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SHORT COMMUNICATION

Fluorometric Titration Assay of Affinity of Tight-Binding Nonfluorescent Inhibitor of Glutathione S-transferase

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Abstract To determine inhibition constant (K_i) of tight-binding inhibitor, the putative method estimated an apparent K_i from the response of initial rates to total concentrations of the inhibitor considering its depletion during binding for conversion into the true K_i, but was impractical with glutathione S-transferase of sophisticated kinetics. A fluorometric titration assay of dissociation constant (K_d) was thus proposed. *Schistosoma japonicum* glutathione S-transferase (SjGST) action on a nonfluorescent divalent pro-inhibitor and glutathione yielded a divalent product in active site to act as a tight-binding inhibitor, whose binding quenched fluorescence of SjGST at 340 nm under the excitation at 280 nm. K_d was estimated from the response of fluorescence of SjGST at 340 nm to total concentrations of the divalent product considering its depletion during binding. By fluorometric titration assay, K_d of two tested nonfluorescent divalent products varied from subnanomolar to nanomolar, but both were resistant to change of SjGST levels and consistent with their apparent K_i estimated via the putative method. Hence, fluorometric titration assay of K_d of nonfluorescent tight-binding inhibitors/ligands was effective to GST and may be universally applicable to common enzymes/proteins; affinities of tight-binding inhibitors of GST can be approximated by their apparent K_i estimated via the putative method.

Keywords Glutathione S-transferase · Dissociation constant · Tight-binding inhibitor · Apparent inhibition constant · Fluorometric titration assay

Abbreviations

BDAA	N,N'- (butane-1,4-diyl)-bis-(4-acryloyl phenoxyacetic amide)
CDNB	2,4-dinitrochlorobenzene
DMF	dimethylformamide
GST	glutathione S-transferase
HDEA	N,N'-(hexane-1,6-diyl)-bis-(ethacrynic amide)
NHS	N-hydroxysuccinimide
SjGST	glutathione S-transferase of *Schistosoma japonicum*
THF	Tetrahydrofuran

Introduction

Glutathione transferase (GST; EC 2.5.1.18) is crucial for catalyzing the conjugation of glutathione (GSH) with electrophilic compounds/xenobiotics. GST has many isozymes with distinctive biological roles. Potent isozyme-selective inhibitors of GST are potential drugs to overcome drug-resistance of cancers and treat the infection of *Schistosoma japonicum*, and are pivotal tools to reveal biological roles of GST isozymes [1–3]. GST is a symmetrical homodimer bearing two active sites. Of GST, a monovalent inhibitor binds to just one active site while a divalent inhibitor binds concomitantly to two active sites; a divalent inhibitor thus usually possesses affinity higher than that of a monovalent inhibitor bearing the same binding moiety. On the other hand, each active site of GST has one subdomain for GSH and another for an electrophilic

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substrate. A monovalent product of GST as a conjugate of one GSH and one electrophilic substrate binds to two subdomains of one active site and has an affinity higher than that of the electrophilic substrate. As a result, symmetrical divalent products of GST bearing suitable linkers can concomitantly bind to two active sites of GST and usually possess inhibition constants (K_i) at nanomolar levels and excellent isozyme-selectivity [4–6]. Therefore, divalent products as potential potent inhibitors of GST are widely studied.

GST activity is usually measured spectrophotometrically with 2,4-dinitrochlorobenzene (CDNB) and GSH. However, GST has limited activity on CDNB and nanomolar levels of GST are needed for activity assay. Symmetrical divalent products of GST bearing nanomolar K_i are thus tight-binding inhibitors [4–6], but it is a great challenge to estimate their K_i. In the putative method to estimate K_i of a tight-binding inhibitor of enzyme acting on single substrate, the response of initial rates to total concentrations of the inhibitor considering its depletion during binding is analyzed to estimate the apparent K_i, which is subsequently converted into the true K_i based on kinetic parameters associated with the inhibition type [7, 8]. Specially, of a noncompetitive tight-binding inhibitor, the apparent K_i is directly taken as the true K_i without conversion. Of any enzyme acting on two substrates, however, no similar methods have been reported for converting the apparent K_i of a tight-binding inhibitor into the true K_i. To date, reported tight-binding divalent inhibitors of GST acted competitively against GSH while noncompetitively against CDNB [4–6]; values of their apparent K_i were directly taken as approximates of true K_i [7, 8], but this approximation was not validated.

Fortunately, enzymes usually have tryptophan residues that emit at 340 nm under the excitation at 280 nm; the binding of a nonfluorescent tight-binding inhibitor to an enzyme can quench fluorescence at 340 nm of tryptophan residues nearby active site [9, 10]. This fact provides the feasibility to estimate dissociation constant (K_d) of a nonfluorescent inhibitor as an equivalent of K_i by fluorometric titration assay, which analyzes response of fluorescence at 340 nm to total concentrations of the inhibitor considering its depletion during binding [9–12]. This fluorometric titration assay of K_d can be applicable to inhibitors bearing affinities over wide ranges and enzymes acting on multiple substrates. In fact, this fluorometric titration assay had already been successfully applied to classical inhibitors of GST bearing micromolar K_d [11], but had not been tested with any tight-binding inhibitor.

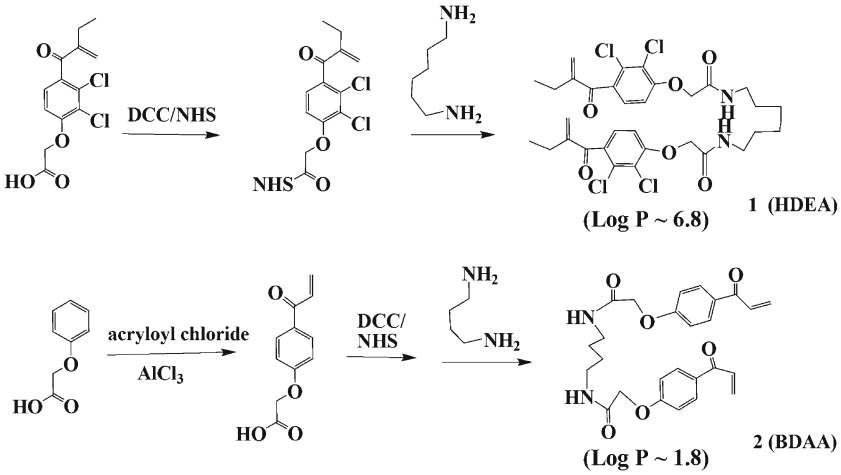
Divalent products of GST as tight-binding inhibitors are difficult to prepare [13], but divalent pro-inhibitors of GST can be easily prepared and bind to GST rapidly to generate divalent products as tight-binding inhibitors in-situ in active site in the presence of GSH in excess. Recently, we designed some new divalent pro-inhibitors of GST that effectively sensitized cisplatin-resistant ovarian cancer SK-OV-3 [14], but how to estimate true K_i of their divalent products was still a challenge. GST of *Schistosoma japonicum* (SjGST) as an anti-parasite target is rich in tryptophan residues [15, 16]. Herein, with the binding of divalent products generated in-situ in active site from nonfluorescent divalent pro-inhibitors to SjGST as models, fluorometric titration assay of K_d of tight-binding inhibitors was proved effective and supported the approximation of the true K_i of tight-binding inhibitors by apparent K_i estimated via the putative method.

Materials and Methods

GSH, GSH-sepharose 4B, ethacrynic acid (EAA), and CDNB were from Sigma-Aldrich. Other chemicals were domestic reagents of analytical grade. The vector pGST-MOLUC carrying on SjGST was a blank tag-expression system with no inserted sequence. Recombinant expression of SjGST followed routine procedure [17]. After induced expression of SjGST for 20 h at 16 °C, transformed *Escherichia coli* BL21 (DE3) cells were harvested and lyzed by sonication treatment. SjGST was purified by affinity chromatography via GSH-Sepharose 4B equilibrated with 10 mM sodium phosphate at pH 7.0 containing 0.14 M NaCl and 2.7 mM KCl, and eluted with the same buffer plus 10 mM GSH. SjGST contained one band of about 26 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were quantified by the Bradford method [18]. The concentration of SjGST was calculated with the molecular weight of 52 kDa for homodimer, unless otherwise stated. One unit of GST produced one micromole conjugate per min at 1.0 mM of CDNB and GSH, at 25 °C and pH 6.5 in 100 mM sodium phosphate buffer. SjGST had the specific activity >10 U/mg.

Syntheses of 1,4-butanediamine-bis-(4-acryloyl phenoxyacetic amide) (BDAA) and 1,6-hexanediamine-bis-(ethacrynic amide) (HDEA) as divalent pro-inhibitors followed those described in Scheme 1 [5, 14]. At first, 4-acryloyl phenoxyacetic acid as the intermediate of BDAA was prepared. In brief, phenoxyacetic acid was converted into 4-acryloyl phenoxyacetic acid via Friedel-Crafts acylation with acryloyl chloride in dichloromethane. After acid hydrolysis with aqueous HCl solution and extraction twice of the resulting mixture with dichloromethane, organic layer was extracted with 5 % $NaHCO_3$, and then acidified to pH 2.0 to yield white precipitate that was subjected to silica gel column eluting with ethyl acetate, petroleum (3:1) plus final 5 % of glacial acetic acid. To prepare HDEA or BDAA (Scheme 1), carboxyl group of EAA or 4-acryloyl phenoxyacetic acid was activated with N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC), and reacted with 1,6-hexanediamine or 1,4-butanediamine in tetrahydrofurane. After the removal of dicyclohexylurea and solvent, residuals were dissolved in dichloromethane for repetitive wash in order

Scheme 1 Synthetic routes of HDEA and BDAA



with 5 % $NaHCO_3$, 0.5 M HCl and a large amount of water. After the removal of solvent, the resulting powder was purified by silica gel chromatography (ethyl acetate: triethylamine = 20:1) to yield a divalent pro-inhibitor with purity over 98 % (reverse-phase HPLC, detected by absorbance at 254 nm). Data supporting their structures were in Spectral Data to Support Expected Structures of HDEA and BDAA and Figure S1, Figure S2 in ESI. 4-substitution of BDAA was supported by NOESY 1D as follows (Figure S3, ESI). There were crossed peaks among δ 7.15 of α-H on acryloyl, δ 6.43 and δ5.90 of β-H on acryloyl, δ 7.90 of aromatic H in vicinity, and crossed peaks among δ 4.55 of 2-H on 2-phenoxyacetyl, δ 6.66 of amide H and δ 6.99 of neighboring aromatic H.

Stock solutions of CDNB and divalent pro-inhibitors were made in dimethylformamide (DMF); final DMF in reaction mixtures was <1.3 % to mitigate alteration of enzyme activity or fluorescence of SjGST. Sodium phosphate buffer at 100 mM and pH 6.5 was pre-incubated in water-bath thermo-stated at 25 °C for more than 30 min before use. MAPADA UV-1600 PC spectrophotometer was used to measure absorbance. Absorbance at 340 nm was recorded at 10-s intervals after 20-s lag in an isolated small room air-conditioned at 25 °C and initial rates were estimated with data from 30 to 90 s since the final addition of CDNB. To ensure the formation of divalent product from HDEA or BDAA, it was pre-incubated with SjGST plus GSH in great excess for 10 min before the addition of CDNB. Except the determination of inhibition types, both CDNB and GSH were fixed at 1.0 mM. The nonenzymatic reaction indexed by absorbance change was about 0.003 per min and corrected.

Carry Eclipse fluorospectrometer was used with band widths of 10 nm for both excitation and emission, unless otherwise stated. To measure fluorescence, the reaction of SjGST with a divalent pro-inhibitor plus GSH took place in 100 mM sodium phosphate buffer at pH 7.0 and 25 °C. With any pro-inhibitor, fluorescence at 340 nm under the excitation at 280 nm was recorded after fluorescence became stable in 5 to 10 min; the contributions of a divalent pro-inhibitor in DMF below 1.3 % were corrected before analysis. To estimate the binding ratio, the responses of fluorescence at 340 nm to concentrations of a divalent inhibitor, at levels much smaller than that of homodimer and at levels much higher than that of homodimer, gave two linear plots whose intersection point indicated the binding equivalent.

In the putative method to estimate the apparent K_i of a tight-binding inhibitor considering its depletion during binding, Eq. (1) was fit to response of initial rates to total concentrations of the inhibitor (I_t) [7, 8]. In Eq. (1), P_0 was the total concentration of binding site; v_0 was the initial rate in the absence of inhibitors; v was the initial rate in the presence of an inhibitor; I_t was the total concentration of the inhibitor. Data were processed with CFtool in Matalab 6.5; only results with R^2>0.98 were accepted.

$$\frac{v}{v_0} = \frac{(P_0 - I_t - K_i) + \sqrt{(P_0 - I_t - K_i)^2 + 4 \times P_0 \times K_i}}{2 \times P_0} \quad (1)$$

Results and Discussion

Theoretical Basis for Fluorometric Titration Assay of K_d

For fluorometric titration assay of K_d of a tight-binding inhibitor, two structural states of GST, *i.e.*, the free one and the divalence-bound complex, are considered. A divalent pro-inhibitor at levels in great excess to GST homodimer can not be completely converted into the divalent product due to its potent inhibition on GST. Thus, for fluorometric titration assay of K_d of a tight-binding divalent product of GST, there are the following prerequisites. (a) For either of those two structural states of GST, there is consistent slope for linear response of fluorescence at 340 nm to its concentrations over

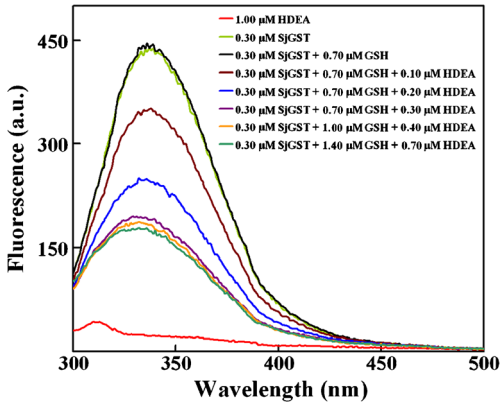


Fig. 1 Fluorescence spectra of the complexes of SjGST and HDEA. SjGST was mixed with HDEA plus GSH in 100 mM sodium phosphate buffer at pH 7.0

tested ranges. (b) The divalent pro-inhibitor and the divalent product have negligible signals at 340 nm under the excitation at 280 nm. (c) There is negligible competition of the divalent pro-inhibitor against the divalent product for GST, which is not considered when K_d of a tight-binding inhibitor itself is estimated. To this end, there should be negligible alteration of

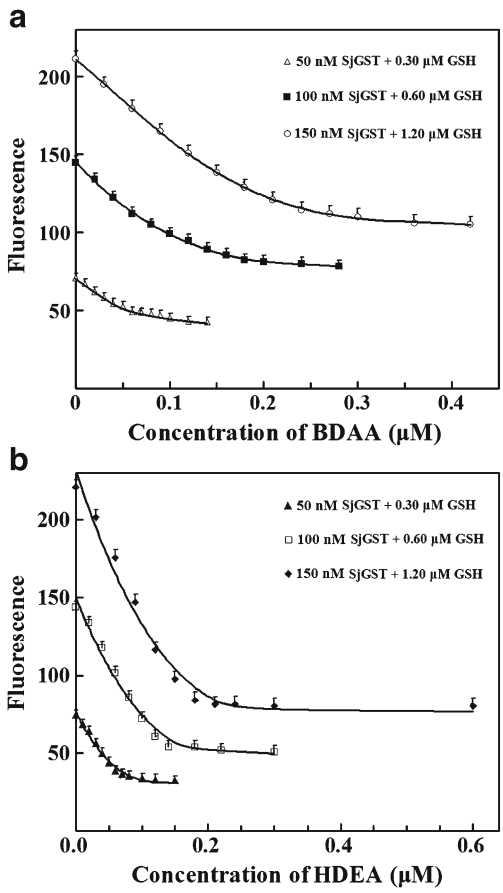


Fig. 2 Responses of fluorescence to total concentrations of (**a**) BDAA and (**b**) HDEA. With each pro-inhibitor, fluorescence at 340 nm under the excitation at 280 nm was recorded after pre-incubation with SjGST and GSH in excess for 10 min

fluorescence of GST by a divalent pro-inhibitor alone while manifest quench of fluorescence of GST by the divalent product, which is achieved with the divalent product bearing much stronger affinity for GST than the divalent pro-inhibitor. (d) The maximum concentration of any divalent pro-inhibitor/product is limited to a reasonable threshold for the following causes. The first is negligible dynamic quench of GST fluorescence by the free divalent product. Namely, only static quench of fluorescence of GST in complexes with the divalent product is considered and thus limited levels of the divalent product should be used. The second is the approximation of the concentrations of the divalent product by those of the divalent pro-inhibitor after pre-incubation of the divalent pro-inhibitor, GST and GSH in excess for a practical time; this approximation requires complete conversion of the divalent pro-inhibitor into the divalent product within a limited period. When all those prerequisites are satisfied, there are the following equations.

Letting F_t be fluorescence at 340 nm of binding solution minus that of the buffer, I_f be the concentration of the free divalent inhibitor, P_0, P_f and P_b be the concentrations of total SjGST (homodimer), the free GST and the divalence-bound GST, s_f and s_b be the slopes for linear responses of fluorescence at 340 nm of the free GST and divalence-bound GST to their concentrations, respectively, there is Eq. (2) for the divalence-binding of a divalent inhibitor to GST in equilibrium while the definition of K_d gives Eq. (3).

$$F_t = s_b \times P_b + s_f \times P_f \tag{2}$$

$$P_b \times K_d = P_f \times I_f \tag{3}$$

According to 1:1 stoichiometry for divalence-binding of the divalent inhibitor to SjGST, there are Eqs. (4) and (5), and thus Eq. (6). According to Eq. (3), there is Eq. (7), which together with Eq. (2) leads to Eq. (8) when all those assumptions are validated. In Eq. (8), I_t was the independent variable and P_0 was a parameter for fitting to titration curve. To estimate K_d, the response of fluorescence to concentrations of an inhibitor at a fixed P_0 was analyzed with CFtool in Matlab 6.5 according to Eq. (8) when all assumptions were valid.

$$I_t = I_f + P_b \tag{4}$$

$$P_0 = P_f + P_b \tag{5}$$

$$P_f = P_0 - I_t + I_f \tag{6}$$

$$I_f = \frac{I_t - P_0 - K_d}{2} + \frac{\sqrt{(I_t - P_0 - K_d)^2 + 4 \times I_t \times K_d}}{2} \tag{7}$$

Table 1 Comparison of K_d, IC_{50} and K_i of divalent pro-inhibitors

Methods	SjGST (nM)	HDEA			BDAA		
		IC_{50} (nM)	K_i (nM)	K_d (nM)	IC_{50} (nM)	K_i (nM)	K_d (nM)
Eq. (1)	5	6.6±1.0	0.3±0.1	/	/	/	/
	10	11±2	0.5±0.2	/	45±5	25±6	/
	15	17±2	0.4±0.2	/	69±7	28±8	/
Eq. (8)	50	/	/	0.4±0.2	/	/	18±4
	100	/	/	0.5±0.2	/	/	23±5
	150	/	/	0.5±0.2	/	/	16±4
	300	/	/	1.9±0.5	/	/	19±5
	500	/	/	2.4±0.6	/	/	20±5

Concentrations for SjGST stood for homodimer. HDEA and BDAA were pre-incubated with SjGST and GSH in excess for 10.0 min before the addition of CDNB. Data were from two or three independent series of assays. Student's *t*-test indicated there was no significant difference between the apparent K_i and K_d for a divalent product as the tight-binding inhibitor, at SjGST levels below 0.20 μM

$$F_t = s_f \times P_0 - \frac{(s_f - s_b)}{2} \times \left(I_t + P_0 + K_d - \sqrt{(I_t - P_0 - K_d)^2 + 4 \times I_t \times K_d} \right) \quad (8)$$

Comparison of the Fluorometric Titration Assay to the Approximation Approach

GSH alone at levels up to 3.0 mM and BDAA at levels below 4 μM had no signals at 340 nm under the excitation at 280 nm. Meanwhile, HDEA at 1.0 μM had very weak signals at 340 nm under the excitation at 280 nm (Figure S4, ESI). On the other hand, HDEA or BDAA alone, or GSH alone, at tested levels caused negligible alteration of SjGST fluorescence at 340 nm; the quench of fluorescence of SjGST was manifest by HDEA or BDAA after its pre-incubation for 10 min in the presence of GSH in excess (Fig. 1). Moreover, under the action of SjGST on BDAA or HDEA plus GSH in excess, the fluorescence of reaction solutions became stable in 2.0 min, when the concentrations of BDAA or HDEA were below those of SjGST homodimer. There was quick binding of BDAA or HDEA to active sites of SjGST and its rapid conversion into the divalent product in-situ in active sites. On the other hand, the binding ratio of HDEA or BDAA to SjGST homodimer was estimated from the change of fluorescence at 340 nm during titration by taking concentrations of a divalent pro-inhibitor as approximates of the concentrations of its divalent product; the binding ratios were consistently (1.06± 0.06) (n=4) for HDEA and BDAA to SjGST homodimer (Fig. 2a and b), supporting divalence-binding of either divalent product to SjGST. As a result, Eq. (8) may be valid with the product of HDEA or BDAA as long as there was consistent linear response of fluorescence of either structural state of

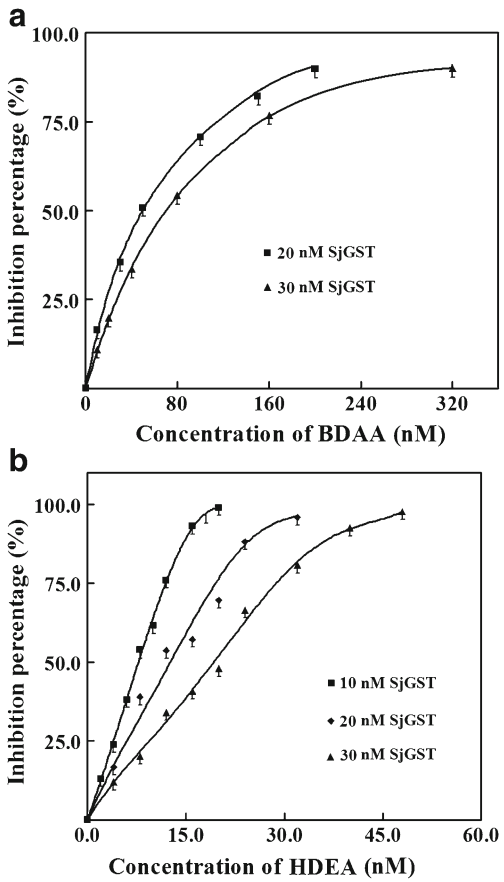


Fig. 3 Response of initial rates to total concentrations of (**a**) BDAA and (**b**) HDEA. Initial rates were measured by recording absorbance at 340 nm at 10-s intervals after a lagging time of 20 s; absorbance from 30 to 90 s since the initiation of reaction between CDNB and GSH was analyzed to get initial rates. Any inhibitor was pre-incubated with SjGST and GSH in excess for 10.0 min before the addition of CDNB to measure the change of absorbance at 340 nm. Apparent inhibition constant was derived from the changes of apparent Michaelis-Menten constants (K_{ik}) or maximum rates (K_{iv}) according to Lineweaver-Burk plot. A ratio of the apparent inhibition constant as the larger one of K_{ik} and K_{iv} to the smaller one was used to determine the inhibition type as described in reference 19

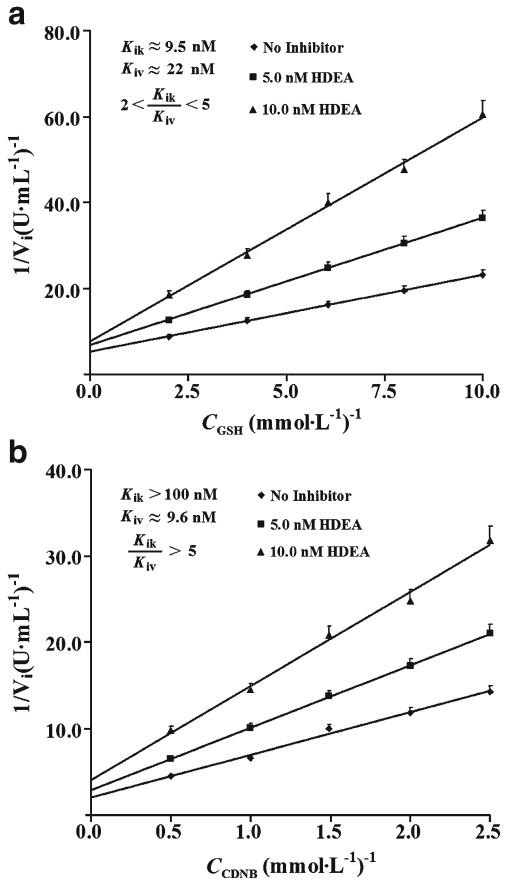


Fig. 4 Inhibition type of the divalent product of HDEA against (**a**) GSH and (**b**) CDNB. Final concentration of SjGST was about 20 nM. The inhibition type was determined as described in reference 19

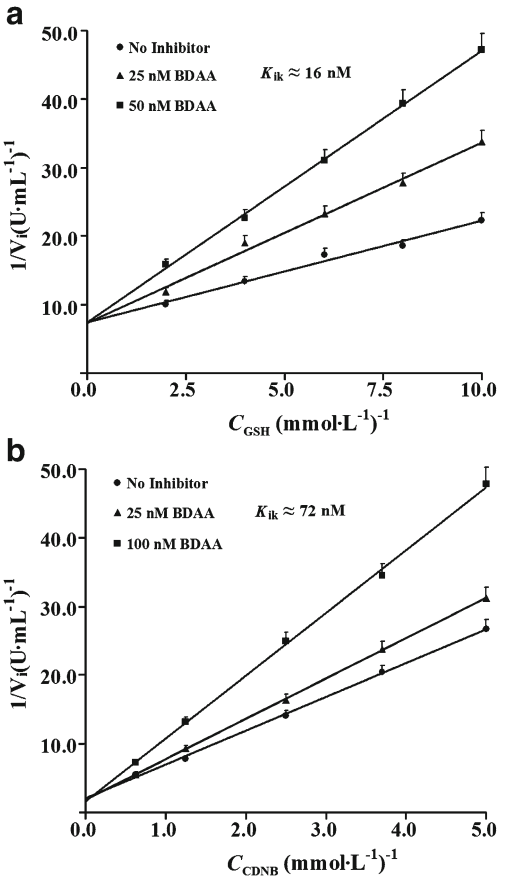


Fig. 5 Inhibition type of the divalent product of BDAA against (**a**) GSH and (**b**) CDNB. Final concentration of SjGST was about 20 nM. The inhibition type was determined as described in reference 19

SjGST to its concentrations over tested ranges, but such a linear response can not be easily proved with any divalent product since its preparation was a great challenge.

When Eq. (8) was validated, K_d of the divalent product of HDEA or BDAA should be consistent over a reasonable range of SjGST; the validity of Eq. (8), and thus reliability of K_d, were examined from the effects of final concentrations of SjGST on K_d of the divalent products of HDEA and BDAA. By fitting of Eq. (8) to response curve of fluorescence at 340 nm to total concentrations of a divalent pro-inhibitor, values of K_d from subnanomolar to nanomolar levels were estimated with R^2>0.98 (Fig. 2a and b; Table 1). More importantly, K_d of the divalent product of BDAA showed good consistence at levels of SjGST homodimer from 0.05 to 0.50 μM, completely matched the expectation (Table 1). Unexpectedly, K_d for HDEA showed good consistence at SjGST levels from 0.05 to 0.15 μM, but displayed some positive deviations at levels of SjGST homodimer over 0.15 μM (Table 1); these results indicated there was not satisfaction to all prerequisites of Eq. (8) with HDEA and SjGST homodimer at higher levels. At higher levels of SjGST, there may be interference from weak scattering signals of HDEA or its divalent product, as we observed before [9]. In fact, as calculated with ACDfree (V11.0), Log P of HDEA was much higher than that of BDAA (Scheme 1). Strong hydrophobicity of HDEA may result in the formation of its aggregates, whose scattering signals at 340 nm under excitation at 280 nm were detected at levels over 0.30 μM (Figure S4, ESI). Moreover, as supported by the consistent K_d estimated with SjGST at levels below 0.15 μM, the divalent product of HDEA displayed subnanomolar affinity for SjGST; such a strong affinity may result in complete inhibition of SjGST after the levels of HDEA exceeded that of SjGST homodimer and thus incomplete conversion of HDEA into the divalent product. These two factors invalidated Eq. (8) and gave false positive deviation in K_d with HDEA at higher levels of SjGST. Hence, fluorometric titration assay of K_d was effective to tight-binding inhibitors bearing no signals at 340 nm under the excitation at 280 nm.

For revealing inhibition types, CDNB or GSH was fixed at 1.0 mM to determine response of initial rates to the other substrate; apparent Michaelis-Menten constants and maximum reaction rates were estimated from initial rates according to Lineweaver-Burk plot. The inhibition type was judged from the response of apparent kinetic parameters to inhibitor concentrations [19]. The divalent product generated in-situ from

HDEA inhibited SjGST competitively against GSH and noncompetitively against CDNB (Fig. 3a and b), but the divalent product generated in-situ from BDAA inhibited SjGST competitively against both GSH and CDNB (Fig. 4a and b). By fluorometric titration assay, the divalent products of HDEA and BDAA belonged to tight-binding inhibitors (Table 1). Such sophisticated inhibition kinetics of those two divalent products challenged the conversion of their apparent K_i into their true K_i of [7, 8]. On the other hand, to approximate the concentrations of each divalent product by the concentrations of its divalent pro-inhibitor at tested levels of SjGST, there should be some residual activity of SjGST to enable the complete conversion of the divalent pro-inhibitor into the divalent product. As a result, data from 10 to 90 % inhibition at 1.0 mM of GSH and CDNB after pre-incubation of SjGST, GSH and the divalent pro-inhibitor for 10 min were analyzed. In this case, IC_{50} values of each divalent product displayed clear dependence on levels of SjGST, further supporting tight-binding of the divalent product (Fig. 5a and b; Table 1). Moreover, analyses of response curves of activities to total concentrations of divalent pro-inhibitors of SjGST via Eq. (1) gave apparent K_i consistent at different levels of SjGST, and consistent with K_d estimated by fluorometric titration assay (Table 1). Therefore, the apparent K_i of the divalent products of GST estimated via the putative method also served as effective approximates of their affinities.

Conclusions

Fluorometric titration assay of K_d was effective to tight-binding inhibitors of GST, which were nonfluorescent at 340 nm under the excitation at 280 nm; it was universally applicable to enzymes/proteins when their fluorescence at 340 nm was susceptible to the binding of nonfluorescent inhibitors/ligands. The true K_i of tight-binding inhibitors of GST can be directly approximated by the apparent K_i estimated via the putative method.

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Appendix A: Spectral Data to Support Expected Structures of HDEA and BDAA

HDEA: ^{1}H NMR(400 MHz, $CDCl_3$) δ(ppm): 7.27(2H,s), 7.20(2H, d, J=8.0 Hz), 6.87(2H, d, J=8.0 Hz), 5.96(2H, s), 5.59(2H, s), 4.57(4H, s), 3.38(4H, q, J=6.4 Hz), 2.47(4H, dd, J=7.2 Hz, J=14.8 Hz), 1.59(4H, m), 1.36(4H, m), 1.15(6H, t, J=7.2 Hz); ^{13}C NMR(400 MHz, $CDCl_3$) δ(ppm): 195.5(2C), 166.6(2C), 154.5(2C), 150.2(2C), 134.1(2C), 128.7(4C), 127.2(2C), 110.9(2C), 127.4(2C), 68.2(2C), 38.9 (2C), 33.9(2C), 29.3 (2C), 26.3(2C), 12.4(2C). ESI-HRMS m/z for $C_{32}H_{36}Cl_5N_2O_6$ [M + Cl^-]: calculated 719.1021, found 719.1005.

BDAA: ^{1}HNMR(500 MHz, DMSO) δ(ppm):, 7.98(4H,d, J=8.4 Hz), 7.16(2H,dd, J=10.2 Hz, J=16.4 Hz), 7.01(4H,d, J=8.4 Hz), 6.67(2H, s), 6.46(2H,d, J=16.8), 5.91(2H,d, J=9.6 Hz), 4.55(4H,ds), 3.39(4H,s), 1.63(4H,s); ^{13}C NMR(500 MHz, DMSO) δ(ppm): 187.9(2C), 166.9(2C), 161.8(2C), 132.1(2C), 130.8(4C), 130.1(2C), 129.4(2C), 114.7(4C), 66.9(2C), 38.0(2C), 26.5(2C). ESI-HRMS m/z for $C_{26}H_{28}N_2O_6Na$ [M + Na^+]: calculated 487.1840, found 487.1879.

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