Free Radical Biology and Medicine 108 (2017) 741–749

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

# Challenges in the evaluation of thiol-reactive inhibitors of human protein disulfide Isomerase

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ARTICLE INFO

*Keywords:*
Assay
Binding
BODIPY FL-L Cystine
Disulfide
Dithiothreitol
Glutathione
Inhibitor
Protein disulfide Isomerase
Reductase
Ribonuclease A
Thiols

ABSTRACT

This paper addresses how to evaluate the efficacy of the growing inventory of thiol-reactive inhibitors of mammalian protein disulfide Isomerase (PDI) enzymes under realistic concentrations of potentially competing thiol-containing peptides and proteins. For this purpose, we introduce a variant of the widely-used reductase assay by using a commercially-available cysteine derivative (BODIPY FL L-Cystine; BD-SS) that yields a 55-fold increase in fluorescence (excitation/emission; 490/513 nm) on scission of the disulfide bond. This plate reader-compatible method detects human PDI down to 5–10 nM, can utilize a range of thiol substrates (including 5 μM dithiothreitol, 10 μM reduced RNase thiols, and 5 mM glutathione; GSH), and can operate from pH 6–9.5 in a variety of buffers. PDI assays often employ low micromolar levels of substrates leading to ambiguities when thiol-directed inhibitors are evaluated. The present work utilizes 5 mM GSH for both pre-incubation and assay phases to more realistically reflect the high concentration of thiols that an inhibitor would encounter intracellularly. Extracellular PDI faces a much lower concentration of potentially competing thiols; to assess reductase activity under these conditions, the pre-reduced PDI is treated with inhibitor and then fluorescence increase upon reduction of BD-SS is followed in the absence of additional competing thiols. Both assay modes were tested with four mechanistically diverse PDI inhibitors. Two reversible reagents, 3,4-methylenedioxy-β-nitrostyrene (MNS) and the arsenical APAO, were found to be strong inhibitors of PDI in the absence of competing thiols, but were ineffective in the presence of 5 mM GSH. A further examination of the nitrostyrene showed that MNS not only forms facile Michael adducts with GSH, but also with the thiols of unfolded proteins ($K_d$ values of 7 and < 0.1 μM, respectively) suggesting the existence of multiple potential intracellular targets for this membrane-permeant reagent. The inhibition of PDI by the irreversible alkylating agent, the chloroacetamide 16F16, was found to be only modestly attenuated by 5 mM GSH. Finally, the thiol-independent flavonoid inhibitor quercetin-3-O-rutinoside was found to show equal efficacy in reoxidation and turnover assay types. This work provides a framework to evaluate inhibitors that may target the CxxC motifs of PDI and addresses some of the complexities in the interpretation of the behavior of thiol-directed reagents in vivo.

## 1. Introduction

In 1963, Anfinsen and Straub independently described an oxidative catalyst present in vertebrate liver and pancreas homogenates, that was later identified as protein disulfide Isomerase [1,2]. About 20 protein disulfide Isomerases have been described in humans [3,4]; the best understood, PDI, is found at nearly millimolar concentrations in the lumen of the ER and at much lower levels on the mitochondrial outer membrane, within the nuclear matrix and cytosol, and at the cell surface [5–8]. PDI contains four thioredoxin-like domains (**a**, **b**, **b′** and **a′**) with the outer **a** and **a′** modules containing CxxC motifs that catalyze both disulfide oxidoreductase and redox-neutral disulfide shuffling modes [9–12]. Additionally, PDI has been shown to function as a chaperone during the folding of reduced lysozyme, glyceraldehyde-3-phosphate dehydrogenase, green fluorescent protein, and procollagen [13–16]. Extracellular PDI has been implicated in a range of key cellular events including integrin activation, viral fusion, adhesion, invasion and thrombus formation [17–20]. Although the Isomerase is now a target for therapeutic intervention [21,22], the similarities in the chemical reactivities of many of the PDI family members and their multiple cellular locales presents significant challenges in the design of specific reagents that can target a single Isomerase at a unique cellular

*Abbreviations:* APAO, 4-amino-phenylarsine oxide; BD-SH, reduced BODIPY FL-L Cystine; BD-SS, BODIPY FL-L Cystine; DTNB, 5,5-dithio-bis-(2-nitrobenzoic acid); GSH, glutathione; MNS, 3,4-methylenedioxy-β-nitrostyrene; NEM, N-ethylmaleimide; PDI, protein disulfide Isomerase; $PDI_{ox}$, oxidized protein disulfide Isomerase; $PDI_{red}$, reduced protein disulfide Isomerase; RNase, ribonuclease A; -SH and -SS-, generic thiol and disulfide; TCEP, tris(2-carboxyethyl)phosphine; THP, tris(hydroxymethyl)phosphine

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http://dx.doi.org/10.1016/j.freeradbiomed.2017.04.367
Received 26 January 2017; Received in revised form 12 April 2017; Accepted 28 April 2017
Available online 30 April 2017

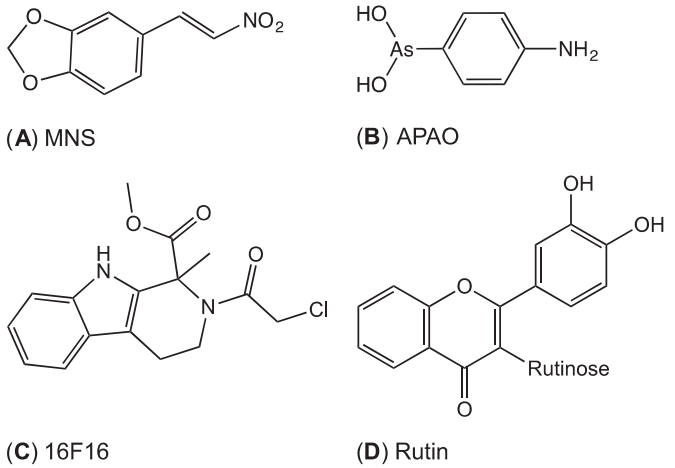


**Fig. 1.** A sampling of inhibitors of PDI. (A) 3,4-methylenedioxy-β-nitrostyrene (MNS). (B) 4-amino-phenylarsine oxide (APAO). (C) 16F16. (D) Quercetin-3-O-rutinoside (rutin).

location.

A large number of small molecule inhibitors of PDI have now been described, including 3,4-methylenedioxy-β-nitrostyrene (MNS) (Fig. 1) [19], 4-amino-phenylarsine oxide (APAO) [23], a chloroacetamide analog, 16F16 [24], and a flavonoid, quercetin-3-O-rutinoside (rutin) [25]. Compounds A-C in Fig. 1 are expected to be overtly reactive towards thiolate nucleophiles. While the enone functionality of rutin (Fig. 1D) is a potential weak Michael acceptor, its mode of inhibition appears to be unrelated to the CxxC motifs of PDI [26]. The observation that a sizable proportion of PDI inhibitors are thiol-reactive raises important issues concerning their mode of action and specificity in vivo, and how PDI inhibition should be best assessed in vitro.

In terms of intracellular targeting of PDI, the high concentration of sulfhydryl groups in mammalian cells, notably the multimillimolar levels of GSH and exposed protein thiols [27,28], could markedly attenuate the effectiveness of membrane-permeant thiol-reactive small molecules. When these reactions involve reversible thiol adducts, the effectiveness of an inhibitor will reflect $K_d$ values combined with the prevailing concentrations of PDI and competing thiols in the cell (Fig. 2A). For irreversible chemistry (Fig. 2B) the determinant for selectivity of labeling would be a strong kinetic preference for reaction with PDI over the competing intracellular thiols. In marked contrast to the intracellular case, cell surface PDI [29] is likely to encounter a much lower concentration of competing thiols (e.g. ~10 μM glutathione and cysteine [28,30,31]). Here, the susceptibility of PDI to inhibition by thiol directed reagents is expected to be strongly influenced by the presence of additional exofacial reductive pathways and thiol-containing proteins [32,33].

Extrapolating the in vivo effectiveness of thiol-reactive inhibitors of PDI using in vitro assays presents significant challenges. Most current steady-state assays of PDI involve thiol-containing substrates or products, with or without the inclusion of redox buffers generated from

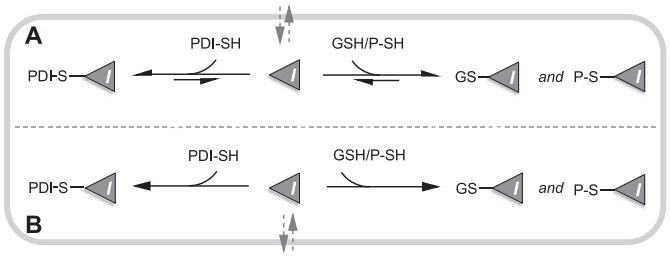


**Fig. 2.** Intracellular competition between glutathione (GSH), reduced PDI (PDI-SH) and other protein thiols (P-SH) for a membrane-permeant inhibitor, *I*. Panels A and B represent reversible and irreversible thiol-directed inhibition, respectively. PDI is likely to be largely reduced intracellularly.

-SH/-SS- mixtures. We suggest that an appraisal of the likely in vivo effectiveness of thiol-reactive inhibitors should include in vitro assessment of activity under realistic concentrations of potentially competing thiols. For example, PDI reduction assays are often conducted at low thiol concentrations (e.g. 5 μM DTT), making them potentially susceptible to artifacts arising from substrate depletion following the addition of thiol-reactive inhibitors at micromolar concentrations.

In view of the increasing inventory of thiol-reactive inhibitors of PDI emerging from high-throughput screening and rational design strategies, we wanted to develop general approaches to assess whether the Isomerase is a prospective target of such inhibitors under conditions that may prevail intracellularly. At the outset we chose the compound 3,4-methylenedioxy-β-nitrostyrene (MNS; Fig. 1A), as it was recently suggested to suppress the growth of human triple negative breast cancer cells (MDA-MD-231) by inhibiting PDI [19]. MNS is one of many nitrostyrene derivatives that have been considered for therapeutic use [34–37], and has also been reported to suppress platelet aggregation and activation of the NLRP3 inflammasome activation [38,39], to inhibit protein tyrosine kinase [39] and monoamine oxidase B [40], to attenuate tumor growth [41], and to slow mobility and colony formation in osteosarcoma cells [42].

In exploring the interaction between human PDI and MNS, we initially employed a very sensitive reductase assay developed by Raturi and Mutus that utilizes a self-quenched dieosin derivative they synthesized by conjugating oxidized glutathione with eosin-isothiocyanate (Fig. 3A) [43]. Subsequently, we found that BODIPY FL-L-cystine (BD-SS) represents a very convenient commercially-available substitute for the dieosin derivative. Herein, we first validate the use of BD-SS in both turnover and reoxidation assays for the general assessment of PDI enzymatic activity. We then use these assays, and additional spectroscopic experiments, to explore the complexities and potential pitfalls in the evaluation of PDI inhibitors that are themselves thiol-reactive.

## 2. Materials and methods

### 2.1. Materials

BODIPY FL L-cystine was from Thermo Fisher Scientific or Setareh Biotech. *4*-amino-phenylarsine oxide was synthesized as described previously [44]. Glutathione, 3,4-methylenedioxy-β-nitrostyrene, N-ethylmaleimide, 16F16, aldolase (rabbit muscle) and pyruvate kinase (rabbit muscle) were obtained from Sigma Aldrich. Quercetin-3-O-rutinoside was from Acros Organics (Fisher Scientific). Tris(hydroxypropyl)phosphine was from Calbiochem (EMD Millipore). Dithiothreitol and tris-(carboxyethyl) phosphine hydrochloride were purchased from Gold Biotechnology. Bovine pancreatic ribonuclease A was from Fisher Scientific.

### 2.2. General methods

Agilent 8452A or 8453 instruments were used to record UV–Vis spectra. Fluorescence spectra were acquired using an Aminco Bowman 2 luminescence spectrometer. Stock solutions of BD-SS were prepared in phosphate buffer and stored in small aliquots at −20 °C. Reductant solutions were made fresh daily from concentrated solutions stored at −20 °C. Thiol and phosphine concentrations were confirmed using 5,5′-dithiobis(2-nitrobenzoic acid). Unless otherwise stated, the buffer used in this work was 50 mM potassium phosphate containing 1 mM EDTA, pH 7.5. BD-SH fluorescence was negligibly affected in a range of buffers spanning pH 6–9.5 (citrate, Tris, HEPES, PBS). Stock solutions of 16F16 were prepared in DMSO with a final solvent concentration of 1.5% in inhibitor and control wells. Stock solutions of MNS were prepared in ethanol and diluted ~100-fold in water before use. Rutin stocks were prepared directly in water.