Review article

Advanced Glycation Endproducts, (AGEs): Formation, complication and pharmacological evaluation to its inhibition.

Abstract:-

Glycation sometimes called non enzymatic glycosylation is result of sugar molecules bonding to a protein or lipid molecule without controlling action of an enzyme. During the process of glycation, early glycation products are formed first, which subsequently rearrange into final AGE structures through a series of very complex chemical reactions and formed (MOLD), (GOLD) and (DOLD). AGEs are implicated in many age related diseases such as type–II (diabetic mellitus), cardiovascular disease (the endothelial cell, collagen, fibrinogen are damaged), Alzheimer diseases (amyloid protein are side product of the reaction progressing to AGEs), Cancer (acryl-amide and other side product are related), peripheral neuropathy (the myelin is attached), and other sensory losses such as deafness (due to demyelination),and blindness (mostly due to micro-vascular damage in the retina),this range of diseases is the result of very basic level at which glycation interfere with molecular and cellular functioning throughout the body. Pharmacologically influence the process of non-enzymatic glycation and AGE product formation Inhibit the formation of AGEs are purported to have therapeutic potentials in patients with diabetes and age-related diseases. The oxidation process is believed to play an important role in AGEs formation The best cross-link inhibitors currently available are carnosine, aminoguanidine, metformin and acarbose, whereas others are now becoming available. No cross-link breakers are commercially obtainable as yet, but these will be marketed within 2-3 years. Soon after, combinations of inhibitors and breakers are due to follow.

Key words: - AGEs, MOLD, GOLD, Amadori reaction, NEG, CML (carboxyl methyl lysine), β-amyloid.

INTRODUCTION:-

The aldehyde or ketone groups of reducing sugars react non-enzymatically with the free amino groups of proteins, lipids and nucleic acids leading to the formation of advanced glycation end products (AGEs)[1]. In this reaction, sugars react reversibly with the free amino group of proteins to form unstable Schiff bases, which then undergo an intra molecular rearrangement to form a stable Amadori product. These Amadori products are believed to undergo a series of reactions to form heterogeneous complex fluorophores and chromophores collectively referred to as advanced Maillard products or advanced glycation end products (AGEs)[2]. The production of these AGEs are implicated in many age related diseases such as type–II (diabetic mellitus), cardiovascular disease (the endothelial cell, collagen, fibrinogen are damaged), Alzheimer diseases (amyloid protein are side product of the reaction

progressing to AGEs), Cancer (acryl-amide and other side product are related), peripheral neuropathy (the myelin is attached), and other sensory losses such as deafness (due to demyelination),and blindness (mostly due to micro-vascular damage in the retina),this range of diseases is the result of very basic level at which glycation interfere with molecular and cellular functioning throughout the body[3] . An important part of tissue damage and of cell death associated with chronic hyperglycemia, and diabetes is mediated by free radicals. E.C.M. (Extra cellular matrix), proteins such as collagen, elastin, actin, and myosin are the backbone for architectural and functional stability of tissues cell and organs. When AGEs accumulations particularly high in E.C.M., proteins are result in intra and inter molecular cross-linking and later has been hypotized to stiffening of these proteins and believed to play an important role in etiology of various AGEs related diseases[4]The present review will focus on AGEs, related complications and on their inhibition by various therapeutic compounds.

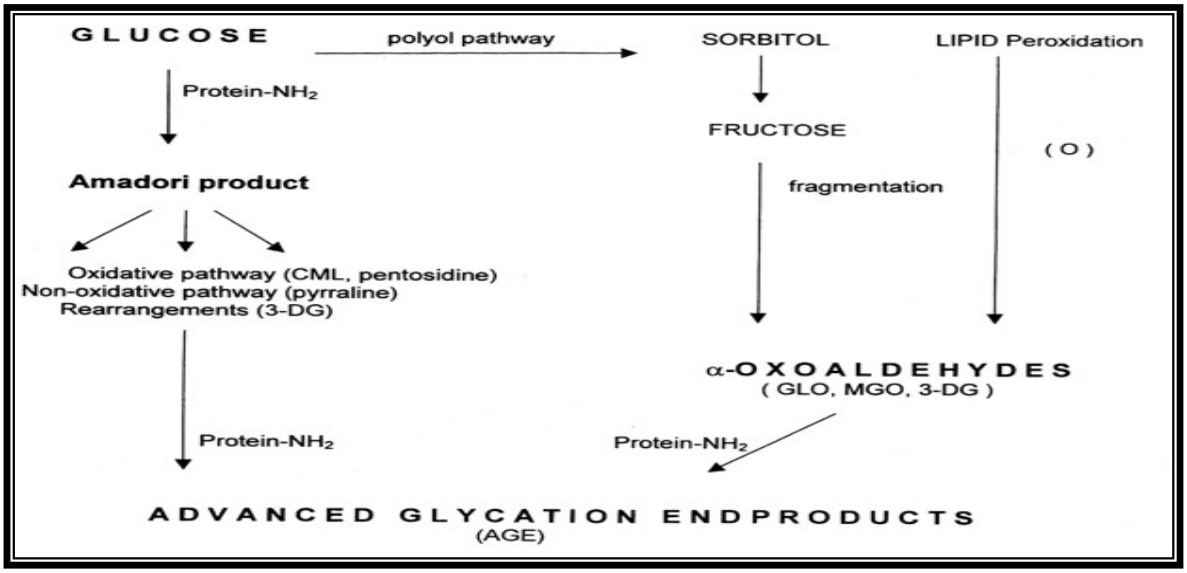
Biochemistry of Non-enzymatic glycation

Non-enzymatic glycation is a process by which glucose is chemically bound to amino groups of proteins but without the help of enzymes. It is a classical covalent reaction in which, by means of N-glycoside bonding, the sugar-protein complex is formed through a series of chemical reactions described by a chemist Maillard. Maillard reactions are complex and multi-layer, and can be analyzed in three steps. The sugar-protein complex is formed first (Amadori rearrangement). It is an early product of non-enzymatic glycation, an intermediary which is a precursor of all later compounds. The second step includes the formation of numerous intermediary products, some of which are very reactive and continue with glycation reaction. The third, final phase consists of polymerization reaction of the complex products formed in the second step, whereby heterogeneous structures named advanced glycation end products (AGE) are formed[5]. It was believed that the primary role in Maillard reactions was exclusively played by high glucose concentration. However, recent data show that, in spite of the fact that sugars are the main precursors of AGE compounds, numerous intermediary metabolites[6], i.e. alpha-oxo-aldehydes, also creatively participate in nonenzymatic glycation reactions. Such intermediary products are generated during glycolysis (methylglyoxal) or along the polyolic pathway, and can also be formed by auto-oxidation of carbohydrates (glyoxal). Alpha-oxo-aldehydes modify AGEs surprisingly fast, in contrast to classical Maillard reactions, which are very slow (Fig. 1).

Figure 1:-. **Schematic presentation of potential pathway leading to AGE formation**

a. AGE arise from decomposition of Amadori products
b. fragmentation products of polyol pathway
c. as glycol-oxidative products,
d. which all react with amino groups of protein
e. which all react with amino groups of protein

Fig no -1 Glycation reaction



GLO=glyoxal; MGO=methylglyoxal; 3-DG=3-deoxyglucosone; CML=carboxymethyl-lysine

In physiological conditions, glycation can be detected in the process of aging, and the reactions are significantly faster and more intensive, with frequently increased glucose concentrations. In diabetology, the importance of these processes manifests in two essential issues: 1) effect of protein glycation on the change of their structure and function, and 2) use of glycated protein level as a parameter of integrated glycemia[7]. A classical example of non-enzymatic glycation is the formation of glycated hemoglobin, or more precisely, HbA1c. As the degree of non-enzymatic glycation is directly associated with the level of blood glucose, the percentage of HbA1c in diabetes can also be greatly increased. HbA1c was the first glycated protein studied, however, soon it was discovered that other various structural and regulatory proteins also are subject to non-enzymatic glycation to form glycation end products[8].

Types of Advanced glycation end products (AGEs)

During the process of glycation, early glycation products are formed first, which subsequently rearrange into final AGE structures through a series of very complex chemical reactions. Protein modification with AGE is irreversible, as there are no enzymes in the body that would be able to hydrolyze AGE compounds[9]. These structures then accumulate during the lifespan of the protein on which they have been formed. In some cases oxidation is also involved, so that it is possible to distinguish between compounds formed by glycation by others formed by glycoxydation. From glucose the non oxidative pathway could give rise to pyrraline; in the oxidative pathway to pentosidine and N6-carboxymethyllysine (CML) [10]. Glyceraldehyde can also be involved. It is formed from glyceraldehyde-3-phosphate, an intermediate of glycolysis, through the polyol pathway, or from fructose, during its transformation by fructokinase. A glyceraldehyde derived AGE is the so called glyceraldehyde-derived pyridinium compound (GLAP), a compound that has been seen to induce oxidative cellular dysfunction. Glyceraldehyde derived AGEs have been shown initially in AD brain and in the cytosol of neurons [11]. Later, GLAP has been detected in the plasma protein and in collagen obtained from streptozotocin-induced diabetic rats [12]. When glycol-oxidation occurs, new compounds are formed, such as MG and glyoxal. These in turn can also react with proteins. In this case MG reacts mainly with Arg, less so with Lys and Cys (contrary to what occurs in the glycation with glucose). One compound obtained is CML, formed from fructoselysine, one of the Amadori products, in the presence of metal ions. However, now CML is suggested to be a marker of oxidation rather than of glycation, as it can also be formed during lipid per-oxidation besides malondialdehyde and hydroxynonenal adducts to lysine. Moreover, the methylglyoxal-lysine dimer (MOLD), the glyoxallysine dimer (GOLD) and the deoxyglucosone-lysine dimer (DOLD), argpyrimidine and its tetrahydroderivative) are also formed (fig-2). Other compounds formed are pentosidine and vesperlysines (A, B, C). Pentosidine derives from lysine and arginine. It has been found in several tissues, such as plasma and erythrocytes. The pentose which is mainly used appears to be ribose. Vesperlysines A has been shown in the lens of diabetic subjects [13]. It derives from ascorbate, ribose and threose. Pyrraline is also formed from 3-deoxyglucosone and lysine.

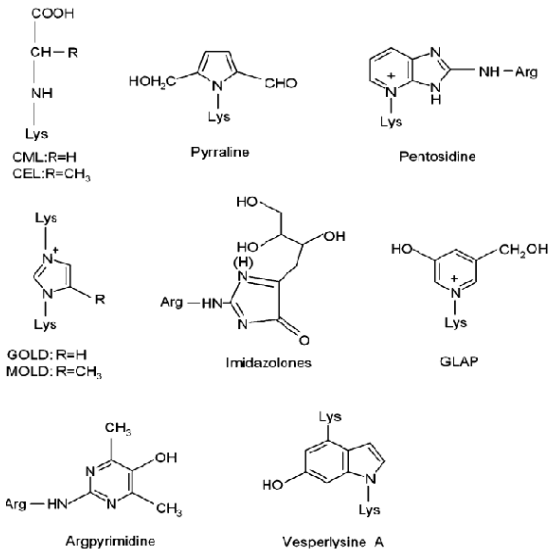


Fig. 2. Chemical structure of various AGEs: *CML* (N-carboxymethyllysine); *CEL* (N-carboxyethyllysine); *GOLD* (glyoxal-lysine dimer); *MOLD* (methylglyoxal-lysine dimer); *GLAP* (glyceraldehyde-derived pyridinium compound); vesperlysine A

AGEs, and oxidation

An important part of tissue damage and of cell death associated with chronic hyperglycemia, and diabetes is mediated by free radicals. In hyperglycemic diabetic patients, exaggerated oxidative stress is due both to an excess in free oxygen species production, secondary to increased oxidation of substrates (sugars, non-saturated fats, and glycated proteins), to increased glucose auto-oxidation, and to a decrease in antioxidants. In animal models of diabetes, hyper-production of free radicals is responsible for endothelial dysfunction, *via* a decrease in NO (nitric oxide) production, thus decreasing vasorelaxation of smooth muscle cells [14]. The links between oxidative stress and AGEs may explain in part the relation between hyperglycemia and both endothelial dysfunction and tissue damage. Oxidized LDL is responsible for decreased NO production, by a reduction in NO synthtase [15]. AGEs quench the NO, and thus contribute to defective vasodilatation observed in animal models. AGEs induce apoptosis in cultured human umbilical vein endothelial cells [16]. Experimentally, we have shown that the interaction between AGEs and RAGE induces an activation of oxidative stress, and stimulates the production and release of cytokines, which amplifying thus tissue damage [17].

AGE receptors

The level of AGE proteins reflects kinetic balance of two opposite processes: the rate of AGE compound formation, and the rate of their degradation by means of receptors. AGE receptors participate in the elimination and change of aged, reticular and denatured molecules of extracellular matrix as well as of other AGE molecules. However, in diabetes mellitus AGE protein accumulation may exceed the ability of their elimination due to chronic hyperglycemia and excessive glycation process[18].

The first structures were identified as possible AGE receptors using radiolabelled AGE proteins. Human and murine monocytes, lymphocytes bind specifically AGEs with a dissociation coefficient between 50 and 200 nmol/L. Receptor proteins which bind AGEs, have been isolated from cell membrane and have been purified. They have different apparent molecular weights according to the cell type: 40 KD for kidney, 36-83 KD for macrophage cell line, 60-90 KD for liver cells. AGEs binding protein have been purified from endothelial cells and characterized. Two polypeptides were obtained from pulmonary endothelial cells, one was described as the receptor for AGEs (RAGE) and the second has a very high homology to lactoferrin (LFl) [19] .RAGE in a truncated form has a molecular weight of 35 Kd and belongs to the immunoglobulin super-family. RAGE gene is located on chromosome six in the MHC region (6p 21-3).Human, rat and bovine RAGE have a high degree of homology, but slight differences in glycosylation sites and susceptibility to proteases may explain their different pharmacological parameters [20]. RAGE has also some homology with molecules of the immunoglobulin super-family (MUC, CD20). RAGE is expressed by different cell types: monocyte/ macrophage, T-lymphocytes, endothelial cells, smooth muscle cells, mesangial cells, neuronal cells. RAGE expression is potentiated by hyperglycemia or TNF-α treatment. RAGE binds different ligands such as amphoterin, β-amyloid substances or calgranulin polypeptides[21]. Carboxylmethyl lysine (CML) is the AGE which after binding to RAGE, is a stronger inducer of vascular cell adhesion molecule (VCAM-1) [22].

Consequences of engagement of the receptor RAGE

The finding that enhanced expression of tissue factor in AGEs-stimulated macrophages retrieved from (gp91phox) null mice was suppressed compared to wild-type macrophages, strongly suggests important roles for NADPH oxidase in AGEs-mediated processes [23]. Importantly, recent studies indicating that endothelial cells express a gp91phox-containing NADPH oxidase support our hypothesis that activation of this enzyme provides source of ROIs upon AGEs engagement of RAGE in endothelial cells. In those studies by Gorlach *et*

al., it was shown that NADPH oxidase was a major source in the arterial wall, as its activation was associated with impaired bioavailability of endothelium-derived NO [24].RAGE is a multi-ligand receptor of the immunoglobulin super-family. In addition to AGEs, RAGE serves as a cell surface receptor for amyloid β- peptide (A, β), a cleavage product of the β-amyloid precursor protein which accumulates in Alzheimer's disease and β sheet fibrils [25, 26]. *In vivo*, blockade of RAGE in a murine model of systemic amyloidosis suppressed amyloid induced nuclear translocation of NF-kB and cellular activation. RAGE is also a signal transduction receptor for EN-RAGES, and related members of the S100/cal granulin family of pro-inflammatory cytokines. The S100/cal granulin family is comprised of closely-related polypeptides released from activated inflammatory cells, including polymorphonuclear leukocytes, peripheral blood-derived mononuclear phagocytes and lymphocytes. Their hallmark is accumulation at sites of chronic inflammation, such as psoriatic skin disease, cystic fibrosis, inflammatory bowel disease, and rheumatoid arthritis. Ligation of RAGE by ENRAGEs mediated activation of endothelial cells, macrophages and lymphocytes. In parallel with suppression of the inflammatory phenotype, inhibition of RAGE-S100/cal granulin interaction decreased NF-kB activation and expression of pro-inflammatory cytokines in tissues, suggesting that receptor blockade changed the course of the inflammatory response. Previous studies further indicated that RAGE was likely a receptor for amphoterin, a molecule linked to neurite outgrowth in developing neurons of the central and peripheral nervous system [27]. These studies suggested that amphoterin-RAGE was linked to cellular migration and invasiveness. Consistent with this concept, the expression of amphoterin and RAGE is increased in murine and human tumors. Blockade of RAGE *in vivo* suppressed local growth and distant spread of implanted tumors, as well as the growth of tumors forming endogenously in susceptible mice. Consistent with an important role for RAGE-mediated signal transduction in these processes, blockade of RAGE/RAGE signaling on amphoterin coated matrices suppressed activation of p44/42, p38 and SAPK/JNK kinases [28]. In settings characterized by increased accumulation and expression of RAGE and its ligands, such as diabetic atherosclerotic lesions and periodontium, chronic disorders such as rheumatoid arthritis and inflammatory bowel disease, and Alzheimer disease, enhanced inflammatory responses have been linked to ongoing cellular perturbation. One consequence of ligand-RAGE-mediated activation of MAP kinases and NF-kB is increased transcription and translation of vascular cell adhesion molecule (VCAM-1). At the cell surface, endothelium stimulated by a range of mediators, such as endotoxin, tumor necrosis factor α (TNF α), AGEs display increased adhesion of pro-inflammatory mononuclear cells, at least in part, *via* VCAM-1. Recent studies have suggested that the pro-inflammatory effects of VCAM-1 are not limited to cellular adhesion events, as binding of ligand to VCAM-1 in endothelial cell lines and primary cultures induced activation of endothelial NADPH oxidase, a process shown to be essential for lymphocyte migration through the stimulated cells. These findings suggest that activation of RAGE at the cell surface may initiate a cascade of events including activation of NADPH oxidase and a range of pro-inflammatory mediators such as VCAM-1.In diabetes, although oxidant stress responses are essential to eliminate pathogenic periodontal pathogens, ongoing AGE/EN-RAGE-mediated cellular activation in infected periodontium has been linked to increased generation of pro-inflammatory cytokines and tissue-destructive matrix metallo-proteinases, processes leading to destruction of alveolar bone [29]. The various role of AGEs receptors in the pathogenesis of later diabetic complications summarized in table-1.

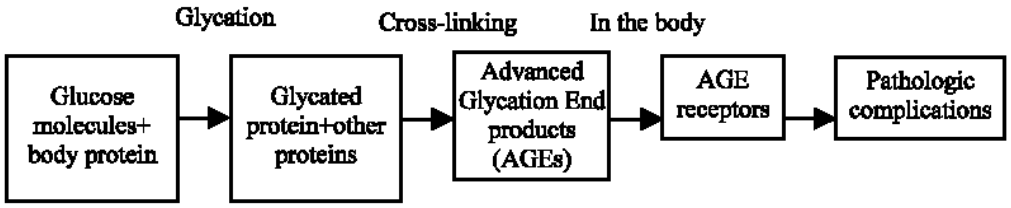


Fig-3:-Formation of AGEs from glycation

Table -1:- Role of AGEs and AGE receptors in the pathogenesis of diabetic complications

Diabetic atherosclerosis

Vascular tissue AGE accumulation → protein crosslinking → oxidative damage
Increased vascular matrix → thickening and narrowing of lumen
Increased endothelial cell permeability and procoagulant activity → thrombosis
Mononuclear cell chemotaxis/activation → cytokine and growth factor release
Increased macrophage uptake of AGE-LDL → atheroma

Diabetic kidney disease

Increased mesangial matrix secretion
Increased basement membrane deposition
Increased vascular permeability
Increased growth factor secretion
Glomerular hypertrophy → glomerulosclerosis

Diabetic retinopathy

Increased cell permeability → vascular leakage and retinal damage
Increased vessel wall thickening → occlusion → retinal
ischemia → neovascularization
Increased intravascular coagulation → occlusion → retinal
ischemia → neovascularization

Diabetic neuropathy

Increased AGEs in vasa nervorum → wall thickening and occlusion
Increased vascular permeability and thrombosis → occlusion → neuronal ischemia
Increased AGE myelin accumulation → myelin damage
Increased macrophage activity → myelin and vascular degeneration

AGEs in diabetic vasculopathy and atherosclerosis

Atherosclerotic cardiovascular disease is the major cause of morbidity and mortality in diabetes. The mechanisms by which diabetes so dramatically increases atherosclerosis are yet poorly understood. AGEs also play a significant role in atherosclerosis. For instance, reticulated and irreversible LDL from the circulation binds to AGE-modified collagen of the blood vessel walls. In the majority of blood vessels, such reticular binding delays normal outflow of LDL particles that have penetrated the vessel wall, thus enhancing cholesterol deposition in the intima. Such AGE reticulation increases lipoprotein deposition regardless of the plasma LDL level. This is followed by an accelerated development of atherosclerosis. [30].It has been well documented that lipids and lipoproteins are deeply involved in the atherogenic process. Diabetes can lead to several lipoprotein modifications that can affect their interaction with arterial wall cells, thereby contributing to the increased risk of atherosclerosis. The modifications of lipoproteins include oxidation and glycation. Approximately 2% to 5% of apo B in the plasma of diabetic persons are glycated, compared with about 1% in the plasma from non diabetic control subjects. AGEs have recently been reported to be associated with LDL, and an elevated level of AGE-LDL was found in patients with diabetes and renal insufficiency as compared with the LDL obtained from normal controls. This observation suggests that the formation of AGE might occur more rapidly than previously believed, or that AGE-LDL may enter plasma from extravascular tissues such as arterial wall. The presence of AGEs on apo B stimulated investigation of the consequences of this modification on LDL metabolism. Glycated LDL interacts poorly with LDL receptor, thereby increasing its residence time in plasma and presumably in the extracellular space of the arterial wall. Furthermore, there is a significant relationship between the extent of apo B-AGE and impairment in the plasma LDL clearance[31]. AGE lipoproteins, like other advanced glycation modified proteins, bind to specific receptors on macrophages and other cell types, and can stimulate the release of cytokines and growth factors which may play a role in atherogenesis. Thus, a reduction in the level of glycation of lipoproteins as well as of the arterial wall extracellular matrix might alter the interaction of lipoproteins with the matrix and reduce their retention in the arterial wall where they are able to exert their atherogenic damage[32].

AGEs and renal failure

Persistent hyperglycemia has a central role in the development of diabetic nephropathy that is clinically manifested by proteinuria progressing to renal insufficiency, and histopathologically by mesangial expansion and glomerular basement membrane thickening30. A possible link between elevated glucose level and diabetic nephropathy

resides in the glycation process producing AGEs. This modification may impair the original function of either protein and may affect normal processes of turnover and clearance. AGEs can induce an excess crosslinking of collagen molecules in the glomerular plasma membrane affecting the assembly and architecture of the glomerular basement membrane and mesangial matrix, and can potentially act on mesangial cells *via* growth factors, causing cells to synthesize more extracellular matrix. All these processes may lead to enhanced deposition of extracellular matrix proteins in the mesangial, interfere with the mesangial clearance of macromolecules, and alter macrophage function, thus contributing to mesangial expansion and glomerular occlusion[33].

Circulating serum AGE level is markedly increased in patients with diabetes and renal insufficiency. Serum AGEs include both serum proteins that have been modified by advanced glycation and low molecular weight AGE peptides. Using specific immunoassay, serum AGE peptide levels have been found to correlate with renal function. In fact, close correlation has been demonstrated between serum AGE levels and creatinine clearance. In normal controls, AGE peptide clearance has been estimated to 0.72 ml/min. Diabetic persons with normal glomerular filtration rate can clear AGE peptides at the same rate. However, progressive loss of renal function is associated with increasing circulating AGE peptide levels. Current renal replacement therapies, hemo-dialysis or peritoneal dialysis, are relatively inefficient in removing AGEs from the serum of diabetic patients. In these patients, AGE peptides persist at up to 8-fold normal level. In contrast, serum AGE peptide levels rapidly decrease and remain within the normal range in patients undergoing kidney transplantation[34].

Skin AGEs levels detected by immunochemistry correlate with severity of nephropathy and increase in early stages of renal involvement[35]. A longitudinal study in type 1 diabetic patients followed during 2.5 years has indicated the predictive value of AGE serum levels for the development of the morphological changes in the kidney[36]. AGEs infusion in normal rats during 5 months results in increased AGEs renal tissue content and in alterations similar to diabetic nephropathy: increase in glomerular volume, in basement membrane thickness and in mesangial extracellular matrix[37]. An effect of AGEs on renal gene expression has been evidenced[38] . Administration of AGE-modified albumin during 4 weeks to normal mice induces glomerular hypertrophy as well as an increase in glomerular extracellular matrix, α1 (IV) collagen, laminin B1 and transforming growth factor β1 (TGF β1) mRNA levels. This response seems to be specific to AGEs because all these changes can be prevented by aminoguanidine co-administration. The role of AGEs in diabetic nephropathy development has been investigated in streptozotocin-induced diabetic rats compared to non diabetic

317 control rats, and diabetic rats co-treated with aminoguanidine [39]. After thirty two weeks, diabetic rats
318 exhibit increased fluorescencein glomeruli and renal tubes, which was prevented by
319 aminoguanidine[40]. Diabetic rats develop albuminuria over the 32-week period[41]. This increase was
320 attenuated by aminoguanidine, but not by antioxidant and by aldose reductase inhibitor[42]. Other
321 inhibitors of renal AGEs accumulation, as ALT-946, are also effective in preventing and retarding
322 diabetic nephropathy in animal models [43]. However, studies with aminoguanidine (pimagedine) are no
323 more in progress in human diabetics at the present time. Treatment with ALT-711 and
324 aminoguanidine, which both attenuate renal AGE accumulation, abrogated these increases in
325 PKC expression. However, translocation of phosphorylated PKC-alpha from the cytoplasm to
326 the membrane was reduced only by ALT-711. ALT-711 treatment attenuated expression of
327 vascular endothelial growth factor and the extracellular matrix proteins, fibronectin and
328 laminin, in association with reduced albuminuria. Aminoguanidine had no effect on VEGF
329 expression, although some reduction of fibronectin and laminin was observed. These findings
330 implicate AGEs as important stimuli for the activation of PKC, particularly PKC-alpha, in the
331 diabetic kidney, which can be directly inhibited by ALT-711.

332 **AGEs and diabetic retina**

333 Diabetic retinal complications result from retinal capillaries functionnal and morphological
334 alterations: increased permeability to albumin and macromelecules, vascular dysfunction,
335 loss of pericytes, and basement membrane thickening. The arguments in favor of a central
336 role for AGEs in these alterations have been discussed above. These alterations lead to
337 macular edema secondary to the leakage of macromolecules, and progressive capillary
338 closures related to microthrombosis. Capillary closures are responsible for non-perfused areas
339 (ischemic retinopathy), which induce the secretion of Vascular Endothelial Growth Factor
340 (VEGF) and the development of neo-vessels (proliferative retinopathy). In diabetic patients,
341 pentosidine skin concentrations have been shown to be associated with the development of
342 proliferative retinopathy[44]. The oxidatively formed CML is increased in diabetic rats both in
343 neuroglial and vascular retinal components, while imidazole-type AGEs are restricted to
344 microvessels, co-localizing with the expression of RAGE[45]. In rats with streptozotocin-
345 induced diabetes, treatment with aminoguanidine prevents diabetic retinopathy, resulting in
346 an 80% reduction in pericytes loss, in an absence of micro-aneurysms development, and of
347 endothelial cell proliferation. The accumulation of AGEs in pre-capillary arterioles is
348 inhibited by treatment with aminoguanidine[46]. Aminoguinidine prevents the development of
349 retinopathy in the diabetic spontaneous hypertensive rat (SHR), and completely suppresses

the deposit of PAS positive material in arterioles,and microthrombosis formation [47]. Evidence of this role relies on the results of studies indicating that the deleterious effects of AGEs on retinal capillary pericytes and endothelial cells are inhibited by RAGE-antibodies [48]. The role of AGEs mediated by VEGF in vascular dysfunction related to pseudo-hypoxemic changes has been suggested by recent experiments [49]. These effects are prevented by neutralizing VEGF antibodies and markedly reduced by aminoguanidine. Moreover, an association between accumulation of CML in human diabetic retina, proliferative and non-proliferative retinopathy, and expression of VEGF has been reported [50].

AGEs in diabetic neuropathy

The major causative link between clinical diabetic neuropathy and peripheral nerve changes is hyperglycemia. One of the important biochemical pathways involved, with a potential role in diabetic neuropathy, is glycation leading to AGE modification of nerve proteins64. AGEs have been stained in the endoneurial, particularly on the axons, endoneurial capillaries, and perineurium of diabetic patients with neuropathy. Axonal cytoskeleton proteins have essential roles in axonal structure and function[51]. Nonenzymatic glycation of axonal proteins causes alteration in structure and transport, leading to axonal atrophy and degeneration. Additionally, studies have shown that glycation of myelin occurs in both peripheral nerve and brain. The AGEs are accumulated in the perinurium, endothelial cells and pericytes of endoneurial microvessels, as well as myelinated and fibers. At the sub microscopic level, the AGEs deposit appear focally as irregular aggregates in the cytoplasm of endothelial cells , pericytes ,axoplasm and Schwann cells of both myelinated and unmyelinated fibebres68. Diabetic polyneuropathy is a complication that affects most patients with long standing hyperglycemia, deteriorating their quality of life. In the last few years , new therapeutic approaches have been developed that can improve symptoms and neutralize function and which may prevent and in some cases stop nerve damage and even promote nerve fiber regeneration[52].

Non-receptors AGEs complication

AGEs, extracellular matrix, and vessel wall components

Capillary basement membrane thickening and hypertrophy of extra vascular matrix are common features of diabetic microvascular complications. The link between high plasma glucose levels and tissue damage is due, at least in part, to the formation and accumulation of

AGEs in tissues [53.] AGEs accumulate in extracellular matrix proteins as a physiological process during aging . However, this accumulation happens earlier, and with an accelerated rate in diabetes mellitus than in non-diabetic individuals [54]. Increased serum and tissue levels of AGEs, due to a reduced removal by kidney, have been evidenced in end-stage renal failure and are more important in diabetic than in non-diabetic patients . A highly significant correlation has been shown between the importance of the AGEs deposits and the severity of diabetic complications . *In vitro* and *in vivo* studies have indicated that AGEs induce irreversible cross-links in long-living matrix structural proteins, such as type IV collagen, laminin, and fibronectin . AGEs are implicated in the basement membrane thickening through these alterations, *via* a reduction in susceptibility of matrix proteins to proteolytic degradation. These architectural changes alter also the functional properties of the basement membrane, including permeability. Advanced glycation of proteoglycans induces a decrease in electronegative charges and therefore modifies selective filtration properties of the basement membrane[55]. Mesangial expansion is an important part of diabetic nephropathy. The role of AGEs in the over expression of TGF-_1, which has been implicated in the pathogenesis of diabetic vasculopathy and of vascular remodeling, has been studied in a model of mesenteric vessels of streptozotocin-induced diabetic rat . Vascular hypertrophy was observed, together with an increase in TGF _1 and in α1 (IV) collagen gene expression. AGEs and extracellular matrix were present in abundance in diabetic, but not in control rats. Treatment of diabetic rats with the AGEs formation inhibitor aminoguanidine results in a significant reduction in pathological changes and in over expression of TGF β1 and α1 collagen genes.[56]

Pharmacologic inhibition of AGE

Attempts have been made, with greater or lesser efficacy, to pharmacologically influence the process of non-enzymatic glycation and AGE product formation [57]. Inhibit the formation of AGEs are purported to have therapeutic potentials in patients with diabetes and age-related diseases. The oxidation process is believed to play an important role in AGEs formation. Further oxidation of Amadori product leads to the formation of intermediate carbonyl compounds that can react with the nearby lysine or arginine residues to form protein crosslink and AGEs. The reactive carbonyl compounds may also be generated from the metal ion-catalyzed auto-oxidation of glucose[58](Rahbar and Figarola, 2003; Voziyan *et al.,* 2003) Therefore, agents with antioxidative or metal-chelating property may retard the process of

AGEs formation by preventing further oxidation of Amadori product and metal-catalyzed glucose oxidation. In addition, they block soluble receptors (sRAGEs) or specific receptors (RAGEs) which recognize AGEs. Some soluble receptors circulate freely, whereas specific ones can be found on macrophages, fibroblasts and endothelial cells. When an AGE molecule interacts with a RAGE it forms an adduct which is then prone to create more damage through oxidation and increased metal toxicity. In this regard, several natural and synthetic compounds known to possess antioxidative property which, have been shown to prevent AGEs formation *in vitro* and *in vivo* [59]

Medicinal plants based AGEs inhibitors

several phytochemicals known to possess anti-oxidative property, such as, curcumin, rutin, garcinoland flavonoid-rich extracts, have been shown to prevent AGEs formation *in vitro* and *in vivo* [60]. Arbutin (hydroquinone-β-D-glucopyranoside) is a naturally occuring compound found in various plant species of diverse family such as Ericaceae (*Arctostphylos* spp.)[61], Betulaceae(*Betula alba*) and Rosaceae (*Pyrus communis* L.) (Petkou et al., 2002)69 in right reffernce]. Arbutin, arbutin possessed an *in vitro* antiglycation activity [62].(Aroma J., 2005).70 Babu et al. (1994)[63], Sheikh et al. (2004)[64,] and Choi et al. (2006)[65] were under taken studies in Glycation inhibitory reaction particularly in medicinal plants like *W. Somnifea*[63], *Allium sativam*[64], and Plantago *asiatica*[65]. Puerariafuran[66], a New Inhibitor of advanced glycation end products (AGEs) Isolated from the roots of *Pueraria lobata* was reported by JANG et al. (2006)[66]. Chaiyasut *et al*. (2007) was observed that *P. emblica* extract showed higher inhibitory effect on AGEs formation than *K. parviflora* and *G. wintii* extracts[67]. Rebecca et al. (2008) were tested whether poly-phenolic substances in extracts of commercial culinary herbs and spices would inhibit fructose-mediated protein glycation. Extracts of 24 herbs and spices were tested for the ability to inhibit glycation of albumin. The most potent inhibitors included extracts of cloves, ground Jamaican allspice, and cinnamon. Potent herbs tested included sage, marjoram, tarragon, and rosemary. The concentration of phenolics that inhibited glycation by 50% was typically 4–12 μg/ml. Relative to total phenolic concentration extracts of powdered ginger and bay leaves were less effective than expected, and black pepper was more effective[68].

Commercial AGEs inhibitors

There are several commercially available inhibitors of cross-linking. Examples of these include carnosine, aminoguanidine, metformin, acarbose, and pyridoxamine. Some of these (like acarbose and metformin) are already in use as anti-diabetic drugs but new research

coming to light is now emphasizing their additional anti-cross-linking effects.Other not yet widely available inhibitors are Tenilsetam, OPB9195, phenazinediamine (2,3-diaminophenazone), and several hundred others still in development[69]. The Alteon Corporation alone has identified over 850 separate cross-link inhibitors.

Carnosine

The dipeptide carnosine (beta alanyl- L-histidine) is a naturally-occurring agent found in muscle and nervous tissue. Carnosine has been hailed as one of the most promising cross-link inhibitors. It has multiple actions and as such it has been called a pluripotent agent. One way carnosine works is by scavenging for free carbonyl groups. Carnosine is one of the few cross-link inhibitors that is not only active against protein-to-protein cross-linking but also against protein-to-DNA cross-linking [70].Another important carnosine activity is 'carnosinylation', which is a process whereby carnosine attaches to the protein bearing a carbonyl group, thus blocking the carbonyl from attaching to another protein. It is just like placing a piece of paper (carnosine) between two proteins bearing glue (carbonyls). In other words, carnosine reacts with carbonylated proteins to form carnosine-carbonyl-protein adducts. These adducts are then removed by proteolysis and degradation. Conveniently, carnosine also stimulates and enhances the process of proteolysis .Carnosine has a direct antioxidant action, and it also has a sparing effect on other antioxidants such as glutathione. It is a strong chelator of copper thereby reducing the copper-mediated damage during AGE activity. Finally, it has a possible, yet unconfirmed, bond-breaking capability by dissolving certain bonds (S-S bonds) on cross-linked proteins[71].At the clinical level, carnosine reduced urinary products of free radical and glycosylation metabolism in humans . One of the most important developments regarding carnosine is its ability to prevent and cure age-related cataract, and possibly glaucoma and other age-related eye conditions.People taking 50 mg-100 mg of carnosine a day have not reported any side effects whereas those taking higher doses (1000 mg to 1500 mg a day) have reported occasional histamine-related allergic reactions[72].

Metformin

Metformin (brand names Glucophage ®, Metforal ®) is a standard anti-diabetic drug (dimethyl-biguanide) used worldwide both against insulin-dependent and against non-insulin-dependent diabetes. Metformin lowers cholesterol, reduces body fat, stimulates antioxidant defenses[73] and it is also an effective inhibitor of glycation. It reduces the formation of AGEs,

particularly those affecting collagen. In that respect, it prevents diastolic stiffness in the myocardium of diabetic dogs. It has direct anti-glycation effects and improves cross-linking induced damage to nerves in diabetic rats. Its main mechanism of action is its carbonyl trapping ability, as will be explained below. In a clinical trial examining 57 people with type 2 diabetes, treatment with metformin was shown to reduce the concentration of methylglyoxal in a dose dependent manner [74]. Methylglyoxal, and the related compound, glyoxal, are both reactive carbonyl agents (alpha-dicarbonyls) which are blocked by the quanidine molecule, (remember that metformin is a guanidine-containing drug). Specifically, the guanidine moiety of metformin combines with methylglyoxal dicarbonyls to form guanidine-dicarbonyl adducts which are then eliminated from the tissues [75]. With reduced amounts of carbonyl groups in the tissues, the likelihood of cross-linking is reduced. This mechanism of action is similar to that of aminoguanidine (below), which, as the name suggests, it is also a guanidine-containing molecule.More recent experiments show metformin to have widespread activities as a cross-link inhibitor. It reduces cross-linking of fibrin proteins which take part in the clotting of blood. Metformin reduces fibrin cross-linking and therefore, ultimately, reduces the risk of thrombosis[76].In summary, with regards to glycation, metformin has a dual effect. It lowers blood glucose, (a well-known and established activity) plus, as new research is revealing, it is an effective inhibitor of cross-linking through carbonyl trapping.

Aminoguanidine

As with the case of metformin, aminoguanidine is also a guanidine-containing agent, and it therefore acts as a carbonyl trapping agent [77].Aminoguanidine too works by forming guanidine-dicarbonyl adducts, thereby reducing the numbers of free carbonyl groups. In particular, it is active against certain aldehydes which contribute to cross-linking, (e.g. alpha-oxoaldehyde, and malondialdehyde). Aminoguanidine is active mainly during the early stages of glycosylation.It is an effective inhibitor of cross-linking initiated by glucose molecules, but not as effective in situations involving ribose-related cross-linking. In any case, it prevents collagen cross-linking in tendons and skin [78] which shows its potential for prevention of muscle and joint age-related stiffness, and skin ageing (wrinkles). It limits the development of diabetic complications in animals and it has shown promising actions in improving diabetic nephropathy in double blind human trials . In addition, it is a weak copper chelator. Copper chelation is important in AGE induced damage, as high amounts of free copper are

more likely to increase AGE-induced injury.Aminoguanidine prevents cardiac enlargement in animal studies by reducing the risk of glycation-induced damage to cardiac collagen. Also, it prevents cross-linking between lipoproteins, (proteins carrying fat molecules) and therefore reduces the risk of blockage of the arteries, particularly the small arteries that feed the nerves [79].It is such a strong carbonyl scavenger that it can sometimes result in excessive removal of carbonyl groups (which, in small quantities, are necessary for the normal functioning of the metabolism). Side effects are rare and mild and include nausea or headache. There are two main varieties of aminoguanidine, the hydrochloride and the bicarbonate variety. Although the bicarbonate variety is more commonly available, the hydrochloride version is believed to be the most active (bioavailable) as it is more soluble. Aminoguanidine may be used together with carnosine which is active both in early and late stages of glycosylation, or together with metformin, particularly in diabetics.

Acarbose

Alpha-glocosidases are enzymes which facilitate the breakdown of complex carbohydrates, (such as starch) into smaller sugar molecules which are then absorbed through the intestinal wall. Acarbose blocks this, therefore inhibiting the absorption of certain sugar molecules such as maltose and sucrose, while allowing the absorption of glucose and lactose, which are needed for energy. In this way the overall absorption of carbohydrates is reduced and this lessens the risk of glycation-induced damage and AGE formation. Acarbose's main activities include a reduction of blood lipids (reduced uptake of triglycerides), an aid to weight loss, as well as being an important anti-glycation activity [80].Several studies have shown that Acarbose reduces the formation of glycated proteins (including the glycated haemoglobin A1c which is a marker for diabetes). Animal models show an ability of acarboseto slow down the rate of protein glycation and delay renal, brain and eye complications of diabetes [81].Other studies confirm its effectiveness in protecting against nephropathy, neuropathy and retinopathy in diabetes, by its ability to lower AGE formation [82]. With regard to the kidney-protecting effects of acarbose, it was shown that one possible mechanism could be its ability to protect the glomerular membranes, (where filtering of urine takes place in the kidney) against the effects of cross-linking [83].Acarbose is safe but it may have side effects such as abdominal pain and cramps, bloatedness and diarrhea. These are due to excessive amounts of unabsorbed carbohydrates in the bowel. The usual dose is 50 mg to 100 mg daily but the maximum should be kept to 300 mg a day to prevent these side effects. For greater benefits, it

may be worth using acarbose together with other cross-link inhibitors such as carnosine. (Ed.- Acarbose is best taken by chewing the tablets, usually just before or during meals).

Tenilsetam (3-2-thienyl-2-piperazinone)

This is manufactured by Cassella, a subsidiary of Aventis, and has traditionally been used as a brain stimulant (nootropic). New research has examined its anti-AGE actions and its significant glycosylation-inhibiting benefits. It works like most cross-link blockers, namely by carbonyl trapping. In addition, Tenilsetam has antioxidant activities and copper chelating properties[84] .A double blind, placebo-controlled trial performed over a decade ago showed that Tenilsetam increases brain performance, (increased rate of information processing, improved cognition and memory) [85]. Re-evaluation of these results shows that the effectiveness of Tenilsetam may be due to a reduction of AGEs in the brain. Particularly, it blocks the reactive sites on glycated proteins and does not allow these to be cross-linked. With a low rate of AGE formation in the brain, the damage caused by inflammation is reduced and brain activities improve[86].More recent experiments show that Tenilsetam reduces AGEs in diabetic rats, reduces amyloid aggregates (amyloid is the result of brain protein cross-linking), prevents oxidation injury to the brain and has an overall anti-dementia effect [87]. Due to its brain protective effects it may be used by diabetics who are concerned about age-related dementia or those who want to improve brain function plus cover them against cross-linking.

Pyridoxamine

All of these are naturally occurring. Pyridoxamine (brand name Pyridorin(, made by BioStratum) is found in animal sources, whereas pyridoxine is also found in plant sources. All three variants have a certain degree of anti-cross-linking actions, but pyridoxamine is the strongest and most significant. Trials are in progress to evaluate the product's safety and efficacy in preventing diabetic complications. Pyridoxamine prevents the formation of AGEs by 25-50% and ameliorates diabetes-related kidney dysfunction, (it improves albuminuria, plasma creatinine and hyperlipidemia). It works by trapping reactive carbonyl groups[88] and exhibits free radical scavenging properties [89]. It is most effective in the later stages of glycosylation and therefore, for full protection, it may be used together with aminoguanidine

which is active in the early stages of glycosylation. In fact, comparison studies with aminoguanidine suggest that, although both are effective against AGEs, pyridoxamine may be a more versatile agent to use against glycosylation, in order to avoid the low risk of potential toxicity problems with aminoguanidine mentioned above[90].Pyridoxamine does not affect the levels of blood glucose. It inhibits both methylglyoxal and glycoaldehydes which are most active following lipid peroxidation. It forms methylglyoxal-pyridoxamine dimers which are inactive and eliminated easily [91].There have been reports of neurotoxicity from using very high doses of pyridoxine, but the use of pyridoxamine is thought to be free from these side effects. The reason is that pyridoxamine needs to be phosphorylated (i.e. it needs the addition of phosphate on the main molecule) before it can become active.

OPB-9195(2-isopropyli-denehydrazono-4-oxo-thiazolidin-5-ylacetanilide)

A relatively new compound, first described in 1997, this carbonyl-trapping agent is a synthetic thiazolium derivative which inhibits cross-linking and improves kidney function. It is made by a Japanese company, Otsuka Pharmaceuticals Ltd. It works by blocking carbonyl groups, reducing the overall rate of AGE formation and, in addition, it reduces lipoxidation end-products such as malondialdehyde (MDA) [92].It was studied in relation to diabetic complications in rats. It reduced AGEs, restored nerve conduction velocity, limited free radical formation and reduced the rate of DNA damage [93]. OPB-9195 modulates the production of toxic cytokines (TNF alpha and interleukin 6), and increases the rate of elimination of abnormal proteins [94]. OPB-9195 protects against vascular tissue damage and prevents intimal (internal arterial) thickening [95].Other experiments showed it to be active in protecting against diabetic nephropathy in rats, through an AGE inhibiting action. It does not reduce blood glucose levels, and therefore it may need to be taken with metformin or acarbosewhen it becomes available.

Other potential cross-link inhibitors are:

- Pentoxifylline (brand name Trendal$^{(@)}$ which is normally used to improve circulation to the extremities .
- Pioglitazone , This is used in diabetes, to sensitise the cells to the actions of insulin, and it is best used together with Metformin. It has weak activity during early glycation but it becomes more active in the end stages[96].

- Kinetin (furfuriladenine) brand name Kinerase(. In a study, kinetin inhibited carbonyl activity and reduced AGEs by up to 68% [97].

Cross-link Breakers

The most important cross-link breaker is the drug ALT-711, an orally active compound. This is a thiazolium product (dimethyl-3-phenacyl-thiazolium chloride) manufactured by the Alteon Corporation in the US. A related compound is PTB (dimethyl-Phenacyl-Thiazolium Bromide), which has actions similar to the chloride variety. ALT-711 is not an enzyme as such, but it has enzymatic properties. It has been shown to actually break the covalent bonds between cross-linked proteins and free the proteins which are then able to function again normally.Particularly, ALT-711 breaks the bonds between -O=C - C=O- , (the first -O=C group belonging to one protein and the second C=O- belonging to another). When the bond between C-C is broken, the first protein has a -COOH group and the second protein has a -CHO group. Although, in theory, the bonds may then re-form, (because the carbonyl group is still active on the freed protein), ALT-711 has benefits which persist after the drug is stopped (Alteon Corporation, personal communication). In other words, if the proteins are cross-linked again, ALT-711 will divide them once more, and if they are then rebound, it will keep on separating them. For this reason, it may be necessary to use a combination of the cross-link inhibitor carnosine together with ALT-711 for full protection against cross-linking. In that situation, when the C-C bond is broken, carnosine will immediately bind to the carbonyl group (i.e. it will 'carnosinylate' the protein) and therefore cross-linking of that particular protein will not take place for the second time. The ALT-711 molecule will then be free to seek out other cross-linked proteins to work on.ALT7-11 can reverse aortic stiffening in rodents, canines and primates. A 40% reduction on age-related left ventricular stiffness (in dogs) was reported after just one month of treatment [98]. Other experiments support its effectiveness against hypertension, cardiovascular stiffness and heart failure [99].It has also been studied in a number of human clinical trials. It was found to be effective in reversing some of the complications of diabetes, improving myocardial and arterial stiffness, heart failure, and reducing blood pressure.In July 2001 Alteon has started the placebo-controlled SAPPHIRE (Systolic And Pulse Pressure Haemodynamic Improvement Restoring Elasticity) phase IIb clinical trial for systolic hypertension. It includes 450 patients aged over 50 years, and it involves 40 centres throughout the United States. The results are expected during 2003. A second, phase IIb SILVER (Systolic hypertension Interaction with Left Ventricular

Remodelling) trial is a companion to the first and has enrolled 180 patients with left ventricular hypertrophy[100].

Preliminary reports are optimistic, showing that ALT711 is effective at reducing clinical symptoms, (statistically significant reduction of blood pressure and an increase in large artery compliance, achieved after an eight week treatment period). The drug was well tolerated and few side effects were reported. Other trials are in progress aiming to study ALT711 in relation to diabetes and skin ageing. Far from being unique, ALT711 is in a group of 375 other cross-link breakers developed by Alteon in near future.

Conclusion

Increased non enzymatic protein glycation, formation of AGEs and their accumulation in tissue and serum have an important role in the pathogenesis diabetic complication. Long lived extra cellular matrix (E.C.M.) proteins have highlighted importance of intra cellular glycation. The diabetic complication can be reduced by reducing glycation synthesis, crosslink formation and tissue accumulation of AGEs or by blocking AGEs receptors blocker.The best cross-link inhibitors currently available are carnosine, aminoguanidine, metformin and acarbose, whereas others are now becoming available. No cross-link breakers are commercially obtainable as yet, but these will be marketed within 2-3 years. Soon after, combinations of inhibitors and breakers are due to follow.

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