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Preclinical paper

Differential effects of UCN-01, staurosporine and CGP 41 251 on cell cycle progression and CDC2/cyclin B_1 regulation in A431 cells synchronized at M phase by nocodazole

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UCN-01 (7-hydroxystaurosporine) and CGP 41 251 (4′-*N*-benzoyl staurosporine), both of which were discovered as protein kinase C selective inhibitors, have entered in phase 1 clinical trials as anti-cancer drugs. In this study, we have directly compared the effects of these drugs as well as staurosporine (STP) on cell cycle progression of A431 human epidermoid carcinoma cells synchronized at M phase by treatment with nocodazole. The nocodazole-synchronized cells progressed from M to G_1 phase in the absence of the drug, which was accompanied by a decrease of cyclin B_1 protein expression, disappearance of the complex formation of CDC2 with cyclin B_1 and reduction of the kinase activity. Treatments of the M phase cells with UCN-01, STP and CGP 41 251 at 80% growth-inhibitory concentrations (IC_{80}s) resulted in specific G_1 block, G_2M block and polyploidy, respectively. Decrease of cyclin B_1 protein expression was partially prevented by treatments with STP and CGP 41 251 but not with UCN-01 at IC_{80}s. Reductions of active complex and kinase activity of CDC2/cyclin B_1 were also observed in the presence of the three drugs. In addition, augmentation of CDC2 protein tyrosine phosphorylation was induced only when the cells were treated with STP. These observations demonstrated that higher concentrations of UCN-01, STP and CGP 41 251 showed different effects on cell cycle progression as well as CDC2/cyclin B_1 regulation in A431 cells synchronized at M phase. The data suggest that UCN-01 and CGP 41 251 may act at quite different points on the cell cycle.

Key words: Cell cycle, CDC2, CGP 41 251, cyclin B1, staurosporine, UCN-01.

Introduction

Since our re-discovery of staurosporine (STP) as a potent inhibitor of protein kinase C (PKC),[1] a lot of novel potent and highly selective PKC inhibitors have been generated from the chemical lead provided by STP.[2] Among these novel PKC selective inhibitors, UCN-01 (7-hydroxystaurosporine) and CGP 41 251 (4′-*N*-benzoyl staurosporine) are presently in phase 1 clinical trials as anti-cancer agents.[3,4] See Figure 1.

UCN-01 was originally isolated from the culture broth of *Streptomyces* sp. as a PKC selective inhibitor.[5,6] The compound was found to exhibit potent antitumor activity in several human tumor xenograft models as a single agent and in combination with pre-existing DNA-damaging agents and anti-metabolites *in vitro* and *in vivo*.[7–9] Further studies revealed that UCN-01 showed G_1 accumulation in several human tumor cell lines tested irrespective of its concentration,[10–12] and that it induced apoptosis in leukemia and colon carcinoma cell lines.[13,14] Until recently, however, the true mechanism of action of antitumor activity of UCN-01 remains to be elucidated.

CGP 41 251 was synthesized from STP as a PKC selective inhibitor and was also shown to exert antitumor activity as a single agent and in combination with adriamycin.[15,16] Flow cytometric analysis revealed that CGP 41 251 preferentially induced G_2M arrest and DNA polyploidy in target tumor cells.[17–19] However, it should be also pointed out that the true mechanism of action of CGP 41 251 was as uncertain as that of UCN-01.

Previous studies of STP, a non-selective protein kinase inhibitor without any antitumor activity *in vivo*, on cell cycle regulation revealed that the compound showed G_1 accumulation at a lower concentration around 10 nM, and exerted preferential G_2M arrest and/or DNA polyploidy at higher concentrations than 50 nM.[11,20–23] Altogether, these studies on cell cycle regulation with STP, UCN-01 and CGP 41 251 suggested that lower concentrations of STP might

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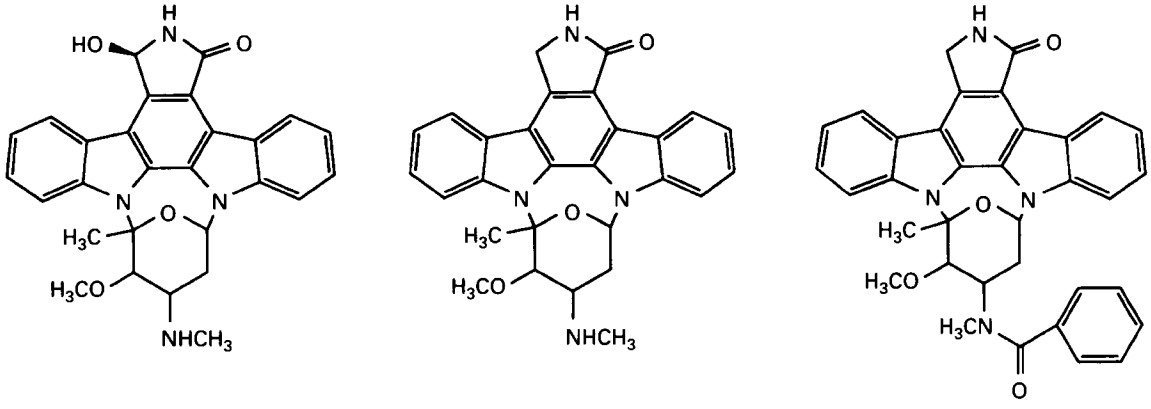

Figure 1. Chemical structures of UCN-01, STP and CGP 41 251.

have common features of mechanism of action with UCN-01 and that higher concentrations of STP might exert the same effect as CGP 41 251.

Cell cycle progression in mammalian cells is regulated by members of a cyclin-dependent kinase (CDK)/cyclin family.[24] G_1 to S phase progression is regulated by CDK4/cyclin D, CDK6/cyclin D and CDK2/cyclin E through phosphorylation of their target, the retinoblastoma (Rb) protein. G_1 to S phase progression is also regulated by CDK inhibitor proteins which bind to and inactivate CDKs. CDK inhibitor proteins are divided into two groups, a CDK4/CDK6-specific inhibitor group including $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, and a universal inhibitor group including $p21^{Cip1/WAF1}$, $p27^{Kip1}$ and $p57^{Kip2}$. Recently, G_1 accumulation by STP at a lower concentration has been shown to be mediated through inhibitions of CDK2 activity and Rb protein phosphorylation in tumor cells.[25-27] In addition, we have also shown that G_1 accumulation induced by UCN-01 at about 50% growth-inhibitory concentration (IC_{50}) was mediated through inhibition of CDK2 activity and Rb protein phosphorylation in A431 human epidermoid carcinoma cells.[28]

G_2 to M phase progression is regulated by one of the members of a CDK/cyclin family, CDK1 (CDC2)/cyclin B, which has been reported to target proteins such as histone H1, lamins and p53.[29] CDC2 protein, which is constitutively expressed throughout the cell cycle, is activated by binding to its partner cyclin B during S phase.[24] The kinase activity of CDC2/cyclin B complex is suppressed by phosphorylation at Tyr15 of CDC2 protein by WEE1 kinase during S to G_2 phase.[30,31] Dephosphorylation of the Tyr15 by CDC25C phosphatase at M phase leads to activation of CDC2.[32,33] From M to G_1 phase, CDC2 kinase activity is reduced concomitantly with a rapid degradation of its partner cyclin B protein and this degradation is thought to be a rate limiting factor for M to G_1 phase progression.[34-36]

To directly assess the differential aspects of STP, UCN-01 and CGP 41 251 on cell cycle regulation, we have examined the effects of these drugs on cell cycle progression of A431 cells synchronized at M phase by pretreatment with nocodazole. In addition, we have examined the effects of the compounds on the kinase activity, protein expression level and complex formation of CDC2/cyclin B1.

Materials and methods

Reagents

UCN-01 and STP were produced by fermentation in our institute as described previously.[6] CGP 41 251 was synthesized in our institute. The drugs were dissolved in dimethyl sulfoxide (DMSO), and freshly diluted with cell culture medium [Dulbecco's modified Eagle medium (DMEM); Life Technologies, Rockville, MD; and 10% fetal bovine serum; Life Technologies]. Tris and Tween 20 were purchased from BioRad (Hercules, CA). NaCl and sodium fluoride were from Kanto Chemical (Tokyo, Japan). EDTA and dithiothreitol were from Wako Pure Chemical (Osaka, Japan), and Triton X-100 was from Yoneyama Yakuhin Kogyo (Osaka, Japan). HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), β-glycerophosphate, sodium *o*-vanadate, phenylmethylsulfonyl fluoride, aprotinin, leupeptin and the other reagents were purchased from Sigma (St Louis, MO).

Antibodies

Anti-cyclin B_1 and anti-CDC2 monoclonal antibodies and anti-CDC25C polyclonal antibody were purchased from Santa Cruz (Santa Cruz, CA). Anti-phosphotyrosine monoclonal antibody was obtained from Kyowa Medex (Tokyo, Japan).

Cell culture

Human epidermal carcinoma, A431 was obtained from ATCC through Dainippon Pharmaceutical (Osaka, Japan). The cell cultures were performed at 37°C in a humidified atmosphere of 5% CO_2.

Cell cycle analysis

A431 cells (3×10^5/dish) were cultured in DMEM with 10% fetal bovine serum in Falcon 3003 plastic dishes (Becton Dickinson, Lincoln Park, NJ) overnight and treated with 0.5 μg/ml nocodazole for 16 h. The nocodazole-pretreated cells were washed with phosphate-buffered saline (PBS) twice and cultured in fresh medium with or without drug. After the indicated time, the cells were harvested by treatment with 0.25% trypsin and fixed with ice-cold 70% ethanol solution. The fixed cells were hydrolyzed with 250 μg/ml ribonuclease A (type 1-A; Sigma) at 37°C for 30 min and stained with propidium iodide (Sigma) for 20 min. The DNA content of the cells was analyzed by an Epics Elite flow cytometer (Coulter, Hialeah, FL). The cell cycle distribution was calculated by a Multicycle program (Coulter).

Immunoprecipitation and kinase assay

For cyclin B_1-associated kinase assay in a cell-free system, nocodazole-pretreated A431 cells were harvested by treatment with 0.25% trypsin, washed with PBS and stored at −80°C. The cells were lysed in lysis buffer (50 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM dithiotreitol, 50 mM sodium fluoride, 50 mM β-glycerophosphate, 1 mM sodium *o*-vanadate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin) for 20 min at 4°C. The cell lysate was clarified by centrifugation at 15 000 *g* for 10 min at 4°C and its protein content was determined using the protein assay kit (BioRad). Then 2 mg of protein was added to 1.5 ml of Protein A-Sepharose CL-4B (20% v/v; Pharmacia, Uppsala, Sweden) preassociated with anti-cyclin B_1 antibody and mixed gently for 2 h at 4°C. The immunoprecipitate was washed with lysis buffer twice and wash buffer (50 mM HEPES/NaOH, pH 7.4, 10 mM $MgCl_2$ and 1 mM dithiothreitol) two times and separated into 15 tubes. Cyclin B_1-immunoprecipitate was mixed with 40 μl of wash buffer containing 16 μg of histone H1 (Boehringer, Mannheim, Germany), 50 μM ATP, 2.5 μCi [γ-^{32}P]ATP (5000 Ci/mmol; Amersham, Amersham, UK) and various concentrations of drugs in DMSO and incubated for 10 min at 30°C. Each sample was mixed with 30 μl of 3 × SDS sample buffer[37] to stop the reaction, heated for 5 min at 95°C and subjected to SDS-polyacrylamide gel electrophoresis. The gel was dried, stained with Coomassie brilliant blue and analyzed by a BAS2000 image analyzer (Fuji Photo Film, Tokyo, Japan). For cyclin B_1-associated kinase assay in culture, A431 cells were pretreated with nocodazole for 16 h and with drugs for 3 h, and harvested and lysed as described above. Each cyclin B_1 immunoprecipitate was prepared from 200 μg of protein of the lysate and incubated in the reaction mixture as described above but not containing drugs for 10 min at 30°C. The following analyses were performed as described above.

Western blotting

Whole cell lysate or cyclin B_1 immunoprecipitate was heated in 1 × SDS sample buffer for 5 min at 95°C and subjected to SDS-polyacrylamide gel electrophoresis. The protein was transferred to a P-membrane (Atto, Tokyo, Japan), probed with primary antibodies followed by secondary antibodies conjugated with horseradish peroxidase (Amersham), and detected by the enhanced chemiluminescence system (Amersham).

Results

Effects of UCN-01, STP and CGP 41 251 on cell cycle progression of A431 cells synchronized at M phase

As shown in Figures 2 and 3, A431 cells were synchronized at M phase by pre-incubating with 0.5 μg/ml of nocodazole for 16 h. Analysis of cell cycle distribution with a multicycle program revealed that more than 80% of the cells were arrested in M phase in every experiment. After intensive washing of the drug and re-addition of fresh medium, the cells progressed into G_1 phase within 3 h and entered into S phase thereafter at 6–9 h (Figures 2 and 3). There was

a substantial difference in the degree of synchronization and the timing for S phase entry between experiments (Figures 2 and 3, and data not shown).

Addition of UCN-01 just after the nocodazole release of the cells resulted in preferential G_1 block, irrespective of its concentration [at IC_{50} of 260 nM and 80% growth-inhibitory concentration (IC_{80}) of 1560 nM, respectively] (Figure 2), which is essentially consistent with our previous study using asynchronous A431 cells.[11] The same treatment of the cells with STP exhibited G_1 block at IC_{50} (5.8 nM); however, the drug caused apparent G_2M block at IC_{80} (58 nM) (Figure 2). These results are also consistent with our previous study with the same asynchronous cells.[11] In another set of experiments, addition of CGP 41 251 after nocodazole release of the cells resulted in transient G_2M arrest within 6 h followed by induction of polyploidy thereafter at

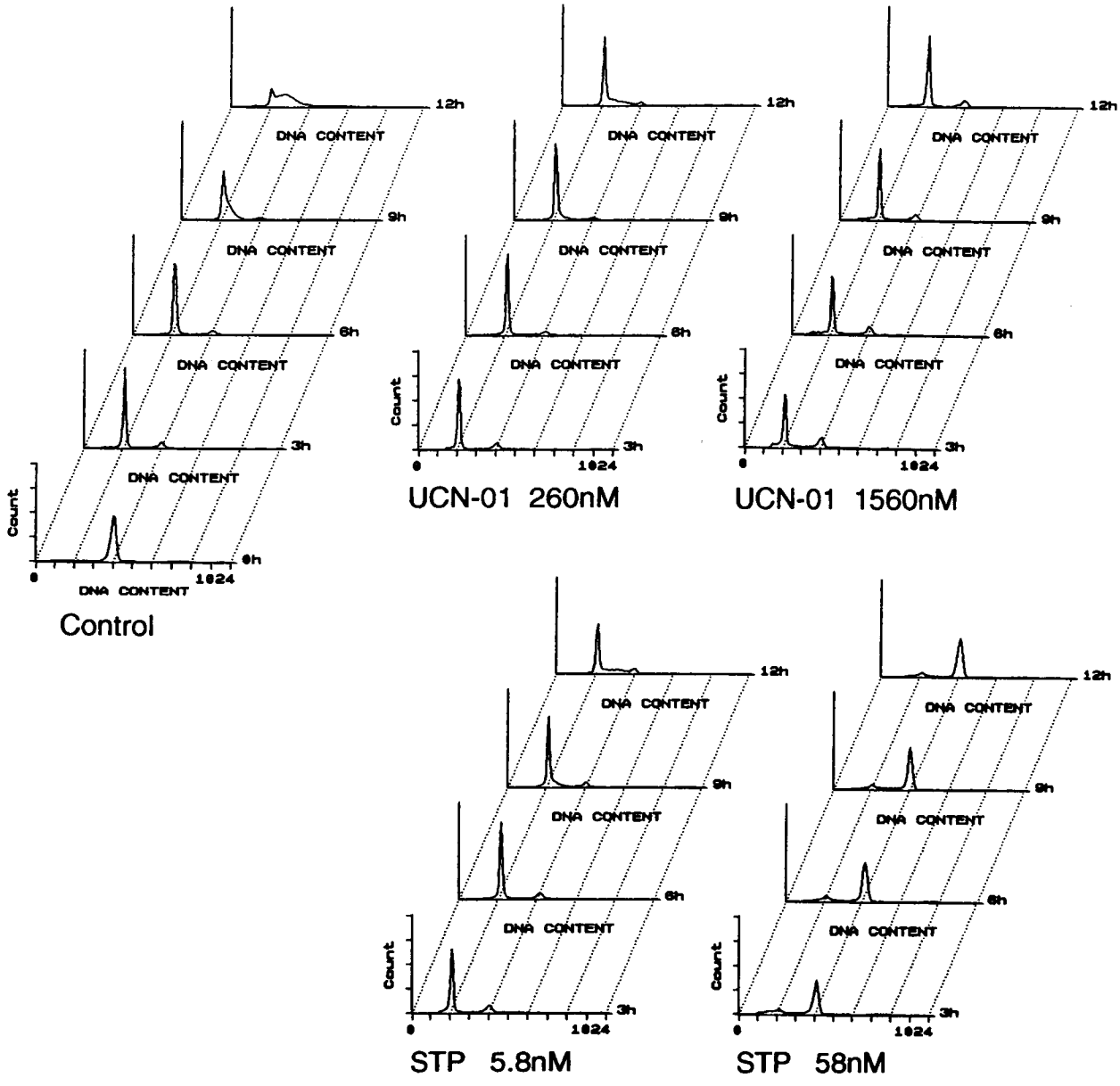

Figure 2. Effects of UCN-01 and STP on cell cycle progression of M-arrested A431 cells. Cells were synchronized at M phase by treatment with nocodazole, washed with PBS, and cultured in fresh medium containing UCN-01 and STP for the indicated times. Cell harvest and cell cycle analysis were performed as described in Materials and methods.

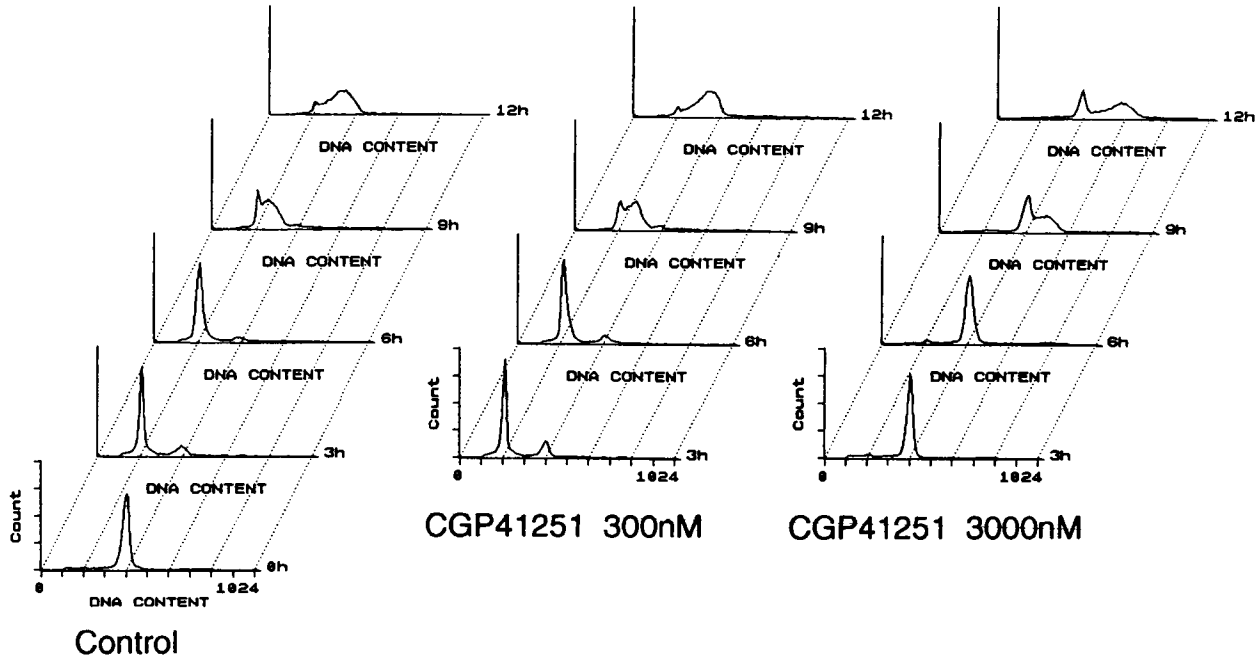

Figure 3. Effect of CGP 41 251 on cell cycle progression of M-arrested A431 cells. Cells were synchronized at M phase by treatment with nocodazole, washed with PBS and cultured in fresh medium containing CGP 41 251 for the indicated times. Cell harvest and cell cycle analysis were performed as described in Materials and methods.

IC_{80} (3000 nM), while the drug at IC_{50} showed no effect on cell cycle progression of the cells (Figure 3). These results clearly demonstrated that UCN-01 and CGP 41 251, both of which are known as PKC selective inhibitors, showed quite different effects on cell cycle progression from M to G_1 phase in A431 cells at both IC_{50} and IC_{80}. In addition, the effect of lower concentrations of STP on cell cycle progression of A431 cells might resemble that of UCN-01, while higher concentrations of STP exhibited a unique G_2M block which was never seen in UCN-01- and/or CGP 41 251-treated cells.

Inhibitory effects on cyclin B_1-associated kinase activity in a cell-free system

Cell cycle progression from M to G_1 phase is known to be strictly regulated by CDC2/cyclin B kinase and the reduction of the kinase activity is thought to be essential for the progression.[24,34-36] Since STP was reported to inhibit CDC2/cyclin B kinase in a cell-free system,[38,39] we have examined the effect of these indolocarbazole drugs on the kinase activity using a cell-free kinase assay. CDC2/cyclin B kinase was recovered from the nocodazole-treated A431 cells as cyclin B_1 immune complexes by an immunoprecipitation technique and its kinase activity was determined by [γ-^{32}P]ATP incorporation into histone H1 as a substrate. As shown in Figure 4, STP, UCN-01 and CGP 41 251 showed a concentration-dependent inhibition of cyclin B_1-associated kinase activity, giving 50% inhibitory concentrations of 25, 2800 and 31 000 nM, respectively. The inhibitory activity of STP is consistent with previous reports,[38,39] thus validating our assay system. As shown in Figures 2 and 3, 3 h after drug addition at IC_{80}, there is a clear difference between the effect induced by each drug, i.e. UCN-01-treated cells progressed into G_1 phase just like untreated control, STP-treated cells were blocked in G_2M phase and CGP 41 251-treated cells transiently arrested in G_2M phase. Our results suggested that the addition of 80% growth-inhibitory concentrations of STP, UCN-01 and CGP 41 251, i.e. 58, 1560 and 3000 nM, would inhibit cyclin B_1-associated kinase (which is probably CDC2/cyclin B_1 kinase) in the M-arrested A431 cells by 70, 40 and 20%, respectively. Regarding the effect of STP, the data suggested that the G_2M block induced by the drug might be mediated through the direct inhibition of the kinase before mitotic maturation. However, this was unlikely because UCN-01 showed no G_2M arrest under the condition that the drug inhibited 40% of cyclin B_1-associated kinase activity and CGP 41 251 exerted transient but apparent G_2M arrest by inhibiting only 20% of the kinase activity.

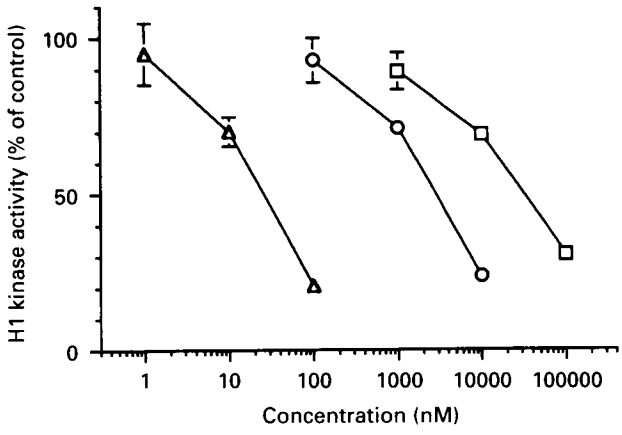

Figure 4. Effect of STP analogs on cyclin B_1-associated kinase activity in a cell-free system. Cyclin B_1 immunoprecipitates were prepared from nocodazole-pretreated A431 cells, and reacted with [γ-^{32}P]ATP, histone H1 and various concentrations of UCN-01 (○), STP (△) and CGP 41 251 (□) for 10 min at 30°C as described in Materials and methods. Means ± SD of triplicates in the experiment are shown as symbols and error bars, respectively.

These results suggested that there would exist another mechanism than direct inhibition of CDC2/cyclin B_1 kinase for G_2M arrest induced by CGP 41 251 as well as G_2M block induced by STP.

Cyclin B_1 and CDC2 protein expression in cells

Since cyclin B protein expression itself was reported to significantly regulate the G_2 to M phase cell cycle progression,[29,34] we determined the effects of these drugs on the expression level of cyclin B_1 as well as CDC2 protein in A431 cells pretreated with nocodazole for 16 h and then treated with IC_{80} of each drug for 3 h using Western blotting. As shown in Figure 5 (A and C), the expression level of cyclin B_1 protein in untreated control cells in G_1 phase (3 h after nocodazole release) was decreased by about 50% compared to its level in M phase. UCN-01 treatment did not affect this decrease of cyclin B_1 protein level; however, STP and CGP 41 251 treatments apparently blocked the decrease of the cyclin B_1 protein level which was different from control cells. The cyclin B_1 level in the cells pretreated with STP and CGP 41 251 remained as high as in M phase cells (Figure 5A and C). Thus, the decrease of cyclin B_1 protein level during M to G_1 phase progression was prevented by STP and CGP 41 251 but not UCN-01. These results suggested that G_2M block or arrest induced by STP or CGP 41 251 might be mediated at least in part through the inhibition of the degradation of cyclin B_1 protein in the cells. In contrast to its partner protein, i.e. cyclin B_1, the CDC2 protein level was not altered in the cells in the absence or presence of the drugs (Figure 5B and D). Although the amount of CDC2 protein analyzed in Figure 5(B) appeared to be excessive for loading to the gel, the application of less amount of the protein to the gel showed essentially the same result, in which only a lower band of doublet of the protein was detected (data not shown).

CDC2 tyrosine phosphorylation in cells

Previous reports have revealed that CDC2 kinase is inactivated by phosphorylation at Tyr15 by WEE1

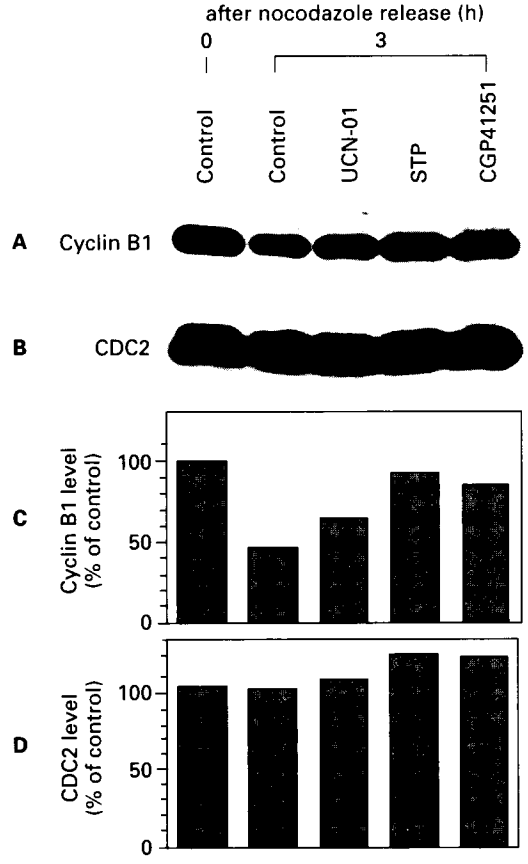

Figure 5. Effects of STP analogs on cyclin B1 and CDC2 protein expressions in M-arrested A431 cells. Cells were synchronized at M phase by treatment with nocodazole, and harvested at 0 h (Control) and after 3 h culture in the absence (Control) or presence of UCN-01 (1560 nM), STP (58 nM) and CGP 41 251 (3000 nM). Cell lysis and Western blotting were performed as described in Materials and methods. Cyclin B_1 (A) and CDC2 (B) proteins in whole cell lysate were detected by their specific antibodies. The quantitations of the proteins are shown in C (cyclin B_1) and D (CDC2).

kinase and that Tyr15-phosphorylated CDC2 protein shows the mobility shift of the band in an SDS-polyacrylamide gel from the slower to the faster one.[40,41] However, as described above and shown in Figure 5(B), Tyr15-phosphorylated and Tyr15-unphosphorylated forms of CDC2 protein were not clearly separable under our Western blotting conditions. To elucidate the effects of the drugs on the tyrosine phosphorylation state of CDC2 protein in the cells more precisely, we purified the kinase as cyclin B_1 immune complex from A431 cells pretreated with the drugs after nocodazole release and detected CDC2 protein in the immune complex using Western blotting. As shown in Figure 6(A), we could detect CDC2 protein in the cyclin B_1 immune complex as two major bands (top and bottom) and a single minor band (middle) in M phase cells. In untreated cells progressed into G_1 phase, the top band remained and the bottom band apparently disappeared (Figure 6A). This disappearance of the bottom band was also observed in the cells pretreated with all three drugs (Figure 6A). These results and the previous reports suggested that the top band and the bottom band of CDC2 protein in Figure 6(A) might be the Tyr15-phosphorylated inactive form and the Tyr15-unphosphorylated active form, respectively. The top band (which is probably inactive CDC2 kinase) in cells pretreated with STP was more intensive than that in cells pretreated with UCN-01 or CGP 41 251, or without the drugs (control) (Figure 6A), suggesting that Tyr15 phosphorylation of CDC2 kinase in A431 cells might be augmented by treatment with STP. Furthermore, this experiment also showed that the active form of CDC2 protein bound to cyclin B_1 in M-arrested A431 cells was dissociated from cyclin B_1 when the cells exited from M phase. In addition, this dissociation was also observed in the cells pretreated with the three drugs, although these drugs showed quite different cell cycle effects under this condition (Figures 2 and 3).

To confirm that the top band of CDC2 protein bound to cyclin B_1 is actually the tyrosine phosphorylated form, the same cyclin B_1 immune complex described above was further analyzed by Western blotting using anti-phosphotyrosine antibody. As shown in Figure 6(B), the tyrosine phosphorylated band was clearly detected as a single band corresponding to the top band of CDC2 protein in an SDS-polyacrylamide gel, confirming that the top band of CDC2 protein was the tyrosine phosphorylated form. Although this tyrosine phosphorylated band was also detected in all the cells irrespective of drug treatment, the most prominent tyrosine phosphorylated CDC2 protein band was detected in the cells pretreated with STP (Figure 6B). These results suggested that inactivation of CDC2 kinase through tyrosine phosphorylation could play an important role in STP-induced G_2M block but not in CGP 41 251-induced transient G_2M arrest.

CDC25C protein expression and phosphorylation in cells

It has previously been reported that CDC25C phosphatase which dephosphorylates Tyr15 of CDC2

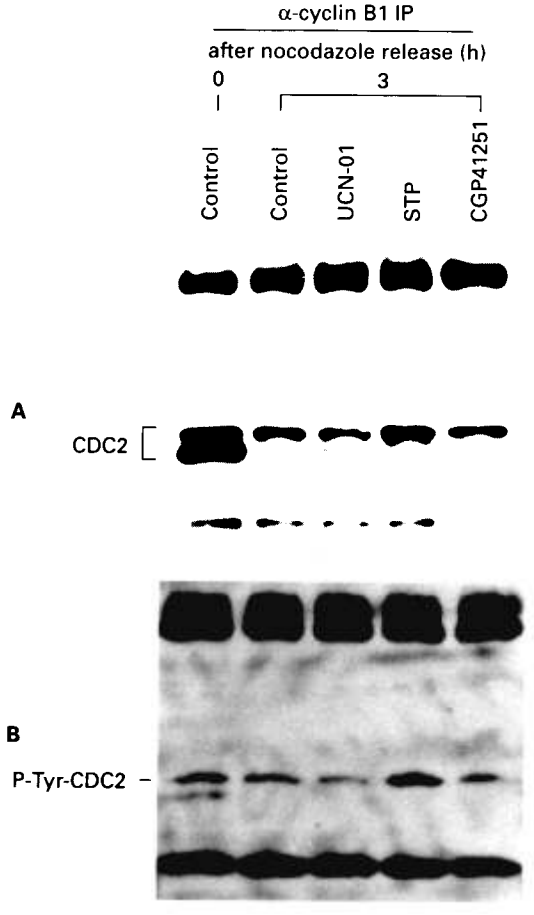

Figure 6. Effects of STP analogs on tyrosine phosphorylation of CDC2 protein bound to cyclin B_1. A431 cells were synchronized at M phase by treatment with nocodazole, and harvested at 0 h (Control) and after 3 h culture in the absence (Control) or presence of UCN-01 (1560 nM), STP (58 nM) and CGP 41 251 (3000 nM). Cyclin B_1 immunoprecipitates were prepared from the cells and analyzed by Western blotting as described in Materials and methods. Proteins in the cyclin B_1 immunoprecipitates were detected by anti-CDC2 (A) and anti-phosphotyrosine antibodies (B).

protein is activated by hyperphosphorylation.[42] To determine the effect of STP on protein expression and the phosphorylation state of CDC25C, we have detected the protein by Western blotting in A431 cells pretreated with STP for 3 h after nocodazole release. As shown in Figure 7, in a SDS-polyacrylamide gel, CDC25C protein was detected as the slower migrated several bands in M-arrested cells and the faster migrated single band in G_1-progressed control cells. These results were consistent with the previous report that CDC25C was hyperphosphorylated and activated at M phase, and dephosphorylated and inactivated during the other cell cycle phases.[42] The dephosphorylated form of CDC25C protein was detected in STP-pretreated cells (Figure 7), suggesting that CDC25C phosphatase might be inactivated in the cells. These results suggested that the inactivation of CDC25C phosphatase might contribute to the augmentation of tyrosine phosphorylation of CDC2 in the STP-pretreated and G_2M-blocked cells.

Cyclin B_1-associated kinase activity in cells

Furthermore, to elucidate the effects of UCN-01, STP and CGP 41 251 on cellular CDC2/cyclin B1 kinase activity, we determined the kinase activity as cyclin B1-associated kinase activity in the cells pretreated with the drugs at IC_{80}s for 3 h after nocodazole release. As shown in Figure 8, cyclin B_1-associated kinase activity in M-arrested A431 cells was very high and was substantially decreased after the release from nocodazole treatment by more than 80%. The kinase activity of the cells pretreated with UCN-01, STP and CGP 41 251 was also decreased in the same manner as untreated control cells (Figure 8). These results suggested that G_2M block induced by STP and transient G_2M arrest induced by CGP 41 251 might not be caused by prevention of reduction of cellular cyclin B_1-associated kinase activity which is a common feature of G_1-progressed cells. It also suggested that this reduction of the kinase activity in A431 cells in the absence or presence of the drugs might be caused by the decrease of the active form of the CDC2/cyclin B_1 complex and/or tyrosine phosphorylation of CDC2 (Figure 6).

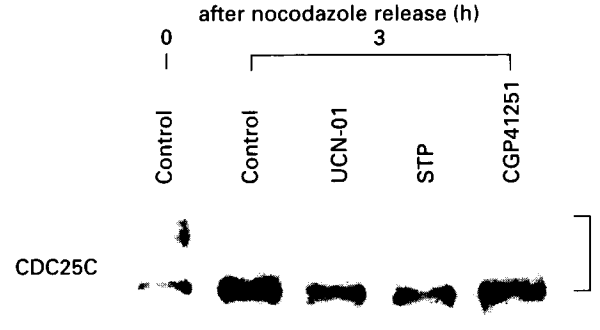

Figure 7. Effects of STP analogs on CDC25C protein expression in M-arrested A431 cells. Cells were synchronized at M phase by treatment with nocodazole, and harvested at 0 h (Control) and after 3 h culture in the absence (Control) or presence of UCN-01 (1560 nM), STP (58 nM) and CGP 41 251 (3000 nM). Cell lysis and Western blotting were performed as described in Materials and methods. CDC25C protein in whole cell lysate was detected by its specific antibody.

Discussion

In our study, we directly compared the effects of a non-selective protein kinase inhibitor (STP) and PKC-selective inhibitors (UCN-01 and CGP 41 251) on cell cycle progression as well as the regulation of CDC2/cyclin B_1 complex using M-arrested A431 cells. Our results clearly demonstrate that these three indolocar-

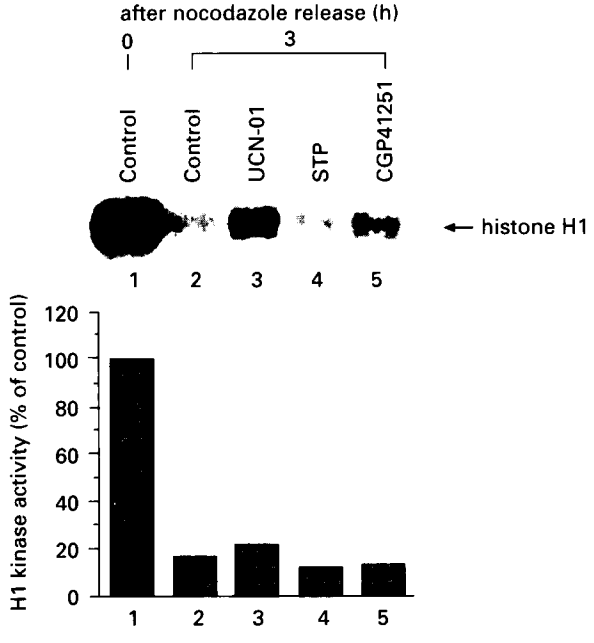

Figure 8. Effect of STP analogs on cyclin B_1-associated kinase activity in M-arrested A431 cells. Cells were synchronized at M phase by treatment with nocodazole, and harvested at 0 h (lane 1) and after 3 h culture (lanes 2–5) in the absence (Control, lanes 1 and 2) or presence of UCN-01 (1560 nM, lane 3), STP (58 nM, lane 4) and CGP 41 251 (3000 nM, lane 5). Cyclin B_1 immunoprecipitates were prepared from the cells, and reacted with [γ-^{32}P]ATP and histone H1 for 10 min at 30°C as described in Materials and methods.

bazole compounds show quite different effects on cell cycle progression and the regulation of CDC2/cyclin B_1 complex in nocodazole-pretreated A431 cells, despite their structural similarity.

At 50% growth-inhibitory concentration (IC_{50}), both STP and UCN-01 induced preferential G_1 blocks with little, if any, effect on M to G_1 phase progression in A431 cells, which was consistent with our previous study using asynchronous A431 cells.[11] On the other hand, CGP 41 251 at IC_{50} showed no effect on cell cycle progresion of M-arrested A431 cells, which was inconsistent with the previous reports using asynchronous A549 cells.[43] The reason for this discrepancy is presently uncertain; however, this could result from a difference in the method used or in the cell type, e.g. A431 cells harbor mutated p53 and A549 cells harbor wild-type p53. G_1 block induced by UCN-01 or STP has recently been reported to be mediated through the dephosphorylation of Rb protein by the direct inhibition of CDK2 as well as direct inhibition of the kinase through the induction of CDK inhibitor proteins, $p21^{Cip1/WAF1}$ and $p27^{Kip1}$.[25-28] Therefore, G_1 blocks induced by UCN-01 and STP at IC_{50}s in nocodazole-arrested A431 cells might be also mediated through the same mechanism of action.

At 80% growth-inhibitory concentration (IC_{80}), UCN-01 induced G_1 block in the same manner as at its IC_{50}. This G_1 block might also be mediated through the dephosphorylation of Rb protein, because UCN-01 at IC_{80} could induce the dephosphorylation of Rb protein and the induction of $p21^{Cip1/WAF1}$ protein in asynchronous A431 cells (data not shown). In contrast, CGP 41 251 at IC_{80} induced putative DNA synthesis without cytokinesis, i.e. polyploidy in the cells (Figure 3), which was also reported in other reports.[17-19] The mechanism of this inhibitory effect on cytokinesis is still unclear, although we examined the effects of CGP 41 251 on CDC2/cyclin B_1 in the cells in this study. On the other hand, STP at IC_{80} induced an apparent G_2M block that was in sharp contrast to its effect at IC_{50} (Figure 2), and this phenomenon is consistent with our previous report using asynchronous A431 cells and other reports using other cell lines.[11,20-23] The STP-pretreated G_2M-blocked cells might already be exited from M phase and progressed into G_1 phase, because the molecular markers such as the activity level of CDC2/cyclin B_1 kinase and phosphorylation state of CDC25C protein in the G_2M-blocked cells were consistent with those in G_1 phase cells but not in M phase cells. This idea was strongly supported by the report by Hall *et al.*[44] that a brief treatment of M-arrested cells with 100 ng/ml (approximately 0.21 μM) STP resulted in the induction of G_1 reset followed by polyploidy. The G_1-reset cells were continuously exposed to higher concentrations of STP in our experimental condition. There is a possibility that the G_1-reset and STP-exposed cells might be prevented from entry into S phase through the mechanism just like as at IC_{50} or the inhibitions of wide variety of protein kinases.[1,38] Taken together, G_2M block in M-arrested A431 cells after treatment with STP might be mediated through G_1 reset followed by G_1 block.

To elucidate the effects of UCN-01, STP and CGP 41 251 at 80% growth-inhibitory concentration (IC_{80}) on cell cycle machinery, we have determined the effects of these drugs on CDC2 and cyclin B_1 protein expressions in M-arrested A431 cells. Our results indicated that partial decrease of cyclin B_1 protein in the cells during the progression from M to G_1 phase in the absence of drug was prevented by treatments with higher concentrations of STP and CGP 41 251, but not UCN-01 (Figure 5A and C). Previous study reported that the degradation of cyclin B protein was required for CDC2 binding and for its kinase activity.[35] In our study, the complex formation of CDC2 with cyclin B_1 and its kinase activity were shown to be reduced in the cells pretreated with STP and CGP 41 251, suggesting that these effects might induce the inhibition of the degradation of cyclin B_1 protein in the cells. However, the complex formation of CDC2 with cyclin B_1 and the kinase activity were also shown to be reduced in the cells pretreated with UCN-01 and without drug, suggesting that there might exist other mechanisms of inhibition of the degradation of cyclin B_1 protein induced by STP and CGP 41 251. Previous studies have revealed that degradation of cyclin B protein may be regulated by ubiquitin-proteasome pathways.[36,45] The effects of STP and CGP 41 251 on these pathways should therefore be determined.

To elucidate the difference of action site of STP and CGP 41 251 in cells further, we examined the effect of both the drugs on tyrosine phosphorylation of CDC2 protein bound to cyclin B_1. Our result demonstrated that CDC2 protein was intensively tyrosine phosphorylated only when the cells were pretreated with STP (Figure 6), suggesting that CDC2 kinase might be inactivated through tyrosine phosphorylation by STP. Previous studies revealed that CDC2 tyrosine phosphorylation occurred in rapid response to DNA damage, which may play an important role for G_2 checkpoint function.[46,47] Very recently, it has been reported that CDC2 tyrosine phosphorylation could be induced by treatment with adriamycin or UV irradiation in nocodazole-arrested M phase cells.[48] In the adriamycin-pretreated cells, the inactivation of CDC25C was also induced.[48] The same phenomena, i.e. tyrosine phosphorylation of CDC2 and inactivation

of CDC25C, were also observed in STP-pretreated and G_2M-blocked cells in our study. Thus, interestingly, a higher concentration of STP apparently acts on CDC2 kinase in just the same manner as DNA-damaging agents, although STP itself is shown not to directly act on DNA.[49] Further studies are needed to elucidate whether STP may directly affect CDC25C phosphatase, WEE1 kinase or their regulatory factor such as chk1 or polo-like kinase.[50,51]

Our data suggested that G_2M block induced by STP (58 nM) might be associated with the inhibition of the degradation of cyclin B1 protein and tyrosine phosphorylation of CDC2 kinase. Our data also suggested that the transient G_2M arrest induced by CGP 41 251 (3000 nM) might be associated with the inhibition of the degradation of cyclin B_1 protein. We did not examine the effects of lower concentrations of STP (5.8 nM) and CGP 41 251 (300 nM) on these regulatory proteins of M to G_1 phase transition because they did not affect M to G_1 phase transition in the cell cycle study (Figures 2 and 3). In addition, since a higher concentration (1560 nM) of UCN-01 which blocked the cell cycle at G_1 phase, as was the case for a lower concentration (260 nM), did not affect the biochemical characteristics of these M to G_1 phase regulatory proteins, we did not see the effect of a lower concentration of UCN-01 (260 nM) on these regulatory proteins.

It is generally known that CDC2 kinase activity is reduced concomitantly with a rapid degradation of its partner cyclin B protein.[34] Interestingly, in A431 cells, G_1 phase cells progressed from M phase after nocodazole release was shown to contain a high level of cyclin B_1 protein (Figure 5A). The high expression level of cyclin B_1 protein in G_1 phase in A431 cells was also observed in our experiment using a double-staining method with propidium iodide and anti-cyclin B_1 antibody (data not shown). Gong *et al.*[52] reported unscheduled expression of cyclin B_1 protein in tumor cell lines such as T47D human breast carcinoma and HL60 human leukemia cells using a double-staining method, suggesting that A431 cells might be included in this type of tumor cell line. In these tumor cell lines, cyclin B_1 degradation does not appear to be a rate-limiting factor for M to G_1 phase progression. These observations led to the question what would bring about the reduction of CDC2/cyclin B1 kinase activity, and what would be a rate-limiting factor of M to G_1 phase progression in A431 cells. Our study proposes a possible answer to these questions. We found that the active form of CDC2 protein was dissociated from cyclin B_1 protein at M to G_1 phase progression in the cells. This event may play an important role and be a rate-limiting factor of M to G_1 phase progression in A431 cells. The mechanism of the dissociation, however, remains to be elucidated. In this study, the dissociation of CDC2/cyclin B_1 complex was also observed in cells after treatments with STP-pretreated G_2M-blocked cells and CGP 41 251-pretreated G_2M-arrested cells. Whether treatment of the cells with STP or CGP 41 251 would affect the process of dissociation of CDC2/cyclin B_1 complex and would be related to the inhibition of cycokinesis also remains to be elucidated.

STP, UCN-01 and CGP 41 251 are potent PKC inhibitors. Recent studies suggest that PKC may be associated with the regulations of cell cycle progressions from G_1 to S phase and from G_2 to M phase.[53] Further studies are needed to elucidate the contribution of PKC to the differential effects of these indolocarbazole compounds on cell cycle progression, and the precise mechanisms of antitumor activities of UCN-01 and CGP 41 251.

Conclusion

Three indolocarbazole compounds, i.e. UCN-01, STP and CGP 41 251, exhibited different effects on cell cycle progression of A431 cells synchronized at M phase by nocodazole. In addition, at higher concentrations (80% growth-inhibitory concentrations), these compounds showed quite different effects on CDC2/cyclin B_1 regulation in the cells. Thus, these results suggested that two PKC selective inhibitors, UCN-01 and CGP 41 251, which are under clinical investigation as anti-cancer drugs, might have different action points on the cell cycle.

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