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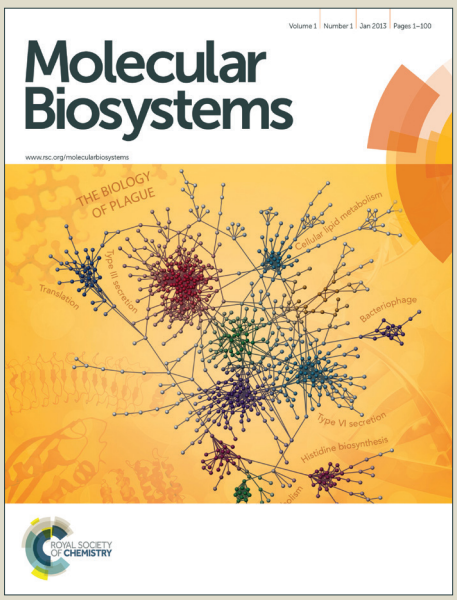

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ARTICLE TYPE

The efficiency of compounds with α-amino-β-mercapto-ethane group in protection of human serum albumin carbonylation and cross-linking with methylglyoxal

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α-oxoaldehydes, produced in higher quantities in diabetes, uremia, oxidative stress, inflammation and aging, react with proteins amino, guanidine and thiol groups and cause the formation of advanced glycated end-products and protein cross-linking. To prevent these reactions, the efficiency of low molecular mass thiols with α-amino-β-mercapto-ethane group (Cys, penicillamine and N-acetylcysteine (NAcCys, with blocked amino group)) as scavengers of methylglyoxal, compared to glutathione (GSH) and biguanidine derivative metformin, was investigated. Time courses of the mentioned compounds reactions with methylglyoxal were assayed. The reactivity of their thiol and amino groups decreased in order: Cys>penicilamine>GSH>NAcCys and penicilamine>Cys>GSH, resp. Human serum albumin (HSA) carbonylation in the absence or presence of methylglyoxal scavengers were monitored by determination of amino, guanidine and thiol groups content, as well as by spectrofluorimetry, CD and native and SDS PAGE. Cys and penicillamine were very efficient in the prevention of carbonylation of HSA-amino (for 80%) and guanidne (for 84% and 55%, resp.) groups and formation of fluorescent AGEs. GSH and metformin exhibited medium efficiency (reduction of amino group's carbonylation for 60%, and guanidine for about 30%). The least efficient was NAcCys. The presence of Cys, penicillamine and NAcCys led to almost complete protection of the HSA-thiol group carbonylation, while metformin was unefficient. The efficiency in prevention of protein crosslinking increased in order: metformin, NAcCys<GSH<penicilamin<Cys. Thus, the substances with α-amino-β-mercapto-ethane group as pharmacophore have great potential to be efficient methylglyoxal scavangers and thus are promising compounds for medicinal chemistry. In addition, they protect HSA-SH group and preserve its antioxidative potential, which is very important for HSA`s function *in vivo*.

1. Introduction

The reactions of non-enzymatic glycation lead to development of numerous pathological conditions: micro- and macrovascular complications in diabetes, Alzheimer's disease, cirrhosis, uremia, arthritis and changes in the aging process.[1-3] α-dicarbonyls such as methylglyoxal (MG) are 20,000-fold more reactive than glucose in glycation reactions.[4] They react with thiol, amino and guanidine groups of proteins, causing protein modification, formation of advanced glycated end-products (AGEs) and protein cross-linking.[5-7] The most considered, as factors involved in development of micro- and macrovascular complications in diabetes are AGEs, their biochemical nature and their mode of action.[8, 9] Because it is difficult to achieve and maintain normal glycemia in diabetes,[10] inhibition of the formation of AGEs is very important in the prevention and treatment of complications, in diabetes and other pathological conditions where carbonyl stress is present.

To prevent above mentioned reactions many natural and synthetic inhibitors of glycation and AGEs formation were developed, a few of them are in the final phases of clinical trials. Aspirin, diclofenac, inositol, pioglitazone, metformin and pentoxifylline are listed as inhibitors of early glycation products formation. They interfere with the initial reaction between reducing sugars and amino groups, thus inhibiting the formation of Schiff base.[11] The substances, that bind reactive carbonyl compounds and radicals that are generated in the processes of oxidation and rearrangement of primary glycation reactions products, are suitable to prevent AGEs formation. The aminoguanidine, piridoxamine, metformin, carnosine and benfotiamine were proposed for that purpose. Aminoguanidine (Pimagedine) was the first AGEs inhibitor studied.[12] Because of its side effects, which included flu-like symptoms, abnormalities in liver function test, gastrointestinal disturbances, rare vasculitis and anaemia,[13] aminoguanidine is unlikely to be used for therapeutic purposes. Pyridoxamine (a vitamin B6 derivative) cleaves 3-deoxyglucosone(3-DG) -reactive carbonyl intermediates[14] and in diabetic nephropathy (in combination with B9 and B12 vitamins) accelerates decline in renal function.[15] However, it is unlikely that this effect is due to vitamin B6 and thereby compromises the

use of pyridoxamine.[16]

Metformin (dimethyl-biguanide) is a good dicarbonyl compounds scavenger,[17] it reacts with glyoxal and MG (but not with 3-DG). It is applied in the treatment of metabolic syndrome and diabetes.[18] Carnosine (β-alanyl-L-histidin) is competitive glycation target. Therefore, it is used to prevent and treat cataract (resulting from the aging process), glaucoma and other eye diseases caused by the formation of AGEs.[19] Benfotiamine, a pro-drug of thiamine monophosphate, reduced circulating AGE levels [20] by preventing dicarbonyl formation.

Although low molecular mass thiols can be also used as competitive targets for α-dicarbonyls, their scavenging capacity has not been investigated enough. During the examination of the mechanism between thiol group and MG it was shown that the substances containing α-amino-β-mercapto-ethane as pharmacophore could be successful scavengers of MG.[21] MG was chosen because of its high reactivity, high production (from spontaneous decomposition of triose phosphates, autoxidation of carbohydrates and glucose degradation, and also by Maillard reaction and lipid peroxidation) in diabetes, oxidative stress, uremia, aging process and inflammation.[3, 22, 23] MG has been identified as the major precursor in the formation of intracellular AGEs in endothelial cells [24] and it was proposed to be a candidate metabolite that causes neuropathic pain in metabolic disorders.[25] It also causes posttranslational modifications of regulatory proteins, and therefore influences gene expression regulation and modifies cellular functions.[26] Recently, it has been shown that MG modifies major proteasome proteins, resulting in impaired proteasome function.[27] Therefore, in this paper, the efficiency of low molecular mass thiols with α-amino-β-mercapto-ethane group (Cys, penicillamine and N-acetylcysteine (NAcCys, with blocked amino group) and glutathione (physiological thiol) as protective agents of protein carbonylation reaction with MG, e.g. their influence on the decrease of carbonylation levels of amino, guanidine and thiol groups, on the electrophoretic properties and the structure of HSA molecules, on the MG-induced HSA-cross-linking and antioxidant capacity of HSA Cys 34 group, was investigated. The scavenger potency of metformin (drug used in the treatment of metabolic syndrome and diabetes in clinical practice), that has been stated as a good scavenger of dicarbonyl compounds being a biguanidino reagent, was also tested and compared with the potency of thiol compounds.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma, Steinheim, Germany, unless otherwise noted. The 20% solution of HSA was purchased from Baxter, Vienna, Austria. Glacial acetic acid, acrylamide, bis-acrylamide, N,N,N',N'-tetramethyl-ethylenediamine (TEMED), glycerol, β-mercaptoethanol, thymol, bromine and Folin-Ciocalteu reagent were purchased from Merck, Darmstad, Germany.

2.2. Monitoring of the reactivity of scavengers` thiol, amino and guanidine groups with MG

The solutions of scavenger (Cys, penicillamine, NAcCys, GSH and metformin) were prepared in 0.1 M sodium phosphate buffer (pH 7.4) and incubated with MG in 1:1 ratio, in capped vials at 37 ºC for 6 h. All solutions were sterile-filtered prior to incubation. Aliquots of the reaction mixtures were taken at predetermined intervals and the content of scavengers` thiol, amino and guanidine groups was determined.

2.3. Monitoring of changes of the HSA molecules during the incubation with MG and MG scavengers

HSA (0.5 mM) solutions were prepared in 0.1 M sodium phosphate buffer (pH 7.4) and incubated in capped vials at 37 ºC for 8 h without (control), with 42 mM MG (equimolar with the total content of reactive amino, guanidine and thiol groups at the surface of HSA molecules,[28] without and in the presence of 21 mM inhibitor of glycation (Cys, pencillamin, NAcCys, GSH, or metformin). All solutions were sterile-filtered prior to incubation. Aliquots of the reaction mixtures were taken at predetermined intervals during incubation. The excess of MG and glycation inhibitors in those aliquots was removed by ultrafiltration with 0.02 mM phosphate buffer (pH 7.4), (Amicon Ultra -15 cut off 30,000, Millipore, Carrigtwohill, Ireland).

The reaction of HSA carbonylation was monitored by spectrophotometric determination (in triplicate) of amino, guanidine and thiol group content. Also, protein changes during the incubation experiments were monitored using native and SDS PAG (10 % acrylamide) electrophoresis, according to the protocol of Hoeffer scientific instruments,[29] as well as by spectrofluorometric and CD analysis. For electrophoresis, 7 μL of protein samples (0.05–0.15 mg/mL) were applied. Gels were stained by Coomassie Brilliant Blue G-250 (CBB). The concentration of proteins was determined by Biuret method, according to Slater.[30]

2.4. The amino groups assay

The spectrophotometric determination of HSA amino groups content[31] was performed as follows: 100 μL of the sample, 1360 μL of 0.1 M potassium phosphate buffer (pH 7.4) and 40 μL of 0.1 M p-benzoquinone in dimethylsulfoxide were mixed and incubated at 37 ^{o}C for 15 min. The absorbance at 480 nm was then measured against the sample blank promptly after incubation. The standard curve was created with HSA in the amino group concentration range from 0.2 to 2.0 mM (in probe), which corresponded to the real sample levels of amino groups of 3 to 30 mM (Y=0.0218X +0.0261, r=0.9988, p<0.0001).

The amino group content in the reaction mixtures of scavengers and MG were determined by a ninhydrin spectrophotometric method,[32] as follows: 800 μL of the sample and 400 μL of 0.028 M ninhydrin buffered (0.28 M Na_2HPO_4 and 0.44 M KH_2PO_4, pH 6.8) reagent were mixed and boiled for 16 min in boiling water, then cooled in a water bath for 15 min at 20 ºC. After the addition of 2 mL of 0.12 M KI in 38 % ethanol, the absorbance at 570 nm was measured against the reagent blank. The standard curve was generated with alanine in the concentration range from 0.1 to 1 mM (Y=0.9101X – 0.0722, r=0.9991, p<0.0001).

2.5. The guanidine group assay

For the determination of guanidine group, a spectrophotometric method was used:[33] to 10 μL of sample, 2 mL of thymol solution (0.01 % thymol in 0.5M NaOH) is added. Absorbance at 480 nm is measured (on Beckman DU 50

spectrophotometer) after addition of 100 μL sodium hypobromite (2 % bromine in 5 % NaOH) against reagent blank. The standard curve was prepared with the standard arginine solutions in the concentration range of 4.8 to 190 μM (in probe), which corresponded to the real sample levels of guanidine groups of 1 to 40 mM (Y=0.0308X+0.0019, r=0.9997, p<0.0001).

The calibration curve with metformin as standard in the concentration range from 0.2 to 3 mM was used (Y=0.0571X+0.0751, r=0.9928, p<0.0001) in experiments of investigation of its reactivity with MG.

2.6. The thiol group assay

The content of thiol group on the surface of HSA molecules in the reaction mixture with GSH and amino acids was determined by Ellman's spectrophotometric method.[34] Sample (100 μL) was mixed with equal volumes of 2 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 1M TRIS buffer (pH 8.0) and brought up to 1000 μL with water. All reagents and samples were kept at room temperature 30 min before determination. Absorbance at 412 nm was recorded after 30 minutes, against the sample blank. The content of thiol group was then calculated using the value of 13600 M^{-1} cm^{-1} as extinction coefficient of the reagent.

2.7. Circular dichroism (CD) spectroscopy

HSA samples were ultrafiltrated (Amicon Ultra Microcon, 0.5 ml, cut off <10 kDa) with MiliQ water. The final concentration of proteins in all samples was adjusted to 1 mg/mL. The CD spectra were recorded on a J-815 spectrometer (Jasco Corporation, Tokyo, Japan). The far-UV region (190–260 nm) measurements were made in a 0.01 cm path length cell at 25 C. Data were collected at 0.1 nm intervals (100 nm/min) with a sensitivity of ±200 mdeg. Spectra represent the average of four accumulations and were baseline-corrected by subtraction of blank (MiliQ water).

2.8. Fluorescence spectroscopy

Fluorescence measurements were performed on spectrofluorimeter Fluoromax-4 Jobin Yvon (Horiba Scientific, Japan). Prior the measurement, HSA samples were ultrafiltrated (same as for CD spectra). The HSA concentration used for fluorescence was 0.5 μM. The fluorescence emission spectra were recorded in the wavelength range of 290 to 450 nm with excitation at 290 nm. Quartz cell (1 cm path-length) and slit widths (4 nm), were used for all measurements. The each spectrum was the average of two scans and respective blanks MiliQ water of sodium phosphate buffer were used for the correction of all fluorescence spectra. The experiment was repeated and found to be reproducible within experimental errors.

The fluorescent emission spectra of AGEs were obtained using λexc/λem=330/(340-500) and λexc/ λem= 360/370-550.

3. Results

3.1. Time course of MG reaction with low molecular mass thiols and metformin

To investigate the potential of Cys, penicillamine and NAcCys, thiols with α-amino-β-mercapto-ethane group, as MG scavengers, the time courses of MG reactions with their reactive groups (amino, tiol i guanidine) were monitored and compared with the potential of GSH, and biguanidine derivative metformin.

The shapes of time course reaction curves of amino group were significantly different in the case of Cys, penicilamine and GSH (Figure 1A). The amino group of penicillamine is the most reactive, about 80 % reacted immediately, after mixing the reactants and there were no additional significant changes during further incubation. During the first 30 min of incubation, 43 % Cys amino group reacted, reaching 60% by the end of incubation. The slowest reaction was observed for the GSH amino group. After mixing with MG, only 12 % of amino groups reacted, without any changes during further incubation.

However, the time course reaction curves of Cys and penicillamine thiol groups have the similar shapes (Figure 1B). In the first minutes of reaction, the thiol group reacted in high percent in the case of Cys (78 %) and in significant percent in the case of penicillamine (50 %). During further incubation, the percentage of reacted Cys and penicillamine thiol groups decreased, so after four hours of incubation, when equilibrium is reached, it was 40 % for both compounds. The reactivity of NAcCys and GSH thiol group was significantly lower. During six hours of incubation, only 10 % of NAcCys-SH and 30 % of GSH-SH groups reacted. Thus, the reactivity of SH groups of investigated compounds decreased in the order Cys>penicilamine>GSH>NAcCys.

During the reaction of metformin with MG, equilibrium was reached after two hours of incubation, when about 50 % of metformin reacted (Figure 1 C).

Insert Figure 1

3.2. The efficiency of thiol-compounds containing α-amino-β-mercapto ethane group in inhibition of HSA carbonylation with MG

Carbonylation of protein with MG, without and with presence of Cys, pencillamine, NAcCys, GSH and metformin as carbonyl scavengers, was monitored *in vitro* by incubation (at 37 °C, during 8 hours) of HSA (0.5mM) with 42 mM MG (concentration equimolar to the total sum of reactive groups, at protein surface, n=84[28]). The concentration of MG, much higher than physiological, was chosen in order to monitor the effects of scavengers on reaction of carbonylation with more reliability. The aliquots of the reaction mixtures were taken at predetermined intervals and the level of HSA carbonylation was monitored by quantification of amino, guanidine and thyol groups' content. Without the presence of scavengers, both guanidine and amino groups reacted in high percent. Guanidine group reaction rate is higher than the rate for amino group (Figure 2 A and B). In the first 30 min of the reaction 43 % of available guanidine groups reacted (9.0 mM of guanidine groups/mM HSA), and that percentage was increased to 58 % for the next 7.5 hours of incubation. On the other hand, 7.4 % of available amino groups (3.9 mM of amino groups/mM HSA) reacted in the first 30 min and after 2 hours that percentage doubled and was rising gradually (to 28 %, 16 mM of amino groups/mM HSA) during 8 hours incubation (Figure 2B).

Insert Figure 2

Thus, during the reaction of HSA carbonylation with MG (at mentioned conditions) guanidine and amino groups on the protein surface are significantly modified. All the tested MG scavengers reduce the level of guanidine and amino group carbonylation, but they differ significantly in terms of efficiency (Figure 2). Cys and penicillamine prevented the reaction of carbonylation amino groups very effectively during the entire incubation period (2B). At applied concentration of scavenger, as a half as the concentration of reactive groups (the sum of amino and guanidine), almost no carbonylation of amino groups was observed. In the presence of Cys, guanidine groups reacted in negligible extent, only 10 % by the end of the incubation (Figure 2A). Penicillamine decreased the level of guanidine groups' carbonylation by 55 % in comparison to the reaction without its presence.

The efficiency of GSH and metformin as MG scavangers was similar. In their presence, carbonylation of amino groups was reduced by 60 % and for guanidine groups by about 30 %. The lowest inhibitory effect in preventing carbonylation of HSA (especially amino groups) was obtained with NAcCys, which is consistent with the results of the examination of its reactivity with MG (Figure1).

In the presence of low molecular mass thiols as scavengers, there was no reaction of HSA-SH group with MG. However, in the presence of metformin, the reaction of thiol groups is taking place at approximately same extent as in the absence of inhibitors (Figure 2C).

As it mentioned above, in order to monitor the effects of scavengers on reaction of carbonylation with more reliability we used much higher concentrations of MG (42 mM) and scavangers (21 mM) than physiological ones (for example, serum concentrations of free and bound Cys are 131 and 202 μM [35]. To perceive what is the relevance of these *in vitro* results to *in vivo* effects and to the treatment of human disease the concentration (dose)-response dependence was investigated. HSA (0.5 mM) was incubated at 37 ºC for 1 h with 42 mM MG in the absence or presence of different concentrations of Cys (from 5 to 40 mM), and the guanidine group content was determined. The guanidine group was chosen as its reaction rate with MG is higher than the rate for amino and thiol group. Very strong negative correlation ($r=-0,996$, $p< 0.0001$) between Cys concentration (from 5 to 30 mM) and the percent of reacted guanidine group was found (Figure 3).

Insert Figure 3

3.3. The effects of carbonylation reaction with MG on HSA conformation changes and AGEs formation

The effects of carbonylation reaction (with and without MG scavengers) on HSA conformation changes were monitored by recording the fluorescent emission spectra. An internal HSA fluorescence (which originates from the Trp 214) at the emission maximum λem338 nm was measured after excitation of HSA molecule at λeksc290 nm. Quenching of internal fluorescence at λem338 by 45% compared to untreated HSA (control) (Figure 4A, Table 1) was observed, due to HSA carbonylation. In the presence of Cys or penicillamine, a decrease of fluorescence after two hours of incubation was only 4 % and 11 %, respectively. In the presence of metformin and NAcCys, it was very high (31 and 37 %, resp.) (Table 1). The GSH exerted the middle efficiency in preventing fluorescence quenching.

To determine whether the observed differences in fluorescence intensity of HSA (control) and HSA-MG arise from differences in their secondary structure, far-UV CD spectra were recorded. As shown in Fig. 4B, the far-UV CD spectra of the HSA (control) and HSA-MG were overlapped and exhibited two negative bands in the UV region at 208 and 222 nm, which are typical for α-helical proteins.[36]

Insert Figure 4

Insert Table 1

HSA-AGEs generated in the reaction of HSA with MG could contribute to tryptophan (intrinsic) fluorescence quenching. AGEs show fluorescence after excitation at the λexc higher than 290 nm, i.e. they have characteristic excitation at wavelengths in the range of 328 to 370 nm and fluorescence emission from 378 to 440 nm.[28, 37-39] Therefore, the fluorescent emission spectra of HSA carbonylated with MG (without and with MG-scavangers) were recorded, after excitation at 330 and 360 nm (Figure 5). The lowest relative increase in the fluorescent emission intensity at maxima (λem=417 and 440 nm, Table 2) in comparison to untreated HSA (control) was observed in the presence of Cys and penicillamine. So, when in the reaction mixture, in addition to HSA and MG, Cys and pencillamine were present they significantly prevented the formation of fluorescent AGEs. Contrary, in the presence of metformin, the protection of the fluorescent AGEs formation was the least.

Insert Figure 5

Insert Table 2

3.4. The efficiency of thiol-compounds with α-amino-β-mercapto-ethane group in protection of protein cross-linking with methylglyoxal

The efficiency of low molecular mass thiols and metformin in protection of protein cross-linking was examined by native and SDS PAG electrophoresis (Figure 6). The carbonylation of HSA with MG leads to changes in HSA charge and formation of cross-links between HSA molecules (Figure 6, lines b). The mobility of HSA-MG adduct (band b, Figure 6A) is greater in comparison to HSA carbonylated with MG in the presence of MG-scavangers (bands C, G, P, M and N). The changes in HSA`s relative electrophoretic mobility (REM) (Figure 7), i.e. the changes of HSA monomer charge during incubation with MG, are in accordance with the changes in the reacted HSA amino groups content (Figure 2B). The lowest charge changes occurred in the presence of Cys and penicillamine, and the highest in the presence of NAcCys. In addition, besides HSA monomer the band with REM corresponding to BSA dimer was observed (Figure 6A, band m).

Insert Figures 6 and 7

During SDS PAGE (Figure 6B) a band of MG-HSA monomer with expressed tailing to higher molecular mass values was obtained. In addition, the bands of dimers and oligomers appeared. The approximate contents of the HSA monomer, dimer, and oligomers in the incubation mixtures were determined using the program Image J.[40] The percentage of cross-linked species generated in the reaction mixture of MG and HSA, after two hours of incubation, was approximately 25 % (Table 3). In the presence of Cys and penicillamine the content of cross-linked species was the lowest (from 9 to 11 %). The least effective in preventing protein cross-linking were metformin and NAcCys (with about 20 % of generated dimers and oligomers).

Insert Table 3

4. Discussion

Reactive dicarbonyl species are generated in higher quantities in diabetes, uremia, oxidative stress, inflammation and aging.[3, 22] The elevated levels of reactive carbonyl compounds characterize the phenomenon known as "carbonyl stress".[41] Carbonyl compounds react with amino, guanidine and thiol protein groups, leading to the changes of protein molecule conformation, and thereby alter their function.[42, 43] Molecules modified with MG and their derivatives can affect cellular functionality *via* gene expression,[26] lead to micro- and macro-vascular complications in diabetes,[44] cause neuropathic pain in metabolic disorders[25] and contribute to the upregulation of inflammatory and tissue injury-provoking molecules through the interaction of AGEs and receptors for advanced glycation end products,[45] protein cross-linking and apoptosis.[46]

To prevent nonenzymatic carbonylation of proteins with α-oxoaldehydes and above noted consequences it is necessary to design and synthesize compounds-scavangers of these reactive species. Many natural and synthetic inhibitors of glycation and AGEs formation were developed,[11, 12, 17-20] but the scavenging capacity of low molecular mass thiols has not been investigated enough. Investigation of the reaction's mechanism of thiol group (with different microenvironment) and MG[21] showed that the low molecular mass thiols containing α-amino-β-mercapto-ethane group as a pharmacophore could be a successful α-oxoaldehyde scavangers. Therefore, the potential of Cys, penicillamine and NAcCys (thiols containing above-mentioned structural element) as MG scavangers, was firstly investigated by monitoring of the reactions time courses of their reactive groups (amino, thiol and guanidine) with MG. For the purpose of comparison, the potential of GSH (small physiological thiol) and biguanidino derivative metformin were also investigated. It was found that the reactivity of SH groups decreased in the order Cys>penicillamine>GSH>NAcCys (Figure 1B). The reaction curves of the Cys and penicillamine thiol groups have similar shapes, but those of their amino groups are significantly different. It was previously found[21] that shapes of reaction curves of Cys-thiol and Cys-amino groups with MG are the consequence of rapid hemithioacetal formation reaction, and then slower reaction of intramolecular thiazolidine intermediate and Cys-MG-glycosylamine adduct formation (Scheme 1).

Insert Scheme 1

The presence of α-amino-β-mercapto-ethane structure in the molecule of both Cys and pencillamine contributes to the high potential of their thiol group for scavenging MG. If the amino group is blocked (acetylated in the case of NAcCys) or protected (as a peptide bond in GSH) the thiol-group reactivity is significantly reduced (in the case of NAcCys only 10 % of it reacts while in the case of GSH 30%). It should be underlined that the presence of two additional methyl groups in the β-position of penicillamine (3,3-dimethyl-D-cysteine) partially reduce the reactivity of SH group in comparison to Cys (50 % reacted thiol compared to 78 %), but contributes to a significant increase in penicillamine amino group reactivity at the beginning of reaction (80 % reacted compared to 16 % of Cys) (Figure 1A). The sum of the percentages of reacted penicillamine amino and thiol groups, throughout the entire incubation period exceeds the amount of MG in the reaction mixture, indicating the presence of intermolecular product thiazolidine.[21, 47] High reactivity of Cys and penicillamine amino- and SH-groups in the reaction with MG, especially at the beginning of the reaction, indicates that the compounds with α-amino-β-mercapto-ethane structure have high potential in capturing reactive dicarbonyls. In addition, the time course reaction curves of amino and thiol groups with MG show that changes in the microenvironment of this structure can significantly alter the compounds scavenging properties.

Metformin, a drug used in the treatment of metabolic syndrome and diabetes,[18] as biguanidino reagent may capture dicarbonyl compounds.[17] It was found that metformin is the potent inhibitor of glycation.[48, 49] The low percentage of reacted metformin guanidino group at the start of the reaction (less than 10%) and after the establishment of the equilibrium (50 %) indicates that metformin is less potent MG scavanger compared to pencillamine and Cys.

The reaction of non-enzymatic glycation of proteins, by a series of reactions lead to formation a class of heterogeneous adducts that are called AGEs.[50] Numerous studies have indicated that the formation of AGEs in long-lived connective tissue and matrix components is a causative factor in the development of diabetic complications, including diabetic cardiovascular dysfunction and diseases associated with aging, as well as that chemical inhibition of AGEs formation results in attenuation of diabetic complications. A number of natural or synthetic compounds that target AGEs, including AGEs inhibitors and breakers, have been discovered and are being further developed[51-53]).

To examine the prevention of protein carbonylation in the presence of small thiols as MG scavengers, i.e. the prevention of AGEs formation and protein crosslinking, HSA as a model system was used. HSA was chosen because it is the most abundant protein in blood plasma and is significantly exposed to the influence of carbonyl compounds (half-life 18 days). The number of amino acid side-chains on the HSA surface, i.e. the number of reactive groups that MG can react with, is known (59 Lys, 24Arg and 1Cys side-chains).[28] On the basis of proteomic profiling, it was found that glucose attaches at HSA *in vivo* by the 31 glycation sites.[54] In normal adult individuals, nearly of 10 % HSA is modified by glycation, which increased 2-3 fold under hyperglycemic conditions such as diabetes.[31, 55] In addition, beside many HSA functions, it may play a protective role *in vivo* as an antioxidant. It was shown that more than 70 % of the free radical trapping activity of serum was due to HSA Cys34 thiol

group.[56] The antioxidant property of HSA was modified following *in vitro* glycation by MG[57] and also *in vivo* in patients with diabetes.[58] As Amadori-glycated proteins may contribute to the pathogenesis of diabetic vascular diseases, agents that neutralize or prevent their formation in diabetes may offer a new therapy against early changes leading to diabetic vascular complications.

During the incubation of HSA with MG (without MG scavengers) 58.3 % of guanidino, 27.1 % of amino and 65 % of thiol groups of HSA were modified. Our data suggest that Cys and penicillamine can effectively compete for MG with HSA lysine and arginine side chains (Figure 2 A and B) and, thus, almost completely protect HSA amino residues from carbonylation and very efficiently HSA-guanidine residue (especially Cys). On the other side, GSH (physiological small thiol) and drug metformin, exhibit middle efficiency (of 30 to 60 %). The presence of small thiols as scavenger led to almost complete inhibition of the HSA-SH group reaction with MG, as opposed to metformin which had no effect. The efficiency of Cys as inhibitor of glycation with MG was also demonstrated by Sharma and Santhoshkumar.[58] In addition, it was found that low molecular mass thiols are better glycation inhibitors than carnosine (beta-alanyl-histidine; competitive glycation target; lead to the decomposition of aldosamines) and pirydoxamine (which prevents the formation of AGEs from Amadori-intermediates and cleaves 3-DG-reactive carbonyl intermediates and exhibits therapeutic effects in clinical trials for diabetic nephropathy).[17, 49]

The relevance of these obtained *in vivo* results to *in vivo* effects (to the treatment of human disease) was shown through the dose (Cys concentration)-response (guanidine group carbonylation) relationship. Very strong negative correlation ($r=-0{,}996$, $p< 0.0001$) between Cys concentrations and the levels of carbonylated guanidine group shows that substances with α-amino-β-mercapto-ethane group as pharmacophore have great potential to be efficient MG scavangers and thus are promising compounds for medicinal chemistry.

HSA carbonylation with MG, i.e. formation of HSA-MG adducts leads to decrease of fluorescence intensity at λem338, which originates from Trp 214 residue, for about 45 % in comparison to nontreated HSA. Due to above underlined efficiency of MG scavengers with α-amino-β-mercapto-ethane group (Cys and penicillamine), quenching of fluorescence of HSA modified with MG in their presence exists in significantly lower extent (only 4 % and 11 %, resp.). The Trp fluorescence quenching of HSA-MG could be consequence of the changes in HSA's tertiary and/or secondary structure (which could lead to changes in Trp solvent accessibility) or of the changes in Trp 214 micro environment. Mendez et al[59] investigated fluorescence of HSA glycated with glucose and suggested that quenching of Trp fluorescence is due to modification of Lys residues (of Lys 199, and probably of Lys 212 and Lys 195) in Trp 214 environment, so this modification additionally stabilizes secondary structure and local structure around Trp. UV CD spectra of HSA (control) and HSA modified with MG (Figure 4B) were overlapped, indicating that no changes had occurred in the conformation of the polypeptide backbone (secondary structure) after MG binding. Decrease in the intensity of fluorescence at the peak wavelength (λem 338 nm) could be the consequence of changes in tertiary structure. The structural changes in the HSA molecule lead to the changes of its physical-chemical properties[60] and affinity to bind different ligands (warfarin, furosemide, phenylbutazone, bilirubin, lipoic acid, fatty acids, ibuprofen, dansyl sarcosine, copper, iron ...) for which HSA is transporter.[61-63] In the presence of MG scavenegers with α-amino-β-mercapto-ethane group the structural changes of HSA molecules are the smallest.

HSA–AGEs could contribute to the quenching of Trp fluorescence intensity (at λem 338 nm), as they show fluorescence after excitation at wavelengths λ>290 nm. Thus, AGEs could be Trp fluorescence quenchers. Pentozidine (adduct formed by crosslinking of Lys and Arg residues) and Arg-pyrimidine are fuorescent AGEs with characteristic λeksc/λem=328/378.[37, 64] HSA modified with glucose shows the characteristic fluorescence λeksc/λem=335/406,[28] which originates from pentosidine and Arg-pyrimidine.[65] Monitoring of the fluorescence at λeksc/λem=360/430, and 365/440[38] or 370/430[39] was suggested as indicator of protein glycation level. The changes in fluorescent emission spectra (recorded after excitation at λ 330 and 360 nm) of HSA carbonylated with and without presence of MG scavengers, that is increase of fluorescence intensity at emission maxima (λem=417 and 440 nm, Table 2) in comparison to untreated HSA (control) was the least in the presence of Cys and penicillamine. Thus, Cys and penicillamine prevent formation of fluorescent AGEs. That is why Trp emission at λem338 (after excitation at λeksc=290nm) was quenched at least in their presence. Wondrak et al[47] were, also, established that NAcCys, Cys and GSH lead to quenching of fluorescence which originates from AGEs. In the presence of metformin, preventing of generation of fluorescent AGEs was the lowest.

One of the consequences of AGEs accumulation is increased cross-linking of protein molecules (AGE cross-links) in cardiovascular tissue with a resulting increase in vascular stiffness, which is an important risk factor for cardiovascular morbidity and mortality. During the investigation of protein modifications with MG it was proposed that protein Lys side chains participate in HSA crosslinking via protein-SH-MG (hemithioacetal) or protein-NH_2-MG products formed during the first step.[5, 66] The MG-HSA monomer bands, that on SDS electrophoregram spread over larger molecular masses as well as bands of dimers and oligomers (Figure 6), indicate fragmentation and the cross-linking of fragments, as well as cross-linking of HSA molecules during the carbonylation. In the presence of Cys and penicillamine the percentage of cross-linked species was about 2.5 times lower in comparison to the percentage obtained during carbonylation of HSA without presence of MG scavengers. The least effective in the protein cross-linking inhibition were metformin and NAcCys.

5. Conclusions

Based on the all it can be concluded that by trapping of the reactive dicarbonyl MG, Cys and penicillamine efficiently inhibit reaction of HSA carbonylation (in dose dependent manner), formation of AGEs and cross-linking of protein molecules, and they are more effective in comparison with metformin (drug used

in the treatment of metabolic syndrome and diabetes). Penicillamine, classically used in the treatment of Wilson disease, rheumatoid arthritis, cystinuria and lead poisoning, is relatively stable *in vivo*.[67] Cys and penicillamine have additional advantage because they enable HSA thiol group protection, and maintenance of its antioxidative potential, which is essential for HSA function *in vivo*. Thus, the substances containing α-amino-β-mercapto-ethane group as pharmacophore may be used as effective scavengers of MG. Introduction of Cys as scavenger could be possible only via intravenous injection as its metabolism is tightly controlled and oral ingestion would not significantly change the circulating levels. On the other hand, the highly reactive MG produces in all cells and its intracellular concentrations are much higher than its circulating levels. Therefore, design and synthesis of the compounds with α-amino-β-mercapto-ethane pharmacophore, as well as with the hydrophobic part (in order to facilitate the passage of compound through the membrane) could provide effective scavenge of intracellular MG and protection of cell damage.

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Figure legends

Fig. 1. Time course curves of: amino (A) and thiol (B) groups of the amino acids (Cys, penicillamine, NAcCys) and GSH, as well as guanidine (C) group of metformin with MG (1:1), during the incubation in 0.1 mM of sodium phosphate buffer (pH 7.4) at 37 ˚C, over a 6 h period. Data presented are means from three determinations with RSD lower than 3%.

Fig. 2. The quantification of the guanidine (A), amino (B) and thiol (C) groups during the incubation of 0.5 mM HSA with 42 mM MG in the presence of 21 mM inhibitors (Cys, penicillamine, GSH, metformin and NAcCys) and without them, in 0.1 M sodium phosphate buffer (pH 7.4) at 37 ^{o}C for 8 hours. Data presented are means from three determinations with RSD lower than 3 %. Slika za izreagovale SH grupe nije dobra i trebala bi da se promeni, jer ce neko detaljnije da cita ovaj rad pa ce se pitati, kako je ovo moguce. Sve vreme radi se sa komercijalnim HSA koji ima najvise 40% HSA-SH grupa, a ovde iz grafika ispada da je 70 % HSA-SH grupa redukovano na pocetku i da jos pri inkubiranju raste sadrzaj HSA-SH grupa do skoro 1. Ovako nacrtana slika ukazuje da mali tioli nisu potpuno uklonjeni iz reakcione smese.

Fig. 3. Relationship between the concentration of Cys (MG scavenger) and the protection of the guanidine group reaction with MG. HSA (0.5 mM) was incubated at 37 ºC for 1 h with 42 mM MG without and with the presence of different concentrations of Cys (from 5 to 40 mM). Data presented are means from three determinations with RSD lower than 3%.

Fig. 4. A. Fluorescent emission spectra of HSA (control), HSA after 2 hours of incubation with MG in the presence of MG scavangers (Cys, penicillamine, GSH, NAcCys and metformin), and without them in 0.1 M sodium phosphate buffer (pH 7.4) at 37 ºC. The concentration of HSA was 0.5 μM in all samples. (λexc=290nm)
B. Far-UV CD spectra of HSA (control) and HSA modified with MG.

Fig. 5. The fluorescent emission spectra (λexc 330 nm (A) and λexc 360 nm (B)) of HSA (control), HSA carbonylated with MG during 6 hours incubation in the presence of MG-scavangers (Cys, penicillamine, GSH, NAcCys, metformin), and without them, in 0.1 M sodium phosphate buffer (pH 7.4) at 37 ºC.

Fig. 6. Native (A) and SDS-PAGE (B) showing the changes of HSA (0.5 mM) mobility and cross-link formation, resulted from the reaction with methylglyoxal (41 mM), without (line b) and in the presence of inhibitors (21 mM): C -Cys, G -GSH, P -penicillamine, M -metformin, N -NAcCys. Lane h shows the non-treated HSA, and lane m –BSA marker (A) and SDS markers (B). Incubation was performed in 0.1 M phosphate buffer (pH 7.4) at 37 ^{o}C for 2 hours. The electrophoresis was performed on 10 % polyacrylamide gels and bands were visualized by CBB.

Fig. 7. The changes in HSA relative mobility values (REM) during the incubation of 0.5 mM HSA with 42 mM MG in the presence of 21 mM inhibitors (Cys, penicillamine, GSH, NAcCys and metformin), and without them, in 0.1 M phosphate buffer (pH 7.4) at 37 ^{o}C for 8 hours.

Scheme 1. Chemical reactions occurring in the reaction mixture of Cys and MG (1 : 1) .

Table 1. The reduction of fluorescence intensity at λem 338 nm and the shift of fluorescence maximum, during incubation of HSA with MG in 0.1 M sodium phosphate buffer (pH 7.4) at 37º, for 2, and 6 hours, without and in the presence of MG scavangers (Cys, penicillamine, GSH, NAcCys and metformin).

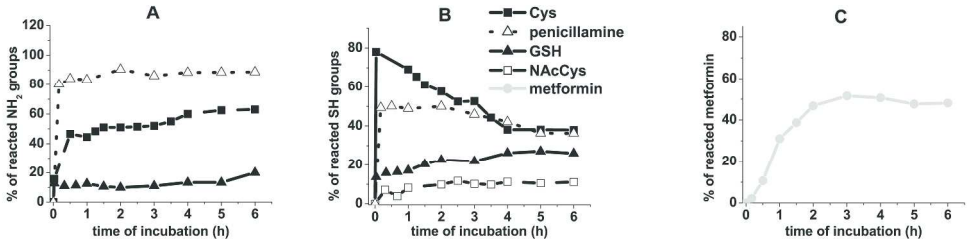
HSA + MG +... MG scavanger	The reduction of fluorescence at λem 338 (%)		Fluorescence maximum shift
	2 h	6 h	
-	45.67	44.49	332
Cys	4.31	15.27	340
Penicillamine	10.99	22.97	335
GSH	19.21	28.36	336
Metformin	30.81	47.16	336
NAcCys	37.20	52.46	335

Table 2. The relative increase of fluorescence intensity at λem 417 and 440 nm (obtained after excitation at 330 and 360 nm, recpectively) of carbonylated HSA (after incubation with MG during 6 hours) with and without MG scavangers in comparison to untreated HSA (control).

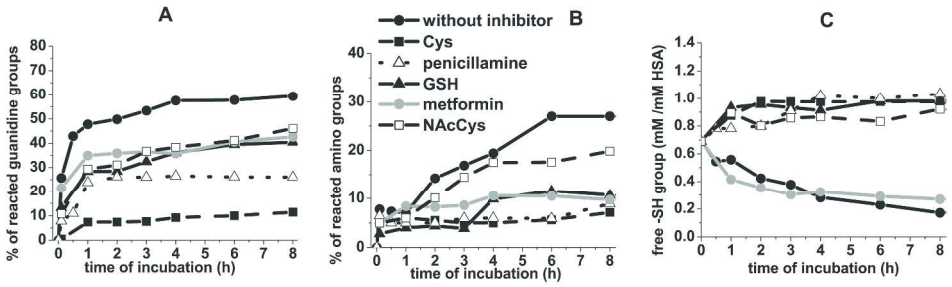
HSA + MG +... MG scavenger	Relative increase of fluorescence intensity at	
	λem=417 nm	λem=440 nm
-	24.93	13.82
Cys	5.79	3.20
Penicillamine	8.36	5.43
GSH	11.27	6.59
NAcCys	11.30	6.39
Metformin	20.85	11.37

Table 3. Distribution of HSA monomer, dimer and oligomer contents (analysed by Image J of SDS PAGE electrophoregram) in reaction mixtures of HSA (0.5 mM) and MG (42 mM) (with and without presence of scavengers, 21 mM), obtained after two hours of incubation in 0.1 M phosphate buffer (pH 7.4) at 37 ^{o}C.

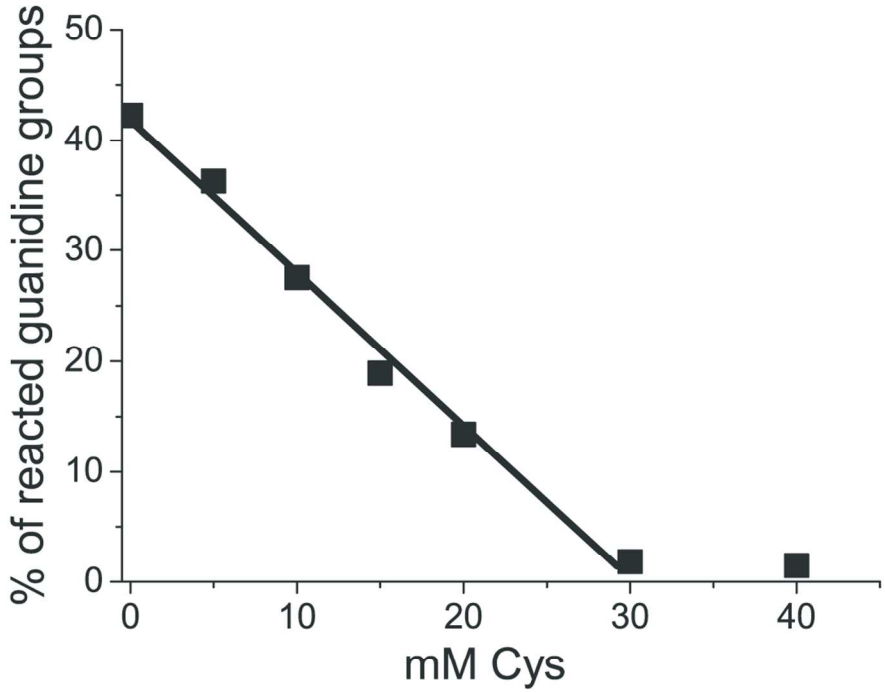
HSA + MG +... MG scavenger	Monomer (%)	Dimer (%)	Oligomer (%)
-	74.87	16.2	8.5
Cys	89.30	9.69	
Penicillamin	87.58	11.15	
GSH	83.96	14.56	
Metformin	79.97	15.7	4.17
NAcCys	79.2	17.72	3.76



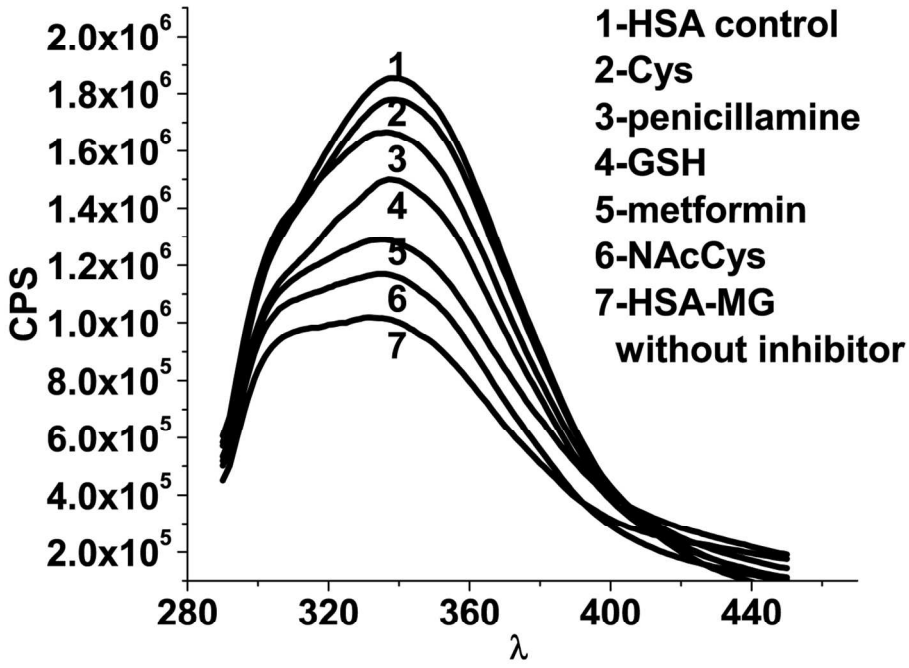
Time course curves of: amino (A) and thiol (B) groups of the amino acids (Cys, penicillamine, NAcCys) and GSH, as well as guanidine (C) group of metformin with MG (1:1), during the incubation in 0.1 mM of sodium phosphate buffer (pH 7.4) at 37 °C, over a 6 h period. Data presented are means from three determinations with RSD lower than 3%.
334x78mm (300 x 300 DPI)



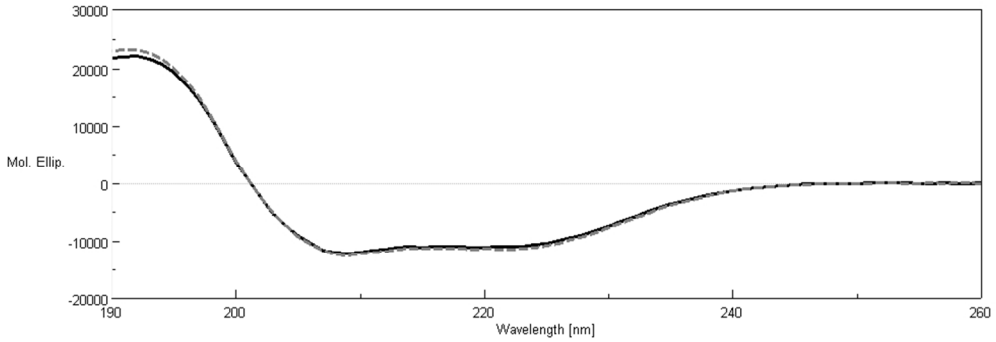
The quantification of the guanidine (A), amino (B) and thiol (C) groups during the incubation of 0.5 mM HSA with 42 mM MG in the presence of 21 mM inhibitors (Cys, penicillamine, GSH, metformin and NAcCys) and without them, in 0.1 M sodium phosphate buffer (pH 7.4) at 37°C for 8 hours. Data presented are means from three determinations with RSD lower than 3 %.
302x88mm (300 x 300 DPI)



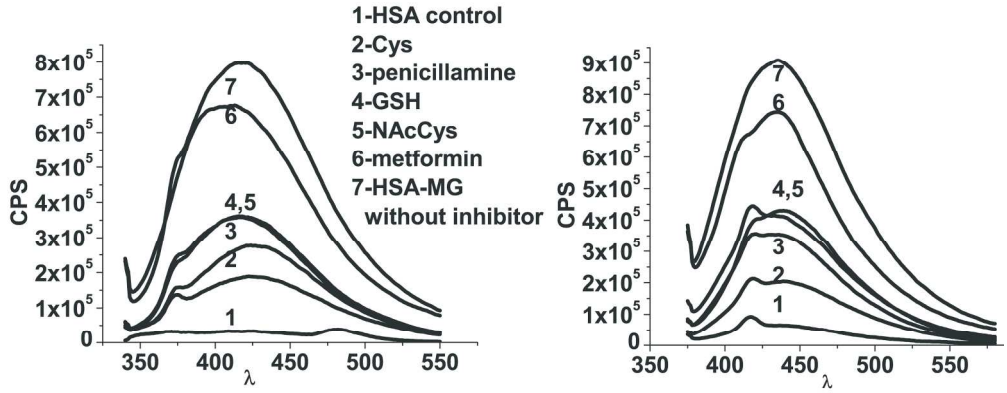
Relationship between the concentration of Cys (MG scavenger) and the protection of the guanidine group reaction with MG. HSA (0.5 mM) was incubated at 37 °C for 1 h with 42 mM MG without and with the presence of different concentrations of Cys (from 5 to 40 mM). Data presented are means from three determinations with RSD lower than 3%
104x86mm (300 x 300 DPI)



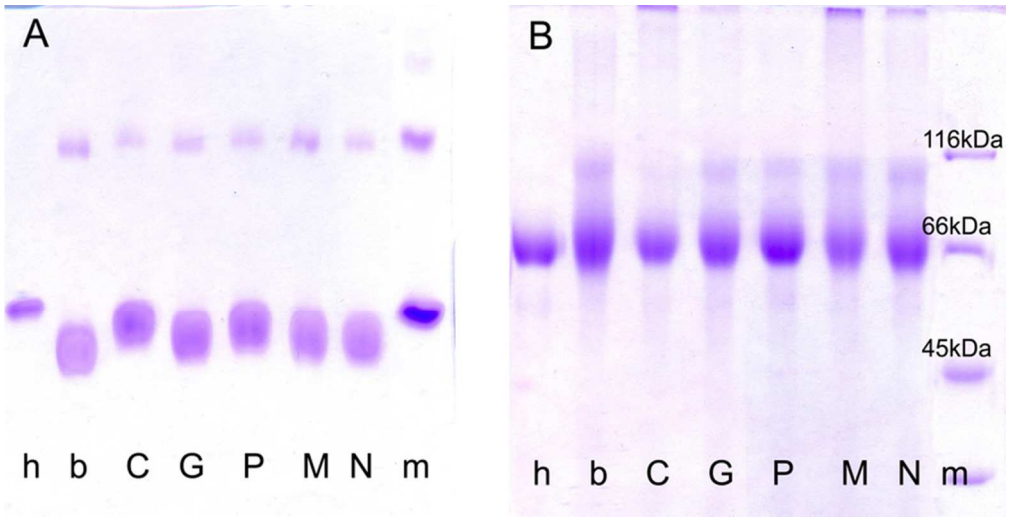
Fluorescent emission spectra of HSA (control), HSA after 2 hours of incubation with MG in the presence of MG scavangers (Cys, penicillamine, GSH, NAcCys and metformin), and without them in 0.1 M sodium phosphate buffer (pH 7.4) at 37 °C. The concentration of HSA was 0.5 μM in all samples. (λexc=290nm)
122x94mm (299 x 299 DPI)



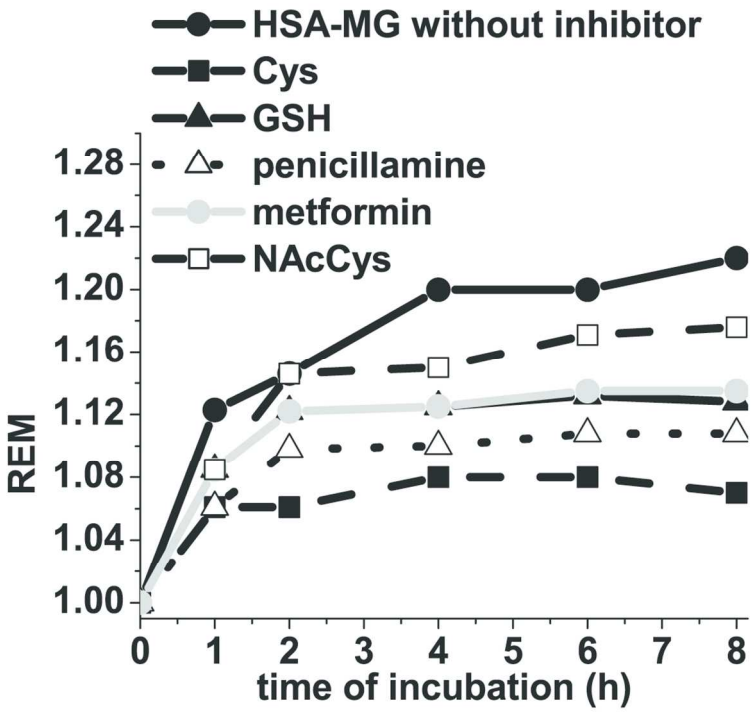
Far-UV CD spectra of HSA (control) and HSA modified with MG.
83x28mm (300 x 300 DPI)



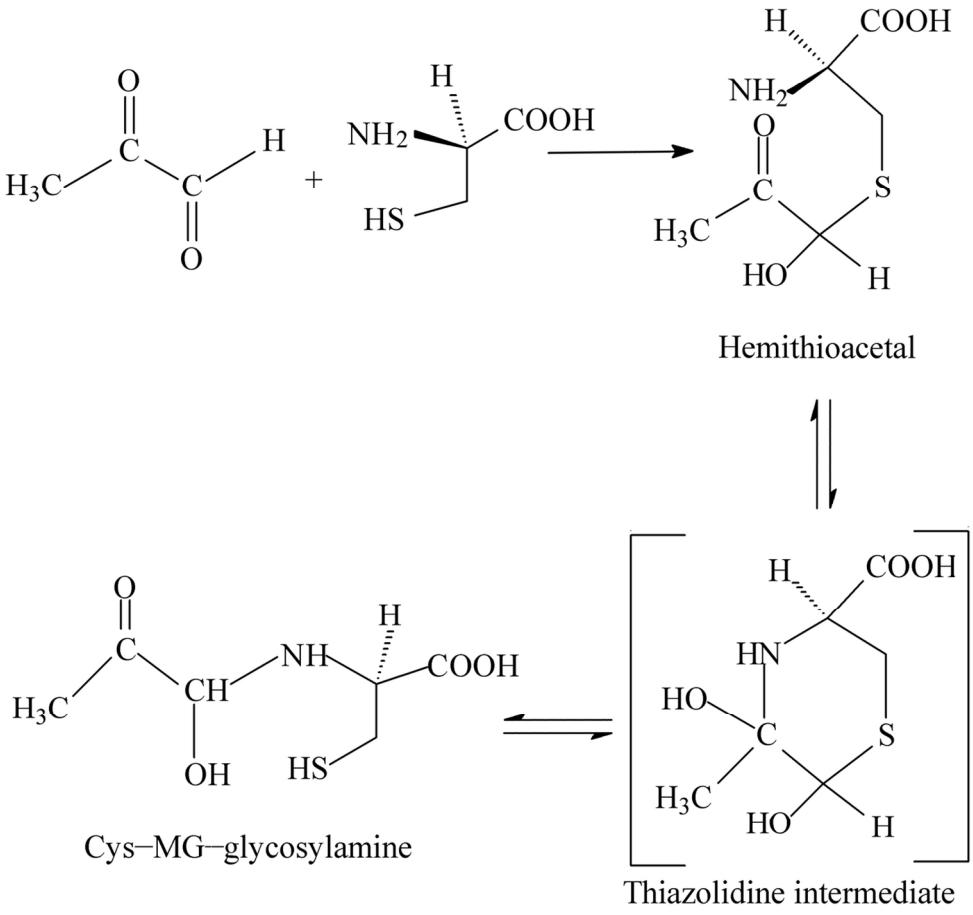
The fluorescent emission spectra (λexc 330 nm (A) and λexc 360 nm (B)) of HSA (control), HSA carbonylated with MG during 6 hours incubation in the presence of MG-scavangers (Cys, penicillamine, GSH, NAcCys, metformin), and without them, in 0.1 M sodium phosphate buffer (pH 7.4) at 37 °C.
197x88mm (300 x 300 DPI)



Native (A) and SDS-PAGE (B) showing the changes of HSA (0.5 mM) mobility and cross-link formation, resulted from the reaction with methylglyoxal (41 mM), without (line b) and in the presence of inhibitors (21 mM): C -Cys, G -GSH, P -penicillamine, M - metformin, N -NAcCys. Lane h shows the non-treated HSA, and lane m –BSA marker (A) and SDS markers (B). Incubation was performed in 0.1 M phosphate buffer (pH 7.4) at 37 oC for 2 hours. The electrophoresis was performed on 10 % polyacrylamide gels and bands were visualized by CBB.
74x38mm (300 x 300 DPI)



The changes in HSA relative mobility values (REM) during the incubation of 0.5 mM HSA with 42 mM MG in the presence of 21 mM inhibitors (Cys, penicillamine, GSH, NAcCys and metformin), and without them, in 0.1 M phosphate buffer (pH 7.4) at 37 °C for 8 hours.
121x99mm (300 x 300 DPI)



Chemical reactions occurring in the reaction mixture of Cys and MG (1 : 1).
61x58mm (600 x 600 DPI)