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# Antioxidant Activity and Neuroprotective Effects of Zolpidem and Several Synthesis Intermediates

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Structural relationship between the antioxidant melatonin and the non-benzodiazepine hypnotic zolpidem (ZPD) suggests possible direct antioxidant and neuroprotective properties of this compound. In the present work, these effects were analyzed for zolpidem and four of its synthesis intermediates. *In vitro* assays include lipid peroxidation and protein oxidation studies in liver and brain homogenates. Intracellular antioxidant effects were analyzed by evaluation of free radical formation prevention in HT-22 hippocampal cells treated with glutamate 10 mM and measured by flow cytometer DCF fluorescence. The neuroprotective effect of these compounds was evaluated as neuronal death prevention of HT-22 cells treated with the same concentration of glutamate. Zolpidem was found to prevent induced lipid peroxidation in rat liver and brain homogenates showing figures similar to melatonin, although it failed to prevent protein oxidation. ZPD-I was the most effective out of the several zolpidem intermediates studied as it prevented lipid peroxidation with an efficiency higher than melatonin or zolpidem and with an effectiveness similar to estradiol and trolox. ZPD-I prevents protein oxidation, which trolox is known to be unable to prevent. When cellular experiments were undertaken, ZPD-I prevented totally the increase of intracellular free radicals induced by glutamate 10 mM in culture medium for 12 h, while zolpidem and ZPD-III partially prevented this increase. Also the three compounds protected hippocampal neurons from glutamate-induced death in the same conditions, being their comparative efficacy, ZPD–III > ZPD–I = ZPD.

*Keywords*: Antioxidants; Zolpidem; Zolpidem synthesis intermediates; Melatonin; Neuroprotection; Intracellular peroxides

## INTRODUCTION

Zolpidem is a non-benzodiazepine related hypnotic with a imidazopyridine structure [N,N,6-trimethyl-2-*p*-tolyl-imidazo (1,2-a) pyridine-3-acetamide L-(+)].[1] It is known to bind to the omega I (BZ-1) site of the GABA benzodiazepine chloride channel receptor complex with a Kd of 6–8 nM.[2–4]

The activity of zolpidem as antioxidant or neuroprotector has never been reported although structural similarities with melatonin—amine heterocycle structure (see Fig. 1)—suggest that zolpidem might well have the same properties. Indole heterocycle has previously been reported as antioxidant pharmacophore and trials in search of new synthetic antioxidants carrying this group were published.[5,6] Gozzo *et al*.[7] found that changes in the aromatic ring of the indolamine melatonin could reduce its antioxidant properties. Although both the 5-methoxy group and the acetamidoethyl side chain are important in the antioxidant properties, it appears that it is balance between both substitutes—rather than their particular peculiarities—that is responsible for these hallmarks, given that the absence of both side chains implies an antioxidant ability higher than the lack of each of them separately.[8]

No reference to any possible direct or indirect antioxidant properties of benzodiazepines has ever been reported, although Carayon *et al*.,[9] showed a clear correlation between benzodiazepine

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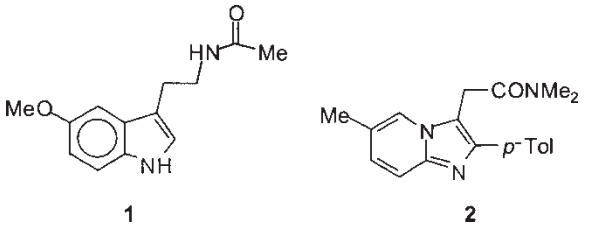


FIGURE 1 Chemical structures of melatonin (1) and zolpidem (2).

peripheral receptor expression and resistance of hematopoietic cell lines to oxidant citotoxicity, involving this receptor in the antioxidant pathway. Nevertheless, zolpidem has been reported not to be a ligand of peripheral benzodiazepine receptors.[10,11] Neuroprotective effects of benzodiazepines are being currently studied yielding controversial results. Diazepam appears to protect hippocampal neurons against cerebral ischemia[12] although most of its effect relates to induced hypothermia.[13] A family of atypical benzodiazepines has also been reported to function as non-competitive AMPA-receptor antagonists. Besides the fact they have been shown to attenuate excitotoxicity by AMPA in hippocampal cultures[14], their *in vivo* effectiveness remains divisive.[15] Other AMPA antagonists recently synthesized seem to be more promising.[16,17] Also agents fostering GABA-A responses[18,19] or peripheral benzodiazepine receptor[20] have been shown to present neuroprotective effects.

In the present report, antioxidant properties and neuroprotective action against glutamate-induced neurotoxicity of the well known hypnotic zolpidem and four of its synthesis intermediates carrying several differences between their side chains, were evaluated *in vitro* and in cell culture. Cells used for the neuroprotective studies were HT-22, known to lack of AMPA and NMDA receptors for glutamate[21]. Comparative studies with melatonin and other antioxidants are also shown.

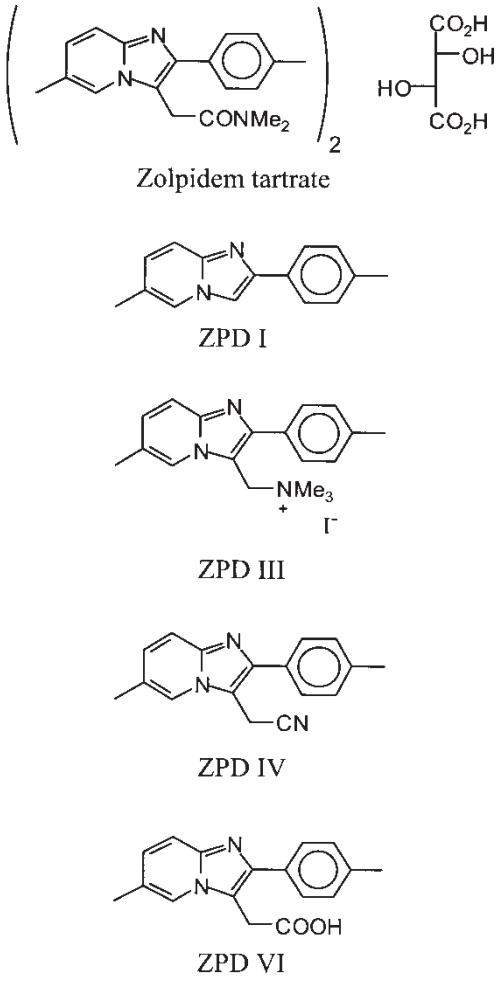


FIGURE 2 Chemical structures of Zolpidem tartrate (CAS # 99294-93-6), Zolpidem I (ZPD-I) (CAS # 88965-00-8), Zolpidem III (ZPD-III) (CAS # 106961-34-6), Zolpidern IV (ZPD-IV) (synthesized as described in the patent # GB1076089), and, Zolpidern VI (ZPD-VI) (CAS # 189005-44-5).

## MATERIAL AND METHODS

### Materials and Chemicals

The kit for the lipid peroxidation assay was obtained from OXIS International Incorporation, Portland, OR, USA (–LPO586–). Melatonin was purchased to Aldrich (Sigma Aldrich, Milwaukee, WI, USA). Zolpidem tartrate (ZPD), CAS # 99294-93-6, and zolpidem intermediates were synthesized in Asturpharma, S.A. Zolpidem intermediates used (Fig. 2) are the following: zolpidem I (ZPD-I), CAS # 88965-00-8; zolpidem III (ZPD-III), CAS # 106961-34-6; zolpidem IV (ZPD-IV), which has been synthesized as described in the patent # GB1076089; and zolpidern VI (ZPD VI), CAS # 189005-44-5. Purity of all of them was verified by high performance liquid chromatography (HPLC) (Fig. 3). All other reagents were obtained from Sigma Chemicals (St. Louis, MO).

### Animals

Liver and brain from male Wistar rats (1 month old) were used for the lipid peroxidation assays. They were kept in the animal room of the University of Oviedo, fed ad libitum with an equilibrated standard diet and sacrificed by decapitation 24 h after food restriction. These studies have been carried out in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institute of Health.

### Cell Culture

Rat hippocampal cells (HT22) were grown in 90% Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/till penicillin,

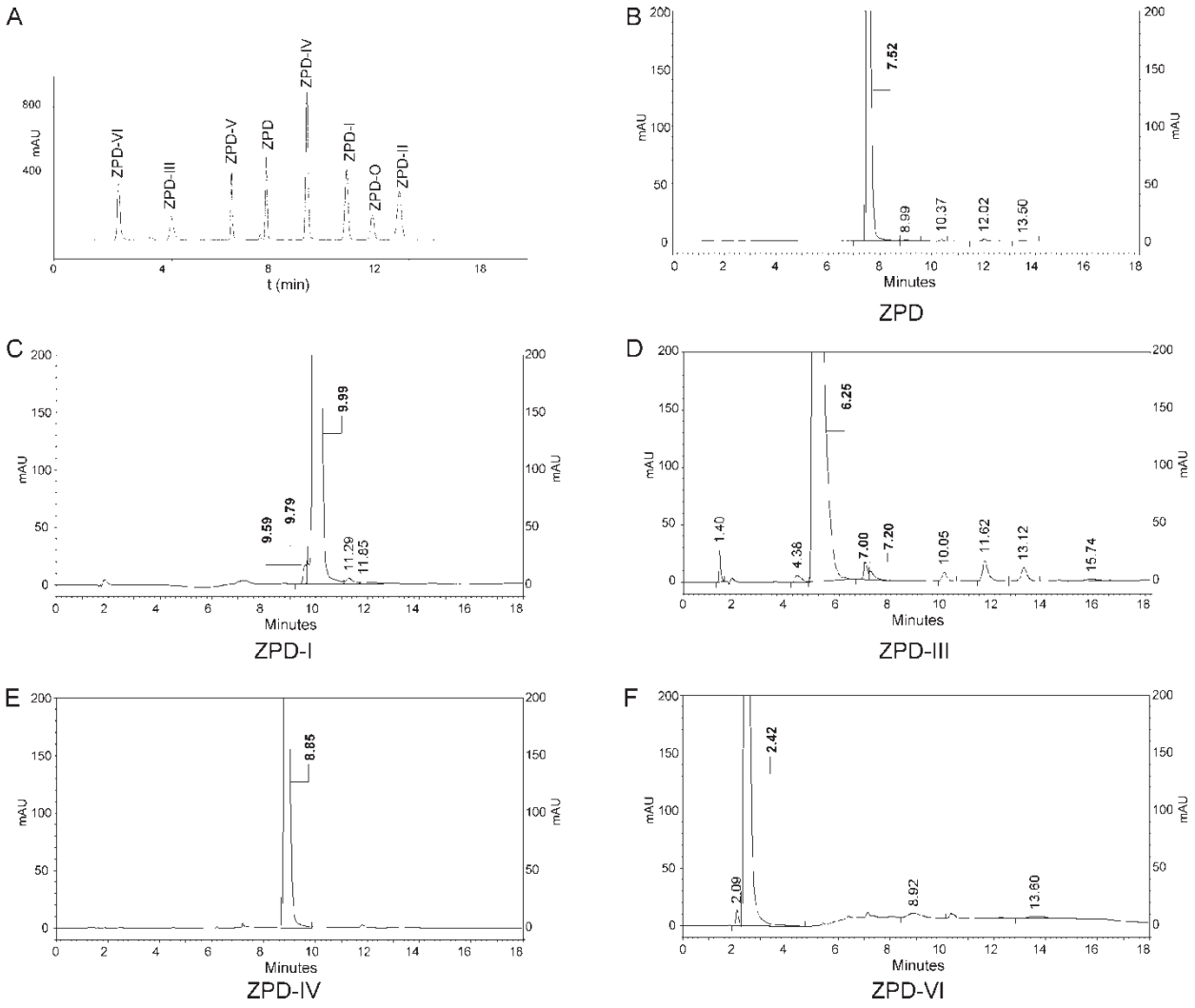


FIGURE 3 HPLC data of all Zolpidem synthesis intermediates used in the study (A), and each of them separately: (B) Zolpidem (ZPD); (C) ZPD-I; (D) ZPD-III; (E) ZPD-IV; and (F) ZPD VI.

100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were maintained in T-75 flasks in 5% $CO_2$ at 37°C and medium was changed every other day.

### Lipid Peroxidation Evaluation

Tissues (liver and brain) were homogenized in 10% (w/v) Tris–HCl buffer (20 mM, pH 7.4, 40°C). After centrifugation at 2500 *g* and 4°C for 5 min, supernatant was collected and used for the LPO study. Treatment of samples and assay were undertaken as described previously.[22]

### *In Vitro* Protein Oxidation Induction

Free radical protein damage was induced using $Cu^{2+}$ and $H_2O_2$. Bovine serum albumin (BSA) (Fraction V) solved in phosphate buffer (150 mM, pH 7.3) at 0.5 mg/ml was incubated for 3 h with or without 100 μM $Cu^{2+}$ and 2.5 mM $H_2O_2$ in presence or absence of zolpidem tartrate, melatonin or zolpidem intermediates. These drugs were solved in 100% DMSO and diluted to the desired concentrations. Final concentration of DMSO was 1% being this included in control and $Cu^{2+}/H_2O_2$ groups. Protein damage was estimated by running protein with the different treatments in a polyacrylamide gel and staining the gel with Comassie blue. Differences in the band intensity are due to damage in protein integrity.

Protein samples were mixed with loading buffer (10% glycerol, 2% SDS, 62.5 mM *Tris*–HCl pH 6.8, 5% β-mercaptoethanol, 0.05% bromophenol blue) and boiled for 5 min. Four μg of protein were loaded in a 12% polyacrylamide gel and electrophoresed at 125 V during 1 h. Gels were stained with 0.1% Comassie blue R-250 for 30 min, washed and dried in a gel drier (Bio-Rad). Experiment was repeated 6 times. Band intensity was estimated using the Scion Image Beta 4.02 for Windows™ analysis software,

downloaded at the web site address. URL: http://www.scioncorp.com. The density of each band was estimated and standardized with respect to the control group. A total of six experiments were accomplished, being each of them analyzed three times. Data show the median ± standard error of six independent experiments.

### Carbonyl Content Determination

To measure the formation of carbonyls in the proteins the method described by Levine *et al.*[23] was used. This method is based in the reaction with 2–4 dinitrophenylhydrazine (DNPH). The oxidative reaction with $Cu^{2+}$ and $H_2O_2$ was stopped with 0.2 ml of 1 M manitol and 0.5 M EDTA solution. One mg of protein was mixed with 0.55 ml of 10 mM DNPH (final concentration 2 mM). After 1 h at 37°C 0.55 ml of 20% trichloroacetic acid was added and the mixture was incubated on ice for 10 min. Samples were then centrifuged at 11,000*g* during 5 min. Pellets were washed three times with ethanol/ethyl acetate (1:1 v/v), resuspended in 1 ml of 6 M guanidine in 2 N HCl (pH 2) and incubated at 37°C for 15 min. After centrifugation at 13,000*g* for 5 min the absorbance of supernatants was read at 375 nm in an automatic microplate reader (μQuant, Bio-Tech Instruments Incoporation).

### Measurement of Intracellular ROS

Intracellular production of ROS was evaluated by the fluorescent probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) following a modification of the technique described by Bass *et al.*[24] DCFH-DA is a compound that enters cells freely. It is cleaved by intracellular esterases giving the non-fluorescent compound DCFH which is oxidized by intracellular oxidants to the fluorescent DCF. Cells were seeded in 60 min plates at a density of 6,500 cells/cm$^2$ in 4.5 ml of complete medium. Afterwards cells were not treated or treated for 12 h with one of the following drugs: glutamate (10 mM), melatonin (1 mM), zolpidem tartrate (500 μM), ZPD-I (25 μM), ZPD-III (500 μM), glutamate plus melatonin, glutamate plus zolpidem tartrate, glutamate plus ZPD-I, or glutamate plus ZPD-III. Thereafter, cells were trypsinized and pelleted at 200*g* for 5 min. Pellets were resuspended in 500 μl DCFH-DA 10 μM in serum free medium and incubated during 15 min at 37°C. Afterwards, cells were again centrifuged at 200*g* during 5 min, and pellets were resuspended in 500 μl of PBS after discarding the supernatant cells. Ten microlitres of 50 μg/ml propidium iodide were added to each tube. After shaking, DCF fluorescence of 10,000 alive cells per group (cells without propidium iodide uptake) was measured in a Beckman Coulter FC500 flow cytometer. All samples were performed by triplicate. Data showed are the mean ± standard error of three independent experiments.

### Measurement of Cell Death

HT-22 cells were plated in 96 well plates at a density of $2 \times 10^3$ cells in 100 μl of complete medium. Cells were non treated or treated with glutamate 10 mM or glutamate plus each of the several antioxidant tested during 12 or 24 h. Once the treatment was completed, the method described by Denizot and Lang[25] was followed. Absorbance at 540 nm was determined in an automatic microplate reader (μQuant, Bio-Tek Instruments Incorporation, Winooski, VT, USA).

### Statistical Analysis

Results shown are the average of at least three independent experiments. Data are represented as the mean ± S.E. Significance was tested by one-way ANOVA followed by a Student-Newman-Keuls multiple range test. Statistical significance was accepted when $p < 0.05$.

## RESULTS

### *In Vitro* Prevention of Lipid Peroxidation

Liver and brain homogenates were used to study the ability of zolpidem and its intermediates to prevent lipid peroxidation *in vitro*. Lipid peroxidation was induced by $Fe^{2+}$ sulphate. A previous dose-response study (0.1 μM–1 mM) with this compound was carried out in order to determine the correct dose for the antioxidant experiments. One hundred micro molar of $Fe^{2+}$ sulphate was the choice given it showed a 223% average increase of lipid peroxidation vs. basal peroxidation in control samples in the four trials conducted (data not shown).

Dose-response studies in liver homogenates were carried out to determine the most efficient dose for each of the several antioxidants used, including zolpidem and its intermediates (data not shown). The optimal concentration of each compound was used in a single experiment to compare the antioxidant activity of the drugs under study.

Zolpidem tartrate (1 mM) prevented lipid peroxidation induced by 100 μM of $Fe^{2+}$ sulphate by 50%; Melatonin (1 mM) by a 52%; Trolox (200 μM) and estradiol (200 μM) totally prevented induced lipid peroxidation also reducing basal peroxidation by more than 65% (Fig. 4A).

Out of the five zolpidem intermediates evaluated, ZPD-I (100 μM) fully prevented $Fe^{2+}$ sulphate induced lipid peroxidation and could—in part—limit basal peroxidation; ZPD-III (1 mM) reduced induced peroxidation by 26%; ZPD-IV (1 mM) fully prevented induced peroxidation; whereas ZPD VI

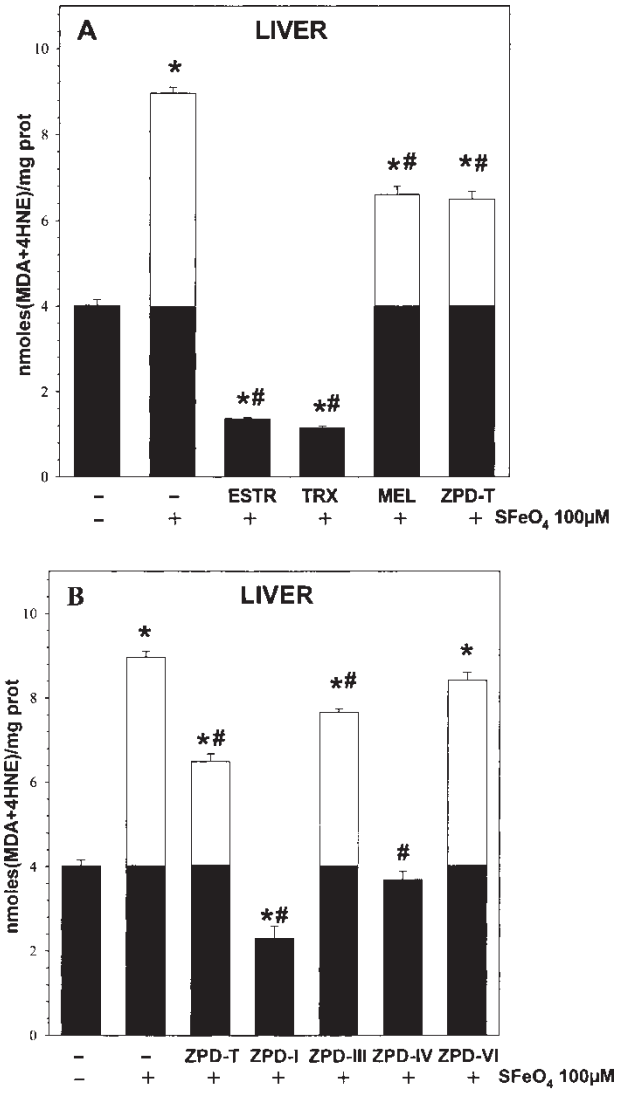


FIGURE 4 Prevention of iron sulphate ($SFeO_4$) 100 μM induced lipid peroxidation in rat liver homogenates by (A) Zolpidem tartrate 1 mM (ZPD-T) and several antioxidants: Estradiol 200 μM (ESTR), Trolox 200 μM (TRX) and Melatonin 1 mM (MEL); (B) Zolpidem tartrate 1 mM (ZPD-T) and several of its synthesis intermediates: Zolpidem I 100 μM (ZPD-I), Zolpidem III 1 mM (ZPD-III), Zolpidem IV 1 mM (ZPD-IV) and Zolpidem VI 1 mM (ZPD-VI). Filled part of the columns indicates basal peroxidation obtained in the control group. Data represented are the mean ± standard error of four independent experiments. *$p < 0.05$ vs. non-treated group; #$p < 0.05$ vs. $SFeO_4$ treated group.

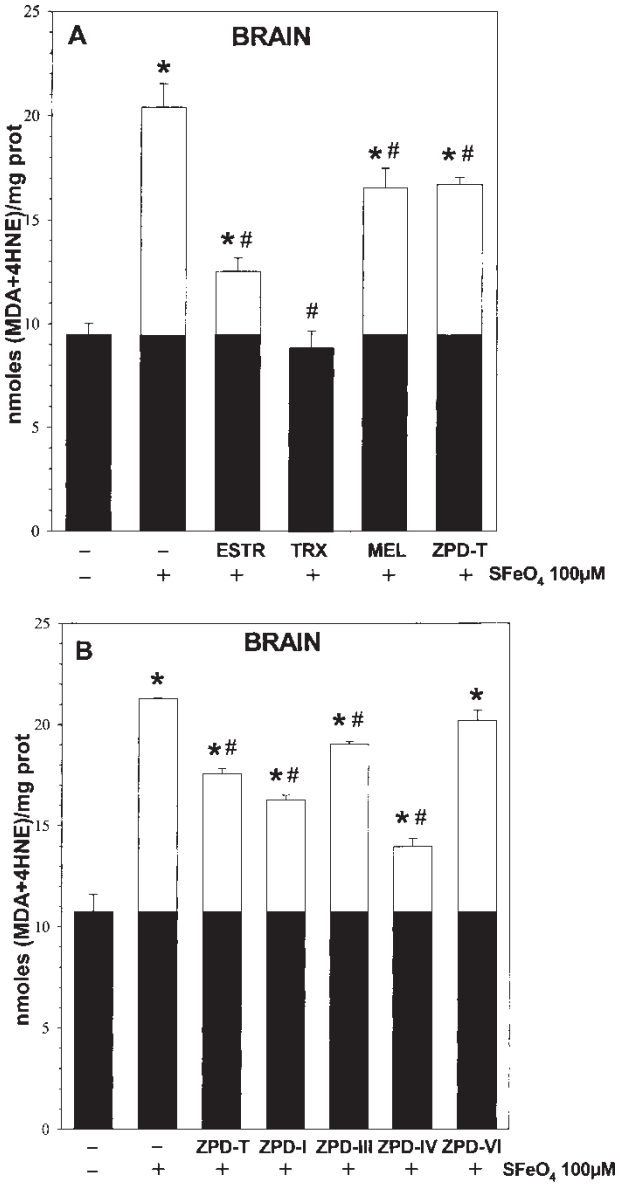


FIGURE 5 Prevention of iron sulphate ($SFeO_4$) 100 μM induced lipid peroxidation in rat brain homogenates by (A) Zolpidem tartrate 1 mM (ZPD-T) and several antioxidants: Estradiol 200 μM (ESTR), Trolox 200 μM (TRX) and Melatonin 1 mM (MEL); (B) Zolpidem tartrate 1 mM (ZPD-T) and several of its synthesis intermediates: Zolpidem I 1 mM (ZPD-I), Zolpidem III 1 mM (ZPD-III), Zolpidem IV 1 mM (ZPD-IV) and Zolpidem VI 1 mM (ZPD-VI). Filled part of the columns indicates basal peroxidation obtained in the control group. Data represented are the mean ± standard error of four independent experiments. *$p < 0.05$ vs. non-treated group; #$p < 0.05$ vs. $SFeO_4$ treated group.

(1 mM) failed to provide protection against $Fe^{2+}$ sulphate as oxidant (Fig. 4B).

When ZPD, its intermediates and other antioxidants were tested in brain, the protective effect was lesser although all of them paralleled the effects in liver as mentioned above. Zolpidem tartrate and melatonin 1 mM cut induced peroxidation by 34% and 35% respectively; β-estradiol (200 μM) by 72%; and trolox (200 μM) prevention of the induced peroxidation was total (Fig. 5A). Zolpidem intermediates were assayed in separate experiments together with zolpidem at 1 mM in all cases. Zolpidem reduced induced lipid peroxidation by 35%; ZPD-I by 47%: ZPD-III by 21%; ZPD-IV by 70% and ZPD-VI did not prevent in a significant way lipid peroxidation (Fig. 5B).

### *In Vitro* Protein Protection against Metal-catalyzed Oxidative Damage

*In vitro* protection by zolpidem tartrate and its intermediates vs. protein damage was assayed by using the combination of $CuSO_4$ plus hydrogen

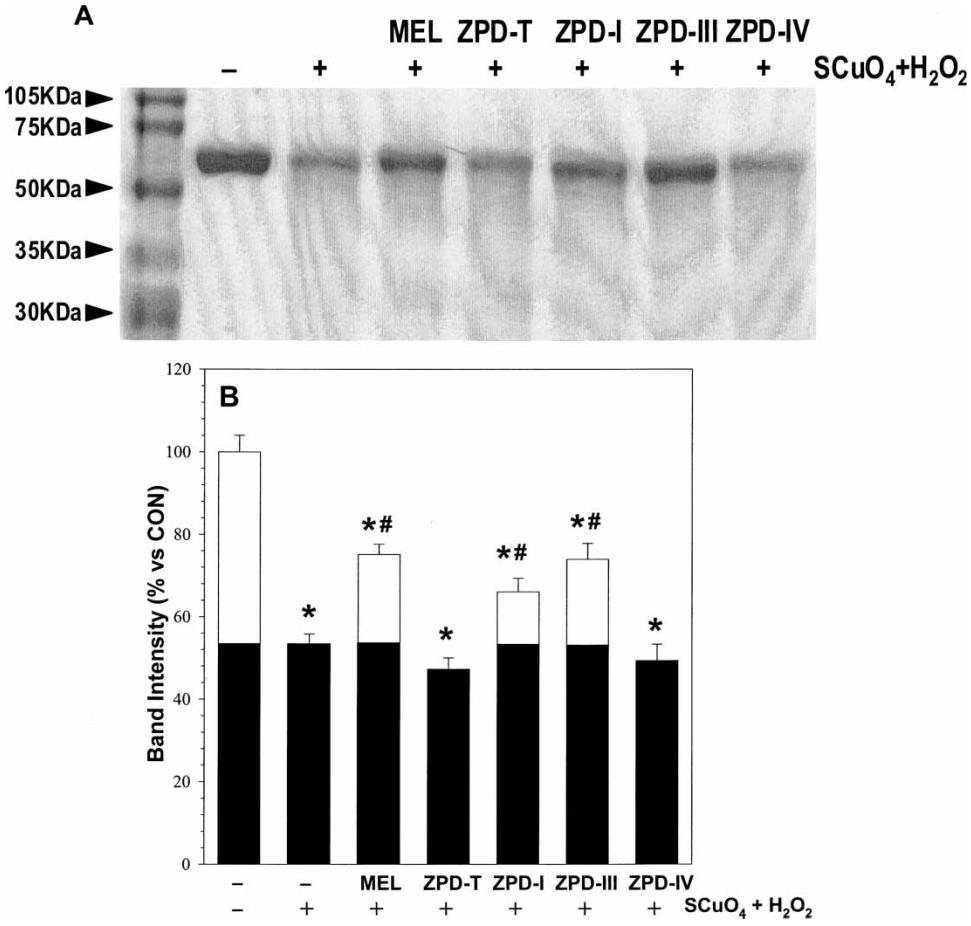


FIGURE 6 Protection of proteins (bovine serum albumin) against metal-catalyzed oxidative damage ($SCuO_4$ 100 μM + $H_2O_2$ 2.5 mM) by Melatonin 1 mM (MEL), Zolpidem tartrate 1 mM (ZPD-T), Zolpidem I 250 μM (ZPD-I), Zolpidem III 1 mM (ZPD-III), and Zolpidem IV 1 mM (ZPD-IV). (A) Representative gel out of six independent experiments. (B) Densitometric analysis of the protein bands from the gels. Data represented are the mean ± standard error of six independent experiments. *$p < 0.05$ vs. non-treated group; #$p < 0.05$ vs. $SCuO_4 + H_2O_2$ treated group.

peroxide. These oxidants were added to the BSA mixture at 100 μM and 2.5 mM, respectively. Melatonin, zolpidem tartrate, ZPD-III and ZPD-IV were added 1 mM. ZPD-I was added to saturation in DMSO 1%: 250 μM. Melatonin, ZPD-III and ZPD-I showed protection against protein degradation caused by the oxidants used. The degree of protection was Melatonin > ZPD-III > ZPD-I. Zolpidem tartrate and ZPD-IV did not prevent protein damage. Figure 6 shows a representative gel out of the six independent experiments carried out (A) and the result of the densitometric analysis of the protein bands of six independent experiments (B).

Figure 7 represents the carbonyl content (nmol/mg protein) increase induced by $CuSO_4$ plus hydrogen peroxide at the concentrations used for the previous experiments vs. the effects of melatonin (1 mM), zolpidem tartrate (1 mM), ZPD-I (250 μM), ZPD-III (1 mM), and ZPD-IV (1 mM). $CuSO_4$ induces a carbonyl content 14.5 fold vs. control Melatonin and ZPD-I cut this augment by 30% and 22%, respectively. ZPD-IV reduced carbonyl content increase by 12%. Although the prevention by ZPD-IV is not significant, it was reproducible in the six experiments conducted. Zolpidem tartrate did not prevent carbonyl formation. Finally, ZPD-III increased—quite strikingly—in a significant and reproducible way the carbonyls formed (40%).

### Decrease in Intracellular ROS Production

The assay on the intracellular antioxidant effect of these compounds and their influence on free radicals induced by glutamate was performed on hippocampal neurons. Rat hippocampal cells (HT22) were treated with glutamate, melatonin, ZPD and ZPD intermediates and with glutamate plus each of these drugs as described in "Material and Methods" section. Both melatonin 1 mM and ZPD-I 25 μM were able to prevent in full glutamate (10 mM) induced oxidative stress on the experimental cells. Zolpidem tartrate 1 mM and ZPD-III 1 mM also had a good effect on the control of free radicals induced by this neurotoxin. Nevertheless, these compounds can increase DCF fluorescence by themselves that translates in a 50% reduction of

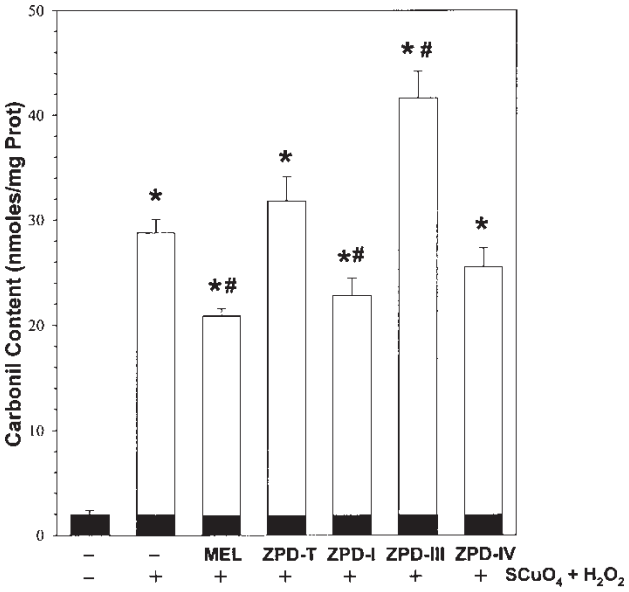


FIGURE 7 Prevention of protein oxidation (bovine serum albumin) induced by $CuSO_4$ (100 μM) + $H_2O_2$ (2.5 mM) by Melatonin 1 mM (MEL); Zolpidem tartrate (ZPD-T) 1 mM; Zolpidem I (ZPD-I) 1 mM; Zolpidern III (ZPD-III) 1 mM; and Zolpidem IV (ZPD-IV) 1 mM. Filled part of the columns indicates basal carbonyl content in the control group. Data represented are the mean ± standard error of six independent experiments. *$p < 0.05$ vs. non-treated group; #$p < 0.05$ vs. $SCuO_4 + H_2O_2$ treated group.

fluorescence in the presence of glutamate. Results are shown in Fig. 8A–F.

### Neuroprotective Effects of Zolpidem and Some of its Intermediates

Several antioxidants have been proved to prevent neuronal death induced by oxidative stress.[26–29] HT22 cells do not have ionotropic glutamate receptors[21] being excitotoxicity not responsible of neuronal death by glutamate in these cells. Oxidative stress has been reported to be involved in neurotoxicity by glutamate in HT22 cells.[30] For the study of the neuroprotective effect of these compounds, the model described above was used. After treatment with high concentration of glutamate (10 mM) during 12 h, melatonin 1 mM, zolpidem 500 μM, ZPD-III 500 μM, and ZPD-I 25 μM—concentration limited by solubility—fully prevented neuronal death induced by the neurotoxin (Fig. 9A). When glutamate 10 mM concentration was maintained in the culture for 24 h, melatonin 1 mM and ZPD-III 500 μM were still able to prevent neuronal death by 70% (Fig. 9B).

## DISCUSSION

This work focuses on the antioxidant and neuroprotective effect of the non-benzodiazepine hypnotic zolpidem and four of its synthesis intermediates. Comparison with the protection given by the indolamine melatonin—which shares structural similarities—and other antioxidants was also undertaken. Zolpidem tartrate was as effective as melatonin in preventing lipid peroxidation in liver and brain tissue in terms of concentration range and percentage of protection, although zolpidem did not show *in vitro* protein protection against oxidation, contrarily to melatonin. Both compounds were however less effective than trolox and estradiol in preventing lipid peroxidation.

We found that some zolpidem intermediates present an effect better than zolpidem. The most effective of them, regarding protection against lipid peroxidation, protein degradation and carbonyl formation, was ZPD-I. This zolpidem intermediate was able to prevent induced lipid peroxidation at 50 μM with figures similar to melatonin or zolpidem tartrate at 1 mM (data not shown). Higher doses managed to prevent in full induced lipid peroxidation showing an efficiency similar to estradiol and close to trolox. When the protein oxidation model was assayed, we found that ZPD-I was nearly as effective as melatonin in preventing metal-catalyzed protein degradation and carbonyl formation induced by $SCuO_4$.

Although ZPD-I is almost as effective as trolox in preventing lipid peroxidation, the specific action of trolox on lipid membranes must be kept in mind. Although this antioxidant has previously been shown to protect greatly against lipid peroxidation showing an effect better than melatonin,[22] it has been found to lack any effect on protein oxidation. Trolox has even been reported to present prooxidant activity at concentrations in the range of 25 μM–4 mM, losing effect at lower doses when the oxidative model was protein damage induced by metal-catalyzed oxidation.[31]

The working concentrations of zolpidem are similar to the effective doses for the direct antioxidant effect of melatonin previously reported and confirmed in the present work. Given the structural homology, similar effects are not surprising. Nevertheless, the fact that zolpidem failed to prevent protein damage, similarly to trolox,[31] indicates that structural differences are hiding this particular action. Copper binds albumin eagerly. Copper-catalyzed protein damage to serum albumin by hydrogen peroxide works by producing hydroxyl radical which oxidizes albumin immediately since it forms just close to the protein. Very few antioxidants are capable to protect albumin under these particular assay conditions. Mayo *et al.*[31] reported that ascorbic acid and trolox behave like prooxidants under such experimental conditions and only high doses of GSH or melatonin can eventually prevent albumin oxidation. Not even antioxidants like resveratrol and mannitol were able to protect albumin from oxidation

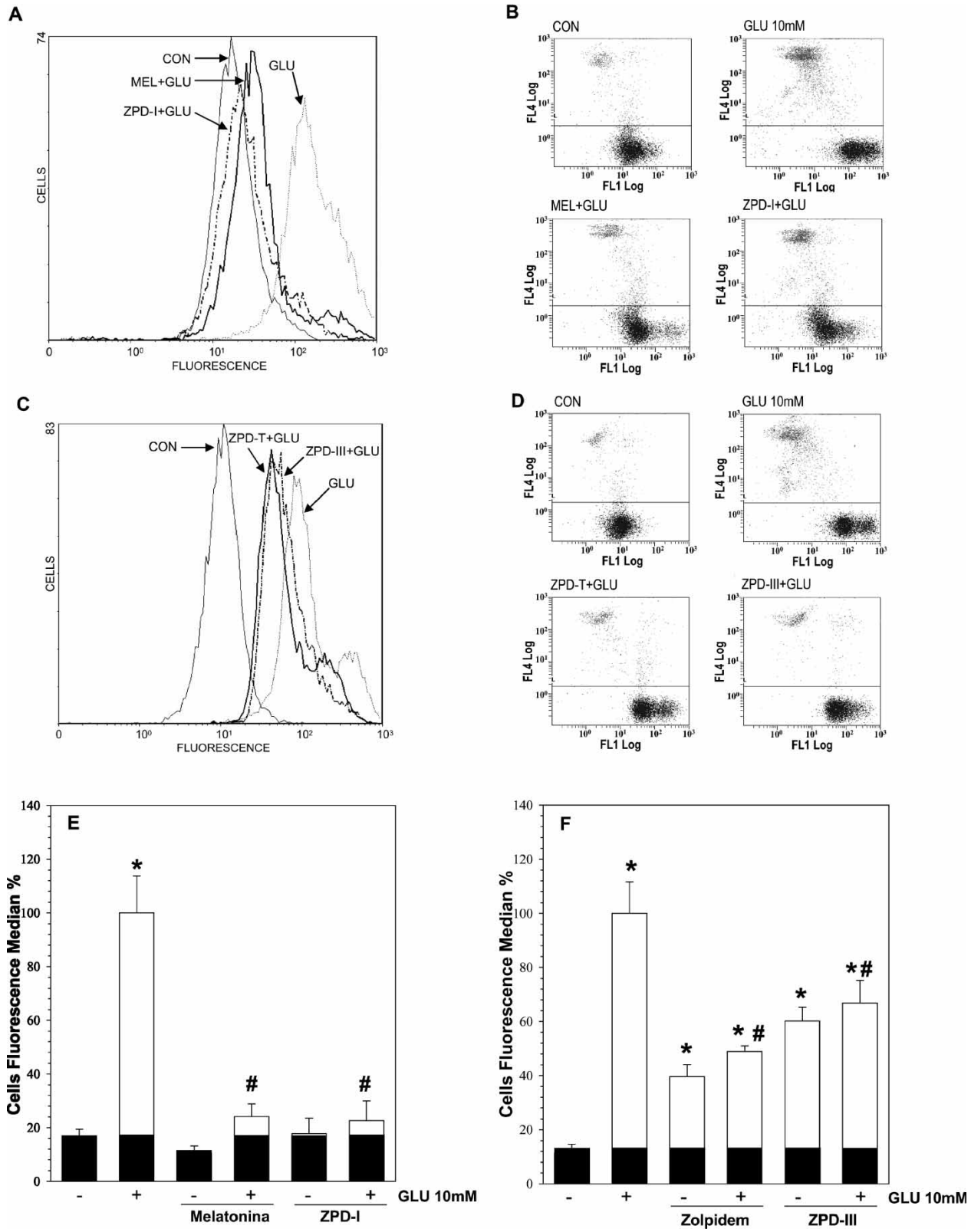


FIGURE 8 ZPD-I and melatonin totally prevent the intracellular increase of free radicals induced by glutamate and measured by flow cytometry using the fluorescent probe DCF as described in "Material and Methods Section". Zolpidem tartrate (ZPD-T) and Zolpidem III (ZPD-III) also prevent the increase of free radicals induced by glutamate but themselves induce increase of DCF fluorescence. (A,C) Cell fluorescence of HT22 cells non-treated (CON) or treated with: glutamate 10 mM (GLU); glutamate 10 mM + melatonin 1 mM (MEL + GLU); glutamate 10 mM + ZPD-I 25 μM (GLU + ZPD-I) (A); or with: glutamate 10 mM (GLU); glutamate 10 mM + Zolpidern tartrate 500 μM (ZPD-T + GLU); glutamate 10 mM + ZPD-III 500 μM (ZPD-III + GLU) (C). The data patterns shown are representative of three experiments. (B,D) Histograms show both, death cells—propidium iodide fluorescence—(Y axis) and level of cellular oxidation—DCF fluorescence—(*X* axis). Botton box corresponds to live cells (cells which do not show propidium iodide uptake). (E,F) Graphs showing the DCF fluorescence mean ± standard error of three independent experiments. Filled part of the columns indicates basal intracellular free radicals present in the control group. *$p < 0.05$ vs. non-treated group; #$p < 0.05$ vs. glutamate-treated group.

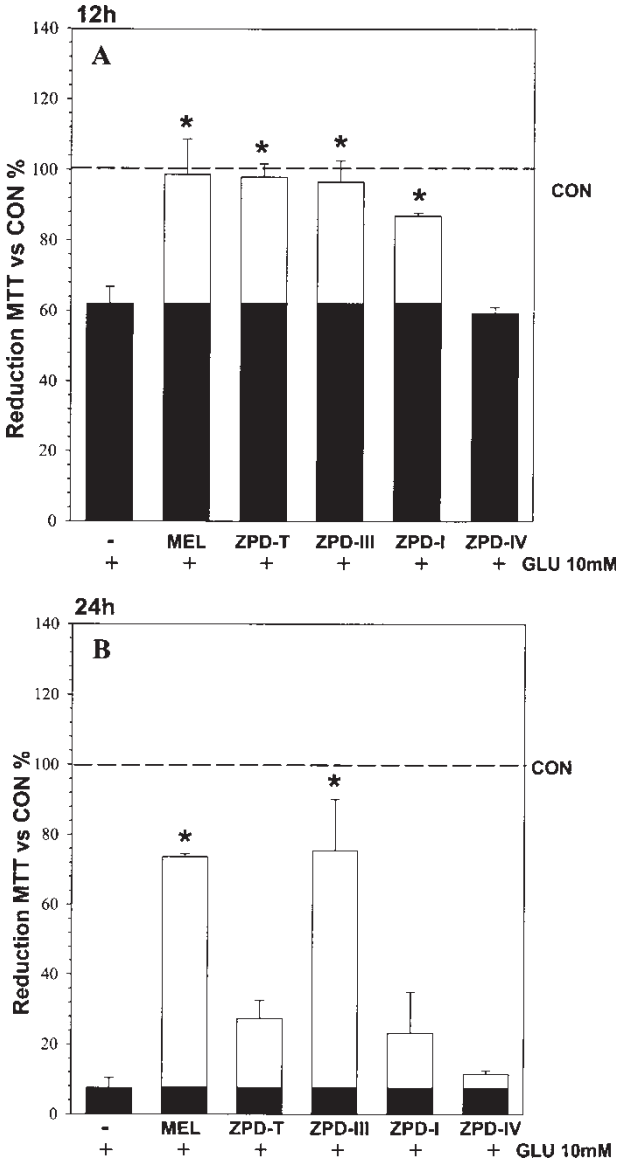


FIGURE 9 Zolpidem tartrate (ZPD-T) 500 μM, melatonin (MEL) 1 mM, Zolpidem III (ZPD-III) 500 μM and Zolpidem I (ZPD-1) 25 μM but not Zolpidem IV (ZPD-IV) 50 μM protect hippocampal HT-22 cells against cell death induced by glutamate 10 mM present in the cell culture during 12 h (A). Melatonin 1 mM and ZPD-III 500 μM still protect hippocampal neurons after 24 h of glutamate 10 mM present in the culture (B). Filled part of the columns indicate MTT reduction after treatment with glutamate; CON = MTT reduction in not treated groups. Data represented are the mean ± standard error of at least three independent experiments. *$p < 0.05$ vs. glutamate-treated group.

in that report. The fact that melatonin and not zolpidem can protect albumin from metal-catalyzed oxidation indicates that structural differences make zolpidem—similarly as trolox—unable to scavenge the *in situ* produced hydroxyl radicals.

The structural difference between zolpidem and ZPD-I is the lack of the CNmetoxy group in position 1. This difference would explain a higher antioxidant effect of ZPD-I when compared to zolpidem in terms of both, lipid and protein oxidation. ZPD-IV is also more efficient than zolpidem in preventing lipid peroxidation and in this case only the oxygen of the carbonyl group and the metilene group were left, indicating that these groups are—alone or in combination—responsible for the smallest antioxidant properties of zolpidem as compared to some of its intermediates. The indole carbonyl group in the 3-lateral chain has been previously shown to decrease the antioxidant activity of the compound.[5] This could be hypothetically explained by a prooxidant activity of this group. ZPD-IV, as well as zolpidem, were not however efficient in preventing protein oxidation, indicating than the CN group formed is also affecting negatively its antioxidant properties. The hidroxy group of ZPD-VI must be responsible for its lack of antioxidant effect. This group was previously reported to have prooxidant activity when present in the melatonin molecule.[32,33]

As summary, the heterocyclic amine structure seems to be critical when referring to the antioxidant properties of both melatonin and zolpidem. Both of these compounds were equally potent in preventing lipid peroxidation, although melatonin protected proteins more efficiently against oxidation. *In vivo* administration of vitamin E to patients with Alzheimer's disease improves systemic oxidative stress parameters and ameliorates cognitive performance,[34] indicating that potent antioxidants may change the future of diseases involving oxidative stress condition. The antioxidant properties of the ZPD molecule may be improved by removing the lateral chain in position 1. The resulting compound would be as potent as β-estradiol and trolox in the prevention of lipid peroxidation but indead it would be able to prevent protein oxidation.

Besides the *in vitro* antioxidant properties, these compounds also prevented intracellular formation of free radicals induced by glutamate in hippocampal HT22 cells. ZPD-I, at a concentration of 25 μM—lower than those in *in vitro* studies—was able to fully prevent the increase of intracellular free radicals induced by glutamate in hippocampal cells. This hints of a potential use in the prevention of oxidative stress caused by neurotoxins although further studies have yet to be performed in this field. Zolpidem and ZPD-III also prevented formation of free radicals in hippocampal cells, although they can increase DCF fluorescence by themselves. The intracellular prevention of the raise in free radicals by these compounds agree well with their efficacy *in vitro*: ZPD-I > ZPD-III = zolpidem. The antioxidant properties of zolpidem and ZPD intermediates are therefore also quite evident in cellular systems, where intracellular free radicals rise induced by glutamate was totally prevented. Although further research is necessary in order to clarify

the intracellular mechanisms of these compounds as well as to evaluate *in vivo* toxicity and absorption, these potent antioxidants should be considered as a potential drug in the study of therapeutic approaches to chronic diseases where free radical toxicity is implied.

Neuroprotective effect on neuronal death induced by glutamate in HT-22 cells is a good model for the study of protection against free radical neurotoxicity. The mechanism involved in toxicity of glutamate in these cells is independent of glutamate receptors and is related to shut down of the high affinity glutamate transporters on nerve and glia,[35] which normally clear extracellular glutamate.[36] Cystine entrance to the cell through the cystine/glutamate antiporter does not occurs, and intracellular glutathione decreases, producing an increase of intracellular ROS.[30] This kind of glutamate toxicity has been shown to occur in prooxidant conditions such as ischemia and trauma[35] which increase levels of extracellular glutamate up to 500 μM[37,38] making it possible for glutamate to induce neurotoxicity, even in cells not expressing functional glutamate receptors. All of the three compounds tested, zolpidem, ZPD-I and ZPD-III, can prevent totally cell death after 12 h of exposure to this toxin in the culture medium. Considering the lack of AMPA receptors in HT-22 cells[21] and the proven involvement of oxidative stress on glutamate induced cell death,[30] it is reasonable to think that the high antioxidant properties of the compounds studied are involved on neuroprotection. The fact that ZPD-III is clearly more efficient than zolpidem and ZPD-I after 24 h of glutamate exposure—besides their similar intracellular antioxidant activity—indicates that further studies should be done to rule out other mechanisms behind HT22 neuronal death prevention by this compound.

It may be concluded that zolpidem presents antioxidant and neuroprotective effects against the oxidative stress and neuronal death induced by glutamate in hippocampal cells. Its synthesis intermediate ZPD-I has much better *in vitro* antioxidant properties and similar neuroprotective effects in the model studied. The intermediate ZPD-III also prevents glutamate-induced free radical formation similarly to zolpidern, but it shows better neuroprotective effects. *In vivo* studies are worth to find out more on the pharmacological properties of zolpidem intermediates in terms of absorption, ability to enter the blood brain barrier, or toxicity, given the involvement of high levels of extracellular glutamate in the neuronal damage caused by several conditions and the proven ability of zolpidem to cross the blood brain barrier together with the low toxicity of this drug.



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