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Antioxidative Properties of Organotellurium Compounds in Cell Systems

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ABSTRACT. The protective/antioxidative properties of diaryl tellurides were demonstrated in cellular systems of increasing complexity. In the presence of glutathione, bis(4-hydroxyphenyl) telluride (**1a**), bis(4-aminophenyl) telluride (**1d**) and bis(2-carboxyphenyl) telluride (**1h**) reduced by more than 50% *t*-butyl hydroperoxide-induced cell death in lung fibroblast cultures at concentrations below 2 μM. Bis(2,6-dimethyl-4-hydroxyphenyl) telluride (**2b**) reduced by more than 50% leukocyte-mediated and phorbol-12-myristate-13-acetate-stimulated damage to Caco-2 cells at 0.1 μM concentration. As judged by their abilities to reduce formation of thiobarbituric acid reactive substances at concentrations close to 1 μM, diaryl tellurides **1a**, **1d** and **2b** protected rat kidney tissue against oxidative damage caused by anoxia and reoxygenation. The organotellurium compounds also offered protection after systemic administration. In the presence of diaryl telluride **2b** (0.1–1 μM), the ischemia/reperfusion-induced vascular permeability increase in the hamster cheek pouch was significantly reduced as compared with the control. Some of the most active organotellurium cell protectants were evaluated for their ability to inhibit formation of the inflammatory mediators leukotriene B_4 and interleukin-1β. An inhibitory effect on the secretion of these species was seen for compounds **1a** and **2b** at or above 10 μM concentrations. The protective effects of diaryl tellurides against *t*-butyl hydroperoxide-induced cell injury can be ascribed mainly to the peroxide-decomposing, glutathione peroxidase-like capacity of the compounds. The chain-breaking, electron- or hydrogen atom-donating ability of diaryl tellurides seems to be the main reason for their protection against leukocyte-mediated cell damage in Caco-2 cells and in the oxidatively challenged rat kidney and hamster cheek pouch. BIOCHEM PHARMACOL **55**;5:573–584, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. organotellurium compounds; antioxidant; lung fibroblasts; Caco-2 cells; anoxia and reoxygenation; ischemia/reperfusion

It is now well established that oxidants, many of which are free radicals [1], are important in normal physiology. For example, in the defence mechanism of phagocytic cells, superoxide anion radicals, $O_2^{-\bullet}$, are produced [2]. These can dismutate to form hydrogen peroxide, H_2O_2, and, via the enzyme myeloperoxidase, be further converted to hypochlorous acid (HOCl). If suitable transition metal reductants are present, hydrogen peroxide may also form highly reactive hydroxyl radicals (HO•). The dangers of hosting free radicals in physiological processes are obvious. Due to their high reactivity, they may cause damage to cellular constituents or important biomolecules, such as DNA, proteins, lipids, or carbohydrates. Under certain reaction conditions, even small amounts of free radicals may initiate chain reactions which would chemically modify a significant number of membrane lipids in a short time (lipid peroxidation) [3]. In order to prevent undesired, radical-induced damage, the organism is equipped with elaborate antioxidant systems [4]. The most important of these act either by interception of free radicals (vitamin E, ascorbate, glutathione, and uric acid) or by destruction of precursors to free radicals (catalase, glutathione peroxidases, and superoxide dismutases). The term "oxidative stress" is used vaguely by biologists and biochemists to describe a situation characterized by an elevation in the cellular steady-state concentration of reactive oxygen-derived species [5]. This condition occurs if the balance between oxidants and the various antioxidant defences is impaired. The imbalance could either be caused by exogenous sources (air pollutants, tobacco smoke, and radiation), through metabolism of xenobiotics, or by oxidative stress from endogenous sources.

"Antioxidant pharmacotherapy" [6, 7] has emerged as a remedy for pathological conditions characterized by oxidative stress. For example, excessive radical formation has

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been implicated in chronic inflammatory disorders, adult respiratory distress syndrome, atherosclerosis, ischemia/reperfusion injury, shock, and cataract. However, the nature of the active damaging species and the details of the processes leading to the radical-dependent pathology are rarely known. Often, alteration of intracellular calcium signalling and impairment of ATP-generating pathways seem to be the causes of cell dysfunction and cell death rather than the direct interaction of peroxides and free radicals with biological structures [8]. Considering the great efforts made to improve antioxidant pharmacotherapy [9], surprisingly few drugs have been developed where the pharmacological effect is clearly associated with antioxidative properties alone. Many drugs already in use, though, such as probucol, lipoic acid, captopril, *N*-acetyl cysteine, flavonoids, various antiinflammatory drugs, or desferrioxamine, show antioxidant properties in various model systems. The therapeutic efficiencies of some newly developed drugs seem to be better correlated to their antioxidative properties. Thus, allopurinol has been found to significantly reduce the incidence of poor renal function following transplantation [10], and the lazaroids have been developed for the acute treatment of injury to the central nervous system, for ischemia and for subarachnoid haemorrhage [11].

We have previously evaluated the antioxidative properties of organotellurium compounds in various cell-free systems. The capacity of these materials to retard oxidation could be ascribed to the ready redox cycling of the heteroatom between the oxidation states +II and +IV. It was recently demonstrated that diaryl tellurides act as catalytic decomposers of hydrogen peroxide and organic hydroperoxides in the presence of thiols as stoichiometric reducing agents [12, 13]. Thus, they mimic the properties of the glutathione peroxidase enzymes. The ability of some of these materials to inhibit stimulated peroxidation in rat hepatocytes and rat liver microsomes [14, 15] and azo-initiated peroxidation of linoleic acid in methanol [16] suggested that organotellurium compounds may also act as chain-breaking antioxidants. Recent studies in a two-phase model of lipid peroxidation have provided further evidence in support of a chain-breaking capacity of the compounds and shown that the effect could be catalytically expressed in the presence of a thiol reducing agent [17].

To elucidate the importance of the glutathione peroxidase-like effects of the organotellurium compounds in living cells, a simple cell-based model, involving TBH[‖]-induced damage, was initially used. Since activated inflammatory cells are also capable of producing damaging principles other than hydrogen peroxide (superoxide, hydroxyl radicals, and hypochlorite) which are likely to be intercepted by organotellurium compounds, the protective activity was also probed in more complex coculture systems with activated leukocytes and tissue cells as targets. Finally, the *in vivo* effects of a few selected compounds were evaluated in an animal model where inflammation was induced partly by production of reactive oxygen species.

MATERIALS AND METHODS

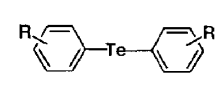
Organochalcogen compounds **1a** [18], **1b** [19], **1c** [20], **1d** [20], **1e** [21], **1f** [22], **1g** [22], **1h** [21], **1i** [13], **1j** [21], **2a** [23], **2b** [16], **4a** [24], **4b** [25], **5** [26], **6a** [27], **6b** [28], **7** [29], **10a** [13], **10b** [13], **11** [13], **12a** [17], **12b** [17], **13** [13] and **14** [30] (see Fig. 1) were prepared according to methods described in the literature. BWA4C (**16**) was kindly donated by the Wellcome Research Laboratories. UK. Zileuton (**17**) and indenoindoles **8** and **19** were kindly provided by Astra Hässle AB. Budesonide (**18**) is an Astra Draco product. All other compounds were of the highest grade available from commercial suppliers.

Human embryonal lung fibroblasts (F2002) were obtained from Flow Laboratories and the human colonic carcinoma cell line, Caco-2, obtained from ATCC. The cells were seeded in microplates (Nunc) and allowed to reach confluency in appropriate medium (F2002 cells were grown in MEM including 10% FCS and 1 IU/1 μg/mL of PEST, respectively; Caco-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 1% NEAA and 1 μg/mL PEST, all from Life Technologies. Cell culture was performed at 37° and 5% CO_2 in air.

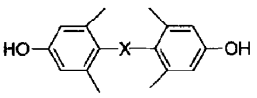
Oxidant-Mediated Cytotoxicity

For the study of oxidant-mediated damage, confluent cultures of F2002 were incubated with 12–15 μM TBH in HH for 20 hr. For the assessment of protective effects, various combinations of test compound (0.2–20 μM) in DMSO and glutathione (300 μM) were also added to the wells. These materials were added separately, immediately prior to the addition of TBH. DMSO in the concentrations used was without effect on the target cells (data not shown). Cultures in HH alone, with or without test compound, served as controls. After the overnight incubation, the plates were washed and incubated with serum containing medium with 50 μg/mL NR for 3 hr. Then, the cells were fixed, the dye eluted and the absorbance at 540 nm (A_{540}) measured. The reduction in A_{540}, in comparison with the control, was used as a measure of cytotoxicity. NR is taken up into lysosomes of living cells but not of dead ones. Xenobiotics that injure cell or lysosomal membranes will decrease uptake and retention of the dye. Dead cells cannot

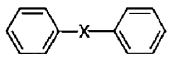
[‖] *Abbreviations:* BHT, butylated hydroxytoluene; CD_{25}, concentration of the compound which causes 25% cell death; EC-SOD, extracellular superoxide dismutase; FCS, fetal calf serum; FIA, flow injection analysis; HH, Hanks buffer with 25 mM HEPES; IC_{50}, concentration of the compound needed to reduce LTB_4 production/TBARS formation by 50%; IL-1β, interleukin 1β; I/R, ischemia/reperfusion; LPS, lipopolysaccaride; NEAA, non-essential amino acids; NR, neutral red; PC_{50}, concentration of the compound required to reduce cell damage by 50%; PDT, photodynamic therapy; PEST, penicillin/streptomycin; PMA, phorbol-12-myristate-13-acetate; PMN, human blood polymorphonuclear leukocytes; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TBH, *t*-butyl hydroperoxide; TCA, trichloroacetic acid.



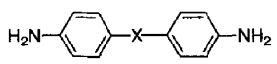
1 a R=4-OH
b R=4-OMe
c R=4-NMe$_2$
d R=4-NH$_2$
e R=4-NO$_2$
f R=4-H
g R=4-F
h R=2-COOH
i R=2-COOMe
j R=2-CN



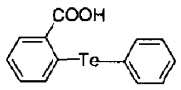
2 a X=S
b X=Te



3 a X=O
b X=S
c X=Se
d X=NH



4 a X=S
b X=Se



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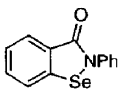
6 a X=Se
b X=Te



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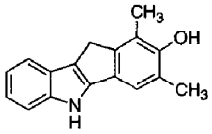

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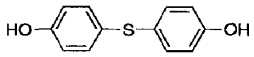

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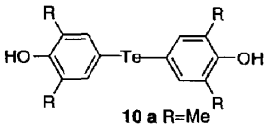
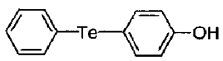


10 a R=Me
b R=*t*-Bu



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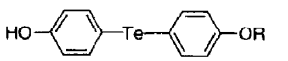
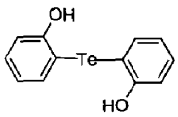


12 a R=CH$_2$COOH
b R=C$_{14}$H$_{29}$



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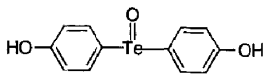

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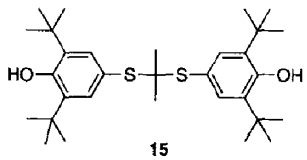

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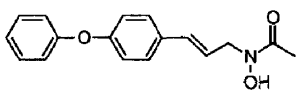

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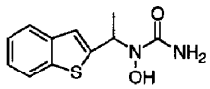

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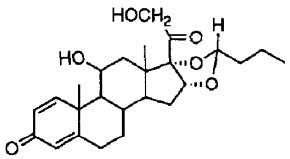

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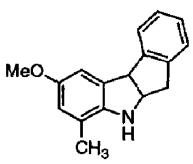

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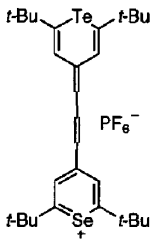

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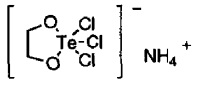

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FIG. 1. Structures of organochalcogen compounds investigated.

retain the dye after the washing/fixation procedure. The amount of NR correlates linearly with the number of viable cells [31]. The toxicity of the compounds (CD_{25}) was determined in the absence of externally added GSH. However, glutathione itself did not show any toxic effects in the concentrations used as demonstrated in separate experiments (not shown).

Cell-Mediated Cytotoxicity

In an effort to study the effects of antioxidants on oxidant-mediated injury, cytotoxicity in confluent cultures of Caco-2 cells or human embryonal fibroblasts was induced in microtiter plates by coincubation with either human blood PMN or monocytes in the presence or absence of test compound. Surviving target cells after 20 hr were quantified by the uptake of NR. In detail: Human blood polymorphonuclear leukocyte and monocyte effector cells were obtained from healthy volunteers and purified by density centrifugation on Ficoll (Pharmacia). The experiments were started by adding effector cells (3.2×10^5 PMN or 3×10^5 monocytes/well; approximately 10:1 ratio of effector and target cells) to the target cell cultures. In PMN cocultures, PMA 20 ng/mL was added as stimulus 30 min after the addition of phagocytes. Monocytes were purified by adherence for 3 hr, whereafter nonadherent cells were removed by washing twice with PBS. After incubation overnight, the medium was changed to Hanks buffer and stimuli (PMA) added. Survival of target cells was quantified by NR uptake as described above. Test compounds (0.02–20 μM in DMSO) were added together with the PMN. The DMSO was without effect on the target cells (data not shown). When monocytes were used as effector cells, the test compounds were added just before the addition of PMA. The inhibition of cytotoxicity by test compounds, expressed in %, was calculated as: $100 \times (C\text{-}B)/(A\text{-}B)$, where A, B and C are defined in Fig. 3.

Inflammatory Mediators

Peripheral venous blood samples were obtained from healthy volunteers. Mononuclear cells were isolated from EDTA-treated blood by centrifugation on Mono-Poly Resolving Medium (Flow Laboratories, Scotland) for 35 min at $400 \times g$. The mononuclear cell layer was removed and the cells were washed three times with PBS. The cells were then resuspended in growth medium RPMI 1640 (Flow Laboratories), supplemented with 5% FCS and gentamycin (50 μg/mL) and aliquots of cell suspension (5×10^6 cells in 2 mL) were seeded in tissue culture multidishes (24 wells, Nunc). The cells were incubated for 3 hr at 37° and the nonadherent cells were removed by washing with Ca^{2+} and Mg^{2+}-free PBS. The adherent cells were further incubated in fresh medium overnight. The final yield of monocytes (May-Grünwald Giemsa stain) was 97–99% and the cell viability (Trypan blue exclusion) was 98 ± 1%.

For the studies of LTB_4 secretion, the cells were stimulated with human serum-opsonized zymosan (1 mg/mL) suspended in Hanks buffer in the presence or absence of test compound for 90 min at 37°. The tested compounds were dissolved in DMSO (final concentration 0.1 %) and then in Hanks buffer. Cell viability was not altered after treatment with the test compounds. The incubation was stopped by rapid cooling to 4° and the supernatants were removed and stored at −70°. The concentration of LTB_4 in the culture medium was assayed using specific radioimmunoassays (Advanced Magnetic Inc.) and related to the content of DNA in the monocyte cultures. The DNA was analyzed by means of the reaction of bisbenzimidazol, essentially as described previously [32]. The effects of the tested compounds were studied in four separate experiments.

For the studies of IL-1β secretion, the cultured, FCS and gentamicin supplemented monocytes were stimulated with LPS (10 μg/mL) from *Escherichia coli*, serotype 026:B6 (L 2762, Sigma) and incubated in the presence or absence of test compounds for 20 hr at 37°. The IL-1β in culture supernatants was assayed by specific radioimmunoassay (Amersham Int.) and related to the cell numbers in monocyte cultures as described above.

Anoxia and Reoxygenation of Rat Kidney

Male Sprague–Dawley rats were anaesthetized with Mebumal and the kidneys excised. The kidneys were cut in ≈2 mm slices, gently dried off and weighed and two slices per flask put into wide-necked 50 mL flasks containing 3.9 mL argon-saturated buffer. The stoppered flasks were put in a 37° waterbath for 20 min and 0.1 mL of either vehicle, test compound or 400 μg desferrioxamine was added. The kidney slices were cut, under argon, with a pair of scissors and a knife homogenizer into pieces of ≈1 mm. The flasks were then incubated for another 10 min at 37° and reoxygenated by vigorous bubbling of oxygen for 1 min. After another 30 min of incubation at 37° in a shake waterbath, 100 μg desferrioxamine was added to all flasks to quench ongoing reactions. The contents of the flasks were transferred to plastic tubes and homogenized for 20 sec. Samples (50 μL) of the homogenates were added to 500 μL 2% TCA in Eppendorf vials and spun for 2 min. The supernatant (300 μL) was then added to a vial containing 550 μL 14 mM TBA in 2% TCA and the samples incubated for 1 hr at 95° and analyzed for TBARS with a FIA system (200 μL was injected into a mobile phase, MilliQ-water/acetonitrile 50/50, at a flow rate of 1.5 mL/min. Peak heights were determined with a fluorescence detector).

In the *ex vivo* experiments, male rats were injected with vehicle or test compounds 1 hr before the kidneys were excised. Anoxia, reoxygenation and analysis of TBARS followed the above *in vitro* procedure.

Ischemia and Reperfusion in the Hamster Cheek Pouch

Golden hamsters (male, 90–120 g, Harlan Sprague-Dawley Inc.) were anaesthetized intraperitoneally with sodium

pentobarbital which was supplemented with i.v. chloralose (2.5% solution) through a right femoral vein catheter. Another catheter (PE 10) in the left femoral vein was used for the FITC-dextran infusion. The hamster cheek pouch was prepared for intravital microscopy according to Duling [33] as modified by Svensjö [34] and Erlansson *et al.* [35]. Briefly, the cheek pouch was everted and mounted on a microscope stage and an area of about 1 cm^2 was prepared for intravital microscopy. Thirty min after completed preparation, fluorescein-labeled dextran (FITC-dextran, MW = 150000, Bioflor HB) was injected intravenously (25 mg/100 g b.w.) as a macromolecular tracer. Ischemia was introduced with an inflatable cuff placed around the neck of the hamster cheek pouch [35]. The cheek pouch was subjected to a 30-min period of ischemia after which reperfusion was initiated by releasing the pressure of the cuff. The microvascular permeability increase for large molecules or plasma leakage following I/R was quantified by counting fluorescent spots (leaky sites = leaks) at postcapillary venules. The number of leaks was counted before and at 2, 5, 7, 10, 15, 20 and 30 min after the end of ischemia and start of reperfusion. Topical application of bradykinin, final concentration 4×10^{-7} M, was made after I/R to verify a normal vascular permeability response. The maximal number of leaks at 5–15 min was used for statistical calculations.

Compounds **1a**, **2b**, **8** and **19** were dissolved in a small volume of ethanol and then diluted in a 5% cyclodextrin vehicle (final concentration of ethanol <0.1%). These solutions were given locally into the superfusing buffer resulting in final concentrations in the cheek pouch tissue between 0.1–10 μM, starting 10 min prior to the ischemic period and continuing throughout the experiment. Compound **1a** previously showed glutathione peroxidase-like activity *in vitro* [12]. It was therefore supplemented with GSH at a final concentration of 1 μM.

RESULTS

TBH-Mediated Cytotoxicity

TBH caused a substantial reduction in the number of viable cells, which was not affected by the coincubation with either glutathione or antioxidant alone (Fig. 2). However, when the organotellurium compound (2 or 20 μM) was added together with glutathione, the TBH-mediated cell damage was significantly reduced. In Table 1, the protective effect of a variety of synthetic compounds in the above-mentioned system is described in the form of PC_{50} values. Among symmetrical 4,4′-disubstituted diaryl tellurides tested, the hydroxy (**1a**), methoxy (**1b**), dimethylamino (**1c**) and amino (**1d**) derivatives turned out to be the most efficient compounds with PC_{50} values close to 2 μM. A sterically more hindered analogue **2b** of the organotellurium compound **1b** was inefficient in the assay used. Also, diaryl chalcogenides with other heteroatoms (O, S, Se) and diphenylamine were poorer reductants of cytotoxicity than the corresponding organotellurium compounds (*cf.* compound **1f** with the four compounds **3** and compound **1d** with the two compounds **4**). Among symmetrical 2,2′-disubstituted diaryl tellurides tested, the carboxy (**1h**) and carbomethoxy (**1i**) derivatives were particularly efficient with PC_{50} values in the low micromolar range. The corresponding monosubstituted carboxy compound **5** was also tested and found to be efficient (PC_{50} = 2–10 μM). Two organoselenium compounds with documented glutathione peroxidase-like capacity—the diselenide **6a** [27] and Ebselen (**7**) [27]—were also tested for their ability to reduce TBH-mediated injury. Both compounds were efficient, the former with a PC_{50} value (2 μM) in the same range as the most efficient organotellurium compounds. In contrast, the organotellurium compound **6b** was without effect in the assay. A recently developed, chain-breaking antioxidant of the indenoindol type (compound **8**) also did not offer any protection against TBH-mediated cell injury.

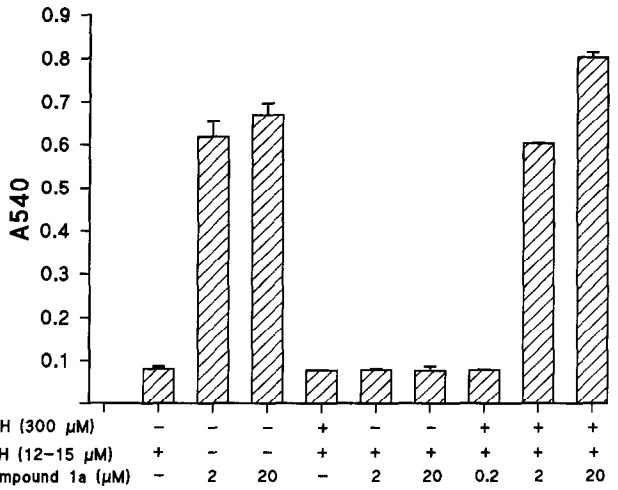

FIG. 2. Survival of human embryonal lung fibroblasts (F2002) measured as absorption (A_{540}) of incorporated Neutral Red. Effects of TBH combined with GSH (300 μM) and/or compound 1a (0.2–20 μM) as indicated in the figure. Mean ± SEM. N = 8 wells/treatment.

For compound **1a**—one of the most active test compounds—glutathione was replaced by sodium ascorbate (300 μM) in the standard assay. A protective effect of the compound was still observed, but at the highest concentration tested (20 μM), 50% protection was not reached (data not shown).

Also listed in Table 1 are toxicity data for the compounds investigated. These are expressed as CD_{25} values and were determined by treatment of the fibroblasts with test compound of different concentrations as exemplified in Fig. 2. Ebselen (**7**) showed a less favorable ratio between PC_{50} and CD_{25} than many organotellurium compounds.

Cell-Mediated Cytotoxicity

As shown in Fig. 3, the organotellurium compound **2b** dose-dependently inhibited the PMN-mediated injury to Caco-2 cells. Inhibition data (calculated as described in

TABLE 1. Protective effect of synthetic compounds against TBH-mediated cytotoxicity

Compound	PC_{50} (μM)[a]	CD_{25} (μM)[b]	Compound	PC_{50} (μM)[a]	CD_{25} (μM)[b]
1a	<2	>20	3a	≫50	>50
1b	2	>50	3b	≫50	>50
1c	2–10	>50	3c	≫50	>50
1d	<2	>50	3d	≫50	>50
1e	>50	<10	4a	≫50	>50
1f	30	>50	4b	≫50	>50
1g	>50	—	5	2–10	>10
1h	<2	20	6a	2	20
1i	2–10	>10	6b	≫50	2
1j	≫50	>50	7	20	10
2b	>50	>50	8	>50	10–50

[a] Concentration of the compound required to afford a 50% protection.
[b] Fibroblast toxicity given as the concentration of the compound which causes 25% cell death.

"Materials and Methods") for investigated compounds are presented in Table 2. Among organotellurium compounds tested, diaryl telluride **2b** was the most active. Figure 4 represents a comparison of the inhibiting effect by compound **2b** and the structurally related organotellurium compound **1a**. Their corresponding organosulfur derivatives, compounds **2a** and **9**, respectively, also showed protective effect in the assay used. However, as shown in Fig. 5, the organotellurium compounds usually seemed to be superior to their respective organosulfur analogues. A derivative **10a** of the active telluride **2b**, with the four methyl groups *meta* to tellurium, showed low protective effect at the 2 μM level. This was also true for the corresponding *t*-butyl derivative **10b** (Table 2). As judged from the results with monosubstituted compound **11**, both hydroxyl groups of compound **1a** seem to be essential for the protective effect. Alkylation of one of them with a hydrophilic group (CH_2COOH) had little effect on the activity (compound **12a**). In contrast, alkylation with a hydrophobic group ($C_{14}H_{29}$) significantly reduced the protective effect (compound **12b**). The *ortho* dihydroxylated diaryl telluride **13** offered significantly less protection against PMN-induced cell damage than its corresponding *para* substituted compound **1a**. The product resulting from oxidation of compound **1a**—telluroxide **14**—showed inhibition characteristics very similar to those recorded for the divalent compound.

Ebselen (**7**) was a poor inhibitor of cell damage at the level tested (2 μM). At this concentration, some conventional antioxidants were also without effect (vitamin E, vitamin C, ethoxyquin, and BHT; data not shown for the latter two compounds). Probucol (**15**) showed 25–50% inhibition at the 2 μM level. Among lipoxygenase inhibitors tested, BWA4C (**16**) was significantly more active than Zileuton (**17**). The corticosteroid budesonide (**18**) inhibited 25–50% of the cell damage at the 1 μM level. Diaryl tellurides **1a** and **2b** both inhibited >50% of PMA-induced monocyte mediated cytotoxicity to human lung fibroblasts at 2–10 μM concentrations. Figure 6 shows a comparison with the indenoindol derivatives **8** and **19**.

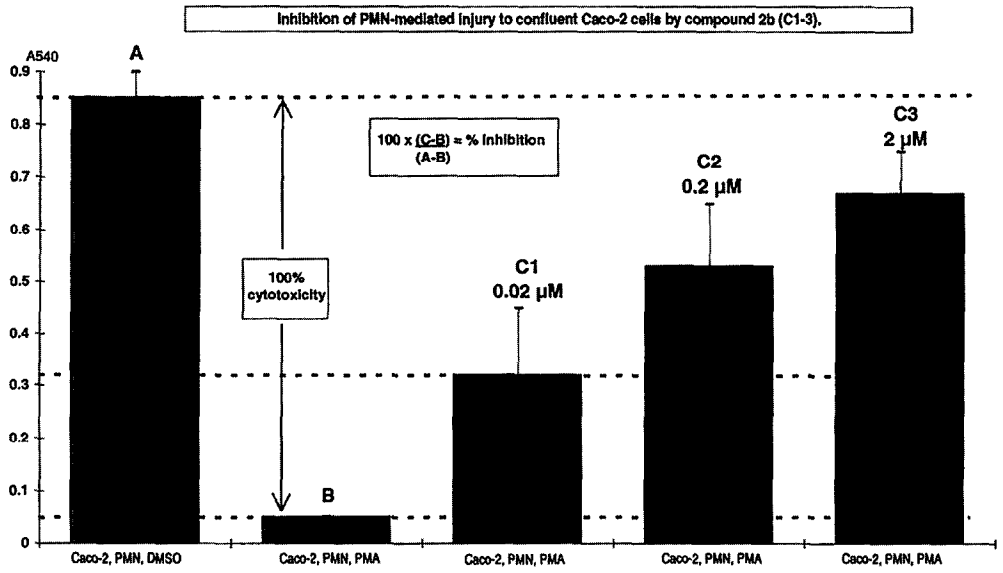

FIG. 3. Example of calculation of cell-mediated cytotoxicity. A = A_{540} (incorporation of Neutral Red) of untreated Caco-2 cells. B = A_{540} of Caco-2 cells cocultured with PMA-stimulated PMN. C1–3 = A_{540} of Caco-2 cells cocultured with PMA-stimulated PMN in the presence of compound 2b (0.0, 0.2, 2 μM). Percent inhibition was calculated as indicated. Mean ± SEM. N = 8 wells/treatment.

TABLE 2. Protective effect of synthetic compounds against PMN-mediated injury to Caco-2 cells

Compound	Concentration (μM)	Score for inhibition[a]
1a	0.2	0
1a	2	++
1a	10	++
1f	10	0
2a	0.02	+
2a	0.2	++
2a	2	++
2b	0.02	+
2b	0.2	++
2b	2	++
7	2	0
9	0.2	0
9	2	+
10a	2	0
10b	2	0
11	2	0
12a	2	++
12a	10	++
12b	10	0
13	2	0
vitamin E	2	0
vitamin E	10	++
vitamin C	2	0
vitamin C	10	+
15	2	+
15	10	+
16	2	++
17	2	0
18	1	+

[a] <25% inhibition = 0; 25–50% inhibition = +; >50% inhibition = ++.

When PMN was used as an effector with these target cells, compound **2b** was also the better inhibitor at 0.02–2 μM concentrations but a poorer one at higher concentrations (data not shown).

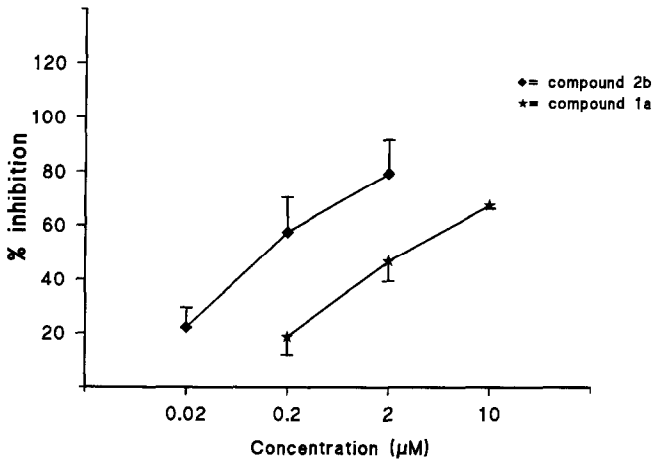

FIG. 4. Effects of compounds 1a and 2b (0.02–2 μM) on inhibiting PMA stimulated PMN-mediated cytotoxicity to human colonic carcinoma cells (Caco-2). Percent inhibition of cytotoxicity calculated as indicated in Fig. 3. Mean ± SEM. N = 12–13 experiments.

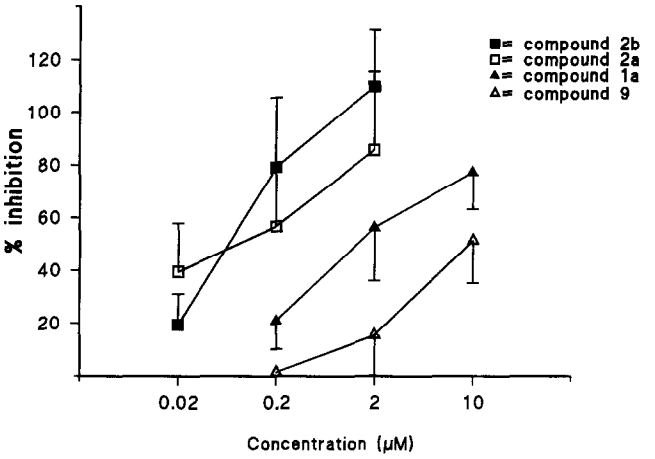

FIG. 5. Effects of compounds 1a, 2a, 2b and 9 on inhibiting PMA stimulated PMN-mediated cytotoxicity to confluent human colonic carcinoma cells (Caco-2). Percent inhibition of cytotoxicity calculated as indicated in Fig. 3. Mean ± SEM. N = 4–5 experiments.

Inflammatory Mediators

Lipid peroxidation, induced by oxidants or by PMN, is known to cause the release of cytokines and membrane-bound arachidonic acid [36]. The latter compound is then metabolized to leukotrienes, which are potent chemoattractants and endothelial-permeabilizing stimulants. The wide variety of events triggered by these and other bioactive lipid metabolites may very well culminate in the induction of tissue injury. We therefore decided to study the effects of two of the most active organotellurium cell protectants on the secretion, as monitored by radio-immunoassay, of the inflammatory mediators leukotriene B_4 (LTB_4) and interleukin-1-β (IL-1β). Compounds **1a** and **2b** were evaluated in comparison with indenoindol **19** for their ability to inhibit secretion of LTB_4 in human isolated monocyte cultures stimulated with opsonized zymosan. As shown in Fig. 7, all three compounds inhibited LTB_4 production,

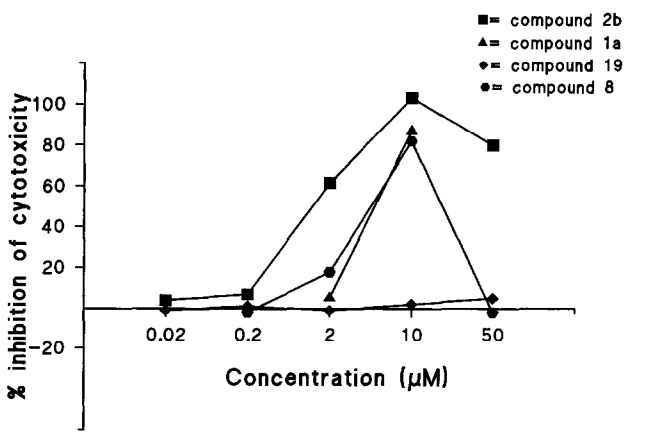

FIG. 6. Effects of compounds 1a, 2a, 8 and 19 on inhibiting PMA stimulated monocyte-mediated cytotoxicity to confluent human lung fibroblasts (F002). Inhibition of cytotoxicity calculated as indicated in Fig. 3. Mean of 2–3 experiments.

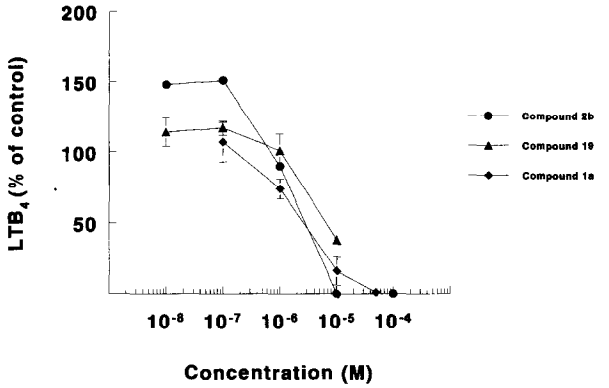

FIG. 7. Effects of compounds 1a, 2b and 19 on inhibiting LTB_4 release from peripheral blood mononuclear cells stimulated with serum opsonized zymosan. Values represent mean ± SEM of cells from four different blood donors.

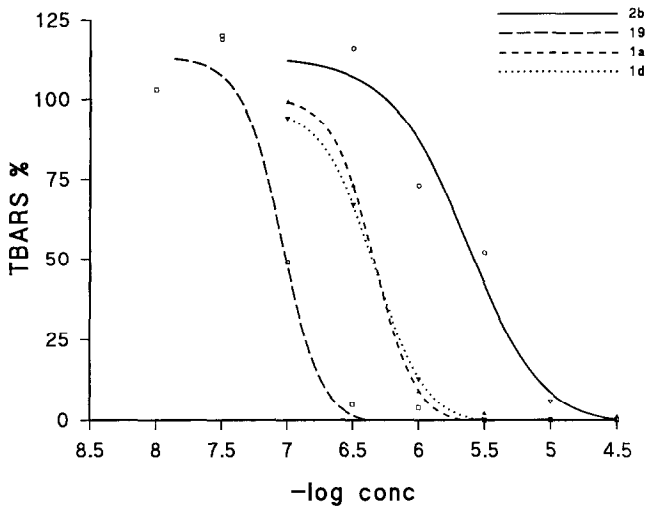

FIG. 8. TBARS formation (in % of control) in rat kidney damaged by anoxia/reoxygenation. Data simulation (sigmoidal E_{max} model) of dose-response curve (N = 1) on *in vitro* addition of the compounds 1a, 1d, 2b and 19.

with IC_{50} values ranging from 5 to 10 μM. For comparison, the potent 5'-lipoxygenase inhibitor BWA4C (**16**) had an IC_{50} value of 0.03 μM in the assay used. Both organotellurium compounds also inhibited the secretion of IL-1β in cultures of human blood monocytes stimulated with LPS. The IC_{50} values recorded were 10 μM for both compounds.

Anoxia and Reoxygenation of Rat Kidney

It was apparent early on that hypoxia produces many profound changes in the function and structure of the affected organ. It appears likely that the endogenous antioxidant defenses are overwhelmed during such conditions and that free-radical production/lipid peroxidation make a major contribution to the progression of the injury [37]. We therefore decided to study the protective effect of some organotellurium compounds on anoxia reoxygenation-induced damage in rat kidney. In the assay used, rat kidney slices were incubated in flasks under anoxic conditions for 20 min at 37°. After addition of test substances and mincing of the kidney slices to ≈1 mm pieces, the anoxic condition continued for another 10 min. The samples were then reoxygenated by vigorous bubbling of oxygen for 1 min and incubated for 30 min at 37°. After addition of desferrioxamine, the kidney residues were homogenized and analyzed for TBARS in a FIA system. As shown in Fig. 8 and summarized in Table 3, low micromolar concentrations of diaryl tellurides **1a**, **1d** and **2b** were able to inhibit 50% of the TBARS formation in the control incubation. Indenoindol derivative **19** was the most efficient protective agent tested, with an IC_{50} value of 0.1 μM. The IC_{50} values recorded for Ebselen (**7**) and Probucol (**15**) were more than three orders of magnitude higher.

Some of the test compounds were injected intravenously into the rats 1 hr before the kidneys were excised. Anoxia, reoxygenation and analysis of TBARS were then performed according to the standard procedure. As seen in Fig. 9 for compounds **1a**, **2b** and **19**, the relative inhibiting capacities *in vitro* were largely reproduced in the *ex vivo* experiments. Thus, 20 μmol/kg of compound **1a** inhibited almost 50% of the TBARS formation of the control experiment.

Ischemia/Reperfusion in the Hamster Cheek-Pouch

The hamster cheek pouch has been used in several studies for the observation of microvascular responses to several inflammatory mediators. Local I/R is known to cause an increase in the permeability of macromolecular compounds, e.g. dextran, which can be conveniently quantified as leaks of fluoroscein-labelled material in postcapillary venules. I/R resulted in reversible increases in the number of postcapillary leaks in all hamsters. In the untreated control group, the I/R-induced permeability increase reached a maximum number of 153 ± 20 leaks, which was comparable to results in previous studies [35]. Of the tested compounds, **1a**, **2b**, **8** and **19**, all inhibited I/R-induced leakage, but **1a** did not (Fig. 10). Indenoindol derivative **19** reduced vascular permeability increase already at 0.1 μM. Bradykinin stimulation following normalization after the I/R-induced re-

TABLE 3. Protective effect of synthetic compounds against oxidative damage in rat kidney

Compound	pIC_{50}[a]
1a	6.35
1d	6.34
2b	5.71[b]
7	<4[c]
10b	5.42
15	<4[c]
19	7.02

[a] ^{10}Log of the concentration required to inhibit 50% of the TBARS formed in the control experiment.

[b] 1% hydroxypropyl cyclodextrin was present in the incubation.

[c] Extrapolated value, 50% inhibition was never observed with the concentrations used.

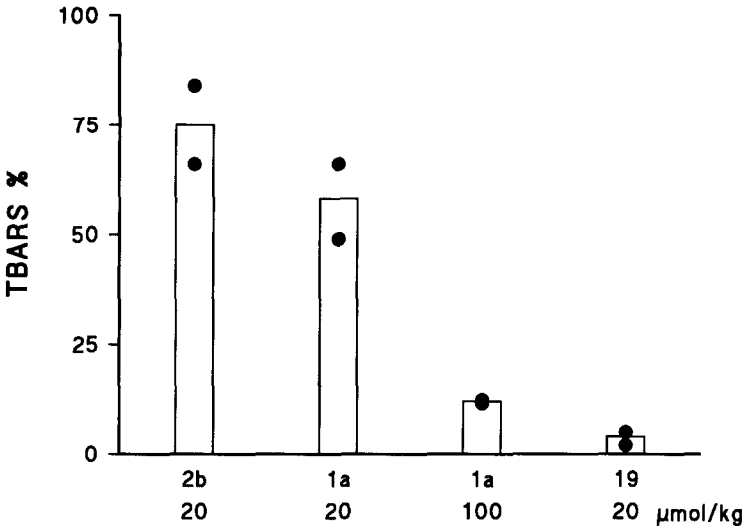

FIG. 9. TBARS formation (in % of control) in rat kidney (N = 2) damaged by anoxia/reoxygenation. Effects of compounds 1a, 2b, and 19 when administered intravenously before tissue collection.

sponse was normal and showed that the antioxidants selectively inhibited oxidant-induced changes in vascular permeability.

DISCUSSION

Evidence for the participation of reactive oxygen species and other free radicals in human disease states is mounting. However, surprisingly few model studies are known where the severity of disease has been correlated to depletion and subsequent supplementation with natural or synthetic antioxidants. Considering the complexity of the systems studied, it is always problematic to establish beyond doubt that any pharmacodynamic effect is a function solely of antioxidant activity.

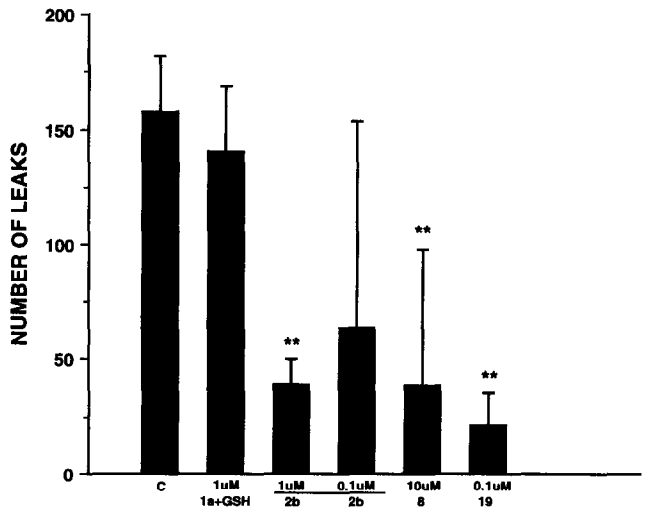

FIG. 10. The number of macromolecular leaks at reperfusion after 30 min ischemia in the hamster cheek pouch. The control group (N = 14) had no pharmacological treatment. The other groups were treated locally with compound 1a (N = 4) and glutathione, compound 2b in high (N = 5) and low (N = 5) concentration, compound 8 (N = 6) and compound 19 (N = 5), respectively. Error bars represent SEM (**$P < 0.01$).

Naturally occurring oxidative damage can be mimicked by exposing cells *in vitro* to redox-active xenobiotics such as paraquat, menadione, or adriamycine. Alternatively, hydroperoxides may be used as inducers of cell damage [38]. TBH is an attractive model oxidant for several reasons. Its lipid solubility affords rapid delivery to the cytosolic milieu and, unlike hydrogen peroxide, it is not a substrate for catalase. The metabolism of TBH as well as hydrogen peroxide is glutathione-dependent. Thus, the system is amenable to interventions which can either increase or decrease the effects of the oxidant. If the capacity of the natural antioxidant system is overwhelmed, free radical decay may give rise to reactive *t*-butoxy or *t*-butylperoxy radicals which may oxidize membrane lipids and cause cell death [39, 40]. In another toxicity hypothesis, less emphasis is placed on oxidative damage as such, and more on detrimental effects caused by draining the cell of reducing equivalents (GSH, NADPH). Rising GSSG levels would result in the oxidation of protein thiols and elevation of cytosolic calcium, leading to Ca^{2+}-dependent cell injury [41–43].

In the presence of GSH, TBH-mediated cytotoxicity in lung fibroblast cultures was inhibited by a variety of diaryl tellurides with PC_{50} values in the low micromolar range. However, when added in more than stoichiometric amount relative to the oxidant in the absence of supplemented thiol, the organotellurium compounds did not offer any protection. This seems to indicate slow delivery of the organotellurium compounds to the cytosolic milieu and the need for a stoichiometric reducing agent that could serve to regenerate diaryl telluride from the diaryltelluroxide formed in the reaction with hydroperoxide. This glutathione peroxidase-like activity of diaryl tellurides has previously been demonstrated in several *in vitro* model systems. In fact, the relative protective effects of compounds **1a–1g** in the cytotoxicity model studied largely reflect their thiol peroxidase activities as determined by using a ^1H NMR method [13] or the coupled reductase method [12]. The poor

protection offered by the sterically hindered (and thus poorly glutathione peroxidase-mimicing [13, 17]) diaryl telluride **2b** and the good protection seen with the organoselenium compounds **6a** and **7** (both with well-documented glutathione peroxidase-like effects) also suggest the catalytic peroxide decomposing capacity as the main contributor to the cytoprotective effects of organotellurium compounds in this system. It was recently shown that TBH is much more toxic to selenium-deficient murine leukemia cells than to selenium-supplemented ones [44]. The cytoprotection is probably mediated by glutathione peroxidase. Similarly, ebselen and some glutathione peroxidase mimetics of the cyclic selenenamide type protected vascular endothelial cells against damage on exposure to linoleic acid hydroperoxide [45]. The potent cytoprotective effects of diaryl tellurides **1h** and **5** could not be anticipated from the studies in cell-free models. Their good protective effects may be a result of more rapid transport to the cytosolic milieu.

When stimulated, neutrophils and monocytes are known to damage a wide spectrum of malignant and normal cells. Out of the compounds tested, telluride **2b** and its corresponding sulfur derivative **2a** turned out to be by far the most efficient protectants against PMN-mediated damage to Caco-2 cells. These compounds were active already at the 0.2 μM level. Due to steric hindrance around the tellurium atom, compound **2b** has a very limited peroxide decomposing capacity, whereas compound **2a** lacks glutathione peroxidase-like activity. However, since they are phenolic, both compounds could be expected to act as chain-breaking donating agents. Although at significantly higher concentrations, other chain-breaking antioxidants tested (vitamin E, vitamin C and probucol) also offered protection in the system investigated. Ebselen (**7**)—a peroxide decomposer, but a poor chain-breaking agent [46]—was an inefficient cytoprotectant even in micromolar concentrations.

The potent 5′-lipoxygenase inhibitor BWA4C (**16**) inhibited more than 50% of PMN-mediated damage to Caco-2 cells when present at 2 μM concentration. As redox active and peroxide-decomposing compounds [47], organotellurium compounds could also be expected to affect the release of inflammatory mediators. Diaryl tellurides **1a** and **2b** did indeed inhibit both LTB_4 and IL-1β production, but the effect was substantial only at concentrations exceeding 10 μM. Thus, we conclude that the cytoprotective effect of organotellurium compounds observed can only be marginally ascribed to their capacity to inhibit the formation of these inflammatory mediators.

As judged by the results with Ebselen (**7**), the oxidative damage in rat kidney caused by anoxia and reoxygenation is only slightly affected by the presence of a peroxide decomposer. On the other hand, the indenoindol antioxidant [48, 49] **19** inhibited 50% of the TBARS production already at the 0.1 μM level. The cytoprotective effects of diaryl tellurides **1a**, **1d**, **2b** and **10b** at concentration close to 1 μM can probably also be ascribed to their chain-breaking capacities. It is interesting that the compounds are also effective after systemic administration.

Although there are multiple components to clinical ischemic and reperfusion injury, free radical production is likely to make a major contribution to the metabolic and morphologic changes and oedema formation observed in the progression of the damage [50]. Reperfused tissues are protected in a variety of laboratory models by scavengers of superoxide radicals and by allopurinol and other inhibitors of the superoxide-generating xanthine oxidase. Therefore, it is believed that superoxide is produced as a source of more reactive radicals shortly after the onset of ischemia. The hamster cheek pouch preparation has been used in many studies for the observation of microvascular responses to several inflammatory mediators [51] and it has been shown that the I/R-induced injury which was used in the present study was completely inhibited by active EC-SOD but not by inactivated EC-SOD [35]. Furthermore, it has been shown that I/R-induced permeability increase is closely related to the number of firmly adherent leukocytes in the postcapillary venules [52]. The protective effects of compounds **2b** and **19** in the oxidatively damaged rat kidney were also seen *in vivo* in the hamster cheek pouch at similar concentrations. In contrast, compound **1a**, even when supplemented with glutathione, did not show any significant reduction in vascular permeability at 1 μM. Again, this seems to indicate that the oxidative damage caused by neutrophils or by ischemia/reperfusion is more efficiently prevented by an efficient chain-breaking antioxidant than a preventive (peroxide-decomposing) one.

With few exceptions, the effects of organotellurium compounds in biological systems have not been previously investigated. PDT is an emerging area of medicine that utilizes light, oxygen and a sensitizer to generate a cytotoxic species. Due to their capacity to generate singlet oxygen on irradiation at 775 nm, tellurapyrylium dye **20** and similar organotellurium compounds have been used for treatment of cancers by PDT [53]. Ammonium trichloro(dioxoethylene-O,O′)tellurate, AS101 (**21**) has been shown to act *in vitro* and *in vivo* as an immunomodulator. Thus, it stimulates the production of IL-1, IL-2, CSF, tumor necrosis factor, and other cytokines. AS101 has recently been shown in clinical trials to improve the result of conventional chemotherapy of cancers [54].

We have demonstrated, for the first time, potent protective/antioxidative activity of organotellurium compounds in complex cellular systems. Of the peroxide-decomposing and chain-breaking activities previously demonstrated in cell-free systems, the latter capacity seems to be more important in the cell-based assays studied. With the assumption that potential problems such as toxicity, lack of bioavailability, chemical instability, etc. can be coped with, compounds of this sort may in future be useful tools for mechanistic studies and possibly for antioxidant therapy.

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