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On the cytotoxicity and status of oxidative stress of two novel synthesized tri-aza macrocyclic diamides as studied in the V79 cell lines

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Abstract—Two tri-aza macrocycles as diamide derivatives of macrocyclic compounds possess a hydrophilic cavity surrounded by hydrophobic ring, which enables them to diffuse cell membrane and interfere with different living systems. In this study, we comparatively evaluated cytotoxicity effects of tri-aza dibenzo sulfoxide (TSD) and dibenzo sulfide (TTS) macrocyclic diamides in a range of doses (0.5–8 mM) and the role of oxidative stress in V79 cell culture.
We assessed the effects of these substances on ROS level, cellular viability, apoptosis events, activity of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and on some macromolecules' oxidative damage end-products: malondialdehyde (MDA), dityrosine, and 8-hydroxy-deoxyguanosine (8-OH-dG) that were assessed by spectrometry and HPLC methods. Both compounds revealed cytotoxicity effects on cell culture particularly at doses >1 mM after 24-h incubation. They decreased cellular viability and significantly promoted ROS generation, increased enzyme activities, and enhanced oxidative damages in which TSD was more effective. Treatment of cells with each compound alone increased significantly the percent of apoptotic events at 2 and then 4 mM. Co-treatment with alpha-tocopherol (α-TCP) drastically reduced these events. Cells' exposure with mixture of 30 μM α-tocopherol and 8 mM of each compound exerted significant decrease in the levels of ROS, enzyme activities, and oxidative damage biomarkers.
As conclusion, our study documented the oxidative radical forming ability of the studied compounds and further strengthened the documentation of their cytotoxicity effects through lipids, proteins and DNA oxidation damages.

1. Introduction

Macrocycles as diamide derivatives are macrocyclic compounds, which possess similar properties to those of crown ethers. A plethora of these compounds has been synthesized since Pedersen reported the ability of these macrocyclic ethers to bind alkali metal cations.[1] Macrocyclic diamides are valuable intermediates formed during the preparation of aza-crown compounds.[2] They have high capability in selective and effective complexation with a variety of transition and heavy metals, molecular ions, and neutral molecules. Accordingly, macrocyclic diamides and corresponding aza-crown compounds have gained a great deal of attention due to their wide applications in chemistry, biology, molecular recognition, medical and industrial uses, and agriculture. These compounds structurally possess a hydrophilic cavity surrounded by a hydrophobic ring and are characterized by having a different number of ethylene oxide units and amide groups.[3] Due to their ionophoretic properties that enable them to transport across cell membranes and their interference as xenobiotics with different living systems, there is an increasing interest in their biochemical stability and cytotoxicity effects. The cytotoxicity effect of many xenobiotic compounds to cells often occurs through the induction of oxidative stress and apoptosis with possible involvement of overproduction of reactive oxygen species (ROS). Apoptosis, the physiological mode of cell death, is related to the regulation of development and homostasis.[4,5]

Reactive oxygen species are normally produced in aerobic growth conditions within cells, but they are elevated

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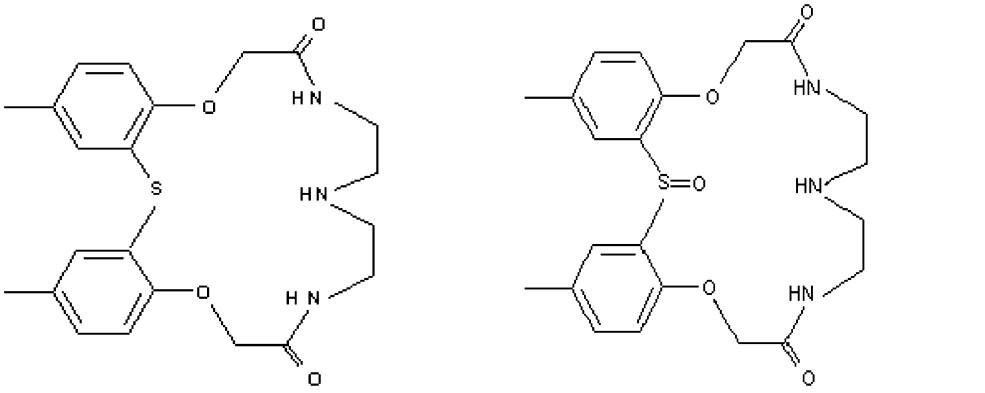

Dibenzo sulfide (TTD) macrocyclic diamide **Dibenzo sulfoxide (TSD) macrocyclic diamide**

Figure 1. Structural formulas of tested compounds.

under the influence of exogenous treatments. ROS attack on cellular membrane lipids, proteins, and even DNA may cause oxidative injury. The alteration of membrane phospholipids through lipid peroxidation is considered to be one of the primary key events in oxidative damages.[6] Free oxidative radicals can also cause extensive chemical modifications and alterations in DNA and nucleoproteins, including modified bases and sugars and even strand breaks.[7] Since half-lives of ROS are extremely short, their stable end product of oxidative damages to cellular macromolecules can be used for oxidative stress monitoring.[8] Dityrosine, a hallmark of oxidized proteins, 8-OH-deoxyguanosin (8-OH-dG), a biomarker of oxidative DNA damage, and malondialdehyde (MDA), a stable product of lipid peroxidation, are, closely correlated with level of oxidative stress.[9]

On the other hand, antioxidant defense systems have co-evolved with aerobic metabolism to counteract their oxidative consequences. Among them, superoxide dismutase (SOD) catalyzes dismutation of the superoxide anion to hydrogen peroxide and molecular oxygen.[10] Catalase inactivates hydrogen peroxide to oxygen and water.[11] Glutathione peroxidase protects the membrane lipids from oxidative damage and detoxifies the organic peroxide.[12]

Since, no study has yet been performed on cytotoxicity of two novel synthesized tri-aza macrocyclic diamides, the aim of our study was to check their cytotoxicity on V79 Chinese hamster cell line as a model of mammalian cells. We also investigated the possibility whether this effect was mediated by apoptosis in which ROS may be involved. Accordingly, ROS generation potential of these compounds and the levels of oxidative damage biomarkers of DNA, lipids, and proteins were determined (Fig. 1).

2. Results

The effects of two kinds of macrocyclic diamides on incubated V79 cells were assayed using the MTT test over a range of doses (0.5–8 mM). Our basic results in present study showed that both compounds were cytotoxic in the studied range (Fig. 2). As comparison, TSD macrocyclic diamide inhibited markedly and significantly the viability after 24-h incubation with respect to TTD macrocyclic diamide. After exposure of cells to 8 mM of TSD macrocyclic diamide, around 24% of viability was observed.

Treatment of cells with each macrocyclic diamide demonstrated that they were capable of promoting ROS generation in V79 cell culture in a dose-dependent manner (Fig. 3). On the other hand, marked increase in ROS was only significant at 2 and then at 8 mM of TSD against TTD. In this condition, exposure of cells to each of macrocyclic diamides at 8 mM caused the formation of ROS, elevating up to 3.9- and 2.5-fold of control for TSD and TTD, respectively.

Data of morphological analysis for apoptosis detection are revealed in Table 1. Both compounds caused apoptosis in 2 and 4 mM treatment, in which TSD caused significantly greater percent of apoptotic events with respect to TTD. On the other hand, the effect of α-tocopherol on TSD and TTD-induced apoptosis was then investigated. Results showed that co-treatment with α-tocopherol reduced drastically the percent of macrocyclic diamide-induced apoptosis and could rescue cell viability. For each compound, the percentage of apoptotic and/or necrotic cells obtained after treatment with 4 mM alone was significantly higher than that of 2 mm.

Table 2 demonstrates antioxidative enzyme activities in cell culture after 24 h incubation. Exposure of cells to each compound significantly increased these enzyme activities at concentrations of 2 mM and higher with respect to control. Among these enzymes, only the significant increase of SOD was observed at 1 mM in treatments for each compound as compared with control. However, the SOD and CAT activities in cells associated with TSD macrocyclic diamide exposure were insignificantly higher than TTD macrocyclic diamide treatments. On the other hand, there were insignificant

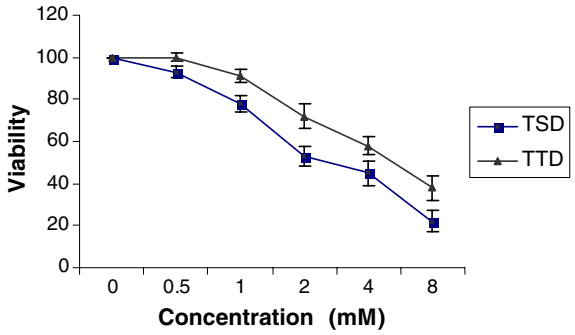

Figure 2. Cytotoxicity of TSD and TTD in V79 cells. Viability was assessed by MTT assay after 24 h treatments. Results are expressed as percent of corresponding control and representative of at least three duplicated independent experiments.

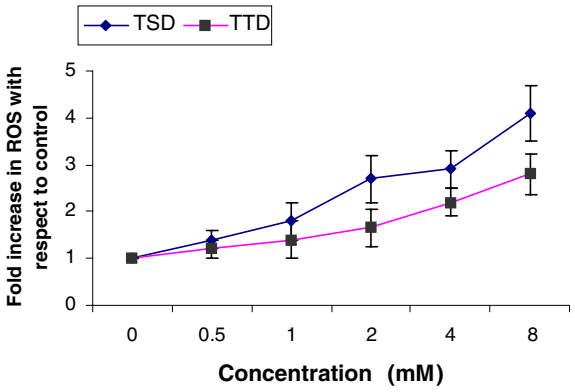

Figure 3. ROS production levels in V79 Cells, treated for 24 h with different concentrations of TTD and/or TSD. Results are the means ± SD of at least three replicated independent experiments.

decreases in GPX activities in cells treated with each concentration of TSD as compared with TTD macrocyclic diamide.

Table 3 reveals that the formation of MDA, dityrosine, and 8-OH-dG as lipids, proteins and DNA damage parameters occurred in control condition, although their levels were elevated significantly in culture cells exposed to the studied macrocyclic diamides. These increases were significant for all parameters when cells were exposed to each compound at 2 mM and over. On the other hand, dityrosine in treated cells with TTD and MDA in treated cells with TSD revealed significant increase as compared with controls at 1 mM and over. Moreover, among these parameters, there was only significant increase in the level of 8-OH-dG in TSD treated cells with respect to TTD. Besides, dityrosine concentrations were slightly and insignificantly lower in cells exposed to TSD as compared with TTD. Cells treated with TSD revealed insignificant increase in MDA levels in comparison with TTD.

Whether the studied compounds induced oxidative stress was investigated by using α-tocopherol as antioxidant, and cells were treated in the presence and absence of α-TCP. Table 4 demonstrates that antioxidative enzyme activities along with the levels of MDA, dityrosine, and 8-OH-dG diminished significantly in each macrocyclic diamide (8 mM) treated cells in the presence of α-tocopherol (30 μM) when compared with treated cultures in the absence of this antioxidant. The presence of α-tocopherol during each compound treatment of cells altered these parameter levels insignificantly against their control levels and also with respect to each other. Antioxidant alone induced an insignificant decrease in the studied parameters in this table as compared with control.

3. Discussion

Many xenobiotics or exogenous chemicals may exert toxic effects when animal cells metabolize them. Cytotoxicity effects of these compounds have different biological mechanism patterns among which the interference with ROS generation process within cells is of most interest in toxicological studies. Cytotoxicity results for crown ethers have been tested and confirmed in some mammalian cell cultures. However, our study was the first in its kind that investigated cytotoxicity effects of two novel synthesized macrocyclic diamides following V79 cell treatment. V79 cell line was considered as a living system model that has been widely used to clarify the mechanism of cytotoxicity and ROS effects in response to various compounds.[13] In this study, we have demonstrated that both tested compounds potentially inhibit the proliferation of V79 cells through inducing a cytotoxic effect and triggering apoptosis in a dose-dependent manner. In accordance with our results, Arenaz et al.[14] showed that substituted and unsubstituted forms of two hetero macrocyclic crown ethers: 18-crown-6 and/or 15-crown-5 were cytotoxic for V79 cell culture after

Table 1. Percent of viable, apoptotic, and necrotic cells detected morphologically on the basis of the differential uptake of acridine orange and ethidium bromide in V79 cells incubated with TTD, TSD in the presence (30 μM) and absence of α-TCP[a]

Characteristic	Control	TSD (mM)		TTD (mM)	
		2	4	2	4
Viable	98.1 ± 3.1	54.4 ± 1.9[b]	41.2 ± 2.8	71.4 ± 3.2[b]	58.2 ± 2.4[c]
Apoptotic	0.0 ± 0.0	40.2 ± 2.3[b]	51.4 ± 2.7	25.1 ± 0.9[b]	37.3 ± 1.7[c]
Necrotic	1.4 ± 0.0	4.6 ± 0.2[b]	6.3 ± 0.4	2.6 ± 0.1[b]	4.1 ± 0.3[c]
Apoptotic in α-TCP	0.0 ± 0.0	28.5 ± 1.2[b]	32.7 ± 1.8	17.4 ± 0.8[b]	22.6 ± 0.9[c]
Viable in α-TCP	96.3 ± 2.9	71.2 ± 2.3[b]	52.6 ± 2.6	80.5 ± 3.1[b]	69.1 ± 2.3[c]

[a] Data are presented as means ± SD.
[b] Significant difference from treatments with 4 mM macrocyclic diamide ($p < 0.05$).
[c] Significant difference from treatments with 4 mM TSD.

Table 2. Activities of antioxidant enzymes in V79 cells after 24-h treatments[a]

Concentration (mM)	TSD			TTD		
	SOD (U/mg protein)	CAT (μM/min/mg)	GPX (nM/min/mg)	SOD (U/mg protein)	CAT (μM/min/mg)	GPX (nM/min/mg)
0	17.15 ± 4.3	6.12 ± 2.4	19.14 ± 4.2	17.15 ± 4.3	6.12 ± 2.4	19.14 ± 4.2
0.5	14.41 ± 4.8	6.81 ± 1.9	16.61 ± 3.2	18.82 ± 3.1	5.48 ± 1.6	18.63 ± 3.7
1	22.14 ± 5.6[b]	9.71 ± 3.8	22.14 ± 4.4	21.32 ± 5.4	8.47 ± 3.8	23.34 ± 5.6
2	36.41 ± 7.1[b]	15.26 ± 5.7[b]	26.34 ± 6.5[b]	31.25 ± 7.2[b]	12.17 ± 5.4[b]	30.65 ± 7.6[b]
4	48.68 ± 10.5[b]	27.24 ± 7.4[b]	33.41 ± 8.3[b]	40.12 ± 9.2[b]	22.37 ± 4.4[b]	37.55 ± 9.1[b]
8	56.36 ± 12.4[b]	35.73 ± 8.7[b]	41.14 ± 9.3[b]	49.25 ± 10.2[b]	27.17 ± 6.2[b]	47.61 ± 10.2[b]

[a] Data are presented as means ± SD (number of replicates = 5).
[b] Significant difference from control ($p < 0.05$).

Table 3. The levels of lipid peroxidation, protein and DNA oxidative damage biomarkers of V79 cells after 24 h treatment with TTD and/or TSD[a]

Concentration (mM)	TSD			TTD		
	MDA (nM/mg protein)	Dityrosine[b] (nM/mg protein)	8-OH-dG/10^5dG	MDA (nM/mg protein)	Dityrosine[b] (nm/mg protein)	8-OH-dG/10^5dG
0	9.37 ± 4.12	0.59 ± 0.21	5.2 ± 0.22	9.37 ± 4.12	0.59 ± 0.21	5.2 ± 0.24
0.5	8.72 ± 3.46	0.54 ± 0.17	4.7 ± 0.15	11.17 ± 3.28	0.74 ± 0.26	4.4 ± 0.13
1	16.53 ± 5.84[c]	0.75 ± 0.29	5.5 ± 0.19	13.77 ± 3.44	1.19 ± 0.45[c]	5.0 ± 0.21
2	25.16 ± 6.12[c]	1.61 ± 0.57[c]	7.4 ± 0.27[c]	21.46 ± 3.71[c]	2.45 ± 0.75[c]	5.8 ± 0.38
4	36.63 ± 9.19[c]	3.88 ± 1.67[c]	8.5 ± 0.48[c]	35.61 ± 5.17[c]	4.31 ± 1.44[c]	7.3 ± 0.34[c]
8	55.12 ± 11.58[c]	6.41 ± 2.15[c]	9.1 ± 0.39[c]	52.55 ± 5.79[c]	8.27 ± 2.30[c]	6.9 ± 0.39[c]

[a] Data are presented as mean ± SD (number of replicates = 5).
[b] Number of replicates = 4.
[c] Significant difference from control ($p < 0.05$).

Table 4. Activities of antioxidant enzymes and the levels of oxidative damage parameters after 24-h treatments with 8 mM TSD and/or TTD in the presence and absence of 30 μM α-tocopherol[a]

Treatment	GPX (nM/min/mg)	SOD (U/mg protein)	CAT (μM/min/mg)	MDA (nM/mg protein)	Dityrosine[b] (nM/mg protein)	8-OH-dG/10^5dG
None	19.14 ± 4.2	17.15 ± 4.30	6.12 ± 2.4	9.37 ± 4.12	0.59 ± 0.21	5.2 ± 0.22
TSD	41.14 ± 9.3[c]	56.36 ± 12.4[c]	35.73 ± 8.7[c]	55.12 ± 11.58[c]	6.41 ± 2.15[c]	9.1 ± 0.39[c]
TSD + α-TCP	22.41 ± 4.28	23.54 ± 4.41	10.15 ± 2.81	17.63 ± 3.47[d]	2.21 ± 0.14[d]	6.40 ± 0.31[d]
TTD	47.61 ± 10.2[c]	49.25 ± 10.2[c]	27.17 ± 6.2[c]	52.55 ± 5.79[c]	8.27 ± 2.30[c]	6.9 ± 0.39[c]
TTD + α-TCP	20.79 ± 3.65	21.80 ± 4.32	8.77 ± 2.19	15.01 ± 3.28	1.43 ± 0.23	5.76 ± 0.28
α-TCP	14.09 ± 2.58	13.24 ± 3.36	4.50 ± 0.79	7.51 ± 1.27	0.42 ± 0.14	3.51 ± 0.14

[a] Data are presented as means ± SD (number of replicates = 5).
[b] Number of replicates = 4.
[c] Significant difference from control and from treatments in the presence of α-TCP ($p < 0.05$).
[d] Significant difference from control.

24 h. On the other hand, the exhibition of survival profile was similar to both compounds in our study, suggesting that they may have similar toxicity mechanism, in which differences in the observed cytotoxicity intensity may be a function of their ability to induce perturbations in cell membrane or to their individual chemical characteristics in metabolism by cells.[15] Moreover, many studies documented that alteration in viability of different cultures is associated with a significant enhancement of oxygen metabolism and is accompanied by the formation of reactive by-products including ROS.[16,17] In agreement with these studies, our V79 cell culture incubation with each of macrocyclic diamides caused induction of oxidative stress leading to elevation in ROS level as evaluated directly. Accordingly, both compounds were potent in ROS generation. Reactive oxygen species formed within cells can oxidize biomolecules and this may lead to cell death. Oxidative stress is also known to induce apoptosis in a variety of cell types by activating intracellular cell death signaling cascades following ROS elevation.[18] An alternative approach to clarify the mechanisms for cellular oxidative damage by ROS is to quantify stable end products of ROS reactions with macromolecules. Malondialdehyde (MDA) appears when ROS attack lipids by peroxidation mechanism and is the most frequently used test for lipid damages.[19] In addition, *o*,*o*′-dityrosine is produced when hydroxyl radicals cross-link two molecules of tyrosyl radicals and is suggested as a promising biomarker for oxidative damage to proteins.[20] Among different DNA base adducts, the most abundant and oxidatively stable is 8-OH-dG, whose level represents the net effects of the rates of oxidative DNA damage and repair.[18] In particular, many studies confirmed that decrease in cell viability after exposure to ROS generator compounds was accompanied by marked increases in formation of

MDA,[19] 8-OH-dG,[21] and *o*,*o*′-dityrosine.[22] In this study, we found significant rises in these biomarkers, providing indirect evidences that both tri-aza macrocyclic diamides' exposure was relevant sources of oxidative stress. For cells to avoid or limit damages in an oxidative environment, elaborate antioxidant systems are necessary.[23,24] Up gradations of antioxidant defense system through overexpression of antioxidative enzymes have also been shown after oxidative exposure in V79 fibroblasts.[25]

Superoxide radicals are accumulated by SOD, whereas, hydrogen peroxides can be scavenged by CAT and GPX. Our results also showed considerable increase in all antioxidative enzyme activities in treated cells after 24 h in which TSD was more effective than TTD macrocyclic diamides.

Alpha-tocopherol (α-TCP) as a naturally occurring substance is one of the most important inhibitors of free-radical chain reactions and is included in non-enzymatic defense system against ROS by scavenging them. With regard to the importance of this substance, we, along with other authors, observed considerable decrease effect of α-TCP on biomarker's levels of key cellular macromolecules' oxidative damage; 8-OH-dG, dityrosine, MDA and also on enzymatic defense system.[19,25,26]

4. Conclusion

The present study revealed that both tri-aza macrocyclic diamides were able to elevate ROS levels in V79 cells and further strengthen the documentation of their cytotoxicity effects through proteins, lipids, and DNA oxidative damages. The involvement and initiation pattern of our studied compounds in these processes may possibly be attributed to their ionophoric characteristics and membranes permeability. Each tri-aza macrocyclic diamide has its own cavity size that is directly compatible with the physiologically important ions, Na^+, K^+, and Ca^{2+}. On the other hand, the presence of additional oxygen atom in sulfoxide group of TSD macrocyclic diamide causes considerable increase in its hydrogen binding capacity. It is our conviction that the obvious difference in their biological intensity effects may partially be due to these discriminative structural characteristics. A number of other features have also been shown to determine the potential with which a macrocyclic diamide molecule contracts with living processes. The most important include the ratio of the size of macrocyclic diamides' cavity to the size of an ion, number and position of oxygen atom in the ring, kind and number of repeating units' arrangement.[27] However, the precise mechanisms in detail through which macrocyclic diamides increase cellular level of ROS and the pattern of their oxidative effects on V79 cells are unclear and need further investigations.

5. Experimental

5.1. Chemicals

Highly pure methylthiazoldiphenyl-tetrazoliumbromide and dihydroethidium were purchased from Sigma Chemical Company (St. Louis, MO). Culture medium and fetal bovine serum were from Gibco Laboratories (Paisley, Scotland). Culture medium was diluted in distilled water. Two newly synthesized and crystallized macrocyclic diamides were kindly gifted by Professor A. Shockravi, from laboratory of organic chemistry, University of Tarbiat Moallem.[2,28]

Dibenzo sulfide macrocyclic diamide was 7,10,13-triaza-1-thia-4,16-dioxa-20,24-dimethyl-2,3:17,18-dibenzo-cyclooctadecane-6,14-dione (TTD), and dibenzo sulfoxide macrocyclic diamide was 7,10,13-triaza-1-sulfoxo-4,16-dioxa-20,24-dimethyl-2,3:17,18-dibenzo-cyclooctadecane-6,14-dione (TSD).

Macrocyclic diamides were utilized without further purification. Other chemicals were of the highest quality and purity, commercially available. On the day of exposure, the standards of macrocyclic diamides were dissolved in ethanol, to prepare stock solutions. For each treatment, the stock solution was added to the culture media solution to provide the final favorite treatment concentration. Culture media solutions were sterilized by filtration through a 0.22-μ filter (Acrodisc, Gelman). The slight amount of ethanol in the incubating solution was less than 0.1%. Subsequent experiments presented equivalent amount of ethanol, however, had no apparent effect on biological parameters.

5.2. Cell cultures

V79 Chinese hamster cells (V79-UL) were maintained in minimal essential medium (MEM) with Earle's salts, supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics. Cells were cultivated in a humidified incubator at 37 °C with 5% CO_2 at pH 7.2 and harvested with 0.15% trypsin and 0.08% EDTA.[29] Cell culture media were obtained from Biochrom (Berlin, Germany). Potassium bromate ($KBrO_3$) and potassium superoxide (KO_2) were purchased from Sigma (Munich, Germany) and dissolved immediately before use in Hanks' balanced salt solution. $KbrO_3$ concentrations between 5 and 20 mM and KO_2 concentrations between 0.15 and 0.6 mM were tested according to published experiments with mammalian cells.

For MTT-assay, cells were plated in flat-bottomed 96-microtiter plates (Nunclon, Nunc) in 100 μl growth medium at densities of 5000 and 10,000 cells per well and allowed to attach for 24 h at 37 °C in 5% CO_2. At both densities cells remained in exponential growth phase during the exposure time. 100 μl of culture medium (control) or culture medium containing test substance was added to each well with six replicas for each concentration. The plates were then incubated for further 24 h. Wells without cells (background) were always examined in parallel.

5.3. MTT assay

The assay was performed essentially as described by Alley et al.[30] Briefly, cells grown in 96-well tissue culture plates were treated with various doses of crown ethers

for 24 h and at the end of the incubation period 50 μl of MTT [3(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; Sigma–Aldrich Co.] solution in PBS was added to each well (final concentration 0.4 mg/ml) and cultures were incubated at 37 °C in 5% CO_2 for 4 h. Then medium was carefully removed by pipetting and formazan crystals were dissolved in 150 μl DMSO. After 10 min agitation on a shaker, the absorbance was measured using a microtiter plate reader (SLT-Labinstruments, Germany) at a wavelength of 550 nm (test) and 620 nm (reference), respectively.

5.4. Spectrophotometric assay of ROS production

Cells treated with different concentrations of macrocyclic diamides and untreated control cells were centrifuged and incubated with 2 μM of dihydroethidium (λ_{ex} = 360 nm, λ_{em} = 420 nm) for 10 min, washed with phosphate-buffered saline, and then analyzed by spectrofluorometry in RPMI-1640 medium without phenol red. In the presence of ROS, dihydroethidium is oxidized to ethidium and fluoresces in red (λ_{em} = 640 nm). The 640/420 nm fluorescence intensity ratio permits one to evaluate the production of ROS in living cells. Raw data were normalized with respect to control value and results expressed as the fold increase of 640/420 fluorescence intensity ratios. Results are given as means ± SD of three independent experiments.[31]

5.5. High-performance liquid chromatography (HPLC) assay of TBA + MDA

After macrocyclic diamide incubation, the V79 cells were scraped off, using a cell craper, and centrifuged (5 min, 800*g*) and were washed twice in nominally calcium and magnesium free MEM. The cells were resuspended in 0.8 ml NaCl (0.9%, w/v) at 4 °C. Aliquots were taken for protein analysis (Bio-Rad),[32] and the cells were lysed and proteins precipitated with 40% trichloroacetic acid (TCA), w/v. The MDA assay is based on the condensation of one molecule malondialdehyde with two molecules of thiobarbituric acid in the presence of reduced reagent volumes to increase sensitivity, generating a chromogen with UV absorbance.[33]

The TBA + MDA complex was analyzed by HPLC essentially as described by Bird et al.[34] Briefly, the HPLC system consisted of a Hewlett + Packard 1050 gradient pump (Avondale, PA) equipped with an automatic injector, a 1050 diode-array absorption detector, and a personal computer using Chem Station Software from Hewlett + Packard. Aliquots of the TBA + MDA samples were injected on a 5 mm Supelcosil LC-18 reversed phase column (30 × 4.6 mm). The mobile phase consisted of 15% methanol in double-distilled water degassed by filtering through a 0.5 μm filter (Millipore, Bedford, MA). The flow rate was 2 ml/min. MDA + TBA standards were prepared using tetraethoxypropane.

The absorption spectra of standards and samples were identical with a characteristic peak at 540 nm. Measurements were expressed in terms of malondialdehyde (MDA) normalized to the cell protein content.

5.6. SOD activity assay

SOD (E.C. 1.15.1.1) activity assay was performed according to the Spitz's method.[35] Cells were homogenized in 50 mM potassium phosphate buffer (pH 7.8). Total SOD activity was assayed at 25 °C by the nitroblue tetrazolium (NBT) reduction assay with bathocuproine sulfonate. The rate of reduction of NBT by superoxide, which was generated from xanthine and xanthine oxidase, was monitored spectrophotometrically at 560 nm. One unit of SOD was defined as the amount of protein, which causes a 50% inhibition of the rate of NBT reduction.

5.7. CAT activity

CAT (E.C. 1.11.1.6) activity was measured by the method of Beers and Sizer with slight modifications.[36] For both CAT and GPX activity assays, same preparation of samples was used by homogenizing cells in 50 mM phosphate buffer (pH 7.4). Supernatant from 1000*g* centrifugation of cell homogenates was used for assays. The assay reaction for CAT consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.02 M H_2O_2, and samples in a total volume of 1 ml. The reaction was carried out at 25 °C. The rate of absorbance change (ΔA/min) at 240 nm was recorded, which indicated the decomposition of H_2O_2. Activities were calculated using the molar extinction coefficient of H_2O_2 at 240 nm, 43.59 L/mol cm.

5.8. GPX activity assay

Activity assays of selenium-dependent GPX (E.C. 1.11.1.9) were performed as previously described.[37] The coupling reagent consisted of 50 mM Tris–ClH buffer (pH 7.7), 100 μl of 0.01 M/L glutathione, 100 μl glutathione reductase (0.24 U), and 100 μl of 1.05 mM/L NADPH. The coupling reagent in 875 and 100 μl of sample was incubated for 2 min at 25 °C and hydrogen peroxide (final 25 μM) was added to initiate the reaction. ΔA/min at 340 nm was recorded. ΔA/min of blank, in which sample was replaced by Tris–ClH buffer, was also recorded. The net ΔA/min of samples after subtracting the blank rate was used to calculate the GPX activity using the molar extinction coefficient of NADPH at 340 nm, 6220 L/mol cm.

5.9. Measurement of dityrosine

Purification of *o*′,*o*′-dityrosine was accomplished by preparative HPLC. *o*′,*o*′-dityrosine was recovered by gradient elution from the C-18 column (Econosil C18, 250 mm × 10 mm).[8] The composition of eluent varied linearly from acetonitrile/water/TFA (1:99:0.02) to acetonitrile/water/TFA (20:80:0.02) over 25 min. The gradient was started 5 min after the injection. A flow rate of 4 ml/min was used. *o*′,*o*′-dityrosine was analyzed by reversed-phase HPLC with simultaneous UV-detection (280 nm) and fluorescence-detection (ex. 280 nm, em. 410 nm). A phenomenex inertsil ODS 2 (150 mm × 4.6 mm, 5 μm) HPLC column (Bester, Amsterdam, The Netherlands) equipped with a guard column was

used for these analyses. A gradient was formed from 10 mM ammonium acetate, adjusted to pH 4.5 with acetic acid, and methanol, starting with 1% methanol and increasing to 10% over 30 min. The flow rate was 0.8 ml/min. A standard solution of dityrosine was prepared according to the method of Amado et al.[38] Dityro sine was quantified by assuming that its generation from the reaction of tyrosine with horseradish peroxidase in the presence of H_2O_2 was quantitative (using the extinction coefficient $e_{315} = 4.5\ mM^{-1}\ cm^{-1}$ at pH 7.5).

5.10. Determination of 8-hydroxydeoxyguanosine (8-OH-dG)

A sensitive analytical technique, described elsewhere,[39] was used to measure the amount of 8-OH-dG by HPLC (Unicam; Ultrasphere-ODS; 5 μm, 4.6 × 250 nm) coupled to an electrochemical detector (ESA Coulochem II: guard cell, 0.35 V; detector 1, 0.15 V; and detector 2, 0.30 V). Briefly, the nuclear DNA from cells was extracted using the DNA Extractor WB Kit (Wako Biochemicals, Osaka, Japan). The extracted DNA samples were digested with nuclease P1 (0.8 U, Yamasa, Chiba, Japan) and acid phosphatase (1U, Sigma Chemical) in a solution of 1 mM EDTA and 10 mM sodium acetate (pH 4.5). After incubation at 37 °C for 30 min, the iron exchange resin Muromac was added to remove the NaI and the mixture was centrifuged at 15,000 rpm for 5 min. The supernatant was transferred to an Ultrafree Probind filter (Milipore, Bedford, MA) and then centrifuged at 10,000 rpm for 2 min. The filtered deoxynucleoside was injected onto the HPLC column. Standard solutions of dG (0.5 mg/ml) and 8-OH-dG (5 ng/ml) were used for measuring of these compounds in cells that were treated or were in control condition. The molar ratio of 8-OH-dG to dG was calculated based on the integrated peak area of authentic 8-OH-dG with an electrochemical detector and UV absorbance of dG using a millennium software (Waters, Milford, MA). The titer of 8-OH-dG is shown as the number per 10^5 guanine residues.

5.11. Apoptosis detection

Apoptosis detection was performed by morphological analysis after treating sub-confluent cells (24 h after seeding) with 2 and/or 4 mM of each of tested compounds for 24 h. For morphological observation, untreated (control) and treated cells were stained with 0.1 mg/ml acridine orange/ethidium bromide (AO/EB) solution and 5 μl of cell suspension was placed onto a Thoma-Zeiss and examined under fluorescence microscope (Axiophot, Zeiss). Based on the differential uptake of fluorescent DNA binding dyes acridine orange and ethidium bromide, viable cells (bright green chromatin) can be discriminated from early apoptotic cells (bright green highly condensed or fragmented chromatin), late apoptotic cells (bright orange highly condensed or fragmented chromatin), and necrotic cells (bright orange chromatin).[40] Quantification was done by counting a minimum of 250 cells/condition in four independent experiments. Morphological cell death quantification was also performed in V79 cell line co-treated at sub-confluence with 2 mM of each of macrocyclic diamides and 30 μM α-tocopherol.

5.12. Statistical analysis

Each experiment ($n \geqslant 3$) was run at least in duplicate and the data presented are given as means ± SD. Statistical analysis of data was performed by analysis of variance (ANOVA) using the SPSS-PC1 version 4.01 (SPSS INC., Chicago, IL). A level of $P < 0.05$ was considered statistically significant for all experiments.

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