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Suppressive effect of salicylaldehyde benzoylhydrazone derivatives on ferrous iron-induced oxidative molecular damage – evaluation of the structure-protection activity relationship *via* Raman spectral analysis

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In the present research are included the iron chelator salicylaldehyde benzoylhydrazone (SBH) and two methoxy bearing derivatives - (3-methoxysalicylaldehyde benzoylhydrazone 3mSBH and 4-methoxysalicylaldehyde benzoylhydrazone 4mSBH). The goal was to correlate the experimental data concerning the vibrational characteristics (Raman spectra) of the tested hydrazone with its antioxidant potency in ferrous iron-induced oxidative damage model systems and others. The suppressive effect of the compounds on Fe-induced oxidative damage using as oxidisable substrates lecithin, egg yolk homogenate and deoxyribose was investigated and their potency to protect the deoxyribose molecules from UV-induced damage were tested. All the compounds demonstrated protection effect in the system of Fe-induced molecular damage. The extent of the witnessed effect is depending on the used oxidisable substrate and the position of the structural modification in the lead compound. All hydrazones have demonstrated better effectiveness in the more biologically plausible egg yolk lipoprotein containing model system compared to the lecithin containing one. The hydrazones proved that they can significantly decrease not only the iron-induced damage of deoxyribose but also reduce the "% molecular damage" of this oxidisable substrate upon experimental conditions of UV-irradiation. The calculated coefficient of correlation denoted R^2 ranges from 0.877 (lecithin system) to 0.98 (Fe-induced oxidative damage of deoxyribose) when seeking for relationship with the displacement of the peak around 1160 cm^{-1}, where are expected ν(Φ-OH) and δ(O-H) vibrations. Only the C-50 values from the iron free deoxyribose model system had R^2 higher than 0.5 (0.997) with a shift to higher frequencies of the band around 1290 cm^{-1}, which is corresponding to the vibrations of the C-O in the phenol nucleus.

Keywords: Fe-induced oxidative damage, hydrazones, antioxidant activity, SAR, Raman spectrum

INTRODUCTION

Rational drug design is a continuous process which comprises several multidisciplinary approaches. Each year the medical chemists synthesize hundreds of series of biologically active molecules possessing desired pharmacological effects. The process of ranking the candidate compounds and disclosing the optimal structural parameters proving optimized biological effects, increased selectivity, and lessened toxicity requires significant capital and technical resources [1, 2].

The evaluation of the structure – activity relationship (SAR) is a primary tool optimizing the early points of the process of drug development and discovery. One used approach is to investigate the vibrational characteristics of the compounds which are molecular specific and determined by the compounds chemical structure. The obtained information by studying the infrared and the Raman spectra help optimizing the structure [3].

One of the possible ways of researching the relationship between structure and effects is studying separately the vibrational spectra and the behaviour in biologically relevant systems [3]. Then the possible correlation between the changes in the vibrational energy of the molecules and their biological activities can be investigated.

The performed experiments with different groups of organic compounds including hydrazones denote that different biologically significant properties are associated with different structural modifications and the extent of the observed effect is determined by the type and the position of the substituent in the molecules of the newly synthesized compounds [4, 5].

Hydrazones are a group of organic compounds known with their exceptionally wide pharmaceutical activities. In the literature data have been reported numerous experiments proving their antimicrobial, analgesic, anti-inflammatory, antioxidant and anticancer activity [5]. As a result of the increased scientific interest the parallel synthesis of a series (structural derivatives of lead biologically active hydrazones) expanded significantly the diversity of the compounds available for study as new drugs [6, 7].

A key point in the overall process of

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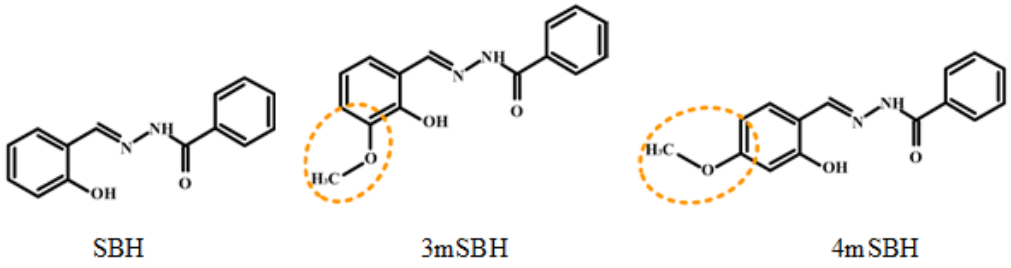
investigation of the capability of new designed compounds to become clinically effective drugs is the pre-clinical estimation of their possible influence on free radical processes in the human body using *in vitro* and *in vivo* biologically relevant model systems. The collected experimental data are used to evaluate the possible therapeutic value of new designed compounds in the treatment of pathological conditions with increased generation of free radicals and change of the viable for the living organisms antioxidant balance like cancer, inflammatory and neurodegenerative disease.

The goal of the present investigation was to correlate the experimental data concerning the vibrational characteristics (Raman spectra) of methoxy derivatives of the iron chelator salicylaldehyde benzoylhydrazone (SBH) with their suppressive effect on ferrous iron-induced oxidative damage of biologically important molecules and their properties to scavenge free radicals. There are multiple pathophysiological conditions that are associated with disturbance of iron homeostasis and tissue iron accumulation – cancer, cardiovascular and neurodegenerative disease [8, 9]. The multiple side effects and described adverse reactions of the available at the moment pharmaceutical agents used to perform long-term treatment and prevention of iron-mediated toxicity of patients with these conditions have implicated the need of development of new improved oral remedies.

In the research are included the initial compound, the iron chelator SBH and two methoxy bearing compounds (3-methoxysalicylaldehyde benzoylhydrazone 3mSBH and 4-methoxysalicylaldehyde benzoylhydrazone 4mSBH) – Scheme 1. All compounds were synthesized and structurally characterized by a research group from the Faculty of Pharmacy of the Medical University of Sofia. Anticancer drug screen was performed using human tumor cell lines in order to provide their cytotoxic effects and their ability to act as chelating agents coordinating different metal ions are estimated in order to determine compounds' capability for reduction of their toxic effects [10-12]. In the present investigation we aimed to evaluate the suppressive effect of these three aroylhydrazones on ferrous iron-induced oxidative molecular damage using as oxidisable substrates lecithin, egg yolk homogenate and deoxyribose. Their capability to protect the deoxyribose molecules from UV-induced oxidative damage was tested. Furthermore, readings of the Raman spectra of the three compounds in solid phase were taken and evaluation of the structure-protection activity relationship was performed.



Scheme 1. Chemical structure of the investigated hydrazones.

EXPERIMENTAL

Raman spectroscopy

Raman spectroscopy is an efficient, fast and non-destructive technique. The method is based on the measurement of the oscillations of the atoms in the studied molecules and provides information about the molecular vibrational energy levels which are related to the molecular structure, its conformational characteristics, and intermolecular interactions. Due to this fact it has proven itself as a useful screening method for structural characterization of new-designed compounds.

The Raman spectra of the investigated hydrazones were recorded using micro-Raman spectrophotometer Lab RAM HR 800. The probe excitation was achieved at room temperature using the 632 nm line of an argon ion laser. The spectra were collected with Raman microscope equipment with 50× objective resolution 1 cm^{-1}.

Antioxidant potency

Iron-induced lipid peroxidation (LP) – the determination of the level of Fe-induced LP was performed using systems containing egg yolk homogenate or lecithin [13, 14]. Hydrazones with different concentration were added to 1 ml of homogenate (diluted v/v 1:100). The reaction was initiated with 50 μL of $FeCl_2$ with final concentration of 1 mmol/L. Samples were incubated for 30 min at 37°C. Then to each sample was added 0.5 ml of 2.8% trichloroacetic acid solution and 0.5 ml of 1% thiobarbituric acid solution (TBA). After 20 min incubation at 100°C

the samples were centrifuged and the absorbance of the supernatant was measured at 532 nm. The obtained results are presented as percentage of the control sample.

Iron-induced deoxyribose oxidative damage – the assay was carried out using one mL samples of phosphate saline buffer (PBS) – 50 mM K_2HPO_4/KH_2PO_4, pH 7.4, containing 0.3 mM of 2-deoxy-D-ribose, 0.5 mmol/L of H_2O_2, 50 μmol/L of ascorbate, 50 μmol/L of Fe(III) and 52 μmol/L of EDTA [15]. The extent of deoxyribose oxidative degradation by the produced hydroxyl radicals was measured using TBA test. After the addition of the tested hydrazones at the investigated concentration the experimental procedure follows the same protocol used for determination of the TBARS products in the lipid containing model systems. The obtained results are presented as percentage of the control samples.

UV-induced deoxyribose damage – the deoxyribose assay was performed as described by Halliwell *et al*., with small modifications [15]. The tested hydrazone derivatives and 0.6 mmol/L of 2-deoxy-D-ribose were added in phosphate buffer. In the control sample, hydrazones were omitted. After 30 min of balanced UV irradiation (UV 220 – 400), 0.6 ml of 1% TCA and 0.6 ml of 0.6% of thiobarbituric acid were added to 1 ml of the irradiated sample solution. Again the experimental procedure follows the same protocol used for determination of the TBARS products in the lipid containing model systems.

RESULTS AND DISCUSSION

Nowadays, from a scientific perspective, the *in vitro* model systems comprising evaluation of protection effect against different mechanisms of molecular damage of biologically important molecules enjoy increasing popularity as first-line experiments in the novel drugs discovery process. Due to this fact the first step of our investigation comprises evaluation of the effect of the tested hydrazones against oxidative damage in model systems containing lecithin, egg yolk homogenate and deoxyribose molecules. The used model systems have proven their applicability in determining the protection effect of aroyl hydrazones in our previous investigations. [16].

In both lipid-containing model systems the initial compound and its methoxy derivatives have demonstrated a protection effect. This is evident from the diminishment of both - the absorbance and the subsequently calculated by its values "degree of molecular damage" for the hydrazone containing samples compared to the control probes where hydrazones were omitted and maximal molecular damage is being observed. The extent of the witnessed protection from Fe-induced oxidative damage is depending from the used oxidisable substrate – all compounds have demonstrated better effectiveness in diminishment of the generation of TBA-RS products in the egg yolk lipoprotein containing a model system.

In the lecithin-containing samples the basic compound SBH has decreased the "degree of molecular damage" to 71% compared to the control samples. The compounds possessing modification associated with incorporation of methoxy group at 3- and 4- position in the aldehyde part of the molecule also possess capability to diminish the generation of TBA-RS products but the "degree of molecular damage" is the same (4mSBH) or slightly elevated (3mSBH) compared with this of SBH.

In the egg yolk homogenate the SBH chelator has diminished the "degree of molecular damage" with approximately 60% compared to the control samples – the diminishment is approximately 30% more compared to the lecithin model system. The additional modifications, i.e. incorporation of methoxy groups in the aldehyde part of the molecule at third and fourth position, ameliorates the denoted by the initial compound protection effect and the "degree of molecular damage" is lessened to one/third compared to the controls.

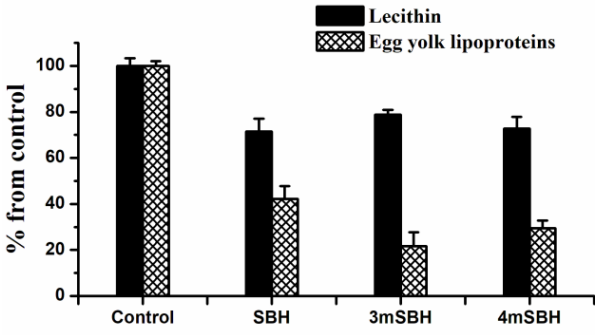


Figure 1. Degree of molecular damage in percentage during iron-induced peroxidation in lipid containing model systems in presence of the initial compound SBH and its 3- and 4-methoxy derivatives at a concentration of 120 μmol/L.

The experiments on the evaluation of the capability of the tested hydrazones to decrease the deoxyribose molecular damage have also proven protection of the oxidisable substrate. The tests were performed in alternative model systems – in the first one we have used Fe (II) to induce oxidative damage and in the second - UV irradiation. In both systems we observed a statistically significant decrease of the degree of molecular damage at the lowest tested concentration in all samples containing hydrazones.

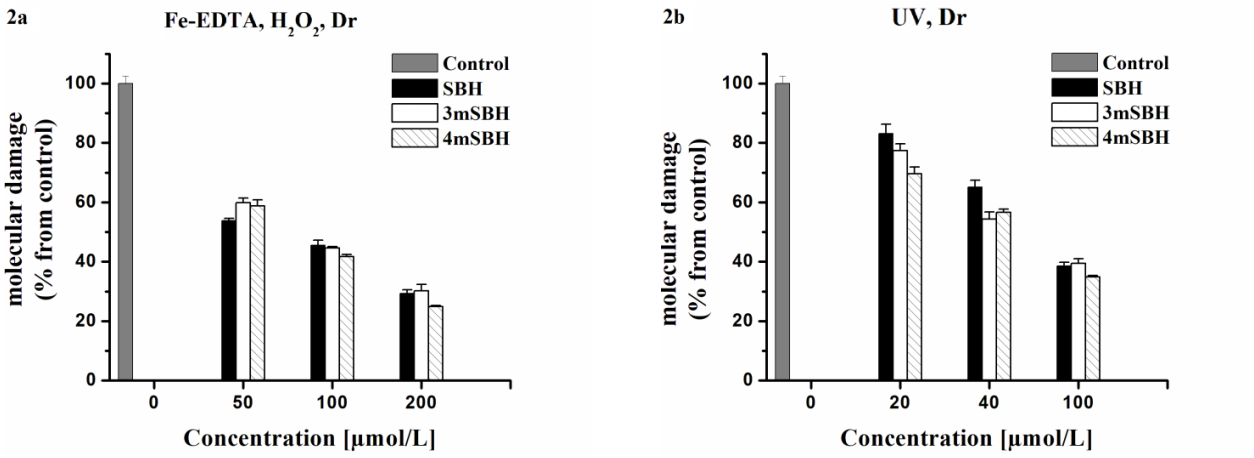


Figure 2. Effect of the chelator SBH and its 3- and 4-methoxy derivatives in spectrophotometric model systems with different mechanism of initiation of deoxyribose oxidative damage, respectively – 2a. Ferrous iron-induced oxidative molecular damage and 2b UV – induced deoxyribose damage.

Table 1. Comparison between the concentrations of hydrazones leading to 50% decrease of the deoxyribose molecular damage in the model system of iron-induced deoxyribose oxidative damage and UV initiated deoxyribose oxidative damage

Compound	C-50 [µmol/L] UV, Dr	C-50 [µmol/L] Fe-EDTA, H_2O_2, Dr
SBH	67.06 ± 0.06	66.88 ± 2.26
3mSBH	58.37 ± 2.36	78.81 ± 0.02
4mSBH	51.23 ± 0.22	71.38 ± 0.02

The observed effect of decrease of deoxyribose molecular damage is concentration-dependent – increasing the hydrazone concentration decreases the sample absorbance and respectively the molecular damage. In order to compare the influence of the structural modifications in the aldehyde part of the molecule on the properties evaluated in each system and to perform comparative evaluation of the influence of each hydrazone on the deoxyribose molecule oxidative damage in both systems we have calculated the C-50 values - hydrazone concentration inducing 50% inhibition of the % molecular damage using the data presented in Figure 2. The presented in Table 1 C-50 values denote that there is no statistically significant difference in the concentration which is needed to decrease with 50% the deoxyribose molecular damage for SBH. The structural modifications associated with incorporation of methoxy group have ameliorated the protection effect in the system where we have used UV induced deoxyribose damage (lower C-50 values compared to SBH) and slightly decreased the initial compound protection effect in the system of Fe-induced oxidative damage (elevated C-50 values compared to SBH). In both tested model systems the 4-methoxy bearing compound demonstrated better ability to protect the deoxyribose molecules compared to the 3-methoxy derivative.

The Raman spectra of the substances, taken from samples in a solid phase at room temperature, are shown in Figure 3. The most intense bands were observed about 1600 cm^{-1}, the less intense ones are about 1300 cm^{-1} and 1160 cm^{-1}. Typical for all spectra is the narrow band about 1000 cm^{-1}. The latter can be interpreted as ν deformation of the carbon atoms bands in the ring plane [17]. These oscillations are active in the Raman spectrum only if there are substitutions in the benzene nucleus. The position of the peak is not affected by the type of substitution, nor by the position of the group.

According to the literature, the vibrations affecting the hydroxyl group and its bonds with the core are located around 1300 cm^{-1} [18], varying between different sources – 1025 cm^{-1} [19], 1170 cm^{-1} [20], ν(C-O) – 1290 cm^{-1} [21]. The vibrations associated with bending deformations are in the range 1100-1400 cm^{-1} [20]; Phenol-O stretching is about 1200 cm^{-1} [22].

We found that addition of a methoxy group results in a shift of the peak around 1160 cm^{-1} (SBH), where oscillations ν(Φ-OH) and δ(O-H) are expected and the one at 1290 cm^{-1}, where oscillation ν(C-O) is expected.

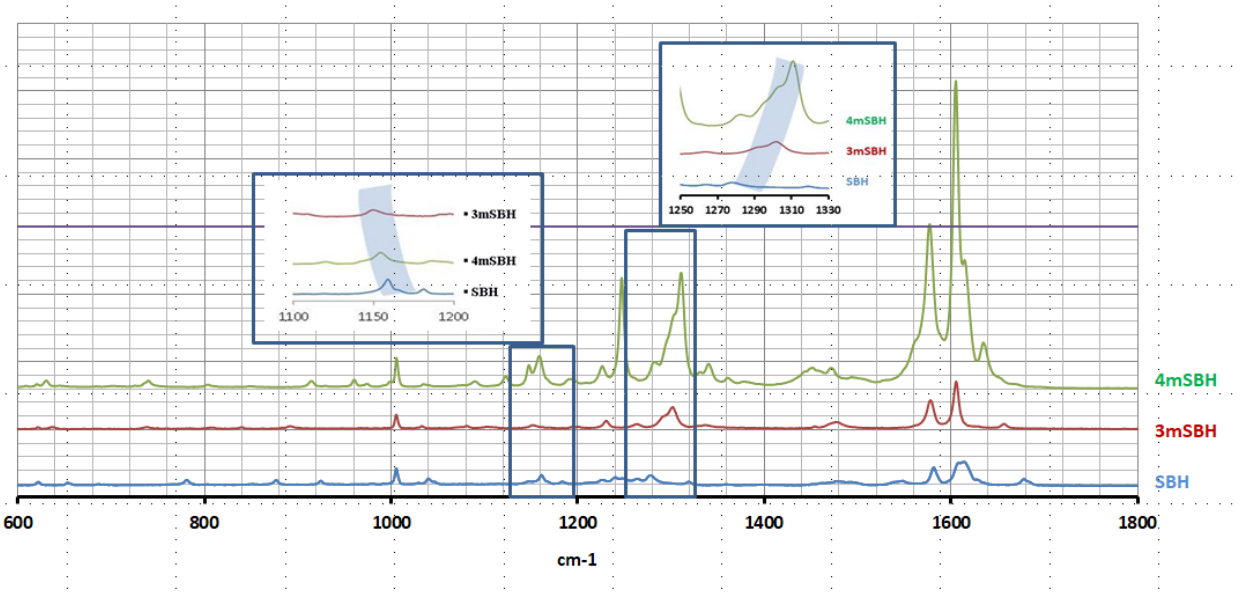


Figure 3. Raman spectra of the solid-state investigated hydrazones – excitation at 632 nm. The labeled bands illustrate the shift of the peaks in the frequency regions 1100-1200 and 1250-1350 cm^{-1}.

The Raman spectra of the tested substances were searched for frequency areas which could correlate with AOA. Since the AOA of substances with cyclic structure is determined by the presence and amount of hydroxyl groups, our attention was focused in the area where vibrations related to the OH group occur.

We evaluated the degree of correlation between the denoted antioxidant potential in the studied spectrophotometric model system (3 with iron induced oxidative damage and one lacking iron – the system with UV irradiation) and the Raman spectra of the compounds in the area of the mentioned vibrations. For this purpose we used the "molecular damage" for the lipid containing systems and C-50 values for the deoxyribose containing ones. The obtained data denoted linear correlation between the observed antioxidant potency in the systems and the shift of the peaks located around 1290 cm^{-1} and 1160 cm^{-1} when changing position with a constant step of cm^{-1}.

The calculated coefficient of correlation denoted R^2 is ranging from 0.877 (lecithin system) to 0.98 (Fe-induced oxidative damage of deoxyribose) when seeking for relationship with the displacement of the peak around 1160 cm^{-1}, where stretching ν(Φ-OH) and bending δ(O-H) vibrations are expected. Only the C-50 values from the iron-free deoxyribose model system had R^2 higher than 0.5 (0.997) with the shift to higher frequencies of the band around 1290 cm^{-1}, which is corresponding to the vibrations of the C-O in the phenol nucleus. No correlation was observed between the observed protection effect in this system and the shift of the peak around 1160 cm^{-1} toward lower frequencies.

CONCLUSIONS

In conclusion we can assume that the compounds demonstrated a protective effect in the system and have the capability to diminish molecular damage of biologically important molecules. The extent of the witnessed effect is depending on the used oxidisable substrate, the used methods for initiation of the oxidative damage and the position of the structural modification in the lead compound. The analysis of the Raman spectra of the investigated compounds revealed that the incorporation of a methoxy group leads to a displacement of the peak at 1160 cm^{-1}, which, according to literature data, is assigned to ν(Φ-OH) and δ(O-H) vibrations. A linear dependence was established between the position of the spectrum bands and the magnitude of the observed antioxidant potency of the compounds.

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