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Bivalent inhibitors of glutathione *S*-transferase: The effect of spacer length on isozyme selectivity

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Abstract—Glutathione *S*-transferases (GSTs) are cytosolic enzymes that catalyze the conjugation of glutathione with a variety of exogenous and endogenous electrophiles. High affinity, isozyme-specific inhibitors of GST are required for use as pharmacological tools as well as potential therapeutics. The design of selective inhibitors is hindered due to the broad substrate binding capabilities of the GST enzymes. GSTs are dimeric enzymes, and therefore offer a unique discriminator for achieving inhibitor selectivity: the distance between binding sites on each monomer unit as a function of its quaternary organization. Bivalent analogs of the non-selective GST inhibitor ethacrynic acid were prepared, and selectivity for the GST A1-1 isozyme over GST P1-1 (IC_{50} values of 13.7 vs 1022 nM, respectively) was achieved through the optimization of the spacer length between the ethacrynic acid ligand domains.

Glutathione *S*-transferases represent a family of cytosolic enzymes that catalyze the conjugation of glutathione (GSH) to a variety of exogenous and endogenous electrophiles,[1,2] and are implicated in tumor cell resistance toward chemotherapy agents.[3] While recent studies have focused on the GST P1-1 enzyme and its involvement in cell proliferation,[4] considerable interest has been paid to the study of the related isozyme GST A1-1 and its role in the regulation of lipid peroxides,[5,6] which are important regulators of apoptosis.[7] A potent and selective inhibitor of GST A1-1 would help further characterize its biochemical role in acquired tumor cell resistance and apoptosis, and potentially result in a useful adjuvant to restore chemotherapeutic sensitivity in resistant tumor cells.

The difficulty in the rational design of selective inhibitors for GST lies in their inherent ability to bind and conjugate a wide variety of hydrophobic substrates. GSTs are dimeric proteins and therefore provide a unique discriminator for selectivity: the differences in distance and environment between binding sites on each monomer unit. This would allow selectivity to be engineered through the optimization of the spacer length between ligand domains in a bivalent inhibitor. 'Selective bivalency' describes this concept of imparting isozyme selectivity through spacer optimization to an otherwise non-selective inhibitor. The concept of selective bivalency is illustrated in Figure 1. In example I, dimeric proteins A and B differ by the spatial

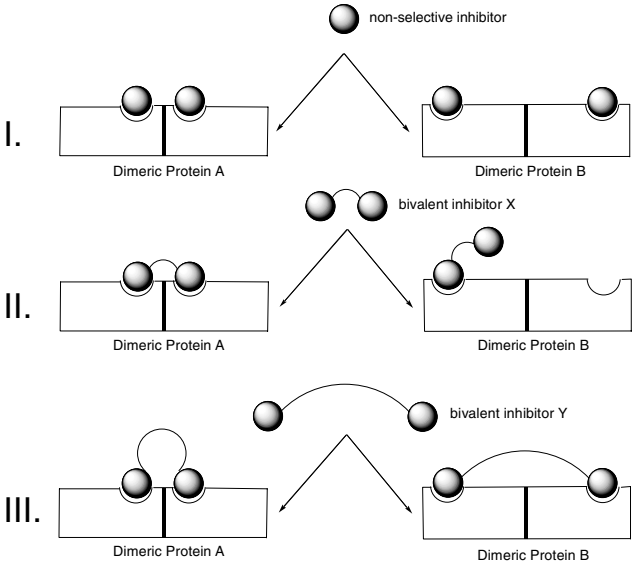

Figure 1. The concept of selective bivalency.

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orientation of the binding sites on the individual monomer units. The non-selective monovalent inhibitor will bind to each monomer unit binding site with equal affinity, and thus exhibit no selectivity. In example II, two non-selective inhibitors are bound together by a spacer of optimized length, resulting in bivalent inhibitor X with enhanced affinity to dimeric protein A. The spacer length chosen is too short to span the gap between the binding sites of dimeric protein B, therefore bivalent ligand X does not exhibit the same increase in affinity for dimeric protein B, resulting in an overall increase in selectivity for dimeric protein A. In example III, the spacer length in bivalent inhibitor Y is increased such that it is able to bind both dimeric proteins A and B. In this case, selectivity for either dimeric protein is not observed.

While the concept of selective bivalency was utilized to prepare potent and selective inhibitors of human glyoxalase I,[8] its use as a method to develop GST inhibitors was first investigated by Lyon et al.[9] in which bivalent inhibitors based on *S*-nitrophenyl glutathione conjugates exhibited a 100-fold increase in affinity and a 10-fold increase in isozyme selectivity for GST P1-1. In order to develop selective bivalent inhibitors for GST A1-1, the crystal structures of GST A1-1[10] and GST P1-1[11] co-crystallized with the non-selective GST inhibitor ethacrynic acid **1**[12] (EA, Fig. 2) were analyzed. We hypothesized that GST A1-1 selectivity could be achieved by exploiting the spatial orientation differences between the EA binding sites of GST A1-1 and P1-1. Bivalent inhibitors comprised of EA ligand domains coupled to various amino acid spacers linked to a bivalent core were prepared and evaluated for inhibition against GST A1-1 and GST P1-1.

The method used to prepare the bivalent inhibitors based on EA was adapted to a solid-phase strategy to allow for synthetic ease as well as the potential for future combinatorial library development. This was accomplished by utilizing 3,5-diaminomethylbenzoic acid as the bivalent core, which was synthesized in its protected form as shown in Scheme 1. Methyl 3,5-diaminomethyl benzoate **2** was treated with *N*-bromosuccinimide and catalytic amounts of benzoyl peroxide, followed by diethyl phosphite and diisopropylethylamine to yield dibromo ester **3**.[13] Treatment with sodium azide,

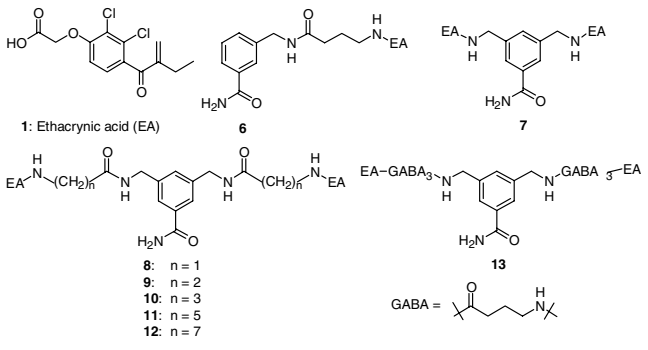
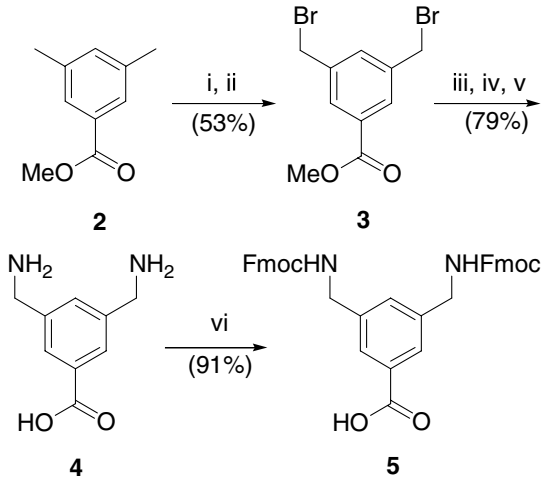


Figure 2. Ethacrynic acid and bivalent inhibitors based on ethacrynic acid.



Scheme 1. Synthesis of bivalent core **5**. Reagents and conditions: (i) *N*-bromosuccinimide, benzoyl peroxide; (ii) diethyl phosphite; (iii) sodium azide; (iv) triphenylphosphine; (v) 2 N aqueous HCl, reflux; (vi) Fmoc-OSu.

followed by reduction with triphenylphosphine[14] and subsequent acid-catalyzed ester hydrolysis resulted in diamino acid **4**. The free amines were then protected using *N*-(9-fluorenylmethoxycarbonyl)oxysuccinimide (Fmoc-OSu) to yield the protected bivalent core **5**. The bivalent core was then loaded onto Tentagel SRAM resin using *O*-benzotriazole-*N*,*N*,*N*,*N*′-tetramethyluronium hexafluorophosphate (HBTU) as the coupling agent with *N*-hydroxybenzotriazole (HOBt) as an additive. Initial synthetic efforts to prepare the bivalent inhibitors used Fmoc-protected spacer amino acids, but HPLC and mass spectrometric analyses of the crude products indicated that there were substantial amounts of by-products containing one or two additional spacer amino acid residues. A phthaloyl-based protection scheme resulted in higher product purities, and this protection strategy was then adopted for the bivalent inhibitor series. Phthaloyl-protected amino acid spacers of different lengths were then coupled to the bivalent core using HBTU and HOBt. The phthaloyl group was then removed using 1 M hydrazine in EtOH, and the carboxyl group of EA was coupled to the resulting free amine using HBTU and HOBt. In bivalent inhibitor **7**, EA was directly coupled to the bivalent core molecule to investigate the effects of having no spacer amino acid present. In contrast, bivalent inhibitor **13** was synthesized with a spacer consisting of three repeating 4-aminobutyric acid units to measure the effects of a lengthy spacer between the EA ligand domains. As a control, the monovalent analog **6** was also synthesized using commercially available Fmoc-3-aminomethyl benzoic acid as the monovalent core. The inhibitors were then cleaved from the solid support by treatment with 95% TFA/H_2O and purified by semi-preparative HPLC. The purified compounds were then evaluated for inhibition of GST A1-1 and GST P1-1 mediated GSH conjugation to 1-chloro-2,4-dinitrobenzene (CDNB)[15]. GST A1-1 and GST P1-1 were expressed in *Escherichia coli* and purified as described previously.[9] The results are shown in Table 1.

Table 1. Evaluation of compounds **1**, **6**–**13** on the inhibition of GST A1-1 and GST P1-1

Compound	IC_{50} ± SE (nM)		Selectivity A1-1/P1-1[a]	Distance (Å)[b]
	GST A1-1	GST P1-1		
1[c]	5000	4000	0.8	N.A.
6	13,850 ± 1475	15,046 ± 1149	1.1	N.A.
7	32.7 ± 4.6	992.4 ± 180	30	19.28
8	24 ± 4	704 ± 38	29	26.97
9	13.7 ± 1.8	1022 ± 136	75	27.91
10	14.3 ± 1.4	624 ± 109	44	32.04
11	39.3 ± 5.0	46.1 ± 2	1.2	37.06
12	52.1 ± 5.3	31.0 ± 0.3	0.6	42.03
13	98.9 ± 7.8	142 ± 7	1.4	56.23

IC_{50} values are the average ± SEM of three independent experiments.

[a] Selectivity ratio = (GST P1-1 IC_{50}) ÷ (GST A1-1 IC_{50}).

[b] Distances between the aromatic groups of the EA ligand domains were estimated using ChemDraw 3D (CambridgeSoft, Cambridge, MA) with the bivalent inhibitors drawn and rendered in the fully extended state.

[c] IC_{50} values for EA obtained from Ploemen et al. (Ref. 12).

Upon evaluation, the monovalent EA-based inhibitor **6** exhibited non-selective inhibition of GST A1-1 and GST P1-1 with IC_{50} values of 13,580 and 12,500 nM, respectively. The higher IC_{50} values seen for the monovalent control would suggest that any improvement in inhibition observed for the bivalent analogs can be attributed to factors other than favorable interaction between the linker core with the enzyme. While bivalent inhibitors **7**–**10** all exhibited increased selectivity for GST A1-1 over GST P1-1, bivalent inhibitor **9** exhibited the highest degree of selectivity (75-fold), as well as the lowest IC_{50} for GST A1-1 (13.7 nM). Incubation of GST A1-1 in the presence of inhibitor **9** for a period of 2 h did not yield any conjugation products by mass spectrometric analysis (data not shown), suggesting that bivalent ligand **9** is not inhibiting GST A1-1 via covalent modification.

The estimated distance between the aromatic rings of the pendant EA ligand domains in **9** corresponds well to the measured separation distances of the bound EA molecules in the crystal structure of GST A1-1 (27.91 vs 26.0 Å, respectively). As depicted in Figure 1, we hypothesize that bivalent inhibitor **9** is able to bind in a bivalent manner to GST A1-1, resulting in a large increase in binding affinity in regard to both the parent EA and the monovalent control. Due to its length, it is postulated that bivalent inhibitor **9** is unable to bridge the distance between the GST P1-1 binding sites, therefore precluding bivalent (but not monovalent) interaction with GST P1-1. The increase in binding affinity to GST A1-1 resulted in a large increase in overall selectivity for GST A1-1 over GST P1-1. This selectivity is lost as the spacer is lengthened, as depicted in the IC_{50} values for bivalent inhibitors **11**, **12**, and **13**. In these cases, we postulate that the distance between the EA ligand domains was long enough to allow bivalent interaction with GST P1-1, resulting in a dramatic increase in GST P1-1 binding affinity and subsequent loss of GST A1-1 selectivity.

In summary, the concept of selective bivalency was applied to the non-selective inhibitor EA to impart selectivity for GST A1-1. Bivalent inhibitors with varying spacer lengths between the EA ligand domains were prepared and evaluated for GST A1-1 and P1-1 inhibition. Bivalent inhibitor **9** exhibited markedly increased selectivity for GST A1-1 with respect to EA as well as the monovalent analog. To our knowledge, bivalent inhibitor **9** represents a GST A1-1 inhibitor with the highest affinity and selectivity reported in the literature to date, as well as an example in which the concept of selective bivalency was used to impart isozyme selectivity to a non-selective inhibitor between two dimeric proteins belonging to the same enzyme family.

Acknowledgments

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15. Briefly, the inhibitors were dissolved in DMSO to yield a 2 mM stock solution and then serially diluted with water to yield the final test concentrations (final DMSO concentration <1%). The GST enzymes (20 nM) were incubated in the presence or absence (vehicle control) of test inhibitors with GSH and CDNB. The concentrations of GSH and CDNB used were at their respective K_M values (A1-1: 350 and 720 μM; P1-1: 150 and 820 μM). Rates of product formation were obtained by measuring absorption at 340 nM for 1 min on Beckmann DU 7400 spectrophotometer. Inhibitor concentrations spanned four orders of magnitude and experiments were carried out in triplicate. The resulting data were then fitted to sigmoidal dose–response curves using GraphPad Prism (GraphPad Software Inc., San Diego, CA) to determine the 50% inhibitory concentration.