Combination Treatment of Prostate Cancer Cell Lines with Bioactive Soy Isoflavones and Perifosine Causes Increased Growth Arrest and/or Apoptosis

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Abstract Purpose: To determine whether targeting the androgen receptor (AR) and Akt pathways using a combination of genistein combined polysaccharide (GCP) and perifosine is more effective at inducing growth arrest/apoptosis in prostate cancer cells compared with treatment with GCP or perifosine as single agents.

Experimental Design: The effect of GCP and perifosine treatment was assessed in five prostate cancer cell lines: LNCaP (androgen sensitive), LNCaP-R273H, C4-2, Cds1, and PC3 (androgen insensitive). A clonogenic assay assessed the long-term effects on cell growth and survival. Flow cytometry and Western blot analysis of poly(ADP)ribose polymerase cleavage were used to assess short-term effects. Preliminary studies to investigate mechanism of action included Western blot for P-Akt, Akt, P-p70S6K, p70S6K, p53, and p21; prostate-specific antigen analysis; and the use of myristoylated Akt and AR-specific small interfering RNA.

Results: Combination treatment with GCP and perifosine caused a decrease in clonogenic potential in all cell lines. In short-term assays, growth arrest was observed in the majority of cell lines, as well as increased inhibition of Akt activity and induction of p21 expression. Increased apoptosis was only observed in LNCaP. Knockdown of AR caused a further increase in apoptosis. GCP is prepared by fermentation of soybean extract cultured with basidiomycete mushrooms, a process that converts the isoflavones to their bioactive form allowing them to be readily absorbed through the gut lining into the bloodstream. Our group has previously shown that in prostate cancer cells, GCP reduces androgen receptor (AR) and prostate-specific antigen (PSA) levels, inhibits mammalian target of rapamycin (mTOR) activity, and also induces growth arrest and a modest increase in apoptosis (6). One hypothesis suggested by our data is that GCP mediates growth inhibition and apoptosis through molecular mimicry of androgen ablation.

Akt plays an important role in cancer cell proliferation and survival (7). This is often due to amplification of Akt caused by loss of PTEN, a key regulator of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (8, 9). Loss of PTEN, and the resulting hyperactivation of the PI3K/Akt pathway, is common in prostate cancer, making the PI3K/Akt pathway a key survival mechanism. Akt activity is known to increase upon androgen withdrawal (10, 11). In vitro inhibition of the PI3K/Akt pathway using LY294002 or wortmannin results in the inhibition of prostate cancer cell growth. Unfortunately, LY294002 and wortmannin have not proved clinically useful as they have too many off-target and cytotoxic side effects. Perifosine is a strong inhibitor of Akt activity and is currently being evaluated as an anticancer agent in phase 1 and phase 2 clinical trials (1, 12–17). Perifosine is an alkylphospholipid with promising antineoplastic activity. Clinical studies have
shown that perifosine concentrations of 10 to 18 μmol/L are well tolerated by patients (16, 18). The precise details of how perifosine inhibits Akt activity remain unclear. Perifosine appears to modulate a number of processes involved in plasma membrane synthesis and function. For example, perifosine can affect inositol metabolism and inhibit phospholipase C and protein kinase C activity (19–23). However, inhibition of Akt activity is thought to be the predominant mechanism by which this drug promotes growth inhibition in cancer cells (24). Perifosine is able to interfere with the pleckstrin homology domain of Akt and thereby block recruitment (25). Cells that express myristoylated Akt, which causes permanent anchorage of Akt to the plasma membrane and therefore constitutive activation of Akt, remain unaffected by perifosine treatment (25).

Using a combination of therapeutic agents to target the pathways that cause cancer has been shown in many cases to enhance the effectiveness of treatment while minimizing cytotoxic side effects. Targeting components of the same pathway with different drugs has proved particularly effective. Perifosine has been combined with UCN-01, a PDK1 inhibitor, to increase apoptosis in both PC-3 and A549 cells (26). PDK1 is an upstream effector of Akt. Synergy has also been seen with other treatments, such as cyclophosphamide, cisplatin, and γ-irradiation in leukemia models (24, 27–29). Our previous study showed that GCP is able to inhibit mTOR activity, a downstream effector of Akt, providing further rationale for combining GCP and perifosine treatment (6). mTOR can also be inhibited by rapamycin; however, unlike rapamycin, GCP treatment does not increase AR expression. The clinical efficacy of perifosine and rapamycin is currently being evaluated in clinical trials (1, 12–17, 30).

It is known that both the AR pathway and Akt pathways promote the growth and survival of prostate epithelial cells (31–33). There is sound rationale for targeting these pathways simultaneously in prostate cancer. In the current study, we show that combining GCP and perifosine treatment can increase the effectiveness of these drugs in inducing growth arrest and/or apoptosis of prostate cancer cells. We show that treatment with a combination of GCP and perifosine causes increased inhibition of Akt activity levels. In LNCaP, we show that increased inhibition of Akt activity and inhibition of the AR pathway is important.

Materials and Methods

Cell lines and culture. LNCaP, C4-2, and PC-3 cells were purchased from the American Type Culture Collection. Cds1 were generated in house; Cds1 is an androgen-independent cell line that was derived by culturing LNCaP in androgen-free conditions for >1 year (34). LNCAP-R273H, LNCaP cells that are stably transfected with a dominant-negative p53 gain-of-function mutant, were also generated in house (35). Cell lines were maintained in RPMI 1640 (Invitrogen/Life Technologies) supplemented with 5% fetal bovine serum (FBS) for LNCaP, LNCAP-R273H, C4-2, and PC3 (Omega Scientific, Inc.) or 5% charcoal-stripped serum (CSS) for Cds1 (Omega Scientific). Cells were maintained in RPMI 1640 (without phenol red) supplemented with 5% CSS.

Reagents. Perifosine was provided by Keryx Biopharmaceuticals. GCP was provided by Amino Up Chemical Company, Ltd., and stock solutions were made in 50% DMSO/50% ethanol. Mouse monoclonal anti-AR, PSA, and anti–poly(ADP)ribose polymerase (PARP) antibodies were purchased from Santa Cruz Biotechnology, and p53 (clone DO-1) was from BD PharMingen. Rabbit polyclonal anti–phospho(Ser473)-Akt, anti-Akt, anti–phospho(Thr389)-p70S6K, and anti-p70S6K antibodies were purchased from Cell Signaling Technology. The mouse monoclonal antibody against β-actin was obtained from Sigma-Aldrich Corporation.

Growth curves. Prostate cancer cell lines (LNCaP, LNCAP-R273H, C4-2; Cds1, and PC3) were plated at 5,000 per well in 96-well plates in the presence of RPMI 1640 containing 5% FBS or 5% CSS. Each experimental group consisted of five replicate wells, and all experiments were repeated at least thrice. Cells were allowed to attach overnight before treatment with GCP, perifosine, or a combination of GCP and perifosine. 3-(4,5-Dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assays were done to assess growth (36) after drug treatments. MTT is converted to a colored formazan by mitochondrial dehydrogenases in viable cells and is a dependable correlate of cell proliferation. MTT [0.5 mg/mL in PBS (pH 7.4)] was added to each well (10% v/v) and incubated for 3 h at 37°C/5% CO2. After 3 h, the medium was aspirated and 175 μL of DMSO (Sigma) were added to lyse cells and solubilize the formazan crystals. Plates were then placed on an orbital shaker for 15 min and read at 570 nm using a microplate reader.

Clonogenic assay. Cell survival after treatment with GCP and perifosine alone or in combination was measured by clonogenic assay. For all experiments, single cells were seeded into 60-mm culture dishes on day 0 and allowed to attach for 24 h at 37°C in 5% FBS medium. For LNCaP, LNCAP-R273H, C4-2, and Cds1, 12,000 cells were plated per dish; for PC3, 6,000 cells were plated per dish. The cells were then treated with GCP (50 μg/mL), perifosine (8 μmol/L), or a combination of GCP and perifosine (50 μg/mL and 8 μmol/L, respectively). Drugs were washed out 24 h posttreatment and fresh 5% FBS medium was added. After 14 days, colonies were fixed in 1% crystal violet and 0.5% glacial acetic acid in ethanol, and visible colonies containing ~50 or more cells were counted.

Flow cytometry. Cells (7.5 × 10⁵) were seeded into 10-cm dishes and allowed to adhere overnight. Cells were then treated with GCP (50 μg/mL), perifosine (8 μmol/L), or a combination of GCP and perifosine. At 24 or 72 h posttreatment, cells were harvested by trypsinization for cell cycle analysis. Cells were resuspended in 0.5 mL PBS (pH 7.4) then fixed in a final concentration of 70% ethanol and stored at -20°C. Before analysis, cells were washed once with PBS (pH 7.4) and resuspended in 0.9 mL PBS (pH 7.4) with 20 μL of DNase-free RNase (Fermentas) then incubated at 37°C for 30 min. Propidium iodide (Boehringer Mannheim Corp.) was added to a final concentration of 50 μg/mL, and samples were allowed to stand at room temperature, protected from light, for 10 min. To facilitate removal of cell aggregates, samples were filtered through cell strainers into polystyrene tubes (BD Falcon). DNA content/cell number was measured by using propidium iodide fluorescence using a Coulter Epics XL flow cytometer (Beckman Coulter). Cells (5 × 10⁴) were analyzed per sample. The percentage of cells in the sub-G1 phase was determined from the DNA histogram obtained from each cell sample using Phoenix Multicycle software (Phoenix Flow Systems).

Immunoblot analysis. Protein was extracted from cells using radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 8.0), 5 mmol/L EDTA, 1% Triton X-100; Sigma] containing 10 mg/mL leupeptin, 0.1 mmol/L aprotinin, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 0.1 mmol/L NaVO₄ (Sigma). Following extraction, protein samples were sonicated then stored at -70°C. Protein samples were electrophoresed on a Mini-PROTEAN Tetra Cell (Bio-Rad), transferred to nitrocellulose membranes, and probed with specific antibodies to either phospho-Akt, total Akt, PARP, phospho-p70S6K, total p70S6K, AR, p53, p21, PSA, or B-actin (see Reagents for details). After transferring, membranes were blocked, washed, and probed with specific antibodies.
Washing with TBST, membranes were incubated with a horseradish peroxidase–linked anti-mouse secondary antibody (1:5,000 dilution; Promega), washed again, and then incubated with enhanced chemiluminescence reagent (Amersham Pharmacia). Labeling was detected using X-ray film (Kodak).

**Transient transfection.** Cells were plated overnight in the absence of antibiotics. Following the manufacturer’s instructions, LipofectAMINE 2000 (Invitrogen) was used to transfect cells with myristoylated Akt (pCMV-6-myr-Akt-HA, a kind gift from Dr. Paramita Ghosh, University of California, Davis, Sacramento, CA) versus a vector-only control or with siRNA that specifically down-regulates AR expression (Invitrogen) versus a nonsilencing control siRNA (Invitrogen). The final concentration of plasmid added to the cultures was 0.5 μg/mL. The final concentration of siRNA added to cultures was 60 nmol/L. To minimize toxicity of the transfection reagent, the medium was replaced 5 h after transfection. A green fluorescent protein control plasmid or nonsilencing FITC-conjugated control siRNA was used to assess transfection efficiency. To monitor down-regulation of AR and expression of Akt, Western blot analysis was done 96 h after transfection.

**Statistical analysis.** At least three independent experiments were completed for each analysis described in this article. When appropriate, Student’s *t* test was conducted to determine whether the treatment

![Graphs of perifosine concentration on prostate cancer cell proliferation as assessed by MTT analysis.](image)

**Table 1. IC50 values 3 d post-treatment with perifosine**

<table>
<thead>
<tr>
<th>Prostate cancer cell line</th>
<th>Perifosine IC50 (μmol/L)</th>
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<tbody>
<tr>
<td>LNCaP, FBS, 72 h</td>
<td>~8</td>
</tr>
<tr>
<td>R273H, CSS, 72 h</td>
<td>~22</td>
</tr>
<tr>
<td>C321, CSS, 72 h</td>
<td>~58</td>
</tr>
<tr>
<td>C42, CSS, 72 h</td>
<td>~64</td>
</tr>
<tr>
<td>PC3, CSS, 24 h</td>
<td>~1</td>
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*Fig. 1.* Effects of perifosine concentration on prostate cancer cell proliferation as assessed by MTT analysis. LNCaP, an androgen-sensitive cell line, was moderately sensitive to treatment with perifosine. The IC50 for LNCaP treated with perifosine for 72 h was ~8 μmol/L (A). Patients can tolerate doses of between 10 and 18 μmol/L perifosine. The androgen-independent cell lines were more resistant to perifosine treatment. The IC50 values for LNCaP-R273H, C4-2, and C321 treated with perifosine for 72 h were ~22 μmol/L (B), ~58 μmol/L (C), and ~64 μmol/L (D), respectively. The other androgen-independent cell line, PC3, was very sensitive to treatment with perifosine. The IC50 value for PC3 treated with perifosine for 24 h was ~1 μmol/L (E). Transient transfection of LNCaP with myristoylated Akt prevented perifosine-mediated growth inhibition (F).
Results

Growth inhibition of prostate cancer cell lines by perifosine. The IC\(_{50}\) of LNCaP cultured in medium containing FBS was achieved at a dose of ~8 \(\mu\)mol/L perifosine (the calculated IC\(_{50}\) value lies between 5 and 10 \(\mu\)mol/L; Fig. 1A; Table 1). This dose is below the level that is reportedly well tolerated by patients (10-18 \(\mu\)mol/L; ref. 26). As we were interested in determining the effect of these drugs at a physiologically relevant dose, treatment with 8 \(\mu\)mol/L perifosine was chosen for use in all subsequent experiments. PC3 cells were extremely sensitive to perifosine. The 24 h IC\(_{50}\) value for PC3 was 1 \(\mu\)mol/L (the calculated IC\(_{50}\) value lies between 0.1 and 5 \(\mu\)mol/L; Fig. 1E; Table 1). In contrast, the other androgen-independent cell lines were less sensitive to perifosine treatment. The 72 h perifosine IC\(_{50}\) value for various androgen-independent sublines of LNCaP, LNCaP-R273H, C4-2, and Cds1 was 22, 64, and 58 \(\mu\)mol/L, respectively (the calculated IC\(_{50}\) values lie between 10 and 100, 20 and 100, and 20 and 100 \(\mu\)mol/L, respectively;

![Fig. 2](image_url)

**Fig. 2.** Effects of perifosine, GCP, and a combination of GCP and perifosine treatment on Akt activity in prostate cancer cell lines. Cells were treated with GCP (50 \(\mu\)g/mL), perifosine (Peri; 8 \(\mu\)mol/L), or GCP and perifosine (G+P; 50 \(\mu\)g/mL and 8 \(\mu\)mol/L, respectively). At various time points, protein was harvested and Western blot analysis was done to assess phospho-Akt (Ser\(^{473}\)) and total Akt levels. GCP treatment alone did not affect Akt activity significantly at any of the time points for any of the cell lines (A-F). Perifosine treatment resulted in a marked decrease in Akt activity in LNCaP, LNCaP-R273H, C4-2, and PC3 cells 24 h posttreatment (A-D and F). Cds1 seemed to be resistant to perifosine treatment, and no decrease in phospho-Akt levels was observed at 24 or 72 h posttreatment (E). Treatment with a combination of GCP and perifosine further inhibited Akt phosphorylation in the LNCaP, LNCaP-R273H, and C4-2 cell lines (A, B, and D). In LNCaP, this inhibition was maintained for as long as 72 h for the combination treatment. B: Cds1 was resistant to treatment with a combination of GCP and perifosine (E). As treatment of PC3 cells with perifosine alone resulted in near-maximal inhibition of Akt, further increase resulting from treatment with a combination of GCP and perifosine was hard to discern (F).
Fig. 1C and D; Table 1). It is possible that survival pathways other than the PI3K/Akt pathway are active in these cell lines and this is why perifosine treatment is less effective. In C4-2 cells, this added resistance may also be due to these cells having particularly active PI3K/Akt pathway (7). The 72 h IC_{50} value for LNCaP treated with GCP has been previously published as ~70 µg/mL (6). On average, patients given GCP in oral form achieve a dose range of 5 to 20 µg/mL genistein in their serum. GCP contains ~10% genistein (4). To achieve a physiologically relevant dose, we therefore used 50 µg/mL GCP, equivalent of 5 µg/mL genistein, in all of our in vitro experiments. Transfection of LNCaP with myristoylated Akt, which is constitutively active, prevented perifosine-mediated growth inhibition even at the 100 µmol/L dose (Fig. 1F).

A combination of GCP and perifosine causes a further decrease in Akt activity compared with GCP or perifosine alone. GCP treatment alone did not affect Akt activity significantly at any of the time points in any of the cell lines (Fig. 2A-F). As expected, perifosine treatment resulted in a marked decrease in Akt activity in the majority of the sublines (LNCaP, LNCaP-R273H, C4-2, and PC3). Interestingly, no inhibition was observed in the Cds1 subline (Fig. 2E). In LNCaP, LNCaP-R273H, and C4-2, treatment with a combination of GCP and perifosine further increased the level of Akt activity inhibition (Fig. 2A-D). In LNCaP and LNCaP-R273H, this increased inhibition was maintained for as long as 72 h (Fig. 2A-C). The data indicate that combination treatment with GCP and perifosine is able to cause further inhibition of the Akt pathway in some prostate cancer cell lines. PC3 cells were very sensitive to treatment with perifosine as a single agent at the 8 µmol/L dose. As such, Akt activity was analyzed at 8 and 24 h, as by 72 h no living cells remained. By 24 h, perifosine treatment caused maximal inhibition of Akt activity in PC3 cells (Fig. 2F). Further inhibition of Akt activity by the combination of GCP and perifosine treatment was therefore difficult to discern.

A combination of GCP and perifosine decreases clonogenic potential further when compared with GCP and perifosine alone. To determine the long-term effect of GCP and perifosine treatment on cell growth, a clonogenic assay was used. The clonogenic assay assesses the ability of a cell to undergo several rounds of mitosis over a 14-day period after an initial 24-h treatment time. As such, the clonogenic assay represents a more appropriate way to measure long-term effects on growth inhibition than the classic MTT assay. In addition, it allows for assessment of the ability of prostate cancer cells not only to survive but also to retain reproductive potential after treatment, a characteristic that facilitates tumor recurrences in patients. It should be noted that although the clonogenic assay allows for the measure of long-term effects of a drug on cell growth, it does not discern the mechanism by which the drug affects growth. As an individual agent, GCP caused up to a 40% reduction in clonogenic potential in LNCaP and LNCaP-R273H.
C4-2 and PC3 cells were more sensitive to GCP treatment, showing an ~80% and ~60% reduction, respectively (Fig. 3A and B). Cds1 cells were very resistant, showing only an ~10% reduction in clonogenic potential (Fig. 3A and B). All of the cell lines, except LNCaP-R273H and PC3, had a similar level of sensitivity to treatment with perifosine alone, with the reduction in clonogenic potential in LNCaP, C4-2, and Cds1 ranging from ~40% to 50%. LNCaP-R273H cells were more resistant, showing only an ~30% decrease in clonogenic potential. PC3 were very sensitive to treatment with perifosine, with almost 100% inhibition. Treatment with a combination of GCP and perifosine resulted in a further decrease in clonogenic potential when compared with treatment with GCP and perifosine as single agents in all of the prostate cancer cell lines except PC3. As perifosine treatment alone caused such a dramatic reduction in the clonogenic potential of PC3, any additive effect from the combination of GCP and perifosine treatment was not seen. LNCaP showed the most dramatic difference in response, with nearly a 100% inhibition in response to treatment with the combination of GCP and perifosine. It is possible that LNCaP are more sensitive to the combination treatment compared with the other cell lines because GCP can cause molecular mimicry of androgen ablation and LNCaP was the only androgen-sensitive cell line being assessed. C4-2 and Cds1 cells showed an ~90% and ~85% level of inhibition, respectively, when treated with a combination of GCP and perifosine. LNCaP-R273H cells were more resistant to the combination treatment compared with the other cell lines, and only an ~65% inhibition was observed. Stable transfection of LNCaP with the R273H p53 mutant allele has been shown to confer several gain-of-function characteristics (35, 37). One of these gain-of-function characteristics is resistance to drug treatment, and the multiple drug resistance gene, MDR-1, has been shown to be up-regulated in the LNCaP-R273H subline (35). This may explain the relative resistance of the LNCaP-R273H subline to treatment with GCP and perifosine. Alternatively, other survival pathways may be active in this subline.

**Treatment of LNCaP with a combination of GCP and perifosine causes a significant increase in the sub-G₁ population.** As single agents, GCP (30 μg/mL) and perifosine (8 μmol/L) caused relatively little or no increase in the sub-G₁ population by 3 days posttreatment of LNCaP, LNCaP-R273H, C4-2, or Cds1 (Fig. 4A.i-A.v and B). The main effect of the single agents was to induce a growth arrest. Treatment with GCP as a single agent resulted in a decreased S-phase population for all of the cell lines except LNCaP (Fig. 4A.i-A.v and B). The LNCaP-R273H, C4-2, and Cds1 cell lines, which are androgen independent, were more resistant to the combination treatment and there was relatively little or no increase in sub-G₁ levels in response to treatment with a combination of GCP and perifosine. In support of this, the effect of the combination of GCP and perifosine treatment was greater in LNCaP cultured in CSS versus FBS medium (Fig. 4A.i, A.ii, and B). The LNCaP-R273H, C4-2, and Cds1 cell lines, which are androgen independent, were more resistant to the combination treatment and there was relatively little or no increase in sub-G₁ levels in response to treatment with a combination of GCP and perifosine (Fig. 4A.iii-A.v and B). Treatment with GCP and perifosine caused growth arrest in these cell lines, however, explaining why treatment with GCP and perifosine affected their long-term growth. PC3 cells were very sensitive to treatment with perifosine (Fig. 4A.vi and B). At 24 h posttreatment with 8 μmol/L of perifosine, a big increase in sub-G₁ levels (~22%) was observed. Treatment with a combination of GCP and perifosine actually caused a decrease in sub-G₁ levels (sub-G₁ levels dropped to ~12%). Surprisingly, treatment of PC3 cells with a combination of GCP and perifosine caused an increase in the S-phase population. It should be noted that treatment with a combination of GCP and perifosine did not cause a further decrease in S-phase levels compared with treatment with GCP or perifosine as single agents, in any of the cell lines studied. The increase in sub-G₁ levels observed in LNCaP in response to treatment with a combination of GCP and perifosine resulted from decreased G₁ and G₂-M levels.

**Treatment of LNCaP with a combination of GCP and perifosine increases levels of apoptosis.** The cell cycle analysis data implied that treatment of LNCaP with a combination of GCP and perifosine could result in a dramatic increase in apoptosis when compared with treatment with GCP or perifosine alone (sub-G₁ levels are often used as a surrogate marker of apoptosis). Analysis of PARP cleavage was used to verify these data. PARP is a target of active caspases and its cleavage can be used to detect apoptosis. Treatment of LNCaP with a combination of GCP and perifosine caused significant cleavage of PARP, indicating that these cells are undergoing apoptosis (Fig. 5A). In accordance with the flow data, increased PARP cleavage was observed in LNCaP treated with the combination of GCP and perifosine in CSS medium compared with FBS medium (Fig. 5A). A minor increase in PARP cleavage was observed in LNCaP treated with the single agents alone when LNCaP cells were cultured in CSS medium. No increase in PARP cleavage was observed in LNCaP-R273H, C4-2, or Cds1 treated with combination of GCP and perifosine or as single agents (Fig. 5B-D). Again, these data are in accordance with our flow data. At 24 h posttreatment, treatment with GCP or perifosine alone was able to induce PARP cleavage in PC3 cells (Fig. 5E). Treatment of PC3 cells with a combination of GCP and perifosine was unable to cause a further increase in PARP cleavage compared with treatment with perifosine alone. As
mentioned before, this is likely due to maximal inhibition of Akt activity by perifosine alone. The level of PARP cleavage in perifosine-treated PC3 cells versus PC3 cells treated with combination of GCP and perifosine was approximately the same, indicating that levels of apoptosis were similar in response to these treatments. Transfection of LNCaP with myristoylated Akt prevented perifosine and GCP/perifosine–mediated inhibition of Akt activation (Fig. 5F), and prevented the combination treatment from causing increased apoptosis. These data confirm that perifosine-mediated inhibition of Akt activity can cause increased apoptosis.

Treatment with GCP and perifosine causes induction of p53 and p21 in some prostate cancer cell lines. To determine whether p53/p21 induction could be responsible for causing increased growth arrest and/or apoptosis, we analyzed p53 and p21 levels in the prostate cancer cell lines treated with GCP and/or perifosine. Treatment with GCP or perifosine alone caused increased p53 and p21 expression in LNCaP and Cds1 cells (Fig. 6A and D) but not in LNCaP-R273H or C4-2 (Fig. 6B and C). p21 levels were also increased in PC3 cells (Fig. 6E). Treatment with a combination of GCP and perifosine resulted in an ~20% increase (A.i and B). In CSS medium, the combination treatment caused a ~30% increase in sub-G1 (A.ii and B). Little or no increase in sub-G1 levels was observed for the combination treatment in the other cell lines when compared with treatment with GCP or perifosine as single agents (A.iii, A.iv, A.v, and B). In fact, sub-G1 levels dropped by ~15% in PC3 cells treatment with a combination of GCP and perifosine compared with treatment with perifosine alone (A.vi and B). *, P < 0.05; **, P < 0.005; ****, P < 0.0005.

![Figure 4](image_url)

Fig. 4. Effects of GCP, perifosine, and a combination of GCP and perifosine treatment on the cell cycle. Prostate cancer cell lines were treated with GCP (50 μg/mL), perifosine (8 μmol/L), or GCP and perifosine (50 μg/mL and 8 μmol/L, respectively). After 24 h (PC3) or 72 h (all of the other cell lines), cells were harvested, fixed, treated with RNase, and then labeled with propidium iodide to facilitate analysis of DNA content. As a single agent, the main effect of GCP was to cause a decrease in the S-phase population in all of the prostate cancer cell lines. Very minimal or no increase in the sub-G1 population was observed in all of the cell lines except LNCaP, in which GCP caused ~5% to 7% increase (A.i, A.ii, and B). Treatment with perifosine as a single agent resulted in a small increase in the sub-G1 population in LNCaP and C4-2 (~10% increase for both cell lines; A.i, A.ii, A.iv, and B). LNCaP-R273H and Cds1 were relatively insensitive to treatment with perifosine (A.iii, A.v, and B). PC3 were very sensitive to treatment with perifosine, showing an ~22% increase in the sub-G1 population (A.ii and B). A dramatic G2/M arrest was also observed. A significant increase in sub-G1 in response to treatment with a combination of GCP and perifosine was only seen in LNCaP (A.i, A.ii, and B). In FBS medium, the combination treatment induced an ~20% increase (A.i and B). In CSS medium, the effect was even more dramatic, and treatment with a combination of GCP and perifosine resulted in an ~30% increase in sub-G1 (A.ii and B). Little or no increase in sub-G1 levels was observed for the combination treatment in the other cell lines when compared with treatment with GCP or perifosine as single agents (A.iii, A.iv, A.v, and B). In fact, sub-G1 levels dropped by ~15% in PC3 cells treatment with a combination of GCP and perifosine compared with treatment with perifosine alone (A.vi and B). *, P < 0.05; **, P < 0.005; ****, P < 0.0005.

\[ \text{Formula} \]
treatment does not affect Akt activity in Cds1 (Fig. 2D), it is possible that activation of p53 and p21 in this cell line may explain the GCP/perifosine–mediated decrease in clonogenic potential that we observed (Fig. 3A and B). Induction of p21 by perifosine treatment has been reported previously (38).

**Treatment of LNCaP with a combination of GCP and perifosine causes a further decrease in AR and PSA levels and in p70S6 kinase phosphorylation.** Western blot analysis showed that treatment of LNCaP with GCP alone or in combination with perifosine caused a decrease in AR and PSA levels (Fig. 7A). PSA levels were further decreased by treatment with a combination of GCP and perifosine (Fig. 7A). Treatment with perifosine caused little or no decrease in AR levels but decreased PSA expression levels. PSA analysis was also done on culture supernatants taken from LNCaP cultured in FBS that had been treated with GCP, perifosine, or a combination of GCP and perifosine in addition to pretreatment with control or AR-specific siRNA. These data confirmed that treatment with GCP or perifosine alone or in combination could decrease PSA expression (Fig. 7B). Treatment with GCP alone was more potent than treatment with perifosine alone (a 4.4-fold decrease with GCP treatment, compared with a 1.8-fold decrease with perifosine treatment). Treatment with a combination of GCP and perifosine cause a slight further decrease in PSA levels (a 5.5-fold decrease), confirming the Western blot analysis data. As expected, treatment of LNCaP with AR-specific siRNA resulted in a dramatic reduction in PSA levels even in the absence of drug treatment (a 9.4-fold decrease compared with LNCaP treated with control siRNA; Fig. 7B). Treatment with GCP and perifosine, alone or in combination, caused a further decrease in PSA levels (~2.3-fold further decrease compared with treatment with AR siRNA alone for all treatments; Fig. 7B).

The combined data show that GCP and perifosine treatment can affect the AR signaling pathway and indicate that this increased inhibition of AR signaling may in part explain the increase in apoptosis that is observed in LNCaP treated with a combination of GCP and perifosine. As previously reported by our group (6), GCP treatment caused a decrease in p70S6 kinase phosphorylation, indicating a decrease in mTOR activity (Fig. 7C). Treatment with a combination of GCP and perifosine caused a further decrease in p70S6 kinase phosphorylation (Fig. 7C). These data show that in combination GCP and perifosine can cause increased inhibition of both Akt and mTOR activity.

**Knockdown of AR causes increased apoptosis in LNCaP treated with perifosine.** Transient transfection of LNCaP with siRNA specific for AR was used to knockdown AR expression (Fig. 8A). Twenty-four hours posttransfection, LNCaP were then treated with GCP and/or perifosine for 72 h. Knockdown of AR caused an increase in phospho-Akt levels; however, treatment with perifosine or a combination of GCP and perifosine was still able to reduce phospho-Akt levels to similar levels as seen in LNCaP transfected with a siRNA control (Fig. 8A). Sub-G₁ levels were 1.35%, 1.89%, 5.90%, and 12.16%, respectively, for LNCaP treated with vehicle, GCP, perifosine, or GCP and perifosine in combination (Fig. 8B). Treatment with AR siRNA caused increased inhibition of both Akt and mTOR activity.
increase in sub-G1 levels (~12% increase compared with control siRNA; Fig. 8B-C). Treatment of LNCaP with siRNA specific for AR followed by treatment with perifosine caused a dramatic increase in sub-G1 levels (~40%; Fig. 8B and C). No further increase was observed in LNCaP treated with a combination of GCP and perifosine and with AR-specific siRNA. Based on these data, we hypothesize that inhibition of the AR pathway is the main mechanism by which GCP is able to synergize with perifosine to cause increased apoptosis in LNCaP. The fact that the sub-G1 levels achieved by treatment of LNCaP with a combination of AR-specific siRNA and perifosine caused a statistically significant drop in PARP cleavage compared with treatment with GCP or perifosine alone or in combination (Fig. 8B and C) suggests that the mechanism by which GCP and perifosine induce apoptosis in LNCaP is likely due to the more complete inhibition of AR signaling by AR-specific siRNA compared with treatment with GCP or perifosine alone or in combination. The combination of GCP and perifosine was also more effective in inducing growth arrest in the LNCaP-R273H, C4-2, and Cds1 cell lines when compared with treatment with either agent alone. The clonogenic assay allows for assessment of the ability of prostate cancer cells not only to survive but also to retain reproductive potential after treatment, a characteristic that facilitates tumor recurrences in patients. The increased response was observed in all androgen-dependent and androgen-independent cell lines, indicating that treatment with a combination of GCP and perifosine could represent a valid treatment option for patients with either localized or metastatic disease. Our data show that GCP treatment can enhance perifosine-mediated inhibition of Akt activity. As the PI3K/Akt pathway plays a key role in prostate cancer growth and survival, we hypothesize that increased inhibition of the Akt pathway is responsible for the growth inhibition and/or apoptosis observed in response to treatment with these agents. In support of this hypothesis, we have previously shown that GCP can inhibit mTOR activity (6); here, we show that treatment with a combination of GCP and perifosine can cause a further decrease in mTOR activity. mTOR is a downstream effector of Akt. It is possible that the simultaneous inhibition of Akt and mTOR activity contributes to the increase in apoptosis levels that are observed in response to the combination treatment. Targeting two components of the same signaling pathway has proved to be a successful strategy in the treatment of several prostate cancer cell lines (24, 26–29). In short-term studies, treatment with GCP and perifosine induced growth arrest in the LNCaP-R273H, C4-2, and Cds1 cell lines and caused little if any increase in apoptosis.

Discussion

In this study, we show that treatment with a combination of GCP and perifosine at physiologically relevant doses can further inhibit the clonogenic potential of several prostate cancer cell lines when compared with treatment with either agent alone. The clonogenic assay allows for assessment of the ability of prostate cancer cells not only to survive but also to retain reproductive potential after treatment, a characteristic that facilitates tumor recurrences in patients. The increased response was observed in both androgen-dependent and androgen-independent cell lines, indicating that treatment with a combination of GCP and perifosine could represent a valid treatment option for patients with either localized or metastatic disease. Our data show that GCP treatment can enhance perifosine-mediated inhibition of Akt activity. As the PI3K/Akt pathway plays a key role in prostate cancer growth and survival, we hypothesize that increased inhibition of the Akt pathway is responsible for the growth inhibition and/or apoptosis observed in response to treatment with these agents. In support of this hypothesis, we have previously shown that GCP can inhibit mTOR activity (6); here, we show that treatment with a combination of GCP and perifosine can cause a further decrease in mTOR activity. mTOR is a downstream effector of Akt. It is possible that the simultaneous inhibition of Akt and mTOR activity contributes to the increase in apoptosis levels that are observed in response to the combination treatment. Targeting two components of the same signaling pathway has proved to be a successful strategy in the treatment of several prostate cancer cell lines (24, 26–29). In short-term studies, treatment with GCP and perifosine induced growth arrest in the LNCaP-R273H, C4-2, and Cds1 cell lines and caused little if any increase in apoptosis. In
In comparison, the combination treatment induced a dramatic increase in apoptosis in LNCaP in short-term experiments as well as caused nearly 100% inhibition of growth and survival in long-term assays. As LNCaP was the only androgen-sensitive cell line assessed in this study and as we already know that GCP can inhibit AR signaling (6), we hypothesize that this dramatic effect on apoptosis and clonogenic potential is due to blockage of the AR pathway by GCP in addition to the increased inhibition of Akt signaling resulting from treatment with a combination of GCP and perifosine. Our preliminary analyses to determine a mechanism of action support the importance of blocking the AR pathway in facilitating GCP/perifosine-mediated apoptosis in LNCaP. LNCaP cultured in CSS medium, and therefore in which the AR signaling pathway is less active, were more sensitive to treatment with a combination of GCP and perifosine compared with LNCaP cultured in FBS medium. We also showed that treatment with a combination of GCP and perifosine further decreased PSA levels when compared with treatment with GCP or perifosine alone. A definitive role for the AR pathway was confirmed by demonstrating that blocking AR signaling by using AR-specific siRNA in combination with perifosine treatment was able to cause a dramatic increase in apoptosis. As the AR-specific siRNA knocked down AR levels by nearly 100%, we were not surprised that treatment of LNCaP with a combination of AR siRNA, perifosine, and GCP did not cause a further increase in apoptosis, or that the level of apoptosis was higher than that seen when LNCaP were treated with a combination of GCP and perifosine in CSS medium.

These differences simply reflect the fact that the AR siRNA was able to cause a more complete knockdown of AR signaling when compared with treatment with GCP or culture in CSS. Both the AR and Akt pathways play key roles in promoting growth and survival of prostate epithelial cells (31–33). The AR pathway can be important even in metastatic prostate cancer (39, 40). In fact, up-regulation of AR is a common event after androgen ablation (31, 32), as is increased Akt activity. Although the effect of GCP and perifosine treatment on AR pathway activity in the androgen-independent prostate cancer cell lines was not assessed in this study, it is possible that the inhibition of the AR pathway also plays a role in causing the increased effect of the combination treatment on clonogenic potential in these cell lines. The absence of the AR survival pathway may also explain why PC3 cells are extremely sensitive to treatment with perifosine alone, as they have to rely on the Akt pathway alone for survival. These data suggest that blocking both the AR and Akt pathways in prostate cancer patients should promote further inhibition of growth and apoptosis.

The LNCaP-R273H subline was more resistant to the combination treatment when compared with the other androgen-independent cell lines. This subline is stably transfected with a dominant-negative p53 allele that has been shown to confer several gain-of-function characteristics, including the ability to grow in the absence of androgen (35). Mutations in p53 are common in prostate cancer (~71% in androgen-refractory prostate cancer patients). The R273H p53 mutation is

**Fig. 6.** Effects of GCP, perifosine, and a combination of GCP and perifosine treatment on induction of p53 and p21. Prostate cancer cell lines were treated with GCP (50 μg/mL), perifosine (8 μmol/L), or GCP and perifosine (50 μg/mL and 8 μmol/L, respectively). Treatment with GCP and perifosine alone or in combination induced an increase in both p53 and p21 in LNCaP, C4-2, and PC3 (A, D, and E). The most dramatic increase was observed in C4-2 (D). The drug treatments did not seem to cause any significant increase in p53 or p21 levels in LNCaP-R273H, a subline that is stably transfected with a dominant-negative mutant p53 allele.
a hotspot mutation associated with a particularly aggressive phenotype. As such, the study of the effect of GCP and perifosine on prostate cancer sublines that harbor p53 mutations is clinically relevant and this is the reason the LNCaP-R273H subline was chosen for use in this study. It is unlikely that loss of p53 function is responsible for the lesser effect of the combination of GCP and perifosine treatment on the clonogenic potential of this subline, as p53-null PC3 cells are very sensitive to treatment with perifosine alone or in combination with GCP. Instead, we hypothesize that other pathways, besides the Akt pathway, that support cell survival are activated in the LNCaP-R273H subline, and that these pathways provide a partial rescue of LNCaP-R273H from the combination therapy. Alternatively, it is possible that resistance to treatment with GCP and perifosine is a novel gain-of-function characteristic of the LNCaP-R273H subline. In support of this, the LNCaP-R273H subline expresses elevated levels of the multiple drug resistance gene (MDR-1) and the R273H allele has been shown to confer resistance to other drug treatments (35, 41–43). If p53 status affects the effectiveness of the GCP/perifosine treatment, patients with advanced disease may not respond well because p53 mutations are frequent at this stage of prostate cancer.

Interestingly, perifosine did not inhibit Akt activity in the Cds1 cell line. However, treatment with perifosine alone or in combination with GCP did cause inhibition of clonogenic potential in this cell line. These findings indicate that perifosine may affect other pathways that can affect long-term growth and survival. Induction of p21 was particularly strong in Cds1 treated with a combination of GCP and perifosine. We hypothesize that induction of p21 may play a role in the response of some prostate cancer cell lines to perifosine. It has previously been reported that perifosine induces p21 expression; however, the exact mechanism by which this occurs remains unclear (38).

In conclusion, we show that the treatment of prostate cancer cells with a combination of GCP and perifosine can further inhibit their long-term growth and survival when compared with treatment with GCP or perifosine alone. We hypothesize that in androgen-independent cells this is due to the increased inhibition of the Akt pathway, possibly through the simultaneous inhibition of both mTOR and Akt. In the androgen-dependent cell line LNCaP, the more dramatic effect of the combination treatment on long-term growth and on apoptosis is likely due to the inhibition of the AR pathway by GCP in addition to the increased inhibition of the Akt signaling pathway mediated by both GCP and perifosine. A further increase in apoptosis was achieved by combining inhibition of the AR pathway using AR-specific siRNA and treatment with perifosine and/or GCP, suggesting a synergistic effect of this combination. Last, our data imply that the response of cells to the combination treatment with GCP and perifosine may depend on p53 status, and that induction of p21 may play a role. Further studies to confirm these hypotheses are under way.

From a clinical standpoint, the combination of androgen deprivation with these targeted therapies may have a significant effect on the management of prostate cancer. Perifosine as a single agent has marginal effects in patients with hormone-independent (16) and hormone-sensitive prostate cancer (44). Our data suggest that the combination of androgen deprivation and perifosine/GCP is more effective than androgen deprivation alone. Currently, we are developing two clinical trials. In one study, we will treat patients with low-risk prostate cancer (defined as ≤T2a, Gleason ≤6, and PSA <10) with 6 months of 5a reductase inhibitor, GCP, and perifosine. These patients are presently considered candidates for active surveillance. We hope that this combination would cause more prostate cancer cells to undergo apoptosis, thus reducing tumor volume and increasing the time to disease progression. The second group would be patients with hormone-naive metastatic prostate cancer requiring hormonal therapy and patients with biochemically
Fig. 8. Effect of combining perifosine, GCP, and a combination of GCP and perifosine treatment of LNCaP with knockdown of AR. Cells were transiently transfected with siRNA specific to either AR for 24 h before drug treatments. After 24 h, LNCaP were then treated with GCP (50 \( \mu \text{g/mL} \)), perifosine (8 \( \mu \text{mol/L} \)), or GCP and perifosine (50 \( \mu \text{g/mL} \) and 8 \( \mu \text{mol/L} \), respectively). After 72 h, cells were collected for both flow and protein analysis. Knockdown of AR was confirmed by Western blot analysis (A). Knockdown of AR did not affect the ability of perifosine or the combination of GCP and perifosine to inhibit Akt activity (A). Flow cytometry analysis showed that treatment of LNCaP with a combination of perifosine and AR-specific siRNA resulted in a dramatic increase (~40%) in sub-G1 levels (B and C). This effect was not enhanced by the addition of GCP.

\({ }^*, P < 0.05; **, P < 0.005; ***, P < 0.0005.

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Combination Treatment of Prostate Cancer Cell Lines with Bioactive Soy Isoflavones and Perifosine Causes Increased Growth Arrest and/or Apoptosis

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