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Review

Gamma-glutamyltransferase of Cancer Cells at the Crossroads of Tumor Progression, Drug Resistance and Drug Targeting

ALESSANDRO CORTI, MARIA FRANZINI, ALDO PAOLICCHI and ALFONSO POMPELLA

Department of Experimental Pathology BMIE, University of Pisa, Italy

Abstract. *Gamma-glutamyltransferase (GGT) is a key enzyme involved in glutathione metabolism and whose expression is often significantly increased in human malignancies. In the past years, several studies focused on the possible role of GGT in tumor progression, invasion and drug resistance. The involvement of a pro-oxidant activity of GGT, besides its early recognized contributions to cellular antioxidant defenses, has been repeatedly documented. GGT-derived pro-oxidants can modulate important redox-sensitive processes and functions of the cell, with particular reference to its proliferative/apoptotic balance, which has obvious and important implications in tumor progression and drug resistance. In addition, the specificity of the enzymatic reaction carried out by GGT suggests that suitable pro-drugs could be selectively metabolized (activated) by GGT expressed in tumor tissue. This paper is a review of the recent investigation in the field, focusing on the potential role of GGT as a diagnostic/prognostic marker, as well as a target for anticancer treatments.*

Gamma-glutamyltransferase (GGT) is a membrane-bound enzyme involved in the metabolism of glutathione (gamma-glutamyl-cysteinyl-glycine; GSH), and is expressed by a wide number of cell types. GGT catalyzes the transfer of the glutamyl moiety, linked through the glutamate gamma-carboxylic acid to cysteine, to acceptor molecules including peptides, amino acids and water. Being located on the outer aspect of the cell membrane, GGT in the first place catalyzes the degradation of extracellular GSH, thus favouring the recovery of constituent amino acids for subsequent intracellular GSH resynthesis. As GSH is the main water-soluble antioxidant within the cell, GGT has been traditionally regarded as a component of the cell protection system against oxidative stress (1). On the other hand, other pathophysiologically relevant compounds are also GGT substrates, in particular all GSH conjugates, including leukotriene C4 (2), *S*-nitroso-glutathione (GSNO; 3) and GSH adducts of xenobiotics formed by the action of glutathione-*S*-transferases (4).

GGT expression varies considerably among normal tissues. In particular, high GGT activities are present on the luminal surface of secretory and absorptive cells, including those of bile ducts, bile canaliculi and proximal tubules of the kidney, and in endothelial cells of nervous system capillaries (1, 5). A dysregulated expression of GGT has been detected in several tumor types (6), and several papers have suggested a role for GGT in GSH-dependent drug-resistance mechanisms (7). On the other hand, recent findings have documented that redox processes ensuing from GGT-mediated metabolism of extracellular GSH may be implicated in the modulation of critical aspects of tumor cell biology (8, 9), and the possibility of exploiting tumor GGT as a means for local activation of anticancer pro-drugs has also been recently explored (10). Details of the several aspects involved are illustrated in the following pages.

Pathways of GGT Induction

Early reports showing the appearance of GGT-positive foci in laboratory animals exposed to chemical carcinogens first suggested the hypothesis of GGT as an early marker of neoplastic transformation (1, 7). The increased expression of GGT in actively proliferating pre-neoplastic foci in the liver was recently confirmed (11). The mechanisms underlying the increased GGT expression induced by carcinogens remained however unidentified. Several studies showed that GGT is up-regulated in different cell types after acute exposure to oxidative stress (12-16), and the involvement of activator protein-1 (AP-1)-like transcription factor(s) (17), or of electrophile response element/nuclear factor erythroid 2-

Correspondence to: Alessandro Corti, Ph.D., Dipartimento di Patologia Sperimentale BMIE, Scuola Medica, Via Roma 55, 56126 Pisa, Italy. e-mail: a.corti@biomed.unipi.it

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related factor 2 (EpRE/Nrf2) signalling through activation of the extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways were suggested (18). The involvement of a *ras*-dependent transduction pathway was recently proposed (11), and indeed, a connection between GGT expression and activation of Ras-MAPK pathways has been demonstrated in colon cancer cells following gamma-irradiation (19), as well as exposure to oxidative stress (20). Reactive oxygen species (ROS) have been implicated in the process of carcinogenesis, and at the same time, the redox regulation of many genes in response to ROS/electrophiles seems to modulate GGT expression; this could altogether explain the increased GGT expression described in tumors.

Interestingly, *GGT* mRNA was shown to be induced also by cytokines, including tumor necrosis factor alpha (TNF-alpha) (21), and interferon (IFN)-alpha and -beta (22), and evidence was obtained that TNF-alpha is able to induce GGT expression through nuclear factor-kappaB (NF-κB)-dependent signaling, specificity protein 1 (Sp1) transcription factor and RNA polymerase II recruitment to the GGT promoter (23). These results seem to connect inflammation to GGT expression, not just as a response to inflammation-related oxidative stress, but rather as the effect of specific inflammatory cytokines. From this perspective, the biological significance of an increased GGT expression could thus be twofold, *i.e.* i) a defensive mechanism against oxidative stress, as well as ii) a regulatory mechanism, possibly through GGT-mediated metabolism of leukotrienes and GSNO.

GGT Expression in Neoplasia

The distribution and concentration of GGT in human tumors present several differences from what is observed in normal tissues. Increased levels of GGT have been observed in cancer of ovary (24), colon (25), liver (26), astrocytic glioma (27), soft tissue sarcoma (28), melanoma (29, 30) and leukemias (31). A large study by Hanigan *et al.* (6) of 451 human tumors showed that most tumors deriving from GGT-positive tissues were positive themselves, and that carcinomas of lung and ovary were also generally GGT-positive despite deriving from GGT-negative epithelia. In studies on melanoma cells *in vitro* and *in vivo*, elevated GGT activity was found to accompany an increased invasive growth (29, 32, 33), and a positive correlation was described between GGT expression and unfavourable prognostic signs in human breast cancer (34). Nevertheless, a constant relationship between malignant transformation and the expression of GGT was not demonstrated (1). Besides the studies reported above, other works did not find any correlation between GGT expression and standard clinical-pathological parameters in models of prostatic (35), colorectal (36) and breast cancer (37). These differences can be explained as the result of the high variability present in cancer cells, as well as the effect of other factors, such as the environment, drugs and diet, which may alter the phenotype of neoplastic lesions, including GGT expression (38). A summary of the available data concerning GGT expression in a series of important human neoplasms has been recently provided (7).

GGT Functions in the Cancer Cell

Several studies have addressed the relationships of GGT activity with the malignant phenotype, in particular the question of whether an increased GGT expression itself plays any active role in neoplastic transformation (1). The involvement of GGT in cellular resupply of GSH, and the increased resistance to pro-oxidant drugs observed in several GGT-expressing cell lines suggested the inclusion of GGT among the components of cellular defensive systems. On the other hand, a number of recent findings indicate that, under particular conditions, the metabolism of GSH by GGT can exert pro-oxidant effects, with modulatory effects on several redox-sensitive processes (7-9).

GSH is synthesized inside cells and transported in the extracellular milieu through plasma-membrane transporters (39), down a concentration gradient (millimolar *vs.* micromolar). Extracellular metabolism of GSH by GGT, in concert with cell surface dipeptidases, promotes the release and recovery by cells of constituent amino acids, among which glutamic acid (40) and essential cysteine (41). Indeed, studies performed both *in vitro* and *in vivo* showed that GGT-overexpressing cells are able to utilize extracellular GSH as a source of cysteine more efficiently (42-44), resulting in a selective growth advantage both at physiological and at limiting cysteine concentrations (45, 46). It was in fact observed that a short (2 h) inhibition of GGT is able to lower intracellular cysteine in GGT-positive cervical carcinoma cell lines (47). Thus, the favouring action of GGT in tumor growth is twofold, in that it operates as a source of essential amino acids both for protein synthesis and for the maintenance of intracellular levels of GSH (Figure 1).

Adequate levels of GSH are the basis of cellular resistance against several electrophilic/alkylating compounds and indeed, GGT-overexpressing cells were shown to be more resistant to hydrogen peroxide (48), and chemotherapics such as doxorubicin (49), cisplatin (45, 50-51) and 5-fluorouracil (52). In melanoma cells, GSH depletion and GGT inhibition significantly increased cytotoxicity of oxidative stress conditions (53). Interestingly, the same treatments were also shown to induce GGT expression (49-52), possibly a protective adaptation induced by oxidative stress itself. As such, GGT expression would perfectly fit in the so-called 'resistance phenotype', *i.e.* a common pattern of biochemical changes exhibited by chemically transformed, pre-neoplastic

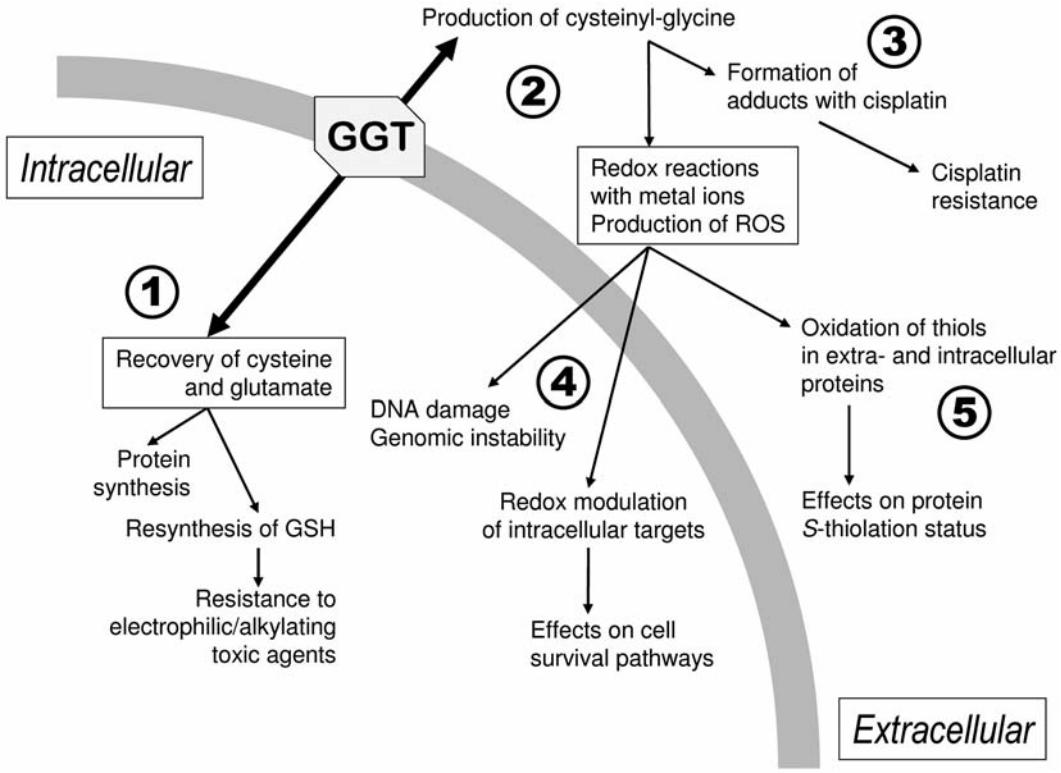

Figure 1. *Intra- and extracellular reactions promoted by GGT in cancer cells: resupply of cysteine for protein and glutathione synthesis (1), production of cysteinyl-glycine giving rise to redox reactions (2) and formation of adducts with cisplatin (3), oxidative DNA damage and genomic instability (4), modulatory effects on protein thiol residues (5). ROS, Reactive oxygen species; GSH, glutathione.*

cells, allowing them a better defense against injury by oxidants and xenobiotics (7).

Conflicting results were however reported on the supposed roles of GSH and GGT in protection against cell injury. In the first place, a decrease rather than an increase of intracellular GSH in different cancer cell lines transfected with *GGT* cDNA was described, both *in vitro* (46, 54-55) and in tumors obtained by transplantation in nude mice (45, 46, 56). An inverse relationship between GGT activity and intracellular GSH levels was even described in cisplatin-resistant melanoma (57, 58) and A2780 ovarian carcinoma (59) cell lines. Finally, no significant correlations were found between GSH levels and cisplatin resistance in a study with different human tumor xenografts (60), and in human patients with germ cell tumors, there was no evidence of increased resistance to cisplatin in GGT-positive tumors (61). Several pieces of evidence suggest that these apparent inconsistencies can be explained taking into account additional aspects of GGT activity which lead to extracellular detoxication of platinum-based drugs, but also to pro-oxidant effects catalyzed by metal ions present extracellularly (9).

GGT, Extracellular Thiols and Cisplatin Resistance

It has been documented that sulfur amino acids, in particular cysteine (62, 63), and other small peptides containing cysteine, such as cysteinyl-glycine and GSH (64), are able to form adducts with cisplatin (*cis*-diamminedichloroplatinum (II)), and that such complexes are poorly transported across plasma membrane. Similar complexes were also described in plasma of patients treated with oxaliplatin (65). The final effects of such extracellular interactions are a decreased intracellular accumulation and a reduced toxicity of cisplatin towards treated cells (62, 64). Interestingly, it was also shown that cisplatin adducts with cysteinyl-glycine are formed 10 times faster than those with GSH, and that such adducts are present in the extracellular medium of GGT-overexpressing HeLa cells treated with cisplatin (66). This effect can be explained by the fact that the pK_a of cysteinyl-glycine thiol is significantly lower than that of GSH (6.4 *vs.* 8.56, respectively) (67), which causes its more rapid dissociation at physiological pH and its more efficient interaction with cisplatin. GGT activity, by converting poorly

reactive GSH into highly reactive cysteinyl-glycine, is in fact able to trigger the formation of cisplatin/thiol complexes in the extracellular space (66), resulting in lower cellular accumulation of cisplatin, reduced DNA platination and reduced cytotoxicity (46, 64). Thus, the protective effect of GGT expression against cisplatin cytotoxicity is dependent on the extracellular detoxication of cisplatin, rather than supposedly higher intracellular GSH levels. It is likely, however, that the relevance of GGT-mediated detoxication may depend on the specific biological context, in which the concomitant expression of other resistance mechanisms has also to be considered. Cellular resistance to toxic agents can be seen as a multifactorial phenomenon, involving not only defense mechanisms, but also cellular response to genotoxic stress (DNA repair efficiency, DNA damage tolerance, stress response and susceptibility to apoptosis) (7).

Dosage of cisplatin *in vivo* is limited by its nephrotoxicity (68, 69), and the mechanism by which cisplatin kills proximal tubule cells has been the object of intense investigation for many years. Based on the fact that a high level of GGT activity is expressed on the luminal surface of proximal tubule cells, a series of papers focused on the possibility that cisplatin may be bioactivated to a nephrotoxin through the action of GGT. The hypothesis was thus proposed that cisplatin–GSH complexes reaching the tubular lumen with the glomerular filtrate may undergo a sequential extracellular hydrolysis by tubular GGT and membrane dipeptidase activities, resulting in the formation of cysteine–cisplatin complexes. These *S*-conjugates would be then converted to a toxic, highly reactive thiol by any of several enzymes that catalyze the cysteine *S*-conjugate beta-lyase reactions (70). In agreement with this hypothesis, no CDDP nephrotoxicity was observed in *GGT* knockout mice (71), while pre-treatments with GGT inhibitor acivicin or cysteine *S*-conjugate beta-lyase inhibitor amino-oxyacetic acid allowed a protection in wild-type mice (72, 73). On the other hand, in a recent paper, which even confirmed the key role of GGT, the same authors reported that the inhibition of aminopeptidase *N* or renal dipeptidase did not reduce cisplatin toxicity, and that cysteine *S*-conjugate beta-lyase inhibition did not prevent nephrotoxicity *in vivo* or cytotoxicity *in vitro* (74). These conflicting data suggest that the mechanisms of cisplatin nephrotoxicity may involve other factors. In particular, it has to be taken into account that both animal studies and clinical trials demonstrated that pre-treatment with exogenous GSH reduced cisplatin-induced nephrotoxicity without reducing its antitumor activity (75, 76). Moreover, GGT inhibition by acivicin (77), as well as *GGT* knockout mice (78), resulted in several-fold increases in plasma GSH concentrations as compared to controls; this in turn resulted in increased glomerular filtration of GSH, up to concentrations of 5-30 mmol/l in preurine (79). Such high levels are expected to provide a direct protection against cisplatin cytotoxicity, irrespective of both GGT activity and the mechanism ultimately responsible for nephrotoxic damage (64).

Other factors could also concur with the discrepancies present in the literature. Experiments performed *in vitro* showed the formation of symmetrical *bis*-bidentate adducts between GSH and cisplatin, consistent with a time-dependent formation of high molecular weight 2:1 complexes (80, 81). More recently, it was suggested that the reaction of cisplatin with GSH *in vitro* proceeds *via* the formation of at least 11 distinct glutathione-platinum adducts, but that only two of those are still present in the reaction mixture after 24 h of incubation (82). Finally, Townsend *et al*. have described the formation of two different GSH cisplatin conjugates, a GSH monoplatinum conjugate (a possible GGT substrate) and a diplatinum GSH conjugate. The latter may not be a GGT substrate due to the presence of a second cisplatin molecule bound to the free amino group of glutamate (83). It can be envisaged that factors such as the *ratio* of GSH to cisplatin, the time of GSH cisplatin incubation, and the composition of media used for incubations in fact modulate the observed effects. Actually, the type of adducts formed and their relative abundancy can likely account for the differences reported with respect to the toxicity of GSH cisplatin conjugates (64, 83, 84).

GGT Pro-oxidant Effects and Tumor Progression

In recent years, several studies documented that GGT can exert pro-oxidant effects at the membrane surface level and in the extracellular microenvironment. This phenomenon was explained with the high reactivity of cysteinyl-glycine, the GGT product of GSH cleavage. As described above, the lower pKa of the cysteinyl-glycine thiol makes it able to dissociate more rapidly at physiological pH, and to reduce extracellular transition metal cations (in particular Fe^{3+} and Cu^{2+}) more efficiently than GSH itself. Iron reduction by GSH, in fact, might be limited by the chelating properties of the alpha-carboxyl group of the glutamate residue, affecting sterical and redox interactions of the cysteine thiol (85). GGT-catalyzed removal of glutamic acid causes a decrease of the cysteine thiol pKa and makes it free to interact with iron (67). The 'redox cycling' started following iron reduction was shown to produce ROS (superoxide anion, hydrogen peroxide) and thiyl radicals, *i.e*. reactive species capable of promoting several intra- and extracellular biomolecular effects (Figure 1).

The possible pro-oxidant effects of GGT were first highlighted in preneoplastic hepatic foci induced in rats by chemical carcinogens, where the appearance of lipid peroxidation in GGT-rich nodules was demonstrated after exposure of fresh tissue sections to an incubation mixture containing GSH and complexes of ferric iron. The effect was

inhibited by removal of iron or GSH, as well as by addition of free radical scavengers or inhibition of GGT activity (86). Subsequent studies showed that incubation mixtures containing purified GGT and transition metal ions were mutagenic in *Salmonella typhimurium* strains (87, 88). It was suggested that such GGT-induced damage could play an active role in the processes by which cells of preneoplastic foci progress to malignancy (86). The production of ROS, in particular of hydrogen peroxide, following iron reduction induced by the GGT-mediated catabolism of GSH has been repeatedly documented (89-92), and GSH/GGT-dependent iron reduction was confirmed to result in the promotion of lipid peroxidation in chemically induced preneoplastic lesion in rat liver (93), in rat liver microsomes and isolated hepatocytes (94), and in isolated human plasma low-density lipoproteins (LDL) (95). The pro-oxidant activity of GGT was also recently shown to promote the iron-dependent oxidative damage of DNA in *GGT*-transfected melanoma cells, thus potentially contributing to genomic instability and increased mutation risk in cancer cells (96).

It appears clear that metal ion redox cycling with production of reactive oxygen species is a critical step in the phenomena described. In respect to this, it was demonstrated that iron transport proteins transferrin and ferritin, as well as copper-binding ceruloplasmin, can act as sources of metal ions for the reactions described (90, 97-98). Indeed, it was demonstrated that GGT activity is able to promote the release of free iron from transferrin, thus promoting the uptake of iron by cancer cells (99). This effect may play an additional role in supplying iron to malignant cells, and the role of iron in carcinogenesis is well established.

The findings described so far suggest that the pro-oxidant reactions produced by GGT could serve as an additional source of (low levels of) endogenous ROS in cancer cells, possibly contributing to the 'persistent oxidative stress' described as a factor in genomic instability and carcinogenesis (100). It is now well established that low ('physiological') levels of pro-oxidants can exert regulatory roles within the cell by acting on targets sensitive to the redox state of the cell (101, 102). A major role in such regulation is played by cysteine thiols, which can undergo different redox modifications, all of which possibly reflecting a distinct functional state of a protein. A number of such phenomena have been described in proteins participating in crucial cell functions such as cell proliferation, apoptosis, cell adhesion and gene expression, whose alterations are of primary importance in progression of cancer and other diseases. It was documented that GGT activity can promote the oxidation of thiol groups in cell surface proteins, a process involving hydrogen peroxide and formation of mixed disulfides ('protein *S*-thiolation'; 103, 104). In particular, a study performed on melanoma cells expressing different levels of GGT activity showed a corresponding GGT-dependent oxidation of cysteine thiols in the cell surface tumor necrosis factor receptor-1 (TNFR1), with possible consequences on receptor–ligand interaction and signal transduction (105). Through production of hydrogen peroxide, which freely diffuses across the plasma membrane, GGT/GSH-dependent pro-oxidant reactions can also involve crucial intracellular targets. It was shown that GGT-dependent pro-oxidants can induce the binding of NF-κB and AP-1 to DNA (103, 106-108), and modulate the balance between protein kinase/phosphatase activities (109). It is well known that redox processes can play modulatory roles in the transduction of proliferative/apoptotic signals, due to interactions with growth factor receptors, protein kinases and transcription factors (110). GGT/GSH-dependent pro-oxidant reactions were in fact shown to exert an antiproliferative action in ovarian cancer cells (111), while other studies in U937 lymphoma cells showed that basal GGT-dependent production of hydrogen peroxide can instead represent an anti-apoptotic signal (91).

The modulatory effects of GGT-mediated pro-oxidant reactions could contribute to the resistance phenotype of GGT-expressing cancer cells, by regulating both signal transduction pathways involved in proliferation/apoptosis balance, as well as by inducing protective adaptations in the pool of intracellular antioxidants. For example, GGT-expressing melanoma cells were shown to display a twofold higher expression of catalase as compared to cells with low expression of GGT (112), likely as a result of the continuous GGT-dependent low level production of pro-oxidants. GGT/GSH-dependent pro-oxidant reactions were also shown to increase intracellular levels of vitamin C, by promotion of the extracellular oxidation of ascorbic acid and uptake of its oxidation product, dehydroascorbate (97).

GGT as a Target for Anticancer Treatments

The envisaged roles of GGT activity in the resistance phenotype of cancer cells suggests the potential advantages of associating GGT inhibitors with chemotherapeutics, in order to deplete intracellular levels of GSH and/or to inhibit extracellular drug detoxication. Different GGT inhibitors are known, such as glutamine analogs acivicin (AT125), 6-diazo-5-oxo-L-norleucine and azaserine (113, 114); boronate derivatives (115); L-glutamic acid derivatives (116); gamma-(monophenyl) phosphonoglutamate analogs (117). Unfortunately, the above mentioned molecules are toxic and cannot be used in humans (117-119). Acivicin was recently used in combination with aggressive therapy to deplete tumor GSH, and complete cure of metastatic melanoma in the liver was achieved in 90% of test animals (120). Recently, a novel class of uncompetitive inhibitors of GGT, structurally distinct from and less toxic than glutamine

analogs, were described (121). The development of GGT inhibitors with low toxicity remains an interesting perspective of pharmacological research, and could have an important impact on cancer therapy.

As discussed above, the antioxidant adaptations associated with GGT expression are the basis for an increased cellular tolerance against oxidative stress, which itself is a factor of resistance to the effects of pro-oxidant drugs. Association of more agents in therapy can however overcome such resistance; in a recent paper, for example, the combination of arsenic trioxide with subtoxic concentrations of ascorbic acid resulted in a sensitization to apoptotic cell death of GGT-transfected/arsenic trioxide-resistant melanoma cells (122).

Another line of evidence points to the relevance of GGT expression and activity in the pathophysiology of cellular processes involving nitric oxide (NO) and related compounds, GSNO in the first place. It has been shown that treatments of human cancer cells with NO and NO mimetics can effectively restore the sensitivity of resistant cell populations to the cytotoxic effects of chemotherapeutics. NO thus acts as a 'chemosensitizing agent', likely by modulating processes associated with prevention or inhibition of cellular drug resistance mechanisms, including those induced by hypoxia in solid tumors (123). Reactivation of NO signalling might in some way counteract the effects produced by hypoxia. The mechanisms by which NO restores sensitivity to anticancer agents are not clearly understood. Critical roles in NO chemosensitizing action might be played by vascular changes (promotion of blood perfusion and tumor oxygenation), radical scavenging/antioxidant effects, down-regulation of the GSH detoxification/redox buffering system, inhibition of key transcription factors such as hypoxia inducible factor 1 (HIF-1) and NF-κB, as well as inhibition of drug efflux transporters and DNA repair enzymes (124). NO mimetics glyceryl trinitrate (GTN) and isosorbide dinitrate attenuated hypoxia-induced resistance to doxorubicin and paclitaxel, and GTN patches increased the antitumor efficacy of doxorubicin in nude mice (125). Growth inhibition and chemosensitization in favour of carboplatin treatments were observed after exposure of glioma cells to NONOates (126), while significant chemosensitization to cisplatin cytotoxicity was observed in cells transfected with inducible nitric oxide synthase (iNOS) gene (127).

S-Nitrosothiols, GSNO initially, are considered physiologic NO metabolites, capable of transporting NO in blood and tissues in a stable form. On the basis of its gamma-glutamyl structure, GGT selectively metabolizes GSNO, thus promoting the release of its NO load (3, 128). This fact may well be exploited in order to selectively target NO to GGT-expressing cancer cells, by treating them with GSNO. By investigating the kinetics of GGT with respect to GSNO, a K_m of approximately 0.4 mM was found, comparable with that K_m value for GSH, which confirms the feasibility of using GSNO as an efficient pro-drug in order to perform selective NO treatment of GGT-expressing tumors (128). Future studies will substantiate the applicability and usefulness of such approach to therapy.

Besides GSNO, other gamma-glutamyl compounds can be selectively cleaved by GGT expressed in cancer cells, and the development of gamma-glutamyl pro-drugs is therefore an attractive possibility. One such agent, 4-(*N*-(*S*-glutathionylacetyl)amino) phenylarsonous acid (GSAO), a hydrophilic derivative of phenylarsenoxide obtained by attaching it to the cysteine thiol of reduced GSH (129), has been recently shown to possess notable antiproliferative/antiangiogenic action (130). This compound can inactivate the mitochondrial inner membrane adenine nucleotide translocase, thus inducing an increase in superoxide levels, proliferation arrest, ATP depletion, mitochondrial depolarization and finally apoptosis, both in endothelial and cancer cells (130-132). Being a GSH derivative, GSAO is an efficient substrate for GGT, and the product of the reaction, 4-(*N*-(*S*-cysteinylglycylacetyl)amino) phenylarsonous acid, is accumulated much more rapidly in cells and has greater antiproliferative activity than GSAO itself. GSAO therefore appears to be a promising GGT-activated pro-drug. Preclinical toxicology studies in mice and rats showed that high GSAO dosages resulted in damage to kidney distal tubules, possibly as a result of GSAO activation by high GGT activity expressed by cells in proximal tubules. This is one major aspect of GSAO pharmakokinetics in need of thorough investigation in view of future applicability of the compound in human therapy.

One additional aspect related to a role of GGT as therapeutic target is given by the fact that soluble GGT may effect a cytokine-like function. It was in fact recently observed that the structure of GGT includes the chemokine-like CX3C motif (133) and that GGT is able to modulate bone resorption independently of its catalytic activity (134, 135). Moreover, it was demonstrated that urinary excretion of GGT changes in parallel with established biochemical markers of bone resorption, and therefore could reflect bone resorptive activity (136). The possibility therefore exists that the overexpression and release of GGT by human tumors may have a role in establishment and development of bone metastasis.

GGT Macromolecular Complexes: Novel Biomarkers for Cancer and Other Pathologies

Serum GGT is widely used as a biomarker of liver dysfunction and excessive alcohol use, as it is thought to derive exclusively from the liver (1). On the other hand, studies of the past decade have revealed that GGT serum

levels are positively associated with the risk of cardiovascular events (137), hypertension, type II diabetes and metabolic syndrome (138-140), renal failure (141) and cancer, even unrelated to hepatic involvement (142). This raises the suspicion that diseased tissues other than the liver might contribute to serum GGT activity, thus explaining its broad predictive value.

The release of GGT from cancer cells was described in several types of neoplasia, but the mechanisms by which cellular GGT is released in blood are still poorly characterized. Several papers investigated the possible specificity of serum GGT complex for certain tumors, in particular hepatocellular carcinoma, focusing on parameters such as GGT post-translational modifications and lipoprotein association, in the attempt to identify parameters exploitable in diagnosis, monitoring or even prevention of cancer. Specific GGT macroforms with clinical significance have been reported in patients with primary hepatocellular carcinoma, but the origin or structures of these complexes were not established (143-145). Recently, in an *in vitro* study on melanoma and prostate cancer cells, the release of a GGT-containing soluble complex with a MW >2000 kDa, and corresponding to a specific GGT fraction (b-GGT) found in human plasma of healthy individuals was described (146). This fraction, despite having the same MW as VLDL, displays a higher density, thus showing that b-GGT found in plasma is not simply due to the absorption of GGT onto VLDL, but corresponds rather to a specific particle, with properties similar to the b-GGT obtained *in vitro*. The component molecules of b-GGT are still to be identified. Variations in GGT glycosylation have been described when comparing the enzyme from malignant and normal tissues. These changes appear however to vary with the type of tumor analysed (147-152), and the amount of tumor-derived GGT forms in serum may be affected by a rapid clearance rate (153).

Other studies are needed to better understand the properties of serum GGT fractions and the way they are released from cancer cells, in view of a clinical utilization of GGT as a biomarker of disease. In a retrospective study, total serum GGT was significantly increased in patients with metastatic renal cell carcinoma, but was normal in those with localized primary growths (154). Similar results were obtained in more recent work, where both alkaline phosphatase and GGT activities were normal in a majority of patients with localized renal cell carcinoma, but increased in most of the patients with metastatic disease involving liver and/or bones (155). In both cases, GGT appeared to be a sensitive marker of metastatic renal cell carcinoma, even though not specific for the site of metastasis. Significantly higher serum GGT levels were also found in hepatocellular carcinoma patients with poorly differentiated tumours, as compared to those with well- and moderately differentiated tumours (156). Total serum GGT was shown to increase with the growth of transplantable melanoma cells in inbred mice (157). Nevertheless, serum GGT levels seem to be at least partly independent of GGT expression in tumors (6, 30), and their specificity as marker of cancer has been questioned (155).

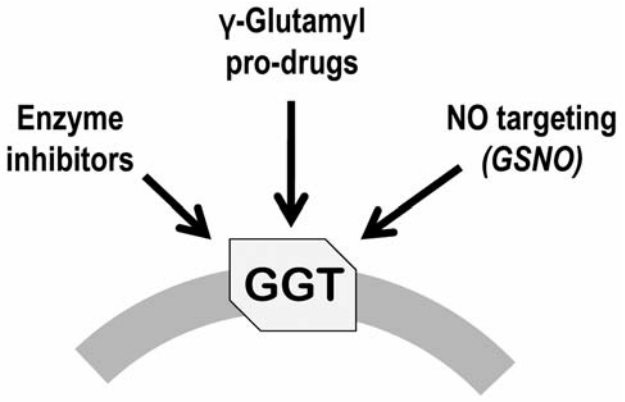

Figure 2. *Three distinct (complementary) approaches for exploiting GGT of cancer cells as a target for pharmacological treatments. NO, nitric oxide.*

On the other hand, epidemiologic studies have sparked further interest in elevated GGT as an independent predictor for morbidity and mortality from causes other than liver disease. In a recent study, the relationship of GGT with the risk of death was examined in a cohort of 283,438 patients of the Vienna General Hospital, and, in both sexes, GGT levels above the reference values (GGT>9 U/l in women, >14 U/l in men) was significantly ($p<0.001$) associated with all cause, cancer, hepatobiliary, and vascular mortalities (142). The association between GGT and risk of overall and site-specific cancer incidence was subsequently investigated in two large population-based cohort studies of 79,279 healthy Austrian men (158) and 92,843 women (159). Elevated GGT significantly increased overall cancer risk and, in site-specific cancer models, GGT was significantly associated with malignant neoplasms of digestive and respiratory/intrathoracic organs in both genders. GGT was also associated with malignant neoplasms of breast, female genital organs, lymphoid and hematopoietic cancer (women) and urinary organs (men). Altogether, the described studies suggest that a better understanding of serum GGT properties can be of use for the early identification of high-risk patients, thus allowing for optimization of therapeutic procedures during both the acute phase and at follow-up.

Conclusion

The findings discussed in this review clearly indicate that GGT functions in cancer cells may be more complicated than

previously thought. It is likely that a fine equilibrium exists between antioxidant *vs*. pro-oxidant functions of GGT, and that the latter may prevail under selected conditions, *e.g*. in GGT-overexpressing cells and in the presence of redox catalysts (metal ions). The pro-oxidant activity of GGT may contribute to the persistent oxidative stress described in cancer and modulate processes involved in tumor progression, such as cell proliferation/apoptosis and protective adaptation against electrophilic/alkylating compounds. The heterogeneous expression of GGT in different tumor types, and even different tumors of the same type, may become an important determinant for selection of therapeutic approaches, in view of its role as a factor for targeting of NO to tumor tissue or for activation of gamma-glutamyl pro-drugs (Figure 2). At the same time, the potential significance of serum GGT complexes suggests the additional application of GGT as diagnostic/prognostic marker of cancer in the optimization of therapeutic procedures.

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