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Investigation of antioxidant properties of some 6-(α-aminobenzyl) thiazolo[3,2-b]-1,2,4-triazole-5-ol compounds

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Abstract
Promising antiinflammatory activity together with low ulcerogenic properties of some Michael addition products of thiazolo[3,2-b]-1,2,4-triazole-5(6H)-ones which have been synthesized in our previous study, prompted us to investigate their antioxidant properties. Since compound **Ib** has both antioxidant and antiinflammatory activities beside the lowest ulcerogenic incidence, it was selected for investigation of its inhibitory effect on various cyclooxygenase ezymes. It was found that while it did not inhibit cyclooxygenase-1 (COX-1) enzyme, there was a small inhibitory effect (17%) on COX-2 enzyme. We concluded that the diminished harmful effects on the stomach of this novel antiinflammatory compound were related to its antioxidant properties since it is ineffective on COX-1 enzyme. In conclusion, the compounds having both antioxidant and antiinflammatory activities with a lack of COX-1 enzyme inhibitory effect may improve the gastrointestinal safety profile of such compounds.

Keywords: *Antiinflammatory, antioxidant, condansed thiazolo-triazole compounds, ethanol, cyclooxygenase, COX-1/2*

Introduction

It is well known that the major limitation concerning the use of nonsteroidal antiinflammatory drugs (NSAIDs) in various medical disorders is their adverse effects on gastrointestinal mucosa. In recent years, new antiinflammatory agents have become of increasing interest because they cause less gastric ulceration than do the acidic agents [1]. Although initial studies indicate that some of these compounds may have limited gastrointestinal toxicity compared to traditional NSAIDs such as indomethacin, their safety has not been clearly established.

Several investigations point out to the possibility that lipid peroxidation mediated by free radical formation are related to several pathological conditions including inflammation and gastric ulceration [2,3]. Many NSAIDs are known to act either by inhibiting the production of free radicals or by scavenging them [1,4]. Since COX enzymes are believed to involve oxygen radical intermediates (5), treatments with some NSAIDs at therapeutic doses significantly diminish free oxygen radical dependent injuries at the inflammation site by inhibiting COX enzymes (5, 6).

Previous studies have shown that modifications of the structures of known NSAIDs that yield an antioxidant, neutral molecule or a molecule with greatly reduced acidic character, can reduce the gastrointestinal toxicity of some NSAIDs [5,7]. The results obtained from the above studies indicated that 6-(α-aminobenzyl)thiazolo[3,2-b]-1,2,4-triazole-5-ols, which have been synthesized and demonstrated to posses moderate antiinflammatory activities against carrageenin induced mice paw edema in our previous studies [8,9, Table I], may be capable of inhibiting the reactive oxygen radicals.

Our investigations have focused therefore on the role of this new synthesized compounds on oxidative stress and on the various COX activities. To evaluate

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Table I. The structures of 6-(α-aminobenzyl)thiazolo[3,2-b]-1,2,4-triazole-5-ols and their antiinflammatory activities and ulcer scores [8,9].

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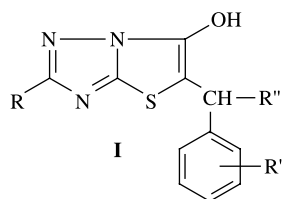
Compounds (Antiinflammatory activity, % inh.)	Ulcer incidence	R	R'	R''
Ia (66%) [9]	2/4	$-CH_3$	3,4,5-t-OCH$_3$	(piperidine with CH$_3$)
Ib (41%) [9]	0/4	$-CH_3$	3,4,5-t-OCH$_3$	(piperidine with CH$_3$)
Ic (52%) [8]	6/8	$-CH_3$	3-NO$_2$	(piperazine N-CH$_3$)
Id (63%) [8]	5/8	$3-NO_2C_6H_4-$	$-H$	(piperazine N-CH$_3$)

the antioxidant properties of the compounds, thiobarbituric acid reactive substances (TBARS) as an indicator of lipid peroxidation, total thiol groups (T-SH) and non-protein thiol groups (NP-SH; GSH) were studied. Inhibitory activity on the COX isoforms of the chosen active compound was also investigated by using *in vitro* human whole blood assay.

Materials and methods

Chemicals

All used chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). Test compounds were chemically synthesized as described previously [8,9] and are shown in Table I.

Animals and treatment

Locally bred Swiss Albino male mice (Refik Saydam Hıfzısıhha Institute, Animal Care Unit, Ankara, Turkey) weighing approximately 22 g were used. Throughout the experiments, animals and their care conformed to institutional guidelines, in compliance with national and international laws and guidelines for the use of animals in biomedical research (Hacettepe University Ethical Council, protocol no: 2000/35-1).

In the first step of our investigation, the animals received the compounds at the doses of exhibiting the best antiinflammatory activity (**Ia** and **Ib**: 20 mg/kg; **Ic**

and **Id**: 50 mg/kg) by a gastric tube, for 3 days to determine whether they could have any oxidative effect in tissues. To evaluate the peroxidative injury, at the end of 3rd day, stomach, liver and kidney were removed and lipid peroxidation was measured as nmol TBARS/g wet weight of tissue.

In the second step of our investigation, the compounds -which were found safe on the peroxidative tissue injury- were suspended in 0.5% carboxymethylcellulose and administered 60 min before the absolute ethanol (0.1 mL/mice) to mice by using gastric gavage needle. One hour after the application of ethanol, under ether anaesthesia the stomach, liver and kidney were removed. Ethanol was used as a pro-oxidant agent and indomethacin (10 mg/kg), a non-spesific NSAID was used as a reference control. Antioxidant properties of compounds were evaluated by measuring the tissue T-SH and NP-SH (GSH) levels in addition to tissue TBARS levels.

The compound -which was found active in terms of antioxidant activity and with the lowest ulcer incidence- was chosen to investigate its *in vitro* inhibitory activity on COX-1 and COX-2 enzymes.

Lipid peroxidation in tissues [10,11]

The method was based on the formation of a red chromophore which absorbed at 532 nm, following

the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) and other breakdown products of peroxidized lipids. Results were expressed as TBARS (nmol/g wet weight tissue).

T-SH and NP-SH (GSH) groups in tissues [12]

Tissues were homogenized in $0.02\,M$ ethylenediaminetetraacetic acid disodium (EDTA-Na$_2$). For determination of total-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 3000xg at room temperature for 15 min. The absorbance of supernatants was read at 412 nm.

For determination of GSH, aliquots of $5.0\,mL$ of the homogenates were mixed with $4.0\,mL$ distilled water and $1.0\,mL$ of 50% TCA. Tubes were centrifuged for 15 min at approximately $3000 \times g$. $2.0\,mL$ 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.

COX-1 and COX-2 enzyme assays [13]

The compound **1b** examined in this study that doesn't cause peroxidative cell injury and has the lowest ulcerogenic properties (Table II) in applied doses, was sent to CEREP (France, www.cerep.com) for COX assays.

The assays were performed using the general procedures of Miralpeix et al. (1997).

The experimental conditions are summarized below:

Statistical analysis

Statistical significance of data (expressed as mean $\pm$ SEM) was assessed by the Tukey-Kramer post hoc test using INSTAT computer software.

Results and discussion

The compounds evaluated in this study have been synthesized and investigated for their antiinflammatory activities against carrageenin induced mice paw edema in our previous studies [8,9]. It has been reported in literature that compounds showing less ulcerogenic activity also showed reduced TBARS content. Since these compounds possessed promising antiinflammatory activity together with low ulcerogenic properties, we attempted to make a correlation between their ulcerogenic activity and their lipid peroxidation effect. In the first step of the study, the compounds were evaluated for pro-oxidative activity. The test compounds **Ic** and **Id** exhibited extremely high potency for induction of oxidative stress in different tissues (Table–II). Although compound **Ic** did not show any significant differences in stomach TBARS levels, it significantly increased the peroxidative tissue injury in kidney ($p < 0.001$). Test compound **Id** also caused significant peroxidative tissue injury in stomach and liver ($p < 0.001$ and $p < 0.05$ respectively). Thus, only compounds **Ia** and **Ib** were studied in the second step of this study to evaluate their antioxidant effects on ethanol-induced oxidative stress. The extent of tissue damage and the results of treatment with the compounds in ethanol-induced oxidative stress were shown in Tables III–V.

It can be clearly seen that compounds **Ia** and **Ib** have a protective effect on ethanol-induced GSH consumptions in stomach ($p < 0.001$; $p < 0.05$

Assay	Substrate/Stimulus	Incubation	Reaction product	Method of detection
COX-1 (*h*)	AA ($0.3\,\mu M$)	15 min./37°C	PGE$_2$	RIA
COX-2 (*h*)	PMA ($100\,nM$)	20 h/37°C	PGE$_2$	RIA
(Whole cells-activity) for COX-2 induction				
	AA ($50\,\mu M$) for COX-2 activity	30 min./37°C		

Abbreviations: AA: arachidonic acid; COX: cyclooxygenase; HUVEC: human umbilical vein endothelial cells; PGE$_2$: prostaglandin E$_2$; PMA: phorbol 1,2-myristate 13-acetate.

Results are expressed as a percent of control activity and as a percent inhibition of control activity obtained in the presence of the compound. The IC$_{50}$ value obtained for the reference compounds has passed the required inspections. They are within accepted limits of historic averages obtained ± 0.5 log unit.

Diclofenac and NS 398 were used as reference standards for selective inhibition of COX-1 and COX-2, respectively.

respectively). However, they were not found to be effective on ethanol-enhanced TBARS levels in stomach tissue. Unlike stomach and kidney tissues, ethanol-induced TBARS level in liver was significantly increased by the test compounds (Tables III–V). It can be concluded that the protective effects of the compounds **Ia** and **Ib** on the ethanol-induced oxidative stress occured in liver are more obvious.

At the end of the evaluations of the antioxidant properties of the compounds, compound **Ib** with the

Table II. Effects of the test compounds administered by a gastric tube (for 3 days) on lipid peroxidation (as mean $\pm$ SEM nmol TBARS/g wet weight) levels in stomach, liver and kidney tissues of mice.

Compounds (n = 4–6)	Dose mg/kg	Stomach	Liver	Kidney
Control		67.3 $\pm$ 3.8	182.8 $\pm$ 7.1	138.2 $\pm$ 3.8
Ia	20	73.4 $\pm$ 5.7	173.9 $\pm$ 4.3	168.5 $\pm$ 3.9
Ib	20	62.8 $\pm$ 2.9	212.8 $\pm$ 6.5	175.6 $\pm$ 7.2
Ic	50	62.7 $\pm$ 2.9	212.6 $\pm$ 12.0	323.9 $\pm$ 13.7 ★★★
Id	50	213.1 $\pm$ 18.0 ★★★	251.7 $\pm$ 9.9 ★	148.0 $\pm$ 3.2
Indomethacin	10	127.1 $\pm$ 6.9 ★★★	315.0 $\pm$ 12.8★★★	147.0 $\pm$ 3.8

★ $p < 0.05$; ★★ $p < 0.01$;★★★ $p < 0.001$.

Table III. Effects of the synthesized compounds on the alteration of TBARS, T-SH and GSH in ethanol-induced oxidative stress in mouse stomach.

Groups (n = 5)	TBARS	T-SH (μmol/g wet wt.)	GSH (μmol/g wet wt.)
Control	64.4 $\pm$ 4.6	168.7 $\pm$ 0.7	24.1 $\pm$ 1.5
EtOH[a]	130.9 $\pm$ 5.2 ★★★	116.7 $\pm$ 1.2★★	6.0 $\pm$ 0.5 ★★★
Ia + EtOH[b]	122.7 $\pm$ 9.7	129.9 $\pm$ 2.9★★	12.2 $\pm$ 0.8★★★
Ib + EtOH[b]	121.8 $\pm$ 5.9	117.9 $\pm$ 1.9	9.8 $\pm$ 0.4★
Indo + EtOH[b]	167.2 $\pm$ 2.3★★	119.3 $\pm$ 3.2	2.7 $\pm$ 0.5★

EtOH, ethanol; Indo, indomethacin.
a: compare to the control group; **b:** compare to the EtOH group. ★ $p < 0.05$; ★★ $p < 0.01$; ★★★$p < 0.001$.

lowest ulcer incidence was chosen to investigate for COX enzyme inhibitory activity. *In vivo* active compound **Ib** on oxidative stress resulted in little COX-2 inhibition (17%) while it did not produce inhibitory activity on the COX-1 enzyme (Table VI).

It is well known that traditional NSAIDs are non-selective COX inhibitors, associated with gastrointestinal problems. However, COX-2 specific inhibitor NSAIDs are potentially less harmful compared to the non-selective NSAIDs [1,13]. Thus, the development of selective COX-2 inhibitors was thought to be a reasonable target for safer NSAIDs. Contrary to the classic NSAIDs, this new class of enzyme inhibitors is lacking a carboxylic acid group. It has been considered that it is interesting to modify the compounds structure in such a way that it would lead to an antioxidant, neutral molecule or a molecule with greatly reduced acidic character [14–16]. The combination of two properties, anti-inflammatory and antioxidant activity, with a simultaneous drastic

Table IV. Effects of the synthesized compounds on the alteration of TBARS; T-SH and GSH levels in ethanol-induced oxidative stress in mouse liver.

Groups (n = 5)	TBARS (nmol/g wet wt.)	T-SH (μmol/g wet wt.)	GSH (μmol/g wet wt.)
Control	220.5 $\pm$ 4.7	324.6 $\pm$ 7.2	38.6 $\pm$ 1.6
EtOH[a]	309.6 $\pm$ 6.6★★★	179.6 $\pm$ 11.2★★★	10.9 $\pm$ 0.3 ★★★
Ia + EtOH[b]	233.5 $\pm$ 9.3★★★	290.4 $\pm$ 13.5★★★	18.3 $\pm$ 0.7★★★
Ib + EtOH[b]	267.1 $\pm$ 10.4★	265.9 $\pm$ 10.9★★★	15.0 $\pm$ 0.7★
Indo + EtOH[b]	315.4 $\pm$ 9.4	216.9 $\pm$ 13.2	7.4 $\pm$ 0.9

EtOH, ethanol; Indo, indomethacin.
a: compare to the control group; **b:** compare to the EtOH group. ★ $p < 0.05$; ★★ $p < 0.01$; ★★★$p < 0.001$.

Table V. Effects of the synthesized compounds on the alteration of TBARS, T-SH and GSH levels in ethanol-induced oxidative stress in mouse kidney.

Groups (n = 5)	TBARS (nmol/g wet wt.)	T-SH (μmol/g wet wt.)	GSH (μmol/g wet wt.)
Control	146.1 $\pm$ 5.9	131.4 $\pm$ 0.4	21.9 $\pm$ 1.0
EtOH[a]	164.7 $\pm$ 2.6	129.8 $\pm$ 2.1	18.7 $\pm$ 0.6★
Ia + EtOH[b]	147.3 $\pm$ 9.6	126.3 $\pm$ 2.6	19.2 $\pm$ 0.7
Ib + EtOH[b]	187.6 $\pm$ 17.6	122.8 $\pm$ 1.2	16.6 $\pm$ 0.4
Indo + EtOH[b]	214.6 $\pm$ 10.9★	109.9 $\pm$ 2.1★★★	3.1 $\pm$ 0.3★★★

EtOH, ethanol; Indo, indomethacin.
a: compare to the control group; **b:** compare to the EtOH group. ★ $p < 0.05$; ★★ $p < 0.01$; ★★★$p < 0.001$.

Table VI.   COX inhibitory activity of the compound **Ib** and IC_{50} values for the reference compounds.

Test compound	COX-1 inhibition (%)*, 10 μM	COX-2 inhibition (%)*, 10 μM
Ib	–	17
Reference compounds	IC_{50} (μM)	*(nH)*
Diclofenac	0.015	(0.6)
NS 398	0.34	(1.2)

* For the test compound, the result is expressed as a percent inhibition of control activity. The symbol (−) indicates an inhibition of less than 10%. IC_{50} values and Hill coefficients (*nH*) were determined by non-linear regression analysis of the inhibition curves. These parameters were obtained by Hill equation curve fitting.

reduction of acidic character, may lead to the development of novel, useful antiinflammatory and cytoprotective pharmacomolecules, with potentially important therapeutic applications. The rational is that these structures: (i) are almost neutral and are not acidic, (ii) could present improved anti-inflammatory activity compared with the parent NSAIDs due to the added free radical scavenging activity, and (iii) should present reduced ulcerogenicity due to their action against oxidative stress [5,17–19].

Since compounds **Ia** and **Ib** produced antioxidant activity, the antiinflammatory effects might be depend on their free radical scavenging properties. Although compound **Ib** has a small inhibitory effect (17%) on COX-2 enzyme, it cannot be definitively concluded that this compound exerts its antiinflammatory activity through COX inhibition. The results of the present study support the assumption that it may be important to further investigate whether a series of NSAIDs with diminished acidic character and antioxidant properties combined in a single molecule could be of value in a strategy to prevent the ulcerogenic properties.

In conclusion, potent antioxidant properties may be useful for the development of a novel class of therapeutic agents for the inflammatory diseases. Further studies are necessary to explain the antiinflammatory mechanism(s) of these compounds as well as their efficacy and safety under long-term conditions.

Acknowledgements

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