Biochemical Pharmacology, Vol. 58, pp. 1713–1721, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00258-0

Comparison of the Efficacy of 7-Hydroxystaurosporine (UCN-01) and Other Staurosporine Analogs to Abrogate Cisplatin-Induced Cell Cycle Arrest in Human Breast Cancer Cell Lines

*Sang I. Lee, Mary Kay Brown and Alan Eastman**

Department of Pharmacology and Toxicology, and the Norris Cotton Cancer Center, Dartmouth Medical School, Hanover, NH 03755, U.S.A.

ABSTRACT. DNA-damaging agents such as cisplatin arrest cell cycle progression at either the G_1, S, or G_2 phase, although the G_1 arrest is seen only in cells expressing the wild-type p53 tumor suppressor protein. Caffeine has been shown to abrogate the S and G_2 arrest in p53-defective cells and to enhance cytotoxicity, but at concentrations too toxic to administer to humans. We have reported that 7-hydroxystaurosporine (UCN-01) also overcomes S and G_2 phase arrest and enhances the cytotoxicity of cisplatin. We show here that UCN-01 at non-cytotoxic concentrations abrogated S and G_2 arrest induced by cisplatin in two p53-defective human breast cancer cell lines. UCN-01 pushed the cells through S phase and mitosis, with subsequent apoptosis. Inhibition of mitosis with nocodazole reduced the apoptosis induced by UCN-01 plus cisplatin. Seven staurosporine analogs were compared for their ability to abrogate cell cycle arrest. Staurosporine was as effective as UCN-01 at abrogating S and G_2 arrest, but the concentrations required were cytotoxic. K252a abrogated S phase arrest but failed to abrogate G_2 arrest because alone it induced G_2 arrest. Hence, K252a did not enhance cisplatin-induced cytotoxicity because it failed to push the cells through a lethal mitosis. None of the other analogs influenced cell cycle progression at the concentrations tested. Accordingly, UCN-01 was the only analog that overcame cell cycle arrest and enhanced the cytotoxicity of cisplatin while exhibiting no cytotoxicity of its own. Hence, UCN-01 remains the most promising candidate for testing clinically in combination with cisplatin. BIOCHEM PHARMACOL **58**;11:1713–1721, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. staurosporine; UCN-01; K252a; cisplatin; cell cycle checkpoints

It has been known for almost 30 years that caffeine and other methylxanthines can enhance the cytotoxicity of DNA-damaging anticancer agents [1]. In 1982, Lau and Pardee [2] showed that caffeine abrogates the G_2 arrest elicited by DNA damage, thereby limiting the time available for DNA repair. Many subsequent papers, including our own [3], have confirmed this observation. With the discovery that the *p53* tumor suppressor gene regulates the G_1 checkpoint in damaged cells [4, 5], a strong justification was provided for further investigating the effects of caffeine. It seemed that normal cells, when damaged, would arrest in G_1, whereas tumor cells that had lost this checkpoint control would progress to G_2, where caffeine could kill them preferentially. This strategy was given further impetus by the recent observation that p53 also influences the G_2 checkpoint, such that caffeine preferentially abrogates G_2 arrest and enhances cytotoxicity only in the absence of functional p53 [6–8]. Considering that more than 50% of tumors are defective in p53 [9, 10], caffeine seems like an excellent therapy for these tumors, as all of its desired actions appear to occur preferentially in the absence of p53. Unfortunately, the concentration of caffeine required to achieve these effects in cell culture (5 mM) exceeds by more than 50-fold that tolerated by a patient.

Caffeine has many known actions including the ability to inhibit protein kinases. Accordingly, we have asked whether other protein kinase inhibitors might mimic this effect. We recently established that 7-hydroxystaurosporine (UCN-01) dramatically enhances the cytotoxicity of cisplatin in CHO† cells, and that this occurs at exactly the same concentrations that bypass the S and G_2 checkpoint [11, 12]. We have also shown that UCN-01 enhances the activity of cisplatin in human cell lines, and, furthermore, that this occurs preferentially in cells with disrupted p53 function [13]. Abrogation of S and G_2 arrest occurs at 25–100 nM UCN-01, a concentration that can be achieved readily in animal studies. A single intravenous injection

* Corresponding author: Dr. Alan Eastman, Department of Pharmacology, 7650 Remsen, Dartmouth Medical School, Hanover, NH 03755. Tel. (603) 650-1501; FAX (603) 650-1129; E-mail: Alan.Eastman@Dartmouth.edu

Received 10 July 1998; accepted 5 May 1999.

† *Abbreviations*: CDK, cyclin-dependent kinase; CHO, Chinese hamster ovary cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; and PKC, protein kinase C.

into mice at 10 mg/kg led to a peak plasma concentration in excess of 5 μM, and concentrations above 100 nM were maintained for at least 6 hr [14]. Toxicology experiments have shown that this dose is well tolerated in both mice and dogs [15]. Accordingly, UCN-01 would appear to have great potential to be used effectively in combination with DNA-damaging agents to enhance cell killing specifically in the tumor.

To gain further knowledge on the mechanism by which UCN-01 abrogates cell cycle arrest, we have compared the activity of other staurosporine analogs. These studies used two breast cancer cell lines that are defective for p53 and that are shown here to be responsive to the effect of UCN-01. Only two other staurosporine analogs were found that overcame S phase arrest, and only one of these overcame G_2 arrest. However, UCN-01 was the only analog that abrogated both S and G_2 arrest at concentrations that were not cytotoxic. Hence, UCN-01 remains the best candidate for therapeutic trials in combination with cisplatin or other DNA-damaging agents.

MATERIALS AND METHODS

The breast cancer cell lines MDA-MB-231 and T-47D were obtained from the American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and fungizone (0.25 μg/mL).

Cisplatin, in the form of Platinol (which contains saline and mannitol), was obtained from Bristol-Myers Squibb, and redissolved in water. UCN-01 was provided by Dr. Edward Sausville (National Cancer Institute). N-Benzoylstaurosporine (CGP 41251) was provided by Novartis Pharma AG. Staurosporine and caffeine were purchased from the Sigma Chemical Co. The other staurosporine analogs were purchased from Biomol Research Laboratories, Inc. All analogs were dissolved in DMSO. Incubations of cells with cisplatin were for 2 hr, and incubations with the staurosporine analogs were for 6–24 hr.

Cell cycle analysis was performed according to a previously described procedure in which both adherent and detached cells are harvested, fixed in ethanol, incubated with ribonuclease, and stained with propidium iodide [3]. Then DNA content was determined on a Becton Dickinson FACScan flow cytometer.

For growth inhibition assays, 500-1000 cells were added to each well of a 96-well plate. After 12 hr for attachment, a range of cisplatin concentrations were added for 2 hr; then the cells were washed and incubated in fresh medium for 4 days. In the case of staurosporine analogs, the drugs were added for 24 hr, and the cells were incubated in fresh medium for 7 days. The medium was removed, and 100 μL of 1 mg/mL of MTT in medium was added to each well. After a 3-hr incubation at 37°, 100 μL of DMSO was added to each well to solubilize the dye. Absorbance was read at 550 nm on a microtiter plate reader.

For colony forming assays, 1000 cells were plated into 60-mm culture dishes and allowed to attach for 12 hr. A range of drug concentrations were added for 24 hr; then the cells were washed and incubated in fresh medium for 9 days. Cells were fixed in methanol and stained with Giemsa, and colonies were counted. The plating efficiency of undamaged cells was 15–20%.

RESULTS

Impact of UCN-01 on Cisplatin-Mediated Cell Cycle Arrest

Our previous experiments on the effects of UCN-01 involved analysis of cell cycle progression in CHO cells [11, 12]. We established that cisplatin caused both an S and a G_2 phase arrest, depending upon time and concentration, and that 50 nM UCN-01 rapidly overcame this arrest and enhanced apoptosis. In the current experiments, we performed similar experiments in two breast cancer cell lines, both of which are mutant for p53. The initial experiments investigated the impact of cisplatin alone on cell cycle perturbation. The cells were incubated with cisplatin for 2 hr; then the drug was removed, and the cells were incubated in fresh medium for up to 72 hr. Next the cells were fixed and analyzed by flow cytometry for cell cycle phase. Results for incubation with 10 μg/mL of cisplatin are shown, as this concentration caused pronounced cell cycle perturbation (Fig. 1). Eighteen hours following cisplatin, a large number of cells were seen emerging from the G_1 phase. By 24 hr, there was a marked accumulation of cells in S phase, and these cells accumulated in G_2 at 48 hr. At 72 hr, the majority of cells remained viable but arrested in the G_2 phase. Both cell lines showed very similar cell cycle perturbations in response to cisplatin. However, when assayed for cytotoxicity using the MTT assay 4 days after treatment, it appeared that MDA-MB-231 cells were more sensitive than T-47D cells (2 vs 20 μg/mL). Considering the lack of cell death observed as sub-G_1 cells by flow cytometry or by microscopic assessment of cell detachment, this probably reflects differences in mitochondrial response to cisplatin rather than a marked difference in cytotoxicity.

Considering the above results, it was decided to investigate the impact of UCN-01 on cell cycle progression when added 24 hr after 10 μg/mL of cisplatin. In the absence of UCN-01, there was little movement of cells through S phase between 24 and 30 hr (Fig. 2). The addition of UCN-01 during this time period caused a concentration-dependent shift of the cells to G_2; concentrations of 50–200 nM caused most of the S phase cells to progress to G_2, whereas concentrations as low as 12.5 nM clearly showed partial abrogation of S phase arrest.

Cells were also harvested 24 hr after the addition of UCN-01 (48 hr after cisplatin). In this case, concentrations of 50–200 nM UCN-01 abrogated the G_2 arrest, although the consequences were slightly different for the two cell lines (Fig. 2). In MDA-MB-231 cells, there was a significant population of cells with sub-G_1 DNA content, indic-

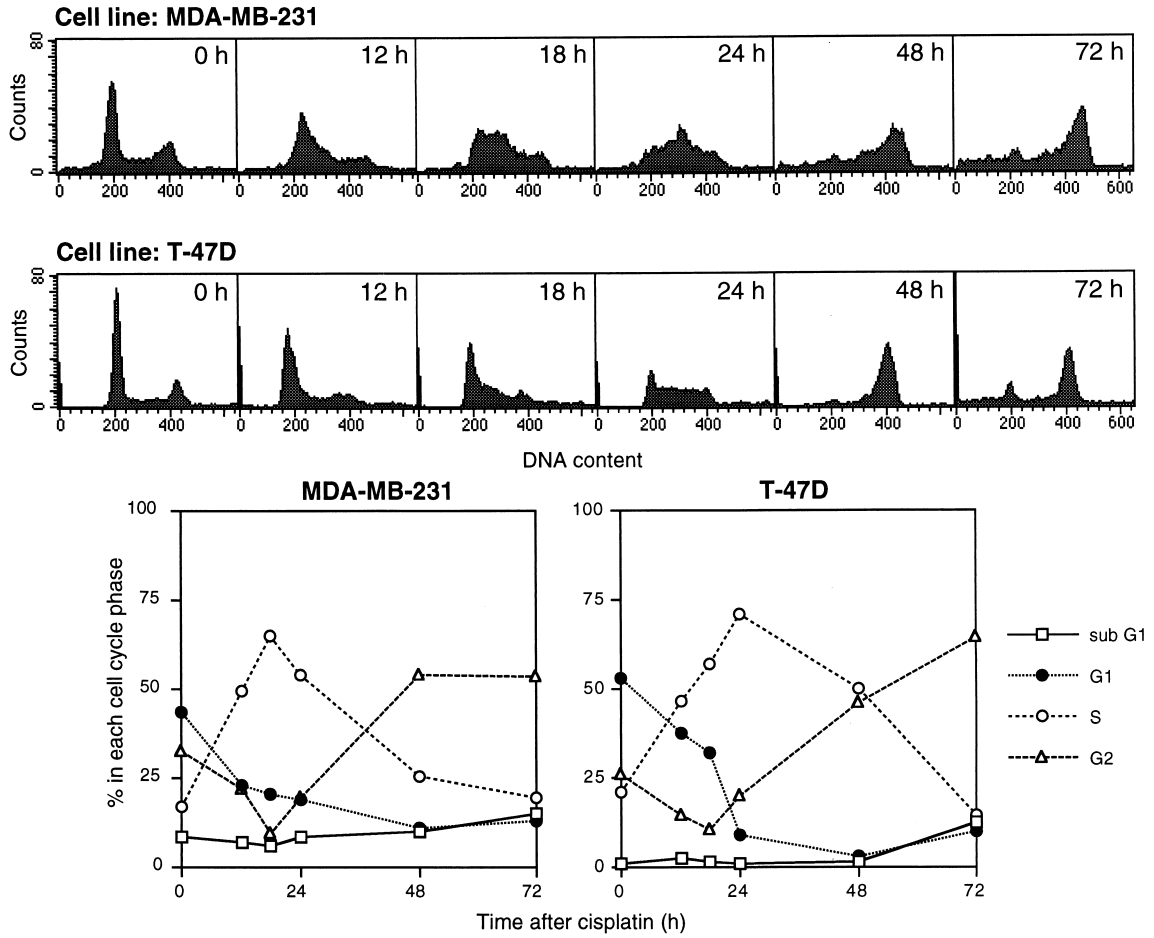

FIG. 1. Cell cycle perturbation in two human breast cancer cell lines following incubation with cisplatin. The MDA-MB-231 and T-47D cell lines were incubated with 10 μg/mL of cisplatin for 2 hr. The drug was removed, and cells were incubated for up to 72 hr prior to analysis of cell cycle phase by flow cytometry. Similar profiles were obtained in at least three separate experiments.

ative of dead cells, at concentrations as low as 25–50 nM UCN-01. There was only a slight increase in the G_1 peak, suggesting that most of the cells passed from G_2 into apoptosis; evidence will be presented below that these cells did pass through mitosis. In the case of T-47D cells, abrogation of the G_2 arrest appeared to be incomplete, and most of the cells that did undergo mitosis had a G_1 rather than a sub-G_1 DNA content. Hence, whereas MDA-MB-231 cells appeared to pass from G_2 to apoptosis as judged by the sub-G_1 population, the T-47D cells appeared to enter G_1, and higher concentrations of UCN-01 were required before cells were observed with sub-G_1 DNA content.

We further investigated the ability of UCN-01 to abrogate G_2 arrest in T-47D cells by adding UCN-01 48 hr after cisplatin treatment, a time at which the majority of cells had already accumulated in G_2 (Fig. 3). By 72 hr, cells again appeared with G_1 DNA content, but the higher concentrations of UCN-01 were more effective at abrogating G_2 arrest, and a larger number of cells with sub-G_1 DNA content were observed.

In all these experiments, the combination of cisplatin and UCN-01 induced apoptosis in up to 20% of the cells as judged by the sub-G_1 population. However, the experiments with T-47D cells suggest that the calculation of sub-G_1 population may underestimate the level of cytotoxicity. For example, the computer fit of the profiles in Fig. 3 suggests that UCN-01 also caused an increase of up to 40% in S phase cells. Some of this population, as well as some of those in G_1, may represent apoptosis of G_2/M cells. Although these results may not show the absolute level of cytotoxicity, they clearly showed an enhanced cytotoxicity when UCN-01 was combined with cisplatin.

We have shown previously that passage through mitosis is required for apoptosis following incubation of CHO cells with cisplatin plus caffeine [3]. To extend those studies, we determined whether passage through mitosis was required for apoptosis following abrogation of G_2 arrest by UCN-01. At 24 hr following cisplatin, UCN-01 and nocodazole were added simultaneously to the cells, the latter drug to prevent completion of mitosis (Fig. 4). Analysis of cell cycle distribution at 48 hr showed that nocodazole effectively retained most of the cells in G_2/M and prevented the appearance of sub-G_1 cells. Hence, passage through mitosis appeared to be necessary for the induction of apoptosis.

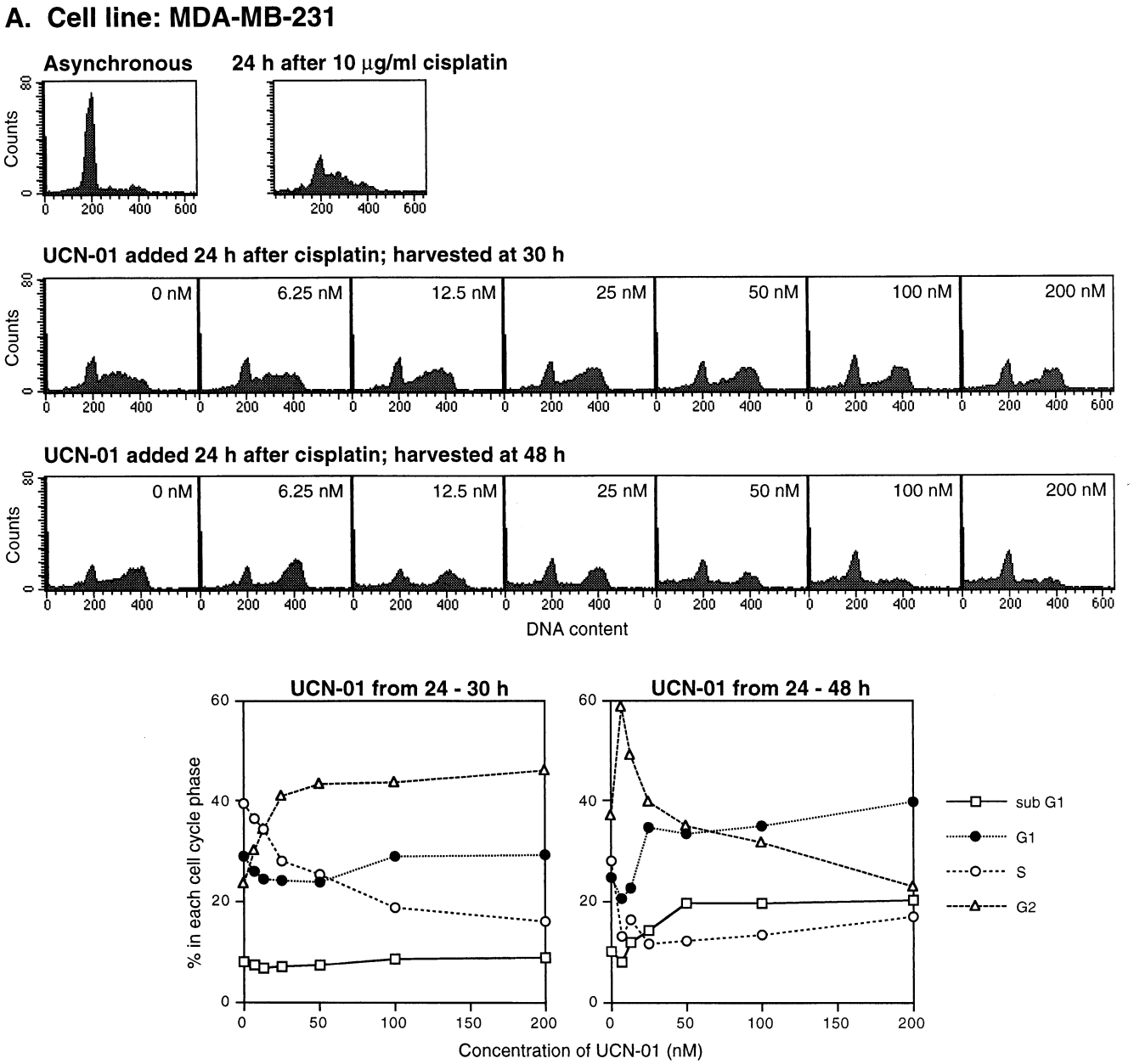

FIG. 2. Abrogation of cell cycle arrest by UCN-01 in two human breast cancer cell lines following incubation with cisplatin. (A) MDA-MB-231 and (B) T-47D cells were incubated with 10 μg/mL of cisplatin for 2 hr. The drug was removed, and cells were incubated for an additional 24 hr (top row). At that time, UCN-01 was added at the indicated concentrations, and the cells were incubated for an additional 6 hr (middle row) or 24 hr (bottom row) prior to analysis of cell cycle phase by flow cytometry. Similar profiles were obtained in at least three separate experiments.

Abrogation of Cell Cycle Arrest by Staurosporine Analogs

Parallel experiments were performed with a range of concentrations of seven analogs of UCN-01. These analogs were added at 24 hr, and S phase abrogation was scored after 6 hr (Table 1). Results are expressed relative to 25 nM UCN-01, which caused partial abrogation of S phase arrest (considered to be about 50% abrogation of S phase arrest as shown in Fig. 2). Staurosporine was as effective as UCN-01 at abrogating arrest. The only other analog that abrogated S phase arrest was K252a, but at a much higher concentration. All of the other analogs were tested up to 1 μM, but failed to have any impact on S phase arrest in either cell line. Caffeine also was tested and found to abrogate S phase arrest at 625 μM. The effective concentrations for each analog were similar in both cell lines.

Staurosporine and caffeine were similar to UCN-01 in their ability to abrogate G_2 arrest when assayed 24 hr after the addition (i.e. 48 hr after cisplatin; data not shown). However, K252a did not abrogate G_2 arrest. This led to the

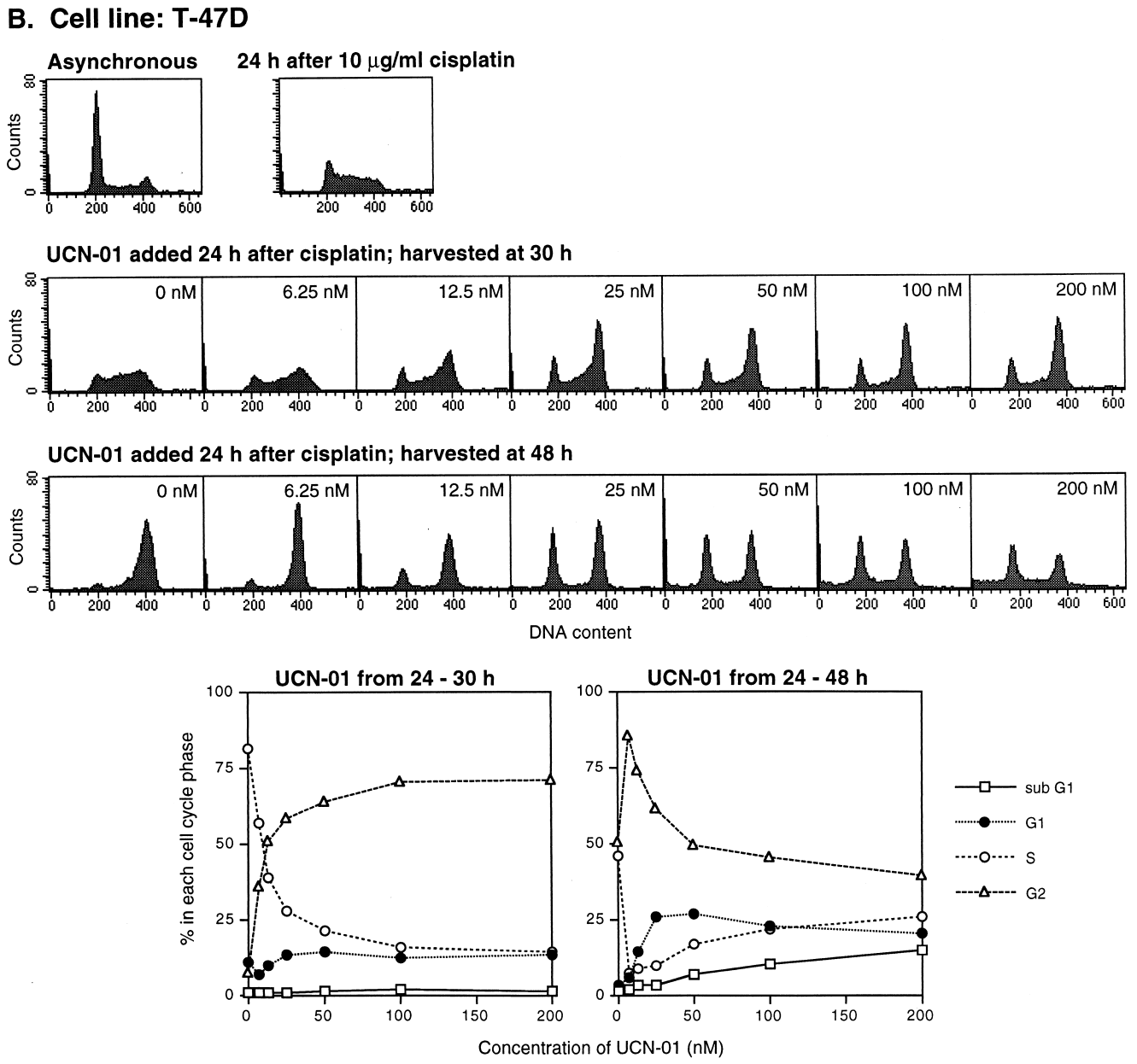

FIG 2. (Continued)

concern that if some analogs could only abrogate S phase arrest, others might only abrogate G_2 arrest. Accordingly, we retested the analogs in T-47D cells that first had been allowed to accumulate in G_2 by a 48-hr incubation following cisplatin (as in Fig. 3). The results are expressed relative to 50 nM UCN-01, which caused about 50% abrogation of G_2 arrest (Table 1). Again, staurosporine was as effective as UCN-01 at abrogating the G_2 arrest, whereas K252a and all the other analogs failed to abrogate the arrest. Caffeine also abrogated G_2 arrest, although at a higher concentration than those required to bypass the S phase arrest.

The inability of K252a to abrogate G_2 arrest led us to investigate the ability of each of these analogs to cause G_1 and/or G_2 arrest, a property well known for staurosporine [16, 17]. Cells were incubated with each analog for 24 hr prior to analysis of cell cycle distribution. Staurosporine (100–200 nM), CGP 41251 (250–500 nM), and K252a (250–500 nM) caused G_2 accumulation in both cell lines. None of the other analogs caused G_2 arrest. Furthermore, none of the analogs caused G_1 arrest consistent with the cell lines being defective in p53. Hence, K252a failed to overcome G_2 arrest because the concentration required, as judged from that required to abrogate S phase arrest, would itself cause a G_2 arrest. We also found that 1 μM K252a prevented UCN-01-mediated abrogation of G_2 arrest and apoptosis (data not shown), suggesting that K252a inhibits cell cycle progression at a point downstream from the action of UCN-01.

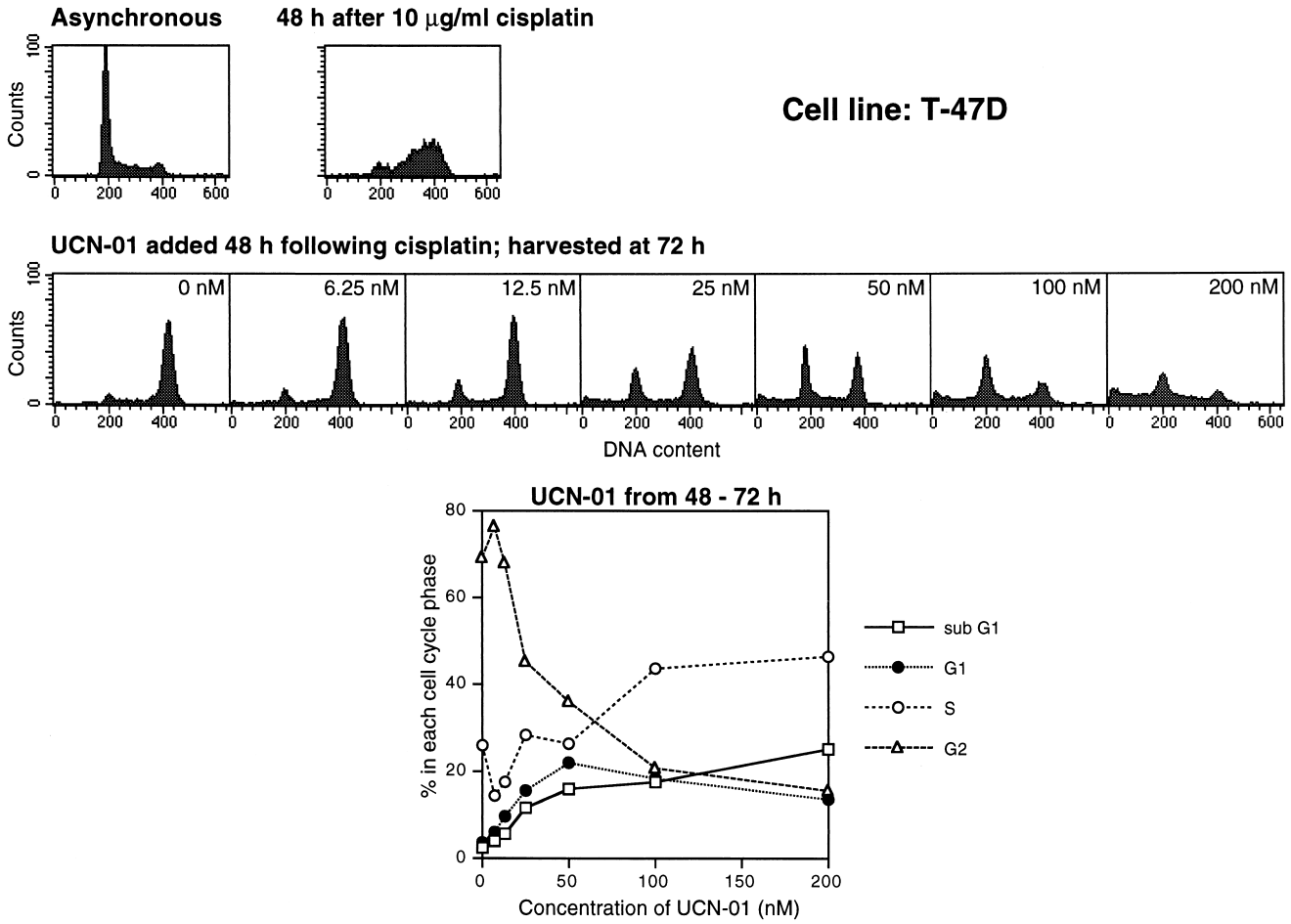

FIG. 3. Abrogation of G_2 arrest by UCN-01 in the human breast cancer cell line T-47D following incubation with cisplatin. The T-47D cells were incubated with 10 μg/mL of cisplatin for 2 hr. The drug was removed, and cells were incubated for an additional 48 hr (top row). At that time, UCN-01 was added at the indicated concentrations, and the cells were incubated for an additional 24 hr prior to analysis of cell cycle phase by flow cytometry (bottom row). Similar profiles were obtained in two separate experiments.

Comparison of the Cytotoxicity of Staurosporine Analogs

One important characteristic of UCN-01 is that it abrogates S and G_2 arrest at concentrations that alone are not cytotoxic. We initially analyzed colony formation following a 24-hr incubation with each analog; the results are shown in Table 1 for T-47D cells, but MDA-MB-231 cells produced diffuse colonies that were not countable. Subsequently, we analyzed cytotoxicity in both cell lines using the MTT assay. Cells were incubated with each analog for 24 hr, and then were incubated for an additional 7 days before assay (Table 1). The IC_{50} for staurosporine was very close to the concentration that abrogated cell cycle arrest. In contrast, much higher concentrations of UCN-01 were required for cytotoxicity than for abrogating arrest. Of the other analogs, only CGP 41251 exhibited cytotoxicity below 1 μM. K252a caused transient inhibition of cell growth consistent with its ability to cause G_2 arrest, but this arrest was reversible after removal of the analog; accordingly, the values in Table 1 do not reflect any cytotoxicity of this analog. Caffeine was also non-toxic up to 10 mM. Hence, UCN-01 and caffeine were the only two drugs that, at non-cytotoxic concentrations, abrogated cell cycle arrest and enhanced apoptosis induced by cisplatin.

DISCUSSION

Pharmacological agents that abrogate cell cycle arrest and enhance cytotoxicity of DNA-damaging agents have been known for many years. The prototype drug in this regard is caffeine [2, 18]. Unfortunately, patients cannot tolerate the levels of caffeine required for this effect. We have reported that UCN-01 is 100,000 times more potent than caffeine at abrogating cell cycle arrest [11, 12]. Both caffeine and UCN-01 have been shown to abrogate G_2 arrest preferentially in cells defective for the p53 tumor suppressor protein [6–8, 13]. This is an interesting observation, as p53 is not required for arrest of cells in S or G_2. The mechanism for this differential effect of mutant versus wild-type p53 is not known, although it is possible that the p53-mediated increase in $p21^{waf1}$ may inhibit cyclin-dependent kinases that are required for abrogating cell cycle arrest. To compare the action of other analogs, we have focused on

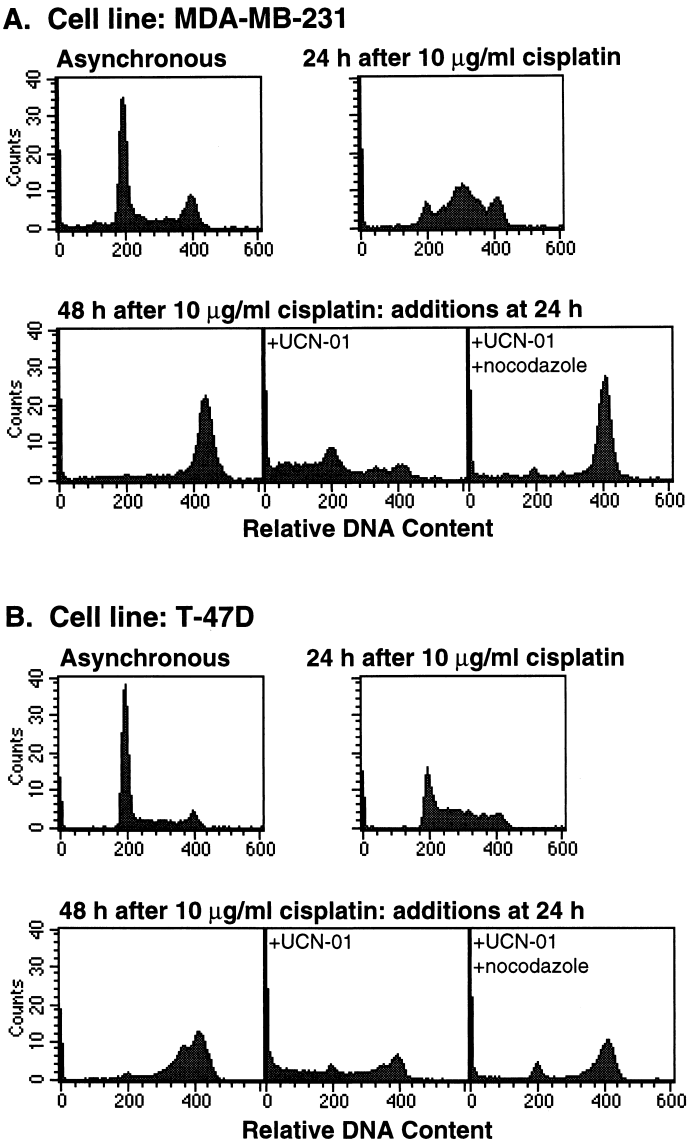

FIG. 4. Role of mitosis in the induction of apoptosis by cisplatin plus UCN-01. (A) MDA-MB-231 and (B) T-47D cells were incubated with 10 μg/mL of cisplatin for 2 hr. The drug was removed, and cells were incubated for an additional 24 hr (top row). At that time, 0 or 200 nM UCN-01 was added, and the cells were incubated for an additional 24 hr prior to analysis of cell cycle phase by flow cytometry (bottom row). In one sample, 0.4 μg/mL of nocodazole was added simultaneously with UCN-01 to arrest cells in mitosis; this prevented the appearance of sub-G_1/apoptotic cells (bottom right panel). Similar profiles were obtained in two separate experiments.

two breast cancer cell lines that are defective for p53. These cells accumulated in S phase 24 hr after cisplatin, and then in G_2 after 48 hr. The addition of 25–50 nM UCN-01 overcame arrest and drove the cells rapidly through S phase, and then through mitosis, which was lethal as judged by the appearance of apoptotic cells. One important property of UCN-01 is that it appears to be completely non-cytotoxic at the concentrations that overcome cell cycle arrest and enhance apoptosis. This property makes UCN-01 a promising candidate for testing in combination chemotherapy.

Staurosporine also abrogated both S and G_2 arrest at concentrations close to those of UCN-01. However, these concentrations of staurosporine were cytotoxic, which would make it a poor analog to combine with cisplatin in a clinical trial. Staurosporine initially generated considerable interest when it was characterized as an inhibitor of PKC. However, it is also active against numerous other protein kinases, including protein kinase A, protein kinase G, and myosin light-chain kinase [19]. Recent interest has focused on the potent activity of staurosporine against CDK2, including resolution of the crystal structure of a CDK2–staurosporine complex [20]. This interaction is likely to explain the ability of staurosporine to cause G_2 arrest at 200 nM, although the cytotoxicity of staurosporine is probably attributable to a different target, as cells died at

TABLE 1. Efficacy of staurosporine analogs to abrogate cisplatin-induced S and G_2 phase arrest

	MDA-MB-231 cells		T-47D cells			
Drug	S phase bypass	Toxicity (MTT)	S phase bypass	G_2 bypass*	Toxicity (MTT)	Toxicity (CFA)
Staurosporine	16 nM	25 nM	16 nM	50 nM	42 nM	42 nM
UCN-01	25 nM	1.55 μM	25 nM	50 nM	1.7 μM	>1 μM
CGP 41251	>1 μM	800 nM	>1 μM	>1 μM	400 nM	350 nM
K252a	500 nM	>1 μM	800 nM	>1 μM	>1 μM	>1 μM
K252b	>1 μM	ND†	>1 μM	ND	ND	>1 μM
KT5823	>1 μM	>1 μM	>1 μM	>1 μM	>1 μM	>1 μM
KT5926	>1 μM	>1 μM	>1 μM	>1 μM	>1 μM	>1 μM
KT5720	>1 μM	>1 μM	>1 μM	>1 μM	>1 μM	>1 μM
Caffeine	625 μM	>10 mM	625 mM	5 mM	>10 mM	ND

Cells were incubated with 10 μg/mL of cisplatin for 2 hr. The drug was removed, and the cells were incubated for an additional 24 hr. Then each analog was added for 6 hr, and S phase bypass was assessed. G_2 bypass was assessed in T-47D cells by adding each analog 48 hr after cisplatin, and assessing changes in cell cycle arrest after an additional 24 hr. Cytotoxicity was determined by either the MTT assay or the colony formation assay (CFA) following a 24-hr incubation with each analog alone. The majority of points reflect the mean of duplicate experiments; results with active compounds have been repeated at least three times.

*Note that 200 nM staurosporine, 500 nM CGP 41251, and 500 nM K252a also caused G_2 arrest (see text).

†ND, not determined.

concentrations considerably lower than those that cause G_2 arrest.

UCN-01 was discovered during a search for more specific inhibitors of PKC [21, 22]. This raises the question as to whether PKC is the critical target for abrogation of cell cycle arrest. We compared the efficacy of UCN-01 and CGP 41251, another analog considered selective for inhibition of PKC [23]. The inefficacy of CGP 41251 appears to rule out the possibility that PKC is critical for abrogation of cell cycle arrest. CGP 41251 did elicit an effect in the cells, as judged by its ability to cause G_2 arrest and cytotoxicity when used alone. Hence, the inability of CGP 41251 to abrogate cisplatin-induced cell cycle arrest was not a consequence of its failing to enter the cells.

The only other analog that was observed to abrogate cisplatin-induced S phase arrest was K252a, but it failed to abrogate G_2 arrest. This is explained by the observation that K252a when used alone caused G_2 arrest at concentrations lower than those expected to abrogate cisplatin-induced arrest. K252a is a broad-spectrum kinase inhibitor, but is somewhat more inhibitory to Ca^{2+}/calmodulin kinase II [19]. Staurosporine has little inhibitory activity against this kinase, so this is unlikely to be the mechanism by which these drugs overcome cisplatin-induced cell cycle arrest.

Four of the analogs failed to have any impact on cell cycle progression or cytotoxicity. One likely possibility is that these analogs failed to cross the cytoplasmic membrane in concentrations adequate to elicit an effect. Most of these analogs have been compared previously for cytotoxicity in a CHO-derived cell line [24]. When incubated with staurosporine continuously for 4 days, an IC_{50} value in the MTT assay was reported as 2.5 nM; values for other analogs were KT5926 = 0.25 μM; K252b = 1.6 μM; KT5720 = 5 μM; and KT5823 = 6 μM. Considering that our incubations with each analog were for 24 hr, it is likely that a 10-fold higher concentration of each analog may be required to elicit cytotoxicity (i.e. compare 2.5 nM staurosporine in the 4-day assay to 25 nM reported here after a 24-hr treatment). This would have required testing the other analogs at concentrations up to 50 μM, which unfortunately was cost-prohibitive. Hence, it is possible that some of these other analogs might retain the ability to abrogate cell cycle arrest at higher concentrations.

Our results have identified three separable consequences of incubating cells with these analogs: cell cycle arrest, cytotoxicity, and abrogation of DNA damage-induced cell cycle arrest. Cell cycle arrest is likely to be due to direct inhibition of CDKs. Cytotoxicity may be due to inhibition of PKC or other unidentified targets. As for abrogation of cell cycle arrest, it can be concluded that neither PKC nor any CDK is the critical target. Interestingly, it appears that the simple addition of a hydroxyl at the C7-position of staurosporine (to give UCN-01) inhibits both the ability to cause cell cycle arrest at G_2 and cytotoxicity. Both K252a and CGP 41251 also lack a hydroxyl at the C7-position, and both caused G_2 arrest. Analysis of the crystal structure of staurosporine binding to the ATP binding site in CDK2 suggests that there is no room to accommodate this hydroxyl group [20], thereby providing a reason as to why UCN-01 does not cause cell cycle arrest. This appears to be a significant property of UCN-01, as lethal mitosis seems important for enhancing cisplatin-induced cytotoxicity. In contrast, the ability of K252a to cause G_2 arrest prevents this lethal mitosis, and furthermore, K252a even seems to protect cells from the combination of cisplatin plus UCN-01. Accordingly, K252a would not appear to be a promising candidate to improve the therapeutic index of cisplatin.

Another possible target for UCN-01 may be CHK1, a protein kinase that is responsible for DNA damage-induced G_2 arrest by inhibiting CDK1 activity [25, 26]. However, we have shown previously that CDK1 is not activated in CHO cells undergoing abrogation of S phase arrest [12], suggesting that CHK1 is not the target for UCN-01 in S phase. Alternately, CHK1 could function in S phase

through an alternate effector, although this would require that CDK1 remain suppressed by a different mechanism when UCN-01 is added. A recent paper has suggested that S phase arrest in yeast may be mediated by the combination of two protein kinases, CHK1 and CDS1 [27]. However, in these experiments, S phase arrest was induced by hydroxyurea, rather than DNA damage, and the resulting lack of deoxyribonucleotides prevented completion of DNA synthesis. We suspect that abrogation of the S phase arrest in CHK1/CDS1-defective cells led to premature mitosis rather than progression to G_2 as occurs upon incubation with UCN-01.

In summary, the results presented here confirm the ability of UCN-01 to abrogate cisplatin-induced S and G_2 arrest in p53 mutant cell lines and to enhance cytotoxicity. The enhanced cytotoxicity was dependent upon passage through mitosis; hence, K252a, which failed to abrogate G_2 arrest, did not enhance cytotoxicity. The only other analog found to abrogate both S and G_2 arrest is staurosporine, but it is far too cytotoxic to be considered as a potential therapeutic agent. Hence, UCN-01 remains the most promising candidate for testing clinically in combination with cisplatin.

The authors thank Dr. Edward Sausville for providing UCN-01 and Novartis Pharma AG for providing CGP 41251. This work was supported by American Cancer Society Grant DHP161. Flow cytometry was performed in the Herbert C. Englert Cell Analysis Laboratory of the Norris Cotton Cancer Center, which is supported by National Cancer Institute Cancer Center Core Grant CA23108.

References

1. Rauth AM, Evidence for dark-reactivation of ultraviolet light damage in mouse L cells. *Radiat Res* **31**: 121–138, 1967.
2. Lau CC and Pardee AB, Mechanism by which caffeine potentiates lethality of nitrogen mustard. *Proc Natl Acad Sci USA* **79**: 2942–2946, 1982.
3. Demarcq C, Bunch RT, Creswell D and Eastman A, The role of cell cycle progression in cisplatin-induced apoptosis in Chinese hamster ovary cells. *Cell Growth Differ* **5**: 983–993, 1994.
4. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B and Craig RW, Participation of p53 protein in the cellular response to DNA damage. *Cancer Res* **51**: 6304–6311, 1991.
5. Hartwell LH and Kastan MB, Cell cycle control and cancer. *Science* **266**: 1821–1828, 1994.
6. Russell KJ, Wiens LW, Demers GW, Galloway DA, Plon SE and Groudine M, Abrogation of the G_2 checkpoint results in differential radiosensitization of G_1 checkpoint-deficient and G_1 checkpoint-competent cells. *Cancer Res* **55**: 1639–1642, 1995.
7. Powell SN, DeFrank JS, Connell P, Eogan M, Preffer F, Dombkowski D, Tang W and Friend S, Differential sensitivity of $p53^{(-)}$ and $p53^{(+)}$ cells to caffeine-induced radiosensitization and override of G_2 delay. *Cancer Res* **55**: 1643–1648, 1995.
8. Fan S, Smith ML, Rivet DJ II, Duba D, Zhan Q, Kohn KW, Fornace AJ Jr, O'Connor PM, Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. *Cancer Res* **55**: 1649–1654, 1995.
9. Osborne RJ, Merlo GR, Mitsudomi T, Venesio T, Liscia DS, Cappa APM, Chiba I, Takahashi T, Nau MM, Callahan R and Minna JD, Mutations in the *p53* gene in primary human breast cancers. *Cancer Res* **51**: 6194–6198, 1991.
10. Moll UM, Riou G and Levine AJ, Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. *Proc Natl Acad Sci USA* **89**: 7262–7266, 1992.
11. Bunch RT and Eastman A, Enhancement of cisplatin-induced cytotoxicity by 7-hydroxystaurosporine (UCN-01), a new G_2-checkpoint inhibitor. *Clin Cancer Res* **2**: 791–797, 1996.
12. Bunch RT and Eastman A, 7-Hydroxystaurosporine (UCN-01) causes redistribution of proliferating cell nuclear antigen and abrogates the cisplatin-induced S phase arrest in Chinese hamster ovary cells. *Cell Growth Differ* **8**: 779–788, 1997.
13. Wang Q, Fan S, Eastman A, Worland PJ, Sausville EA and O'Connor PM, UCN-01: A potent abrogator of G_2 checkpoint function in cancer cells with disrupted p53. *J Natl Cancer Inst* **88**: 956–965, 1996.
14. Hill DL, Tillery KF, Rose LM and Posey CF, Disposition in mice of 7-hydroxystaurosporine, a protein kinase inhibitor with antitumor activity. *Cancer Chemother Pharmacol* **35**: 89–92, 1994.
15. Sausville EA, *A Phase I Trial of Continuous Infusion UCN-01 in Patients with Refractory Neoplasms (NCI Protocol T95–0052)*. National Cancer Institute, Bethesda, MD, 1996.
16. Crissman HA, Gadbois DM, Tobey RA and Bradbury ME, Transformed mammalian cells are deficient in kinase-mediated control of progression through G_1 phase of the cell cycle. *Proc Natl Acad Sci USA* **88**: 7580–7584, 1991.
17. Bruno S, Ardelt B, Skierski JS, Traganos F and Darzynkiewicz Z, Different effects of staurosporine, an inhibitor of protein kinases, on the cell cycle and chromatin structure of normal and leukemic lymphocytes. *Cancer Res* **51**: 470–473, 1992.
18. Schlegel R and Pardee AB, Caffeine-induced uncoupling of mitosis from the completion of DNA replication in mammalian cells. *Science* **232**: 1264–1266, 1986.
19. The BIOMOL Catalog and Handbook, Edn 6, p, 63. BIOMOL Research Laboratories, Plymouth Meeting, PA, 1997.
20. Lawrie AM, Noble MEM, Tunnah P, Brown NR, Johnson LN and Endicott JA, Protein kinase inhibition by staurosporine revealed in details of the molecular interaction with CDK2. *Nat Struct Biol* **4**: 796–801, 1997.
21. Takahashi I, Asano K, Kawamoto I and Nakano H, UCN-01 and UCN-02, new selective inhibitors of protein kinase C. I. Screening, producing organism and fermentation. *J Antibiot (Tokyo)* **42**: 564–570, 1989.
22. Takahashi I, Saitoh Y, Yoshida M, Sano H, Nakano H, Morimoto M and Tamaoki T, UCN-01 and UCN-02, new selective inhibitors of protein kinase C. II. Purification, physico-chemical properties, structural determination and biological activities. *J Antibiot (Tokyo)* **42**: 571–576, 1989.
23. Meyer T, Regenass U, Fabbro D, Alteri E, Rosel J, Muller M, Caravatti G and Matter A, A derivative of staurosporine (CGP 41 251) shows selectivity for protein kinase C inhibition and *in vitro* anti-proliferative as well as *in vivo* anti-tumor activity. *Int J Cancer* **43**: 851–856, 1989.
24. Abraham I, Wolf CL, Sampson KE, Labordie AL, Shelly JA, Aristoff PA and Skulnick HI, K252a, KT5720, KT5926 and U98017 support paclitaxel (taxol)-dependent cells and synergize with paclitaxel. *Cancer Res* **54**: 5889–5894, 1994.
25. Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H and Elledge SJ, Conservation of the Chk1 checkpoint pathway in mammals: Linkage of DNA damage to Cdk regulation through Cdc25. *Science* **277**: 1497–1501, 1997.
26. Furnari B, Rhind N and Russell P, Cdc25 mitotic inducer targeted by Chk1 DNA damage checkpoint kinase. *Science* **277**: 1495–1497, 1997.
27. Boddy MN, Furnari B, Mondesert O and Russell P, Replication checkpoint enforced by kinases Cds1 and Chk1. *Science* **280**: 909–912, 1998.