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New potential anti-cancer agents synergize with bortezomib and ABT-737 against prostate cancer

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Abstract

We previously described the identification of a transcriptional inhibitor ARC and FoxM1 inhibitors, Siomycin A and thioistrepton that were able to induce potent p53-independent apoptosis in cancer cell lines of different origin. Here, we report the characterization of these drugs on a panel of prostate cancer cell lines. We showed that ARC inhibited the viability of prostate cancer cells and induced apoptosis in low nanomolar concentration. It potently downregulated the expression of Mcl-1 and showed synergistic combination effect with Bcl-2 inhibitor ABT-737. Thiazole antibiotics, Siomycin A and thioistrepton inhibited growth and induced cell death in prostate cancer cells in low micromolar concentrations. The inhibition of the oncogenic transcription factor FoxM1 by Siomycin A and thioistrepton correlated with the induction of apoptosis. In addition, thioistrepton and ARC synergistically induced apoptosis in prostate cancer cells following combination treatment with proteasome inhibitor bortezomib. Furthermore, we found that all tested drug combinations were able to induce apoptosis selectively in transformed, but not normal cells of the same origin. Overall, these compounds represent potential candidates for drug development against prostate cancer.

Keywords

ARC; thiazole antibiotics; apoptosis; bortezomib; ABT-737

Prostate cancer (PC) is the most commonly diagnosed cancer accounting for 25% of total cases and the second leading cause of cancer related mortality in American males {Jemal, 2008 #5551}. Hormone (androgen) withdrawal therapy usually works as a first-line treatment option in PC patients {Tammela, 2004 #5555}, but most patients develop hormone-refractory PC (HRPC) resulting in disease progression and death {Chowdhury, 2007 #5552}. Until recently chemotherapy demonstrated only restricted use for the treatment of HRPC {Lin, 2007 #5553} with docetaxel and prednisone as the preferred treatment for most patients with metastatic HRPC {Oudard, 2005 #5554}. Since a majority

of patients eventually progress to docetaxel-resistant status, there is still a need for development of novel therapeutics against prostate cancer.

In this paper, we describe two types of drugs that were identified in our laboratory earlier {Radhakrishnan, 2006 #3008; Radhakrishnan, 2006 #3095} that demonstrate proapoptotic activity against prostate cancer (PC) cells *in vitro*. First, a nucleoside analog, ARC, a global transcription inhibitor {Radhakrishnan, 2006 #3008; Radhakrishnan, 2008 #5385; Stockwin, 2009 #5556} that targets the expression of short-lived, antiapoptotic protein Mcl-1 {Radhakrishnan, 2008 #3298; Bhat, 2008, Bhat, 2008}, which is overexpressed in PC {Krajewski, 1995 #5557; Krajewska, 1996 #5558}. Second, thiazole antibiotics (TAs), Siomycin A and its structural analog thiostrepton that were identified as inhibitors of Forkhead Box M1 (FoxM1) protein {Radhakrishnan, 2006 #3095; Bhat, 2008 #5518; Bhat, 2009 #5529} and may also act as the proteasome inhibitors {Bhat, 2009 #5548}. FoxM1 is an oncogenic transcription factor that is overexpressed in the majority of human carcinomas, including PC {Pilarsky, 2004 #3050}. Increased levels of FoxM1 have demonstrated to accelerate development and progression of PC in mouse models, while knockdown of FoxM1 by small interfering RNAs (siRNAs) led to anchorage independent cell growth on soft agar of PC cell lines {Kalin, 2006 #3047}. In addition, it has been shown that FoxM1 is one of the only few genes to be consistently upregulated in androgen ablation resistant metastatic PC {Chandran, 2007 #5381}.

In this study we characterize the activity of ARC, and the TAs alone or in combination with ABT-737 (Bcl-2/Bcl-X_L/Bcl-w inhibitor; Abbott) {Oltersdorf, 2005 #5482} or bortezomib (Velcade) (proteasome inhibitor; Millenium/Takeda) {Adams, 2000 #5559} on PC cell lines DU145, LNCaP and PC-3. Our data suggest that ARC and the TAs have potent proapoptotic activity against PC cells alone or in combination with other anticancer agents.

Material and Methods

Cell lines and reagents

BJ, PC-3, LNCaP, and DU145 cells were obtained from American Type Culture Collection. PC cells were cultured in DMEM supplemented with 10% fetal bovine serum (Atlanta Biological) and 1% penicillin-streptomycin (Invitrogen). p53shRNA-LNCaP cells with knockdown of p53 were cultured as described before {Radhakrishnan, 2006 #3013}. Immortal BJ cells were a kind gift from William Hahn, Harvard Medical School {Hahn, 1999 #5536}. BJ wild type and immortal cells were cultured in knockout DMEM + medium 199 supplemented with 10% fetal bovine serum (Atlanta Biological), 5 mM glutamine and 1% penicillin-streptomycin (Invitrogen). The cells were maintained under standard cell culture conditions at 37°C and 5% CO₂ in a humid environment. Thiostrepton was purchased from Sigma, Siomycin A and ARC were obtained from NCI, bortezomib (Velcade) was kindly provided by Millennium pharmaceuticals/Takeda and ABT-737 was received from Abbott. All drugs were dissolved in dimethylsulfoxide (DMSO; Sigma).

Cell Viability assay

The effect of ARC and thiopeptides Siomycin-A and thiostrepton was determined by the MTT assay. 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was procured from Sigma. Cells were plated at a density of 1×10^4 per well in 200 μ L of complete culture medium and treated with varying concentrations of the drugs in 96-well micro titer plates. After incubation for 72 hours at 37°C in a humidified incubator, 10 μ L MTT (5 mg/mL in PBS) was added to each well, after which the plate was centrifuged briefly. After careful removal of the medium, 0.1 mL buffered DMSO was added to each well. The absorbance was recorded on a microplate reader at the wavelength of 540 nm.

Annexin V-PE/7AAD staining

PC cells were grown at a density of 50% confluence in 100-mm culture dishes and were treated with varying concentrations of the drugs for 48 hours. Cells were stained using Annexin V-PE apoptosis detection kit (BD-Pharmingen) according to the manufacturer's recommendations. Briefly, the cells were washed in PBS and resuspended in binding buffer (1×10^6 per ml). 5 μ L of AnnexinV-PE and 5 μ L of 7-AAD (7-aminoactinomycin-D) were added and incubated for 15 minutes at room temperature in the dark and analyzed by flow cytometry.

Immunoblotting

PC cells were seeded to 100 mm plates at a density of 7.5×10^5 cells. Cells were treated with single agents or their combinations for 48 hours after which the cells were lysed. BJ (wt and immortal) cells were seeded to 100 mM plates and treated with single agents or their combinations for 24 hours following which they were lysed. Cells were lysed in IP buffer (20 mM HEPES, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 100 mM NaF, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM sodium orthovanadate, 0.2 mM PMSF supplemented with protease inhibitor tablet (Roche Applied Sciences) and the protein concentration was determined using the Bio-Rad protein assay reagent. Fifty micrograms of the cell lysates were separated by electrophoresis on SDS-polyacrylamide mini gel and transferred to PVDF membrane. Immunoblotting was performed with specific antibodies for cleaved caspase-3 (Cell Signaling), Mcl-1 (Lab Vision), β -actin (Sigma), FoxM1 {Radhakrishnan, 2006 #3095}, p53 and PARP-1/2 (Santa-Cruz Biotech.),

Measurement of Combination Index by using Chou-Talalay median-effect method

We studied the effect of drug combinations on cell viability by changing the doses of the two agents while monitoring the percentage of viable cells {Chou, 2006 #5560}. Serial dilutions of these drugs were prepared and combined with each other from the lowest to the highest concentration, while assessing the cell fraction affected. In our experiments, the IC_{50} (fractional effect 0.5), IC_{60} (fractional effect 0.6) and IC_{80} (fractional effect 0.8) values (i.e., the drug concentrations needed to cause 50, 60 and 80% reduction in cell viability) were chosen for measurements. Percent viability of cells was determined using standard MTT assay. Combination index (CI) values were calculated using the formula:

$$\text{CI} = \frac{\text{CA}, x}{\text{IC}_{x, A}} + \frac{\text{CB}, x}{\text{IC}_{x, B}} \quad \{\text{Chou, 2006 \#5560}\}.$$

CA,x and CB,x are the concentrations of drug A

and drug B used in combination to achieve x% drug effect. $IC_{x,A}$ and $IC_{x,B}$ are the concentrations for single agents to achieve the same effect. CI values of <1 indicate synergy, a value of 1 indicates additive effects and a value of >1 indicates antagonism.

Results

Thiazole antibiotics, Siomycin A and thiostrepton inhibit the expression of FoxM1 in prostate cancer cells and induce p53-independent apoptosis

We showed earlier that TAs inhibited the growth of liver cancer, melanoma and leukemia cells {Bhat, 2008 #5518; Bhat, 2009 #5529} in vitro. To determine how thiopeptides inhibit the growth of PC cells we performed an MTT cell viability assay. PC cell lines (LNCaP, DU145 and PC-3) were treated with different concentrations of Siomycin A and thiostrepton for 72 hours. As shown in the Fig. 1A thiazole antibiotics potently inhibited the viability of PC cells in a dose dependent manner with IC_{50} values of about $2\mu M$. Anti-proliferative activity of the thiopeptides was independent of the androgen dependence of the PC cells, because LNCaP, an androgen dependent cell line had similar sensitivity to thiazole antibiotics as androgen independent DU145 and PC-3 cells.

To examine the ability of thiazole antibiotics to induce apoptosis in PC cells, LNCaP, p53shRNA-LNCaP, DU145 and PC-3 cells were treated with $5\mu M$ of Siomycin A and thiostrepton, respectively, and apoptosis was assessed by the appearance of caspase-3 cleavage after immunoblotting (Fig 1B). We found that treatment of cells with $5\mu M$ of the TA for 48 hrs led to potent caspase-3 cleavage indicating that these cells underwent programmed cell death (Fig 1B). The induction of apoptosis by the TAs was almost identical in the wt LNCaP cells and in p53 knockdown LNCaP cells (p53shRNA-LNCaP) suggesting that TAs induce p53-independent apoptosis in PC cells (Fig 1B). In addition, we found that caspase-3 cleavage correlated with the downregulation of FoxM1 in prostate cancer cells (Fig 1B) similarly to our previous results with other cancer cell lines treated with TAs {Radhakrishnan, 2006 #3095; Bhat, 2008 #5518; Bhat, 2009 #5529}. To quantitate the degree of apoptosis induced by the thiopeptides in PC cells, we performed annexin V-PE staining of DU145 cells treated with TAs. Treatment with $5\mu M$ of thiopeptides in DU145 cells resulted in ~50% of apoptotic cells, while only ~10% of control cells underwent apoptosis (Fig 1C).

ARC inhibits proliferation, induces apoptosis and suppresses Mcl-1 in prostate cancer cells

To determine the anti-proliferative activity of ARC in prostate cancer cells, we performed an MTT cell viability assay. Prostate cancer cells (LNCaP, DU145 and PC-3) were treated with various concentrations of ARC for 72 hours. As shown in Fig 2A, ARC very potently inhibited the viability of PC cells with IC_{50} values between 30 nM (DU145) and 80 nM (PC-3). The cytotoxic activity of ARC was independent of the androgen dependence of the cells. To investigate the mechanism of the anti-proliferative activity of ARC in PC cells, these cells were treated with concentrations from 100 nM to $2\mu M$ of ARC and apoptosis was assessed by the appearance of caspase-3 cleavage after immunoblotting (Fig 2B). ARC demonstrated a concentration-dependent downregulation of Mcl-1 expression that correlated

with caspase-3 cleavage in the drug-treated cells (Fig 2B). Additionally, the degree of apoptosis was identical between the wt-p53 LNCaP cells and their p53 knockdown counterparts (data not shown). To quantitate the degree of apoptosis induced in PC cell lines by ARC we performed annexin V-PE staining of these cells after exposure to ARC or DMSO control and confirmed that all treated cell lines underwent potent apoptosis in 48 hour (Fig 2C).

Combined treatment of ARC and thiostrepton demonstrates synergy with novel anti-cancer agents, ABT-737 and bortezomib

To evaluate the interactions between ARC and two novel anti-cancer agents, ABT-737 (a Bcl-2 inhibitor) {Oltersdorf, 2005 #5482} and bortezomib (proteasome inhibitor) {Adams, 2000 #5559}, prostate cancer cells DU145 and PC-3 were co-treated with sub-lethal doses of ARC and ABT-737, or ARC and bortezomib. Exposure of cells to sub-apoptotic concentrations of the drug combination resulted in potent apoptosis as indicated by induction of caspase-3 cleavage in western blot analysis, in contrast to no apoptosis after treatment with either drug individually (Fig. 3A, B). Similarly, to assess the interaction between thiostrepton and bortezomib, prostate cancer cells DU145 and PC-3 were treated with sub-apoptotic concentrations of thiostrepton or bortezomib or co-treated with thiostrepton and bortezomib. Exposure of cells to sub-apoptotic concentrations of combination of these drugs resulted in apoptosis indicated by induction of caspase-3 cleavage after western blot analysis as compared to no apoptosis demonstrated by treatment with these drugs separately (Fig 3A, B).

To quantitatively evaluate cell death induced by individual drug treatments or by the combinations of these drugs we performed annexin V-PE staining. The percent of DU 145 cells undergoing apoptosis after treatments with combinations of sub-lethal concentrations of ARC and bortezomib and ARC and ABT-737 was 22–27% (Fig 3C). Co-treatment of sub-apoptotic doses of thiostrepton and bortezomib also resulted in more than 30% of apoptotic cells. The percent of apoptotic cells induced by combination treatments was 20–30%, while number of apoptotic cells after single drug treatments was the same as in DMSO treated control cells (Fig 3C), demonstrating strong synergistic killing effects of these drug combinations.

To validate the synergistic nature of the interaction of these drug treatments, we analysed the cell viability after single and combination drug treatments by using the Chou/Talalay median-effect equation method {Chou, 2006 #5560}. The CI represents a quantitative measure of the interaction of the different drug tested, for example, CI values below 1 indicate synergistic anti-proliferative effect. In prostate cancer cell lines ARC and thiostrepton induced a synergistic growth inhibitory effect in combination with bortezomib and ABT-737 (Table 1). The CI range values for the combined treatments were 0.3–0.8. As an example, ARC and bortezomib demonstrate combination indices for cell growth inhibition with CI values of 0.6, 0.5 and 0.3 for IC₅₀, IC₆₀ and IC₈₀ (concentrations required to inhibit viability of 50, 60 and 80% of cells). Together, these data indicate that co-treatment of ARC and thiostrepton with ABT-737 or bortezomib results in synergy and strong increase in apoptosis.

In order to further test the specificity of used combination treatments towards transformed and tumor cells, we employed two isogenic cell lines derived from human foreskin fibroblasts for these experiments: wild-type BJ (wt-BJ) and BJ cells immortalized by the overexpression of SV-40 large-T antigen and hTERT (Imm-BJ). 24 hours after treatments with ARC (1.5 μ M), ABT-737 (350 nM), bortezomib (10 nM) and thiostrepton (1.5 μ M) both transformed (Imm-BJ) (Fig 3D) and wild-type (wt-BJ) cells (data not shown) did not undergo any visible programmed cell death. In contrast, combination treatment of transformed (Imm-BJ) cells with the same concentrations of ARC/ABT-737, ARC/bortezomib and thiostrepton/bortezomib induced very potent apoptosis as indicated by PARP and caspase-3 cleavage (Fig 3D). At the same time the untransformed normal (wt-BJ) cells did not exhibit any signs of cell death after identical combination treatments (Fig 3D), suggesting that these combination treatments specifically induce apoptosis in transformed, but not in normal cells.

Discussion

Previously, we have reported anti-cancer activities of a nucleoside analog ARC {Radhakrishnan, 2006 #3008; Radhakrishnan, 2008 #3298; Bhat, 2008 #3300; Bhat, 2008 #5518} and thiazole antibiotics Siomycin A and thiostrepton in cancer cell lines of different origin {Radhakrishnan, 2006 #3095; Bhat, 2008 #5518; Bhat, 2009 #5529}. In this study, we demonstrated that ARC and thiazole antibiotics, Siomycin A and thiostrepton hinder the viability and induce apoptosis in prostate cancer cell lines, LNCaP, DU-145 and PC-3. The pro-apoptotic activity of these drugs was independent of the p53 and androgen status of PC cells.

The nucleoside analog, ARC was identified in our laboratory as a general transcriptional inhibitor that could induce apoptosis in a variety of cancer cell lines of different origin, while not targeting normal cells {Radhakrishnan, 2006 #3008; Radhakrishnan, 2008 #3298; Bhat, 2008 #3300; Bhat, 2008 #5518}. Recently, in addition to our results another group has validated the strong growth inhibitory activity of ARC against a panel of 12 human cancer cell lines {Stockwin, 2009 #5556}. In the current study we have demonstrated potent proapoptotic activity of ARC against PC cells with IC₅₀ of 30–80 nM. These data suggest that PC cells in vitro are much more sensitive to ARC (Fig 2) than other epithelial cancer cell lines that we studied before {Radhakrishnan, 2006 #3008; Bhat, 2008 #3300; Bhat, 2008 #5518}. The degree of ARC-induced apoptosis in PC cells correlated with the degree of downregulation of short half-life, anti-apoptotic protein Mcl-1 (Fig 2B), which was also repressed by ARC in melanoma {Bhat, 2008 #5518}, neuroblastoma {Radhakrishnan, 2008 #3298} and colon cancer cells {Bhat, 2008 #3300}. Furthermore, overexpression of Mcl-1 in melanoma cells and neuroblastoma cells protected them from apoptosis induced by ARC {Bhat, 2008 #5518; Radhakrishnan, 2008 #3298}, suggesting that Mcl-1 is a general target of ARC in human cancer cells. Since Mcl-1 is overexpressed in 81% of prostate adenocarcinomas {Krajewska, 1996 #5558} and ARC in low concentrations targets Mcl-1 in prostate cancer, ARC may have a potential as a drug against PC.

Previously, we reported that TA induce apoptosis in human cancer cells, suppress transcriptional activity of FoxM1 and downregulate FoxM1 expression in melanoma,

neuroblastoma, leukemia and liver cancer cells {Radhakrishnan, 2006 #3095; Bhat, 2008 #5518; Bhat, 2009 #5529}. In addition, Kwok et. al. recently showed that thiostrepton represses FoxM1 expression and induces apoptosis in breast cancer cells {Kwok, 2008 #5455}. In this paper, we showed that TA inhibited the viability of PC cells in a dose dependent manner with similar IC₅₀ values, about 2 μ M. Downregulation of FoxM1 by Siomycin A and thiostrepton correlated with induction of apoptosis by TA in PC cells (Fig 1B). Since FoxM1 expression contributes to the progression of prostate cancer in mouse models {Kalin, 2006 #3047}, targeting FoxM1 by TA in prostate cancer may have therapeutic benefits.

In the present study we also evaluated combinations of ARC and thiostrepton with two recently introduced anticancer agents ABT-737 and bortezomib against human prostate cancer cell lines using the Chou-Talalay method {Chou, 2006 #5560}. Combination of anticancer agents may be more effective in inducing apoptosis and additionally this regimen would enable the use of sub-lethal doses of individual drugs, thus reducing adverse side effects. Bortezomib is currently approved for the treatment of patients with mantle cell lymphoma and multiple myeloma {Raab, 2009 #5561} and ABT-737 analog (orally bioavailable ABT-263) is currently in clinical trials {Tse, 2008 #5483}. Bortezomib has demonstrated weak activity as a single agent against prostate cancer {Papandreou, 2004 #5562}, but potent anti-prostate cancer activity in combination with other chemotherapeutic agents {Cao, 2008 #5563; Christian, 2009 #5564}. Antiapoptotic short half-life protein, Mcl-1 gets turn over rapidly at steady state due to degradation by the 26S proteasome and we showed that proteasome inhibitors and TA induce stabilization of Mcl-1 {Bhat, 2008 #5518; Bhat, 2009 #5548}. Therefore, suppression of Mcl-1 by ARC {Radhakrishnan, 2008 #3298; Bhat, 2008 #3300; Bhat, 2008 #5518} may explain synergistic interaction between bortezomib and ARC in prostate cancer cells (Fig 3; Table 1). It has been shown previously that Mcl-1 inhibitor, obatolax may synergize with bortezomib to induce apoptosis in human cancer cells {Nguyen, 2007 #3309}.

Not long ago, a novel Bcl-2/Bcl-xL/Bcl-w inhibitor (ABT-737), synthetic small-molecule produced by structure-based drug design, has been developed by Abbott {Oltersdorf, 2005 #5482}. It has been shown that ABT-737 competes with BAD for docking to the hydrophobic groove of Bcl-2/Bcl-X_L, thus increasing activity of proapoptotic BH3 proteins {Oltersdorf, 2005 #5482}. However, ABT-737 has a low affinity for another member of the Bcl-2 family protein, Mcl-1 {Oltersdorf, 2005 #5482}, which is highly expressed in prostate cancer cells {Krajewska, 1996 #5558} and a critical survival factor for various malignancies {Adams, 2005 #5484}. Consequently, it has been shown that ABT-737 had low capacity to induce apoptosis in cancer cells with high levels of Mcl-1 {Dai, 2007 #3310; Lin, 2007 #5487}, while down-regulation of Mcl-1 drastically increased ABT-737 lethality in human cancer cells of different origin {van Delft, 2006 #5488; Konopleva, 2006 #5489; Chen, 2007 #3310}. As a single agent, ABT-737 was largely ineffective at promoting cell death in prostate cancer cells {Song, 2008 #5497}. In our hands combination of ARC with ABT-737 resulted in synergistic induction of PC cell death (Fig 3; Table 1). Our data suggest that downregulation of Mcl-1 by ARC may contribute to its synergy with bortezomib and ABT-737 and these combinations both look very promising against PC.

We showed recently that TAs may act as proteasome inhibitors and well-known proteasome inhibitors also inhibit FoxM1 {Bhat, 2009 #5548}. It has been demonstrated before that combination of two proteasome inhibitors Lactacystin and MG132 synergized against PC cells in vitro {Shirley, 2005 #5565}. Similarly, we confirmed here that co-treatment of PC cells with sub-lethal concentration of proteasome inhibitors, thioistrepton and bortezomib strongly increased the apoptotic cell population comparatively to the sum of single agent treatments (Fig 3C) and displayed combination index 0.5–0.75 (Table 1). Since combination index values between 0.3 and 0.7 were graded as a relatively strong form of synergy {Chou, 2006 #5560}, our data suggest that proteasome inhibitors thioistrepton and bortezomib have strong synergistic relationship against human cancer cells. However, the mechanisms of interactions between different proteasome inhibitors need additional investigation.

To prove that all drug combinations that were synergistic in prostate cancer cells are specific for cancer, but not normal cells, we used two isogenic human foreskin fibroblast BJ cell lines: wt-BJ and Imm-BJ (Fig 3D). We found that in immortal cells all drug combinations display synergy to induce apoptosis similar to effect of these drugs in prostate cancer cells. However, normal BJ cells were completely resistant to individual or combination treatment (Fig 3D). These data suggest that described drug combinations are specific for cancer cells and may be attractive candidates for further anticancer drug development against PC.

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Abbreviations

PC	prostate cancer
TA	thiazole antibiotic
BOR	bortezomib
Sio A	Siomycin A
Thio	thioistrepton
HRPC	hormone-refractory prostate cancer

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Figure 1. TAs induce apoptosis and inhibit cell growth of PC cells

A. Growth inhibition curves of Siomycin A and thiostrepton in PC cells. Mid log LNCaP, PC-3 and DU145 cells were treated with DMSO or various concentrations of Siomycin A or thiostrepton (8.33, 5, 2.5, 1.25, 0.625 and 0.32 μM) for 72 hours and IC_{50} values were determined by using MTT cell viability assay as described in materials and methods. The values shown are mean $\pm$ SD for three separate experiments. IC_{50} values were 2.1 ± 0.1 , 3.1 ± 0.5 (LNCaP), 2.5 ± 0.7 , 2.9 ± 0.2 (PC-3) and 1.8 ± 0.4 , 2.2 ± 0.3 (DU145) μM against Siomycin-A and thiostrepton, respectively.

B. Thiazole antibiotics induce p53-independent apoptosis in PC cells. Siomycin A and thiostrepton downregulate FoxM1 expression in LNCaP, p53shRNA-LNCaP, PC-3 and

DU145 PC cells and induce apoptosis as indicated by appearance of cleaved caspase-3. DU145, PC-3, LNCaP and p53shRNA-LNCaP cells were treated with 5 μ M of Siomycin A and thiostrepton for 48 hours, lysed and subjected to immunoblotting analysis with antibodies against FoxM1, cleaved caspase-3, p53 and β -actin.

C. Quantitation of TA-induced apoptosis by flow cytometry. DU 145 cells were treated with 5 μ M of Siomycin A and thiostrepton for 48 hours, stained with annexin V-PE and 7-AAD and analyzed by flow cytometry. The values shown are mean of at least two experiments.

Figure 2. Evaluation of effects of ARC on PC cells

A. Growth inhibition curves of ARC in PC cells. ARC inhibits the proliferation of PC cell lines. Mid-log cells were treated with various concentrations of ARC (10, 2, 0.4, 0.08, 0.02, 0.004 μM) for 72 hours and IC₅₀ values were determined as described in materials and methods. IC₅₀ values of ARC were calculated to be 0.05±0.01(LNCaP), 0.08±0.02(PC-3) and 0.03±0.01(DU-145) μM respectively. The values were measured as mean ± SD for three separate experiments.

B. ARC inhibits the expression of anti-apoptotic protein Mcl-1 and induces apoptosis in PC cell lines. The cells were treated with increasing concentrations of ARC as indicated for 48 hours, lysed and subjected to immunoblotting analysis with antibodies against Mcl-1, cleaved caspase-3 and β-actin.

C. Induction of apoptosis by ARC in PC cells confirmed by annexin V-PE/7-AAD staining. PC cell lines were grown at a density of 50% confluence in 100-mm culture dishes and were treated with varying concentrations of the drugs for 48 hours, stained with annexin V-PE /7-AAD and analyzed by flow cytometry. The values shown are average of at least two experiments.

Figure 3. ARC and thiothrepton synergize with ABT-737 and Bortezomib in induction of apoptosis in human PC cells

A. Immunoblotting analysis of cell death induced by combinations of drugs in DU145 cells. DU145 cell line was treated with DMSO, 25 nM ARC, 1.5 μM thiothrepton, 350 nM ABT-737, 7.5 nM bortezomib or combination of 25 nM ARC and 350 nM ABT-737 or 25nM ARC and 7.5 nM bortezomib or 1.5 μM thiothrepton and 7.5 nM bortezomib respectively for 48 hrs and apoptosis was assessed by immunoblotting for cleaved caspase-3 and β-actin.

B. Immunoblotting analysis of cell death induced by combinations of drugs in PC-3 cells. PC-3 cells were treated with 100 nM ARC, 1.5 μ M thiostrepton, 350 nM ABT-737, 7.5 nM bortezomib or combination of 100nM ARC and 350 nM ABT-737 or 100 nM ARC and 7.5 nM bortezomib or 1.5 μ M thiostrepton and 7.5 nM bortezomib respectively for 48hrs and apoptosis was assessed by immunoblotting for cleaved caspase-3 and β -actin.

C. Flow cytometry analysis of cell death induced by combinations of drugs in DU145 cells. Synergistic induction of apoptosis was measured by treating DU145 cells with DMSO (control), single agents (25 nM ARC, 1.5 μ M thiostrepton, 350 nM ABT-737, 7.5 nM bortezomib) and their combinations (25nM ARC and 350 nM ABT-737 or 25nM ARC and 7.5 nM bortezomib or 1.5 μ M thiostrepton and 7.5 nM bortezomib) for 48 hrs followed by staining the cells with annexin V-PE and 7-AAD and analysis by flow cytometry. Percent of cells undergoing apoptosis are shown as mean $\pm$ S.D of three experiments.

D. Immunoblotting analysis of cell death induced by combinations of drugs in normal and immortalized BJ cells. Wt-BJ and Imm-BJ cell lines was treated with DMSO, 1.5 μ M ARC, 1.5 μ M thiostrepton, 350 nM ABT-737, 10 nM bortezomib or combination of 1.5 μ M ARC and 350 nM ABT-737 or 1.5 μ M ARC and 10 nM bortezomib or 1.5 μ M thiostrepton and 10 nM bortezomib respectively for 24 hrs and apoptosis was assessed by immunoblotting for cleaved caspase-3 and PARP with β -actin as a control.

Table 1
**Synergistic combination index was confirmed for ARC/thiostrepton with ABT-737/
 bortezomib in DU145 cells PC cells**

Viability of cells treated with the single agents' alone and combination of the two drugs was determined using standard MTT assay. Combination index values were measured for fractional effects of 0.5, 0.6 and 0.8 as described in the materials and methods.

Combination of	Fractional effect	Combination Index
ARC+ABT-737	0.5	0.8
	0.6	0.8
	0.8	0.7
ARC +BOR	0.5	0.6
	0.6	0.5
	0.8	0.3
Thio+BOR	0.5	0.7
	0.6	0.5
	0.8	0.75