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ROS inhibitor *N*-acetyl-L-cysteine antagonizes the activity of proteasome inhibitors

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Abstract

NAC (*N*-acetyl-L-cysteine) is commonly used to identify and test ROS (reactive oxygen species) inducers, and to inhibit ROS. In the present study, we identified inhibition of proteasome inhibitors as a novel activity of NAC. Both NAC and catalase, another known scavenger of ROS, similarly inhibited ROS levels and apoptosis associated with H_2O_2. However, only NAC, and not catalase or another ROS scavenger Trolox, was able to prevent effects linked to proteasome inhibition, such as protein stabilization, apoptosis and accumulation of ubiquitin conjugates. These observations suggest that NAC has a dual activity as an inhibitor of ROS and proteasome inhibitors. Recently, NAC was used as a ROS inhibitor to functionally characterize a novel anticancer compound, piperlongumine, leading to its description as a ROS inducer. In contrast, our own experiments showed that this compound depicts features of proteasome inhibitors including suppression of FOXM1 (Forkhead box protein M1), stabilization of cellular proteins, induction of ROS-independent apoptosis and enhanced accumulation of ubiquitin conjugates. In addition, NAC, but not catalase or Trolox, interfered with the activity of piperlongumine, further supporting that piperlongumine is a proteasome inhibitor. Most importantly, we showed that NAC, but not other ROS scavengers, directly binds to proteasome inhibitors. To our knowledge, NAC is the first known compound that directly interacts with and antagonizes the activity of proteasome inhibitors. Taken together, the findings of the present study suggest that, as a result of the dual nature of NAC, data interpretation might not be straightforward when NAC is utilized as an antioxidant to demonstrate ROS involvement in drug-induced apoptosis.

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AUTHOR CONTRIBUTION

Marianna Halasi, Ming Wang, Tanmay Chavan, Vadim Gaponenko, Nissim Hay and Andrei Gartel designed the experiments and analysed the data. Marianna Halasi, Ming Wang, Tanmay Chavan and Vadim Gaponenko performed the experiments. Marianna Halasi, Ming Wang, Nissim Hay and Andrei Gartel wrote the paper.

Keywords

catalase; Forkhead box protein M1 (FOXM1); *N*-acetyl-L-cysteine (NAC); reactive oxygen species (ROS); proteasome inhibitor

INTRODUCTION

Both proteasome inhibitors and ROS (reactive oxygen species) inducers are important emerging classes of anticancer drugs. In general, proteasome inhibitors hinder proteasome function resulting in cancer cell death, and ROS inducers increase ROS-related stress leading to ROS-dependent apoptosis in cancer cells [1,2]. Proteasome inhibitors were initially used to study the catalytic activity of the proteasome [3]. The proteasome itself affects so many different cellular pathways that it was highly questionable whether it could be an appropriate target for anticancer therapy. However, accumulating experimental data suggested that tumour cells exhibit increased sensitivity to proteasome inhibition compared with normal cells [3–5]. This observation and the advancement in the mechanism of proteasome function led to the generation of effective proteasome inhibitors against human malignancies. Proteasome inhibitors reversibly or irreversibly bind to the catalytic sites of the proteasome inhibiting its chymotrypsin-like, trypsin-like and/or caspase-like activities [3]. The mechanism of the anticancer activity of proteasome inhibitors is still debated, but FOXM1 (Forkhead box protein M1) inhibition, increase in pro-apoptotic proteins, induction of ER (endoplasmic reticulum) stress and inhibition of angiogenesis are suggested, among others, as underlying mechanisms for their antitumour properties [3,5]. Currently, bortezomib (Velcade) is the only proteasome inhibitor in clinical practice, but several others are in clinical development including carfilzomib, NPI-0052, MLN9708, CEP-18770 and ONX0912 [1,3].

ROS are produced either as normal by-products of aerobic metabolism or upon environmental stresses [6]. ROS can be generated at many different organelles, but the main sites of ROS production are the mitochondria, the ER, plasma membrane and cytosol. ROS are important for several physiological processes, but due to their highly reactive nature, ROS can damage proteins, lipids and DNA [2,6,7]. When the production of ROS exceeds its deactivation it leads to oxidative stress [6,8]. Interestingly, previous publications have suggested that oxidative stress could be utilized to preferentially kill cancer cells [2,9,10]. For this reason, several ROS inducers, including PEITC (β-phenylethyl isothiocyanate), 2-ME (2-methoxyestradiol), ATN-224 and Imexon, are already in Phase I/II clinical trials against different types of human cancer [2]. Cells possess many naturally occurring antioxidants to detoxify the generation of damaging oxidizing agents. This antioxidant defence system includes antioxidant enzymes [catalase, SOD (superoxide dismutase) and Gpx (glutathione peroxidase)] and non-enzymatic antioxidants (glutathione and thioredoxine) [6]. There are also several artificial antioxidants available that mimic the effects of the natural antioxidants. One of them is NAC (*N*-acetyl-L-cysteine), which is known to inhibit ROS-dependent apoptosis [6]. NAC is a synthetic precursor of intracellular cysteine and glutathione, and its anti- ROS activity results from its free radical scavenging property either directly via the redox potential of thiols, or secondarily via increasing

glutathione levels in the cells [11]. ROS scavengers and especially NAC are commonly used to confirm the involvement of ROS in drug-induced apoptosis.

In the present study, we determined that the known ROS scavenger NAC directly binds to proteasome inhibitors and consequently inhibits their activity. Our findings demonstrate that the alleged ROS inducer PL (piperlongumine) also displays proteasome inhibitory properties and induces ROS-independent apoptosis. PL was previously validated as a ROS inducer by using NAC as a ROS inhibitor [12], but according to the results of the present study, NAC inhibits the activity of PL as a proteasome inhibitor and not by scavenging ROS. In addition, the results of the present study suggest that, due to the dual function of NAC, ROS-related drug activity cannot be validated by NAC alone.

MATERIALS AND METHODS

Cell culture and chemical compounds

MIA PaCa-2 human pancreatic cancer cell line (A.T.C.C.), U2OS osteosarcoma (A.T.C.C.) and U2OS-derived C3-luc cells [13] were grown in DMEM (Dulbecco's modified Eagle's medium; Invitrogen). The MDA-MB-231 (A.T.C.C.) human breast cancer cell line was grown in RPMI medium (Invitrogen). The media were supplemented with 10% FBS (Atlanta Biologicals) and 1% penicillin/streptomycin (Gibco). All of the cells were maintained at 37°C in 5% CO_2. Bortezomib (Velcade; Millenium Pharmaceuticals), MG132 (Calbiochem), thiostrepton (Sigma), PL (TimTec), Trolox (Sigma) and PEITC (Sigma) were dissolved in DMSO (Fisher Scientific), H_2O_2 (Fisher Scientific) was dissolved in culture media, NAC (Sigma) and lactacystin (Cayman Chemical) were dissolved in deionized water, and catalase (Sigma) was dissolved in PBS.

Immunoblot analysis

Treated cells were harvested and lysed using IP (immunoprecipitation) buffer [20 mM Hepes, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate tetrabasic, 1 mM sodium orthovanadate and 0.2 mM PMSF supplemented with a protease inhibitor tablet (Roche Applied Sciences)]. The protein concentration was determined using Bio-Rad Protein Assay reagent. Isolated proteins were separated by SDS/PAGE (8–15% gradient gel) and transferred on to PVDF membrane (Millipore). Immunoblotting was carried out with antibodies specific for Mcl-1 (Lab Vision), cleaved caspase 3 (Cell Signaling Technology), PARP1/2 [poly(ADP-ribose) polymerase 1/2; Santa Cruz Biotechnology], ubiquitin (Santa Cruz Biotechnology), FOXM1 (the rabbit polyclonal antibody against FOXM1 has been described previously [14]), p21 (BD-Pharmingen), catalase (Abcam) and β-actin (Sigma).

Luciferase assay

Cells were pretreated with 1 μg/ml doxycycline in the presence or absence of 3 mM NAC or 500 units/ml catalase for 2 h. Then, the cells were treated with the indicated concentrations of the drugs overnight. The luciferase activity was determined by the Luciferase Assay System (Promega) according to the manufacturer's recommendations. Briefly, the culture media was removed and the cells were rinsed with PBS, which was also aspirated. Then, the

cell culture lysis reagent provided in the assay was added and the cells were lysed at room temperature (25 °C) for 15 min. Cell lysates were assayed with the luciferase assay substrate. Light intensity was measured by a luminometer (Bio-Tek). The data were normalized to the amount of protein in the samples.

ROS measurement

At the end of the treatment schedule, cells were incubated with 10 μM DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate dye; Molecular Probes) in culture media for 30 min. Then, cells were washed with PBS, trypsinized, resuspended in PBS supplemented with FBS and analysed for intracellular ROS production by flow cytometry.

20S proteasome activity assay

20S purified proteasome (~0.5 μg) (R&D Systems) was pre-incubated with PL or PLN (NAC-modified PL) in 10 × assay buffer [10 μl; 250 mM Hepes (pH 7.5), 5 mM EDTA, 0.5% Nonidet P40 and 0.01% SDS] and water (~70 μl) for 30 min at room temperature. Then, the Ac-YVAD-AMC (*N*-acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin) fluorogenic substrate (~10 μl, 0.5 mM) (Enzo Life Sciences) was added and the assay mixture was further incubated for 2 h at 37 °C. Fluorescence was measured using a fluorometer (Bio-Tek).

Adenoviral infection

Human cancer cells were infected with Ad5-CMV (cytomegalovirus)-catalase adenoviral particles (Gene Transfer Vector Core, University of Iowa, Iowa City, IA, U.S.A.) at a MOI (multiplicity of infection) of 100 in FBS-free medium for 1 h. Then, the FBS concentration was brought up to 10% and additional media was added. At 48 h after infection, the cells were treated as indicated.

NMR experiments

The ^{1}H-^{13}C NMR HSQC (heteronuclear single-quantum coherence) experiments were carried out on an 800 MHz Bruker Avance Spectrometer. Spectra were recorded at different concentrations of antioxidants and drugs. The ratios for NAC to either bortezomib or lactacystin were kept to 300:1. Trolox was mixed with either lactacystin or bortezomib at a molar ratio of 5000:1. Similarly, catalase was also mixed with either lactacystine or bortezomib at a molar ratio of 500:1. The mixtures with Trolox and the drugs were prepared using DMSO as a solvent. All of the other mixtures were in water. The experiments were carried out at 25°C. Data analysis was performed using NMRPipe.

HPLC separation of PL and PL–NAC conjugates

To prepare PL–NAC conjugates, PL (40 μmol) was incubated with NAC (8 mmol) in Hepes (pH 7) for 24 h at 37°C. For analytical HPLC, PL and PL–NAC solutions were analysed by LC-MS (Shimadzu HPLC system with Class-VP v.5.x software, Nucleodur 100-5 CN-RP column), using a separation gradient of 0–100% ACN (acetonitrile) in 0–15min, where detection was with a multi-wavelength UV detector. For separation of PL–NAC conjugates, PL–NAC conjugation solutions were separated by preparative HPLC, using a preparative

C_{18} column (Supelco Discovery BIO wide pore C_{18}, 15 cm × 21.2 mm), with a separation gradient of 0–5 min [10% H_2O, 90% ACN and 0.1% TFA (trifluoroacetic acid)], 5–65 min (60% H_2O, 40% ACN + 0.1 % TFA), 65–70 min (80% H_2O, 20% ACN + 0.1% TFA), 70–75 min (80% H_2O, 10% ACN + 0.1% TFA), 75–76 min (10% H_2O, 90% ACN + 0.1% TFA) and 76–90 min (10% H_2O, 90% ACN + 0.1% TFA). Detection was performed at λ = 280 nm. Post-separation, collected separations were freeze-dried and weighed. After analysis by MS, compounds were dissolved in DMSO to form concentrations of 10 μM.

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison post test. *P* values of <0.05 were considered to be statistically significant.

RESULTS

NAC is an inhibitor of proteasome inhibitors

Down-regulation of FOXM1, a mammalian oncogenic transcription factor, by proteasome inhibitors has been recognized recently as one of the hallmarks of proteasome inhibition [3]. Our previous studies demonstrated that all proteasome inhibitors thus far tested down-regulate the transcriptional activity of FOXM1 [13,15,16]. Using the previously generated and described U2OSderived C3-luc osteosarcoma cells [13], which stably express the doxycycline-inducible FOXM1–GFP and firefly luciferase under the control of multiple FOXM1-responsive elements, we show in the present study (in agreement with our earlier findings) that elevation of doxycycline-inducible FOXM1–GFP fusion protein expression after the addition of doxycycline to the culture media correlates with a severalfold increase in firefly luciferase activity (Figure 1A). However, *bona fide* proteasome inhibitors bortezomib and MG132 effectively decreased the firefly luciferase activity close to basal levels. Presumably, proteasome inhibitors suppress FOXM1 transcriptional activity via the stabilization of a negative regulator of FOXM1 [17]. To our great surprise, NAC, a well-known inhibitor of ROS, reversed the inhibitory effect of proteasome inhibitors on the transcriptional activity of FOXM1 (Figure 1A). This was the first evidence that NAC may negatively affect the activity of proteasome inhibitors. In addition, we found that in comparison with other known ROS scavengers, such as catalase [18] and Trolox [19], only NAC interfered with proteasome inhibitor-related apoptosis and with other features of proteasome inhibition, such as protein stabilization and accumulation of ubiquitin conjugates (Figures 1B–1D). These data suggest that only NAC, but not catalase or Trolox, disrupts the activity of proteasome inhibitors.

NAC, catalase and Trolox similarly inhibit ROS levels and apoptosis associated with H2O2

To compare NAC, catalase and Trolox as ROS scavengers in our cell system, we evaluated their activity against H_2O_2. First, we assessed ROS levels after H_2O_2 treatment in the absence and presence of the antioxidants by flow cytometry and found that NAC, catalase and Trolox efficiently quenched the ROS associated with H_2O_2 (Figures 2A–2D). Next, H_2O_2-mediated apoptosis in the absence and presence of the scavengers was determined by immunoblotting for cleaved caspase 3. We found that both NAC and catalase fully abolished ROS-dependent cell death induced by H_2O_2 (Figure 2E). In addition, H_2O_2 did not inhibit

proteasome activity as assessed by the lack of accumulation of ubiquitin conjugates (Supplementary Figure S1 at http://www.biochemj.org/bj/454/bj4540201add.htm). Although NAC, catalase and Trolox equally inhibited ROS levels and ROS-induced apoptosis (Figure 2), only NAC antagonized the activity of proteasome inhibitors (Figure 1). These data suggest that while NAC, catalase and Trolox are all inhibitors of ROS, only NAC is an inhibitor of proteasome inhibitors.

Novel ROS inducer PL is also a proteasome inhibitor

Recently, a novel anticancer compound termed PL, that increases the level of ROS and kills cancer cells, was identified by Raj et al. [12]. PL was proposed to be a ROS inducer with unique anticancer properties. Our own experiments, however, suggest that this compound is a conventional proteasome inhibitor that targets FOXM1, stabilizes protein expression and induces apoptosis in cancer cells by a ROS-independent mechanism.

Since inhibition of FOXM1has been suggested as a new readout for proteasome inhibition [3,17], first we evaluated how PL affects FOXM1 compared with known proteasome inhibitors. Using the cell-based system discussed above (Figure 1A) we found that PL inhibited FOXM1 transcriptional activity as strongly as the FOXM1/proteasome inhibitor thiostrepton (Figure 3A) [15,16]. In addition, only NAC was able to alleviate the inhibition on FOXM1 transcriptional activity by PL and thiostrepton, further supporting the notion that NAC, but not catalase, interferes with proteasome inhibitor activity. In line with previously published data, PL also suppressed FOXM1 protein level and stabilized protein expression as efficiently as other proteasome inhibitors (Figures 3B and 3C) [15,16,20]. To test whether PL directly inhibits the proteasome *in vitro*, we measured 20S proteasome activity after PL treatment. We found that PL inhibited 20S proteasome activity in a concentration-dependent manner, further confirming the idea that PL has proteasome inhibitory activity (Figure 3D).

NAC, but not catalase or Trolox, antagonizes the activity of *bona fide* proteasome inhibitors and PL

To provide additional evidence that NAC, but not other scavengers such as catalase or Trolox, is an inhibitor of proteasome inhibitors and that PL exhibits proteasome inhibitory activity, breast (Figures 4A and 4B, and Supplementary Figure S2A at http://www.biochemj.org/bj/454/bj4540201add.htm) and pancreatic (Figures 4C–4E, and Supplementary Figures S2B and S2C) human cancer cell lines were treated with PL or proteasome inhibitors including thiostrepton, MG132, bortezomib and lactacystin [21] in the presence and absence of NAC, catalase or Trolox. We found that PL displayed several properties similar to the proteasome inhibitors thiostrepton, MG132 and bortezomib, including stabilization of p21 and Mcl-1 expression [5,16,22], induction of robust apoptosis and enhanced accumulation of ubiquitin conjugates (Figures 4A and 4C). These data suggest that PL truly has proteasome inhibitory activity. In addition, NAC, but not catalase or Trolox, reversed all effects linked to proteasome inhibition by PL and other proteasome inhibitors (Figures 4A–4E, and Supplementary Figure S2) confirming that NAC indeed impedes the activity of proteasome inhibitors. To ensure that insufficient cellular uptake of catalase is not the underlying mechanism for catalase not interfering with proteasome inhibitors, MIA PaCa-2 pancreatic cancer cells were infected with catalase carrying

adenoviral particles and were treated with PL and thiostrepton, and H_2O_2 as the positive control (Figure 4F). As the Western blot analysis shows, catalase was expressed adequately, but it was still unable to affect the activity of proteasome inhibitors thiostrepton and PL, including protein stabilization (results not shown) and induction of cell death in comparison with that of H_2O_2 (Figure 4F).

Taken together, we conclude that PL inhibits proteasome activity, which may lead to the accumulation of ROS as a secondary effect, as was demonstrated previously for other proteasome inhibitors [23,24]. Since proteasome inhibitors may elevate intracellular ROS by inducing ER stress [25] and by inhibiting FOXM1 [16], which has been shown to induce the expression of ROS scavengers [26], their essential proteasome inhibitory activity may be overlooked. However, the results of the present study suggest that the predominant pro-apoptotic activity of PL is through its proteasome inhibitory activity because cell death induced by PL is only inhibited by NAC, which is an inhibitor of both ROS and proteasome inhibitors, but not by catalase or Trolox, which are only ROS scavengers (Figures 4A, 4C and 4E). Also, PL and other proteasome inhibitors did not elevate ROS levels in the cell system used in the present study (Supplementary Figure S3 at http://www.biochemj.org/bj/454/bj4540201add.htm). Taken together, these results imply that PL induces ROS-independent apoptosis. In contrast with the results of the present study, Raj et al. [12] claimed that PL is a novel ROS inducer, but this conclusion was largely based on the inhibition of PL activity by NAC.

NAC directly binds to proteasome inhibitors

To investigate whether NAC binds to well-known proteasome inhibitors of different structure, we performed ^{13}C-edited HSQC NMR experiments on mixtures of NAC with either bortezomib or lactacystin. Both experiments were performed at a molar ratio of 300:1 of NAC to the respective inhibitors. When the NMR signals obtained on these mixtures were compared with those of NAC alone, we observed chemical-shift perturbations (Figures 5A and 5B). This suggests that NAC directly binds to bortezomib as well as lactacystin. To show that other scavengers such as catalase and Trolox did not interfere with the activity of proteasome inhibitors because they do not bind to proteasome inhibitors, we performed additional binding experiments with Trolox and catalase. Trolox was mixed with either bortezomib or lactacystin at a molar ratio of 5000:1 and the spectra were recorded. Upon overlaying these signals with those of Trolox alone, there was no chemical-shift change observed (Figures 5C and 5D). Similarly, catalase was also mixed with either lactacystin or bortezomib at a molar ratio of 500:1. No chemical-shift change was observed when compared with the signals of catalase alone (Figures 5E and 5F). All of these results suggest that NAC binds to proteasome inhibitors of different structure, whereas other ROS scavengers, such as catalase and Trolox, do not.

NAC is a small molecule with a free thiol group that is readily available for nucleophilic attack. We also noticed that in solution NAC could react with PL through nucleophilic addition to both α,β unsaturated carbonyl sites of PL. Purification of this reaction by HPLC showed products with molecular masses of 644 g/mol and 479 g/mol (as analysed by MS), corresponding to covalent conjugates of two NACs and one NAC to PL (PLN) respectively

(Figure 5G). In contrast with non-modified PL that inhibited 20S proteasome activity *in vitro* (Figure 3D), induced cell death, stabilization of cellular protein p21 and ubiquination (Figures 4A, 4C and 4E), administration of PLN to cancer cells and purified proteasome showed no such effects (Figures 5H and 5I). These data suggest that the inhibitory activity of NAC towards PL is through direct conjugation to PL that leads to the inhibition of the activity of PL as a proteasome inhibitor and not by scavenging ROS.

DISCUSSION

In the present study, we re-evaluated the activity of known ROS inhibitor NAC and hypothetical ROS inducer PL. We showed that NAC directly binds to proteasome inhibitors including bortezomib, lactacystin and PL (Figure 5), and inactivates their proteasome inhibitory function (Figures 1, 3 and 4). We also demonstrated that ROS inhibitors catalase and Trolox do not interact with proteasome inhibitors (Figure 5) and consequently do not affect their activity (Figures 1, 3 and 4, and Supplementary Figure S2). These data suggest that only NAC, and not other ROS scavengers, antagonizes the activity of proteasome inhibitors. To our knowledge, NAC is the first known inhibitor of proteasome inhibitors and presumably it will be useful in future studies of these drugs. In addition, we found that PL, which was previously identified as a novel ROS inducer by using NAC as a ROS inhibitor [12], exhibits proteasome inhibitory activities. The findings of the present study are supported by a recently published report suggesting that ROS induction by PL is a secondary effect of PL as a proteasome inhibitor [27]. Taken together, because of the dual activity of NAC, in order to avoid false identification of new ROS inducers in the future, multiple different antioxidants should be used to show the actual involvement of ROS in drug-induced apoptosis.

Proteasome inhibitors bortezomib, MG132 and lactacystin generally inhibit proteasome activity through direct covalent attachment to active sites within the proteasome. What these proteasome inhibitors have in common is that they all contain an electrophilic site through which a nucleophile, such as the hydroxy group of a threonine residue, can attack. Structural studies have shown that the proteasome inhibitors mentioned can covalently attach to proteasomes at the Thr^1 position of the β5 subunits of the proteasome, resulting in the inhibition of proteasome activity. The electrophilic site of bortezomib lies at the borate moiety [28], whereas the electrophilic site of MG132 is presented at the aldehyde [29]. Lactacystin poses as a special case as it must undergo spontaneous intramolecular rearrangement to form its active electrophilic site, a β-lactone, before its activity as a proteasome inhibitor is exhibited [30].

Like threonine, NAC is an amino acid (although acylated at the N-terminus) with a nucleophilic side chain. In the case of NAC, the nucleophilic moiety is a thiol, which is a stronger nucleophile than hydroxy groups, which means it can react with electrophiles with greater efficiency. This means that if bortezomib, MG132 and lactacystin can react with the hydroxy groups of threonine, they will more readily react with the thiol group of NAC. Lactacystin is particularly special in that, as a molecule, it already contains a NAC-component within its structure. Lactacystin itself is an inactive precursor, and only becomes active upon molecular rearrangement, particularly when the NAC-component is removed to

form ‘clasto-lactacystin β-lactone’ [31]. Clasto-lactacystin β-lactone becomes inactive again once NAC is re-attached. Studies have shown that at high concentrations of glutathione (contains active thiols, similar to NAC), this clasto-lactacystin β-lactone molecule becomes inactive and cannot inhibit proteasome activity. In the present study, using NMR and MS we showed that NAC directly interacts with bortezomib, lactacystin and PL (Figure 5), thus inhibiting their proteasome inhibitory function (Figures 1B, 4A, 4C and 4E, and Supplementary Figures S2A and S2C). Our own data and the data available in the literature [30,31] suggest that NAC reacts with common electrophilic sites of proteasome inhibitors with high efficiency, consequently it can bind to proteasome inhibitors regardless of their structure and it can equally inhibit their activities. However, additional experiments are needed to prove the universal nature of these interactions and the general inhibitory activity of NAC against proteasome inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

ACN	acetonitrile
DCFH-DA	2′,7′-dichlorodihydrofluorescein diacetate
ER	endoplasmic reticulum
FOXM1	Forkhead box protein M1
HSQC	heteronuclear single-quantum coherence
NAC	*N*-acetyl-L-cysteine
PARP	poly(ADP-ribose) polymerase
PEITC	β-phenylethyl isothiocyanate
PL	piperlongumine
PLN	NAC-modified PL
ROS	reactive oxygen species
TFA	trifluoroacetic acid

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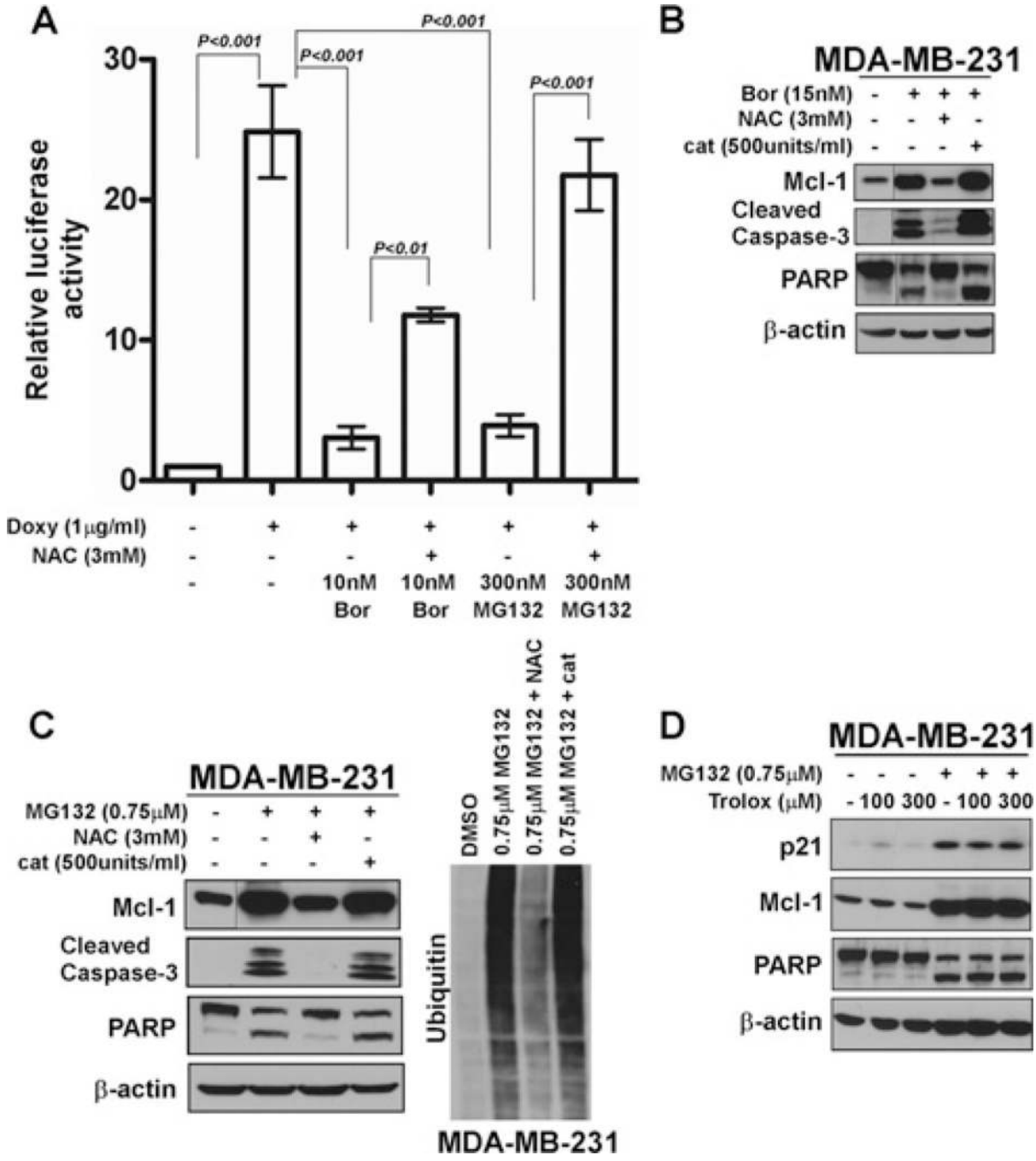

Figure 1. NAC inhibits proteasome inhibitory activity of bortezomib and MG132

(**A**) C3-luc cells were treated as indicated overnight and luciferase activity was measured using the Luciferase Assay System kit (Promega). Values are means ± S.D. for a representative triplicate experiment. Doxy, doxycycline. (**B**) MDA-MB-231 human breast cancer cells were treated with bortezomib (Bor) after a 2 h pre-incubation with 3 mM NAC or 500 units/ml catalase (cat). Immunoblot analysis of Mcl-1, cleaved caspase 3, PARP and β-actin as the loading control was carried out 24 h after treatment. (**C**) MDA-MB-231 human breast cancer cells were treated with MG132 after a 2 h pre-incubation with 3 mM

NAC or 500 units/ml catalase. Immunoblot analysis of Mcl-1, cleaved caspase 3, PARP, ubiquitin and β-actin as the loading control was carried out 24 h after treatment. (**D**) MDA-MB-231 human breast cancer cells were pre-incubated with the indicated concentrations of Trolox for 2 h and then treated with MG132 for 24 h. Immunoblotting was carried out with antibodies specific for p21, Mcl-1 and PARP. β-Actin was used as the loading control.

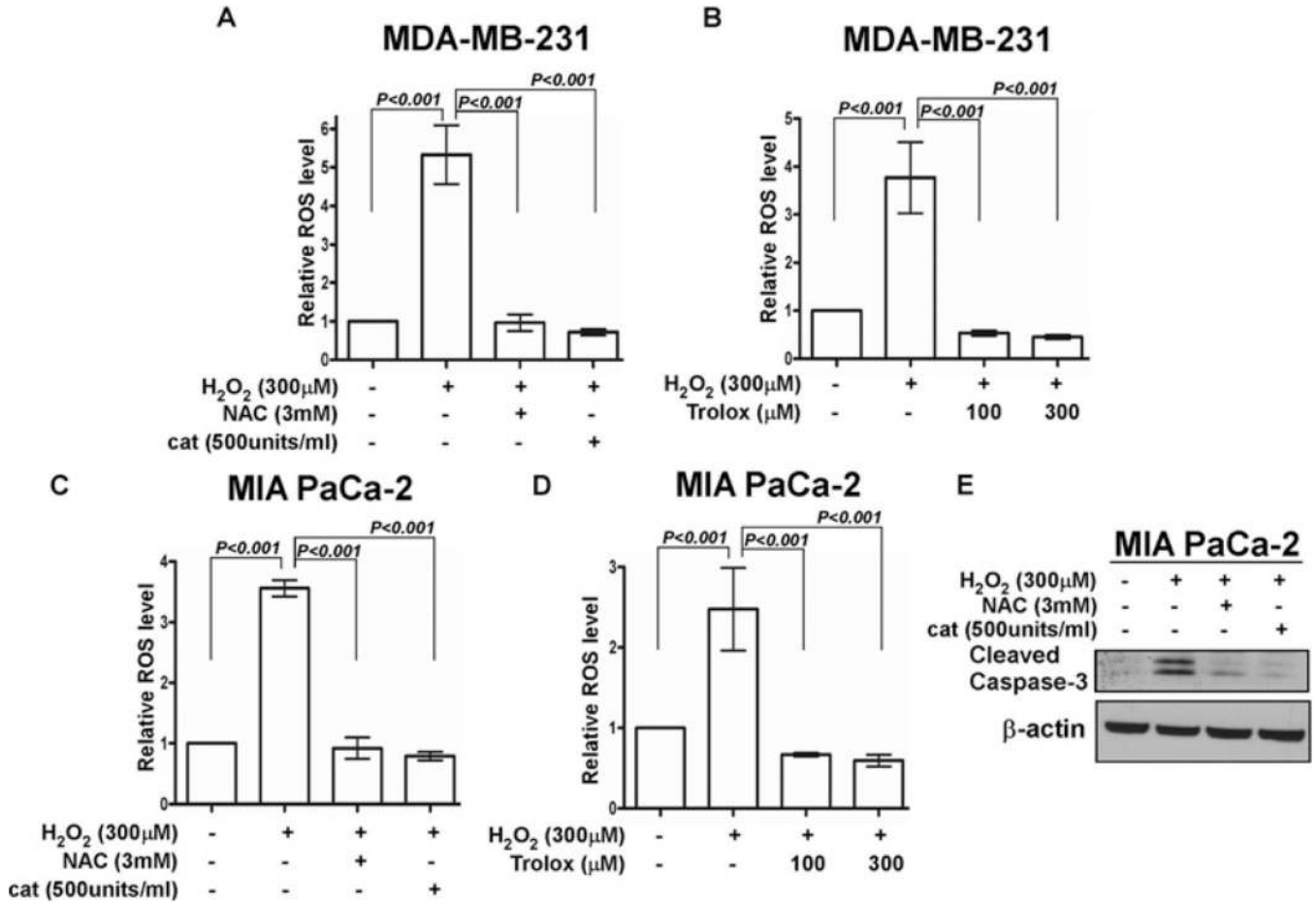

Figure 2. NAC, catalase and Trolox inhibit ROS and ROS-induced apoptosis

(**A–D**) MDA-MB-231 breast and MIA PaCa-2 pancreatic cancer cells were pre-incubated with 3 mM NAC, 500 units/ml catalase (cat), or 100 and 300 μM Trolox for 2 h and then treated with H_2O_2. Intracellular ROS production was measured by flow cytometry following staining with 10 μMDCFH-DA dye. Values are means ± S.E.M. for three independent experiments (**A** and **C**) or means ± S.D. for a representative triplicate experiment (**B** and **D**). (**E**) Following treatment with the indicated concentrations of H_2O_2 for 24 h, MIA PaCa-2 cells were harvested and immunoblotting was performed for cleaved caspase 3. β-Actin was used as the loading control.

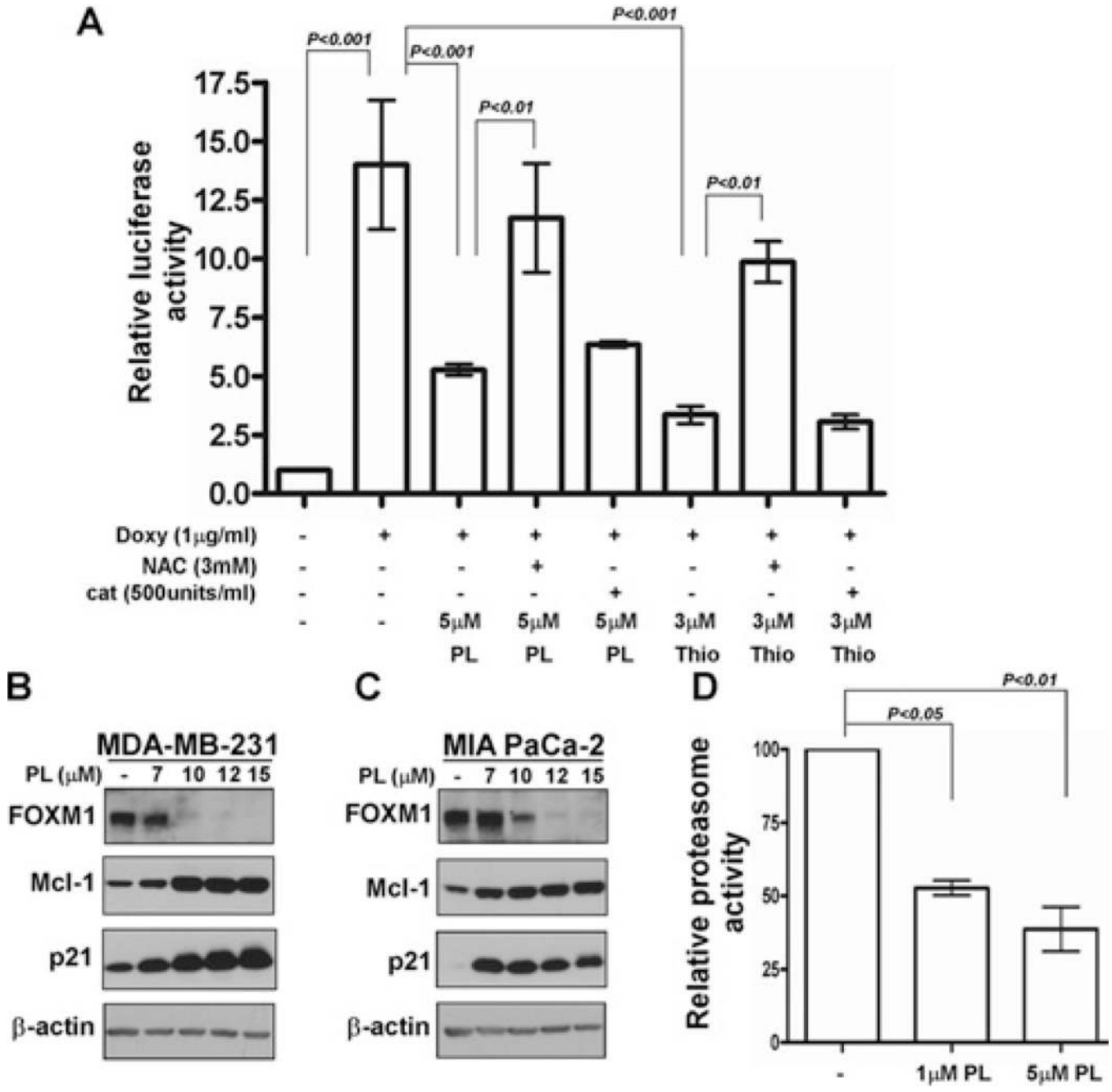

Figure 3. PL acts as a proteasome inhibitor

(**A**) C3-luc cells were treated as indicated overnight and luciferase activity was measured using the Luciferase Assay System kit (Promega). Values are means ± S.D. for a representative triplicate experiment. cat, catalase; Doxy, doxycycline; Thio, thiostrepton. (**B** and **C**) MDA-MB-231 breast and MIA PaCa-2 pancreatic human cancer cells were treated as indicated for 24 h. Immunoblotting was carried out for FOXM1, Mcl-1 and p21. β-Actin was used as the loading control. (**D**) 20S proteasome activity of purified proteasome was measured after PL treatment as described in the Materials and methods section. Values are means ± S.E.M. for two independent experiments.

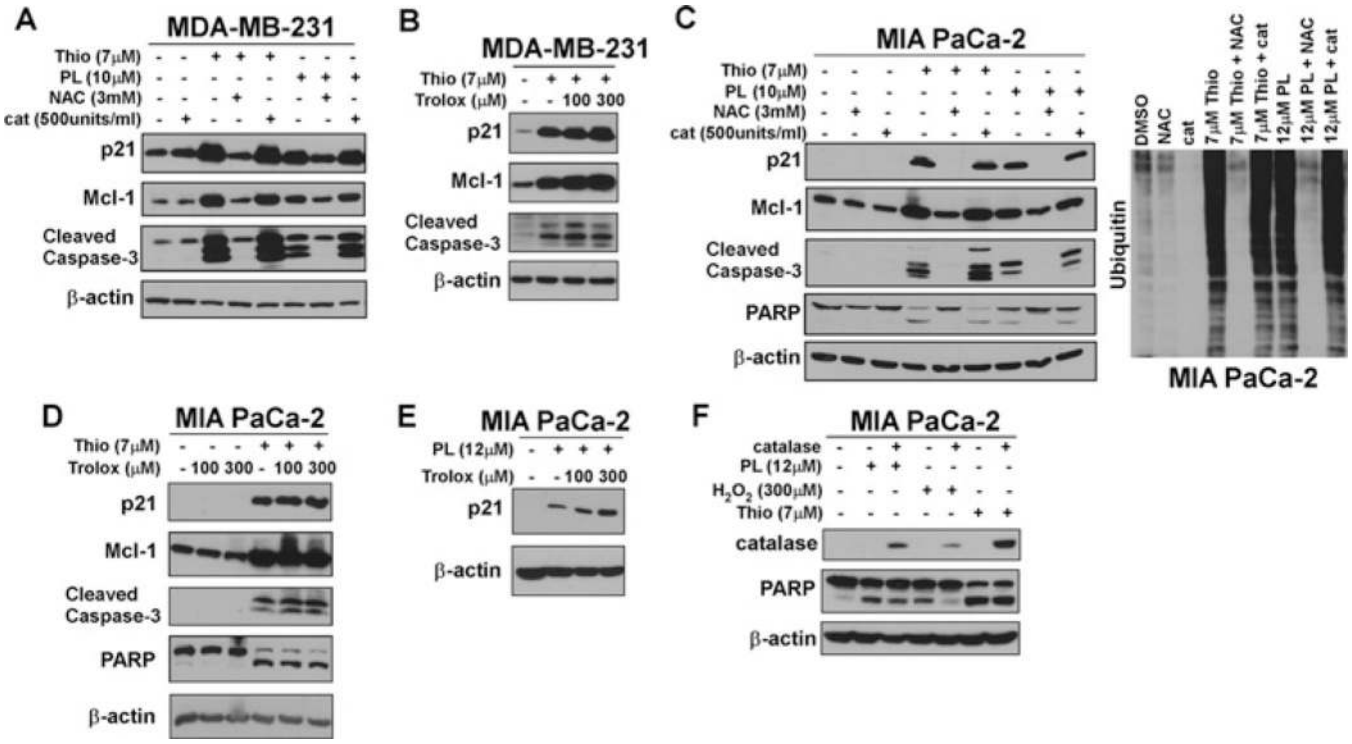

Figure 4. NAC, but not catalase or Trolox, inhibits proteasome inhibition by thiostrepton and PL

(**A**) MDA-MB-231 human breast cancer cells were treated with thiostrepton (Thio) and PL as indicated after a 2 h pre-incubation with 3 mM NAC or 500 units/ml catalase (cat). Immunoblot analysis of p21, Mcl-1 and cleaved caspase 3 was carried out 24 h after treatment. β-Actin was used as the loading control. (**B**) MDA-MB-231 human breast cancer cells were pre-incubated with the indicated concentrations of Trolox for 2 h and then treated with thiostrepton for 24 h. Immunoblotting was carried out with antibodies specific for p21, Mcl-1 and cleaved caspase 3. β-Actin was used as the loading control. (**C**) MIA PaCa-2 pancreatic cancer cells were pre-incubated with 3 mM NAC or 500 units/ml catalase for 2 h and then treated as indicated with thiostrepton and PL. At 24 h after treatment, cell lysates were immunoblotted for p21, Mcl-1, cleaved caspase 3, PARP and ubiquitin. β-Actin was used as the loading control. (**D** and **E**) MIA PaCa-2 pancreatic cancer cells were pre-incubated with Trolox for 2 h and then treated with thiostrepton (**D**) and PL (**E**) as indicated for 24 h. Immunoblotting was performed with antibodies against p21, Mcl-1, cleaved caspase 3 and PARP. β-Actin was used as the loading control. (**F**) MIA PaCa-2 pancreatic cancer cells were infected with catalase adenoviral particles for 48 h and then treated for 24 h as indicated. Immunoblotting was performed with antibodies specific for catalase and PARP. β-Actin was used as the loading control.

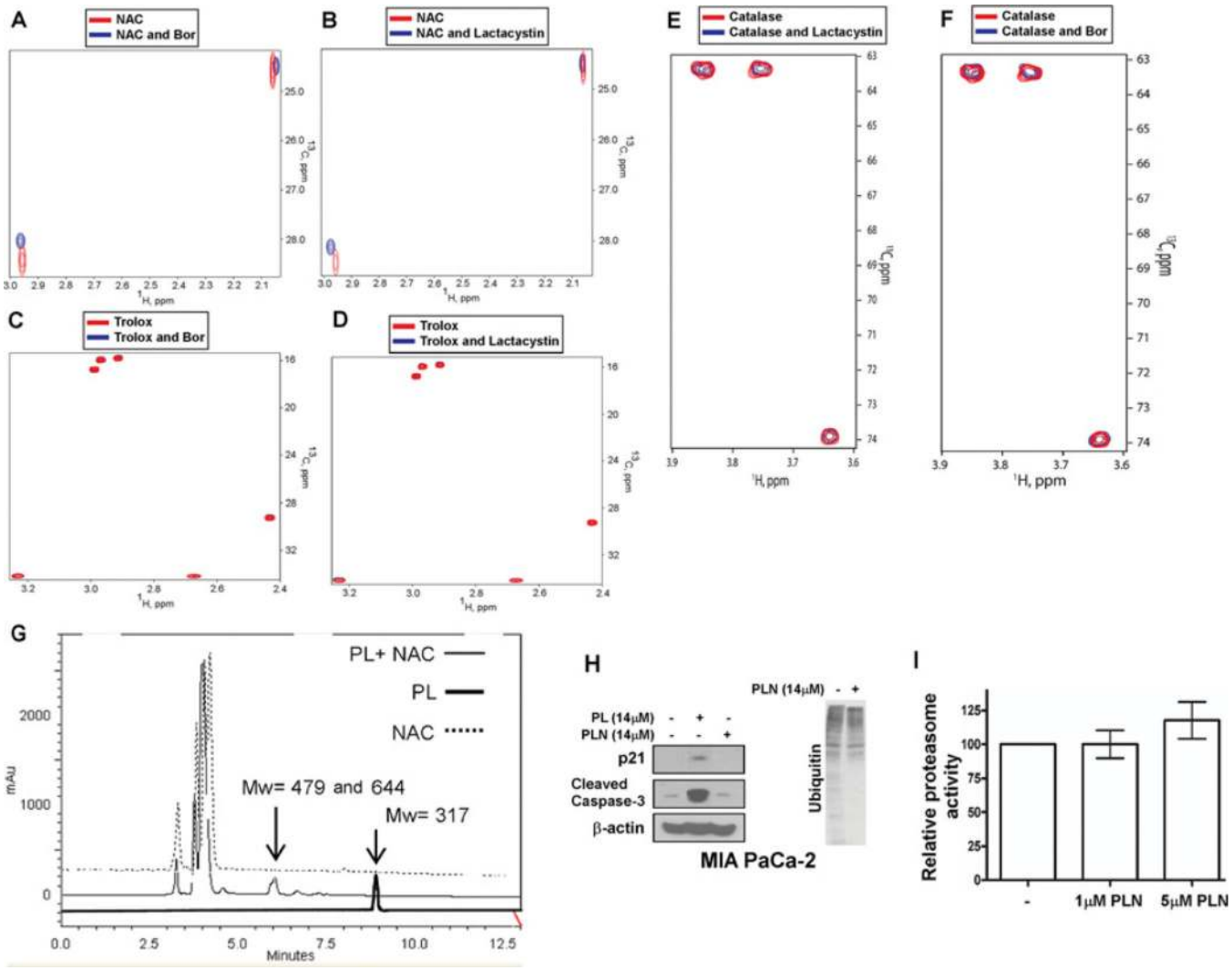

Figure 5. NAC directly interacts with proteasome inhibitors

(**A–F**) All NMR experiments were carried out at 25°C and scanned four times. The Figures show a superimposition of ^{1}H-^{13}C HSQC spectra of the following. (**A**) NAC (red) and a mixture of NAC and bortezomib (Bor) (blue). The ratio of NAC to bortezomib was 300:1 (300 mM:1 mM). (**B**) NAC (red) and a mixture of NAC and lactacystin (blue). The ratio of NAC to lactacystin was 300:1 (30 mM:100 μM). (**C**) Trolox (red) and a mixture of Trolox and bortezomib (blue). The ratio of Trolox to bortezomib was 5000:1 (50 mM:10 μM). (**D**) Trolox (red) and a mixture of Trolox and lactacystin (blue). The ratio of Trolox to lactacystin was 5000:1 (50 mM:10 μM). (**E**) Catalase (red) and a mixture of catalase and lactacystin (blue). The ratio of catalase to lactacystin was 500:1 (5 mM:10 μM). (**F**) Catalase (red) and a mixture of catalase and bortezomib (blue). The ratio of catalase to bortezomib was 500:1 (5 mM:10 μM). (**G**) PL (40 μmol) was incubated with NAC (8000 μmol) in Hepes (pH 7) for 24 h at 37°C, after which the reaction was analysed by LC-MS (C_{18} column, 0–100% ACN in 0–15 min, multi-wavelength UV detector) and separated by preparative HPLC. After the reaction with NAC, the peak associated with the PL species is shifted towards a shorter retention time, indicating a decrease in hydrophobic nature. The

new peak consisted of a mixture of compounds with molecular masses of 479 and 644, corresponding to the nucleophilic addition of one and two NACs respectively to the electrophilic sites of PL. (**H**) MIA PaCa-2 cells were incubated with 14 μM of either PL or NAC-modified PL (PLN) for 24 h, after which cell lysates were analysed for p21, cleaved caspase 3 and ubiquitin expression. PLN had no effect on cell death or ubiquination. (**I**) 20S proteasome activity of purified proteasome was measured after PLN treatment as described in the Materials and methods section. Values are means ± S.D. for a representative duplicate experiment.