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α,β -Unsaturated Carbonyl System of Chalcone-Based Derivatives is Responsible for Broad Inhibition of Proteasomal Activity and Preferential Killing of Human Papilloma Virus (HPV)-Positive Cervical Cancer Cells

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Abstract

Proteasome inhibitors have potential for the treatment of cervical cancer. We describe the synthesis and biological characterization of a new series of 1,3-diphenylpropen-1-one (chalcone)-based derivatives lacking the boronic acid moieties of the previously reported chalcone-based proteasome inhibitor 3,5-bis-(4-boronic acid-benzylidene)-1-methyl-piperidin-4-one and bearing a variety of amino acid substitutions on the amino-group of the 4-piperidone. Our lead compound **2** (RA-1) inhibits proteasomal activity and has improved dose-dependent anti-proliferative and pro-apoptotic properties in cervical cancer cells containing human papillomavirus. Further, it induces synergistic killing of cervical cancer cell lines when tested in combination with an FDA approved proteasome inhibitor. Exploration of the potential mechanism of proteasomal inhibition by our lead compound using in silico docking studies suggests that the carbonyl group of its oxopiperidine moiety is susceptible to nucleophilic attack by the γ -hydroxy threonine side chain within the catalytic sites of the proteasome.

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Supporting Information Availability. Analytical data of intermediates and products **2–10**, specificity and reversibility data are available free of charge at <http://pubs.acs.org>.

Keywords

Cervical Cancer; Proteasome Inhibitors; Chalcones; Ubiquitin Proteasome; System (UPS); UPS-stress

Introduction

The 26S proteasome is composed of two 19S regulatory sub-units, termed “caps” and of one 20S catalytic sub-unit, referred to as the “proteolytic core”^{1, 2}. The targeting of a protein for degradation by the proteasome occurs via its enzymic conjugation to the small protein ubiquitin. Chains of ubiquitin are recognized by the proteasome caps to facilitate the entrance of the targeted protein into the proteolytic chamber wherein the actual degradation occurs. The 20S proteasome comprises four stacked rings; two α - (outer) and two β - (inner) rings. Each β -ring is composed of seven sub-units containing three catalytic sites: the $\beta 1$ sub-unit is associated with a peptidylglutamyl peptide hydrolyzing-like (PGPH-like) activity the $\beta 2$ sub-unit is associated with the trypsin-like activity (T-like) while the $\beta 5$ sub-unit is associated with the chymotrypsin-like activity (CT-like). All three proteolytic activities utilize the γ -hydroxyl group of an N-terminal threonine residue within each catalytic site for nucleophilic attack of the α -amine proton donor/acceptor within the targeted protein³.

The polypeptide targets of the proteasome include proteins involved in cell cycle progression, survival and inflammation and while the ubiquitin-dependent proteasomal degradation is crucial for both, normal and malignant cells the higher demand for metabolic/catabolic activity associated with the malignant phenotype renders the ubiquitin-proteasome pathway a suitable tool for cancer treatment^{4, 5}.

Inhibition of the catalytic activities of the proteasome can be achieved by compounds that covalently bind the N-terminal threonine residue in the catalytic sites of the β -subunits; this includes Bortezomib⁶ (PS-341), Salinosporamide A⁷ (NPI-0052) and Carfilzomib⁸ or by compounds that bind to the catalytic sites of the β -subunits in a noncovalent fashion^{9, 10} like in the case of TMC-95A^{11, 12}, Ritonavir¹³ and lipopeptides¹⁴.

Undoubtedly, members of both classes have shown to have potential as antineoplastic agents with Bortezomib, a covalent slowly reversible proteasome inhibitor¹⁵, been the first FDA approved for the treatment of multiple myeloma and mantle cell lymphoma¹⁵.

Proteasome inhibitors may be particularly efficacious for certain cancers types with critical pathways that are dependent upon proteolytic degradation. Human papillomavirus (HPV) causes 5% of all cancers worldwide and the actions of only two of its oncoproteins, E6 and E7 are necessary to maintain the transformed state in cervical cancer¹⁶. Because E6 and E7 oncoproteins target p53 and pRb tumor suppression proteins for proteasomal degradation, proteasome inhibitors may have utility in the treatment of cervical and other HPV-related cancers^{17–19}.

Chalcones (1,3-diphenylpropen-1-ones) are naturally occurring compounds belonging to the flavonoid family, and include curcumin and green tea-derived polyphenols and flavonoids. While several chalcones represent promising tools for cancer treatment^{20–22}, their mechanism of action as anti-proliferative and anti-angiogenic agents remains unknown. However, recent data suggests that the carbonyl carbon of tea polyphenols and flavonoids confers proteasome-inhibitor properties upon them^{21, 23, 24}. Herein we report an effort to further optimize the previously described chalcone-based proteasome inhibitor 3,5-bis-(4-boronic acid-benzylidene)-1-methyl-piperidin-4-one (AM-114)²⁰ carrying an α, β -

unsaturated carbonyl system and two boronic acid moieties, compound **1**. In here, we describe the synthesis and the biological characterization of a new series of α,β -unsaturated carbonyl system compounds lacking the boronic acid moieties of our previously described proteasome inhibitor and bearing various amino acid substitutions on the amino-group of the 4-piperidone. In this new series of compounds, we explore whether the carbonyl group of the α,β -unsaturated system might function as a substrate for the γ -hydroxy threonine side chain within the catalytic sites of the proteasome, as has been previously suggested for curcumin²³. The amino group of the 4-piperidone is functionalized with either aromatic (compound **2**, **3**), hydrophobic (compound **4**), acidic (compound **5**) or basic (compound **6**) mono-aminoacidic substitutions in position P. These mono aminoacidic substitutions were chosen because they are predicted to direct the selectivity of proteasome inhibitors towards chymotrypsin-like (compound **2–4**), peptidylglutamyl peptide hydrolyzing-like (compound **5**) and trypsin-like (compound **6**) activities of the proteasome respectively^{25, 26}. In compounds **7–10** the length of the aminoacid portion was extended by introduction of dipeptide sequences Phe-Tyr, (compound **7**), Glu-Asp (compound **8**), Lys-Arg (compound **9**) or Arg-Lys (compound **10**) to increase the potential affinity of the newly synthesized inhibitors for chymotrypsin-like, peptidylglutamyl peptide hydrolyzing-like and trypsin-like of the proteasome respectively as previously reported^{25–28}.

On the basis of our results we propose that, unlike our previously identified chalcone-based proteasome inhibitor, the presence of the boronic acid groups is not required for conferring proteasome inhibitory capacity to these new series of compounds and that while the presence of single aromatic aminoacid substitution is capable of equally inhibiting the three catalytic activity of the proteasome, the presence of tyrosine or leucine decrease the overall proteasome inhibitor capacity. Further, the combination of our proteasome inhibitor lead compound and the FDA approved proteasome inhibitor Bortezomib induces synergistic killing of cervical cancer cell cells. Finally, docking simulation conducted on our new lead compound suggest that the α,β -unsaturated carbonyl system may represent the functional group for nucleophilic attack from the N-terminal threonine residue in the catalytic sites.

Results and Discussion

Synthesis

The compounds **2–10** were synthesized following the schematic strategy reported in Scheme-I and II by the classical solution and solid phase reactions. 3,4-dichloro benzaldehyde **1** (2.0 mmol) was added to a suspension of 4-piperidone hydrochloride monohydrate **2** (1.0 mmol) in glacial acetic acid (15 ml). Dry hydrogen chloride gas was passed through this mixture for 0.5 h during which time a clear solution was obtained. After standing at room temperature for 24 h, the precipitate (AcOH salt of bischalcone) was collected and dried under the vacuum. Compound **3** was treated with triethyl amine/ dichloro methane and the resultant mixture was stirred for 30 min. Solvents were removed under rotary evaporator and the crude compound was extracted with ethyl acetate/ water. Organic layer was washed again with water (50 ml), and evaporated and dried to get bischalcone **4**. p-Nitrophenyl chloroformate (10 mmol) and diisopropylethylamine (10 mmol) were added to the amino acid loaded trityl linked polystyrene-1% divinylbenzene resin swollen in THF under nitrogen in a glass vial. After 45 min the resin was washed with THF (3 × 10 mL), bischalcone **4** (10 mmol) and NMM (10 mmol) in THF were added and after 16 – 24 h stirring a yellow suspension was obtained. After washing the resin with DCM and methanol each three times the polymer was dried. The resin bound compounds were cleaved using 20% trifluoroacetic acid in DCM (30 min) yielded compounds **2–10**. All the compounds were purified by HPLC and characterized by MS, NMR.

Biological Results

To explore the feasibility of the use of new chalcone-based derivatives for the treatment of cervical cancer, compounds **1–10** (Table 1) were tested for their cell growth inhibitory capacity in the respectively HPV18- and HPV16-positive HeLa and CaSki cervical cancer cell lines in a range of concentration from 100 to 0.01 μM . The IC_{50} values by XTT assay in HeLa and CaSki (Table 2) show that compounds **2–4** have an anti-proliferative activity that is the same range of concentration as our previously reported proteasome inhibitor compound **1**, while compounds **5–10** showed a dramatic decreased of the anti-proliferative activity with $\text{IC}_{50} > 50 \mu\text{M}$. Interestingly, compound **2** was found 15-fold more potent than compound **1** with IC_{50} values of 0.32 μM and 1.5 μM for HeLa and CaSki respectively. To test whether the decrease in cell viability following exposure to compound **2–4** was due to perturbation of proteasomal function we examined the impact of compounds **2–4** treatment on accumulation of poly-ubiquitinated proteins by immunoblot analysis in HeLa cervical cancer cells. As shown in Figure 1, eight hours treatment of HeLa cells with compounds **2–4** produced an increase in the levels of poly-ubiquitinated proteins similar to the one obtained with the FDA approved proteasome inhibitor Bortezomib here used as positive control²⁹. The rapid accumulation of poly-ubiquitinated proteins observed following exposure to compounds **2–4** is consistent with impairment of proteasomal functions. Notably, no changes in the levels of poly-ubiquitinated proteins were observed in cells exposed to compound **5**, here used as negative control, which also showed an $\text{IC}_{50} > 50 \mu\text{M}$ in HeLa and CaSki cell lines. Taken together these results suggest that the toxicity exerted by compounds **2–4** on cervical cancer cells is associated with in-vivo proteasomal inhibition and that, when tested in the same range of concentrations, compounds that fail to affect cell viability also fail to cause impairment of proteasomal functions. To gain insight on the ability of compounds **2–4** to inhibit specific catalytic sub-units within the 20S proteasome we measured residual fluorogenic activity in 20S purified proteasome pre-exposed to 10 μM of compounds **2–4** for a period of 8 hours following addition of fluorogenic substrates specific for the three major proteolytic activities of the proteasomes²⁶. Specifically, compounds **2–4** as well as the FDA approved proteasome inhibitor Bortezomib were tested for their capacity to inhibit the chymotrypsin-like (CT-like), trypsin-like (T-like) and peptidylglutamyl peptide hydrolyzing-like (PGPH-like) activities in vitro of the proteasome purified from lymphoblastoid cell lines (LCLs)³⁰. The profile of proteasome inhibition of compounds **2–4** (Figure 2) shows that 10 μM of compound **2** nearly fully abrogated the chymotrypsin-like (CT-like), trypsin-like (T-like) and peptidylglutamyl peptide hydrolyzing-like (PGPH-like) activities of 20S proteasome while compounds **3** and **4** only induced partial inhibition of the chymotrypsin-like (CT-like), trypsin-like (T-like) and peptidylglutamyl peptide hydrolyzing-like (PGPH-like) activities with a selective inhibition pattern toward the chymotrypsin-like (CT-like) activity. The IC_{50} values are reported in Table 3. The rapid accumulation of poly-ubiquitinated proteins within 8 hours and as early as 2 hours (data not shown) observed following compound **2** exposure together with its profile of inhibition on the catalytic sub-units of the 20S proteasome suggest that cell death following compound **2** exposure is primarily due to impairment of proteasomal functions. However, to exclude the possibility that lead compound **2** inhibited other proteases we tested for its capacity to inhibit Cathepsin D and Calpain *in vivo* by measuring the residual Cathepsin D and Calpain activity in HeLa cells exposed to various concentrations of compound **2**. We found that **2** had no inhibitory capacity toward Cathepsin D or Calpain activities when tested at concentrations up to 50 μM (Supporting Information Fig.1). Taken together, these results suggest that while the three-carbon α,β -unsaturated carbonyl carbons system structure may represent the functional group for nucleophilic attack from the N-terminal threonine residue in the catalytic site of the proteasome the nature of the amino acid inserted in the functionalized amino group of the oxo-piperidine play a role in determining both the potency and the selectivity of this class of compounds. In particular, compound **2** containing

the aromatic amino acid phenylalanine is, capable of equally inhibiting the three catalytic activity of the proteasome (IC_{50} 5 μ M), while the presence of tyrosine (compound **3**) or leucine (compound **4**) seems to correspond to a general decrease of proteasomal activity and specifically towards trypsin-like and peptidylglutamyl peptide hydrolyzing-like activities. The higher demand for metabolic/catabolic activity associated with the malignant phenotype renders proteasome inhibitors a suitable tool for cancer therapy^{4,5}. The toxicity profile observed in the HeLa and CaSki cervical cancer cells following chalcones treatment suggest that the high demand for metabolic activity of highly proliferating cancer cell lines may render them more sensitive to proteasomal inhibition as compared to the normal counterpart. To test this hypothesis, the effect on cell viability following lead compound **2** exposure was compared in two additional cervical cancer cell lines SiHa and ME180 versus primary human Keratinocytes. As shown in Fig. 3, compound **2** treatment produced a dose-dependent drop in the viability of SiHa and ME180 but minimal effects on the viability of primary human Keratinocytes. These findings suggested that compound **2** induces dose-dependent cell toxicity in a variety of HPV+, but not in normal cells and that its toxicity may be associated with transformation by HPV, regardless of the oncogenic type (HeLa are HPV18+, SiHa and CaSki are HPV16+, ME180 are HPV58+).

Because our lead compound **2** had a different profile of selectivity towards the catalytic activities of the proteasome as compared to Bortezomib we tested whether combination of the latter and compound **2** would be able to generate a broader and therefore more potent inhibition of proteasomal functions as compared to single agents alone at lower doses. To test this hypothesis, we utilized the engineered Ubiquitin-Firefly (Ub-FL) reporter in which four copies of mutant ubiquitin (ubiquitin G76V) are fused to the N-terminus of firefly luciferase (FL)³¹. The Ub-FL and the FL control expression vectors were transiently transfected in HeLa cervical cancer cell line and expression levels of Ub-FL and FL proteins were confirmed by Western Blot analysis (data not shown). Following 42 hours from transfection cells were mock treated or treated with sub-optimal doses of either Bortezomib (5nM), compound **2** (1 μ M) or the combination of both. The results shown in Fig. 4 indicate that sub-optimal doses of the compounds were able to significantly enhance the stabilization of the Ub-FL degron compared to single agents alone.

Recent studies indicate that inhibition of multiple catalytic activities of the proteasomes by proteasome inhibitors with distinct spectra of activity results in potent antitumor activity at lower and potentially less toxic doses. Because combination of compound **2** and Bortezomib triggers a broader and more potent inhibition of proteasomal functions in living cells as compared to single agents alone we tested whether the combination of the latter and compound **2** would induce synergistic killing in HeLa cervical cancer cells. Isobologram analysis (Figure 5) indicates that rather than a simple additive killing the combination is highly synergistic, consistent with inhibition of complementary catalytic functions of the proteasomes. The optimal Combination Index (CI) was achieved at the following concentrations: (CI=0.6) Bortezomib 3.95 nmol/L/compound **2** 2.5 mM/L; (CI=0.6) Bortezomib 6.125 nmol/L/compound **2** 1.64 mM/L; (CI=0.59) Bortezomib 7.5 nmol/L/compound **2** 1.06 mM/L. We then wanted to test whether the reduction in the cell viability observed in HeLa cells following exposure to Bortezomib and compound **2** alone or in combination was consistent with cell death via apoptosis. To test this hypothesis HeLa cells were mock-treated or treated with sub-optimal doses of Bortezomib (5nM), lead compound **2** (1 μ M) or combination of Bortezomib (5nM)+compound **2** (1 μ M) and their morphology evaluated 48 hours post treatment. As shown in Figure 6 combination treatment resulted in greater apoptotic-associated morphological changes in HeLa cells as compared to single treatment alone.

In order to obtain a deeper insight into the molecular basis of the enzyme-inhibitor recognition process, the molecule exhibiting the highest affinity, compound **2**, was chosen for performing docking simulations to 20S proteasome, co-crystallized with the $\beta 5$ -inhibitors homobelactosin C and 2-spiro-lactacystin, whose structures were retrieved from the Protein Data Bank, PDB³². The Lowest-Unoccupied-Molecular-Orbital (LUMO) of compound **2** was found by DFT calculations to be localized mainly around the carbonyl group of the oxo-piperidine moiety, as shown in Figure 7A; this finding, according to the fact that a nucleophilic reaction is usually predicted to occur on the atom having the largest LUMO, could be taken as an indication that this group is the most reactive towards an attacking nucleophile. Actually, in the first docking simulation, out of ten “poses” with high docking scores, four have the “active” carbonyl group located at 3–4 Å far away from the nucleophilic threonine residue, a distance that is typical of a hydrogen bonding interaction in this type of emulation with rigid protein frame. The pose with the highest score and a schematic view of the ligand-enzyme interaction are reported in Figure 7B and C respectively. The binding pocket appears to have T shape, resembling the molecule’s conformation; part of the inhibitor, *i.e.* the two chlorine atoms of phenyl A (upper part of Figure 7C) and some of the carbon atoms belonging to the terminal phenyl group C, are placed toward the inner proteasome channel. Many of the aminoacid residues, which we suggest, are participating in the formation of the ligand-binding pocket, *i.e.* Ser129, Gln131, Gly128, Lys33 are found to be involved in homobelactosin-enzyme interactions as well. A π - π short contact (distance between the centroids of the phenyl rings 3.6 Å) was suggested between Tyr168 residue and phenyl group C. Most interestingly, the oxygen of the oxo-piperidine was located at a distance of 3.1 Å from the active Thr residue, positioning in this way the carbonyl group in a suitable place to undergo a possible nucleophilic attack. Furthermore, the ‘docked’ compound **2** accommodated in the $\beta 5$ binding site of the proteasome/ 2-spiro-lactacystin structures shows a very similar interaction pattern with the surrounding residues. To better visualize how the present compound could act inside the enzyme, a superposition of the three inhibitors compound **2**, homobelactosin C and 2-spiro-lactacystin, is shown in Fig. 7D. Compound **2** although quite different from a chemical point of view, is able to efficiently ‘occupy’ the binding pocket, in particular in proximity of the active threonine residue. Moreover, the CO-phenylalanine arm seems to be well superimposable to homobelactosin molecule, even with regard to the spatial disposition of N/O atoms.

The molecular docking studies suggest a nucleophilic attack from the N-terminal threonine residue of the β -subunits to the carbonyl group of compound **2** as a mechanism for proteasomal inhibition. To test whether the binding of compound **2** to the proteasome is reversible we performed washout experiment in HeLa cells briefly treated with compound **2** or Bortezomib, here used as reversible inhibitor positive control, and evaluated for residual cell viability. Our results (Supplemental Information Fig.2) indicate that similarly to Bortezomib, compound **2** washout leads to a partial recovery in cell viability as compared to non-washout cultures. Taken together this suggests that compound **2** is a covalent and reversible proteasome inhibitor.

Conclusion

In this report we have designed, synthesized and defined the biological properties of a new series of chalcone-based compounds containing an α , β -unsaturated carbonyl system and bearing amino acid substitutions on the amino-group of the 4-piperidone. The lead within this series, compound **2**, is an inhibitor of proteasomal function *in vitro* and in living cells, is cell permeable and acts upon all three known proteolytic activities in purified proteasomes. Nucleophilic susceptibility suggested by *in silico* docking studies conducted with compound **2** suggest that the α , β -unsaturated carbonyl system may represent the functional group for

nucleophilic attack from the N-terminal threonine residue in the catalytic sites of the proteasome. Moreover, a single neutral amino acid substitution with tyrosine or leucine results in a decrease in the overall inhibition proteasome activity in all three known catalytic activities and reduced killing of HPV+ cervical cancer cells. Importantly, the different profile of proteasome inhibition of our lead compound **2** versus Bortezomib results in synergistic killing of cervical cancers indicating that that simultaneous inhibition in cervical cancer cells of multiple proteasome activities results is a prerequisite for cervical cancer cells killing. These findings provide the rationale for combining compound **2** with Bortezomib to potentially achieve broader proteasome inhibition and improved antitumor activity while possibly allowing for the use of lower doses of Bortezomib to minimize toxic effects.

Experimental Section

General

All reagents and solvents were obtained from Aldrich. Anhydrous solvents were used as received. Reaction progress was monitored with analytical thin-layer chromatography (TLC) plates carried out on 0.25 mm Merck F-254 silica gel glass plates. Visualization was achieved using UV illumination. ¹H NMR spectra were obtained at 400 MHz on a Bruker Avance spectrometer and are reported in parts per million downfield relative to tetramethylsilane (TMS). EI-MS profiles were obtained using a Bruker Esquire 3000 plus. All tested compounds were >95 % purity as determined by a waters HPLC system and one of the following methods, unless explicitly noted. Method 1 (at both 214 and 254 nm): used semi-prep RP-HPLC column from Phenomenex: Luna C18, particle size 10u, 250×10 mm with the flow rate of 1–6 mL/min with a gradient of 5–95 % acetonitrile/water with 0.1 % TFA over 30 min. Method 2: (at both 214 and 254 nm): used semi-prep RP-HPLC column from Phenomenex: Luna C18, particle size 10u, 250×10 mm with the flow rate of 1–6 mL/min with a gradient of 5–95 % acetonitrile/water with 0.1 % TFA over 40 min.

Chemistry

Synthesis of library compounds was based on Scheme I and II. 3,5-Bis-(arylidene)-4-piperidones **3** have been synthesized utilizing a Claisen-Schmidt condensation of 4-piperidone monohydrate hydrochloride **2**, and 3,4-dichloro benzaldehyde **1** by passing dry HCl gas through a glacial acetic acid solution³³. The AcOH salt of bischalcone **3** was treated with triethyl amine to get free amine **4** (Scheme I). *p*-Nitrochloroformate **5** was treated with the amine functionality of appropriate amino acid **6** bound to chlorotrityl resin in the presence of *N,N*-diisopropylethylamine (DIEA) resulting in the formation of compound **7** (*in situ*) (Scheme II) which has been further coupled to compound **4** in the presence of NMM resulted in the formation of compound **8**, which then detached from the chlorotrityl resin by using 20% trifluoroacetic acid yielded the target compounds **2–10**. The dipeptides were synthesized using *tert*-butoxycarbonyl manual stepwise solid-phase peptide synthesis (SPPS) on chlorotrityl resin using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/(DIEA).

Cell culture

Cervical cancer cell lines HeLa, CaSki, SiHa and ME180 were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 5% CO₂. Keratinocytes were obtained from Invitrogen (Carlsbad, CA) and cultured in defined Keratinocyte-SFM.

Cell viability assay

Cell viability was determined by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay (Roche Diagnostics GmbH, Mannheim, Germany). Cells seeded at the concentration of 1,000 per well in 100 μ L medium in 96-well plate were treated with chalcone-based derivatives at specified concentrations. After the indicated periods, cells were incubated according to the manufacturer's protocol with the XTT labeling mixture for 4 hours. Formazan dye was quantified using a spectrophotometric plate reader to measure the absorbance at 450nm (ELISA reader 190; Molecular Devices, Sunnyvale, CA). All experiments were done in triplicate.

Plasmids

The plasmids p-CMV-FL and Ub-FL containing four tandem copies of ubiquitin G76V were a generous gift of Dr. D. Piwnica-Worms (Washington University School of Medicine, St. Louis, MO).

Luciferase Assay

HeLa cells were cultured in DMEM medium with 10% heat-inactivated FBS, 1% glutamine and 0.1% penicillin/streptomycin solution. 2×10^5 cells/6well were transfected using Effectine transfection reagent (Qiagen) with either p-CMV-FL or Ub-FL plasmids. 24 hours post-transfection cells were transferred into 96 well plates at 1×10^4 cells/well in 100 μ L culture volume. After cells attached (18 hours), culture medium was replaced with 100 μ L medium containing drugs at the indicated concentrations. After 6 hs of treatment, luciferase activity in cell lysate was determined with a luciferase assay kit (Promega). Values are calculated as Relative Luminescence Units (RLU) and expressed as % of control.

Cellular morphology analysis

Leica DM IL LED inverted microscope was used for the imaging with phase contrast for cellular morphology.

In Vitro Effect of Proteasome Inhibitors

Cells (5×10^8) were washed in cold PBS and resuspended in buffer containing 50 mM TRIS-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT (Sigma), 2 mM ATP and 250 mM sucrose. Glass beads equivalent to the volume of the cell suspension were added, and the mixture was vortexed for 1 min at 4°C. Beads and cell debris were removed by 5 min centrifugation at 1,000g, followed by 20 min centrifugation at 10,000g^{34, 35}. Lysates were cleared by ultracentrifugation for 1 hr at 100,000g and supernatants were further ultracentrifuged for 5 hr at 100,000g. Proteasome-containing pellets were resuspended in 0.5 ml of homogenization buffer [50 mM TRIS-HCl (pH 7.5), 100 mM KCl, 15% glycerol]. Protein concentration was determined using the BCA protocol (Pierce, Rockford, IL). Fluorogenic substrates Suc-LLVY-AMC, Boc-LRR-AMC and Ac-YVAD-AMC were used to measure chymotryptic-like, tryptic-like and caspase-like activities, respectively. Semipurified proteasomes were pretreated or not with inhibitors for 30 min at 37°C, were assayed at 37°C for 45 min using the different peptide substrates in a buffer containing 50 mM TRIS-HCl (pH 7.5), 5 mM $MgCl_2$ and 1 mM DTT (final volume 100 l). The reaction was quenched with 1 ml 1% SDS and fluorescence determined by fluorimeter (Perkin-Elmer, Beaconsfield, UK) with excitation at 380 nm and emission at 440 nm. Data are expressed as the percent inhibition relative to untreated proteasomal preparations.

Docking simulation

The equilibrium structure of compound **2** was obtained by DFT calculations at the PW91/DN level, using Dmol³ code of the Material Studio system program³⁶. At first, the molecule

was docked to the crystal structure of the yeast 20S proteasome in complex with the inhibitor homobelactosin C covalently bound to the terminal threonine of the $\beta 5$ active site [PDB code: 3E47]³⁷; then, the molecule in the same conformation was docked to the crystal structure of 2-spiro-lactacystin/proteasome complex [PDB code: 3DY4]³⁸. The structures have been retrieved from the Protein Data Bank (PDB) archive (www.pdb.org) and the docking simulation has been performed using the MOE/Dock procedure integrated in the MOE system of programs (Chemical Computing Group Inc., MOE 2006.08). Before the simulation, hydrogen atoms were added to the inner part of the enzyme and the energy of the structure was minimized using the Amber99 molecular mechanics force field³⁹. During the first step of the docking application, the ligand was treated in a flexible manner by rotating rotatable bonds and a number of the ligand's configurations were generated and scored in an effort to determine favorable binding modes by the application of the Alpha Triangle placement method. The ligand was docked restricting the search for binding modes to a specific, small region of the subunit called the $\beta 5$ -binding site, in which the terminal Thr1 is the active residue.

Statistical analysis

Results are reported as mean $\pm$ Standard Error (SE). Statistical significance of differences was assessed by two-tailed Student's *t* using Prism (V.5 Graphpad, San Diego, CA) and Excel. The level of significance was set at $p \leq 0.05$. The combination index (CI) of PS-341 and compound **2** was calculated by the median-effect analysis according to the method of Chou and Talaly⁴⁰. CI<1 indicates synergism, CI=1 indicates additivity, and CI>1 indicates antagonism. Further regression analyses were performed to stabilize estimates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AcOH	Acetic Acid
THF	Tetrahydro Furan
DCM	Dichloro Methane
NMM	N-Methyl Morpholine
HPV	Human Papilloma Virus
DMEM	Dulbecco's Modified Eagle Medium
Keratinocyte-SFM	Serum Free Medium

References

1. Ciechanover A, Finley D, Varshavsky A. The ubiquitin-mediated proteolytic pathway and mechanisms of energy-dependent intracellular protein degradation. *J Cell Biochem.* 1984; 24:27–53. [PubMed: 6327743]
2. Ciechanover A. The ubiquitin-mediated proteolytic pathway. *Brain Pathol.* 1993; 3:67–75. [PubMed: 8269085]
3. Orłowski M, Wilk S. Catalytic activities of the 20 S proteasome, a multicatalytic proteinase complex. *Arch Biochem Biophys.* 2000; 383:1–16. [PubMed: 11097171]
4. Bazzaro M, Lin Z, Santillan A, Lee MK, Wang MC, Chan KC, Bristow RE, Mazitschek R, Bradner J, Roden RB. Ubiquitin proteasome system stress underlies synergistic killing of ovarian cancer cells by bortezomib and a novel HDAC6 inhibitor. *Clin Cancer Res.* 2008; 14:7340–7347. [PubMed: 19010849]
5. Bazzaro M, Lee MK, Zoso A, Stirling WL, Santillan A, Shih Ie M, Roden RB. Ubiquitin-proteasome system stress sensitizes ovarian cancer to proteasome inhibitor-induced apoptosis. *Cancer Res.* 2006; 66:3754–3763. [PubMed: 16585202]
6. Curran MP, McKeage K. Bortezomib: a review of its use in patients with multiple myeloma. *Drugs.* 2009; 69:859–888. [PubMed: 19441872]
7. Fenical W, Jensen PR, Palladino MA, Lam KS, Lloyd GK, Potts BC. Discovery and development of the anticancer agent salinosporamide A (NPI-0052). *Bioorg Med Chem.* 2009; 17:2175–2180. [PubMed: 19022674]
8. Kuhn DJ, Chen Q, Voorhees PM, Strader JS, Shenk KD, Sun CM, Demo SD, Bennett MK, van Leeuwen FW, Chanan-Khan AA, Orłowski RZ. Potent activity of carfilzomib, a novel, irreversible inhibitor of the ubiquitin-proteasome pathway, against preclinical models of multiple myeloma. *Blood.* 2007; 110:3281–3290. [PubMed: 17591945]
9. Genin E, Reboud-Ravaux M, Vidal J. Proteasome inhibitors: recent advances and new perspectives in medicinal chemistry. *Curr Top Med Chem.* 2010; 10:232–256. [PubMed: 20166955]
10. Basse N, Montes M, Marechal X, Qin L, Bouvier-Durand M, Genin E, Vidal J, Villoutreix BO, Reboud-Ravaux M. Novel organic proteasome inhibitors identified by virtual and in vitro screening. *J Med Chem.* 2010; 53:509–513. [PubMed: 19919035]
11. Kaiser M, Groll M, Renner C, Huber R, Moroder L. The core structure of TMC-95A is a promising lead for reversible proteasome inhibition. *Angew Chem Int Ed Engl.* 2002; 41:780–783. [PubMed: 12491334]
12. Kohno J, Koguchi Y, Nishio M, Nakao K, Kuroda M, Shimizu R, Ohnuki T, Komatsubara S. Structures of TMC-95A-D: novel proteasome inhibitors from *Apiospora montagnei* sacc. TC 1093. *J Org Chem.* 2000; 65:990–995. [PubMed: 10814045]
13. Laurent N, de Bouard S, Guillamo JS, Christov C, Zini R, Jouault H, Andre P, Lotteau V, Peschanski M. Effects of the proteasome inhibitor ritonavir on glioma growth in vitro and in vivo. *Mol Cancer Ther.* 2004; 3:129–136. [PubMed: 14985453]
14. Basse N, Papapostolou D, Pagano M, Reboud-Ravaux M, Bernard E, Felten AS, Vanderesse R. Development of lipopeptides for inhibiting 20S proteasomes. *Bioorg Med Chem Lett.* 2006; 16:3277–3281. [PubMed: 16630721]
15. Dick LR, Fleming PE. Building on bortezomib: second-generation proteasome inhibitors as anti-cancer therapy. *Drug Discov Today.* 2010; 15:243–249. [PubMed: 20116451]
16. Hawley-Nelson P, Vousden KH, Hubbert NL, Lowy DR, Schiller JT. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *EMBO J.* 1989; 8:3905–3910. [PubMed: 2555178]
17. Reinstein E, Scheffner M, Oren M, Ciechanover A, Schwartz A. Degradation of the E7 human papillomavirus oncoprotein by the ubiquitin-proteasome system: targeting via ubiquitination of the N-terminal residue. *Oncogene.* 2000; 19:5944–5950. [PubMed: 11127826]
18. Narisawa-Saito M, Kiyono T. Basic mechanisms of high-risk human papillomavirus-induced carcinogenesis: roles of E6 and E7 proteins. *Cancer Sci.* 2007; 98:1505–1511. [PubMed: 17645777]

19. Boulet G, Horvath C, Vanden Broeck D, Sahebali S, Bogers J. Human papillomavirus: E6 and E7 oncogenes. *Int J Biochem Cell Biol.* 2007; 39:2006–2011. [PubMed: 17768080]
20. Achanta G, Modzelewska A, Feng L, Khan S, Huang P. A boronic-chalcone derivative exhibits potent anticancer activity through inhibition of the proteasome. *Mol Pharmacol.* 2006; 70:426–433. [PubMed: 16636137]
21. Bonfili L, Cecarini V, Amici M, Cuccioloni M, Angeletti M, Keller JN, Eleuteri AM. Natural polyphenols as proteasome modulators and their role as anticancer compounds. *FEBS J.* 2008; 275:5512–5526. [PubMed: 18959740]
22. Cuccioloni M, Mozzicafreddo M, Bonfili L, Cecarini V, Eleuteri AM, Angeletti M. Natural occurring polyphenols as template for drug design. Focus on serine proteases. *Chem Biol Drug Des.* 2009; 74:1–15. [PubMed: 19519739]
23. Milacic V, Banerjee S, Landis-Piowar KR, Sarkar FH, Majumdar AP, Dou QP. Curcumin inhibits the proteasome activity in human colon cancer cells in vitro and in vivo. *Cancer Res.* 2008; 68:7283–7292. [PubMed: 18794115]
24. Mozzicafreddo M, Cuccioloni M, Bonfili L, Eleuteri AM, Fioretti E, Angeletti M. Antiplasmin activity of natural occurring polyphenols. *Biochim Biophys Acta.* 2008; 1784:995–1001. [PubMed: 18456009]
25. Bogyo M, Shin S, McMaster JS, Ploegh HL. Substrate binding and sequence preference of the proteasome revealed by active-site-directed affinity probes. *Chem Biol.* 1998; 5:307–320. [PubMed: 9653549]
26. Kessler BM, Tortorella D, Altun M, Kisselev AF, Fiebigler E, Hekking BG, Ploegh HL, Overkleeft HS. Extended peptide-based inhibitors efficiently target the proteasome and reveal overlapping specificities of the catalytic beta-subunits. *Chem Biol.* 2001; 8:913–929. [PubMed: 11564559]
27. Marastoni M, Baldisserotto A, Trapella C, Gavioli R, Tomatis R. Synthesis and biological evaluation of new vinyl ester pseudotriptide proteasome inhibitors. *Eur J Med Chem.* 2006; 41:978–984. [PubMed: 16713026]
28. Baldisserotto A, Marastoni M, Fiorini S, Pretto L, Ferretti V, Gavioli R, Tomatis R. Vinyl ester-based cyclic peptide proteasome inhibitors. *Bioorg Med Chem Lett.* 2008; 18:1849–1854. [PubMed: 18294845]
29. Chauhan D, Hideshima T, Anderson KC. Targeting proteasomes as therapy in multiple myeloma. *Adv Exp Med Biol.* 2008; 615:251–260. [PubMed: 18437898]
30. Baldisserotto A, Destro F, Vertuani G, Marastoni M, Gavioli R, Tomatis R. N-terminal-prolonged vinyl ester-based peptides as selective proteasome beta1 subunit inhibitors. *Bioorg Med Chem.* 2009; 17:5535–5540. [PubMed: 19577931]
31. Luker GD, Pica CM, Song J, Luker KE, Pownall-Worms D. Imaging 26S proteasome activity and inhibition in living mice. *Nat Med.* 2003; 9:969–973. [PubMed: 12819780]
32. Groll M, Huber R. Purification, crystallization, and X-ray analysis of the yeast 20S proteasome. *Methods Enzymol.* 2005; 398:329–336. [PubMed: 16275340]
33. McElvain SM, McMahon RE. Piperidine derivatives. 4-piperidone, 4-piperidinol and certain of their derivatives. *J. Am. Chem. Soc.* 1949; 71(3)
34. Gavioli R, Vertuani S, Masucci MG. Proteasome inhibitors reconstitute the presentation of cytotoxic T-cell epitopes in Epstein-Barr virus-associated tumors. *Int J Cancer.* 2002; 101:532–538. [PubMed: 12237893]
35. Hendil KB, Uerkevitz W. The human multicatalytic proteinase: affinity purification using a monoclonal antibody. *J Biochem Biophys Methods.* 1991; 22:159–165. [PubMed: 2061562]
36. Belley B. Massive thermostattins in isothermal density functional molecular dynamics simulations. *J. Chem. Phys.* 1990; 50:1.
37. Groll M, Larionov OV, Huber R, de Meijere A. Inhibitor-binding mode of homobelactosin C to proteasomes: new insights into class I MHC ligand generation. *Proc Natl Acad Sci U S A.* 2006; 103:4576–4579. [PubMed: 16537370]
38. Groll M, Balskus EP, Jacobsen EN. Structural analysis of spiro beta-lactone proteasome inhibitors. *J Am Chem Soc.* 2008; 130:14981–14983. [PubMed: 18928262]
39. Ponder JW, Case DA. Force fields for protein simulations. *Adv Protein Chem.* 2003; 66:27–85. [PubMed: 14631816]

40. Chou TC, Talalay P. Quantitative analysis of dose-effect relationship: the combined effects of multiple drugs or enzyme inhibitors. *Advances in Enzyme Regulations*. 1984; 22:27–55.

Figure 1. Effect of compounds 2–5 treatment on the levels of poly-ubiquitinated proteins in HeLa cervical cancer cells

Immunoblot analysis of ubiquitinated proteins in HeLa cervical cancer cells eight hours after treatment with or without compounds 2–5 at the indicated concentrations. Bortezomib was used as positive control. Equal protein loading in each lane, was verified by using and antibody against β -actin.

Figure 2. Inhibition of the 20S proteasome activity by compounds 2–4

20S purified proteasomes were treated for 30 min. with or without compounds 2–4 and with positive control at the indicated concentrations following addition of the specific fluorogenic substrates for chymotrypsin-like, trypsin-like and peptidylglutamyl peptide hydrolyzing-like hydrolytic proteasome capacities. Representative examples of two independent experiments.

Figure 3. Effect of compound 2 treatment upon cervical cancer cell lines versus normal cells
Cultures of HPV-transformed cervical cancer cells (SiHa and ME180) or primary human Keratinocytes were treated with the indicated concentrations of compound 2 for 48 hours. Cell viability was determined by XTT assay and plotted as a fraction of the untreated control cultures.

Figure 4. Sub-optimal doses of Bortezomib and lead compound 2 results in an increased bioluminescence from Ub-FL as compared to single agent alone

Transiently transfected firefly luciferase (CMV-FL) and Ub-FL HeLa cervical cancer cells were either mock treated or treated with Bortezomib, lead compound 2 or combination at the indicated concentrations for 8 hours. Luciferase activity on cell lysate was quantified in Relative Luminescence Units (RLU) and expressed as % of control. Error bars are Standard Errors (SE) for three independent experiments.

Figure 5. Bortezomib and compound 2 synergistically kill HPV-transformed HeLa cervical cancer cells

HeLa cells were treated with checker board dilution series of Bortezomib and lead compound **2**. Cell viability was measured by XTT assay and calculated as percent of control untreated cultures. Synergy is shown by plotting the interaction between drugs in isobologram. The dotted diagonal corresponds to an additive effect while the points below indicate synergy. CI=combination index.

Figure 6. Morphological changes in HeLa cervical cancer cells treated with combination of Bortezomib and lead compound 2

Phase contrast microscopy of HeLa cultures exposed to Bortezomib, compound **2** or combination at the indicated concentration for 48 hours.

Figure 7. Docking simulation of lead compound 2 to 20S proteasome

A, LUMO (*in blue*) is localized around the carbonyl group of the oxo-piperidine of lead compound 2. B, Schematic view lead compound 2 docked to the crystal structure of 20S proteasome. C, lead compound 2 depicted in the 20S proteasome active site. D, superposition of the three inhibitors, compound 2, homobelactosin C and 2-spiro-lactacystin.

Scheme I.

Reagents and Conditions: (a) dry HCl gas, glacial AcOH, 24 h, (b) Triethyl amine/
Dichloromethane

Scheme II.

Reagents and Conditions: (a) DIEA/THF, 0°C-rt, 45 min, (b) NMM/THF, 0°C-rt, (c) 20 % Piperidine-DMF, rt, 30 min

Table 1

Summary of the structure of compounds 1–10.

No.	Compound	R ₁	R ₂	R ₃	R ₄	P
1	AM-114	H	B(OH) ₂	H	H	Me
2	RA-1	Cl	Cl	H	H	-CO-Phenylalanine
3	RA-2	Cl	Cl	H	H	-CO-Tyrosine
4	RA-3	Cl	Cl	H	H	-CO-Leucine
5	RA-4	Cl	Cl	H	H	-CO-Glutamic acid
6	RA-5	Cl	Cl	H	H	-CO-Lysine
7	RA-6	Cl	Cl	H	H	-CO-Phenylalanine-Tyrosine
8	RA-7	Cl	Cl	H	H	-CO-Glutamic acid-Aspartic acid
9	RA-8	Cl	Cl	H	H	-CO-Lysine-Arginine
10	RA-9	Cl	Cl	H	H	-CO-Arginine-Lysine

Table 2
Cell killing activities of compounds 1–10 on HeLa and CaSki cervical cancer cell lines

IC₅₀ values were determined by XXT assay. The IC₅₀ value reported are average of three independent determinations.

No	IC ₅₀ (μ M) HeLa cells	IC ₅₀ (μ M) CaSki cells
1	8	9
2	0.32	1.5
3	10	10
4	10	20
5	>50	>50
6	>50	>50
7	>50	>50
8	>50	>50
9	>50	>50
10	>50	>50

Table 3
Inhibition of the proteolytic activities of proteasomes isolated from LCLs

The IC₅₀ values reported are the average of three independent determinations.

No	Isolated enzyme IC ₅₀ (μ M)		
	CT-like	T-like	PGPH-like
2	4.8	6.6	6.2
3	4.2	8.4	>10
4	9.3	>10	>10