Antioxidant and Antifungal Properties of Benzimidazole Derivatives

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Antioxidant and radical scavenging properties of a series of 2-[4-(substituted piperazin-/piperidin-1-ylcarbonyl)phenyl]-1*H*-benzimidazole derivatives were examined. Free radical scavenging properties of compounds **11**–**30** and **33** were evaluated for the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide anion radical. In addition the inhibitory effects on the NADPH-dependent lipid peroxidation levels were determined by measuring the formation of 2-thiobarbituric acid reactive substances (TBARS) using rat liver microsomes. Compound **33** which has a *p*-fluorobenzyl substitutent at position 1 exhibited the strongest inhibition (83%) of lipid peroxidation at a concentration of 10^{-3} M, while the nonsubstituted analogue **13** caused 57% inhibition. This result is fairly consistent with the antimicrobial activity results against both *Staphylococcus aureus* and *Candida albicans*.

Key words: Benzimidazole, Lipid Peroxidation, Superoxide Anion

Introduction

Antioxidants can act as direct scavengers of free radicals and reactive oxygen species, or they can indirectly metabolize free radicals or their intermediates into harmless products. Some of indirect antioxidants are enzymes that remove toxic molecules either before they damage the cell or prevent more toxic agents from being formed. Some of the antioxidative enzymes include the superoxide dismutases (SOD), the subspecies of which are located throughout the cell, and glutathione (GSH), peroxidases (GPx), GSH reductase (GRd), and catalase (CAT). The GPx and CAT catalytically remove H_2O_2 and lipid hydroperoxides from the cell, thereby reducing the generation of $^{\cdot}OH$.

Oxidative damage to DNA and other macromolecules appears to have a major role in aging, degenerative diseases, and cancer (Ames *et al*., 1993; Hagen *et al*., 1997, 1998; Beckman and Ames, 1998). Oxidative lesions accumulate with age in DNA (Helbock *et al*., 1998) and protein (Berlett and Stadtman, 1997). In fact, both oxidant by-products of normal energy metabolism (superoxide, hydrogen peroxide, and hydroxyl radical) and mutagens produced by radiation are the same. DNA is oxidized in the normal metabolism because antioxidant defenses, though numerous, are not perfect. Because of the oxidation of lipids, aldehydes are produced (Yeo *et al*., 1994; Liu *et al*., 1997), some of which are mutagenic (Marnett *et al*., 1985; Burcham, 1998). White cells and other phagocytic cells of the immune system combat bacteria, parasites, and virus-infected cells by destroying them with the mutagenic oxidizing agents NO, HOCl, and H_2O_2 (Ames *et al*., 1995; Christen *et al*., 1999). The burst of oxidants, and consequent inflammation, from phagocytic cells is a major source of NO_x (a mixture of reactive nitrogen oxides) and contributes to both cancer and heart disease (Ames *et al*., 1995). These oxidants protect humans from immediate death caused by infections, but they also cause oxidative damage to DNA, chronic cell killing with compensatory cell division, and mutation (Yamashina *et al*., 1986; Shacter *et al*., 1988). Chronic infections cause new cancer cases in the world (Pisani *et al*., 1997).

Antioxidants could affect the membrane function (Simonetti *et al*., 2001), which may in turn regulate the fluconazole susceptibility (Simonetti *et al*., 1991). Bacteria and yeasts demonstrated resistance to azole action because of outer mem-

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brane modification (Simonetti *et al.*, 2001). Simonetti *et al.* (2002) reported that the combination of antioxidant and triazole brought a marked increase in the inhibitory activity of fluconazole in susceptible isolates in which the MIC was reduced from 1 to 0.125 mg L^{-1} (data not shown). There was no change in metabolic activity in contact experiments. The interaction of phenolic antioxidants with membrane phospholipids could affect the molecular organization and promote the drug passage into the membrane bilayer (Vaara, 1992), and could increase the *in vitro* antimicrobial activity of fluconazole. In another study, it was published that the fungistatic effect of ketoconazole, another antifungal azole drug, associated with its membrane-stabilizing effects on *Candida* species is indicated by inhibition of lipid peroxidation, and it inhibits lipid peroxidation in both microsomal and liposomal systems, significantly (Wiseman *et al.*, 1991).

Owing to not only antifungal but also other biological effects, benzimidazole derivatives have always aroused the researchers' interests. Considering the research of Wiseman *et al.* (1991) we were tempted to study whether there is any correlation between the antimicrobial and antioxidant activities or not. The 4-(1*H*-benzimidazol-2-yl)benzamides **11**–**30** and 5-chloro-1-(*p*-fluorobenzyl)-2-{4-[(4-methylpiperazin-1-yl) carbonyl]phenyl}-1*H*-benzimidazole (**33**) were already reported to have antimicrobial activities (Kuş *et al.*, 2009). In addition to this, we evaluated the antioxidant and radical scavenging properties of these compounds and compared them in terms of their antifungal activities.

Experimental

Lipid peroxidation assay

Male albino Wistar rats (200–225 g) were used. The animals were fed with standard laboratory rat chow and allowed to drink tab water *ad libitum*. The animals were starved for 24 h prior to be sacrificed, and then killed by decapitation under anaesthesia. The livers were removed immediately, washed in ice-cold distilled water, and microsomes were prepared as described previously (Iscan *et al.*, 1984).

NADPH-dependent lipid peroxidation (LP) was determined using the optimum conditions determined and described by Iscan *et al.* (1984). In this assay, the control activity has been regarded as the activity measured in the presence of the pure diluent for the chemicals tested [dimethylsulfoxide (DMSO) for synthesized compounds and butylated hydroxytoluene (BHT)]. Thus, the assay has been carried out in the presence of only solvent, as a control, or at the indicated concentrations of compounds. NADPH-dependent LP was measured spectrophotometrically by estimation of 2-thiobarbituric acid reactive substances (TBARS). The amounts of TBARS were expressed in terms of nmol malondialdehyde (MDA) mg $protein^{-1}$. The assay was essentially derived from the methods of Wills (1966, 1969), and modified by Bishayee and Balasubramanian (1971). A typical optimized assay mixture contained 0.2 nM Fe^{2+}, 90 mM KCl, 62.5 mM potassium phosphate buffer, pH 7.4, the NADPH-generating system (0.25 mM $NADP^+$, 2.5 mM $MgCl_2$, 2.5 mM glucose-6-phosphate, 1.0 U glucose-6-phosphate dehydrogenase, and 14.2 mM potassium phosphate buffer, pH 7.8), and 0.2 mg microsomal protein, in a final volume of 1.0 mL. The reaction was initiated by addition of the NADPH-generating system to the microsomal mixtures. The reaction was carried out at 37 °C for 30 min, and trichloroacetic acid was added to stop the reaction. The denatured proteins were then removed by centrifugation. Finally, the supernatant was mixed with thiobarbituric acid (TBA) and boiled for 15 min. The absorbance was measured spectrophotometrically at 532 nm. Each experiment was performed in triplicate. The protein contents of liver microsomes were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Superoxide radical scavenging activity

The capacity of compounds to scavenge superoxide anions was determined spectrophotometrically on the basis of inhibition of cytochrome c reduction, according to the modified method of McCord and Fridowich (1969).

Superoxide anions were generated in the xanthine/xanthine oxidase system. The reaction mixture contained 0.05 M phosphate buffer, pH 7.8, 0.32 U xanthine oxidase, 50 μM xanthine, 60 mM cytochrome c, and 100 μL of different concentrations of the synthesized compounds in a final volume of 1 mL. DMSO and BHT were used as the control solution and reference compound, respectively. The absorbance was measured spec-

trophotometrically at 550 nm to determine the cytochrome c reduction. Each experiment was performed in triplicate, and the results expressed as percentage of the control.

DPPH free radical scavenging activity

The free radical scavenging activities of the compounds were tested by their ability to bleach the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), as described by Blois (1958). This assay has often been used to estimate the antiradical activity of antioxidants. Because of its odd electronic structure DPPH gives a strong absorption band at 517 nm in the visible region. DPPH was dissolved in methanol to give an 100-μM solution. 0.1 mL of the test compounds and BHT, dissolved in DMSO, were added to 1.0 mL of the methanolic DPPH solution. The absorbance at 517 nm was determined after 30 min at room temperature, and the scavenging activities calculated as percentage of the radical reduction. Each experiment was performed in triplicate. DMSO was used as a control solution and BHT as a reference compound. The radical scavenging activity was obtained as follows:

$$\text{radical scavenging activity (\%)} = [(OD_{control} - OD_{sample})/OD_{control}] \cdot 100,$$

where $OD_{control}$ is the absorption of the blank sample, and OD_{sample} is the absorption of tested compounds.

Results and Discussion

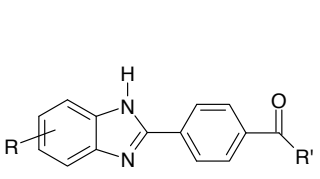
The inhibitory effects of compounds **11**–**30** and **33** on the NADPH-dependent LP levels were determined by measuring the formation of TBARS using rat liver microsomes. The effects of the compounds and BHT on rat liver LP levels are shown in Table I. Free radical scavenging properties of these compounds were examined *in vitro* by determining the capacity to scavenge superoxide anion formation and the interaction with the stable free radical DPPH; results of which are displayed in Table II.

Previously, we reported that compound **19** had the best antifungal activity against *Candida albicans* similar to that of fluconazole (MIC = 3.12 μg mL^{-1}). Compounds **13**, **14**, **18**, **19**, and **33** exhibited good antifungal activity (MIC = 3.12 μg mL^{-1}) against *C. krusei* close to that of fluconazole (MIC = 6.25 μg mL^{-1}). When we searched for antioxidant properties of **12**, **14**, **19**, and **33**, meaningful inhibition results on LP were observed. Compound **33**, the only one having a *p*-fluorobenzyl substitutent at position 1, showed the best results on all *Candida* subspecies and LP levels. Almost all 5-nitro-substituted derivatives, **14**, **19**, **24**, and **29**, displayed better antimicrobial activity and inhibition on LP than the others. By the way, almost none of the piperidine derivatives showed any significant activity against either bacteria or fungi, except **29** and **30** which have 5-nitro and 5,6-dichloro substituents, respectively. Similarly, almost none of the piperidine derivatives was effective on liver LP levels and on superoxide anion radical scavenging activity. The results displayed that, when there are nitro or dichloro substitutents at 5- and/or 6-position of the benzimidazole moiety and *p*-fluorophenylpiperazine or *N*-methylpiperazine, the antifungal activity against *Candida* species is especially improved. Compounds **13**, **14**, **19**, and **25** displayed both good antifungal activity and antioxidant effects on liver LP levels. Compound **33**, which is the 1-(*p*-fluorobenzyl)-substituted analogue of the nonsubstituted compound **13**, had better antifungal activity against *Candida* sp. (MIC = 3.12 and 6.25 μg mL^{-1}) than **13** (MIC = 6.25 and 12.5 μg mL^{-1}). At the same time, **13** showed 57% inhibition and **33** displayed the best inhibition (83%) on liver LP. Interestingly, convenient biological activity results were obtained in both antifungal activity tests against *Candida* species and in inhibition tests on liver LP levels test. That the antimicrobial activity of benzimidazole derivatives is improved by N^1-substitution was confirmed one more time in this series of compounds, already reported in other studies (Kuş *et al.*, 2009; Göker *et al.*, 1995). By the way, substitution at position 1 of the benzimidazole ring enhanced both antifungal and antioxidant activity results. Therefore, there could be a possible correlation between *N*-substitution and biological activity; so further studies are in progress.

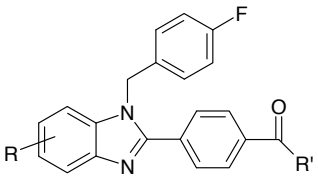
Although, the piperidine-substituted derivatives **27** (109%) and **30** (106%) increased the LP level, others like **26** (12%), **28** (21%), and **29** (38%) showed weak LP inhibition at 10^{-3} M concentration.

Compounds **13**, **14**, **19**, and **25** exhibited the highest lipid peroxidation inhibition, in the range of 50–57%, and these inhibitions were close to

Table I. Effects of the compounds on liver LP levels *in vitro*[a].



11 – 30



33

Compound[b]	R'	R	LP level [nmol mg^{-1} min^{-1}]	% of control
11	*N*-Methylpiperazine	5(6)-H	12.02 ± 1.32	74
12	*N*-Methylpiperazine	5(6)-CH_3	13.43 ± 0.72	83
13	*N*-Methylpiperazine	5(6)-Cl	7.04 ± 0.83	43
14	*N*-Methylpiperazine	5(6)-NO_2	7.60 ± 0.44	47
15	*N*-Methylpiperazine	5,6-dichloro	12.96 ± 1.65	78
16	*p*-Fluorophenylpiperazine	5(6)-H	10.71 ± 1.52	66
17	*p*-Fluorophenylpiperazine	5(6)-CH_3	10.61 ± 1.53	65
18	*p*-Fluorophenylpiperazine	5(6)-Cl	10.61 ± 1.79	65
19	*p*-Fluorophenylpiperazine	5(6)-NO_2	7.25 ± 1.79	45
20	*p*-Fluorophenylpiperazine	5,6-dichloro	11.18 ± 0.07	69
21	*o*-Methoxyphenylpiperazine	5(6)-H	11.65 ± 0.20	72
22	*o*-Methoxyphenylpiperazine	5(6)-CH_3	10.24 ± 0.93	63
23	*o*-Methoxyphenylpiperazine	5(6)-Cl	11.46 ± 0.01	71
24	*o*-Methoxyphenylpiperazine	5(6)-NO_2	11.45 ± 0.04	69
25	*o*-Methoxyphenylpiperazine	5,6-dichloro	8.17 ± 0.13	50
26	Piperidine	5(6)-H	14.28 ± 1.25	88
27	Piperidine	5(6)-CH_3	17.65 ± 0.40	109
28	Piperidine	5(6)-Cl	12.87 ± 2.13	79
29	Piperidine	5(6)-NO_2	10.14 ± 0.47	62
30	Piperidine	5,6-dichloro	17.19 ± 0.21	106
33	*N*-Methylpiperazine	5-Cl	2.73 ± 0.48	17
Control[c]			16.25 ± 1.45	100
BHT			5.68 ± 0.22	35

[a] Each value represents the mean ± S.D. of 2–4 independent experiments.
[b] Concentration in incubation medium (10–3 M).
[c] Dimethylsulfoxide only, control for compounds and BHT.

that obtained with 10^{-3} M BHT (65%), one of the well known classical antioxidant compounds, which was used as a positive control for comparison with the benzimidazole derivatives. The other compounds had no meaningful effects on the level of LP (Table I). The most active compound **33** showed the best inhibition (83%) on liver LP, and the inhibitory potencies (IC_{50}) against LP of **33** and BHT were $4.40 \cdot 10^{-4}$ M and $4.08 \cdot 10^{-4}$ M, respectively.

The superoxide anion radical scavenging activities of the compounds were evaluated using the xanthine/xanthine oxidase system (Table II). The *N*-methylpiperazine derivatives, especially, were more potent than the others. *p*-Fluorophenyl- and *o*-methoxyphenylpiperazine derivatives induced the superoxide radical production only weak, while *N*-methylpiperazine and piperidine derivatives showed moderate inhibitory effects on superoxide radicals. The superoxide anion radical scavenging activities of the most active compounds **14**, **15**, and **28** depended on their concentration, and these compounds appeared to have similar inhibitory effects on superoxide anion formation, *i.e.* 88%, 93%, and 70% at 1 mM, respectively, like vitamin E (90% at 1 mM) that was used as a positive control. Compounds **11**, **13**, **18**, **26**, and **27** showed very limited inhibitory ef-

Table II. Effects of compounds **11**–**30** and **33** on superoxide anion radical scavenging activity[a].

Compound	Superoxide radical scavenging activity (%)					IC_{50} [mM]
	0.0625 mM	0.125 mM	0.25 mM	0.5 mM	1 mM	
11	NE	NE	NE	5 ± 0.3	6.0 ± 0.7	
12	NE	NE	NE	NE	NE	
13	NE	NE	8.0 ± 2.1	14 ± 0.7	25 ± 1.4	
14	22 ± 2.4	32 ± 3.5	52 ± 4.2	65 ± 4.2	88 ± 2.1	0.282
15	22 ± 2.1	48 ± 1.4	62 ± 0.7	63 ± 2.8	93 ± 3.5	0.242
16	NE	NE	NE	NE	P	
17	NE	NE	NE	P	P	
18	NE	NE	NE	NE	19 ± 1.4	
19	P	P	P	P	P	
20	NE	NE	NE	NE	NE	
21	NE	NE	NE	NE	NE	
22	NE	NE	NE	NE	P	
23	NE	NE	NE	NE	P	
24	NE	NE	NE	NE	NE	
25	NE	NE	NE	NE	NE	
26	NE	NE	NE	NE	10 ± 0.7	
27	NE	NE	NE	NE	11 ± 1.4	
28	29 ± 2.4	60 ± 2.8	71 ± 1.4	69 ± 2.1	70 ± 0.7	0.176
29	NE	NE	NE	NE	NE	
30	NE	NE	NE	P	P	
33	-	-	-	-	-	
Vitamin E	33 ± 1.4	79 ± 2.1	82 ± 3.5	85 ± 2.8	90 ± 3.5	0.126

[a] Each value represents the mean ± S.D. of 2–4 independent experiments.
NE, not effective; P, prooxidant.

fects on superoxide anion formation, in the range of 6–25% at 1 mM. Compounds **12**, **20**, **21**, **24**, **25**, and **29** had no effect on superoxide anion formation. By the way, **16**, **17**, **19**, **22**, **23**, and **30** acted as prooxidants.

All tested compounds were found to have no interaction with the stable free radical DPPH.

In both of our studies with compounds **11**–**30** and **33**, similar activity results were obtained, namely a remarkable antifungal effect against *Candida* species and inhibition levels on LP. Compound **33**, the only one having a *p*-fluorobenzyl substitutent at position 1, showed the best results on all *Candida* subspecies and LP levels.

The activity patterns of compounds on LP, superoxide anion formation, and DPPH radical scavenging activity were dissimilar because each method relates to the generation of a different radical, acting through a variety of mechanisms, and the measurement of a range of end points at a fixed time point or over a time period. It should also be realized that the analytical methods of measurement and the conditions can lead to variable results for the same compound (Frankel and Meyer, 2000). Distinct antioxidant effects of chemicals have already been noted in different *in vitro* assay systems (Olgen and Coban, 2003; Ates-Alagoz *et al*., 2004). Therefore, it is extremely difficult to compare the results from different assays. The biggest problem is the lack of a validated assay that can reliably measure the antioxidant capacity of foods and biological samples.

In connection with these results, whether there is a correlation between antifungal activity and antioxidant properties requires further research.

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