Glyoxalase I inhibitors in cancer chemotherapy

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Abstract
Several recent developments suggest that the GSH-dependent glyoxalase enzyme system deserves renewed interest as a potential target for antitumour drug development. This summary focuses on the design and development of new classes of tumoricidal agents that specifically target this elementary detoxification pathway in order to induce elevated concentrations of cytotoxic methylglyoxal in tumour cells. Special emphasis is placed on structure- and mechanism-based inhibitors of GlxI (glyoxalase I), the first enzyme in the pathway. A new class of bivalent transition-state analogues is described that simultaneously bind the active site on each subunit of the homodimeric human GlxI, resulting in $K_i$ values as low as 1 nM. Also described is a new family of bromoacyl esters of GSH that function as active-site-directed irreversible inhibitors of GlxI. Newer produgs for delivering the GSH-based inhibitors into tumour cells include reactive sulphoxide esters that undergo acyl exchange with endogenous GSH to give the inhibitors, and polymethacrylamide esters of the inhibitors that are potentially tumour-selective on the basis of the 'enhanced permeability and retention effect'. Finally, a preliminary evaluation of the efficacy of selected GlxI inhibitors in tumour-bearing mice is given.

Introduction
GlxI (glyoxalase I) is a homodimeric Zn$^{2+}$-metalloenzyme that catalyses the GSH-dependent conversion of a variety of aromatic and aliphatic α-ketoaldehydes into α-hydroxy thioesters having the $R$ configuration at C-2 [1,2]:

\[
\begin{align*}
\text{Methylglyoxal (R = CH$_3$)} & \quad \text{GSH} \\
\text{Thiohemiacetal} & \quad \text{5-α-Lactoylglutathione}
\end{align*}
\]

where $R = \text{CH}_3$, $\text{C}_6\text{H}_5$, $\text{C}_6\text{H}_4\text{Cl}$. This enzyme is receiving renewed interest as a potential antitumour target [3].

Several observations suggest that the primary physiological function of GlxI is to work in concert with the thioester hydrolase GlxII (glyoxalase II) to chemically remove cytotoxic methylglyoxal from cells as non-toxic d-lactate. Methylglyoxal arises as a normal byproduct of carbohydrate metabolism as well as from other sources [4]. This elementary α-ketoaldehyde is known to form covalent adducts with proteins and nucleic acids, which is the presumed basis of cytotoxicity [5,6]. The glyoxalase system is well designed to remove methylglyoxal from cells, as both enzymes operate near the diffusion-controlled limit under physiological conditions ([7] and references therein). Presumably, this high level of kinetic efficiency reflects the need to maintain minimal levels of methylglyoxal in cells [8].

Key words: anticancer agent, glyoxalase I, inhibitor, prodrug.

Abbreviations used: GlxI, glyoxalase I; GlxII, glyoxalase II; $IC_{50}$, concentration of drug producing 50% growth inhibition

Inhibitors of GlxI
In 1971, Vince and Daluge [9] first suggested that inhibitors of GlxI might function as antitumour agents by inducing elevated levels of methylglyoxal in cancer cells. Indeed, tumour cells were subsequently found to exhibit exceptional sensitivity to exogenous methylglyoxal [10–13]. For example, methylglyoxal inhibits the growth of human leukaemia 60 cells in culture ($IC_{50} \sim 238 \mu M$), but is much less toxic to differentiated neutrophils [11]. Thus cell-permeable inhibitors of GlxI might exhibit tumour-selective toxicity.

A range of different structure-based and mechanism-based inhibitors of GlxI have now been synthesized that could be used to test this hypothesis (Figure 1). From an historical perspective, simple S-aryl and S-alkyl GSH derivatives were found to be good competitive inhibitors of the enzyme, with p-bromobenzyl-glutathione (Figure 1, structure 1) being one of the strongest inhibitors [14]. Quantitative structure-activity relationships show that the glutathionyl function plays an important role in binding and that binding affinity increases with increasing hydrophobicity of the S-substituent, suggesting the presence of an apolar binding pocket in the active site [1,15]. In anticipation of in vivo testing, a γ-glutamyl transeptidase-resistant peptidomimetic of 1 has been prepared by substituting the γ-glutamyl peptide bond of 1 with a ureido function (Figure 1, structure 2) [16].

Elucidation of both the reaction mechanism and structural chemistry of human GlxI confirmed some of the earlier notions about the mode of inhibitor binding to the active site, and prompted the development of a new generation of interesting and, in some cases, quite novel enzyme inhibitors. The hypothesis that the mechanism of action of GlxI...
involves an ene-diolate intermediate formed by a proton-transfer mechanism inspired the synthesis and testing of a new class of S-(N-aryl-, and S-(N-alkyl-N-hydroxycarbamoyl) esters of GSH (Figure 1, 3a–3d and 4a–4c respectively) as tight-binding transition-state mimics of the enzyme [14,17,18]. Indeed, these compounds are among the strongest competitive inhibitors of the enzyme yet reported.

The X-ray crystal structure of human GlxI, with transition-state mimic 3d complexed to each active site, has been determined [19] (Figure 2). The p-iodophenyl function of the bound ligand is positioned in a hydrophobic binding pocket in the active site, which probably accounts for the general observation that, as the hydrophobicity of the S-substituent increases, binding affinity also increases (Figure 1). Binding appears to be further augmented by direct co-ordination interactions between the catalytically essential Zn$^{2+}$ in the active site and the cis-oid oxygen atoms of an energy-minimized syn-conformation of the S-substituent of bound 3d, calculated using semi-empirical and ab initio methods [18].

The observation that the $\gamma$-glutamyl-NH$_2$ groups of the bound inhibitors are exposed to bulk solvent (Figure 2) has also been exploited by chemically cross-linking two molecules of 3b through the exposed NH$_2$ groups using poly-$\beta$-alanyl tethers of differing length (6a–6d) (Z.-B. Zheng and D.J. Creighton, unpublished work). In principle, binding affinity could increase if the length of the tether allows simultaneous binding to both active sites. Indeed, binding affinity increases dramatically when $n = 6$ ($K_i = 0.96$ nM), corresponding to a maximum separation of 70 Å (Figure 1). This is fully consistent with the separation distance of 30 Å between the amino groups of bound ligands observed in the X-ray structure.

Progress has been made towards identifying active-site-directed irreversible inactivators of human GlxI (Figure 1). For example, both $\alpha$-bromodiketo (H. Fan, M.J. Kavarana,
A major obstacle to using GSH derivatives as antitumour agents is that the charged glutathionyl function precludes rapid diffusion into cells. An important advance in drug delivery was achieved in 1992, when Lo and Thornalley demonstrated that the diethyl ester of S-(4-bromobenzyl)glutathione, I(Et)₂, diffuses into human leukaemia 60 cells and subsequently undergoes de-esterification to give 1 [20]. This general strategy has been successfully used to deliver transition-state mimics 3a–3c into a range of different types of tumour cells in culture (Scheme 1) [21]. The kinetics of drug uptake by L1210 cells in culture indicate that the diethyl ester prodrugs enter cells by passive diffusion across the cell membranes.

A ‘sulphoxide’ prodrug strategy has also been developed that can be used to rapidly introduce 3b into tumour cells via an acyl-interchange reaction between a neutral ethyl sulphoxide, which diffuses very rapidly across cell membranes, and intracellular GSH (Scheme 1) [22]. The apparent rate constant for the appearance of 3b in L1210 cells in the presence of the sulphoxide prodrug is about 35-fold greater than that obtained using 3b(Et)₂. An interesting modification of this strategy has been achieved by incorporating the sulphoxide prodrug into a high-molecular-mass hydroxypropylmethacrylamide co-polymer, which rapidly forms 3b in the presence of GSH in aqueous buffered solution (Scheme 1) (Z.-B. Zheng, J.L. Eiseman and D.J. Creighton, unpublished work). Polymer–drug conjugates have previously been used to selectively deliver antitumour drugs such as doxorubicin to tumours, because blood vessels in rapidly growing tumours are abnormally ‘leaky’ [23,24]. Thus a polymer–drug conjugate that is activated by intracellular GSH could be used for the tissue-specific delivery of GlxI inhibitors.

**Antitumour activities in vitro**

Of the competitive inhibitors shown in Figure 1 that have been tested, all show significant antitumour activity in vitro when delivered as the dialkyl ester or sulphoxide prodrugs. Antitumour activity is most easily explained by inhibition of GlxI. Both the diethyl and dicyclopropyl esters of 1 inhibit the growth of a range of different human tumours in culture [20,25]. Incubation of human leukaemia 60 cells in the presence of 1(cyclopropyl), causes a detectable increase in the concentration of methylglyoxal [26]. The diethyl ester prodrugs of 3a(Et)₂–3c(Et)₂ are potent inhibitors of murine leukaemia L1210 and murine B16 melanoma cells in vitro, with GI₅₀ (concentration of drug producing 50% growth inhibition) values (~100 to ~7 µM) that closely parallel the Kᵢ values of the diacid form of the inhibitors for human GlxI (the diethyl esters themselves do not inhibit GlxI) [21]. Moreover, preincubation of L1210 cells with 3b(Et)₂ at its GI₅₀ concentration increases the sensitivity of L1210 cells to exogenous methylglyoxal by about 5-fold. The sulphoxide prodrug of 3b is approx. 10-fold more potent to L1210 cells in culture (GI₅₀ = 0.5 µM) than the corresponding diethyl ester prodrug 3b(Et)₂ (GI₅₀ = 7 µM) [25]. This probably reflects the greater rate at which the sulphoxide delivers the inhibitor into cells.

The diethyl ester prodrugs of 1, 3b and 3c also display significant tumour-selective toxicity towards L1210 cells compared with normal murine splenic lymphocytes in vitro [21]. Differential toxicity might reflect the greater intrinsic sensitivity of tumour cells to the toxic effects of methylglyoxal. In the cases of 3b and 3c, selectivity could also arise from the 10-fold lower levels of GlxII activity in L1210 cells compared with splenic lymphocytes, as 3b and 3c are slowly hydrolysed in the presence of the GlxII [14]. Low activity of GlxII is a general characteristic of tumours [27].

**Pharmacokinetics and in vivo efficacy studies**

The dialkyl ester prodrug strategy provides an efficient means of distributing GlxI inhibitors to the tissues of esterase-deficient laboratory mice [28]. Intravenous administration of 3b(Et)₂ to male and female mice bearing murine B16 melanoma near its maximally tolerated dose (120 mg/kg)
resulted in rapid clearance from plasma ($t_{1/2} \approx 8$ min) with the concomitant appearance of 30–60 µM 3b in tumour tissue after 15 min, followed by a decrease to a plateau value of $\sim 7$ µM after 120 min. Compartmental analysis of the data indicated that the rapid loss of 3b(Et)$_2$ from plasma is due primarily to the rapid and extensive extravascular distribution of the prodrug into the tissues of the mouse and not to hydrolytic conversion into 3b(Et) and 3b.

Small-scale efficacy studies indicate that 3b(Et)$_2$, using aqueous hydroxypropyl-β-cyclodextran as a vehicle, can effectively inhibit tumour growth in plasma esterase-deficient mice bearing murine B16 melanoma and in esterase-deficient athymic nude mice bearing androgen-independent human prostate PC-3 tumours or human colon HT-29 tumours. In the murine melanoma study, intravenous bolus administration of 3b(Et)$_2$ or 3b(cyclopentyl)$_2$ at 67% of the maximally tolerated dose (80 mg/kg per day for 5 days over 2 weeks) proved to be as effective as Adriamycin (4 mg/kg for 3 days) administered intraperitoneally near its maximally tolerated dose, reducing tumour growth rates by $>50\%$. Similar results were obtained when comparing the efficacies of 3b(Et)$_2$ and cisplatin with human prostate PC-3 tumours, and 3b(Et)$_2$ and vincristine with human colon HT-29 tumours in athymic nude mice. There were no adverse side effects associated with the administration of 3b(Et)$_2$ in any of the in vivo efficacy studies.

Conclusions/future directions

Major advances in our understanding of the catalytic mechanism and structural chemistry of human GlxI have led to several novel reversible and irreversible inhibitors of the enzyme. For example, the bivalent transition-state analogues are potentially capable of binding extremely tightly to the enzyme, with subnanomolar $K_i$ values. Different prodrug strategies have also been described that are capable of efficiently delivering GlxI inhibitors into tumour cells either in vitro or in vivo. Preliminary efficacy studies provide the necessary proof-of-concept to prompt further evaluation of GlxI as a viable antitumour target, particularly in view of the availability of more powerful inhibitors of the enzyme.

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References


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