Cell Cycle Arrest and Growth Inhibition by the Protein Kinase Antagonist UCN-01 in Human Breast Carcinoma Cells

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ABSTRACT

UCN-01 is a derivative of staurosporine, initially developed as a potentially selective inhibitor of the Ca\(^{2+}\)- and phospholipid-dependent protein kinase C, but with the capacity to inhibit a number of tyrosine and serine/threonine kinases. UCN-01 inhibits the growth of 5 breast carcinoma cell lines with a 50% inhibitory concentration range of 30–100 nM during 6 days of continuous exposure. In MCF-7, MDA-MB453, and SK-BR-3 cells, UCN-01 is 5-fold more potent in growth inhibition than its diastereomer UCN-02, but the 2 compounds are equipotent in the inhibition of MDA-MB468 and H85787 cell growth. A differential sensitivity to a 24-h period of exposure to UCN-01 followed by drug removal and growth for 5 subsequent days was observed. The rank order for persistent inhibition of cells by UCN-01 was MCF-7, MDA-MB453 > SK-BR-3 > H85787 > MDA-MB468. MCF-7 and MDA-MB453 cells did not resume proliferation within the 5 days after brief exposure to UCN-01. In contrast, MDA-MB468 and H85787 cells showed no net growth inhibition after a 24-h pulse of UCN-01, followed by 5 more days of growth in drug-free medium. In MDA-MB468 cells, 150 nM UCN-01 retards but does not prevent cell cycle progression through S phase, but the cells are clearly blocked from exit of G\(_1\) and entry into S. Progression through S phase is completely inhibited by 600 nM UCN-01. The development of a G\(_1\) to S block by UCN-01 in MDA-MB468 cells occurs in conjunction with inhibition of \(^{32}\)P|orthophosphate labeling and decreased phosphotyrosine mass of discrete cellular phosphoproteins.

INTRODUCTION

Protein kinases are critical mediators of signal transduction from stimulated growth factor receptors that lead to cell proliferation. Clinical and experimental studies suggest the importance of protein kinase expression in the growth of human breast carcinoma (1, 2). For example, an adverse prognosis has been described for breast cancer patients whose breast carcinoma cells express the epidermal growth factor receptor and HER-2/new oncogene product, both protein tyrosine kinases (3, 4). A potential regulatory role also exists for the serine/threonine kinase PKC\(\alpha\) in breast cancer growth. Elevated PKC activity has been found in breast tumor biopsies compared to normal breast tissue (5). Furthermore, the growth-inhibitory effect of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate in breast carcinoma cells (6, 7) is closely related to proteolysis of the PKC enzyme. Resumption of cell growth in these cells after removal of 12-O-tetradecanoylphorbol-13-acetate is coincident with a gradual up-regulation of PKC (6). A positive correlation has been observed between the number of epidermal growth factor receptors and the level of PKC activity (8), whereas an inverse correlation of PKC activity with the number of epidermal growth factor receptors that lead to cell proliferation. Clin...
exposure to nocodazole (0.4 µg/ml), a reversible inhibitor of mitotic spindle formation, for 12 h (18). After synchronization, the cells were washed drug-free, transferred to fresh growth medium with or without UCN-01 as indicated in the Figure legends, and further incubated until assay. At indicated times, nuclei were isolated as described previously (19). DNA content was measured using a FACScan flow cytometer calibrated with Autocomp beads and stained chicken RBC. Data acquisition and analysis were carried out using CellFIT software (Becton-Dickinson Immunocytometry Systems). Log fluorescence (FL2) height, area, and width as well as forward angle and side scatter were collected for 10,000 events. Duplicate ungated data were collected for all samples. Control G0/G1 DNA content was obtained using normal human lymphocytes. The coefficient of variation for lymphocytes averaged 2%, the Go/G1 coefficient of variation of the samples averaged 4.9%. Flow-cytometric data were analyzed using Multicycle (Phoenix Flow Systems).

**SDS/PAGE Electrophoresis and Immunoblot Analysis.** Cellular proteins of nocodazole-synchronized MDA-MB468 cells were labeled with 1.0 mCi [32P]orthophosphate/100-mm dish for the last 90 min before harvesting. Cells were then washed twice with phosphate-buffered saline, pH 7.2, and scraped into 0.6 ml of a 20 mM sodium phosphate (pH 7.0) lysis buffer (1% Triton X-100, 12 mM sodium deoxycholate, 100 mM NaCl, 3.8 mM NaN3, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin, 20 µg/ml leupeptin, 1 mM sodium vanadate) and centrifuged (10,000 × g, 15 min). The same protein load for each sample (50 µg) was boiled for 5 min in SDS-PAGE sample buffer and subjected to SDS-PAGE, followed by transfer of the resolved proteins to polyvinylidene difluoride (20, 21).

For phosphotyrosine immunoblotting, membranes were blocked in a buffer of 20 mM Tris, pH 7.4, 0.05% Tween 20, 0.9% NaCl, 0.01% NaN3, 3% bovine serum albumin for 3 h. Blots were probed with a monoclonal mouse anti-phosphotyrosine antibody, which was detected by rabbit anti-mouse IgG antibodies followed by 125I-labeled protein A.

### RESULTS

**Effect of UCN-01 and UCN-02 on Cell Growth.** Continuous exposure to UCN-01 for 5 to 6 days potently inhibits breast carcinoma cell growth (IC50 = 22–100 nM). Table 1 shows the IC50s for growth inhibition by UCN-01, its diastereomer UCN-02, and the ratio of the respective IC50 values for the different breast carcinoma cell lines. Both MDA-MB468 and H85787 cells are equally sensitive to UCN-01 and UCN-02. In contrast, SK-BR-3, MDA-MB453, and MCF-7 cells show greater sensitivity to growth inhibition by UCN-01 compared to UCN-02. The concentration dependence of UCN-01 cell growth inhibition of MDA-MB468 or MCF-7 cells after 1-, 3-, or 6-day exposure with a 5-, 3-, or 0-day recovery period, respectively, is shown in Fig. 1. Also included in Fig. 1 for comparison are the same experiments performed with UCN-02. It can be seen in Fig. 1A that after 24-h exposure to UCN-01, MDA-MB468 cells show little growth inhibition after removal of drug, even up to 400 nM. In contrast, 24-h exposure to UCN-01 at the IC50 is enough to suppress growth of MCF-7 cells during the subsequent 5-day period of observation (Fig. 1C). Only after exposure of MDA-MB468 cells for 72 h to UCN-01...
was persistent growth inhibition observed. UCN-02 displays qualitatively similar behavior, but at lower potency (Fig. 1, B and D).

**Minimum Exposure Time for Growth Inhibition.** The kinetic features for recovery from growth inhibition by MCF-7 and MDA-MB468 (Fig. 1) suggest important differences in the capacity of different cell lines to recover from brief exposure to UCN-01. Fig. 2A describes the experimental design to examine this point in detail. One group of cells was exposed to different concentrations of UCN-01 continuously for 6 days until MTT assay. Other groups, at 1, 2, or 3 days, had drug-containing medium replaced with drug-free medium for the remaining time until MTT assay on day 6. The extent of growth inhibition at the IC_{50} for those cells continuously exposed to UCN-01 (6 days) is compared with the growth inhibition of cells exposed to the same UCN-01 concentration for shorter periods of time (Fig. 2B). It can be seen that MCF-7 and MDA-MB453 cells are inhibited to a similar extent, regardless of whether they are exposed to UCN-01 for only 1 or 6 days. In contrast, MDA-MB468 and H85787 cells show a large reduction in growth inhibition when the period of exposure to UCN-01 is reduced. For example, after a 2-day exposure to UCN-01, less than 20% inhibition of H85787 growth is detected when assayed on day 6. SK-BR-3 recovers growth better than MCF-7 and MDA-MB453 cells, but to a lesser extent than either H85787 or MDA-MB468. Thus, the response of breast carcinoma cell lines to brief exposure to the protein kinase antagonist UCN-01 is heterogeneous. In some cases (e.g., MCF-7 and MDA-MB453), brief exposure appears to inhibit irreversibly cell growth for the period observed. In other cases (e.g., MDA-MB468), prolonged and perhaps continuous exposure to UCN-01 is necessary to effect persistent growth inhibition.

**UCN-01 Delays MDA-MB468 Progression through Cell Cycle.** To characterize the mechanism for the reversible inhibition of MDA-MB468 breast carcinoma cell growth by UCN-01, additional studies focused on these cells. Initial experiments indicated that although a 24-h exposure of MDA-MB468 cells to UCN-01 does not affect the net growth over a subsequent 5-day growth period (Figs. 1 and 2), \(^{[3H]}\)thymidine, \(^{[3H]}\)uridine, and \(^{[3H]}\)leucine incorporation are inhibited within 24 h, in that order of sensitivity (Fig. 3A). Using a sub-maximal concentration of UCN-01, the inhibition of \(^{[3H]}\)thymidine incorporation develops gradually over 12 h, after an initial lag period of 2-3 h (Fig. 3B). The continued incorporation of \(^{[3H]}\)thymidine for this initial 2-3-h period suggests that UCN-01 might affect entry into or exit from S phase. This point was examined in greater detail with cells that were synchronized in early S phase by exposure to aphidicolin.
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MDA-MB468 cells released into drug-free medium from aphidicolin block have largely progressed through S phase by 6 to 8 h (Fig. 4). In contrast, UCN-01 (150 nm)-treated cells are delayed in progression through S phase. This inhibition is not absolute, inasmuch as a fraction of the population does appear to divide between 8 and 10 h after release from aphidicolin block. At a higher concentration, UCN-01 (600 nm) clearly inhibits progression through S phase (Fig. 4). These results indicate a concentration-dependent effect of UCN-01 to either delay or inhibit progression through S phase but not completion of M phase. This was further examined using cells synchronized in M phase with nocodazole. Following release from nocodazole block, both UCN-01 (150 nm)-treated and control cells complete mitosis, but although control cells have entered S phase by 12–14 h, the UCN-01-treated cells do not enter S phase (Fig. 5).

Overall, these results indicate that in MDA-MB468 cells, UCN-01 can block entry into S (Fig. 5) and progression through S phase (Fig. 4). Block of entry into S phase is apparent at a lower drug concentration than is inhibition of S phase progression. Completion of mitosis does not appear to be affected by the drug (Fig. 5).

Effect of UCN-01 on Protein Phosphorylation. Inasmuch as UCN-01 is a protein kinase antagonist (12, 14), determination of its effect on protein phosphorylation may provide information about the mechanism of its growth-inhibitory action and cell cycle effects. In MDA-MB468 cells released from nocodazole block into UCN-01 containing medium, there is clear reduction in [32P]orthophosphate labeling of 2 proteins (M, 16,000 and 38,000), and virtual abolition of [32P]-labeling of a M, 33,300 protein (Fig. 6). The phosphotyrosine Western blot of lysates from nocodazole-synchronized cells (Fig. 7) shows decreased phosphotyrosine mass in 4 proteins (approximately M, 33,000, 57,000, 83,000, and 175,000 species) in MDA-MB468 cells exposed to UCN-01 compared to control cells. The reduction of phosphotyrosine content in these proteins is observed at all time points. Phosphotyrosine in the M, 83,000 species is detected in control cells at 10 and 12 h after release from nocodazole block, which corresponds with transition into S phase, whereas the signal is lacking in treated cells (Fig. 7). The tyrosine phosphorylation of the M, 175,000 species is most evidently decreased at 10 and 12 h after release from nocodazole block.
Thus, UCN-01, an inhibitor of protein kinases, caused decreased $[^{32}\text{P}]$orthophosphate labeling of at least 3 discernable protein species and decreased tyrosine phosphorylation of at least 4 proteins as arrest in G1 becomes apparent (Fig. 5).

DISCUSSION

Here we have demonstrated that UCN-01 acts to inhibit potently breast carcinoma cell growth and that the minimum time of drug exposure required for irreversible growth inhibition is particular to each cell line. In MDA-MB468, a cell line that does not appear to be adversely affected by a 24-h pulse exposure to UCN-01, we define each cell line. In MDA-MB468, a cell line that does not appear to be

UCN-01 inhibits cell cycle progression in a manner distinctive from other protein kinase antagonists. For example, staurosporine (2.2 nm) is capable of blocking cell cycle progression at G1 in nontransformed cells but not in transformed cells (22). At higher concentrations, staurosporine (110–220 nm) blocks both nontransformed and transformed cells in G2 (22, 23). Two other staurosporane analogues, K-252a and RK-286C, isolated from the culture broth of Nocardiosis sp. and Streptomyces sp., respectively, cause inhibition in G2, without inhibition of DNA synthesis leading to polyploid cells (24). In MDA-MB468 cells, UCN-01 blocks cell cycle progression most potently in G1 (Fig. 6), similar to the effect of staurosporine in nontransformed cells. However, at slightly higher concentrations, UCN-01 inhibits cell cycle progression clearly within S phase (Fig. 5) in marked distinction to other staurosporine-like protein kinase inhibitors described to date. These observations raise the possibility of a causal relationship between the protein species with diminished phosphorylation after exposure to UCN-01 (Fig. 6) and cell cycle block by UCN-01. Several protein species in MDA-MB468 cells have decreased incorporation of $[^{32}\text{P}]$orthophosphate after exposure to UCN-01. Whether these proteins are PKC substrates, and the relationship of these species with the several recently described regulators of cell cycle progression including p34$^\text{cdc2}$ (25) and cdk-like kinases (26) remain to be defined. Most notable is the decrease of phosphorylation in the $M$, 33,500 protein species, and the inhibition of tyrosine phosphorylation in the approximately $M$, 83,000 protein at a time when the transition from G1 to S is occurring in these cells.

It has become clear that specificity of protein kinase antagonists for one particular kinase has been difficult to achieve. K252a has claimed selectivity for diverse kinases such as the trk tyrosine kinases (27), Ca$^{2+}$/calmodulin-dependent kinase (28), phosphorylase kinase (29), and myosin light chain kinase (30). Yet it also has demonstrated nanomolar potency for inhibition of myosin light chain-, PKC-, PKA-, and cGMP-dependent kinase (IC$^{50}$ 20, 25, 18, and 20 nm, respectively) (31). In considering protein kinase antagonists as inhibitors of cell growth, inhibition of a single kinase entity is potentially not the explanation for inhibition of cell growth. However, our data clearly indicate selective inhibition of $[^{32}\text{P}]$orthophosphate labeling or decreased phosphoryrosine mass in discrete cellular proteins.

In considering the development of UCN-01 as a potential therapeutic agent, our demonstration that brief exposure to the drug may not be effective in persistently inhibiting cell growth is of interest (Figs. 1 and 2). Nonetheless, cell lines exist (MCF-7, MDA-MB453) in which a relatively brief exposure to drug, achievable by a 24-h infusion, may have persistent growth-inhibitory effect. To maximize the in vivo activity of this compound already observed in some models (13), protracted exposure of the tumor model to drug and specific dosing schedules to ensure maintenance of minimal plasma levels should be sought. Consideration could be given to combinations of UCN-01 with other agents active in either S or G2 phase of the cell cycle. The basis for different minimal exposure times required for persistent inhibition of breast carcinoma cell growth (Figs. 1 and 2) is of interest and clearly needs further investigation, particularly with respect to protein kinase(s) activity or kinase cascades that might distinguish relatively sensitive cells (e.g., MCF-7) compared to more resistant cells (e.g., MDA-MB468).

REFERENCES

growth factor-, insulin-, and serum-induced mitogenesis with stimulation of phos-


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