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Protection by tropolones against H_2O_2-induced DNA damage and apoptosis in cultured Jurkat cells

PASCHALIS-THOMAS DOULIAS[1], LAMBROS NOUSIS[1], BEN-ZHAN ZHU[2], BALZ FREI[2], & DIMITRIOS GALARIS[1]

[1]*Laboratory of Biological Chemistry, University of Ioannina Medical School, 451 10, Ioannina, Greece, and* [2]*Linus Pauling Institute, Oregon State University, Corvallis, OR, 97331, USA*

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Abstract
Tropolones, the naturally occurring compounds responsible for the durability of heartwood of several cupressaceous trees, have been shown to possess both metal chelating and antioxidant properties. However, little is known about the ability of tropolone and its derivatives to protect cultured cells from oxidative stress-mediated damage. In this study, the effect of tropolones on hydrogen peroxide-induced DNA damage and apoptosis was investigated in cultured Jurkat cells. Tropolone, added to the cells 15 min before the addition of glucose oxidase, provided a dose dependent protection against hydrogen peroxide induced DNA damage. The IC_{50} value observed was about 15 μM for tropolone. Similar dose dependent protection was also observed with three other tropolone derivatives such as trimethylcolchicinic acid, purpurogallin and β-thujaplicin (the IC_{50} values were 34, 70 and 74 μM, respectively), but not with colchicine and tetramethyl purpurogallin ester. Hydrogen peroxide-induced apoptosis was also inhibited by tropolone. However, in the absence of exogenous H_2O_2 but in the presence of non-toxic concentrations of exogenous iron (100 μM Fe^{3+}), tropolone dramatically increased the formation of single strand breaks at molar ratios of tropolone to iron lower than 3 to 1, while, when the ratio increased over 3, no toxicity was observed. In conclusion, the results presented in this study indicate that the protection offered by tropolone against hydrogen peroxide-induced DNA damage and apoptosis was due to formation of a redox-inactive iron complex, while its enhancement of iron-mediated DNA damage at ratios of [tropolone]/[Fe^{3+}] lower than 3, was due to formation of a lipophilic iron complex which facilitates iron transport through cell membrane in a redox-active form.

Keywords: *Apoptosis, hydrogen peroxide, iron, single cell gel electrophoresis (comet assay), single strand breaks, tropolones*

Introduction

Oxidants such as superoxide anion and hydrogen peroxide are continuously generated in aerobic organisms and it is likely to play important physiological roles, especially in signal transduction processes [1–3]. However, elevated steady state of these species is believed to contribute to the progress of many diseases, including ageing, cardiovascular disease and cancer [4–6]. Although the molecular mechanisms underlying the contribution of reactive oxygen species to disease development remains largely unknown, epidemiological evidence indicates that diets rich in fruit and vegetables are associated with a lower risk of several degenerative diseases [7,8]. This is mainly attributed to the fact that these foods may provide phytochemicals with antioxidant capacity. The antioxidant properties of a vast number of food constituents and other plant derived compounds have been extensively studied by using mainly *in vitro* chemical systems [9]. However, in many cases, the antioxidant (electron donating) capacity of a number of compounds did not predict their ability to

Correspondence: D. Galaris, Linus Pauling Institute, Oregon State University, Corvallis, OR, 97331, USA. Tel.: +30-26510-97562. Fax: +30-26510-97868. E-mail: dgalaris@cc.uoi.gr

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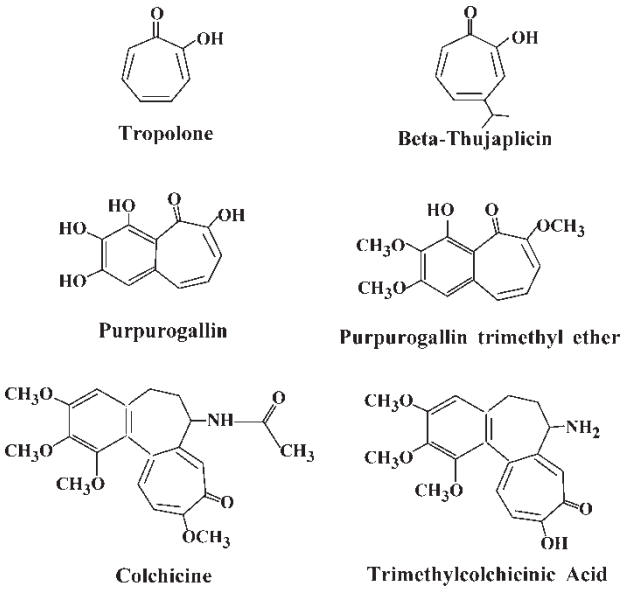
protect cells exposed to oxidative stress. This observation indicates that other properties, i.e. chelation of metals, inhibition or activation of enzymes, induction of specific proteins, membrane stabilization and others, may be crucial for the cell protecting activities of various compounds.

DNA represents one of the most sensitive components in cells exposed to conditions of oxidative stress. DNA damage is widely believed to be mediated by transition metal ions, mainly iron and/or copper, which are able to catalyze the formation of hydroxyl radicals (˙OH) by Fenton-type reactions [10–12]. However, the exact intracellular location of redox-active metal ions is likely to be of utmost importance for the ultimate effect, because hydroxyl radicals, due to their extreme reactivity, interact exclusively in the vicinity of the bound metal [13,14]. Formation of ˙OH close to DNA (due to bound Fe or Cu ions) results in its damage, including base modifications, single and double strand breakage, and sister chromatid exchange [10–12]. However, there are indications that location of iron at positions other than DNA may contribute indirectly to DNA damage and ensuing apoptosis. Extensive recent work has shown that lysosomal iron may be a key player in peroxide-dependent cell damage and apoptosis [15–17]. In general, it looks like, that prevention of formation of free radicals from hydroperoxides rather than scavenging them after their formation, may represent a superior strategy in order to counteract their action. In this respect, transition metal chelators with appropriate additional properties (membrane permeability, antioxidant capacity, etc), may be of particular interest.

Tropolone, along with several of its analogues (Scheme 1), are natural products, mainly of plant origin that have been proposed to possess both strong metal chelating and antioxidant properties.[18–22] These naturally occurring substances have been proposed to be responsible for the resistance of fungal decay and insect attack on heartwood of several cupressaceous trees such as *Thuja plicata* D. Don., *Thujopsis dolobrata* Sieb. et Zucc and *Thuja standishii* Carr [21,22]. However, little is known about the ability of tropolone and its derivatives to protect cultured cells from oxidative stress-mediated damage.

In the present study, some tropolone derivatives were tested for their ability to protect intact cells in culture from H_2O_2-induced DNA damage and apoptosis. The results indicate that, in the absence of exogenous iron, several tropolone derivatives provided dose-dependent protection. However, in the presence of exogenous iron, tropolone exerted protective action only when its ratio to iron was higher than 3, while at lower ratios it exerted cytotoxic effects. The nature of tropolone–iron complex is likely to determine the mode of action of this compound.



Scheme 1. Chemical structures of tropolone and its derivatives.

Materials and methods

Materials

RPMI 1640 growth medium supplemented with L-glutamine and gentamicin, SDS (sodium dodecyl sulfate), glucose oxidase (from *Aspergillus niger*, 18,000 units/g), catalase (from bovine liver), tropolone, colchicine, trimethyl-colchicinic acid, purpurogallin, purpurogallin trimethyl ether, β-thujaplicin and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were from Sigma-Aldrich Corporation (St Louis, MO, USA). Propidium iodide was purchased from Molecular Probes (Eugene, OR). Fetal bovine calf serum, Nunc tissue culture plastics, low melting point agarose, penicillin/streptomycin and proteinase K were obtained from Gibco BRL (Grant Island, NY, USA). Normal melting point agarose was obtained from Serva GmbH (Heidelberg, Germany). Microscope glass super frosted slides were supplied by Menzel-Glase and 4,6-diamidine-2-phenylindole dihydrochloride (DAPI) and RNase A by Boehringer Mannheim (Mannheim, Germany). Ethidium bromide and H_2O_2 was from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

Cell culture and treatment

Jurkat cells (ATCC, clone E6-1) were grown in RPMI-1640 containing 10% heat inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 ng/ml streptomycin, at 37°C in 5% CO_2 in air. Cells in the log phase were harvested by centrifugation (250*g*, 10 min, room temperature), resuspended in a density of 1.5×10^6 cells per ml and allowed to stay for 1.5 h under standard culturing conditions. Cells were then treated with the indicated concentrations of tropolone and/or iron before the addition of H_2O_2 or glucose oxidase for the doses and times indicated.

Finally, cells were collected and their viability checked by Trypan blue exclusion before any further analysis. In order to assess whether the product of the reaction of glucose oxidase (D-glucono-δ-lactone) or the oxygen during the reaction affected the results, cells were incubated with both glucose oxidase and excess of catalase. No significantly different results compared to non treated cells were observed (not shown).

Single cell gel electrophoresis (comet assay)

RPMI 1640 growth medium of 100 μl containing 1.5×10^5 Jurkat cells were placed into each of 96 wells of ELISA plastic plates and treated with glucose oxidase (0.6 μg/ml, able to generate about 12 μM H_2O_2 per min) in the presence or absence of the indicated amounts of tropolone and/or iron as described.

The comet assay performed in this work was essentially the same as previously described [23]. Cells were suspended in 1% low melting point agarose in PBS, pH 7.4, and pipetted onto super frosted glass microscope slides precoated with a layer of 1% normal melting point agarose (warmed to 37°C prior to use). The agarose was allowed to set at 4°C for 10 min and then the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris at pH 10, 1% Triton X-100 v/v) at 4°C for 1 h in order to remove cellular proteins and lipids. Slides were then placed in single rows in a 30-cm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM EDTA, pH >13 at 4°C for 40 min in order to allow for the separation of the two DNA strands (alkaline unwinding). Electrophoresis was performed in the unwinding solution at 30 V (1 V/cm), 300 mAmps for 30 min. The slides were then washed three times for 5 min each with 0.4 M Tris, pH 7.5 at 4°C before staining with DAPI (5 mg/ml).

Image analysis and scoring

DAPI stained nucleoids were examined under a UV-microscope with an excitation filter of 435 nm and a magnification of 400. The damage was not homogeneous and visual scoring of the cellular DNA on each slide was based on characterization of 100 randomly selected nucleoids. The comet-like DNA formations were categorized into 5 classes (0, 1, 2, 3 and 4) representing an increasing extent of DNA damage seen as a "tail". Each comet was assigned a value according to its class. Accordingly, the overall score for one hundred comets ranged from 0 (100% of comets in class 0) to 400 (100% of comets in class 4). In this way the overall DNA damage of the cell population can be expressed, in arbitrary units (see Ref. [23]). Visual scoring expressed in this way correlated near-linearly with other parameters such as percent of DNA in the tail estimated after computer image analysis using a specific software package (Comet Imager, MetaSystems) (results not shown). The same linear correlation between visual scoring and computer image analysis has also been reported from other laboratories [24,25]. Visual observations and analyses of the results were always carried out by the same experienced person, using a specific pattern when moving along the slide.

ESR studies

The basic system used in this study was comprised of Fe^{2+} (0.2 mM), H_2O_2 (5 mM) and the spin-trapping agent DMPO (100 mM), with or without tropolone, in chelex-pretreated phosphate buffer (100 mM, pH 7.4). Tropolone was added to the reaction mixture one minute before H_2O_2 was added. ESR spectra were recorded at room temperature on a Bruker ER 200 D-SRC spectrometer operating at 9.8 GHz and a cavity equipped with a Bruker Aquax liquid sample cell. Typical spectrometer parameters were: scan range, 100 G; field set, 3470 G; time constant, 200 ms; scan time, 100 s; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; receiver gain, 1.25×10^4; and microwave power, 9.8 mW.

UV-visible spectral analysis

The interaction between tropolone and iron was studied in a Beckman DU-640 spectrophotometer. Tropolone/iron complexes were prepared by mixing Fe (III) with tropolone at different molar ratios. The spectra were recorded between 400 and 700 nm at room temperature.

Partition studies

Phosphate buffer solution (100 mM, pH 7.4) containing ferric ion (0.05 mM) and tropolone at varying concentrations was mixed with an equal volume of *n*-octanol. The solution was vigorously shaken for 2 min and equilibrated for 1 h. After settling, the layers were separated. The concentration of tropolone–iron complex in *n*-octanol phase was measured at 418 nm.

Flow cytometric analysis of cellular DNA content

For flow cytometric analysis, cells cultured and treated with H_2O_2 in the presence or absence of tropolone, as described above, were fixed overnight in 70% ice cold ethanol at 4°C. After one washing, cells were resuspended in 0.5 ml PBS and 1 ml of DNA extraction buffer (192 mM Na_2HPO_4, 8 mM citric acid, pH: 7.8) was added. Cells were incubated for 5 min at room temperature and centrifuged before addition of 0.3 ml of staining solution (0.7 μg/ml

propidium iodide (PI), 70 μg/ml RNAase A) for 30 min in the dark. Finally, 1.5×10^6 cells were suspended in 0.2 ml PBS and analyzed on a FACScan Becton Dickinson flow cytometer (Mountain View, CA, USA).

Extraction of DNA and fragmentation analysis

After the indicated treatment, cellular DNA was isolated from 3×10^6 cells for each sample. Cells were collected and washed twice in cold PBS (450*g*, 4°C), resuspended in 500 μl lysis buffer (50 mM Tris–HCl, pH 8.0, 100 mM EDTA, 1% SDS, 0.1 M NaCl) and proteinase K was added to a final concentration of 0.6 mg/ml. The cell suspension was allowed to digest overnight at 37°C. DNA samples were then extracted with an equal volume of phenol:chloroform (v/v, 1:1) for 1 h and centrifuged at 16,000*g*, for 10 min. This step was repeated two more times before the aqueous phase was mixed with 500 μl chloroform and centrifuged again for 5 min at 16,000*g*. The upper phase, containing the DNA was then transferred to new Eppendorf tubes and precipitated from the supernatant with 1/10 volume of 3 M sodium acetate, pH 5.2 and 2 volumes of ethanol at −20°C overnight. The samples were then centrifuged at 16,000*g* for 15 min, the supernatant was removed, and 1 ml of 70% ethanol was added to the DNA pellet. The samples were centrifuged again at 16,000*g* for 15 min and the supernatant removed before the DNA pellet was allowed to dry at room temperature. After that, DNA was solubilized in Tris–EDTA buffer (10 mM Tris–Cl, 1mM EDTA, pH 8.0) containing boiled RNAase (1.7 mg/ml). The samples were incubated for 2 h at 37°C. The resulted solution of DNA was quantitated spectrophotometrically at 260/280 nm and mixed with loading buffer (0.02% bromophenol blue, 40% glycerol in Tris, boric acid, EDTA (89:89:2, pH 8.0)) before loaded in 2.0% agarose gel containing 0.5 μg/ml ethidium bromide. After electrophoresis, gels were illuminated with ultraviolet light for examination and photography.

Measurement of hydrogen peroxide generation

The amount of hydrogen peroxide generated by the action of glucose oxidase in PBS containing 5.0 mM glucose was estimated either by following the increase in the absorbance at 240 nm (Molar Extinction Coefficient = $43.6\ M^{-1}\ cm^{-1}$), or polarographically by using an oxygen electrode (Hansatech Instruments, Norfolk, UK.) detecting the liberation of O_2 following the addition of excess of catalase.

Statistical analysis

A Student's paired *t*-test was used in order to examine statistically significant differences.

Results

Protection against H_2O_2-induced DNA damage

We have shown previously that H_2O_2 rapidly and efficiently induces formation of single strand breaks in the nuclear DNA of exposed [26,27]. Likewise, as shown in Figure 1, addition of glucose oxidase (0.6 μg/ml), which was able to generate about 12 μM H_2O_2 per min, directly to 100 μl of culture medium (containing 1.5×10^5 cells) increased the level of single strand breaks in DNA from 101 ± 2 to 279 ± 15 arbitrary units within 10 min ($p < 0.001$). Tropolone, added to the cells 15 min before the addition of glucose oxidase, offered a dose dependent protection in the induced DNA damage (Figure 1A). The protective effects offered by tropolone were apparent at concentrations higher than 0.1 μM, while no further protection was observed at concentrations higher than 100 μM. The IC_{50} value observed was around 15 μM for tropolone.

Other tropolone derivatives were also tested and some of them found to be able to protect the cells from H_2O_2-induced DNA damage. However, the IC_{50} for β-thujaplicin, (Figure 1B), trimethylcolchicinic acid (Figure 1C) and purpurogallin (Figure 1D) were found to be somewhat higher than tropolone, about 75, 34 and 70 μM, respectively. In contrast, colchichine and purpurogallin trimethyl ether, the corresponding methyl ether form of trimethyl-colchicinic acid and purpurogallin, did not show any protective effects (Figure 1E and F). These results suggest that the free tropolone structure is critical for the protection by the tropolone derivatives against H_2O_2-induced DNA damage. All tropolone derivatives when tested under the same conditions, did not affect significant the basal level of DNA single strand breaks in the absence of exogenous H_2O_2. Since tropolone was the most effective, it was used in the rest of experiments of this work.

Biphasic effect of tropolone on H_2O_2-induced DNA damage in the presence of exogenous iron

Ferric iron, in the form of $FeCl_3$ (0.1 mM), when added to a suspension of Jurkat cells (1.5×10^5 per 100 μl) in complete growth medium did not affect the number of single strand breaks in the nuclear DNA (Figure 2). Surprisingly, the simultaneous presence of tropolone and Fe^{3+} in ratios lower than 3 to 1 (100 μM Fe^{3+}) dramatically increased the formation of single strand breaks, indicating that, under these conditions, the iron tropolone complex is toxic for the cells in the absence of exogenously added H_2O_2. However, when the ratio increased over 3, no toxicity was observed. A plausible explanation of these results is that Fe is rendered redox inactive only when it forms a complex with three molecules of tropolone. Moreover, ferric

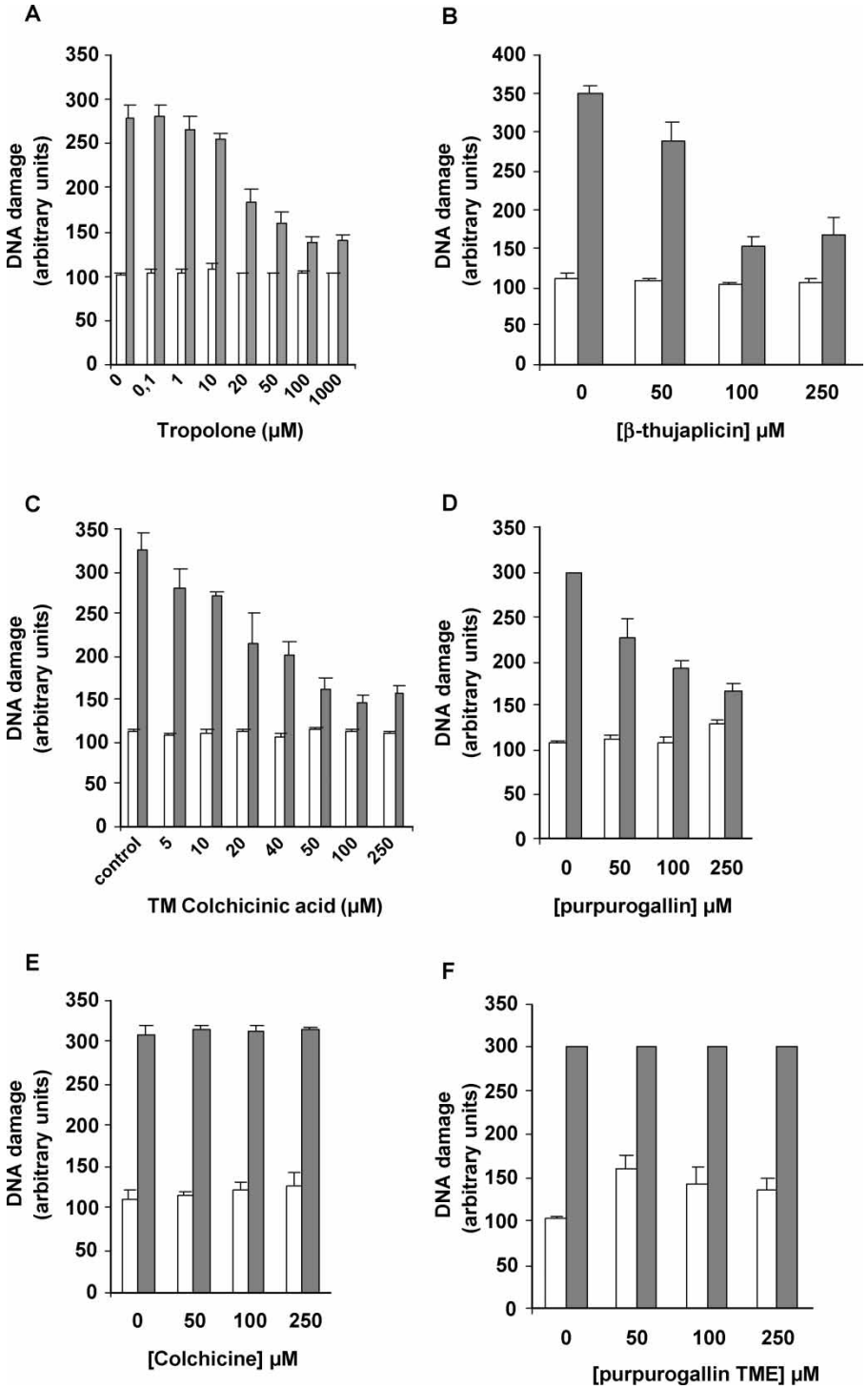


Figure 1. Protective action of tropolone and tropolone-derivatives on H_2O_2-induced DNA damage. Jurkat cells (1.5×10^5 cells/0.1 ml) were incubated for 15 min with the indicated concentrations of tropolone (A) or the tropolone-derivatives, β-thujaplicin (B), trimethylcolchicinic acid (C), purpurogallin (D), colchicine (E) and purpurogallin trimethyl ether (F), followed by addition of 60 ng/well glucose oxidase (filed bars) or PBS (open bars) for 10 min. The DNA damage of individual cells was estimated by single cell gel electrophoresis (comet assay) and expressed in arbitrary units, as described in "Materials and methods" section. Each value represents the mean ± SD ($n = 3$).

Fe^{3+} has to be reduced to ferrous Fe^{2+} in order to be able to participate in Fenton type reactions. This reduction may be accomplished after transport of the complex into the cells where it may be reduced by intracellular reducing agents. In this case, the H_2O_2 needed for Fenton reaction may be provided by Jurkat cells, which are known to continuously generate considerable amounts of H_2O_2 [28].

The above conclusions were further substantiated by the following experiments in which H_2O_2 and

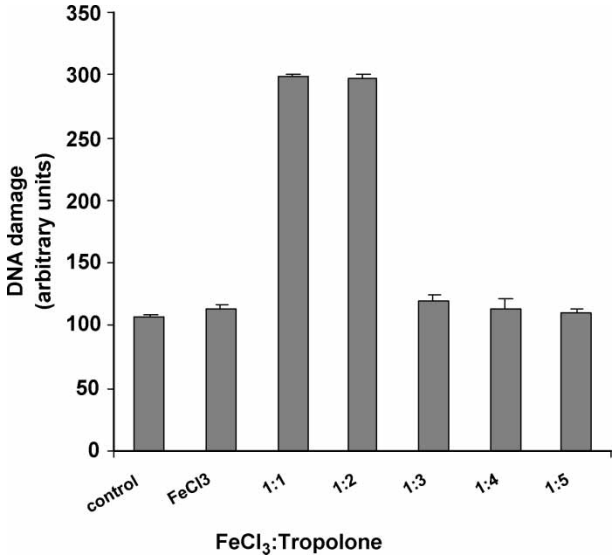


Figure 2. Effects of the ratio of Fe^{3+}–tropolone on induction of DNA damage in cultured cells. Jurkat cells (1.5×10^5 cells/0.1 ml) were incubated for 15 min with 100 μM Fe^{3+} alone or together with the indicated concentrations of tropolone. The DNA damage of individual cells was estimated by using single cell gel electrophoresis (comet assay) and expressed in arbitrary units, as described in "Materials and methods" section. Each value represents the mean ± SD ($n = 5$).

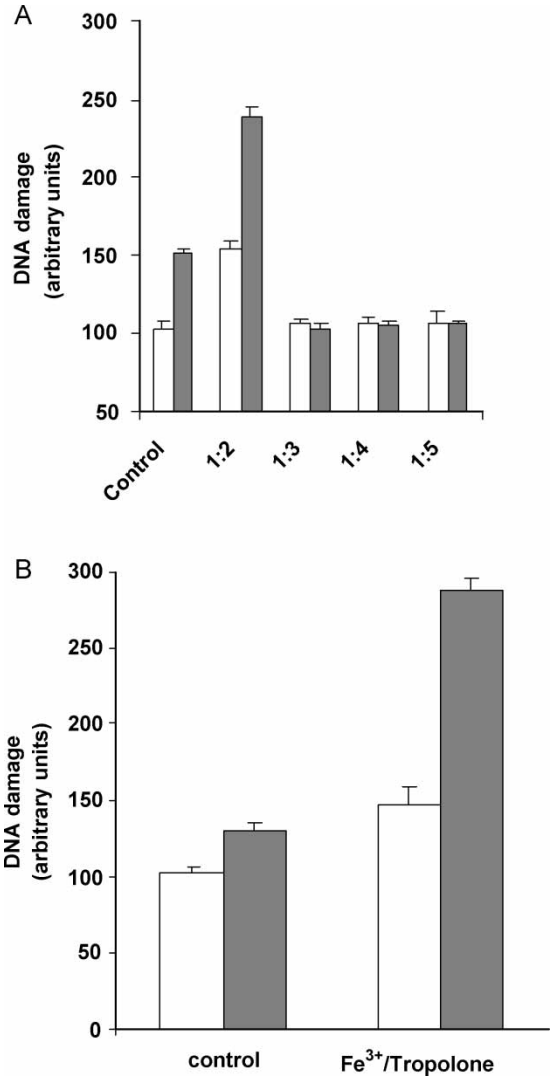


Figure 3. Effects of the ratio of Fe^{3+}–tropolone on H_2O_2-induced nuclear DNA damage. (A) Jurkat cells (1.5×10^5 cells/0.1 ml) were incubated for 15 min with 20 μM Fe^{3+} and tropolone at the indicated ratios, before the addition of PBS (open bars) or 5 ng of glucose oxidase in PBS (filed bars). (B) Cells (1.5×10^5 cells/0.1 ml) were incubated for 15 min in the presence or absence of Fe^{3+}–tropolone complex 1:2 (15 μM Fe^{3+}). Cells were then treated with PBS (open bars) or 1 ng of glucose oxidase in PBS (filled bars). The DNA damage of individual cells was estimated 10 min after the addition of glucose oxidase by using single cell gel electrophoresis (comet assay) and expressed in arbitrary units, as described in "Materials and methods" section. Each value represents the mean ± SD ($n = 5$).

tropolone/Fe^{3+} complexes were used together. Addition of an amount of glucose oxidase (able to generate about 1 μM H_2O_2 per min) to 100 μl of growth medium, containing 1.5×10^5 cells induced an increased number of single strand breaks (152 ± 2 compared to 103 ± 5 of controls) (Figure 3A). Pretreatment with complexes at ratios of tropolone to Fe^{3+} (20 μM) lower than 3 to 1, increased the toxicity in H_2O_2-exposed cells, while at ratios 3:1 or higher offered complete protection. Moreover, when even lower amounts of glucose oxidase was added (able to generate about 0.2 μM H_2O_2 per min), in order to be too low to induce considerable DNA damage by itself, the presence of tropolone to Fe^{3+} complexes in a ratio 2:1 (15 μM Fe^{3+}) induced an increase in DNA damage far exceeding the sum of the separate treatments (Figure 3B). These results clearly indicate that although tropolone is able to chelate iron, the ratio of tropolone/iron complex is decisive on whether this iron is redox-active or not. At ratios of tropolone to Fe^{3+} higher than 3:1, tropolone probably render iron redox-inactive and is protective against H_2O_2-induced nuclear DNA damage, while at ratios lower than 3:1, the iron may be still redox-active and tropolone may facilitate iron transport into the cells, where it may exert toxic effects by catalyzing Fenton-type reactions.

Inhibition of hydroxyl radical formation by tropolone

To further test the above hypothesis, we studied the effect of tropolone on hydroxyl radical production by Fenton reaction. Since hydroxyl radicals produced by Fenton reaction have been proposed to be responsible for H_2O_2-induced cellular DNA damage [29], it is interesting to know whether tropolone can inhibit its production from Fenton reaction. The most direct technique available for the detection of $^{\cdot}OH$ is ESR spectroscopy coupled with spin-trapping approach [30,31]. Hydroxyl radicals can be detected by ESR spectroscopy by using DMPO as a spin trapping agent. As shown in Figure 4, tropolone

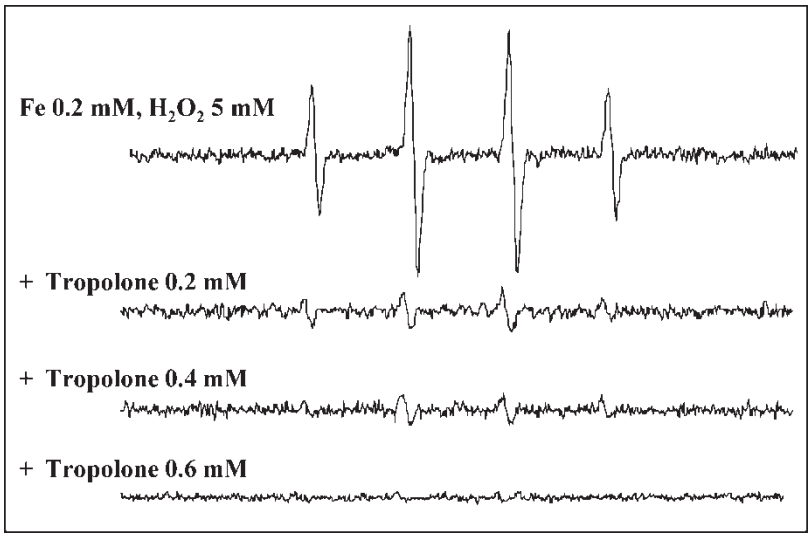


Figure 4. Effect of tropolone on DMPO/˙OH formation after interaction of Fe^{2+} and H_2O_2. Tropolone was added to the reaction mixture one minute before the addition of H_2O_2. Reactions were carried out at room temperature in chelex-pretreated phosphate buffer (100 mM, pH 7.4). All reaction mixtures contained 100 mM DMPO, 0.2 mM Fe^{2+} and 5 mM H_2O_2.

progressively decreased the formation of hydroxyl radicals as its ratio to Fe^{2+} increased. When the ratio of tropolone/Fe^{2+} was 3:1 or higher, no hydroxyl radical formation was observed. These results indicate that tropolone may bind iron and form a redox-inactive tropolone–iron complex at higher tropolone/Fe^{3+} ratios (≥ 3), while at lower ratios (≤ 2) most ferric ion is still redox-active and only partial inhibition was observed.

Formation and distribution of tropolone–iron complex between organic and aqueous phases

Although it has been shown that tropolone can bind iron and form tropolone–iron complex [32], little is known about its solubility and lipophilicity, especially at physiological pH. Therefore, to better understand the protective mechanism of tropolone, the interaction between tropolone and iron were studied by UV-visible spectrometry. A new absorbance peak with characteristic absorption at 525 nm was observed only when tropolone and iron were added together (results not shown). These spectral lines of evidence indicate that a new complex between tropolone and iron was formed. The solubility of the tropolone and Fe(III) complex in phosphate buffer is dependent on the ratio between tropolone and Fe(III). At 1:1 and 2:1 ratio (tropolone/Fe (III)), the complex remained soluble for longer than 10 min, but at ratios 3:1 and higher, the complex will be precipitated in less than 6 min. The higher the tropolone:Fe(III) ratios, the faster they precipitated.

The interaction between tropolone and iron was further studied by investigating the distribution of these compounds between organic (*n*-octanol) and aqueous phases (phosphate buffer, 100 mM, pH = 7.4). We found that the tropolone–iron precipitate is quite extractable into the organic layer. The absorbance maximum of the complex in *n*-octanol phase is found at 418 nm. These data are consistent with the interpretation that the positively charged ferric cation and the negatively charged tropolone anion indeed form a neutral and lipophilic complex. The simplest explanation is that the anions of tropolone (a weak mono-valent acid, pKa = 6.7) [32], neutralizes the positive charges of ferric cation and forms a strong hydrophobic complex with the latter. Since the anionic tropolone has one negative charge, while the cationic ferric ion has three positive charge, the composition of the extractable tropolone–iron complex was tentatively assumed to be $[tropolone]_3[Fe(III)]$.

The formation of a lipophilic complex should enhance its distribution into the organic phase, and consequently increase the iron ions content in the organic phase. This was confirmed by distribution experiments. The $[tropolone]_3[Fe(III)]$ complex could be readily partitioned into the organic phase. The higher the initial ratio of tropolone/Fe(III), the more $[tropolone]_3[Fe(III)]$ chelate was subsequently formed and partitioned into the organic phase (Figure 5). Interestingly, at ratios higher than 5, there are insoluble precipitates even in the *n*-octanol phase. Thus, the formation of a lipophilic complex from the negatively-charged tropolone and the positively-charged ferric ion possibly served as the driving force for the transport of the ferric ion into the organic phase.

Protection by tropolone against H_2O_2-induced apoptosis

Since it is known that H_2O_2, apart from inducing DNA damage, is able to induce apoptosis, we also tested the

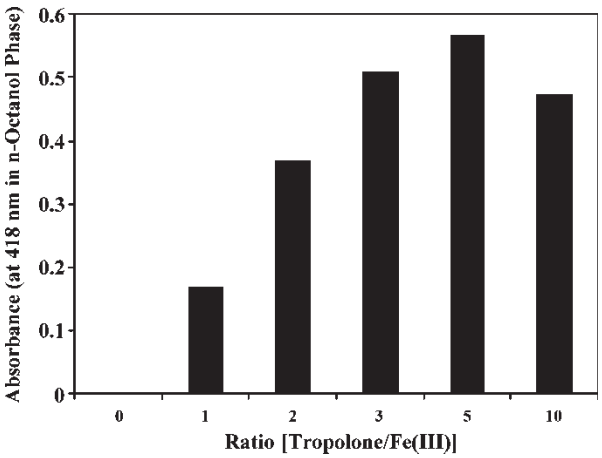


Figure 5. Partition of tropolone–iron complex into organic phase in the presence of increasing amounts of added tropolone. Results are presented as the absorbance of tropolone–iron complex at 418 nm in *n*-octanol phase.

ability of tropolone to protect cells from H_2O_2-induced apoptosis. For this kind of experiments a bolus addition of 250 μM H_2O_2 was added to the cells since continuous generation of H_2O_2 was unable to induce any significant degree of apoptosis [33]. This treatment resulted in massive apoptosis 6 h later, as indicated by the amount of cellular DNA measured by flow cytometry after staining with propidium idodide (Figure 6). Control, untreated cells contained a number of apoptotic cells (16.0%), as decided by DNA content lower than G_0/G_1 phase (Figure 6A). This number increased to 48.5% 6 h after the addition of 250 μM H_2O_2 (Figure 6B). Tropolone alone at 100 μM did not increase significantly the number of apoptotic cells (18.0%) (Figure 6C), while its presence inhibited the H_2O_2-induced apoptosis (20.0%) (Figure 6D). These results clearly indicate the ability of tropolone to protect Jurkat cells exposed to H_2O_2 from apoptotic cell death in analogy with the protection of cellular DNA.

This conclusion was further substantiated from the results shown in Figure 7. In this case, apoptosis was estimated by the formation of a ladder pattern, after electrophoresis of DNA extracted from treated cells. Lane 1 represents control untreated cells without apparent cleavage of their DNA. Intense ladder formation in lane 2 indicates the cleavage of DNA to parts multiple to 180 bp denoting clearly the apoptotic cell death induced by exposure to 250 μM H_2O_2 for 6 h. Tropolone alone at increasing concentrations 50, 100, 250 and 500 μM (in lanes 3, 5, 7 and 9, respectively) induced a slight increase of ladder pattern formation, possibly by restricting cells from

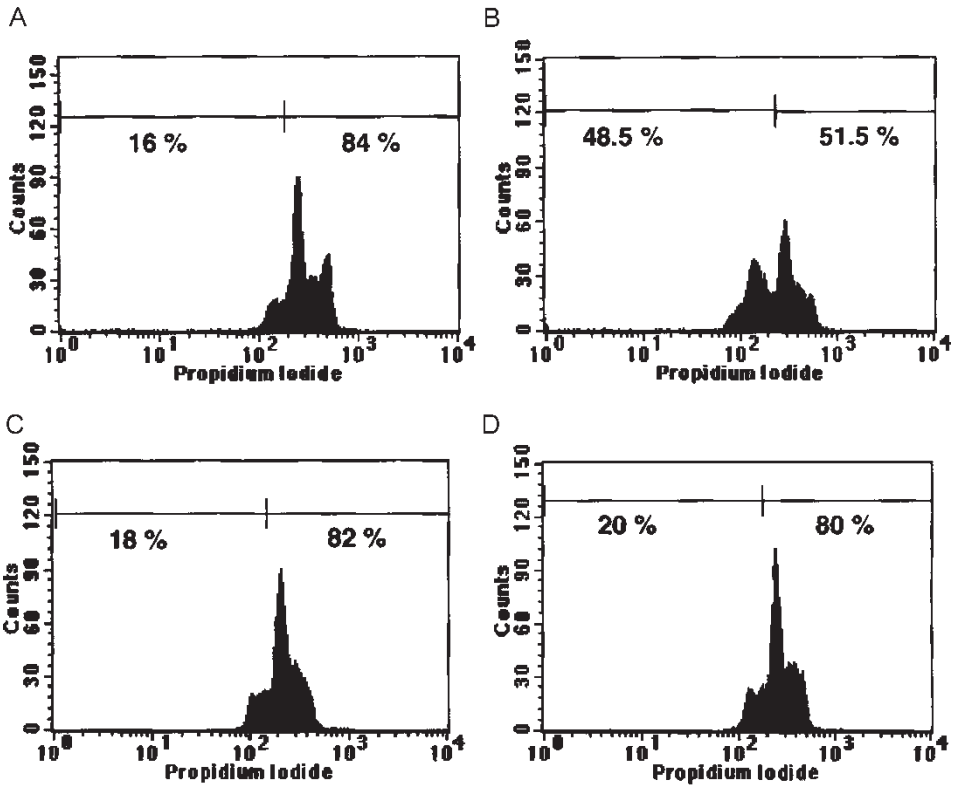


Figure 6. Effects of tropolone on H_2O_2-induced apoptosis in Jurkat cells as assessed by flow cytometric analysis. Cells (1.5×10^6 per ml) were treated for 6 h with PBS (A), 250 μM H_2O_2 (B), 100 μM tropolone (C), and 250 μM H_2O_2 plus 100 μM tropolone (D). At the end of the treatment, cellular DNA was stained by propidium iodide (0.7 μg/ml) and the cellular DNA content was analyzed on a FACScan Becton Dickinson flow cytometer as described in "Materials and methods" section. Cells containing less DNA than that of G_0/G_1 phase were regarded as apoptotic.

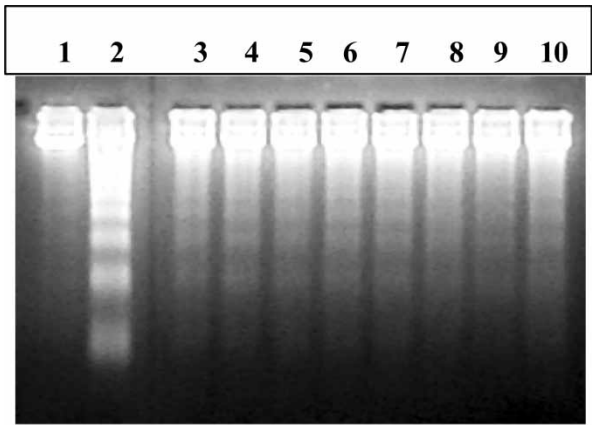


Figure 7. Effects of tropolone on H_2O_2-induced apoptosis in cultured cells as assessed by detection of DNA laddering. Cells (1.5×10^6 per and thinsp;ml) were treated with 250 μM H_2O_2 in the presence or absence of increasing concentrations of tropolone for 6 h. At the end of the treatment, DNA was isolated from 3×10^6 cells per sample and the isolated DNA was electrophoresesed in 2.0% agarose gels as described in "Materials and methods" section. After electrophoresis, gels were illuminated with ultraviolet light for examination and photography. The numbers on the lanes represent: (1) control, non treated cells; (2) 250 μM H_2O_2; lanes (3), (5), (7) and (9) represent 50, 100, 250 and 500 μM tropolone, respectively; lanes (4), (6), (8) and (10) represent samples treated with 250 μM H_2O_2 in the presence of 50, 100, 250 and 500 μM tropolone, respectively. This experiment was repeated one more time with essentially the same results.

needed iron as previously reported [34]. However, at the same time all these concentrations were able to inhibit the apoptotic cell death induced by 250 μM H_2O_2 (lanes 4, 6, 8 and 10).

Discussion

The molecular mechanism(s) of H_2O_2-induced DNA damage, although intensively studied, is still controversial. The prevailing idea is that H_2O_2 interacts with transition metal ions bound on the DNA by a Fenton-type reaction, leading to hydroxyl radical ($^{\cdot}OH$) formation. The extremely reactive $^{\cdot}OH$ damages mostly the nearby DNA chains [35]. The existence of a redox-active iron pool has been recently identified [36]. However, the exact intracellular locations of these redox-active metal ions (mainly iron), although of utmost importance for the ultimate effect, remain unknown (see Refs. [13,14]). In this regard, the use of metal chelators with chemical characteristics that allow them to reach specific cellular compartments may be useful tools in studies aiming to identify the location of the initial interaction of peroxides with iron ions. Moreover, such agents may be valuable tools in developing metal chelating drugs with specific pharmacological properties.

Tropolones represent a group of compounds the chemical structure of which—seven carbon rings with conjugated single-double bonds and the (C=O, C–OH) moiety—suggests that they are good antioxidant agents as well as metal chelators. The protection offered by tropolone against H_2O_2-induced DNA damage is most probably due to chelation of intracellular iron and formation of a redox-inactive iron complex, as demonstrated by the ESR spin-trapping (Figure 4) and UV-VIS spectral studies. One of the most interesting finding of this study is that tropolone, when added to cell suspensions at levels that are near stoichiometric with respect to the iron concentration, markedly *potentiated rather than inhibited* the iron-mediated toxicity of H_2O_2. In the present study, the potentiation by low concentrations of tropolone might be mainly due to its ability to form a lipophilic Fe^{3+}/tropolone complex and thus transport more iron ions across the membrane into the cell. Often, metal-mediated biological damage is a result of a deleterious process occurring inside the cell. Thus, for the expression of an intracellular injury, metal must act within the cell. Since Fe(III)/tropolone complex is lipophilic, this should favour the penetration of the Fe(III) complex through the lipid cytoplasmic membrane of lymphocytes. Once inside the cell, Fe(III)/tropolone complex was expected to dissociate and form new chelate with cellular ligands such as DNA and proteins. A part of the iron ions will be sequestered in non-reactive forms as they are transported into the cells and moves through cellular compartments. However, the sequestering ability of cell could be overwhelmed, since the liposoluble tropolone complex should readily penetrate cell membranes in a way which the cell cannot regulate [37].

Since iron still remains redox active at low tropolone/Fe(III) ratios, one would expect that the excess of iron which is transported into the cells by tropolone participates in oxygen radical-generating reactions, i.e. the Fe(III) or Fe(II) chelates with cellular ligands (DNA and proteins) would reduce oxygen to superoxide and/or H_2O_2 followed by hydroxyl radical formation, probably at or near iron binding sites. This will lead to the destruction of critical proteins, nucleic acids and lipids, and eventually to the cell death. Maximum potentiation was observed when the tropolone/Fe(III) ratio is in the range of 1–2, i.e. when tropolone concentration exists at levels which are below or near stoichiometric with respect to the iron concentration. The toxicity of iron to the cell will depend mainly on two factors: (1) the bioavailability of iron; and (2) the redox activity of iron. We have shown that tropolone exerted its effect by two opposite ways. On the one hand, tropolone could form a lipophilic complex with iron, the higher the ratio of tropolone/Fe(III), the more Fe(III)/tropolone chelate was formed. Since Fe(III)/tropolone chelate is lipophilic, we can expect that more iron could be transported into the cell at higher tropolone/Fe(III) ratios. However, the potentiation by tropolone was not further enhanced at high tropolone/Fe(III) ratios but rather diminished and finally reversed. This has to do with another competing factor, the redox activity of

Fe(III)/tropolone complex. At low tropolone/Fe(III) (≤ 2), tropolone only partly inhibited the iron-catalyzed formation of hydroxyl radicals (Figure 4), suggesting that iron was still partly redox-active under these conditions. At high tropolone/Fe(III) (≥ 3), however, tropolone completely inhibited the iron-catalyzed production of hydroxyl radicals (Figure 4), suggesting that the redox activity of iron was completely inhibited by high concentrations of tropolone. The biphasic effect of tropolone should be the outcome of the competition between the above two competing factors. It has been shown[38] that tropolone forms three distinct complexes with ferric ions with different stoichiometries and stabilities: Fe(III)(Tropolone)$_1$, Fe(III)(Tropolone)$_2$ and Fe(III)(Tropolone)$_3$. It is reasonable to assume that the toxic species of tropolone is the 1:1 or 1:2-iron complex because it is unsaturated, in other words it has the unused combining power that is necessary in a catalyst. On the other hand, the 3:1-complex, is saturated and consequently is unlikely to act catalytically. The decrease of the potentiation effect of tropolone and its complete reversal could be attributed to progressive and exclusive formation of Fe(III)(Tropolone)$_3$ with increasing tropolone concentrations.

In conclusion, the results of the present study indicate that compounds with the chemical structure of tropolone (seven carbon ring with conjugated single-double bonds and a C=O, C–OH moiety, may be important protecting agents in cases that cells are exposed to increased oxidative stress. However, the concentration of tropolone in relation to the available labile iron is the main determinant of the protective or toxic action of the formed complex.

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