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Enhancement of Cisplatin-induced Cytotoxicity by 7-Hydroxystaurosporine (UCN-01), a New G2-Checkpoint Inhibitor

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Abstract

DNA-damaging agents arrest cell cycle progression at either G1 or G2. A variety of agents such as caffeine have been shown to abrogate the DNA damage-dependent G2 checkpoint and enhance cytotoxicity. Unfortunately, this strategy has not enhanced therapeutic activity because adequate concentrations of these modulators are not tolerated in vivo. Here, using Chinese hamster ovary cell lines, we show that the potent protein kinase inhibitor 7-hydroxystaurosporine (UCN-01) abrogates the G2 arrest induced by the DNA-damaging agent cisplatin. UCN-01 not only was effective at inducing mitosis when added to G2-arrested cells but also prevented cells from arresting in G2 when added to S-phase cells. Furthermore, UCN-01 did not cause premature mitosis of S-phase cells; rather, the cells progressed to G2 before undergoing mitosis. These effects were observed at nontoxic concentrations of UCN-01 that alone had no effect on cell cycle passage. Furthermore, the same concentrations of UCN-01 that resulted in abrogation of the cisplatin-induced G2 arrest also enhanced cisplatin-induced cytotoxicity, as determined by a colony formation assay. UCN-01 enhanced cisplatin cytotoxicity up to 60-fold and reduced by 3-fold the concentration of cisplatin required to kill 90% of the cells. The concentrations of UCN-01 required for this enhancement have been shown to be well tolerated in animal models, suggesting that this combination may represent an effective strategy for enhancing cisplatin-based chemotherapeutic regimens.

Introduction

Cisplatin is a widely used cancer chemotherapeutic agent that is highly effective against a number of tumor types, including testicular, ovarian, and small cell lung carcinoma. Although cisplatin interacts with a number of cellular components, its antitumor properties are thought to be mediated via its ability to interact with DNA to form DNA adducts, including intra- and interstrand DNA cross-links (1, 2). Several nucleotide excision repair-deficient cell lines have been developed that are hypersensitive to cisplatin (3, 4). Also, cisplatin-resistant cell lines have been developed that exhibit increased DNA excision repair capacities (5, 6). These two general observations support the role of DNA as a critical target for cisplatin.

Although a number of cellular responses have been observed after cisplatin treatment, it has been shown that cisplatin is highly effective in perturbing cell cycle progression. For example, CHO3 cells exhibit both a delay in S phase and a prominent G2 arrest (4, 7). The current paradigm suggests that the G2 arrest provides time for DNA repair to occur before mitosis, thereby protecting the cells from a lethal mitosis (8). This hypothesis is supported by results obtained in yeast in which cells deficient in the RAD9 protein are unable to arrest in G2 after DNA damage and are thereby sensitized to DNA-damaging agents (9).

It has been known for almost 30 years that caffeine and other methylxanthines can enhance cytotoxicity of DNA-damaging agents (10). Initially, this was thought to be due to inhibition of postreplication repair, a phenomenon that remains elusive. However, it was shown subsequently that caffeine caused bypass of the G2 arrest elicited by DNA damage (11, 12), again supporting the idea that sufficient G2 arrest, and presumably DNA repair, can prevent or at least limit the number of lethal mitoses. These methylxanthines inhibit phosphodiesterase activity, thereby raising intracellular cyclic AMP levels and activating PKA. However, cyclic AMP analogs do not mimic the ability of caffeine to abrogate the G2 checkpoint (13). In fact, others have suggested that inhibition of PKA leads to mitosis (14). Thus, it is unlikely that these agents abrogate the G2 checkpoint by enhancing a PKA-dependent pathway. Other agents shown to be effective at abrogating this DNA damage-dependent G2 checkpoint include 2-aminopurine, 6-dimethylaminopurine, and staurosporine (15, 16). Like caffeine, all of these agents are reportedly protein kinase inhibitors, but the specific protein kinase involved remains unknown. These agents provide potentially useful therapeutic modulators, but they all suffer from an inability to attain sufficiently high concentrations in plasma that can be tolerated by a patient.

Staurosporine was identified during the course of a screen for inhibitors of PKC, yet it is nonspecific, exhibiting similar potencies against several other protein kinases. A search for more selective inhibitors of PKC resulted in the discovery of UCN-01 (17). UCN-01 inhibits both conventional and novel PKC isoforms and is 10-fold less effective at inhibiting, for

1 The abbreviations used are: CHO, Chinese hamster ovary; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; UCN-01, 7-hydroxystaurosporine.
example, PKA (18, 19). In contrast, staurosporine is only about 2-fold more selective for PKC than PKA. Thus, this differential protein kinase inhibitory profile suggests that staurosporine and UCN-01 may have different effects in vivo.

Here we present the results of studies performed to examine the ability of UCN-01 to abrogate the cisplatin-induced G2 checkpoint and enhance the cytotoxicity of cisplatin in CHO cells. UCN-01 alone has been shown to alter cell cycle kinetics (20, 21), exhibit antiproliferative activities against several tumor models (22), and enhance the cytotoxicity of another DNA-damaging agent, mitomycin C (23). Recently, it was shown that UCN-01 cytotoxicity was associated with a shortened G2 phase in several T-lymphoblast cell lines (24). This led us to question whether UCN-01 might also abrogate the G2 arrest occurring in cells incubated with DNA-damaging agents. We show here that UCN-01 is a potent inhibitor of the cisplatin-induced G2 checkpoint and can enhance cisplatin-induced cell death by up to 60-fold, as measured by a clonogenic assay. Furthermore, this enhancement is achieved at noncytotoxic concentrations of UCN-01 and at concentrations that are well tolerated in animals (25). These findings suggest that UCN-01 may represent a new agent by which to improve the effectiveness of cisplatin-based chemotherapeutic regimens.

Materials and Methods

Materials. Cell culture reagents, including α-MEM, sodium bicarbonate, horse serum, fetal bovine serum, trypsin, penicillin, and streptomycin, were obtained from Life Technologies, Inc. (Grand Island, NY). All tissue culture plastics were obtained from Corning-Costar Corp. (Cambridge, MA). UCN-01 was provided by Dr. Edward Sausville (National Cancer Institute, Bethesda, MD).

Cell Culture. CHO/UV41 and CHO/AA8 cells (obtained from American Type Culture Collection) were maintained as exponentially growing monolayer cultures in α-MEM supplemented with 2.5% fetal bovine serum, 2.5% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37°C with 5% CO2.

Cell Synchronization and Treatment. CHO/UV41 cells were synchronized at the G1-S border as described previously (7). Briefly, mitotic cells were collected by mitotic shake and incubated in medium containing 2 μg/ml aphidicolin for approximately 16 h. This resulted in a highly synchronized population arrested at the beginning of S phase. Cells were released from the aphidicolin arrest by washing in complete medium. Cells were then incubated with 0.5 μg/ml cisplatin for 1 h, washed, and incubated in drug-free medium. UCN-01 was added at various time points after the platination. Control experiments omitted incubation with either cisplatin, UCN-01, or both.

Cell Cycle Analysis. Cell cycle analysis was performed according to a procedure described previously (7). Briefly, cells were harvested and fixed in 70% ethanol. DNA was then stained by incubating cells in PBS containing 100 μg/ml propidium iodide and 1 mg/ml RNase A for 30 min at 37°C. DNA content was determined on a Becton Dickinson FACScan flow cytometer. The cell cycle distribution was quantitated using the Cell Fit or Cell Quest software.

Results

UCN-01 Abrogates the Cisplatin-induced G2 Checkpoint. We have previously studied the ability of caffeine to abrogate the cisplatin-dependent G2 arrest in CHO/UV41 cells (7). This model was chosen specifically because of its deficiency in DNA excision repair. Accordingly, the level of cisplatin-induced DNA damage does not vary during subsequent incubations. Furthermore, these cells are hypersensitive to cisplatin and enter a G2 arrest when incubated with very low concentrations of the drug that do not cause any slowing of passage through S phase.

CHO/UV41 cells were synchronized by mitotic shake followed by aphidicolin to produce a G1-S phase-arrested population. Upon release from aphidicolin, these cells progress through S phase, reach G2 after 8 h, and undergo mitosis around 10 h (see flow cytometry analysis in Fig. 1 of Ref. 7; see also Fig. 2, discussed below). Immediately after release from aphidicolin, cells were incubated with 0.5 μg/ml cisplatin for 1 h. These cells also reach G2 by 10 h but then arrest. We have shown previously that the addition of 5 mM caffeine at 10 h causes rapid onset of mitosis, followed by cytokinesis and eventual apoptosis (7). In a similar experiment, we added UCN-01 to G2-arrested cells at 10 h and then analyzed the number of cells in either G1 or G2 at 16 h (Fig. 1A). UCN-01 caused a dose-dependent abrogation of the G2 arrest, with 5 nM being ineffective and 50 nM having maximum effect.

We next investigated the time required for UCN-01 to abrogate the G2 arrest. UCN-01 was added to cells arrested in G2 by incubation with cisplatin as above and then harvested and analyzed for cell cycle distribution at various times. Results are expressed as the percentage of cells in G1 and, therefore, reflect the number of cells that have undergone mitosis and cytokinesis (Fig. 1B). Cells incubated only with cisplatin remained arrested in G2 for at least an additional 6 h before a few began to enter mitosis and appear as G1 cells. The addition of 50 nM UCN-01 at 10 h resulted in the majority of cells passing through mitosis during the following 4–6 h. The addition of 300 nM UCN-01 caused most of the cells to undergo mitosis and cytokinesis within 2 h. These observations compare to previous results in which 5 mM caffeine induced mitosis within 4 h (7). Hence, UCN-01 is about 100,000-fold more potent than caffeine at abrogating the G2 arrest.

UCN-01 Prevents the Onset of the Cisplatin-induced G2 Arrest. In the experiments described above, UCN-01 was added after the cells had reached G2. To determine whether a prior G2 arrest was necessary for the action of UCN-01, we added 50 nM UCN-01 to cells in early S phase immediately after removal of cisplatin. Cells were then analyzed for cell cycle...
passage of cells through S phase but caused the cells to undergo

cisplatin and UCN-01, the cells were in late S phase by 6 h,
distribution at times thereafter (Fig. 2). In the absence of both
cisplatin and UCN-01, the cells were in late S phase by 6 h,
mitosis soon after they reached G2; this can be seen as cells
to undergo cisplatin cytotoxicity. We initially assessed this possibility in the
CHO/UV41 cells. The cells were incubated with cisplatin for 2 h,
cytotoxicity was assessed as the inhibition of colony
formation. The addition of UCN-01 after cisplatin caused no
additional cytotoxicity (data not shown). This was the antici-
panied result because CHO/UV41 cells are DNA repair deficient,
regardless of the duration of the G2 arrest, repair cannot
occur. Thus, to fully determine the potential of this combination
chemotherapy of cisplatin plus a mitosis-inducing agent, studies
were performed in a DNA repair-proficient cell line, CHO/AA8,
where the consequences of a reduced DNA repair period can be
examined.

The effect of a continuous UCN-01 incubation on colony
formation was examined. UCN-01 alone exhibited little toxicity
up to 100 nM but reduced colony formation by >99% at 300 nM
(Fig. 3A). Based on this dose response, 0–100 nM UCN-01 were
added to CHO/AA8 cells immediately after a 2-h incubation
with 0–10 μg/ml cisplatin, and colony formation was assessed
(Fig. 3B). Even at these nontoxic concentrations, UCN-01
caused a marked sensitization of the cells to cisplatin. The
results show that 10 nM UCN-01 caused only a slight sensitiza-
tion, whereas 50 nM produced a dose enhancement factor of 3,
calculated as the difference in cisplatin concentration required
to reduce colony formation by 90%. Increasing the UCN-01 con-
centration to 100 nM caused little additional sensitization to
cisplatin. These values can also be expressed with respect to the
enhanced killing elicited by UCN-01; for example, 50 nM
UCN-01 increases killing by about 10-fold at 2 μg/ml cisplatin
and 60-fold at 10 μg/ml cisplatin. The values for 2 μg/ml
cisplatin, together with additional experimental data, have been
replotted in Fig. 3A. In this case, the data have been normalized
for the toxicity attributed to cisplatin alone so that the curve
represents only the additional toxicity elicited by the addition of
UCN-01. These results clearly show approximately a one log
increase in cell killing at nontoxic concentrations of UCN-01;
further sensitization possibly occurred as UCN-01 was in-
creased to concentrations that were toxic alone.

We also investigated the effects of a 12-h incubation with
UCN-01 (Fig. 3C). Under these conditions, 300 nM UCN-01
was far less toxic than if it were left on continuously. The
addition of UCN-01 for 12 h after cisplatin produced only
slightly less sensitization than when incubated continuously; for
example, 50 nM UCN-01 reduced by 2.4-fold the concentration
of cisplatin required to reduce colony formation by 90% (Fig.
3D). Incubation with 300 nM UCN-01 caused little increase in
sensitization.

The above results indicate that cisplatin-induced cytotox-
icity can be significantly enhanced by relatively low concentra-
tions of UCN-01. Alone, these concentrations of UCN-01 have
little effect on colony formation, even when given continuously
for 6 days. Furthermore, these concentrations of UCN-01 are the
same as those that effectively abrogate the DNA damage-dependent G2 checkpoint. This correlation suggests that abrogation of this checkpoint by UCN-01 is the mechanism underlying the dramatic enhancement of cisplatin cytotoxicity observed here.

Discussion

In the present study, we have examined the ability of the staurosporine analogue, UCN-01, to abrogate the G2 arrest induced by cisplatin and to enhance cisplatin cytotoxicity. These studies used two CHO cell lines, CHO/UV41 and CHO/AA8, which are DNA repair deficient and proficient, respectively. The synchronized UV41 cell line provides a good model with which to examine responses to cisplatin-induced DNA damage (7). These cells, when treated with cytotoxic doses of cisplatin at the beginning of S phase, proceed normally through S phase, experience a protracted G2, and eventually enter mitosis. During mitosis, a large proportion of the cells produce multipolar mitotic spindles. These cells subsequently complete cytokinesis but with unequal chromosome segregation and formation of micronuclei. Finally, the cells undergo apoptosis from the subsequent G1. We have also shown that caffeine can hasten the onset of this lethal mitosis and subsequent apoptosis (7).

Previously, UCN-01 has been shown to alter cell cycle distribution, and in particular, to shorten the length of G2 (20, 21, 24). However, those studies were performed at relatively high concentrations of UCN-01. The concentrations of UCN-01 required to abrogate the DNA damage-dependent G2 checkpoint in the studies presented here are much lower and produce no cell cycle alterations in the absence of DNA damage. A concentration of 50 nM UCN-01 neither caused cell cycle perturbation nor elicited any cytotoxicity. This is important as it discriminates between the various cell cycle checkpoints. Cells contain at least two different G2 checkpoints; one checkpoint prevents mitosis.
Fig. 3  UCN-01 enhances the sensitivity of CHO/AA8 cells to cisplatin in a colony formation assay. Asynchronous cells were incubated with or without cisplatin for 2 h and then continuously (A and B) or for 12 h (C and D) with UCN-01, and colonies were counted after 6 days. In A and C, the UCN-01 concentration was varied after incubation with either 0 or 2 µg/ml cisplatin; the values for the cisplatin/UCN-01-treated cells are corrected for the cytotoxicity of cisplatin alone. In B and D, cells were incubated with 0–10 µg/ml cisplatin, followed by 0–300 nm UCN-01 as indicated; the values for the cisplatin/UCN-01-treated cells are corrected for the cytotoxicity of UCN-01 alone. A and B, each point represents the mean of at least three separate experiments; bars, SE. C and D, each point represents the average value obtained from at least two independent experiments.

until satisfactory completion of replication, and the second checkpoint prevents mitosis if the replicated DNA is damaged. The latter checkpoint, at least in yeast, is not essential for survival (9); however, it is activated when DNA damage is detected. It is clear from our studies that UCN-01 is preferentially abrogating only the DNA damage-dependent G2 checkpoint. For example, when cells are incubated with UCN-01 immediately after cisplatin, the cells complete S phase before undergoing mitosis. This is in contrast to other checkpoint inhibitors such as 2-aminopurine and fostriecin, which seem to cause premature mitosis while the cells are still in S phase and even in the absence of DNA damage (26, 27).
The specific target for this action of UCN-01 is unknown. The most potent known action of UCN-01 is on PKC, where it has an IC_{50} of 4.1 nM (18). Other PKC inhibitors have been reported to enhance the sensitivity of cells to cisplatin and other DNA-damaging agents (28). In contrast, PKC activators have also been shown to enhance sensitivity, but this requires pretreatment of cells with the activator, and this seems to be mediated by enhanced cisplatin accumulation (29, 30). PKC is probably not the target for UCN-01 because another PKC inhibitor did not accelerate progression through G_{2} (24). It has also been reported that UCN-01 may activate cyclin-dependent kinases 1 and 2 (24), but this only implies that UCN-01 probably modifies an upstream regulator of these kinases. Accordingly, the target for UCN-01-mediated abrogation of the DNA damage-dependent checkpoint remains to be identified.

Here, we have shown that concentrations of UCN-01 that are optimal for inhibition of the G_{2} arrest can markedly enhance cisplatin cytotoxicity in the DNA repair-proficient CHO/AA8 cell line. Specifically, UCN-01 caused a 3-fold reduction in the concentration of cisplatin needed to kill 90% of the cells and enhanced cisplatin killing by more than 10-fold. This enhancement in cisplatin cytotoxicity was seen at nontoxic concentrations of UCN-01. As with caffeine, it is believed that the premature induction of mitosis reduces the period for DNA repair and hence causes the enhanced cytotoxicity. This hypothesis was confirmed in the DNA repair-deficient CHO/UV41 cell line in which UCN-01 was unable to enhance cisplatin-mediated cytotoxicity; the lack of DNA repair in this cell line prevents recovery from DNA damage during G_{2} arrest.

The synergy between caffeine and DNA-damaging agents has been observed frequently. Two recent studies showed a caffeine-induced dose enhancement ratio of <1.5 for \gamma\text{-irradiation (31, 32). These lower enhancement ratios could be due to differences in cell line, DNA-damaging agent, or modifier. However, in other data, we have observed that UCN-01 caused only slight enhancement of \gamma\text{-irradiation-induced toxicity in two human cell lines (33).} Hence, the combination of UCN-01 with cisplatin may be a more effective and clinically applicable combination. The clinical potential for this combination therapy is enhanced by observations relating to the p53 tumor suppressor protein. The discovery that p53 is required for the DNA damage-dependent G_{1} arrest suggests that many tumor cells will preferentially accumulate in G_{2} because they lack functional p53. Hence, agents that abrogate the G_{2} checkpoint would be preferentially active on the tumor. This strategy has been given further encouragement by the recent observation that p53 may also regulate the G_{2} checkpoint such that caffeine works preferentially in the absence of p53 (31, 32, 34). Whether UCN-01 will also function preferentially in the absence of p53 remains to be determined [CHO cells are defective in p53 function (35)]. Current studies are aimed at determining which tumor phenotypes respond to the UCN-01-mediated enhancement of cisplatin-induced cell death. This will establish which tumor types are best suited for this potentially promising combination chemotherapy.

These findings indicate an excellent correlation between the ability of UCN-01 to abrogate the cisplatin-induced G_{2} arrest and enhance cisplatin toxicity. Thus, these results substantiate the general observation that the G_{2} checkpoint is an important cellular response to DNA damage that ensures propagation of only undamaged, faithfully replicated chromosomes. Furthermore, pharmacokinetic experiments have shown that mice can tolerate plasma concentrations well in excess of 100 nm for at least 6 h (25), a concentration that is well above those required to abrogate the G_{2} checkpoint. These results suggest a clinically applicable combination chemotherapy that has not been achievable with previous agents that abrogate the DNA damage-dependent G_{2} checkpoint.

References

15. Schlegel, R., Belinsky, G. S., and Harris, M. O. Premature mitosis induced in mammalian cells by the protein kinase inhibitors 2-aminopu-


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