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Hepatoprotective activity of a vinylic telluride against acute exposure to acetaminophen

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ABSTRACT

Acetaminophen (APAP) hepatotoxicity has been related with several cases of cirrhosis, hepatitis and suicides attempts. Notably, oxidative stress plays a central role in the hepatic damage caused by APAP and antioxidants have been tested as alternative treatment against APAP toxicity. In the present study, we observed the hepatoprotector activity of the diethyl-2-phenyl-2-tellurophenyl vinylphosphonate (DPTVP), an organotellurium compound with low toxicity and high antioxidant potential. When the dose of 200 mg/kg of APAP was used, we observed that all used doses of DPTVP were able to restore the –SH levels that were depleted by APAP. Furthermore, the increase in thiobarbituric acid reactive substances levels and in the seric alanine aminotransferase (ALT) activity and the histopathological alterations caused by APAP were restored to control levels by DPTVP (30, 50 and 100 μmol/kg). On the other hand, when the 300 mg/kg dose of APAP was used, DPTVP restored the non-proteic –SH levels and repaired the normal liver morphology of the intoxicated mice only at 50 μmol/kg. Our *in vitro* results point out to a scavenging activity of DPTVP against several reactive species, action that is attributed to its chemical structure. Taken together, our results demonstrate that the pharmacological action of DPTVP as a hepatoprotector is probably due to its scavenging activity related to its chemical structure.

1. Introduction

Acetaminophen (APAP) is a widespread and very effective drug used as analgesic and antipyretic. However, recent epidemiological studies have shown that the hospitalization rate due accidental or intentional APAP overdose is estimated to be over than 26,000 cases per year (Nourjah et al., 2006), being considered as the major cause of liver failure (Larson et al., 2005), hepatic transplant (Nourjah et al., 2006) and often used in suicides attempts (Watson et al., 2003). The toxicity mechanism in the liver is not fully understood, however several studies in the literature demonstrate that the metabolization of this drug is responsible by its toxic effects. At therapeutic doses, APAP mainly undergoes glucuronidation and sulfation in the liver, which does not cause toxic effects (Prescott, 1980). On the other hand, at over dosages the cytochrome P-450 system also metabolizes APAP, generating N-acetyl-*p*-benzoquinone imine (NAPQI), a very toxic metabolite (Dahlin et al., 1984) that is able to alkylate mitochondrial proteins (Jaeschke et al., 2003; James et al., 2003) and to conjugate with GSH, depleting it from the hepatic stores (Mitchell et al., 1973).

These events trigger a cascade of events, starting with mitochondrial dysfunction and generation of reactive oxygen/nitrogen species (Cover et al., 2005). The oxidative stress results in the opening of the mitochondrial membrane permeability transition pore, causing rapid loss of ATP, inhibition of the calcium pumps and, finally, a massive Ca^{++} influx into the mitochondria (Kass, 2006). This cellular collapse ends with hepatocytes death, which is characterized by the centrilobular necrosis with inflammatory cell proliferation, increase in plasmatic alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and GSH depletion (Kon et al., 2004; Nelson, 1990; Oliveira et al., 2005). In addition, APAP hepatotoxicity may cause haemostatic disorders such as the development of thrombosis and atherosclerosis once coagulation and anticoagulation factors are produced in the liver (Hsu et al., 2006).

In view of the fact that the imbalance of the oxidant/antioxidant system is the main consequence of APAP-induced hepatotoxicity, many authors have described the action of several antioxidants in different models of APAP intoxication in rodents. They demonstrated that different plant extracts (Kupeli et al., 2006; Yen et al., 2007) and synthetic compounds (Srinivasan et al., 2001) are able to diminish the

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hepatic biochemical and histological alterations caused by APAP. Of particular interest, organoselenium compounds as ebselen and diphenyl diselenide were also evaluated, being the selenide ebselen a good agent against APAP-induced hepatotoxicity (Rocha et al., 2005).

In this context, a variety of molecules containing selenium or tellurium have been evaluated due to the pharmacological properties that they have shown in several models *in vitro* and *ex vivo*. Despite of the higher antioxidant potential of organotellurides over organoselenides (Engman et al., 1995; Tiano et al., 2000; Wieslander et al., 1998), organotellurium compounds are commonly very toxic to animals (Goodrum, 1998; Laden and Porter, 2001; Nogueira et al., 2001). On the other hand, we recently reported that a vinylic telluride, diethyl-2-phenyl-2-tellurophenyl vinylphosphonate (DPTVP), possess an interesting antioxidant activity, displaying low toxicity to mice exposed by intraperitoneal or subcutaneous routes for 12 days at doses reaching 500 μmol/kg (Avila et al., 2007; de Avila et al., 2006). Besides that, the vinylic telluride is probably metabolized in the liver of the rats, once it was already demonstrated that the liver weight of treated mice increases. Nevertheless, the biochemical parameters of toxicity evaluated in this tissue were not affected, indicating that even at high concentrations the compound did not cause any biochemical damage to the liver (Avila et al., 2007). These characteristics make this vinylic telluride interesting in the research for useful antioxidant agents against the hepatic oxidative damage caused by APAP.

Notably, several reports have shown that the antioxidant activity of some organotellurium compounds relies on a distinct mechanism based on the redox cycling of the heteroatom between oxidation states II and IV (Andersson et al., 1994; Briviba et al., 1998; Droge, 2002; Engman et al., 1997). This may suggest a mechanistic action for DPTVP; nevertheless it still needs to be determined.

Hence, in this study we evaluated the effects of the organotellurium compound against the biochemical and histological hepatic damage caused by APAP. Furthermore, to elucidate the possible mechanisms of the antioxidant action we also aimed to investigate the scavenging ability of DPTVP against different form of reactive species such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), nitric oxide radical (NO•), and 1,1-diphenyl-2-picrylhydrazyl(DPPH•).

2. Materials and methods

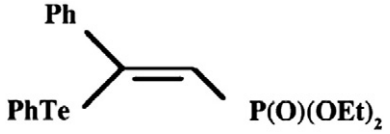
2.1. Chemicals

Diethyl 2-phenyl-2-tellurophenyl vinylphosphonate (Scheme 1) was synthesized by the addition of alkynylphosphonates to a solution of sodium organyl tellurolate, prepared by the reduction of diorganyl ditellurides with sodium borohydride in ethanol at room temperature (Braga et al., 2000).

All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Animals

Adult male albino mice (25–35 g) from our own breeding colony were maintained in an air conditioned room (20–25 °C) under natural lighting conditions with water and food (Guabi-RS, Brasil) *ad libitum*. All experiments were conducted in accordance with the Guiding Principles of the Animal Care and Wellness Committee of the Universidade Federal de Santa Maria.



Scheme 1. Chemical structure of DPTVP.

2.3. Animals treatment and ex vivo analysis

APAP was dissolved in warm saline and DPTVP in canola oil. A total of 50 animals were used in the experiments ($n=5$ mice per group).

1) 200 mg/kg of APAP ($n=5$ each group)
   I) vehicle (saline 0.9%, p.o.) + DPTVP vehicle (canola oil i.p.);
   II) APAP (200 mg/kg, p.o.) + DPTVP vehicle;
   III) APAP(200 mg/kg, p.o.) + DPTVP (30 μmol/kg, i.p.);
   IV) APAP (200 mg/kg, p.o.) + DPTVP (50 μmol /kg, i.p.);
   V) APAP (200 mg/kg, p.o.) + DPTVP (100 μmol /kg, i.p.).
2) 300 mg/kg of APAP ($n=5$ each group);
   I) vehicle (saline 0.9%, p.o.) + DPTVP vehicle (canola oil i.p.);
   II) APAP (300 mg/kg, p.o.) + DPTVP vehicle;
   III) APAP(300 mg/kg, p.o.) + DPTVP (30 μmol /kg, i.p.);
   IV) APAP (300 mg/kg, p.o.) + DPTVP (50 μmol /kg, i.p.);
   V) APAP (300 mg/kg, p.o.) + DPTVP (100 μmol /kg, i.p.).

Mice received a single dose of APAP or vehicle, and after 30 minutes DPTVP or oil were administered. Twenty-four hours after DPTVP injection, mice were anesthetized with ether for heart blood puncture. Blood was collected in tubes containing heparin and plasma was obtained by centrifugation. Liver was removed and homogenized in 10 mM Tris–HCl buffer (pH = 7.4) and then centrifuged to obtain the supernatant (S1) for the biochemical analysis. The APAP doses were chosen after dose responses curves that were previously performed in our lab. The DPTVP doses used here do not have any toxic effects by the i. p route, as reported in a previous study (Avila et al., 2007).

2.3.1. Thiobarbituric acid reactive substances (TBARS) formation

Lipid peroxidation in liver S1 was assessed by the measurement of thiobarbituric reactive substances (Ohkawa et al., 1979). Thiobarbituric acid reactive substances were determined spectrophotometrically at 532 nm after 1 h of pre-incubation at 37 °C, followed by 1 h incubation with SDS 8.1%, Acetic acid/HCl buffer and thiobarbituric acid (TBA) 0.6% at 95 °C , using Malondialdehide as standard.

2.3.2. GSH content

Liver reduced glutathione content was estimated using Ellman's reagent (DTNB 10 mM) after S1 deproteinization with trichloroacetic acid (TCA 10%), according with the standard methodology (Ellman, 1959).

2.3.3. δ-ALA-D activity

Liver δ-ALA-D activity was assayed according to the method of Sassa (1982) (Sassa, 1982), by measuring the rate of product (porphobilinogen, PBG) formation, except that 84 mM potassium phosphate buffer, pH 6.4, and 2.5 mM ALA were used. All experiments were carried out after a 15 min preincubation of S1 with the medium, starting the reaction by adding the substrate, aminolevulinic acid. Incubation was carried out for 1 h at 37 °C. The reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of $6.1\times104\ M^{-1}$ for the Ehrlich-porphobilinogen salt. The reactions rates were linear with respect to time of incubation and added protein for all the experimental conditions. Simultaneously, a set of tubes was assayed in the presence of 8 mM of dithiothreitol (DTT) to observe the possible reversion of the δ-ALA-D inhibition.

2.3.4. Superoxide dismutase (SOD) activity

SOD was determined in S1 of liver according to the methodology of Misra and Fridovich (1972) (Misra and Fridovich, 1972). The adrenochrome production was measured spectrophotometrically at 480 nm. One unit of the enzyme was defined as the amount of enzyme required to inhibit the rate of adrenaline auto-oxidation by 50%.

2.3.5. Catalase activity

Catalase activity was assessed according to Aebi (1984) (Aebi, 1984) in S1 of liver, measuring the rate of disappearance of H2O2 spectrophotometrically at 240 nm. One unit of the enzyme is considered as the amount which decomposes 1 μmol H2O2/min at pH 7.

2.3.6. Plasmatic AST and ALT activities

Plasmatic AST and ALT were quantified as biochemical endpoints of hepatotoxicity using commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, BR) and according to the method of Reitman and Frankel (1957) (Reitman and Frankel, 1957).

2.3.7. Histopathology

The liver of treated mice was carefully removed, washed in saline solution and then immersed in formalin 10%. For light microscopy examination, tissues were embedded in paraffin, sectionated in 5 μm and stained with hematoxylin and eosin ($n=3$ per group).

2.4. In vitro analysis

2.4.1. Chemioluminescence assay

The scavenging of hydrogen peroxide (H_2O_2) was assessed by chemiluminescence assay according to (Singh et al., 1988). Briefly, a range of DPTVP concentrations were incubated with 10 μM luminol. The reaction was started by the addition of H_2O_2 (50 mM). Chemiluminescence values were measured in counts per minutes (cpm). The DPTVP effect is expressed as % of control (without DPTVP).

2.4.2. Deoxyribose degradation

The deoxyribose degradation assay was performed according to Puntel et al. (2005). Briefly, the reaction medium was prepared containing the following reagents at the final concentrations indicated: DPTVP (concentrations indicated in the figures), deoxyribose 3 mM, ethanol 5%, potassium phosphate buffer 0.05 mM, pH 7.4, $FeSO_4$ 50 μM and H_2O_2 500 μM. Solutions of $FeSO_4$ and H_2O_2 were made prior to use. Reaction mixtures were incubated at 37 C for 30 min and stopped by the addition of 0.8 ml of TCA 2.8% followed by the addition of 0.4 ml of TBA 0.6%. Next, the medium was incubated at 100 C for 20 min and the absorbance was recorded at 532 nm (Gutteridge, 1981; Halliwell and Gutteridge, 1981). Standard curves of malondialdehyde (MDA) were made in each experiment in order to determine the MDA generated by the deoxyribose degradation. The values are expressed as percentage of control values (without DPTVP).

2.4.3. DPPH radical scavenging activity

The measurement of the scavenging activity of the DPTVP against the radical DPPH• was performed in accordance with (Choi et al., 2002). Briefly, 85 μM DPPH• was added to a medium containing different DPTVP concentrations. The medium was incubated for 30 min at room temperature. The decrease in absorbance measured at 518 nm depicted the scavenging activity of the DPTVP against DPPH•. The values are expressed in percentage of inhibition of DPPH• absorbance in comparison to the control values without the DPTVP.

2.4.4. NO• scavenger activity

The scavenging of NO• was assessed by incubating sodium nitroprusside (SNP) (5 mM, in PBS) with different DPTVP concentrations at 25 °C. After 120 min, 0.5 ml of incubation solution was sampled and mixed with 0.5 of Griess reagent (Green et al., 1982). The absorbance was measured at 550 nm. The amount of nitrite was calculated using a standard curve with different concentrations of sodium nitrite. A curve of sodium nitrite, constructed in the presence of the DPTVP in order to verify its interaction with nitrite depicted no interference of the DPTVP in the color development after Griess reagent addition. The values were compared to control to determine the percentage of inhibition of nitrite reaction with Griess reagent depicted by the telluride as an index of its NO• scavenging activity (Marcocci et al., 1994).

2.4.5. Protein carbonylation content

The whole brain of treated mice were homogenized 1:10 in Tris–HCl buffer (10 mM, pH 7.4) for protein carbonyls content according to a modified method by (Levine et al., 1990). Briefly, 1 ml aliquots were mixed with 0.2 ml of 2,4 dinitrophenylhydrazine (10 mM DNPH) or 0.2 ml HCl (2 M– blank). After 1 h incubation at room temperature in a dark environment, 0.5 ml of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing 3% of SDS), 1.5 of ethanol and

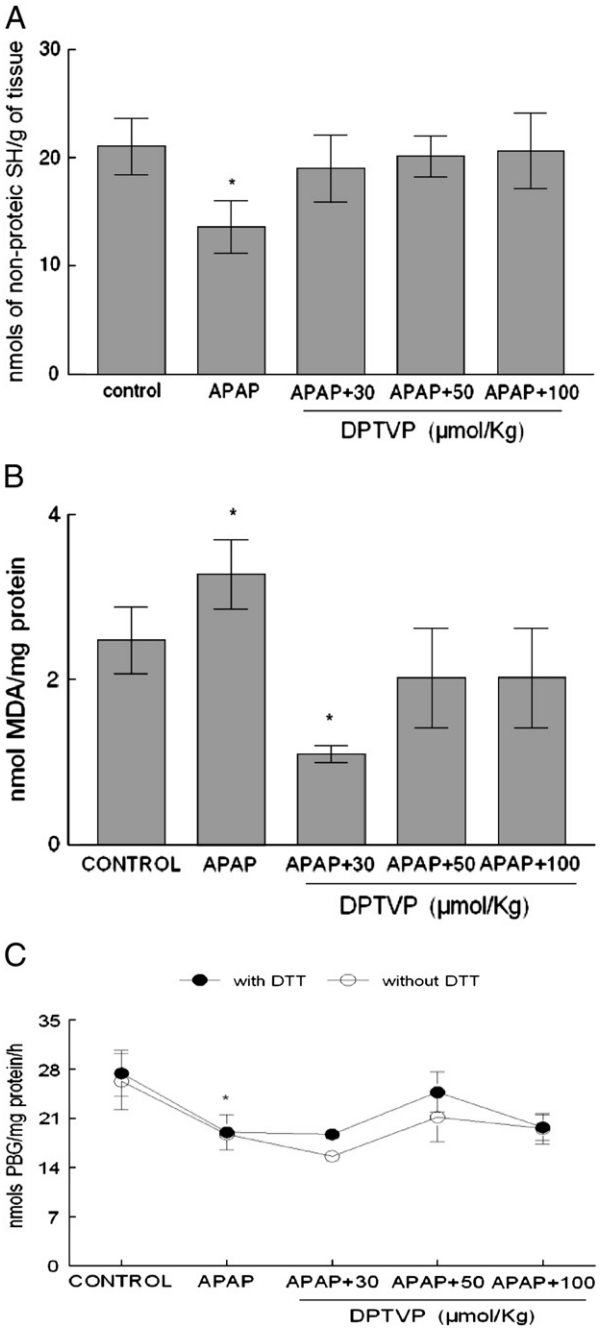


Fig. 1. Biochemical parameters evaluated in the liver of mice treated with APAP 200 mg/kg p.o. and post-treated after 30 min with DPTVP (0, 30, 50, 100 μmol/kg, i.p.). A) non-proteic –SH levels; B) thiobarbituric acid reactive substances levels; C) δ-ALA-D activity. Each bar represents mean ± S.E.M ($n=5$). * Indicates statistical difference from control group by one-way ANOVA, following by SNK post-hoc test ($P<0.05$).

Table 1
Seric ALT and AST activities in mice after 24 h of the pre-treatment with APAP 200 mg/kg (p.o) followed by administration of 0,30, 50 or 100 μmol/kg of DPTVP (i.p).

Dose (μmol/kg)	AST (U/ml)	ALT (U/ml)
Control	55 ± 0.5	34.1 ± 0.07
APAP	56.3 ± 0.5	45.5 ± 0.01[a]
APAP + DPTV (30)	48.1 ± 0.5	40 ± 0.3
APAP + DPTV (50)	59.2 ± 0.5	38.3 ± 0.3
APAP + DPTV (100)	55.4 ± 0.5	38.8 ± 0.3

Data are expressed as mean ± S.E.M for five animals each group.
[a] Indicates significant difference from control group ($P<0.05$).

1.5 ml of heptane were added and mixed for 40 s by vortex agitation, and subsequently centrifuged for 15 min at 2000 ×*g*. Next, the isolated protein in the interface ethanol:heptane was washed twice with ethyl acetate/ethanol 1:1 (v/v) and suspended in 1 ml of denaturing buffer. Each sample was measured at 370 nm in a Hitachi U-2001 spectrophotometer and compared to a corresponding blank sample. Total carbonylation was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1}.

2.5. Protein content determination

Aliquots from the homogenates were separated to protein measurements that were assessed according to Lowry et al., 1951 (Lowry et al., 1951).

2.6. Statistical analysis

Statistical significance was assessed by one-way ANOVA, followed by Student–Newman Keuls (SNK) test for post hoc comparison. Results were considered statistically significant at values of $P<0.05$.

3. Results

3.1. Hepatotoxicity induced by APAP 200 mg/kg

The exposure of 200 mg/kg APAP leads to an impoverishment of GSH levels in the liver, observed by the reduction of non proteic-SH groups ($P<0.05$, Fig. 1A). In addition, a significant increase in thiobarbituric acid reactive substances levels was observed (Fig. 1B), as well as inhibition of hepatic δ-ALA-D activity, which was not reverted by DTT ($P<0.05$, Fig. 1A). Also, there was a leakage of ALT activity in the plasma, reflecting the failure of the liver function due to APAP treatment ($P<0.05$, Table 1). On the other hand, the enzymatic biomarkers used as SOD and catalase activities were not altered by 200 mg/kg APAP administration (data not shown).

Notably, DPTVP was able to overturn the biochemical parameters in APAP-induced hepatoxicity. GSH depletion caused by APAP was restored by DPTVP at all tested doses ($P<0.05$, Fig. 1A). In accordance, the oxidative stress caused by GSH depletion was reverted by the organotellurium compound, as indicated by decreased MDA levels ($P<0.05$, Fig. 1B). Besides, ALT leakage was not observed from the dose of 30 μmol/kg ($P<0.05$, Table1). The only parameter altered by

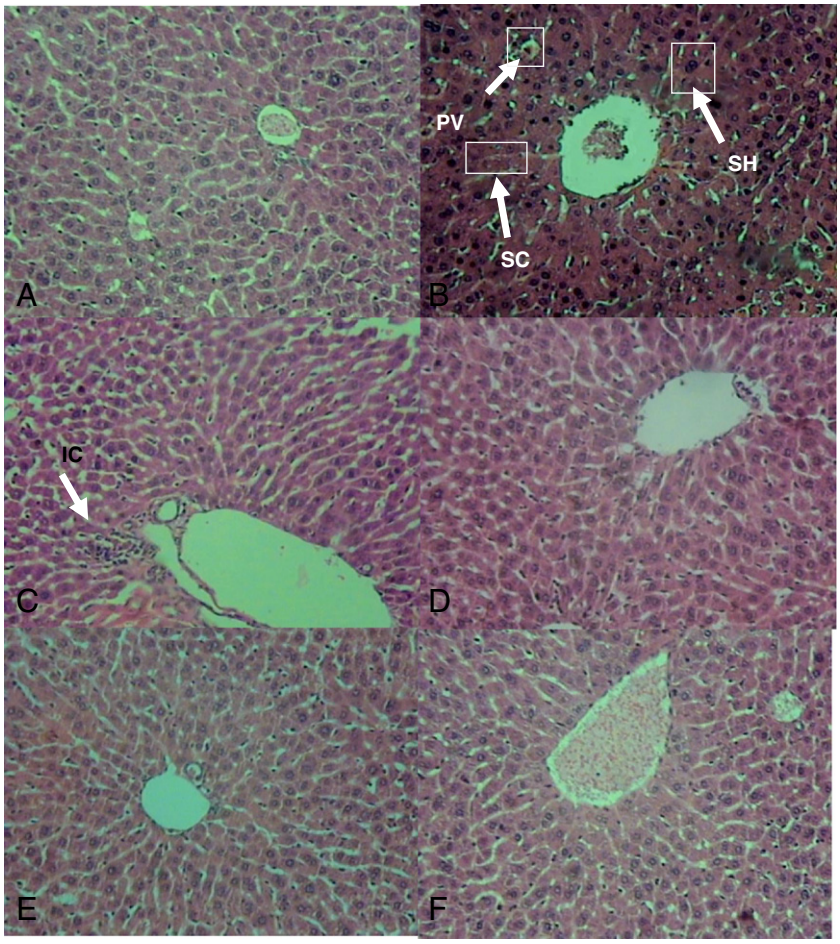


Fig. 2. Histopathological analysis of the liver of mice treated with APAP 200 mg/kg p.o. and post-treated after 30 min with DPTVP (0, 30, 50, 100 μmol/kg, i.p.). A) control B) APAP C) APAP + DPTVP 30 D) APAP + DPTVP 50 E) APAP + DPTVP 100. F) DPTVP 100 μmol/kg. Arrows indicate APAP damage PV – perinuclear vacuolization; IC – inflammatory cells accumulation; SH – swollen hepatocytes; SC – sinusoidal congestion (magnification 200×).

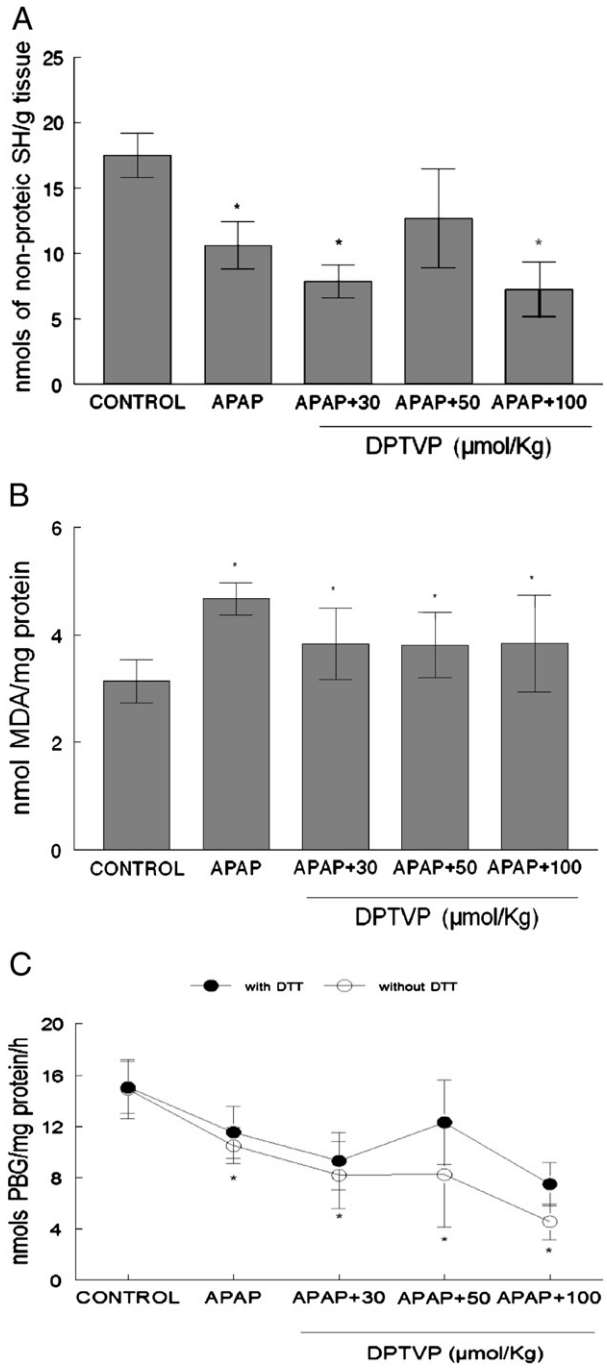


Fig. 3. Biochemical parameters evaluated in the liver of mice treated with APAP 300 mg/kg p.o. and post-treated after 30 min with DPTVP (0, 30, 50, 100 μmol/kg, i.p.). A) non-proteic –SH levels; B) thiobarbituric acid reactive substances levels; C) δ-ALA-D activity. Each bar represents mean ± S.E.M ($n = 5$). * Indicates statistical difference from control group by one-way ANOVA, following by SNK post-hoc test ($P<0.05$).

Table 2
Seric ALT and AST activities in mice after 24 h of the pre-treatment with APAP 300 mg/kg (p.o) followed by administration of 0, 30, 50 or 100 μmol/kg of DPTVP (i.p).

Dose (μmol/kg)	AST (U/ml)	ALT (U/ml)
Control	93 ± 8.8	31.4 ± 5.1
APAP	202.4 ± 14.7[a]	115.3 ± 12.1[a]
APAP + DPTV (30)	165.7 ± 5[a]	100.3 ± 17[a]
APAP + DPTV (50)	161.1 ± 10.1[a]	113.2 ± 30[a]
APAP + DPTV (100)	206.6 ± 25[a]	139.1 ± 1.7[a]

Data are expressed as mean ± S.E.M for five animals each group.
[a] Indicates significant difference from control group ($P<0.05$).

APAP that was not restored by the compound was the activity of δ-ALA-D (Fig. 1C).

Fig. 2A–E displays the histology of mice's liver after different treatments. Light microscopic evaluation showed regular morphology of the liver parenchyma with well-designed hepatic cells and sinusoids in control group (Fig. 2A). The administration of the DPTVP alone did not cause any histological damage to liver (data not shown). The APAP treated group showed mild liver injury with accumulation of inflammatory cells, perinuclear vacuolization and swollen hepatocytes (Fig. 2B). Post-treatment with DPTVP (30, 50 and 100 μmol/kg) evidenced the morphological improvement of liver after APAP exposure. Still, it is possible to observe little inflammatory cells infiltration, some vacuolization but no sinusoidal congestion, indicating regeneration (Fig. 2C–E).

3.2. Hepatotoxicity by APAP 300 mg/kg

The liver injury caused by APAP was strongly enhanced by the rise of the dose administered to mice. This was demonstrated by the failure of DPTVP to completely revert the biochemical parameters that were changed by APAP. The decrease in GSH levels was restored only when the DPTVP used was 50 μmol/kg ($P<0.05$, Fig. 3A). The APAP-induced thiobarbituric acid reactive substances increment was not decreased by the treatment with the compound (Fig. 3B). The injured hepatic function indicated by AST and ALT leakage to the plasma was not improved by the vinylic telluride at any of the tested doses (Table 2). Furthermore, the inhibition in hepatic δ-ALA-D activity was impaired by the treatment with DPTVP at 100 μmol/kg ($P<0.05$, Fig. 3C). Once more, we did not observed alterations in hepatic SOD and catalase activities, even when the high dose was employed (data not shown).

In the histology of the livers in APAP-treated mice, we observed severe liver injury with congestion of the sinusoidal space and high accumulation of inflammatory and red blood cells. Besides, swollen hepatocytes and extensive necrosis in periportal region were observed (Fig. 4B). The treatment with 30 μmol/kg of the vinylic phosphonate did not show any improvement. On the other hand, the images from APAP + 50 or 100 μmol/kg of DPTVP treated livers displayed the significant improvement of the morphological alterations caused by APAP alone. It is possible to note that there is still some polimorphonuclear infiltration and ballooning type necrosis, but the decreased sinusoidal congestion and absence of necrotic sites strongly indicate that the hepatocytes could be starting a regenerating process (Fig. 4C–E).

3.3. In vitro evaluations

Fig. 5A shows the scavenger activity of H_2O_2 by DPTVP using the chemioluminescence assay. It is possible to observe a biphasic curve, once the scavenging effect was observed at 1 μM and then only from 50 μM ($P<0.05$). In order to evaluate whether the compound could interfere with the Fenton reaction, we used the deoxyribose degradation assay (Fig. 6). We observed again the H_2O_2-scavenger activity by DPTVP (from 50 μM) when only this ROS was added to the medium ($P<0.05$). Furthermore, in the Fenton's reaction conditions ($Fe^{2+} + H_2O_2$), the deoxyribose degradation was higher than in the H_2O_2 condition, due to the formation of OH•. DPTVP was able to decrease deoxyribose degradation caused by H_2O_2 and by the Fenton reaction at the concentration of 100 μM.

To confirm the OH• scavenging ability by DPTVP, we used the DPPH• assay. We observed an inhibition of the DPPH• reduction, indicating a scavenging activity from 50 μM ($P<0.05$, Fig. 5B). Furthermore, the vinylic telluride was also able to scavenge NO• from 50 μM, once it was able to ($P<0.05$, Fig. 5C).

Fig. 7A–C illustrates the antioxidant effect of DPTVP against SNP-induced protein carbonylation in liver, kidney and brain, respectively. In the liver (Fig. 7A), DPTVP showed a per se effect from 4 μM,

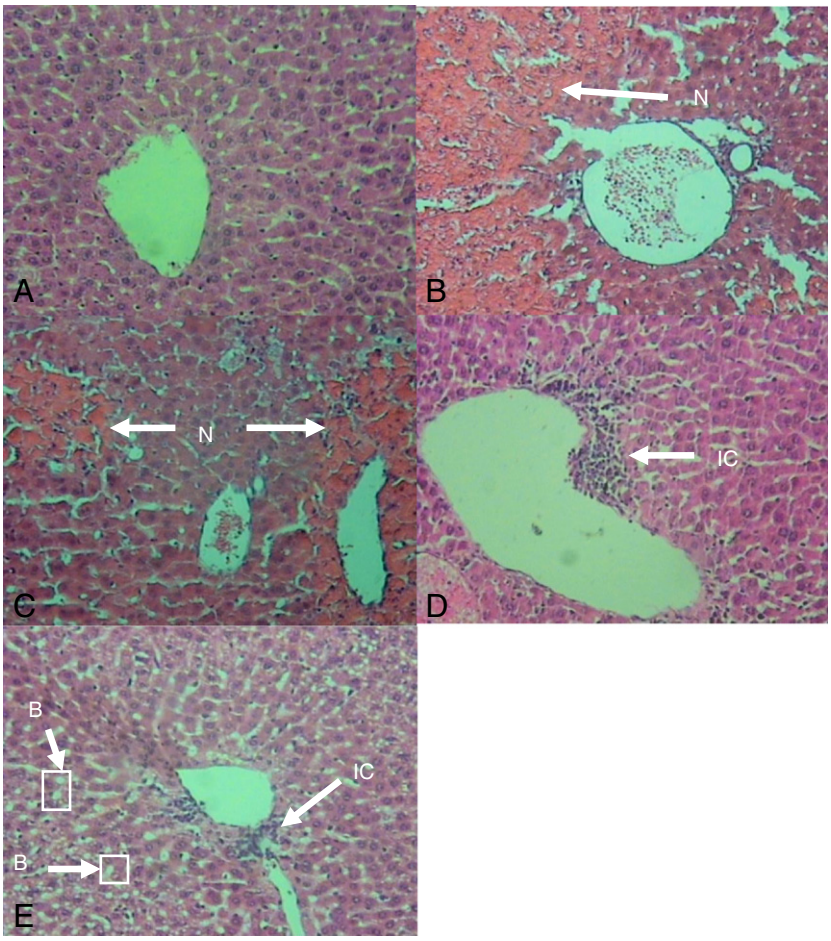


Fig. 4. Histopathological analysis of the liver of mice treated with APAP 300 mg/kg p.o. and post-treated after 30 min with DPTVP (0, 30, 50, 100 μmol/kg, i.p.). A) control B) APAP C) APAP + DPTVP 30 D) APAP + DPTVP 50 E) APAP + DPTVP 100. CV-central vein. Arrows indicate APAP damage N – necrosis; IC – inflammatory cells accumulation; B – ballooning type necrosis (magnification 200×).

protecting against the carbonylation under normal conditions ($P<0.05$). Furthermore, SNP caused a significant increase in the protein carbonyl content, which was reverted by DPTVP from the concentration of 4 μM ($P<0.05$). In the kidney (Fig. 7B), there was no per se effect by DPTVP, nevertheless the carbonyl content which was increased by SNP returned to the control levels from the concentration of 1 μM ($P<0.05$). In the brain (Fig. 7C), DPTVP decreased the SNP-induced protein oxidation from 4 μM, without any per se effect ($P<0.05$).

4. Discussion

In agreement with previous studies, in the present investigation we detected that acetaminophen administered orally to mice causes biochemical and histological damage to hepatic tissue. Post-treatment with DPTVP, an organotellurium compound with low toxicity to mice, was able to restore all the parameters altered by APAP (200 mg/kg), displaying a hepatoprotective activity. When challenged to a higher dose of APAP (300 mg/kg), DPTVP did not show the same level of protection; however the histological data demonstrated its potential for further screenings. Furthermore, we found strong evidences to support that DPTVP acts as a scavenger, providing decreased oxidative stress in hepatocytes and a prospect for these cells to recover from the damage caused by APAP.

Recently, there has been wide interest in the role of oxidative stress in the pathogenesis and progression of liver diseases, as well as in several drug-induced hepatotoxicity models, particularly in APAP-induced liver injury (Fontana, 2008; Larson, 2007). As the deliberated use of this drug is still increasing, the research for new compounds that act against oxidative stress in intoxicated hepatocytes without causing any damage to these cells and other organs is relevant. DPTVP has arisen as an option to be tested as a hepatoprotector once it does not cause toxicological alterations and does not cause any liver damage to mice, even when receiving high doses as 500 μmol/kg for 12 days (de Avila et al., 2006). In addition, our group has reported its good antioxidant potential *in vitro* and *ex vivo* (Avila et al., 2007, 2008, 2010; de Avila et al., 2006).

In our APAP exposure protocol, we observed that non-proteic-SH groups were affected by 200 mg/kg of APAP. It is known that the APAP metabolite NAPQI reacts rapidly with glutathione (which is 90% of the non-proteic SH content) (Nelson, 1990; Oz et al., 2004), consequently exacerbating oxidative stress as indicated by thiobarbituric acid reactive substances increased levels. Furthermore, we observed a rise in the seric ALT activity, which is strictly related to the hepatocytes degeneration associated to oxidative stress caused by APAP. ALT is a cytoplasmic enzyme that is released into circulation after structural damage to the hepatocytes, being used as a standard biomarker of liver damage (Sallie et al., 1991). Probably because of its antioxidant activity, DPTVP restored APAP-induced SH depletion at all tested doses. Moreover, we obtained decreased MDA levels in the liver of the animals treated with the vinylic telluride, which indicates that the oxidative stress was indeed restricted by the compound. Furthermore, the seric ALT activity increased by APAP was recovered to control levels by DPTVP. Confirming the biochemical evidences, the

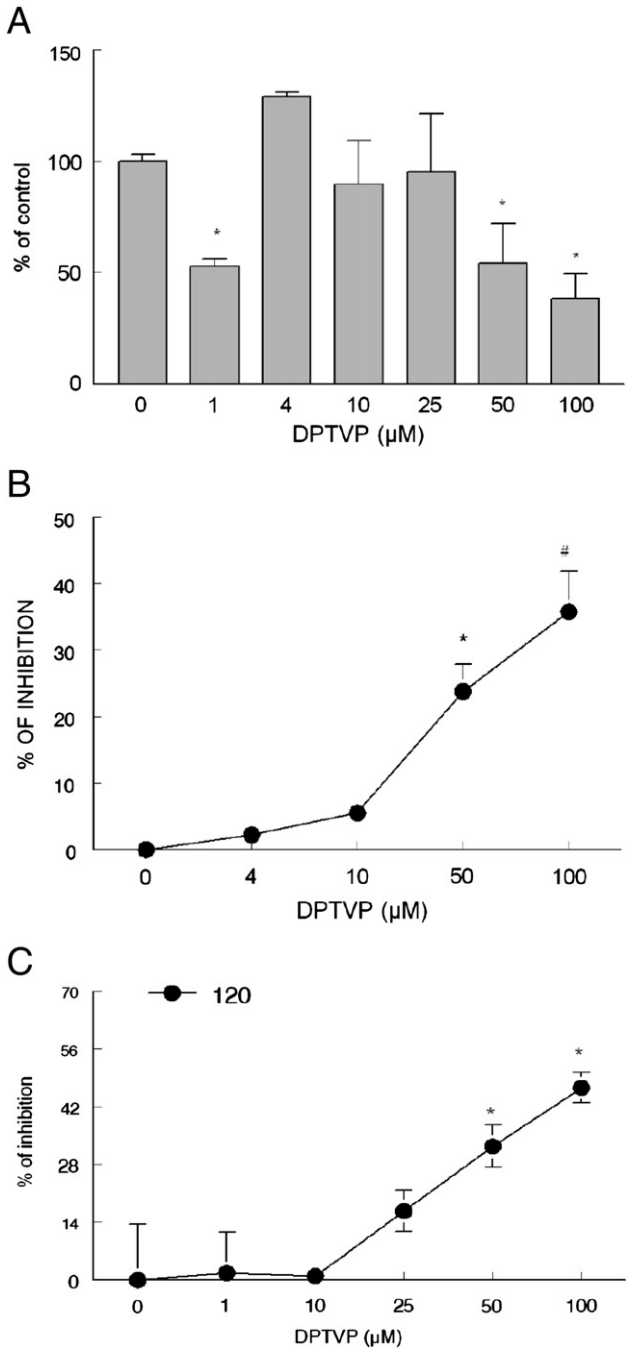


Fig. 5. Scavenger activity of DPTVP against (A) H_2O_2. Results are represented as percentage of control. The mean control value is 902392 ± 110098 cpm; (B) DPPH•. The values are expressed in percentage of inhibition in relation to control without DPTVP. The mean control value is 0.959 ± 0.02 of absorbance (518 nm). (C) Effects of DPTVP on the NO• scavenging assay. The values are expressed in percentage of inhibition. The mean control value is 22.3 ± 0.05 μM of nitrate. Data are expressed as mean ± S.E.M ($n = 4$). * Indicates statistical difference with $P < 0.05$ and # refers to significance with $P < 0.001$ by SNK test.

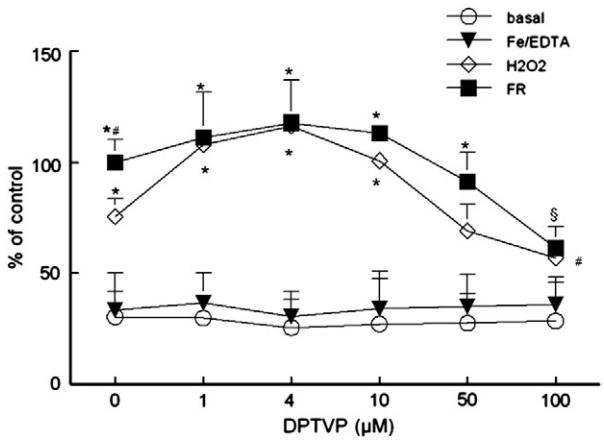


Fig. 6. Effects of DPTVP on deoxyribose degradation. The (O) indicates basal conditions, (▼) Fe^{2+} 5 μM, (◊) H_2O_2 500 μM and (■)Fe^{2+} 5 μM + H_2O_2 500 μM, the Fenton reaction (FR). Results are indicated as percentage of FR. 100% of FR value is 1.102 ± 0.009 μmol MDA/g of deoxyribose. Data are expressed as mean ± S.E.M ($n = 5$). *Indicates statistical difference from basal conditions without DPTVP, # indicates statistical difference from H_2O_2 500 μM without DPTVP and § indicates statistical difference from FR without DPTVP by one-way ANOVA, following by SNK-post-hoc test ($P < 0.05$) for five individual experiments.

histopathological analysis showed the regeneration of the hepatic tissue when compared to APAP treated group, with remarkable signs of regeneration.

On the other hand, the vinylic telluride could not restore the δ-ALA-D inhibition caused by APAP treatment. δ-ALA D is an oxidative stress sensitive enzyme, being used as biomarker. In its active site there are two cisteinyl residues that can be easily oxidized by several agents (Farina et al., 2003; Nogueira et al., 2004; Perottoni et al., 2004). Nevertheless, the inhibition of this enzyme by APAP in our experimental protocol is not only related to the oxidation of the SH groups in the enzyme. We can infer this once the addition of DTT, a thiol donator, did not restore the δ-ALA D activity. This effect is in accordance to Rocha et al., which observed the same pattern of enzyme inhibition in the presence of DTT in a model of APAP exposure in rats. In addition, they observed restoration of the δ-ALA D activity with Ebselen treatment, an organoselenium compound with glutathione-peroxidase-like activity (Rocha et al., 2005). The mechanism underlying the enzyme inhibition by APAP is not fully understood, but probably involves oxidative stress at a level that cannot be restored by DTT and the organotellurium compound.

As all tested doses of DPTVP recovered the livers after pre-exposure to 200 mg/kg of APAP, we decided to challenge the hepatoprotective potential of the compound using the 300 mg/kg dose. This exposure caused severe damage and biochemical alterations in the hepatic tissue, as indicated by the high activity of seric ALT and AST, increased thiobarbituric acid reactive substances levels, non-proteic SH depletion, δ-ALA-D inhibition and remarkable histopathological alterations. We found that only the dose of 50 μmol/kg of the vinylic telluride restored –SH levels and decreased the necrosis in the histological analysis. This finding suggests that normal levels of gluthatione are necessary for the restoration of the hepatic morphology.

The liver damage caused by 300 mg/kg of APAP was so extensive that even the DPTVP dose that restored –SH levels was not able to recover ALT and AST seric activities and increased thiobarbituric acid reactive substances levels. We hypothesize that DPTVP was no effective because it acts as a ROS scavenger, not being able to undo the lipoperoxidation and SH depletion quickly imposed by APAP pre-exposure. As the ROS produced by APAP had produced a significant damage previous to DPTVP treatment, the compound could not restore the molecular damage after only 24 h. On the other hand, we observed that the treatment with 50 and 100 μmol/kg of DPTVP decreased the sinusoidal congestion, the swelling in the hepatocytes and the necrosis observed in the livers of the APAP-treated mice (Fig. 4B). Because of these benefic effects, we believe that a chronic treatment with lower doses of the vinylic telluride could lead to an improvement in the biochemical parameters altered by APAP in the hepatocytes.

It was reported that acute administration of high doses of APAP decreases SOD and catalase activities (Ghosh and Sil, 2007).

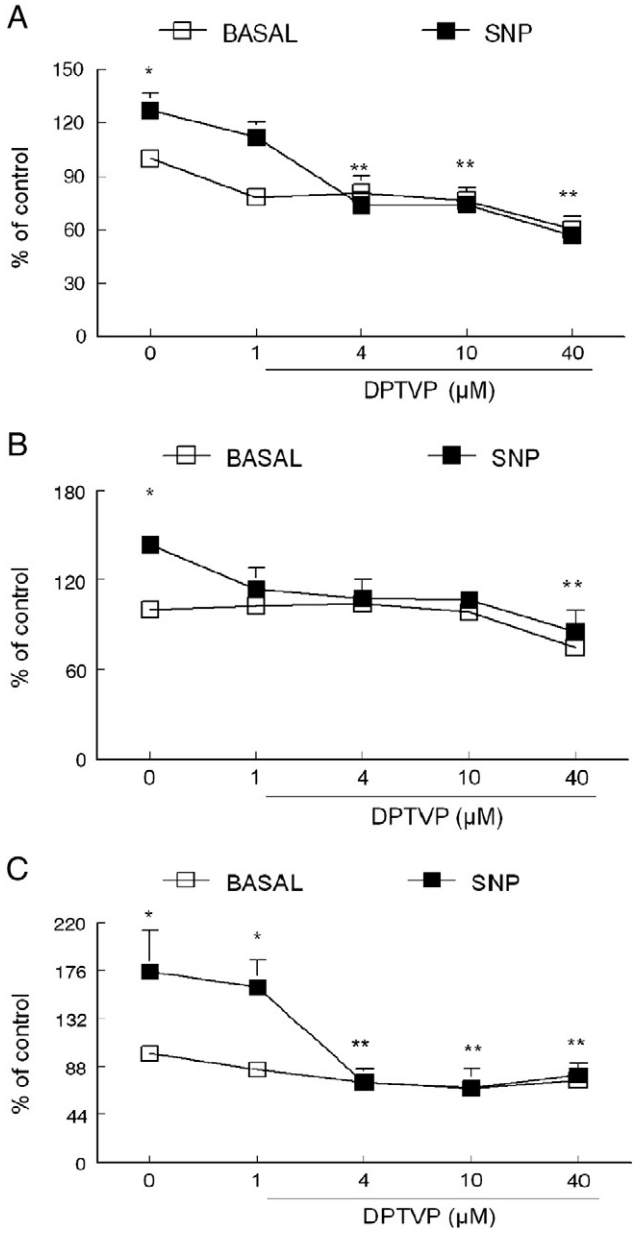


Fig. 7. Effects of the vinylic telluride on basal (□) and SNP-induced (■) protein carbonylation *in vitro* in liver (A), kidney (B), brain (C) of mice. Results are expressed as percent of control. 100% of control corresponds to 24.67 ± 2.4 nmol carbonyls/mg of tissue of tissue (A) to 15.9 ± 2.3 nmol carbonyls/mg of tissue of tissue (B) and 13.9 ± 1.5 nmol carbonyls/mg of tissue of tissue (C). Data are expressed as mean ± S.E.M ($n = 4$). *Indicates statistical difference from the basal without DPTVP, $P<0.05$; ** indicates statistical difference from SNP-induced carbonylation without DPTVP ($P<0.05$) by SNK post-hoc test.

Furthermore, some authors have found the same alterations just several days after APAP exposure (Olaleye and Rocha, 2008; Sabir and Rocha, 2008). In our experimental protocol we noticed the absence of alterations in SOD and catalase activities. These findings are in agreement with a study from Cigremis et al. (2009), demonstrating in rabbits that the mRNA expression levels of SOD and catalase were not altered by a unique and mild dose of APAP that caused thiobarbituric acid reactive substances increase and hepatic histological alterations. They suggested that probably the activity of these enzymes do not have a critical role and may show dual functions in APAP-induced hepatotoxicty.

In order to investigate the possible antioxidant mechanism by which DPTVP would protect against APAP, we performed some *in vitro* experiments. As already mentioned, APAP metabolization generates a large amount of a variety of reactive species such as H_2O_2, NO, O_2^- (Jamil et al., 1999) and the scavenging of such species would be crucial for the protection against the hepatotoxicant.

H_2O_2 is a hazardous molecule produced by APAP (Jamil et al., 1999). In excess it can degrade certain haem proteins such as myoglobin and hemoglobin, releasing iron (Fe^{2+}) (Halliwell and Gutteridge, 2003). This metal can react with H_2O_2 via the well known Fenton reaction, generating OH• radical, the most reactive specie (Halliwell and Gutteridge, 1984). To mimic this reaction, we used the deoxyribose degradation assay (Gutteridge, 1987), which is based in a system containing Fe^{2+} and H_2O_2 to generate OH•. These radicals attack the 2-deoxy-D-ribose, degrading it into a series of fragments that are reactive to thiobarbituric acid. Our results indicate that besides its ability to scavenge H_2O_2, DPTVP interacts with the Fenton reaction, decreasing OH• production.

In order to confirm whether this interaction reaction was due to OH• scavenging activity, we performed the DPPH assay. The DPPH• radical is a one electron radical very similar to OH•, being used to evaluate the OH• scavenging (Choi et al., 2002). The concentration of DPTVP that was able to scavenge the DPPH radical was even lower that the observed in the deoxyribose assay, probably because of the absence of the agents that continue generating the ROS as in the deoxyribose degradation assay's medium.

In addition, DPTVP can scavenge reactive nitrogen species, such as NO•. NO• is a very important signaling molecule, however is harmful in the presence of $O_2^{\bullet -}$. Such effect can be attributable to the formation of the metabolite peroxynitrite ($ONOO^-$), which is highly reactive (Halliwell and Gutteridge, 2003). $ONOO^-$ can be generated *in vitro* by the reaction between the released NO• (from SNP) and $O_2^{\bullet -}$, which is constantly produced by mitochondrial respiration (Murphy, 2009). In the presence of animal tissue, $ONOO^-$ can react with susceptible amino acids such as lysine, cysteine and arginine and oxidize them. (Singh et al., 2007). We observed that DPTVP protected against the protein oxidation induced by SNP, which could indicate a scavenger activity against $ONOO^-$.

Considering previous findings regarding tellurides in the literature, we attribute the scavenger activity depicted by DPTVP against ROS and RNS to its chemical structure (Fig. 8). DPTVP has a phosphonate group which is an electron-withdrawing group. This group causes a decrease in the electronic density in the Te atom, which becomes Te^+, an electrophilic specie. Considering that reactive species have unpaired electron, we purpose that the Te^+ can accept this electron, becoming telluroxide (Te IV), similarly to what is described for several organotellurium compounds with symmetrical structure or not (Andersson et al., 1994; Braga et al., 2009; Briviba et al., 1998). This mechanism can explain the scavenging of H_2O_2 and oxygen singlet, including some RNS as NO• and $ONOO^-$ (Fig. 8).

Another finding which cannot be unnoticed is the difference between the antioxidant concentration in the presence and absence of tissue in the medium reaction. We have previously reported that very low concentrations such as 2 μM can decrease lipid peroxidation in rat

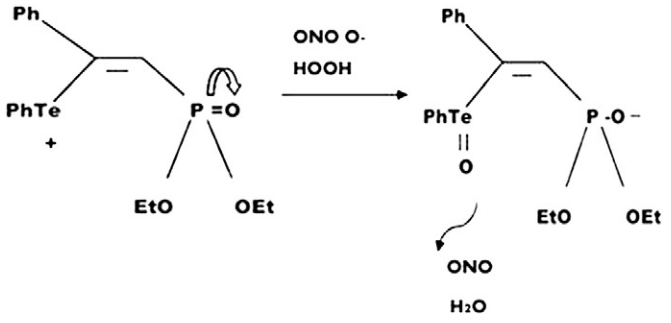


Fig. 8. Hypothesized mechanism of action of DPTVP's scavenging activity against $ONOO^-$ and H_2O_2 (see text for details).

brain, kidney and liver homogenates (Avila et al., 2008; de Avila et al., 2006). Here, we observed that a concentration of 3 μM is already efficient to decrease protein oxidation. Nevertheless, in those assays that do not require tissue addition, we observed that higher DPTVP concentrations as 50 μM were required to reach the scavenger activity. This suggests that probably the components present in the tissues can react with the compound and generate a metabolite which has better activity than the DPTVP by itself. The tissue influence on the antioxidant activity of the compounds needs to be further investigated, once it can also cause a decrease in the scavenger potency, as reported by other authors (Puntel et al., 2009).

Overall, our study demonstrated for the first time the hepatoprotector activity of DPTVP *ex vivo* in rodents. Previously, our lab established the antioxidant potential of this compound in several tissues, including the hepatic (de Avila et al., 2006), and also its low toxicity to rodents. Furthermore, our *in vitro* results allow us to postulate a chemical structure/pharmacological action relation. These results instigate us in the search for other benefic activities of the compound, but also demonstrate that additional studies regarding the elucidation of the DPTVP mechanisms must be assessed.

Disclosure statement

There were no conflicts of interest in the preparation of this manuscript.

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