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Protein disulfide isomerase: a promising target for cancer therapy

Q2 **Shili Xu[1], Saranya Sankar[2] and Nouri Neamati[1]**

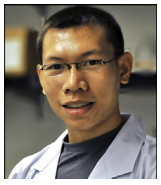
[1] Department of Medicinal Chemistry, College of Pharmacy, University of Michigan, North Campus Research Complex, 2800 Plymouth Road, Building 520, Ann Arbor, MI 48109, USA
[2] Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, 1985 Zonal Avenue, Los Angeles, CA 90089, USA

Protein disulfide isomerase (PDI) has a key role in maintaining cellular homeostasis by mediating oxidative protein folding. It catalyzes disulfide bond formation, breakage and rearrangement in the endoplasmic reticulum and has chaperone protein activity. Increasing evidence suggests that PDI supports the survival and progression of several cancers. During the past decade, robust PDI activity assays have been developed and several PDI inhibitors identified, but none has been approved for clinical use. Herein, we review current knowledge of the role of PDI in cancer and discuss various assays for measuring the activities of PDI, highlighting their sensitivities and usefulness for high-throughput screening. The previously reported PDI inhibitors require further validation to serve as *bona fide* leads and additional optimization to generate novel drug candidates for clinical studies.

PDI is a 57-kDa dithiol-disulfide oxidoreductase and molecular chaperone. It is one of the most abundant soluble proteins in the endoplasmic reticulum (ER), and accounts for up to 0.8% of total cellular protein [1]. It was discovered as the first protein-folding catalyst in 1963 by two independent research groups led by Bruno Straub [2] and Christian B. Anfinsen [3], respectively and, a decade later, it was named PDI [4]. At least 21 other members have been subsequently identified, forming the PDI protein family (Fig. 1a). Different aspects of the biochemistry of the PDI family members have been previously reviewed [5,6].

PDI has a central role as a reductase, an oxidase, an isomerase and a molecular chaperone in the ER (the central organelle for protein folding). It catalyzes disulfide bond oxidation (formation), reduction (breakage) and isomerization (rearrangement) in its protein and peptide substrates, mediating oxidative protein folding. In addition, PDI binds and stabilizes the major histocompatibility complex (MHC) class I peptide-loading complex (PLC) that mediates MHC class I folding and peptide loading [7]. It binds NAD(P)H oxidase subunits and regulates NAD(P)H oxidase activity in vascular smooth muscle cells [8]. PDI is also a subunit of proly-4 hydroxylase (an essential enzyme for the synthesis of collagens) [9] and microsomal triglyceride transfer protein (a central enzyme for the assembly of apolipoprotein B-containing lipoproteins) [10]. To

Corresponding author: Neamati, N. (neamati@umich.edu)



Shili Xu is currently a postdoctoral research fellow in the Department of Medicinal Chemistry at the College of Pharmacy, University of Michigan. He obtained his BSc degrees in Biological Sciences and Economics from Peking University, China. He completed his PhD degree under the supervision of Nouri Neamati at the University of Southern California. The title of his dissertation was 'Discovery of novel small molecules for ovarian cancer therapy'. He has worked on the preclinical development of several novel small molecules for the treatment of ovarian, colorectal, lung and pancreatic cancers. Representative compounds include PACMA31 and SC144 (a first-in-class gp130 inhibitor). He also has a strong interest in developing robust and efficient high-throughput activity assays for drug discovery applications.

Saranya Sankar is currently pursuing her Master's degree in the Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California. Her work is focused on the discovery of novel classes of PDI inhibitors for the treatment of ovarian cancer. She received her BSc degree in Pharmacy from Narsee Monjee Institute of Management Studies, Mumbai, India.

Nouri Neamati is the John G. Searle Professor of Medicinal Chemistry at the College of Pharmacy, University of Michigan and a member of the UM Translational Oncology Program. He obtained his PhD in Biomedical Sciences from the University of Texas Graduate School of Biomedical Sciences and MD from Anderson Cancer Center, Houston, Texas in 1995. From 1995 to 2000, he was a postdoctoral and a research fellow at the National Institutes of Health (NIH). In September 2000, he joined the faculty at the School of Pharmacy, University of Southern California, with a joint appointment at the Norris Comprehensive Cancer Center. Nouri Neamati is the recipient of numerous awards, including the NIH Technology Transfer, STOP CANCER, GlaxoSmithKline Drug Discovery Award, several Awards from the US Department of Defense, LUNGevity Discovery Award from the American Lung Association, and the Littlefield-AACR Award in Metastatic Colon Cancer Research. He has published over 200 peer-reviewed manuscripts, 18 book chapters, and over 30 patents in the area of drug design and discovery. He was the founding Editor-in-Chief of *Current Molecular Pharmacology*, an Associate Editor of *Current Anticancer Drug Targets* and an Editorial Advisory Board member of several journals, including *Expert Opinion on Drug Discovery*, *Expert Opinion on Investigational Drugs*, *Hormones & Cancer* and the *Journal of Medicinal Chemistry*.

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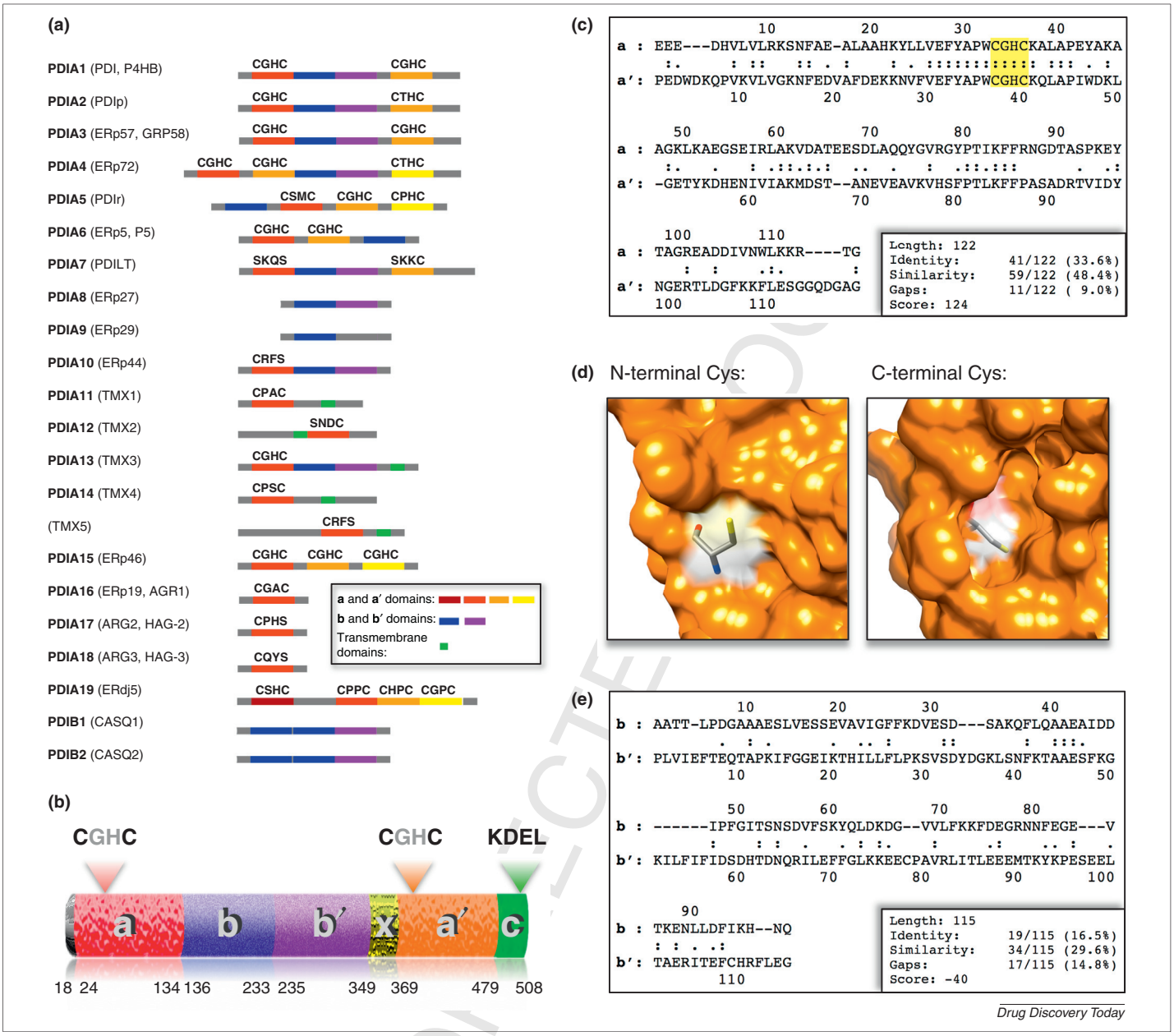

FIGURE 1

Structural properties of protein disulfide isomerase (PDI). (a) Protein members of the PDI family. Amino acid sequences of the active sites are shown. (b) Domain architecture of PDI. (c) Sequence comparison of the **a** and **a′** domains. Sequence comparison was performed using STRETCHER (EMBOSS, Pasteur: http://mobyle.pasteur.fr). The active site CGHC is highlighted. (d) Accessibility of the N-terminal and the C-terminal cysteines in the PDI active site. Structural information was obtained from the **a′** domain of 4EKZ, and analyzed using Chimera 1.7 (UCSF). (e) Sequence comparison of the **b** and **b′** domains.

date, the generation of a PDI global knockout mouse has not been reported. However, given the essential role of PDI, knockout mice might not be viable. Dysregulation of PDI expression and/or enzymatic activity is associated with a series of human diseases [11], such as neurodegenerative [12–15] and cardiovascular diseases [16–19]. Besides its primary location in the ER as a soluble oxidoreductase, PDI is also present on the extracellular side of the plasma membrane [20,21]. Although the mechanism by which PDI is secreted or translocated to the cell surface remains unclear, some evidence suggests that it interacts with the cell membrane via electrostatic charges [22]. PDI mainly functions as a reductase [23] as well as an isomerase [24] on the cell surface. Cell-surface PDI regulates multiple important biological processes, including coagulation [25], injury response [26], platelet activation [27–29] and thrombus formation [30–32], T cell migration [23], glioma cell migration [33], gamete fusion [34] and nitric oxide internalization from extracellular *S*-nitrosothiols [35]. Importantly, cell-surface PDI facilitates viral infection [36], as exemplified by its involvement in HIV-1 fusogenic events. Cell-surface PDI catalyzes the reduction of at least two disulfide bonds in gp120, an HIV-1 envelope glycoprotein, resulting in a major conformational change in gp120 that enhances its binding to the co-receptors chemokine (C-X-C motif) receptor 4 (CXCR4) and C-C chemokine receptor type 5 (CCR5) [37–39]. Besides ER and the cell surface, PDI

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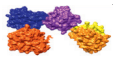
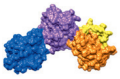
TABLE 1

Currently available structures of isolated domains in PDI

PDB ID	Structure	Domain	Method	Resolution (Å)	Deposition date	Refs
4EL1		abb′xa (oxidized)	X-ray	2.88	2012	[48]
4EKZ		abb′xa (reduced)	X-ray	2.51	2012	[48]
3UEM		b-b′-x-a′	X-ray	2.29	2011	[43]
2K18		b-b′	NMR	N/A[a]	2008	[44]
3BJ5		b′-x	X-ray	2.2	2007	[45]
1X5C		a′	NMR	N/A	2005	N/A
2BJX		b	NMR	N/A	1998	[46]
1MEK		a	NMR	N/A	1996	[47]

[a] N/A: not applicable.

has also been reported at other subcellular locations, including cytoplasm, mitochondria and nucleus [40,41]. However, these observations are not conclusive and the biological functions of PDI at these distinct locations remain unclear.

Increasing knowledge on the involvement of PDI in multiple diseases, the development of various assays and the availability of several crystal structures have led to the development of potential new small-molecule inhibitors. Recent studies have implicated PDI as a novel and promising drug target for several types of cancer. Herein, we discuss current knowledge on the relation between PDI and cancer, the various assays used for the discovery of PDI inhibitors, and currently available PDI inhibitors.

Structural properties of PDI

Encoded by the *P4HB* gene, PDI has a multidomain structure [42]. The full-length PDI contains 508 amino acids, whereas the mature form lacks the 17-amino-acid N-terminal signal peptide. PDI has four distinct domains, **a**, **b**, **b′** and **a′**, with a highly acidic C-terminal extension **c** and a **b′**–**a′** linker **x** (Fig. 1b). A classic ER-retrieval signal sequence (KDEL) lies at the C terminus of **c**. Most PDI family members share the thioredoxin (Trx)-like Cys-X-X-Cys active site and the ER-retrieval signal sequence. Although the structure of the full-length human PDI has not yet been resolved, structures of isolated domains are available (Table 1) [43–47]. Recently, Wang *et al.* solved high-resolution structures of human PDI **abb′xa′** in both reduced and oxidized states, showing that the **a**, **b**, **b′** and **a′** domains are arranged in a horseshoe shape with two CGHC active sites facing each other [48]. This is consistent with the previously published crystal structure of full-length yeast PDI [49]. These structures provide important insights into the structure–activity relation (SAR) of PDI and facilitate the design of PDI inhibitors.

The **a** and **a′** domains share 33.6% identity (Fig. 1c) and each contains an identical active site Cys-Gly-His-Cys (Fig. 1b). The reductase, oxidase and isomerase activities of PDI rely on the thiol groups of these active-site cysteines [50,51]. The **a** and **a′** domains operate independently of one another, because disruption of active-site cysteines in either domain abolished 50% of the catalytic activity of PDI, and disruption of cysteines in both domains completely abolished its oxidoreductase activity [52]. In each thioredoxin-like domain, the N-terminal active-site cysteine is positioned on the protein surface with its thiol group accessible for redox reactions, whereas the C-terminal active-site cysteine has limited solvent exposure (Fig. 1d). The N-terminal cysteine has a pK_a in the range of 4.5–5.6 [53,54], whereas the pK_a of the C-terminal cysteine was calculated to be 12.8 [55]. However, some studies suggest that, during biochemical reactions, the **a** domains undergo conformational changes that cause the pK_a of the C-terminal cysteine to shift from 12.8 to 6.1 [55,56]. In the initial step of a reaction, the N-terminal cysteine forms a transient disulfide bond with a cysteine residue in the substrate to form a heterodimer. This is followed by the 'escape pathway' in which the C-terminal cysteine attacks the N-terminal cysteine to release the substrate [57].

The **b** and **b′** domains do not contain an active site and only share 16.5% sequence identity (Fig. 1e). Although all PDI domains contribute to the binding of misfolded proteins, the **b′** domain constitutes the principal substrate-binding site and displays high affinity and broad specificity [58]. The **b′** domain is essential and sufficient for binding small peptides through hydrophobic interactions [59], but not for binding large peptides or proteins [58]. The substrate-binding site in **b′** is also necessary for the chaperone activity of PDI [60] and its interaction with the α-subunit of prolyl-4-hydroxylase [61].

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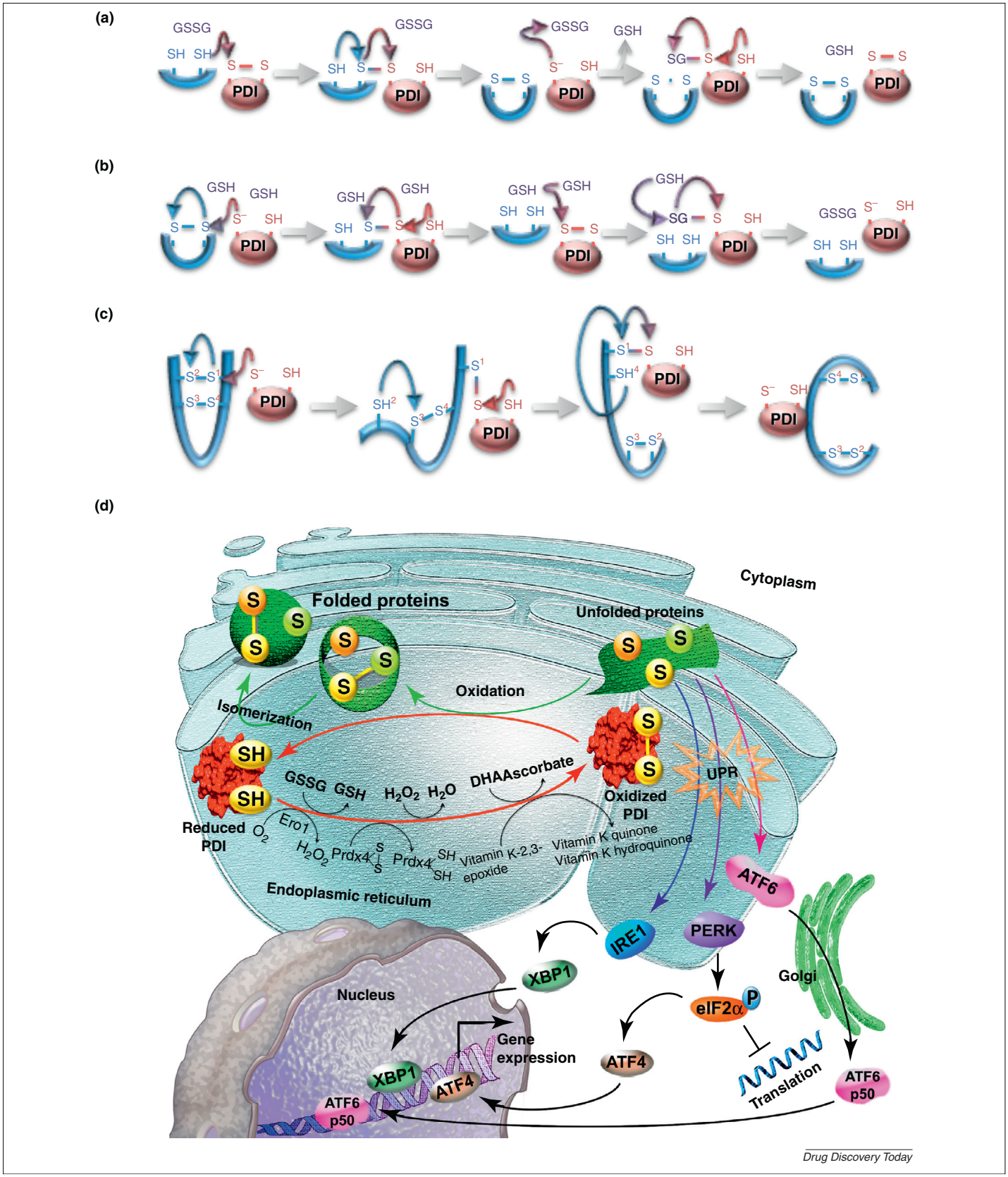

FIGURE 2

In vitro and *in vivo* biochemical reactions involving protein disulfide isomerase (PDI). PDI catalyzes (a) oxidation, (b) reduction and (c) isomerization reactions *in vitro*. In the oxidation reaction, glutathione disulfide (GSSG) represents the terminal electron acceptors, and in the reduction reaction, glutathione (GSH) represents the terminal electron donors. No extra electron acceptor or donor is needed for the isomerization reaction that is initiated by the reduced form of PDI. Arrows indicate the nucleophilic reactions. (d) In endoplasmic reticulum (ER), PDI mainly catalyzes oxidation and isomerization reactions, mediating disulfide bond formation and rearrangement for oxidative protein folding. While catalyzing disulfide bond formation in a substrate, the active-site cysteines of PDI are reduced

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The thiol-disulfide exchange reactions of PDI

It is well established that PDI is able to act as an oxidase, a reductase and an isomerase, depending on the redox state of its active-site cysteines and the properties of the substrates. In an oxidation reaction, a substrate dithiol is oxidized to a disulfide in parallel with the reduction of the active-site disulfide in PDI to the dithiol state. Subsequently, oxidants such as glutathione disulfide (GSSG) act as terminal electron acceptors to oxidize the dithiol back to the disulfide state and to complete the catalytic cycle (Fig. 2a). In a reduction reaction, a substrate disulfide is reduced to the dithiol state with the concomitant formation of an active-site disulfide in PDI. To fulfill the catalytic cycle, reductants, such as glutathione (GSH), NADPH and dithiothreitol (DTT), serve as terminal electron donors to reduce the disulfide in PDI back to its dithiol state (Fig. 2b). In an isomerization reaction, where no additional redox reagents are required, PDI catalyzes a shift of the disulfide-bond position among the substrate cysteines without a net change in the number of disulfide bonds in the substrate or in the redox state of the active sites in PDI (Fig. 2c).

In normal cells and under physiological conditions, PDI activity is tightly regulated. When PDI catalyzes the formation of disulfide bonds in nascent proteins to mediate oxidative protein folding, the active-site disulfides in PDI are reduced and subsequently reoxidized in the oxidizing environment of the ER (Fig. 2d). Previously, GSSG was considered to be a major regulator in oxidizing the active sites of PDI. However, this consensus was challenged by the discovery of an ER flavor-oxidase, endoplasmic reticulum oxidoreductin 1 (Ero1), that preferentially interacts and oxidizes PDI [62,63], especially the **a**′ domain [64]. As part of this reaction, Ero1 uses molecular oxygen as an electron acceptor and produces one molecule of H_2O_2 per disulfide bond [65]. Oxidation of the active-site cysteines in PDI is also contributed by other mechanisms, such as H_2O_2, peroxiredoxin 4 (Prdx4), docosahexaenoic acid (DHA) and vitamin K, and has been recently reviewed in depth [6,66].

Post-translational modifications on the active-site cysteines regulate the activity of PDI. For example, prolonged or acute nitrosative stress induces S-glutathionylation (P-SSG) [67–69] and S-nitrosylation (P-SNO) [12] of the active-site cysteines in PDI, blunting its activity and causing an unfolded protein response (UPR) and ER stress. Carbonylation of PDI (4-HNE-PDI) induced by oxidized low-density lipoproteins (oxLDLs) or 4-hydroxynonenal (4-HNE) was also reported to disrupt the enzymatic activity of PDI and, hence, to potentiate ER stress and apoptosis [70]. Moreover, the intracellular distribution of PDI can be regulated by other cellular events. For example, low levels of ruticulon-4A (Nogo-A), an inhibitor of neurite outgrowth specific to the central nervous system (CNS), cause a diffuse distribution of PDI. By contrast, higher levels lead to a punctate distribution of PDI protecting against neuron degeneration during amyotrophic lateral sclerosis (ALS). This effect is independent of UPR, perturbation of the overall ER structure, or distribution of other ER resident proteins [71]. Reticulon 1-C was also reported to induce PDI redistribution in ER vesicles and to decrease the levels of S-nitrosylated PDI [72].

Efficient regulation of PDI activity is crucial for most cellular functions. Disulfide bond formation occurs in approximately 30% of all proteins, and is essential for their bioactivities. Given that PDI serves as one of the most abundant and essential enzymes for oxidative protein folding, its dysfunction results in rapid accumulation of unfolded and misfolded proteins in the ER lumen. With hydrophobic amino acid side chains exposed to the surface, these proteins form insoluble aggregates and trigger UPR and ER stress [73,74]. Subsequently, three signal-transducing proteins [protein kinase RNA-like endoplasmic reticulum kinase (PERK), Inositol-requiring protein 1 (IRE1) and activating transcription factor 6 (ATF6)] that are positioned on the ER membrane and act as ER stress sensors, are activated to modulate UPR. PERK activation immediately leads to a global translational attenuation through direct phosphorylation of E74-like factor 2 (eIF2), the regulating initiator of mRNA translation, resulting in a decrease of protein influx into the ER lumen [75]. Phosphorylated eIF2α also enhances the translation of ATF4, a transcription factor regulating UPR genes [75]. Activated IRE1, a site-specific endonuclease, stabilizes XBP-1 mRNA by direct removal of a small intron, leading to the upregulation of X-box binding protein 1 (XBP-1) levels [76]. ATF6, when triggered by the UPR, translocates to the Golgi apparatus and is cleaved to yield an active fragment, ATF6 p50 [77]. ATF6 p50, XBP-1 and ATF4 enter the nucleus, bind to ERSE promoters, and activate the expression of proteins involved in UPR regulation. These UPR pathways define the pro-survival mechanism during the early phase of the ER stress to restore ER homeostasis. Failure to restore ER homeostasis results in the initiation of apoptosis.

PDI as a potential drug target for cancer treatment

Although PDI has been extensively studied during the past decades, its role in cancer progression is not well established. However, published data strongly suggest that PDI is significantly associated with cancer progression and is a potential drug target for cancer treatment.

PDI expression is high in a variety of cancer types

Gene expression microarray studies provide an important tool for assessing PDI expression levels in different cancer types. By analyzing published microarray data sets, we compared PDI expression in different cancer types with that in normal tissues. We observed that PDI expression was significantly upregulated ($P < 10^{-5}$, fold change >2) in brain and CNS cancers (Fig. 3a) [78–83], lymphoma (Fig. 3b) [84–86], kidney (Fig. 3c) [87–89], ovarian (Fig. 3d) [90,91], prostate (Fig. 3e) [92,93], lung (Fig. 3f) [94] and male germ cell tumors (Fig. 3g) [95]. Upregulation of PDI in cancer has also been confirmed by proteome analyses. PDI protein levels are increased in tissues from patients with prostate adenocarcinoma compared with benign prostate hyperplasia (BPH), and are significantly correlated with Gleason score [96]. 2-DE/matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) studies showed that, compared with the respective adjacent non-neoplastic tissues, PDI is overexpressed in infiltrating ductal carcinomas of both the female [97] and the male breast

and efficiently reoxidized by different regulators. Cellular molecules involved in PDI reoxidation include: endoplasmic reticulum oxidoreductin 1 (Ero1), GSSG, H_2O_2, peroxiredoxin 4 (Prdx4), docosahexaenoic acid (DHA) and vitamin K. Impairment of PDI activity leads to the accumulation of unfolded and misfolded proteins, causing an unfolded protein response and ER stress.

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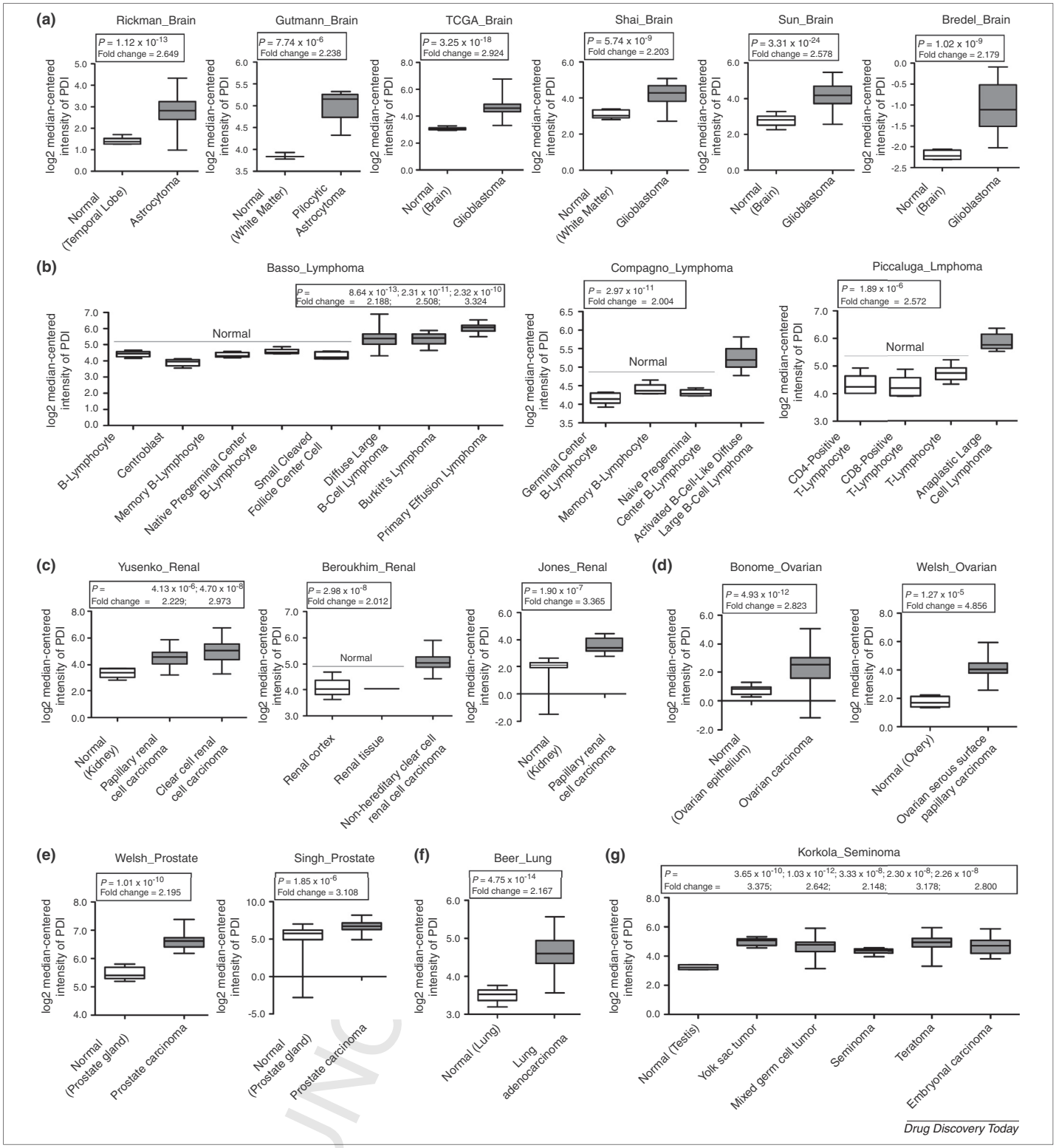

FIGURE 3

Protein disulfide isomerase (PDI) is highly expressed in multiple cancer types compared with respective normal tissues. Cancer types analyzed include (a) brain [78–83], (b) lymphoma [84–86], (c) kidney [87–89], (d) ovarian [90,91], (e) prostate [92,93], (f) lung [94] and (g) male germ cell tumors [95]. Data sets were obtained from Oncomine™ (http://www.oncomine.com) with filtering thresholds as $P < 10^{-5}$ and fold change >2, and analyzed using Prism 5 (GraphPad Software, Inc). The Student t-test was used for statistical analysis to compare gene expression levels between normal and cancer tissues. Box: 25–75%. Whiskers: Min and Max.

[98], and in both primary and metastatic gastric cancer induced by methylnitronitrosoguanidine (MNNG) from a rat model [99]. Another study reported that PDI was one of the most upregulated proteins in breast tumor interstitial fluids and, thus, could serve as a potential serological marker for early detection of breast cancer [100].

Upregulation of PDI protein also correlates with cancer metastasis and invasion. PDI protein levels are significantly higher in

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axillary lymph node metastatic breast tumor compared with primary breast tumor [101]. PDI was also observed to be strongly expressed in migrating glioma cells in an *in vitro* migration assay, and in invasive glioma cells in both xenografts and at the invasive front of human glioblastomas [33]. In addition, PDI serves as a tumor marker for the diagnosis of colorectal cancer [102]. Together, these data suggest that overexpression of PDI could be used as a diagnostic marker for select cancer types. Moreover, PDI was reported as an antibody target during immune-mediated tumor destruction in patients with melanoma or refractory acute myeloid leukemia (AML) who received vaccination with irradiated autologous granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting tumor cells [103].

PDI is associated with clinical outcomes

Resistance to chemotherapy is a major concern in clinical cancer treatment. It is becoming clear that PDI has a role in chemoresistance in some cancers. For example, compared with aplidin-sensitive HeLa cells, the aplidin-resistant HeLa-R cells express significantly higher levels of PDI protein and, in turn, inhibition of PDI by bacitracin sensitized HeLa-R cells to aplidin [104]. These data suggest that combination therapies using PDI inhibitors with traditional anticancer agents could overcome chemoresistance and even achieve synergetic effects. In support of these experimental data, lower PDI expression was observed to be significantly associated with higher overall survival rate of patients with glioblastoma (Fig. 4a) and breast cancer (Fig. 4b) [80,81,105]. Therefore, PDI levels in cancer biopsies could be used as a prognostic marker, in particular, because PDI can be secreted into the extracellular tumor environment, serum PDI levels might reflect tumor PDI expression, facilitating clinical detection.

PDI supports tumor survival and progression

Increasing evidence from functional studies has indicated that PDI has an important role in supporting cancer progression. First, PDI is associated with the resistance to the growth-inhibitory effects of transforming growth factor-β1 (TGF-β1) that frequently occurs in cancer cells. TGF-β1 downregulates the mRNA for PDI in TGF-β1-sensitive cells but not in cells that are insensitive to the growth-inhibitory effects of TGF-β1 [106]. Second, PDI protects cancer cells from apoptosis. In melanoma, inhibition of PDI activity using bacitracin enhanced apoptosis triggered by fenretinide or velcade [107]. Cytosolic PDI is a substrate of caspase-3 and -7 during etoposide-induced apoptosis in AML HL-60 cells, and overexpression of cytosolic PDI (ER retention sequence deleted) suppressed cell death [108]. Human hepatocellular carcinoma (HCC) cells express PDI in a hypoxia-inducible manner, and bacitracin was reported to enhance hexokinase II inhibitor (3-bromopyruvate)-triggered apoptosis in HCC cells via augmenting ER stress and anti-angiogenesis [109]. It was recently shown that silencing PDI in human ovarian cancer cells resulted in substantial cytotoxicity [110]. Similarly, propynoic acid carbamoyl methyl amides (PACMA) 31, a novel irreversible PDI inhibitor, exhibited significant anticancer activity in both *in vitro* and *in vivo* ovarian cancer models [110].

However, the effects of PDI in supporting tumor survival and progression are cell- and cancer-type dependent. Silencing of PDI was reported to induce significant cytotoxicity in cultured MCF-7 (breast cancer) and SH-SY5Y (neuroblastoma) but not in HeLa (cervical cancer) cells, which might result from the different levels of caspase activation in these cells [111]. Moreover, PDI promotes cancer invasion and metastasis. PDI-mediated disulfide bond formation is important for the gelatinolytic activity and secretion of matrix metallopeptidase 9 (MMP-9), a major proteinase digesting extracellular matrix and facilitating tumor metastasis and angiogenesis [112]. Bacitracin or an anti-PDI mAb inhibited *in vitro* migration and invasion of human glioma cells [33], suggesting that cell-surface PDI is also involved in cancer progression. It is also important to note that PDI protein levels change differently in different cell types and in response to different pharmacological agents that induce the UPR. For example, both sarcoma cell lines HT1080 and RD-ES increased PDI levels after tunicarmycin treatment, but only HT1080 upregulated PDI levels after ritonavir treatment [113]. Therefore, it is important and necessary to understand the specific cancer molecular context for the application of PDI targeted therapy.

We hypothesize that ER stress contributes to the cytotoxicity induced by PDI silencing or inhibition. A hallmark of cancer is its ability to maintain growth and proliferation [114], requiring a sufficient supply of proteins for cellular construction and functions,

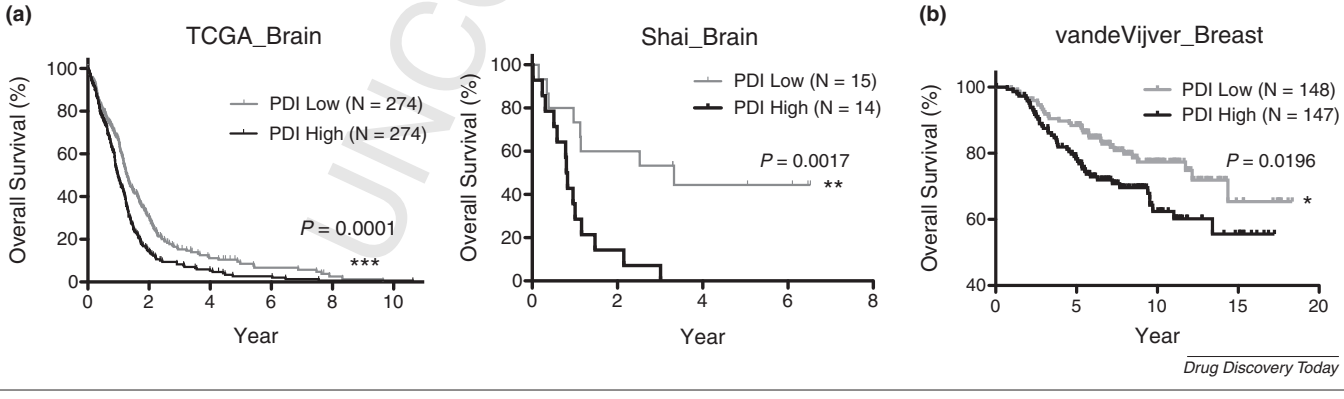

FIGURE 4

Protein disulfide isomerase (PDI) expression is associated with the overall survival rate of patients with either (a) glioblastoma [80,81] or (b) breast cancer [105]. Data sets were obtained from Oncomine™ http://www.oncomine.com) and analyzed using Prism 5 (GraphPad Software, Inc). The Kaplan–Meier survival analysis method was used to generate survival curves. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$.

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intercellular communication, extracellular matrix destruction and angiogenesis stimulation. Compared with normal cells, cancer cells need to express proteins more efficiently by turning on different transcriptional (e.g. c-Myc or Jun) and translational (e.g. eIF2α or eIF4E) machineries [115,116] and, thus, upregulate PDI levels to facilitate excess protein production. Therefore, cancer cells are more sensitive to PDI inhibition and UPR. In addition, PDI inhibition might also suppress cancer invasion and metastasis that cell-surface PDI is involved in.

Approaches to measuring PDI activities and screening for PDI inhibitors

During the past decades, a series of assays has been established for measuring the enzymatic activities of PDI as well as screening for inhibitors. Here, we discuss the currently available PDI activity assays based on the different activities of PDI (reductase, isomerase, oxidase and chaperone). Given that many of these assays are widely used to screen for PDI inhibitors, it is important to understand the advantages and limitations of each one when used to identify specific inhibitors.

Reductase assays

Insulin turbidity assay

As a reductase, PDI disrupts the two disulfide bonds between insulin A and B chains, causing B chain aggregation whereas the A chain remains soluble in solution (Fig. 5a). The turbidity generated by the B chain increases light absorbance (540–650 nm) and, therefore, can serve as a readout to measure the reductase activity of PDI. Commonly used electron donors for this reaction include GSH [117] and DTT [118,119]. Such a reaction can be continuously measured by coupling the formation of GSSG to NADPH oxidation using glutathione reductase [117,120]. The insulin turbidity assay can be converted into an end-point assay by using a stop reagent (e.g. H_2O_2). In this format, the assay can be automated and used for high-throughput screening (HTS) of PDI inhibitors [121]. This relatively simple, efficient and cost-effective method is commonly utilized in the field. The sensitivity of this assay is in the micromolar range. It is important to note that this assay can generate false negatives when screening or testing PDI inhibitors, because compounds that are easily reduced by DTT or GSH can lose their inhibitory activity.

Insulin degradation assay

The insulin degradation assay is a modified version of the insulin turbidity assay [122] (Fig. 5b). In this assay, PDI catalyzes the reduction of disulfide bonds in insulin, resulting in the precipitation of ^{125}I-labeled B chains. The remaining acid-soluble radiation intensity in the supernatant provides an estimate of the reductase activity of PDI [123,124]. However, the assay might underestimate the reductase activity when the system is contaminated by proteases that could add extra soluble radioactive fragments to the supernatant. Additional drawbacks of the assay are the multiple steps involved and the use of radioactive materials limiting its broad application as an HTS platform.

Fluorimetric assay

In a fluorimetric assay, PDI catalyzes the reduction of a fluorescent probe [e.g. di-(o-aminobenzoyl)-GSSG (diabz-GSSG) or dieosin-GSSG (Di-E-GSSG)] where a disulfide bond links two identical fluorescent moieties [(o-aminobenzoyl)-GSSG (abz-GSH) or eosin-GSSG (E-GSH)] to maintain fluorescence self-quenching (Fig. 5c). The detachment of the two fluorescent moieties causes a substantial increase in fluorescence intensity [125,126]. The assay is sensitive and can measure the reductase activity at a PDI concentration as low as 2.5 nM [125]. The fluorimetric assay is adaptable for HTS but the cost is high because of the need to synthesize fluorescent probes.

Oxidation assays

Ribonuclease (RNase) oxidation assay

RNase digests RNA by catalyzing the hydrolysis of phosphodiester bonds. In this assay, PDI catalyzes the oxidative renaturation of RNase from its inactive reduced form (Fig. 5d) [127,128]. Active oxidized RNase subsequently catalyzes the hydrolysis of cyclic cytidine monophosphate (cCMP) into CMP, leading to an increase in absorbance at 296 nm. The rate of cCMP hydrolysis indicates the oxidase activity of PDI. Although convenient, one has to be aware that the product (CMP) can competitively inhibit PDI and alter the reaction rate. In addition, the calculation of active RNase concentration depends on the K_m value for cCMP and the K_i value for CMP, and these parameters can vary depending on the pH and salt concentration of the reaction.

Alternative versions of this assay utilize other enzymes and substrates. For example, PDI catalyzes the oxidation of inactive reduced lysozyme to its active oxidized form that can be used to digest a suspension of the *Micrococcus lysodeikticus* cell wall [118]. Reduced and denatured bovine pancreatic trypsin inhibitor (RBPTI) is also used as an alternative [129]. PDI catalyzes the oxidation of RBPTI to its active form that can be measured by virtue of its ability to inhibit trypsin. However, these alternative versions are less commonly used.

Peptide oxidation assay

This assay uses a decapeptide NRCSQGSCWN comprising two cysteine residues separated by a linker region, with a fluorescent residue (tryptophan) adjacent to one cysteine and a protonatable residue (arginine) adjacent to the other cysteine (Fig. 5e) [53]. These two groups are brought together when PDI catalyzes the formation of the disulfide bond between the two cysteines, resulting in fluorescence quenching. Fluorescence spectroscopy enables rapid and reproducible determination of its oxidase activity.

Isomerization assays

Scrambled RNase (sRNase) assay

The sRNase assay was the first assay used for measuring PDI activity [130]. sRNase is prepared by subjecting RNase to denaturing conditions such that it is in a fully oxidized and inactive state with randomly formed disulfide bonds [120,131]. The addition of PDI catalyzes the exchange of inter- and intramolecular disulfides in sRNase, resulting in native disulfide pairing and recovery of RNase enzymatic activity (Fig. 5f). The isomerase activity of PDI is measured by evaluating RNase activity using RNA as a substrate. Given that PDI is an essential isomerase in cells, this sensitive and relatively easy-to-perform assay is useful for mechanistic studies. However, because it involves aliquot withdrawal at regular intervals, it is inconvenient for HTS. It is also important to note that the substrate sRNase is a complex mixture of species with intramolecular and intermolecular disulfides displaying batch-to-batch variation. Moreover, the RNA hydrolysis can be catalyzed by an

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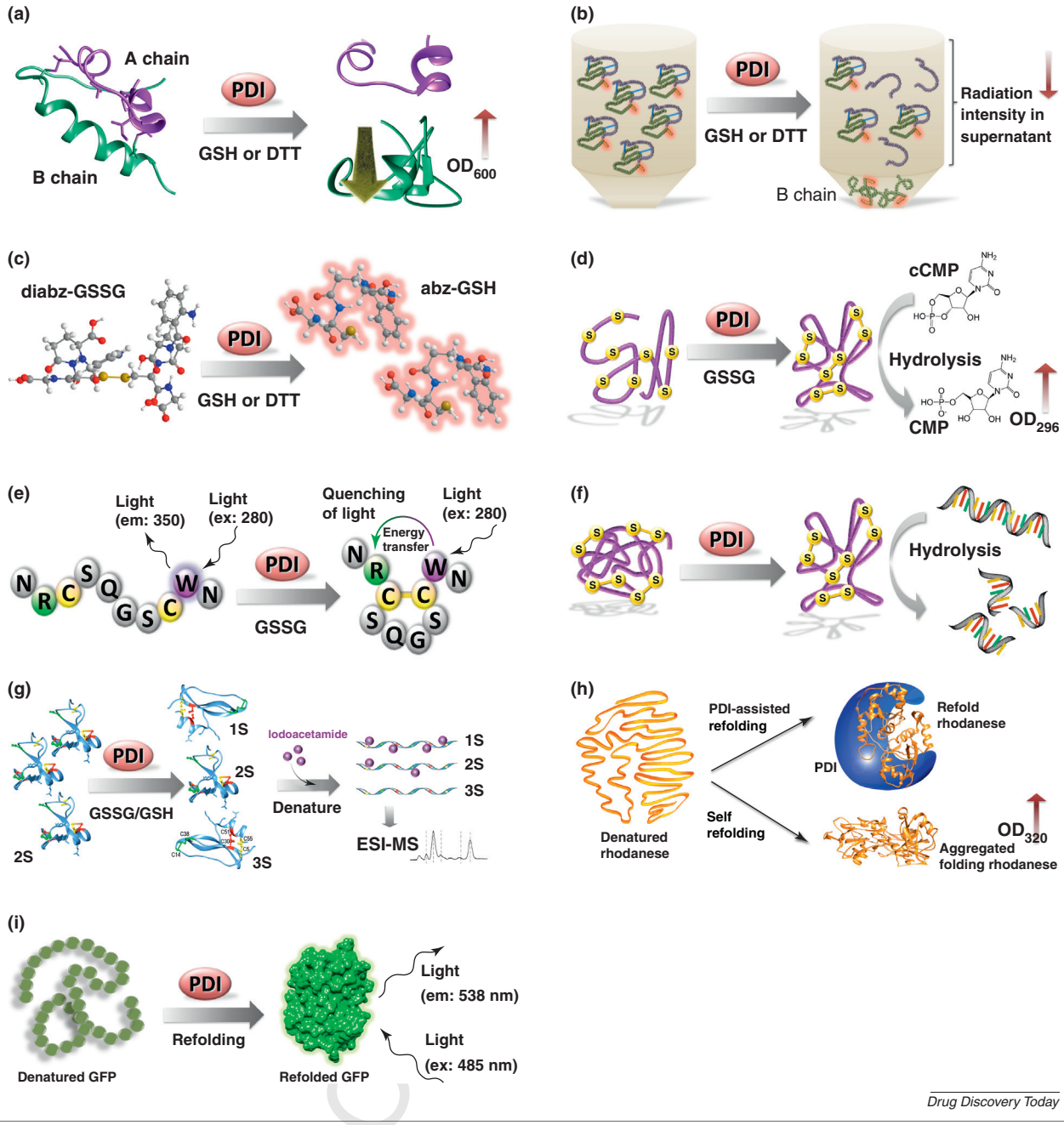

FIGURE 5

Protein disulfide isomerase (PDI) activity assays. (a) Insulin turbidity assay. PDI catalyzes the reduction of disulfide bonds between insulin A and B chains, causing aggregation of the B chain. (b) Insulin degradation assay. PDI catalyzes the reduction of disulfide bonds between the insulin A chain and ^{125}I-labeded B chain, resulting in a decrease in radiation intensity in the supernatant. (c) Fluorimetric assay. PDI catalyzes reduction of the disulfide bond in di-(o-aminobenzoyl)-glutathione disulfide (diabz-GSSG), releasing two (o-aminobenzoyl)-glutathione (abz-GSH) molecules with an increase of fluorescent activity. (d) RNase oxidation assay. PDI catalyzes oxidation of reduced RNase to its active form that hydrolyzes cyclic cytidine monophosphate (cCMP) into CMP, causing an increase in absorbance at 296 nm. (e) Peptide oxidation assay. PDI catalyzes the formation of an intramolecular disulfide bond in NRCSQGSCWN and, hence, brings Trp and Arg residues in close proximity, resulting in quenching of Trp fluorescence by Arg. (f) Scrambled RNase (sRNAse) assay. PDI catalyzes refolding of sRNase to its native active form that digests RNA, leading to a change in A260/A280. (g) Bovine pancreatic trypsin inhibitor (BPTI) refolding assay. PDI catalyzes the refolding of BPTI from its non-native 2S form to the native 3S form. Such a change can be measured using electrospray ionization mass spectrometry (ESI-MS). (h) Protein aggregation assay. PDI mediates denatured rhodanese refolding, preventing folding rhodanese from aggregation. (i) Green fluorescent protein (GFP) assay. PDI catalyzes the folding of nonfluorescent denatured GFP to its native form that regains fluorescent activity.

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TABLE 2

Name	IC_{50} (MM)	Refs	Activity assay	Selectivity	Reversibility	Membrane permeability	*In vitro* PDI inhibition activity	*In vivo* PDI inhibition activity	Refs
PACMA 31	10	[110]	Insulin turbidity assay	Selective for PDI over other proteins like BSA and Grp78.	Irreversible	Permeable	Cytotoxic in ovarian cancer cell lines (OVCAR-8, NCI/ADR-RES, HEY and OVCAR-3)	Accumulates in tumor and suppresses tumor growth in a mouse xenograft model of ovarian cancer; no significant toxicity towards normal tissues	
16F16	63	[15]	Fluorimetric assay	N/A	Irreversible	Permeable	Inhibits apoptosis in P12 cell model of HD	Prevents neurotoxicity in medium spiny neurons in striatal region of brain slices	
RB-11-*ca*	40	[142]	Fluorimetric assay	N/A	Irreversible	Permeable	Cytotoxic in HeLa cells with EC_{50} = 23.9 MM	N/A	
PAO	85	[110]	Insulin turbidity assay	Low specificity Reaction with proteins containing cxxc motif [145,146]	Irreversible	Permeable	Induces rapid shedding of L-selectin from isolated neutrophils, a process negatively regulated by PDI Inhibits PDI-catalyzed reductive release of acid soluble [^{125}I] tyramine-SH from surface bound [^{125}I] tyramine-SS-poly(D-lysine) (IC_{50} = 10 MM) Effective before or during HIV-1 infection, but not after infection progressed in P4, PM1, H9, 1G5 and macrophage-depleted peripheral blood monocytic cells	N/A	[174] [37] [37]
DTNB	100	[124]	Insulin degradation assay	Nonspecific	Irreversible	Impermeable	Inhibits diphtheria toxin activation Prevents H9 cells from HIV-1 infection (IC_{50} = 0.3 mM) Long-lasting host cell protection at late stages of viral cycle	N/A	[147] [147] [175]
Iodoacetaminde	8 (pH 6)	[150]	Insulin turbidity assay	Nonspecific	Irreversible	Permeable	N/A	N/A	
NEM	8	[150]	Insulin turbidity assay	Nonspecific	Irreversible	Permeable	N/A	N/A	

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Acrolein	10 (pH 6.3)	[150]	Insulin turbidity assay	Nonspecific	Irreversible	Permeable	N/A	N/A	
Thiomuscimol	23	[15]	Fluorimetric assay	Nonspecific	Irreversible	Permeable	Prevents polyQ-induced apoptosis in P12 cell model of HD	N/A	
Cystamine	66	[15]	Fluorimetric assay	Nonspecific	Irreversible	Permeable	Prevents polyQ-induced apoptosis in a P12 cell model of HD	N/A	
Juniferdin	0.156	[118]	Insulin turbidometric assay	Selective for PDI over ERp57 and ERp72	Reversible	Permeable	Inhibits PDI-catalyzed reduction of HIV gp120 and viral entry Cytotoxic in HeLa, HepG2, HT1080 and K562 cells	N/A	
Quercetin-3-rutinoside	6.1	[152]	Insulin turbidity assay	Selective for PDI over ERp5, ERp57, ERp72, thioredoxin and thioredoxin reductase	Reversible	Poor permeability	Inhibits platelet aggregation and endothelial cell-mediated fibrin generation	Inhibits thrombus formation	
Bacitracin	90	[153]	Insulin degradation assay	Nonspecific [156]	Irreversible	Poor cell permeability [157]	Increases apoptosis along with other drugs in melanoma cells	Cancels neuroprotective effect of 4-HBA	[107,182]
Bacotracin A (major analog)	590	[155]					Reduces glioma cell migration and invasion		[33]
Bacitracin B	1050	[155]					Inhibits virus entry		[33,147, 176,177]
Bacitracin F	20						Inhibits platelet aggregation		[27,29]
Bacitracin H	40						Increases aggregation of Cu/Zn superoxide dismutase Inhibits VKORC1 activity Increases transcriptional activity of NF-κB Inhibits NAD(P)H oxidase activity Inhibits cytotoxicity of diptheria toxin Inhibits thyroid-stimulating hormone receptor shedding		[178] [179] [180] [8] [124] [181]
Ribostamycin	Sufficient inhibition at 100:1 molar ratio of ribostamycin to PDI (Kd = 3.19×10^{-4} M)	[138]	Protein aggregation assay	Nonspecific	Reversible	Permeable	N/A	N/A	
Estrogens: E_1, E_2, DES and E_3	>30% inhibition at 1 MM	[161]	Insulin degradation assay	Nonspecific	Reversible	Permeable	N/A	N/A	
T_3	3.49	[163]	sRNase assay	Nonspecific	Reversible	Permeable	N/A	N/A	
BPA	3.72	[163]	RNase oxidation assay	Nonspecific	Reversible	Permeable	N/A	N/A	

intermediate instead of a final native RNase product from the isomerization reaction, resulting in miscalculation of the isomerase activity of PDI.

Bovine pancreatic trypsin inhibitor (BPTI) refolding assay

BPTI is a single-chain polypeptide that suppresses trypsin enzymatic activity. In its native conformation, the protein folds upon itself and is held together by three disulfide bonds (3S) [132]. In this assay, PDI catalyzes a disulfide rearrangement in non-native BPTI to refold it from the 2S to the 3S native state (Fig. 5g) [59,133]. At set time points, the reaction is quenched by the addition of iodoacetamide that reacts with free thiol groups and adds 57 Da to the protein mass. Electrospray ionization mass spectrometry (ESI-MS) is subsequently used to analyze the amount of refolded BPTI (3S) and its folding intermediates (1S and 2S). This assay is time consuming because refolded BPTI and its folding intermediates need to be purified for the ESI-MS analysis. Moreover, when this assay is used for testing PDI inhibitors that interfere with the MS analysis, additional steps are required to remove the compounds after the reaction [59]. Therefore, it is not amenable for HTS. In addition, different BPTI protein species (1S, 2S and 3S) can bias their detection by ESI-MS. This assay is semiquantitative [59].

Protein chaperone assays

Protein aggregation assay

Rhodanese comprises a single polypeptide chain folded into two domains of equal size. Guanidine hydrochloride (Gdn-HCl)-denatured rhodanese tends to aggregate during the self-refolding process in the absence of protein chaperones. Based on this property, refolding of denatured rhodanese can be used for studying the chaperone activity of PDI by measuring absorbance at 320 nm (Fig. 5h) [59]. The absence of disulfide bonds in rhodanese makes it suitable for testing the chaperone activity of PDI independent of its isomerase activity [134]. Other proteins have also been used as alternatives in the protein aggregation assay, such as D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, another protein containing no disulfide bonds) [135]. In addition, citrate synthase and luciferase have been used in the protein aggregation assay to study the chaperone activity of DsbG, a protein disulfide isomerase present in the periplasm of *Escherichia coli* [136]. Besides chemical denaturation using guanidine hydrochloride (Gdn-HCl), thermal denaturation is also utilized for the protein aggregation assay. Substrate proteins used for thermal denaturation include alcohol dehydrogenase (ADH) [137] and citrate synthase [138].

Green fluorescent protein (GFP) assay

In the GFP assay, refolding of acid-denatured GFP is promoted by PDI, resulting in an increase in the fluorescence intensity that can be monitored in real time (Fig. 5i) [139]. GFP serves as a model substrate to study the chaperone activity of PDI because not only does it lack disulfide bonds, but also upon acid denaturation (pH 1.5) it exhibits a low fluorescence intensity compared with the active structure.

Small-molecule inhibitors of PDI

Although PDI has been intensively studied over the past few decades, no selective PDI inhibitors have emerged for clinical use. Among the limited number of PDI inhibitors, most are neither potent nor selective, and show significant off-target toxicity. However, increasing knowledge on the protein structure and functions of PDI and its family members will lead to the discovery of potent and selective inhibitors. Recent discoveries of synthetic small-molecule PDI inhibitors, such as propynoic acid carbamoyl methyl amide (PACMA) 31 and 16F16, have provided necessary tools to further understand the role of PDI in human disease. In this section, we provide a comprehensive review of lead PDI inhibitors published to date. Properties of the PDI inhibitors are listed in Table 2 and chemical structures of select lead compounds Q3 are shown in Figs 6,7. Most of these compounds are irreversible inhibitors targeting the active site cysteines of PDI. It is also important to note that all purported PDI inhibitors require further validation to be considered *bona fide* and selective for this target. Further studies will shed more light on the usefulness of these compounds for further development.

Synthetic compounds

Propynoic acid carbamoyl methyl amides

PACMAs are a class of novel small molecules with significant cytotoxicity towards a broad range of cancer cells [140]. A representative compound, PACMA 31 (**1**) was recently reported as an irreversible inhibitor of PDI with potency in *in vitro* and *in vivo* models of ovarian cancer [110]. Its terminal propynoic group covalently reacts with the thiol groups of the active-site cysteines in PDI. This interaction also alters the secondary protein structure of PDI. A marked correlation has also been observed between PDI inhibitory activity and cytotoxicity in ovarian cancer cells among PACMA analogs (S. Xu, S. Saranya, and N. Neamati, unpublished data). A fluorescent analog, PACMA 57 (compound **2**), synthesized via conjugation of a fluorescent molecule BODIPY to compound **1**, showed similar properties [110]. It serves as a useful tool to further study the *in vitro* and *in vivo* properties of PACMAs, and to expand knowledge of PDI-associated cellular cascades.

16F16 (3)

16F16 (**3**) was identified in a HTS of 68 887 compounds for the ability to suppress apoptosis in an *in vitro* P12 cell-based model of Huntington's disease (HD) [15], in which apoptosis is induced by polyglutamine (polyQ) and mediated by PDI. By using 'click chemistry' and MS, PDI isoforms PDIA1 and PDIA3 were identified as cellular targets of compound **3**. Within the 3–12 MM range, compound **3** showed a dose-dependent rescue of polyQ-induced apoptosis that correlated with PDI inhibition, whereas at concentrations >12 MM, compound **3** showed cytotoxicity owing to PDI inhibition as well as potential off-target effects. SAR analysis showed that the chloroacetyl moiety is crucial to its activity. Its ester moiety tolerates small modifications, such as a change from methyl to ethyl, whereas incorporation of biotin or fluorescein affinity tags causes a complete activity loss. Compound **3** might bind to active-site cysteines of PDI in a similar manner to compounds **1** and **2**. It is possible that compounds **1–3** could bind to other proteins, especially those containing free cysteines within similar binding cavity as PDI, but they might not affect the activities of these proteins if the cysteine residues are not related to their function. In addition, radiolabeled compound **3** and its analogs have recently been reported as new potential positron emission tomography (PET) agents for imaging of PDI in cancer [141].

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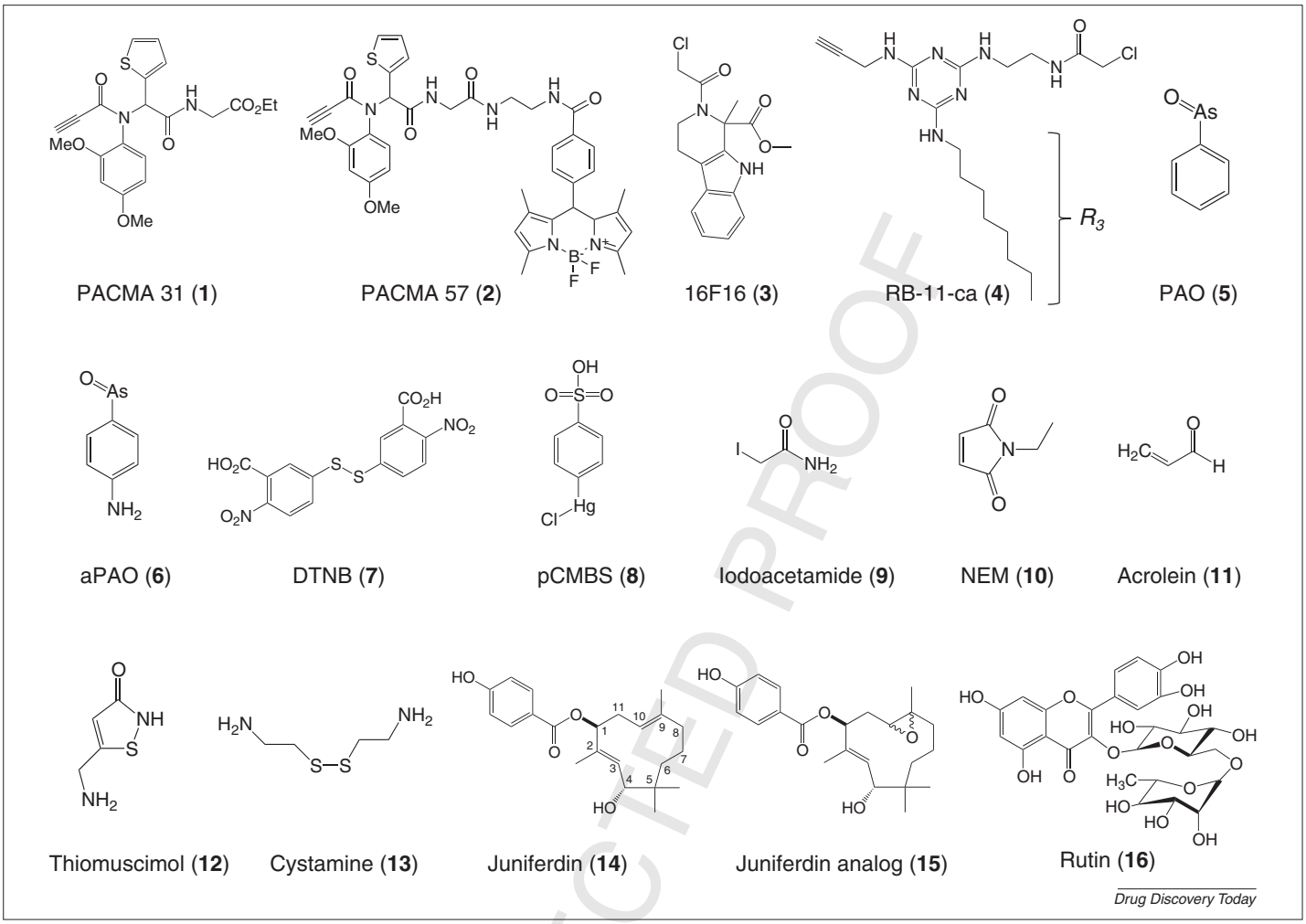

FIGURE 6

Chemical structures of protein disulfide isomerase (PDI) inhibitors from synthetic compounds. *Abbreviations*: DTNB: 5,5′-Dithiobis(2-nitro benzoic acid); NEM: *N*-ethylmaleimide; PACMA: propynoic acid carbamoyl methyl amide; PAO: Phenylarsine oxide; pCMBS: *p*-chloromercuribenzene sulfonate.

RB-11-ca (4)

A trifunctionalized 1,3,5-triazine RB-11-*ca* (**4**) was identified as a covalent PDI inhibitor by using click-chemistry [142]. Interestingly, compound **4** showed a relative specificity for the active-site Cys53 residue in the **a** domain. SAR studies showed that the hydrophobic moiety (R_3) is essential for the binding selectivity of compound **4**, because an analog (RB-20-*ca*) with reduced hydrophobicity in R_3 binds a different cellular protein (approximately 28 kDa) that was not identified in the study.

Arsenic-containing compounds

Phenylarsine oxide (PAO, **5**) is known to crosslink vicinal sulfhydryl groups [143] and form coordination bonds through its As^{13} with the vicinal thiols of the CXXC motif of proteins such as PDI [144]. Its para-amino derivate, aPAO (**6**), was shown to prevent HIV-1 entry into cells [37]. However, this class of PDI inhibitors has low specificity for PDI because they also react with other proteins containing the CXXC motif. For example, compound **6** has been reported to inhibit protein tyrosine phosphatase (PTPase) [145] and Rho GTPase [146]. In fact, it is a widely used PTPase-specific inhibitor.

Sulfhydryl reagents

A series of sulfhydryl reagents were reported to inhibit the catalytic activity of PDI. They react with the free thiol groups in PDI and, therefore, act as irreversible inhibitors. Generally, they exhibit relatively low specificities for PDI.

5,5′-Dithiobis(2-nitro benzoic acid) [DTNB (**7**)], known as Ellman's reagent, is a membrane-impermeable sulfhydryl blocker. It was shown to inhibit the activation of diphtheria toxin, a process involving disulfide bond cleavage mediated by cell-surface PDI [147,148]. The inhibition of diphtheria toxin activation by compound **7** was similar to the inhibition of PDI by bacitracin or anti-PDI antibodies [124]. *p*-Chloromercuribenzene sulfonate (pCMBS, **8**) is another membrane-impermeable sulfhydryl reported to block the thiol groups in PDI and prevent diphtheria toxin activation [148,149].

Alkylators and unsaturated aldehydes, including iodoacetamide (**9**), *N*-ethylmaleimide (NEM; **10**), acrolein (**11**), thiomuscimol (**12**) and cystamine (**13**), were reported to inhibit the reductase activity of PDI in the insulin turbidity assay at low physiological pHs [15,150]. SAR analyses showed that replacement of the sulfur

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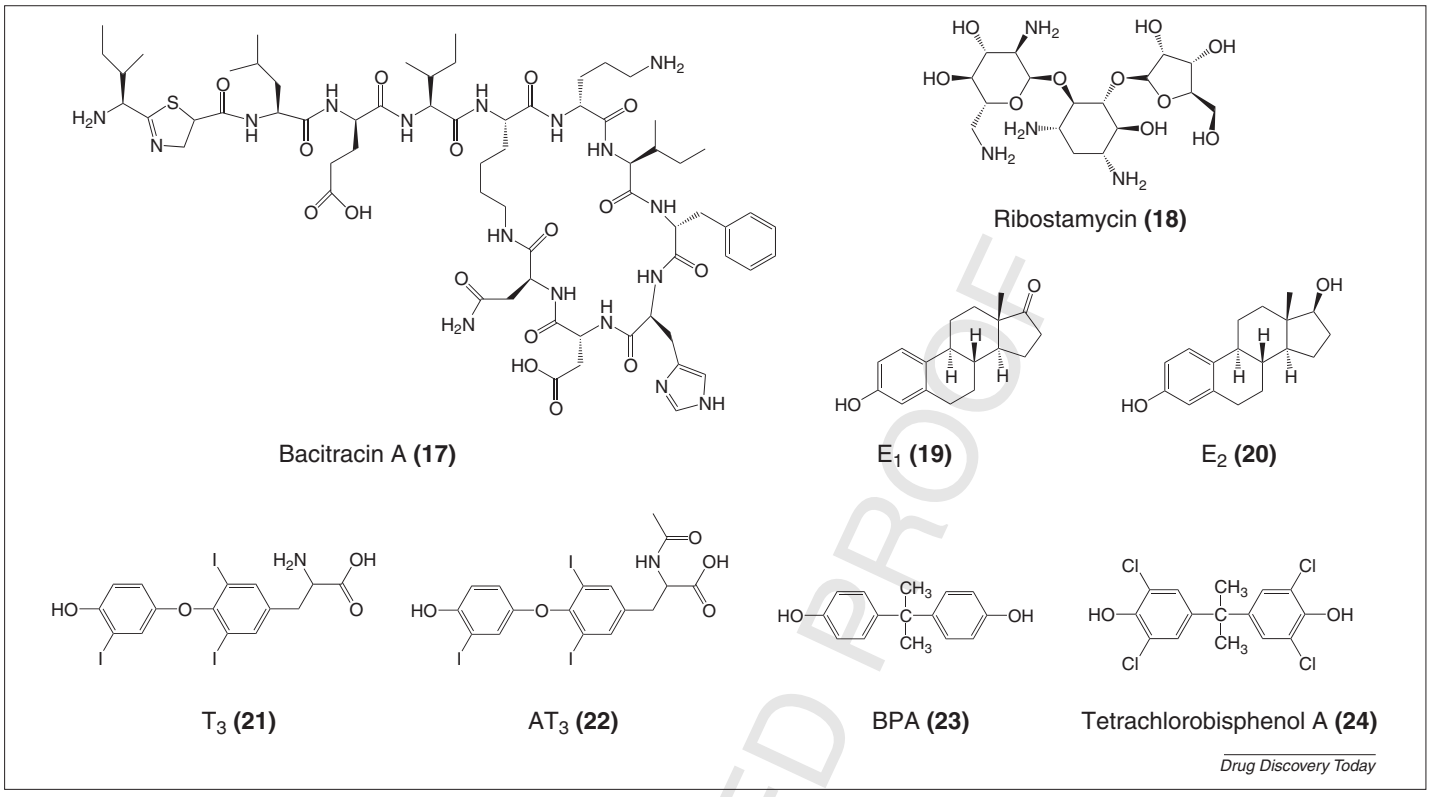

FIGURE 7

Chemical structures of protein disulfide isomerase (PDI) inhibitors from plant metabolites, antibiotics, hormones and xenoestrogens. *Abbreviations*: AT_3: *N* acetylated form of T_3; BPA: bisphenol A; E_1: estrone; E_2: 17β-estradiol; T_3: 3,3′,5-triiodo-L-thyronine.

by oxygen in compound **12** or reduction of the intramolecular disulfide in compound **13** completely abolished their inhibitory activities, confirming that they act as irreversible inhibitors.

Plant metabolites

Juniferdin (14) and its analogs

Juniferdin is a sesquiterpenoid originally isolated from the plant *Ferula juniperina*. This natural product was identified as a hit in an insulin turbidity HTS of 10 000 RIKEN Natural Product Depository (NPDepo) compounds for PDI inhibitors as anti-HIV-1 agents [118]. SAR studies showed that the sesquiterpene ring is essential for the activity of juniferdin, because replacement with a 1-octyl completely abolished its inhibitory activity, whereas other ring structures, such as cyclooctyl, cyclododecyl or (1R)-menthyl, retain modest activity. Modifications on the sesquiterpene ring also affected the activity of juniferdin. Whereas the 9,10-monoepoxide 1:1 stereoisomers showed inhibition similar to juniferdin, the 2,3,9,10-diepoxide or the 4,9,10-trihydroxy showed no inhibition, and the 2,3-monoepoxide and the stereoisomeric 4,9,10-trihydroxy derivative exhibited 15- and 11-fold lower inhibition, respectively. Compound **15** carrying a 9,10-monoepoxide showed activity similar to juniferdin, with an IC_{50} value of 0.167 MM. The *p*-hydroxybenzoate group is also important for the activity. Juniferdin and compound **15** were shown to be specific inhibitors of the reductase activity of PDI without significant inhibition of its oxidase activity. In addition, juniferdin showed negligible inhibition of other PDI family reductases, ERp57 and ERp72, that share the same active site, CGHC. Only juniferdin showed pronounced cytotoxicity. Considering that PDI silencing using small interfering (si)RNA, small hairpin (sh)RNA or PDI inhibitors (e.g. compounds **1**, **2** and **3**) resulted in cytotoxicity [15,110,111,151], it is possible that compound **15** is cell impermeable, unstable or readily metabolized.

Quercetin-3-rutinoside (16)

Quercetin-3-rutinoside is also known as rutin, a natural product belonging to the flavonol family. It is a plant polyphenolic compound widely consumed in daily foods, such as buckwheat, berries, tea and vegetables. An insulin turbidity HTS of a library of 4900 compounds identified rutin as a lead inhibitor of PDI [152]. Rutin does not covalently bind to PDI, because it showed reversible inhibition of PDI in a fluorimetric assay. The K_d value of rutin binding to PDI was 2.8 MM. SAR analysis indicated that the sugar moiety is essential for the activity of the compound. Rutin acts as a relatively specific inhibitor. Although it inhibited PDI by 60% at 30 MM, only negligible inhibition (<10%) was observed for other oxidoreductases sharing the active site CGHC of PDI, including ERp5, ERp57, ERp72, thioredoxin and thioredoxin reductase. Although genetic deletion of PDI is toxic to cells [15,110,111,151], incubation of cultured endothelial cells with rutin at 100 MM for >72 hours showed no toxicity, suggesting that it has poor cell permeability and only targets extracellular PDI. In fact, rutin was tested in two clinical trials. NCT00003365 protocol evaluating the effect of rutin on colon cancer prevention was terminated. The results of protocol NCT01254006 examining the effect of rutin in combination with forskolin, and vitamins B1 and B2 have not yet been released.

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Antibiotics

Bacitracin

The cyclic dodecapeptide antibiotic bacitracin was reported in 1981 to be the first PDI inhibitor [153]. Since then, it has been widely used in studies of the biochemistry of PDI as well as its role in various cellular events and serves as a standard control in testing PDI inhibitors [118]. Natural bacitracin is produced by certain strains of *Bacillus licheniformis* and *Bacillus subtilis* as a mixture of over 22 structurally related peptides [154]. Among them, bacitracin A (**17**) is the major analog [59], and B, F and H are also of relatively high abundance in commercial bacitracin mixtures [155]. MALDI-TOF/TOF MS demonstrated that bacitracin binds PDI through disulfide bond formation between an open thiol form of the bacitracin thiazoline ring and Cys314/345 in the substrate-binding **b**′ domain [155]. Another study confirmed that bacitracin does not inhibit the reductase activity of the isolated catalytic **a** domain [59]. Bacitracin is nonspecific for PDI, because it binds and inhibits other proteins with or without PDI activity. For example, bacitracin (1 mM) completely inhibited the oxidase activity of fibronectin (FN) in the RNase oxidation assay but only 25% in PDI [156]. A recent study extensively examined bacitracin in different PDI activity assays [59], showing that bacitracin did not significantly inhibit the oxidase activity of PDI in the peptide oxidation assay, or the isomerase activity in the BPTI refolding assay. In the protein aggregation assay, bacitracin prevented refolding of rhodanese from aggregation in a PDI-independent manner without a significant effect on its chaperone activity, whereas it inhibited the chaperone activity of BiP, an ER-resident molecular chaperone [59]. However, another study showed that bacitracin (15 MM) inhibited the chaperone activity of PDI without having a substantial effect on substrate aggregation in the absence of PDI in the protein aggregation assay [137]. In the insulin turbidity assay, bacitracin inhibited the reductase activity of PDI as well as *E. coli* DsbC in a dose-dependent manner in the millimolar range *via* competition of substrate binding [59]. Hence, there is a need to re-evaluate the *in vivo* effects of bacitracin and its relation with PDI. The off-target effects of bacitracin should be considered for its future use in PDI studies. Although widely used in research on PDI-associated diseases for decades, bacitracin failed to enter clinical trials primarily because of its poor cell permeability [157] and its nephrotoxicity [158].

Ribostamycin (18)

Produced by *Streptomyces ribosidificus*, ribostamycin is an aminoglycoside antibiotic that is effective against both Gram-positive and Gram-negative strains. Using an affinity column chromatography of proteins in bovine liver, PDI was identified as the main binding protein target of **18** [138]. Ribostamycin was the first reported inhibitor of the chaperone activity of PDI, but has no significant effect on its isomerase activity, indicating that ribostamycin does not bind to the active site. Ribostamycin binds not only PDI, but also other cellular proteins, such as the 16S ribosomal RNA to cause mistranslation [159].

Other antibiotics

Based on the discovery of ribostamycin as a PDI chaperone inhibitor, a panel of other antibiotics was also screened for their ability to inhibit the chaperone activity of PDI [160]. Several were found to bind PDI at remarkably high doses, including vancomycin (K_d = 206 MM), sisomycin (K_d = 392 MM), neomycin (K_d = 872 MM), gentamycin (K_d = 904 MM), kanamycin (K_d = 1.05 mM) and streptomycin (K_d = 1.25 mM). They all inhibited the chaperone activity of PDI. In particular, vancomycin and sisomycin sufficiently inhibited the chaperone activity at a 100:1 molar ratio of antibiotic to PDI. It is still unclear whether these compounds bind and inhibit PDI *in vivo* or if PDI inhibition contributes to their antibiotic actions.

Hormones

Estrogens

At a 1-MM concentration, several estrogens have been shown to inhibit the reductase activity of PDI by over 30%, including estrone (E_1, **19**, 56%), 17β-estradiol (E_2, **20**, 55%), diethylstilbestrol (DES, 45%) and estriol (E_3, 38%). In addition, E_1 and E_2 also inhibited the isomerase activity [161]. No significant inhibition of the chaperone activity was observed [137]. Interestingly, amino acid sequence segments in PDI have high similarity with the estrogen-binding domain in the estrogen receptor (ER), but not with the steroid domains of the progesterone and glucocorticoid receptors or with thioredoxin [161]. It was further confirmed that PDI has one E_2 binding site (K_d = 2.1 ± 0.5 MM) that is distinct from the peptide/protein- and the bacitracin-binding sites [137]. A recent study proposed an E_2-binding model in which the E_2 bound to a hydrophobic pocket comprising mainly the **b**′ domain and partially the **b** domain through the formation of a hydrogen bond between the 3-hydroxyl group of E_2 and His 256 of PDI [162]. In addition, E_2, 17α-E_2 and DES are also potent inhibitors of somatostatin binding to PDI [163].

Thyroid hormones

3,3′,5-Triiodo-L-thyronine is also known as triiodothyronine or T_3 (**21**). It is a thyroid hormone that binds the nuclear receptor c-erbA and has important roles in numerous biological processes, such as cell growth, development and differentiation, energy metabolism, and regulation of body temperature and heart rate [164]. Studies using an affinity-labeling reagent *N*-bromoacetyl-3,3′,5-[^{125}I]triiodo-L-thyronine (BrAc[^{125}I]T_3) identified a 55-kDa polypeptide as a major T_3-binding protein (T_3BP) that was further confirmed to be PDI [165–167]. At equilibrium, T_3 binds PDI at two independent sites [137,168]. Guthapfel *et al.* showed that, whereas the first binding site exhibited high affinity and could be saturated at near physiological T_3 concentrations with a K_d of 21 nM, it had a remarkably low B_{max} (1.8 mmol T_3/mol PDI monomer), implying that T_3 binding is mainly nonspecific; the second binding site had low affinity and was unsaturated at up to 100 MM [168]. Later, Primm *et al.* reported that the two T_3 binding sites in PDI had comparable affinity, with K_d values of 4.3 ± 1.4 MM [137].

PDI also binds a wide variety of T_3 analogs, including D-T_3, 3,3′,5-triiodothyropropionate, 3,3′,5-triiodothyroacetate, 3,5-diiodo-L-tyrosine, L-thyronine, and 3,5-diiodo-L-thyronine. However, these analogs did not show significant inhibition of PDI in mediating RNase refolding; neither did they have any effects on the chaperone activity [137]. By contrast, Guthapfel *et al.* showed that T_3 inhibited PDI in mediating RNase refolding under similar conditions, with an inhibition constant K_i of 1.3 ± 0.5 MM [168]. The ability of T_3 to inhibit PDI (IC_{50} = 3.49 MM) was further supported by Hirol *et al.* [163]. The physiological relevance of the inhibition of PDI redox activity is unclear because T_3 is in

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nanomolar concentration in the cells but has a high K_i for PDI [168]. AT_3 (**22**), an *N*-acetylated form of T_3, inhibited PDI-catalyzed reductive cleavage of the cell surface-bound [^{125}I]tyramine-SS-poly(D-lysine) (IC_{50} = 70 MM) and HIV-1 entry into host cells [37].

Xenoestrogens

Xenoestrogens are chemical compounds that imitate estrogen. Bisphenol A [2,2-bis-(4-hydroxyphenyl) propane; BPA; **23**] is a synthetic versatile industrial monomer for plastic manufacturing [169,170]. As a hormone-disrupting chemical, it strongly binds to, and activates, estrogen-related receptor γ (ERR-γ) (K_d = 5.5 nM) [171]. PDI was identified as another major target in a screen for BPA-binding proteins in the rat brain [163]. BPA binds to both recombinant rat (K_d = 22.6 ± 6.6 MM) and human PDI (K_d = 17.51 ± 3.93 MM). Although BPA has a lower affinity to the hormone-binding site on PDI compared with E_2 (K_d = 2.1 ± 0.5 MM [137]) and T_3 (K_d = 4.3 ± 1.4 MM [137]), it is a competitive inhibitor of PDI binding to E_2 and T_3. An analog, tetrachlorobisphenol A (**24**), was reported to be the most potent inhibitor of T_3 binding to PDI with an IC_{50} value of 0.2 MM, 100-times lower than that of BPA [172]. The BPA-binding site is located within the **a** and **b**′ domains, and the **b**′ domain contributes to its inhibition of PDI [173].

Concluding remarks

Structure and domain architecture, biochemical redox reactions, physiological roles and the involvement of PDI in multiple diseases have been extensively studied during the past decades. Dysregulation of PDI gene expression, post-translational modification or enzymatic activity results in various human diseases. However, only recently has the relation between PDI and cancer been documented. PDI is highly expressed in select cancer types, supports tumor growth and is associated with clinical outcomes. Therefore, PDI is a potential drug target for cancer therapy.

Currently, there are no PDI inhibitors in the clinic. The exact functions of PDI in many diseases need further characterization before clinical trials can be conducted. In addition, there has been a lack of potent and selective PDI inhibitors for clinical development. Bacitracin, the first PDI inhibitor, failed to enter clinical trials because of its off-target toxicity and weak cell permeability. The development of robust PDI activity assays has led to the recent discoveries of a series of novel PDI inhibitors, such as the irreversible inhibitors PACMA 31, 16F16 and RB-11-*ca*, and the reversible inhibitors juniferdin and rutin. These novel PDI inhibitors have proven potent in disease models of cancer, HD, HIV-1 infection and thrombosis, and are useful tools for further exploring PDI biology. Although further evaluation of their specificity for PDI and their off-target effects is needed, they can serve as leads for further optimization to select viable candidates for clinical studies.

PDI function is important for normal cellular homeostasis and, as such, there might be concerns for developing PDI inhibitors as a therapeutic strategy. Under normal physiological conditions, PDI expression is tightly regulated. However, cancer cells require higher levels of PDI to cope with significant ER stress and a global increase in protein synthesis to sustain rapid proliferation. Increased protein synthesis leads to an abundance of misfolded proteins in the ER that need to be refolded by PDI. As such, cancer cells are more vulnerable to PDI inhibition than are normal cells. Moreover, because of this increased energy demand, cancer cells produce high levels of reactive oxygen species (ROS), exacerbating ER stress. This differential activity of PDI between normal and cancer cells can be targeted by small-molecule drugs. In fact, recent studies using PACMAs clearly demonstrated that PDI inhibitors: (i) induce ROS; (ii) accumulate in tumor tissues; (iii) show *in vivo* efficacy in mice xenografts; and (iv) do not exhibit toxicity in whole animal. Therefore, we believe that PDI is an important target for select cancers.

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