Cancer Chemother Pharmacol (1994) 33: 273–280

Cancer Chemotherapy and Pharmacology

Effect of UCN-01, a selective inhibitor of protein kinase C, on the cell-cycle distribution of human epidermoid carcinoma, A431 cells

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Received: 24 May 1993/Accepted: 31 August 1993

Abstract. UCN-01 (7-hydroxy-staurosporine), a selective inhibitor of protein kinase C (PKC), was shown to exhibit antitumor activity in murine and human tumor cell lines in vitro and in vivo. On the other hand, staurosporine, a non-selective protein kinase inhibitor, was not shown to exert antitumor activity in vivo despite its potent antiproliferative activity in vitro. To compare the modes of action of UCN-01 and staurosporine in vitro, the effects of both drugs on the cell cycle progression of human epidermoid carcinoma A431 cells were examined by flow cytometry using propidium iodide (PI) staining. At 50% growth inhibitory concentrations, both UCN-01 and staurosporine induced G1 phase accumulation in the cell cycle. At 80% growth inhibitory concentrations, UCN-01 also induced preferential G1 phase accumulation, but staurosporine mostly induced G2M phase accumulation. Staurosporine also induced higher DNA ploidy when the cells were exposed to the drug for more than one generation time of A431 cells. An analysis of cell kinetics by 5-bromo-2-deoxyuridine incorporation versus DNA content confirmed that the G1 phase block by UCN-01 and the G1 and G2M phase block by staurosporine at the respective doses, as was the case for PI staining. Additionally, DNA synthesis of the cells, which was determined by the uptake of ^{3}H-TdR, was not suppressed at least 8 h after the treatment with UCN-01. These results suggested that UCN-01 could affect the G1 phase of cell cycle in A431 cells in quite different manners from staurosporine. The G1 phase block induced by UCN-01 might be important for the growth inhibitory activity of UCN-01 against A431 cells in vitro and in vivo.

Abbreviations used: PKC, Ca^{2+} and phospholipid-dependent serine/threonine kinase; PBS, phosphate buffered saline; PI, propidium iodide; FITC, fluorescein isothiocyanate; cPKC, conventional PKC including PKC α, β and γ; nPKC, neo PKC including PKC δ, ε, η (L) and θ; IC_{50}, concentration required for 50% growth inhibition; TPA, phorbol 12-myristate 13-acetate

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Introduction

UCN-01 (7-hydroxy-staurosporine) was isolated from the culture broth of *Streptomyces* sp. It has a potent and selective inhibitory activity against PKC [20]. Additionally we have found that UCN-01 has the ability to inhibit the growth of human nd murine tumor cell lines in in vitro and in vivo animal models [4]. In contrast, non-selective protein kinase inhibitor staurosporine [21–23] did not exhibit any antitumor activity in vivo although it had much greater antiproliferative activity than UCN-01 in vitro [4, 23]. These results suggest that the selective inhibition of protein kinases such as PKC is important for the antitumor activity.

In yeast cells, PKC1 gene product, which is a putative cPKC in yeast cells, was shown to participate in cell cycle regulation through G2 to M phase [14]. In addition, nPKC was shown to be important in the cell cycle regulation through G0–G1 to S phase in mouse fibroblast cells [17]. However, little is known about the contribution of cPKC and nPKC on the cell cycle regulation of mammalian cells.

Recently several groups have reported that staurosporine could arrest the cell cycle progression of normal and transformed cells either in G1 or in G2 phase, depending on its concentration [1, 6, 7, 25]. At lower concentrations, staurosporine exhibited a G1 phase arrest in the cells although there were some exceptions [1, 6, 7, 25]. In contrast, at higher concentrations, it exhibited the G2 arrest both in normal and transformed cells [1, 6, 7, 25]. In the case of lymphocytic leukemia cells, higher concentrations of staurosporine induced higher DNA ploidy [6].

Recent studies have indicated that the cell cycle in eukaryotes are regulated by a group of proteins, namely $p34^{cdc2/cdc28}$ and cyclins [12, 13, 16]. These protein complexes, with serine/threonine kinase activity, are thought to play a critical role at the major regulatory points in cell cycle progression, the G1 to S phase and G2 to M phase transitions [12, 13, 16]. Staurosporine was reported to inhibit the activity of $p34^{cdc2}$ kinase with IC_{50} value of 4–5 nM, suggesting that the G2 block caused by staurosporine is due, at least in part, to the inhibition of this kinase [8].

In this study, we examined and compared the effect of UCN-01 with staurosporine on the cell cycle distribution of human epidermoid carcinoma A431 cells, which has been shown to be sensitive to UCN-01, but not to staurosporine, in an in vivo animal study [4].

Material and methods

Drugs. UCN-01 and staurosporine were produced by fermentation in our laboratories as previously described [20, 23].

Cell culture. Human epidermoid carcinoma, A431 [10] was obtained from the American Type Culture Collection through Dainippon Pharmaceutical Co., Osaka, Japan. The cell cultures were performed at 37 °C in a humidified atmosphere of 5% CO_2.

In vitro antiproliferative activity. A431 cells (1.5×10^4/1 ml per well) were precultured in Dulbecco's minimal essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (Gibco Grand Island, NY) for 24 h in Nunclon 24-well multidishes (No. 167008; Nunc, Rosklide, Denmark). Drugs were added to the plates ($n = 3$) in serial dilutions, and the plates were incubated for 72 h. The total cell number was counted by a micro-cell counter (Toa Medical Electronics Co., Kobe, Japan) after detachment of the cells by treatment with 0.05% trypsin (Difco Laboratories, Detroit, Mich.)–0.02% ethylene diammine tetraacetic acid (EDTA; Wako Pure Chemical Industries, Co., Ltd., Osaka, Japan).

DNA content analysis by PI staining A431 cells (3×10^5/dish) were cultured in Falcon 3003 plastic dishes (Becton Dickinson, Lincoln Park, N. J.) on day 0, and were treated with UCN-01 or staurosporine according to the appropriate treatment schedule as shown in Fig. 2. At the times indicated, the cells were harvested by treatment with 0.25% trypsin, fixed with cold 70% ethanol solution, hydrolyzed with 25 μg/ml of ribonuclease A (type 1-A, Sigma Chemical Co., St Louis, Mo.) at 37 °C for 30 min, and stained with PI (Sigma) for 20 min. The DNA content of the cells was analyzed by a EPICS ELITE flow cytometer (Coulter, Hialeah, Fla.). The proportion of cells in a particular phase in the cell cycle was estimated by a MULTICYCLE program (Coulter).

Cell cycle analysis by BrdUrd. A431 cells were cultured as described above on day 0, and were pulse-treated with 10 μM BrdUrd (Sigma) for 30 min at 37 °C on day 1. The cells were washed twice with PBS(–) to remove unincorporated BrdUrd. After the cells had been replenished with fresh prewarmed medium, they were treated with UCN-01 or staurosporine. At the times indicated (shown in Fig. 4) the cells were washed with PBS(–) and were harvested and fixed with 70% cold ethanol solution. BrdUrd immunostaining was performed as follows. After acid denaturation of double helical DNA with 4 *N* HCl for 15 min at room temperature, the cells were neutralized with 0.1 M $Na_2B_4O_7$ solution (pH 8.5) and washed with PBS(–). Then 20 μl of FITC-conjugated monoclonal anti-BrdUrd antibody (Becton Dickinson, San Jose, Calif.) and 80 μl PBS(–) containing 0.5% Tween 20 (Kanto Chemical, Tokyo, Japan) were added to the cells, and they were incubated at 37 °C for 30 min. After the cells had been treated with 25 μg/ml of ribonuclease A (type 1-A, Sigma) for 15 min at 37 °C, they were stained with PI (Sigma) for 20 min on ice. Green fluorescence represented the amount of FITC-conjugated anti-BrdUrd antibody (rate of DNA synthesis), and red fluorescence represented the amount of bound PI (DNA content). From each sample, 20000 events were collected in list mode, and bivariate BrdUrd/DNA (green/red) contour plots were constructed.

Incorporation of ^{3}H-TdR into A431 cells. A431 cells were cultured in 24-well multidishes (Nunc) on day 0, and were treated with various concentrations of UCN-01 for the times as indicated in Fig. 5 on day 1.

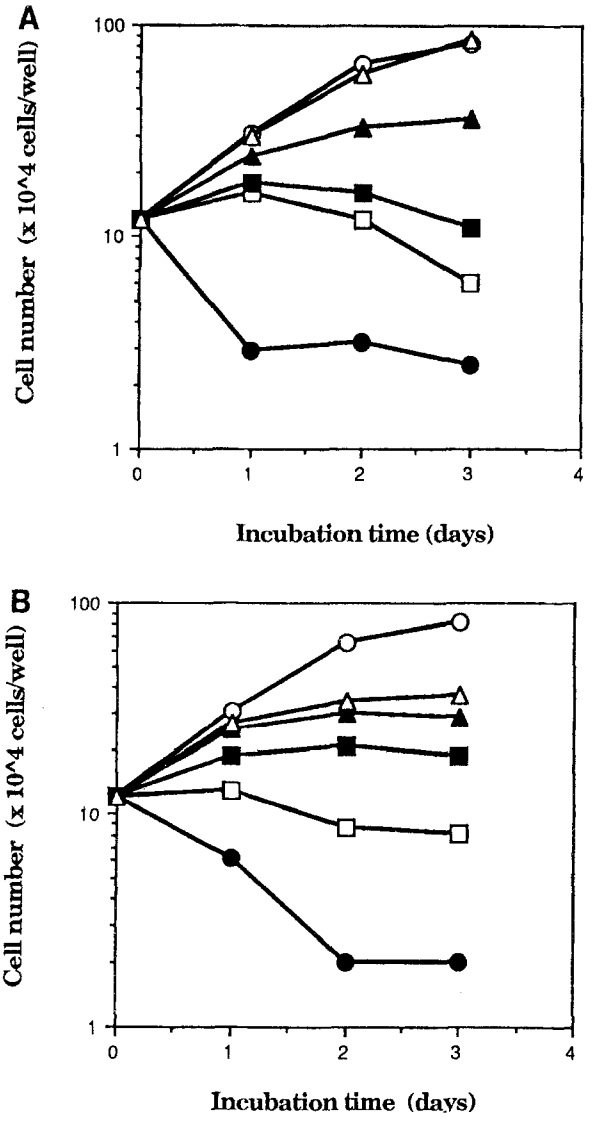

Fig. 1. Inhibition of the growth of A431 cells by **A** UCN-01 or **B** staurosporine. **A** A431 cells were treated with 0 μM (○, control), 0.1 μM (△), 0.25 μM (▲), 0.5 μM (■), 1.0 μM (□) and 2.5 μM (●) UCN-01 for the times indicated. **B** The cells were treated with 0 μM (○, control), 0.005 μM (△), 0.01 μM (▲), 0.025 μM (■), 0.05 μM (□), 0.1 μM (●) staurosporine for the times indicated

Then the cells were labeled with ^{3}H-TdR (0.5 μCi/ml; Amersham, UK) for 1 h at 37 °C and washed once with PBS(–). The radioactivity incorporated into the 0.5% (W/V) trichloroacetic acid (TCA)-insoluble fraction was counted in a liquid scintillation counter (Packard, Meriden, Conn.) after solubilization with 1 *N* NaOH.

Results

Inhibition of cell growth

UCN-01 and staurosporine inhibited the growth of A431 cells in a concentration-dependent manner (Fig. 1). IC_{50} (50% growth inhibitory concentration) of each drug was calculated: 0.26 μM for UCN-01 and 0.0058 μM fo staur-

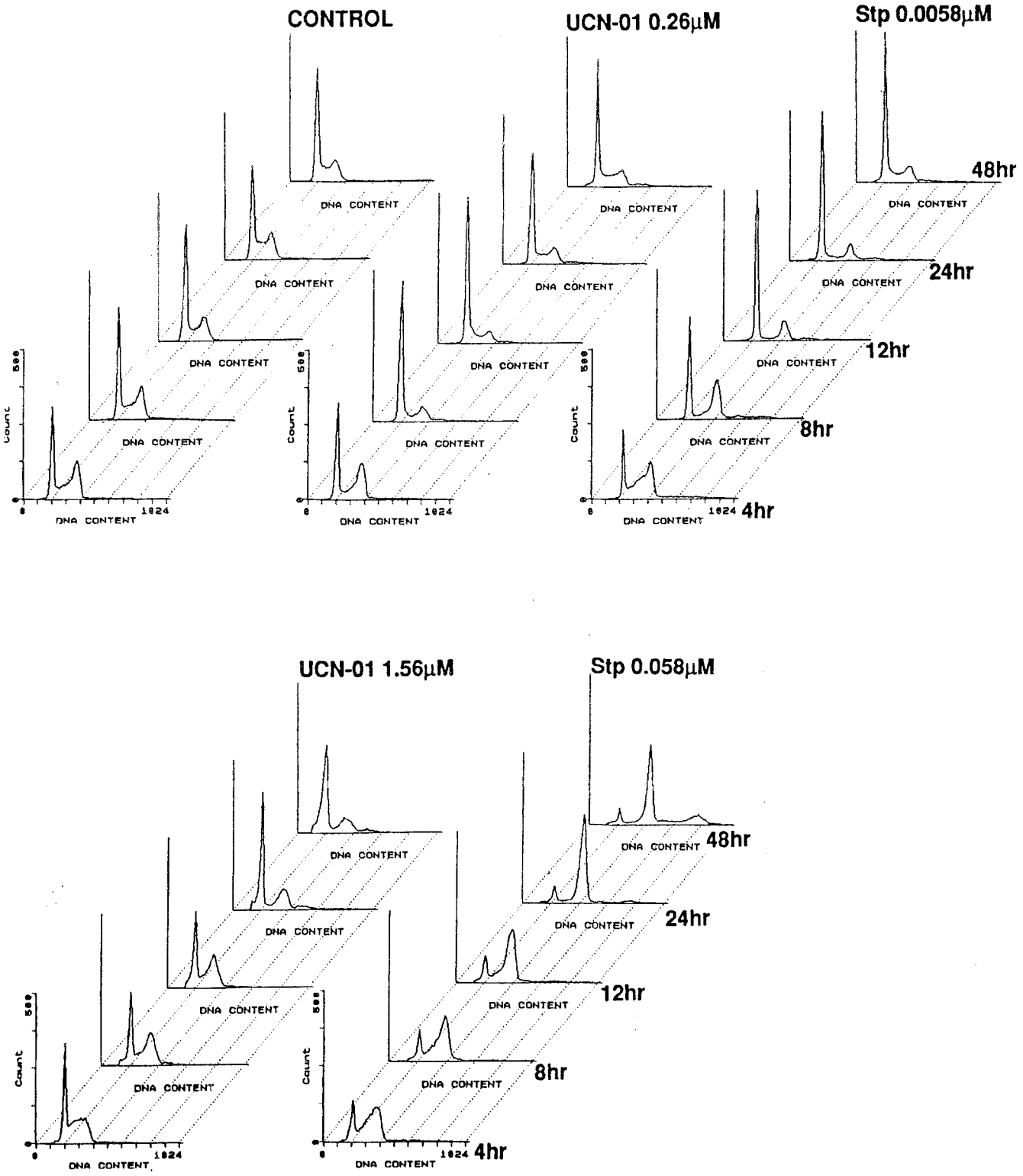

Fig. 2. DNA histogram of A431 cells treated with UCN-01 or staurosporine. A431 cells (3×10^5 cells/20 ml per dish) were cultured on day 0, and treated with UCN-01 (0.26 or 1.56 μM) or staurosporine (0.0058 or 0.058 μM) on day 1. The cells were fixed for flow-cytometric analysis 4, 8, 12, 24 and 48 h after the addition of the drugs. DNA histograms were obtained by flow cytometry as described in "Materials and methods"

osporine. The study of UCN-01 and staurosporine on the cell cycle distribution of A431 cells was performed at the IC_{50} value of each drug and at around 80% growth inhibitory concentration (IC_{80}) of each (6 times the IC_{50} for UCN-01 and 6 or 10 times the IC_{50} for staurosporine).

DNA content analysis by PI staining

A DNA histogram of A431 cells treated with UCN-01 or staurosporine is shown in Fig. 2. Cells treated with UCN-01 at the IC_{50} (0.26 μM) showed an accumulation in G1 phase

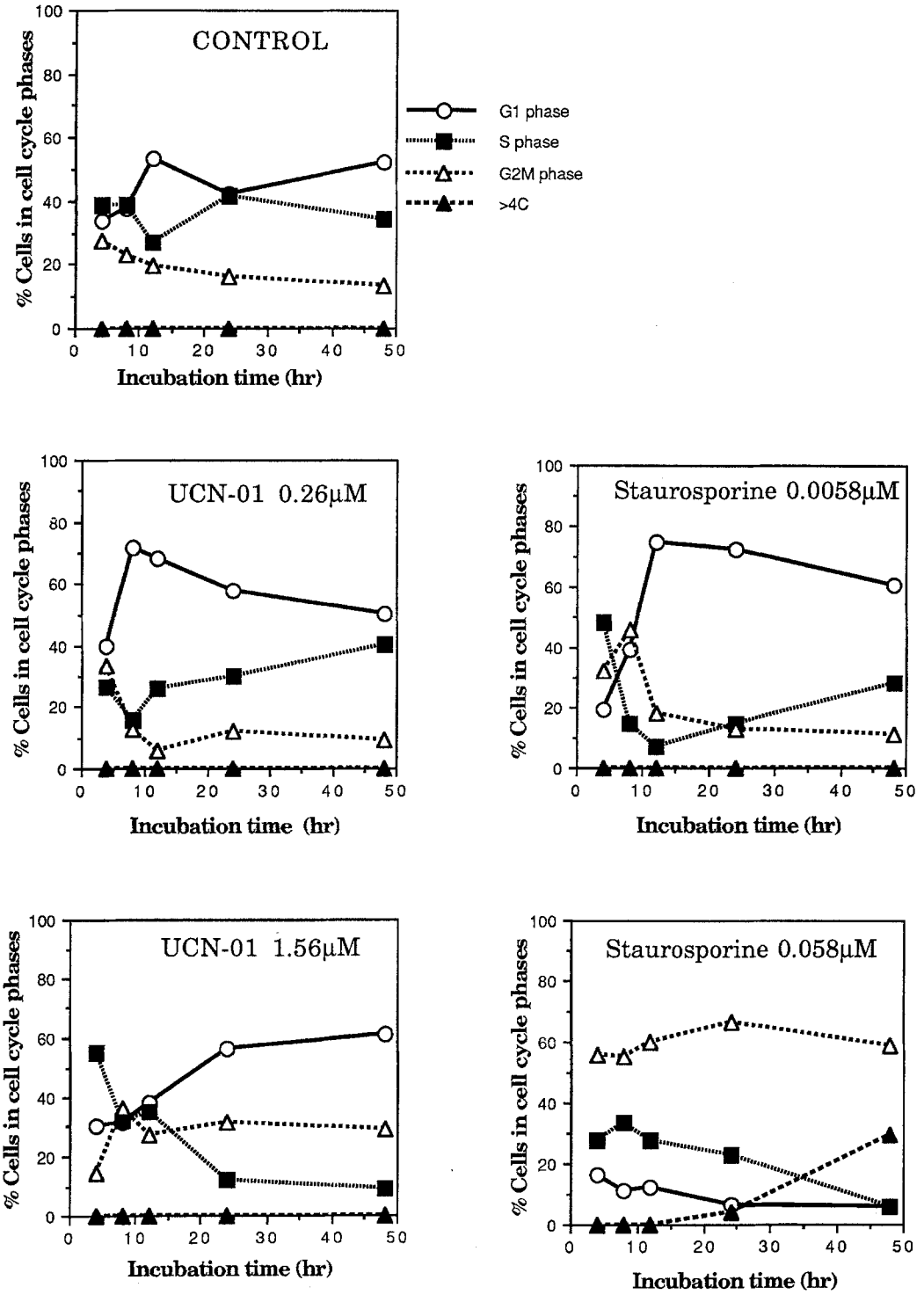

Fig. 3. Effect of UCN-01 or staurosporine on the cell cycle kinetics of A431 cells. The cell cycle distributions in the DNA histograms described in Fig. 2 were calculated by a MULTI CYCLE program

of the cell cycle, which was detected at 8 h after the addition of the drug, and persisted for at least 24 h. Treatment of the cells with the IC_{80} of UCN-01 (1.56 μM) induced a transient delay of G2M progression of the cell cycle from 8 h to 12 h after the drug addition, and then caused a G1 block thereafter. In the case of staurosporine, at the IC_{50} (0.0058 μM) it exerted a transient G2M progression delay from 4 h to 8 h after addition of the drug and then arrested the cells in G1 phase of the cell cycle. In contrast, at the IC_{80} (0.058 μM), staurosporine exhibited a profound G2M block of the cell cycle from 12 h to 24 h. In addition, when the cells were exposed to the drug for more than one generation time of A431 cells (>16 h), a higher DNA ploidy without mitosis was detected. This phenomenon was never detected in the cells treated with UCN-01 at either concentration. These cell cycle effects and the relative cell

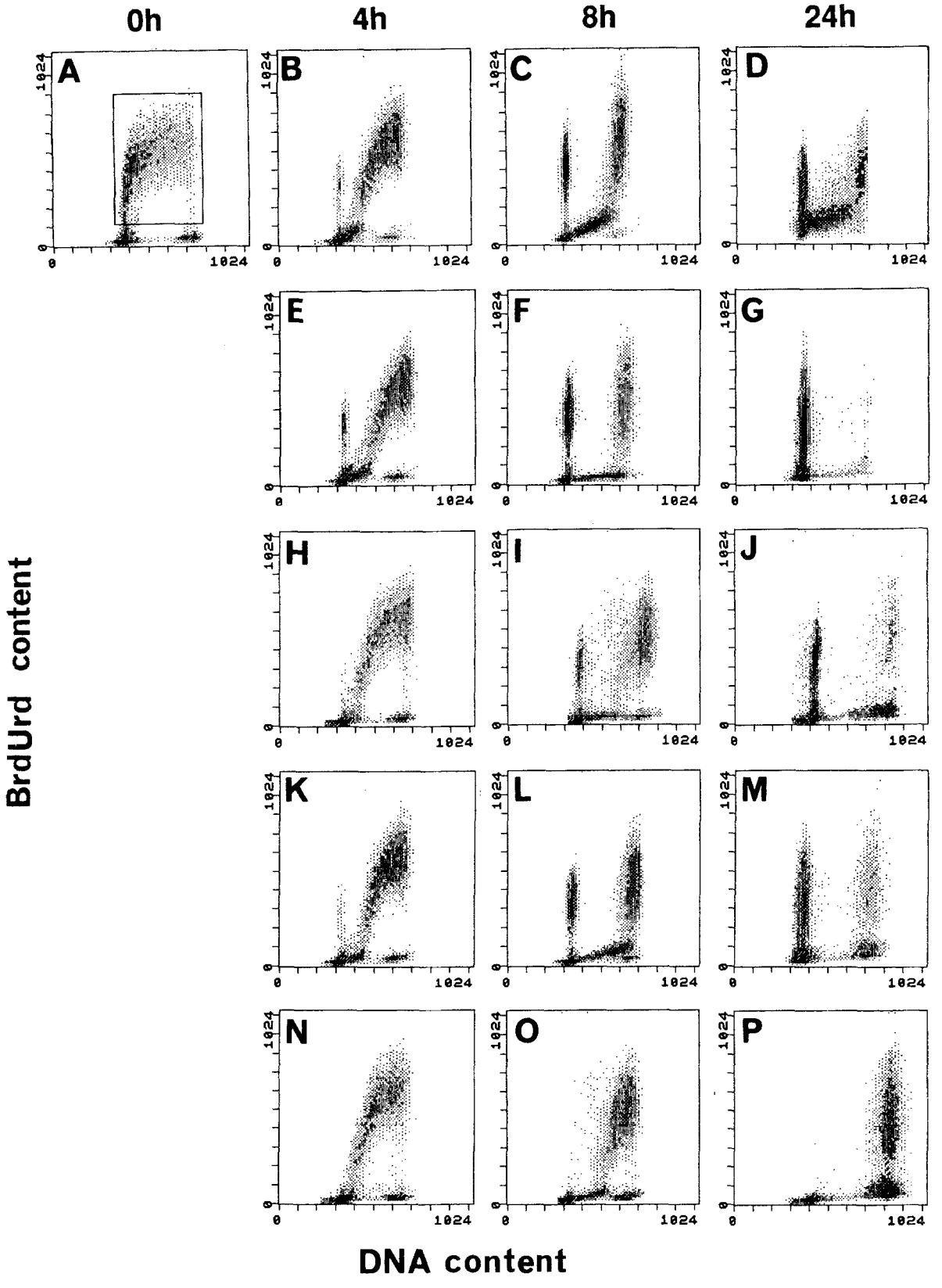

Fig. 4A–P. Effect of UCN-01 and staurosporine on the kinetics of DNA/BrdUrd distribution contour plot cytograms. A431 cells (3×10^5 cells/20 ml per dish) were cultured on day 0, and labeled with BrdUrd for 30 min on day 1. Then the labeled cells were exposed to growth medium (B~D) or UCN-01 (0.26 [E~G] or 1.56 [H~J] μM) or staurosporine (0.0058 [K~M] or 0.035 [N~P] μM) for 4–24 h. A bivariate cytogram for BrdUrd/DNA double-stained contour plots was obtained by a flow cytometry as described in "Materials and methods"

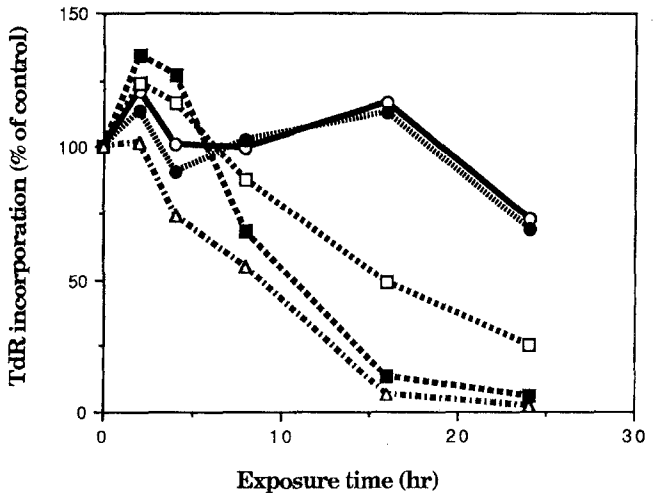

Fig. 5. Kinetics of inhibition of DNA synthesis by UCN-01 on A431 cells. A431 cells (1×10^5 cells/ml per well) were cultured in 24-well dishes on day 0, and treated with 0.05 μM (○), 0.1 μM (●), 0.25 μM (□), 0.5 μM (■) and 1.0 μM (△) of UCN-01 for 0–24 h on day 1. Then the cells were labeled with 3H-TdR for 1 h, and the radioactivity incorporated into the 0.5% trichloroacetic acid-insoluble fraction was counted as described in "Materials and methods"

kinetics were supported by the cell cycle phase analysis using a MULTICYCLE program as shown in Fig. 3.

Cell cycle analysis by BrdUrd/DNA double staining

To further investigate the effects of UCN-01 and staurosporine on the cell cycle perturbations, the cell cycle kinetic was studied by means of BrdUrd/DNA double staining. A typical progression pattern of the cell cycle of untreated A431 cells throug 0 to 24 h is shown in the cytograms at the top of Fig. 4. As time progressed, the S-phase cells moved into a G2M phase of the cell cycle at around 8 h and some of the population were already passing into a G1 phase at this time (Fig. 4B, C). The cells treated with the IC_{50} of UCN-01 moved into a G2M phase through 8 h (Fig. 4E and F) to 12 h (data not shown), in precisely the same manner as untreated cells, and were thereafter arrested in the G1 phase of the cell cycle by 24 h (Fig. 4G). Treatment of the cells with the IC_{80} of UCN-01 caused a transient delay in cell cycle progression at 8 h (Fig. 4I) and then exerted a G1 arrest through the mitotic phase by 24 h (Fig. 4J).

On the other hand, the cells treated with staurosporine at the IC_{50} moved into the G2M phase by 8 h in a similar way to untreated control cells (Fig. 4K), and were arrested in the G1 phase by 24 h except for a few populations arrested in the G2M phase (Fig. 4M). This G1 phase arrest is quite similar to that seen with UCN-01. However, the cells treated with around the IC_{80} value of staurosporine were blocked in the G2M phase by 8 h (Fig. 4O), and were thereafter halted in their progression through cell cycle at the G2M phase. This phenomenon was never seen in the cells treated with either concentration of UCN-01. These cell cycle kinetic patterns caused by UCN-01 and staurosporine supported the results of DNA histogram in Figs. 2 and 3.

Kinetics of inhibition of DNA synthesis by UCN-01

For further assessment of the effect of UCN-01 on the S phase cells, the kinetics of inhibitory effect of UCN-01 on ^{3}H-TdR incorporation into A431 cells was determined. As shown in Fig. 5, UCN-01 inhibited the DNA synthesis of A431 cells in a time-dependent manner. Even at the highest dose examined (1.0 μM), UCN-01 did not affect the DNA synthesis of A431 cells until 4 h after the addition of the drug. At lower doses (0.05–0.25 μM), UCN-01 did not inhibit the DNA synthesis within one generation time (16 h) of the cell cycle.

Discussion

This study clearly demonstrates that UCN-01 (7-hydroxystaurosporine) and staurosporine can affect the cell cycle of A431 cells in quite different manners, at least when used at their 80% growth inhibitory concentrations.

UCN-01, a more selective PKC inhibitor than staurosporine [4, 20, 22], induced transient G1 phase accumulation at the IC_{50} (0.26 μM) (Figs. 2, 3). Under the same condition staurosporine, a nonselective protein kinase inhibitor [22, 23], at 0.0058 μM also caused G1 phase arrest of the cells in a similar manner to UCN-01 (Figs. 2 and 3), as previously reported by others [1, 6, 7, 25]. In addition, staurosporine brought about a transient G2M delay (Fig. 3), which was barely detected in the cells treated with UCN-01 (Fig. 3).

At higher concentrations near IC_{80}, UCN-01 and staurosporine showed quite different effects on the cell cycle perturbations of A431 cells. UCN-01 (1.56 μM) blocked the cell cycle in the G1 phase through mitosis by 24 h (Figs. 2, 3). In marked contrast to this, staurosporine, under the same condition (0.058 μM), blocked the cell cycle in the G2M phase by 24 h (Figs. 2, 3), as previously reported in other cell lines [1, 6, 7, 25]. In addition to this, staurosporine caused the appearance of some cells with higher DNA ploidy, which was reported previously in leukemic cells [6]. These results suggest that UCN-01 and staurosporine could affect the cell cycle of A431 cells in different manners.

Then we analyzed the effect of UCN-01 and staurosporine on the cell cycle progression of BrdUrd-labeled A431 cells (early S phase cells) by a double staining method. At the IC_{50}, the BrdUrd-labeled cells treated with UCN-01 or staurosporine went through all the S phase and the G2M phase within 8 h after the treatment in a similar manner to control cells (Fig. 4B, C (vs) E, F, K, L). Interestingly, these cells entering G1 never progressed to the S phase of the second generation from 16 h (data not shown) to 24 h (Fig. 4G, M). This suggests that cell cycle progression during S, G2 and M phases is not affected by either drug at the IC_{50}, at least for one generation. These results were supported by time-course analysis of ^{3}H-TdR incorporation (Fig. 5).

At concentrations near IC_{80}, UCN-01 and staurosporine showed quite different effects on the cell cycle progression of A431 cells (Fig. 4H–J, N–P). Through 8 h to 24 h, all the BrdUrd-labeled cells treated with staurosporine were completely blocked at the G2M phase (Fig. 4N–P), while

those pretreated with UCN-01 entered the second G1 phase but could not enter the next S phase. These results indicate that UCN-01 and staurosporine may affect progression of A431 cells from the G2M phase in quite different manners in these conditions.

The G1 phase block induced by UCN-01 at a higher concentration is unique for this class of indolocarbazole compounds; all other indolocarbazole compounds, e.g., K252a [25], RK-286C [25], RK-1409 [18], KT5926 [9], and including staurosporine [1, 6, 7, 25], were shown to induce apparent G2M blocks in the target cells at higher concentrations. The G2M block induced by these compounds was thought to be due, at least in part, to the inhibition of cdc2 kinase [8, 25]. The effect of UCN-01 on cdc2 kinase is still unknown.

Then why does UCN-01 cause the apparent G1 phase block in A431 cells and what is critical to the G1 phase block by UCN-01? The exact answer to this question is also not yet known. However, there are several possible targets of UCN-01 and the lower concentration of staurosporine in the G1 phase: (1) cdk2 Kinase, which is reported to play an important role in G1 to S transition of the cell cycle [12, 13, 16]. (2) nPKCs, which have recently been reported to be involved in the G0/G1 to S phase transition of rat 3Y1 cells [17]: UCN-01 and staurosporine are shown to be potent inhibitors of this class of PKCs (K. Mizuno, S. Ohno, T. Tamaoki, K. Suzuki, submitted for publication). (3) Mitogen-activated protein kinases (MAPK), which are shown to be activated in response to TPA and other growth factors [11, 19, 24]; staurosporine was shown to inhibit MAP kinase from EL-4 lymphoma cells [15]. (4) Other unknown protein kinases that might be inhibited by indolocarbazole compounds reported to induce G1 phase accumulation [9].

As we have previously shown, UCN-01, but not staurosporine, displayed antitumor activity against three human xenografts (including A431 cells) and two murine tumor models, all of which possessed certain abberations in cellular signal transduction systems [4]. These results suggest that the G1 phase block, but not the G2M phase block, induced by UCN-01 is important for its potent antitumor activity in vivo. However, there could be other possible explanations for the ineffectiveness of staurosporine in vivo, including differences in pharmacokinetics. Several anticancer agents were reported to exhibit a G1 or G1/S phase accumulation depending on their concentrations [5]. However, at least to our knowledge [5], few anticancer agent cause G1 block, irrespective of the concentration, like UCN-01 in this study. These results, taken together with the unique mechanism(s) of action of UCN-01, suggest that UCN-01 could be used in combination with various anticancer agents. In fact, we reported that UCN-01 showed synergistic combined effects with various anticancer agents on A431 cells in vitro [3] and with mitomycin C in vivo [2]. In addition, this combined effect was confirmed as a specific effect on the cell cycle that is apparently different from the effect of either UCN-01 or the anti-cancer agent alone [2, 3].

In conclusion, our experimental results show that UCN-01 affects the cell cycle of A431 cells in a completely different manner from staurosporine. In addition, the preferential G1 (or G1/S) phase block induced by UCN-01 is thought to be important for its antitumor activity. However, further studies are needed to uncover the mechanism(s) of the G1 arrest by UCN-01.

Acknowledgements. We are grateful to Miss Shinobu Koyama and Miss Yuka Watanabe for their excellent technical assistance. We thank Drs. Tadashi Hirata and Tatsuya Tamaoki for their suggestions and encouragement during the studies.

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