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Research Article

Synthesis and Experimental Validation of New PDI Inhibitors with Antiproliferative Activity

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Protein disulfide isomerase (PDI) is a member of the thioredoxin superfamily of redox enzymes. PDI is a multifunctional protein that catalyzes disulfide bond formation, cleavage, and rearrangement in unfolded or misfolded proteins and functions as a chaperone in the endoplasmic reticulum. Besides acting as a protein folding catalyst, several evidences have suggested that PDI can bind small molecules containing, for example, a phenolic structure, which includes the estrogenic one. Increasing studies indicate that PDI is involved in both physiology and pathophysiology of cells and tissues and is involved in the survival and proliferation of different cancers. Propionic acid carbamoyl methyl amides (PACMAs) showed anticancer activity in human ovarian cancer, both in vitro and in vivo, by inhibiting PDI. The inhibition of PDI's activity may have a therapeutic role, in various diseases, including cancer. In the present study, we designed and synthesized a diversified small library of compounds with the aim of identifying a new class of PDI inhibitors. Most of synthesized compounds showed a good inhibitory potency against PDI and particularly 4-methyl substituted 2,6-di-*tert*-butylphenol derivatives (**8–10**) presented an antiproliferative activity in a wide panel of human cancer cell lines, including ovarian ones.

1. Introduction

Protein disulfide isomerase (PDI) belongs to the thioredoxin superfamily of oxidoreductases and is the founding member of the PDI family, consisting of 20 related mammalian proteins. All members of this family share the thioredoxin-like domain structure, characterized by the $\beta\alpha\beta\alpha\beta\alpha\beta\beta\alpha$ fold [1]. PDI (Figure 1) is a soluble 55-kDa protein with four tandem thioredoxin-like domains, namely, **a**, **b**, **b**′, and **a**′. The homologous **a** and **a**′ domains contain the catalytically active site of PDI, which consists of a CGHC (Cys-Gly-His-Cys) motif. The cysteines can exist either in an intramolecular disulfide (oxidized PDI) or in the dithiol form (reduced PDI) and interact with newly synthesized proteins, mediating thiol-disulfide exchanges. The **b** and **b**′ domains link the active site domains and assist in the binding of protein substrates. The **b**′ domain was identified as the chaperone domain, by NMR and X-ray crystallography. Between the **b**′ and the **a**′ domains there is a short **x**-linker interdomain region, responsible for the U-shape structure of PDI. The acidic **C**-terminus of PDI is followed by an endoplasmic reticulum (ER) retrieval signal, KDEL [1–3]. A recent crystal structure of yeast PDI reveals that this protein has a high flexibility, essential for its enzymatic activity, in vitro and in vivo [4]. Several studies have suggested that PDI can function as an intracellular binding protein. In human breast cancer cells, it has been shown that PDI can modulate the intracellular level of 17β-estradiol (E_2), increase its hormonal activity, and reduce its metabolic availability [5]. Although the E_2-binding site structure of human PDI is still not known, it is located to a hydrophobic pocket between the **b** and the **b**′ domains [6].

PDI is a multifunctional protein, acting as a folding enzyme by catalyzing the formation (oxidation), cleavage (reduction), and rearrangement (isomerization) of the disulfide bonds in unfolded or misfolded proteins [7]. It has also been proposed to function as a molecular chaperone in the refolding of denatured protein in vitro [8, 9]. Depending on

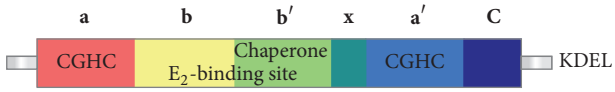


Figure 1: Domain structure of PDI.

its initial concentration, PDI exhibits both chaperone and antichaperone activities [10]. PDI is ubiquitously expressed but is primarily localized in the ER of eukaryotic cells, where it maintains an oxidant environment, contributing to ER homeostasis [11–14]. Despite the presence of the ER retention sequence, PDI has been localized in diverse subcellular places, such as cell surface, cytosol, mitochondria, and extracellular matrix [15]. The mechanism by which PDI gets away from the ER is still unclear. Originally, PDI was considered necessary to maintain healthy cells and tissues, while recent studies indicate that this protein is involved in both their physiology and pathophysiology. In particular, PDI has a protective effect in neurodegenerative conditions and cardiovascular diseases. PDI is also implicated in mediating the entry of pathogens during infectious diseases [3]. In many different cancer types, including brain, lymphoma, kidney, ovarian, prostate, lung, and male germ cell tumors, PDI is highly expressed and upregulated [16]. Although more than four decades of studies exist on PDI, its role in cancer progression is not well understood yet. Acting as an ER chaperone, PDI might play a role in the survival of metastatic breast cancers [17]. Suppression of apoptosis by PDI has been proposed as possible mechanism for tumor growth and metastasis. In fact, knockdown of PDI induces cytotoxicity in human breast cancer and activates apoptotic signaling in MCF-7 cells [18]. Cell surface PDI is also associated with cancer progression and migration of glioma cells [19]. Since PDI is involved in many important cell functions, among which is supporting the growth and invasion of various cancer types; in the past years PDI has received considerable attention as a potential drug target, especially for cancer therapy [16]. The modulation of PDI's activity with function-specific inhibitors may have a therapeutic role in various diseases including cancer. A wide range of drugs, including antibiotics, estrogen, polyphenols, and heterocyclic compounds, have been found to inhibit PDI [20–23]. Propionic acid carbamoyl methyl amides (PACMAs) can inhibit irreversibly PDI, by forming a covalent bond (C-S) with the cysteines in the active site. In particular, PACMA 31 showed anticancer activity in human ovarian cancer, both in vitro and in vivo [24]. As an important considerable drug target, PDI has attracted our interest. With the aim of identifying new PDI inhibitors, considered an attracting approach for the treatment of cancer, we screened a small library of compounds using the insulin turbidimetry assay [16]. Lactone frame was identified as a potential inhibitor of PDI activity, so we modified and optimized the identified scaffold to improve upon its potency (Figure 2).

Based on the structure of known PDI modulators, bearing specific substituents in critical positions, we also designed and synthesized a new class of compounds, 4-methyl substituted 2,6-di-*tert*-butylphenol derivatives. We kept some structural similarities with the known ligands, with the aim of enriching and diversifying our small library (Figure 3).

Table 1: Inhibition of PDI reductase activity (expressed in %) by the synthesized compounds and IC_{50} values of the most potent ones.

Compound	PDI reductase activity inhibition (%)	IC_{50} (μM)
PACMA 31	80.5	**10.0 ± 0.10**
1	57.2	
2	35.9	
3	**80.7**	**>30**
4	**93.3**	**>30**
5	**87.6**	**>30**
6	64.6	
7	22.6	
8	**98.5**	**1.51 ± 0.09**
9	**97.5**	**1.69 ± 0.22**
10	**82.3**	**4.17 ± 0.40**

Each value represents the average of three independent experiments ± SD.

In this study, we investigated and reported the inhibitory effects of all synthesized compounds against the reductase activity of PDI. Compounds **3–5** and **8–10** highly inhibited the PDI reductase activity (see Table 1). In addition, the cytotoxicity of all synthesized compounds was also evaluated against different cancer cell lines, including ovarian tumor (see Table 2). Compounds **8–10** showed a good potency again. These results suggest that the new 4-methyl substituted 2,6-di-*tert*-butylphenol derivatives are potential antiproliferative agents, targeting PDI.

2. Material and Methods

2.1. Chemistry. All reagents were purchased from Sigma-Aldrich and used without further purification. Solvents were dried and distilled according to conventional procedures. Reactions were carried out under nitrogen (N_2) atmosphere, monitored by thin layer chromatography (TLC) on silica gel plates (Merck 60F254, 0.2 mm) and visualized by UV light at 254 and 366 nm of wavelength. Organic solutions were dried over anhydrous $NaSO_4$ and evaporated on a rotary evaporator under reduced pressure. Final compounds were purified by flash chromatography columns, on silica gel (Merck, 60, 230–400 mesh, 0.040–0.063 mm). Melting points were obtained by a GallenKamp21374 apparatus and were uncorrected. ^{1}H-NMR spectra were recorded on a Bruker 300 MHz spectrometer. Chemical shift (δ) was reported in ppm, using tetramethylsilane (TMS) as the internal reference standard. Multiplicities, coupling constants reported as a J value in Hertz (Hz), and number of protons are indicated parenthetically. Mass spectra data were determined after electron impact ionization at 70 eV with HP 5973 MS spectrometer. Yields refer to purified products and are not optimized.

2.1.1. Synthetic Procedures. As known in literature [25], the indole and pyrrole lactones (**1–5**) were synthesized as

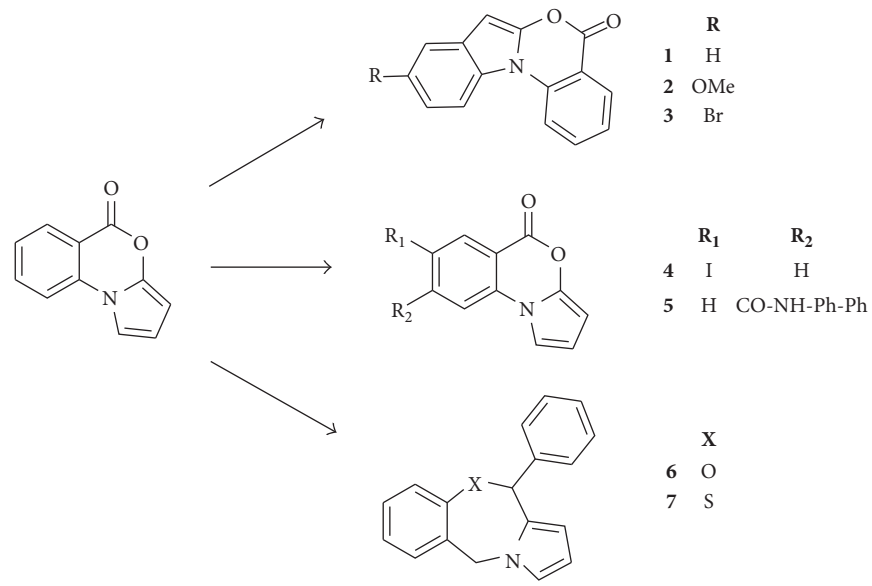


Figure 2: Chemical modifications made to the identified scaffold.

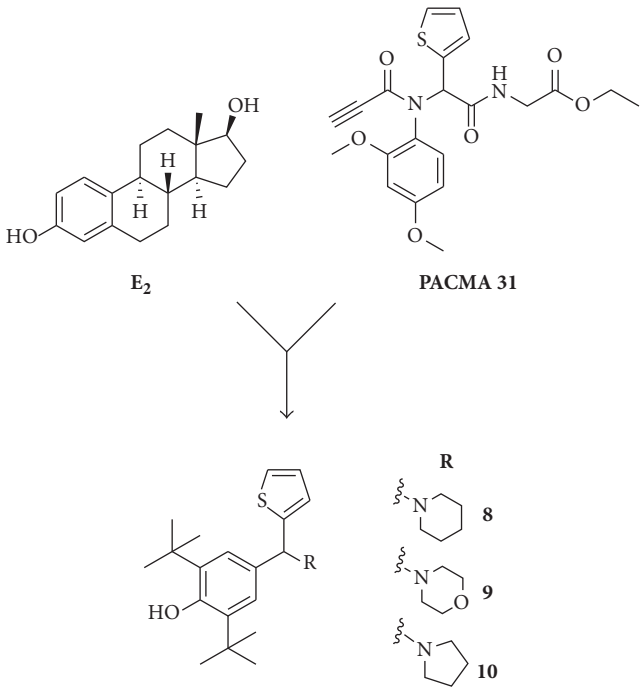


Figure 3: Design of a new class of PDI inhibitors.

depicted in Scheme 1. The further formation of amide **5** was obtained following a classical synthetic method.

Scheme 2 represents the synthetic pathway for 11-phenyl-5H,11H-benzo[f]pyrrolo[2,1-c][1,4]oxazepine (**6**). The starting (1-(2-fluorobenzyl)-1*H*-pyrrol-2-yl)(phenyl)methanol was synthesized following the procedure described by Garofalo et al. [26]. Finally, the cyclization of compound **6** was obtained, according to the reaction conditions described by Effland and Davis [27].

The synthesis of 11-phenyl-5H,11H-benzo[f]pyrrolo[2,1-c][1,4]thiazepine (**7**) was realized following a similar procedure [26].

The 4-methyl substituted 2,6-di-*tert*-butylphenol derivatives (**8–10**) were synthesized by a catalyst-free reaction between thiophene-2-carbaldehyde, a cyclic amine, and 2,6-di-*tert*-butylphenol (Scheme 3).

*2.1.2. General Procedure for the Synthesis of 5H-Benzo[4,5,1,3]oxazino[3,2-a]indol-5-one Derivatives (**1**–**3**) and 7-iodo-5H-benzo[d]pyrrolo[2,1-b][1,3]oxazin-5-one (**4**).* The synthesis of the aforementioned compounds was obtained following the same procedure. Characterization data are in agreement with published data [25].

*2.1.3. 5H-Benzo[4,5,1,3]oxazino[3,2-a]indol-5-one (**1**).* Brown solid (49% yield); mp: 123-124°C; **^{1}H NMR**: known compound [25].

*2.1.4. 9-Methoxy-5H-benzo[4,5][1,3]oxazino[3,2-a]indol-5-one (**2**).* Amorphous yellow solid (32% yield); **^{1}H NMR ($CDCl_3$) δ**: 8.28 (dd, 1H, J = 1.4, 5.1 Hz); 8.09 (d, 1H, J = 8.3 Hz); 7.91–7.78 (m, 1H); 7.37–7.31 (m, 2H); 7.11 (t, 1H); 6.94 (dd, 1H, J = 2.6, 4.2 Hz); 6.16 (s, 1H); 3.39 (s, 3H); **MS (EI. 70 eV)**: m/z 266 $[M + H]^+$.

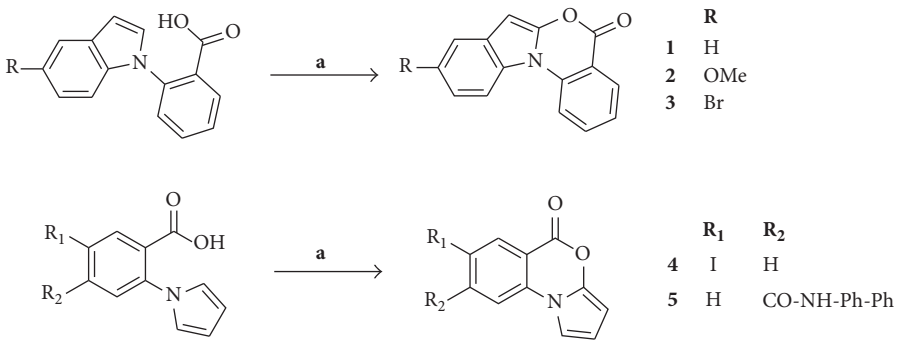
*2.1.5. 9-Bromo-5H-benzo[4,5][1,3]oxazino[3,2-a]indol-5-one (**3**).* Beige solid (54% yield); mp: 162–163°C; **^{1}HNMR**: known compound [25].

*2.1.6. 7-Iodo-5H-benzo[d]pyrrolo[2,1-b][1,3]oxazin-5-one (**4**).* Yellow solid (28% yield); mp: 128°C; **^{1}H NMR ($CDCl_3$) δ**: 8.52 (s, 1H); 8.03 (dd, 1H, J = 2.1, 8.6 Hz); 7.25 (dd, 1H, J = 2.3, 8.6 Hz); 6.97 (s, 1H); 6.42 (d, 1H, J = 2.3 Hz); 5.71 (s, 1H); **MS (EI. 70 eV)**: m/z 312 $[M + H]^+$.

Table 2: Cytotoxicity of the synthesized compounds against a panel of human cancer cell lines.

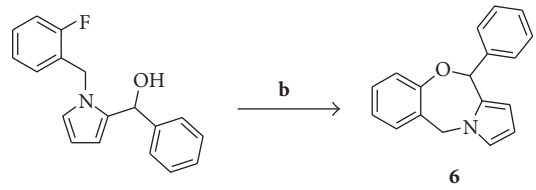
Compound	IC_{50} (μM)						
	MCF-7	MDA-MB 231	U87G	OVCAR-8	Mia PaCa-2	HCT116 $p53^{+/+}$	SKOV3 WT
1	>50	>50	>50	nt	nt	nt	nt
2	>50	>50	>50	nt	nt	nt	nt
3	>50	>50	>50	nt	nt	nt	nt
4	>50	>50	>50	nt	nt	nt	nt
5	>50	42.4 ± 0.6	45.1 ± 0.9	nt	nt	nt	nt
6	>50	>50	>50	nt	nt	nt	nt
7	>50	>50	>50	nt	nt	nt	nt
8	5.8 ± 0.2	8.7 ± 0.1	8.5 ± 0.3	3.4 ± 0.4	5.0 ± 0.3	11.1 ± 0.5	29.2 ± 0.5
9	6.1 ± 0.4	9.7 ± 0.3	8.8 ± 0.3	3.8 ± 0.6	5.1 ± 0.2	13.3 ± 0.6	28.1 ± 0.2
10	7.9 ± 0.9	9.7 ± 0.3	18.3 ± 0.8	6.9 ± 0.9	7.0 ± 0.9	19.3 ± 0.6	40.9 ± 0.6

Each value represents the average of three independent experiments ± SD.



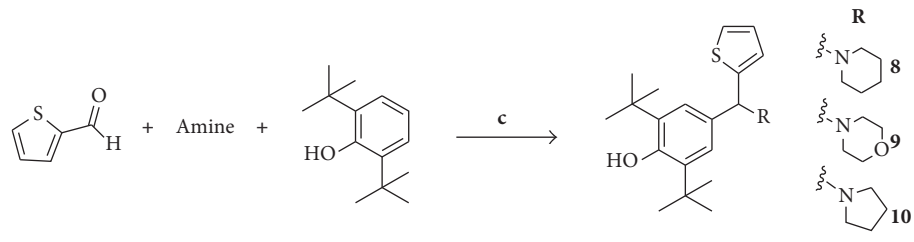
Reagent and conditions.
(a) MnO_2, toluene, reflux

Scheme 1



Reagent and conditions.
(b) NaH, DMF/C_6H_6

Scheme 2



Reagent and conditions.
(c) Dry toluene, reflux

Scheme 3

*2.1.7. General Procedure for the Synthesis of N-([1,1′-Biphenyl]-4-methyl)-5-oxo-5H-benzo[d]pyrrolo[2,-b][1,3]oxazine-8-carboxamine (**5**).* The starting 5-oxo-5*H*-benzo[*d*]pyrrolo[2,1-*b*][1,3]oxazine-8-carboxylic acid for the synthesis of compound **5** was obtained following the procedure mentioned above [25]. A solution of 5-oxo-5*H*-benzo[*d*]pyrrolo[2,1-*b*][1,3]oxazine-8-carboxylic acid (0.044 mmol; 1 eq.) and PCl_5 (0.053 mmol; 1.2 eq.) in dry toluene (0.1 mL) was stirred at rt, for 3 h. To the obtained acyl chloride solution (0.04 mmol; 1 eq.), Et_3N (0.08 mmol; 2 eq.), and [1,1′-biphenyl]-4-ylmethanamine (0.06 mmol; 1.5 eq.) were added and the reaction mixture was kept at rt for 24 h. Then, the solvent was evaporated under vacuum and the residue was solubilized in chloroform and washed with brine. The organic layer was dried over anhydrous $NaSO_4$, filtered, and concentrated under vacuum. The pure compound was crystalized from chloroform.

*2.1.8. N-([1,1′-Biphenyl]-4-methyl)-5-oxo-5H-benzo[d]pyrrolo[2,-b][1,3]oxazine-8-carboxamine (**5**).* Yellow solid (8% yield); mp: 165°C; **^{1}HNMR ($CDCl_3$) δ**: 7.70 (s, 1H); 7.60–7.50 (m, 3H); 7.50–7.30 (m, 9H); 7.15 (dd, 1H, J = 0.8, 2.6 Hz); 6.84 (dd, 1H, J = 0.8, 3.7 Hz); 6.37 (dd, 1H, J = 2.5, 3.7 Hz); 4.71 (d, 2H, J = 5.6 Hz); **MS (EI. 70 eV)**: m/z 386 $[M + H]^+$.

*2.1.9. General Procedure for the Synthesis of 11-Phenyl-5H,11H-benzo[f]pyrrolo[2,1-c][1,4]oxazepine (**6**).* The synthesis of the aforementioned compound was realized following the procedure described by Garofalo et al. [26] and by Effland and Davis [27].

*2.1.10. 11-Phenyl-5H,11H-benzo[f]pyrrolo[2,1-c][1,4]oxazepine (**6**).* Brown solid (41% yield); mp: 124–126°C; **^{1}HNMR (DMSO) δ**: 7.60–7.45 (m, 5H); 7.22 (s, 1H); 6.97–6.83 (m, 4H); 6.80 (s, 1H); 5.90–5.78 (m, 1H); 5.45 (s, 1H); 5.08–4.98 (d, 1H, J = 15.6 Hz); **MS (EI. 70 eV)**: m/z 262 $[M + H]^+$.

*2.1.11. General Procedure for the Synthesis of 11-phenyl-5H,11H-benzo[f]pyrrolo[2,1-c][1,4]thiazepine (**7**).* The title compound was synthesized following the procedure described by Garofalo et al. [26].

*2.1.12. 11-Phenyl-5H,11H-benzo[f]pyrrolo[2,1-c][1,4]thiazepine (**7**).* Beige solid (46% yield); mp: 126–128°C; **^{1}HNMR**: known compound [26].

*2.1.13. General Procedure for the Synthesis of 4-Methyl Substituted 2,6-di-tert-butylphenol Derivatives (**8–10**).* A stirred solution of thiophene-2-carbaldehyde (1.05 eq.) and secondary heterocyclic amine (2.31 eq.) in dry toluene was refluxed (110°C), under N_2 atmosphere, overnight. Then, the reaction mixture was cooled down to 90°C and a solution of 2,6-di-*tert*-butylphenol in dry toluene was added, drop by drop. The reaction mixture was refluxed again, under N_2 atmosphere, overnight. The solvent was evaporated under vacuum; methanol was added to the reaction residue and was stirred and heated at 60°C, for 30 minutes. The obtained precipitate was collected by filtration and purified by crystallization from methanol or by chromatographic column on silica gel (n-hexane/ethyl acetate, different ratios, as eluent).

*2.1.14. 2,6-Di-tert-butyl-4-(piperidin-1-yl(thiophen-2-yl)methyl)phenol (**8**).* Yellow solid (33% yield); mp: 133–34°C; **^{1}HNMR (DMSO) δ**: 7.39–7.36 (m, 1H); 7.14 (s, 2H); 6.92–6.87 (m, 2H); 6.85 (bs, 1H); 4.55 (s, 1H); 2.25 (s, 4H); 1.52 (s, 6H); 1.35 (s, 18H); **^{13}CNMR ($CDCl_3$) δ**: 24.66, 26.26 (x2C), 30.28 (x6C), 34.34 (x2C), 52.26 (x2C), 71.31, 124.40, 124.93, 125.06 (x2C), 125.95, 131.40, 135.15, 148.50, 148.52, 152.57. **HRMS**: m/z 384 $[M - H]^-$, 301 [M - piperidinyl]$^-$.

*2.1.15. 2,6-Di-tert-butyl-4-(morpholino(thiophen-2-yl)methyl)phenol (**9**).* Reddish orange oil (46% yield); **^{1}HNMR (DMSO) δ**: 7.99 (d, 1H, J = 3.0 Hz); 7.85 (s, 1H); 7.68 (s, 1H); 7.58 (d, 1H, J = 3.0 Hz); 7.28–7.24 (m, 1H); 7.18–7.15 (m, 1H); 6.87 (s, 1H); 4.01–3.98 (m, 4H); 2.10–1.98 (m, 4H); 1.30 (s, 9H); 1.24 (s, 9H); **^{13}CNMR ($CDCl_3$) δ**: 31.60 (x6C), 34.72 (x2C), 52.13 (x2C), 66.99 (x2C), 71.65, 125.01 (x2C), 125.51, 126.81, 127.04, 127.96, 136.13, 148.50, 148.52, 152.57. **HRMS**: m/z 301 [M - morpholino]$^-$.

*2.1.16. 2,6-Di-tert-butyl-4-(pyrrolidin-1-yl(thiophen-2-yl)methyl)phenol (**10**).* Brown oil (46% yield); **^{1}HNMR (DMSO) δ**: 8.0 (d, 1H, J = 3.0 Hz); 7.84 (s, 1H); 7.67 (s, 1H); 7.57 (d, 1H J = 3.0 Hz); 7.29–7.24 (m, 1H); 7.20–7.16 (m, 1H); 7.13 (s, 1H); 1.48 (s, 8H); 1.30 (s, 9H); 1.24 (s, 9H); **HRMS**: m/z 372 $[M + H]^+$.

2.2. Biology

2.2.1. Cell Culture. All used tumor cell lines (MCF-7, MDA-MB 231, U87G, OVCAR-8, Mia PaCa-2, HCT116 $p53^{+/+}$, and SKOV3 WT) were grown at 37°C in a humidified incubator with 5% CO_2, in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). The culture medium was changed twice a week.

2.2.2. Treatment with Synthesized Compounds and MTT Colorimetric Assay. Cells were seeded in 180 μL medium in 96-well plates and incubated at 37°C. After overnight attachment, cells were treated with the compounds (final concentration from 30 μM to 0.12 μM, in duplicate). DMSO was used as negative control. Plates were incubated in the presence of compounds for 72 h and then cytotoxicity was measured with colorimetric assay, based on the use of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). 20 μL of MTT solution in DPBS (Dulbecco's phosphate-buffered saline) (3 mg/mL) was added and plates were incubated for 3-4 h. Mitochondrial reductase enzymes in viable cells reduce the yellow tetrazolium MTT in its formazan, which has a purple color when dissolved in DMSO. Media were finally replaced with 125 μL of DMSO and cell viability was determined by measuring absorbance on a multiwell scanning spectrophotometer, using a wavelength of 570 nm. The IC_{50} (half-maximum inhibitory concentration) values were determined using GraphPad Prism and expressed

as average of three independent experiments ± standard deviation (SD) value.

2.2.3. Colony Formation Assay. Cells were seeded in 180 μL medium in 96-well plates and allowed to attach overnight at 37°C. Then, cells were treated with the compounds (final concentrations 10 μM, 5 μM, and 1 μM, in duplicate). DMSO was used as negative control. After 24 h treatment, compound-containing medium was carefully removed and replaced with 200 μL of fresh medium supplemented with 10% FBS. Cells were kept in culture at 37°C until visible colonies were formed in control wells. Then, medium was removed and colonies were stained with 50 μL of 0.5% crystal violet solution for 30 min at rt. Finally, colonies were washed 3 times with 100 μL of water to remove excessive stain and allowed to dry overnight under hood. Colonies were imaged with Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE).

2.2.4. Scratch Wound Healing Assay. Cells were seeded in 96-well plates, at a high density to have 70–80% confluency after overnight incubation at 37°C, and then starved for 24 h. Using 200 μL tip, every well was gently and slowly scratched and washed twice with DPBS. Fresh medium, supplemented with 5% FBS and containing compounds (final concentrations 10 μM and 1 μM), was added and cells were grown at 37°C, until the wound in control wells was closed. Finally, medium was removed and cells were stained with 50 μL of 0.5% crystal violet solution for 30 min at rt. Finally, cells were washed 3 times with 100 μL of water to remove excessive stain and allowed to dry overnight under hood. Wells were imaged with Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE).

2.2.5. Measurement of PDI Reductase Activity. Reductase activity was assayed by measuring the PDI-catalyzed reduction of insulin in the presence of compounds (final concentrations from 30 μM to 0.33 μM, in duplicate) in a 100 μL reaction volume containing 0.5 mM DTT, 100 mM sodium phosphate buffer, pH 7.0, 0.5 mM DTT, 2 mM EDTA, 0.13 mM bovine insulin (Sigma), and 1.3 μM purified human PDI. Then, the aggregation of reduced insulin chains was measured spectrophotometrically at 650 nm. The IC_{50} values were determined using GraphPad Prism and expressed as average of three independent experiments ± standard deviation value.

3. Results and Discussion

Using an insulin-based turbidimetric assay [16], we screened a small library of various chemical scaffolds to identify small molecules suitable to inhibit PDI. The assay identified the lactone as an inhibitory compound of PDI. Heterocycles are widely studied because of their well-known biological activities, among these indole and pyrrole frames. Due to their attractive importance, we designed and synthesized some indole lactones, variously substituted with both electron-donor and attractor groups. We also synthesized pyrrole lactones, bearing different substituents, such as the amide moiety. Finally, we wanted to enlarge the central six-membered ring of the lactone structure into a seven-membered heterocycle, containing either oxygen or sulfur atom and bearing an appended phenyl ring. All the chemical modifications were investigated with the aim of better understanding whether the new synthesized molecules could improve the potency of the lead compound, against PDI.

Recent studies have suggested that PDI can bind small molecules containing, for example, a phenolic structure, which includes endogenous hormones like estrogen. In particular, it has been demonstrated that estradiol fits into a hydrophobic pocket placed between the **b** and the **b′** domains of PDI and its hydroxyl group on C3 interacts with the histidine residue present in position 256, through formation of an H-bond (Figure 4) [6]. A class of PACMAs has exhibited cytotoxic effect in a panel of human cancer cell lines, especially against the resistant ovarian cancer cell line NCI/ADR-RES [28]. A series of PACMAs, electron-deficient compounds, were identified as potent PDI inhibitors, able to react irreversibly with the thiol groups of cysteines in the catalytically active site of PDI, forming covalent adducts. In particular, PACMA 31 showed an anticancer activity in human ovarian cancer, both in vitro and in vivo. It has been suggested that the electrophilic alkyne group of PACMA 31 is essential for its cytotoxicity [24].

Based on the structure of E_2 and PACMA 31, we designed a new class of 4-methyl substituted 2,6-di-*tert*-butylphenol derivatives, simplifying the structure of the known modulators. The phenolic frame, the hydroxyl group, and the appended thiophene ring were kept in the same position of E_2 and PACMA 31, substituting the propionyl group with a *tert*-butyl and the amide moiety with a cyclic amine (Figure 5).

PDI catalyzes the reduction of insulin in the presence of dithiothreitol (DTT); the reduced insulin chains aggregate and turbidimetry is monitored using a spectrophotometer. Reductase activity of PDI was, then, assayed by measuring the reduction of insulin, in the presence of compounds. Firstly, all synthesized compounds were screened in the insulin turbidimetry assay, at a single concentration (100 μM). As shown in Table 1, three lactone derivatives **3–5** and all 4-methyl substituted 2,6-di-tert-butylphenol derivatives **8–10** showed the best inhibition of PDI reductase activity (inhibition > 80%).

Dose-response curves and IC_{50} values were calculated for the most potent compounds. Compounds **8–10** inhibited PDI in a dose-dependent manner; in particular, piperidine and morpholine derivatives showed an IC_{50} lower than 2 μM (Figure 6).

These results suggest that although lactone frame could moderately inhibit the reductase activity of PDI, 4-methyl substituted 2,6-di-tert-butylphenol structure is better suited as potential PDI inhibitor.

Increased levels of PDI have been found in a variety of human cancer cell lines, including ovarian ones [16]. Increasing evidence suggests that PDI supports the survival and proliferation of several cancer types. In particular, it has been showed that PDI activity is essential for human ovarian cancer cells [24]. To demonstrate the PDI inhibitory activity of the synthesized compounds and verify a correlation between the inhibition of both PDI and cancer cell growth, we

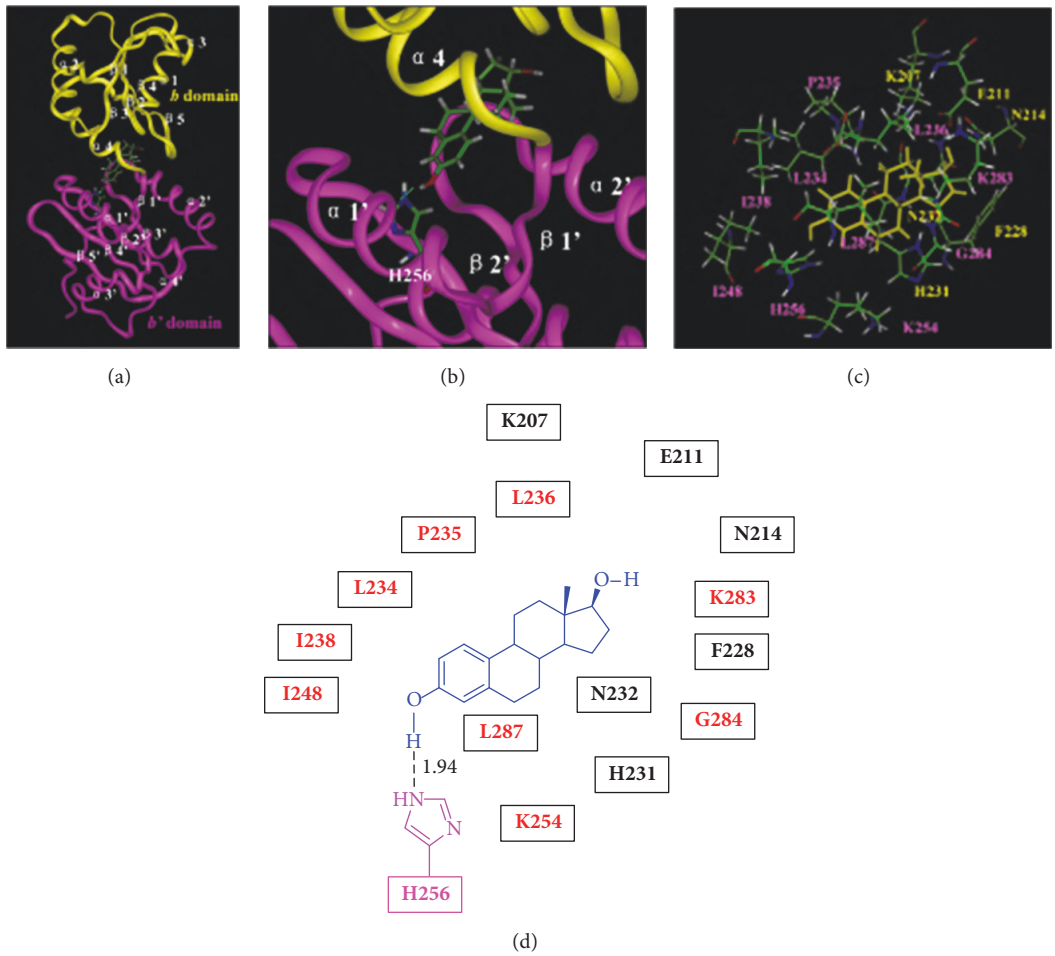


FIGURE 4: Docking analysis of the binding interaction of E_2 inside human PDI b-b′ fragment (Figure by Fu et al. [6]).

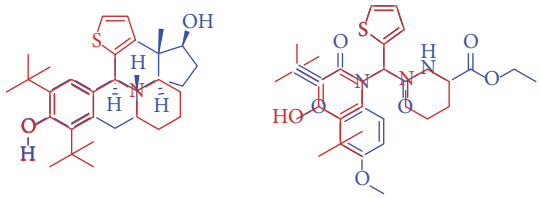


FIGURE 5: Superimposition of new 4-methyl substituted 2,6-di-tert-butylphenol derivatives and known modulators.

determined the cytotoxicity of all derivatives against a panel of human cancer cell lines (Table 2).

We chose seven different human cancer cell lines, which include breast, ovarian, brain, colorectal, and pancreatic cancer and investigated a selectivity of synthesized compounds against a specific cancer type. The synthesized heterocyclic compounds did not show a potent antiproliferative effect in all tested cell lines; only compound **5** exhibited a moderate cytotoxicity against breast and brain cancer cells. On the other hand, the new derivatives **8–10** were cytotoxic in all the chosen cell lines.

It is important to note that they showed the best antiproliferative activity in the human ovarian cancer cells with IC_{50} values lower than 10 μM. Additionally, the same compounds showed the capability to inhibit the formation of colonies and the migration of human ovarian cancer cells (Figure 7).

These results confirm PDI as preferred target of the new 4-methyl substituted 2,6-di-tert-butylphenol derivatives. Although they could bind PDI in a similar way of E_2, by forming an H-bond with a specific residue in the protein structure, additional experiments are ongoing to verify the site of binding and the mode of action of 4-methyl substituted 2,6-di-tert-butylphenol derivatives.

4. Conclusions

Recently, PDI has been considered an attracting drug target especially for cancer therapy. With this purpose, we designed and synthesized a diversified small library of compounds and evaluated their inhibitory effects against the reductase activity of PDI. The 4-methyl substituted 2,6-di-tert-butylphenol derivatives, with an aliphatic cyclic amine, exhibited the best inhibition with IC_{50} values lower than 2 μM (**8** and **9**) and 5 μM (**10**). The possible involvement of PDI inhibition

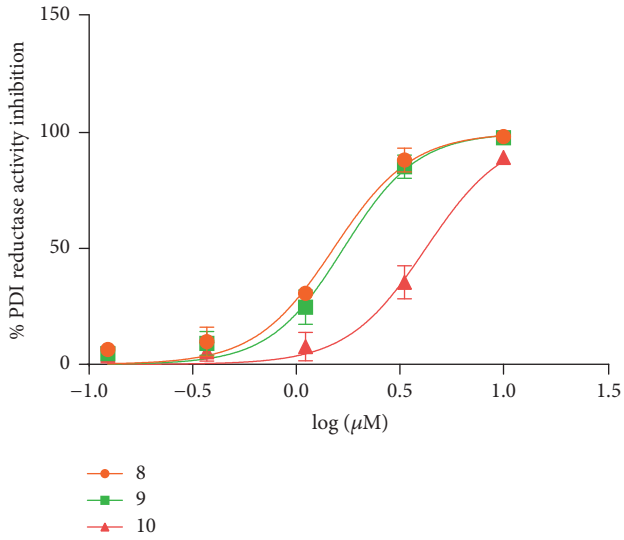


Figure 6: Dose-response curves of the most potent compounds.

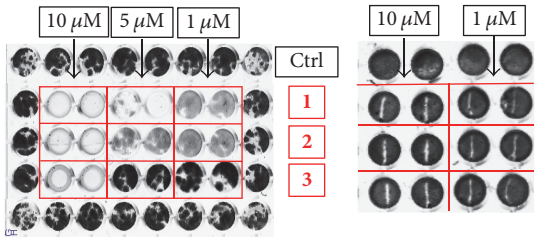


Figure 7: Inhibition of colony formation (a) and migration (b) of OVAR-8 cells.

in the mechanism of action of the mentioned compounds was confirmed with the antiproliferative activity in vitro, in particular against human ovarian cancer cell line. These results encourage further investigation about the binding and mode of action of 4-methyl substituted 2,6-di-tert-butylphenol derivatives and a biological evaluation in vivo, as anticancer PDI inhibitors.

Additional Points

Supplementary Data. Supplementary data associated with this article can be found at https://doi.org/10.1155/2017/2370359.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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