

The Protein Kinase C Inhibitor Gö6976 Is a Potent Inhibitor of DNA Damage-induced S and G₂ Cell Cycle Checkpoints¹

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Abstract

In response to DNA damage, cells arrest progression through the cell cycle at either G₁, S, or G₂. We have reported that UCN-01 (7-hydroxystaurosporine) abrogates DNA damage-induced S and G₂ arrest and enhances cytotoxicity selectively in p53 mutant cells, thus providing a potential, tumor-targeted therapy. Unfortunately, UCN-01 binds avidly to human plasma proteins, limiting bioavailability. Because UCN-01 also inhibits protein kinase C (PKC), we screened other PKC inhibitors, expecting them to be unable to abrogate arrest. However, Gö6976 potently abrogated S and G₂ arrest and enhanced the cytotoxicity of the topoisomerase I inhibitor SN38 only in p53-defective cells. Importantly, Gö6976 was nearly as potent at abrogating S and G₂ arrest in human serum, a property not possessed by UCN-01. Cell viability studies demonstrated that Gö6976 was impressively nontoxic as a single agent. Analysis of proteins that regulate cell cycle arrest suggested that both drugs inhibit the checkpoint kinases Chk1 and/or Chk2. Additionally, Gö6976 abrogated S and G₂ arrest at a concentration substantially lower than that required to inhibit PKC; UCN-01 did not demonstrate this selectivity for checkpoint inhibition. These properties make Gö6976 a promising candidate for preclinical and clinical studies.

Introduction

In the face of DNA damage, the genomic integrity of mammalian cells is maintained by activating signaling pathways called DNA damage checkpoints. These checkpoints arrest cell cycle progression to allow time for repair of DNA lesions before genome duplication in S phase and chromosomal segregation in mitosis and thereby protect against the propagation of progeny cells containing damaged or mutated DNA. Many cancer chemotherapy drugs currently in use (*e.g.*, cisplatin, mitomycin C, camptothecin, and etoposide) are DNA-damaging agents and arrest cells in the G₁, S, or G₂ phases of the cell cycle. The G₁ arrest is dependent upon wild-type p53 activity, whereas S and G₂ arrest do not require p53, so cells mutated for p53 (about 50% of tumors) arrest primarily in S or G₂ in response to damage. Cell cycle arrest protects cells from the toxicity of chemotherapeutic agents and insults such as ionizing radiation. Inhibition of damage-induced checkpoints by pharmacological means leads to abrogation of cell cycle arrest and subsequent lethal mitosis, thereby sensitizing cells to DNA-damaging agents. This was first demonstrated in cell culture with caffeine, which is now recognized to be an inhibitor of the DNA damage checkpoint kinases ATM and ATR (1). Importantly, caffeine selectively sensitized cells with nonfunctional p53 to γ -irradiation and other agents by abrogation of the G₂ arrest but did not abrogate S and G₂ arrest or cause sensitization in cells with wild-type p53 (2, 3). These observations demonstrate selectivity for

enhancing toxicity in cells with mutated p53 (many tumor cells), whereas cells with wild-type p53 (normal cells) are spared.

Despite its efficacy in cell culture, caffeine is not a viable choice as a clinical agent because the concentration required to abrogate arrest is far above the clinically achievable concentration. Recently, we demonstrated that UCN-01 (7-hydroxystaurosporine) is 100,000-fold more potent than caffeine at abrogating S and G₂ arrest in p53-mutant cells and thereby sensitizes cells to DNA damage-induced toxicity (4, 5). Similar results have subsequently been reported by others (6, 7). This effect has since been shown to be due to inhibition of the checkpoint kinases Chk1 and perhaps Chk2 by UCN-01 (8–10). UCN-01 has further been shown to enhance the therapeutic activity of DNA-damaging agents in animal models (7) and has completed Phase I clinical trials as a single agent in both Japan and the United States. Unexpectedly, UCN-01 was found to bind avidly to the human plasma protein α_1 -acid glycoprotein, resulting in an extremely long half-life and greatly decreased bioavailability (11, 12). Indeed, cell culture studies demonstrated that incubation of cells in the presence of 5% human serum instead of bovine serum required nearly 100-fold more UCN-01 to abrogate DNA damage-induced S and G₂ arrest (13). Additionally, UCN-01 has been shown to inhibit kinases other than Chk1 and Chk2, which may produce unwanted side effects in patients (14–16).

Considering the difficulties with UCN-01, we have sought checkpoint inhibitors that abrogate damage-induced arrest but lack these undesirable additional properties (*i.e.*, plasma binding and nonspecific kinase inhibition). In a recent article (17), we described ICP-1, a rationally designed analogue of K252a that we found abrogated DNA damage-induced S and G₂ arrest in MDA-MB-231 breast carcinoma cells and bound less avidly than UCN-01 to human serum proteins. As a negative control in ongoing studies, we used several PKC³ inhibitors and, surprisingly, found that Gö6976, an indolocarbazole with a similar structural backbone to UCN-01 (Fig. 1A), also abrogated S and G₂ cell cycle arrest. Further analyses demonstrated that Gö6976 has other properties such as lower toxicity and greater selectivity that make it a better drug than the current lead compound UCN-01 in abrogation of DNA damage-induced cell cycle arrest. Additionally, and of particular interest, Gö6976 was found to potently abrogate arrest in the presence of human serum.

Materials and Methods

Cell Culture. The breast cell lines used in this study were p53 wild type (MCF-10A) and p53 mutant (MDA-MB-231; American Type Culture Collection, Manassas, VA). The cells were maintained in DMEM:Ham's F-12 supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μ g/ml), and fungizone (0.25 μ g/ml). In addition, the MCF-10A cells were maintained in 8 μ g/ml insulin, 20 ng/ml epidermal growth factor, and 500 ng/ml hydrocortisone.

SN38, the active metabolite of the topoisomerase I inhibitor irinotecan, was

³ The abbreviations used are: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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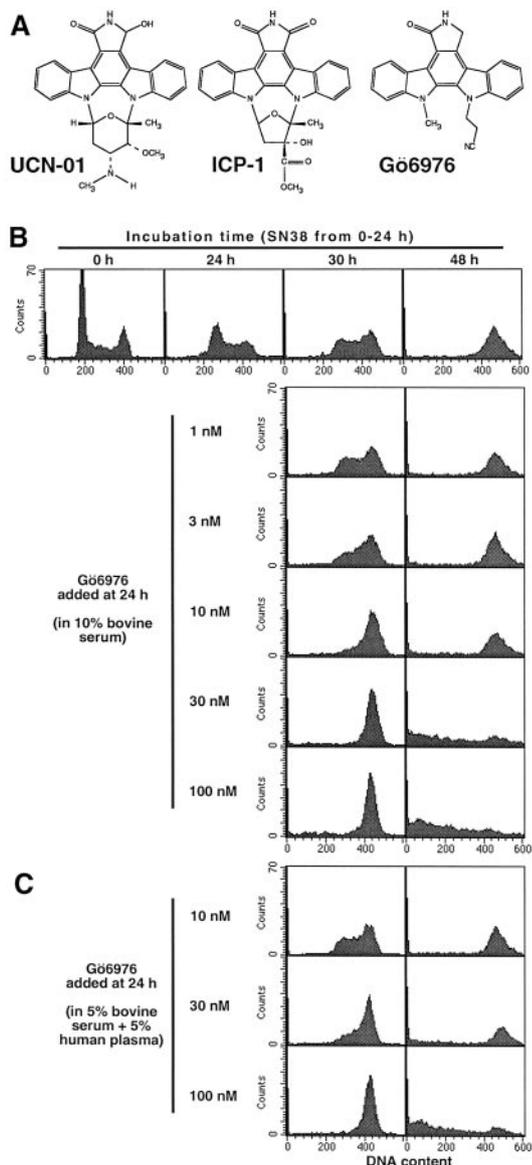


Fig. 1. Gö6976 potently abrogates SN38-induced S and G₂ arrest in MDA-MB-231 cells. A, structures of UCN-01, ICP-1, and Gö6976. B and C, cells were incubated with 10 ng/ml SN38 for 24 h in media containing 10% bovine serum. Media were removed, and fresh media containing 0–100 nM Gö6976 were added for an additional 24 h in either 10% bovine serum (B) or 5% bovine serum plus 5% human plasma (C). Cells were harvested at the indicated times for flow cytometric analysis.

kindly provided by Dr. J. Patrick McGovren (Pharmacia Upjohn Inc., Kalamazoo, MI). UCN-01 was kindly provided by Dr. Edward Sausville (National Cancer Institute, Bethesda, MD). Gö6976 was obtained from Calbiochem (La Jolla, CA). These drugs were dissolved in DMSO. Cells were incubated with SN38 for 24 h, after which time the drug was removed, and the cells were incubated in fresh medium with or without the addition of Gö6976 for up to an additional 24 h.

Cell Cycle Analysis. Cell cycle analysis was performed as described previously, whereby cells were harvested, fixed in ethanol, incubated with RNase, and stained with propidium iodide (18). DNA content was then determined on a Becton Dickinson FACScan flow cytometer. Results are shown as histograms because modeling programs, although good for displaying the number of cells in either G₁, S, or G₂, do not adequately display the progression through S phase (*i.e.*, discriminate early S phase, mid-S phase, and late S phase). Furthermore, the binding of propidium iodide to DNA is dependent on chromatin structure, and extensive DNA breakage, such as occurs upon incubation with SN38, can lead to an apparent increase in DNA content, particularly in G₂-arrested cells, which can confound the modeling programs (13).

Cell Viability. Logarithmically growing cells were incubated with or without 5 ng/ml SN38 for 24 h and then incubated with or without 50 nM UCN-01 or 100 nM Gö6976 for the following 24 h. Cells were harvested daily and scored for viable cell number on the basis of trypan blue exclusion.

Analysis of Cell Growth. MDA-MB-231 (500 cells) or MCF-10A (1000 cells) were plated in 100 μ l in each well of a 96-well plate. The following day, drugs were added at the desired concentrations and with the required schedule to replicate wells (a minimum of 4 wells/concentration). Drugs were removed, and plates were rinsed and then incubated for an additional 6 days. Inhibition of growth was then assessed on the basis of DNA content (17). Briefly, the media were removed, and attached cells were washed in 0.25 \times PBS, followed by the addition of 100 μ l of H₂O. Cells were lysed by freeze/thawing the plates. Hoechst 33258 was added in high-salt buffer, cells were incubated for 2 h, and fluorescence was measured on a CytoFluor II (PerSeptive Biosystems).

Immunoblotting. For immunoblot analysis, cells were rinsed with PBS and then lysed by direct addition of Laemmli sample buffer. Samples were immediately boiled for 5 min and stored at -20° C. Proteins were separated by SDS-PAGE (8%) and transferred to either nitrocellulose (phospho-Chk1/2) or polyvinylidene difluoride membranes (Cdc25C, phospho-threonine-67-Cdc25C, and phospho-serine PKC substrate). Membranes were blocked with 5% nonfat milk in Tris-buffered saline and 0.1% Tween 20 and then probed with the appropriate antibody overnight at 4 $^{\circ}$ C [Cdc25C (Neomarkers); phospho-threonine-67-Cdc25C, phospho-Chk1, phospho-Chk2, and phospho-serine PKC substrate (Cell Signaling)]. Subsequently, membranes were washed in Tris-buffered saline and 0.1% Tween 20 and incubated with secondary antibody conjugated to horseradish peroxidase (Bio-Rad). Proteins were visualized by enhanced chemiluminescence (Amersham).

PKC Activity Assay. Logarithmically growing MDA-MB-231 or MCF-10A cells were incubated with 0–100 nM UCN-01 or 0–1000 nM Gö6976 for 1 h, and then 10 nM TPA was added for an additional 10 min to activate PKC. Lysates were prepared, and immunoblotting was carried out as described above. Two representative bands observed to increase upon incubation with TPA were used as indicators of PKC activity and quantified by densitometric analysis using ImageQuant software. IC₅₀ values were obtained by interpolation of the average curve of the two quantified bands.

Results

Gö6976 Potently Abrogates SN38-induced S and G₂ Arrest in MDA-MB-231 Cells. We have demonstrated previously that incubation of MDA-MB-231 cells with SN38 arrests cells in different cell cycle phases, depending upon the concentration of SN38, with 10 ng/ml causing predominantly an S-phase arrest, and that this S-phase arrest can be abrogated by incubation of cells with UCN-01 at concentrations as low as 7.5 nM (13). Because UCN-01 was originally identified as a PKC inhibitor, we examined the ability of several commercially available PKC inhibitors to abrogate arrest, thinking that they would serve as negative controls to reiterate that abrogation of cell cycle arrest is independent of PKC inhibition. Consistent with this logic, neither chelerythrine chloride nor GF109203X, used at concentrations of up to 2 and 5 μ M, respectively, caused any abrogation of S-phase arrest after incubation for 24 h, as determined by flow cytometric analysis (data not shown). Surprisingly, Gö6976 was found to abrogate S and G₂ arrest. Dose-response studies revealed that 30 nM Gö6976 was sufficient to cause abrogation of S-phase arrest in 6 h and abrogation of G₂ arrest followed by lethal mitosis in 24 h (Fig. 1B). This is only slightly less potent than UCN-01, which exerted these effects at 7.5–15 nM (13).

A major drawback to the use of UCN-01 in patients is its avid binding to human serum proteins. Therefore we examined the ability of Gö6976 to abrogate SN38-arrested MDA-MB-231 cells in the presence of 5% human serum. Impressively, 30 nM Gö6976 caused substantial abrogation of S-phase arrest after 6 h (Fig. 1C). Incubation of cells with 100 nM Gö6976 was sufficient to cause complete abrogation of S and G₂ arrest at 6 and 24 h, respectively, which is only slightly less potent than in bovine serum. In a previous study, we

demonstrated that UCN-01 did not abrogate arrest in 5% human serum until the concentration was raised to 1 μM , almost 100-fold more than that required in bovine serum (17). These observations demonstrate that Gö6976, unlike UCN-01, potently sensitizes MDA-MB-231 cells to SN38-mediated toxicity in the presence of human serum. Importantly, no effect was seen with Gö6976 in the wild-type p53 MCF-10A cells (data not shown); this is consistent with previous findings in which UCN-01 and ICP-1 failed to abrogate SN38-induced arrest in these cells (13, 17). This selectivity for p53-mutant cells has also been observed in other cell lines (4, 6), although the generality of this observation needs to be established in cells of different histological origins.

As an additional measure of toxicity resulting from the combination of SN38 with UCN-01 or Gö6976, MDA-MB-231 and MCF-10A cells were scored for viability using trypan blue exclusion. Incubation of cells with UCN-01 or Gö6976 alone did not decrease viability compared with control at the concentrations used (Fig. 2). Incubation of cells with 5 ng/ml SN38 resulted in cytostasis, and addition of 50 nM UCN-01 or 100 nM Gö6976 to arrested MDA-MB-231 cells caused a dramatic decrease in viable cell number by 96 h (Fig. 2A). Incubation of SN38-arrested MCF-10A cells with these agents had no impact on viability compared with SN38 alone (Fig. 2B), consistent with the resistance of these cells to checkpoint abrogation (13, 17).

In evaluating clinical potential, it is important that agents have low innate toxicity. We therefore examined the impact of Gö6976 alone on proliferation of MDA-MB-231 and MCF-10A cells at various concentrations and compared this with the effects of UCN-01 in the same system. Cells were incubated with either UCN-01 or Gö6976 for 24 h, and proliferation was assessed 6 days later. Gö6976 inhibited proliferation by 50% at 6 μM in MDA-MB-231 cells and at 700 nM in MCF-10A cells. In contrast, UCN-01 inhibited growth by 50% at 60 nM in the MDA-MB-231 cells and at 250 nM in MCF-10A cells. Hence, Gö6976 is much better tolerated as a single agent than UCN-01.

Inhibition of PKC by Gö6976 and UCN-01. Because both Gö6976 and UCN-01 were initially shown to be PKC inhibitors, there is concern that their clinical utility as checkpoint inhibitors could be confounded by the additional inhibition of PKC, which may cause undesirable side effects in patients. Given this, we carried out dose-response experiments with Gö6976 and UCN-01 to determine the concentrations at which these agents inhibit PKC activity in cells. In MDA-MB-231 cells, UCN-01 potently inhibited TPA-stimulated phosphorylation of PKC substrate motif-containing proteins, with an IC_{50} of 10 nM (Fig. 3). This is consistent with previously published

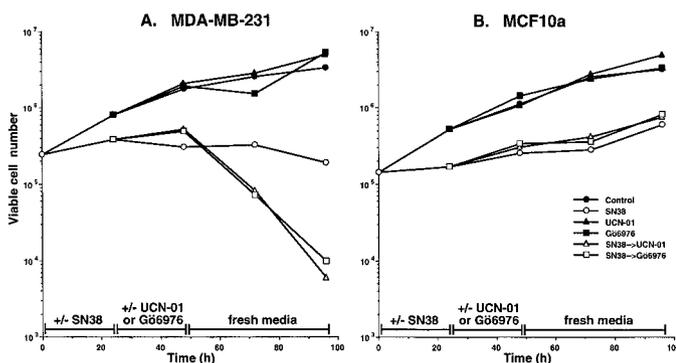


Fig. 2. Impact of UCN-01 and Gö6976 on viability of two human breast cell lines incubated with SN38. MDA-MB-231 (A) or MCF-10A (B) cells were incubated with or without 5 ng/ml SN38 for 24 h and then incubated with or without 50 nM UCN-01 or 100 nM Gö6976 for the following 24 h. Cells were harvested daily and scored for viable cell number on the basis of trypan blue exclusion. A representative experiment is shown (one of two such experiments performed).

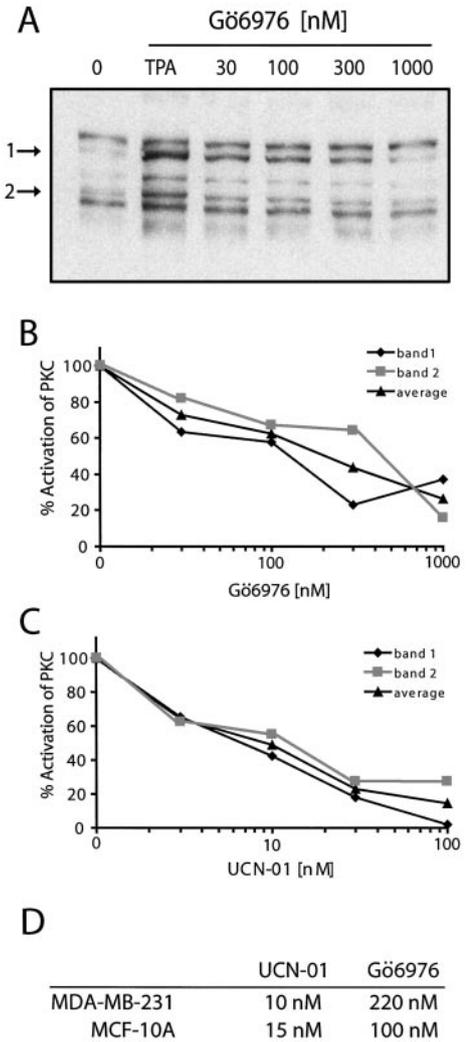


Fig. 3. Inhibition of PKC activity by Gö6976 and UCN-01. A, representative Western blot of Gö6976-mediated inhibition of TPA-stimulated PKC substrate phosphorylation. Arrows indicate bands used for IC_{50} determination. B and C, graphic representation of inhibition of PKC substrate phosphorylation in MDA-MB-231 cells by Gö6976 (B) and UCN-01 (C). D, IC_{50} values obtained by interpolation of densitometric values for MDA-MB-231 cells (B and C) and MCF-10A cells (data not shown). Results of a single experiment are shown, although the values are consistent with initial dose ranging experiments.

findings by Wang *et al.* (14). We found Gö6976 to be markedly less potent, with an IC_{50} of 220 nM in MDA-MB-231 cells. We also examined the effect of these compounds in MCF-10A cells and observed similar results (Fig. 3D). Because UCN-01 abrogates cell cycle arrest in cell culture at 7.5–15 nM (13), these experiments suggest that PKC may additionally be inhibited in this dose range. However, Gö6976, inhibits PKC activity at concentrations substantially higher than those that abrogate cell cycle arrest and sensitize cells to SN38, suggesting the latter drug has a substantial therapeutic window for checkpoint inhibition without PKC inhibition.

Mechanism of Action of Gö6976. It has been shown that the ability of UCN-01 to sensitize cells to DNA-damaging agents is due to inhibition of the checkpoint kinases Chk1 (8, 9) and perhaps Chk2 (10), resulting in abrogation of cell cycle arrest and subsequent death by lethal mitosis. We demonstrated previously that treatment of S phase-arrested MDA-MB-231 cells with 15 nM UCN-01 resulted in abrogation of S and G_2 arrest and entry into mitosis in 9–12 h, which coincided with activation of the mitosis-inducing phosphatase Cdc25C (13). Because Gö6976 is structurally similar to UCN-01 and

abrogates cell cycle arrest with similar kinetics, we thought it possible that it is also acting through inhibition of Chk1/2 kinase activity. Analyses of Chk1 and Chk2 in asynchronously growing MDA-MB-231 cells showed little phosphorylated (active) protein, whereas incubation with SN38 for 24 h led to phosphorylation (activation) of these proteins (Fig. 4). Subsequent incubation of cells for 15 h with 30 nM Gö6976, which abrogated S phase arrest by 6 h and drove cells through a lethal mitosis by 12–15 h, caused no decrease in Chk1 and Chk2 phosphorylation levels, indicating that this compound inhibits downstream of ATM and ATR, the kinases responsible for phosphorylation of Chk2 and Chk1, respectively (19, 20). Analysis of Cdc25C revealed activation in 9–12 h, as evidenced by the band with retarded electrophoretic mobility that represents the hyperphosphorylated form. This was verified using the threonine 67 phospho-specific Cdc25C antibody that recognizes a mitotic form of the protein. This activation preceded cell death, observed as cells with sub-G₁ DNA content by fluorescence-activated cell-sorting analysis at 15 h. These results are identical to those obtained previously with UCN-01 (13) and are therefore consistent with Gö6976 abrogating cell cycle arrest by inhibition of Chk1 and possibly Chk2 kinase activity.

Discussion

One of the major drawbacks of many current cancer therapies is the lack of selectivity for tumor cells. Given this, a promising approach is the generation of molecularly targeted therapies that are selectively toxic to the tumor, sparing normal cells that have a different molecular phenotype. A potential therapeutic target that we and others have investigated is the DNA damage-induced cell cycle checkpoint, which

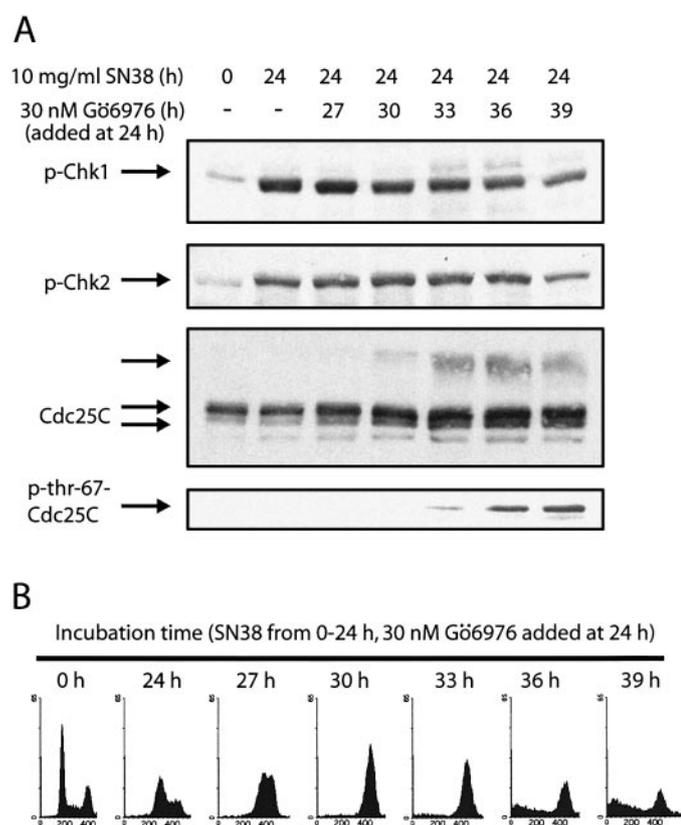


Fig. 4. Analysis of cell cycle checkpoint-regulatory proteins Chk1, Chk2, and Cdc25C. MDA-MB-231 cells were incubated with 10 ng/ml SN38 for 24 h. The drug was removed, and cells were incubated for an additional 3–15 h with 30 nM Gö6976. At the indicated times, cells were harvested, and Western blotting for the indicated proteins (A) or flow cytometric analysis of cell cycle distribution (B) was performed.

can be inhibited (at least in cell culture) by pharmacological means, selectively sensitizing cells that have a disrupted p53 pathway.

UCN-01 has been shown to be a potent checkpoint inhibitor in cell culture, but it also has the undesirable properties of avid binding to human serum proteins and inhibition of other, non-checkpoint kinases (14–16). Therefore, we have been examining analogues of UCN-01 and related compounds to identify those that sensitize cells to DNA damage by checkpoint inhibition but are more selective and do not bind human serum proteins (17, 21). Recent analyses of a novel K252a analogue synthesized at Dartmouth, ICP-1, revealed a compound that lacked the toxicity and avid binding to serum proteins but was less potent than UCN-01 (17). Here we show that Gö6976, a drug originally identified and marketed as a PKC inhibitor, is in fact very effective at abrogating DNA damage-induced cell cycle arrest. It was found to abrogate arrest and cause cell death in 24 h at 30 nM, making it nearly as potent as UCN-01 in the same cell system. Importantly, Gö6976 also demonstrates marginal toxicity as a single agent because 6 μ M was required to inhibit proliferation by 50% in MDA-MB-231 cells; this concentration is 200-fold greater than that needed to abrogate cell cycle arrest. UCN-01 was found to be moderately toxic in the same system, showing 50% inhibition of proliferation at 60 nM, only 4-fold above the concentration used to abrogate arrest. The marginal toxicity of Gö6976 suggests a strong selectivity for checkpoint kinase inhibition, whereas the greater toxicity of UCN-01 may reflect inhibition of other kinases. It was demonstrated recently that 500 nM UCN-01 can inhibit PDK1, which is consistent with observations that UCN-01 causes a G₁ arrest at this concentration (22). We observed that Gö6976 did not cause a G₁ arrest at concentrations up to 10 μ M, so presumably it is ineffective at inhibiting PDK1. Other studies have also demonstrated inhibition of PKC and cyclin-dependent kinase 2 by UCN-01 (14, 16). It was shown here that Gö6976 is a less potent inhibitor of PKC than UCN-01, with an IC₅₀ of 220 nM in MDA-MB-231 cells, as compared with 10 nM for UCN-01. If inhibition of PKC contributes to toxicity in the patient, this substantially increased selectivity for checkpoint inhibition translates to a much larger therapeutic window for Gö6976, avoiding the toxicities provided by UCN-01 and other drugs with lesser selectivity.

Analyses of checkpoint proteins suggest that Gö6976, like UCN-01, abrogates arrest by inhibition of Chk1 and perhaps Chk2 kinases. The substrate of Chk1 and Chk2 in S phase is unclear, but in G₂, inhibition of Chk1/Chk2 leads to activation of Cdc25C, which promotes the onset of mitosis. This activation was observed with Gö6976 after 9–12 h, the time at which cells were entering mitosis; Cdc25C did not become activated in a similar time course in the absence of Gö6976 (data not shown), demonstrating that Cdc25C activation is due to the action of Gö6976. Chk1 and Chk2 remain phosphorylated in the presence of Gö6976, indicating that its site of action is downstream of ATM/ATR, presumably inhibiting at the level of Chk1/Chk2 activity.

In addition to these potency and toxicity characteristics, Gö6976 also demonstrates much greater efficacy than UCN-01 in the presence of human serum. In clinical trials, no responses were observed until plasma concentrations of UCN-01 reached 20–40 μ M, a range that saturates α_1 -acid glycoprotein binding (12). Because levels of α_1 -acid glycoprotein vary considerably, it is difficult to administer UCN-01 at doses sufficient to abrogate cell cycle arrest without causing toxicity. The efficacy of Gö6976 in the presence of human serum suggests little, if any, plasma binding, which should lead to much greater bioavailability and better control of the administered dose, as desired in the clinical setting.

We have demonstrated that Gö6976, although initially identified as a PKC inhibitor, is in fact very effective at sensitizing cells to DNA damage through abrogation of arrest, likely by inhibition of Chk1

and/or Chk2. In comparison to the current lead compound UCN-01, Gö6976 is almost as potent, substantially less toxic, and abrogates arrest at a much lower concentration in the presence of human serum. We therefore believe that Gö6976 is a much better compound than UCN-01, especially as a potential clinical therapeutic, for sensitization of tumor cells to DNA-damaging agents.

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The Protein Kinase C Inhibitor Gö6976 Is a Potent Inhibitor of DNA Damage-induced S and G₂ Cell Cycle Checkpoints

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