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Original article

In vitro Assessment of the Lipid Peroxidation of *N,N′*-Disubstituted Benzimidazole-2-Thiones: Hydrazides vs Esters

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SUMMARY

Introduction: Oxidative stress and resulting lipid peroxidation are involved in numerous pathological conditions. For this reason, the role of antioxidants attracts attention and the radical-scavenging capacity of many natural and synthetic supplements and drugs has been extensively evaluated.
Material and methods: In the present study, seven *N,N′*-disubstituted benzimidazole-2-thiones with ester (1 - 4) and hydrazide (5 - 7) side chains were investigated for *in vitro* antioxidant activity using lipid peroxidation method.
Results: Among the assayed compounds, three hydrazides, 1,3-bis[3-(hydrazinooxy)-3-oxopropyl]-1,3-dihydro-2*H*-benzimidazole-2-thione (5), 1,3-bis[3-(hydrazinooxy)-3-oxopropyl]-5-methyl-1,3-dihydro-2*H*-benzimidazole-2-thione (6) and 1,3-bis[3-(hydrazinooxy)-3-oxopropyl]-5-benzoyl-1,3-dihydro-2*H*-benzimidazole-2-thione (7) showed good antioxidant properties (IC_{50} < 100 μM), with the best lipid peroxidation inhibition values (IC_{50}) shown for compound 5 (64 ± 10 μM) and compound 6 (73 ± 29 μM).
Conclusion: Indicated hydrazide structures may constitute a sort of molecular basis, a promising starting point for the development of compounds for the prevention and treatment of diseases resulting from oxidative damage.

Keywords: **antioxidant activity, lipid peroxidation, *N,N′*-disubstituted benzimidazole-2-thiones**

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INTRODUCTION

Reactive oxygen species (ROS) are continuously generated in cells as normal by-products of aerobic metabolism. While low concentration of ROS is essential for normal cellular signaling, a moderate increase in ROS levels interferes and disturbs normal physiological process and cellular proliferation (1). A significant increase in ROS levels and long-time exposure may provoke a state of imbalance between oxidant production and antioxidant defense capacity of a cell, ultimately inducing an oxidative stress. It has long been recognized that high levels of oxidants such as free radicals and ROS can cause direct damage to lipids by attacking carbon-carbon double bond(s). The process results in generation of lipid peroxyl radicals and hydroperoxides and is known as lipid peroxidation. Among diverse reactions triggered by peroxyl radicals induced by lipid peroxidation, those contributing to the degradation of cell membranes and organelles leading to tissue dysfunction and damage, are of particular importance. Moreover, oxidative stress and resulting lipid peroxidation are involved in numerous pathological states including inflammation, atherosclerosis, diabetes, cancer, neurodegenerative and cardiovascular disorders, and other chronic conditions (2). For this reason, the role of antioxidants continues to attract much attention and the radical-scavenging capacity of many natural and synthetic supplements and drugs has been extensively evaluated (3).

As a pharmacophore, being an isostere to purine bases, benzimidazole scaffold exhibits a wide range of biological activities (4), with a number of structurally modified compounds having antioxidant properties (5 - 7). Moreover, structural resemblance between N-substituted benzimidazoles and powerful antioxidants melatonin and melatonin N-substituted derivatives (8, 9) have shown promising results, giving impetus to research of 1,3-disubstituted benzimidazole-2-thiones (10 - 13) (Figure 1). Starting from these considerations, we decided to examine whether the synthetized 1,3-disubstituted benzimidazole-2-thiones 1-7 could show direct antioxidant effect and this was assessed by *in vitro* lipid peroxidation method.

Apart from the current study, compounds 1-7 were subject of two previous researches (11, 14). Anastassova et al. (11) evaluated the hepatoprotective and antioxidant properties using a model of *tert*-butylperoxide induced oxidative stress, measuring the parameters characterizing the functional-metabolic status of isolated rat hepatocytes, while Kolarević et al. (14) tested the *in vitro* inhibitory potential against bovine pancreatic deoxyribonuclease I (DNase I). Both studies have revealed that 1,3-benzimidazole-2-thiones (ester and hydrazide) containing benzoyl moiety could represent promising candidates for potential therapeutic applications.

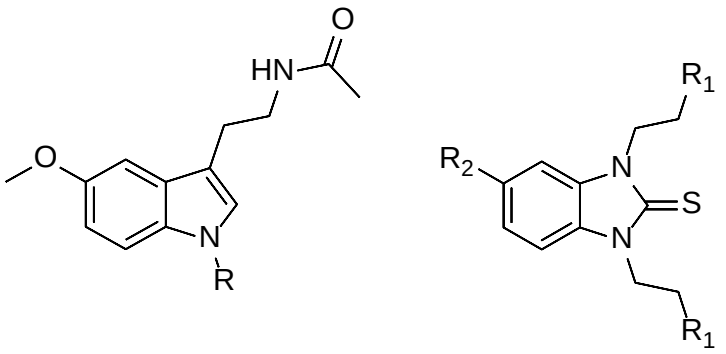

Figure 1. *Chemical structures of potent antioxidants: melatonin (R= H), melatonin N-substituted derivatives (R= $COCH_3$, COC_6H_5) and N,N'-disubstituted benzimidazole-2-thiones*

MATERIALS AND METHODS

Reagents and Materials

All inorganic and organic reagents, including standards and solvents, were of analytical grade, obtained from commercial sources. Phospholipids (Phospholipon® 90 - PL90) were obtained by courtesy of Phospholipid GMBH, Cologne, Germany. According to the manufacturer, the PL90 mixture is composed of phosphatidylcholine 98 % and lysophosphatidylcholine 2.1 %, with phospholipids' fatty acid composition: palmitic acid 12 ± 2%, stearic acid 3 ± 1%, oleic acid 10 ± 3%, linoleic acid 66 ± 5% and linolenic acid 5 ± 2%; the peroxide maximum value is 1.3. Thiobarbituric acid (TBA), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), trichloroacetic acid (TCA), methanol, sodium hydroxide and standards of caffeic acid, quercetin and Trolox® were purchased from Sigma-Aldrich (St. Louis, MO).

Chemistry

The melatonin analogues, *N,N′*-disubstituted benzimidazole-2-thiones (1-7), were synthesized following the method based on aza-Michael addition, a procedure described in Anastassova et al. (11) (Figure 2). The chemical structures and the purity of all products (purity > 98%) was verified by IR-, ^{1}H and ^{13}C NMR, DEPT, COSY and HSQC measurements (11).

Lipid peroxidation inhibition by thiobarbituric acid-malondialdehyde assay

Lipid peroxidation and the lipid peroxidation inhibition in the presence of the tested compounds (1 - 7) were measured by thiobarbituric acid-malondialdehyde (TBA-MDA) assay according to the procedure given by Lazarević et al. (15).

The absorbance of TBA-MDA complex in the supernatant, measured at 530 nm, was used to calcu-

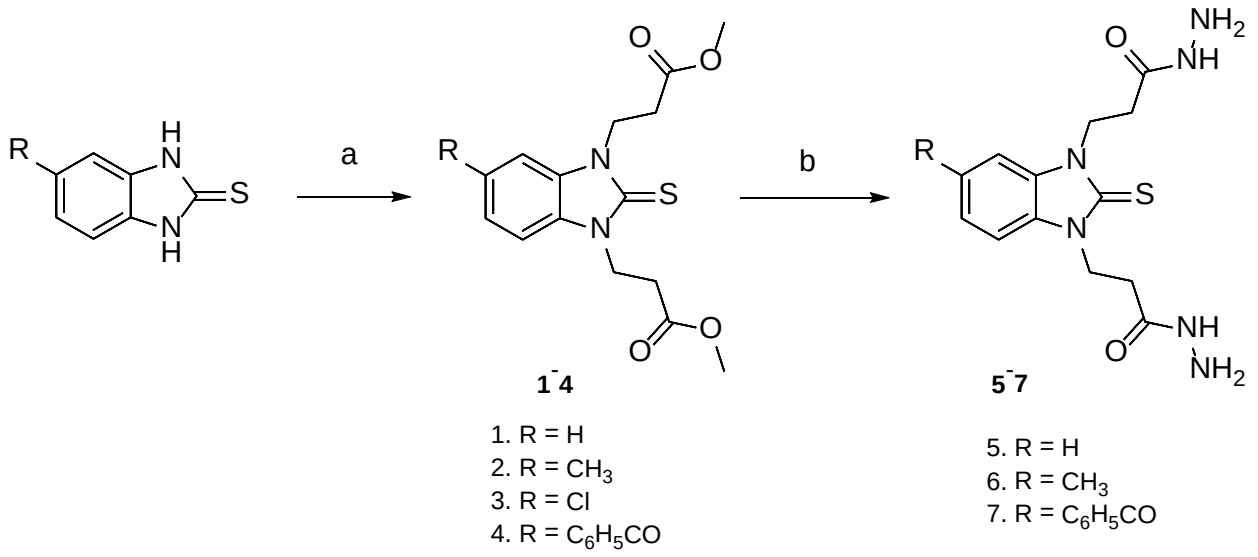

Figure 2. *Synthesis of the assayed N,N′-disubstituted benzimidazole-2-thiones (1-7). Reagents and conditions: a) methyl acrylate, DMF, refluxing; b) hydrazine hydrate, ethanol solution, refluxing*

late the inhibition percentage of lipid peroxidation given by the equation:

Inhibition of lipid peroxidation (%) = 100 × (Ac-As)/(Ac-Ab)

Ac - the absorbance of control (PL90 in methanol treated with the AAPH and TBA solution), As -the absorbance of sample (compounds 1 - 7 dissolved in PL90 solution, afterwards treated with the AAPH and TBA solution) and Ab - the absorbance of blank (PL90 in methanol, not treated with AAPH, but with TBA solution).

Samples were evaluated for lipid peroxidation inhibitory activity, and only those showing inhibition greater than 50% at 500 μM were investigated further in a broader concentration range to allow the calculation of IC_{50} values. The same type of analysis was done by using either Trolox, quercetin or caffeic acid (frequently used antioxidants) as standards. The standards were evaluated for lipid peroxidation inhibitory activity at concentrations of 50 μM (caffeic acid) and 80 μM (quercetin and Trolox) in the final reaction mixture. The measurements were done in triplicate.

RESULTS AND DISCUSSION

The results of the *in vitro* lipid peroxidation assay indicated that the tested *N,N'*-disubstituted benzimidazole-2-thione hydrazides 1,3-bis[3-(hydrazinooxy)-3-oxopropyl]-1,3-dihydro-2*H*-benzimidazole-2-thione (5), 1,3-bis[3-(hydrazinooxy)-3-oxopropyl]-5-methyl-1,3-dihydro-2*H*-benzimid-azole-2-thione (6) and 1,3-bis[3-(hydrazinooxy)-3-oxopropyl]-5-benzoyl-1,3-dihydro-2*H*-benzimidazole-2-thione (7) had good antioxidant properties, being fairly good radical scavengers (IC_{50} < 100 μM). Among the hydrazides, the most potent lipid peroxidation inhibition was expressed by compounds 5 and 6 (IC_{50} = 64 ± 10 μM and IC_{50} = 73 ± 29 μM, respectively), followed by compound 7 (IC_{50} = 92 ± 3 μM). The IC_{50} values are presented in Table 1 and lipid peroxidation inhibitory activities for the most potent compounds are given in Figure 3 as IC_{50} curves generated using four different concentrations, with three replicates at each. However, the tested reference antioxidants (caffeic acid, Trolox and quercetin) gave better results in inhibiting the lipid peroxidation process (Table 1). None of the ester derivatives (1 - 4) showed antioxidant properties, having IC_{50} > 200 μM.

It should be considered that the chemical mechanisms characterizing hydrogen atom transfer and single electron transfer reactions are also the mechanisms that play a dominant role in biological redox reactions, reflecting the antioxidative, preventive action at physiological level (16). Based on experimental data, melatonin and its *N*-substituted derivatives react with free radicals preferably by hydrogen atom transfer (HAT) and single electron transfer (SET) mechanism (9). While the SET mechanism of melatonin for direct radical scavenging is the most favorable one in aqueous solution, HAT is prevailing in nonpolar aprotic medium (17). Based on the structure similarities, these mechanisms are also expected to contribute to the overall free radical-scavenging activity of 1,3-disubstituted benzimidazole-2-thiones herein studied (1-7). Additionally, the theoretical model for the radical scavenging mechanisms of 1 - 7 established in our previous density-functional theory (DFT) study (11) can also be used for interpreting the experimental results of the current study. As it was observed in our research before, the ability of 1,3-disubstituted benzimidazole-2-thione hydrazides to inhibit the process by HAT mechanism is greater compared to the ester derivatives. While the studied esters could possibly react in HAT fashion, either by abstraction of hydrogen atoms from the alkyl groups next to the N-atoms in the benzimidazole ring or from the C-H bonds next to the ester carbonyl groups, the hydrazide derivatives have two additional sites for the H atom abstraction: the amide N-H bonds and the amino N-H bonds. Not only based on statistical probability, but also based on bond dissociation enthalpy (BDE) calculations (11), the 1,3-disubstituted benzimidazole-2-thione hydrazides (5 - 7) have greater ability to inhibit the lipid peroxidation process than the ester derivatives in lipid phase (HAT). This is what can also be observed by analyzing the current experimental data (Table 1). On the other hand, it is possible for the studied benzimidazole-2-thiones to transfer an electron to lipid radicals (expected to be a less selective process), forming radical cations, that may undergo molecular rearrangement and form a wider variety of products by giving metabolites that may subsequently act as radical scavengers (11). Nevertheless, based on preliminary experimental data, the 1,3-disubstituted benzimidazole-2-thione hydrazides provide more efficient protection against lipid peroxidation than the corresponding esters.

Table 1. *Lipid peroxidation inhibition effects of the studied N,N′-disubstituted benzimidazole-2-thiones **1**-**7** (IC_{50} values are given in μM).*

Compound No.	1	2	3	4	5	6	7	Caffeic acid	Trolox	Quercetin
lipid peroxidation inhibition IC_{50} (μM) ± SD	>200 μM	>200 μM	>200 μM	>200 μM	64 ± 10	73 ± 29	92 ± 3	15 ± 3	22 ± 6	23 ± 6

Figure 3. *Lipid peroxidation inhibitory activity (IC_{50}, μM). Each IC_{50} curve was generated using four different concentrations, with three replicates at each concentration*

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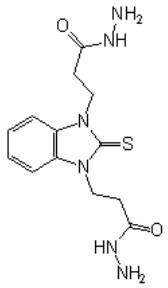
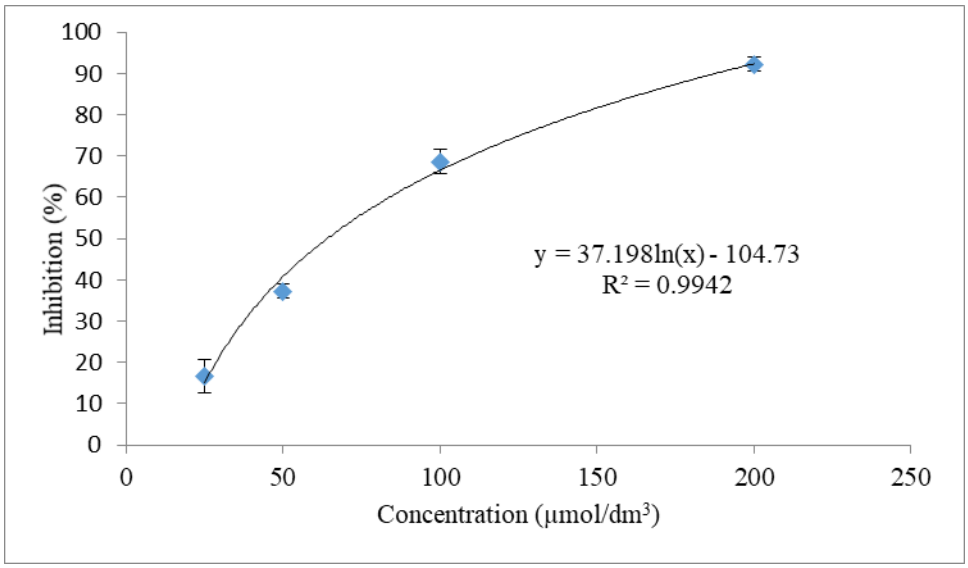


IC_{50} = 64 ± 10



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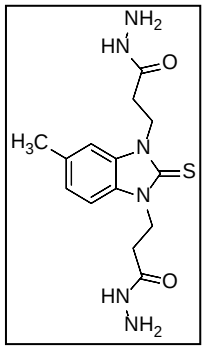
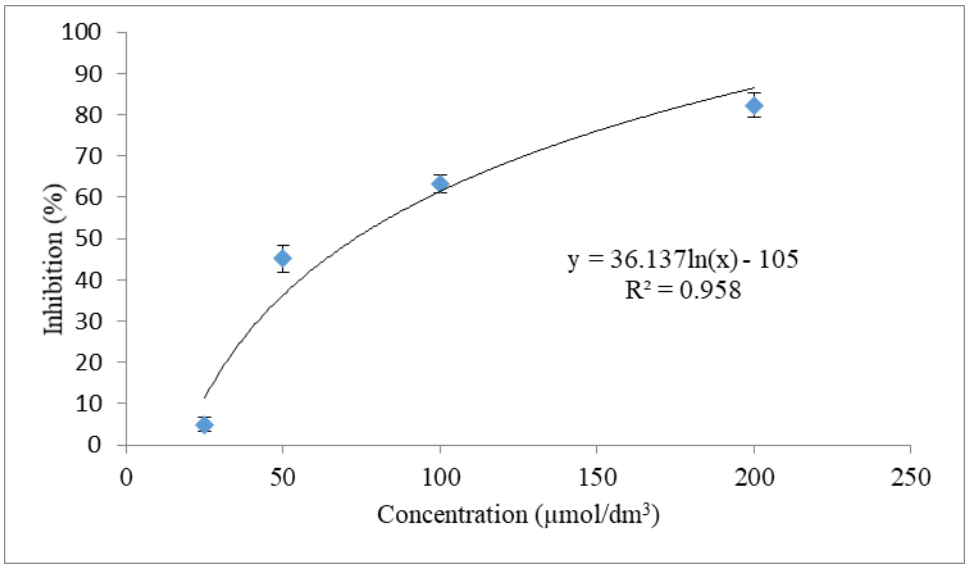
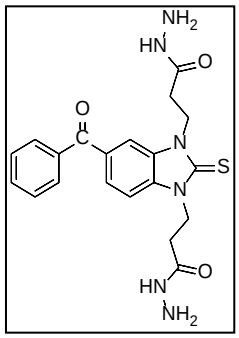
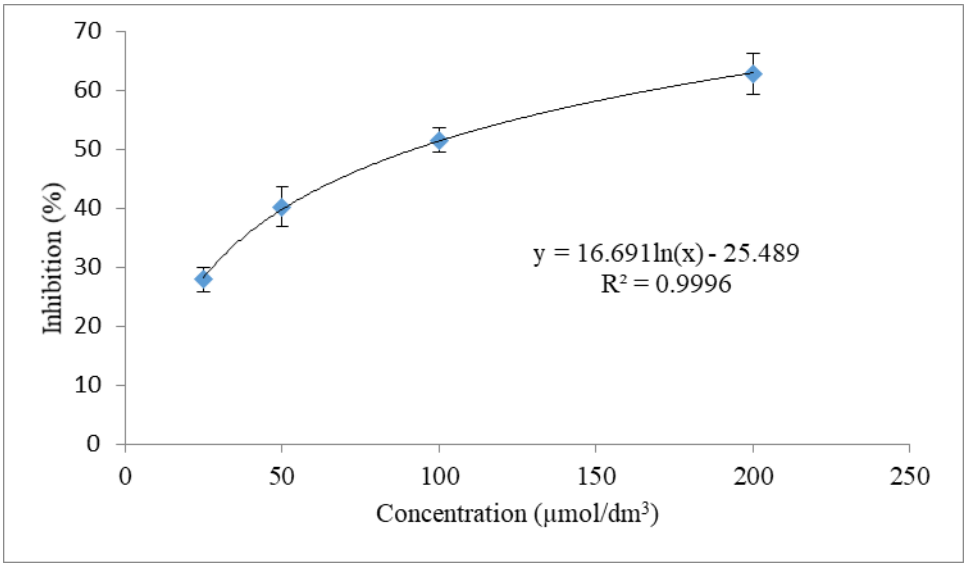


IC_{50} = 73 ± 29



7  $IC_{50} = 92 \pm 3$	

CONCLUSION

In this study we evaluated the antioxidant activity on lipid peroxidation of seven benzimidazole-2-thiones with *N,N'*-disubstituted ester (1 - 4) and hydrazide (5 - 7) side chains. The *N,N'*-disubstituted benzimidazole-2-thione hydrazides (compounds 5, 6 and 7) have good antioxidant properties being fairly good radical scavengers (IC_{50} < 100 µM), with compounds 5 and 6 (IC_{50} = 64 ± 10 µM and IC_{50} = 73 ± 29 µM) being the most effective *in vitro* antioxidants. The *N,N'*-disubstituted benzimidazole-2-thione hydrazides 5 and 6 can be regarded as model compounds and a promising starting point for the development of more potent radical scavengers and oxidative stress inhibitors.

Acknowledgements

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Antioksidativna aktivnost derivata *N,N′*-disupstituisanih benzimidazol-2-tiona: *in vitro* studija

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SAŽETAK

Uvod. Oksidativni stres i lipidna peroksidacija dovode do nastanka brojnih patoloških stanja: upale, ateroskleroze, dijabetesa, kancera, neurodegenerativnih i kardiovaskularnih poremećaja. Iz tog razloga, uloga antioksidanasa i dalje privlači veliku pažnju, a kapacitet uklanjanja radikala mnogim prirodnim i sintetičkim suplementima i lekovima i dalje se intenzivno proučava.
Metode. Antioksidativna aktivnost sedam *N,N′*-disupstituisanih benzimidazol-2-tiona, sa funkcionalizovanim bočnim nizovima u položajima 1- i 3- estarskim (1 – 4), odnosno hidrazidnim (5 – 7) grupama, ispitana je *in vitro*, metodom lipidne peroksidacije.
Rezultati. Hidrazidi 1,3-bis[3-(hidrazinooksi)-3-oksopropil]-1,3-dihidro-2*H*-benzimidazol-2-tion (5), 1,3-bis[3-(hidrazinooksi)-3-oxopropil]-5-metil-1,3-dihidro-2*H*-benzimidazol-2-tion (6) i 1,3-bis[3-(hidrazinooksi)-3-oxopropil]-5-benzoil-1,3-dihidro-2*H*-benzimidazol-2-tion (7) pokazuju dobra antioksidativna svojstva (IC_{50} < 100 μM), sa najboljim vrednostima inhibicije lipidne peroksidacije (IC_{50}) za jedinjenja 5 (64 μM ± 10 μM) i 6 (73 μM ± 29 μM).
Zaključak. Na osnovu *in vitro* dobijenih IC_{50} rezultata, pomenuta dva hidrazida predstavljaju dobru polaznu molekularnu osnovu i obećavajući strukturni element u razvoju jedinjenja za prevenciju bolesti, nastalih kao posledica oksidativnog oštećenja.

Ključne reči: **antioksidativna aktivnost, lipidna peroksidacija, *N,N′*-disupstituisani benzimidazol-2-tioni**