

Small-Molecule Inhibitors of Glutathione S-Transferase P1-1 as Anticancer Therapeutic Agents

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1. INTRODUCTION

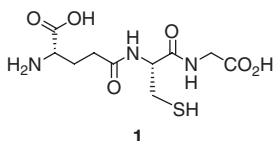
The Glutathione S-Transferase family of enzymes (EC 2.5.1.18), also referred to as glutathione transferases or GSTs, comprises a group of isoenzymes present in most aerobic eukaryote organisms. Discovered in 1961, GSTs were initially thought to act as carrier proteins and were originally called ligandins [1,2]. GSTs are found in mammalian tissues (e.g., muscle, liver, brain, testis, kidney, spleen, skin, placenta) and constitute approximately 1–4% of the total cellular protein content [3–5]. GSTs play a role in the synthesis of prostaglandins [6], steroids [7,8] and leukotrienes [9,10], as well as in the intracellular transport of molecules including metabolites [11], hormones [12–18], neurotransmitters [12], bilirubin [14,19,20], hemin [21,22], heme [22], thyroid [23], bile acids [24–26], and steroids [16,17,27,28].

One of the most important functions of GSTs is the detoxification of endogenous as well as exogenous substances via conjugation with glutathione

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(γ -L-glutamyl-L-cysteinylglycine, GSH, **1**). GSH is a thiol-containing tripeptide found in 0.5–10 mM concentration in the cytosol of a vast number of cell types [29] and in micromolar concentration in plasma [30].



GSTs contain a site that accommodates GSH (“GSH binding domain”) [31], where the proton of the GSH’s thiol group is abstracted, promoting the nucleophilic conjugation of the thiolate to electrophilic substrates. The resulting adducts become more water-soluble and are then eliminated by a phase II detoxification mechanism.

GSTs are of therapeutic interest because their overexpression has been associated with diseases such as chronic renal failure [32], neurodegeneration [33–37], multiple sclerosis [33,38,39], asthma [33,40–44], and particularly prostate [45], colon [46], and ovarian cancers [47].

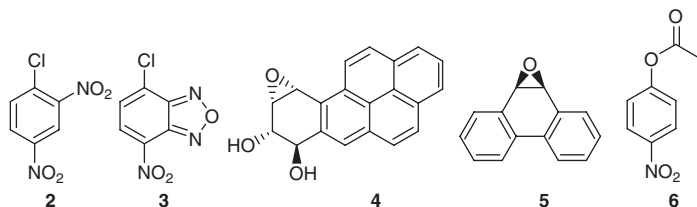
To date, 16 GST isozymes have been found in humans [48]. Studies of several cancer tissues have revealed the overexpression of different GST isozymes, with GST P1-1 (GST Pi, GST π) being the most predominant. For this reason, GST P1-1 is regarded as a potential tumor marker [5,49–53]. The high expression levels of GST P1-1 (up to 2.7% of the total cytosolic protein [52]), combined with its detoxification role against xenobiotics, make GST P1-1 a major player responsible for drug resistance in patients undergoing anticancer chemotherapy [49].

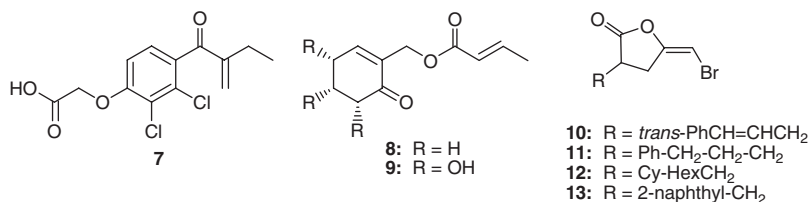
This report will cover inhibitors of GST P1-1 based on their mode of action.

2. TYPES OF INHIBITORS

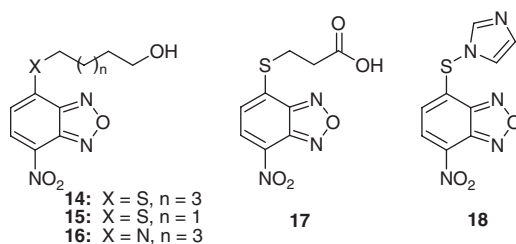
2.1 Suicide inhibitors

Suicide or irreversible inhibitors of GST P1-1 include agents that bind covalently to glutathione, thereby forming thioether adducts that are stabilized at the active site of the enzyme. These agents include activated aromatic systems (**2**, **3**), epoxides (**4**, **5**), esters (**6**), and Michael acceptors such as ethacrynic acid (**7**), cycloalkenones (**8**, **9**), and haloenol lactones (**10–13**), among others [3,48,54–57].

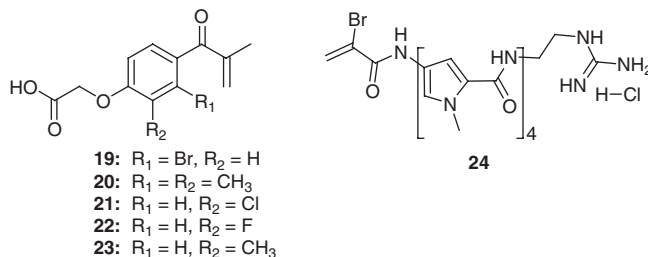




Recently, 7-nitro-2,1,3-benzoxadiazole thioethers (**14**, **15**, **17**, **18**) have been reported as a new class of suicide GST P1-1 inhibitors. GST P1-1 catalyzes the nucleophilic addition of GSH to C-4 of the benzoxadiazole ring forming a sigma-complex intermediate that is stabilized at the active site of the enzyme. This results in the dissociation of the complex between JNK and GST P1-1, inducing apoptosis in K562, HepG2, and GLC4 cancer cell lines. The requirement of the thioether group for activity became clear as amino analog **16** did not inhibit GST P1-1 at concentrations as high as 100 μ M. The observed SAR indicates that thioether hexanol **14** inhibits GST P1-1 at submicromolar concentrations ($IC_{50} = 0.8 \mu$ M), while **15**, **17**, and **18** inhibit GST P1-1 in the single-digit micromolar range ($IC_{50} = 2$, 5.7, and 6.3 μ M, respectively) [53,58].



Several Michael acceptors have been studied as irreversible GST P1-1 inhibitors. A recent SAR study of analogs of **7** in HL-60 cells revealed that substitution at the R₁ position is essential for GST P1-1 inhibition (**7**, **19**, **20**: 89–94% inhibition at 40 μ mol/L), while substitution at the R₂ position did not contribute significantly towards activity (**21**–**23**: <19% inhibition at 40 μ mol/L) [59].



The DNA minor groove binder brostallicin (PNU-166196, **24**), currently in Phase 2 clinical trials for the treatment of soft-tissue sarcoma, exhibited potent

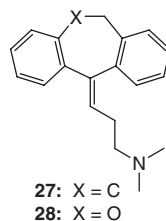
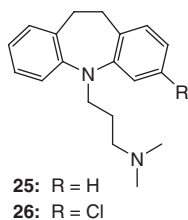
activity against GST P1-1 ($IC_{50} = 103 \text{ nM}$) in GST-transfected MCF-7 cells where the intracellular GST P1-1 activity is high (78 nmol/min/mg) [30,60].

2.2 Competitive inhibitors

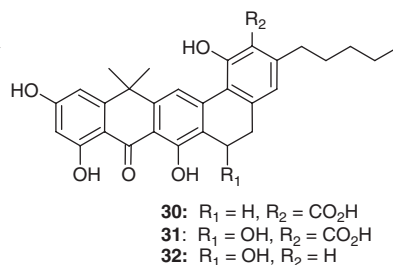
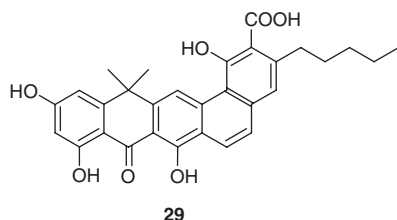
Competitive inhibitors of GST P1-1 fall under two categories: non-glutathione- and glutathione-based compounds. The former group covers a broad range of chemical structures such as tricyclic-based dibenzazepines, polyphenolic natural products, alkaloids, pyrimethamine, and dyes. The latter group, as its name indicates, covers compounds whose main structure or backbone is that of GSH.

2.2.1 Non-glutathione-based small molecules

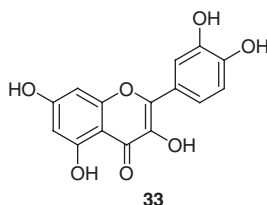
Tricyclic antidepressants such as imipramine (**25**), clomipramine (**26**), amitriptyline (**27**), and doxepine (**28**) were found to be weak inhibitors of GST P1-1 *in vitro*. Inhibition of GST P1-1 was enhanced with the introduction of a chloro group on the dibenzazepine ring (**25**: 40% inhibition at 15 mM; **26**: 70% inhibition at 10 mM). The same result was observed with the substitution of an oxygen for a carbon in the heptadiene ring (**27**: 18% inhibition at 10 mM; **28**: 48% inhibition at 15 mM) [35].



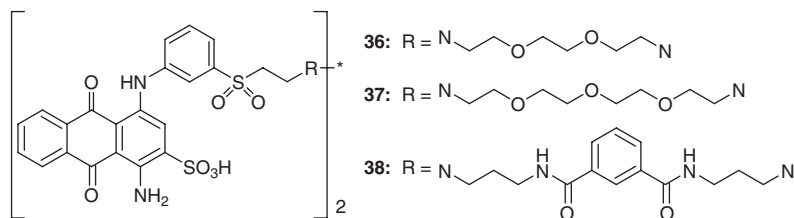
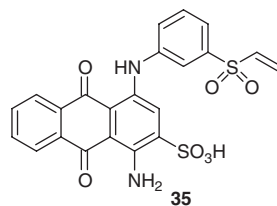
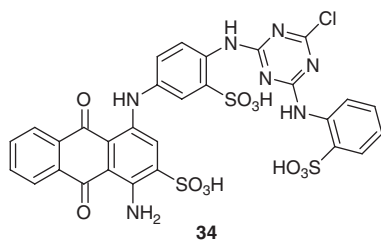
Natural products such as benastatin A (**29**), benastatin B (**30**), bequinostatin A (**31**), and bequinostatin B (**32**), all isolated from the culture broth of *Streptomyces* sp. MI384-DF12, were tested for inhibition of GST P1-1. Compounds **29** and **30** exhibited similar binding affinity towards murine-derived GST ($K_i = 3.5 \times 10^{-6} \text{ M}$ and $4.2 \times 10^{-6} \text{ M}$, respectively). Studies with compounds **31** and **32** indicate that the carboxylic acid group is required for inhibition of human-derived GST P1-1 (**31**: $IC_{50} = 0.6 \mu\text{g/mL}$; **32**: $IC_{50} = 100 \mu\text{g/mL}$). Compounds **29–32** exhibited low toxicity when tested in mice at 100 mg/kg i.p. [61,62].



Phase 1 clinical trials of the natural polyphenol quercetin (**33**) against several types of cancer have shown that this compound is well tolerated when administered at a dose of 70 mg/kg by i.v. bolus [63,64]. Further studies revealed that **33** inhibits GST P1-1 completely after 2 h at a concentration of 25 μM ; however, addition of GSH partially restores activity. HPLC and LC-MS studies indicate that **33** inhibits GST P1-1 through the formation of a covalent yet reversible bond with GST P1-1 cysteine residue at position 47.

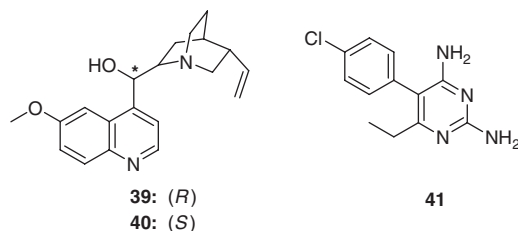


The anthraquinone analog cibacron blue (**34**) has been co-crystallized with GST P1-1 (PDB entry: 20GS). Using the vinyl sulfone Uniblue A (**35**) as the binding moiety, bivalent-based inhibitors **36–38** were synthesized incorporating different linkers. It was observed that analogs **36** and **37**, both containing flexible PEG-based linkers, exhibited higher affinity towards GST P1-1 ($\text{IC}_{50} = 44$ and 72 nM, respectively) than the more rigid isophthalamide-linked analog **38** ($\text{IC}_{50} = 440$ nM). It is worth noting that compounds **36–38** were highly selective GST P1-1 inhibitors, as none of them inhibited GST A1-1 at concentrations as high as 100 μM [65].



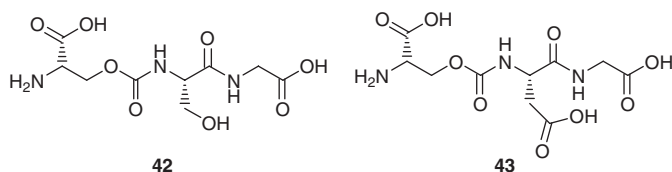
Alkaloids **39** and **40**, which are enantiomers of one another and are both used as antimalarial agents, inhibited GST P1-1 in the single-digit micromolar range ($\text{IC}_{50} = 4$ and 1 μM , respectively), indicating that the chirality of the secondary alcohol does not play a significant role in this activity. Pyrimethamine (**41**),

another antimalarial agent, also inhibited GST P1-1 in the single-digit micromolar range ($IC_{50} = 1 \mu M$) [66].

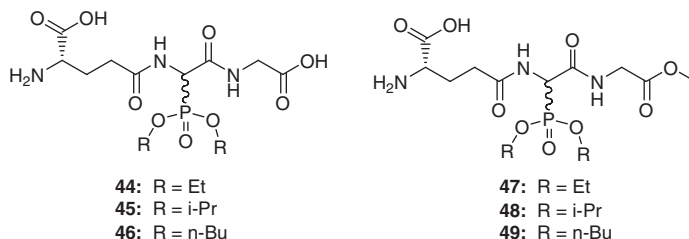


2.2.2 Glutathione-based inhibitors

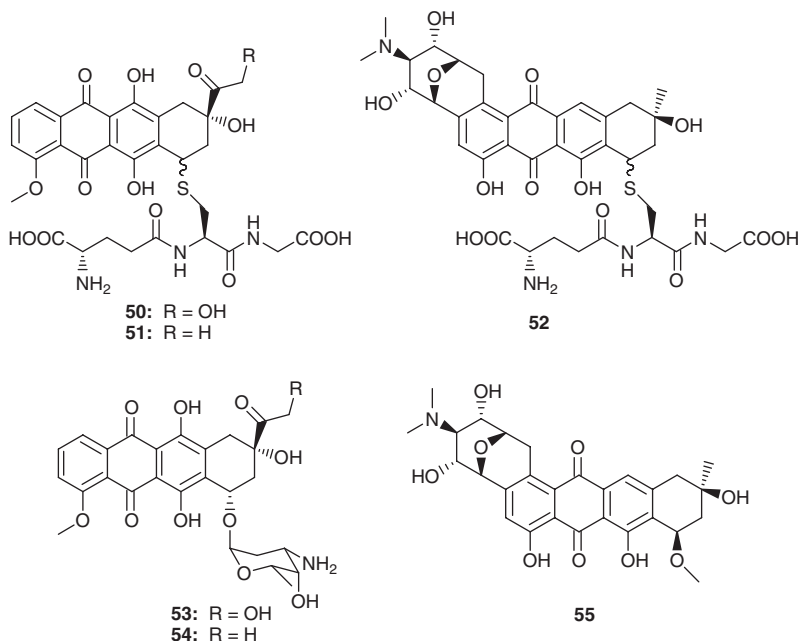
Among the strategies used for the development of GST P1-1 inhibitors is the modification of the GSH backbone to leverage its inherent affinity for GST P1-1. One approach centered on the incorporation of a carbamate group as an isosteric replacement of the γ -carboxylic Glu linkage in GSH. Synthesis and *in vitro* testing of 42 and 43 showed that this carbamate-replacement approach was not well tolerated [67].



A similar approach consists of replacing of the methylene thiol group of the cysteine residue in 1 with phosphonate esters. Phosphonodiacid analogs 44–46 inhibited GST P1-1 ($IC_{50} = 145, 61, \text{ and } 15 \mu M$, respectively) more potently than their corresponding monomethyl esters 47–49 ($IC_{50} = 288, 201, \text{ and } 98 \mu M$, respectively). The SAR of these compounds indicates that an increase in the lypophilicity of the phosphono ester groups increases potency against GST P1-1 ($nBu > i\text{-Pr} > Et$), and that the more hydrophilic dicarboxylic acid analogs are more potent than their less hydrophilic monomethyl esters. Cellular uptake experiments using HT29 and EPG-257 cell lines confirmed that diacid analogs 44–46 did not enter the cells (unchanged extracellular compound concentration), while the monomethyl esters 47–49 were transported into the cells where their corresponding free acids 44–46 were detected. These data indicate that 47–49 act as prodrugs of 44–46 for GST P1-1 inhibition. Metabolic stability experiments of 44–49 against γ -glutamyl transpeptidase (γ -GT) did not produce proteolytic products, which indicates that the phosphonate ester groups increase peptide stability against enzymatic degradation [68].

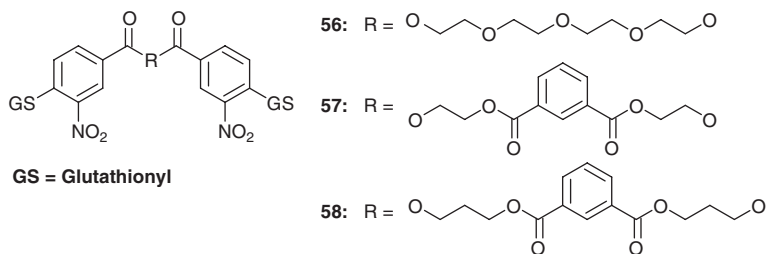


The most explored strategy for the development of GST P1-1 inhibitors involves the conjugation of the thiol group of the GSH cysteine residue with electrophilic moieties. Studies with anthracycline-based GSH conjugates **50–52** conducted in doxorubicin-resistant human breast cancer MCF-7/DOX cells, where the GST P1-1 activity is 14 times higher than in non-resistant MCF-7 cells, revealed that the epimers of **50–52** not only exhibited high GST P1-1 affinity ($K_i = 1.0\text{--}2.2\ \mu\text{M}$), but were also less cytotoxic than the respective anti-tumor anthracyclines doxorubicin (adriamycin, hydroxydaunorubicin, **53**), daunorubicin (daunomycin, **54**), and menogaril (**55**). These studies also showed that GST P1-1 did not catalyze the conjugation of GSH to **53–55** under physiological conditions [69].

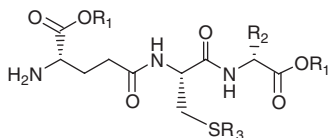


Another approach studied the influence of bivalent conjugates of GSH on GST P1-1 inhibition. It was observed that bis-glutathionyl nitrophenyl analogs **56–58** exhibited single-digit micromolar to submicromolar activity ($\text{IC}_{50} = 4.5, 2.9$ and $0.3\ \mu\text{M}$, respectively) against GST P1-1, but they were not as potent as analogs

36–38. Interestingly, the most potent analog **58** contains both the more rigid isophthalate linker and the longer propane-1,3-diol linker [65].



Another approach to the development of glutathione-based inhibitors involves analogs of GSH bearing different alkyl substituents on the cysteine sulfur. Compounds **59–63** revealed that inhibitory activity against GST P1-1 increased with the incorporation of linear alkyl chains on the cysteine sulfur (**59**, **60**: $K_i = 10$ and $1.9 \mu\text{M}$, respectively), as well as bulky hydrophobic substituents on the glycine residue (**61**: $K_i = 0.85 \mu\text{M}$). When the *n*-hexyl group in **61** was replaced with a bulkier, more hydrophobic benzyl group, potency was increased 2-fold (**62**: $K_i = 0.42 \mu\text{M}$). Interestingly, it was found that the increase in hydrophobicity and bulkiness was moderately tolerated, as the incorporation of a naphthyl group resulted in only a 3-fold loss of activity compared with **62** (**63**: $K_i = 1.2 \mu\text{M}$) [70,71].



59: $R_1 = R_2 = \text{H}$, $R_3 = n\text{-hexyl}$

60: $R_1 = R_2 = \text{H}$, $R_3 = n\text{-octyl}$

61: $R_1 = \text{H}$, $R_2 = \text{Ph}$, $R_3 = n\text{-hexyl}$

62: $R_1 = \text{H}$, $R_2 = \text{Ph}$, $R_3 = \text{benzyl}$

63: $R_1 = \text{H}$, $R_2 = \text{Ph}$, $R_3 = \text{naphthyl}$

64: $R_1 = \text{Et}$, $R_2 = \text{H}$, $R_3 = n\text{-octyl}$

65: $R_1 = \text{Et}$, $R_2 = \text{Ph}$, $R_3 = n\text{-hexyl}$

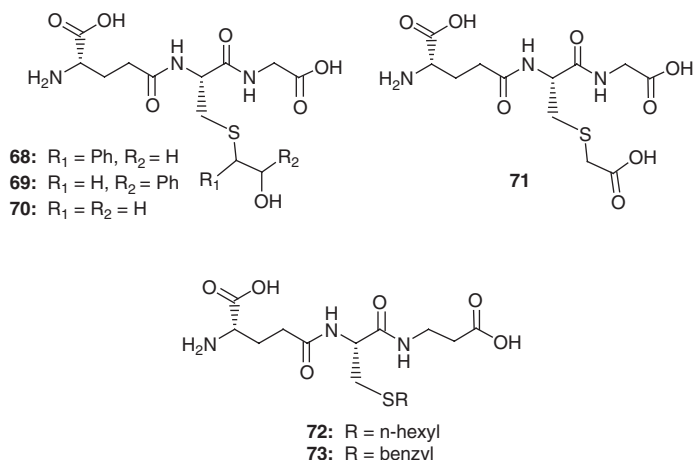
66: $R_1 = \text{Et}$, $R_2 = \text{Ph}$, $R_3 = \text{benzyl}$

67: $R_1 = \text{Et}$, $R_2 = \text{Ph}$, $R_3 = \text{naphthyl}$

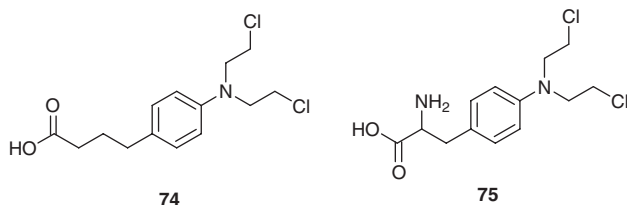
Attempts to increase hydrophilicity by incorporating a phenyl-containing 2-hydroxyethyl moiety on the cysteine sulfur resulted in further loss of potency against GST P1-1 (**68**, **69**: $K_i = 9$ and $4.7 \mu\text{M}$, respectively). Removal of the phenyl group on the thioethyl chain resulted in a dramatic loss of activity (**70**: $K_i = 280 \mu\text{M}$). Replacement of both phenyl and hydroxyl groups with a more hydrophilic carboxylic acid group retained activity (**71**: $K_i = 5.5 \mu\text{M}$) [72]. Replacing the glycine residue of the peptidic backbone with a β -alanine residue was detrimental for activity (**72**, **73**: $K_i = 550$ and $710 \mu\text{M}$, respectively) compared to the corresponding glycine-containing analogs (**59** and **62**, respectively).

Further studies with **60–63** showed that these compounds lacked cell membrane permeability. However, as observed in the previously discussed case of phosphonodiacid derivatives **44–46**, the diethyl ester analogs **64–67** possessed

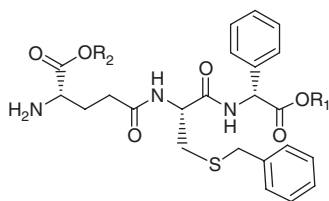
improved cell permeability. Cytotoxicity studies with **64–67** in HT-29 cells showed that these diethyl esters had IC_{50} values between 22 and 47 μ M, while their corresponding diacid analogs **60–63** did not exhibit cell cytotoxicity at concentrations up to 200 μ M [71].



Owing to the favorable activity profile of **66**, which acts as a prodrug of the active species **62**, additional studies were conducted on **66** to establish its cell-based profile. It was determined that **66** potentiated chlorambucil (**74**) toxicity in cell lines expressing GST P1-1, namely HT-29, HT4-1, SK OV-3, and SK VLB. Also, while **66** alone did not prevent tumor growth in the HT4-1 xenograph model, **66** increased by 56% the tumor growth inhibitory effect of melphalan (**75**).



Metabolic stability studies with **66** in HT-29 cells showed that about 30% of **66** was converted into its phenyl glyceryl monoethyl ester analog **76** after 20 min of incubation, and about 70% after 18 h. No traces of the glutamyl monoethyl ester **77** were detected at any time [71]. Although other diester analogs of **66** have exhibited promising profiles for cancer treatment (**78–82**, particularly **81**) [73], **66** (TLK199, TER199, Telintra[®]) is the only inhibitor in clinical trials. This compound is being tested for the treatment of myelodysplastic syndrome (MDS), a bone-marrow neoplastic disease that can eventually progress to acute myeloid leukemia (AML) [74].



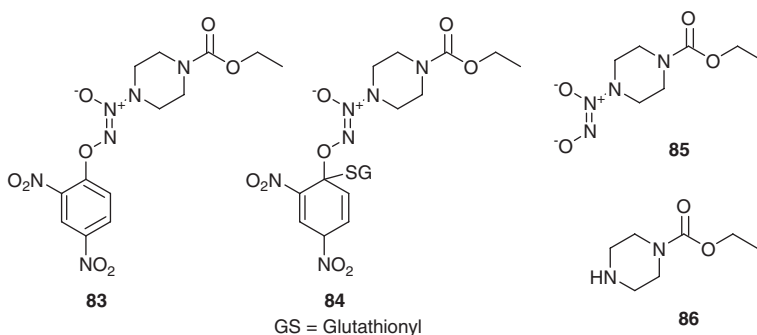
76: $R_1 = \text{Et}$, $R_2 = \text{H}$
 77: $R_1 = \text{H}$, $R_2 = \text{Et}$
 78: $R_1 = \text{C}_8\text{H}_{17}$, $R_2 = \text{Et}$
 79: $R_1 = \text{C}_{12}\text{H}_{25}$, $R_2 = \text{Et}$

80: $R_1 = \text{C}_{14}\text{H}_{29}$, $R_2 = \text{Et}$
 81: $R_1 = \text{C}_{16}\text{H}_{33}$, $R_2 = \text{Et}$
 82: $R_1 = \text{C}_{18}\text{H}_{37}$, $R_2 = \text{Et}$

2.3 GST P1-1-activated prodrugs

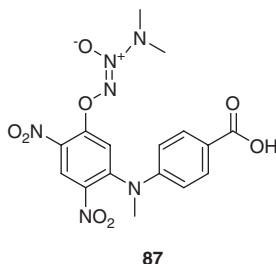
Rather than inhibiting the catalytic function of GST P1-1, GST P1-1-activated prodrugs undergo GST P1-1 catalyzed breakdown to release a molecule that is the active species responsible for the anticancer effect.

Based on the fact that nitric oxide (NO) induces apoptosis and inhibits growth in HL-60 cells, diazeniumdiolate analog **83** was developed as an NO prodrug. After establishing that **83** is stable at physiological pH, thereby ruling out the possibility of spontaneous degradation, conjugation of GSH catalyzed by GSTs (A1-1, M1-1, and P1-1) produced a Meisenheimer complex intermediate **84** that released the diazeniumdiolate intermediate **85** which, in turn, decomposed producing **86** and NO (two equivalents) [75].



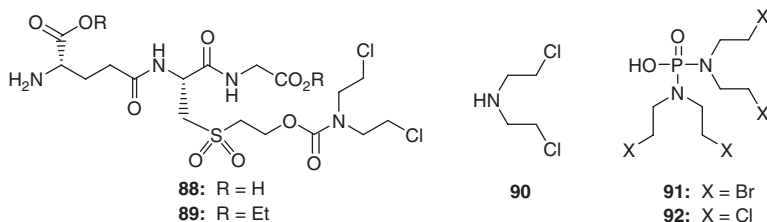
Molecular modeling of **84** with GST A1-1, M1-1, and P1-1 indicated that while the complex could be accommodated in the catalytic sites of the A1-1 and M1-1 isozymes, steric constraints were expected in the catalytic site of GST P1-1. Measurements of NO release from **83** revealed that GST P1-1 weakly catalyzed the conjugation of GSH producing a slow NO release, confirming molecular modeling predictions. Cell growth inhibition experiments showed that **83** inhibited growth of HL-60 and U937 leukemic cells with submicromolar activity ($\text{IC}_{50} = 0.5$ and $0.3 \mu\text{M}$, respectively). In a PPC-1 cancer cell xenograph model in NOD/SCID mice, **83** inhibited tumor growth when given at $4 \mu\text{mol/kg}$, i.v., three times/week [75].

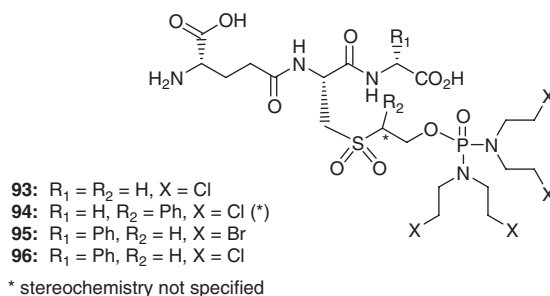
Molecular modeling of **84** suggested that it would be beneficial to replace the piperazine ring with a smaller amino group to improve a fit in the GST P1-1 catalytic site. With this in mind, analog **87** was developed. Measurements of NO release indicate that **87** releases more NO in the presence of GST P1-1 than in the presence of GST A1-1 (30% and 5%, respectively), validating the molecular modeling predictions. Compound **87** (3.36 mg/kg, twice/week) significantly delayed tumor growth when administered to SCID mice implanted with A2780 human ovarian cancer cells. After 45 days of treatment, no significant body weight loss was observed and renal activity remained normal as indicated by unchanged serum creatinine levels [76].



Another class of GST P1-1-activated prodrugs includes analogs of GSH with a β -linked alkylating agent-containing sulfonyl group. GST P1-1 recognizes the GSH backbone of this class of compounds (e.g., **88**, **89**, **93–96**). After being properly oriented in the enzyme, the Tyr 7 residue abstracts one of the alpha protons next to the sulfonyl group promoting release of the alkylating agent (**90–92**) and formation of a vinyl sulfone derivative of the parent compound [4,51,77–79] by a β -elimination/decarboxylation sequence.

In vitro incubation of **88** with GST P1-1 and GST A1-1 showed that this compound was selectively metabolized by GST P1-1. Cell-based toxicity assays using GST P1-1 transfected MCF-7 cells (2-h exposure) revealed that the phenyl-containing analogs **94–96** were more cytotoxic ($IC_{50} \sim 10 \mu M$) than the more hydrophilic analogs **88** and **89** ($IC_{50} = 86$ and $69 \mu M$, respectively) [4,51]. Cytotoxicity studies of **96** in 11 human malignant cell lines showed that this compound had IC_{50} values ranging from 6 to $67 \mu M$ [80]. Xenograph experiments using five cell lines in nude mice showed that **96** was more effective when administered at 200 mg/kg, q.d. $\times 5$, than at a single 400 mg/kg dose. Tumor growth was delayed significantly in the MX-1 and DLD-2 cell lines [80].





From this pro-drug class, **96** (TLK286, TER286, Telcyta[®]) is in clinical trials for the treatment of cancer.

3. CONCLUSIONS

Cancer is one of the most devastating diseases. Even though a few cancer types are associated with a particular gender (e.g., ovarian cancer, prostate cancer), the onset usually takes place regardless of age, sex, and race. Although cancer prevention remains the ideal approach to eradicate the disease, the best second option is early detection.

The overexpression of GSTs in some cancer cells, particularly of GST P1-1, offers an opportunity to detect and treat some cancer types (e.g., ovarian cancer). Recent developments in the design of small molecules that either inhibit the catalytic activity of GST P1-1 or use GST P1-1 catalytic site to release the actual anticancer agent, have shown promising results in preclinical studies, with the graduation of **66** and **96** as potential anticancer drug candidates currently undergoing clinical trials.

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