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A Vinyl Sulfone-Based Fluorogenic Probe Capable of Selective Labelling of PHGDH in Live Mammalian Cells

Sijun Pan, Se-Young Jang, Si Si Liew, Jiaqi Fu, Danyang Wang, Jun-Seok Lee, and Shao Q. Yao**

Abstract: *Chemical probes are powerful tools for interrogating small molecule-target interactions. With additional fluorescence Turn-ON functionality, such probes might enable direct measurements of target engagement in live mammalian cells. Herein, we report* ***DNS-pE*** *(and its terminal alkyne-containing version* ***DNS-pE2****) as the first small molecule that can selectively label endogenous 3-phosphoglycerate dehydrogenase (PHGDH) from various mammalian cells. Endowed with an electrophilic vinyl sulfone moiety that possesses fluorescence-quenching property,* ***DNS-pE/DNS-pE2*** *became highly fluorescent only upon irreversible covalent modification of PHGDH. With inhibitory property (in vitro* K_i *= 7.4* μ*M) comparable to that of known PHGDH inhibitors, our probes thus offer a promising approach to simultaneously image endogenous PHGDH activities and study its target engagement in live-cell settings.*

3-Phosphoglycerate dehydrogenase (PHGDH), a key enzyme in the biosynthesis of serine, is up-regulated in many rapidly proliferating cancer cells.[1,2] Therefore it is a potential anticancer target. To date, only several small-molecule PHGDH inhibitors, e.g. NCT-503 and CBR-5884, are reported in the literature (Figure S1).[3] Both were serendipitously discovered from high-throughput screening (HTS) assays, and possess moderate micromolar *in vitro* and cellular inhibitory activities against PHGDH. While useful for studying serine metabolism, these two compounds are not "tractable" *in situ* and cannot be used to study direct PHGDH-ligand engagement. In fact, despite remarkable advances in screening technologies,[4] very few assays can reveal direct small molecule-target interactions in intact cellular environments.

Activity-based protein profiling offers a versatile approach to interrogate small molecule-protein interaction at a proteome-wide scale, and could be made live-cell compatible with cell-permeable small molecule probes.[5] Recently, this approach has been combined with fragment-based screening to discover ligandable cysteines targeted by small-molecule covalent modifiers in live-cell settings.[6] Inspired by this study, we have taken a step further to develop chemical probes capable of covalently and selectively labelling of PHGDH, with novel "reaction-based" fluorescence Turn-ON properties that enabled, for the first time, simultaneous imaging of endogenous PHGDH activities and studying its target engagement in live mammalian cells (Figure 1).

Such so-called dual-purpose probes should contain several essential components: 1) a PHGDH active site-binding group, 2) an electrophilic moiety for covalent labelling of PHGDH, 3) a reporter with significant Turn-ON fluorescence upon covalent reaction with PHGDH, and finally 4) a chemically tractable tag (i.e. terminal alkyne shown in **DNS-pE2**; Figure 1C) for *in situ* target identification and enrichment.[7] Previously, quenched activity-based probes were designed based on similar criteria, but were only applicable to hydrolytic enzymes.[8] Recent advances in the development of reaction-based small molecule biosensors provided a clue to design the corresponding "reaction-based" PHGDH sensor,[9] whose fluorescence would be turned on only upon covalent reaction with PHGDH (Figure 1C). Based on the available X-ray structure of PHGDH (PDB ID: 2G76) and a previous report,[3a] several cysteine residues located within the PHGDH active site, i.e. Cys^{234}, Cys^{281} and Cys^{295} (Figure 1A) could be potentially targeted by electrophilic compounds. Indeed, some of these residues were reported as hyper-reactive cysteines that were readily labelled by iodoacetamide (IA, a common cysteine-alkylating agent),[6] and Cys^{234} was previously reported to be important for NCT-503/PHGDH interaction.[3a] Gratifyingly, upon carrying out a preliminary docking experiment, we discovered commercially available phenyl vinyl sulfone (PVS) and phenyl vinyl sulfonate (PVSN), two neutral and electrophilic phosphotyrosine mimics,[10] could fit snugly into the PHGDH

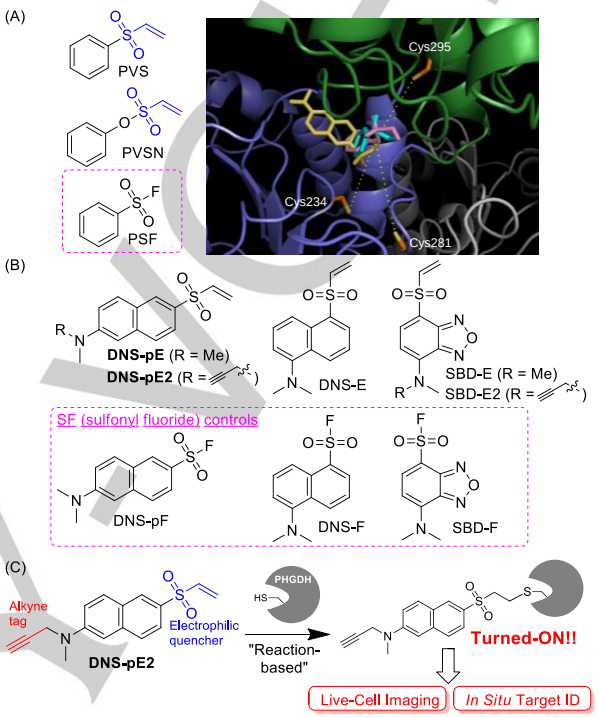

Figure 1. (A) Molecular docking of PVS (cyan), PVSN (pink) and **DNS-pE** (yellow) in the active site of PHGDH (PDB ID: 2G76), where the nucleotide- and substrate- binding domains are shown in slate and green, respectively. The structures of the compounds are shown on the left, with vinyl sulfone highlighted in blue. (B) Chemical structures of small-molecule probes designed and tested in this study. Sulfonyl fluoride (SF) controls are boxed in pink. (C) Proposed fluorescence Turn-ON mechanism of the dual-purpose probe, **DNS-pE2**, upon covalent labelling with PHGDH, leading to subsequent live-cell imaging and *in situ* target identification.

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active site with predicted binding affinities (pK_d) of -5.3 and -6.3 kcal/mol respectively (Figure 1A); all three cysteine residues were shown in close proximity to the docked ligands (< 10 Å). We therefore hypothesized that both PVS and PVSN, as previously reported mechanism-based inhibitors of protein tyrosine phosphatases (PTPs),[10] might also be electrophilic irreversible inhibitors of PHGDH (a phosphate-binding protein). Since both molecules contain a vinyl sulfone, we envisaged this group could be used as both an electrophile and a fluorescence quencher in our probe design (Figure 1B). Several cysteine-reactive, electrophilic α,β-unsaturated moieties, including ketones, amides and vinyl sulfonamides, are known fluorescence quenchers in suitably designed small molecule fluorophores.[11-13] We took note of the structural and electronic similarities between vinyl sulfone and sulfonamide, an acceptor moiety commonly used in push−pull fluorophores, including 2,6-dansyl amide, dansyl amide and 4-sulfamoyl-7-aminobenzoxadiazole (Figure S1).[12b,14] By replacing sulfonamide in these fluorophores with vinyl sulfone (giving **DNS-pE**, DNS-E and SBD-E, respectively; Figure 1B), our newly designed probes might provide a unique starting point to develop novel fluorogenic PHGDH probes. We further noted that our probes are also structural analogs of two well-known thiol-reactive fluorogenic small molecules, i.e. acrylodan and DNS-D (Figure S1).[11,12b] As controls, probes containing a sulfonyl fluoride (SF) were also examined (PSF in Figure 1A, DNS-pF/DNS-F/SBD-F in Figure 1B); with numerous reports on biocompatible SF-based compounds as emerging electrophilic probes capable of modifying different nucleophilic residues in proteins,[15] these probes may help to expand the potential ligandable region around the PHGDH active site. Docking experiments were again carried out amongst these newly designed PHGDH probes, and **DNS-pE** emerged as the best PHGDH binder with an improved pK_d value of -6.9 kcal/mol and a potential interaction between **DNS-pE** and Cys^{234} (Figure 1A). Finally, a terminal alkyne was installed in this probe, providing the dual-purpose **DNS-pE2** (Figure 1C).

Upon successful synthesis of these probes (Scheme S1), we first investigated their thiol-dependent fluorescence properties. As shown in Figure 2A, **DNS-pE** showed a significant fluorescence increase upon incubation with cysteine but not any other nucleophilic amino acids (i.e. lysine, serine or tyrosine). Its fluorescence Turn-ON ratio was comparable to that of acrylodan (21x *vs* 14x), but markedly higher than that of DNS-D (6x), under identical conditions. In contrast, negligible cysteine-responsive fluorescence changes were observed with other probes, including DNS-E and SBD-E, as well as all three SF-based control probes (DNS-pF, DNS-F and SBD-F). These results offered the first clue that vinyl sulfone could indeed act as a fluorescence quencher when properly positioned in a suitable fluorophore (compare **DNS-pE** *vs* DNS-E). Even more significant Turn-ON fluorescence was observed when **DNS-pE** or **DNS-pE2** was reacted with 2-mercaptoethanol (BME) under physiological conditions (Figures 2B & S2); 215- and 185-fold increases in fluorescence were quantitatively recorded at the probe's absorption/emission maxima ($\lambda_{abs}/\lambda_{em}$ = 333/453 nm and 324/437 nm, respectively). The complete Michael addition reaction (**DNS-pE2** + BME) was unequivocally confirmed by LCMS (Figure S2B). For **DNS-pE2**, both the quantum yield (Φ_F) and extinction coefficient (ε) went from 0.0073 and 3900 $M^{-1}cm^1$ in the "OFF" state to 0.88 and 6000 $M^{-1}cm^{-1}$ in the "ON" state. Similar increases were recorded for (**DNS-pE** + BME) (Figure S2A). Since acrylodan is a well-known polarity-sensitive dye that can label cysteines in a protein,[16] the emission spectra of cysteine-reacted acrylodan and **DNS-pE** were further investigated in different solvents (Figure S3A-C); unlike the fluorescence of the acrylodan adduct which was highly solvent polarity-dependent, fluorescence of the **DNS-pE** adduct was only marginally solvent-sensitive. To gain better insights into the fluorescence Turn-ON property of **DNS-pE** (and **DNS-pE2**),

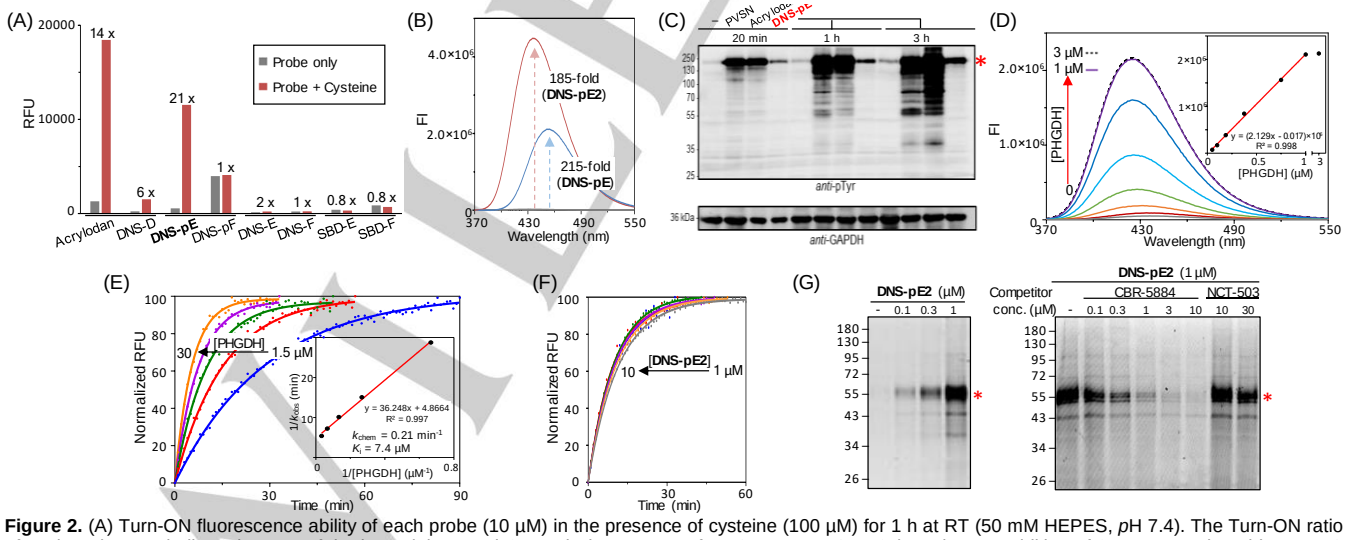

Figure 2. (A) Turn-ON fluorescence ability of each probe (10 μM) in the presence of cysteine (100 μM) for 1 h at RT (50 mM HEPES, *p*H 7.4). The Turn-ON ratio of each probe was indicated on top of the bars. (B) Turned-ON emission spectra of **DNS-pE** or **DNS-pE2** (1 μM) upon addition of 2-mercaptoethanol in HEPES buffer (50 mM, *p*H 7.4). (C) Western blotting analysis of endogenous tyrosine phosphorylation levels (*anti*-pTyr) of COS-7 cells upon treatment with 200 μM of probe at 37 °C (20 min, 1 h, 3 h). The 170-kDa band (*) was determined to be from EGFR phosphorylation at Tyr^{1068} (Figure S4F), consistent with previous report that PTP inhibitors up-regulated EGFR phosphorylation.[10] (Bottom gel): GAPDH loading control. (D) Fluorescence emission spectra (λ_{ex} = 324 nm) of **DNS-pE2** (1 μM) with increasing concentrations of purified PHGDH (0 to 3 μM) in HEPES buffer. (Inset): fitted graph showing a linear relationship between 0 to 1 μM protein concentration. (E) Kinetics of the emergence of fluorescence upon **DNS-pE2** (1 μM) labelling of PHGDH at different concentrations (1.5, 3.8, 7.5, 15, 30 μM) in HEPES buffer. (Inset): fitted graph showing a linear relationship at tested concentrations. (F) Same as (E) except that PHGDH (5 μM) was incubated with different concentrations of **DNS-pE2** (1, 2, 4, 5, 7.5, 10 μM). (G) Probe concentration-dependent (left; 0-1 μM **DNS-pE2**) and competitive (right) *in vitro* labelling of PHGDH (*) overexpressed in bacterial lysates with **DNS-pE2** (1 μM) at RT for 1 h, followed by click chemistry with Rh-N_3, separation and visualization with in-gel fluorescence scanning.

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time-dependent density functional theory (TD-DFT) calculations were conducted (Figure S3D/E), which suggested that **DNS-pE** was likely quenched by intramolecular charge transfer (ICT) from vinyl sulfone. Upon thiol addition, the resulting adduct was able to effectively eliminate such an ICT state and attained a planarized conformation capable of emitting strong fluorescence.[17] Thus, both **DNS-pE** and **DNS-pE2** are reaction-based biosensors.[9]

Since PVS/PVSN are reported irreversible PTP inhibitors,[10] we next evaluated the potential cross-reactivity of our probes toward PTPs. By using PTP1B as a model,[10] we carried out PTP1B inactivation assay and *in vitro* protein labelling (Figure S4A-D). Interestingly, while all probes showed similar PTP1B inactivation profiles, those except SF-based probes could covalently label PTP1B in an activity-dependent manner. The inability of SF-based probes to label PTP1B suggests such probes might not form stable covalent complexes with the protein under SDS-PAGE conditions. Next, by using the 'clickable" **DNS-pE2** to more quantitatively compare its PTP class selectivity with various PTPs,[18] we observed some preferences toward labelling of PTP1B, followed by TC-PTP and SHP1, and insignificant labelling toward SHP2, LMW-PTP and MPtpB. In addition, we observed only a moderate 4.4-fold increase in fluorescence when **DNS-pE** (1 μM) was incubated with purified PTP1B (3 μM) (Figure S4E). In a cell-based assay, unlike relatively strong effects from both PVSN and acrylodan in inhibiting endogenous PTP activities, the effect from **DNS-pE**-treated cells was minimal (Figure 2C).[10] These results indicated that despite noticeable *in vitro* cross-reactivity of **DNS-pE** toward some PTPs, they might not be its major cellular targets. Remarkably, when the same amount of **DNS-pE2** (1 μM) was incubated with different concentrations of purified PHGDH (0 to 3 μM), very strong fluorescence increases with peaked emission at 420 nm were observed (Figure 2D), which followed a linear relationship with increased PHGDH concentrations that reached saturation at 1 μM (1:1 protein/probe; inset), resulting in an 50-fold increase in Turn-ON fluorescence. This indicates **DNS-pE2** only labelled a specific cysteine in PHGDH. Such linearity can be used to quantify the labelling kinetics between **DNS-pE2** and PHGDH.[13] By using a fixed concentration of **DNS-pE2** (1 μM) and an excessive amount of PHGDH, time-course experiments were performed to measure the emergence of fluorescence upon mixing of the probe and PHGDH (Figure 2E); the obtained curves followed single-turnover pseudo-first-order reaction kinetics, which provided a maximum rate constant for the chemical labelling (k_{chem}) of 0.21 min^{-1} and a concentration of PHGDH that achieved a half-maximal rate of labelling (K_i) of 7.4 μM (inset). This value is comparable to the *in vitro* IC_{50} of NCT-503 (2.5 μM) and CBR-5884 (33 μM).[3] On the contrary, when a fixed concentration of PHGDH (5 μM) with different concentrations of **DNS-pE2** (1-10 μM) was used, no apparent change in k_{obs} was observed (Figure 2F). We further performed time-course experiments to monitor the reaction between **DNS-pE2** (3 or 10 μM) and 5 mM of glutathione (a highly abundant endogenous thiol), which revealed a pseudo-first-order rate constant (k) of 0.14 $M^{-1}s^{-1}$ (Figure S4G). In comparison, acrylodan showed a much faster k_{obs} of 1.94 $M^{-1}s^{-1}$. We reasoned that the conversion from enone (acrylodan) to vinyl sulfone (**DNS-pE**/**DNS-pE2**) was able to attenuate the thiol reactivity in **DNS-pE**/**DNS-pE2**, and with their preferential PHGDH binding, led to selective labelling of cellular PHGDH over other endogenous thiol sources (e.g. glutathione, PTPs and others). Indeed as shown in Figure 2G, **DNS-pE2** was able to selectively label PHGDH overexpressed in bacterial lysates in both dose- (left gel) and activity-dependent manner (right gel). Competitive labelling experiments showed effective but incomplete inhibition of **DNS-pE2** in the labelling of PHGDH with CBR-5884 (an allosteric covalent PHGDH inhibitor [3b]), while such effect was comparatively poorer for NCT-503 (a reversible inhibitor [3a]). Similar labelling experiments were done with the active-site mutants of PHGDH (Figure S4H); significant attenuation in labelling of C234S mutant by **DNS-pE2** indicates that Cys^{234} might be the probe-labelled cysteine residue in PHGDH. We thus concluded **DNS-pE**/**DNS-pE2** were indeed suitable fluorogenic probes of PHGDH.

To further evaluate whether **DNS-pE**/**DNS-pE2** could selectively label endogenous PHGDH from live mammalian cells, we first carried our competitive proteome labelling with "clickable" IA, previously reported to label ligandable cysteines from a variety of endogenous proteins including PHGDH (Figure 3A);[6] live MCF-7 cells treated with the IA probe (1 μM, 1 h) showed a large number of labelled bands (lane 1) including a 56-kDa band whose fluorescent intensity was progressively weakened with increasing concentrations of competing **DNS-pE** (0-50 μM). This band subsequently matched a major fluorescent band from MCF-7 cells labelled directly with **DNS-pE2** (Figure 3B; labelled with *). Further competitive labelling by pre-treatment of the cells with a competitor followed by pull-down (PD)/Western blotting (WB) with *anti*-PHGDH unequivocally confirmed that this band was indeed covalently labelled, endogenous PHGDH. Next, the direct probe-PHGDH engagement in MCF-7 cells was studied by using cellular thermal shift assay (CETSA; Figure 3C);[19] both NCT-503 and CBR-5884 induced an increase in the thermal stability of cellular PHGDH, resulting in a positive shift in the melting temperature of the CETSA curve (ΔT_m = 1.8 and 1.5 °C, respectively). On the contrary, **DNS-pE2** induced a negative thermal shift (ΔT_m = -3.8 °C) which might have resulted from the destabilization of cellular PHGDH.

Having confirmed that **DNS-pE**/**DNS-pE2** were able to covalently and selectively label endogenous PHGDH in an activity-based manner, as exemplified by the nearly exclusive, single-band labelling profile in lane 1 of Figure 3B, and that both probes had excellent fluorescence Turn-ON properties, we next investigated their ability in live-cell imaging of endogenous PHGDH. As shown in Figure 3D, strong dansyl fluorescence signals were detected in **DNS-pE2**-treated live MCF-7 cells, most of which was retained after cell fixation and permealization (compare panels 1 & 2), indicating successful Turned-ON fluorescence via covalent target labelling. Highly colocalized rhodamine fluorescence was obtained on cells further "clicked" with Rh-N_3 (Figure S5). Competitive imaging was also performed by pre-treatment of cells with a PHGDH inhibitor (10x; 1 h) prior to probe addition (panels 3 & 4); significantly reduced fluorescence signals were observed in the competitor-treated cells, especially in the nuclear region, with more pronounced effect from CBR-5884. Immunofluorescence (IF) by using *anti*-PHGDH was used to further confirm the Turned-ON fluorescence from **DNS-pE2**-treated cells was indeed from the PHGDH-bound probe (compare panels 1 & 2 in Figure 3E). Finally, in order to unequivocally confirm the highly selective PHGDH labelling

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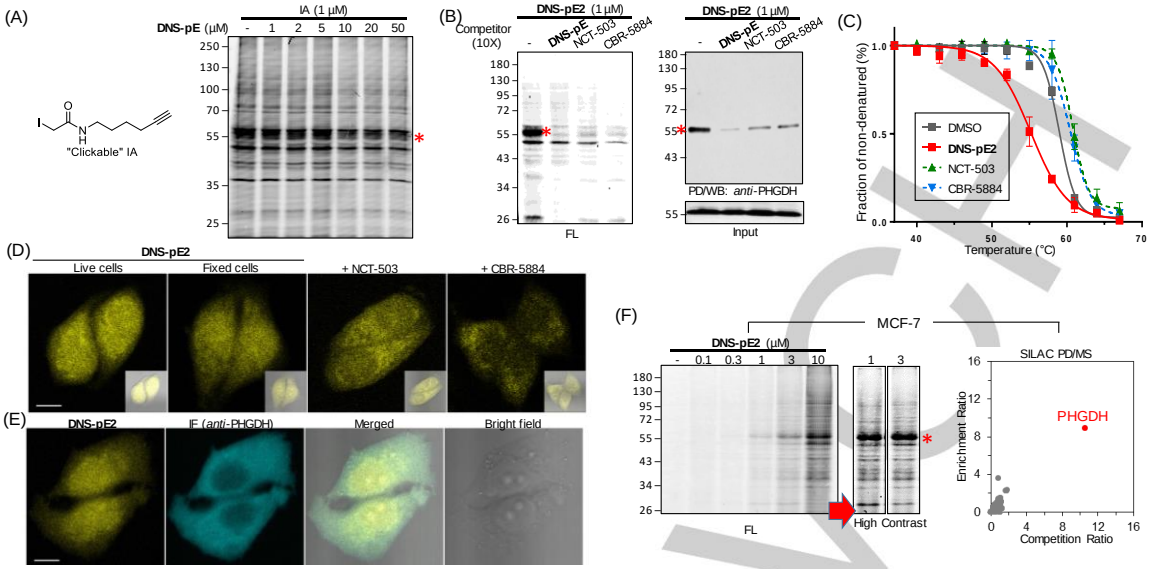

Figure 3. (A) *In situ* proteome profile of IA-labelled MCF-7 cells pre-treated with different concentrations of **DNS-pE** (0 to 50 μM, 3 h). Upon IA probe labelling (1 μM), cells were clicked reaction with Rh-N_3 followed by in-gel fluorescence scanning. The 56-kDa fluorescent band was marked with *. (B) In-gel fluorescence scanning profile (FL; left) of live MCF-7 cells labelled with **DNS-pE2** (1 μM for 3 h, ± 10× competitor), and the corresponding gel upon PD/WB analysis (right). The 56-kDa labelled endogenous PHGDH (*) was unequivocally enriched/identified. (C) CETSA curves of endogenous PHGDH from MCF-7 cells upon treatment with an indicated compound (10 μM; 3 h). (D) Confocal fluorescence images of MCF-7 cells treated with **DNS-pE2** (3 μM for 2 h, ± 10X competitor). (Panels 1/3/4): live cells. (Panel 2): fixed cells. (Inset): merged images with bright-field. Scale bar = 10 μm. (E) Same as (D) except that immunofluorescence (IF) was performed with the fixed cells. (F) *In situ* proteome profiles of MCF-7 cells labelled with **DNS-pE2** (0-10 μM, 3 h). (Left): in-gel fluorescence scanning (FL; higher-contrasted lanes 1 & 3 were shown as indicated); (Right): SILAC ratio plots of **DNS-pE2**-labelled proteins identified from PD/MS studies of (1 μM, ± 10× **DNS-pE**). PHGDH band (*) was highlighted, and shown in red in the SILAC plots.

profile by **DNS-pE2** at a proteome-wide scale, *in situ* proteome labelling followed by large-scale PD/quantitative mass spectrometry (stable isotope labelling with amino acids in cell culture, or SILAC[6,20]) was done on MCF-7, COS-7 and HepG2 cells (Figures 3F & S5); selective PHGDH labelling by the probe was observed in all three cell lines, at 1 to 3 μM probe concentration. As shown in Figure 1F (SILAC ratio plot), of the > 1000 proteins identified, PHGDH was the only hit that exhibited a significant increase in SILAC ratios (H/L ratio > 5). Competitive SILAC experiments, carried out with either **DNS-pE** or PVSN, further showed that **DNS-pE2** labelled endogenous PHGDH with good selectivity from different live mammalian cells.

Our rational design of reaction-based probes has led to the identification of **DNS-pE2**, the first fluorescence Turn-ON probe capable of highly selective, covalent labelling of endogenous PHGDH from different mammalian cells. The PHGDH labelling was presumably through one of the key active-site cysteines in PHGDH. With its dual-purpose feature, our probe thus offers a promising approach to simultaneously image endogenous PHGDH activities and study its active-site engagement in live-cell settings.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: fluorescent probe • chemical proteomics • live-cell imaging • inhibitor • PHGDH

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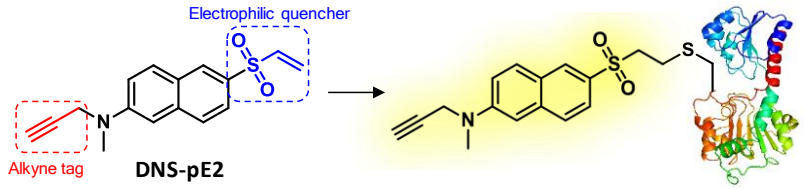

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S. Pan, S.-Y. Jang, S. S. Liew, J. Fu, D. Wang, J.-S. Lee,* S. Q. Yao*

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A Vinyl Sulfone-Based Fluorogenic Probe Capable of Selective Labelling of PHGDH in Live Mammalian Cells

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