Multiple roles of glyoxalase 1-mediated suppression of methylglyoxal glycation in cancer biology – involvement in tumour suppression, tumour growth, multidrug resistance and target for chemotherapy

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ABSTRACT

Glyoxalase 1 (Glo1) is part of the glyoxalase system in the cytoplasm of all human cells. It catalyses the glutathione-dependent removal of the endogenous reactive dicarbonyl metabolite, methylglyoxal (MG). MG is formed mainly as a side product of anaerobic glycolysis. It modifies protein and DNA to form mainly hydroimidazolone MG-H1 and imidazopurinone MGdG adducts, respectively. Abnormal accumulation of MG, dicarbonyl stress, increases adduct levels which may induce apoptosis and replication catastrophe. In the non-malignant state, Glo1 is a tumour suppressor protein and small molecule inducers of Glo1 expression may find use in cancer prevention. Increased Glo1 expression is permissive for growth of tumours with high glycolytic activity and is thereby a biomarker of tumour growth. High Glo1 expression is a cause of multi-drug resistance. It is produced by over-activation of the Nrf2 pathway and GLO1 amplification. Glo1 inhibitors are antitumour agents, inducing apoptosis and necrosis, and anoikis. Tumour stem cells and tumours with high flux of MG formation and Glo1 expression are sensitive to Glo1 inhibitor therapy. It is likely that MG-induced cell death contributes to the mechanism of action of current antitumour agents. Common refractory tumours have high prevalence of Glo1 overexpression for which Glo1 inhibitors may improve therapy.

Taxonomy: protein glycation, cancer.
Key words: cancer prevention; gene amplification; cancer chemotherapy; inhibitor; apoptosis; replicative stress

1. Introduction: the glyoxalase system

The glyoxalase system is a metabolic pathway in the cytoplasm of all human cells which catalyses the detoxification of the endogenous reactive metabolite, methylglyoxal (MG) – Fig. 1A. It consists of two enzymes which catalyse successive reactions, glyoxalase 1 (Glo1) and glyoxalase 2 (Glo2), and a catalytic amount of reduced glutathione (GSH). Glyoxalase 1 (Glo1) catalyses the isomerisation of the hemithioacetal, formed spontaneously from MG and GSH to S-D-lactoylglutathione: $CH_3COCHO + GSH \rightleftharpoons CH_3COCH(OH)\text{-}SG \rightarrow CH_3CH(OH)CO\text{-}SG$. Glyoxalase 2 (Glo2) catalyses the conversion of S-D-lactoylglutathione to D-lactate and reforms GSH consumed in the Glo1-catalysed reaction step [1]. The glyoxalase system metabolises other reactive, acyclic α-oxoaldehydes such as glyoxal and hydroxypyruvaldehyde. MG is the most reactive dicarbonyl and of highest endogenous flux and so is often the primary concern for health [2]. The primary function of the glyoxalase system is to metabolise MG and/or other reactive acyclic α-oxoaldehyde metabolites, and thereby suppress them to low steady-state concentrations.

MG is formed mainly by the degradation of triosephosphates, and also by the metabolism of ketone bodies, threonine degradation and the fragmentation of glycated proteins – reviewed in [3]. The estimated flux of formation in healthy adults is *ca.* 3 mmol MG per day. Little MG is absorbed from the diet and *ca.* 0.1 μmol MG per day is excreted in urine. The typical concentrations of MG are 100 – 200 nM in plasma and in 1 – 4 μM in cells and tissues [4].The glyoxalase system is the major pathway for metabolism of MG. When Glo1 activity is decreased *in situ* by gene silencing [5, 6], cell permeable Glo1 inhibitors [6, 7] and depletion of GSH [8], the cellular concentration of MG increases markedly. MG is a potent glycating agent, reacting non-oxidatively mainly with arginine residues of proteins to form the hydroimidazolone adduct MG-H1 and reacting with DNA to form mainly imidazopurinone MGdG [9, 10] – Fig. 1, B & C. MG-H1 and MGdG are quantitatively and functionally important protein and nucleotide-derived advanced glycation endproducts (AGEs). Urinary excretion of protein and nucleotide MG-derived glycation adducts is *ca.* 10 μmol per day or *ca.* 0.3% of the flux of MG formation [10, 11], indicating that the protection against protein and DNA glycation afforded by metabolism of MG by the glyoxalase system is highly effective. Protein and DNA contents are 0.1 – 0.8 mmol/mol arg and *ca.* 9 adducts per 10^6 nucleotides, respectively. Abnormally high concentrations of MG, and/or other reactive α-oxoaldehyde metabolites, is a dysfunctional metabolic state called dicarbonyl stress where dicarbonyl glycation is increased [12]. The protein adduct MG-H1 is frequently directed to functional sites of cellular and extracellular proteins. There is loss of positive charge of modified arginine residues resulting in protein inactivation and dysfunction [2, 13] – including induction of apoptosis and anoikis [14, 15]. The DNA adduct MGdG is mutagenic and linked to malignant transformation when Glo1 is down regulated [10].

The glyoxalase system has been historically linked to cancer research through early studies of tumour metabolism and the cytotoxic activity of high exogenous concentrations of MG [16]. It is one of the oldest and well-developed links of glycation to carcinogenesis, tumour growth and cancer chemotherapy. The historical development of the glyoxalase research, molecular characteristics of glyoxalase enzymes, molecular physiological of glyoxalase metabolites, including in cancer [17], and techniques available to study the glyoxalase system – the glyoxalase researchers "toolkit" – have been reviewed elsewhere recently [3, 16, 18-20]. The aim of this review is to address recent advances – including development of small molecule inducers of Glo1 expression or "Glo1 inducers" and their potential use in cancer prevention, association of Glo1 expression to tumour growth and multidrug resistance (MDR) in cancer chemotherapy, GLO1 amplification in cancer and MDR, barriers to developing Glo1 inhibitor anticancer agents and mechanism of their

antitumour activity, and a distinct tumour type, neuroendocrine tumours (NETs), where Glo1 biomarkers and inhibitors may be beneficial.

2. Methylglyoxal in carcinogenesis - glyoxalase 1 is a tumour suppresser protein and thereby glyoxalase 1 inducers may have a role in cancer prevention

The presence of MG and glyoxal-derived nucleotide AGEs in DNA is associated with increased mutation frequency, DNA strand breaks and cytotoxicity. The mutagenicity of methylglyoxal was improved markedly in the presence of hydrogen peroxide. Mutation is suppressed by (NER) - reviewed in [21]. MGdG is the major nucleotide adduct formed spontaneously in human cells, exceeding the oxidative damage adduct 7,8-dihydro-8-oxo-2'-deoxyguanosine in DNA of peripheral blood mononuclear cells by *ca.* 3-fold [10]. Frameshift mutations were induced by high concentrations of MG in human HepG2 cells *in vitro* and prevented by a Glo1 inducer [22]. Until recently, however, it has been difficult to gain evidence for a role of MG in carcinogenesis and tumour prevention by Glo1.

In studies of a p53 knockout Ras overexpression model of liver carcinogenesis in mice, an unfocused genome-wide scan for tumour suppressor genes was made. Hits were identified by increased tumour development with selective gene silencing achieved by introduction of pools of short hairpin RNAs into premalignant progenitor cells and selection for those that promote tumour formation after transplantation. Thirteen tumour suppressor genes (in addition to p53) were identified: one was GLO1 [23]. If this model translates to clinical carcinogenesis, increasing Glo1 expression is expected to be associated with decreased cancer risk – at least for hepatocellular carcinoma (HCC). Glo1 inducers are now in clinical evaluation for other therapeutic applications [24]. Future studies may explore cancer prevention by Glo1 inducers in high risk subject groups.

The association of decreased Glo1 expression with increased tumour development is likely through genetic instability induced by increased formation of MG-derived adducts of DNA, MGdG and CEdG [10, 25]. Other contributions may be from disturbance in dicarbonyl stress of signal transduction producing overstimulation by mediators of cell proliferation and inflammation. Of great interest in this regard is the overexpression of cyclo-oxygenase-2 or prostaglandin synthetase-2 (COX-2 or PTGS2) in most human neoplasia. COX-2 inhibitors have cancer chemopreventive effects in experimental and clinical carcinogenesis [26, 27]. Recent phase 1 clinical valuation of a Glo1 inducer, *trans*-resveratrol-hesperetin (tRES-HESP) combination, showed potent down regulation of COX-2 expression in peripheral blood mononuclear cells (PBMCs) [24]. If this translates to tissues, this suggests that indirect suppression of COX-2 by Glo1 inducers may also make available an expected cancer chemoprevention effect. COX-2 inhibitors have been considered and evaluated clinically for prevention of colorectal adenocarcinomas but cardiotoxic adverse effects linked to off-target effects of COX-2 inhibitors have prevented their use. Nevertheless, studies of siRNA silencing of COX-2 and other studies provide compelling evidence for its involvement in carcinogenesis and metastasis. Targeting upstream regulation of COX-2 by tRES-HESP, likely linked to increased expression of β-Klotho and down-regulation of monocyte chemoattractant pritein-1 (MCP-1), may be a better option – Fig. 2. Further research is required to identify pre-cancerous lesions that have increased COX-2 expression and functionality which may be sensitive to Glo1 inducer down regulation of COX-2 [28]. Cancer prevention effects of tRES alone have been reviewed elsewhere in this journal with beneficial responses mainly achieved at concentrations and doses that are not clinically translatable [29]. tRES and HESP do not shown potent down regulation of COX-2 individually; rather synergism of the tRES-HESP combination is required for potent anti-inflammatory response [24].

Clinical treatment with the Glo1 inducer, tRES-HESP combination, also decreased expression of the receptor for advanced glycation endproducts (RAGE) in healthy human subjects [24]. Decreased expression of RAGE is expected to be linked to decreased tumour development, growth and metastasis [30] which may also contribute to a cancer chemopreventive response.

3. Association of increased Glo1 expression with robust tumour growth and multidrug resistance (MDR)

Increased expression and activity of Glo1 in established human tumours has been found in many studies – reviewed in [17]. The association of increased Glo1 expression with tumour growth may suggest GLO1 is an oncogene [31]. However, increased expression of Glo1 or mutation of Glo1 does not drive malignant transformation *in vitro* or *in vivo* [32, 33]. This suggests that GLO1 is not an oncogene [32, 34]. Indeed, in the non-malignant state, Glo1 is a tumour suppressor gene – see above. Rather, increase Glo1 expression contributes to robust growth – particularly in conditions of high glycolytic activity and adaptation to hypoxia [35, 36]. Increased Glo1 expression is likely due to an increased requirement to protect the tumour proteome against a local, relatively high flux of MG formation. Many tumours display high rates of anaerobic glycolysis even when well-perfused and oxygenated. This has been called the "Warburg effect". It has been proposed that this is an adaptation to intermittent hypoxia in pre-malignant lesions and may offer growth advantage [37]. Pentosephosphate pathway activity is also increased, providing pentoses for nucleotide synthesis and increased flux of NADPH for biosynthesis – which also sustains a reducing cytoplasm and resistance to oxidative stress [38]. The Warburg effect is now viewed as part of a broader spectrum of metabolic reprogramming in tumours to provide for growth, tissue invasion and metastasis with a changing nutrient supply [39]. A consequence of this is related relatively high flux of MG formation and requirement for high expression of Glo1 [40]. Increased Glo1 activity is permissive for survival and growth of tumours with high glycolytic rates and related high fluxes of formation of MG [10].

Hypoxia in tumours could pose a threat to tumour cell survival through dicarbonyl stress since the precedent from proliferating non-malignant stem cells suggests that Glo1 expression is down-regulated and flux of formation of MG is increased through switch to increased anaerobic glycolysis in hypoxia [41]. Down regulation of Glo1 is driven by hypoxia-inducible factor 1-alpha (HIF1α) [42]. However, tumour-derived stem cells have adapted to circumvent this. Bcr-Abl+ leukaemia-derived stem cells exhibited a counter response – increased Glo1 expression in response to hypoxia [36]. This is a growth advantage: evidenced by transfection of the HEK293 tumour cell line to overexpress Glo1 gave improving adaptation to growth in hypoxic conditions [35]. The dependence of Glo1 for hypoxia adaptation is a metabolic weakness or "Achilles heel" to exploit since hypoxia-adapted tumour stem cells were vulnerable to cytotoxicity induced by cell permeable Glo1 inhibitors [36]. This suggests tumour stem cells may be more sensitive to than non-malignant hematopoietic and mesenchymal stem cells to Glo1 inhibitors. Since cancer stem cells survive many commonly employed cancer therapies, this could provide a significant advantage in cancer chemotherapy.

Transcriptome-wide study of gene expression in cell lines sensitive and resistant to anticancer drugs revealed increased Glo1 expression was associated with MDR in cancer chemotherapy [43]. This suggests that current antitumour drugs may increase the cellular concentration of MG as part of their mechanism of action for antitumour cytotoxic effect. Subsequent studies have shown that the cell permeable Glo1 inhibitor, S-p-bromobenzyl-glutathione cyclopentyl diester ($BBGCp_2$), provides effective treatment for tumour cell *in vitro* and tumour-bearing mice with Glo1-linked MDR [6, 31, 44]. Tumours with high expression of

Glo1 were also sensitive to toxicity of short hairpin RNA knock-down of Glo1 in primary cultures and xenograft tumours established from HCC cells. This suggests Glo1 inhibition is a target for HCC chemotherapy with expected high response rate [45]. It is likely that Glo1 overexpression contributes to MDR in prevalent refractory tumours in clinical therapy.

As high expression of Glo1 supports robust tumour growth then poor treatment outcomes may not be limited to chemotherapy but to radiotherapy and surgical interventions. In radiotherapy dicarbonyl stress is induced by GSH depletion and decreased *in situ* activity of Glo1 contributes to the tumour cell kill [46]. Increased Glo1 expression was also associated with tissue invasiveness, lymph node metastasis and pathological stage – for example, in gastric cancer [47] – making the effectiveness and success of surgical therapy more challenging.

If Glo1 expression is permissive for high glycolytic rates and growth of tumours then Glo1 mRNA and protein measurements could provide useful clinical biomarkers to assess risk of tumour progression, metastasis and survival. There is high prevalence (79%) of increased Glo1 expression in breast cancer, compared to control non-malignant tissue, and in a cross-sectional study Glo1 expression increased progressively with tumour grade [48]. In clinical HCC, increased Glo1 expression had prevalence of 48% [45]. Despite recent advances in treatment, clinical HCC remains refractory to current clinical chemotherapy [49].

The association of Glo1 expression of tumours with clinical outcome of treatment at follow-up has been examined in few studies. In colorectal cancers, increased Glo1 protein assessed by immunohistochemistry (IHC) was associated with rapid tumour progression and poor survival [50]. In gastric cancer, increased Glo1 protein by IHC was associated with decreased 5-year survival (43% versus 70%) [47] and survival hazard ratio 2.5 [31].

Increased Glo1 expression may occur through dysfunctional transcriptional regulation. Several promoter elements in GLO1 are known and some have increased functional activity in tumours – particularly Nrf2 interaction with the functional antioxidant response element (ARE) [19, 22]. Nrf2 is often over-activated in lung cancer and HCC [51, 52].

4. Glyoxalase 1 gene amplification in cancer

A further mechanism contributing to increased Glo1 expression in tumours is GLO1 gene amplification [6]. In 2010 working with the Cancer Genome project investigators, we discovered increased GLO1 copy number (gene copy >2) and Glo1 expression in human tumours. Human GLO1 is located at locus 6p21.2 with low level duplication in the healthy population of 2% prevalence [53]. The DNA segment copied in tumour GLO1 copy number increase was larger than in gene duplication in the healthy population [54]. In clinical tumours GLO1 copy number was functional; increased copy number correlated with increase Glo1 mRNA and protein. In our study of 225 human tumours of different types, the highest prevalence of GLO1 copy number increase in breast cancer (22%), sarcomas (17%) and non-small cell lung cancer (NSCLC) (11%) – assessed by qPCR [6]. *In vitro* studies suggest any functional increase in GLO1 copy number produces MDR, severity of MDR increasing with level of copy number increase. Other investigators have followed up with studies on GLO1 copy number in human tumours. GLO1 amplification has very high prevalence in breast cancers that do not express oestrogen receptor, progesterone receptor and HER-2 genes, triple negative breast cancer (TNBC) [55]. TNBCs respond poorly to current chemotherapy [56]. Glo1 copy number increase also had high prevalence in advanced malignant melanoma [57]; it was moderate in gastric cancer [31] and low in hepatocellular carcinoma (HCC) [45] – Table 1. Further data on GLO1 copy number in human tumours is provided at "Tumourscape" (http://portals.broadinstitute.org/tumorscape/pages/portalHome.jsf) – a compendium of copy number alterations across multiple types of tumour [58]. The

association of Glo1 copy number increase with clinical follow-up has been examined in few studies. In gastric cancers**,** increased GLO1 copy number assessed by qPCR was linked to decreased survival [31]. There is a requirement for further validation of the association of increased GLO1 copy number with tumour progression and patient survival as clinical measurement of GLO1 copy number could be easily implemented and a potentially useful biomarker to guide patient treatment and care.

We also studied GLO1 copy number in 750 human tumour cell lines [6]. In human tumour cell lines *in vitro* GLO1 copy number increase is not always functional and hence there is a weak correlation of GLO1 copy number with Glo1 expression. For example, using data from the compendium of gene copy number of expression data in human cancer cell lines – the Broad-Novartis Cancer Cell Line Encyclopedia (CCLE) (https://portals.broadinstitute.org/ccle/home) [59], for all breast cancer cell lines we found Glo1 copy number correlates positively with Glo1 expression level ($r = 0.57$, $P<0.001$, $n = 59$, *Spearman*), supporting the likely importance of change in GLO1 copy number as a determinant of Glo1 expression in human breast cancer cell lines.

The mechanism of GLO1 amplification in cancer is unknown but a suggestion came from a study of GLO1 duplication in mouse embryonic stem cells (mESCs) [41]. Low level copy number increase of GLO1 was found in hypoxia, suggesting a role for hypoxia-activated histone demethylase KDM4A/JMJD2A. Exploring the possibility that GLO1 may suffer copy number increase induced by exposure to high concentrations of MG, we studied prolonged, 12-day, exposure of mESCs to exogenous MG *in vitro* [12]. For physiological relevance we studied Glo1 activity and copy number changes also under an atmosphere of 3% oxygen – equivalent to ambient oxygen concentration for embryonic stem cells *in vivo* [60]. We found low level increased copy number focussed to the GLO1 genomic domain induced to a similar level by addition of exogenous MG, 3% oxygen, and exogenous MG and 3% oxygen together.

The Glo1 gene is present in a large transcriptional domain of the genome at the flanking region of *Btbd9* in which copy number increases are enriched. Large transcription units drive locus-specific genomic instability during DNA replication. They are replicated late in the cell cycle and organize copy number duplications in their flanking regions. This is thought to occur through transcription-dependent double-fork failure in DNA replication [61].

Black *et al*. reported genetic domain specific increase of gene copy number in primary human T-lymphocytes and tumours – including genes linked to MDR [62]. This was driven by KDM4A [63]**.** Increased histone demethylation is hypothesised to create more open chromatin which promotes inappropriate recruitment of mini-chromosome maintenance (MCM) proteins and DNA polymerases and thus promote re-replication for copy number gain [63]. Copy number change at the GLO1 locus was not studied. We proposed that hypoxia and/or high concentrations of MG activate KDM4A demethylation to drive GLO1 copy number increase in tumours. KDM4A is highly expressed in many tumours where it is also involved in metabolic reprogramming for increased tumour anaerobic glycolysis [64]. Alternatively, increased glycation of histones may achieve a similar functional change in histone proteins to produce increase GLO1 copy number. This would explain why hypoxia and MG treatment achieve the same level of induced Glo1 copy number increase individually and together – Fig. 3. Increased Glo1 expression was found in hypoxia-adapted tumour stem cells but copy number increase was not investigated as a possible mediating factor [36].

KDM4A may likely drive low level GLO1 copy increase in the healthy population and also in tumours post-carcinogenesis. GLO1 copy number increase in the early stages of tumour development may become dominant through clonal selection and lead to MDR. This is supported by observations in malignant melanoma, for example, where GLO1 copy

number increase has low prevalence (2%) in early stages and high prevalence (80 - 89%) in advanced stages [6, 57, 58]. Antitumour drug treatment may also increase MG exposure and be an additional pressure for GLO1 copy number increase.

5. Barriers to developing Glo1 inhibitor anticancer agents

Development of cell permeable Glo1 inhibitors for cancer chemotherapy was recently reviewed [17]. Treatment with MG was initially proposed and evaluated for cancer chemotherapy. It was ineffective, likely due to the high capacity of non-malignant and some tumour tissue to metabolize MG by Glo1 [65]. To circumvent this Vince and Wadd proposed development of Glo1 inhibitors which were initially based on substrate analogues, glutathione S-thioethers such as S-p-bromobenzylglutathione [66, 67]. These were not effective antitumor agents *in vitro* because they lacked cell permeability and stability to degradation by cell surface ɣ-glutamyl transferase in the extracellular compartment. These problems were circumvented by our team when we prepared S-p-bromobenzylglutathione diester prodrugs. Diesterification blocked extracellular degradation, provided cell permeability and was hydrolyzed when delivered inside cells by cellular non-specific esterase to reveal the active inhibitor, S-p-bromobenzylglutathione. The inhibitor constant K_i value of S-p-bromobenzylglutathione for human Glo1 is 160 nM [68]. The most potent diester derivative to date is the cyclopentyl diester, $BBGCp_2$, which resists hydrolysis by serum or plasma esterase before delivery into cells [7, 69, 70]. Creighton and co-workers developed more potent Glo1 inhibitors [71]. They also recognized that for evaluation in tumour-bearing mice, esterase-deficient mice were required as conventional wild-type strains have markedly higher plasma esterase activity than human subjects. Use of conventional wildtype strains of laboratory mice would underestimate clinical potency of the prospective new drugs by rapid de-esterification in plasma [72]. For the National Cancer Institute panel of leukaemia, non-small lung cancer, colon cancer, central nervous system cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer cell lines, S-p-bromobenzylglutathione diethyl ester gave median growth inhibitory concentration GC_{50} values in the range 7 - 20 μM [73]. $BBGCp_2$ had antitumour activity in tumour bearing mice and was particularly effective against tumour with high Glo1 expression and resistance to established anticancer drugs [7, 44]; and similar Glo1 substrate studies were performed with Glo1 competitive inhibitor S-(N-p-chlorophenyl-N-hydroxycarbamoyl)-glutathione (CHG) administered as prodrug CHG ethyl diester ($CHGEt_2$) and cyclopentyl diester [74]. From efficacy studies, dosing schedules have been achieved that give similar potency to current clinical antitumour agents [7, 44, 74]. So why are cell permeable Glo1 inhibitors not in clinical use? The following are the likely reasons:

5.1 Limited potency

Whilst good efficacy of treatment has been achieved with $BBGCp_2$ and similar compounds in tumour-bearing mice, the doses required for potent therapeutic effect were high, 50 – 200 mg/kg. For $BBGCp_2$ these doses gave no indication of toxicity other than to the tumour but for $CHGEt_2$ the doses used were close to the maximum tolerable dose [7, 44, 74]. The reason for the high dose requirement may be that the inhibitor is cleared relatively rapidly from the tumour. The active inhibitors are substrate analogues - GSH conjugates - and so are rapidly cleared from cells by the mercapturic acid pathway [74]. For $CHGEt_2$ administered to mice intravenously, 120 mg/kg, the peak concentration of 30 - 60 μM in the tumour occurred 15 min after injection and thereafter decreased with a half-life of 10 min to an extended plateau phase of 6 μM [74].

5.2 Metabolic resistance

MG is also metabolised by aldoketo reductase isozymes, AKR isozymes 1A4, 1B1 (aldose reductase) and 1B3 to mainly hydroxyacetone [75]. Expression of these enzymes is

upregulated by Nrf2 which is often over-activated in tumours – see above. Consequently, relatively high capacity metabolism of MG by AKR may be available to some tumours. AKRs are enriched in both squamous cell carcinomas (SCCs) and adenocarcinomas that contain somatic alterations in the Nrf2 pathway; and in the case of SCC, AKRs were also enriched in most other tumours [51]. Early evidence of the effect of AKR activity on potency of Glo1 inhibitors was that an aldose inhibitor Sorbinil potentiated the growth inhibitory response of $BBGCp_2$ in HL60 cells *in vitro* [7]. There is also the risk that increase of MG may induce Glo1 expression [22, 76] but this would be circumvented with a high dose of Glo1 inhibitor that kills cells before significant induction of Glo1 expression can occur.

5.3 Tumour sensitivity markers

From surveying activity of $BBGCp_2$ activity in tumour cell lines it was clear that some tumour cell lines are sensitive to Glo1 inhibitors and some are resistant. The lack of tumour sensitivity markers for Glo1 inhibitors stymies development for cancer treatment because tumours that will likely give strong therapeutic response cannot be easily identified. This impacts particularly on commercial pharmaceutical development. Team leaders in the pharmaceutical industry seeking to develop new antitumour drugs often have limited time to identify a type of tumour sensitive to a particular drug target before strategic decisions are taken to more to a different drug target. Many studies have found potent cytotoxicity to tumour cells by siRNA silencing of Glo1 – for example [6, 31]. This increases cellular and extracellular levels of MG driving apoptosis and anoikis [7, 15]. There is little doubt that Glo1 is a valid target for antitumour development from the cell biology and pharmacology viewpoint. To identify tumours that are likely sensitive to Glo1 inhibitors, we need to identify biomarkers indicative of high risk of increase of MG to a cytotoxicity level when Glo1 is inhibited.

A reasonable assumption for the key criterion of cytotoxicity by a Glo1 inhibitor is attainment of a critical threshold steady-state level of MG-derived protein or nucleotide AGEs that activates programmed cell death – see below. The ability to increase this above this threshold level for cytotoxicity by Glo1 inhibition will be linked positively to <u>both</u>: (i) steady level of MG-derived AGE in the tumour, and (ii) Glo1 activity of the tumour. MG-H1 and MGdG are major adducts quantitatively in tumour cells so these are the best analytes to determine, and Glo1 protein is an easily measurable surrogate of Glo1 activity in the setting of the clinical chemistry laboratory. Both MG-derived AGE and Glo1 protein could be measured by frozen tissue by Western blotting if the clinical sample was thought to be homogenously malignant or in frozen tissue section by immunohistochemistry whereby analyte intensities in tumour and non-tumour tissue could be spatially discriminated. Use of formalin-fixed paraffin-embedded (FFPE) tissues would compromise the AGE content measurement. This hypothesis predicts that tumours most sensitive to Glo1 inhibitor chemotherapy are tumours with high steady-state level of MG-H1 residues in cell protein concurrent with high expression and activity of Glo1. This is a surrogate of a high flux of MG formation in the presence of a high Glo1 activity – mathematical modelling of the glyoxalase pathway confirms this [19]. The high activity of Glo1 indicates there is a high concentration of receptor for the inhibitor, Glo1 protein, to achieve high metabolic impact through rapid accumulation of MG and related increased protein and nucleotide MG-derived AGE formation. This remains to be evaluated clinically. For tumour cell lines *in vitro*, HL60 cells had relatively high Glo1 activity and high AGE content and was a cell line sensitive cell lines to $BBGCp_2$: GC_{50} = 4.2 μM [7, 10, 77], and sensitivity to $BBGCp_2$ also reflected this for other selected cell lines [6, 10]. Sensitivity of tumour cell liens to $BBGCp_2$ is not reflected in GLO1 copy number alone as GLO1 copy number increase is not always functional in tumour cell lines and is not always associated with high levels of MG-derived AGEs [6, 10].

Cell permeable Glo1 inhibitors employed in cancer chemotherapy and other therapeutic agents and prospective therapeutic agents with Glo1 inhibitor activity are given in Table 2.

6. Cytotoxic mechanism of action of glyoxalase 1 inhibitors: mechanism of cytotoxicity induced by methylglyoxal accumulation in tumour cells

The cytotoxic mechanism of action of accumulation of cellular MG by inhibition of Glo1 may be investigated by studying responses to siRNA silencing of Glo1 and indirectly by addition of high concentrations of exogenous MG. The latter approach has the drawback that there is a steep negative gradient of MG concentration from outside to inside cells when the reverse applies in Glo1 inhibitor chemotherapy. There have also been many studies that have used MG from commercial sources which is known to have major formaldehyde contamination [4, 78]. How this contamination may compromise the outcomes is not known; where there are corroborate studies with Glo1 siRNA knockdown, the outcomes are secure.

To identify key processes mediating MG-induced inhibition of tumour cell growth, study of changes occurring early in the time course of cytotoxicity under MG-concentration limiting conditions are critical. From initial studies with human leukaemia 60 (HL60) cells we found growth inhibition required exposure to MG for 24 h. This was approximately equivalent to one cell growth cycle duration [79]. It is likely therefore that MG-treated cells commit to cell death mainly at one point of the cell growth cycle and that MG concentration has to be maintained at a cytotoxic level for 24 h for effective antitumour effect. Analysis of the cell growth cycle in MG-treated HL60 cells showed that by 12 h there was a decrease of cells in the S-phase, accumulation of cells in the G_0-G_1 phase and induction of apoptosis, suggesting cells are growth arrested and die at entry to the S-phase and DNA synthesis is inhibited. Indeed, from MG concentration-response curves, inhibition of cell growth was closely linked to inhibition of DNA synthesis rather than inhibition of RNA or protein synthesis. Time course studies with [^{14}C]MG where 98% inhibition of cell growth was achieved showed intriguingly MG adducts with protein maximized at 30 min and thereafter decreased, MG adducts with DNA increased to 1 h and remained at that level and MG adducts with RNA increased slowly throughout the initial 2 h period studied at very low levels. This peak protein adduct content was *ca.* 35 pmol per 10^6 cells, equivalent to an increment of *ca.* 1 mmol/mol arg MG-H1 adducts. This is only an approximate doubling of MG-H1 adduct content, compared to control. The decrease in protein adducts beyond 30 min indicate cellular proteolysis is activated rapidly in response to MG treatment [14]. The maximum level of MG-derived DNA adducts attained, was *ca.* 40 adducts per 10^6 nucleotides cells. This was only a 2-fold increase with respect to untreated cells [10]. Similar studies with the cell permeable inhibitor $BBGCp_2$ (10 μM, inhibiting cell growth by 97%) increased cellular DNA adducts to *ca.* 80 adducts per 10^6 nucleotides. This was a 4-fold increase with respect to untreated cells [10]. It is likely that the increases in steady-state levels of MG-derived protein and DNA adducts are relatively modest because the MG and $BBGCp_2$ treatments are activating cell proteolysis and DNA damage repair mechanisms. It cannot be inferred that 2-fold and 4-fold increase of protein and DNA adducts in other tumour cells lines and primary cultures will induce toxicity. Tumours cells maintain markedly different steady-state levels of MG-H1 and MGdG which will tolerate different extents of change in MG concentration before cytotoxicity. For example, tumour cell lines had over 10-fold difference in steady-state level of MGdG [10]. This relates to both glyoxalase pathway metabolism and activity of NER.

To predict the cellular concentration of MG sustained by Glo1 inhibition we developed a mathematical model of the glyoxalase pathway [19]. Currently this is one-compartment that does not take into account cellular export MG but provides initial insights

into how large the cellular increase in MG is likely to be for effective tumour cell kill. A key aspect of MG metabolism accommodated in the model is the large capacity of reversible binding sites for MG provided by protein thiols. Note that experimental estimates of MG concentration represent the sum of free MG, GSH-MG hemithioacetal and MG-protein thiol adducts. Most (>99%) MG *in situ* is reversibly bound to protein thiols. Modifying the model to predict change in MG concentration when Glo1 is inhibited by 90% (10-fold inhibition), the predicted steady-state increase in cellular MG was increased 6.3 fold and the decrease in D-lactate formation was 31% [19]. The increase in MG is less than might be expected on first thoughts but the less than 10-fold increase in MG concentration is due the effect of increased MG and hemithioacetal concentrations reacting more effectively with the residual Glo1 activity. In cell culture studies where 97% inhibition of HL60 cell growth was achieved by 10 μM $BBGCp_2$, the cellular concentration of active inhibitor S-p-bromobenzylglutathione was *ca.* 13 μM [7]. Given that the K_i = 160 nM, this is predicted to inhibit Glo1 by 97%. Re-running the metabolic mathematical model for 97% inhibition of Glo1 (30-fold inhibition), the predicted increase in cellular MG concentration is *ca.* 12-fold. So a sustained 12-fold increase in steady-state MG concentration in HL60 cells for 24 h or a cell cycle is likely to produce potent antitumour effect. The very high concentrations of MG required to inhibit HL60 cell growth in the absence of Glo1 inhibition was probably required to maintain cellular MG concentration to at least 12-fold higher than control for 24 h. This remains to be confirmed.

Exogenous MG, siRNA silencing and cell permeable Glo1 inhibitors activate apoptosis of tumour cells under concentration/dose limited conditions [6, 7, 14] and also necrosis at very high concentrations of MG [80]. Characteristics of this have been investigated in many studies – see Table 3. Key features are: growth arrest at entry into the S-phase of the cell cycle; decline of mitochondrial membrane potential and activation of the mitochondrial apoptotic pathway with release of cytochrome c; activation of caspase-3 and caspase-9 and decreased anti-apoptotic factors XIAP, survivin, cIAP1, Bcl-2, and Bcl-xL; and activation of kinase pathways ATM-Chk1 and Chk2 kinases, p38 kinase, MAPK and ASK1-JNK, kinase pathways. Findings that MG induced cell cycle arrest at G_2/M phase using the nocodazole-cell synchronization technique [81] are now thought insecure as cells treated this way do not progress round the cell cycle normally on release from nocodazole treatment [82].

MG likely activates the mitochondrial pathway of apoptosis by modification of the mitochondrial permeability transition pore (MPTP), a high conductance channel in mitochondria. MG-H1 residue formation was produced rapidly, producing membrane depolarization, swelling, and cytochrome c release [83]. Downstream of this is activation of caspase-9 and caspase 3, and interaction with BCL2, XIAP and Bcl-xL [84]. These are responses to increased protein damage by MG. Activation of ATM, Chk1 and Chk2 kinases is indicative of a DNA damage response and is likely the response to increased levels of DNA damage by MG. By analogy with other types of DNA damage, primary MGdG lesions are likely converted into single strand DNA (ssDNA), which is rapidly coated by the ssDNA-binding protein replication protein A (RPA), other proteins and finally Chk1/2 [85]. They are intra-S phase protein activated in response to DNA damage and delay replication origin firing to provide time to deal with repair and preventing under-replicated DNA regions being taken beyond S-phase [86]. When the DNA damage is too high for repair capacity, DNA replication stalls and there is replication catastrophe and apoptosis or necrosis – as reviewed [87]. So, MG-induced cell death likely has both mitochondrial and DNA damage response pathways. Other features of MG-treated cells were: decreased cell migration, invasiveness and tubule formation [88]. This is likely linked to MG modification of integrins and extracellular matrix (ECM) proteins, blocking migration and angiogenesis [15, 32]. MG

modification of ECM protein induces cell detachment-activated cell death where the apoptosis is activated by the extrinsic pathway - lack of ECM contact or the engagement with inappropriate ECM leads to the activation of death receptors, and the intrinsic pathway - mitochondria apoptotic pathway [89].

N-Acetyl-cysteine (NAC) was found to inhibit MG-induced apoptosis and this was considered to be evidence of oxidative stress mediating cell death by increased reactive oxygen species (ROS) [81, 90, 91]. Use of NAC in this way is misleading, however, and this interpretation is probably incorrect. NAC binds MG in the culture medium and blocks apoptosis and related cell response by preventing MG entering cells [92]. Consistent with lack of critical involvement of ROS in MG-induced cells death was the observation that overexpression of cytoplasmic and mitochondrial superoxide dismutases did not inhibit MG-induced apoptosis [93].

In studies of MG-modified proteins, there was apparent preferential modification of heat shock protein-27 (HSP27; also known as heat shock protein beta-1) in tumour cells on arg-188. HSP27 has anti-apoptosis activity and MG modification of this may block its suppression of the mitochondrial apoptotic pathway [94]. The modification was detected by immunoblotting with monoclonal antibody mAb3C which recognises the MG-derived AGEs, argpyrimidine, and with lower affinity also MG-H1 [95]. The modification was claimed to be argpyrimidine but this is unlikely as it is a minor MG-derived AGE [9]. In recent studies of MG-modified proteins by direct detection with high resolution mass spectrometry we were able to detect MG-H1 residue modification of HSP27 at arg-188 in cell protein incubated with exogenous MG but it was one of 344 proteins modified by MG in a total of 1366 proteins detected in the cell cytosol [96]. One possibility for this disparity may be that the antibody is recognizing a specific MG-modified peptide. The MG-modified, arginine containing tripeptide motif residue in HSP27 is ser-arg-ala. This same peptide motif residue occurs in multiple domains in keyhole limpet haemocyanin – the protein used for MG modification to produce the immunogen to raise the mAb3C antibody. This antibody may detect only this MG-modified tripeptide motif rather than all MG-modified proteins in cells, giving an impression of selective MG-modification of HSP27 in cells when this is not the case. This requires further investigation but blocking of anti-apoptotic activity of HSP27 by MG may contribute to cytotoxicity [94].

The mechanism of cytotoxicity of cell permeable Glo1 inhibitors through cellular accumulation of MG accumulation in tumour cells is summarized – Fig. 4. From the observation that several current antitumour drugs suffer MDR with high expression of Glo1, it is likely that MG-induced apoptosis and anoikis contribute in part to the mechanism of action of current clinical antitumour drugs.

7. Neuroendocrine tumour as target for GLO1 amplification and MDR

Malignant neuroendocrine tumours (NETs) comprise only ~2% of all malignant tumours diagnosed in the western world [97] but incidence is increasing [98]. The majority of NETs are well differentiated and consequently often insensitive to chemotherapy [97, 99], with the exception of pancreatic NET where response rates are highly varying. However, lack of objective response to chemotherapy has been reported in up to 60-70% of patients even with pancreatic NETs [100, 101]. This has been attributed to low mitotic rates, presence of high levels of the anti-apoptotic protein Bcl-2 and increased expression of the multi-drug resistance gene P-glycoprotein 1 [99]. A further factor, however, may be amplification of GLO1. Overexpression of Glo1 confers resistance to streptozotocin which are first choice treatments for chemotherapy of pancreatic NETs [102]. It is therefore of interest and important to study GLO1 amplification and Glo1 expression in NETs. This may in future inform for optimum antitumour drug selection for decreased sensitivity to resistance by Glo1

overexpression. The prevalence of GLO1 copy-number increase in patients with GEP-NET has not been investigated to date but Nrf2 has been found over-activated in some NETs due to mutation that block its proteolysis [103]. This is expected to increase the expression of Glo1 and confer growth advantage, MDR and possibly poor treatment outcome. We are currently performing related research at our local NET Centre.

8. Concluding remarks

Methylglyoxal metabolism and glyoxalase are frequently overlooked in cancer research but new advances suggest oncologists will find renewed interest. Glo1 inducers may provide a safe ane effective way to clinically suppress COX-2 and RAGE for cancer prevention. Glo1 response to hypoxia may provide the basis of selective cytotoxic chemotherapy for removal of tumour stem cells. Glo1 copy number, mRNA and protein and MG-modified protein assessments clinically may provide biomarkers of tumour growth, MDR and sensitivity to cell permeable Glo1 inhibitors. Finally, if the barriers to development of cell permeable Glo1 inhibitors can be overcome, there may be effective therapy for refractory tumours with high Glo1 expression - possibly including NETs.

Conflict of interest

The authors declare no competing financial interests.

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Table 1. GLO1 Copy number increase in human tumours.

Tumour type(s) and prevalence	Link to functionally, tumour growth and survival	Reference
Breast cancer (22%), sarcomas (17%), NSCLC (11%), bladder (9%), gastric (6%), renal (6%) colon (3%), glioma (1%)	Linked to increased Glo1 expression (mRNA and activity).	[6]
TNBC (83%). Non-TNBC (9%)	Linked to Glo1 expression.	[55]
Malignant melanoma, advanced stage (80 – 89%)	Not investigated	[57]
Gastric cancer (33%)		[31]
Hepatocellular carcinoma (6%)	Linked to increased Glo1 expression	[45]

Table 2. Inhibitors of glyoxalase 1 and related compounds.

Inhibitor	K_i (nM)	Comment	References
S-p-Bromobenzyl-glutathione	160	Delivered into tumour cells and tissue by diester esterification	[7, 44, 69, 70]
S-(N-p-chlorophenyl-N-hydroxycarbamoyl)-glutathione	40	Delivered into tumour cells and tissue by diester esterification	[74, 104]
Curcumin	5,100	High doses of curcumin, 8 g per day, achieved peak plasma concentrations of *ca.* 1.8 μM. Curcumin is unlikely to provide potent inhibition of Glo1 *in vivo* unless it can be actively accumulated in tumours.	[105, 106]
Methotrexate	20,000	Peak plasma concentrations of methotrexate in cancer chemotherapeutic use is *ca.* 16 μM, so some antitumur activity of methotrexate may be linked to inhibiton of Glo1.	[107, 108]

For other potent Glo1 inhibitors not yet evaluated for cancer treatment see [17].

Table 3. Mechanism of methylglyoxal induced cell death

Cell type	[MG] (μM)	Characteristics	Reference
HL60 (acute myeloid leukemia)	238	GC_{50}: 282 μM. 24 h exposure period required for growth inhibition and toxicity. Growth inhibition increased with medium serum content (growth rate)	[79]
	33 – 524	Apoptosis. Growth inhibition linked concentration-dependently to inhibition of DNA synthesis; early decrease in S-phase cells. Protein adducts maximized at 30 min and DNA adducts at 1 h.	[14]
LNCaP (androgen-sensitive prostate adenocarcinoma)	1,000	Activates mitochondrial apoptotic pathway (cytochrome c release form mitochondria), potentiated by Glo1 silencing	[109]
HEK393 cells	400 & 800	Activation of ATM-Chk1 and Chk2 kinases, p38 kinase, MAPK and ASK1-JNK, kinase pathways.	[81]
Jurkat (T-cell leukemia), MOLT-4 (T-cell leukemia), and HeLa (cervical adenocarcinoma)	250 & 500	Apoptosis (250 μM) with necrosis (500 μM). JNK and caspase-3 driven apoptosis	[80]
Jurkat cells	250	MG-induced activation of caspase-3 and caspase-9, release of cytochrome c, decline of mitochondrial membrane potential and JNK activation;	[91]
SW480 colonic cancer cells	100 - 500	MG suppressed the expression of anti-apoptotic factor XIAP, survivin, cIAP1, Bcl-2, and Bcl-xL. Potentiated TRAIL apoptosis. GLO1 siRNA had the same effect.	[93]
MCF7, MDA-MB-231, T47D	100 – 1,600	Growth inhibition, decreased invasiveness and tubule formation, MAPK activation and decreased Bcl-2 at ≥ 800 μM MG, increasing apoptosis; decreased cell migration at 400 μM ; decreased colony formation at 100 – 200 μM. GLO1 siRNA had the same effect.	[88]

Figure legends.

Figure 1. The glyoxalase system and glycation of protein and DNA by methylglyoxal. **A.** Metabolism of methylglyoxal by the glyoxalase system. **B.** Glycation of arginine residues by methylglyoxal to form hydroimidazolone MG-H1. The peptide amide binds are omitted for clarity Other hydroimidazolone structural isomers may be formed [110]. Other minor MG-derived AGEs are: Nε-(1-carboxyethyl)lysine (CEL), argpyrimidine, methylglyoxal-derived imidazolium cross-link (MODIC), and methylglyoxal-derived lysine dimer, 1,3-di(N^{ε}-lysino)-4-methyl-imidazolium salt (MOLD). In mammalian tissues MG-H1 usually represents > 90% total MG-derived adducts [111]. **C.** Glycation of deoxyguanosine to form imidazopurinone isomers, MGdG. The nucleotide base is shown only for clarity. Lower amounts of 2-(1,R/S-carboxyethyl)-deoxyguanosine (CEdG) are also formed [10].

Fig. 2. Proposed mechanism of anti-inflammatory mechanism of action of Glo1 inducer formulation. Key: yellow filled arrows – mechanism of health improvement by; red filled arrows – damaging processes suppressed. See also [112]. Abbreviations: KLB, β-klotho; CBP, CREB binding protein; and maf, small maf protein – accessory proteins for Nrf2 activation.

Fig. 3. Mechanism proposed for gene amplification of GLO1. See also [41, 62, 63]. Abbreviations: CNV, copy number variation; mini-chromosome maintenance (MCM) proteins; Me_3, histone lysine triple methylation.

Fig. 4. The mechanism of cytotoxicity of cell permeable Glo1 inhibitors through cellular accumulation of methylglyoxal accumulation in tumour cells is summarized. Yellow filled arrows, key reactions of MG. Red arrows: cell death response. Green elements: anoikis; purple elements, mitochondrial apoptotic pathway. Abbreviations: Apaf-1, apoptotic protease activating factor 1; BAX, bcl-2-like protein 4; BID, BH3 interacting-domain death agonist; Fas, CD95 cell death receptor; FADD, Fas-associated death domain adaptor protein; MPTP, mitochondrial permeability transition pore; ss, single strand; tBID, truncated (activated) BID; TNFR1, tumour necrosis factor receptor-1. See also [83, 87, 89].

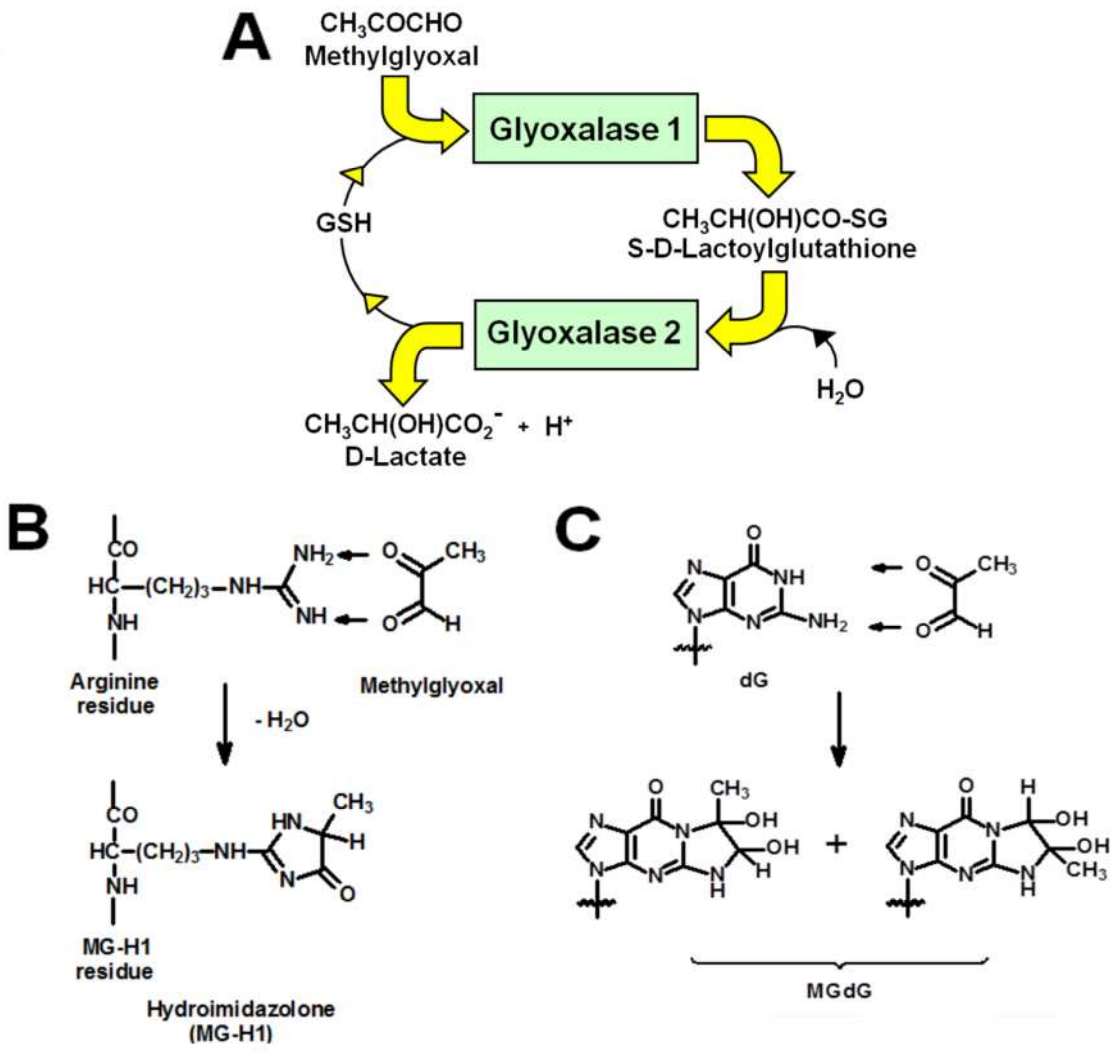

A
CH3COCHO
Methylglyoxal
Glyoxalase 1
CH3CH(OH)CO-SG
S-D-Lactoylglutathione
GSH
Glyoxalase 2
H2O
CH3CH(OH)CO2- + H+
D-Lactate
B
Arginine residue
Methylglyoxal
- H2O
MG-H1 residue
Hydroimidazolone (MG-H1)
C
dG
MGdG

Figure 1

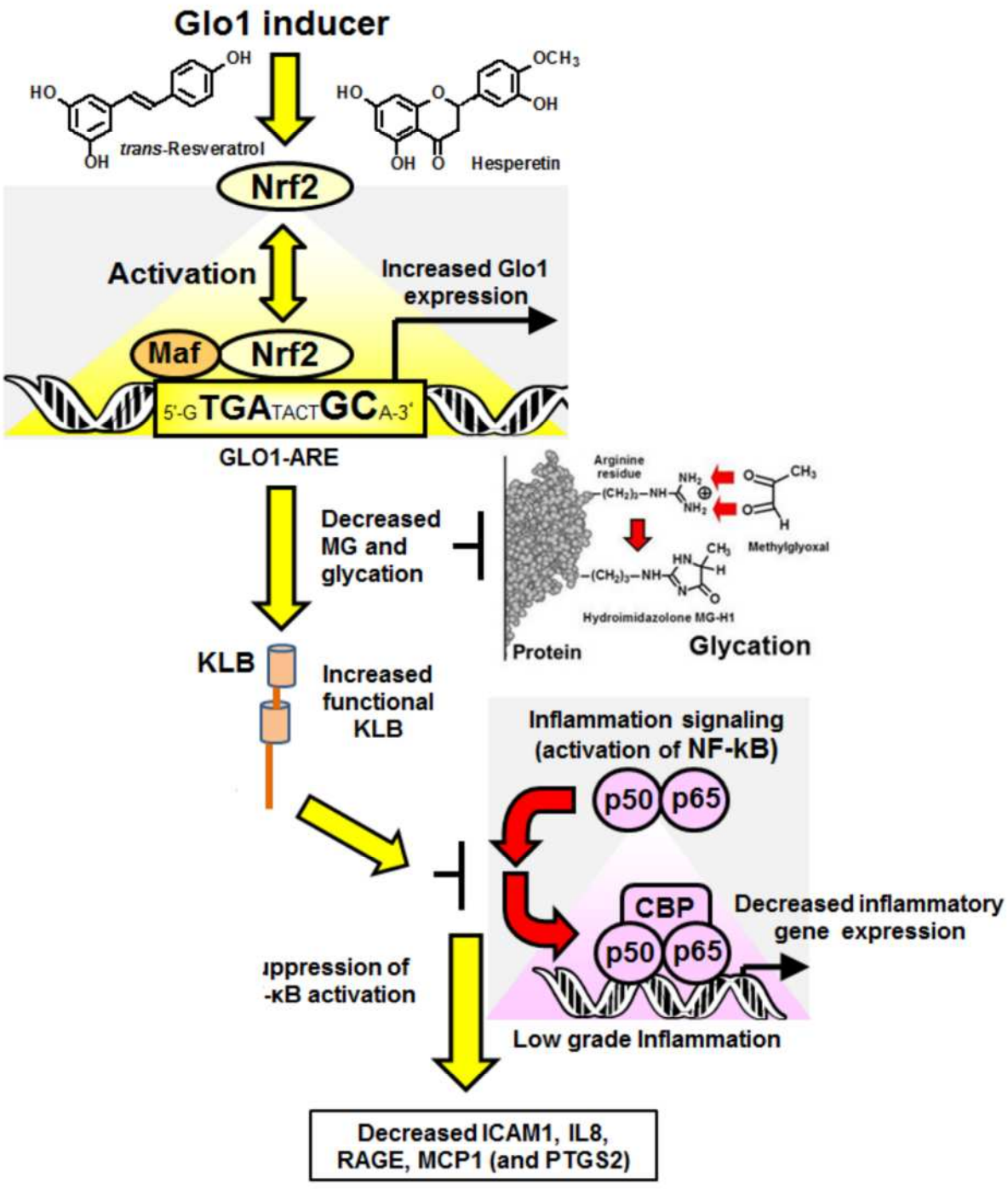
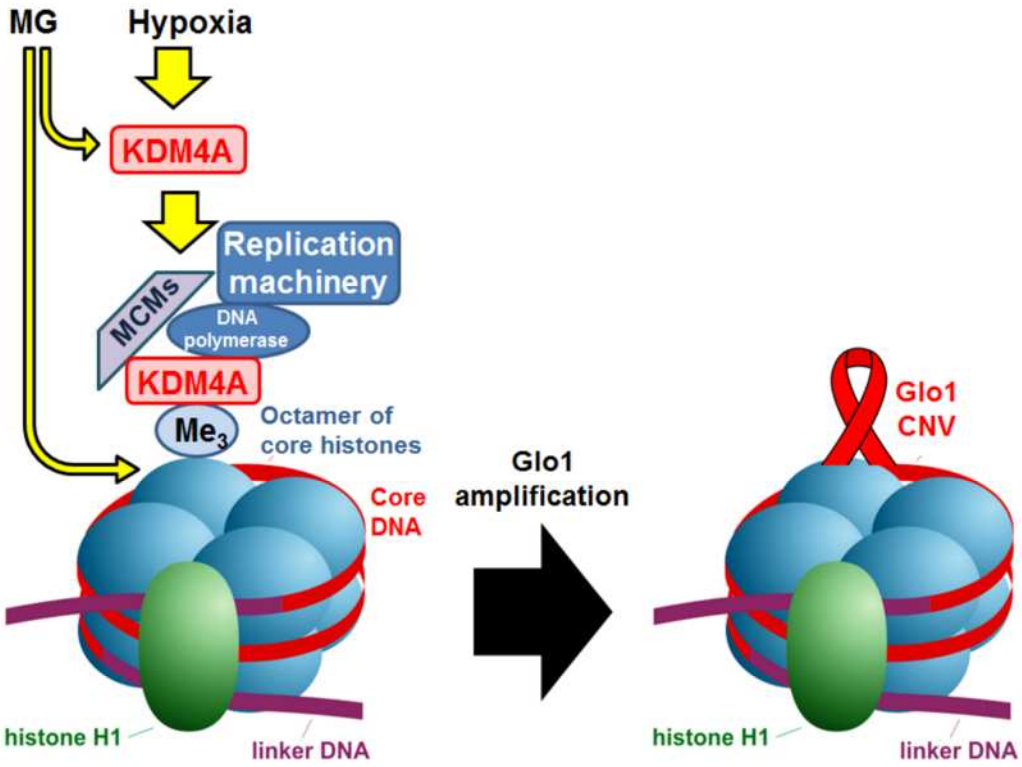

Glo1 inducer
trans-Resveratrol
Hesperetin
Nrf2
Activation
Increased Glo1 expression
Maf
Nrf2
5'-GTGATACTGCA-3'
GLO1-ARE
Decreased MG and glycation
Arginine residue
Methylglyoxal
Hydroimidazolone MG-H1
Protein
Glycation
KLB
Increased functional KLB
Inflammation signaling (activation of NF-kB)
p50
p65
CBP
p50
p65
Decreased inflammatory gene expression
ıppression of -κB activation
Low grade Inflammation
Decreased ICAM1, IL8, RAGE, MCP1 (and PTGS2)

Figure 2


MG
Hypoxia
KDM4A
Replication machinery
MCMs
DNA polymerase
KDM4A
Me3
Octamer of core histones
Core DNA
histone H1
linker DNA
Glo1 amplification
Glo1 CNV
histone H1
linker DNA

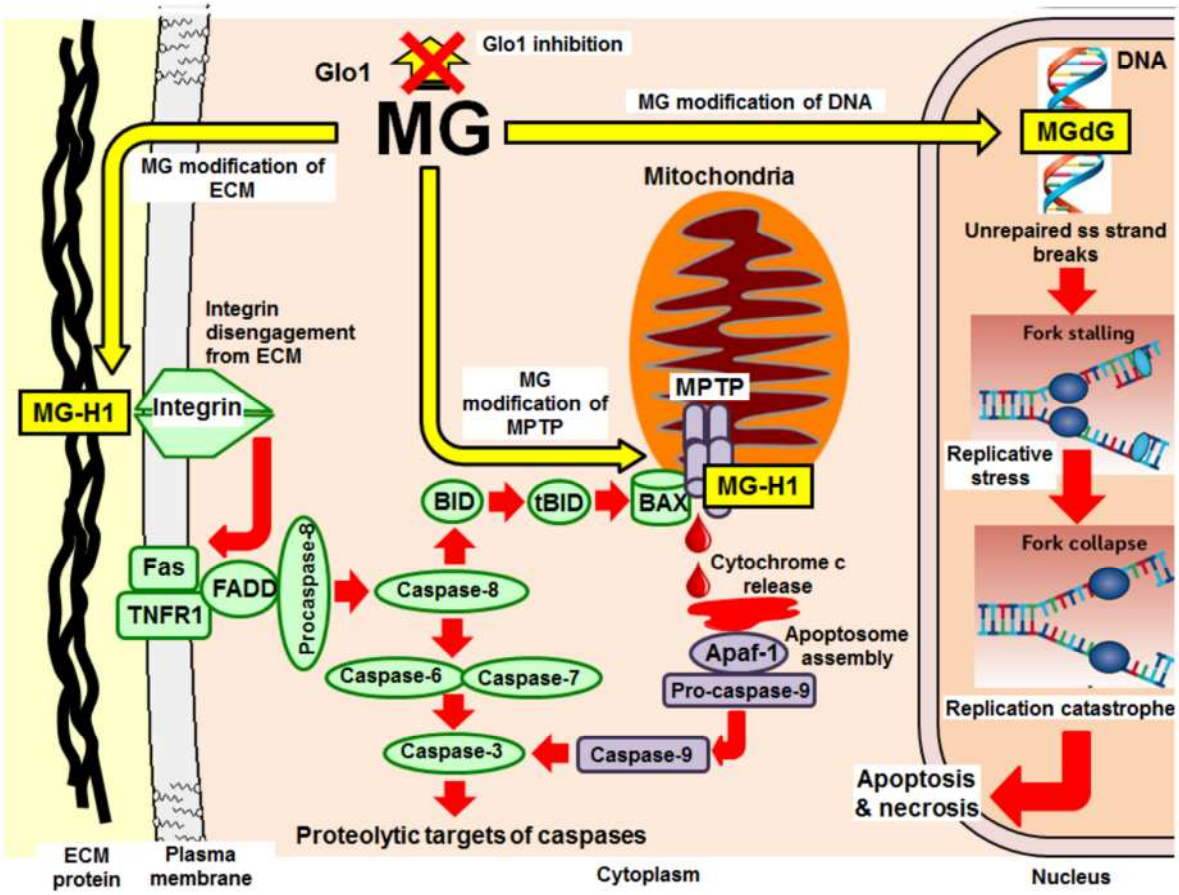

Glo1
Glo1 inhibition
MG
MG modification of DNA
DNA
MGdG
MG modification of ECM
Mitochondria
Unrepaired ss strand breaks
Integrin disengagement from ECM
Fork stalling
MG-H1
Integrin
MG modification of MPTP
MPTP
MG-H1
Replicative stress
BID
tBID
BAX
Fork collapse
Fas
TNFR1
FADD
Procaspase-8
Caspase-8
Cytochrome c release
Apoptosome assembly
Apaf-1
Caspase-6
Caspase-7
Pro-caspase-9
Replication catastrophe
Caspase-3
Caspase-9
Apoptosis & necrosis
Proteolytic targets of caspases
ECM protein
Plasma membrane
Cytoplasm
Nucleus

Figure 4