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REVIEW

THE GLYOXALASE SYSTEM – A LINK BETWEEN CARBONILIC STRESS AND HUMAN THERAPY

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Several pathological states are characterized by an increased oxidative and carbonyl stress. Cells developed different strategies for the inactivation of reactive oxygen and nitrogen species, and respectively reactive carbonyl species. To date the best-known enzymatic system implicated in the inactivation of carbonyl species is the glyoxalase system. It comprises two enzymes, glyoxalase I and II, which use glutathione for the conversion of acyclic α-oxoaldehydes into corresponding α-hydroxyacids. Using glutathione this enzymatic system underline the strong link between oxidative and carbonyl stress: augmentation of oxidative stress depletes glutathione leading to the accumulation of carbonylic compounds. These compounds are able to modificate all classes of biomolecules: protein glycation is associated with enzymatic inactivation, protein denaturation and cellular-mediated immune response, nucleotide glycation is associated with mutagenesis and apoptosis, and excessive lipid glycation with membrane lipid bilayer disruption. Inhibition of the glyoxalase system leads to the accumulation of toxic carbonyl species. This strategy is used for the design of different drugs with anti-tumor, anti-protozoal and anti-bacterial properties. The present paper will focus on the mechanistical aspects of the glyoxalase system, reviewing some of the therapeutical strategies used for its inhibition.

INTRODUCTION

Tissue and organ damage during the normal process of aging and in several pathological conditions, such as diabetes, Alzheimer's disease, atherosclerosis and chronic renal failure is associated with an increased oxidative and carbonyl stress.

Oxidative stress is defined as an imbalance between the production of free radicals (reactive oxygen and nitrogen species, ROS and RNS respectively) and antioxidant defense mechanisms. The concept of "carbonyl stress" was introduce for the first time by Miyata and colleagues in 1999.[1] They defined "carbonyl stress" as an imbalance between reactive carbonyl species (RCS) production and carbonyl-scavenging mechanisms. This originates from an increased formation and/or decreased clearance or detoxification of reactive carbonyl compounds.[2]

RCS will accumulate in the body leading to the modification of virtually all classes of biomolecules (nucleic acids, proteins and basic phospholipids like phosphatidilethanolamine). The most prominent modification made by RCS is the generation of advanced glycation end-products (AGEs) and advanced lipoxidation end-products (ALEs). AGEs and ALEs are involved in the pathogenesis of severe and frequent diseases and their fatal vascular/cardiovascular complications, i.e. diabetes mellitus and its complications (nephropathy, angiopathy, neuropathy and retinopathy), renal failure and uraemic and dialysis-associated complications, atherosclerosis, neurodegenerative diseases, rheumatoid arthritis etc.

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REACTIVE CARBONYL SPECIES

Reactive carbonyl species are highly reactive aldehydes (-CHO) or α,β-dicarbonyl compounds (-CO-CHO). RCS can be derived from carbohydrates, lipids and aminoacids both by oxidative and non-oxidative pathways and they are detoxified by several enzymes and excreted by the kidneys. Oxidative pathways includes (i) auto-oxidation of sugars and ascorbat to glyoxal, glycoaldehyde and dehydroascorbat, (ii) peroxidation of polyunsaturated fatty acids to malondialdehyde, 4-hydroxy-2-trans-nonenal, acrolein and glyoxal,[3] and (iii) oxidation of aminoacids to glyoxal, methylglyoxal, acrolein and glycoaldehyde.[4] Non-oxidative pathways include degradation of fructose-3 phosphate and triosophosphates (intermediates of glycolitic pathway) to 3-deoxyglucosone and methylglyoxal.[5] The compounds mentioned above are by-products of metabolic pathways in normal conditions, but in situations associated with an increase oxidative stress their generation will be enhanced. Additionally, some of the RCS mentioned above (glyoxal, methylglyoxal) are further metabolized using glutathione. If glutathione concentration is low, due to its excessive consumption in increased oxidative stress conditions, the metabolism of these RCS is inhibited, with a subsequent rise in their concentration in the body.

These carbonyl compounds react non-enzymatically with the amino groups of proteins giving rise to reversible Schiff bases. The Schiff bases can be rearranged to Amadori products, which can undergo condensation, dehydratation, fragmentation and cyclization giving rise to AGEs and ALEs.[6] This sequence of reactions is known as **the Maillard reaction** and it was described for the first time by the French chemist Louis-Camille Maillard (1912).

After their formation *in vivo* AGEs and ALEs can be degraded only if the proteins from which they had been formed are degraded. Although, all proteins and lipoproteins that contains lysine, hydroxylysine and arginine residues are prone to glycation and AGEs/ALEs generation, the most extensive AGEs/ALEs accumulation will occur in tissues with long-lived proteins, i.e crystalline in the lens, collagen in the extracellular matrix of connective tissues (cartilage, bone, tendon and skin).[7,8] RCS can also interact with enzymes involved in ROS and RNS inactivation, rendering them less active. Thus, RCS accumulation leads to enhanced oxidative stress.

AGEs and ALEs interact with specific and non-specific plasma membrane receptors. The best known receptors are **RAGE** (**r**eceptors for **AGE**) which have a ubiquitous expression in the body (endothelial cells, neurons, pericytes from retina, macrophages, mesangial cells and podocites in kidneys).[9] The interaction of AGE with RAGE results in activation of different signaling pathways, which converge in the activation of transcription factors such as NF-kB and CREB.[10] NF-kB controls the expression of almost 100 pro-inflammatory genes (genes that codes for cytokines, adhesion molecules, and ROS and RNS generating enzymes).[11] Thus, in phagocitic cells, like macrophages, RAGE activation leads to induction of ROS and RNS generating enzymes (NADPH-oxidase, superoxide dismutase, inducible nitric oxide synthase and myeloperoxidase).[12] This way can be understood why an over-production of RCS results in enhanced oxidative stress.

Of major interest is also the fact that some AGEs and ALEs can complex redox active metals, thus creating redox active centers that generate free radicals, leading to increase oxidative stress.[13] This is another link between the accumulation of RCS and subsequent oxidative stress augmentation.

Another targets of RCS are nucleotides from RNA and DNA. Glyoxal and methylglyoxal react with deoxyguanosine, the most reactive nucleotide under physiological conditions. At higher concentrations of glyoxal and methylglyoxal, interstrand cross-links are formed in the duplex of DNA,[14] multi-base deletions, base-pair substitutions and transversions are also induced. Patients with end stage renal disease, a pathological state characterized by an increased generation of RCS, have a high incidence of tumors, probably due to the modification of DNA by RCS.[15]

RCS can also react with basic lipids (phosphatidilethanolamine) from biological membranes. An excessive lipid glycation is leading to membrane lipid bilayer disruption.[16]

In addition to their damaging properties to macromolecules, α-oxoaldehydes also exert direct cellular effects. For example, methylglyoxal is cytotoxic by an apoptotic mechanism and is also able to disturb signaling pathways by activation of different kinases including p38 MAPK (mitogen activating protein kinase) and cJun N-terminal kinase.[17,18]

The relationship between oxidative stress and carbonyl stress can be summarized taking into account the following: (1) increased oxidative stress can lead to formation of RCS, and (2) increased generation of RCS can enhance oxidative stress due to (a) AGE-RAGE interaction with subsequent activation of NF-kB, (b) interaction of AGEs with transition metals, rendering them redox active, and (c) inactivation of enzymes involved in ROS and RNS neutralization. Thus, there is a vicious circle between RCS generation and oxidative stress.

ENZYMATIC DEFENCE AGAINST CARBONYL STRESS

AGEs, ALEs and their precursors can be detoxified by specific enzymes, degraded by macrophages, excreted by the kidneys or may accumulate in the tissues leading to their damage. Similarly to the enzymatic defence against oxidative stress, it has been recognized recently that there is an enzymatic defence against glycation protecting against glycation-mediated cell damage.

The enzymatic defense against RCS includes enzymes that suppress the formation of glycation adducts by acting at distinct "sites" of this process: glyoxalases, aldehyde reductase and dehydrogenases, and amadoriases. "Amadoriases" are a group of deglycating enzymes and comprises fructosyl-amine-oxidases and fructosyl-amine kinases.[19] Both recognize Amadori products as substrates but have different deglycating mechanisms (i) fructosyl-amine-oxidases, produced by microorganisms, generate fructosamine/glucosone, aldehyde/amine and hydrogen peroxide and (ii) fructosamine kinase converts fructoselysine to fructoselysine-3-phosphat which in turns decompose to free lysine, inorganic phosphate and 3-deoxyglucosone.[20]

The glyoxalase system:

The glyoxalase system comprises two enzymes located in the cytosol of all cells: glyoxalase I (E.C. 4.4.1.5) and glyoxalase II (E.C. 3.1.2.6). Glo I catalyses isomerization of the hemithioacetal, formed spontaneously from α-oxoaldehydes and GSH, into *S*-2-hydroxyacylglutathione derivates. Glo II catalyses the conversion of *S*-2-hydroxyacylglutathione derivates into α-hydroxyacids with subsequent regeneration of GSH consumed in the reaction catalyzed by Glo I (Fig.1). The physiological substrates of Glo I are methylglioxal, glyoxal, hydroxypiruvaldehyde, 4,5-dioxovalerate and other α-oxoaldehydes.

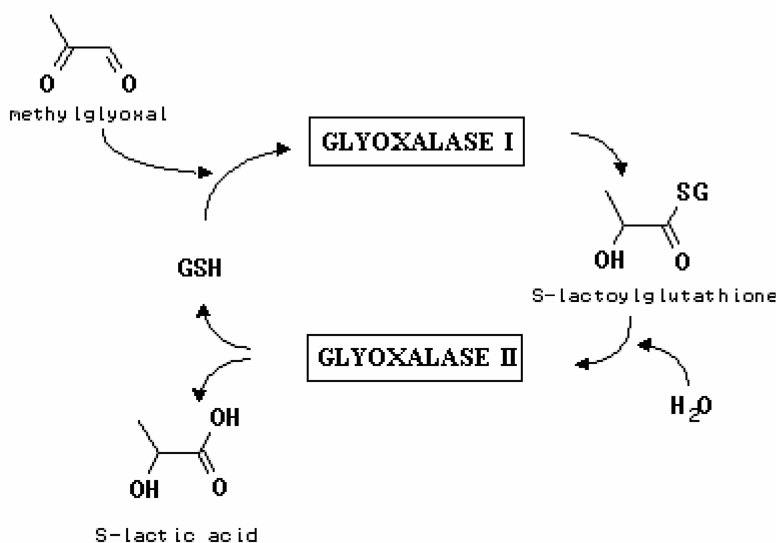


Fig. 1 – The reactions catalyzed by glyoxalase system.

The biological significance of the reactions catalyzed by the glyoxalase system has been long-time uncertain. Initially it was suggested that this system is involved in the regulation of cell growth,[21] and even

in the assembley of microtubules.[22] Mannervik and Thornalley suggested the fundamental function in detoxification of α-oxoaldehydes.[23,24] Experimental data support the link between the activity of glyoxalases and different pathological states like cancer and diabetes. Moreover, in the past years Glo I has been targeted for the development of novel anti-tumor, anti-protozoal and anti-bacterial agents that by inhibition of this system would raise concentrations of α-oxoaldehydes to toxic levels.[25]

Glyoxalase I is a ubiquitous dimeric enzyme, being present in all human tissues. The diallelic GLO locus, located on the chromosome 6, encodes for two similar subunits in heterozygotes, giving rise to three isoenzymes GLO 1-1, GLO 1-2 and GLO 2-2. The gene promoter contains an insulin response element and a metal response element.[26] The molecular weight of the three isoenzymes differs from 42 kDa (sequencing) to 46 kDa (gel filtration), with pI values between 4,8 and 5,1. Each subunit contains one Zn^{2+} ion essential for the catalytic mechanism.[27]

Glo I catalyses the isomerisation of the hemithioacetal, formed spontaneously from an α-oxoaldehyde and glutathione, into *S*-2-hydroxyacylglutathione derivates which in turn will be converted by Glo II in α-hydroxyacids and free glutathione.

The X-ray diffraction study of the complex of Glo I with the analogue substrate *S*-benzylglutathione able to determine the structure with a resolution of 2,2 Å. Each monomer has two structurally equivalent domains, the active site being situated on the interface of the two monomers. In the catalytic active site, the Zn^{2+} ion has an octahedral coordination with (i) two water molecules and (ii) two structurally equivalent residues from each domain Gln-33A and Glu-99A, respectively His-126B and Glu-172B. The γ-carboxy group of each Glu residue is acting as a bidentate ligand.[25] It has been suggested that due to the unusual octahedral coordination, the Zn^{2+} ion can be replaced with essentially no reduction of the activity of the enzyme, by Mg^{2+}, an ion that generally adopts an octahedral environment.

In the initial step of bounding the substrate, the *R*-hemithioacetal, to the catalytic site of enzyme two water molecules are removed. The Zn^{2+} ion conserves its octahedral coordination because the hemithioacetal is acting as a bidentate ligand with its oxo and hydroxy groups. The proposed reaction mechanism for Glo I consists of extraction of a proton from C1 atom of substrate by a shielded base (Glu-99 residue) leading to a cis-ene-diolate intermediate.[25] The second step consists of the rapid reprotonation of this cis-ene-diolate at the C2 atom to the thioester product (Fig.2). The subsequent reprotonation occurs stereospecifically to generate the *S*-2-hydroxyacylglutathione derivates. Both enantiomers (*R* and *S*) of the hemithioacetal are bound to the catalytic site of Glo I;[28] if the *S*-hemithioacetal is substrate the shielded base is the Glu-172 residue.

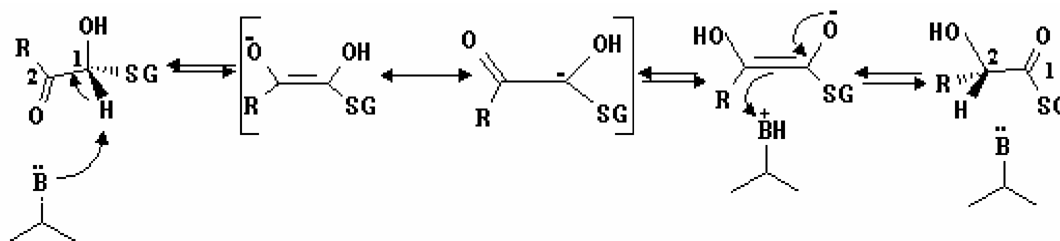


Fig. 2 – Proposed reaction mechanism for Glo I. A shielded base (B) abstracts a proton from the C1 atom of the hemithioacetal and then reprotonate at C2. Intermediate is an cis-ene-diolate anion
[modified after reference 25].

Glyoxalase II belongs to the superfamily of zinc metallo-hydrolases with an αβ/βα fold along with class B β-lactamases, arylsulfatases, A-type flavoprotein, enzymes involved in processing of mRNA 3'-ends, some proteins involved in the repair of DNA interstrand cross-link, etc.[29] The hallmark of these proteins is the presence of the αβ/βα fold, consisting of a core unit of two β-sheets sandwich surrounded by

solvent-exposed helices toward the external faces. Interestingly, although having an identical fold, all these proteins catalyze distinct reactions, without apparent common features.[30]

Glo II is a thiolesterase that converts *S*-2-hydroxyacylglutathione, result under the action of Glo I, to a α-hydroxyacid with subsequent liberation of glutathione.

The enzyme is composed of two distinct structural domains and has a binuclear metal-binding site. Like all species of Glo II for which the sequence and structure are available, the human Glo II has a highly conserved metal binding domain (THXHXDH), that is also present at proteins belong to the superfamilly of zinc metallo-hydrolases.[31] Determination of the crystal structure of human Glo II reveals that the two Zn^{2+} ions are coordinated by five histidine residues, two aspartic acid residues and a bridging water molecule.[32]

Although the available structural data indicates the presence of a di-zinc metal-binding site, it has been found recently that both Zn^{2+} and Fe^{2+} can occupy the site and, even more interestingly, that a binuclear zinc-iron center is essential for substrate binding and catalysis.[31] β-lactamases bind only zinc and have in the catalytic center a histidine residue.

This versatility in metal content of the glyoxalase could be explained in terms of susceptibility of ions to experience a charge shift in their electron shell through interactions with a coordination partner. The substitution of a soft ligand (histidine) by a harder one (aspartate) in the catalytic center of Glo II, in contrast with other members of metallo-hydrolases family, is responsible for the versatility in metal content.[30]

In the first stept of reaction, the binding of *S*-2-hydroxyacylglutathione is mediated through the participation of additional amino-acid residues (Arg, Lys, Asn). The two metal ions are implicated in the orientation of the substrate for the nucleophilic attack that takes place in the reaction catalyzed by Glo II.[31]

Glyoxalases as targets in different therapeutical strategies

Due to their implication in detoxifying RCS, the understanding of reaction mechanisms for both glyoxalases is essential for rational design and implementation of different inhibitors for human use. Due to the better understanding of reaction mechanism of Glo I, the studies in the case of this enzyme are more advanced than GloII.

In certain disease states, such as cancer and microbial infections, we may wish to induce pharmacologically, cytotoxicity to tumor cells and microbial organisms. This is achieved by cell-permeable Glo I inhibitors. Using Glo I inhibitors, RCS can accumulate, and this accumulation of cytotoxic compounds will result in decreased cell viability. Taking into account the fact that targeted cells in this kind of therapy are harmful for the body, RCS accumulation with subsequent cell viability decrease will have a benefic overall effect.

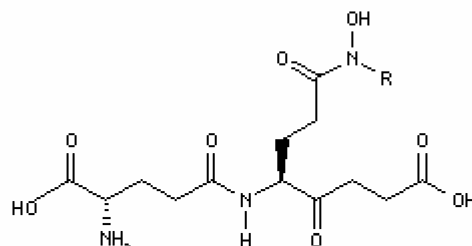
Also, it is very often the phenomena of multi-drug resistance of tumor cells that is followed by the over-expression of the glyoxalase system. Over-expression will ensure a very efficient detoxification of RCS enabling cancer cell to proliferate. Glo I inhibitors will render cancer cells unable to detoxify RCS leading finally to their destruction.

For the first time, Vince and Daluge suggested that inhibitors of Glo I might act as anti-tumor agents by inducing elevated levels of methylglyoxal in cancer cells.[32]

The first generation of Glo I competitive inhibitors were simple *S*-aryl and *S*-alkyl GSH derivates, with ***p*-bromobenzyl-glutathione** being one of the strongest inhibitors.

The diethyl ester of *p*-bromobenzyl-glutathione had also potent antimalarial activity in the red blood cell stage of *Plasmodium falciparum*. In this stage the parasite has only an anaerobic glycolysis and hence a very high flux of methylglyoxal. So, the inhibition of Glo I leading to accumulation of methylglyoxal to cytotoxic levels, is a good therapeutical approach.[33]

Elucidation of both reaction mechanism and structural chemistry of human Glo I allow the rational design of the second generation of competitive inhibitors. The fact that the reaction catalyzed by Glo I involves an *cis*-ene-diolate intermediate, formed by a proton-transfer mechanism inspired the synthesis of *S*-(*N*-aryl-*N*-hydroxycarbamoyl) esters and *S*-(*N*-alkyl-*N*-hydroxycarbamoyl) esters of glutathione as competitive inhibitors that mimics the transition-state of the physiological substrates (Fig. 3). Indeed, these compounds are among the strongest competitive inhibitors of the enzyme yet reported.[34]



R	K_i (nM)	R	K_i (nM)
C_6H_5 (phenyl)	160	CH_3CH_2 (ethyl)	1180
C_6H_4Cl (p-cloro-phenyl)	46	$CH_3(CH_2)_3$ (n-butyl)	180
C_6H_4Br (p-bromo-phenyl)	14	$CH_3(CH_2)_5$ (n-hexyl)	16
C_6H_4I (p-iodo-phenyl)	10		

Fig. 3 – Structure of competitive inhibitors that mimics the transition-state of physiological substrate of glyoxalase I [modified from reference 35].

By linking two molecules of the transition-state analogue *S*-(*N*-4-chlorophenyl-*N*-hydroxycarbamoyl) glutathione (CHG) through their free amino group with poly β-alanyl groups of different length and a suberate diamide bridge were generated the bivalent transition-state analogue inhibitors for human glyoxalase I (Fig.4).[35]

The advantage of this type of inhibitors is the capacity to link simultaneously to the both catalytic sites of the glyoxalase I dimer. K_i values obtained are small as 0,96 nM for the compound with n=6.

Experimental data suggested a general observation that an increase in the hydrophobicity of the S-substituent leads to the increase of the binding affinity of enzyme for the substrate indicating the presence of a hydrophobic binding pocket in the active site of the enzyme. Also, the binding of competitive inhibitor is stabilized by the coordination of the Zn^{2+} ion from the catalytic center with the two-cisoid oxygen atoms from the syn-conformation of the C-N bond from inhibitor.

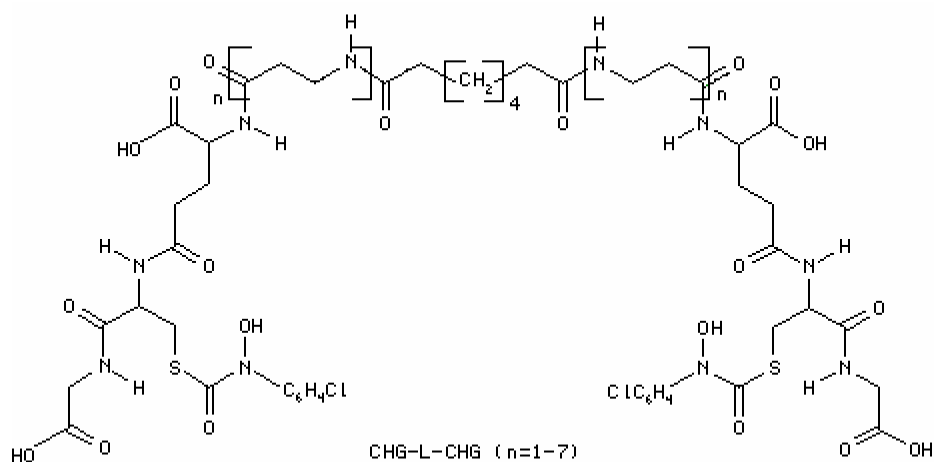


Fig. 4 – General structure of the bivalent transition-state analogue inhibitors of human glyoxalase I [modified from reference 35].

A major difficulty in using GSH derivates as anti-tumor agents is the presence in GSH of two carboxy-ionized groups that preclude rapid diffusion into cells. An important achievement was made by Lo and Thornalley which converted *S*-(4-bromobenzyl)-glutathione into diethyl ester with a high rate of diffusion into cells were it undergoes de-esterification (second reaction, Fig.5). The same strategy was successfully applied to deliver transition state-mimics.

Another strategy developed uses an acyl-interchange reaction between a neutral sulphoxide, which diffuses very rapidly across cell membranes, and intracellular GSH (first reaction, Fig.5). The sulphoxide

prodrug is approximately 10-fold more potent to L1210 cells in culture (GI_{50}=0,5 μM) than the corresponding diethyl ester prodrug (second reaction from Fig. 5) (GI_{50}=7 μM).[36]

Finally, another approach is to incorporate pro-drugs into polyacrylamide conjugates leading to the improvement of the tumor targeting via so-called "enhanced permeability and retention" effect. The uptake of these polymers by cells is realized via pinocytosis.[37]

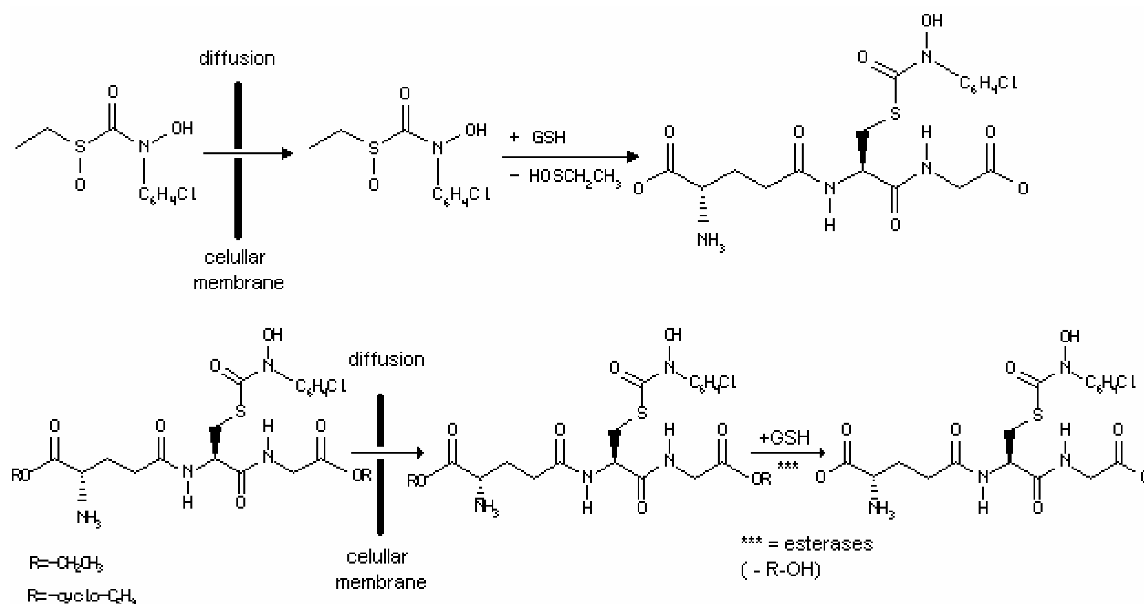


Fig. 5 – Two different strategies for prodrug delivery to cells; sulfoxide strategy (up); Thornalley dialkyl ester (down) [adapted from reference 38].

There were also developed some irreversible inhibitors of Glo I: α-bromodiketo and α-bromodiester derivates of GSH.[38] These compounds modify by bromination amino-acid residues from the catalytic site of the enzyme.

Inhibitors of Glo I are synthetic or natural microbial originating compounds. One natural compound is COTC [2-crotonyloxymethyl-(4R,5R,6R)-4,5,6-trihydroxycycloxe-2-enone] isolated from culture broth of *Streptomyces griseosporeus*. Kamiya and colleagues found that depletion of GSH and inhibition of Glo I by COTC increase the chemotherapy-mediated apoptosis, leading to new strategies for treatment of pancreatic carcinoma.[39]

The crotonyloxy group can be displaced very easy by SH-compounds like glutathione, cysteine, 2-mercaptoethanol and thiophenol. In cells this is achieved by glutathionyl transferase that can utilize COTC and COMC [2-crotonyloxymethyl-2-cyclohexenone] as substrates giving rise to electrophilic exocyclic enone intermediates.[40,41]

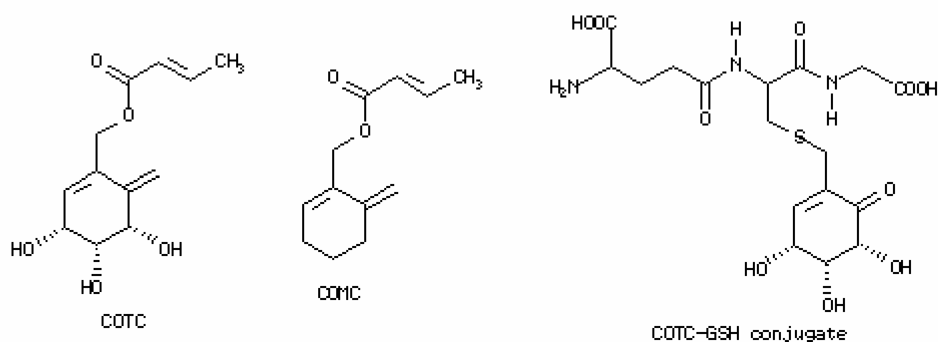


Fig. 6 – Chemical structures of COTC, COMC and COTC-GSH conjugate.

There is still debate concerning the molecular mechanism by which COTC and COMC exerts their anti-tumor effects. Some data suggests that electrophilic exocyclic enone intermediates generated from these

two compounds can react with amino groups from proteins and nucleic acids modifying them.[42] So the anti-tumor activity is not achieved by the competitive inhibition of Glo I by COTC-GSH and COMC-GSH aducts but rather by the reaction of enone intermediates with cellular proteins and nucleic acids.

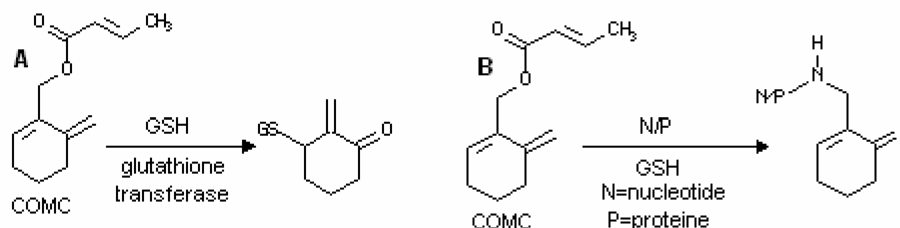


Fig. 7 – Generation of the electrophilic exocyclic enone intermediat in the reaction catalyzed by glutathione transferase (A) and the reaction of this intermediate with $–NH_2$ of proteins and nucleic acids (B) [modified from references 40 and 41].

Huntley and colleagues found that the COTC-GSH conjugate can competitively inhibit the human Glo I (K_i=183±6 μM), which is not inhibited by COTC itself in the absence of GSH.[43,44]

CONCLUDING REMARKS

Glycation of proteins, nucleotides and basic phospholipids by reactive carbonyl species is potentially damaging to the proteome and mutagenic. It is now recognized that, like in the case of oxidative and nitrosative stress, there is an enzymatic defence against glycation, with the glyoxalase system being the most important. The two-enzyme glyoxalase system detoxifies α-oxoaldehydes preventing the negative effects of carbonyl stress. The fact that over-expression of the glyoxalase system is associated with the phenomena of multi drug resistance has lead to the development of different drugs that act as anti-tumor agents by inhibition of the glyoxalase system, and subsequent accumulation of toxic carbonyl compounds. The goal is to develop drugs that act specific on one of the two enzymes of the glyoxalase system, and that have very side effects on the patient.

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