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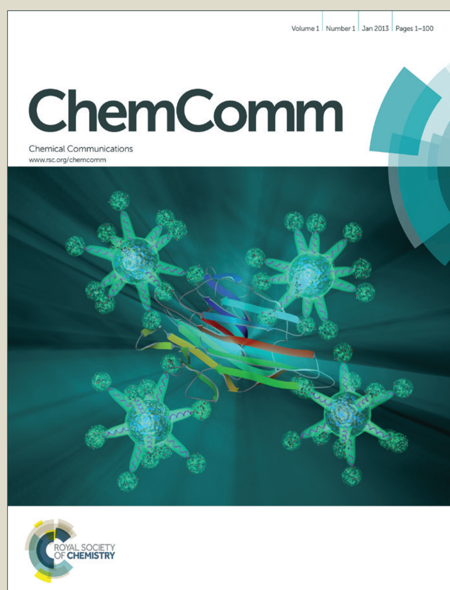

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COMMUNICATION

A practical fluorogenic substrate for high-throughput screening of glutathione S-transferase inhibitors†

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Yuuta Fujikawa,*[a] Fumika Morisaki,[a] Asami Ogura,[a] Kana Morohashi,[b] Sora Enya,[b] Ryusuke Niwa,[bc] Shinji Goto,[d] Hirotatsu Kojima,[e] Takayoshi Okabe,[e] Tetsuo Nagano,[e] and Hideshi Inoue [a]

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We report a new fluorogenic substrate for glutathione S-transferase (GST), 3,4-DNADCF, enabling assay with a low level of nonenzymatic background reaction. Inhibitors against Noppera-bo/GSTe14 from *Drosophila melanogaster* were identified by high throughput screening using 3,4-DNADCF, demonstrating utility of this substrate.

Glutathione S-transferases (GSTs) are multifunctional proteins, mainly known as phase II detoxifying enzymes, widely distributed from prokaryotes to eukaryotes. They are classified into three superfamilies: cytosolic, mitochondrial, and microsomal. The cytosolic GST family consists of various isoenzymes, which are grouped into six classes in humans: alpha, mu, pi, sigma, theta, zeta, and omega[1]. In invertebrates and plants, other classes have been identified, for example delta and epsilon in insects. In addition to their roles in drug metabolism, the cytosolic GSTs are involved in pathways for steroid hormone biosynthesis[2], eicosanoid biosynthesis[3], amino acid catabolism[4], and oxidative stress resistance[5,6]. The well-investigated reaction catalysed by GSTs is addition of reduced glutathione (GSH) to endogenous and exogenous electrophiles. The resulting glutathione conjugates are excluded from cells by glutathione-conjugate (GS-X) transporters[7,8]. Some GSTs, e.g. GSTP1-1, GSTO1-1, are overexpressed in many types of cancer cells and are involved in chemotherapeutic resistance[9-11]; therefore, GST inhibitors have been investigated as potential anti-tumour drugs[12,13]. Noppera-bo(Nobo)/GSTe14 is an epsilon class GST expressed in the prothoracic gland of the fruit fly *Drosophila melanogaster*, and plays an essential role in ecdysteroid biosynthesis[14,15]. Knockout mutants of the *D. melanogaster nobo* gene (*nobo-Dm*; also known as *GSTe14*) result in embryonic lethality. The *nobo* family of GSTs is well conserved in some insects including the mosquito *Anopheles gambiae,* one of the malaria vectors. Therefore, inhibitors of *A. gambiae nobo* (known as *GSTe8*) are potential insecticidal agents to control malaria.

Substrates used in a high-throughput screen (HTS) for GST inhibitors should fulfil the following criteria: (1) nonenzymatic reaction with GSH (background) is kept as slow as possible to achieve a high signal-to-background (S/B) ratio; i.e. the ratio of the GST activity-dependent signal to the background signal; (2) the substrate and its product are respectively non-fluorescent and highly fluorescent to enable sensitive measurement; (3) enzymatic reaction parameters for the substrate are good (e.g. high k_{cat}/K_M value), enough to avoid assay in the presence of a high concentration of protein; (4) the substrate can be acted on by multiple GSTs to evaluate subtype specificity of hit compounds. Previously, we reported novel fluorogenic substrates for GST, DNAFs, which exhibit a large increase in fluorescence upon GSH conjugation concomitant with de-nitration[16]. However, assays using DNAFs must be done at a pH as high as 7.4 to obtain a large increase in fluorescence and a high S/B ratio, because the pK_a of the phenolic group of the fluorophore's xanthene moiety is around 6.4[17]. At that pH, the nonenzymatic background reaction is too fast to use this substrate in HTS. Therefore, to avoid an unfavourable compromise in the sensitivity of assay, a fluorogenic substrate that has a fluorophore with a lower pK_a is desirable. Such a fluorogenic substrate would afford a practical assay system, e.g. one with a high

Scheme 1. Reaction of a fluorogenic substrate, 3,4-DNADCF, that is less sensitive to pH.

fluorescence activation and high S/B ratio, for HTS. Hence, a new fluorogenic substrate, 3,4-DNADCF, was designed and synthesised (Scheme S1; for synthesis details, see the ESI†).

Here we show characterisation of fluorogenic substrate 3,4-DNADCF and its utility in identification of GST inhibitors against

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nobo-Dm/GSTe14. This is the first report of a practical fluorogenic substrate for large-scale HTS for GST inhibitors.

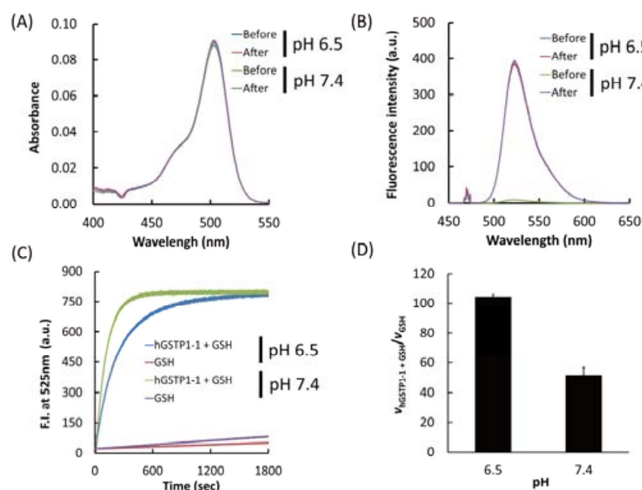

Fig. 1. Optical and kinetic properties of 3,4-DNADCF.
(A) UV-Vis and (B) fluorescence spectra of 3,4-DNADCF before and after GSH conjugation catalysed by recombinant 6xHis-hGSTP1-1 in 100 mM sodium phosphate buffer at pH 6.5 or 7.4. (C) Time course of fluorescence increase ($\lambda_{ex}/\lambda_{em}$ = 505/525nm) of 3,4-DNADCF (1 μM). Assay was performed in 100 mM sodium phosphate buffers (at the indicated pH) containing 0.1% DMSO as a cosolvent and 0.1 mM GSH in the presence or absence of 6xHis-hGSTP1-1. (D) pH dependence of increase in reaction rate by 6xHis-hGSTP1-1; ratio of initial velocity of the enzymatic and nonenzymatic reactions.

Table 1. Optical properties of 3,4-DNADCF

	λ_{ex} (nm)	λ_{em} (nm)	apparent QE[b]
3,4-DNADCF	504	524	0.005
3,4-DNADCF + 6xHis-hGSTP1-1/GSH	505	524	0.27
DNAF1	493[a]	512[a]	0.002
DNAF1 + 6xHis-hGSTP1-1/GSH	492[a]	516[a]	0.11

[a] Data from Reference 16 [b] Measured in a sodium phosphate buffer (100 mM, pH 7.4) with excitation at 490 nm, and calculated using QE of fluorescein (0.85) as a standard.

First, the optical properties and reactivity of 3,4-DNADCF were evaluated. Using 0.1% DMSO as a cosolvent, at least 1 μM 3,4-DNADCF was soluble enough to be dissolved in 100 mM sodium phosphate buffer. The UV-Vis and fluorescence spectra were both characterised by an absorption peak at 504 nm and an emission peak at 524 nm (Fig. 1A and B, Table 1). Based on the pH dependence of the spectra, the pK_a values of the phenolic group were 4.7 for 3,4-DNADCF and 4.4 for the fluorescent product (Figs. S1 and S2 ESI†). The pK_a value of the fluorescent product is low enough to use this substrate for GST activity measurement without loss of sensitivity, even at a pH as low as 6.5. HPLC and LC-MS analyses of the reaction mixture at pH 6.5 demonstrated that the increase in fluorescence could be ascribed to generation of a glutathione conjugate, 4-GS-3-NADCF, similar to one described previously[16] (Figs. S3 and S4 ESI†). 3,4-DNADCF was a compound with very low fluorescence, with a quantum efficiency (QE) determined to be 0.005 (Fig. 1B, Table 1). By incubation of 3,4-DNADCF with recombinant hGSTP1-1 and 0.1 mM GSH, fluorescence intensity was quickly enhanced, resulting in a high QE (up to 0.27 after the reaction) (Fig. 1C, Table 1). The change in QE was 54-fold, comparable to the 56-fold change in the case of DNAF1 (QE 0.002 and 0.111 for before and after the reaction, respectively; Table 1). However, QE of the fluorescent product 4-GS-3-NADCF was twice that of the product 4-GS-3-NAF from DNAF1. The relationship between fluorescence intensity and concentration of 4-GS-3-NADCF was linear up to 5 μM, indicating that a concentration of 3,4-DNADCF up to 5 μM is suitable for high throughput screening (Fig. S5 ESI†). Compared to a currently available chromogenic assay with 1-chloro-2,4-dinitrobenzene (CDNB), which is typically used at 1 mM, it is noteworthy that assay with 3,4-DNADCF is practicable even at a 1000-fold lower concentration. Moreover, the detection limit of 6xHis-hGSTP1-1 activity for 3,4-DNADCF (1.26 ng/ml enzyme) was lower than that for CDNB (6.63 ng/ml enzyme) (Fig. S6 ESI†). Thus, 3,4-DNADCF has superior optical properties and reactivity for high throughput screening.

Second, we examined the pH dependence of the time course of fluorescence increase by enzymatic and nonenzymatic reactions. The fluorescence increase by hGSTP1-1 was only slightly slower at pH 6.5 than at pH 7.4, as in the case of hGSTP1-1 activity toward CDNB[18], while the logarithm of the nonenzymatic reaction velocity is proportional to pH in this range. In consequence, the initial velocities of the enzymatic and nonenzymatic reactions gave a higher S/B ratio at pH 6.5 (Fig. 1D). In the presence of 0.07 μg/ml hGSTP1-1, fluorescence of the reaction mixture increased and reached a plateau within 30 min at both pH 6.5 and 7.4 (Fig. 1C), while the nonenzymatic increase in fluorescence at pH 6.5 at 30 min was at most one-fiftieth of the enzymatic increase. These results suggest the utility of 3,4-DNADCF as a fluorogenic substrate for measurement of GST activity with a high fluorescence activation and S/B ratio.

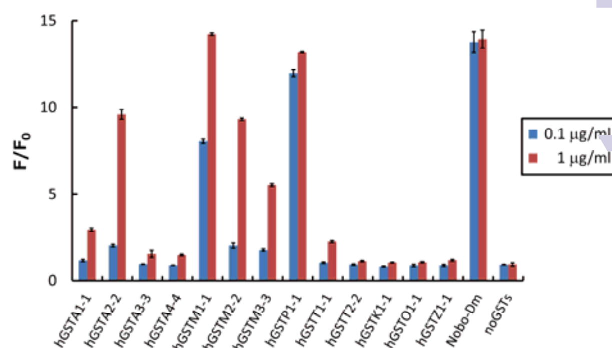

Fig. 2. Relative fluorescence intensity (F/F_0) of various GST isoenzymes. 3,4-DNADCF was incubated with GSH (0.1 mM) in the presence (0.1 or 1.0 μg/ml) or absence of GST in sodium phosphate buffer (100 mM, pH 6.5) at 28°C for 30 min.

To assess applicability of 3,4-DNADCF to GSTs of various classes, recombinant N-terminal hexahistidine (6xHis)-tagged GSTs including 13 human GSTs (hGSTs) and C-terminal 6xHis-tagged Nobo-Dm/GSTe14, were assayed with this substrate (Fig. 2). After 30 min of incubation at pH 6.5, fluorescence enhancement (F/F_0) was evaluated. In the presence of 0.1 mM GSH, F/F_0 of the nonenzymatic reaction was close to 1.0, while the F/F_0 values of enzymatic reactions with 1.0 μg/ml of hGSTM1-1, hGSTP1-1, and Nobo-Dm/GSTe14 were larger than 10. Further, specific activities of various GSTs toward this substrate were determined and are listed in Table S2 (ESI†) for a more quantitative comparison, which indicated that all the GSTs tested except for hGSTT1-1 and hGSTK1-1 exhibit measurable specific activity.

In order to assess applicability of 3,4-DNADCF to screening for GST inhibitors, GSH conjugations of 3,4-DNADCF catalysed by hGSTA1-1, hGSTM1-1, hGSTP1-1, and Nobo-Dm/GSTe14 were kinetically analysed using 0-1.0 μM 3,4-DNADCF in the presence of 0.1 mM GSH. As shown in Fig. S7, the initial velocity versus substrate concentration curves given by hGSTM1-1 and Nobo-Dm/GSTe14 followed the Michaelis-Menten equation. The

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calculated K_M values toward 3,4-DNADCF were 314 ± 16 and 162 ± 18 nM for hGSTM1-1 and Nobo-Dm/GSTe14, respectively (Table S3 ESI†). In contrast, the K_M value was estimated at around 2 μM for hGSTA1-1, and was too large to be estimated for hGSTP1-1. The K_M value of 162 ± 18 nM for Nobo-Dm/GSTe14 was lower than values for various combinations of GST isoenzymes and substrates. This is thought to reflect high affinity of 3,4-DNADCF for Nobo-Dm/GSTe14, which can be an advantage in screening for effective inhibitors[19]. As a positive control inhibitor, we examined ethacrynic acid, known to inhibit various GST isoenzymes[20,21] as well as hGSTP1-1 activity by 50% at 0.13 μM in our assay (data not shown), and was found to inhibit Nobo-Dm/GSTe14 by 50% at 31.9 ± 3.3 nM (Fig. S8 ESI†).

To perform HTS for inhibitors of Nobo-Dm/GSTe14, we tested a 384-well format (20 μl/well) assay with various concentrations of Nobo-Dm/GSTe14. A significant increase in fluorescence occurred in a manner dependent on both time and enzyme concentration, while it was negligible in the absence of the enzyme or in the presence of 20 μM ethacrynic acid (Fig. 3A). The variability of fluorescence intensity values obtained after 70 min from two independent assay plates is shown on a scatter plot (Fig. 3B). They reproducibly gave a Z'-factor value greater than 0.75, which is a performance index for screening, and Z' > 0.5 indicates a high quality assay[22]. Furthermore, the S/B ratio (ratio of signal increases that were dependent on and independent of GST) was determined greater than 29. Thus, the reliability and robustness of the 384-well format assay was demonstrated.

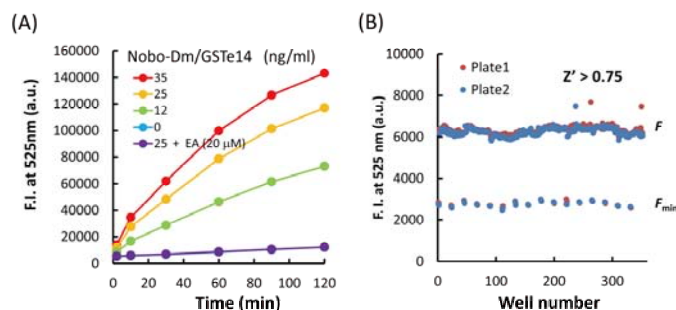

Fig 3. Validation of HTS system for Nobo-Dm/GSTe14 in a 384-well format.
(A) Time- and concentration-dependent fluorescence increase by Nobo-Dm/GSTe14. Nobo-Dm/GSTe14 activity was fully inhibited by 20 μM ethacrynic acid (EA), with only the background level of increase (no Nobo-Dm/GSTe14). (B) Scatter plot of fluorescence intensity in two distinct microtitre plates at 70 min. Z'-factor was calculated from the means of F and F_{min}. Reproducible results with consistent Z'-factor values were obtained. Detailed experimental procedures are described in supplementary materials and methods.

Using the assay system, 9,600 small molecules from a chemical library (Open Innovation Centre for Drug Discovery, The University of Tokyo) were screened for inhibitors against Nobo-Dm/GSTe14. The assay was performed in 200 mM sodium phosphate buffer (pH 6.5, 0.005% Tween 20, and 1.1% DMSO as a cosolvent) containing 0.1 mM GSH and 35 ng/ml Nobo-Dm/GSTe14-6xHis in the presence or absence of a 20 μM test compound from the chemical library at 25°C. Fluorescence intensity after a 90-min incubation was measured and percentage inhibition was calculated after subtraction of background fluorescence. In the primary screening, 81 hit compounds with potent inhibitory activity (>80% inhibition) were picked up, all of which were confirmed to act in a concentration-dependent manner. Interestingly, three steroid compounds were included.

In contrast, no steroid has been hit in counter-screening against hGSTP1-1, suggesting that steroids are not general GST inhibitors but rather, specific against Nobo-Dm/GSTe14. One of the three steroids is 17β-oestradiol, an oestrogen, with an IC_{50} estimated to be 1.2 ± 0.1 μM at pH 6.5 by dose-response analysis (Fig. 4). Nobo-Dm/GSTe14 plays an important role in ecdysteroid biosynthesis[14,15]. While 17β-oestradiol itself is not thought of as an endogenous hormone in insects[23,24], these results suggest the possibility that ecdysteroid biosynthesis is regulated by direct interaction between Nobo-Dm/GSTe14 and a steroid compound.

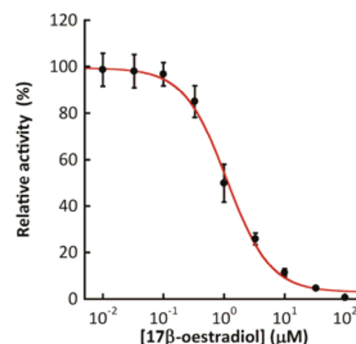

Fig. 4. 17β-oestradiol, a hit compound from primary screening, is a potent inhibitor of Nobo-Dm/GSTe14.
Dose-response relationship of 17β-oestradiol against Nobo-Dm/GSTe14 activity. Relative activity represents the percentage of Nobo-Dm/GSTe14 activity in the presence of 17β-oestradiol with respect to that in its absence. The dots and error bars respectively represent means and standard error of the mean (n = 3). IC_{50} was determined to be 1.2 ± 0.1 μM.

In conclusion, 3,4-DNADCF, a new fluorogenic substrate for GSTs, enables a highly sensitive and reliable assay, e.g. with high fluorescence activation and S/B ratio, applicable to HTS for GST inhibitors. We performed HTS with this substrate and found that 17β-oestradiol was a potent inhibitor against Nobo-Dm/GSTe14. Since 3,4-DNADCF is also a good substrate for several human GST isoenzymes (Fig. 2), this substrate is potentially applicable to HTS for inhibitors against those GSTs, and thus may be helpful in drug development.

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Notes and references

[a] School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan.
E-mail: yfuji@toyaku.ac.jp; Fax and Tel: +81-42-676-5391
[b] Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8572, Japan.
[c] PRESTO, JST, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan.
[d] Department of Stem Cell Biology, Atomic Bomb Disease Institute, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.
[e] Open innovation Center for Drug Discovery, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.

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† Electronic supplementary information (ESI) available: Figs. S1-S8, Tables S1-S3 and experimental details. See DOI: 10.1039/c000000x/

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