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

ÖZ

GİRİŞ ve AMAÇ: Bu çalışmada, bazı 2-(2-fenil)-1H-benzo[d]imidazol-1-il)-N’-(arilmetilen) asetohidrazit türevlerinin in vitro antioksidan özellikleri araştırılmıştır.

YÖNTEM ve GEREÇLER: 1-12 numaralı bileşiklerin in vitro antioksidan aktiviteleri, lipid peroksidasyon (LPO) seviyelerinde sıçan karaciğer mikrozomal NADPH-bağlı inhibisyonunun ve mikrozomal etoksiresorufin O-deetilaz (EROD) aktivitesinin belirlenmesiyle incelenmiştir.

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

TARTIŞMA ve SONUC: Benzimidazol halkasının 2. konumunda p-bromofenil sübstitüenti taşıyan bileşik 3, LPO seviyesinde %57 inhibisyonuna neden olan en aktif bileşik iken, butilli hidroksitoluen (BHT) %65 inhibisyon göstermiştir. Sentezlenen bileşiklerin hiçbir EROD aktivitesi üzerinde belirgin bir inhibisyona sahip değildir.

Anahtar Kelimeler: Antioksidan, benzimidazol, imin, lipid peroksidasyon

ABSTRACT

INTRODUCTION: In this study, the in vitro antioxidant properties of some 2-(2-phenyl)-1H-benzo[d]imidazol-1-il)-N’-(arylmethylene) acetohydrazide derivatives (1-12) were investigated.

METHODS: The in vitro antioxidant activity of compounds 1-12 were explored by determination of rat liver microsomal NADPH-dependent inhibition on lipid peroxidation (LPO) levels and microsomal ethoxyresorufin O-deethylase (EROD) activity.

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

DISCUSSION AND CONCLUSION: The most active compound 3 bearing p-bromophenyl substituent at the 2nd position of benzimidazole ring led to 57% inhibition on LPO level while butylated hydroxytoluene (BHT) showed 65% inhibition. None of the synthesized compounds had a marked inhibitory effect on EROD activity.

Keywords: Antioxidant, benzimidazole, imine, lipid peroxidation

Rahman Basaran, School of Chemistry, University of Leeds, LS2 9JT, Leeds, United Kingdom
INTRODUCTION
Antioxidant-defence mechanisms are present in living cells to maintain cellular homeostasis and survival by preventing cellular damages caused by oxidative stress in various diseases. 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 the diseases induced by insufficient antioxidant capacity. It is well recognized that lipid peroxidation (LPO) is a free-radical-mediated chain process whereby results in oxidative damage to cell membranes and other lipid-containing structures. It is an important tool to probe the antioxidant capacity of a novel compound. Almost all LPO products have been long reported to possess carcinogenic and/or mutagenic effects. Moreover, reactive oxygen species (ROS) are generated by a variety of cellular mechanisms including Cytochrome P450 (CYP450) enzymes which catalyse a wide range of endogenous and exogenous substances, particularly CYP1A1/2 have great importance in NADPH-dependent LPO. Probing the effects of synthesised compounds on LPO levels and CYP450 system is, therefore, crucial.

Benzimidazoles have become an attractive pharmacophore in drug design and discovery and exhibit a wide range of biological activities e.g. antimicrobial, antiparasitic, antihistaminic, anticaner, antiallergic, and antioxidant. The synthesis, characterisation, and antioxidant capacities of some benzimidazole derivatecs containing thiaizole, triazole, oxadiazole and thiazolidinone rings at the 1st position have been reported in the previous studies, and most of these compounds have been shown to possess substantial antioxidant properties. In the present study, antioxidant properties of some benzimidazole derivatives having aryl-methylene amino acetamide (1-12) (Table 1) which have previously been shown Epidermal Growth Factor Receptor (EGFR) kinase inhibitory activities were investigated.

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

Treatment of Animals
Albino male Wistar rats with 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 this study were approved by the Ethics Committee for Animal Experiments of Ankara University. 

**Isolation of Rat Liver Microsomes**

The rat liver tissues were weighed and homogenized with 1.15% KCl (w/v) at 3000 rpm on ice and centrifuged at 11,000 g for 25 mins. Once the supernatant fractions were then centrifuged again at 108,000 g for 60 mins, the pellets were mixed with 20% glycerol and were immediately stored at -80°C until use. Total protein levels of liver microsomes were measured by the method of Lowry et al. using bovine serum albumin as a standard.

**In vitro Antioxidant Activity**

**Lipid Peroxidation (LPO) Assay**

The NADPH-dependent LPO level was carried out based on the optimum conditions described previously. In this protocol, the control activity was determined as the pure diluent in which the chemicals were dissolved. Dimethyl sulfoxide (DMSO) was used as a control for synthesized 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 with some modifications by Bishayee and Balasubramanian. The measurement of thiobarbituric acid reactive substances (TBARS) is the well-establish 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 nanomole of malondialdehyde (MDA)/mg protein. 1 mL reaction mixture contains 0.2 mg microsomal protein, 62.5 mM potassium phosphate buffer (pH: 7.4), 0.2 mM Fe²⁺, 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 MgCl₂, 0.25 mM NADP⁺, and 1.0 U glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of NADPH-generating system and then allowed to incubate at 37 °C for 30 mins 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 (TCA), then centrifuged at 5000 rpm for 20 mins to remove denatured proteins. 1 mL supernatant was combined with 0.5 mL of thiobarbituric acid (TBA) and the mixture was then boiled for 20 mins in a hot water bath. Finally, the absorbance was read spectrophotometrically at a wavelength of 532 nm. Whilst BHT was used as a standard, the control used in this assay was DMSO.

**7-Ethoxyresorufin O-deethylase (EROD) Assay**

7-Ethoxyresorufin O-deethylase (EROD) activity in rat liver microsomes was assayed as previously described by Burke et al. 7-Ethoxyresorufin is a substrate for CYP1A1, and this enzyme converts it to resorufin that can be measured by spectrofluorimetrically. 1 mL typical optimized reaction mixture contains 0.2 mg 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 NADPH-generating system consisting of 2.5 mM glucose-6-phosphate, 14.2 mM potassium phosphate buffer (pH 7.8), 2.5 mM MgCl₂, 0.25 mM NADP⁺, and 1.0 U glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of NADPH-generating system and then allowed to incubate at 37 °C for 5 mins. The reaction was then stopped by the addition of 3 mL ice-cold methanol, then centrifuged at 5000 rpm for 20 mins to remove denatured proteins. Finally, the absorbance was then 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, DISCUSSION and CONCLUSION**

The antioxidant effects of synthesized compounds on the rat liver microsomal NADPH-dependent LPO levels were ascertained by quantifying the amount of 2-thiobarbituric acid reactive substances (TBARS) formed in the reaction (Table 1). The results indicated that all
synthesized compounds at a concentration of $10^{-3}$ M had LPO inhibitory activities except compound 6 which contains thiophene that well-known isoster of the phenyl ring as 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 $p$-bromophenyl substituent at the second position of benzimidazole ring led to 57% inhibition on LPO level while butylated hydroxytoluene 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 synthesized 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 is 85% (Table 1).

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. When compared with the results obtained from these studies, benzimidazoles carrying 4-chloro phenyl ring at the second position were found to be more effective than benzimidazole counterpart carrying non-substituted phenyl rings for both assays.

![Scheme 1. Synthetic route to compounds 1-12.](image-url)
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.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Ar</th>
<th>EROD (pmol/mg/min)</th>
<th>% of Control</th>
<th>LPO (nmol/mg/min)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4-chlorophenyl</td>
<td>33.41 ± 1.64</td>
<td>80</td>
<td>11.67 ± 0.89</td>
<td>72</td>
</tr>
<tr>
<td>2</td>
<td>4-fluorophenyl</td>
<td>42.76 ± 2.34</td>
<td>103</td>
<td>10.51 ± 1.88</td>
<td>65</td>
</tr>
<tr>
<td>3</td>
<td>4-bromophenyl</td>
<td>38.91 ± 1.55</td>
<td>94</td>
<td>6.97 ± 0.65</td>
<td>43</td>
</tr>
<tr>
<td>4</td>
<td>3-nitrophenyl</td>
<td>38.55 ± 1.07</td>
<td>93</td>
<td>8.94 ± 2.13</td>
<td>55</td>
</tr>
<tr>
<td>5</td>
<td>2-naphtyl</td>
<td>38.87 ± 1.44</td>
<td>93</td>
<td>9.40 ± 2.13</td>
<td>58</td>
</tr>
<tr>
<td>6</td>
<td>3-methylthiophene-2-yl</td>
<td>35.91 ± 4.36</td>
<td>86</td>
<td>82.58 ± 1.23</td>
<td>508</td>
</tr>
<tr>
<td>7</td>
<td>4-benzyloxyphenyl</td>
<td>37.61 ± 0.68</td>
<td>91</td>
<td>13.81 ± 0.32</td>
<td>85</td>
</tr>
<tr>
<td>8</td>
<td>2-chloro-5-nitrophenyl</td>
<td>42.98 ± 3.49</td>
<td>103</td>
<td>11.84 ± 0.66</td>
<td>73</td>
</tr>
<tr>
<td>9</td>
<td>3,4-dibenzzyloxyphenyl</td>
<td>37.29 ± 0.98</td>
<td>90</td>
<td>10.10 ± 1.31</td>
<td>62</td>
</tr>
<tr>
<td>10</td>
<td>3-bromo-4-fluorophenyl</td>
<td>34.50 ± 1.13</td>
<td>83</td>
<td>12.89 ± 0.33</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>2,4-dichlorophenyl</td>
<td>4-chloro-3-nitrophenyl</td>
<td>BHT</td>
<td>Caffeine</td>
<td>DMSO</td>
</tr>
<tr>
<td>---</td>
<td>-------------------</td>
<td>-----------------------</td>
<td>-----</td>
<td>----------</td>
<td>------</td>
</tr>
<tr>
<td>11</td>
<td><img src="image1" alt="2,4-dichlorophenyl" /></td>
<td><img src="image2" alt="4-chloro-3-nitrophenyl" /></td>
<td>40.65 ± 1.02</td>
<td>38.26 ± 1.52</td>
<td>41.53 ± 0.99</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>92</td>
<td>5.68 ± 0.22</td>
<td>6.41 ± 0.36</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>12.08 ± 1.47</td>
<td>11.15 ± 0.98</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>69</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

REFERENCES
28. Iscan M, Arinc E, Vural N, Iscan MY. In vivo effects of 3-methylcholanthrene, phenobarbital, pyretrum and 2,4,5-T isooctylester on liver, lung and kidney microsomal


