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UCN-01-mediated G1 arrest in normal but not tumor breast cells is pRb-dependent and p53-independent

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In this study we investigated the growth inhibitory effects of UCN-01 in several normal and tumor-derived human breast epithelial cells. We found that while normal mammary epithelial cells were very sensitive to UCN-01 with an IC_{50} of 10 nM, tumor cells displayed little to no inhibition of growth with any measurable IC_{50} at low UCN-01 concentrations (i.e. 0–80 nM). The UCN-01 treated normal cells arrested in G1 phase and displayed decreased expression of most key cell cycle regulators examined, resulting in inhibition of CDK2 activity due to increased binding of p27 to CDK2. Tumor cells on the other hand displayed no change in any cell cycle distribution or expression of cell cycle regulators. Examination of E6- and E7-derived strains of normal cells revealed that pRb and not p53 function is essential for UCN-01-mediated G1 arrest. Lastly, treatment of normal and tumor cells with high doses of UCN-01 (i.e. 300 nM) revealed a necessary role for a functional G1 checkpoint in mediating growth arrest. Normal cells, which have a functional G1 checkpoint, always arrest in G1 even at very high concentrations of UCN-01. Tumor cells on the other hand have a defective G1 checkpoint and only arrest in S phase with high concentrations of UCN-01. The effect of UCN-01 on the cell cycle is thus quite different from staurosporine, a structural analogue of UCN-01, which arrests normal cells in both G1 and G2, while tumor cells arrest only in the G2 phase of the cell cycle. Our results show the different sensitivity to UCN-01 of normal compared to tumor cells is dependent on a functional pRb and a regulated G1 checkpoint.

Keywords: UCN-01; cell cycle; p53; pRb; E6; E7

Introduction

Protein kinases are essential for cellular signal transduction leading to differentiation, gene expression, and tumor progression. Clinical and experimental studies have already established the importance of protein kinase expression in the proliferation of human breast cancer (Boorne *et al*., 1998), suggesting that drugs that interrupt signaling pathways mediated by protein kinases could be useful cancer therapeutic agents. UCN-01 (7-hydroxystaurosporine), a staurosporine analogue initially developed as a selective protein kinase C (PKC) inhibitor, was isolated from the culture broth of *Streptomyces* sp. in 1987 (Takahashi *et al*., 1987). Subsequent studies have shown that in addition to PKC this compound inhibits a variety of other kinases at nanomolar concentrations, including PKA, CDK1, CDK2, CDK4, MAPK, $p60^{v\text{-}Src}$ and protein tyrosine kinase (Kawakami *et al*., 1996; Takahashi *et al*., 1989; Wang *et al*., 1995). Studies with cultured cells revealed that UCN-01 exhibited potent anti-tumor activity against several human cancer cell lines such as human epidermoid carcinoma A431, fibrosarcoma HT1080, acute myeloid leukemia HL-60, human lung carcinoma A549, and breast carcinoma MDA-MB-468 cell lines (Kawakami *et al*., 1996; Shao *et al*., 1997; Wang *et al*., 1995). UCN-01 also exhibited significant anti-tumor activities in several experimental animal models *in vivo* (Kawakami *et al*., 1996; Seynaeve *et al*., 1993; Takahashi *et al*., 1987). In addition, UCN-01 has been shown to enhance anti-tumor activities of chemotherapeutic agents such as cisplatin, 5-fluorouracil, mitomycin C, etc. *in vitro* and *in vivo* (Bunch and Eastman, 1997; Pollack *et al*., 1996; Wang *et al*., 1996).

Studies identifying the cellular pathways affected by UCN-01 resulting in G1 arrest suggest that although UCN-01 possesses potent PKC-inhibitory activity, inhibition of PKC activity is not essential for its growth inhibitory activity (Courage *et al*., 1995). Recent studies on the role of cell cycle regulators in UCN-01-mediated G1 arrest indicate that in human epidermoid carcinoma A431 cells, UCN-01-induced G1 arrest was accompanied by decreased cyclin-dependent kinase 2 (CDK2) activity and induction of CDK inhibitors p21 and p27 (Akiyama *et al*., 1997). p21 and p27 are two members of the CIP/KIP family of cyclin-dependent kinase inhibitors which negatively regulate the CDKs (Harper and Elledge, 1996; Sherr and Roberts, 1995). Although the CDK inhibitor p21 is a p53-regulated gene (El-Deiry *et al*., 1993; Harper *et al*., 1993), both p21 and p27 are also regulated through p53-independent pathways (Kato *et al*., 1994; Michieli *et al*., 1994; Polyak *et al*., 1994; Rao *et al*., 1998; Sheikh *et al*., 1994).

The mechanism of action of UNC-01 in either normal or tumor cells and whether or not such a mechanism involves p53 or pRb remain unresolved. p53 and the retinoblastoma protein (pRb) are two major tumor suppressors that are frequently inactivated in human cancer (Berns, 1994; Friend, 1994; Harbour *et al*., 1988; Horowitz *et al*., 1990). Alterations in p53 are linked to poor prognosis, tumor progression, and decreased sensitivity to chemotherapeutic agents. As an important G1 checkpoint regulator, p53 is involved in controlling the G1 to S phase transition in response to DNA damage. Similar to p53, pRb also functions as a

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negative regulator of cell cycle. Phosphorylation of pRb is necessary for the progression through G1 and is regulated primarily by cyclin D/CDK4/CDK6 complexes. The hypo-phosphorylated pRb serves as a tumor suppressor by interacting with and inhibiting cellular proteins such as E2F-DP heterodimeric transcription factors which activate many genes required for DNA replication pivotal for G1/S transition (Weinberg, 1995).

Even though UCN-01 is currently in phase 1 clinical trials in both the United States and Japan, there are several questions on the growth inhibitory effect of this agent in normal and tumor cells. In this study we examined the growth inhibitory activity and other cell cycle perturbations mediated by UCN-01 in several normal and tumor-derived breast epithelial cells. Our results reveal three novel findings on the sensitivity and mechanism of action of UCN-01 in normal and tumor cells. First, we document a significant difference in sensitivity to UCN-01 between normal and tumor cells. UCN-01 is capable of inducing a G1 arrest in normal cells at very low concentrations (i.e. 10 nM), while in tumor cells concentrations up to 80 nM did not result in a significant growth inhibition. Furthermore, we show that the UCN-01-mediated G1 arrest only occurs in normal cells and is concomitant with inhibition of CDK2 activity, decreased phosphorylation of pRb, and increased binding of p27 to CDK2. Secondly, we show that UCN-01-mediated G1 arrest is p53-independent and pRb-dependent using the E6 and E7 immortalized strains of the normal cells. Lastly, we show that at very high concentrations, there is a major difference in the mechanism by which UCN-01 mediates growth inhibition in normal *versus* tumor cells. Treatment of normal cells with UCN-01, at all concentrations examined (i.e. up to 300 nM), results only in G1 arrest, unlike staurosporine which arrests normal cells in both G1 and G2. Treatment of tumor cells, on the other hand, with high concentrations of UCN-01 results only in an S phase arrest, unlike staurosporine which arrest tumor cells only in G2. Collectively our studies suggest that the mechanism of differential sensitivity of UCN-01 in normal *versus* tumor cells is dependent on a regulated G1 checkpoint involving a functional pRb pathway.

Results

UCN-01 selectively arrests normal, but not tumor, cells in G1

We initially investigated whether UCN-01 has a different growth inhibitory effect in normal *versus* tumor cells. For this purpose we examined the effects of UCN-01 in several normal and tumor-derived breast epithelial cells (Figure 1). The two normal cell strains (81N and 76N) were established from reduction mammoplasties obtained from two different individuals (Band and Sager, 1989). The proliferation of these normal cell strains are dependent on growth factors and strictly regulated by checkpoint controls. Furthermore, at the end of their lifespan, these normal mammary epithelial cells stop proliferating and become senescent (Band and Sager, 1989; Gray-Bablin *et al*., 1997). We also examined MCF-10A, a near diploid immortalized cell line which is a subline of a breast epithelial cell strain, MCF-10. This cell line was derived from human fibrocystic mammary tissue and was immortalized after extended cultivation in medium containing low concentrations of calcium (Soule *et al*., 1990). The MCF-10A cell line also contains a wild-type Rb gene with homozygous deletion of the $p15^{INK4B}$ and $p16^{INK4A}$ genes as described (Iavarone and Massague, 1997). Therefore, MCF-10A has lost its strict growth factor requirement, checkpoint regulation (specifically, G1 to S transition) and the ability to senesce. In addition to these two normal and immortalized cell types we examined four breast cancer cell lines with different p53 and pRb status (Rao *et al*., 1998). Following treatment of cells with 0–80 nM UCN-01 for 48 h, growth inhibition was analysed by the MTT assay (Figure 1a), and the effect of UCN-01 on cell cycle distribution was examined by flow cytometry (Figure 1b). The data clearly shows that normal breast epithelial cell strains 76N and 81N were highly sensitive to UCN-01, revealing a 60–70% growth inhibition following treatment with only 20 nM UCN-01 and an IC_{50} of 10–12 nM. Breast cancer cell lines T47D and MDA-MB-157 cells showed little to no response to UCN-01 over the concentration range examined. MCF-10A, MCF-7 and MDA-MB-436, showed an intermediate response to UCN-01 with a 20–25% growth inhibition at 40 nM, and less than 50% growth inhibition at 80 nM of UCN-01. These results demonstrate that at low concentrations (i.e. 0–80 nM) normal cell strains are much more sensitive to UCN-01 than tumor cells. However at 'iso-effective' doses of UCN-01 (i.e. >300 nM) tumor cells respond by significant inhibition of cell proliferation (data not shown). Flow cytometry analysis revealed that treatment of normal cells (76N and 81N) with UCN-01 resulted in a significant accumulation of cells in the G1 phase of the cell cycle (i.e. an increase of 15% in G1 phase) in a dose-dependent fashion (Figure 1b). The G1 accumulation in normal cells was concurrent with an S phase decrement (Figure 1b), while G2+M phase had no significant change (data not shown). The partial growth inhibition of UCN-01 in MCF-10A and MCF-7 cell lines was due to a slight (less than 5%) accumulation in G1 (data not shown). Lastly, UCN-01 was ineffective in inducing any accumulation in the G1 phase of the cell cycle in MDA-MB-157 or MDA-MB-436 cell lines. In fact treatment of MDA-MB-436 cells with 80 nM UCN-01 resulted in a slight increase in S phase (Figure 1b). Additionally treatment of tumor cells with >300 nM UCN-01 resulted in a significant accumulation of cells in S phase (data not shown). The data from Figure 1 suggests that UCN-01 selectively mediates growth inhibition in normal but not tumor cells by arresting the cells in the G1 phase of the cell cycle.

UCN-01-mediated G1 arrest in normal cells results in inactive cyclin/CDK2 complexes due to increased p27 binding to CDK2

To determine which key cell cycle regulators were required for UCN-01-mediated G1 arrest in normal cell strains, we examined the expression of several positive and negative cell cycle proteins in both 76N and 81N

mortal cell strains. Both normal cell strains were treated with the indicated concentrations of UCN-01 for 48 h at which point cells were harvested and subjected to Western blot analysis with antibodies to p27, p21, pRb, p53, CDK2, CDK4, cyclin D1, and cyclin D3 (Figure 2a). These analyses revealed that the total levels p53 and pRb tumor suppressor proteins decreased significantly in the normal cell strains following UCN-01 treatment. In untreated cells pRb is present in both its phosphorylated (upper band) and unphosphorylated (lower band) forms. However the only form of pRb remaining at 40–80 nM UCN-01 is its hypo-phosphorylated form. The generation of hypo-phosphorylated pRb occurred concomitantly with growth inhibition, G1 arrest, decreased expression of cyclin D1, cyclin D3, CDK2 and CDK4 in a dose-dependent manner following UCN-01 treatment (Figure 2a). Additionally the expression of cyclin A and cyclin E were also down regulated by UCN-01 (data not shown).

The simultaneous decrease in p53 and p21 (Figure 2a) in normal cells suggest that in these cells p21 expression may be strongly influenced by p53 and that the UCN-01-induced G1 arrest in these cells is independent of p21. The levels of p27 were unchanged following UCN-01 treatment. This analysis raised the question whether p21 and p27 play a role in UCN-01-mediated G1 arrest. Does the decrease in the CDK4 and CDK2 levels in response to UCN-01 contribute to this arrest? The Western blot analysis in Figure 2a suggests that a likely explanation for the UCN-01-mediated G1 arrest could be due to a sequence of events initiating with down regulation of CDK4 and cyclin D3 leading to the inhibition of CDK2 activity necessary for cells to overcome the G1 restriction point. We addressed this hypothesis by initially examining the association of p21/p27 with CDK2 or CDK4 in a two step experiment consisting of an immunoprecipitation with anti-CDK2 or anti-CDK4 antibodies followed by Western blot analysis with p21 or p27 (Figure 2b and c). Additionally, we examined the CDK2-associated kinase activity by measuring the phosphorylation of histone H1 in immunoprecipitates prepared from UCN-01-treated cells using an antibody to CDK2 (Figure 2d). Treatment of normal cells with UCN-01 caused a rapid decrease of CDK2 activity. At 40 nM UCN-01 (the concentration causing G1 arrest and pRb hypo-phosphorylation) the level of CDK2 activity reaches its nadir. The decreased CDK2 activity observed (Figure 2d) was coupled with increased

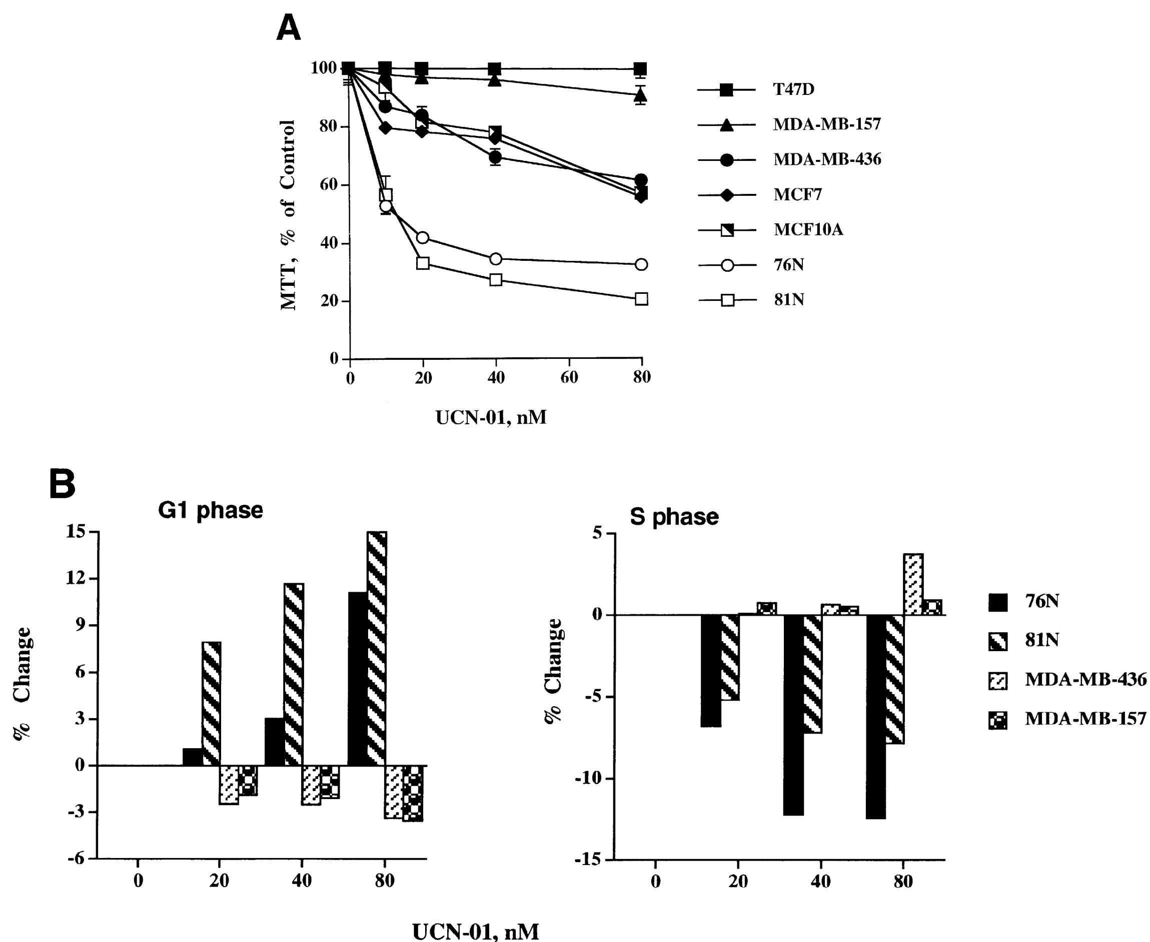

Figure 1 Normal cells are significantly more sensitive to UCN-01 than tumor cells. (**a**) Seven different human breast epithelial cell lines comprised of normal cell strains (76N, 81N), immortalized cell line (MCF10A) and breast tumor cell lines (MCF-7, MDA-MB-157, MDA-MB-436 and T47D) were treated with UCN-01 at 0, 10, 20, 40, and 80 nM for 48 h. Growth inhibition by UCN-01 was measured by the MTT assay. The experiment was repeated three times and error bars are indicated for each condition and each cell line. In most cases the error bars were smaller than the symbol size and cannot be seen. (**b**) Per cent change in cell cycle distribution of cells in G1 and S phase following UCN-01 treatment of normal (76N and 81N) and tumor (MDA-MB-157 and MDA-MB-436) cells. The bar graph reflects the per cent change of G1 and S phases of UCN-01-treated cells relative to the untreated controls, for each cell line

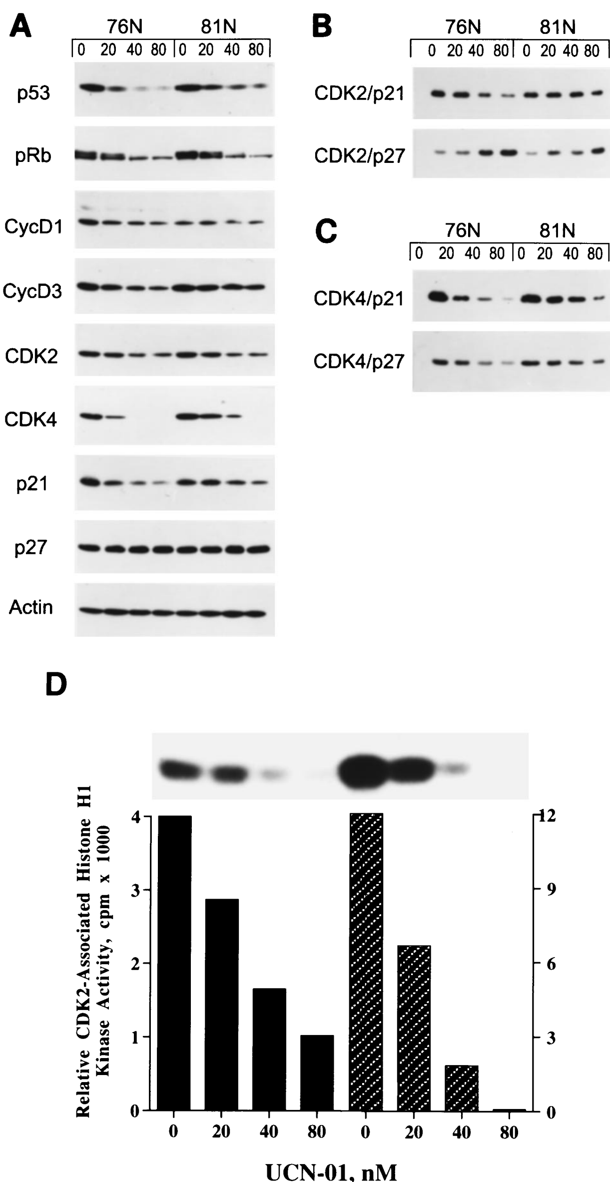

Figure 2 Cell cycle perturbation induced by UCN-01 in normal cell strains. Normal cell strains (76N and 81N) were treated with the indicated concentrations (nM) of UCN-01 for 48 h. Following treatment cells were harvested, cell lysates prepared and subjected to (**a**) Western blot analysis, (**b**) CDK2 immune-complex formation, (**c**) CDK4 immune-complex formation, and (**d**) Histone H1 kinase analysis. For Western blot analysis 50 μg of protein extract from each condition was analysed by Western blot analysis with the indicated antibodies or actin used for equal loading. The blots were developed by chemiluminescence reagents. The same blots were sequentially hybridized with different antibodies (see Materials and methods). The blots were stripped between the antibodies in 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8) and 2% SDS for 10 min at 55°C. For immunoprecipitation followed by Western blot analysis, equal amounts of protein (300 μg) from cell lysate prepared from each cell line were immunoprecipitated with anti-CDK2 (polyclonal) (**b**) or anti-CDK4 (polyclonal) (**c**) coupled to protein A beads and the immunoprecipitates were subjected to Western blot analysis with the indicated antibodies. For kinase activity, equal amounts of protein (300 μg) from cell lysates were prepared from each cell line immunoprecipitated with anti-CDK2 antibody (polyclonal) coupled to protein A beads using histone H1 as substrate. For each cell line we show the resulting autoradiogram of the histone H1 SDS–PAGE and the quantitation of the histone H1 associated kinase activities by Cerenkov counting. The numbers on the left ordinate refer to c.p.m. obtained from H1 kinase assay for 76N cells (solid bars), and the numbers on the right ordinate refer to the c.p.m. obtained from H1 kinase assay for 81N cells (shaded bars)

binding of p27 to CDK2 in the normal cells. The binding of p21 to CDK2 however, decreased in these cells (Figure 2b). These observations raise the question, why is there increased binding of p27 to CDK2 in normal cells (Figure 2b) when the levels of p27 don't change?

Recently several laboratories have proposed that p21 and p27 can function as adaptor molecules, which promote the association of CDK4 with D-type cyclins and increase CDK4 kinase activity (LaBaer *et al*., 1997; Planas-Silva and Weinberg, 1997; Rao *et al*., 1998). Since UCN-01 causes the arrest of cells, apparently by increasing binding of p27 to CDK2 complexes (Figure 2b), it can be hypothesized that this increased binding may be due to the switching of p27 from CDK4 to CDK2, mediated by UCN-01. To test this hypothesis we examined the association of p21 and p27 to CDK4 following UCN-01 treatment (Figure 2c). Our results clearly demonstrate that in untreated normal cells p21 and p27 bind to CDK4, and upon treatment with UCN-01, both p21 and p27 are released from CDK4 in a dose-dependent fashion (Figure 2c) which corresponds to the binding (i.e. switching partners) of p27 to CDK2 (Figure 2b). The decrease in CDK4-associated p21 and p27 (Figure 2c) could further be explained by the decrease in mass of CDK4 (Figure 2a). The total amount of CDK2 and CDK4 immunoprecipitated in Figure 2b and c were also analysed by Western blotting with antibodies to CDK2 and CDK4, and reveal that the fold decrease in the levels of these kinases following UCN-01 treatment were identical to those observed in Figure 2a (data not shown). The residual association of p27 to CDK4 at 40 and 80 nM UCN-01 is a reflection of the sensitivity of the immunoprecipitation assay (Figure 2c) as compared to Western blot analysis (Figure 2a). Our results suggest that UCN-01-mediated G1 arrest in normal epithelial cells is through decreased expression of CDK4 and CDK2. As CDK4 decreases, p27 is released from CDK4 and binds to CDK2, resulting in a decreased CDK2 kinase activity and decreased phosphorylation of pRb.

UCN-01 has no effect on cell cycle regulators in tumor cells

While UCN-01 had a profound affect in inducing G1 arrest and lowering the expression of key cell cycle regulators in normal cells, tumor cells showed no significant change in any of the cell cycle regulators examined (Figure 3). Furthermore, although the expression of some of these regulators was different between the three different tumor cell lines examined, within each tumor cell line the levels remained unchanged. For example, the levels of cyclin D1, CDK2 and CDK4 were the same within and between each cell line, following UCN-01 treatment. Cyclin D3 is overexpressed in MCF-7 cell line, moderately expressed in MDA-MB-157 and not expressed in MDA-MB-436 cells. MCF-7 cells was the only cell line examined which was wild-type for p53 and pRb and no significant change in the levels of these tumor suppressors was observed following UCN-01 treatment. Although pRb is expressed in MDA-MB-157 cells, it is functionally inactive as previously reported (Gray-Bablin *et al*., 1996). Lastly, the levels of p21 and

p27 are elevated in MCF-7 compared to the other two cell lines, while p16 was absent in MCF-7 and overexpressed in MDA-MB-157 and MDA-MB-436 (Figure 3). We also examined the CDK2 kinase activity in these tumor cell lines and as expected the activity of CDK2 was unchanged following UCN-01 treatment in all tumor cell lines examined (data not shown). These results suggest that tumor cells have lost the checkpoint control affected by UCN-01 resulting in no growth inhibition or cell cycle perturbation following treatment.

UCN-01-mediated G1 arrest is through a p53-independent and pRb-dependent pathway

To directly determine if alterations in p53 or pRb mediate G1 arrest in normal but not tumor cells, we examined the effects of UCN-01 in E6 and E7 strains of 76N cells. 76NE6 and 76NE7 are immortalized cell strains derived from normal mammary epithelial cell strain 76N by infection with human papilloma virus (HPV) 16E6 or 16E7 (Band *et al*., 1990, 1991). The E6/p53 and E7/pRb interaction promote degradation/inactivation of p53 and pRb respectively, resulting in the loss of normal phenotype (Band *et al*., 1993; Dyson *et al*., 1992; Werness *et al*., 1990). We initially examined the pattern of growth inhibition of UCN-01 in 76NE6 and 76N-E7 as compared to the parental 76N cells (Figure 4a). This analysis revealed that 76NE6 were as sensitive to the growth inhibitory activity of UCN-01 as 76N parental cell strain with a super-imposable dose-response curve and an IC_{50} of 10 nM. 76NE7 cells were much more resistant to UCN-01; however, their growth was also retarded with an IC_{50} of 75 nM (Figure 4a). Flow cytometry analysis revealed that the UCN-01-mediated growth inhibition in 76NE6 and 76NE7 cells were quite different. Treatment of 76NE6 cells resulted in accumulation of cells in the G1 phase of the cell cycle with a concomitant decrease in S phase cells (Figure 4a) identical to the pattern observed in 76N cells following drug treatment (Figure 1b). However, treatment of 76NE7, pRb deficient, cells resulted in accumulation of cells in S phase at 80 nM UCN-01 with a concomitant decrease in the G1 phase, opposite to the pattern observed with 76NE6, p53-deficient cells (Figure 4b). This data suggests that UCN-01-induced G1 arrest is dependent on a functional Rb, but not a functional p53.

Next we examined the expression of key cell cycle regulatory proteins in 76NE6 and 76NE7 cells following UCN-01 treatment (Figure 5a). 76NE6 cells are devoid of p53, but express pRb at very high levels (Band *et al*., 1990, 1991). Treatment of these cells with UCN-01 resulted in accumulation of the hypo-phosphorylated form of Rb, a decrease in CDK4 levels, and an increase in p21 and p27 levels. The levels of CDK2 and cyclin D1 remained unchanged during the course of treatment and p16 levels were undetectable. 76NE7 cells, on the other hand over express p16, apparently due to pRb inactivation (Khleif *et al*., 1996; Reznikoff *et al*., 1996). Treatment of 76NE7 cells with UCN-01 resulted in no detectable changes in any of the cell cycle regulators examined (Figure 5a). Next we measured the binding of p21 and p27 to CDK2 and CDK4 in 76NE6 and 76NE7 cells (Figure 5b and c). This analysis revealed that in 76NE6 cells the binding of p21 and p27 to CDK2 increased, while in 76NE7 cells the binding of these CKIs to CDK2 or CDK4 remained unchanged. Furthermore the increased binding of both CKIs to 76NE6 cells was concomitant with decreased binding of p21 and p27 to CDK4 suggesting a switching of partners of these CKIs from CDK4 to CDK2 mediated by UCN-01 (Figure 5b and c). Lastly, measurement of CDK2 activity in 76NE6 and 76NE7 cells revealed that treatment of 76NE6 cells with UCN-01 resulted in a dose-dependent decline in the kinase activity with maximum inhibition achieved at 40 nM (Figure 5d). The increased expression and binding of p21 and p27 to CDK2 observed (Figure 5b) contributes to the inhibition of CDK2 kinase activity in 76NE6 cells. The CDK2 activity in 76NE7 cells (Figure 5d) on the other hand, was completely unabated by UCN-01 treatment. The fact that we observe 76NE7 cells arrest in S phase without loss of CDK2 activity could suggest that this arrest occurred in later S phase and not early S phase, a point where CDK2 activity would be required. Furthermore, these results strongly suggest that the UCN-01-mediated G1

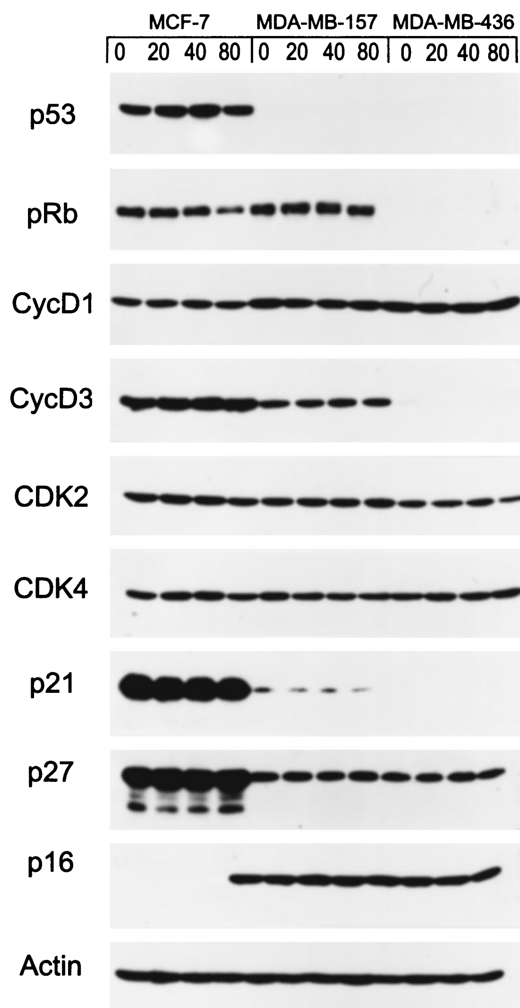

Figure 3 No change in the expression of cell cycle regulators by UCN-01 in tumor cells, MCF-7, MDA-MB-157 and MDA-MB-436 tumor cell lines were treated with the indicated concentrations (nM) of UCN-01 for 48 h. Following treatment cells were harvested, cell lysates prepared and subjected to Western blot analysis as described for Figure 2

arrest, and decreased CDK2 activity are p53-independent, but pRb-dependent.

High concentrations of UCN-01 induces S phase arrest in tumor and 76NE7 but not normal and 76NE6 cells

While examining the cell cycle effects of UCN-01 in tumor (Figure 1) and 76NE7 (Figure 4B) cells we noticed that treatment with 80 nM UCN-01 resulted in a slight increase in S phase and no G1 accumulation. These results raised the question if UCN-01 treatment of cells without a regulated G1 checkpoint and/or functional pRb could lead to only an S phase arrest. To explore this possibility we examined cell cycle phase distribution of 76NE6 and 76NE7 cells at low (i.e. 80 nM) and high (i.e. 300 nM) concentration of UCN-01. At 300 nM the growth of both cell types is completely inhibited (data not shown). We observed a clear difference between the ability of 76NE6 cells and 76NE7 cells to arrest in G1 or S phase (Figure 4b, and data not shown). Treatment of 76NE6 cells, which have an intact pRb, with any concentration of UCN-01 resulted only in a G1 arrest. However, treatment of 76NE7 cells, which have no detectable pRb, results in only an S phase arrest at higher concentrations of UCN-01 (i.e. ⩾80 nM) (Figure 4b, and data not shown). These results suggest that the disruption of the pRb pathway abrogates the ability of cells to arrest in G1 in response to UCN-01 treatment, instead they arrest in the S phase of the cell cycle.

To determine if synchronization of normal cells would sensitize their ability to arrest in S *versus* G1 phase, we synchronized 81N normal cells in the G1/S boundary by double-thymidine block prior to UCN-01 treatment (Figure 6). Under these conditions up to 30% of the cells (i.e. threefold higher than the asynchronous controls) accumulate in S phase following release from this block, and the cells undergo synchronous traverse through the cell cycle for the duration of the experiment (Figure 6). Following the double thymidine block, cells were treated with 300 nM UCN-01 for 3–12 h. We used a high (i.e. 300 nM) concentration of UCN-01, since this concentration was sufficient to arrest 76NE7 cells in the S phase of the cell

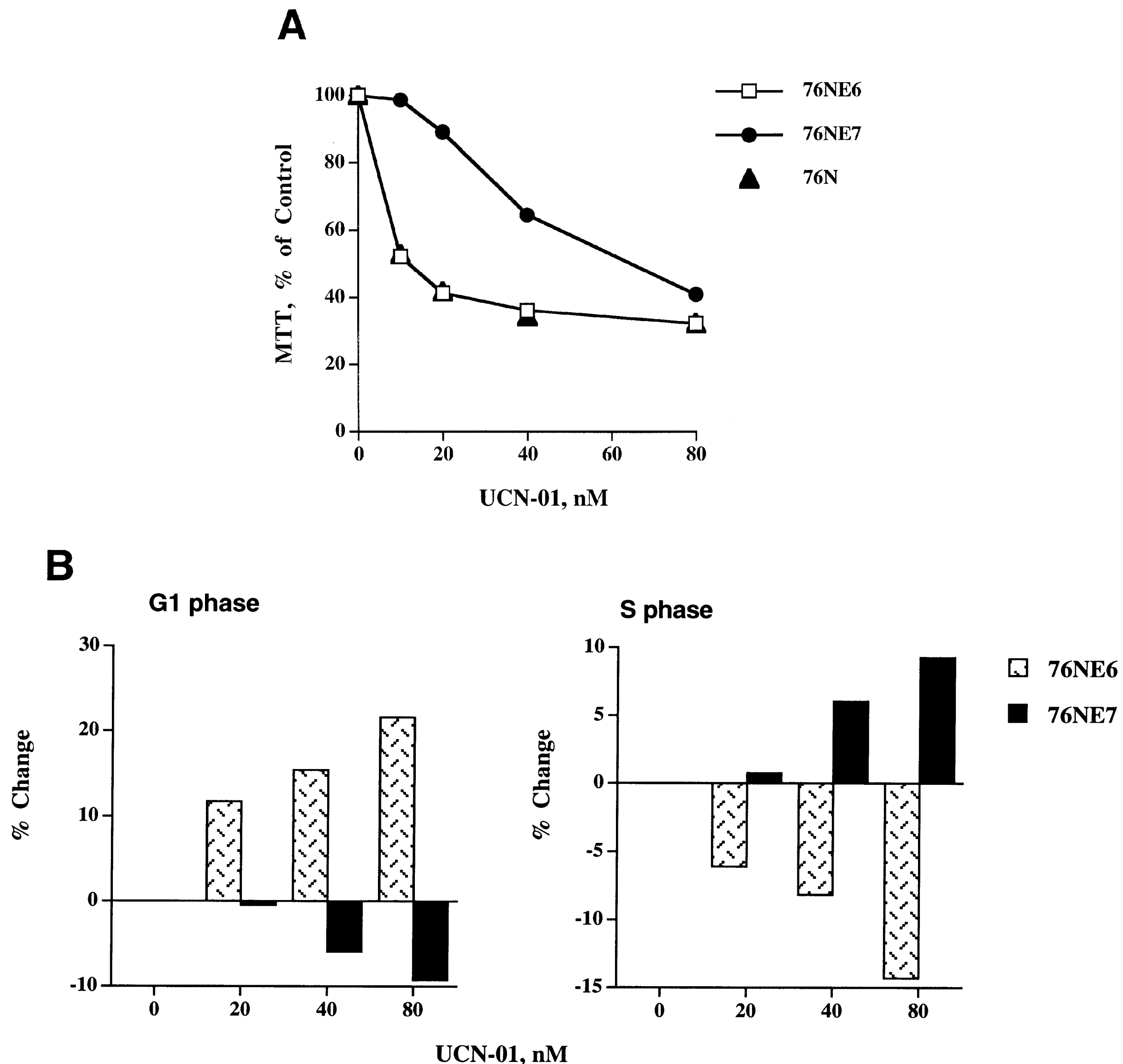

Figure 4 UCN-01-mediated G1 arrest is pRb-dependent and p53-independent. (**a**) 76N, 76NE6, and 76NE7 cells were treated with the indicated concentrations of UCN-01 for 48 h and subjected to growth inhibition analysis as measured by MTT assay and repeated three times. Error bars are indicated for each condition and each cell line. In all cases the error bars were smaller than the symbol size and cannot be seen. (**b**) Per cent change in cell cycle distribution of cells in G1 and S phase following UCN-01-treatment. The bar graph reflects the per cent change of G1 and S phases of UCN-01-treated cells relative to the untreated controls, for each cell line

cycle (Figure 6). At every time interval examined, treatment of 81N with UCN-01 resulted in the accumulation of cells in the G1 but not S phase of the cell cycle (Figure 6). Hence, the UCN-01-mediated G1 accumulation occurred in both asynchronous and synchronized cells. Our results suggest that normal cells, with a functional pRb and G1 checkpoint, are incapable of arresting in any other phase but G1 upon treatment with UCN-01, no matter what their cell cycle distribution is prior to treatment.

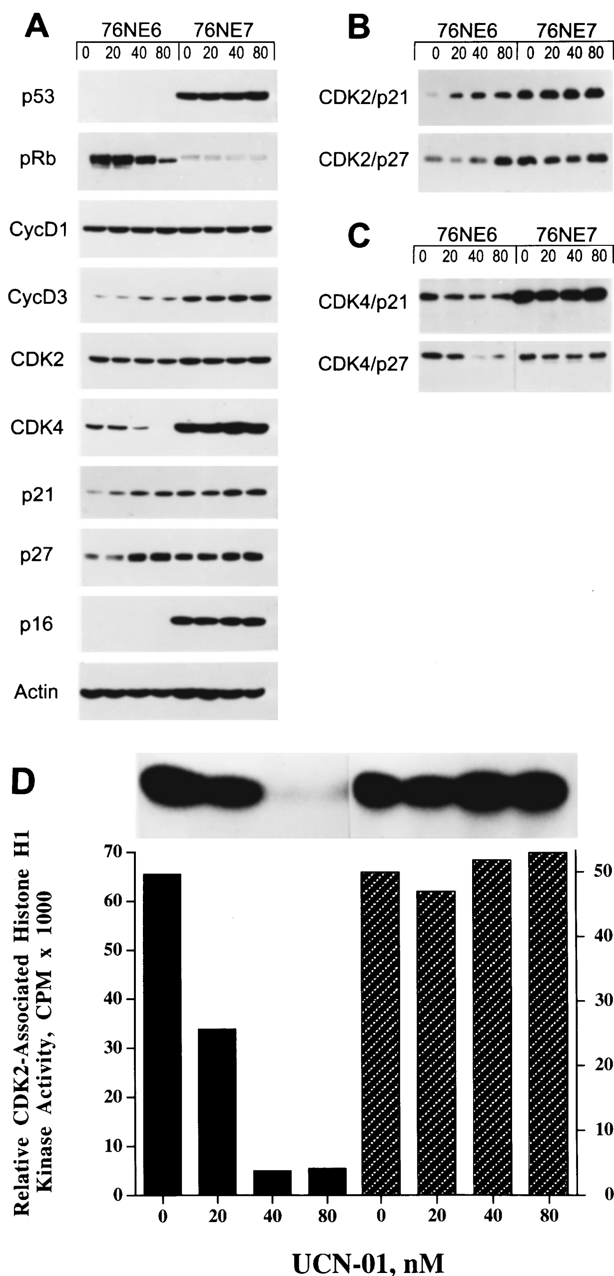

Figure 5 Cell cycle perturbation induced by UCN-01 in 76NE6 and 76NE7 cells. 76NE6 and 76NE7 cells were treated with the indicated concentrations (nM) of UCN-01 for 48 h. Following treatment cells were harvested, cell lysates prepared and subjected to (**a**) Western blot analysis, (**b**) CDK2 immune-complex formation, (**c**) CDK4 immune-complex formation and (**d**) Histone H1 kinase analysis as described for Figure 2. In (**d**) the numbers on the left ordinate refer to c.p.m. obtained from H1 kinase assay for 76NE6 cells (solid bars) and the numbers on the right ordinate refer to the c.p.m. obtained from H1 kinase assay for 76NE7 cells (shaded bars)

The results obtained with UCN-01-treated normal cells are quite different than those with staurosporine-treated cells. It has been well documented that staurosporine, a close structural analogue of UCN-01, can arrest normal cells in both G1 and G2 phases of the cell cycle, and tumor cells in only the G2 phase of the cell cycle. Our results show that UCN-01, also known as 7-hydroxy staurosporine, arrests normal cells in G1 and tumor cells in S phase when used in high concentrations. To compare the effects of staurosporine and UCN-01 in normal *versus* tumor cells we treated two normal cell strains and two tumor cell lines with equally cytotoxic concentrations of UCN-01 and staurosporine (Figure 7). UCN-01 and staurosporine produced quite different effects. These results show that as predicted normal cells respond to high concentrations of staurosporine by arresting in both G1 and G2. However, treatment of these cells with high concentrations of UCN-01 resulted in only a G1 arrest. Furthermore, tumor cells, which have a defect in the pRb pathway, respond to high concentrations of staurosporine (as high as 300 nM, data not shown) by arresting predominately in the G2 phase of the cell cycle. On the other hand, the same tumor cells respond to UCN-01-mediated growth inhibition by arresting in the S phase of the cell cycle (Figure 7). These results clearly indicate that normal and tumor cells respond to these very close structural analogues quite differently. Furthermore, the ability of cells to arrest in either G1 or S phase by UCN-01 is dictated by a functional pRb, while the ability of cells to arrest in G2 by staurosporine is independent of pRb.

Discussion

In this manuscript we investigated the growth inhibitory effects and cell cycle pathways affected by UCN-01 in several normal and tumor-derived breast epithelial cells. This data revealed three novel findings: First, we found that normal cells are significantly more sensitive to UCN-01 than tumor cells. Treatment of normal cells with concentration as low as 10 nM resulted in 50% growth inhibition. Tumor cells were much more resistant to growth inhibitory effects of UCN-01 and concentrations as high as 80 nM resulted in minor to no growth inhibition. The normal cells used in this study were established from reduction mammoplasty samples which undergo senescence after several passages (Band and Sager, 1989). They are normal, diploid, mortal cells with regulated checkpoint control. As shown in Figures 1 and 2 these cells are very sensitive to UCN-01. On the other hand MCF-10A cell line, a near diploid immortalized cell line, was much more resistant to the growth inhibitory activity of UCN-01. In fact the growth inhibitory activity of UCN-01 in MCF-10A was similar to that of MCF-7 and MDA-MB-436, two cancer cell lines used in this study (Figure 1a). These observations suggest that the pathways altered by the immortalization process (i.e. deletions in p15 and p16 genes for example) may compromise the function of the pRb pathway, rendering the MCF-10A cells relatively resistant to low concentrations of UCN-01. Furthermore, the serum concentration in the culture medium of

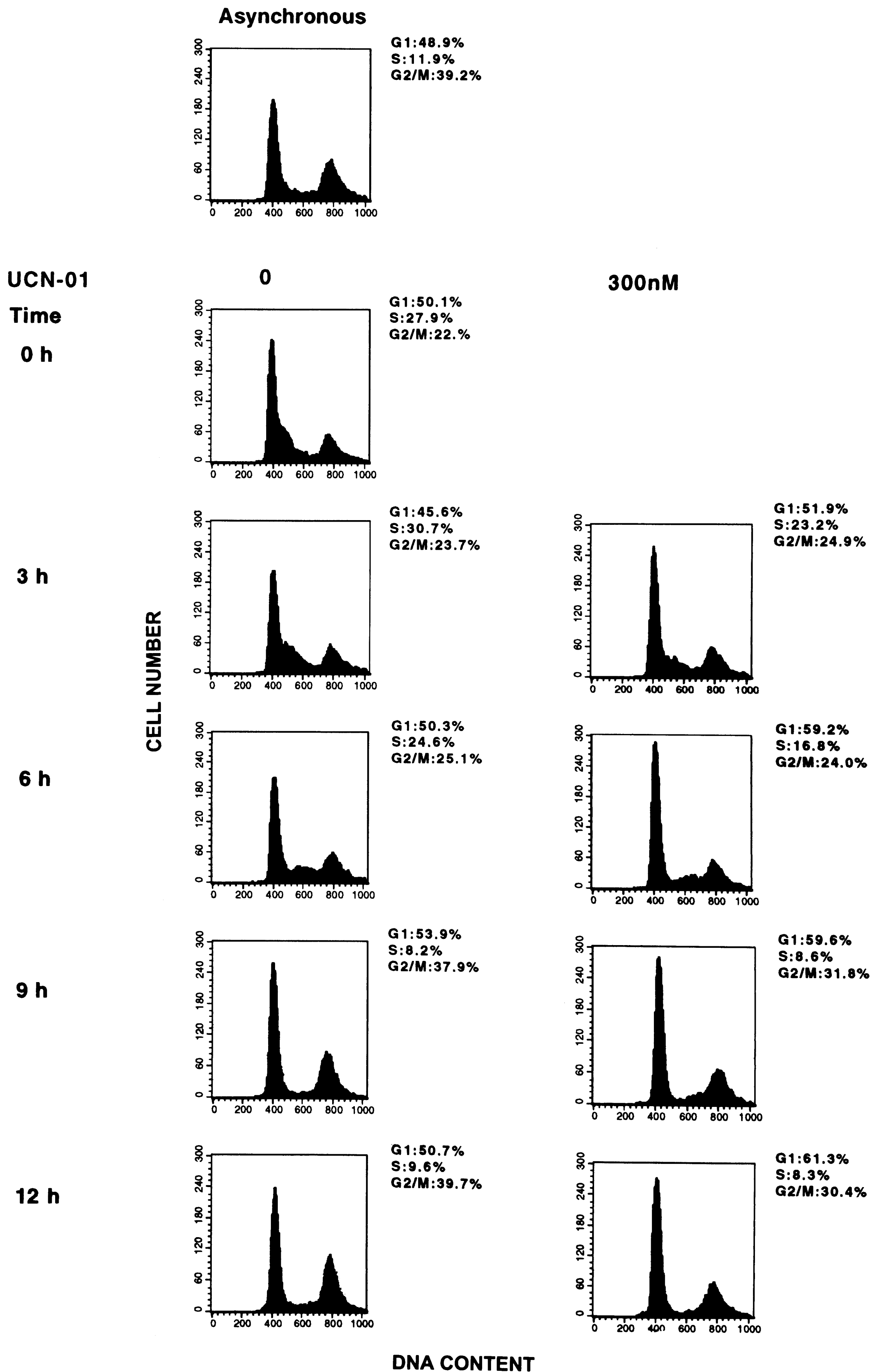

Figure 6 Synchronization of normal cells in the G1/S phase does not abrogate their ability to arrest in G1 by UCN-01. Asynchronously growing 81N cells (top panel) were synchronized at the G1/S boundary by double-thymidine block (see Materials and methods). Synchronized cells were treated with either 0 or 300 nM UCN-01. At the indicated times following UCN-01 treatment cells were harvested for analysis by flow cytometry

normal and tumor cells did not account for the differences in UCN-01-mediated growth inhibition.

The growth inhibitory effects of UCN-01 in normal cells are due to a G1 arrest. We show that such an arrest was concomitant with decreased CDK4 expression, pRb phosphorylation and CDK2 activity, coincident with increased binding of p27 to CDK2 and switching of p27 from CDK4 to CDK2. In addition, UCN-01 treatment also results in down regulation of cyclins D1 and D3 which are usually active in early G1 suggesting that UCN-01 targets an early event in the G1 phase of normal cells. There were no significant cell cycle perturbations observed in tumor cells by UCN-01. Several studies have reported that UCN-01 inhibits cell cycle progression from G1 to S phase in various mammalian transformed cell lines (Akiyama *et al*., 1997; Kawakami *et al*., 1996; Seynaeve *et al*., 1993). However, it is not clear from these studies why some cells respond to UCN-01 by arresting in the G1 phase of the cell cycle while others accumulated in S phase. In this study we clearly show that normal cells with a regulated G1 checkpoint respond to UCN-01 by arresting in G1 while tumor cells, depending on their pRb status arrest either in G1 or S. For example, both MCF-7 cells and MCF-10A which have an intact pRb respond to the growth inhibitory activity of UCN-01 by arresting in the G1 phase of the cell cycle (data not shown). However MDA-MB-436, T47D, and MDA-MB-157 cell lines which are pRb negative (mutations/functional inactivity), arrest in the S phase of the cycle (Figure 7 and data not shown).

Secondly, our studies suggest that the pRb pathway is involved in UCN-01-mediated G1 arrest in normal but not tumor cells. Using the 76NE6 and 76NE7 model system where the HPV E6 binds to and degrades p53, and HPV E7 interacts with pRb and pRb-like proteins and inactivate the pRb pathway. We found that UCN-01 inhibited the growth in G1, and perturbed key cell cycle regulatory proteins in 76NE6 similarly to that of the parental 76N normal cell strain (Figures 2 and 5). However, UCN-01 was incapable of mediating G1 arrest or perturbing the cell cycle regulators not only in tumor cells with a defective pRb pathway such as MDA-MB-157 and MDA-MB-436 (Figure 3) but also in E7 immortalized 76NE7 cells (Figures 4 and 5). These results clearly suggest that the UCN-01-mediated G1 arrest is dependent on a functional pRb and when cells contain a mutant or non-functional pRb, they arrest in the S phase of cell cycle instead.

The mechanism by which UCN-01 mediates G1 arrest through pRb may involve CDK4. We base this hypothesis on the striking decline in CDK4 levels

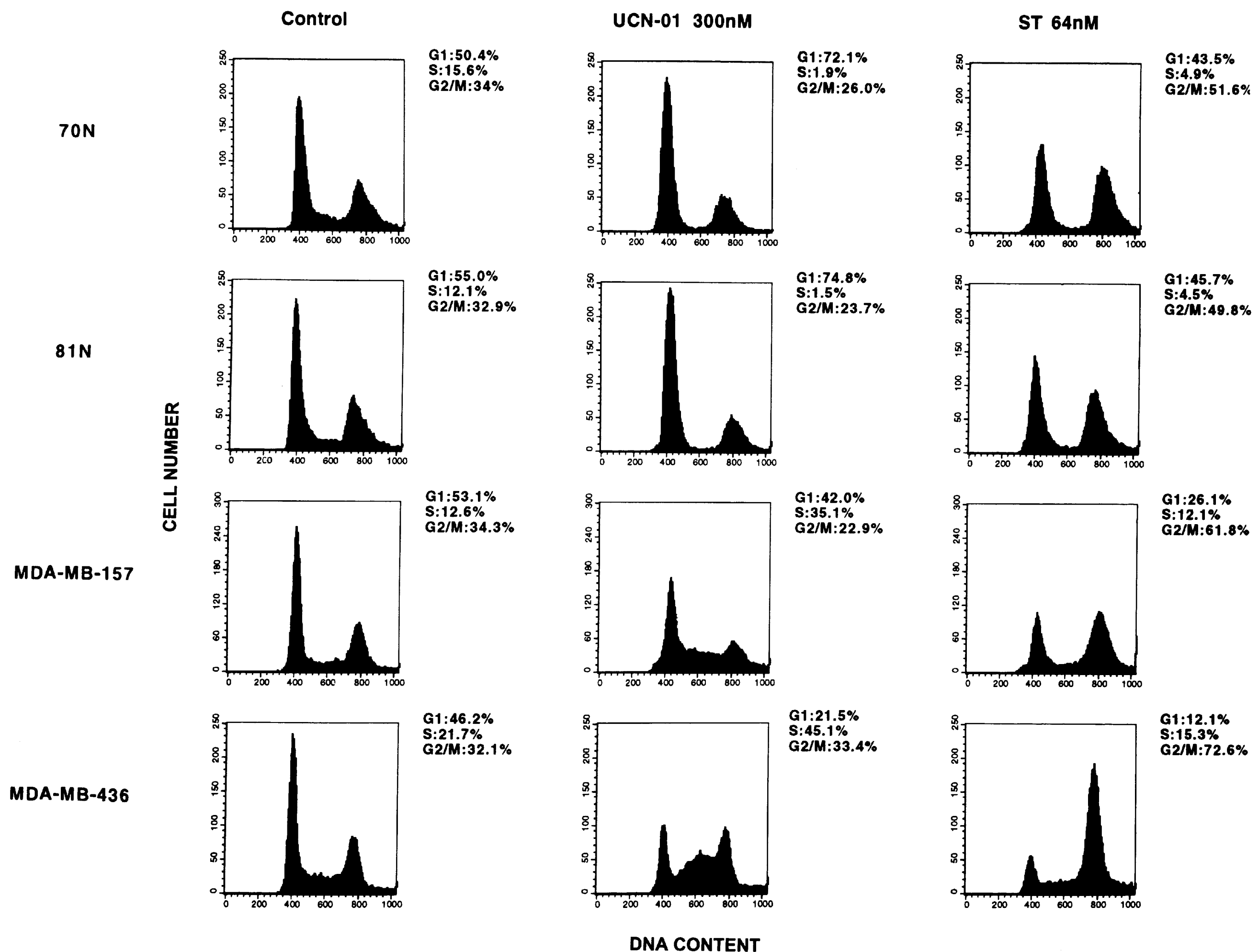

Figure 7 UCN-01 and staurosporine act at different cell cycle checkpoints. Normal breast epithelial (70N and 81N) cell strains and tumor (MDA-MB-157 and MDA-MB-436) cell lines were treated with no drug or equally cytotoxic concentrations of UCN-01 (i.e. 300 nM) or staurosporine (i.e. 64 nM) for 48 h. Following treatment cells were harvested for analysis by flow cytometry

shortly after UCN-01 treatment in the normal cells (Figure 2). The decrease in CDK4 levels occurs only in cells with a functional pRb. Although the immediate downstream effects of UCN-01 responsible for a decline in CDK4 are currently unknown, a likely possibility could involve a feedback regulation between pRb and CDK4. Specifically we suggest that upon the initial hypo-phosphorylation of pRb, a signal is generated to down regulate CDK4 synthesis, resulting in further hypo-phosphorylation of pRb and subsequent G1 arrest.

Lastly, we show that normal and tumor cells respond differently to high concentrations of UCN-01 as compared to staurosporine. Treatment of asynchronous or G1/S-arrested normal cells with either high (300 nM) or low concentrations of UCN-01 results in only a G1 arrest (Figures 6 and 7). On the other hand, treatment of tumor cells with high concentrations of UCN-01 completely inhibits their growth by arresting them in the S phase of the cell cycle (Figure 7). The effect of UCN-01 on normal and tumor cells is very different than its structural analogue, staurosporine, which arrests normal cells in G1 and G2 and tumor cells only in G2 (Figure 7). The G2 (staurosporine) *versus* S (UCN-01) phase arrest seen in the same tumor cells treated with equally toxic concentrations of these two drugs (Figure 7) is very surprising, since these two agents differ only in the presence of a 7-OH group on UCN-01. Although this structural difference is subtle the two agents have different specificity toward CDKs. In a series of experiments (reviewed in Meijer, 1996) aimed at examining the specificity of UCN-01 and staurosporine toward different proteins kinases, it was discovered that even though the IC_{50}s of these two analogues against purified PKC were similar (i.e. 7 nM for UCN-01, and 5 nM for staurosporine), their IC_{50}s toward CDKs were quite different. UCN-01 displayed IC_{50}s of 30–32 nM against purified CDK1, CDK2 and CDK4. On the other hand, staurosporine displayed IC_{50}s of 3–9 nM against CDK1 and CDK2 and $>10\,000$ μM against CDK4. These studies suggest that the mechanism by which UCN-01 and staurosporine mediate their growth inhibitory effects may be different, and that CDKs and not PKC could dictate such difference. It is also important to consider the role of ATP in this process since at high concentrations of ATP in CDK assays (as would occur in living cells), UCN-01 is actually a relatively poor CDK antagonist (Wang *et al*., 1995). Nonetheless, the results presented in this study are consistent with CDK inhibition as the mechanism by which UCN-01 and staurosporine mediate their growth inhibitory potential.

The common link between UCN-01 and staurosporine is that at low concentrations, both agents arrest normal cells in G1 and such an arrest is lost in tumor cells. The mechanistic basis of staurosporine-induced G1 arrest in normal cells and its loss in tumor cells was recently reported to be through pRb since mouse embryonic fibroblasts from pRb knockout mice treated with staurosporine were incapable of arresting in G1 (Orr *et al*., 1998). These and other studies performed on bladder carcinoma cell line 5673 (Schnier *et al*., 1996) provided strong support for the importance of pRb in inducing G1 arrest in cells by staurosporine. The studies we have presented here also provide strong evidence for the role of pRb in UCN-01-mediated G1 arrest in normal cells and loss of G1 arrest in tumor cells lacking pRb. Thus the ability of both UCN-01 and staurosporine to induce G1 arrest seems to be through pRb, independent of p53. The difference between these two agents is twofold: First, these two agents are different in their ability to induce either G2 arrest, with staurosporine, or S phase arrest, with UCN-01. Secondly, staurosporine induces G2 arrest in both normal and tumor cells, while UCN-01 mediates S phase arrest only in tumor cells. The mechanism by which UCN-01 induces S phase arrest in tumor but not normal cells, although unclear at this point, does not involve either p53 or pRb since treatment of tumor cells lacking both p53 and pRb resulted in S phase arrest.

In summary, our results show that UCN-01 can induce G1 arrest in normal cells at very low concentrations while tumor cells are completely resistant to UCN-01-mediated G1 arrest. This G1 arrest is independent of p53, and dependent on pRb. We also show that tumor cells respond to UCN-01-induced growth inhibition by arresting in S phase independent of either p53 or pRb. Understanding the mechanism by which tumor cells arrest in S phase in response to UCN-01 could provide insight into the regulation of the S phase checkpoint in normal and tumor cells.

Materials and methods

Materials, cell lines and culture conditions

UCN-01 was provided by the National Cancer Institute. Serum was purchased from Hyclone Laboratories (Logan, Utah, USA) and cell culture medium from Life Technologies, Inc. (Grand Island, NY, USA). All other chemicals used were reagent grade. The culture conditions for 76N, 81N and 70N normal cell strains, MCF-10A immortalized cell line, and MCF-7, ZR75T, MDA-MB-157, Hs578T, T47D and MDA-MB-231 breast cancer cell lines were described previously (Keyomarsi *et al*., 1995; Keyomarsi and Pardee, 1993). 76N-E6 and 76N-E7 cell lines (gifts from Dr V Band, Tufts Medical Institute Boston, MA, USA) were immortalized and cultured as described previously (Band *et al*., 1990, 1991). All cells were cultured and treated at 37°C in a humidified incubator containing 6.5% CO_2 and maintained free of mycoplasma as determined by Hoechst staining (Hessling *et al*., 1980).

MTT assay

The MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) assay was performed as described (Carmichael *et al*., 1988). Exponentially growing cells were counted by a Coulter Counter (Halieah, FL, USA) and plated at a density of 25 000 cells/ml in the wells of 96-well tissue culture plates (200 μl culture fluid per well) and allowed to recover for 24 h prior to drug treatment. Cells were incubated with the indicated concentration of UCN-01 or staurosporine for 48 h and subjected to the MTT survival assay. Each data point represents the average of six determinations, and the MTT assay for each experimental condition was performed at least three times.

Cell synchronization and flow cytometry

Normal mammary epithelial (81N) cells were synchronized at the G1/S boundary by the double thymidine block procedure as previously described (Keyomarsi *et al*., 1995). UCN-01

(300 nM) was added following the release of cells from thymidine block. Cells were harvested at the indicated times, cell density was measured using a Coulter Counter and flow cytometry analysis was performed as described previously (Rao *et al*., 1998).

Blotting, immunoprecipitation and H1 kinase analysis

Cell lysates from UCN-01-treated cells were prepared and subjected to Western blot analysis as previously described (Rao *et al*., 1998). Primary antibodies used were pRb monoclonal antibody (PharMingen, San Diego, CA, USA), at a dilution of 1 : 100, monoclonal antibody to p16 (a gift from Jim DeCaprio, Dana Farber Cancer Institute) at a dilution of 1 : 20, CDK2, CDK4, and p27, monoclonal antibodies (Transduction Laboratories, Lexington, KY, USA) each at a dilution of 1 : 100, p21 and p53 monoclonal antibodies (Oncogene Research Products/Calbiochem, San Diego, CA, USA) at a dilution of 1 : 100 cyclin D1 monoclonal antibody (Santa Cruz Biochemicals, Santa Cruz, CA, USA) at a dilution of 1 : 100, and actin monoclonal antibody (Boehringer-Mannheim, Indianapolis, IN, USA) at 0.63 μg/ml in Blotto. Following primary antibody incubation, the blots were washed and incubated with goat anti-mouse horseradish peroxidase conjugate at a dilution of 1 : 5000 in Blotto for 1 h and finally washed and developed with the Renaissance chemiluminescence system as directed by the manufacturers (NEN Life Sciences Products, Boston, MA, USA).

For immunoprecipitations followed by Western blot analysis, 300 μg of cell extracts were used per immunoprecipitation with polyclonal antibody to CDK2 (Rao *et al*., 1998) or CDK4 (Santa Cruz Biochemicals, Santa Cruz, CA, USA) as previously described (Rao *et al*., 1998). The immunoprecipitates were then electrophoresed on 13% gels, transferred to Immobilin P, blocked and incubated with the indicated antibodies at dilutions described above. For Histone H1 kinase assay the immunoprecipitates were incubated with kinase assay buffer containing 60 μM cold ATP and 5 μCi of [^{32}P]ATP in a final volume of 50 μl at 37°C for 30 min. The products of the reaction were then analysed on a 13% SDS–PAGE gel. The gel was then stained, destained, dried and exposed to X-ray film. For quantitation, the protein bands corresponding to histone H1 were excised and the radioactivity of each band was measured by Cerenkov counting.

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