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Isozyme specificity of novel glutathione-S-transferase inhibitors

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Abstract. A systematically diversified set of peptide analogs of the reaction product of glutathione with an electrophilic substrate have been tested as isozyme-specific inhibitors of human glutathione-S-transferase (GST). The potency of the best of the inhibitors is in the 0.5 to 20 micromolar range, with kinetics indicative of competitive inhibition with glutathione at the active site. The specificity observed among three recombinant-derived GST isozymes at both low and high potency ranged from negligible to high (at least 20-fold over the next most sensitive isozyme). These results define a novel strategy for the design of drugs targeting cells with elevated levels of particular GST isozymes, such as tumor cells for which elevated levels of GST are believed to be an important cause of chemotherapeutic drug resistance.

Introduction

Current cancer chemotherapy often fails due to the de novo or therapy-induced appearance of tumor cells resistant to chemotherapeutic agents [22, 31]. In some cases, elevated levels of glutathione-S-transferase (GST, E. C. 2.5.1.18) isozymes have been associated with the resistant state [3, 10, 22, 36]. The GST isozymes are a group of enzymes that normally protect cells against toxic electrophiles, particularly those arising from oxidative metabolism, by conjugating the reactive molecules to the nucleophilic scavenger tripeptide glutathione (GSH, γ-glu-cys-gly) [11, 18, 34]. Following conjugation, these generally harmless GSH adducts, or their mercapturic metabolites, are secreted into the bile or urine [23].

GSH is the major soluble intracellular thiol. It is present at a concentration of about 1 m*M* in nearly all cells and is an essential cofactor for many enzymes [7]. In addition to its role in intracellular detoxification, it participates in interconversions of arachidonic acid-pathway metabolites (prostaglandins and leukotrienes) [23] and contributes to regulation of protein and DNA synthesis [39]. All GST isozymes use reduced GSH as an acceptor species, but they differ in the specificity with which different substrates are transferred to the cysteine thiol of GSH. As a result, the absence of one isozyme can render cells unusually sensitive to particular mutagens or toxic agents [41], whereas elevated levels can render cells resistant [44].

At least eight GST isozymes from five gene families have been well documented [29], and DNA analysis suggests the existence of several more isozymes from some of these families [45]. These enzymes are found as homodimers or heterodimers of subunits from the same family, with monomer molecular weights ranging between 23,000 and 27,000 Da. Any one tissue typically expresses one to four isozymes giving a significant tissue specificity for GST content [27]. Tumors often have one or more isozymes elevated, which means that tumors may show an activity profile different from that of normal cells [5, 28]. Transient expression of a recombinant GST in cultured cells can also result in resistance proportional to GST expression [37].

Inhibition of elevated GST levels in cells by the inhibitor ethacrynic acid has been shown to potentiate the cytotoxicity of several cancer drugs in cell culture lines [17, 42, 43], a result that has been extended to an animal xenograft model [9]. Phase I clinical trials of this largely nonselective GST inhibitor have not revealed any severe side effects attributable to transient depression of GST levels by >50% as measured in peripheral white blood cells [34]. Similar results in cell culture have also been obtained with other moderately potent and selective inhibitors, including piriprost, gossypol, and indomethacin [13, 15, 43]. Accord-

Abbreviations: GSH, glutathione; GST, glutathione-S-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; phegly, S(+)phenylglycine; β-ala, 3-aminopropionic acid; 4-ABu, 4-aminobutyric acid; QSAR, quantitative structure/activity relationship; IC_{50}, concentration required for 50% inhibition

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ingly, it is anticipated that GST inhibitors that are isozyme-selective will be clinically usable to potentiate existing chemotherapy preferentially in tumor cells.

Previous work in our laboratory has shown that systematic variation of GSH structure results in the formation of ligands sufficiently selective to be useful for chromatographic separation of GST isozymes from various rat tissues [6, 24]. In the present work, we extend these results to demonstrate that the same ligands are also useful as solution-phase competitive inhibitors of recombinant human GST activity.

Materials and methods

Chemicals. GSH, 1-chloro-2,4-dinitrobenzene (CDNB), and S-alkyl (C1–C10) GSH derivatives were purchased from Sigma Chemical Company (St. Louis, Mo.). Stock solutions of 20 m*M* GSH were made in water. Stock solutions of 20 m*M* CDNB were made in high-performance liquid chromatography (HPLC)-grade ethanol. The novel peptide analogs of GSH compounds [24] and the commercial S-alkyl GSH derivatives were dissolved in 50% methanol and titrated with TRIS base or NaOH until solubilized. In general, the GSH analogs are designated in Tables 1 and 2 by the substituent on the cysteinyl sulfur followed by the amino acid used to replace glycine in the GSH moiety. For instance, S-octyl GSH is designated as octyl/gly. Standard abbreviations are used for the normal set of amino acids and attached moieties. The nonstandard amino acids abbreviated phegly and 4ABu are phenyl glycine and 4-aminobutyric acid, respectively. The additional structural features of certain compounds are as follows:

1. Compound 8, α-aminooctanoic/gly: hexyl-cys is replaced by an analog lacking sulfur.
2. Compound 12, S-(octylmethyl)cysteinyl/gly iodide: the methyl iodide adduct on the sulfur of octyl/gly.
3. Compound 14, *N*-glycidol octyl/gly: an additional glycine is present at the N-terminus.
4. Compound 15, cyclic hexyl/phegly: the N- and C-termini are covalently linked.
5. Compound 44, glutamyl glycinyl hexyl/phegly; an additional gly is present between the glu and the cys.

The small organic molecules tested as inhibitors in Table 4 were obtained from Sigma Chemical Company or Aldrich Chemical Company (Milwaukee, Wis.).

Enzymes. Recombinant human GST isozymes A1-1, M1a-1a, and P1-1 [29] were obtained from Dr. Bengt Mannervik of Uppsala University, Sweden. Protein concentrations were determined using a 96-well microplate Coomassie dye-binding assay (Bio Rad, Richmond, Calif.) with bovine serum albumin as a standard. Absorbencies were recorded in a THERMOmax Plate Reader (Molecular Devices, Menlo Park, Calif.).

Enzyme activity. GST activity was measured at 340 nm in a DU 64 Spectrophotometer using a Soft-Pac Kinetics Module (Beckman Instruments, Fullerton, Calif.) or in a Molecular Devices Plate Reader, in either case using GSH and CDNB as substrates. Standard reaction conditions included 200 m*M* $NaHPO_4$ at pH 6.8 with 1 m*M* GSH and 1 m*M* CDNB at 30° C.

Inhibition potency. Candidate inhibitors were initially tested for their inhibitory effect at a 1 m*M* concentration using standard reaction conditions. If active, the compound was diluted to determine the inhibition at a range of concentrations. The potency of an inhibitor was defined as either the concentration required for 50% inhibition (IC_{50} value) under standard assay conditions or, more precisely, as the disassociation constant (K_i value) obtained by competition experiments using a range of GSH concentrations. In these competition experiments, the apparent Michaelis constant (K_m) for GSH was determined in the presence and absence of the inhibitor and the K_i value was determined using the equation:

$$K^*_m = K_m(1+I/K_i),$$

where I is the concentration of inhibitor used, K_i is the disassociation constant for the inhibitor, and K_m and K^*_m are the apparent K_m values for GSH in the absence and presence of the inhibitor, respectively. K_m and K^*_m values were determined by linear regression of the data points on a Hanes-Woolf plot [40] of [S]/V vs [S], where [S] is the concentration of GSH and V is the specific activity (in units per milligram of protein) as determined from the initial velocity at that substrate concentration. K_m and K^*_m are the negative values of the intercept on the X-axis of the line determined by the linear regression.

Graphical analysis. The multidimensional data (each axis representing the extent of inhibition for one of the recombinant GSTs) were analyzed on an IBM-compatible personal computer (PC) by the principal-components algorithm [32] in Pirouette, a multivariate statistical software package developed for chemical data by Infometrix, Inc. (Seattle, Wash.). The principal components are the eigenvectors of the covariance matrix with the largest associated eigenvalues. As such, they represent composite parameters derived from the original axes to satisfy two conditions: (1) the principal components are independent (orthogonal) dimensions, and (2) they account for the largest portion of the variance in the data set. Consequently, projection of data onto a plane defined by principal components maximizes the clarity of visualization by spreading the data out along dimensions of maximal scatter. Points that are close together in such a projection therefore faithfully display correlations in the original higher-dimensional space. See Cheung et al. [8] for a fuller exposition of the use of multivariate techniques in biochemistry.

Target values are also plotted that represent postulated ideal compounds with 100-fold specificity factors and 0.1-μ*M* K_i values. For this analysis, inhibition potency was defined as log ($1/IC_{50}$), or log ($1/K_i$) when this more accurate value was available.

Results

Screening strategy

Given the variety of nonstandard amino acids that have been substituted for each amino acid in the GSH tripeptide [1, 2] and the numerous other possibilities, combined with the variety of substrates that could be linked to the cysteine sulfhydryl [25], it would be possible to contemplate tens of thousands of GSH analogs as GST inhibitors. Following the previously described paralog strategy [4, 21], a systematic sampling of this potential set has been prepared [24]. Briefly, a representative sampling of the potential diversity was achieved through monomer choices resulting in concurrent variations across a wide range for multiple parameters previously shown to be relevant to binding to GSTs from several mammalian species, primarily the rat [2, 25]. The parameters included hydrophobicity, size, and electronegativity. Variations in properties were created primarily at the C-terminus and sulfhydryl positions.

These compounds were initially screened at 1 m*M* concentrations against three recombinant human GSTs, one from each major isozyme class, to see if they would inhibit the enzyme activity, using CDNB as a spectrophotometrically detectable substrate. To permit the rapid testing of a wide spectrum of compounds, the paralogs used at this stage were crude synthetic samples and were not characterized for purity before testing, although in most cases the intended synthetic compound was the dominant species,

Table 1. Inhibition of GST activity by novel GSH adducts; percentage of inhibition at 1 m*M* and the micromolar concentration required for 50% inhibition

Number	Structure	% Inhibition at 1 m*M*			μ*M* Conc. for IC_{50}		
		P1-1	A1-1	M1a-1a	P1-1	A1-1	M1a-1a
Peptide analogs showing inhibition bias toward P1-1:							
1	S-(Hexylmethyl)cys/Phegly iodide	99	96	83	35	36	450
2	4-Chlorobenzyl/phegly	99	92	89			
3	Octyl/phegly	96	92	90	100	1,000	300
4	Benzyl/phegly	85	68	30			
5	Hexyl/R(−)phegly	75	62	4			
6	Hexyl/S(+)phegly	71	61	48	4	12	100
7	Decyl/phegly	70	60	60	300	300	500
8	α-Aminooctanoic/gly	52	1	8			
Peptide analogs showing inhibition bias toward A1-1:							
9	Octyl/gly	99	99	99	7	1	1
10	Hexyl/gly	93	99	99	75	25	40
11	4-Nitrobenzyl/phegly	90	99	99			
12	S-(Octylmethyl)cys/gly iodide	90	99	99	50	20	50
13	Benzyl/4-ABu	70	70	95	575	575	570
14	*N*-Glycidol octyl/gly	99	91	90	500	100	100
15	Cyclic hexyl/phegly	53	85	80			
16	Benzyl/4-ABu	4	78	44			
17	2,5-Dichlorobenzyl/gly	50	70	90	1,150	570	570
18	Benzyl/val	35	64	74			
19	Benzyl/asp	12	62	57	900	900	
20	Hexyl/4-ABu	25	58	83	3,000	875	1,750
21	Hexyl/val	24	48	47			
22	Hexyl/asp	0	35	30	20,000	2,000	2,000
23	Propyl/his	2	17	3			
24	Hexyl/his	8	11	1			
Peptide analogs showing inhibition bias toward M1a-1a:							
25	4-Chlorobenzyl/gly	85	98	99	200	20	9
26	α-Glu/hexyl/phegly	78	80	99			
27	4-Methylbenzyl/gly	70	90	99	500	150	40
28	Hexyl/β-ala	8	69	99			
29	4-Methylbenzyl/β-ala	55	65	99	800	600	20
30	Benzyl/β-ala	0	25	99	1,000	1,000	25
31	Cyclic benzyl/phegly	11	36	95	6,000	1,500	70
32	3,5-Difluorobenzyl/gly	70	70	95	575	575	570
33	Butyl/gly	91	85	93	48	106	21
34	Trityl/ala	46	30	93			
35	Octyl/β-ala	30	60	90	2,200	1,100	500
36	4-Cyanobenzyl/gly	80	70	90	250	550	500
37	Tertbutyl/gly	88	74	90			
38	4-Bromobenzyl/gly	60	50	90	1,000	1,140	300
39	Benzyl/gly	26	53	84			
40	4-Terbutylbenzyl/gly	49	51	82	2,000	950	200
41	Benzyl/ala	40	30	80			
42	Hexyl/ala	8	30	80			
43	S-(methylbenzyl)cys/β-ala iodide	10	20	80	2,000	2,000	300
44	Glutamyl glycinyl hexyl/phegly	30	45	75			
45	α,α-Dimethylbenzyl/gly	38	25	74	3,000	1,600	400
46	Propyl/ala	13	46	62	ND	900	800
47	Neopentyl/gly	35	36	62	3,000	1,200	300
48	4-Fluorobenzyl/gly	10	40	60	20,000	3,800	640
49	Propyl/asp	ND	33	49			
50	Propyl/val	12	15	28			
51	Benzyl/his	4	2	8	25,000	25,000	10,000

Conc. Concentration; ND, not determined

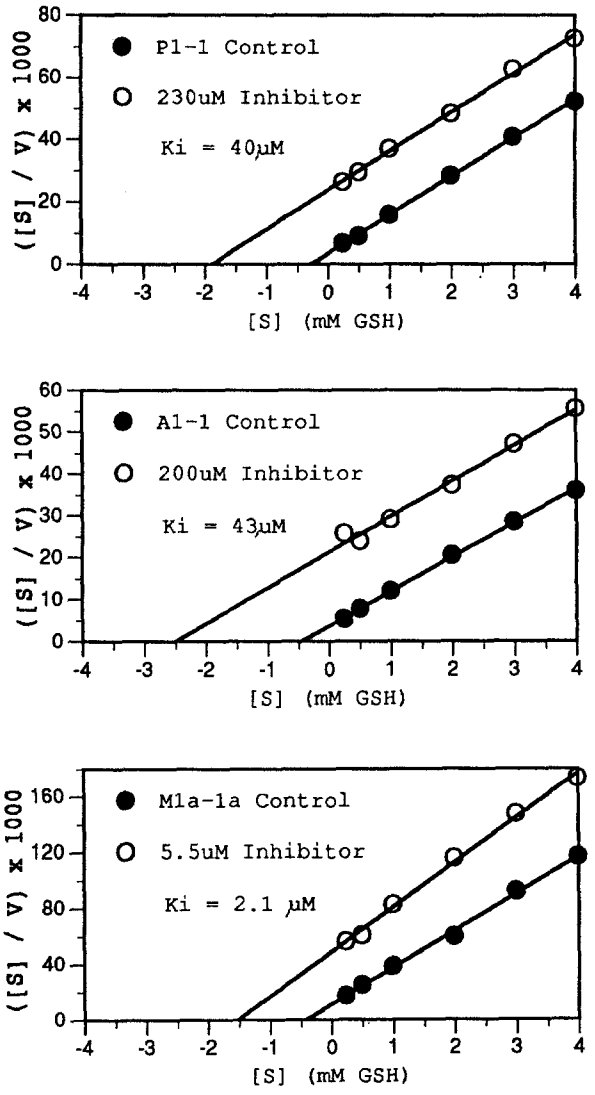

Fig. 1. Hanes-Woolf plots for analyzing the binding of GSH in the presence and absence of a peptide-based competitive inhibitor (4-methylbenzyl/β-ala, compound 29) to GST isozymes; details are given in Materials and methods. ●, Without inhibitor; ○, with inhibitor. The paralog was used at concentrations of 230 μ*M* for the P1-1 isozyme, 200 μ*M* for the A1-1 isozyme, and 5.5 μ*M* for the M1a-1a isozyme

representing about 50% of the mass. The nominal 1 m*M* concentration was based on an assumption of 100% purity. Table 1 shows the results of these inhibition tests for the full series of paralog analogs of GSH.

Within Table 1 the paralogs are grouped by isozyme selectivity, and within each selectivity class they are ranked by decreasing potency. Paralogs that showed strong selectivity for the P1-1 and M1a-1a isozymes were clearly identified in this survey, but no new selective inhibitor for A1-1 was found beyound those previously described [25], namely, certain n-alkyl GSH adducts. The criteria set for proceeding to screening beyound the test of inhibition at the 1 m*M* concentration were: inhibitions of >90% on any GST isozyme or a >6-fold difference in inhibition between an isozyme pair. The IC_{50} value for those paralogs meeting the initial criteria are also presented in Table 1.

Kinetic analysis

A more accurate measure of the binding of paralogs to GST isozymes is obtained by means of GSH competition experiments to determine a K_i value. Such tests were performed on those compounds that had IC_{50} potencies in the 10 to 100 μ*M* range or exhibited >5-fold selectivities between isozymes, and for which >90% pure compounds had been made. If the inhibition by the paralog is competitive, then the K_m values for GSH in the presence and absence of inhibitor can be used to determine the K_i value, or disasociation constant, between the paralog and the GST isozyme [40]. The IC_{50} values were used to establish an appropriate concentration of inhibitor to yield a roughly 50% inhibition at 1 m*M* GSH. Figure 1 presents the data obtained in the GSH competition experiments with the 4-methylbenzyl/β-ala paralog (compound 29). Parallel lines of the inhibited and noninhibited conditions in a Hanes-Woolf plot indicate that the paralog interacts with the GST active site [40]. For most combinations of paralog and GST, strict parallelism was observed. An example of a typical small deviation from parallelism, indicative of a small degree of noncompetitive inhibition, is shown in Fig. 1 (middle panel). The K_m value obtained for GSH alone in this entire series of experiments ranged between 0.4 and 0.6 m*M*, which was approximately 1 to 2 orders of magnitude weaker than that recorded for the most potent paralog.

Experiments similar to those illustrated in Fig. 1 were performed with the other promising paralogs from Table 1 for which purified compounds had been obtained. Table 2 presents the K_i values for this subset of interesting compounds. Most of these compounds appeared more potent in these studies than in the original screening tests because the new preparations used for the GSH competition experiments were at least 90% pure. A further contribution comes from the arithmetic definition of IC_{50} as compared with K_i; the values are equal only as both the GSH and the CDNB concentrations approach zero, which was not the case in our standard protocol. With regard to specificity, the more accurate determinations were generally consistent with the

Table 2. Inhibition of GST activity by purified GSH adducts; micromolar K_i concentration as determined by competition binding with GSH (see Fig. 1)

Number	Structure	GST isozyme		
		P1-1	A1-1	M1a-1a
P1-1-specific paralogs:				
4	Benzyl/phegly	0.45	20	25
6	Hexyl/S(+)phegly	0.85	5.8	41
5	Hexyl/R(−)phegly	3.9	11	44.7
A1-1-specific paralogs:				
9	Octyl/gly	1.9	0.27	1.2
10	Hexyl/gly	10	0.84	2.0
M1a-1a-specific paralogs:				
29	4-Methylbenzyl/β-ala	40	43	2.1
28	Hexyl/β-ala	550	43	11
30	Benzyl/β-ala	710	360	22

Table 3. Inhibition of GST activity by S-(n-alkyl) GSH adducts; micromolar concentration required for 50% inhibition

S-(n-alkyl)-GSH		GST isozyme		
C number	Name	P1-1	A1-1	M1a-1a
C1	Methyl	1,000	2,500	1,300
C2	Ethyl	750	1,800	650
C3	Propyl	500	900	100
C4	Butyl	200	140	20
C5	Pentyl	100	10	5.0
C6	Hexyl	80	2.0	3.0
C7	Heptyl	20	1.5	1.5
C8	Octyl	10	1.5	1.5
C9	Nonyl	1.5	1.0	1.0
C10	Decyl	0.75	1.0	0.75

C, Compound

Table 4. Inhibition of GST activity by nonpeptide (small organic) compounds; micromolar concentration required for 50% inhibition

Abbreviation	Compound	GST isozyme		
		P1-1	A1-1	M1a-1a
cb	Cibacron blue	0.4	4.0	1.5
dx	Doxorubicin	700	80	500
ea	Ethacrynic acid	4.0	2.0	3.0
gs	Gossypol acetic acid	200	4.0	4.0
hm	Hematin	1,000	2.0	5.0
rb	Rose bengal	8.0	0.5	1.0
sp	Sulfobromophthalein	20	25	5.0
in	Indomethacin	>600	300	>600
eb	Eosin b	4.0	2.5	2.0
ey	Eosin y	35	5.0	30

preliminary data, although in several cases relative preferences were changed.

Quantitative structure/activity relationship

The goal of this project was to design potent and selective inhibitors of particular GST isozymes. Following standard medicinal chemistry procedures [16], we next set out to identify trends in the quantitative structure/activity relationship (QSAR) of these compounds. For this purpose, a series of n-alkyl derivatives of GSH were tested. A detailed exploration of this feature is summarized in Table 3. These results show, first, that the inhibitory potency of the compounds increases with the chain length of the n-alkyl group and, second, that this parameter does not generate a regular trend in isozyme selectivity. It is striking that the results obtained for the paralog compounds presented in Table 1 show a much greater diversity in the pattern of inhibition than do those obtained for the set of n-alkyl derivatives of GSH presented in Table 3.

Numerous small organic molecules have also been previously reported to inhibit GST activity [25], although only a few have been carefully characterized with respect to isolated human isozymes. Since comparisons of these compounds with the radically different peptide-based structures were expected to be useful for a more detailed QSAR study, we began testing of such compounds; the results are summarized in Table 4. Several of these compounds showed potency and selectivity comparable with those of the peptide-based compounds.

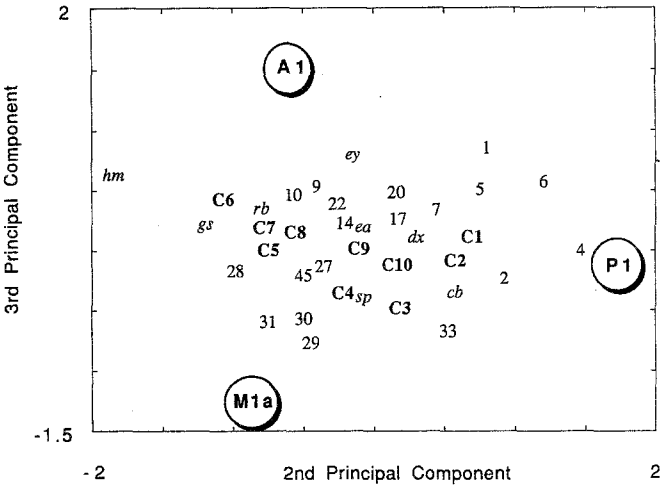

Fig. 2. Multivariate analysis of GST inhibitors. Inhibitor quality is visualized by plotting potency against each of three recombinant GST isozymes (A1-1, M1a-1a, and P1-1) and projecting the points onto a plane defined by the principal components of the distribution. Target values for ideal inhibitors of each isozyme are denoted by *circles* labeled with the isozyme name; the criteria for an ideal inhibitor are 100-fold specificity and $K_i = 0.1$ μM. Three families of compounds are compared: a selection of paralogs from Table 1 (*numbers*), a series of n-alkyl GSH analogs from Table 3 (C_n, *in boldface*), and a selection of the small organic molecules from Table 4 (*italics*). For purposes of clarity, certain data are not plotted, namely, those for compounds of low specificity, which are clustered at the center of the plot

Figure 2 presents a graphical analysis of IC_{50} data from Tables 1, 3, and 4 combined with the K_i data in Table 2. To determine this plot, inhibition properties were first plotted in a three-dimensional space whose axes represent the extent of inhibition for each of the three recombinant isozymes. The principal components of the data distribution were then determined [32]. Principal components are composite factors, derived from the original axes, that are mathematically optimized to display correlations in a multivariate data set by plotting points along axes chosen to maximize scatter. The plot shown is a projection of the data onto the plane defined by the second and third principal components. The first principal component is a dimension largely representing potency, and scatter in this property is well displayed in the tables. Choosing the second and third principal components for Figure 2 highlights the specificity factor, which we believe is an equally important aspect of quality. Such a multivariate plot allows convenient inspection of the quality (both potency and selectivity) of the tested reagents. Also indicated in the plot are target properties defined as 100-fold specificity and 0.1 μM K_i.

Discussion

Paralogs as enzyme inhibitors

The strength of defined-sequence polymer chemistry is its ability to generate a vast number of novel compounds that can be tested for binding to targets of pharmacological interest [12, 14, 19]. Even for short polymers of restricted composition, such as GSH analogs, the number of potential compounds is far too large for detailed characterization, however. The paralog approach [21] for sampling polymer compound libraries varying in multiple parameters has previously been shown to be an effective route for reducing the number of compounds required for testing so as to identify useful chromatographic ligands [4]. Similarly, in the present study, by working with a systematically diversified set of paralogs rather than a complete set of all possibilities, we were capable of undertaking detailed characterization of most of the promising candidates (Table 2) uncovered in the initial screening with crude preparations of the ligands (Table 1). Using this approach, we have now identified potent solution-phase inhibitors that are selective for either the M1a-1a or the P1-1 isozymes, showing 10- to 20-fold selectivity over the tested GST isozymes of other classes. Inhibitors equally strongly selective for the A1-1 GST isozyme have not yet been identified.

An analysis of n-alkyl GSH analogs as probes of the hydrophobicity binding parameter shows that the binding strength increases with the chain length of the alkyl group, confirming previous reports on the preference of GST isozymes for hydrophobic substrates [25]. Interestingly, there is a change in selectivity among these analogs. With S-alkyl chains of three or four carbons, M1a-1a is selectively inhibited. In the range of alkyl groups containing five to eight carbons, the A1-1 and M1a-1a GST isozymes are inhibited to about the same extent and are selectively inhibited as compared with the P1-1 isozyme. Finally, for the S-nonyl and S-decyl GSH derivatives, all the GST isozymes are inhibited to about the same extent. This aspect of the QSAR can be readily seen in the multivariate plot shown in Fig. 2.

The multivariate analysis of the potencies and selectivities of the inhibitors that is presented in Fig. 2 highlights several other interesting features as well. First, it indicates that the benzyl/phegly paralog (compound 4) comes rather close to the goal of a P1-1-specific inhibitor. Second, the benzyl/β-ala (compound 30), 4-methylbenzyl/β-ala (compound 29), and cyclic benzyl/phegly (compound 31) paralogs are the most selective for the M1a-1a enzyme but do not come as close to the goal as achieved for the P1-1 enzyme. Third, no novel A1-1-selective paralog was discovered in this survey. Our analysis of small organic molecules shows that a variety of potencies and specificities are also available from this class of chemical structures. Many of the most effective of such compounds are extremely hydrophobic dyes, however, and are thus unlikely to be clinically useful due to high background binding to proteins in general. Although competition experiments with these inhibitors have not yet been done to determine directly whether they are competitive inhibitors, work is in progress to convert them into GSH derivatives with the hope of achieving potency and selectivity greater than those available from the compounds described in this paper. In the design of more effective inhibitors, the data analysis by multivariate statistics, as summarized in Fig. 2, should be particularly useful for defining structural trends. Finally, the empirically observed trends visualized in this way should provide useful insights for comparison with structural data on GSTs, including studies using X-ray crystallography [38, 47], high-resolution nuclear magnetic resonance (NMR) [35], and site-directed mutagenesis [30, 48].

Pharmacological implications

The diversity of GST activity in tumors, both qualitatively and quantitatively, represents a significant therapeutic opportunity for targeting existing chemotherapy drugs preferentially to tumors [5]. The same diversity, however, poses a major challenge to the pharmaceutical chemist in that potency alone is not sufficient for utility; selectivity among a group of closely related enzymes is also needed. The paralog approach has proved to be well suited to such a challenge. As demonstrated in the present work, crude ligands are useful as initial probes (Table 1). Reliable assessment of inhibitory properties, however, requires about 50 mg of purified compounds (Table 2). Systematic sampling is thus a very useful strategy for limiting the characterization work to a reasonable level.

Furthermore, the range of structures sampled contributes directly to QSAR and enzyme-structure correlation studies aimed at identifying features responsible for isozyme selectivity and for potency. For example, all but one of the most selective inhibitors for the M1a-1a isozyme have beta-alanine as the C-terminal amino acid in the GSH moiety of the paralog. Both of the most selective inhibitors for the P1-1 isozyme have phenylglycine as the C-terminal amino acid of the GSH moiety.

The best inhibitors obtained in this study show the same potency range as the previously studied inhibitor hexyl GSH, which is the standard ligand used for affinity purification of GST isozymes [26]. The potency of these inhibitors is also similar to or better than that of ethacrynic acid, which is being tested clinically as a drug to potentiate chemotherapy [34], and this observation suggests that these novel inhibitors may also be useful for the potentiation of chemotherapy. For instance, preferential expression of P1-1 has been reported in a range of tumors [5, 20, 33], and a potentiator based on the P1-1-selective inhibitor benzyl/phegly (compound 4) may thus be useful in cancer therapy.

Among the paralogs identified by this screen for which a K_i value had been determined (Table 2), all showed competitive inhibition with GSH indicating that they are targeted to the active site of the GST isozymes for their effect and, thus, that they should affect the activity of GST against substrates other than CDNB. In recent years, several reports have documented that a variety of alkylating agents are substrates for GST [44] and that isozymes differ significantly in their specificity for these substrates [42]. Differential expression of particular isozymes in individual

tumors may therefore account for variability in responsiveness to treatment.

Finally, work is in progress to modify the inhibitors identified herein such that they are more likely to permeate the cell membrane. Such derivatives will be needed to test the therapeutic utility of these compounds in cell culture assays since GSH itself is not capable of such permeation [46] and, thus, paralogs containing the GSH moiety are not expected to be directly transported into the cell as intact compounds.

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