Stressing the Ubiquitin-Proteasome System without 20S Proteolytic Inhibition Selectively Kills Cervical Cancer Cells

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Abstract

Cervical cancer cells exhibit an increased requirement for ubiquitin-dependent protein degradation associated with an elevated metabolic turnover rate, and for specific signaling pathways, notably HPV E6-targeted degradation of p53 and PDZ proteins. Natural compounds with antioxidant properties including flavonoids and triterpenoids hold promise as anticancer agents by interfering with ubiquitin-dependent protein degradation. An increasing body of evidence indicates that their α-β unsaturated carbonyl system is the molecular determinant for inhibition of ubiquitin-mediated protein degradation upstream of the catalytic sites of the 20S proteasome. Herein we report the identification and characterization of a new class of chalcone-based, potent and cell permeable chemical inhibitors of ubiquitin-dependent protein degradation, and a lead compound RAMB1. RAMB1 inhibits ubiquitin-dependent protein degradation without compromising the catalytic activities of the 20S proteasome, a mechanism distinct from that of Bortezomib. Treatment of cervical cancer cells with RAMB1 triggers unfolded protein responses, including aggresome formation and Hsp90 stabilization, and increases p53 steady state levels. RAMB1 treatment results in activation of lysosomal-dependent degradation pathways as a mechanism to compensate for increasing levels of poly-ubiquitin enriched toxic aggregates. Importantly, RAMB1 synergistically triggers cell death of cervical cancer cells when combined with the lysosome inhibitor Chloroquine.

Introduction

Ubiquitin-dependent protein degradation via the ubiquitin-proteasome system (UPS) is crucial for the regulation of many cellular processes including cell cycle progression, differentiation and apoptosis in both normal and cancer cells [1]. Aberrant expression of components of the UPS system including ubiquitin-ligases, de-ubiquitinating enzymes and proteasomes has been reported in several cancer settings including cervical cancer [1,2,3], suggesting that in order to sustain their higher levels of metabolic activity cancer cells rely more heavily upon the proper function of the UPS as compared to their normal counterpart [4,5,6,7]. Thus, molecules capable of interfering with ubiquitin-dependent protein degradation, including Bortezomib, show anticancer activity [5]. Human Papillomavirus (HPV) is the primary cause of cervical cancer and responsible for 5% of all cancers worldwide [8]. While HPV vaccines can be an effective preventive measure against cervical cancer, there are currently no virus-specific therapies for it, and the efficacy of standard surgical and chemo/radiotherapies is limited for advanced disease [9]. Expression of two viral oncoproteins, E6 and E7, is necessary for the induction and maintenance of the transformed phenotype [10]. The E6 oncoprotein exerts its oncogenic activity by binding to the E3 ubiquitin ligase E6-AP and redirects its activity towards p53 and other tumor suppressor proteins for their rapid ubiquitin-mediated proteasomal degradation [11,12,13]. This reduces the level of this key cellular cell cycle regulator without its mutation. Therefore, we hypothesized that stabilization of p53 via preventing its ubiquitin-mediated degradation will have therapeutic potential for cervical cancer and possibly for other cancers wild-type for p53.

Natural compounds of the flavonoid and triterpenoids families including curcumin, Celastrol, green tea polyphenols and chalcones have shown promise as antineoplastic agents in a variety of cancer settings including cervical [14], colon [15,16], oesophageal [17], pancreatic [18] and prostate [19,20,21] cancer, linked to pro-apoptotic properties as associated with proteasomal inhibition. We have recently shown that chalcone-derivatives...
Causing UPS Stress for Cervical Cancer Treatment

Materials and Methods

Cell culture
Cervical cancer cell lines HeLa, SiHa, CaSki 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-sulphophenyl]-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 µL medium in 96-well plate were treated with chalone-based derivatives at specified concentrations. After the indicated periods, the cells were fixed and permeabilized with methanol and incubated with the indicated primary antibodies. Fluorescent secondary antibodies were used to visualize protein localization and nuclear DNA visualized by 4,6-diamidino-2-phenylindole (DAPI) staining. Mounted samples were viewed under a Nikon Eclipse TE 2000E inverted microscope and images captured with Spot 3.5.8 acquisition software (Diagnostic Instruments, Sterling Heights, MI).

Antibodies and Western Blot Analysis
Total cellular protein (10–20 µg) from each sample was separated by SDS-PAGE, transferred to PVDF membranes and subjected to Western blot analysis. Antibodies for Western Blot analysis were obtained by following commercial sources: anti-ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p53 clone DO-1 (Calbiochem, Gibbstown, NJ) anti-PARP (BD Pharmingen, San Diego, California), anti-GAPDH (Sigma, St. Louis, MO), peroxi-dase-linked anti-mouse Immunoglobulin G (Amersham, Piscataway, NJ) and utilized at the concentration recommended by the manufacturer. Anti-ubiquitin and anti-vimentin antibodies for immunofluorescence analysis were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Texas red-labeled goat anti-mouse Immunoglobulin G and Fluorescein-labeled horse anti-rabbit Immunoglobulin G were obtained from Molecular Probes (Carlsbad, CA), and Vector Laboratories (Burlingame, CA) respectively and used at the concentration recommended by the manufacturer.

Measurement of Proteasomal activity in 20S Purified Proteasomes
Cells (5×10^5) were washed in cold PBS and resuspended in buffer containing 50 mM TRIS-HCl (pH 7.5), 5 mM MgCl2, 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 1,000 g, followed by 20 min centrifugation at 10,000 g [27]. Lysates were cleared by ultracentrifugation for 1 hr at 100,000 g, and supernatants were further ultracentrifuged for 5 hr at 100,000 g. 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 (10 µl), 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 MgCl2 and 1 mM DTT (final volume 100 µl). The reaction was quenched with 1 ml 1% SDS and fluorescence determined by fluorimeter (Perkin-Elmer, Beach- field, UK) with excitation at 380 nm and emission at 440 nm [18]. Data are expressed as the percent inhibition relative to untreated proteasomal preparations.

Measurement of Proteasomal activity in 26S Proteasomes in living cells
Exponentially growing cells (1×10^5) were plated in 60 mm dishes and either mock treated or treated with different concentration RAMB1 over a period of 4 hours. Proteasomal activity in cell lysates (NP-40 lysis buffer: 0.1% NP-40, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5% glycerol and 1 mM DTT) was determined by measuring residual luminescence activity containing single aminoacid substitutions in their structure act as proteasome inhibitors and that the nature of the aminoacidic portion determines their selectivity toward the different catalytic activities of the 20S proteasome [14]. However other findings suggest that chalone molecules might contain within their α-unsaturated carbonyl system the molecular determinant for inhibition of ubiquitin-mediated protein degradation upstream of the 20S proteasome [22,23,24,25].

We report for the first time that a series of chalone-derivatives lacking aminoacidic components, here termed RAMBs, are ubiquitin-proteasome system (UPS)-stressors via inhibition of ubiquitin-mediated protein degradation upstream of the 20S proteasomal catalytic activities. Specifically, our RAMBs compounds are capable of selective killing of cervical cancer cells via accumulation of poly-ubiquitinated protein followed by triggering of unfolded protein responses including aggresome formation and Hsp90 stabilization. Further, this accumulation of poly-ubiquitinated proteins is accompanied by a compensatory activation of lysosome-dependent protein degradation, stabilization of p53, the destabilization of cyclin D1 and the onset of apoptosis. Our findings suggest that treatment RAMB compound, possibly combined with the lysosome inhibitor Chloroquine, has promise as new avenue for the treatment of cervical cancer.

Cell culture
Cervical cancer cells HeLa, SiHa, CaSki 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-sulphophenyl]-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 µL medium in 96-well plate were treated with chalone-based derivatives at specified concentrations. After the indicated periods, the 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 450 nm (ELISA reader 190; Molecular Devices, Sunnyvale, CA). All experiments were done in triplicate.

Determination of apoptotic cells by flow cytometry
Induction of apoptosis was determined by Annexin-V-7-AAD staining. Annexin-V-7-AAD staining was done using Annexin V-PE Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA) according to manufacturer’s protocol. Briefly, 1×10^6 cells were resuspended in Binding Buffer, 5 µl of Annexin V-PE and 5 µl of 7-AAD were then added into the cells which were then incubated at room temperature for 15 minutes, and analyzed by flow cytometry on a Becton Dickinson FACSCalibur. Data analysis was done with CellQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA).

Antibodies and Western Blot Analysis
Total cellular protein (10–20 µg) from each sample was separated by SDS-PAGE, transferred to PVDF membranes and subjected to Western blot analysis. Antibodies for Western Blot analysis were obtained by following commercial sources: anti-ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA).
following addition of the Suc-LLVY-Glo\textsuperscript{TM} substrate (Promega, Madison, WI) specific for the chymotrypsin-like activity of the proteasome according to the manufacturer’s recommendations.

Assay of 4XUbiquitin-Luciferase degron

The 4Xubiquitin-luciferase fusion construct designated Ub-FL and the control plasmid designed CMV-FL were kindly provided by Dr. David Pwnica-Worms (Washington University, St. Louis, MO) [26]. Sub-confluent cultures of HeLa cells were transfected with plasmids DNA by using Lипofectamine 2000 reagent (Life Technologies, Carlsbad, CA). FL and Ub-FL transfected HeLa cells were seeded at 50,000 cells/well in 24-well plates or 200,000 cells/well in 6-wells plate 24 hours post transfection and incubated with compounds or vehicle (DMSO) at the doses and times indicated. Luciferase activity in cell lysate was determined with a luminometer (Luminometer 2000E inverted microscope and images captured with Spot 3.5.8 acquisition software (Diagnostic Instruments, Sterling Heights, MI). Areas were analyzed using Living Image 2.20 software.

Clonogenic assay

Exponentially growing SiHa, CaSki and HeLa cervical cancer cell lines were seeded at either 1,000 or 100 cells/well in 6 well plates. One day post seeding, the cells were treated with either vehicle alone (mock) or RAMB1 at the indicated doses, and incubated at 37 C in 5% CO\textsubscript{2} for 10 days. At the end of the treatment the medium was removed and the cells were rinsed with PBS prior fixation and staining of the colonies using a mixture of 6.0% glutaradehyde and 0.5% crystal violet as previously described [27]. Colonies were imaged and counted using a Nikon Eclipse TE 2000E inverted microscope and images captured with Spot 3.5.8 acquisition software (Diagnostic Instruments, Sterling Heights, MI). All the experiments were conducted in triplicate.

Statistical analysis

Results are reported as mean ± Standard Deviation (SD). Statistical significance of differences was assessed by two-tailed Student’s using Prism (V.5 Graphpad, San Diego, CA) and Excel. The level of significance was set at p<0.05. The combination index (CI) of RAMB1 and Chloroquine was calculated by the median-effect analysis according to the method of Chou and Talalay [28]. CI<1 indicates synergism, CI = 1 indicates additivity, and CI>1 indicates antagonism. Further regression analyses were performed to stabilize estimates.

Results

RAMB compounds selectively reduce the viability of cervical cancer cells independently of HPV genotype via blockade of proteasomal degradation

Flavonoids and triterpenoids family members including celastrol [19], resveratrol [29,30], curcumin [17], epigallocatechin-3-gallate [20,31,32,33,34] and chalcones [35,36] exhibit anti-cancer properties associated with their activity as proteasome inhibitors [14,15,16,21]. We recently reported that in chalcone-based proteasome inhibitors the nature of the aminoacidic portion of the molecule confers specificity toward catalytic activities of the 20S proteasome [14]. Based upon several prior studies [22,23,37], we hypothesized that the α-β ketone system of chalcones may represent the minimum molecular determinant for inhibition of ubiquitin-mediated protein degradation upstream of proteasomes.

To test this hypothesis we initially screened a library of chalcone-based derivatives carrying various substituents on the aromatic rings adjacent to the α-β ketone system and lacking aminoacidic portions, for their cell growth inhibitory capacity in exponentially growing HPV18-positive HeLa cervical cancer cell line in a range of concentration from 100 to 0.01 μM (not shown). Four chalcone derivatives, hereafter termed RAMB, capable of reducing the cell viability of exponentially growing HeLa cervical cancer cells in a dose-dependent fashion with IC\textsubscript{50} values <5 μM, were chosen for further evaluation (Figure 1). To determine the feasibility of using RAMB compounds for treatment of cervical cancer, we tested whether RAMB1–4 treatment would specifically hinder the cell viability of cervical cancer cells over normal keratinocytes and whether the reduction in cell viability in cervical cancer cells is dependent upon the HPV-genotype. As shown in Figure 2, RAMB1 or RAMB4 treatment produced a dose-dependent reduction in the viability of HPV16-positive SiHa and Caski cells and HPV-39-positive ME180 cervical cancer cell lines respectively with minimal effects on the viability of primary human keratinocytes and with IC\textsubscript{50} similar to the obtained with HeLa. Similar results with slightly higher IC\textsubscript{50} were obtained using RAMB2 and RAMB3 (not shown). To test whether the reduction in cell viability observed in cervical cancer cells following exposure to the RAMB1–4 compounds was due to their capacity of interfere with ubiquitin-mediated protein degradation, we monitored the levels of accumulation of poly-ubiquitinated proteins following treatment. Specifically, HeLa cells were treated with 5 μM of RAMB1, RAMB2, RAMB3, or RAMB4 or 10 nM of Bortezomib, the latter being used as UPS-stressor positive control, over a period of 6 hours. As shown in Figure 3 (left panel), immunoblot analysis of ubiquitinated protein expression levels in HeLa cells revealed a clear pattern of accumulation of poly-ubiquitinated proteins in RAMB -treated HeLa cultures. A semi-quantitative analysis of the polyubiquitinated protein levels shows that the GAPDH-normalized levels of polyubiquitinated proteins are consistently higher (up to 3-fold) in RAMB-treated versus mock-treated cells (Figure 3, right panel). These findings suggest that the decrease in cell viability observed in the cervical cancer cell panel but not in normal cells following RAMB treatment is associated with the perturbation of ubiquitin-mediated protein degradation and occurs regardless of the oncogenic HPV type.

RAMB treatment triggers a Ubiquitin-Proteasome-System (UPS)-stress response without affecting 20S proteasome catalytic activities

To test whether the rapid (six hours or less, unpublished data) accumulation of poly-ubiquitinated proteins following RAMB exposure occurs concomitantly with direct inhibition of the

![Figure 1. Summary of the structure of compounds RAMB1–4 and their IC\textsubscript{50} values in HeLa cervical cancer cell line.](https://doi.org/10.1371/journal.pone.0023888.g001)
catalytic activities of the proteasomes, we tested for the ability of RAMB1 and RAMB4 (which induced a greater accumulation of polyubiquitinated protein than the other RAMBs, Figure 3) to inhibit specific catalytic sub-units within the 20S proteasome. Specifically the RAMBs were tested for their capacities to inhibit the chymotrypsin-like (CT-like), trypsin-like (T-like) and peptidyl-glutamyl peptide hydrolyzing-like (PGPH-like) activities in 20S purified proteasome pre-exposed to escalating doses up to 10 μM of RAMBs for a period of 30 minutes following addition of fluorogenic substrates [38]. The FDA licensed proteasome inhibitor Bortezomib was used as positive control. As shown in Figure 4 the profile of proteasome inhibition shows that unlike Bortezomib, RAMB1 and RAMB4 treatment failed to inhibit proteasomal functions when tested to concentrations up to 10 μM (similar results were obtained with the other compounds of the series, not shown).

In order to assess whether the failure to inhibit proteasomal function in vitro can be recapitulated in the intact proteasome found in living cells, we utilized two different approaches. First, we utilized the ubiquitin-luciferase bioluminescent reporter 4XUb-FL, which resists cleavage by ubiquitin hydrolases [26], to transfet HeLa cells. Using Ub-FL and FL transfected HeLa cells we have monitored luciferase activity following exposure to RAMBs over a period of 6 hours. As shown in Figure 5 (left) unlike Bortezomib or our recently identified proteasome inhibitor RA1 [14], here used as proteasome inhibitors positive controls, RAMB1 or RAMB4 treatment induced a weaker stabilization of the Ub-FL reporter when tested at concentration up to 20 μM than seen for either RA-1 or Bortezomib. Quantification of Ub-FL/FL ratio in mock versus RAMBs exposed culture is provided in Figure 5 (middle panel). Next, the lack of inhibition of 20S proteasomal activity in RAMB-treated cells was confirmed by measuring the residual fluorogenic activity in 20S proteasome purified from CaSki cervical cancer cells pre-exposed to RAMB1 or RAMB4 for 4 hours. As shown in Figure 5 (right panel), unlike Bortezomib, RAMB1 treatment failed to inhibit the chymotryptic activity of proteasomes when tested to concentrations up to 20 μM. Taken together, this suggests that the loss of cell viability in cervical
cancer cells following RAMBs exposure occurs concomitantly with the accumulation of polyubiquitinated proteins without the direct inhibition of 20S proteasomal activity.

Ramb1 treatment induces aggresome formation and triggers heat-shock responses

We and others have shown that inhibition of ubiquitin-mediated protein degradation via proteasomal inhibition triggers heat-shock and unfolded protein responses including formation of cytoprotective structures called aggresomes as a mechanism to compensate for inhibition of proteasomal functions and increasing levels of UPS stress within cancer cells [4,6,39]. To test whether the rapid accumulation of poly-ubiquitinated protein upon Ramb1 treatment occurs concomitantly with heat-shock protein responses (UPR) we monitored the protein expression levels of Hsp90 in HeLa cervical cancer cells exposed to 10 μM Ramb1–3 for 8 hours. As shown in Figure 6A (left panel) immunoblot analysis of Hsp90 protein expression levels revealed that a strong pattern of accumulation of Hsp90 in Ramb1-treated versus mock-treated HeLa cell cultures (similar results were obtained with the other derivative of the series, not shown). A semi quantitative analysis of the Hsp90 protein levels show that the GAPDH-normalized levels of polyubiquitinated proteins are nearly 2-fold higher in treated versus non-treated cells is shown in Figure 6A (right panel).

Next, we hypothesized that accumulation of poly-ubiquitinated proteins following Ramb compound exposure would lead to activation of alternative compensatory pathways to ubiquitin-mediated protein degradation, specifically to lysosomal pathway activation. To test this hypothesis we monitored the sub-cellular localization of ubiquitin by immunofluorescence microscopy analysis in HeLa cervical cancer cells exposed to 10 μM of Ramb1. As shown in Figure 6B immunofluorescence analysis of poly-ubiquitinated proteins in HeLa cells treated with Ramb1 reveals the presence of vimentin-caged, ubiquitin-positive, aggresomes structure consistent with that previously described upon treatment with Bortezomib [6]. Taken together, these findings indicate that in Ramb1-treated cells the accumulation of polyubiquitinated results in similar cytoprotective responses as inhibition of proteasome catalytic activities by Bortezomib, but occurs through a mechanism independent from it.

Ramb1 treatment leads to p53 stabilization, cyclin D1 destabilization and onset of apoptosis

The E6 oncoprotein of HPV exerts its oncogenic activity by targeting p53 and other tumor suppressor proteins for rapid ubiquitin-mediated proteasomal degradation. This reduces the level of this key cellular cell cycle regulator without mutation of p53. To test whether the impairment of ubiquitin-mediated protein degradation following RAMB treatment leads to stabilization of p53 as a potentially contributing mechanism initiating cell death, we examined the expression levels of p53 following 6 hours exposure to compounds 10 μM of RAMBs. As
shown in Figure 7A, 8 hours RAMB1 exposure is associated with dose-dependent accumulation of p53 in CaSki cervical cancer cells as compared to mock control. Importantly, p53 stabilization causes suppression of cyclin D1 promoter resulting in active repression of cyclin D1 transcription [40]. To test whether this results in reduction of cyclin D1 expression levels, CaSki cervical cancer cells were exposed to increasing doses of RAMB1 over a period up to 8 hours. As shown in Figure 7B (left panel) RAMB1 treatment causes time-dependent (top) and dose-dependent (bottom) decrease of cyclin D1 levels suggesting failure of cervical cancer to enter the S-phase of the cell cycle as a cause of cell toxicity [41]. A semi quantitative analysis of β-actin-normalized cyclin D1 levels is given in Figure 7B (right panel top and bottom).

To test whether this results in reduction of cyclin D1 expression levels, CaSki cervical cancer cells were exposed to increasing doses of RAMB1 over a period up to 8 hours. A semi quantitative analysis of β-actin-normalized cyclin D1 levels is given in Figure 7B (right panel top and bottom). As shown in Figure 7B (left panel) RAMB1 treatment causes very rapid (top) and dose-dependent (bottom) decrease of cyclin D1 levels suggesting failure of cervical cancer to enter the S-phase of the cell cycle may contribute to the loss of cell viability [41].

Stabilization of p53 steady-state levels and destabilization of cyclin D1 levels suggest that the reduction in cell viability observed in the panel of cervical cancer cells following RAMBs exposure might trigger the onset of apoptosis. To test this hypothesis, HeLa cervical cancer cells were exposed to 5 μM RAMB1 or 10 nM Bortezomib for 18 hours before fixation and immuno-fluorescent staining of DNA (blue), ubiquitin (green) and vimentin (red) before imaging (60×).

Figure 6. RAMB treatment induces accumulation of Hsp90 and aggresomes. A. Left panel: immunoblot analysis of Hsp90 expression levels in HeLa cervical cancer cells after 8 hours exposure with or without 10 μM RAMBs treatment. Bortezomib was used as positive control. Equal protein loading in each lane was verified by using an antibody against GAPDH. Right panel: Quantification of the Hsp90/GAPDH ratio. B. HeLa cells were incubated with or without 5 μM RAMB1 or 10 nM Bortezomib for 18 hours before fixation and immuno-fluorescent staining of DNA (blue), ubiquitin (green) and vimentin (red) before imaging (60×).

doi:10.1371/journal.pone.0023888.g006

RAMB1 treatment prevents anchorage-dependent tumor-colony formation of cervical cancer cells and synergizes with the lysosome inhibitor Chloroquine

A hallmark of cancer cells is their capacity to form colonies in vitro that is indicative of the loss of contact inhibition typical of normal cells. To test the potential of RAMB1 as a therapeutic agent for cervical cancer, we have examined the effects of RAMB1 treatment on the anchorage-dependent growth of SiHa, CaSki and HeLa cervical cancer cell lines. Specifically, SiHa and CaSki cancer cells were either mock treated or treated with escalating doses of RAMB1 over a period of 10 days and their ability to form colonies was evaluated by crystal violet staining. As shown in Figure 8A, RAMB1 exposure resulted in a dose-dependent inhibition of colony formation by SiHa and CaSki cervical cancer...
cells as compared to mock-treated cultures when used at concentrations as low as 0.62 μM.

We next quantified the impact of RAMB1 treatment on the reduction of colony number and size in 1 × 10^6 HeLa cervical cancer cells treated with or without 0.15 or 0.5 μM RAMB1. As shown in Figure 8B (left panel) RAMB1 exposure resulted in 40% and 60% reduction in colony number when exposed to 0.15 or 0.3 μM RAMB1 respectively. RAMB1 treatment also resulted in 50% and 80% reduction in colony size in mock versus RAMB1 treated HeLa cells as shown in Figure 8B (middle panel). A representative example of crystal violet staining in HeLa cervical cancer cells following RAMB1 exposure is given in Figure 8B (right panel).

The increase in the steady-levels of Hsp90 and formation of aggresome-like structure following RAMB1 treatment suggest compensatory activation of lysosomal pathway of protein degradation (Fig. 6). Therefore, we hypothesized that RAMB1 treatment would result in synergistic cell killing when combined with an inhibitor of this lysosomal pathway. To test this hypothesis, we compared the effect of combined treatment with RAMB1 and the lysosome inhibitor Chloroquine on the viability of HeLa cervical cancer cells. Consistent with our hypothesis, submaximal doses of RAMB1 and Chloroquine act synergistically to cause enhanced cytotoxicity in HeLa cervical cancer cells with a Combination Index (CI) of 0.48 observed using 1.45 μM RAMB1 and 7.23 μM Chloroquine (Figure 7C). Similar results were obtained with SiHa and CaSki cell lines (data not shown). Taken together, this suggest that the combinatorial approach of inhibiting ubiquitin-dependent protein degradation upstream of proteasome and lysosomal pathway could lead to development of combination therapeutic strategies where the side effects of each individual compounds can be reduced without compromising the anti-cancer activity of the treatments.

Discussion
Numerous naturally-derived compounds are either FDA approved or currently under evaluation for the treatment of human malignances. Among these natural compounds with
promising anticancer properties are members of the flavonoid and triterpene families. While the chemical common denominator within these two families capable of inducing preferential killing in cancer cells is yet to be identified, a number of studies support the idea that they share an ability to inhibit one or multiple catalytic activities of the proteasome, and that their anti-cancer activities stems from this inhibition [14,15,17,21].

While we have recently shown that in chalcone-based small molecule inhibitors of proteasome the nature of aminoacidic component determines the selectivity toward individual catalytic activities of the proteasome [14], a substantial body of evidence indicates that chalcones may have within their β-carbons to α,β-unsaturated carbonyls the molecular determinant for inhibition of ubiquitin-dependent protein degradation upstream of proteasomes [22,23,24,25]. Thus, they may exert anticancer activity by induction of UPS stress via a mechanism independent from direct inhibition of the catalytic activities of the 20S proteasome.

Given these observations and based on our recent work [14], we have synthesized a library of chalcone-based small molecules (RAMBs) containing α,β-unsaturated carbonyl systems and lacking aminoacid substitutions and tested their antineoplastic potential via inhibition of ubiquitin-mediated protein degradation.
upstream of proteasomes in the cervical cancer setting. Our results show that, when probed for their capacity to interfere with degradation of poly-ubiquitinated proteins, RAMB treatment is associated with increased levels of poly-ubiquitinated proteins (occurring as early as two hours from exposure) but unaltered 20S proteasomal catalytic activity when tested in vitro and in living cells. Notably, while not statistically significant, RAMB1 treatment led to some stabilization of the Ub-FL degron. This may reflect a feedback inhibition of proteasome function due to increasing levels of poly-ubiquitinated protein content in RAMB1 exposed cultures.

Importantly, when tested against a panel of cervical cancer cell lines versus keratinocytes, RAMBs showed a preferential killing for the malignant versus the normal phenotype. This appears to be consistent with the notion that due to their endogenously high levels of UPS stress cancer cells are more sensitive than normal cells to perturbation of ubiquitin-mediated protein degradation [4].

Activation of p53 via its stabilization is a potential therapeutic approach for treatment of those cancers, including cervical cancer, expressing wild-type p53. E6 oncoprotein transforms cervical cancer cells by targeting p53 for ubiquitin-dependent proteasomal degradation resulting in reduction of the levels of this and other tumor-suppressor proteins. Therefore, stabilization of p53 by preventing its degradation could recover sufficient levels of wild-type p53 to trigger apoptotic cell death as a response to the abnormal growth of the cancer cell. We show here that RAMB1 treatment causes dose-dependent accumulation/stabilization of p53. Because stabilization of the levels of p53 occurs concomitantly with accumulation of high-molecular weight poly-ubiquitin-nated species and as early as 2 hours post-treatment (well before cell death is observed, not shown), this suggests that stabilization of p53 is the cause rather then the consequence of the decrease in cell viability in cervical cancer cells. Induction of p53 via stabilization of its steady state levels correlates with decrease in the levels of cyclin D1 possibly via repression of its transcription [40]. RAMB1 treatment also results in a dose- and time-dependent reduction in the steady state levels of cyclin D1 which could at least partially account for the decrease in cell viability observed in the cervical cancer cell lines. Interestingly, because the decrease in cyclin D1 expression levels are seen as early as 30 minutes following RAMB1 exposure and before detectable stabilization of p53 expression levels, it is possible that cyclin D1 down-regulation is occurring in response to UPS stress rather than p53 activity. In this scenario an arrest in S-phase would precede the onset of apoptosis in RAMBs exposed cervical cancer cells as confirmed by Annexin V staining and PARP-cleavage. This is likely the same mechanism responsible for reduction in colony number observed in the panel of cervical cancer cell lines exposed to RAMB1 treatment.

Interestingly, because the reduction in colony number is accompanied by reduction in colony size we conclude that RAMB1 has an effect in slowing the proliferation rate of cervical cancer cells that are initially resistant to its action. Inhibition of conventional (proteasomal-mediated) ubiquitin-dependent protein degradation triggers activation of alternative pathways to proteasomal degradation as a mechanism to compensate for abnormally increased levels of UPS stress. The increase in the levels of poly-ubiquitinated proteins following RAMB1 exposure corresponds to their sub-cellular localization in vimentin-encaged structures consistent with the formation of aggresomes and occurs concomitantly with up-regulation of Hep90 protein expression levels. This indicates activation of the lysosomal protein degradation pathway as an attempt to “isolate” mis-folded and oxidized proteins and subsequent recycling of the aggresomes via autophagy to cope with increasing levels of UPS stress [6,39]. In this scenario we show that a combinatorial approach of using RAMB1 and the lysosome inhibitor Chloroquine reduce the viability of cervical cancer cells significantly better than either treatment alone. This synergism presumably reflects blockade of the proteasomal and compensatory lysosomal degradation pathways by RAMB1 and Chloroquine respectively, and provides a rational to further explore this new therapeutic approach for the treatment of cervical cancer.

Author Contributions

Conceived and designed the experiments: RKA SRK RBRS MB. Performed the experiments: RKA TS AF YI FD SP MB. Analyzed the data: RKA TS AF YI RG RIV SP RBRS MB. Contributed reagents/materials/analysis tools: SRK RBRS MB. Wrote the paper: MB.

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