Organosulfur Mimics of *S*-allyl Cysteine and Effects on Advanced Glycation End-products

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Abstract—**Diabetes mellitus (DM) is a metabolic disorder characterised by increased blood glucose concentrations resulting from a deficiency of insulin, or insulin resistance. The prolonged hyperglycaemia of DM is extensively recognised as the causal link between diabetes and diabetic complications. Moreover, hyperglycaemia induces protein glycation and the formation of advanced glycation end-products (AGEs). The accumulation of AGE in the body leads to structural and functional modifications of tissue proteins. Herein we evaluate the anti-glycation activities of several inhibitors i.e. *S*-allyl cysteine (SAC), *N*-acetylcysteine (NAC) and synthesised small molecule inhibitors that mimic SAC/NAC (compounds A, B and C) identified as inhibiting the formation of methylglyoxal (MG)-derived AGE. The extent of glycation in the presence and absence of SAC, NAC and compound A were assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). It has been established that SAC, NAC and compound A, are inhibitors of protein glycation.**

Index Terms—**Glycation, Advanced glycation end-product, diabetes, aged garlic extract; S-allyl cysteine, N-acetylcysteine**

I. Introduction

Diabetes Mellitus (DM) is a metabolic, multiple aetiology disease characterised by long-term hyperglycaemia. When untreated, it may lead to severe medical complications such as chronic hyperglycaemia and disturbances in the metabolism of protein, fat and carbohydrate [1], [2]. Hyperglycaemia is defined as a condition when elevated sugar levels are present in the blood. Diabetics are at increased risk of developing macrovascular and microvascular diseases. Diabetes-associated complications include peripheral nerve damage (diabetic neuropathy), renal failure (diabetic nephropathy), retinal damage (diabetic retinopathy), cataract formation, accelerated atherosclerosis leading to increased risk of myocardial infarction and stroke, elevated blood pressure, dyslipidaemia and impaired wound healing [3]. The Maillard reaction or non-enzymatic browning, refers to any chemical reaction involving the interaction between amino acids and carbonyl compounds. The Maillard reaction is linked to hyperglycaemia, which contributes towards the pathogenesis of diabetic complications *via* increased protein glycation and the formation of advanced glycation end-products (AGEs) [4]-[6]. Glycation and AGE formation are accompanied by increased free radical activity, which can induce damage in important biomolecules in cells and induce malignant cell transformation. Moreover, protein glycation occurs gradually in the body's tissues and alters enzyme activity, immunogenicity, decreases ligand binding, and leads to protein cross-linking [4].

Glycation and AGE-mediated complications in diabetes and ageing have assisted the research for identifying the substances which can target glycation and AGE. Two main approaches were adopted to inhibit the formation of AGE and to break the existing protein-protein cross-links. Compounds that inhibit the formation of AGE can act *via* a variety of mechanisms and at different stages of the glycation reaction. Some of these compounds are multifunctional AGE inhibitors working at different stages of the glycation reaction.. Metformin, a widely used hypoglycaemic agent, reduces α-dicarbonyl levels and inhibits AGEs formation by binding to MG *in vivo* [7]. Herbs and dietary supplements have long been used for glycaemic control in diabetes [8]. Recently, there has been greater interest in identifying natural products with anti-glycation properties. *Allium sativum*, commonly known as garlic, has been used as a flavouring agent, functional food and in folklore medicine for many centuries [9]. Aged garlic extract (AGExt) possesses potent antioxidant activity. It is aged for 20 months from natural garlic in order to reduce its harsh, irritating taste and smell. Nevertheless, this AGExt has a higher concentration of sulfur-containing compounds such as alliin and *S*-allyl cysteine (SAC) that are potent antioxidant and free radical scavengers [10]. The essential ingredient from AGExt responsible for the anti-glycation properties of AGEs was SAC, which proved an effective inhibitor of AGEs. Furthermore, studies have shown that four organosulfur compounds derived from garlic, diallylsulfide, SAC, and *N*-acetylcysteine (NAC), protect LDL against oxidation and glycation and may, therefore, explain why garlic protects against cardiovascular disease [11].

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II. Materials and Methods

A. Materials and Chemicals

SAC and NAC were supplied by Wakunaga Pharmaceutical Company, Tokyo, Japan. Three synthesized mimic compounds (**A**, **B**, and **C**) based on the structures of SAC and NAC are shown in Fig 1. Compound A contains a benzyl replacement for the allyl group in SAC. Compound B contains a protected carboxylic acid moiety to compare with NAC. Compound C contains an alternative sulfur protecting group as a comparison to compound A.

Lysozyme and MG were purchased from Sigma, UK. Silver staining kit was obtained from Bio-Rad, UK. Sodium dodecyl sulphate was obtained from ICN Biomedical Incorporation, Ohio, USA.

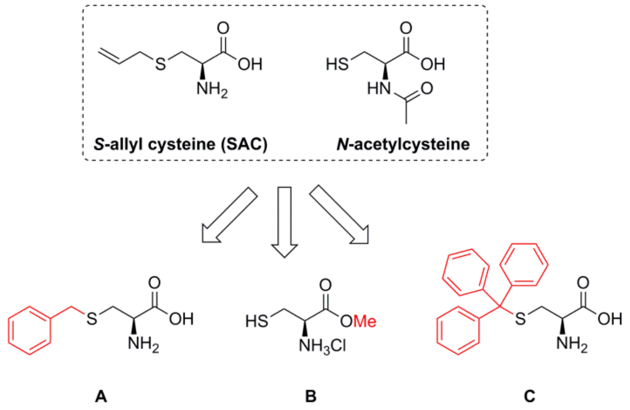

Figure 1. Structure of compounds A, B and C derived from SAC and NAC

B. In Vitro Glycation of Proteins

Lysozyme (10 mg/ml) was incubated in 0.1 M MG ± 0.25 - 250 µg/ml SAC & NAC mixture and the synthesised mimic **compound A** respectively, in 0.1 M sodium phosphate buffer containing 3.0 mM sodium azide, pH 7.4 at 37 ℃ for 1 and 3 days, respectively.

C. Analysis of Crosslinked Advanced Glycation End Products

Glycated proteins were assessed by using 15% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) [12]. Following SDS-PAGE, silver staining was carried out according to the manufacturer's recommendations using the silver stain kit from Bio-Rad. Briefly, the gel was fixed with gentle agitation in a solution of 40% (v/v) methanol, 10% (v/v) acetic acid and 10% (v/v) fixative enhancer concentrate. After decanting the fixing solution, the gel was rinsed in 400 mL deionised H_2O for 40 min with three changes of water after every 10 min. The gel was then stained in the staining and developing solution for 20 minutes. The staining reaction was stopped by placing the gel in a 5% (v/v) solution of acetic acid. This was followed by rinsing the gel in deionised H_2O for 5 minutes. Protein incubated under the same conditions without the addition of sugars and inhibitors was used as a negative control. 1 µL of protein was mixed with 9 µl treatment buffer, and then boiled for 10 min, and then loaded into the wells followed by 2 µL of bromophenol blue and then subjected to electrophoresis using the mini-Protean® 3 apparatus (Bio-Rad Laboratories, Hemel Hempstead, UK).

D. Imaging SDS-PAGE Gels

The gels were photographed using ChemiDoc™ Touch Imaging System (Bio-Rad, UK). All the bands were compared within the same gel. Integrated Density (I.D.) was measured to analyse the one-dimensional electrophoretic gels and computed using the following formula:

I.D. = N × (mean – background). Where N is number of pixels in the selection and the background is the modal grey value (most common pixel value) after smoothing the histogram. Sufficient background was included in the selection to avoid errors.

E. Percentage Inhibition of Cross-linked AGEs

This was calculated using the following formula:

100 × (I.D without inhibitor - I.D with inhibitor) / I.D without inhibitor.

F. Statistical Analysis

Data are reported as the mean ± standard deviation (SD). Student's t-test was performed to test the statistical significance. Values were considered significant with a p-value <0.005. All the values (mean, SD and p-value) were calculated using Microsoft® Excel 2013. Statistical significance between groups was tested by t-tests and one-way analysis of variance (Oneway ANOVA). The analysis was performed by the statistical package IBM SPSS Statistics for Windows, version 22.0 (IBM).

III. Results and Discussion

A. Effect of SAC and NAC on Inhibition of Methylglyoxal-derived AGE Cross-link Formation

Fig. 2 and Fig. 3 showed that the effect of SAC and NAC on inhibition of MG-derived AGEs cross-link formation. SAC and NAC are cysteine-derived amino acids present at a notably higher concentration in AGExt compared to raw garlic. Additional studies were carried out to find whether SAC and NAC could be responsible for the inhibition of AGE formation observed in the glycation studies with AGExt. The results showed that the effects of SAC and NAC are best visible in the dimer band which was used to calculate the percentage inhibition relative to lysozyme glycation. Lysozyme incubated in the presence of glucose produces sufficient cross-linked AGEs. Cross-linking of AGEs causes formation of dimers with an approximate molecular weight of 28.6 kDa. The glycated lysozyme (Fig. 2 A and Fig. 3 A, lane 2) was used as the positive control and native lysozyme was used as negative control (Fig. 2 A and Fig. 3 A, lane 1). The inhibition of AGEs by SAC and NAC showed a reduction in the intensity of the dimerized lysozyme band (Fig. 2 A and Fig. 3 A, lanes 3-9). Image analysis of the gel was performed and the results based on the integrated density of each band are presented in Fig. 2 B and Fig. 3 B. Integrated densities of bands within the same gel were compared for image analysis. SAC inhibited the formation of cross-linked AGEs in a dose-dependent manner (Fig. 2 B). A 44.72% inhibition in the formation of cross-linked AGEs was observed at a 100µg/ml concentration of SAC. This increased to a 56.9, 64.3 and 74.6% inhibition at SAC concentrations of 150, 200 and 250 µg /ml respectively. NAC was a more potent inhibitor of the formation of cross-linked AGEs (Fig. 3 B). A 49.8% of inhibition in the formation of cross-linked AGEs was observed at a 50 µg/mL. This increased to 68.3, 75.9 and 85.8% inhibition at concentrations of 150, 200 and 250 µg/mL of NAC respectively. The inhibition of formation of cross-linked AGEs achieved at 50 µg/ml of NAC was significantly ($p<0.005$) higher than that achieved at 100 µg/mL of SAC. The results represent one of the two independent experiments. The bar chart was made from the average of two independent experiments that showed similar results.

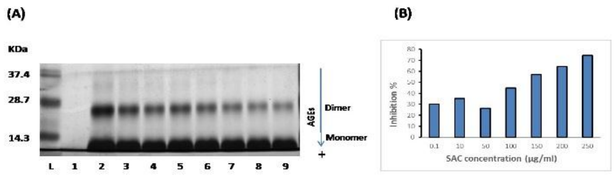

Figure 2. Effect of different SAC concentrations on MG-derived AGE formation; (A) Gel showing lysozyme (10 mg/ml) incubated alone (lane 1) or in the presence of 0.1 M methylglyoxal for 1 day and the effect of 0, 1, 10, 50, 100, 150, 200 and 250 µg /ml of SAC (lanes 2-9 respectively) on dimerization. The cross-linked AGEs were analysed using SDS- PAGE and stained with silver stain. (B) Bar chart show the percentage inhibition of cross-linked AGEs at different concentrations of SAC.

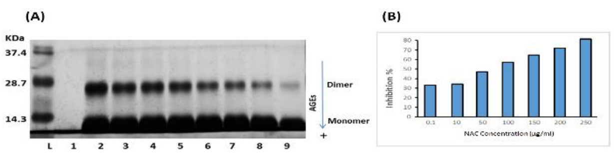

Figure 3. Effect of different NAC concentration on MG-derived AGE formation; (A) Gel showing lysozyme (10 mg/ml) incubated alone (lane 1) or in the presence of 0.1 M methylglyoxal for 3 days and the effect of 0, 1, 10, 50, 100, 150, 200 and 250 µg /ml of NAC (lanes 2-9 respectively) on dimerization. The cross-linked AGEs were analysed using SDS-PAGE and stained with silver stain. (B) Bar chart showing the percentage inhibition of cross-linked AGEs at different concentrations of NAC.

B. *Effect of SAC Alone, NAC Alone and SAC and NAC Mixture on the Formation of Cross-linked AGEs*

The experimental data on the effect of SAC alone, NAC alone and SAC and NAC mixture incubation with different incubation times (1 day and 3 days) on the formation of cross-linked AGEs are shown in (Fig. 4). SAC and NAC mixture was also tested to determine its role in the inhibition of cross-linked AGEs. This experiment was designed to compare in parallel the effects of SAC alone, NAC alone and SAC and NAC mixture *in vitro* cross-linked AGEs formation. Incubation of lysozyme with MG produced sufficient cross-linked AGEs to cause the formation of dimers with an approximate molecular weight of 28 kDa. The glycated lysozyme (Fig. 4 A and C, lane 2) was used as the control and clearly showed reduced electrophoretic mobility with a higher molecular weight as compared with native lysozyme (Fig. 4 A and C, lane 1). The results showed that the SAC and NAC mixture incubated for 3 days provided a more potent inhibition of the formation of cross-linked AGEs (Fig. 4 C, lanes 5 and 8) than SAC alone and NAC alone (Fig. 4 C, lanes 4, 6 and 7). The formation of cross-linked AGEs *in vitro* was significantly ($p< 0.001$) inhibited by the SAC and NAC mixture as compared to SAC alone and NAC alone (Fig. 4 B and D) and this inhibition was dependent on SAC and NAC mixture as optimum (62.7%) inhibition was observed in the sample with 0.5 µg/mL of SAC and NAC mixture while the percentage inhibitions produced by SAC alone were 24% and 17.5% of NAC alone. As a result, the effect of SAC and NAC mixture produces a stronger inhibition and in a dose-dependent manner. The results represent one of two independent experiments. The bar chart was made from the average of two independent experiments that showed similar results.

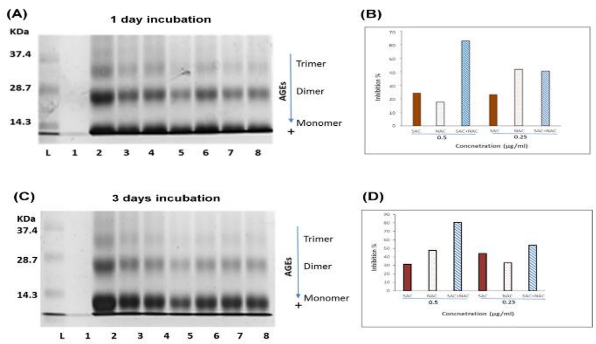

Figure 4. Effect of SAC alone, NAC alone and SAC and NAC mixture for 1 and 3 days' incubation on the formation of cross-linked AGEs; gels showing lysozyme (10 mg/ml) incubated alone (lane 1) or in the presence of 0.1 M MG (lane 2) in 0.1 M sodium phosphate buffer, pH 7.4 at 37 °C for 1 day (A) and 3 days (C). SAC 0.5 µg/ml (lane 3), NAC 0.5 µg/ml (lane 4), SAC and NAC mixture 0.25µg/ml each (lane 5), SAC 0.25 µg/ml (lane 6), NAC 0.25 µg/ml (lane 7) and SAC and NAC mixture 0.125µg/ml each (lane 8). The cross-linked AGEs were analysed using SDS-PAGE and stained with silver stain. Bar charts show the percentage inhibition of cross-linked AGEs in different concentrations of SAC alone, NAC alone and SAC and NAC mixture for 1 day (B) and 3 days (D).

C. *Effect of SAC and NAC Mixture and Compound A Alone on the Formation of Cross-linked AGEs*

Comparison of the effect of SAC and NAC mixture and **compound A** alone on cross-linked AGEs formation *in vitro* is shown in (Fig. 5 A). Incubation of lysozyme with MG generates subunits of cross-linked AGEs that cause the formation of dimers with an approximate molecular weight of 28 kDa as determined by molecular weight markers (Fig. 5 A, lane L). The glycated lysozyme (Fig. 5 A, lane 2) was the positive control and clearly showed reduced electrophoretic mobility as compared with native lysozyme (Fig. 5 A, lane 1). However, proteins that were incubated with MG at 0.1 M for 3 days in the presence of SAC and NAC mixture, and **compound A** alone (Fig. 5 A, lanes 3-8) were found to inhibit AGE formation causing a reduction in the intensity of the dimerized lysozyme band. Image analysis of the gel was performed and the results based on integrated density of each band are presented in Fig. 5 B. Integrated densities of bands within the same gel were compared for image analysis. Results showed that both SAC and NAC mixture and **compound A** alone were significant ($p<0.001$) in reducing MG-derived cross-linked AGEs *in vitro*. **Compound A** alone showed a significant ($p<0.001$) inhibition of AGEs formation at 0.5 and 5 µg/mL. On the other hand, SAC and NAC mixture showed only a non-significant inhibition between concentrations of 0.5 – 5 µg/mL. The percentage inhibitions of 0.25, 5 and 5 µg/mL of compound A alone were 14, 25 and 71.4% respectively, while the percentage inhibitions by SAC and NAC mixture were 6.5, 9.5 and 21.8% respectively, at the same tested concentrations. Compound **B** and **C** did not show any significant inhibition of glycation (data not shown). Therefore, the inhibition of SAC and NAC mixture and **Compound A** alone occur in a dose-dependent manner. At all tested concentrations, **Compound A** showed a stronger inhibitory effect than SAC and NAC mixture in MG-derived cross-linked AGEs in vitro, (Fig. 5 B). The results represent one of the two independent experiments. The bar chart was made from the average of two independent experiments that showed similar results.

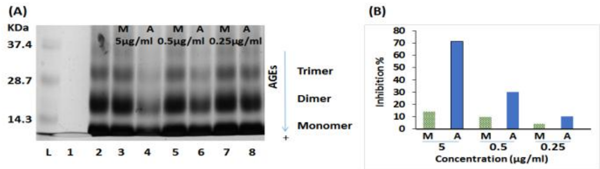

Figure 5. Effect of different concentrations of SAC and NAC mixture and mimic **compound A** alone on MG-induced cross-linked AGEs formation; (A) Lysozyme (10 mg/ml) was incubated for 3 days alone (lane 1) and in the presence of 0.1 M MG (lane 2) in 0.1 M sodium phosphate buffer, pH 7.4 at 37 ℃ and the effect of different concentrations of SAC and NAC mixture and Mimic **compound A** alone (5, 0.5 and 0.25 µg/ml, lanes 3-4, 5-6 and 7-8 respectively). The cross-linked AGEs were analyzed using SDS-PAGE followed with silver stain. (B) Bar chart shows the percentage inhibition of cross-linked AGEs in different concentrations of SAC and NAC mixture and Mimic **compound** A alone. (M: SAC and NAC mixture, A: Mimic **Compound A** alone).

IV. Conclusion

In the present study, protein glycation was established *in vitro* by incubation of proteins with glycating agents in order to accelerate the reaction rates to detect AGEs formation over a short period of time at physiological pH. MG was used as the glycating agent in this study because α-oxoaldehydes are formed by fragmentation and dehydration of hexoses and Amadori products, and the concentration of MG is increased in diabetes. Lysozyme is a good model protein for the measurement of cross-linked AGEs formation, as oligomerisation occurs immediately and is easily detectable by SDS-PAGE. The data presented in this study indicate that anti-glycation activity of AGExt is mainly due to organo sulfur compounds present. SAC, NAC are inhibitors of glycation, and they may prevent the oxidative stress associated with the formation of AGEs and implicated in the diabetic complications. In this study, we chose a mimic **compound A**, due to its similar structure but greater effectiveness than SAC and NAC. The anti-glycation properties of **Compound A** may offer greater therapeutic potential compared to other AGEs inhibitors.

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