Protective Effects of Thiazolo[3,2-b]-1,2,4-Triazoles on Ethanol-Induced Oxidative Stress in Mouse Brain and Liver

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A series of 3-[1-(4-(2-methylpropyl) phenyl) ethyl]-1,2,4-triazole-5-thione (I) and its bicyclic condensed derivatives 6-benzylidenethiazolo[3,2-b]-1,2,4-triazole-5(6H)-ones (IIa-IIf) were investigated for the prevention of ethanol-induced oxidative stress in liver and brain of mice. Administration of ethanol (0.1 mL/mice, p.o.) resulted in a drop of total thiol groups (T-SH) and non-protein thiol groups (NP-SH), and an increase in thiobarbituric acid reactive substances (TBARS) in both liver and brain tissue of mice ($p < 0.001$). Among the compounds investigated (at a dose of 200 mg/kg, p.o.), I and IId ameliorated the peroxidative injury in these tissues effectively. Compounds IIa, IIc and IIe improved the peroxidative tissue injury only in brain. These findings suggest that certain condensed thiazolo-triazole compounds may contribute to the control of ethanol-induced oxidative stress in an organ selective manner.

Key words: Antioxidant activity, Condensed thiazolo-triazole compounds, Ethanol toxicity, Non-steroidal antiinflammatory drugs, Oxidative stress

INTRODUCTION

The intracellular sources contributing to reactive oxygen species (ROS) generation in inflammatory diseases are several, including prostaglandins, cyclooxygenases (COX) and lipoxygenases (Brooks and Day, 1991; Barbieri et al., 2003). It is now known that ethanol exposure results in increased prostaglandin synthesis and COX activity in brain and liver (Mathurin et al., 2000; Sanchez-Moreno et al., 2003). Treatment with nonsteroidal antiinflammatory drugs (NSAIDs) at therapeutic doses significantly diminishes ROS dependent injuries at inflammation sites by inhibiting COX enzymes (Kontogiorgis and Hadjipavlou-Litina, 2002).

The new generation of NSAIDs, such as celecoxib and rofecoxib, treats inflammation by selectively blocking the COX-2 enzyme and has greatly reduced gastrointestinal toxicity. Thus, they are ideal therapeutic agents that have suitable antiinflammatory and analgesic properties (Mitchell et al., 1994; Dannhardt and Kiefer, 2001). It has been reported that some thiazolone derivatives have potent and selective COX-2 inhibitor activity (Song et al., 1999),

and are also used as therapeutic agents in the treatment of diseases involving free radicals (Weber et al., 1982; Wlodek and Rommelspacher, 1994).

In a previous study, we had synthesized 3-[1-(4-(2-methylpropyl)phenyl)ethyl]-1,2,4-triazole-5-thione (I) and its bicyclic condensed derivatives 6-benzylidenethiazolo[3,2-b]-1,2,4-triazole-5(6H)-ones (IIa-IIf) (Fig. 1) and demon-
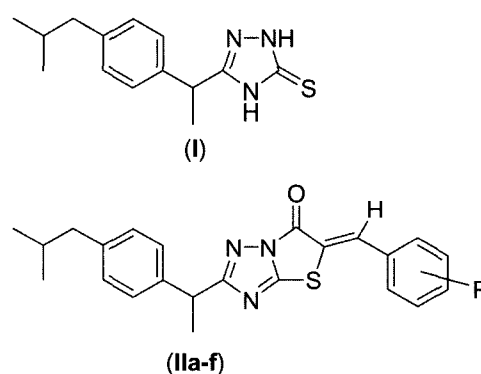
R : 4-Cl(IIa), 3-CH₃(IIb), 4-CH₃(IIc), 2-OCH₃(IId), 3,4,5-trimethoxy(IIe), 4-CF₃(IIf)

Fig. 1. 3-[1-(4-(2-methylpropyl)phenyl)ethyl]-1,2,4-triazole-5-thione (I) and its bicyclic condensed derivatives 6-benzylidenethiazolo[3,2-b]-1,2,4-triazole-5(6H)-ones (IIa-f) which have been synthesized and demonstrated to possess moderate antiinflammatory activities.

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strated that they possess moderate antiinflammatory activities (27% to 63%, Table I) against carrageenan-induced mice paw edema (Tozkoparan *et al.*, 2000). Promising antiinflammatory activity together with low ulcerogenic properties of these compounds prompted us to investigate further with the aim of clarifing their anti-oxidant properties.

To determine the antioxidant effects of these compounds, TBARS (mainly known as oxidative stress marker), T-SH and NP-SH were assayed in the liver and brain of mice.

MATERIALS AND METHODS

Chemicals

The synthesis of compounds was given in our previous study (Tozkoparan *et al.*, 2000). The chemical reagents used for antioxidative activity in the current sdudy were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Animals and treatment

Locally bred Swiss Albino male mice (Refik Saydam Hifzisihha Institute, Animal Care Unit, Ankara, Turkey) weighing approximately 22 g were used. Animals were divided into one control group and ten treatment groups. In the control group there were seven mice and in the each treatment group there were five or six mice. We administered the compounds **I** and **IIa-IIf** at a dose of 200 mg/kg based on a study by Tozkoparan et al, which found high antiinflammatory activity and low ulcerogenicity at a dose of 200 mg/kg. The compounds were suspended in 0.5% carboxymethylcellulose at a dose of 200 mg/kg, and administered 60 min before the injection of absolute ethanol (0.1 mL/mice) using a gastric gavage needle. One hour after the application of ethanol, the livers and brains of the mice were removed, under ether anesthesia. Aspirin, a non-selective COX inhibitor, (ASA, 200 mg/kg) and celecoxib, a selective COX-2 inhibitor, (CEL, 100 mg/kg) were used as reference NSAIDs.

TBARS levels in tissues

The method of Ohkawa *et al.* as modified by Jamall and Smith was used to determine TBARS in tissue samples. The method is based on the formation of a red chromophore which absorbs at 532 nm, following the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) and other breakdown products of peroxidized lipids.

T-SH and NP-SH levels in tissues

T-SH and NP-SH were determined according to the methods employed by Sedlak and Lindsay. Tissues were homogenized in 0.02 M ethylenediamine tetraacetic acid disodium (EDTA-Na_2). For determination of T-SH groups, aliquots of 0.5 mL of the homogenates were mixed with 1.5 mL of 0.2 M Tris buffer, pH 8.2, and 0.1 mL of Ellman's reagent. The mixture was brought to 10.0 mL with 7.9 mL of absolute methanol. Color was developed for 15 min and the reaction mixtures centrifuged at approximately 3000×g at room temperature for 15 min. The absorbance of supernatants was read at 412 nm.

For the determination of NP-SH groups, aliquots of 5.0 mL of the homogenates were mixed with 4.0 mL distilled water and 1.0 mL of 50% trichloroacetic acid. Tubes were centrifuged for 15 min at approximately 3000×g. Two ml of supernatant was mixed with 4.0 mL of 0.4 M Tris buffer pH 8.9 and 0.1 mL Ellman's reagent added. The absorbance was read within 5 min, at 412 nm against a sample blank.

Percent changes were calculated according to following formula:

$$(n-n')/ n \times 100$$

n: average level of TBARS, T-SH, NP-SH in the control or ethanol groups

n': average level of TBARS, T-SH, NP-SH in the test groups

Statistical analysis

All values are expressed as the mean ± SEM. The data were analyzed by a one-way analysis of variance (ANOVA) followed by a Tukey-Kramer post hoc test.

RESULTS AND DISCUSSION

Our findings demonstrated that ethanol significantly increased the TBARS (40%; Fig. 2) and decreased the thiol levels (T-SH: 45% and NP-SH: 72%; Table I) of liver tissue in treatment group compared to control group. None of the compounds **I,IIa, IIb, IIc, IIe** and **IIf** could prevent the increase in TBARS level (Fig. 2), and decrease in thiol levels with the exception of the compound **I** compared to ethanol group in liver tissue (Table I). It is clear that compound **IId** exhibited the most prominent effect on both TBARS (23%) and thiol (TSH: 63%; NP-SH: 44%) levels on ethanol-induced oxidative stress in liver tissue.

It is well known that ROS, which are known to be generated by several mechanisms including oxygenases, can damage DNA, inactivate enzymes, and peroxidize lipids and proteins. Therefore, they are critical in the maintenance of cellular viability (Logani and Davies, 1980; Nanji *et al.*, 1997). Reduced glutathione (GSH), which is the major compound containing NP-SH in mammalian cells, can act as a direct free radical scavenger. It has been estimated that more than 90% of the NP-SH of cells is in the form of GSH (Ravinder, 2002). Most of the studies suggest that depletion of GSH following acute ethanol
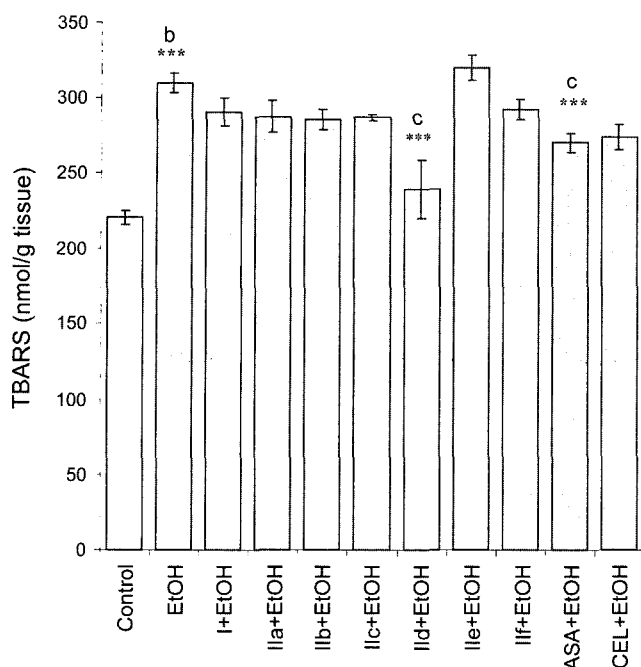
Fig. 2. Effects of synthesized compounds on the alteration of TBARS levels[a] in ethanol-induced oxidative stress in mouse liver. Six mice were included in the control group and five mice in each treatment group. [a]Significantly different from the control or ethanol group (ANOVA followed by Tukey-Kramer post hoc test); *p<0.05, **p<0.01, ***p<0.001; [b]Compared to control group; [c]Compared to ethanol group. ASA: aspirin, CEL: celecoxib, EtOH: ethanol.

radicals, including oxygen-radicals. GSH can quickly be converted to cysteine which is then used for protein synthesis and for the biosynthesis of other thiol metabolites (Logani and Davies, 1980; Shan *et al.*, 1990).

The brain is particularly susceptible to free radical attack since it consumes a large amount of oxygen and is rich in polyunsaturated, highly peroxidable fatty acids (Omodeo-Sale *et al.*, 1997). In our study, we observed that there was a meaningful increase in TBARS (21%) and a decrease in thiol levels (T-SH:20%; NP-SH:85%) in the brain tissue of the ethanol treated group (Fig.e 3, Table II). The compounds I, IIa, IId, and IIe effectively decreased the TBARS levels in the treatment groups compared to the ethanol group (31%, 28%, 19% and 19%, respectively). Although the compounds IIa, IIc, IId and IIe ameliorated only the levels of NP-SH, compound I exhibited greater changes in the levels of both T-SH and NP-SH (Table II). Unlike liver tissue, thiol content in brain tissue was affected by the compounds IIa, IIc and IIe, suggesting that these derivatives affect the redox status of different organs in a selective manner.

The results indicated that compound I, which is the main structure of our compounds, and IId have potent antioxidant activity in both liver and brain tissues. It appears that 3-[1-(4-(2-methylpropyl) phenyl)ethyl]-1,2,4-triazole-5-thione ring may play a significant role in the management of peroxidative injury. Nevertheless, it was not clearly explained why the chlore group in position 4 (IIa), methyl group in position 3 (IIb), methyl group in position 4 (IIc), methoxy groups in position 3,4,5 (IIe) and the trifluoromethyl group in position 4 (IIf) could not be effective on the ethanol-induced free radical production while compound IId with methoxy group in position 2

intoxication is a result of increased TBARS level. Thus, the drop in NP-SH and T-SH tissue levels is a result of the oxidation of GSH and proteins, respectively (Zentella-de-Pina *et al.*, 1993; Wlodek and Rommelspacher, 1994). In general, thiols are very reactive toward free oxidizing

Table I. Effects of synthesized compounds on the alteration of T-SH and NP-SH levels[a] in ethanol-induced oxidative stress in the liver of mice together with antiinflammatory activities (% inhibition) against carrageenan-induced hind paw edema model.

GROUPS (n=5-7)	TSH (μmol/g tissue)	% change	NP-SH (μmol/g tissue)	% change	Antiinf. activity (% Inh) [b]
Control	324.6 ± 7.2		38.6 ± 1.6		
EtOH [c]	179.6 ± 11.2***	-45	10.9 ± 0.3***	-72	
I+EtOH [d]	238.9 ± 12.7*	+33	16.9 ± 0.4**	+55	%50
IIa+EtOH [d]	229.2 ± 9.8	+28	11.6 ± 1.9	+6	%63
IIb+EtOH [d]	192.8 ± 11.6	+7	10.9 ± 0.5	-	%35
IIc+EtOH [d]	200.4 ± 14.5	+12	7.5 ± 0.5	-31	%27
IId+EtOH [d]	292.5 ± 12.7***	+63	15.7 ± 0.4*	+44	%42
IIe+EtOH [d]	177.6 ± 10.7	-	7.6 ± 0.9	-30	%33
IIf+EtOH [d]	197.3 ± 13.9	+10	9.0 ± 0.4	-17	%53
ASA+EtOH [d]	229.2 ± 11.8	+28	17.1 ± 0.8**	+57	
CEL+EtOH [d]	297.2 ± 9.4***	+65	17.4 ± 0.9**	+60	

[a]Statistical significance of data (expressed as mean ± SEM) was assessed by ANOVA followed by the Tukey-Kramer post hoc test. Significantly different from the control or ethanol group; *p<0.05, **p<0.01, ***p<0.001; [b]Tozkoparan *et al.*, 2000; [c]Compared to control group; [d]Compared to ethanol group. ASA: aspirin, CEL: celecoxib, EtOH: ethanol.
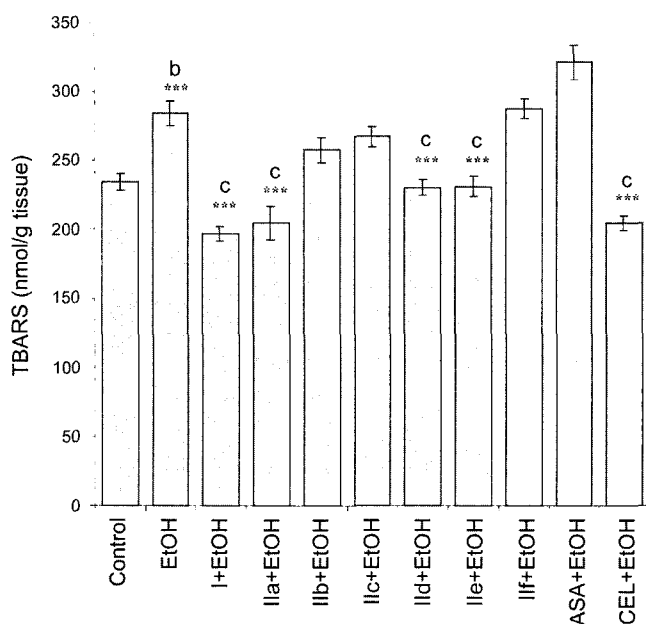
Fig. 3. Effects of synthesized compounds on the alteration of TBARS levels[a] in ethanol-induced oxidative stress in the mouse brain. Six mice were included in the control group and five mice in each treatment group. [a]Significantly different from the control or ethanol group (ANOVA followed by Tukey-Kramer post hoc test); *p<0.05, **p<0.01, ***p<0.001; [b]Compared to control group; [c]Compared to ethanol group. ASA: aspirin, CEL: celecoxib, EtOH: ethanol.

enhanced the prevention of oxidative reaction in livers. The mechanism underlying their antioxidant activity remains to some degree unknown. Further investigation to determine antioxidative effects of substituted groups on

Table II. Effects of synthesized compounds on the alteration of T-SH and NP-SH levels[a] ethanol-induced oxidative stress in the mouse brain.

GROUPS (n=5-7)	T-SH (mmol/g tissue)	% change	NP-SH (mmol/g tissue)	% change
Control	221.9 ± 2.4		29.2 ± 1.7	
EtOH[b]	178.3 ± 3.2***	-20	4.5 ± 0.5***	-85
I+EtOH[c]	202.3 ± 2.8***	+13	23.5 ± 0.9***	+422
IIa+EtOH[c]	186.4 ± 2.4	+5	17.7 ± 0.6***	+293
IIb+EtOH[c]	180.7 ± 1.9	-	8.6 ± 0.6	+91
IIc+EtOH[c]	177.9 ± 2.9	-	10.4 ± 0.6***	+131
IId+EtOH[c]	187.3 ± 3.0	+5	12.2 ± 0.4***	+171
IIe+EtOH[c]	176.2 ± 2.8	-	9.2 ± 0.5*	+104
IIf+EtOH[c]	186.2 ± 2.1	+4	5.6 ± 0.5	+24
ASA+EtOH[c]	195.4 ± 2.6**	+10	9.2 ± 0.5*	+104
CEL+EtOH[c]	200.3 ± 3.5***	+12	9.8 ± 0.6***	+118

[a]Statistical significance of data (expressed as mean ± SEM) was assessed by ANOVA followed the Tukey-Kramer post hoc test. Significantly different from the control or ethanol group; *p<0.05, **p<0.01, ***p<0.001; [b]Compared to control group; [c]Compared to ethanol group. ASA: aspirin, CEL: celecoxib, EtOH: ethanol.

peroxidative reactions is warranted.

According to our results, some of the compounds investigated in this study have varying degrees of antioxidative effects in addition to their moderate antiinflammatory effects. Low ulcerogenic properties of these compounds may also be a result of their anioxidative properties.

In conclusion, among the compounds investigated in this study, I and IId greatly reduced ethanol-induced oxidative injuries in liver and brain tissues. Although the compounds IIa, IIc, and IIe also inhibited the cellular injury in an organ selective manner, further investigations are essential to ascertain the mechanism involved in the antioxidative properties of the ring system. We suggest that compounds I and IId may be a new approach in the treatment of the toxic effects of ethanol ingestion.

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