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Assessment of cytotoxic and cytogenetic effects of a 1,2,5-thiadiazole derivative on CHO-K1 cells. Its application as corrosion inhibitor

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ABSTRACT

This work focuses on the possible use of phenanthro[9,10-*c*]-1,2,5-thiadiazole 1,1-dioxide (TDZ) as a harmless corrosion inhibitor. TDZ range-dose providing minimum adverse effects to the environment and human health, with satisfactory corrosion-inhibiting properties was evaluated. Cytotoxicity and genotoxicity of TDZ at 0.57–12.50 μM concentration range were tested by neutral red, chromosomal aberrations, mitotic index, and colony formation assays. Results showed a significant increase of chromatid-type aberrations for the highest concentration of TDZ assayed (12.50 μM). Additionally, a reduction in the proliferative rate for lower concentrations was detected by the MI assay. We concluded that TDZ should be used at concentrations lower than 1.16 μM. Corrosion assays performed showed good inhibition effect (ca. 50%) at low (0.65 μM) TDZ concentration. Consequently, our results indicated that TDZ induced a time- and dose-dependent genotoxic and cytotoxic response on CHO-K1 cells. Short assays should be complemented with long exposure tests to simulate chronic contact with TDZ since lower threshold levels may be found for shorter exposures and a wrong safety range could be determined.

1. Introduction

Heterocyclic organic compounds have several applications according to their structure. They are often found in natural products and several compounds of pharmaceutical [1–3], biocidal [4] and agrochemical [5] use. Triaromatic rings are the support of some of the most efficient and selective ligands for asymmetric catalysis [6]. Polyaromatics also have characteristic physical properties which could lead to their application as organic conductors or semiconductors [7,8]. Moreover, heterocyclic structural moieties are also found in some biomaterials, colorants and pigments [9]. Several investigations report corrosion prevention by the use of heterocyclic compounds containing nitrogen, oxygen, and/or sulfur in various corrosive environments [10–13].

The use of heterocyclic compounds in a variety of applications is conditioned by environmental and health concerns. Accordingly, during the last years, industrial requirements for chemical compounds refer not only to their efficacy but to safety as well. The requisites for these compounds should focus on non-mutagenic, non-carcinogenic products with characteristics more environmentally acceptable than systems currently in use [14]. They are also particularly important in medicine, for which chemical compounds must necessarily be biocompatible.

It has been reported that heterocyclic compounds such as benzotriazole and its derivatives, which have long been known to protect copper ant its alloys surfaces from corrosion, should be replaced by new environment-friendly inhibitors because of their toxicity [15,16]. Some non-toxic 1,3,4-thiadiazole derivatives have been proposed as efficient inhibitors for copper or bronze corrosion [10,16]. However, other 1,3,4-thiadiazole derivatives with antimicrobial properties showed cytotoxic effects on mammalian cells [4]. Consequently, cytotoxic properties change according to the particular composition and structure of the derivative.

A new and efficient synthesis for the heterocyclic compound phenanthro[9,10-*c*]-1,2,5-thiadiazole 1,1-dioxide, TDZ, has been recently reported [17] (see Fig. 1 for chemical structure). This new route for the preparation of TDZ has proved to be less expensive than the traditional one at laboratory scale [18]. However, there is no information about the toxic level of 1,2,5-thiadiazole derivatives. The objective of the present research study is to demonstrate that TDZ can be used as a harmless corrosion inhibitor when adequate TDZ concentrations are applied. In this sense, it is essential to identify the threshold concentration value of TDZ in order to guarantee minimum adverse effects on the environ-

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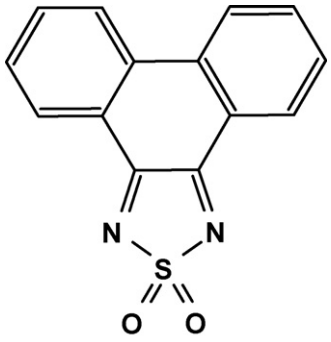

Fig. 1. Chemical structure of phenanthro[9,10-*c*]-1,2,5-thiadiazole 1,1-dioxide (TDZ).

ment and human health, with satisfactory corrosion inhibition efficacy.

We proposed to determine the toxic limit of TDZ and evaluate its corrosion-inhibiting efficacy at a concentration value below this toxic limit. Thus, DNA damage (genotoxicity) and non-specific cell damage (cytotoxicity) caused as a result of chemical effects of TDZ on a mammalian cell line (Chinese hamster ovary cells (CHO-K1)) were evaluated. TDZ cytotoxicity and genotoxicity have not been previously reported in literature. These toxic properties were tested using neutral red (NR), chromosomal aberrations (CA), mitotic index (MI), and colony formation (CF) assays. NR is the cytotoxicity assessment frequently used to evaluate a possible interference of a given chemical compound with the lysosome activity [19]. This interaction could be one of the causes of other cellular events. Genotoxicity endpoints as CA and MI have been selected as biomonitoring markers to measure the biological exposure to genotoxic compounds both in human and other species [20,21]. Additionally CF assay is used for the analysis of cell survival and proliferation after chronic exposure to the action of specific agents [22].

2. Materials and methods

2.1. Cells culture

CHO-K1 cell line was originally obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). Cells were grown as monolayer in Falcon T-25 flasks containing 10 ml Ham-F10 medium (GIBCO-BRL, LA, USA) supplemented with 10% inactivated fetal calf serum (Natocor, Carlos Paz, Córdoba, Argentina), 50 IU/ml penicillin and 50 μg/ml streptomycin sulfate (complete culture medium) at 37 °C in a 5% CO_2 humid atmosphere. Cells were counted in an improved Neubauer haemocytometer, and viability was determined by the exclusion Trypan Blue (Sigma, St. Louis, MO, USA) method; in all cases viability was higher than 95%.

2.2. Chemicals

TDZ was synthesized according to Svartman et al. [17] and solubilized in dimethyl sulfoxide (DMSO) (Merck Química Argentina SAIC, BA, Argentina). The investigated concentration range of TDZ was selected so as to include the concentration used for corrosion tests (0.65 μM) and higher concentrations that may be used to improve the corrosion inhibition efficacy. Doses of 0.57, 1.16, 1.72, 2.94, 5.63, and 12.50 μM were employed.

The final solvent concentration was <1% for all treatments in the different experiments. Negative control (untreated cells) and DMSO-control (solvent vehicle-treated cells) were run simultaneously with TDZ-treated cultures.

All the chemicals used in the experiments were of analytical grade. Milli Pore-MilliQ water was used to prepare solutions.

2.3. Neutral red assay (NR)

TDZ cytotoxicity was estimated in CHO-K1 cells using the NR (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) assay according to Borenfreud and Puerner [23]. This assay measures the cellular transport based on the dye uptake by living cells. Absorbance change is directly proportional to the number of viable cells.

For this analysis 2.7×10^3 cells/well were cultured in 96 multiwell plate and grown at 37 °C in 5% CO_2 humid atmosphere in complete culture medium for 4 h. Then, the medium was replaced by another one with TDZ. After 24 h, the liquid medium was removed and fresh medium containing 40 μg/ml RN dye (Sigma, St. Louis, MO, USA) was added. After 3 h incubation, cells were washed with a phosphate buffer solution (PBS). Color was developed by the addition of 0.1 ml 1% acetic acid in 50% ethanol. The plate was shaken for 10 min and the absorbance was measured at 540 nm using an automatic ELISA plate reader (7530 Microplate Reader Cambridge Technology, Inc., USA). Ethanol (7%) was used as positive control. Cytotoxicity percentage was calculated as $[(A - B)/A] \times 100$, where A and B are the absorbance of control and treated cells, respectively. Three independent assays were performed for each experiment, each including 16 wells (48 wells for each concentration tested). Data were analyzed using one-way ANOVA test and multiple comparisons were made using p values corrected using the Bonferroni method.

2.4. Structural chromosome aberration test (CA) and mitotic index (MI)

CHO-K1 cells were cultured for 12 h at different TDZ concentrations in order to analyze CA frequencies at the first post-treatment metaphase. Colchicine (0.1 μg/ml final concentration) was added to all cultures 2 h before fixation. Air dried slides were prepared following routine protocols [24]. Three experiments were performed in independent trials to assess reproducibility. A total of 300 metaphases per treatment (100 per repetition) were scored in coded slides. Statistical analysis was performed using χ^2 test.

The MI was determined by scoring 3000 cells from each experimental point, expressed as number of mitoses among 1000 nuclei. MI changes were expressed as a factor (f) defined as the relation between the mean MI from treated cultures (MI_t) and the mean MI from controls (MI_c) ($f = MI_t/MI_c$) [25].

2.5. Colony formation assay (CF)

Colony formation assay or clonogenic assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony [26]. For this analysis 50 cells/Petri dish were grown at 37 °C in 5% CO_2 humid atmosphere in complete culture medium at different TDZ concentrations. After 7–10 days incubation, colonies of acceptable size were selected for scoring. They were fixed with methanol:acetic acid (3:1) and stained with Giemsa. Colonies consisting of cell clusters containing more than 50 cells were counted under an inverted light microscope with a 40× objective (Carl Zeiss, Jena, Germany). Three experiments were performed in independent trials to assess reproducibility.

2.6. Corrosion tests

The investigation on TDZ corrosion-inhibiting properties was carried out using commercial 99.99% electrolytic metal copper (Merck Química Argentina SAIC, BA, Argentina).

Cyclic voltammetry (CV) studies were carried out in 0.25 M H_2SO_4 as control corrosive solution (CCS) and in presence of

0.65 μM TDZ in the same corrosive medium (TDZs), at room temperature (25 ± 2 °C).

A conventional undivided gas-tight glass cell with dry nitrogen gas inlet and outlet was used. The working electrode was a 10 mm × 10 mm × 1 mm copper foil (geometrical area exposed to the corrosive media = 1.8 cm^2); the counter-electrode was a 2 cm^2 Pt foil. A mercury/mercurous sulfate ($Hg/Hg_2SO_4(s)$), $K_2SO_4(s)$ reference electrode was used. A computer controlled PAR 273A potentiostat was employed for CV experiments.

Prior to each electrochemical measurement, the copper working electrode was initially polished using successively SiC 600, 1000, and 2500 mesh emery papers. In order to obtain a scratch-free mirror-finish surface, a final polishing and cleaning sequence was performed as follows: polishing with 1.0, 0.5, and 0.3 μm alumina; rinsing with water; degreasing using Cl_2CH_2; thoroughly ultrasonically rinsing with water; final drying with dry nitrogen gas.

Cyclic voltammograms (CVs) of the copper electrode exposed to the corroding acid solution (CCS) without or with TDZ (TDZs) were obtained in the conventional way. Potentiodynamic scans were made at 5 mV/s sweep rate (v), starting at −0.80 V in the anodic direction. At −0.33 V the scan was reversed and ended at the initial −0.80 V potential. Prior to each measurement, the electrode was subjected to a cathodic pre-treatment by holding it potentiostatically at −0.80 V for 60 s to reduce oxide film formed in air. Each CV experiment was repeated at least three times in order to check reproducibility. CVs were registered immediately after immersion of the working electrode into the sulfuric acid solution. The decrease of copper corrosion in the presence of TDZ in the corrosive medium was quantified through the inhibition efficiency according to:

$$IE(\%) = \left[\frac{j_{CCS} - j_{TDZ}}{j_{CCS}}\right] \times 100$$

where j_{CCS} and j_{TDZ} are the current densities in absence and in presence of the corrosion inhibitor, respectively.

3. Results

3.1. Neutral red assay (NR)

The lysosomal activity observed with TDZ at concentrations ranging from 0.57 to 12.50 μM was significantly higher than that of untreated control cultures ($p < 0.001$), but no significant differences were found among cells treated with the different TDZ concentrations with respect to DMSO-control (Fig. 2). Considering these results, 0.57–12.50 μM concentration range was used for CA, MI, and CF assays.

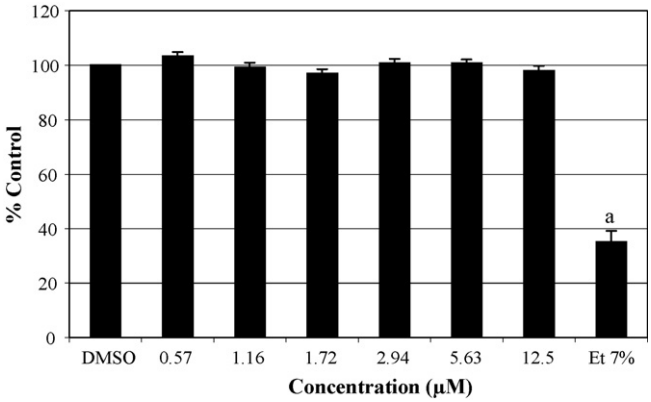

Fig. 2. Effect of TDZ treated CHO-K1 cells after 24 h evaluated with neutral red assay. a. Significant difference at $p < 0.001$.

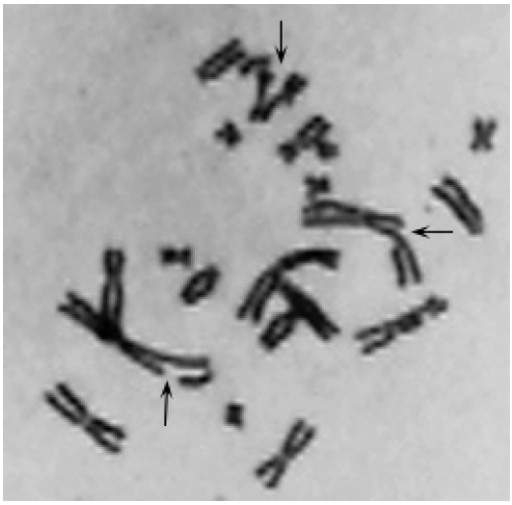

Fig. 3. Optical microphotograph of CHO-K1 metaphases with chromatid-type aberrations (arrows) after treatment with TDZ for one cell cycle. Magnification: 1000×.

3.2. Structural chromosome aberration test (CA) and mitotic index (MI)

Results obtained from CA analysis in CHO-K1 cells treated with different TDZ concentrations are shown in Table 1. TDZ treatment induced the increase of achromatid lesions and chromatid-type aberrations in relation to DMSO-control cells (Fig. 3). However, only

Table 1
Structural chromosome aberrations frequencies in CHO-K1 cells treated with different TDZ concentrations.

Treatment (μM)	Abnormal metaphases (%)[a]	Abnormal metaphases (%)[b]	Chromosomal aberrations per 100 cells			
			AL[c]	B′[d]	B″[e]	RB′[f]
Control	–	0.3	0.3 (0.06)	–	–	–
DMSO	0.3	1.0	1.0 (0.08)	0.3 (0.06)	–	–
0.57	0.6	1.3	0.6 (0.08)	1.0 (0.08)	–	–
1.16	0.6	1.6	1.0 (0.08)	0.6 (0.08)	–	–
1.72	1.3	2.3	1.0 (0.08)	1.3 (0.08)	–	–
2.94	1.3	2.3	1.66 (0.09)	1.0 (0.08)	0.3 (0.06)	–
5.63	0.5	3.0	2.5 (0.15)	–	0.5 (0.07)	–
12.5	2.6	5.0	3.0 (0.17)	3.3 (0.17)	–	0.3 (0.06)

Mean standard error is indicated between brackets.

[a] Abnormal metaphases: metaphases with at least one chromosomal aberration. Metaphases exhibiting only achromatic lesions were not scored as abnormal.

[b] Abnormal metaphases: metaphases with at least one chromosomal aberration. Metaphases exhibiting only achromatic lesions were scored as abnormal.

[c] AL: achromatid lesions.

[d] B′: chromatid breaks.

[e] B″: isochromatid breaks.

[f] RB′: chromatid exchanges.

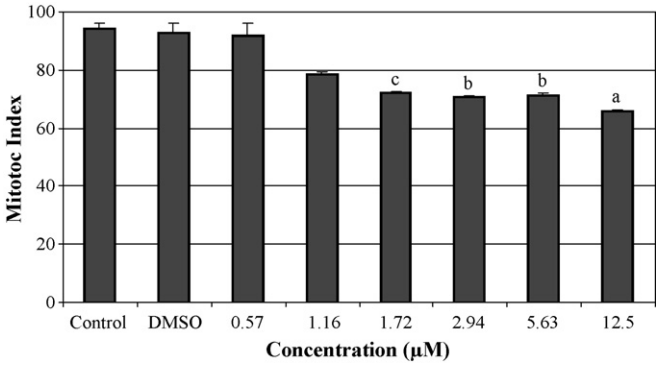

Fig. 4. Mitotic index in CHO-K1 cells treated with different TDZ concentrations. Significant difference at: a. $p < 0.001$; b. $p < 0.01$ and c. $p < 0.05$.

for 12.50 μM concentration a statistically significant difference was observed, with the corresponding increase of abnormal metaphases ($p < 0.01$).

MI decreased significantly at TDZ concentrations ≥1.72 μM (Fig. 4). A marked reduction in relation to the mitotic activity of the control was observed in cultures treated with 12.50 μM TDZ. At this concentration, the mitotic activity of cultures decreased to $f = 0.29$ ($f = 1$ for the control).

3.3. Colony formation assay (CF)

Survival data (percentage of DMSO-control cells) are shown in Fig. 5, reflecting that colony formation rate did not change significantly for 0.57 and 1.16 μM TDZ solutions.

According to the value of the least significant difference (LSD) at $p < 0.001$ level, the lower concentration that induces a significant decrease in the CF is 1.72 μM, depicting a reduction of 38% in the number of colonies with respect to the DMSO-control. Moreover, a decrease of 80% was observed for 2.94 and 5.63 μM, while 100% was detected for 12.94 μM TDZ, with significant differences at $p < 0.001$ level.

3.4. Corrosion tests

Current density (j/A cm^{-2})/potential (E/V) curves for copper in 0.25 M H_2SO_4 without (CCS) or with 0.65 μM TDZ were recorded. CVs of copper foil immersed in the CCS (Fig. 6) showed the typical behavior of copper in H_2SO_4 medium [27] exhibiting an increasing faradaic current at ca. −0.40 V in the anodic scan due to the copper electrodissolution, and a reduction peak at −0.45 V.

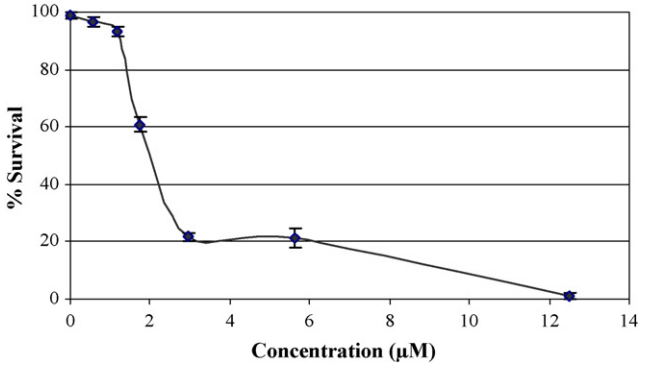

Fig. 5. Survival rates of CHO-K1 cells established by the colony-formation assay in the continuous presence of different TDZ concentrations. Data indicate survival as a percentage of DMSO-control treated cells.

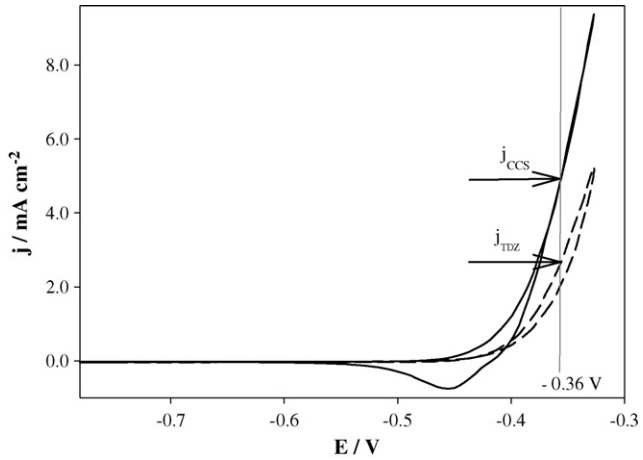

Fig. 6. Typical cyclic voltammetry (CV) of the copper sample in 0.25 M H_2SO_4 without (——) and with 0.65 μM TDZ (– – –) added. CVs conditions: copper foil; $v = 5$ mV/s; at 25 °C.

The comparison between CVs registered for CCS and in presence of TDZ showed considerable differences. The anodic current intensity in TDZs was lower than that measured without TDZ for applied potentials higher than −0.45 V. Interestingly, no electroreduction peak was detected in TDZs, even though in the previous anodic scan copper dissolution occurred. The IE was calculated using the j_{CCS} and j_{TDZ} values at −0.36 V that are indicated in Fig. 6 and resulted in IE ca. 50%.

4. Discussion

Organic compounds present different levels of toxicity according to their composition, structural characteristics, exposure time, and concentration [4,10,28]. Many 1,2,5-thiadiazole 1,1-dioxide derivatives are non-toxic and show diverse pharmacological properties [29–33]. However, other chemicals like 1,3,4-thiadiazole derivatives have been proposed as efficient corrosion inhibitors but they may be cytotoxic [4]. Recently, the use of 1,3,4-thiadiazole derivative as corrosion inhibitors of C-steel in acid media has been reported [34]. A new efficient synthesis of another possible corrosion inhibitor, TDZ, has also been informed [17]; however, the use of this compound is conditioned by environmental and health concerns. Unfortunately, since to the best of our knowledge there is no previous reference about TDZ cytotoxicity, it should be investigated in order to evaluate its potential use as non-toxic corrosion inhibitor.

Electrochemical measurements in Fig. 6 show that TDZ inhibits the corrosion process on copper, probably due to their adsorption on the metallic surface which hinders the anodic dissolution of copper in acid media [34]. Consequently, a promissory future for TDZ as a harmless corrosion inhibitor could be expected if the concentration needed for corrosion protection is low enough to be under the toxic limit. Findings on TDZ cytotoxicity and genotoxicity reported in the present work allowed to identify the threshold value of TDZ able to guarantee minimum adverse effects on mammalian cells.

In vitro short-term tests were used to evaluate cytotoxicity, that is the intrinsic ability of a compound to cause cell death as a consequence of damage to several cellular functions. On the other hand, tests detecting genetic damage provided information about the carcinogenic risk of the chemical evaluated [35]. On this respect, a compound is considered genotoxic if it is capable of inducing genetic damage at non-cytotoxic concentrations or if it is associated with low cytotoxicity.

NR assays showed that 0.57–12.50 μM concentration range could be considered non-cytotoxic. In view of these results, this

concentration range was used for the evaluation of genotoxicity through CA, MI, and CF.

No reduction in the proliferative rate was revealed by MI assay for 0.57 and 1.16 μM TDZ treatments. However, a moderate decrease in proliferation was detected for 1.72–5.63 μM, and an important decrease of about 30% ($p < 0.001$) of the control value was found in cultures treated with 12.5 μM TDZ. Additionally, a significant increase in chromatid-type aberrations was observed for this concentration.

In order to investigate the effect of chronic exposure to TDZ, CF assay was selected because it provides significant information about cells reproductive capacity after chronic exposure to chemicals. After 7 days treatment with 0.57 and 1.16 μM TDZ, cells did not show significant survival decrease. However, a dramatic fall from c.a. 40 to 1% survival was observed at 1.72–12.50 μM concentration range.

In summary, results that refer to a short time of exposure (CA, MI) reflected a clastogenic effect with a critical MI decrease for the highest concentration assayed (12.50 μM). However, CF assays that simulate chronic exposure showed that concentrations higher than 1.16 μM present inhibition higher than 40% for survival and cellular proliferation.

Through the comparison of results it can be inferred that TDZ induced a time- and dose-dependent cyto-genotoxicity, at least in CHO-K1 cells. They also point out that short tests must be complemented with long exposure tests to simulate chronic exposure, since lower toxic levels may be found for shorter exposures and a wrong safety range could be determined.

Even though corrosion inhibition on copper by TDZ was demonstrated for H_2SO_4 acid medium, further research is currently being developed in order to elucidate the mechanism of this electrochemical process.

Results reported here show that TDZ could be used as copper corrosion inhibitor in acid media with good IE at 0.65 μM, which is below the toxic limit. A better efficiency is expected at higher concentrations. However, when used in different environments and applications (biocide, decontamination, antifouling, pharmaceutical and agrochemical compounds), contact with TDZ concentrations higher than 1.16 μM should be avoided. Otherwise, dilution of TDZ solutions before contact with the environment is mandatory.

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