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Design, synthesis and evaluation of protein disulfide isomerase inhibitors with nitric oxide releasing activity

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ABSTRACT

Protein disulfide isomerase (PDI), a chaperone protein mostly in endoplasmic reticulum, catalyzes disulfide bond breakage, formation, and rearrangement to promote protein folding. PDI is regarded as a new target for treatment of several disorders. Here, based on the combination principle, we report a new PDI reversible modulator **16F16A-NO** by replacing the reactive group in a known PDI inhibitor **16F16** with nitric oxide (NO) donor. Using molecular docking experiment, **16F16A-NO** could embed into the active cavity of PDI. From newly developed fluorescent assay, **16F16A-NO** showed rapid NO release. Furthermore, it is capable to moderately inhibit activity of PDI and S-nitrosylate the protein, indicating by insulin aggregation assay and biotin-switch technique. Finally, it displayed a dose-dependent antiproliferative activity against SH-SY5Y and HeLa tumor cells. Our designed hybrid compound **16F16A-NO** showed a reasonable activity and might offer a promising avenue to develop novel PDI inhibitors for disease treatments.

Protein disulfide isomerase (PDI, also known as PDIA1) is a 57-kDa thiol oxidoreductase which catalyzes the thiol-based reactions with their interested substrate proteins, including formation (oxidation), breakage (reduction) and rearrangement (isomerization) through a disulfide bond interchange.[1–3] It mainly localizes at endoplasmic reticulum (ER) through a C-terminal KDEL signal sequence and acts as a chaperone for proper protein folding (Fig. 1A) to inhibit protein aggregation and correct protein misfolding. The catalytic domains (**a** and **a'**), containing redox catalytic Cys-Gly-His-Cys (CGHC, $Cys^{53/56}$ and $Cys^{397/400}$, respectively) motifs, as shown in Fig. 1B, are responsible for the oxidoreductase activity. Whereas, the other two noncatalytic domains (**b** and **b'**), with a large hydrophobic pocket are essential for the recognition and binding of incompletely folded protein substrates.[4–6]

Recently, as PDI is a crucial enzyme participating in protein folding, the investigations of its functions have been revealed that it is linked with a large number of disease states, including cancer, neurodegenerative diseases and cardiovascular diseases.[7–9] The growth of cancer requires continued protein synthesis, giving rise to ER stress by activating unfolded protein response (UPR).[10,11] Consequently, PDI has been upregulated and its evaluated expression have been observed in a wide range of human cancers.[12–14] Besides, resistance of tumor cells to chemotherapeutic agents was also reported to be associated with the elevated PDI expression levels. Furthermore, dysfunction of PDI is also involved with neurological disorders. Upregulated PDI and mutations in the gene encoding PDI was observed in amyotrophic lateral sclerosis (ALS).[15] A wide range of synthetic and natural product-based PDI inhibitors have been explored in recent years, including **16F16**,[9] **KSC-34**,[16] **RB-11-ca**,[17] **PACMA-31**,[18] **P1**,[19] ***S*-CW3554**[20] (structures shown in Fig. S1) and so on. All these compounds contain a cysteine-reactive electrophile group and exhibit elegant irreversible inhibition against PDI. However, it was also reported that irreversible genetic silencing of PDI is cytotoxic to cells and animal models because of its essential chaperone role.[21] Moreover, knockout of PDIA3, with similar features as PDI, implicated the embryonic death in mice.[22] Therefore, irreversible inhibitors of PDI may exhibit cytotoxicity *in vivo* and reversible, non-covalent inhibitors might bring new opportunities for improved therapeutic purposes. In 2015, Stockwell group identified a reversible inhibitor **LOC14** (structure shown in Fig. S1) from screening of

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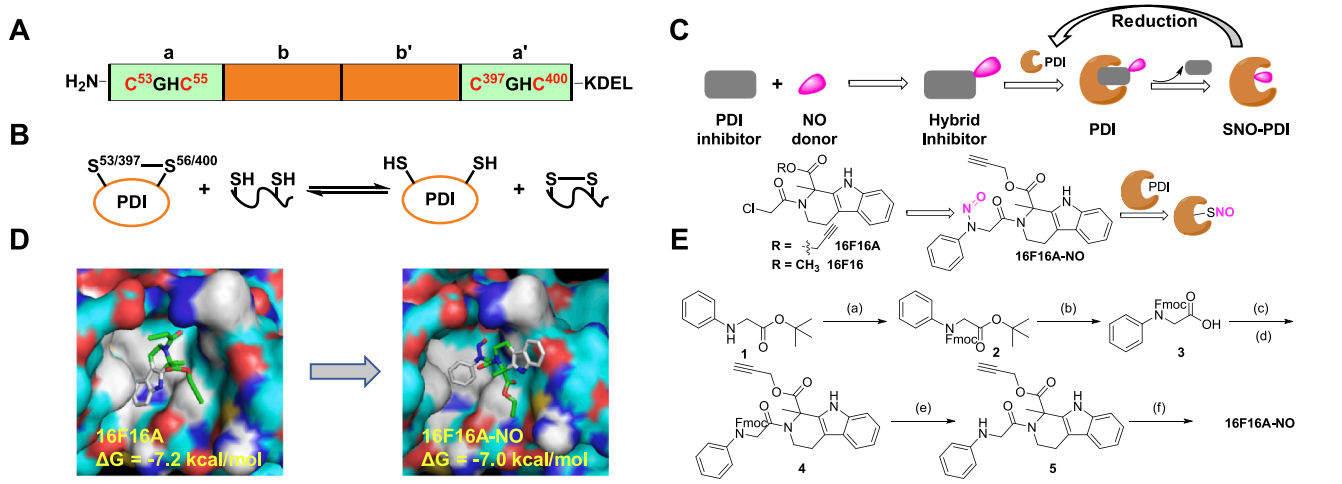

Fig. 1. (A) Domain overview of protein disulfide isomerase, with **a** and **a'** catalytic domain, **b** and **b'** domains and C-terminal ER signal sequence. (B) PDI-catalytic reaction. (C) Our strategy and the hybrid compound. (D) Docking analysis of **16F16A-NO** and **16F16A** against PDI. (E). Synthetic scheme of **16F16A-NO**. (a) Fmoc-Cl, 10% Na_2CO_3/H_2O, 1,4-dioxane, 0 °C to r. t.; (b) 20% TFA/DCM, 0 °C to r. t.; (c) $(COCl)_2$, DMF, DCM, 0 °C to r. t.; prop-2-yn-1-yl 1-methyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-*b*]indole-1-carboxylate, DMF, 0 °C to r. t.; (d) 20% piperidine/$CHCl_3$, 0 °C to r. t.; (f) $NaNO_2$, 20% AcOH/H_2O, 0 °C to r. t.

~10,000 compounds.[23] **LOC14** has promising inhibition (K_d = 62 nM) and protects neuron-like PC12 cells expressing toxic huntingtin proteins.

One of promising strategies to achieve reversible inhibitors is based on the use of nitric oxide (NO).[24] NO is prone to make amines, aromatic rings, alcohols and reductive thiols on protein nitrosylation modification, thus changing protein conformation and affecting its activity and function. Among them, NO-mediated S-nitrosylation of cysteine residues is one of typical post translational modifications (PTM), which is a reversible modification.[25] It is estimated that ~70% of the universal proteome may be subjected to post translational regulation by S-nitrosylation, including transcription factors, kinases, channel proteins.[26] In 2006, Lipton et al reported that PDI was S-nitrosylated *in vitro* and *in vivo* to form an S-nitrosylated protein, causing decrement of its activity.[8]

Herein, we designed a new reversible modulator (**16F16A-NO**, shown in Figs. 1C and S1C), which is a hybrid compound between a potent PDI inhibitor **16F16** and a nitric oxide donor. **16F16** (IC_{50} = 1.5 μM), an irreversible inhibitor with a chloroacetyl electrophile (Fig. S1B), was discovered by Stockwell group through a small-molecule screening approach.[9] The electrophile on **16F16** was replaced with phenyl nitrous amide and erased its covalent linkage properties.[27] The attachment of alkyne group would not affect the proper but provides a tool to understand the binding type of the hybrid. **16F16A-NO** could recognize and embed into the active cavity of PDI. We also found NO release from **16F16A-NO** was rapid *in vitro* and the protein could be S-nitrosylated by **16F16A-NO** resulted from a biotin-switch assay. We further found the inhibition of PDI activity and the proliferation of SH-SY5Y cells and HeLa cells by **16F16A-NO**. These findings would represent a new class of reversible inhibitors of PDI that can probe its potential as a drug for treatment of human cancers.

Molecular docking studies

First, it is important to evaluate the influence of NO donor modification on the binding toward PDI protein. The new inhibitor was converted from **16F16**, which is the first PDI inhibitor generating from high-throughput screening[9] and investigated to localize in the "**a**" catalytic domain of PDI.[23] Hence, using together with the alkyne version **16F16A** as control, **16F16A-NO** was examined for their ability to enter the active activity of PDI (PDB code 4EKZ[28]) using Autodock software. The docking protocol ranks the output values indicating a stronger ligand binding affinity. The output was analyzed for the predicted binding interactions. The best scoring pose and results are shown in Fig. 1D. It showed that **16F16A-NO** had similar affinity value compared with **16F16A**. The docking pose confirmed **16F16A** was in the "**a**" catalytic domain and the aromatic ring of it was π-π interaction with residues W52. Furthermore, **16F16A-NO**, also bind in the C-terminal active site pocket, forming similar interactions. It is important to note that the NO donor groups was embedded deeper into pocket with the additional interactions with other amino acids and closer to the cysteine in the active site. Therefore, the result indicated that **16F16A-NO** could be recognized by PDI.

Synthesis of 16F16A-NO

The synthetic scheme was shown in Fig. 1E with introduction of alkyne at the carboxylic acid site and replacement of chloroacetamide with phenyl nitrous amide. A terminal propargyl alkyne modification, biorthogonal and not causing to lose its biological activity, was designed to validate whether there is any covalent bound to proteins. First, Fmoc protected with phenylaminoacetic acid was prepared through alkylation and protecting group exchanges. Next it was attached with three-ring key core structure with propargyl ester under basic condition. After deprotection with Fmoc group, sodium nitrite in acidic condition was used to oxidate secondary amine to obtain the final product **16F16A-NO**. The product was kept in the dark and always fresh prepared in DMSO. Detailed synthetic procedures and NMR spectra were enclosed in electronic supporting information.

Nitric oxide measurement via a fluorescent assay

Next, we analyzed whether **16F16A-NO** could release NO in water solution working as a NO donor. A fluorescence probe **BT-NH** developed by our group was used to detect NO production.[29–31] Fluorescent signal is turned on when **BT-NH** (λ_{ex} = 520 nm) reacts with NO to convert a fluorescent product (λ_{em} = 620 nm). (Basic mechanism shown in Fig. 2). S-nitrosylglutathione (GSNO), a commercial stable NO donor, known to be decomposed under light, metal ion catalysis and aqueous solution, and subsequently released NO, has been the subject as a control to compare. Hence, quantification of NO release was carried out with 50 μM **BT-NH** in PBS at 37 °C. Different concentrations of **16F16A-NO** and GSNO respectively, upon mixing with the reaction, led to a fluorescent increase. From Fig. 2B, with 600 μM **16F16A-NO**, there

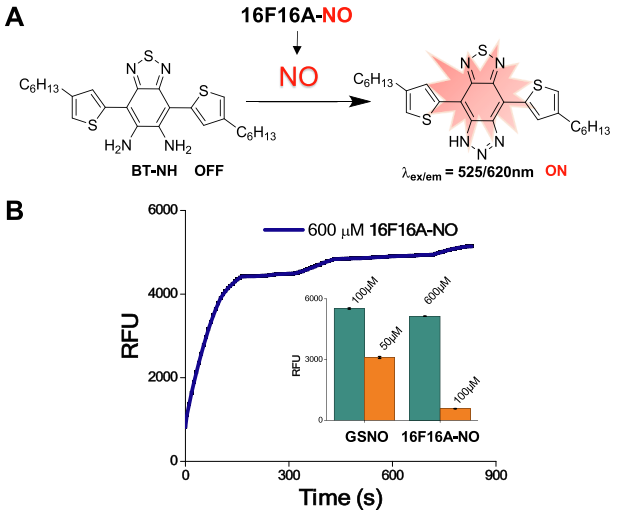

Fig. 2. (A) Schematic representation of the fluorogenic probe **BT-NH** used in detection of NO release. (B) Kinetic profile of NO level was evaluated after adding **16F16A-NO** (600 μM) in PBS buffer. Inset is end-point NO level for incubation of different concentrations of GSNO and **16F16A-NO** with **BT-NH**. Experiments were performed in triplicate with data plotted as mean ± SEM.

was a rapid rise of NO to concentrations in 10 min, which has a stabilized NO release rate after 30 min of reaction. In comparison, GSNO showed similar NO release quality at 100 μM, demonstrating superior NO release capability than **16F16A-NO**. Only one fifth of maximal relative theoretical NO release was obtained. It might be too fast to capture NO by *o*-diamine in the fluorophore. Nevertheless, NO was clearly released from the hybrid to generate detectable NO, encouraging us to test the compound in protein and cell-based assays.

S-nitrosylation of PDI

Next, we sought to determine whether NO release from **16F16A-NO** could lead S-nitrosylation on PDI protein (SNO-PDI). A well-known assay, biotin-switch technique (BST), was proceeded.[26] S-nitrosylation was identified by anti-Biotin after substitution of SNO group with a biotin group by chemical reduction with sodium ascorbate. Based on results of nitric oxide release, in this assay, 600 μM **16F16A-NO** and 50 μM GSNO were chosen to treat with PDI protein, respectively. From Fig. 3, it is obviously found that exposure to both compounds successfully led to generation of SNO-PDI, although **16F16A-NO** appeared weaker. Moreover, under dithiothreitol (DTT) reducing condition, S-nitrosylation is abolished, indicating the reversible ability of this modification under such reduction conditions. Besides, from results of protein profiling using click chemistry,[32–35] there is no significant labeling to any protein (data not shown), indicating there is no covalent binding occurred.

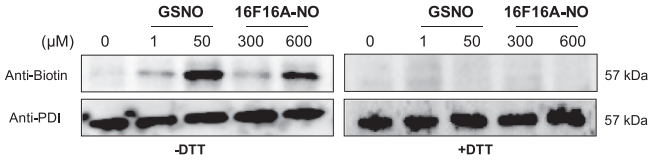

Fig. 3. *In vitro* S-nitrosylation (SNO) of recombinant PDI. Purified protein SNO-PDI were detected by reacting 400 ng protein with in absence or presence of concentrations of GSNO (1, 50 μM) or **16F16A-NO** (300, 600 μM) for 30 min. Purified protein was subjected to BST assay and were analyzed by western blot with anti-biotin and anti-PDI.

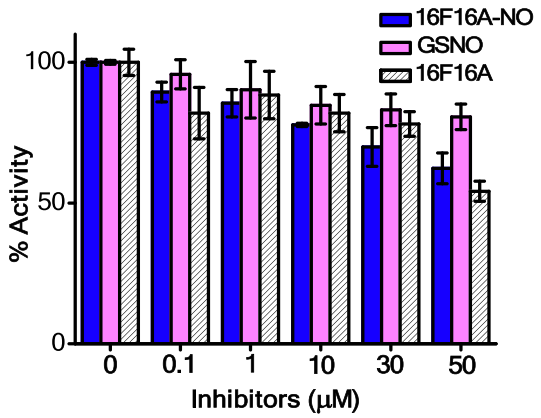

Fig. 4. Effect of PDI inhibition against different concentrations of **16F16A-NO**, GSNO and **16F16A** using insulin aggregation assay. 50 ng PDI was treated with inhibitors (0.1, 1, 10, 30 and 50 μM) in buffer (100 mM Na_3PO_4, 0.2 mM EDTA, pH = 7.0) at 37 °C for 30 min followed by adding with 0.16 mM insulin, reacting at room temperature for 1 h. The absorbance was measured at 650 nm. All experiments were repeated three times.

PDI inhibition assay

To determine whether this NO releasing hybrid affects PDI function, we further validated **16F16A-NO** as a PDI inhibitor using insulin aggregation assay at 50, 30, 10, 1, 0.1 and 0 μM.[19] As shown in Fig. 4, it is observed that the hybrid inhibited PDI reductase activity in a dose-dependent manner. At 50 μM, it showed approximately 50% inhibitory. The original **16F16A** inhibited PDI activity more potently compared to the derivative. Furthermore, the NO donor GSNO, as the control, did not strongly affect PDI activity at 50 μM. It is indicated that although the hybrid inhibitor **16F16A-NO** is also without covalent affinity, it is more effective and potent in combination of an inhibitor-like structure and NO donor, in comparison with **16F16-DC** which is a **16F16** analog lacking with chloro-substituent (structure shown in Fig. S1).

Antiproliferation activity of 16F16A-NO on cancer cells

Cancer cells require increased protein synthesis and thus respond to oxidative stress which is mediated by PDI. PDI is responsible for the isomerization, reduction, and oxidation of nonnative disulfide bonds in unfolded proteins entering the ER, so PDI is usually up-regulated in cancer cells. Previous study found that S-nitrosylation of PDI could abrogate its activity and function. Having successfully confirmed that **16F16A-NO** as NO donor and an *in vitro* PDI inhibitor, we further investigated its cellular activity by evaluating its growth inhibition effect against two cancer cell lines (human neuroblastoma cell line SH-SY5Y and human cervical cancer cell line HeLa) in the presence of DMSO control or at different dosages of **16F16**, **16F16A-NO** and GSNO. The results were shown in Figs. 5 and S2. All three of them showed noticeable notable cell toxicity under high concentrations. At 600 μM, **16F16A-NO** and GSNO showed 40% inhibition against both cancer cell lines, although less potent than **16F16** (5 μM). SH-SY5Y cells were more sensitive than HeLa cells to both NO releasing compounds **16F16A-NO** and GSNO treatment. While, in HeLa cells, **16F16A-NO** cause more significant potency at a lower dose than GSNO, indicating that core structures of **16F16A** moiety also enhanced the inhibition. Furthermore, we have tested them on a normal cell line (human embryo kidney cell HEK293T). The inhibition of 16F16A-NO and GSNO to this cell line is slightly weak. These results suggest that this hybrid analogs may be effective and selective to cancer cells.

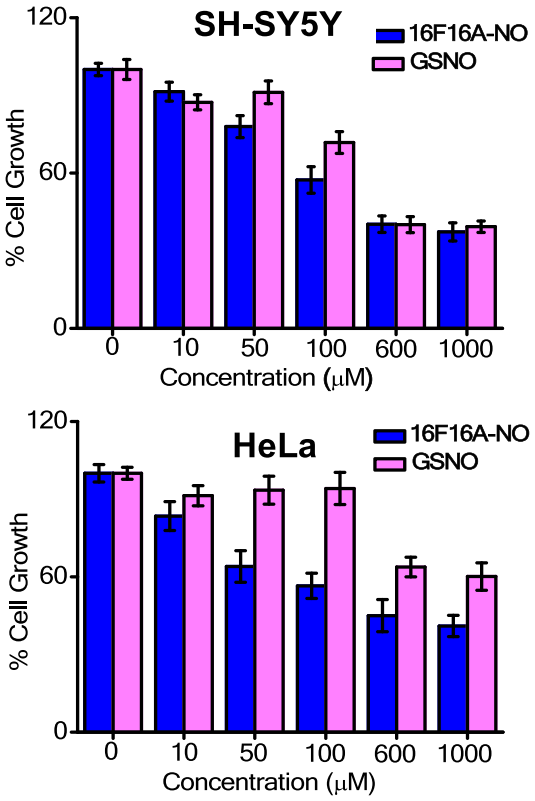

Fig. 5. Cell viability of SH-SY5Y and HeLa cells with different concentrations of GSNO and **16F16A-NO** assessed by CCK-8 assay.

Conclusions

We have developed a reversible modulator, **16F16A-NO**, which could enter into the active cavity of PDI. We further showed that **16F16A-NO** could release NO rapidly *in vitro*, inhibit PDI enzymatic activity effectively and cause S-nitrosylation of PDI. Moreover, we also found that **16F16A-NO** exhibited antiproliferation activity to cancer cell lines. However, further studied are needed to substantiate our speculation. This hybridization may open new complementary or alternative approaches to discover and develop more effective and safer PDI reversible inhibitors for therapeutic applications to understand and treat cancer diseases.[36]

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.126898.

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