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Evaluation of the genotoxic properties of dibenzo sulfide (DBS) and dibenzo sulfoxide (DBSO) macrocyclic diamides (DSOMD) in the human lung cell line A549

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ABSTRACT

Background: Macrocyclic diamides are derivatives of two Tri-Aza macrocyclic crown ethers, poses the ability to transport across membrane and interfere with different living systems. The genotoxic potential along with cytotoxicity of dibenzo sulfoxide (DBSO) and dibenzo sulfide (DBS) macrocyclic diamides were comparatively checked by various biochemical tests in A549 cell line.

Methods: We assessed the effects of these substances in a range doses (0.1 to 8 mMol) on ROS level, cellular viability, colony efficiency, micronucleus (MN), comet test and on HPRT gene mutation.

Results: Both compounds revealed cytotoxicity effects on cell culture particularly at doses >1 mMol after 24 h incubation. They decrease colony efficiency and cellular viability of cells and significantly promoted ROS generation. There were also marked increase in; MN frequencies, DNA migration in comet assay and gene mutation induction at the HPRT locus, in which DBSOMD was more effective. Genotoxicity appeared at concentrations that were cytotoxic.

Conclusion: We documented ROS generation potential of the studied crown ethers and their possible consequence cytotoxicity. We also presented indications of genotoxicity effects through DNA damages.

KEYWORDS

Crown ether;
Genotoxicity;
A549 cells;
Reactive oxygen species;
Micronucleus;
HPRT;
Comet assay.

INTRODUCTION

The crown ethers were the first neutral synthetic compounds, which were found to be able to from stable complexes with alkali metal ions[1]. Crown rings with spatial conformation will endow functional molecular with novel performance and character for its hydrophobicity of outer ethylene group (substituted or unsubstituted) and orderly arrangement of inner oxa atom. The structural simplicity of crown ether molecules allows a wide variety of analogs to be synthesized with varying ring sizes and number of oxygen atoms[2]. Be-

cause of their characteristics, considerable attention has been paid to the effects of their new synthesized kinds on biological systems. In spit of cytotoxic effects of known crown ethers on prokaryotic and eukaryotic organisms, many studies suggested they are not genotoxic in mammalian cells[3]. On the other hand, crown ethers have many derivatives including macrocyclic diamides (as Tri-Aza heteromacrocycles) that their possible effects on biological systems are of recent scientific interests and under investigation.

These materials as exogenous compounds may interfere with oxidative-mediated toxicity within cells via free radicals over production. Oxidative stress results when reactive oxygen species (ROS) are not adequately removed. This can happen if the formation of ROS is increased beyond the ability of antioxidant defense to cope with them and/or if antioxidants are depleted[4]. Free radicals can cause extensive chemical modifications in DNA including modified bases and strand breaks[5]. Since oxidative DNA damage is continuously induced and repaired, a steady-state level of oxidative DNA damage is expected under normal conditions. Oxidative stress causes an increase in oxidative DNA damage that the comet assay is particularly suited for measuring and detected them as DNA breaks and alkali-labile sites with high sensitivity. Some of these alterations represent pre-mutagenic lesions and are supposed to be directly involved in the process of carcinogenesis[6]. Comparative studies of ROS-producing substances allow correlating the pattern of oxidative damages to pattern of induced genotoxic and mutagenic effects. In this way the mutagenic potential of various types of ROS can be evaluated[7]. The genotoxic invitro effects of organic compounds can be evaluated by comet assay and micronucleus test, and mutagenicity can be determined by the established HPRT-mutagenicity test in mammalian cells. Since, no study has not yet been performed on genotoxicity of two novel synthesized Tri-Aza heteromacrocycles, the aim of our study was to check their genotoxicity on the human lung epithelial cell line A549 as a model of human type II alveolar cells. We also investigated their ROS generation potential and determined whether the induced damages also leaded to the formation of gene mutations at the HPRT gene. Furthermore, we tested the ability of these compounds to induce DNA effects in the comet assay that detects DNA strand breaks and alkali-labile sites with high sensitivity[8].

MATERIAL AND METHODS

Chemicals

High pure methylthiazoldiphenyl-tetrazoliumbromide and dihydroethidium were purchased from Sigma Chemical Company (St. Louis, MO). Culture medium and fetal bovine serum Gibco Laboratories (Paisley, Scotland). Culture medium was diluted in distilled water. Two new synthesized and crystallized macrocyclic diamides were kindly gifted by Professor A. Shockravi, from laboratory of organic chemistry, University of Tarbiat Moalem[10, 11].

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, 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.

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.

Cell cultures

The human lung carcinoma epithelial cell line A549 was obtained from Pasteur Institute Cell Bank, Iran). The cells were maintained in 75 cm^2 tissue culture flasks (Becton Dickinson) in culture medium: Dulbecco's modified Eagle medium (DMEM; Biochrom) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and streptomycin (Gibco BRL) at 37 ^oC in a humidified atmosphere with 5% CO_2. For the assays and the continuous cell propagation adherent monolayers in exponential growth

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phase (about 80% confluence) were harvested with trypsin/EDTA (0.05%/0.02%) in phosphate buffered saline (PBS; Biochrom).

For MTT-assay, cells were plated in flat bottom 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 were 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.

MTT assay

The assay was performed essentially as described by Alley et al.,[11]. 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.

Relative cloning efficiency CE as a measure for long term survivability was determined by plating 200 cells into four replicate petri dishes (Ø 60 mm). After cultivation over night, cells were treated as indicated, washed twice and then cultivated for another 7 days in fresh medium. Colonies were fixed, stained and counted. Survival was determined in relation to the corresponding control.

Spectrophotometric assay of ROS production

Cells treated with different concentration of macrocyclic diamides, and untreated control cells were centrifuged and incubated with 2 µM of dihydroethidium (λ_{ex}=360nm, λ_{em}=420 nm) for 10 min, washed with phosphate-buffered saline, and then analysed by spectrofluorometry in RPMI-1640 medium without phenol red. In the presence of ROS, dihydroethidium is oxidized to ethidium and fluorescence in red (λ_{em}=640 nm). The 640/420 nm fluoresces intensity ratio permit to evaluate the production of ROS in living cells. Raw data were normalized with respect to control value and results expressed as the folds increase of 640/420 fluorescent intensity ratio. Results are given as mean $\pm$ SD of three independent experiments[12].

Micronucleus test

About 3×10^5 cells were seeded into flasks (T25) and treated with the test substances the next day (about 24 h) for 1 h. A sampling time of 18 h was chosen and the preparation of the cultures was the same as in the chromosome aberration test with the exception that after adding the hypotonic solution the cells were centrifuged immediately. Slides were stained briefly (about 10 s) in acridine orange (125 mg/ml), rinsed in PBS and evaluated at a fluorescence microscope. Cells (1500) were scored per slide and the frequency of cells with micronuclei was determined. Only mononucleated cells with well-preserved cytoplasm containing five or fewer micronuclei (smaller than one third of the area of the main nucleus, not linked to the main nucleus) were considered.

Chromosome aberration test

About 10^6 cells were treated for 2 h and analyzed 18 h later. Chromosomes were prepared according to standard procedures. Hypotonic treatment was performed with 0.4% KCl (37°C) for 25 min. The cells were fixed with methanol: acetic acid 3:1 and the fixative was changed twice. Air-dried slides were stained with Giemsa 5% in Sorensen buffer and scored for chromosome aberrations according to the following criteria: Chromatid-type aberrations breaks and exchanges and chromosome-type aberrations breaks, rings and dicentrics. Gaps and polyploid cells were also recorded, but not considered for the evaluation of mutagenicity. One hundred metaphases per culture were analyzed for the presence of chromosomal aberrations. Metaphases with more than 5 aberrations were classified as cells with multiple aberrations.

HPRT gene mutation test

About 5 million cells were treated for 1 h in 75 cm^2

cell culture flasks. Survival (relative cloning efficiency, CE) was determined by plating 200 cells into 4 replica Petri dishes at the end of the treatment. The treated cultures (a minimum of 2×10^6 cells) were transferred as needed during the expression period. After 7 days, 2×10^5 cells were plated into 5 replica Petri dishes with selective medium (10 µg/ml 6-thioguanine. At this time, the cloning efficiency (CE 2) was determined in non-selective medium four 60-mm replica Petri dishes with 200 cells each. After one week, colonies were fixed with methanol, stained and counted. The results are expressed as mutants per million surviving cells.

Molecular analysis of HPRT mutations

Multiplex PCR was performed as described earlier[13]. Exons 2–9 of the HPRT gene were amplified simultaneously, and a separate reaction was made for exon 1. 15 µl of PCR product each were subjected to separation by electrophoresis on a 2% agarose gel (Biozym, Berlin, Germany) and stained with ethidium bromide. Mutants were classified into three different categories according to their HPRT amplification pattern: (1) total deletion: mutants without amplification product for any of the HPRT exons; (2) partial deletion: mutants lacking amplification products for one or more exons; (3) wild-type pattern: mutants with undiscoverable changes of the normal amplification pattern. Sequence analysis was performed for all mutants with PCR wild-type pattern according to the method of Zhang et al.[14].

Cytoplasmatic RNA was isolated and used to synthesize HPRT cDNA, which was used as template for PCR. Gel-purified PCR product was used in a second round of PCR with one of the primers being 5'-biotinylated. The biotinylated PCR product was bound to magnetic streptavidin-coated Dynabeads (Dynal, Hamburg, Germany) in order to obtain single-stranded DNA as substrate in the dideoxy sequencing reaction (Pharmacia T7 sequencing kit; Pharmacia, Freiburg, Germany). Sequence analysis using [α-^{32} P]dATP was performed according to the protocol of the company.

Comet assay

For the comet assay, 2×10^5 cells were seeded into multiwells (six wells, Ø 36 mm), cultivated overnight and then treated for 1 h with the test substances. At the end of the treatment, cells were washed with ice-cold PBS and trypsinized with 50 ml trypsin (0.15%). After 2 min, 50 ml complete medium were added, the cells gently resuspended and 15 µl of the cell suspension were immediately used for the test. The comet assay was performed as described by Speit et al.[15]. Microscope slides (with frosted ends) were coated with 1.5% agarose (Roth, Karlsruhe, Germany) and air dried. Cells were mixed with 120 µl low melting agarose (0.5%, LMA; Biozym, Germany) and added to the slides. The slides were covered with a coverslip and placed in a refrigerator for 3 min to allow solidification of the LMA. Afterwards slides were carefully immersed in lysing solution (100 mM Na–EDTA, 10 mM Tris, 2.5 M NaCl, 1% Triton X-100 and 10% DMSO, 1% Na–lauroyl–sarcosinate, pH 10). The slides were kept at 48 °C for at least 1 h to lyse the cells. After lysis, the slides were placed in a horizontal gel electrophoresis chamber (in an ice bath) with alkaline buffer (1 mM Na–EDTA, 300 mM NaOH, pH 13). The cells were exposed to alkali for 25 min to permit DNA unwinding and expression of alkali-labile sites. Electrophoresis was performed for 25 min at 25 V (0.86 V/cm) and 300 mA. All of these steps were conducted under dim light to prevent the occurrence of additional DNA damage. After electrophoresis the slides were rinsed with neutralization buffer (0.4 M Tris, pH 7.5). Finally, they were stained with ethidium bromide (20 µg/ml) and covered with a coverslip. Images of 50 randomly selected cells were analyzed using a fluorescence microscope (200× magnification) with an excitation filter of 515–560 nm and a barrier filter of 590 nm. Measurements were made by image analysis (Comet Assay II, Perceptive Instruments, Haverhill, UK), determining the mean tail moment of the 50 cells. The presence of oxidative DNA base damage was determined with a modified protocol using the bacterial formamidopyrimidine-DNA glycosylase (FPG protein)[15]. After lysis, slides were washed three times in enzyme buffer, drained and the agarose covered with 200 µl of either buffer or FPG protein (1 µg/ml) in buffer, sealed with a coverslip and incubated for 30 min at 37°C. All other steps were as described above.

HPLC analysis of 8-oxodeoxyguanosine

Levels of 8-oxodGuo were measured in DNA hydrolysates using HPLC with electrochemical detection

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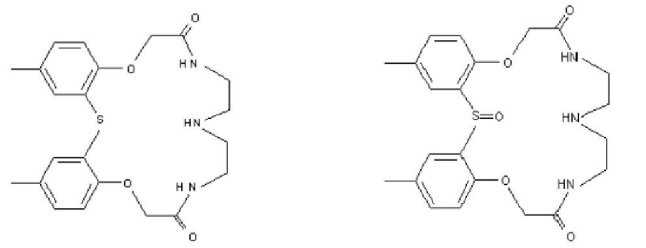
(HPLC/EC)[16]. DNA was isolated according to the method described by Topinka et al.[17]. The isolated DNA was hydrolyzed to nucleosides by sequential incubations with nuclease P1 and alkaline phosphatase (Sigma, Munich, Germany). The hydrolysates were purified by filtration (Microcon-3 microconcentrators, Amicon) and analysed by reversed-phase HPLC/EC with maximally 5 h after hydrolysis, essentially as described by Sodum et al.[18]. Analysis was performed on a System Golde HPLC system (Beckman) using two 0.46×25 cm Ultrasphere ODS columns, particle size 5 µm, in series with a 0.46×4.6 cm Ultrashere ODS guard column eluted with a buffer containing 12.5 mM citric acid, 25 mM sodium acetate and 9.5% methanol (pH 5.1). The flow rate was 1 ml/min. Separations were monitored with a photodiode array detector (Model 168, Beckman) at 254 nm and an amperometric electrochemical detector (Model 41000, Chromsystems). The potential of the detector was +700 mV vs. the Ag/AgCl/3 M KCl reference electrode. Data obtained were processed using a special HPLC software (System Golde, Beckman). For quantitation of 8-oxodGuo, calibration curves were constructed, relating the concentration of 8-oxodGuo to the integrated response of the EC detector and the concentration of dGuo to its integrated UV absorbance.

Statistic analysis

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

RESULTS

The basic results in the present study showed that both compounds were cytotoxic in the range of 0.5 to 8 mMol/ml. As illustrated by Figure 2, in the presence of each crown ethers, the cell survival decreased significantly after 24 h incubation as compared with control in which dibenzosulfoxide macrocyclic diamide (DBSOMD) markedly inhibited the viability and cloning efficiency of cells with respect to dibenzosulfide macrocyclic diamide (DBSMD). The extent of each of



Dibenzo sulfide macrocyclic diamide Dibenzo sulfoxide macrocyclic diamide

Fig 1: Structural formulas of tested compounds

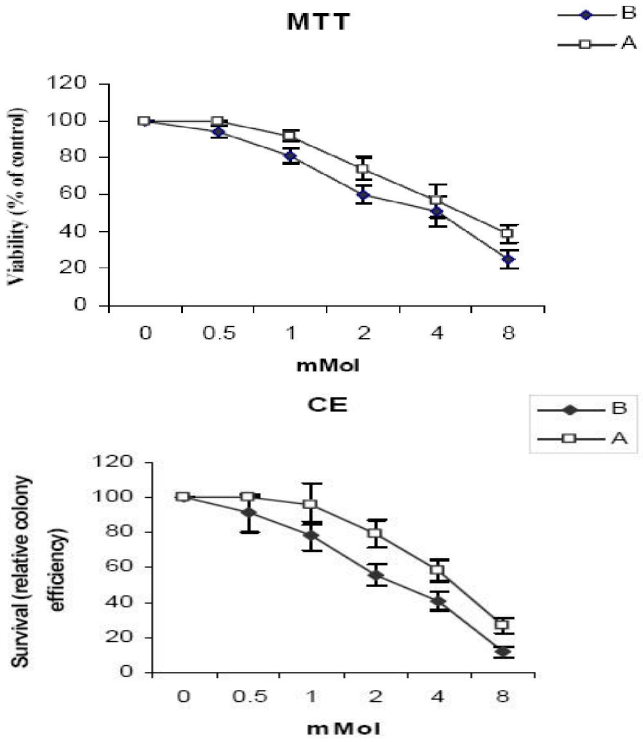


Fig 2: The effects of dibenzosulfide (A) and dibenzosulfoxide (B) macrocyclic diamides on viability (assessed by MTT) and survival (relative colony efficiency) of A549 cells. Each curve represents the mean of two independent experiments.

macrocyclic diamides cytotoxicity, exhibited as viability, increased significantly at concentration up 1mMol with respect to this concentration and lower.

Treatment of cells with each macrocyclic diamide demonstrated that they were capable of promoting ROS generation in A549 cell culture in a dose dependent manner (Figure 3). On the other hand, marked increase in ROS was only significant at 4 and 8 mMol/ml of DBSOMD against DBSMD. In this condition, exposure of cells to each of macrocyclic diamides at 8 mMol/ml, caused the formation of ROS, elevated up to 4.1 and 2.5 fold of control for DBSOMD and DBSMD respectively.

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Table 1: The effects of dibenzosulfide (A) and dibenzosulfoxide (B) macrocyclic diamides on the frequency of chromosome aberration.

	treatment	aberrated cells (%)	chromatid breaks	chromatid exchanges	chromosome breaks	dicentric	rings	Multiple aberration
B	Control	1	1	-	-	-	-	-
	1 (mMol)	3	3	-	-	-	-	-
	4 (mMol)	10	8	-	-	-	-	-
	8 (mMol)	43	37	2	4	1	-	14
A	Control	1	1	-	-	-	-	-
	1 (mMol)	1	1	-	-	-	-	-
	4 (mMol)	4	3	-	-	-	-	-
	8 (mMol)	18	12		1	-	-	4

Table 2: The effects of dibenzosulfide (A) and dibenzosulfoxide (B) macrocyclic diamides on DNA migration (tail moment) in the comet assay with A549 cells and the effect of post-treatment with FPG protein. Each data represents the mean of two independent experiments.

macrocyclic diamide (B) (TSD)			macrocyclic diamide (A) (TTD)		
concentration (mMol)	with FPG	without FPG	concentration (mMol)	with FPG	without FPG
0	0.18	0	0	0.14	0
0.5	0.84 ± 0.24 ¶	0.46 ± 0.11	0.5	0.54 ± 0.13 ¶	0.31 ± 0.08
1	1.24 ± 0.21 ¶	0.87 ± 0.17	1	1.06 ± 0.22 ¶	0.61 ± 0.14
2	2.21 ± 0.29 ¶	1.56 ± 0.26	2	1.83 ± 0.29 ¶	1.18 ± 0.23
4	-	3.47 ± 0.48 *	4	3.29 ± 0.62	2.51 ± 0.39
8	-	4.52 ± 0.54 *	8	4.57 ± 0.82	3.23 ± 0.64

¶ significant difference with respect to comet assay modified without FPG protein

*** significant difference with respect to dibenzosulfide macrocyclic diamide (TTD).**

Figure 4 demonstrated mean MN frequencies ± SD, calculated from four experiments. This indicator was induced in the same range of concentrations but

Table 3: The effects of dibenzosulfide (A) and dibenzosulfoxide (B) macrocyclic diamides on HPRT gene mutation in A549 cells.*

treatment	macrocyclic diamide (B)		macrocyclic diamide (A)	
	CE1(%)#	mutants/10^6	CE1(%)	mutants/10^6
control	100	4	100	3
1 (mMol)	81	6	93	8
4 (mMol)	63	27	46	14
8 (mMol)	18	66	28	23

*** mean of two independent experiments**

 # relative cloning efficiency at the end of the treatment

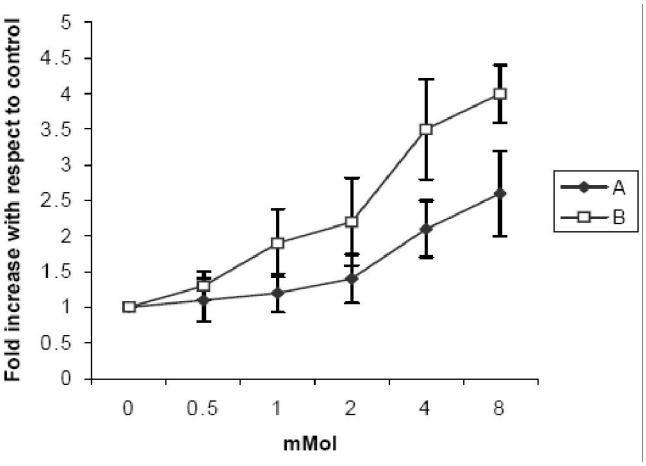

Fig 3: ROS production levels in A549-cells. Cells were treated for 24 h with different concentrations of dibenzosulfide (A) and dibenzosulfoxide (B) macrocyclic diamides and then ROS was evaluated. Results are the mean + S.D of at least three replicated independent experiments.

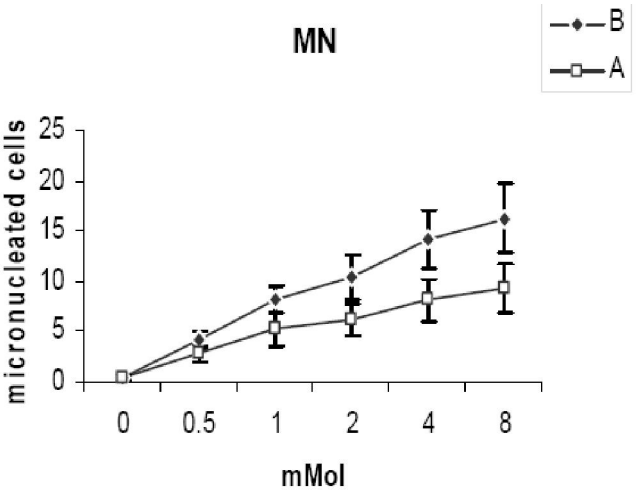

Fig 4: The clastogenic effects of dibenzosulfide (A) and dibenzosulfoxide (B) macrocyclic diamides on A549 cells assessed by the frequency of cells with micronuclei.

DBSOMD induced significant higher frequencies of micronucleated cells with respect to DBSMD when the cells were treated with these compounds up to 2 m

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Mol. A level of 0.5% spontaneous MN was found in the non-exposed control sample. In addition, a concentration-dependent significant increase of micronuclei was observed for both substances ($P<0.5$). After cyclophosphamide treatment used as a positive control for S9-mix metabolic activation, MN frequency was increased from 0.3 to 4.8% (single experiment). Chromosome aberrations were also analysed after treatment with these substances (TABLE 1). A slight increase in the frequency of aberrations was seen at 1 mMol and then at 4 mMol, but there was a strong induction at 8 mMol for both substances. At 8 mMol exposure, DBSOMD caused around 3 and 2.6 folds increase in percentage of aberrated cells and chromatid breaks respectively as compared with DBSMD. The majority of the chromosome aberrations that observed were chromatid breaks and chromatid exchanges. Chromosome aberrations were induced stronger by DBSOMD than DBSMD.

Under standard conditions, both substances caused a significant and dose-dependent manner increase in DNA migration in the comet assay (TABLE 2). In different doses of treatment, DBSOMD not only showed higher effects on this parameter, but also revealed significant increase at 4 mMol and then at 8 mMol with respect to DBSMD. The modified version of the comet assay with the FPG protein markedly enhanced DNA migration for each experiment with respect to the absence of FPG protein in the tests. The higher concentrations up to 2 mMol of DBSOMD were not evaluated with FPG due to the occurrence of apparently dead cells (clouds).

FPG treatment did not enhance significantly the effect of DBSOMD in the comet assay with respect to DBSMD.

Both substances induced gene mutations at the HPRT locus particularly at high concentration of exposure, leading to reduction in survival to 18% and 28% for treatment with DBSOMD and DBSMD respectively (TABLE 3).

Cell viability at the end of the expression period (CE2) was about 100% in all experiments. Ethyl methanesulfonate (EMS) served as appositive control in the HPRT test. Treatment (2 h) with 5×10^3 Mol EMS induced 160 mutants per million. Survivors with a relative CE at the end of the treatment (CE1) of 90%.

DISCUSSION

Exposure of cells to various xenobiotic agents or exogenous compounds may interfere with their living systems leading to cytotoxicity effects. Our results showed that the A549 cell line as a model of living system could be used to assess two macrocyclic diamides crown ethers cytotoxicity in different assays. The cells were sensitive enough to obtain the concentration response curves, although, two macrocyclic diamides were not equally effective among which DBSOMD appeared more cytotoxic. This finding was in agreement with the study of Arenaz et al. that showed cytotoxicity of derivatives of crown ethers on Chinese hamster V79 cells, as another mammalian cell line, after 24 h treatment[19]. In addition, the survival profile was similar to both macrocyclic diamides in our study, suggested that they may have similar cytotoxicity mechanism. However, differences in the observed cytotoxic intensity may be a function of their hydrophobic cavity properties, or the ability of these crown ether derivatives to induce perturbations in the cell membrane. Other investigators also suggested differences in the ability of cells to metabolize these compounds as a main factor affected their living processes[20]. Moreover, there are some documents in the studies of Carolina et al. and Foyouzi et al. revealed that the alteration of viability in cell cultures, exposed to chemical active agents was associated with significant enhancement of oxidative process[21, 22]. In agreement with these studies, our A549 cell culture exhibited high level of ROS after 24 h treatment with these crown ethers derivatives. Another striking feature of macrocyclic diamides toxicity is determination of their ability to induce genotoxic effects. Accordingly, we discriminated between genotoxic and cytotoxic effects of chemicals by careful assessment of cytotoxicity via evaluation of colony efficiency. On the other hand, to better characterize whether bulk DNA were damaged by ROS, genotoxicity tests should be evaluated, however, genotoxicity is relevant if it occurs in cells capable of surviving the damages[23].

There are direct and indirect evidences indicating that crown ethers derivatives are DNA interactive molecules, resulting genotoxicity effects. As direct document, Mark et al. reported the ability of a series of Bis (Propargylic) sulfon crown ethers to cleave supercoiled

DNA in the presence of alkali metal ions. Many other crown ethers exhibited growth inhibitory effects on leukemia cell line that may contribute to their effects on DNA[24,25].

As indirect documentation, the combination of methods (comet assay, MN and HPRT tests) allowed us to determine that macrocyclic diamides induced genotoxicity particularly at concentrations where cytotoxic effects were considerable. Our results were in contrast with another study on V79 cells treatment with 15-crown-5 and/or 21-crown-7, reflected no genotoxic effect when sister-chromatid exchanges were quantitated. The author suggested the metabolic breakdown of these compounds did not involve a putative genotoxic intermediate[3]. Furthermore, the mutagenicity of various superoxide-generating chemicals is determined in a complex manner and not solely related to the production of ROS. However, ROS clearly induced HPRT mutation in different cells[26].

On the other hand, in many studies on genotoxicity of some biochemical reactive substances, the strong cytotoxic and clastogenic effects pointed to DNA strand breaks as the predominated primary lesion in which ROS may involve by different mechanisms[5]. In accordance with these documents, both macrocyclic diamides were ROS generator and induced HPRT gene mutations in A549 cells particularly at high concentrations. In addition, these concentrations which induced chromosome aberrations in A549 cells under the same treatment conditions killed all cells in the course of the HPRT test. Besides, they were clastogenic as tested by micronuclei (MN). The MN test has the advantage to detect in interphase both acentric fragment (due to DNA breakage) and chromosome loss and offers[27].

The comet assay is increasingly used as a rapid and sensitive genotoxicity test. In its alkaline version as used here, DNA strand breaks and alkali-labile sites become apparent[28]. In addition, ROS, particularly, H_2o_2 are potent inducer of DNA migration in the comet assay and of FPG-sensitive sites[29]. Our study detected DNA-breakage after various concentration treatments, leading to increase DNA migration even at higher concentrations. This finding indicated that there was considerable induction of DNA strand breaks and alkali-labile sites under our study conditions. In agreement with our documents, as partially, Paraquat (a ROS generator) induced significant increase in DNA migration in human lymphocytes at high concentration in which superoxide anions were supposed to be involved[30].

Since ROS are expected to be the cause of macrocyclic diamides-induced genotoxicity, we used a modification of the comet assay to detect oxidative DNA base damage. Oxidative base modifications can be determined by converting oxidized DNA bases to strand breaks using bacterial FPG, as DNA repair enzyme[8]. However, post treatment with FPG leading to increase DNA migration in response to the effect of each studied compound in a dose dependent manner, suggesting that both compounds induced oxidative DNA base damage under the test conditions. As comparison, two macrocyclic diamides used in this study have different cavity sizes that are directly compatible with the biochemical important ions, Na^+, K^+ and even Ca^{2+}. On the other hand the presence of additional oxygen atom in sulfoxid group of DBSO macrocyclic diamide cause 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 diamides molecule contract 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[31].

As conclusion, our comparative study of the genotoxic properties of macrocyclic diamides revealed a specific genotoxicity profile in which superoxide generating process is involved. However, the precise mechanisms in detail through which macrocyclic diamides increase cellular level of ROS and the pattern of their oxidative effects on A549 cells are unclear and need further investigations.

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