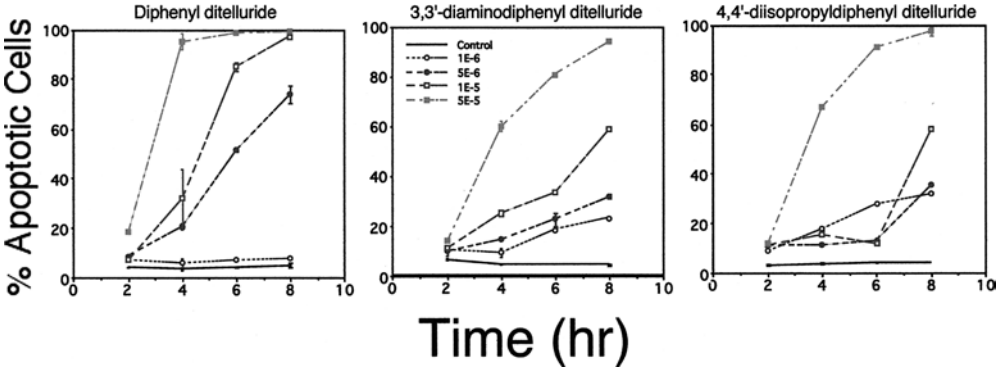

Fig. 2 The induction of apoptosis in HL-60 cell cultures following treatment with the indicated compounds, as determined by direct visualization of ethidium bromide stained nuclei. The *solid lines* represented no treatment, *open circles* represent treatment with 1×10^{-6} M, *closed circles* with 5×10^{-6} M, *open squares* with 1×10^{-5} M, and *closed squares* with 5×10^{-5} M. All three compounds induced a significant increase ($P<0.05$) in the apoptotic cell population within 2 h of treatment at all concentrations. All three compounds induced apoptosis in both a time- and dose-dependent manner. *Error bars* represent ±SD of the mean

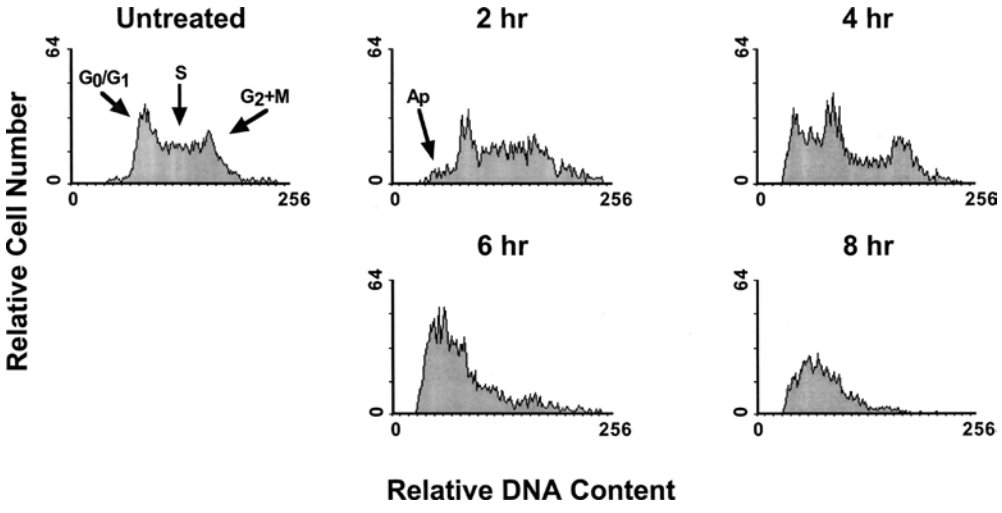

Fig. 3 Flow cytometric DNA content distributions illustrating the cell cycle effects of 1×10^{-5} M diphenyl ditelluride treatment on HL-60 cell cultures over an 8-h time span. Apoptotic cells (Ap) were evident as a sub-G_0/G_1 peak as soon as 2 h post treatment, and continued to increase in number throughout the 8-h treatment time. The preferential loss of cells from the S and G_2+M phases indicates that this compound may be altering DNA synthesis or cell division processes

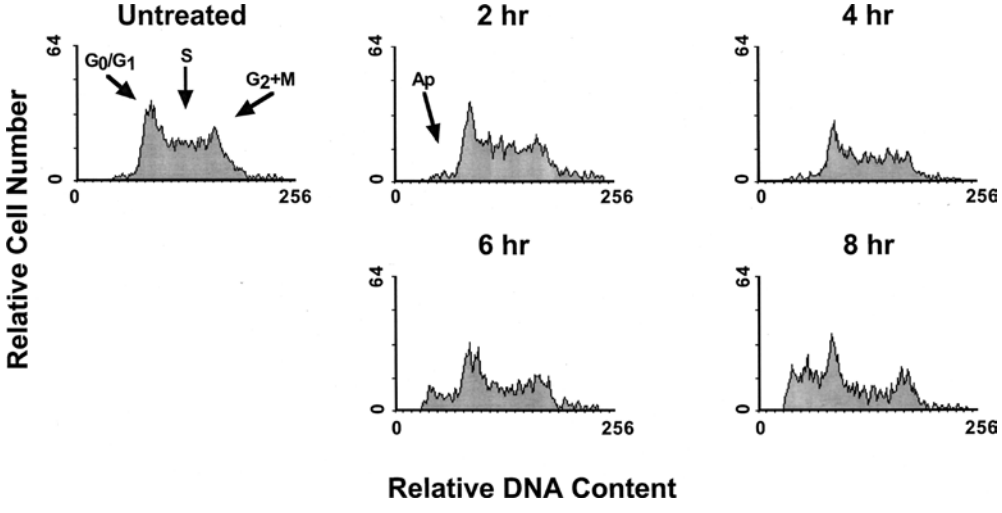

Fig. 4 Flow cytometric DNA content distributions illustrating the cell cycle effects of 1×10^{-5} M 3,3′-diaminodiphenyl ditelluride on HL-60 cell cultures over an 8-h time span. Apoptotic cells were evident as soon as 2 h post treatment, and continued to increase in number throughout the treatment time. There was no indication as to which cell cycle was preferentially affected by the treatment

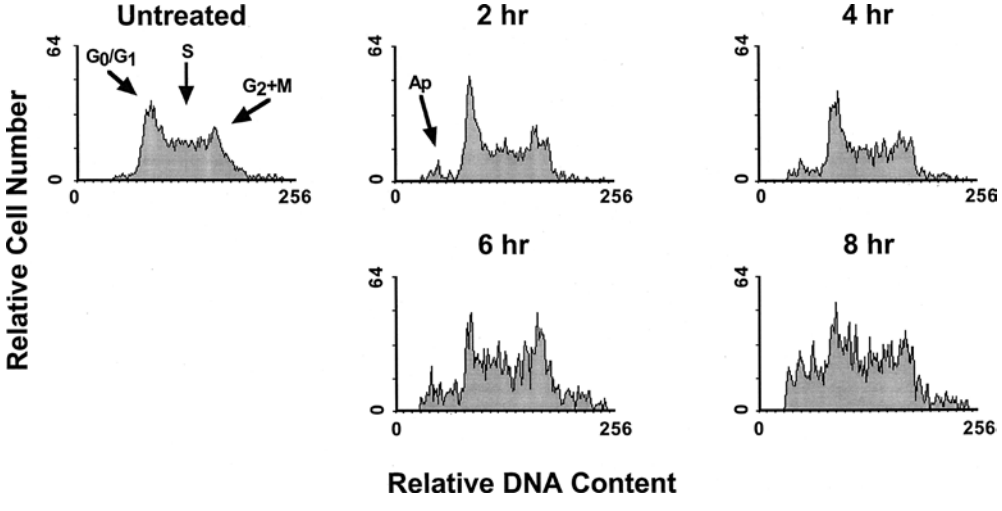

Fig. 5 Flow cytometric DNA content distributions illustrating the cell cycle effects of 1×10^{-5} M 4,4′-diisopropyldiphenyl ditelluride on HL-60 cell cultures over an 8-h time period. Apoptotic cells were evident 2 h post treatment, and continued to increase in number throughout the 8-h treatment time. No cell cycle specificity was noted

molecular weight DNA fragments produced during the apoptotic process. The sub-G_0/G_1 peaks in the samples treated with diphenyl ditelluride (Fig. 3), 3,3′-diaminodiphenyl ditelluride (Fig. 4), or 4,4′-diisopropyldiphenyl ditelluride (Fig. 5) are indicative of apoptotic cells with a lower DNA content following the removal of low molecular weight fragments of DNA by the PC buffer treatment.

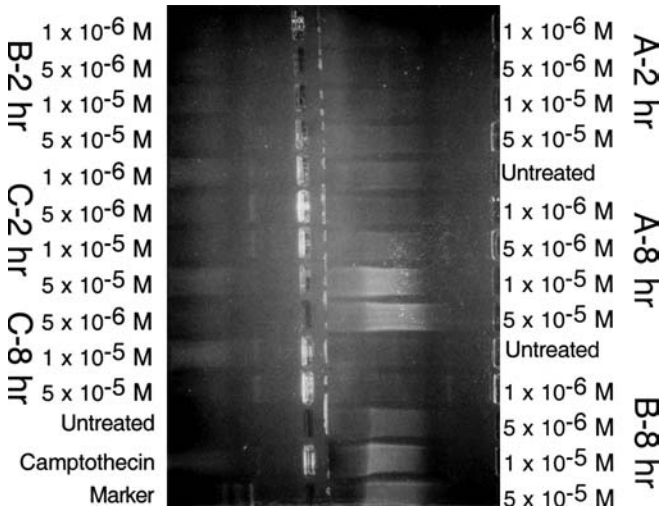

Fig. 6 Detection of apoptotic DNA fragments by gel electrophoresis. Treatment of HL-60 cell cultures with diphenyl ditelluride (*A*), 3,3′-diaminodiphenyl ditelluride (*B*), or 4,4′-diisopropyldiphenyl ditelluride (*C*) at the indicated doses and times resulted in the presence of "apoptotic ladders;" this confirms that the apoptotic cells visualized by fluorescence microscopy and flow cytometry were the result of apoptotic endonuclease activity. Camptothecin was used as a positive control at a concentration of 0.15 μM

Samples treated with diphenyl ditelluride (Fig. 3) demonstrated a time-dependent increase in the number of apoptotic cells present; this is in accord with the fluorescence microscopy (Fig. 2) and gel electrophoretic data (Fig. 6). The cells becoming apoptotic appear to be from the S and G_2+M portions of the cell cycle; this possibly indicates that this compound interferes either with DNA replication, or possibly cell division processes. Samples treated with 3,3′-diaminodiphenyl ditelluride (Fig. 4) also demonstrated a time-dependent increase in the number of apoptotic cells present. But, there did not appear to be any cell cycle specificity for this compound, since the same relative proportions of cells were present in G_0/G_1, S, and G_2+M. Samples treated with 4,4′-diisopropyldiphenyl ditelluride (Fig. 5) also demonstrated a time-dependent increase in the number of apoptotic cell present. Treatment with this compound did not provide evidence as to which phase of the cell cycle was being affected.

Discussion

Diphenyl ditelluride, 3,3′-diaminodiphenyl ditelluride, and 4,4′-diisopropyldiphenyl ditelluride all demonstrate the capability to induce apoptotic cell death in eukaryotic HL-60 cells. Doses of all three compounds as low as 1×10^{-6} M were able to induce apoptotic cell death within 2 h of toxicant addition. The induction of apoptosis induced by the compounds was time- and dose-dependent in intensity.

The toxicity of these compounds may to a large degree depend on the relative solubilities of these compounds in aqueous solutions, or their hydrophobicity, and therefore attraction to the cell membranes. Of the three compounds, 3,3′-diaminodiphenyl ditelluride has the greatest solubility in aqueous solution, while diphenyl ditelluride has the lowest. On the basis of their abilities to induce apoptosis (Figs. 2, 3, 4, 5), diphenyl ditelluride appears to be the most toxic, and, therefore, its hydrophobicity may play a role in its ability to cross the plasma membrane and enter the cytoplasm.

Like selenium, tellurium may be incorporated into amino acids, but, unlike selenium, no human-required tellurium-containing amino acids have, so far, been reported (Stadtman 1974, 1980). Workers have reported the induction of tellurium-containing amino acids in fungi grown on tellurite-rich medium in the absence of sulfur (Ramadan et al. 1989). Production of tellurium-containing proteins has been forced in *Eschericia coli* using telluromethionine and methionine-free protein expression conditions (Budisa et al. 1995). Like selenomethionine (Pickering et al. 1999), telluromethionine is very unstable with respect to oxygen, and expression experiments had to be carried out quickly in anoxic conditions before degradation of the tellurium-containing amino acids occurred (Budisa et al. 1995).

The ultimate fate of tellurium that passes into cells that do not immediately undergo apoptosis may be reduction and protein incorporation via metallothionein (Nordberg 1998), free-radical-scavenging enzymes (Leung 1998), or other cellular components that effect the redox potential in the cell (Gius et al. 1999; Turner et al. 1999). Some toxic metal and possibly some metalloids have been associated with the induction of metallothionein complexes that can act to inhibit cellular free radicals (Leung 1998), sequester metals/metalloids inside cells (Moore and Kaplan 1992), and act as a detoxification method.

Because of chemical similarities between tellurium and selenium compounds, the induction of apoptosis by diphenyl ditelluride, 3,3′-diaminodiphenyl ditelluride, or 4,4′-diisopropyldiphenyl ditelluride may occur through pathways similar to those proposed for selenium-induced apoptosis. Lu et al. (1994) have proposed a general model for the induction of apoptosis by selenium compounds. Treatment with selenium or selenium-containing compounds leads to an induction of DNA single strand breaks. The presence of the DNA single strand breaks activates apoptotic endonucleases, further cleaving the DNA through double-strand breaks. The presence of these subsequent DNA double-strand breaks is then responsible for the oligonucleosomal fragmentation indicative of apoptotic cells. Intracellular metabolism of selenium involves a reaction between selenium and glutathione (Harrison et al. 1997; Spallholz 1994; Stewart et al. 1997; Zhu et al. 1996). As selenium is reduced by glutathione, selenide supplies an electron to oxygen to generate a superoxide anion. The generated superoxide anion can exert toxic effects, such as lipid peroxidation, or the DNA single-strand breaks, which may initiate the apoptotic process (Darzynkiewicz et al.

1997; Harrison et al. 1997; Spallholz 1994; Stewart et al. 1997; Zhu et al. 1996). Very recent work has shown that diphenyl ditelluride inhibited glutamate binding in vitro and in vivo in rats (Nogueira et al. 2001).

The replacement of selenium by tellurium in thioredoxin is another current hypothesis as to how the organotellurium compounds exert their cytotoxicity. Thioredoxin is a redox protein found to be important in cancer prevention in humans. Cell transfected with thioredoxin cDNA show decreased apoptosis in vivo and when exposed to toxicants in vitro (Powis et al. 1997). Thioredoxin reductase has selenocysteine as an essential component. If tellurium can replace selenium in selenocysteine or disrupt the process of its synthesis, then the co-dependent synthesis of thioredoxin can be disrupted (Engman et al. 2000). This would provide a working mechanism for the apoptotic processes seen in the work reported here. Future experiments will involve the evaluation of cell death in the presence of organotellurium compounds in cultures with selenium added, in an effort to evaluate the antagonistic or synergistic effects of these metalloids on a human cell line. Currently, studies are underway to determine the in vivo effects of these compounds on the male reproductive system, as well as the kidneys and liver.

The closest completely organic (that is metalloid-free) analogs of diphenyl ditelluride (tested in this study) are biphenyl (C_6H_5-C_6H_5) or diphenyl disulfide (C_6H_5-S-S-C_6H_5). The first has so far not been classified as a human carcinogen through a lack of data, and relatively high gas-phase exposure concentrations (ca. 18 ppm in volume) for long periods (10 years) ultimately caused coma and death in one human subject (Gosselin et al. 1984); this suggests a relatively low toxicity for biphenyl. Of the three compounds examined by us in this study, diphenyl ditelluride appeared quite toxic (onset of apoptosis at 1×10^{-6} M) and is therefore apparently more toxic than the simpler, tellurium-free biphenyl compound described above. No apoptotic data, however, are available for biphenyl, but it seems reasonable to posit that tellurium appears to play a role in diphenyl ditelluride's toxicity.

The second tellurium-free analog, diphenyl disulfide, is an enzyme-sulfhydryl blocking agent and has been seen to exhibit bio-antimutagenic activity in *E. coli.* (Nakamura et al. 1997), so it is reasonable to propose that the bridged Group 16 (S, Se, Te) analogs' structures may indeed play a part in the toxicology.

Along these same lines, the diphenyl ditelluride molecule is the unbridged version of telluranthrene, a compound examined earlier (Sailer et al. 1999), and, as reported here, it also induces apoptosis in the same human cell line at even lower concentrations, though only by a factor of two. This points to the molecule's tellurium moiety as playing a role in the cytotoxicology.

Polychlorinated biphenyls have, of course, a checkered history of toxicities, and 3,3′,4,4′-tetrachlorobiphenyl (PCB-77) has been shown to increase apoptosis in rats (Tharappel et al. 2002). The diphenyl ditelluride compound is the "chlorine-free", but tellurium-containing analog of the tetrachloro compound examined in our previously published apoptosis work, 3,3′,4,4′-tetrachlorodiphenyl ditelluride (Sailer et al. 1999). The comparable or higher toxicity of diphenyl ditelluride reported here as compared to earlier data for 3,3′,4,4′-tetrachlorodiphenyl ditelluride, using onset of apoptosis as a measure, suggests that the chlorine moieties are *not* the source of the apoptotic effects, since chlorine is present in the latter but not in the former, yet both compounds cause apoptosis at very low concentrations. We feel that this again implicates the molecule's tellurium moiety in the cytotoxicology of these compounds.

Diisopropyl biphenyl (tellurium-free) has been found to be relatively nontoxic, with an oral rat LD_{50} in the 5 g/kg range; this again suggests that the metalloid's presence in the tellurium analog compound is involved in the toxicological effects since the tellurium-containing analog examined in this work produced apoptotic cells at doses of 1×10^{-6} M and higher. No toxicity data are available for metalloid-free analogs of 3,3′-diaminodiphenyl ditelluride.

Regardless of the mechanism of action, the compounds studied have been demonstrated to be potent cytotoxic and apoptosis-inducing agents. That said, there is little information about the actual mode of toxicity of tellurium. Very recent work with trout erythrocytes showed that three diaryl tellurides exhibited protection against oxidative stress or genotoxicity depending on the tellurium compound dosage (Tiano et al. 2000). Growing *E. coli* in a medium with tellurite has shown that cellular reduction potential plays an important role in tellurium resistance in these bacteria (Turner et al. 1999). In reality, and like selenium's role in cellular oxidative reactions, for many tellurium compounds the balance between cell proliferation and apoptosis may be concentration-dependent.

Acknowledgements The research presented was funded by the Sam Houston State University Faculty Research Council, and the Departments of Biological Sciences and Chemistry at Sam Houston State University. Additional support by a Robert A. Welch Departmental Grant to the Chemistry Department is gratefully acknowledged. The research described complies with the current laws of the United States, where the experiments were carried out.

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