In vitro Combination Treatment with Perifosine and UCN-01 Demonstrates Synergism against Prostate (PC-3) and Lung (A549) Epithelial Adenocarcinoma Cell Lines

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ABSTRACT

Purpose: Antineoplastic agents often achieve antitumor activity at the expense of close to unacceptable toxicity. One potential avenue to improve therapeutic index might combine agents targeting distinct components of the same growth regulatory pathway. This might lead to more complete modulation of the target pathway at concentrations lower than those associated with limiting adventitious toxicities from either agent alone. The protein kinase antagonist UCN-01 is currently used in Phase I/II trials and has recently been demonstrated to inhibit potently PDK1 (S. Sato et al., Oncogene, 21: 1727–1738, 2002). We have recently documented that the alkylphospholipid perifosine potently also inhibits Akt kinase (PKB) activation by interfering with membrane localization of Akt (S. Kondapaka et al., Mol. Cancer Ther., 2: 1093–1103, 2003). This leads to the hypothesis that these two agents might act synergistically through distinct mechanisms in the PI3K/Akt proliferation and survival-related signaling pathway.

Experimental Design: The synergistic effects of UCN-01 and perifosine, on two cell lines (A-549 and PC-3), were examined using various long-term in vitro assays for cell growth, cell cycle distribution, clonogenicity, survival morphology, and apoptosis. Along with Western blotting experiments were performed to determine whether this synergistic combination of two drugs has significant effect on their downstream targets and on biochemical markers of apoptosis.

Results: After 72 h, perifosine at concentrations of 1.5 and 10 μM UCN-01 at 40 and 250 nM did not significantly affect the growth of PC-3 and A459 cells, respectively. However, in combination at the same respective individual concentrations (1.5 μM and 40 nM of perifosine and UCN-01, respectively, in PC-3 cells and 10 μM perifosine and 0.25 μM UCN-01 in the somewhat more resistant A549 cells), virtually complete growth inhibition of both the cell lines resulted. Supra-additive inhibition of growth was also demonstrated in independent clonogenic assays. Mechanistic studies in cell culture models suggest enhanced depletion of the S-phase population in cells treated by the combination. This correlated with enhanced inactivation of Akt along with activation of caspases 3 and 9 and poly(ADP-ribose) polymerase cleavage. Evidence of synergy was formally demonstrated and occurred across a wide range of drug concentrations and was largely independent of the order or sequence of drug addition.

Conclusions: As the concentrations of UCN-01 and perifosine causing synergistic inhibition of cell growth are clinically achievable without prominent toxicity, these data support the development of clinical studies with this combination.

INTRODUCTION

Alkylphospholipids have been synthesized with the goal of modifying membrane-related signal transduction events. Hexadecylphosphocholine (miltefosine; Miltelex), one of the first of this class to undergo clinical evaluation, had evidence of immunomodulatory and antitumor activity in model systems (1–3). Although low oral bioavailability, gastrointestinal, and hemolytic toxicities limited its clinical development as an anticancer agent, it is approved for use in Europe for topical treatment of cutaneous lymphomas and breast cancer metastases (4) and has activity against visceral leishmaniasis despite low oral absorption (5).

Perifosine [octadecyl-(1,1-dimethyl-piperidino-4-yl) phosphate; D-21266; NSC639966] is an analogue of miltefosine with greater oral bioavailability currently undergoing clinical evaluation (6). Alkylphospholipids are known to alter several aspects of cell membrane synthesis and function, including inhibition of phospholipase C, guanine nucleotide-binding protein, protein kinase C (PKC) activity, and phosphatidylcholine synthesis (7).

We recently documented that one consequence of perifosine action is cell cycle arrest with induction of p21WAF1/CIP1 in a p53-independent fashion (8). In seeking to define further the pathway leading to p21WAF1/CIP1 induction, others and we have recently demonstrated that perifosine causes rapid decrease in the phosphorylation of Akt (PKB), with loss of Akt activity (9, 10) and decreased translocation of Akt to the plasma membrane (10).

UCN-01 (7-hydroxyxystaurosporine) is a PK antagonist originally defined as a potent selective inhibitor of PKC isoforms α, β, and γ (11–14). It also causes cell cycle arrest in a way that appears to be independent of effects on PKC, with evidence of antitumor activity in model systems (15, 16). It currently is being evaluated in early-phase clinical trials (17). Additional
cellular targets of UCN-01 include the cell cycle checkpoint kinases chk1 (18, 19) and possibly chk2 (20, 21) with sensitization of treated cells to DNA damage. Recently, Sato et al. (22) documented that an additional target of UCN-01 is the phosphatidylinositol-dependent kinase 1 (PDK1). This finding is of interest because PDK1 phosphorylates Akt Ser^473 and Thr^308 and contributes to the activation of Akt activity after growth factor stimulation of phosphatidylinositol-3'-kinase (PI3K; Refs. 23, 24).

Combining antineoplastic agents has proven to result in several effective regimens in cancer therapy. Current cytotoxic regimens emerged from efforts to match agents with nonoverlapping toxicity to the host. In contrast, combining agents, several effective regimens in cancer therapy. Current cytotoxic agents are from Calbiochem.

Tissue Culture. Cell lines were grown in their respective culture medium as recommended by the American Type Culture Collection. A549 non-small cell lung carcinoma cells are grown in DMEM, and human prostate adenocarcinoma PC-3 cells were maintained in RPMI at 37°C in an atmosphere containing 5% CO_2. In both cases, the media are supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin medium, and 10% fetal bovine serum (Grand Island Biological Co., Grand Island, NY).

In Vitro Cytotoxicity Assay. Tumor cells were cultured in 100-mm Petri dishes with initial cell number so that 40–50% confluence would be achieved after 24 h. These exponentially growing cells were exposed to different concentrations of drug for 24 h. The adherent cells were harvested with trypsin, washed with PBS, and collected by centrifugation at 1500 rpm for 5 min. Trypan blue (0.5%), excluding cells, was enumerated by hemocytometer. CalcuSyn software was obtained from BIO- SOFT, Inc. (Cambridge, MA). Three sets of experiments for each drug combinations were carried out, one with the combination of perifosine and UCN-01 and one set with each drug alone. The proportions of dead cells in treated plates were entered into the software, and the degree of synergy assessed by the combination index (CI) value was computed automatically to indicate the degree of synergy or antagonism according to the algorithms described by Chou and Talalay (25).

Soft Agar Colony Formation Assays. Bilayer soft agar colony formation assays of A-549 and PC-3 cell lines were performed using RPMI 1640 containing 10% fetal bovine serum and 10,000 cells/2 ml culture on day 0, as previously described by Alley et al. (26). For drug sensitivity assays, 0.1 ml of culture medium containing drug (n = 3/each of 8 drug concentrations spanning the effective drug concentration range) and/or drug vehicle (n = 3) were applied to cultures on day 1. Cultures were then incubated until day 7, stained with methylthiazol tetrazolium, stabilized, and clarified with protease enzyme buffer and then analyzed by computerized image analysis. Percentage of vehicle control (V/C) values were calculated for each drug concentration as well as the IC_{50} (mean ± SD).

Cell Cycle Analysis. Cells were cultured in a 100-mm Petri dish and allowed to grow to 75–80% confluency. Then cells are treated with drugs of interest for 24 h and compared with control samples not exposed to drug. After drug exposure, cells were harvested with trypsin, followed by centrifugation at 1000–1500 rpm in a 15-ml tube. Harvested cells were washed twice with 1× PBS and then resuspended and then fixed by adding 4 ml of cold 100% ethanol added in pulses of 1 ml each while vortexing. Samples were stored –20°C for a minimum of 24 h and up to 1 month before analysis. When samples were to be analyzed, these are centrifuged, the ethanol removed, and cell pellets washed twice with 1× PBS and resuspended in 0.5–1 ml of 50 μg/ml propidium iodide (PI) solution in PBS plus 2 μl/ml RNase, and incubated for 1 h at 37°C. Cell cycle distribution was analyzed by flow cytometry and analyzed using FACScaliber (Becton Dickinson Immunocytometry Systems, San Jose, CA), using MODFIT software (Verity Software, Topsham, ME).

Bromodeoxyuridine (BrdUrd) Incorporation. A549 cells were cultured, drug treated, and collected as mentioned above. Before collection, cells were incubated with 1× PBS and then resuspended and then incubated by adding 4 ml of cold 100% ethanol added in pulses of 1 ml each while vortexing. Samples were stored –20°C for a minimum of 24 h and up to 1 month before analysis. When samples were to be analyzed, these are centrifuged, the ethanol removed, and cell pellets washed twice with 1× PBS and resuspended in 0.5–1 ml of 50 μg/ml propidium iodide (PI) solution in PBS plus 2 μl/ml RNase, and incubated for 1 h at 37°C. Cell cycle distribution was analyzed by flow cytometry and analyzed using FACScaliber (Becton Dickinson Immunocytometry Systems, San Jose, CA), using MODFIT software (Verity Software, Topsham, ME).

Materials and Methods. Antibodies and Reagents. Antibodies against total and phosphorylated Akt (Ser^473 and Thr^308), phos-PDK1 (Ser^241) and total PDK, and cleaved caspase-3 and caspase-9, caspase-6, and caspase-8, phos-GSK-3α/β (Ser^21/9 , phos-Chk1 (Ser^317), phos-Chk2 (Thr^68)) are purchased from Cell Signaling Technologies (Beverly, MA). Two other antibodies namely Total Chk1 and Chk2 are purchased from Upstate Biotechnology, (Lake Placid, NY). All cell culture medium and reagents were from Life Technologies, Inc. (Rockville, MD). Perifosine was obtained from Asta Medica and UCN-01 from Kyowa Hakko Kogyo, through the Developmental Therapeutics Program, National Cancer Institute. Unless otherwise noted all other chemicals are from Calbiochem.

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Bromodeoxyuridine (BrdUrd) Incorporation. A549 cells were cultured, drug treated, and collected as mentioned above. Before collection, cells were incubated with 10 μl of BrdUrd (BrdUrd kit; Sparta Labs, Biocarta San Diego, CA) for 2 h.

Following the manufacturer’s instructions, cells were harvested and washed with PBS. Cells were then photolyzed with UVB light for 5 min. Seventy percent ethanol was then used to fix the cells for 1 h. Cells were washed with PBS and incubated at room temperature for 5 min. Subsequently, cells were resuspended in PBS, distilled water, and fluorescence-activated cell sorting buffer (provided with kit). To each sample, 10 μl of anti-BrdUrd FITC and 20 μl of 7-aminocinomycin D DNA staining reagent were added. Lastly, samples were incubated at room temperature and in the dark for 1 h and then analyzed with flow cytometry.

Apoptosis Assays. Cells were stained using an Apo- DETECT Annexin V-FITC kit (Zymed Laboratories, Inc.) fol-
Following the manufacturer's instructions. Briefly, cells were cultured in a 90-mm tissue culture plates (Falcon BD) for the confluency to arrive ~35–40% and then treated with UCN-01 (250 nm), perifosine (10 μm), or a combination of the two for 48 h. A549 cells were released with Trypsin-EDTA 0.25% and were collected and washed with PBS. Following the manufacturer's instruction, cells were stained with Annexin V-FITC and PI. Samples were then processed by flow cytometry. Untreated A549 cells were used as a control. Data acquisition and analysis were done on a BD (Becton Dickinson) FACSCaliber using CellQuest software (BD Biosciences). Annexin V is a calcium dependent phospholipid-binding protein with a high affinity for phosphatidylserine. Phosphatidylserine is normally present in the inner lipid bilayer but becomes exposed on the cell surface within the first few hours of the onset of apoptosis (27). PI is a red DNA-binding dye that can only enter cells whose membranes are disrupted such as in cells undergoing necrosis. Apoptotic cells are stained positive with Annexin V-FITC but are resistant to PI staining. To assess nuclear morphology, A549 cells were grown on a single well-chambered slide (Labtek) and incubated with UCN-01 (250 nm) or perifosine (10 μm) or a combination of the two for 48 h. Protocol was followed as stated in (28). Combination treatment included both drugs given at the same time, one given for 24 h and then the second added and vice versa. Control (nondrug-treated) and drug-treated cells were fixed in 100% cold methanol for 20 min, washed with ice-cold PBS, and then stained for 15 min with 3 μM of Hoechst 33342 dye (Molecular Probes, Eugene, OR). Fluorescent nuclei were visualized in fluorescence microscope (Olympus). Under these conditions, nuclei from living, apoptotic, and necrotic cells could be clearly distinguished. Cells with condensed and deformed nuclei that...
Table 1  Analysis of combined treatment of perifosine and UCN-01 in A549 cells: combination index

<table>
<thead>
<tr>
<th>Perifosine (μM)</th>
<th>UCN-01 (μM)</th>
<th>Simultaneous</th>
<th>Sequential-I</th>
<th>Sequential-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.15</td>
<td>0.62 ± 0.017</td>
<td>0.60 ± 0.035</td>
<td>0.32 ± 0.045</td>
</tr>
<tr>
<td>8</td>
<td>0.20</td>
<td>0.55 ± 0.02</td>
<td>0.62 ± 0.021</td>
<td>0.31 ± 0.025</td>
</tr>
<tr>
<td>10</td>
<td>0.25</td>
<td>0.51 ± 0.013</td>
<td>0.58 ± 0.037</td>
<td>0.34 ± 0.023</td>
</tr>
</tbody>
</table>

NOTE. A constant drug concentration ratio was added to A549 cells (sequential-I–UCN-01 added for 48 h after 24 h of exposure to perifosine; sequential-II–perifosine added for 48 h after 24 h of exposure to UCN-01). The fraction of surviving cells in drug-treated plates is calculated compared with controls. CalcuSyn software is used as described in “Materials and Methods” and the combination index of the different combinations is computed.

Fig. 2  Sequential treatment of perifosine and UCN-01 on growth of A549 cells for 24 h. Log-phase growing cells were grown in plates as described in “Materials and Methods.” Perifosine (5 μM) and an increasing concentration of UCN-01 were added at 50% cell confluence. After 24 h of drug exposure, cells were trypsinized, and viable cells were counted.

showed patches of compact chromatin were considered apoptotic.

Cell Lysis and Immunoblot Analysis. After trypsinization and washing twice with PBS, cells were centrifuged at 1500 rpm and lysed with 50 mM HEPES (pH 7.4), 20 mM EDTA, 0.5 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 1 mM sodium fluoride, 10% glycerol, 0.5% NP40, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM 4-(2-aminoethyl) benzene-sulfonylfluoride. Twenty to 40 μg of total protein were resolved by 4–20% Tris-glycine SDS-PAGE (Novex) gel. The separated proteins were transferred to polyvinylidene difluoride membranes (Immobilon; Millipore Corp.) in a Hoofer transblotter using 25 mA Tris, 192 mM glycine, and 20% methanol. After the transfer is completed (2–3 h), the blots were blocked for an hour in a blocking buffer containing 5% (w/v) boleto (Santa Cruz Biotechnology, Santa Cruz, CA) in TTBS [10 mM Tris-HCl, 140 mM NaCl (pH 7.4), and 1% (v/v) Tween 20]. The membranes were washed three times extensively in TTBS. The blots were then placed in their respective primary antibodies at optimal concentrations for 1 h. After three washes with TTBS, the horseradish peroxidase-conjugated specific secondary antibodies were added and additionally incubated for 1 h in presence of 5% (w/v) blotto in TTBS. The membranes were washed extensively in TTBS, and detection was performed with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

RESULTS

Growth Inhibition by Perifosine and UCN-01. The effect of perifosine and UCN-01 as single agents on the growth of the A549 and PC-3 cells was first assessed after 24 and 72 h. Perifosine inhibits A549 cell growth with 50% growth inhibition (IC50) of 40 and 10 μM (Fig. 1A) for 24 and 72 h exposures, respectively. Treatment with UCN-01 causes growth inhibition with an IC50 of 2 and 0.25 μM (Fig. 1B) for the same intervals. In PC-3 cells, the IC50 for growth of perifosine is 1.2 μM after 72 h (Fig. 1C), whereas after 24 h the IC50 is 5 μM [data not shown (10)]. UCN-01 has an IC50 for growth of 100 and 40 nM for PC-3 cells in 24 and 72 h, respectively (Fig. 1D).

Combination of Perifosine and UCN-01 on the Growth of A549 Cells. To evaluate potential synergy, we added combinations of perifosine and UCN-01 at constant ratios with respect to their respective IC50 concentrations. Both drugs were added simultaneously and in separate experiments with one drug preceding the other by 6–8 h. We evaluated synergy using CalcuSyn software to evaluate the combination index originally described by Chou and Talalay (29, 30). Combination index < 1 is evidence for synergy, whereas combination index > 1 is evidence of antagonism, and combination index = 1 indicates simple additivity of drug effect. Both simultaneous as well as

Table 2  Analysis of combined treatment of perifosine and UCN-01 in A549 and PC-3 cells; combination index

<table>
<thead>
<tr>
<th>Perifosine (μM)</th>
<th>UCN-01 (μM)</th>
<th>Combination index</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>2.0</td>
<td>0.05</td>
<td>0.58 ± 0.025</td>
</tr>
<tr>
<td>5.0</td>
<td>0.05</td>
<td>0.40 ± 0.038</td>
</tr>
<tr>
<td>8.0</td>
<td>0.05</td>
<td>0.41 ± 0.031</td>
</tr>
<tr>
<td>10.0</td>
<td>0.05</td>
<td>0.42 ± 0.048</td>
</tr>
<tr>
<td>B</td>
<td>2.0</td>
<td>0.05</td>
</tr>
<tr>
<td>2.0</td>
<td>0.1</td>
<td>0.52 ± 0.045</td>
</tr>
<tr>
<td>2.0</td>
<td>0.15</td>
<td>0.45 ± 0.021</td>
</tr>
<tr>
<td>2.0</td>
<td>0.20</td>
<td>0.49 ± 0.029</td>
</tr>
<tr>
<td>2.0</td>
<td>0.25</td>
<td>0.50 ± 0.037</td>
</tr>
<tr>
<td>C</td>
<td>1.2</td>
<td>0.04</td>
</tr>
<tr>
<td>0.9</td>
<td>0.03</td>
<td>0.52 ± 0.035</td>
</tr>
<tr>
<td>0.6</td>
<td>0.02</td>
<td>0.50 ± 0.025</td>
</tr>
<tr>
<td>0.3</td>
<td>0.01</td>
<td>0.42 ± 0.028</td>
</tr>
</tbody>
</table>

NOTE. A, a nonconstant drug concentration ratio was added in sequential treatment schedule (perifosine added for 48 h followed by UCN-01 after 24 h in A549 cells); B, a nonconstant drug ratio and sequential treatment schedule (UCN-01 added for 48 h followed by perifosine after 24 h in A549 cells); C, constant drug ratio and sequential treatment schedule (UCN-01 is added after 24 h of exposure to perifosine in PC-3 cells). The fraction of cell survival in drug-treated plates is calculated with respect to control, and CalcuSyn software was run with relevant data as per “Materials and Methods,” and the combination index of the different combinations is computed.
Synergistic Effect of Perifosine and UCN-01 in the IC50 concentration have been observed. However, the sequential addition of UCN-01 followed by perifosine shows a trend toward improved synergy compared with the reverse sequence of addition or simultaneous exposure to the drugs.

We have also evaluated the combination indexes for combined treatment with UCN-01 and perifosine with a nonconstant drug ratio. In this design, the concentration of one drug is kept constant at its IC10 concentration, and the concentration of other drug is then added in successive increments (i.e., in a nonconstant drug ratio). Table 2, A and B, shows that the combination index, when drugs are added in sequence (perifosine followed by UCN-01 or in reverse sequence), also suggests a synergistic effect of similar magnitude to that seen in the experiments with a constant drug ratio. An example of one result, with perifosine at its IC10 (5.0 μM), combined with varying concentrations of UCN-01 (Fig. 2), demonstrates a 3.5-fold decrease of IC50 for UCN-01 when added in presence of 5.0 μM perifosine. A similar series of experiments, keeping UCN-01 concentration constant at IC10 value (40 nM) with variable concentrations of perifosine has been carried out (data not shown) and comparable decreases in the IC50 concentration have been observed.

As perifosine and UCN-01 consistently demonstrated evidence of synergy in the A549 lung carcinoma cells [wild-type p53 and intact PTEN gene (31)], we extended a similar set of analyses to PC-3 cells which have mutated p53 (32, 33) and deletion of the PTEN gene product (34) and therefore constitutive activation of the PI3K/Akt pathway. Table 2C indicates that similar to A549 cells, PC-3 cells exhibit considerable synergistic effect to both simultaneous, as well as sequential treatment of perifosine and UCN-01, because in all such cases combination index < 1 is obtained and very often lies between 0.4 and 0.5.

To assess by an independent assay the potential value of combined UCN-01 and perifosine treatment, clonogenic unit assays were undertaken. Fig. 3 demonstrates that in both A549 cells (p53 and PTEN both wild type) and PC-3 cells (p53 and PTEN mutant), perifosine at clinically achievable concentrations of 1–10 μM (A549 cells) or 30–300 nM (PC-3 cells) in the presence of 3–30 nM (A549 cells) or 15–45 nM (PC-3 cells) UCN-01, respectively, shows clear evidence of supra-additive diminution of clonogenic potential.

**Effect of Combined Perifosine and UCN-01 on Cell Cycle Progression.** To begin to understand the basis for synergistic inhibition of cell growth on the part of UCN-01 and perifosine, we studied the effect of the two agents alone (10 μM perifosine and 250 nM UCN-01) or in combination on cell cycle progression. In A549 cells (Fig. 4A) in untreated controls, cells are present in G1 (74%), G2 (9.4%), and S (16.6%) phases. Concordant with the growth inhibition data, A549 cells are minimally perturbed by either 10 μM perifosine or 250 nM UCN-01, whereas the combination causes a consistent increase in G2-M (to 18.5%) along with virtual loss of the S-phase (1.3%) fraction. Similarly, cell cycle analysis of the PC-3 cells (Fig. 4B) indicates that individual treatment of either perifosine or UCN-01 (at 1.2 and 0.04 μM, respectively) has a marginal effect, whereas combined treatment of perifosine and UCN-01 leads also to virtual loss of the S-phase population and retention of G1 and G2 populations for the duration of this experiment. Independent analyses where A549 were plated at lower initial density so that the S-phase fraction of cell cultures was 27.5% at the time of initial exposure to drugs revealed little change after combined exposure to 250 nM UCN-01 and 10 μM perifosine, we studied the effect of the two agents alone (10 μM perifosine [31.6 nM (A549 cells) or 150 nM (PC-3 cells)] and in combination with 250 nM UCN-01, but marked suppression of S-phase fraction (1 to 4%) after combined exposure to 250 nM UCN-01 and 10 μM perifosine (data not shown).

To demonstrate further the capacity of perifosine and UCN-01 to suppress S-phase progression, assessment of BrdUrd incorporation was undertaken. Fig. 5 demonstrates that while 10 μM and 250 nM perifosine and UCN-01, respectively, have little effect as single agents to suppress BrdUrd incorporation, both
agents together or in either sequence of addition markedly suppress BrdUrd incorporation. No significant difference in the type or pattern of cell cycle arrest is observed when combined treatment occurs with simultaneous or sequential drug addition (data not shown). On the basis of these data, we chose concentrations of perifosine (10 μM) and UCN-01 (250 nM) for use in A549 cells to additionally characterize the biochemical correlates of the combined actions of these anticancer agents.

Effect on Akt on Combination Treatment of Perifosine and UCN-01. Fig. 6A indicates that exposure of A549 cells individually to perifosine (10 μM) or UCN-01 (250 nM) has no
effect at all on the phosphorylation state of Akt at either Ser473 or Thr308. On the other hand, complete dephosphorylation of Akt is observed at both sites when the cells are exposed to the combination of 10 μM perifosine and 250 nM UCN-01 for 24 h when both the drugs are added simultaneously. Similar results are obtained with sequential drug treatment where UCN-01 is added for 18 h followed by perifosine for 6 h of treatment. The level of total Akt remains equivalent to control cells, which suggests that perifosine and UCN-01 cause reduced phosphorylation of Akt without evidence for an effect on the turnover of total Akt.

If Akt dephosphorylation is functionally significant, the phosphorylation or activity of downstream Akt targets such as glycogen synthase kinase (GSK-3) should be diminished. Indeed, we observe considerably less phosphorylation of GSK-3α/β when cells are treated with the combination of perifosine and UCN-01 in comparison to either treatment alone (Fig. 6B). Of interest, Fig. 6C shows that P-Chk1 (Ser345), which occurs in relation to replication fork movement (35), shows no diminution of phosphorylation as result of combined UCN-01 and perifosine exposure, in contrast to Akt and GSK-3 isoforms, arguing against a nonspecific effect on phospho-protein status in general.

**Combined Perifosine and UCN-01: Effect on Cell Death Pathways.** As the Akt pathway is prominently known to affect susceptibility to apoptosis, we assessed whether combined exposure to perifosine and UCN-01 resulted in activation of cell death mechanisms. Fig. 7B demonstrates that A549 cells exposed to 10 μM perifosine or 250 nM UCN-01 show minimal changes in nuclear chromatin condensation yet combined exposure or sequential addition of the two agents together causes abundant alteration of nuclear morphology concordant with initiation of apoptosis. Fig. 7A quantifies the increase in cellular fraction labeling with Annexin V, which increases from 2.5 and 3.0% in the presence of perifosine and UCN-01, respectively, to 34% with combined exposure. Fig. 7C indicates that either perifosine (10 μM) or UCN-01 (250 nM) alone each has no effect on cleaved caspase-3 and caspase-9 production, whereas the combination of both either simultaneously or sequentially at these concentrations causes pronounced cleavage of caspase-3 and caspase-9 after 48 h, suggesting that enhanced cell death by the combination occurs and is mediated through activation of caspase-3 and caspase-9 pathway. In addition, Fig. 7C indicated that no activation of caspase-6 and caspase-8, which suggests that cell death through combination drug treatment occurs predominantly through caspase-3- and caspase-9-related pathways.
As expected, combined exposure also augments poly(ADP-ribose) polymerase cleavage and release of Apaf1, indicating mitochondrial damage, diminution of bcl2 and bclX L, and marked increase in bax expression. Fig. 7D indicates that no significant difference on the expression of cell cycle inhibitor proteins such as p27 and p57 Kip1 proteins but, in relation to p21Cip1/Kip1, a modest change is observed when combined treatment occurs with simultaneous or sequential drug addition.

**DISCUSSION**

In this study, we have shown in cell cultures in vitro as well as soft agar colony formation assays that the combination of UCN-01 and perifosine displays substantial synergy in cell growth inhibition of both the p53 wild-type, PTEN wild-type A549 lung carcinoma, and the p53 mutant, PTEN-negative PC-3 prostate carcinoma cell lines. This occurs over a wide range of drug concentration ratios and appears to be relatively independent of the order or sequence of drug addition, although there is a tendency for greater combination effect when UCN-01 preceded perifosine in certain cases. The synergy is manifested in decreased S-phase fraction of treated cultures, loss of Akt activation, and induction of indicators of apoptosis. We therefore propose that additional exploration of this combination in clinical trials is warranted.

Previous studies had shown that PDK1 is a target very sensitive to UCN-01, with subsequent loss of Akt activation and activity (22). Likewise, our recent studies have identified perifosine as a potent inhibitor of Akt activation without a discernible effect on PDK1 intrinsic activity but with a pronounced effect on the recruitment of Akt to the cell membrane (10). Thus, UCN-01 and perifosine could each affect different steps in the pathway, leading to full activation of Akt. Our studies here demonstrate that at very low concentrations of either drug, essentially at concentrations where as single agents they inhibit growth by no more than 10–20%, there is marked interaction in affecting biochemical correlates of Akt activation. These include Akt Thr308 and Ser 473 phosphorylations and Akt activity as measured by phosphorylation of GSK. These combined effects of perifosine and UCN-01 plausibly could contribute to pronounced cell cycle arrest and evidence for induction of apoptosis.

In the experiments studied here, the range of effective concentrations in combinations demonstrating pronounced biochemical effect was 40 nM and 1.5 μM UCN-01 and perifosine, respectively, in PC-3 cells and up to 0.25 and 10 μM, respectively, in the somewhat more resistant A549 cells. Early clinical studies have demonstrated that free UCN-01 not bound to α1-acid glycoprotein ranged between 50 and 400 nM, with total concentrations of as high as 24 μM at well-tolerated dose levels (36, 37). Likewise, perifosine achieves concentrations of 10–18 μM at well-tolerated doses (38). Thus, the concentrations studied here are clearly within clinically achievable range. Moreover, the relative schedule independence conveys confidence that the relatively prolonged elimination half-lives observed in humans for both agents would actually mimic the conditions used in cell culture here. The side effect profiles of the two drugs as single agents are also distinct. At the respective recommended Phase II doses, prominent effects for UCN-01 include headache, low-grade nausea, and a tendency to hyperglycemia (37). Likewise, for perifosine, gastrointestinal toxicities controlled by antiemetics appear to predominate (38). No other serious end-organ toxicities have emerged.

Although in general it is desirable where possible to obtain evidence of synergistic activity in animal models to extend such
Fig. 7  Combined treatment of perifosine and UCN-01 on the effect of apoptosis on A549 cells. Apoptosis measurement through Annexin V staining (A), nuclear morphological evidence (B), effect on biochemical markers of apoptosis (C), effect on the expression of cell-cycle inhibitor proteins (D). A, cells were treated with UCN-01 (250 nM), perifosine (10 μM), or a combination (sequential) of the two with the former given first and the latter given 24 h later, vice versa, or at the same time (simultaneous) for total of 48 h. The number of apoptotic cells was quantified by FACSCaliber flow cytometry using the CellQuest software after the cells were stained with Annexin V and PI. The chart diagram represents the Annexin V-positive and propidium iodide-negative cells, thus accounting for the percentage of apoptotic cells within the population. B, nuclear morphological evidence of apoptosis was demonstrated by using Hoechst 33342 staining after treatment with 10 μM perifosine, 250 nM UCN-01, or a combination of 10 μM perifosine and 250 nM UCN-01 (simultaneous) for 48 h. Other two sequential situations were 10 μM perifosine for 24 h first, followed by 250 nM UCN-01 for another 24 h or vice versa order, i.e., 250 nM UCN-01 first for 24 h followed by 10 μM perifosine for another additional 24 h as described under “Materials and Methods.” Prominent condensed and deformed nuclei of apoptotic cells are evident on either simultaneous and or sequential treatment of both the compounds. C and D, exponentially growing A549 cells in a 100-mm Petri dish were treated with perifosine (10 μM) and UCN-01 (250 nM) for 24 h as indicated in the figure. After the completion of treatment, cells were lysed, equal amounts of protein were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, probed with respective antibodies as indicated in the figure, and as described in “Materials and Methods.”
in vitro results, that goal is problematic in the case of UCN-01 and perifosine because the murine pharmacology of UCN-01 differs considerably from the human because of species-dependent binding to plasma proteins (36, 39). Thus, animal model data of this type would be of uncertain value in reliably modeling the anticipated experience in humans.

There is emerging evidence that several components of the PI3K/Akt/PTEN pathway are involved in oncosogenesis (40, 41). Increased activity of the pathway either through amplification or overexpression of PI3K and Akt, activation of growth factor receptor activity activating PI3K, or loss of PTEN activity have been described in many malignancies (42–45). So, the PI3K/Akt/PTEN pathway is an attractive target for drug development as agents directed at the pathway might inhibit cell proliferation and reverse antiapoptosis pathways conveying resistance to cytotoxic therapy in cancer cells. In this regard, UCN-01 shows some promise in inhibiting PDK1 that causes Akt activation (22). Perifosine also causes Akt inactivation most likely by inhibiting its translocation to the plasma membrane (10) where Akt generally is activated by its PDK1 and PDK2 or other upstream kinases. It has been difficult to define clinically useful drugs to interdict this pathway. Wortmannin and derivatives have been extensively considered as inhibitors of PI3K itself. However, toxicity and specificity concerns have precluded extensive development. Although specific inhibitors of PI3K, PDK1, and Akt have not yet reached the clinic, the rapamycin derivatives CCI-777 and RAD001, which inhibit the additional downstream of Akt, mammalian target of rapamycin, are undergoing clinical evaluation but might be viewed as relatively limited in their influence to the more downstream targets of pathway action. Thus, combined UCN-01 and perifosine may be considered a novel approach to down-modulating PI3K/Akt pathway activation in a way that may obviate concerns with the other currently available strategies. In the event that this combination does receive a clinical test, the end points used here (Akt phosphorylation epitopes, GSK activation) might be of value in assessing PI3K/Akt/PTEN pathway activation in surrogate or tumor cells.

Recently, considerable attention has focused on the use of signal transduction modulators together to enhance the lethal effects of cytotoxic agents, building on the groundbreaking observations of enhancement of Herceptin action when in combination with cytotoxic agents (46–48). In this instance, described here, we provide evidence for the potential value of combining two antisignaling agents. Other combinations of signaling agents have also been recently defined where also the opportunity for enhanced benefit can be discerned. For example, UCN-01 itself has been reported recently to enhance the action of 17-allylamino, 17-demethoxygeldanamycin (49), and of the pharmacological mitogen-activated protein kinase kinase inhibitors (e.g., PD98059 or U0126) in human leukemia (50). Flavopiridol interacts synergistically with imatinib in the inhibition of resistant chronic myelogenous leukemia cell growth (51). All of these efforts are seeking to define a new approach to combination therapy for cancer where the basis for clinical enthusiasm relates not only to acceptable toxicity characteristics of the single agents but where the components of a combination complement each other by modulating distinct steps in a single pathway or in affecting distinct processes important in the development of the neoplasm, e.g., antiapoptorative agents combined with antiangiogenic drugs.

In conclusion, taken together, our results demonstrate that the combined effect of perifosine and UCN-01 clearly results in more greatly enhanced antiproliferative action than is seen with either acting singly and that this occurs coincident with enhanced capacity of the combination to down-regulate Akt signaling. As described here, perifosine and UCN-01 share effects on Akt-mediated signaling that point to their combination as biologically plausible by acting at least partly through effects on different aspects of the same proliferation and survival-related signaling pathway and whose human pharmacological features support the feasibility of a strategy amenable to a relatively straightforward clinical test.

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In vitro Combination Treatment with Perifosine and UCN-01 Demonstrates Synergism against Prostate (PC-3) and Lung (A549) Epithelial Adenocarcinoma Cell Lines

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