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In vitro protective and radical scavenging effect of bromo-salicylaldehyde aroylhydrazones

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The possible applications of aroylhydrazones as bioactive compounds have been widely investigated in the past decades. The performed experiments have proven extensive variety in their biological activities. Two bromo derivatives of the active chelator salicylaldehyde benzoylhydrazone (SBH), in particular 5-bromosalicylaldehyde-4-hydroxybenzoylhydrazone (5BrShBH) and 5- bromosalicylaldehyde isonicotinoylhydrazone (5BrSIH), were included in the investigation. The capability of these compounds to decrease the radical concentration in model systems containing stable free radicals (ABTS and DPPH) was estimated. Their capabilities to decrease the extent of oxidative damage of biologically important molecules in model systems with different oxidisable substrate (lecithin, egg yolk homogenate and deoxyribose) upon diverse experimental conditions of oxidative damage (Fe (II) or UV) were evaluated.

The compounds demonstrated different behavior in both stable free radicals containing systems – lack of scavenging activity against DPPH and necessary concentration to observe 50% antioxidant activity similar or lower to the one of the used reference compound Trolox. The hydrazones exhibited protection effect in all containing important molecules systems. It depended on the method for initiation of oxidative damage, the used oxidisable substrate and the position of the structural modification in the hydrazone molecule.

The observed generation of TBARS products in the egg yolk homogenate system was lower compared to the lecithin containing one – suggesting better effectiveness in the first system.

No influence of the used factor for initiation of deoxyribose damage was observed in the presence of SBH – C-50 varies between 66.88 and 67.06 μmol/L. The C-50 values of the bromo derivatives indicate stronger effectiveness against the Fe (II) induced damage.

Keywords: Hydrazones, TBARS, ABTS, deoxyribose, stable free radicals

INTRODUCTION

The development of most socially significant disease comprises several pathophysiological mechanisms which are less or more associated with free radical generation and oxidative stress processes. This has demanded the development of multi potent substances possessing several biological and pharmacological activities including antioxidant properties [1, 2]. A typical example is cancer - hundreds of drugs including chelating agents and hydrazones, are being evaluated each year in clinical trials.

Searching for new molecules possessing at the same time chelating properties and direct antioxidant activity is due to the fact that cancer cells are known with increased iron uptake and intracellular concentration and its key role as a cofactor for the ribonucleotide reductase enzyme. The last is responsible for both synthesis and repair of the DNA molecule [3]. There is also a scientific proof for increased expression in cancer cells of hepcidin which via interaction with ferroportin (export protein for nonheme iron) regulate iron transport [4, 5]. Another important fact for the combination of chelation and direct antioxidant activity is that the iron is famous with its capability to initiate free radical generation and subsequent molecular oxidative damage. The generated ROS and other peroxidation products are known to be associated with the pathophysiological mechanism of development of oncological disease via influencing signal transduction mechanisms associated with the initiation, angiogenesis and metastasis of cancer cells.

The possible applications of aroylhydrazones as bioactive compounds have been widely investigated in the past decade. Extensive variety in their biological activities have been proven – anti-inflammatory, analgesic, antimicrobial, anti-proliferative and antioxidant activity. This group of compounds is famous with easy and not expensive methodology of synthesis, hydrolytic stability, tendency to yield stereochemistry of higher coordination number and flexible molecular architecture [6].

In the years hydrazones derivatives have demonstrated also direct anti-radical properties

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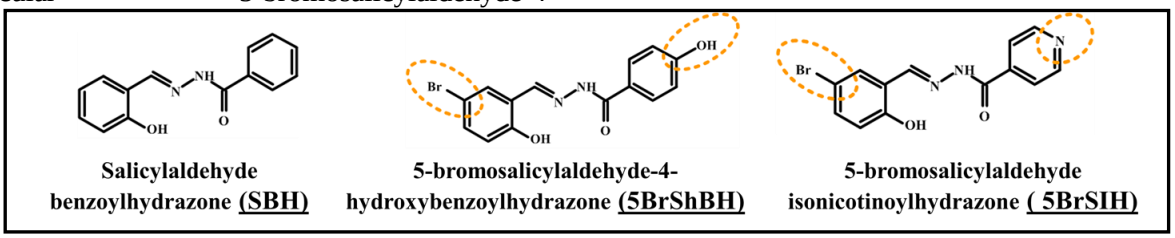
against stable free radicals [7], capability to influence peroxidation process of biologically important molecules [8,9], evaluation of their potency to influence free radical properties using NBT test, FRAP-assay and CUPRAC method have also been reported [10].

The studied by us compounds are bromo derivatives of the active chelator salicylaldehydebenzoylhydrazone (SBH) - in particular 5-bromosalicylaldehyde-4-hydroxybenzoylhydrazone (5BrShBH) and 5-bromosalicylaldehyde isonicotinoylhydrazone (5BrSIH) – structure presented at scheme 1. They are with proven cytotoxic effect in cell lines - model systems for socially significant disease (HL-60, SKW-3) [11]. All of them obey Lipinski's rule of five criteria and the calculated log P are between 1 and 3 which makes them suitable candidates for evaluation as potential new drugs [11, 12].



Scheme 1: Structure of studied compounds - SBH and its two bromo derivatives.

EXPERIMENTAL

Anti-radical properties in stable free radicals containing model systems – for this part of the experiments have been chosen spectrophotometric in vitro systems containing the stable free radicals ABTS and DPPH. Both methods are based on the decolorisation of the prepared fresh working solution of the radicals. Two groups of samples need to be prepared for each assay – samples comprising the potential antioxidant at different concentrations and control samples where the studied substances have been omitted. The observed decrease in the absorbance after 60 min incubation is proportional to the anti-radical effect of the tested compounds. The ABTS assay was performed according to Re et al., [13] - 14 mM ABTS stock solution was mixed with potassium persulfate 2.45 mM (final concentration). After the mixture was allowed to react the obtained suspension was diluted using PBS buffer until obtaining working solution with absorbance 0.70 ± 0.01 units at 734 nm. The DPPH assay was carried out as described by Groupy et al., [14] - DPPH solution in ethanol with initial absorbance of 1 at 518 nm has been prepared. The tested hydrazones at different concentrations were mixed with 2 ml from the working solution. The results have been expressed as antioxidant activity for the ABTS assay and as % from the control for the DPPH method.

Protection effect in spectrophotometric systems containing biologically important molecules – all methods have been based on the formation of TBARS products and their quantification measuring the absorbance at 532 nm. Again two groups of samples have been prepared – controls (containing all reagents except the tested compounds) and samples containing the hydrazones at different concentrations. The obtained results are presented as percentage of the untreated control which was named "% of molecular damage". The experiments in the lipid containing model systems have been performed using two alternative oxidisable substrates – lecithin and egg yolk homogenate (1 mg per mL). The peroxidation process was initiated by adding $FeCl_2$ (0.1 mmol/L – final concentration).

For the deoxyribose model systems have been used different mechanisms for molecular damage. For the iron induced assay were used 1 ml samples comprising 50 mM K_2HPO_4/KH_2PO_4, pH 7.4, containing 0.3 mM 2-deoxy-D-ribose, 0.5 mmol/L H_2O_2, 50 µmol/L ascorbate, 50 µmol/L Fe(III) and 52 µmol/L EDTA. In the case of UV induced damage the derivatives and 0.6 mmol/L 2-deoxy-D-ribose were added in phosphate buffer and 30 min of balanced UV irradiation (UV 220-400) was performed.

The next steps for all biologically relevant molecules containing systems comprise addition of 2.8% thrichloroactetic acid and of thiobarbituric acid. The mixture was vortexed vigorously, heated in 100°C water bath for 20 min and centrifuged at 3000 rpm for 20 min.

RESULTS

As a first step from our investigation we determined the capability of the tested hydrazones to decrease the concentration of stable free radicals using model systems containing ABTS and DPPH. Despite the fact that these two radicals are not presented in the living systems and on the base of the obtained results it is impossible to make

conclusions concerning the capability of the tested compounds to interrupt the chain reactions of autoxidation they are often used as preliminary screening procedures by any authors. The main advantages of both methods are that they are easy, rapid, highly reproducible and economic assays and with proven effectiveness in the evaluation of the radical scavenging activity of non-enzymatic antioxidants.

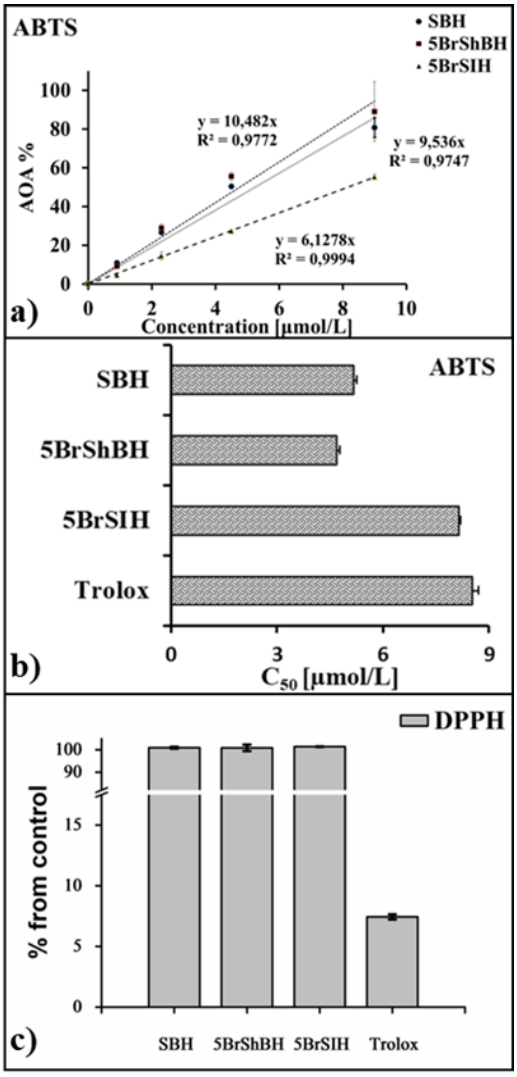


Figure 1. Anti-radical properties of salicylaldehydebenzoylhydrazone and its bromo derivatives determined using stable free radical containing model systems. The results from the ABTS assay are presented as concentration dependence of the AOA% (a) and C-50 values (b) and the experimental data from the DPPH method as "% from the controls" at concentration of the tested hydrazones 90 µmol/L (c).

The tested hydrazones have demonstrated different capability to decrease the concentration of both used stable free radicals. All tested compounds denoted capability to decrease the concentration of the ABTS radical - Figure 1a. The effectiveness of the compounds increases with the increase of their concentration in the sample solution – a linear dependence has been observed. The experiments for the hydrazones and the reference compound – Trolox were performed in the low concentration range from 0 to 9 µmol/L due to the fact that at higher concentrations full decolorisation of the radical solution has been observed. At the highest tested concentration the observed AOA % for SBH was around 80%, for the hydroxyl bearing bromine derivative around 90% and for the pyridine bearing one only 55%. On the base of the obtained results presented on figure 1a have been calculated the C-50 concentrations presented at Figure 1b. It is observed that the calculated C-50 values for SBH and the two bromine bearing derivatives are lower than the one of the reference Trolox. The hydroxyl bearing bromine derivative has the lowest C-50 value which corresponds to lowest concentration of this potential antioxidant needed to observe 50% AOA in this system.

In the DPPH model system the tested hydrazones have demonstrated different effectiveness compared to the ABTS one. Due to this fact only results for the highest tested concentration 90 µmo/L have been presented – Figure 1c. SBH has demonstrated lack of scavenging activity at the mentioned concentration. The subsequent structural modifications associated with incorporation of bromine atom at fifth position in the aldehyde part of the molecule and hydroxyl group (5BrShBH) or hetero atom (5BrSIH) in the hydrazide part didn't ameliorate the studied in the system properties.

The next step of our experiments comprises evaluation of the capability of the tested compounds to decrease the oxidative damage of biologically important molecules upon experimental conditions of ferrous iron induced oxidative molecular damage and UV irradiation.

The evaluation of the potency of newly designed compounds to influence the process of lipid peroxidation is a commonly used approach when differentiating the structural modifications characterized by ameliorated toxic profile and antioxidant activity. This is due to the simplicity of the TBARS method and the importance of the data concerning the potential capability and desire to avoid new substances which via oxidative damage could induce changes in membrane functionality, permeability, membrane potential and generation of cytotoxic products. We estimated the capability of SBH and its bromo derivatives to decrease the formation of TBARS products in two alternative lipid containing model systems. In the first one we have used as oxidisable substrate lecithin and in the second egg yolk homogenate.

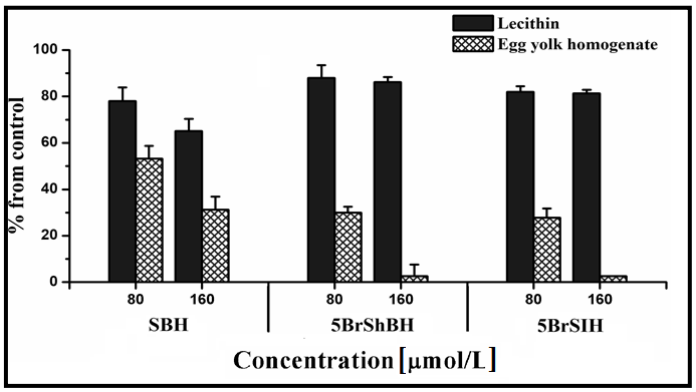


Figure 2: Protection effect of the studied bromo hydrazones in lipid (lecithin and egg yolk homogenate) containing model systems upon experimental conditions of ferrous iron induced oxidative molecular damage.

In the lecithin containing systems SBH had demonstrated concentration dependent protection effect – Figure 2. At the maximal tested concentration the observed decrease of the percentage of molecular damage compared to the controls was around 30%. The subsequent structural modifications in the aldehyde and in the hydrazide part of the molecule of SBH didn't ameliorate its' properties and were associated with lack of concentration dependence of the observed low protection effect.

In the egg yolk homogenate containing system all hydrazones demonstrated concentration dependent decrease of the TBARS products. At the maximal tested concentration SBH decreased the "% molecular damage" to less than 40% compared to the control samples. The bromo derivatives again demonstrated similar effectiveness but this time the structural modifications were associated with amelioration of the studied in the system properties. At concentration of 80 μmol/L their effect was comparable to the one of SBH at twice higher concentration and almost full inhibition of the peroxidation process was observed at the maximal tested concentration.

All the tested compounds demonstrated protection effect and capability to decrease the molecular damage in both systems. The observed effect depended from the used substrate and the structural modifications of the tested compounds. In the egg yolk homogenate containing system all hydrazones have demonstrated better effectiveness. The big difference in the observed protection using the same method for initiation of peroxidation and different substrate suppose lack of possibility the observed results to be due only to chelation properties and suppose also direct antioxidant activity.

Another frequently used target when evaluating the potential of newly designed compounds against oxidative molecular damage is the DNA molecule i.e. its deoxyribose residues. The obtained data are important in view of possible initiation of single and double strand breaks, mutations, genomic instability etc. The evaluation of the capability of the tested compounds was performed in systems using alternative mechanism of oxidative damage initiation – Fe (II) and UV irradiation (figure 3a and 3b). In the first system the decrease of the molecular damage could be explained with chelation activity and/or direct antioxidant properties and in the second – absorbance in UV region and/or direct antioxidant activity.

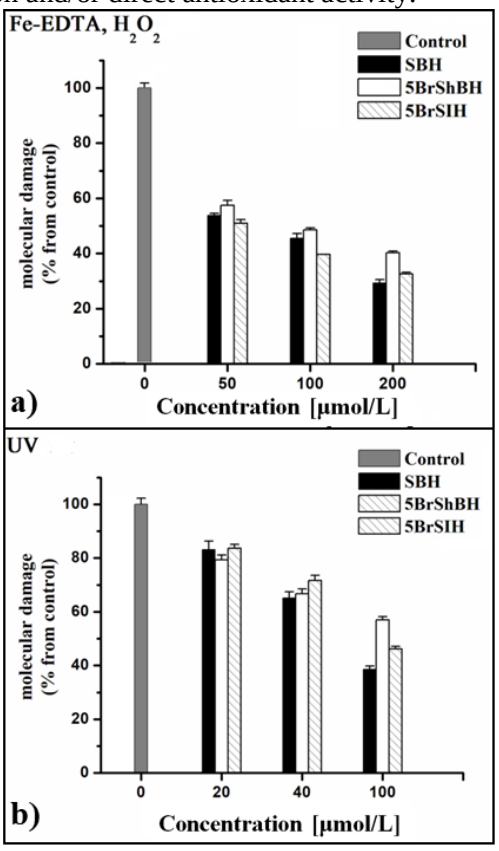


Figure 3. Concentration dependence of the protection effect of SBH and the studied bromo derivatives in deoxyribose containing model systems upon different mechanisms of induction of molecular damage (Fe (II) – a and UV irradiation b).

SBH and its bromo derivatives demonstrated capability to decrease the molecular damage in both systems at all tested concentrations. This indicates less generation of TBARS products in the hydrazones containing samples and protection effect of the tested molecules. The observed effect increased with the increase of the hydrazone concentration in the sample. In order to compare the effect of the investigated hydrazones we have calculated the concentration inducing 50% decrease of the molecular damage assuming that we are observing maximal level under the used experimental conditions in the control samples. For this purpose we used the data from Figure 3a and 3b and the results are presented at Figure 4.

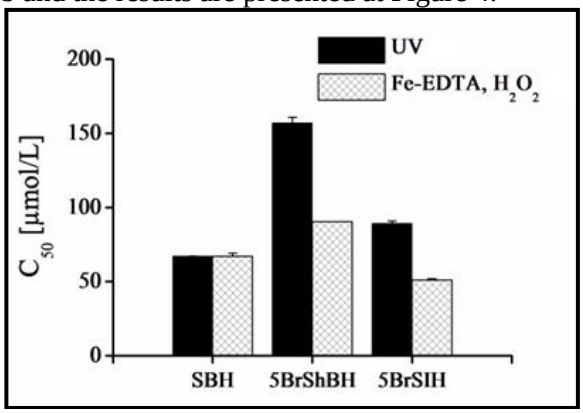


Figure 4:.Comparison between the C-50 values obtained from both spectrophotometric deoxyribose containing systems – Fe-induced hydroxyl radical formation and the assay of UV induced 2-deoxyribose damage.

From the presented at Figure 4 results is evident that there is no influence of the used factor for initiation of deoxyribose damage – ferrous iron induced or UV damage in the presence of SBH. We aren't observing any statistically significant difference between the C-50 values obtained in both systems which is suggesting similar mechanism of action. The C-50 values of the bromo derivatives indicate stronger effectiveness against the Fe(II) induced damage.

CONCLUSION

The tested hydrazones exhibited different behavior in the used stable free radicals model systems – lack of scavenging activity against DPPH and necessary concentration to obtain 50% AOA similar and even lower to the used reference Trolox. All compounds exhibited protection effect in the system containing biologically important molecules. Its extent depended on the method for initiation of the oxidative damage, the used oxidisable substrate and the type of the structural modification in the SBH molecule. The observed generation of TBARS products in the egg yolk homogenate system was lower compared to the lecithin containing one. No influence of the used factor for initiation of deoxyribose damage was observed in the presence of SBH – lack of statistically significant difference between the C-50 values in both systems. The C-50 values of the bromo derivatives indicate stronger effectiveness against the Fe(II) induced damage.

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