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ORIGINAL ARTICLE

Evaluation of the Antioxidant Activity of Some Imines Containing 1*H*-Benzimidazoles

1*H*-Benzimidazol İçeren Bazı İminlerin Antioksidan Aktivitesinin Değerlendirilmesi

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ABSTRACT

Objectives: The *in vitro* antioxidant properties of some 2-(2-phenyl)-1*H*-benzo(d)imidazol-1-yl)-N'-(arylmethylene) acetohydrazide derivatives (**1-12**) were investigated in this study.

Materials and Methods: The *in vitro* antioxidant activity of compounds **1-12** was explored by determination of rat liver microsomal nicotinamide-adenine dinucleotide phosphate dependent inhibition on lipid peroxidation (LPO) levels and microsomal ethoxyresorufin O-deethylase (EROD) activity.

Results: All synthesised compounds had LPO inhibitory activity (15-57%) except compound **6**, which contains a thiophene ring. Almost all the compounds displayed slightly inhibitory activity (2-20%) on EROD.

Conclusion: The most active compound, **3** bearing a p-bromophenyl substituent at the second position of the benzimidazole ring, caused 57% inhibition of LPO level, while butylated hydroxytoluene showed 65% inhibition. None of the synthesised compounds had a marked inhibitory effect on EROD activity.

Key words: Antioxidant, benzimidazole, imine, lipid peroxidation, ethoxyresorufin O-deethylase activity

ÖZ

Amaç: Bu çalışmada, bazı 2-(2-fenil)-1*H*-benzo(d)imidazol-1-il)-N'-(arilmetilen) asetohidrazit türevlerinin *in vitro* antioksidan özellikleri araştırılmıştır.

Gereç ve Yöntemler: **1-12** numaralı bileşiklerin *in vitro* antioksidan aktiviteleri, lipit peroksidasyon (LPO) düzeylerine sıçan karaciğer mikrozomal nikotinamid adenin dinükleotid fosfat bağımlı inhibisyonunu ve mikrozomal etoksirezorufin O-deetilaz (EROD) aktivitesinin belirlenmesiyle incelenmiştir.

Bulgular: Tiyofen halkası içeren bileşik **6** dışında, sentezlenen tüm bileşikler LPO inhibitör aktivite (%15-57) göstermiştir. Hemen hemen tüm bileşikler az miktarda EROD inhibe edici aktivite (%2-20) göstermiştir.

Sonuç: Benzimidazol halkasının ikinci konumunda p-bromo fenil sübstitüenti taşıyan bileşik **3**, LPO seviyesinde %57 inhibisyona neden olan en aktif bileşik iken, butillenmiş hidroksitoluen %65 inhibisyon göstermiştir. Sentezlenen bileşiklerin hiçbiri EROD aktivitesi üzerinde belirgin bir inhibisyon etkisine sahip değildir.

Anahtar kelimeler: Antioksidan, benzimidazol, imin, lipit peroksidasyon, etoksirezorufin O-deetilaz

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INTRODUCTION

Antioxidant-defence mechanisms are present in living cells to maintain cellular homeostasis and survival by preventing cellular damage caused by oxidative stress in various diseases.[1,2] Impairment of antioxidant mechanisms causes the balance between antioxidant defences and oxygen-derived free radicals to shift in favour of free radicals, resulting in oxidative stress. Therefore, the synthesis of novel drugs with antioxidants and free radical scavenging properties can help to treat and/or prevent diseases induced by insufficient antioxidant capacity. It is well recognised that lipid peroxidation (LPO) is a free-radical-mediated chain process that results in oxidative damage to cell membranes and other lipid-containing structures.[3] It is an important tool to probe the antioxidant capacity of a novel compound. Almost all LPO products have long been reported to possess carcinogenic and/or mutagenic effects. Moreover, reactive oxygen species are generated by a variety of cellular mechanisms including cytochrome P450 (CYP450) enzymes, which catalyse a wide range of endogenous and exogenous substances, and particularly CYP1A1/2 have great importance in nicotinamide-adenine dinucleotide phosphate (NADPH)-dependent LPO. Probing the effects of synthesised compounds on LPO levels and the CYP450 system is, therefore, crucial.[4]

Benzimidazoles have become an attractive pharmacophore in drug design and discovery, and exhibit a wide range of biological activities, e.g., antimicrobial,[5-7] antiparasitic,[8] antihistaminic,[9] anticancer,[10-15] antiallergic,[16] and antioxidant.[17-26] The synthesis, characterisation, and antioxidant capacities of some benzimidazole derivates containing thiadiazole, triazole, oxadiazole, and thiazolidinone rings at the first position have been reported in previous studies,[6,18-21,23-25] and most of these compounds have been shown to possess substantial antioxidant properties. In the present study, the antioxidant properties of some benzimidazole derivatives having aryl-methylene amino acetamide (**1-12**) (Table 1), which have previously shown epidermal growth factor receptor kinase inhibitory activity, were investigated.[13]

MATERIALS AND METHODS

General synthetic method

All the desired benzimidazole-derived compounds were synthesised as described in Scheme 1 below. 2-phenyl-1*H*-benzo(d)imidazole (I) was produced via oxidative condensation of *o*-phenylenediamine, benzaldehyde, and sodium metabisulphite. Treatment of *I* with ethyl chloroacetate in KOH/dimethyl sulphoxide (DMSO) yielded the N-alkylated product ethyl 2-(2-phenyl)-1*H*-benzo[d]imidazol-1-yl) acetate (**II**). Hydrazine hydrate and the ester (**II**) in ethanol were refluxed for 4 h to obtain the desired hydrazide compound, 2-(2-phenyl)-1*H*-benzo(d) imidazol-1-yl) acetohydrazide (**III**). Compounds **1-12** were achieved by condensing acyl hydrazide **III** with the corresponding aromatic aldehyde derivatives in the presence of sulphuric acid.[13]

Treatment of animals

Male albino Wistar rats weighing 200-225 g were used throughout the experiments. All animals were housed in single cages under controlled laboratory conditions (22-25˚C room temperature; 12-h light/dark cycle; optimum humidity) and had access to standard rat chow and tap water *ad libitum*. They were deprived of feed for 24-h before sacrifice and then decapitated under anaesthesia. Their liver tissues were carefully dissected and immediately stored in a freezer at -80˚C. All procedures used in the present study were approved by the Ethics Committee for Animal Experiments of Ankara University (2015-8-117).

Isolation of rat liver microsomes

The rat liver tissues were weighed and homogenised with 1.15% KCl (w/v) at 3 000 rpm on ice and centrifuged at 11 000 x g for 25 min. Once the supernatant fractions had been centrifuged again at 108 000 x g for 60 min, the pellets were mixed with 20% glycerol and were then immediately stored at -80˚C until use. Total protein levels of the liver microsomes were measured as described by Lowry et al.[27] using bovine serum albumin as a standard.

In vitro antioxidant activity

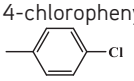
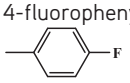
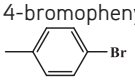
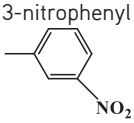
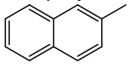
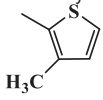
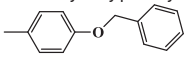
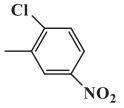
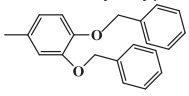
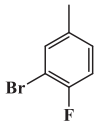
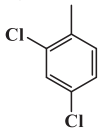
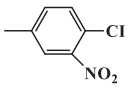
Lipid peroxidation assay

The NADPH-dependent LPO level was determined based on the optimum conditions described previously.[28] In this protocol, the control activity was determined as the pure diluent in which the chemicals were dissolved. DMSO was used as a control for the synthesised compounds. The assay was, therefore, performed only in a solvent as a control or the determined concentrations of compounds. The protocol was carried out as described by Wills[29,30] with some modifications by Bishayee and Balasubramanian.[31] The measurement of thiobarbituric acid reactive substances (TBARS) is the well-established method for quantifying NADPH-dependent LPO levels. This method is based on the principle of spectrophotometrically measuring the coloured product formed by the reaction of TBA with malondialdehyde (MDA) at 532 nm. The amount of TBARS was then indicated as nanomoles of MDA/mg protein; 1 mL of reaction mixture contains 0.2 mg of microsomal protein, 62.5 mM potassium phosphate buffer (pH 7.4), 0.2 mM Fe^{2+}, 90 mM KCl, and cofactor (NADPH-generating system) consisting of 2.5 mM glucose-6-phosphate, 14.2 mM potassium phosphate buffer (pH 7.8), 2.5 mM $MgCl_2$, 0.25 mM $NADP^+$, and 1.0 U of glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of an NADPH-generating system and then allowed to incubate at 37˚C for 30 min in a shaking water bath. At the end of the incubation, the reaction was terminated by the addition of 500 µL of 25% trichloroacetic acid and then centrifuged at 5 000 rpm for 20 min to remove denatured proteins. Next, 1 mL supernatant was combined with 0.5 mL of TBA and the mixture was then boiled for 20 min in a hot water bath. Finally, the absorbance was read spectrophotometrically at a wavelength of 532 nm. Whilst butylated hydroxytoluene (BHT) was used as a standard, the control used in this assay was DMSO.

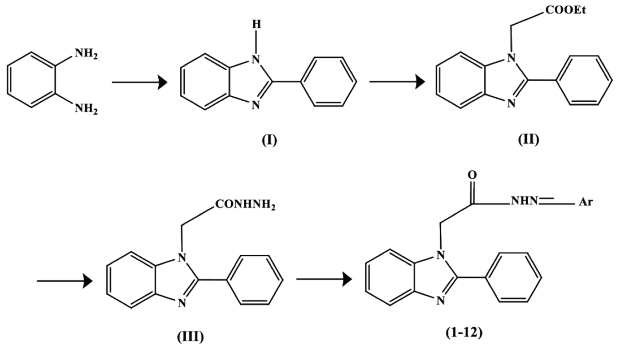
7-Ethoxyresorufin O-deethylase (EROD) assay

EROD activity in the rat liver microsomes was assayed as previously described by Burke et al.[32] 7-ethoxyresorufin is a substrate for CYP1A1, and this enzyme converts it to resorufin,

Table 1. ***In vitro*** **effects of compounds 1-12 on liver LPO levels and EROD enzyme activities. Concentration in incubation medium (10^{-3} M). All the values are means ± SD of three independent experiments**

Compounds	Ar	EROD (pmol/mg/min)	% of control	LPO (nmol/mg/min)	% of control
1	4-chlorophenyl 	33.41±1.64	80	11.67±0.89	72
2	4-fluorophenyl 	42.76±2.34	103	10.51±1.88	65
3	4-bromophenyl 	38.91±1.55	94	6.97±0.65	43
4	3-nitrophenyl 	38.55±1.07	93	8.94±2.13	55
5	2-naphtyl 	38.87±1.44	93	9.40±2.13	58
6	3-methylthiophene-2-yl 	35.91±4.36	86	82.58±1.23	508
7	4-benzyloxyphenyl 	37.61±0.68	91	13.81±0.32	85
8	2-chloro-5-nitrophenyl 	42.98±3.49	103	11.84±0.66	73
9	3,4-dibenzyloxyphenyl 	37.29±0.98	90	10.10±1.31	62
10	3-bromo-4-fluorophenyl 	34.50±1.13	83	12.89±0.33	79
11	2,4-dichlorophenyl 	40.65±1.02	98	12.08±1.47	74
12	4-chloro-3-nitrophenyl 	38.26±1.52	92	11.15±0.98	69
BHT		-	-	5.68±0.22	35
Caffeine		6.41±0.36	15	-	-
DMSO		41.53±0.99	100	16.25±1.45	100

LPO: Lipid peroxidation, EROD: 7-ethoxyresorufin O-deethylase, SD: Standard deviation, BHT: Butylated hydroxytoluene, DMSO: Dimethyl sulphoxide



Scheme 1. Synthetic route to compounds **1-12**

which can be measured spectrofluorimetrically. 1 mL of typical optimized reaction mixture contains 0.2 mg of rat liver microsomal protein, 1.0 mM 7-ethoxyresorufin as a substrate, 100 mM Tris-HCl buffer (pH 7.8), 12 mM albumin, 10^{-3} M test compound, and an NADPH-generating system consisting of 2.5 mM glucose-6-phosphate, 14.2 mM potassium phosphate buffer (pH 7.8), 2.5 mM $MgCl_2$, 0.25 mM $NADP^+$, and 1.0 U of glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of the NADPH-generating system and then allowed to incubate at 37°C for 5 min. After incubation, the reaction was stopped by the addition of 3 mL of ice-cold methanol and then centrifuged at 5 000 rpm for 20 min to remove the denatured proteins. Finally, the absorbance was measured spectrofluorimetrically at the excitation wavelength of 538 nm and the emission wavelength of 587 nm. Whilst caffeine was used as a standard, the control used in this assay was DMSO.

RESULTS

The antioxidant effects of synthesised compounds on the rat liver microsomal NADPH-dependent LPO levels were ascertained by quantifying the amount of 2-TBARS formed in the reaction (Table 1). The results indicated that all synthesised compounds at a concentration of 10^{-3} M had LPO inhibitory activity except compound **6**, which contains thiophene, that well-known isoster of the phenyl ring as an aryl group, and the rates were in the range of 15-57%. Compounds **2**, **4**, **5**, **9**, and **12** have moderate inhibitory activity on LPO levels in the range of 31-45%. The most active compound, **3**, bearing a *p*-bromophenyl substituent at the second position of the benzimidazole ring, caused 57% inhibition of LPO level, while BHT displayed 65% inhibition at the same concentration.

The *in vitro* effects of compounds on rat liver microsomal EROD activity were also tested. The results showed that none of the synthesised compounds had a marked inhibitory effect on EROD activity. Almost all the compounds displayed slightly inhibitory activities (2-20%) on EROD when the value of caffeine was 85% (Table 1).

DISCUSSION AND CONCLUSION

In our previous studies, we described the synthesis and antioxidant effects of 2-[2-(4-chlorophenyl)benzimidazole-1-yl]-N-(2-arylmethylene amino)] acetamides on EROD activity and LPO levels.[21,33] When compared with the results obtained from these studies, benzimidazoles carrying a 4-chloro phenyl ring at the second position were found to be more effective than the benzimidazole counterpart carrying nonsubstituted phenyl rings for both assays.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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