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RESEARCH ARTICLE

Potentiation of the reductase activity of protein disulphide isomerase (PDI) by 19-nortestosterone, bacitracin, fluoxetine, and ammonium sulphate

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Abstract

Protein disulphide isomerase (PDI) in the endoplasmic reticulum catalyzes the rearrangement of disulphide bridges during folding of secreted proteins. It binds various molecules that inhibit its activity. But here, we looked for molecules that would potentiate its activity. PDI reductase activity was measured *in vitro* using di-eosin-oxidized glutathione as substrate. Its classical inhibitor bacitracin was found to exert a biphasic effect: stimulatory at low concentrations (~10^{-6} M) and inhibitory only at higher concentrations (~10^{-4}–10^{-3} M). The weak oestrogenic molecule bisphenol A was found to exert a weak inhibitory effect on PDI reductase activity relative to the strong oestrogens, ethynylestradiol, and diethylstilbestrol. Like 19-nortestosterone, fluoxetine was found to exert a potentiating effect on PDI reductase activity and their potentiating effects could be reversed by increasing concentrations of oestrogens. In conclusion, this paper provides the first identification of potentiators of PDI activity that are potential pharmaceuticals against pathologies affecting protein folding such as Alzheimer's disease.

Keywords: Bacitracin, bisphenol A, ethynylestradiol, Alzheimer's disease

Introduction

Protein disulphide isomerase (PDI) has been known for a long time as a protein-folding catalyst[1] involved in the formation of native disulphide bridges of proteins in the endoplasmic reticulum[2,3]. It binds to numerous ligands including proteins, peptides, and various other molecules. In particular, PDI has been shown to bind oestradiol and this leads to a decrease in its isomerase activity as followed by its activity on RNAse renaturation[4].

Using di-eosin oxidized glutathione (DiE-GSSG) as substrate, it has been previously shown[5] that all the potent oestrogenic molecules tested [oestradiol, E2; 17α-ethynylestradiol (EE2) and diethylstilbestrol (DES)] also exhibited an inhibitory effect on PDI reductase activity. Likewise, the non-steroidal anti-inflammatory molecule indomethacin was also found to inhibit PDI reductase activity in the same assay.

Surprisingly, we found that two non-oestrogenic steroids, medroxyprogesterone acetate (MPA) and 19-nortestosterone (19-NT), in contrast potentiated PDI reductase activity[5]. In order to get a better understanding of the ligands properties responsible for inhibition or augmentation of PDI reductase activity, we performed a preliminary screening with various other molecules in order to get a first guess on the molecular structural features of the ligands exhibiting either inhibitory or stimulatory effects, respectively. In addition to the previously identified 19-NT, three other molecules, bacitacin, fluoxetine (FLX) and ammonium sulphate, were found to potentiate PDI reductase activity using DiE-GSSG as substrate.

Materials and methods

Bacitracin (BAC), bisphenol A (BPA), 17α-EE2, FLX, dithioerythreitol (DTeT), eosin 5-isothiocyanate, GSSG, PDI (E.C. 5.3.4.1) from bovine liver (PDI), were all

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Abbreviations

19-NT, 19-nortestosterone;
BAC, bacitracin;
BPA, bisphenol A;
DES, diethylstilbestrol;
DiE-GSSG, di-eosin oxidized glutathione;
EE2, ethynylestradiol;
FLX, fluoxetine;
MPA, medroxyprogesterone acetate

purchased from Sigma–Aldrich (Isle-d'Abeau, France) and were of the highest available grades.

The PDI substrate, DiE-GSSG, was synthesized and purified as previously described[6] with minor modifications[5].

PDI reductase activity was measured through abolishment of fluorescent self quenching when DiE-GSSG is reduced into two molecules of eosin-reduced glutathione (E-GSH). Initial velocities in fluorescence increase (λexc = 518 nm; λem = 545 nm) were recorded using a Spectra-Max Gemini spectrofluorimeter (Molecular Devices, Sunnyvale, California) and analyzed with SoftMaxPro program (Molecular Devices, Sunnyvale, California). Concentrations of the reagents at t_0 were

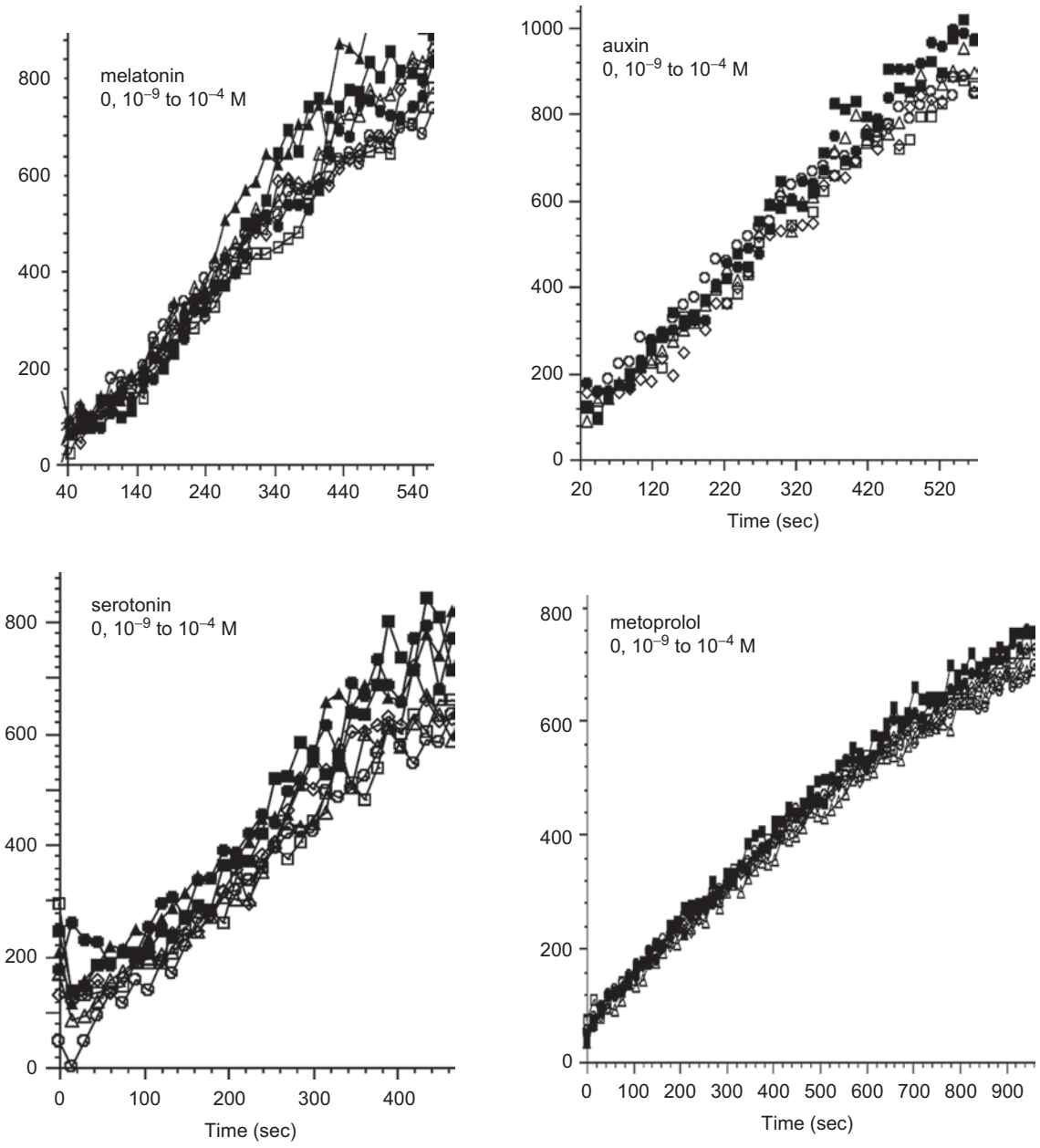

Figure 1. Kinetics of di-eosin oxidized glutathione reduction into fluorescent E-GSH catalyzed by protein disulphide isomerase in the presence of various concentrations (0–10^{-4} M) of melatonin, auxin, serotonin or metoprolol.

333 nM for PDI, 2.4 μM for DiE-GSSG, 33 μM for DteT, and 0.1 nM to 100 μM for the various molecules under study; except ammonium sulphate and sodium chloride (0.5 M).

Results

In the course of screening for molecules affecting PDI reductase activity using DiE-GSSG as substrate we found a number of molecules without any effect including metoprolol, serotonin, melatonin, and auxin (Figure 1).

Since potent oestrogenic molecules were found to inhibit PDI reductase activity, we looked for a possible effect of the weak oestrogen BPA. Figure 2 shows that BPA only exerted a slight inhibitory effect on PDI reductase activity in the 10^{-6}–10^{-5} M range.

In order to get further information on PDI-inhibiting molecules, we included BAC that has been known for a long time as an inhibitor of PDI activity. In fact, Figure 3 shows that BAC exerted a biphasic effect on PDI reductase activity. It potentiated PDI activity at around 10^{-6} M final concentration and inhibited it at around 10^{-4} M final concentration.

Interestingly, we observed two new molecules in addition to 19-NT and MPA[5] exhibiting stimulatory effect on PDI reductase activity. Indeed Figure 4 shows that fluoxetin exerted such a potentiating effect but only in the millimolar range. Figure 5 shows that 0.5 M ammonium sulphate also potentiates PDI reductase activity whereas 0.5 M sodium chloride was without any effect (not shown). We also checked that the increase in fluorescence due to ammonium sulphate was indeed due to a higher production of E-GSH and not to higher fluorescence efficiency.

In order to get a better understanding of the mechanisms of ligands inhibitory and stimulatory effects on PDI reductase activity, we analyzed PDI activity in the simultaneous presence of both potentiating and inhibitory ligands. Figure 6 shows the inhibition of PDI activity by increasing doses of DES in the presence of a potentiating dose of 19-NT whereas Figure 7 shows the inhibitory effect of EE2 in the presence of a potentiating concentration of FLX.

Discussion

PDI is a multifunctional enzyme mainly found in the endoplasmic reticulum of eukaryotes[7] where its main function is to catalyze the rearrangement (isomerization) of disulphide bridges during folding of membrane and secreted proteins. This activity is of utmost importance as over one-third of all human proteins fold in the endoplasmic reticulum[8]. The concentration of PDI in the lumen of the endoplasmic reticulum is known to be very high[9] and it has been reported to act as a high capacity reservoir for various ligands including hormones such as oestradiol (E2) and thyroxine (T3[4,10]).

It has been previously shown that all the potent oestrogenic molecules tested (E2, EE2, DES) exhibited an inhibitory effect on PDI isomerase[4,11] and reductase[5] activities. In the present work, we show that the weaker oestrogen BPA also exerts an inhibitory effect on PDI reductase activity. This result is consistent with previous observation that BPA inhibited the chaperone

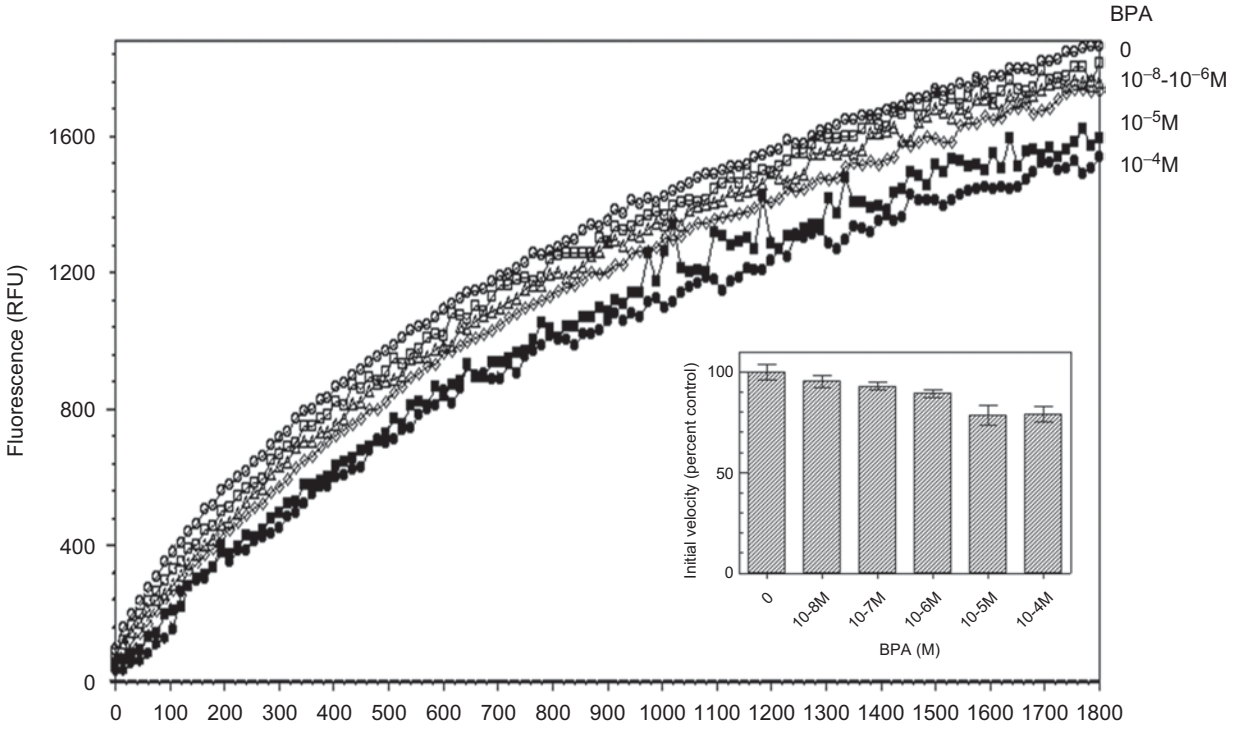

Figure 2. Kinetics of di-eosin oxidized glutathione reduction into fluorescent E-GSH catalyzed by protein disulphide isomerase in the presence of various concentrations (0–10^{-4} M) of bisphenol A. BPA, bisphenol A; RFU, relative fluorescence unit.

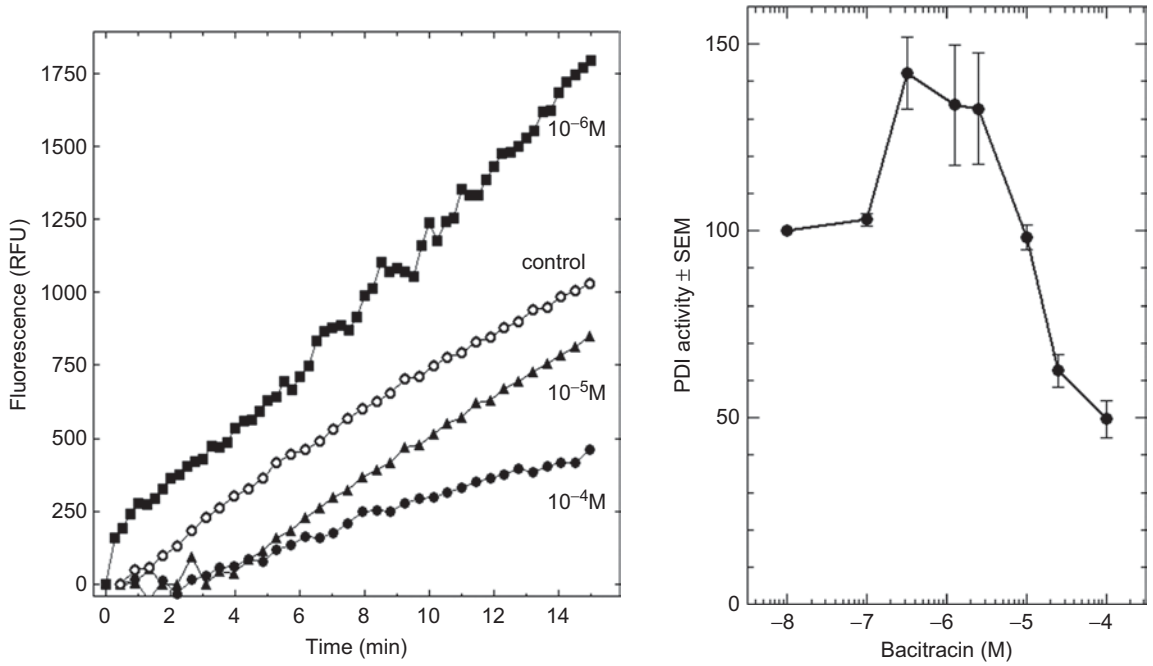

Figure 3. Effect of increasing concentrations of bacitracin (BAC) on protein disulphide isomerase (PDI) reductase activity. Left panel: kinetics of di-eosin oxidized glutathione reduction into fluorescent E-GSH catalyzed by PDI in the presence of various concentrations (0–10^{-4} M) of BAC. Right panel: initial velocity of PDI reducing activity as a function of BAC concentration relative to the velocity in the absence of bacitracin taken as control ($n = 3$). RFU, relative fluorescence unit.

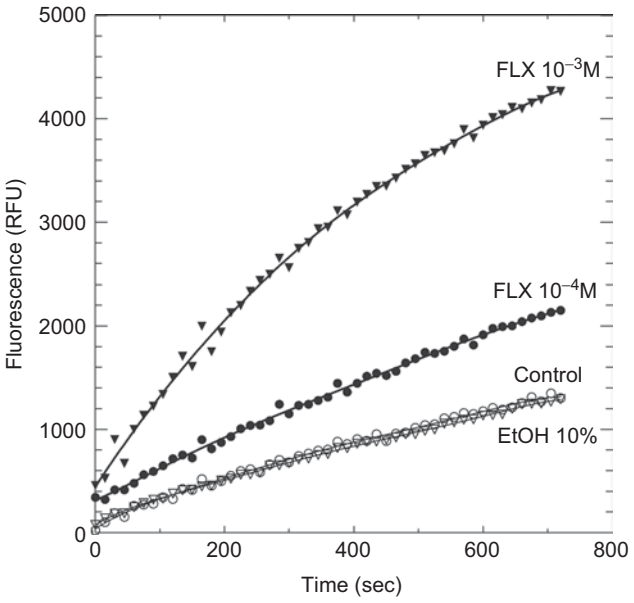

Figure 4. Kinetics of di-eosin oxidized glutathione reduction into fluorescent E-GSH catalyzed by protein disulphide isomerase in the presence of various concentrations (0, 10^{-4}, 10^{-3} M) of fluoxetine (FLX). The figure also shows the effect of 10% ethanol alone as also present with 10^{-3} M FLX.

activity of PDI on RNAse renaturation[12,13]. We found BPA to inhibit only partially (15%) PDI reductase activity and it did so at much higher concentrations (10^{-5}–10^{-4} M) than the more oestrogenic molecules (10^{-9}–10^{-8} M). This difference between the inhibitory doses of BPA and those of the potent oestrogenic molecules (E2, EE2, DES) on PDI reductase activity is roughly in the same order of magnitude as their relative oestrogenic activities[14–21]. It can be that, in some way, the binding sites for these different molecules in the oestrogen receptors (ER) and in PDI, respectively are similar enough to explain their similar relative binding affinities for EE2 and BPA, respectively (although this ratio is variable among species and between males and females). Another more exciting possibility relies on the observations that PDI is also present in locations other than the endoplasmic reticulum[22] and can directly interact with the ERα and modify its functional properties[23]. A provoking but tenable hypothesis is that the binding of oestrogenic molecules to PDI would play a pivotal role in the stimulation of ER through this direct ERα-PDI interaction.

Since serotonin, melatonin, and auxin are derived from the indolic di-cyclic amino-acid tryptophan, we suspected that they could also exert some inhibitory effect on PDI activity. In fact, we found that they all had no effect on PDI activity at concentrations up to up to 10^{-4} M (Figure 1). The β-1 adrenergic receptor antagonists, metoprolol (Figure 1) and atenolol (not shown) also did not exert any effect on PDI activity.

In a previous paper[5], we reported that two non-oestrogenic steroids, MPA, and 19-NT potentiated PDI reductase activity. Even with a somewhat limited screening, in the present study we were able to identify a few other molecules also potentiating PDI reductase activity using the recently developed assay with DiE-GSSG as substrate[6].

Surprisingly, the well-known inhibitor of PDI activity BAC[24,25] was found to exert potentiating activity and it did

so at lower concentrations (around 10^{-6} M) than those leading to the expected inhibition (around 10^{-4} M). BAC is a partly-cyclic polypeptide[26] and to our knowledge, there is no clear structural explanation for its mechanism of inhibition of PDI activities. The present data showing a biphasic dose-response effect of BAC on PDI reductase activity might be helpful in future studies to better understand the PDI mechanism of action.

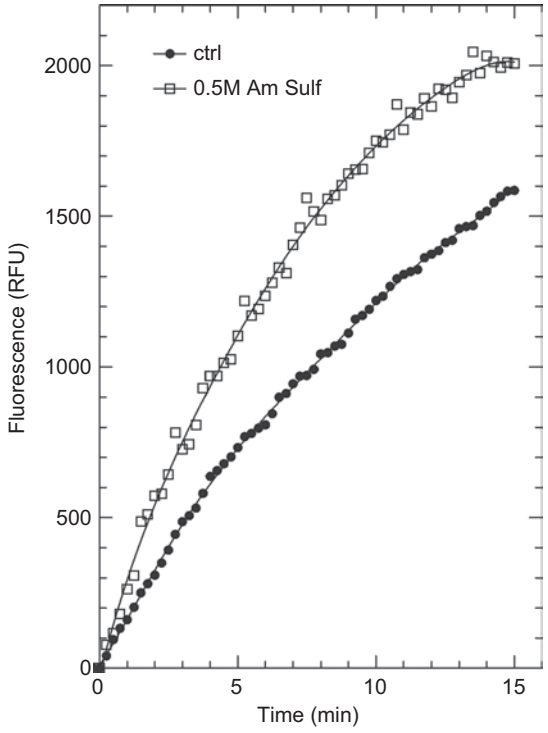

Figure 5. Kinetics of di-eosin oxidized glutathione reduction into fluorescent E-GSH catalyzed by protein disulphide isomerase in the presence or absence of 0.5 M ammonium sulphate. RFU, relative fluorescence unit.

The most efficient potentiating molecule we have identified so far is FLX which is known to act as a selective-serotonin-reuptake inhibitor and is the active component in the antidepressant Prozac™[27]. FLX was found in our study to greatly increase PDI reductase activity but this effect was observed only at millimolar concentration which is largely higher than its active circulating concentrations[28]. The observed augmentation of PDI reductase activity by FLX is therefore not responsible for any of its favourable or unfavourable pharmacological effects[29–33]. Nevertheless, the molecular structure of FLX offers an interesting basis for the search of molecules with higher PDI potentiating activities, in order to better understand the molecular mechanism of this enzyme.

Ammonium sulphate (0.5 M) was also found to potentiate PDI reductase activity in contrast to 0.5 M NaCl. It is likely that the mechanism involved is totally different from those implied for 19-NT, BAC, or FLX. The chaotropic/kosmotropic property of ammonium sulphate is well known and greatly influences protein stability and activity[34,35], and their crystallization capacity[36]. It is therefore possible that ammonium sulphate can influence PDI catalytic efficiency by favouring its active conformation as previously shown for glutamate decarboxylase[37] and consistent with the observation that PDI, like thioredoxins and transgtlutaminases, resist detergents

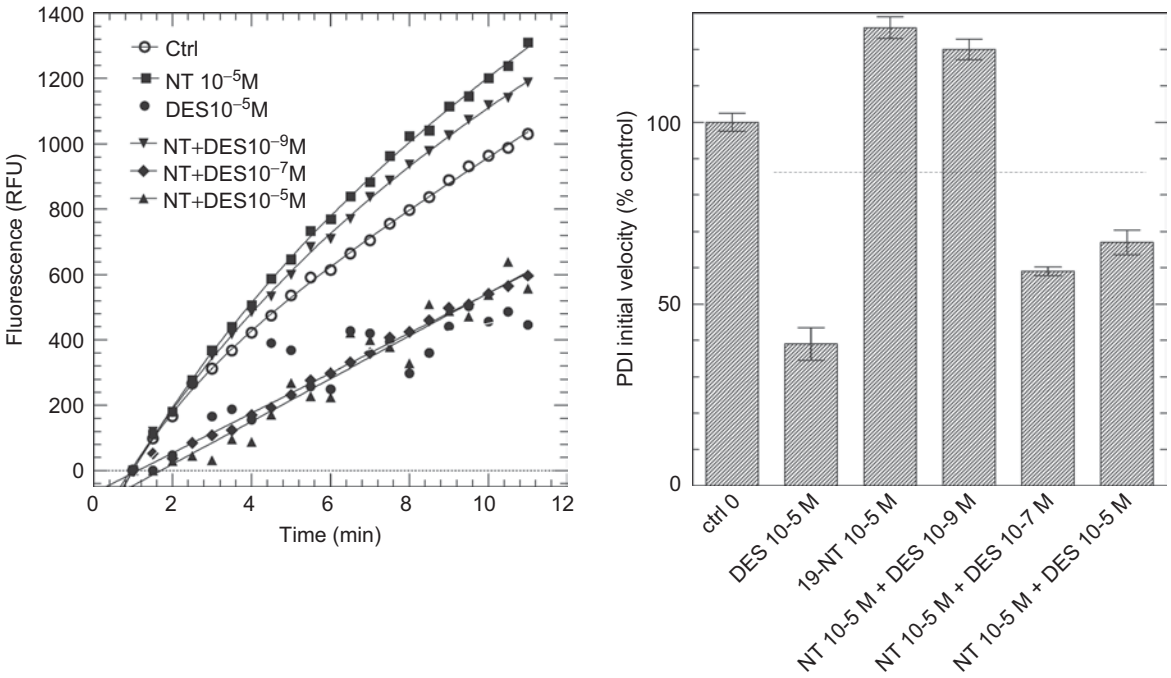

Figure 6. Inhibition of protein disulphide isomerase (PDI) reductase activity by diethylstilbestrol (DES) in the presence of a potentiating concentration of 19-nortestosterone (19-NT). Left panel: kinetics of di-eosin oxidized glutathione reduction into fluorescent E-GSH catalyzed by PDI in the presence of 10^{-5} M of either DES or 19-NT or in the presence of 10^{-5} M 19-NT together with various concentrations (10^{-9}, 10^{-7}, 10^{-5} M) of DES. Right panel: initial velocity of PDI reducing activity in the presence of either DES or 19-NT alone or in combination (data from left panel). RFU, relative fluorescence unit.

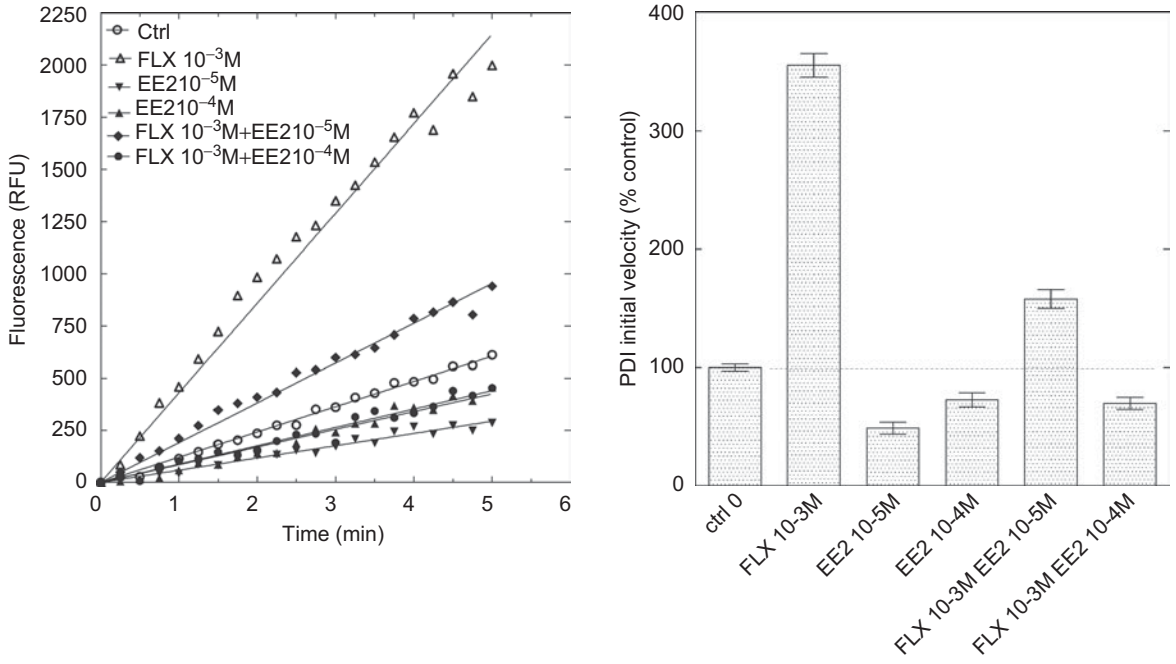

Figure 7. Inhibition of protein disulphide isomerase (PDI) reductase activity by ethynylestradiol (EE2) in the presence of a potentiating concentration of fluoxetine (FLX). Left panel: kinetics of di-eosin oxidized glutathione reduction into fluorescent E-GSH catalyzed by PDI in the presence of 10^{-3} M FLX or 10^{-5} or 10^{-4} M EE2 alone or in the presence of 10^{-3} M FLX. Right panel: initial velocity of PDI reducing activity in the presence of either FLX or EE2 alone or in combination. RFU, relative fluorescence unit.

and chaotropic agents[38]. Another possibility is that ammonium sulphate act by altering water properties and therefore either water-protein interactions[35] or the active concentration of substrate. This latter mechanism would thus be independent of PDI properties.

To our knowledge, this paper and the previous one from our laboratory[5] are the first to describe ligands that potentiate PDI activity. Molecules with such a potentiating effect on PDI activity would be of utmost interest for the treatment of pathologies originating from intracellular protein aggregation. This concerns mainly neurodegenerative diseases such as Alzheimer disease[39,40] but augmentation of PDI activity would also be expected to have favourable effects in situations where its inhibition by millimolar concentrations of BAC was found detrimental such as in angiogenesis41 or stroke protection[42].

The molecules we have shown so far to exert augmentation of PDI reductase activity exhibit dissimilar structures and it is thus likely that they act through different mechanisms. It will be of interest to broaden the screening for PDI activity-potentiating molecules in order to identify highly potent and possibly synergistic drugs favouring correct folding and disulphide bridges formation in proteins.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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