Radical Scavenging Activity of Hydroxy and Methoxy Substituted 1H-Benzimidazole-2-yl Hydrazones against Hypochlorite Ions and Hydrogen Peroxide

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Abstract: *In view of the important role of oxidative stress for human health and pathogenesis, a small series of 1H-benzimidazole-2-yl hydrazones were evaluated for their in vitro efficacy in scavenging hydrogen peroxide and hypochlorite ions. In vitro spectrophotometric system along with luminol-enhanced chemiluminescence were employed to assess the impact of the tested derivatives on the concentration of reactive oxygen species (ROS). The observed effects were compared to those of the known antioxidants Trolox and trans-caffeic acid. The 1H-benzimidazole-2-yl hydrazones demonstrated an ability to scavenge hypochlorite ions and reduce the chemiluminescent signal in greater extend compared to the reference compounds. The hydrazones exhibited a concentration-dependent scavenging activity against H_2O_2, again surpassing those of Trolox and caffeic acid. The two hydrazones, containing hydroxy groups in their structure, showed a potency to chelate iron Fe(II). In all tested in vitro systems, the hydroxy-substituted derivatives were more effective than the methoxy-substituted compounds.*

Keywords: *1H-benzimidazole-2-yl hydrazones, Reactive oxygen species, Hypochlorite ions, Hydrogen peroxide, Iron chelation.*

Introduction

The redox homeostasis, the role of free radicals as secondary mediators of signal transduction, their influence on the transcription of redox responsive genes and protection from infectious agents are important aspects of medicinal chemistry. On one hand, it has been shown that at low and moderate concentrations reactive oxygen species (ROS) are not necessarily damaging products. They play a key role in intracellular signaling, influence gene expression, regulate cell cycle and contribute to defense against infectious agents [14]. At the same time, there is irrefutable evidence demonstrating that the condition associated with imbalance between ROS production and antioxidant defense, which is defined as oxidative stress, leads to physiological dysfunction and underlies the pathogenesis of a number of socially relevant diseases such as cancer, heart and neurodegenerative diseases [19]. In this regard,

antioxidants may protect vital organs against oxidative tissue damage by interfering with ROS production or by scavenging.

In terms of intracellular generation, mitochondria, and more precisely the intermediate steps of the electron-transport chains of the inner mitochondrial membrane, are a major source of free radicals. Other cellular organelles associated with the initiation of oxidative stress are the endoplasmic reticulum (monooxygenase enzymes), peroxisomes (acyl-CoA oxidase, xanthine oxidase, glutaryl-CoA oxidase, and flavin monooxygenase system), and the cytosol (NADPH-oxygenase, dopamine-β-hydroxylase, urate oxidase) [20]. In most cases, the initiating step is the formation of a superoxide anion radical ($O_2^{\bullet-}$). A typical example is the initiation of the phagocytosis process, causing activation of the multicomponent membrane-bound enzyme NADPH-oxidase, which catalyzes the one-electron reduction of molecular oxygen to $O_2^{\bullet-}$. The resulting radical is weakly reactive and cannot independently exert a bactericidal effect, but through a chain of reactions leads to the generation of more potent oxidants that damage cellular structures.

The superoxide anion radical ($O_2^{\bullet-}$) can either be involved in spontaneous dismutation or be efficiently converted by superoxide dismutase (SOD), an enzyme with redox-active metal ions at its catalytic core. The first step in this process involves the oxidation of $O_2^{\bullet-}$ to molecular oxygen by one-electron transfer to the enzyme's oxidized metal center. The same metal atom then reduces a second superoxide molecule to H_2O_2, reestablishing the enzyme's original oxidation state. H_2O_2 is weakly reactive; therefore, it can migrate from the site of production and thus to propagate the radical attack to another site in the cell.

Hydrogen peroxide is a substrate for reactions generating the two strongest oxidants: hypochlorite (in the presence of Cl^- via myeloperoxidase) and the hydroxyl radical ($HO^{\bullet}$) via an ion-catalyzed reaction. The radicals formed are potent oxidizing agents and are consumed instantaneously at the site of their formation, which is associated with oxidative damage to amines, thiols, proteins, nucleic acids, lipids, etc. It is possible that a fraction of HOCl is involved in reactions to form organic chloramines, which are less reactive but have longer lifetimes, leading to dislocation of the oxidative attack at longer distance [26].

Several types of benzimidazole-based compounds often incorporating a hydrazone chain or S-atom in their structure, have shown an antioxidant potential. N-phenyl-5-[(2-phenylbenzimidazole-1-yl)methyl]-1,3,4-oxadiazol-2-amines (Fig. 1 I) exhibited good inhibitory effect on microsomal NADPH-dependent inhibition of lipid peroxidation levels, microsomal ethoxyresorufin O-deethylase activity and scavenge scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals free radicals [10, 11]. A thiol bridged bis-benzimidazole derivative and its dicationic analogue (Fig. 1 II) were recently reported to scavenge DPPH and hydrogen peroxide radicals as well as to protect plasmid DNA from Fenton's reagent radicals [29]. Other 2-mercaptobenzimidazole based derivatives (Fig. 1 III) also have shown good DPPH scavenging activity [1]. N1-(4-arylidene)-1H-benzo[d]imidazole-2-carbohydrazides (Fig. 1 IV) and 2-arylbenzimidazoles, containing hydroxy groups in the phenyl moiety (Fig. 1 V), exhibited promising radical scavenging activity towards DPPH and peroxyl radicals, ferric ion reducing ability and UV protection properties [12, 13, 23]. Benzimidazole-1-arylhydrazones (Fig. 1 VI) and N,N'-disubstituted acylhydrazone derivatives of the benzimidazole-2-thione (Fig. 1 VII) demonstrated potent neuroprotective activity and possess the capability to influence lecithin peroxidation, deoxyribose degradation, scavenge superoxide anion radical in *in vitro* systems with enzymatic (X-XO) and non-enzymatic generation [2, 3].

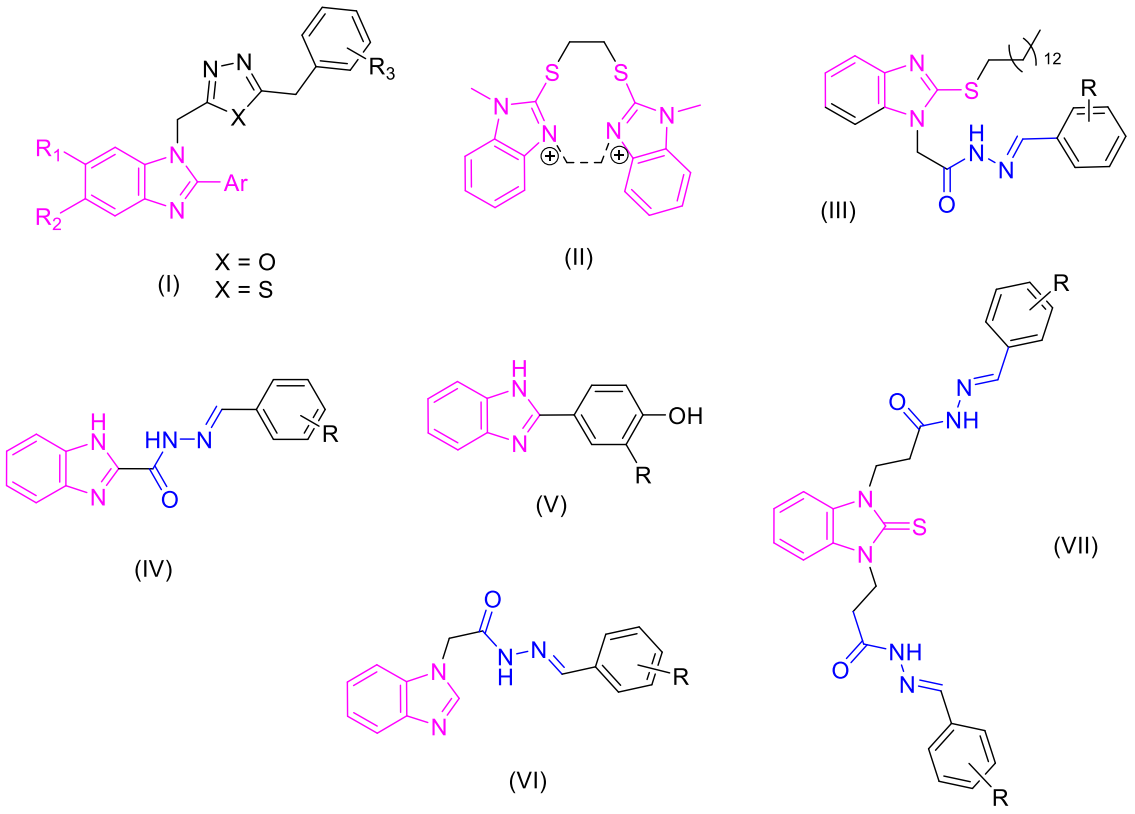


Fig. 1 Benzimidazole derivatives with radical scavenging and antioxidant activity

As part of our broader study of the biological activity of 1*H*-benzimidazole-2-yl hydrazones we have carried out an *in vitro* evaluation on the efficacy of four representative in scavenging hydrogen peroxide and hypochlorite ions. The luminol-dependent chemiluminescence was monitored in model system of NaOCl generated hypochlorite ions. Spectrophotometric assay for scavenging activity against hydrogen peroxide was used. Furthermore, an *ortho*-phenanthroline chelation test was performed to evaluate the compounds' possible chelation properties towards Fe(II). The observed effects were compared to those of the known antioxidants Trolox and *trans*-caffeic acid and rationalized in view of the structural features of the compounds, including hydroxy and methoxy groups in the aryl part linked by a hydrazone chain to an unsubstituted or substituted benzimidazole core.

Materials and methods

Reagents and materials

The methodology for synthesis of 1H-benzimidazole-2-yl-thiols 2.1-2.2, 1H-benzimidazole-2-yl-sulfonic acids 3.1-3.2 as well as 1H-benzimidazole-2-yl-hydrazides 4.1-4.2 was described in previous publications [7, 8].

Hypochlorite scavenging activity

Chemiluminescent (CL) studies are widely used due to their reliable detection capabilities and remarkable detection threshold: 10^{-19} M [21, 24, 25]. In our study, we employed luminol-dependent chemiluminescence to evaluate the ability of the tested hydrazones to decrease the concentration of the hypochlorite ions (OCl^-). An LKB 1251 chemiluminometer (BioOrbit, Turku, Finland) connected to an IBM-PC-compatible computer was used. We utilized the MultyUse program Version 1.08 (BioOrbit) for the data analysis. All experiments were

performed at a constant temperature of 37 °C. The assay involved preparing a "Control" solution without the investigated derivatives and "Sample" solutions containing varying concentrations of the hydrazone under investigation. In each sample run, we included a blank sample. The obtained background signal, measured without the enhancer, was subtracted from both the "Control" and "Sample" measurement compositions. The chemiluminescent response was evaluated by analyzing the area under the resulting CL curve. We calculated the CL ratio in the presence and the absence of the investigated compound, expressed as a percentage. The parameter is termed CL scavenging index (CL-SI, %) and is used to compare the tested substances' scavenging properties. The experiment was conducted using a sodium hypochlorite-based system. Specifically, 1 mL PBS samples containing 0.1 mM luminol, 0.06 mM NaOCl, and either the desired concentration of the test derivatives or buffer (for control) were prepared. The CL signal was then monitored for 1 minute, with readings taken every 50 ms following the addition of NaOCl.

H_2O_2 scavenging activity

The experiment was performed according to Mukhopadhyay et al. [18]. The measurement sample compositions were with a final volume 1 ml and contained the following reagents: 0.05 mM $FeCl_2$, 0.2 mM *ortho*-phenanthroline, 5 mM H_2O_2 and the tested hydrazones. As a preliminary step, the reaction mixture comprised hydrogen peroxide (H_2O_2) and the tested substances at various concentrations. Following a 10-minute incubation at room temperature in the absence of light (in accordance with the method outlined by Ruch et al. [22]), ferrous chloride ($FeCl_2$) at a concentration of 0.05 mM was added to each tube. Subsequently, a second 5-minute incubation at room temperature was carried out. Following this second incubation, *ortho*-phenanthroline was introduced into the mixture. The experiment requires the preparation of one positive and two negative controls. In the positive control ($CTRL^+$) H_2O_2 was included but the tested compounds were omitted. In the first negative control ($CTRL^-$) we excluded both the hydrazones and H_2O_2. The second negative control ($CTRL^-$) involved the tested hydrazones at each tested concentration, but without including H_2O_2. Blank samples containing only the tested derivatives/reference compounds, H_2O_2 and Fe(II) have also been prepared for each tested concentration. The absorbances of all measurement compositions were then measured at a wavelength of 515 nm. The calculation of the ability of compounds to scavenge hydrogen peroxide was performed using following the Eq. (1):

$$\%H_2O_{2\ scavenging\ activity} = \left(\frac{A_{sample}}{A_{CTRL-}}\right).100. \quad (1)$$

Ortho-phenanthroline test

We used the ortho-phenanthroline method to examine the hydrazones' iron-chelating properties. 1 mL sample solution, contains 0.2 mM *ortho*-phenanthroline and 0.05 mM $FeCl_2$, and 0.2 mM tested hydrazones. In controls, the tested compounds were omitted. As the first step, mixtures containing the ferrous iron and the hydrazones were prepared. They were allowed to react for 5 min: the time necessary for the formation of complexes between Fe(II) and the tested compounds if chelation properties are being observed. The second step comprises the addition of the *ortho*-phenanthroline solution. Absorbance was measured at 515 nm via GENESYS 50 UV-VIS in order to determine the colored complexes formation between *ortho*-phenanthroline and the remaining free ferrous irons [17]. The results have been presented as a percentage of the control sample. Absorbance spectra of all the samples, the controls and the measurement compositions containing the tested hydrazones and Fe(II) at the concentrations used in the samples have been taken. In all assays we have used Trolox and caffeic acid as standard reference compounds.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 8 software (v.8; GraphPad software, La Jolla, California, USA). Data are presented as mean ± standard deviation. Differences were analyzed using a one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparisons post hoc test. A threshold of $p < 0.05$ was used to determine statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Results and discussion

Four 1H-Benzimidazole-2-yl hydrazones, bearing 2,3-dihydroxy or 3,4,5-trimethoxyphenyl moiety, were obtained according to the synthetic procedure described earlier [7, 8]. The reaction pathway is briefly outlined in Fig. 2. The obtained benzimidazole derivatives combine two distinctive structural modifications: variation of the arylhydrazone substitution on one hand, and lack or presence of a substituent (methyl group) in the benzimidazole fragment.

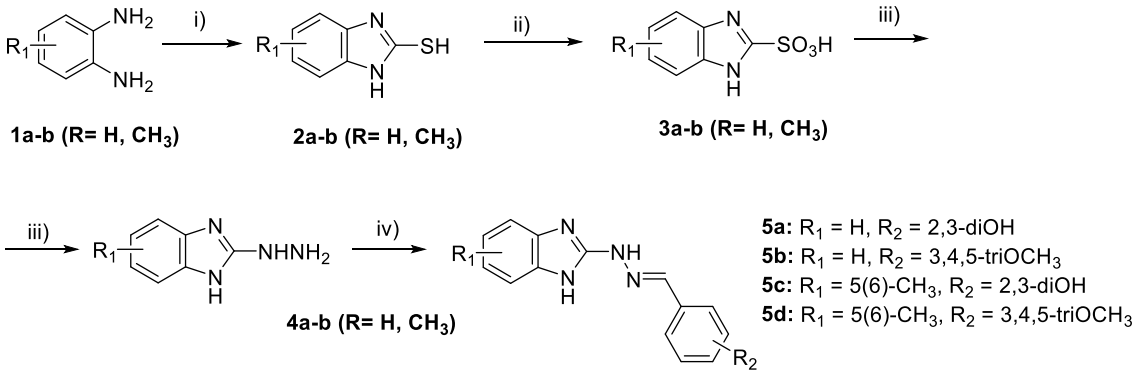


Fig. 2 Synthesis of 1H-benzimidazole-2-yl hydrazones (5a, 5b, 5c, 5d):
i) CS_2, KOH, EtOH, reflux; 50% acetic acid; ii) 50 % $KMnO_4$ in ethanol, 1 h; HCl, pH = 1;
iii) 99% hydrazine hydrate, reflux, 3 h; iv) R_2CHO (1:1), 99 % ethanol, 3-4 h.

We employed an *in vitro* spectrophotometric system along with luminol-enhanced chemiluminescence to assess the impact of the tested derivatives on the concentration of ROS. Initially, we evaluated the compounds' ability to interact with hypochlorite ions. Subsequently, our investigation focused on their efficacy in scavenging hydrogen peroxide.

The scavenging activity of both groups of hydrazones and the reference compounds against the hypochlorite ions is summarized in Fig. 3. The compounds were tested at concentrations below 1 µM, and both groups demonstrated the ability to reduce the CL signal. Data in Fig. 3 are presented from three independent experiments as means ± SD. One-way ANOVA, followed by the Bonferroni post-test, was used for statistical evaluation. As the concentration of hydrazones increased, the scavenging index decreased, indicating a more pronounced scavenging capability. Compounds 5b and 5d exhibited moderate potency in this system compared to 5a and 5c. At the lowest tested concentration of 0.1 µM, the CL-SI for the trimethoxy compounds was higher than 98%. Neither compound significantly decreased the SI compared to the control ($p > 0.05$) at this concentration. Both trimethoxy compounds exhibited statistically identical effects at all tested concentrations except the maximum of 1 µM, where 5d demonstrated superior scavenging activity ($p < 0.01$). Compound 5b had CL-SI of 52.1%, whereas for compound 5d it was 44.3%. Additionally, compound 5d demonstrated a significantly lower IC_{50} value of 0. 90 µM compared to 5b of 1.08 µM ($p < 0.001$), indicating its higher potency in scavenging hypochlorite ions.

The presence of 2,3-dihydroxy moieties rendered these compounds more potent in decreasing the CL signal and enhanced scavenging capabilities. Notably, they significantly reduced the CL ratio even at the lowest tested concentration of 0.1 µM ($p < 0.0001$) compared to the controls. Compounds 5a and 5c exhibited a CL-SI around 85%. These compounds demonstrated nearly identical effects within the system across most tested concentrations. At the highest tested concentration, they managed to decrease the CL ratio to one-third of the control value. Calculated IC_{50} values for 5a and 5c were 0.41 µM and 0.51 µM, respectively, approximately half the values observed for the trimethoxy compounds.

Due to the lower reactivity of Trolox and caffeic acid, the concentration range associated with the evaluation of their potential scavenging capabilities was increased to 5 µM. In the samples containing the reference compounds, we observed a decrease in CL ratio at all tested concentrations, indicating scavenging properties and scavenging potential. Their effectiveness was rising consistently with increasing concentrations. At the maximal tested concentration, a nearly 50% decrease compared to the control has been observed. The SI varied from 80% at the lowest tested concentration of 0.5 µM to a nearly 50% decrease at the maximal concentration of 5 µM. The IC_{50} values, estimated from the concentration CL-SI, were higher compared to the tested benzimidazole derivatives: 4.41 ± 0.04 µM for Trolox and 2.81 ± 0.01 µM for caffeic acid. Lower CL-SI, % values indicate better scavenging activity (Fig. 3).

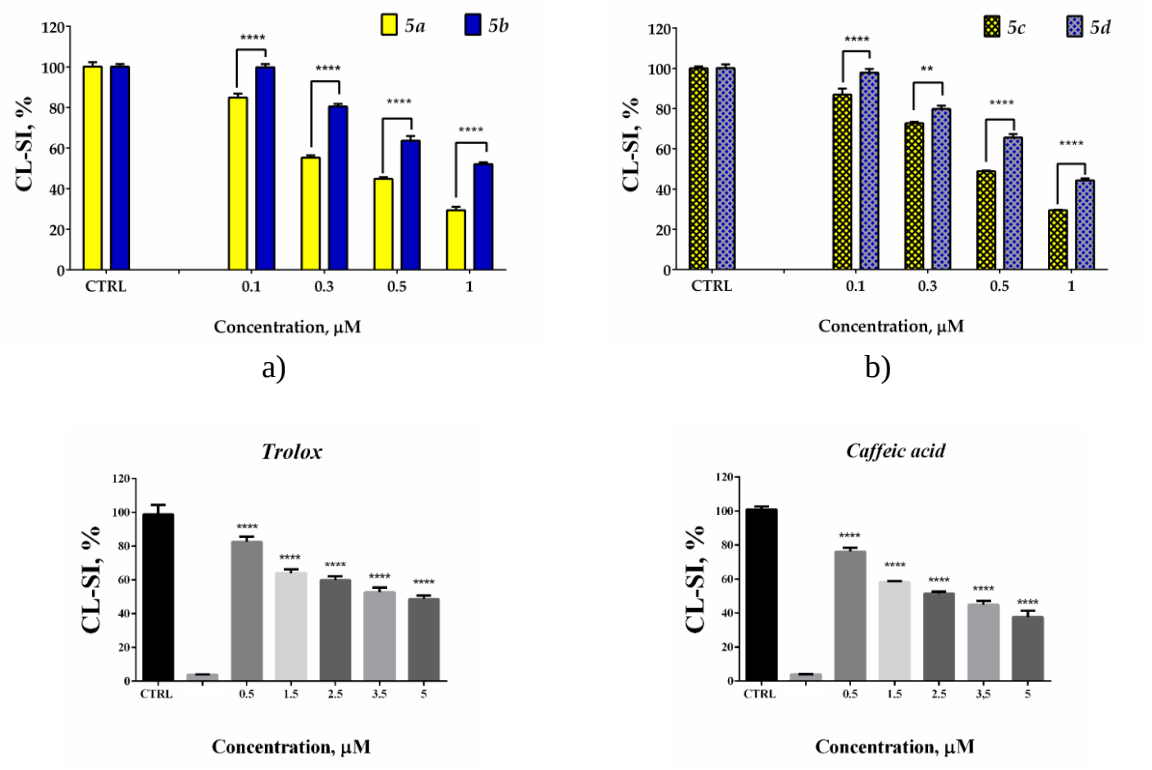
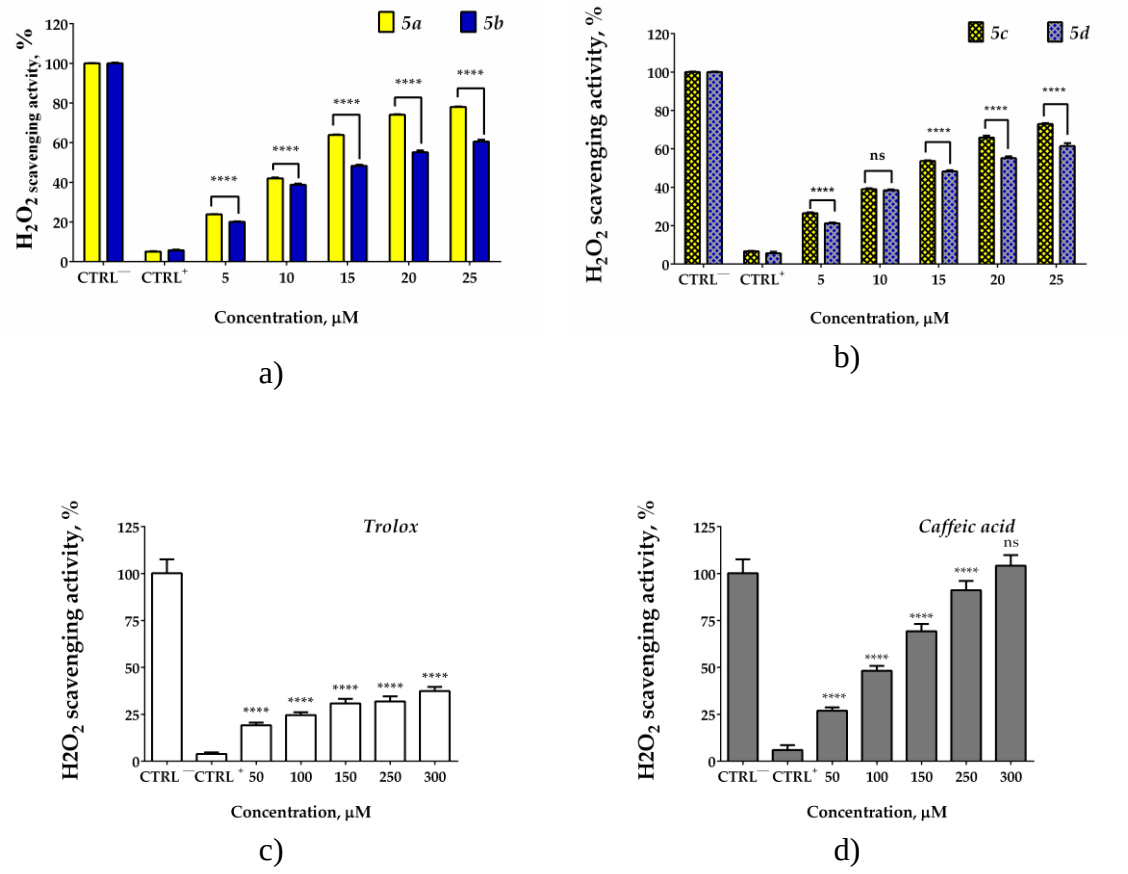


Fig. 3 Reduction of luminol-dependent chemiluminescence in model system of sodium hypochlorite derived hypochlorite: a) unsubstituted benzimidazole derivatives 5a and 5b; b) 5(6)-methylbenzimidazole derivatives 5c and 5d; c) and d) reference compounds Trolox and caffeic acid. Differences were considered statistically significant: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

The potency of the compounds to decrease H_2O_2 concentration is displayed in Fig. 4 where 1 ml reaction mixture contained: 0.05 mM $FeCl_2$, 0.2 mM *ortho*-phenanthroline, 5 mM H_2O_2 and the tested hydrazones. Data in Fig. 4 are presented from three independent experiments as means ± SD. One-way ANOVA, followed by the Bonferroni post-test, was used to perform the statistical evaluation.



a) b)

c) d)

Fig. 4 Hydrogen peroxide scavenging activity: a) unsubstituted benzimidazole derivatives 5a and 5b; b) 5(6)-methylbenzimidazole derivatives 5c and 5d; c) and d) reference compounds Trolox and caffeic acid. Differences were considered statistically significant: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$.

Due to the lower compound effect and the less sensitivity of the spectrophotometric methodology, we increased the concentration range by which we estimated the scavenging capability. We determined the effect of the compound at concentrations above 5 µM. The scavenging activity was concentration-dependent. All hydrazones denoted statistically significant scavenging activity compared to the $CTRL^+$ sample even at the lowest tested concentration. Increasing hydrazone sample concentration corresponded to higher values of the scavenging activity. Notably, among the unsubstituted benzimidazole derivatives, compound 5a demonstrated superior efficiency, exhibiting an absorbance ratio increase of over 75% at the maximal tested concentration. From the 5(6)-methylbenzimidazole derivatives 5c had stronger scavenging activity than 5d at all concentrations except 10 µM. To compare the potency of both dihydroxyl substituted and both 3-methoxy substituted compounds in both cases, the 5(6)-methylbenzimidazole derivatives, we estimated the IC_{50} values from the concentration

radical scavenging activity relationship. Both trimethoxy derivatives 5b and 5d had identical IC_{50} values -17.78 ± 1.18 and 17.61 ± 0.85 µM, respectively. Both, 5a and 5c had significantly lower IC_{50} values: 12.73 ± 0.79 and 14.31 ± 0.18 µM. Again, like in the previous model system, the concentration range for determining the scavenging activity of both reference compounds was increased. The observed results were statistically higher compared to the $CTRL^+$ sample. Despite the maximal tested concentration being 300 µM, the determined H_2O_2 scavenging activity for Trolox did not exceed 40%. At the maximal tested concentration, the caffeic acid had the same effect like the $CTRL^-$ sample. IC_{50} for caffeic acid was 181.79 ± 6.94 µM.

To allow a direct comparison of the scavenging efficacy toward hypochlorite ions and hydrogen peroxide, the corresponding estimated IC_{50} values are summarized in Table 1.

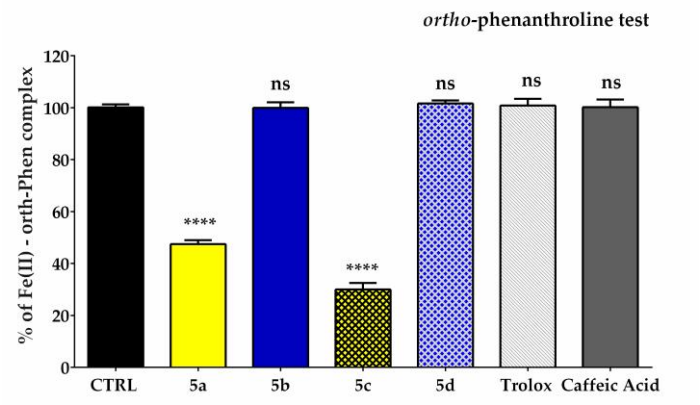
Table 1. IC_{50} values (µM) of the tested derivatives and reference compounds based on the concentration-response relationships obtained in the luminol-dependent chemiluminescence NaOCl-derived hypochlorite model system and the spectrophotometric assay for H_2O_2 scavenging activity

Compound	**Hypochlorite scavenging IC_{50} (µM, mean ± SD)**	**Hydrogen peroxide scavenging IC_{50} (µM, mean ± SD)**
5a	0.41 ± 0.002	12.73 ± 0.79
5b	1.08 ± 0.02	17.78 ± 1.18
5c	0.51 ± 0.03	14.31 ± 0.18
5d	0.90 ± 0.02	17.61 ± 0.85
Trolox	4.41 ± 0.04	> 300
Caffeic acid	2.81 ± 0.01	181.79 ± 6.94

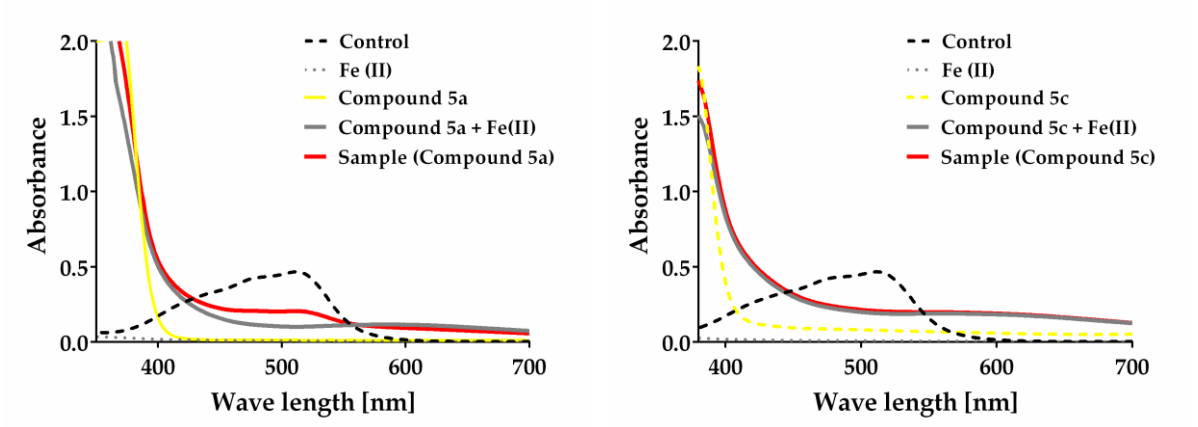
As a next step, we performed an *ortho*-phenanthroline chelation test to evaluate the compounds' possible chelation properties and to explain the observed effect in the H_2O_2-containing system (whether it is due to a direct scavenging effect or possible interference with chelation activity).

From the obtained data (Fig. 5a), it is evident that there are differences in the amount of generated Fe(II)-*ortho*-phenanthroline complex in the presence of the tested compounds. In the samples containing 5b and 5d the generated amount is the same as in the control sample and the references (99.9% and 101.6%), suggesting no chelation activity of these hydrazones. In the samples containing 5a and 5c the amount of Fe(II)-*ortho*-phenanthroline complex formation decreased respectively two times (47.5%) and three times (30.0%) compared to the control and the trimethoxy compounds. The absorbance spectra of 5a and 5c, measured with and without Fe(II), indicate that the presence of iron in the sample mixture is associated with increased absorbance of the solution at a wavelength higher than 400 nm (Fig. 5b and 5c). Data in Fig. 5 are presented from three independent experiments as means ± SD. One-way ANOVA, followed by the Dunnet post-test, was used to perform the statistical evaluation. The absorbance spectra measured in the presence of *ortho*-phenanthroline, hydrazones 5a and 5c, and Fe(II) showed changes compared to the Fe(II)–*ortho*-phenanthroline complex, suggesting a potency of both compounds to chelate iron. In the sample containing 5a, some extent of generation of the Fe(II)–*ortho*-phenanthroline complex has been observed, whereas in the reaction mixture containing 5c, the absorbance spectra of the sample and

the solution containing 5c and Fe(II) are identical. The latter indicated full suppression of the Fe(II)–*ortho*-phenanthroline complex generation in the presence of 5c. The results from the Fe(II)–*ortho*-phenanthroline test comply well with the metal-chelating activity of 5a and 5b found earlier by HORAC assay [9].



a)



b) c)

Fig. 5 a) The extent of formation of the Fe(II)-*ortho* phenanthroline complex;
b) and (c) UV-Vis spectra absorbance spectra of:
control-standard orange-red Fe(II)-1,10-phenantroline complex;
hydrazone [0.2 mmol/L] alone and in the presence of Fe(II) [0.05 mM];
and the sample solution containing 1,10 phenanthroline [0.2 mM],
Fe(II) [0.05 mM] and the hydrazone [0.2 mM].

The extent of the observed effect in the used systems depended on the use in the system ROS and the types of structural modifications of the compounds. In the hypochlorite system 5a had statistically significant higher scavenging activity in the studied concentration range ($p < 0.0001$) compared to 5b at all tested concentrations and behaves as a potent scavenger of hypochlorite ions. Both hydrazones denoted the capability to increase the absorbance signal in the system containing H_2O_2 but have weaker scavenging activity compared to the hypochlorite

containing system. This could be attributed to the lower reactivity of H_2O_2 compared to the hypochlorite ion and the different used method for registration. The principle of the H_2O_2 scavenging assay is based on the formation of the red-orange tri-phenanthroline complex, which absorbs maximally at 515 nm between ferrous ion Fe (II) and 1,10-phenanthroline and the capability of H_2O_2 oxidize all Fe(II) to Fe(III) and prevent complex formation if added before 1,10-phenanthroline to the reaction mixture. In the case of 5b and 5d the effect in the H_2O_2-containing system could be attributed only to scavenging activity due to the lack of chelation potential of this compound. Despite the fact that 5a and 5c have been proven to possess chelation activity against Fe(II). Given the concentration range we studded its H_2O_2 scavenging capability direct interaction with H_2O_2 is more probable.

The comparison of the observed effects with the data for related hydrazone compounds might help outlining the structural factors influencing the radical scavenging activity. For instance, the reported studies on the radical scavenging activity of hydrazone derivatives of 5-methoxyindole carboxylic acid [4], indole-3-acetic acid [5] and N,N'-disubstituted benzimidazole-2-thiones [6] towards hypochlorite ions, comply with the present study and clearly underline the beneficial role of the conjugation between the aromatic core and the hydrazone chain extended with a hydroxyphenyl moiety, and especially dihydroxyphenyl moiety, for effective scavenging of hypochlorite ions. Although direct comparison of the estimated effects is somewhat difficult due to different applied assay protocols, it should be noted that the IC_{50} values of compounds 5a, 5b, 5c, 5d towards hypochlorite ions found in the present study, seems comparable, or even better than those reported for several flavonoid compounds and the corresponding phenols [15, 16, 27, 28].

Conclusion

In this work, four benzimidazole derivatives were evaluated for their radical scavenging properties and chelation. It was found that the studied compounds possess ability to scavenge biologically important ROS like H_2O_2 and hypochlorite ion and some of them to chelate metal atoms such as iron. The data obtained by us provided further evidence for the promising radical scavenging properties of the 1H-benzimidazole-2-yl hydrazones and can provided useful insights in view of further evaluation of their biological activity. This was in accordance with our previous investigations, where it was demonstrated that the hydroxy-substituted derivatives exert better scavenging activity against stable free radicals and antioxidant effect in model systems of iron-induced oxidative damage of biologically important molecules than their methoxy-substituted counterparts. This paper demonstrates that this was also valid for biologically relevant ROS like H_2O_2 and hypochlorite ion. The promising radical scavenging and chelation properties of the studied 1H-benzimidazole-2-yl hydrazones motives further in-depth exploration of their biologicals properties. Having in mind the importance of oxidative stress modulation in various pathological processes, it is noteworthy to explore the potential of the compounds as anthelmintic or anticancer agents, including interaction with biological targets that might be useful in targeted therapies.

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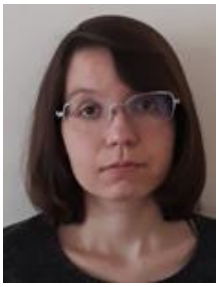

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