

4-Methoxy arylhydrazones – promising agents protecting biologically relevant molecules from free radical damage

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The concomitant toxicity of iron observed in many disorders associated with excess of this essential trace element is mainly due to its ability to promote and participate in free radical generation reactions and the subsequent oxidative damage. The dietary recommendations for some of the patients and the restriction for supplementation with essential antioxidants have led to the necessity of developing novel high-efficiency pharmacologically active substances with multipotent antioxidant properties. The present investigation aimed to determine the protection effect against oxidative stress of three structurally characterized derivatives of the active chelator salicylaldehyde benzoylhydrazone (SBH). The capability of the tested compounds 4-methoxysalicylaldehyde benzoylhydrazone (4mSBH), 4-methoxysalicylaldehyde-4-hydroxybenzoylhydrazone (4mShBH) and 4-methoxysalicylaldehyde isonicotinoylhydrazone (4mSIH) to preserve biologically relevant molecules from oxidative damage was evaluated in *in vitro* spectrophotometric model systems with diverse mechanisms of free radical generation containing deoxyribose and egg yolk homogenate. Additional experiments have been performed in chemiluminescent systems containing different type of reactive oxygen species. The obtained results indicated that the incorporation of methoxy group at 4th position in the aldehyde part of the molecule ameliorates the evaluated properties. The extent of the observed improvement depends on the subsequent modifications in the hydrazide moiety. Greatest protection effect against the oxidative damage of the biologically important molecules was observed in the samples containing hydroxyl bearing compound (4mShBH). This hydrazone also demonstrated higher activity against OH[•] and O₂^{•-} in the chemiluminescent model systems. Comparing the C₅₀ values from the different model systems we suggest that the observed protection effect is associated with antioxidant activity different from Fe-chelation.

Keywords: Hydrazones, Antioxidant properties, Chemiluminescence

INTRODUCTION

Iron is an essential trace element which plays a crucial role in many cellular processes, e.g., oxygen transport, energy generation, DNA synthesis, etc. This transition metal acts as a cofactor in the active center of many enzymes involved in fundamental biochemical pathways [1]. Although its biological importance the excess of iron has pathological consequences which are mainly due to the fact that it easily participates in oxidation-reduction processes implicated in free radical generation like Haber-Weiss and Fenton reactions [2]. The produced hydroxyl radicals are capable to initiate chemical reactions with the main molecular components resulting in their oxidative damage and cell death [3]. Beside this, free iron is able to interact with unsaturated lipid molecules which results in peroxy and alkoxy radical production.

In long-term the increased generation of reactive oxygen species associated with disorders which pathophysiology relates to elevated iron levels such

as β -thalassemia, coronary heart disease, neurodegenerative disease, etc., is accompanied by helplessness of the organism to maintain the vital cellular oxidation/reduction status [4]. This relates with disturbance of the protection of the living organisms against pathological deviation of this parameter. The redox homeostasis is troubled due to the impossibility of the enzymatic, non - enzymatic and daily consumed organic, inorganic and natural compounds enhancing food nutritional qualities to prevent free radical oxidative damage [5, 6].

The dietary recommendations for those patients and the restriction for supplementation with essential antioxidants have led to the necessity of developing novel high-efficiency pharmacologically active substances with multipotent antioxidant properties [7]. The numerous performed investigations indicate that particular attention has been paid to the chelating agents and the possibility to develop on the base on their chemical structure pharmacologically active molecules possessing antioxidant properties [8, 9].

The aim of the present investigation is to estimate the protection effect of three novel

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derivatives of the active chelator salicylaldehyde benzoylhydrazone (SBH) against the damaging effect of different types of ROS and iron-induced oxidation of biologically important molecules. The investigated compounds - 4-methoxysalicylaldehyde benzoylhydrazone (4mSBH), 4-methoxysalicylaldehyde-4-hydroxybenzoylhydrazone (4mShBH) and 4-methoxysalicylaldehyde isonicotinoylhydrazone (4mSIH) have been synthesized and structurally characterized in the Faculty of Pharmacy of the Medical University of Sofia [10].

Our scientific interest associated with the exploration and the evaluation of the protection effect and the antioxidant properties of these SBH derivatives is due to the information available in the scientific literature proving their relatively easy technology of synthesis, chemical stability and variety of biological activities.

EXPERIMENTAL

Chemiluminescent model systems

The evaluation of the chemiluminescent response was done using LKB 1251 luminometer (BioOrbit, Turku, Finland) provided with an automatic injector. The apparatus was connected via AT-serial interface with an AT-type computer and the MultiUse program ver. 10.8 (BioOrbit, Turku, Finland) was used for the collection and the processing of the experimental data. In both used assays the chemiluminescent response was measured using the "flash assay" option of the software and calculated by determination of the area under the chemiluminescent curve. To evaluate the scavenging properties we have used the chemiluminescent scavenging index (CL-SI) – the CL ratio in the presence and absence of the investigated 4-methoxy hydrazone in percentage. Both used chemiluminescent model systems have proven themselves as reliable in our previous experiments concerning the evaluation of the effect of hydrazones with similar structure [11].

Luminol-dependent chemiluminescence in a system of iron-dependent hydroxyl radical formation - The experiment was performed using 1 ml samples of phosphate saline buffer (PBS), containing 0.1 mmol/L luminol, 0.1 mmol/L Fe³⁺, 0.1 mmol/L EDTA, 0.1 mmol/L ascorbate, 1 mmol/L H₂O₂ and the tested compound. In the control samples, the studied compounds were omitted. The chemiluminescence was registered for 1 min. every 50 milliseconds after the addition of hydrogen peroxide.

Luminol-dependent chemiluminescence in a system of KO₂ produced O₂^{•-} - One milliliter samples of 50 mmol/L K₂HPO₄/KH₂PO₄ buffer, pH

7.4, containing 0.1 mmol/L luminol and the tested 4-methoxy SBH derivatives were prepared. Due to the fast release of superoxide, the CL response was measured instantly after the addition of 20 µl of KO₂ dissolved in DMSO. The chemiluminescence was registered for 1 min. every 50 milliseconds.

Spectrophotometric model systems

Protection effect in a lipid containing model system – a colorimetric method was used for the quantitative determination of the thiobarbituric acid reactive substances. The degree of the oxidative molecular damage was presented as percentage of the untreated control. The experiment of iron-induced peroxidation was performed in model systems of egg yolk homogenate. The concentration of the oxidisable substrate was 1 mg/ml. The initiation of the lipid peroxidation was achieved by the addition of 0.1 mmol/L FeCl₂. All samples were incubated for 30 min at 37°C, which was followed by the addition of 0.5 ml of 2.8% trichloroacetic acid and 0.5 ml of 1% thiobarbituric acid. Second 20 min incubation at 100°C was performed and the absorbance was measured at 532 nm.

UV-induced deoxyribose damage – the experiment was performed according to Halliwell *et al.* [12], with some modifications. The investigated hydrazones and the 2' deoxyribose [0.6 mmol/L] were diluted in 50 mmol/L K₂HPO₄/KH₂PO₄, pH 7.4. Control samples in which hydrazones were omitted were prepared. All probes were UV irradiated for 30 min (UV 220-400 nm). To 1 ml of each irradiated sample solution were added 0.6 ml of 1% trichloroacetic acid and 0.6 ml of 0.6% thiobarbituric acid. The obtained mixture was heated for 20 min at 100°C and the absorbance of the samples was measured at 532 nm. Results were presented as percentage of the untreated control and reflect the "damage of 2-deoxyribose"

Determination of C₅₀ values – the concentration of the investigated derivatives decreasing by 50% the chemiluminescent response was named C₅₀. The methodology of determination requires fitting of the obtained experimental data to the "sigmoidal model". Using the same formula we have determined the hydrazone concentration providing 50% AOA in the spectrophotometric model systems.

RESULTS AND DISCUSSION

For the chemiluminescent evaluation of the effect of the 4-methoxy derivatives as inhibitors and/or scavengers we have chosen a model system containing the superoxide anion radical and an assay of hydroxyl radical generation via Fenton

reaction. Choosing these model systems we wanted to provide information concerning: (i) the opportunity the studied by us compounds to possess the capability of tackling the formation of one of the most reactive and potent ROS - the hydroxyl radical. One of its major targets is the DNA molecule and the possible resulting oxidative damages are associated with structural changes of its sugar (deoxyribose); (ii) the capability of the tested hydrazones to reduce the formation of the superoxide anion radical which is the basic step responsible for a cascade of reactions initiating the production of other ROS, which subsequently interact and prompt oxidative damages in biologically important molecules and disturb their functionality.

The evaluation of the radical scavenging activity against $O_2^{\bullet-}$ has been performed in the concentration range from 3 to 100 $\mu\text{mol/L}$. The compounds have demonstrated diverse degree of activity and this effect is more prominent with the increase of the hydrazone concentration in the samples. Comparing these results with previously performed experiments concerning the evaluation of the CL-SI index of the initial compound SBH we have seen that the 4-methoxy derivatives demonstrate better scavenging activity – $\text{CL-SI}\%_{\text{SBH}} = 74.09\%$ at 100 $\mu\text{mol/L}$ [13]. The obtained lower values of the CL-SI index for the samples containing the 4-methoxy derivatives compared to that of the initial compound SBH, proved the favorable effect of the structural modification associated with incorporation of methoxy group at 4th position in the aldehyde part of the molecule on the superoxide anion scavenging activity in this system. 4mSBH demonstrated the lowest inhibition effect from the 4-methoxy derivatives – corresponding to the highest values of the CL-SI% index. The incorporation of hydroxyl group (4mShBH) and substitution with hetero atom (4mSIH) had beneficial effect on the studied properties. The observed diminishment at the highest tested concentration of 100 $\mu\text{mol/L}$ of the CL-SI is 55.53 % (for 4mShBH) and 74.25 % (for 4mSIH) from that of 4mSBH, which denotes the additional favorable effect of these subsequent structural changes on $O_2^{\bullet-}$ scavenging activity. The investigation of the ability of the studied 4-methoxy derivatives to suppress the chemiluminescent response in the hydroxyl radical generating model system indicated that at the lowest tested concentration none of the compounds induced statistically significant diminishment of the CL-SI index compared to the untreated control.

The subsequent increases of the concentration of the hydrazones in the sample induced concentration

dependent inhibition effect on the chemiluminescent response. This correlation is less expressed for the SBH ($\text{CL-SI}\%_{\text{SBH}} = 62.77\%$) [13].

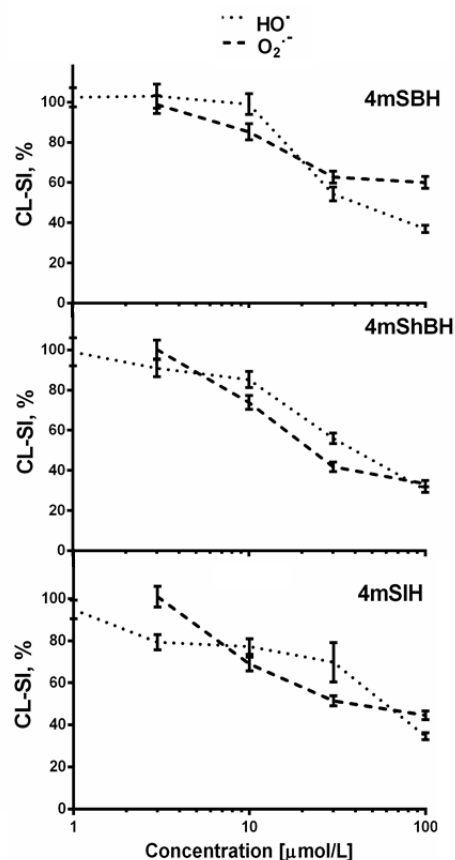


Figure 1. 4-methoxy aroylhydrazones derivatives induced diminishment of the chemiluminescent scavenging index in: (HO^\bullet) – system of iron-dependent hydroxyl radical formation; ($\text{O}_2^{\bullet-}$) – system of KO_2 produced superoxide.

Compared to the initial compound the 4-methoxy derivatives demonstrate higher degree of inhibition of the chemiluminescent lightening. At the highest tested concentration 100 $\mu\text{mol/L}$ the observed effects of 4-methoxy derivatives are similar but statistically different and the CL-SI values are approximately 1/3 of that of the untreated control.

To make possible the comparison of the effect of the investigated compounds we have calculated the hydrazone concentration inducing 50% inhibition of the chemiluminescent scavenging index (C_{50}) using the data presented in Figure 1. The results are shown in Figure 2. The C_{50} values for SBH were calculated from previously performed experiments [13]. In the $\text{O}_2^{\bullet-}$ containing model system the 4-methoxy derivatives exhibited different extent of radical scavenging activity. 4mSBH had the lowest inhibitory effect among the investigated compounds and its C_{50} value was estimated using extrapolation.

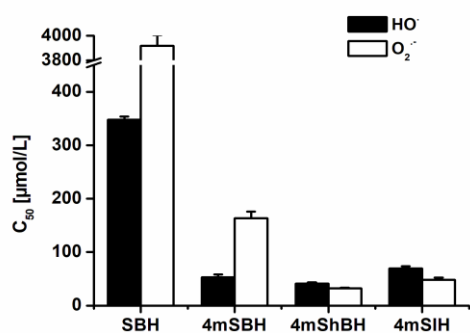


Figure 2. Comparison between the concentrations of hydrazones leading to 50% reduction of the chemiluminescent response (C_{50}) in the chemiluminescent model system containing hydroxyl and superoxide radicals.

The subsequent structural modifications of this compound including incorporation of hydroxyl group (4mShBH) or substitution with hetero atom (4mSIH) reveal the positive effect of the structural changes – which can be seen from the decrease of the C_{50} values. They are respectively 1/5 for 4mShBH and 1/3 for 4mSIH from the C_{50} value of 4mSBH.

The 4-methoxy derivatives express alike behavior in the hydroxyl radical containing model system. The C_{50} values are in the concentration interval from 41.09 to 69.28 $\mu\text{mol/L}$. The incorporation of hydroxyl group in the hydrazide part of the molecule of 4mSBH increases the investigated in this system properties C_{50} $_{4\text{mShBH}} = 41.09$ $\mu\text{mol/L}$, whereas the presence of hetero atom is associated with a slight decrease of the activity.

Figure 2 reveals the comparable behavior of 4mShBH and 4mSIH in the chemiluminescent model systems. Both of them presented themselves as potent inhibitors of the luminol-dependent chemiluminescence created by the ROS generated in the systems, which is a measure for their scavenger activity. The obtained values of C_{50} indicate that the observed effect is more pronounced in the $\text{O}_2^{\bullet-}$ containing model system compared to the one where iron dependent hydroxyl radical formation is expected. The lower C_{50} values of these two compounds suggest that they possess the potential to influence the free radical processes at the initial step of the cascade of reactions, i.e. the $\text{O}_2^{\bullet-}$ generation.

The studied hydrazones display protection effect in both spectrophotometric systems despite the different mechanisms of induction of free radical damage and the use of different oxidisable substrates.

In the system of Fe-induced lipid peroxidation the 4-methoxy derivatives expressed concentration dependent protection of the egg yolk homogenate

(Table 1). With the increase of the hydrazone concentration in the samples the percentage of the oxidized molecules decreases. This effect is more pronounced in the samples containing 4mSBH where we have observed a five-fold decrease of the damages when the concentration of the compound was changed from 80 $\mu\text{mol/L}$ to 160 $\mu\text{mol/L}$. For 4mShBH this decrease is 1.8-fold and for 4mSIH approximately 1.2-fold. Again at the lowest tested concentration of 80 $\mu\text{mol/L}$, 4mShBH demonstrated the highest protection activity.

Table 1. Degree of damage in percentage of the studied 4-methoxy SBH derivatives during iron- induced peroxidation in egg yolk homogenate containing model system. Control damage value was 100 %.

Compound	Hydrazone concentration 80 [$\mu\text{mol/L}$]	Hydrazone concentration 160 [$\mu\text{mol/L}$]
4mSBH	49.115 \pm 3.97	9.786 \pm 2.906
4mShBH	34.637 \pm 2.967	19.367 \pm 4.945
4mSIH	41.516 \pm 5.363	35.547 \pm 2.005

In order to explain more accurately the observed antioxidant effect of the investigated compounds and their interaction with ROS we have evaluated their ability to prevent free radical damage in iron-free spectrophotometric systems where we have used UV-irradiation to generate free radical damage. The method of estimation of ROS generation includes the quantitative determination of the 2'-deoxyribose oxidative product.

The results presented in Figure 3 reveal that the 4-methoxy derivatives possess notable activity in protecting the 2'-deoxyribose molecules. The degree of the oxidative 2-deoxyribose molecules damage was presented as percentage of the control. Their effectiveness was studied in the concentration interval from 0 to 100 $\mu\text{mol/L}$ and at the highest tested concentration all of them decreased the degree of the damaged molecules by more than 60 %.

Comparing the observed effect with the antioxidant efficacy of classical hydroxyl radical scavengers (DMSO – 43.13% \pm 0.61) and antioxidants (Trolox 34.42% \pm 0.38) at a concentration of 100 $\mu\text{mol/L}$ we can conclude that they possess equivalent concentration range of effectiveness in the studied aqueous solutions.

To make a suggestion about the type of the revealed antioxidant effect we have compared the C_{50} values from two model systems where we have used different mechanism for registration of the evaluated properties and for the ROS generation – the chemiluminescent assay of iron-dependent hydroxyl radical formation and the spectrophotomet-

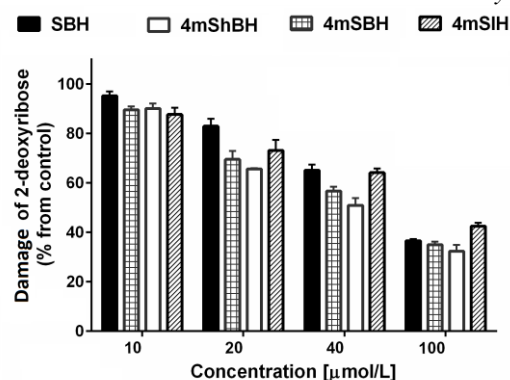


Figure 3. Effect of the chelator SBH and its 4-methoxy derivatives on the UV induced “damage of 2-deoxyribose”.

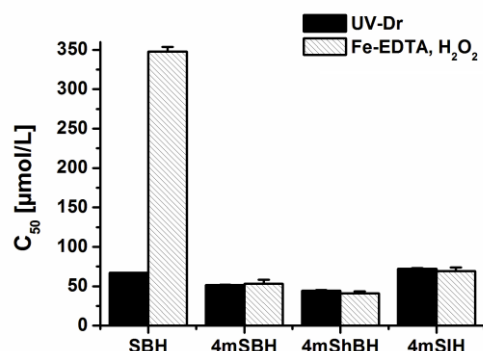


Figure 4. Comparison between the C₅₀ values obtained from the chemiluminescent system of Fe-induced hydroxyl radical formation and from the spectrophotometric assay of UV induced 2-deoxyribose damage.

ric system of UV induced deoxyribose damage (Figure 4). For these experiments we have used as reference compound salicylaldehyde benzoylhydrazone SBH with proved chelation activity.

The obtained results for the C₅₀ concentration in both model systems indicate that the observed effect of the 4-methoxy derivatives in both of them is due to direct antioxidant activity using a similar mechanism of action - different from the Fe-chelation. These data are in accordance with previously published by us results concerning the antioxidant effect of 3-methoxy derivatives of SBH [11]. They exclude the possibility of quenching effect which could be one possible reason for diminishment of the chemiluminescent response and support our statement that the chemiluminescent methods are suitable for the evaluation of the antioxidant properties of this group of compounds.

CONCLUSION

The performed by us experiment indicates that all the studied derivatives of SBH possess radical scavenging activity against OH• and O₂•⁻ and

demonstrate a protection effect against free radical damage of biologically relevant molecules. Comparison with previously performed experiments indicates that the performed structural modification, i.e., incorporation of methoxy group at 4th position in the aldehyde part of the molecule ameliorates the scavenging properties. The extent of the observed improvement depends on the subsequent structural modifications in the hydrazide moiety. The hydroxyl bearing compound (4mShBH) demonstrated higher activity against OH• and O₂•⁻ and better protection effect in all model systems. Comparing the C₅₀ values from model systems where we have used different mechanisms for registration of the evaluated properties and for the ROS generation we suggest that the observed effect is associated with antioxidant activity different from Fe-chelation.

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4-МЕТОКСИ АРОИЛХИДРАЗОНИ – СЪЕДИНЕНИЯ, ПОНИЖАВАЩИ СТЕПЕНТА НА ОКСИДАТИВНО УВРЕЖДАНЕ НА БИОЛОГИЧНО ЗНАЧИМИ МОЛЕКУЛИ

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(Резюме)

При редица заболявания се наблюдава повишаване на плазмените нива на желязото, което води до токсични ефекти. Това се дължи на способността му да инициира свободно-радикални процеси и произтичащото от тях оксидативно клетъчно увреждане. Затова е важно разработването на фармакологично активни вещества, притежаващи наред с хелатиращите си свойства и многостранно проявяваща се антиоксидантна активност. Това би подобрило качеството на живот на пациентите, които трябва да спазват определен хранителен режим и които имат противопоказания за провеждане на съпътстваща терапия с хранителни добавки, съдържащи есенциални антиоксиданти. Настоящото проучване цели да се изследва протективния ефект на три структурно охарактеризирани структурни аналога на активния хелатор салицилалдехидбензоилхидразон (SBH). Способността на тестваните съединения 4-метоксисалицилалдехидбензоилхидразон (4mSBH), 4-метоксисалицилалдехид-4-хидрокси бензоилхидразон (4mShBH) и 4-метоксисалицилалдехидизоникотиноилхидразон (4mSIH)) да понижават степента на оксидативно увреждане на биологично релевантни молекули беше изследвана *in vitro* спектрофотометрични моделни системи с различен механизъм на инициране на оксидативното увреждане, съдържащи дезоксирибоза и жълтъчен хомогенат. Бяха проведени съпътстващи експерименти в хемилуминесцентни системи, доказващи радикалулващи свойства спрямо различни активни форми на кислорода. Получените резултати показват, че заместването с метокси група в алдехидното ядро на изходното съединение SBH подобрява изследваните в тези системи свойства. Степента на подобряване на протективния ефект зависи и от последващите структурни изменения в хидразидната част на молекулата. Оксидативното увреждане на биологично важните молекули се понижава най-силно от хидроксил съдържащия структурен аналог (4mShBH). Това съединение показва и най-силно изразена активност спрямо OH^\bullet и $\text{O}_2^{\bullet-}$ в хемилуминесцентните системи. Сравняването на изчислените C_{50} стойности показва че наблюдаваният ефект на понижение на степента на оксидативно увреждане е свързан с директна антиоксидантна активност, а не с хелатиращи свойства.