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Effective inhibition of protein glycation by combinatorial usage of limonene and aminoguanidine through differential and synergistic mechanisms

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ABSTRACT

Protein glycation is a major mechanism for establishing secondary complication in diabetes mellitus. Effective inhibition of this process can prevent progression of the disorder into secondary complications. Aminoguanidine (AMG) and limonene (LM) are known protein glycation inhibitors. The aim of the present study was to demonstrate their differential mechanisms of action and to study whether combinatorial therapy can act synergistically and lower dosage, and thereby lower toxicity in treatment of secondary complications in diabetes. Glycation in the presence of 2 M urea was inhibited by 23% with AMG and by 66% with LM. AMG is more effective than LM in reducing protein carbonyl formation. SPR studies revealed binding of LM reduces affinity of BSA for glucose. LM demonstrated an increase by 2 °C in thermal transition in DSC studies as against reduction by 0.4 °C by AMG proving that LM can effectively stabilize the protein structure. Combinatorial treatment of AMG and LM prevented α-helix to β-sheet transitions in BSA at 100 μM and inhibited AGE related fluorescence and pentosidine formation by 80 and 90% respectively. The combination can reduce dosage of AMG by almost twenty times, paving the way for effective protein glycation inhibition without toxicity.

1. Introduction

Glycation of proteins is a major cause of establishing secondary complications in diabetes like vasculopathy, retinopathy, nephropathy and neuropathy [1]. Protein glycation leads to generation of advanced glycation end products (AGEs) that disrupt molecular conformation of proteins, alter enzymatic activity, reduce their degradative capacity, and result in abnormal recognition and clearance by receptors [2–5]. Effects of AGEs are mediated through receptors for advanced glycation end products (RAGE). AGE binding to RAGE reportedly induces proinflammatory and procoagulant cellular responses, resulting in activation of NF-κB and it is implicated in the pathogenesis of several diseases including, diabetes, Alzheimer's disease, and renal failure [6].

Considering the pathological implications of glycation, it becomes imperative to design and discover inhibitors of protein glycation that can help to prevent establishment of secondary complications [7]. Protein glycation is a complex process that is influenced by many determinants such as metal ions, α-dicarbonyls and oxidative stress. Several inhibitory mechanisms have been proposed and different compounds are studied as candidates for glycation inhibition [8]. AMG and pyridoxamine are the well studied antiglycating agents and have been tested in clinical trials. AMG is reported to inhibit protein glycation by competition, scavenging α-dicarbonyls, and antioxidant activity by virtue of its guanido group [9]. Placebo-controlled clinical trials were conducted using AMG in Type I and Type II diabetes for the prevention of diabetic nephropathy. In these trials decrease in proteinuria and a delay in progression of retinopathy were observed [10,11]. The trials also noted that upon long term treatment, some patients developed toxic symptoms like myeloperoxidase and anti-neutrophil antibodies [12] and a small number of subjects developed glomerulonephritis [10]; however, similar observations were also noted in the control group. Despite the lack of adequate information on its toxicity, AMG was withdrawn from the market without attempt-

Abbreviations: AMG, aminoguanidine; ALM, aminoguanidine-limonene equimolar combination; AGEs, advanced glycation end products; LM, limonene; RM, reaction mixture.

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ing large scale studies. AMG being a competitive inhibitor, it was required to be used in large quantities for it to be effective and the observed toxicity is mainly due to such very large doses because of which, an effective and useful drug had to be withdrawn from human use.

We have earlier reported LM as a potent AGE inhibitor [13]. LM was found to bind BSA and prevent conformational changes thus preventing glycation of proteins. Chaotropic agents such as urea lead to opening of the protein structure and hence favor protein glycation. In the present study, the differential effects of LM and AMG on protein glycation in the presence of urea is studied. It is also attempted to study the binding kinetics of AMG and LM to BSA and further with glucose using surface plasmon resonance (SPR). Differential scanning calorimetry (DSC) was performed to assess the effect of LM and AMG on thermal stability and to demonstrate their differential mechanism of action.

A reduction in the inhibitor concentration is very desirable and can effectively eliminate the toxic effects observed at higher doses. Further, if LM and AMG have different mechanisms of protein glycation inhibition, a combinatorial therapy could, in principle, effectively lower the doses of both compounds and prove more effective.

2. Materials and methods

2.1. Chemicals and reagents

LM (97% pure) and AMG were purchased from Sigma–Aldrich (St. Louis, MO, USA), bovine serum albumin fatty acid free (fraction V), sodium azide, and other chemicals purchased from Himedia (Mumbai, MS, India) were of analytical grade.

2.2. In vitro glycation of bovine serum albumin

Each 5 ml reaction mixture contained 40 mg ml^{-1} of bovine serum albumin in 50 mM phosphate buffer (pH 7.4), 250 mM D-glucose, 0.01% sodium azide and 25 μM, 50 μM, 100 μM of test molecules i.e. LM and AMG. For combined inhibitory action equimolar concentrations of LM and AMG in the range (25 μM, 50 μM and 100 μM) were used. Each reaction mixture was preincubated with test molecule for 10 min before addition of glucose. Reaction mixtures were incubated in the dark at 37 °C for 3 weeks. At the end of the incubation, unbound glucose was removed by extensive dialysis against the same buffer. Protein concentration was determined by UV–visible spectrometry at λ_{280nm} using BSA as standard. AGE related general fluorescence was measured using 10 mg ml^{-1} protein in 50 mM sodium phosphate buffer (pH 7.4) at excitation wavelength 370 nm and emission wavelength 440 nm while pentosidine specific fluorescence was measured at excitation wavelength 335 nm and emission wavelength 385 nm using a spectrofluorometer Jasco model FP 750 (Jasco Inc., Easton, MD, USA).

2.3. Stabilizing effect of LM against urea denaturation

Stabilizing effect of LM on BSA against physiological concentration of chaotropic agent urea was studied. A total of eight types of reaction mixtures were prepared: reaction mixture **I** native BSA alone as a normal control, reaction mixture **II** [BSA + glucose], reaction mixture **III** [BSA + glucose + AMG], reaction mixture **IV** [BSA + glucose + LM], reaction mixture **V** [BSA + urea + glucose], reaction mixture **VI** [BSA + urea + glucose + AMG], reaction mixture **VII** [BSA + urea + glucose + LM] and reaction mixture **VIII** [BSA + urea + glucose + AMG + LM combination concentration]. For all reaction mixtures, the following concentrations were used: BSA: 10 mg ml^{-1}; glucose: 250 mM; LM: 100 μM; AMG 100 μM; and urea 2 M. Solutions were prepared in phosphate buffer (50 mM, pH 7.4). Reaction mixtures containing LM were preincubated with it for 10 min before addition of glucose and urea. The solutions were incubated for 7 days at 37 °C. After incubation, Na-borohydride reduction of each solution was carried by a previously described method [14]. Briefly, after completion of incubation, reaction mixtures were dialyzed extensively against about 4000 volumes of phosphate buffer (50 mM, pH 7.4) at 4 °C overnight to remove free glucose. Protein concentration of dialysate was determined by Lowry's method using BSA as standard [15]. The dialyzed protein was reduced for 4 h at room temperature by addition of a 500-fold molar excess of Na-borohydride, and the reaction was terminated by slow addition of 1 N HC1 to destroy excess borohydride. Fructosamine was measured by nitro blue tetrazolium (NBT) method.

2.4. Measurement of fructosamine by NBT method

The fructosamine assay was performed by the method of Johnson et al. [16] with slight modifications. The reaction mixture which contained 0.2 ml in vitro glycated sample or diluted serum sample and 0.8 ml nitro blue tetrazolium (NBT) reagent (300 mM) in sodium carbonate buffer (100 mM, pH 10.35) was incubated at room temperature for 15 min, and the absorbance was read at 530 nm against a blank. Results were expressed as fructosamine mM L^{-1} protein using an extinction coefficient of 12,640 M^{-1} cm^{-1} for monoformazan.

2.5. Biacore analysis

2.5.1. Biosensor surface preparation

Binding affinity of LM and AMG interaction was studied using Biacore T200 Instrument (GE Healthcare, USA). Bovine Serum Albumin was immobilized on sensor chip Series CM5 (GE Healthcare, USA). A reference surface, to which no ligand was bound, was included on chip. BSA was immobilized to the CM5 gold surfaces by amine coupling as previously described [17]. Briefly, the BSA to be immobilized (50 μg ml^{-1} in 50 mM sodium acetate, pH 4.5) was injected onto the activated CM5 surface at 10 μl min^{-1} (PBS running buffer: 10 mM phosphate, 130 mM NaCl, 0.27 mM KCl, 0.005% surfactant P20, pH 7.4). The surfaces were then deactivated by passage of 1 M ethanolamine. Biosensor surfaces were coupled to final resonance values of ~12,000 response units (RU) for BSA.

2.5.2. Acquisition of kinetic binding data

Various concentrations of LM and AMG (100–1000 μM) were injected during the association phase for 100 s (30 μl min^{-1}), with PBS as the running buffer. The dissociation phase, initiated by passage of PBS alone, was carried out over a period of 120 s. The biosensor surfaces were regenerated by a 30 s injection of 10 mM Glycine–HCl pH 2. Samples were injected in duplicate in random order in at least three separate experiments. Kinetic data were analyzed using the Biacore T200 evaluation software, version 2.0. All binding curves were corrected for background and bulk refractive index contribution by subtraction the contribution from reference flow cells. Models were fitted both globally across the data sets and for a single concentration. Models used were 1:1 binding interaction describing 1:1 binding between analyte (A) and ligand (B) ($A + B \leftrightarrow AB$), and or a two-state reaction (conformational change) model, based on a 1:1 binding of analyte to an immobilized ligand followed by a conformational change ($A + B \leftrightarrow AB \leftrightarrow AB^*$). The 1:1 stoichiometry for the interaction between BSA and analytes was previously established [18].