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Characterization of Protein Kinase chk1 Essential for the Cell Cycle Checkpoint after Exposure of Human Head and Neck Carcinoma A253 Cells to a Novel Topoisomerase I Inhibitor BNP1350

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ABSTRACT

Cellular topoisomerase I is an important target in cancer chemotherapy. A novel karenitecin, BNP1350, is a topoisomerase I-targeting anticancer agent with significant antitumor activity against human head and neck carcinoma A253 cells in vitro. As a basis for future clinical trials of BNP1350 in human head and neck carcinoma, in vitro studies were carried out to investigate its effect on DNA damage and cell cycle checkpoint response. The treatment of A253 cells with BNP1350 caused biphasic profiles of DNA fragmentation displayed from 0 to 48 h after 2-h exposure. Pulsed-field gel electrophoresis demonstrated that the first wave of DNA damage was mainly megabase DNA fragmentation, but the second wave of DNA damage was 50- to 300-kb DNA fragmentation in addition to megabase DNA damage. The cell cycle checkpoint response was characterized after exposure to 0.07 and 0.7 μM concentrations of BNP1350, the IC_{50} and IC_{90} values, respectively. After exposure to a low concentration of BNP1350 (IC_{50}), A253 cells accumulated primarily in G_2 phase. In contrast, treatment with a high concentration of BNP1350 (IC_{90}) resulted in S phase accumulation. The concentration-associated cell cycle perturbation by BNP1350 was correlated with different profiles of cell cycle-regulatory protein expression. When treated with the low concentration of BNP1350, cyclin B/cdc2 protein expression was up-regulated, whereas with the high concentration, no significant change was observed at 24 and 48 h. In addition, increased phosphorylation of a G_2 checkpoint kinase chk1 was observed when cells were treated with a low concentration of BNP1350, whereas only slight inhibition of chk1 activity was found in the cells treated with the higher concentration. Altered chk1 phosphorylation after DNA damage appears to be associated with specific phases of cell cycle arrest induced by BNP1350. Because A253 cells do not express the p53 protein, the drug-induced alterations of the G_2 checkpoint kinase chk1 are not p53-dependent.

DNA topoisomerase I (topo I) is a ubiquitous enzyme with key roles in DNA replication, transcription, and possibly recombination and repair (Chen and Liu, 1994; Pommier, 1996). It is known to control DNA supercoiling by transiently breaking and resealing DNA strands. Increased expression of topo I was observed in colorectal tumors compared with their normal tissue counterparts (Husain et al., 1994), enhancing the possibility of using a topo I inhibitor as a promising anticancer drug. Camptothecin (CPT) analogs, such as topotecan and irinotecan (CPT-11), are among the most promising anticancer drugs in clinical trials (Potmesil, 1994; Dancey and Eisenhauer, 1996). Karenitecins are novel fourth-generation CPT derivatives that can be administered orally. They are highly lipophilic, they sustain high levels of the active lactone form, and they do not require hepatic activation. BNP1350, the lead karenitecin, exhibits more potent antitumor activity than CPT in in vitro and xenograft studies and has reasonable preclinical safety (Hausheer et al., 1999; Kerr et al., 1999). BNP1350 is currently being evaluated in a phase I clinical trial.

G_2 phase cell cycle arrest is a common cellular response to DNA damage (Rao, 1980; Konopa, 1988) and is also referred to as a checkpoint response to DNA damage (Hartwell and Weinert, 1989; O'Connor and Kohn, 1992). The G_2/M checkpoint helps to prevent further damage and gives the cell time to repair the lesions that have already occurred. This serves to preserve viability and to maintain the integrity of the genome.

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ABBREVIATIONS: topo I, topoisomerase I; CPT, camptothecin; CFGE and PFGE, constant- and pulsed-field gel electrophoresis; PAGE, polyacrylamide gel electrophoresis; NP40, Nonidet P-40; DTT, dithiothreitol; cdk, cyclin-dependent kinase; PI, propidium iodide; chk, checkpoint kinase; HBSS, Hanks' balanced salt solution; NF-κB, nuclear factor-κB; ATM, ataxia telangiectasia mutated.

Down-regulation of cyclin B/cdc2 activity arrests cells in G_2 and presumably allows DNA repair before commitment to M phase (Tsao et al., 1992). A novel protein kinase, $p56^{chk1}$, was identified as a component that interacts with the cell cycle-regulatory kinase cdc2. chk1 protein has been implicated in the regulation of cdc2 activity and is involved in the cell cycle arrest when DNA damage has occurred or when unligated DNA is present (Walworth and Bernards, 1996; Furnari et al., 1997). In most species, the G_2 checkpoint prevents the cdc25 phosphatase from activating the cell division kinase cdc2 by maintaining cdc25 in a phosphorylated form that binds to 14-3-3 proteins. chk1 regulates the interactions between cdc25 and 14-3-3 proteins by phosphorylating cdc25C (Chen et al., 1999; Lopez-Girona et al., 1999). Thus, chk1 is a key molecular mediator of the G_2 checkpoint.

In this study, human head and neck carcinoma A253 cells were used to investigate the effects of BNP1350 on cellular growth inhibition, DNA fragmentation, and cell cycle arrest. These effects were analyzed at cellular and molecular levels to identify determinants for BNP1350 activity, including protein kinases essential for DNA damage checkpoint.

Materials and Methods

Drugs and Chemicals. BNP1350 (Fig. 1) was kindly supplied by BioNumerik Pharmaceuticals, Inc. (San Antonio, TX). The compound was dissolved in DMSO with final dilutions made in the growth medium. Proteinase K and RNase A were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA). SRB and sarkosyl were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Line and Culture Conditions. The human A253 head and neck carcinoma cell line was purchased from American Type Culture Collection (Rockville, MD) and maintained as a monolayer in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO BRL, Life Technologies, Grand Island, NY). The doubling time of the cells is approximately 27 h. All treatments were carried out using exponentially growing cell cultures. The cell line was free from mycoplasma as tested every 2 months with the Mycoplasma T. C. Rapid Detection System (Gen-Probe Inc., San Diego, CA).

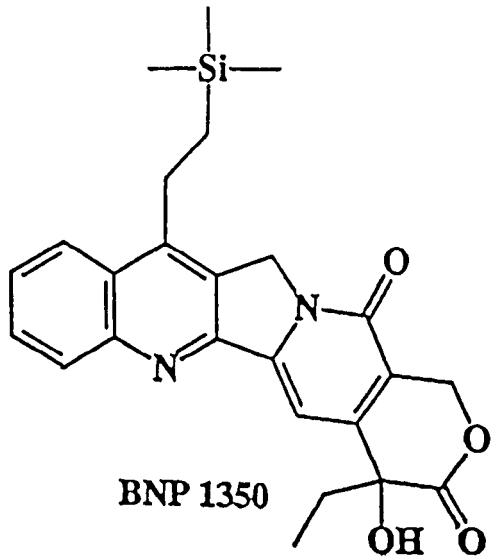

Fig. 1. Chemical structure of BNP1350.

Growth Inhibition Assay. Cell growth inhibition was determined using the total protein SRB assay as described elsewhere (Skehan et al., 1990). Briefly, 600 cells/well were seeded onto 96-well plates. After 24 h, exponentially growing A253 cells were treated with BNP1350, which was diluted in culture medium, for 2 h. At four doubling times after drug exposure, the cells were fixed with 10% trichloroacetic acid and further processed according to the published SRB procedure. The optical density was measured at 570 nm using an automated Bio Kinetics reader (model EL 340; Bio Tek Instruments, Winooski, VT). Antiproliferative activities were expressed as drug concentrations that induced growth inhibition of 50 or 90% compared with growth of untreated controls (IC_{50} and IC_{90} values).

Constant- and Pulsed-Field Gel Electrophoresis (CFGE and PFGE) Analysis for DNA Fragmentation. The procedure for the preparation of DNA plugs was a modification of that described by Schwartz and Cantor (1984) and Giaccia et al. (1991). Approximately 5×10^6 cells were washed using Hanks' balanced salt solution (HBSS) and resuspended in 0.1 ml of HBSS. An equal volume of 2% low-melting-point agarose prepared in HBSS was added at 50°C. The mixture was immediately poured into the molds. Cells embedded in agarose were digested for 24 h in more than 20 volumes of lysis buffer containing 0.5 M EDTA, pH 8.0, 10 mM Tris, 1% sarkosyl, and 1 mg/ml proteinase K and then were incubated for 1 h in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) containing 0.2 mg RNase A/ml. Each plug contained approximately 1×10^6 cells.

For CFGE, DNA plugs were inserted into wells of a 1.8% agarose gel, sealed with a small amount of agarose, and electrophoresed in TAE buffer (40 mM Tris-acetate and 1 mM EDTA) at room temperature as described previously (Panadero et al., 1995). After electrophoresis, gels were stained with ethidium bromide (5 μg/ml in H_2O) and then photographed on a UV-transilluminator. For quantitative analysis of DNA fragmentation, the cells were prelabeled with ^{14}C-dThyd (0.025 μCi/ml) for 24 h. After CFGE, each band was cut out, and the quantity of ^{14}C-labeled DNA was determined by scintillation counting. A 1-kb DNA ladder (Life Technologies) was used to estimate DNA fragment size.

For PFGE, agarose gels were prepared in 0.5× BRL TBE buffer (75 mM Tris, 25 mM boric acid, and 0.1 mM EDTA, pH 8.9) as

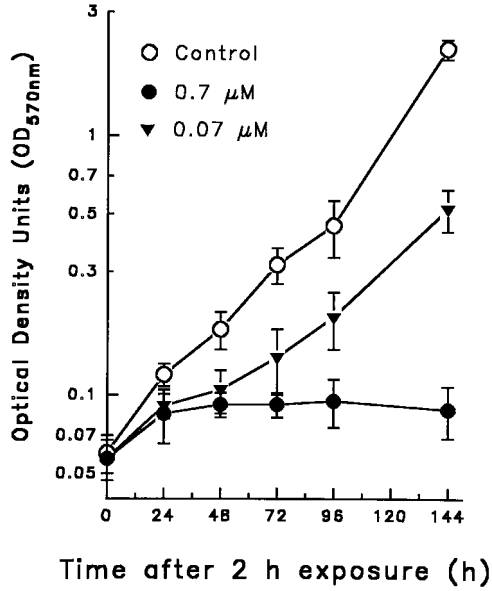

Fig. 2. Dose-time effect of BNP1350 on the growth of A253 cells in culture. Exponentially growing cells were treated with the IC_{50} and IC_{90} concentrations of BNP1350 for 2 h and then incubated in drug-free medium for the indicated times. The growth inhibition was determined by the total protein SRB assay as described in *Materials and Methods*. Symbols represent average ± S.D. of at least three experiments, each with eight wells of culture.

described previously (Panadero et al., 1995). Electrophoresis was carried out using Hex-A-Field horizontal gel electrophoresis apparatus (Life Technologies), which contains a hexagonal array of electrodes with a reorientation angle of 120 degrees. The electrophoresis was performed in TBE buffer at 14°C with buffer circulation. After electrophoresis, gels were stained with ethidium bromide and then photographed on a UV-transilluminator.

Western Blot Analysis. Analysis of cyclin E/cdk2, cyclin A and B/cdc2, and chk1 protein expression was performed by Western blotting. A253 cells were lysed in RIPA buffer [50 mM Tris-HCl, pH 8, 100 mM NaCl, 0.5% SDS, 0.5% sodium deoxycholate, 0.5% Nonidet P-40 (NP40), 10 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, and 0.4 TIU aprotinin], and the protein content was determined by the Bio-Rad DC protein assay (Hercules, CA). Proteins were then transferred onto a nitrocellulose membrane (Bio-Rad). Western blotting was performed as described previously (Li et al., 1994) with the following antibodies: anti-cyclin E monoclonal antibody IgG_{2b} (HE12), anti-cyclin A monoclonal antibody IgG_1 (BF683), anti-cdk2 polyclonal antibody IgG (M2), anti-cdc2 polyclonal antibody (H297), and anti-chk1 polyclonal antibody (FL-476; all from Santa Cruz Biotechnology Inc., Santa Cruz, CA) and anti-cyclin B monoclonal antibody (Transduction Laboratories, Inc., Lexington, KY), using the Renaissance Chemiluminescence Reagent Kit (NEN Life Science Products).

Immunoprecipitation and Histone H1 Kinase Assay. Immunoprecipitation and in vitro histone H1 kinase assay were performed as described previously (Tsai et al., 1993) with minor modification. Briefly, cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, pH 8.0, 0.1% NP40, 5 mM DTT, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.1 mM sodium orthovanadate) and sonicated twice using a SONIC dismembrator (Artek Systems Corp., Farmingdale, NY). After centrifugation, clarified materials were incubated with protein A agarose (Pierce, Rockford, IL) for 1 h at 4°C for preclearing in IP buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, and 0.1% Tween 20). Immunoprecipitations were carried out with 2.5 μg of the indicated antibody, and immunocomplexes were recovered with protein A agarose.

For the in vitro histone H1 kinase activity assay, the protein A agarose beads were washed three times with lysis buffer and once with kinase buffer (50 mM HEPES, pH 7.0, 10 mM MgCl, 1 mM DTT, and 1 μM cold [γ-^{32}P]ATP). The washed beads were then incubated with reaction mixture containing 2 μg of histone H1 and 5 μCi of [γ-^{32}P]ATP in 20 μl of kinase buffer at 30°C for 20 min. After incubation, 20 μl of 2× Laemmli's sample buffer was added to each sample, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Cell Cycle Analysis. Exponentially growing cells were exposed to drug for 2 h, washed twice, and then maintained in drug-free medium. Cells were harvested at the indicated time intervals. Approximately 10^6 cells were resuspended in 1 ml of modified Krishan buffer with propidium iodide (PI; Molecular Probes, Eugene, OR) (0.1% sodium citrate, 0.02 mg/ml RNase A, 0.37% NP40, and 0.05% mg/ml PI, pH 7.4) and kept on ice and protected from light for 30 to 60 min. The cells were pelleted, resuspended in fresh modified Krishan buffer with PI, and filtered. An FACScan flow cytometer was used to analyze DNA content.

Results

Cell Growth Inhibition. The IC_{10}, IC_{50}, and IC_{90} values of 2-h BNP1350 exposure in A253 cells were 0.01, 0.07, and 0.7 μM, respectively (data not shown). To identify the onset of cell growth inhibition after a 2-h drug exposure to BNP1350, kinetic analysis of cell growth inhibition was performed. The data in Fig. 2 show that the growth of BNP1350-treated cells was

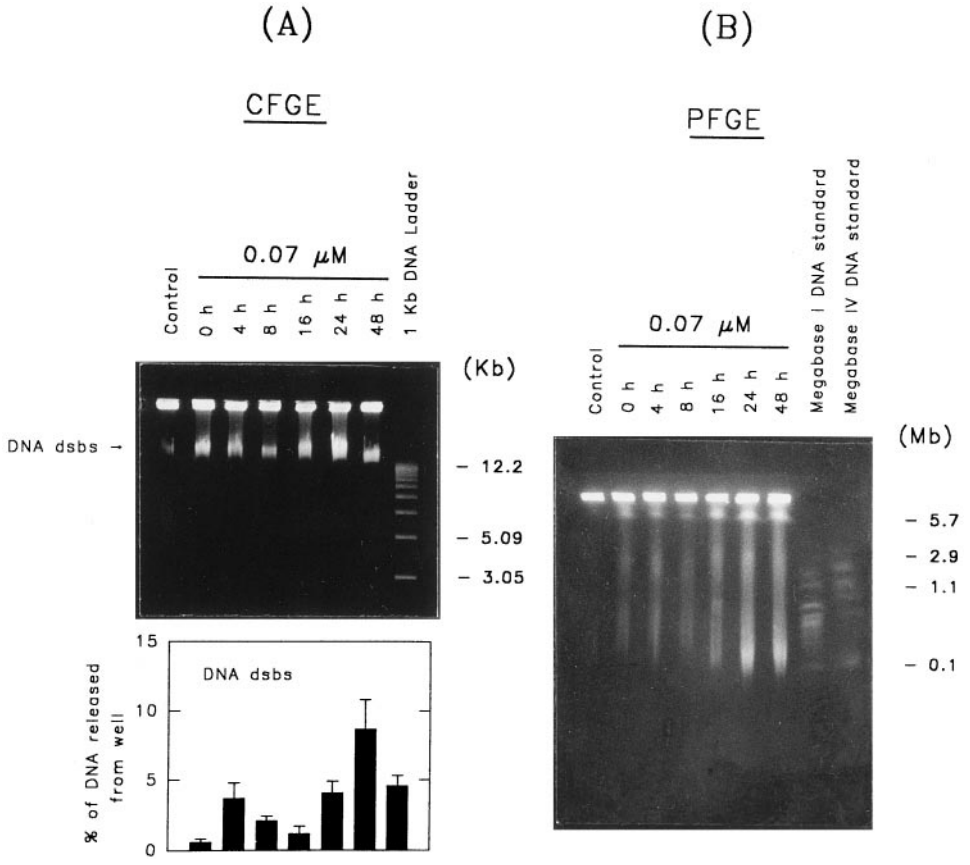

Fig. 3. CFGE and PFGE analyses for BNP1350-induced DNA fragmentation. A, exponentially growing cells were treated with 0.07 μM BNP1350 for 2 h and then incubated in drug-free medium for 0, 4, 8, 16, 24, and 48 h. Preparation of agarose plugs containing DNA were processed as described in *Materials and Methods*. DNA damage assay was performed using CFGE. Bottom, quantification of the percentage of DNA released from the well was determined by scintillation counting. DNA ladder of 1 kb is suitable for sizing linear double-strand DNA. B, cells were harvested for preparation of DNA agarose plugs as described in A. Determination of DNA damage by PFGE was carried out in two phases. Phase I consisted of a 20-min pulse time and a 48-h running time, and phase II consisted of a 6-min pulse time and a 24-h running time, at 14°C in 0.8% agarose. Megabase I DNA standards are suitable for sizing double-strand DNA from 0.1 to 5.7 Mb.

inhibited starting 24 h and persisting from 48 h (low dose) to at least 144 h (high dose) after drug removal.

CFGE and PFGE Analysis for DNA Fragmentation by BNP1350. BNP1350 induced DNA damage as assayed by CFGE and PFGE. In BNP1350-treated cells, the percentage of DNA released from the well was concentration-dependent, with 0.01, 0.07, and 0.7 μM producing DNA breakage (1.0 $\pm$ 0.4%, 7.9 $\pm$ 1.7%, and 12.8 $\pm$ 1.3%, respectively) compared with the control (0.6 $\pm$ 0.2%) (data not shown). Kinetic analysis of DNA damage indicated that exposure of A253 cells to 0.07 μM BNP1350 for 2 h caused a biphasic profile of DNA fragmentation (Fig. 3A). Significant DNA fragmentation (3.7 $\pm$ 1.1%) was produced immediately after 2-h exposure, with a subsequent decrease in DNA breakage between 0 to 8 h after drug removal (1.2 $\pm$ 0.5% at 8 h). A secondary wave of DNA fragmentation (4.1 $\pm$ 0.8%, 8.7 $\pm$ 2.1%, and 4.6 $\pm$ 0.7%) was observed at 16, 24, and 48 h, respectively, after drug treatment. A similar biphasic profile of DNA fragmentation was found in cells treated with another topo I inhibitor, SN-38 (data not shown).

The pattern of BNP1350-induced biphasic DNA fragmentation was analyzed further by PFGE. Figure 3B shows that the first wave of drug-induced DNA damage was primarily associated with megabase DNA fragmentation. However, the secondary wave of DNA damage is associated with the induction of 50- to 300-kb DNA fragmentation in addition to the megabase DNA damage.

Effects of BNP1350 on Expression of Cell Cycle-Regulatory Proteins. To explore the mechanisms of BNP1350-induced cell cycle arrest, the expression of cyclin E/cdk2 and cyclin B/cdc2 proteins was examined by Western blotting. Treatment with IC_{50} and IC_{90} concentrations of BNP1350 produced a dose-dependent increase in cyclin E and cdk2 protein expression in A253 cells. However, the reverse effect was seen with cyclin B/cdc2 protein expression. When treated with the low concentration of BNP1350, the cyclin B/cdc2 protein expression was up-regulated, whereas when treated with the high concentration, the cyclin B/cdc2 protein expression was first increased, followed by down-regulation (Fig. 4).

Cyclin B/cdc2 Kinase Activity Analysis. Because cyclins act by binding to and activating a series of cdks, in vitro cdk kinase activities of the appropriate immunoprecipitates prepared from control and BNP1350-treated A253 cells were evaluated. Cyclin- and cdk-associated kinase activities were assayed using histone H1 as the in vitro substrate. The results indicate that cyclin B/cdc2-associated kinase activities were markedly up-regulated in A253 cells treated with the low concentration of BNP1350, whereas exposure of cells to the higher concentration of BNP1350 slightly suppressed cyclin B/cdc2-associated kinase activity (Fig. 5).

BNP1350-Induced Cell Cycle Effects. Cell cycle response to BNP1350 in A253 cells was examined at various time points after 2-h treatment with the agent. After 2-h exposure to 0.07 μM BNP1350, accumulation of cells in the G_2 phase was observed at 24 h and persisted at 48 h after drug exposure. However, cells treated with 0.7 μM BNP1350 accumulated in S phase at 24 and 48 h after 2-h exposure (Fig. 6). Similar cell cycle perturbations were observed in A253 cells treated with another topo I inhibitor, SN-38: an accumulation of A253 cells at the G_2 phase occurred within 24 h after 2-h exposure to 0.35 μM SN-38 (IC_{50}), whereas the majority of the cells treated with 3.5 μM SN-38 (IC_{90}) accumulated in S phase (Fig. 6B).

Effect of BNP1350 on chk1 Phosphorylation. chk1 encodes the protein kinase $p56^{chk1}$, which is essential for the G_2 DNA damage checkpoint. A gain-of-function caused by overexpression of chk1 can, by itself, elicit a cell cycle arrest in G_2 (Walworth et al., 1993; O'Connell et al., 1997). Inhibition of the human checkpoint kinase, chk1, abrogates G_2 arrest in response to DNA damage (Roshak et al., 1999). Therefore, we evaluated whether G_2 phase arrest induced by a low concentration of BNP1350 is associated with chk1 phosphorylation. The data indicate that chk1 phosphorylation was not significantly changed in cells treated with the

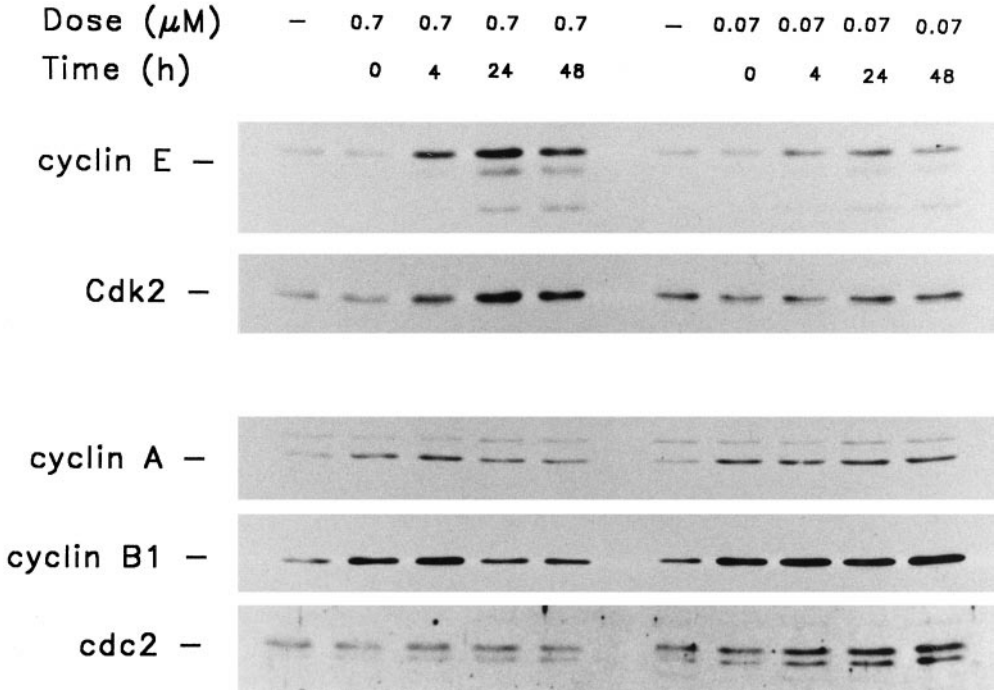

Fig. 4. Western blot analysis of the expression of cell cycle-regulatory proteins. Exponentially growing cells were exposed to 0.07 or 0.7 μM BNP1350 for 2 h and then incubated in drug-free medium for an additional 0, 4, 24, and 48 h. Cell lysates were prepared, and 50 μg of total cell extracts was separated in 10% SDS-PAGE. Protein bands were detected with antibodies to cyclin E, A, and B; cdk2; and cdc2.

higher concentration of BNP1350 (Fig. 7A). However, after low-dose treatment, increased phosphorylation of chk1 was found when cells accumulated at the G_2 phase. Furthermore, chk1 activity was elevated at the low concentration of BNP1350 by histone H1 assay, whereas inhibition of chk1 activity was found in the cells treated with the higher concentration of the agent (Fig. 7B).

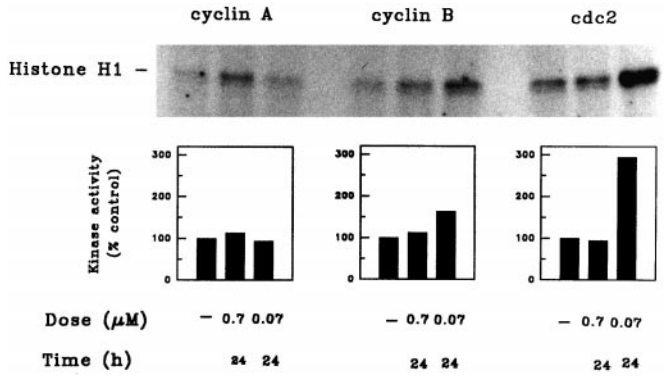

Fig. 5. In vitro assay for cyclin and cdk activities. Cells were exposed to BNP1350 as described in the legend to Fig. 4, and kinase activities were measured 24 h after BNP1350 removal. The preparation of cell lysates is described in *Materials and Methods*. Then, 200 μg of total cell extracts was immunoprecipitated with the indicated antibodies. Two micrograms of histone H1 as the substrate were incubated with each immunoprecipitate plus 5 μCi of [γ-^{32}P]ATP for 20 min at 30°C. The reaction mixture was then subjected to SDS-PAGE, and the extent of histone H1 phosphorylation was detected by autoradiography.

Discussion

The cytotoxicity of CPT and its analogs is predominantly exerted during S phase and is associated with an inhibition of DNA replication. This inhibition is generally thought to be the result of a collision of the advancing replication fork with the CPT/topo I/DNA cleavable complex (Hsiang et al., 1989; Pommier et al., 1994). Such collisions are expected to cause an inhibition in the elongation steps of DNA replication and to kill cells by generating DNA double-strand breaks (Avemann et al., 1988; Ryan et al., 1991). In this study, using the human head and neck carcinoma A253 cells, BNP1350 as a potent inhibitor of topo I was found to be capable of promoting the formation of DNA fragments. However, not all effects of CTP and its analogs can be explained by the collision model. Recent evidence indicates that the sensitivity of cells to CPT might be determined by their ability to activate checkpoints during S and G_2 phases (Tsao et al., 1992; Pommier, 1996). Most cells respond to DNA damage by triggering

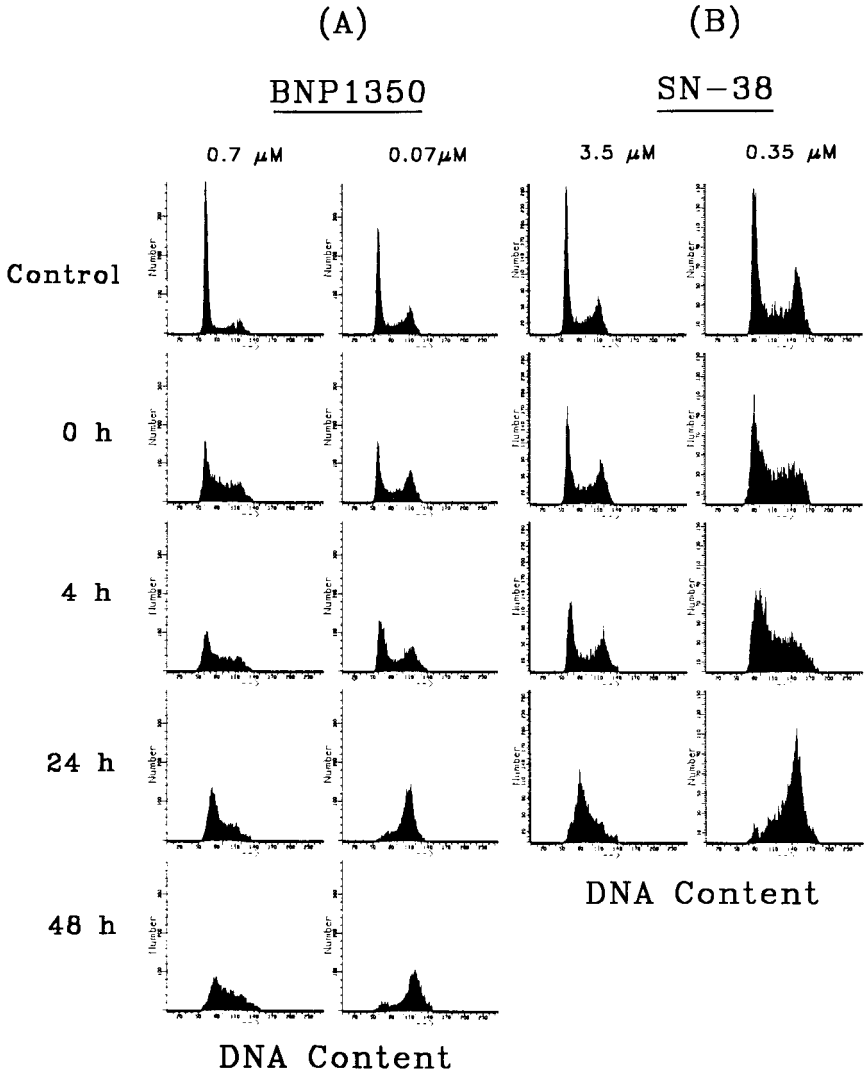

Fig. 6. Effect of BNP1350 and SN-38 on cell cycle distribution. Exponentially growing cells were exposed to 0.7 or 0.07 μM BNP1350 (A) and 3.5 or 0.35 μM SN-38 (B) and then incubated in drug-free medium as described in the legend to Fig. 4. The cells were stained with PI and analyzed by flow cytometry. Abscissa values are proportional stained DNA content, and ordinate values indicate the relative numbers of cell cycle distribution.

signaling pathways that lead to a delay in cell cycle progression, allowing time for repair (Kaufmann, 1995; Murnane, 1995). Thus, the action of CPT and its analogs, including BNP1350, may involve cell cycle checkpoint response.

To analyze the relationship between DNA damage and cell cycle arrest, the kinetics of DNA damage by BNP1350 were first characterized by CFGE and PFGE. The data demonstrated that exposure of A253 cells to 0.07 μM (IC_{50}) BNP1350 resulted in a biphasic profile of DNA fragmentation: a first wave of DNA fragmentation was observed between 0 and 8 h, followed by a second wave of DNA fragmentation observed from 16 to 48 h after 2-h exposure (Fig. 3A). It is unlikely that the initial DNA damage reduction is due to damaged cell loss from the culture, in that direct observation of cells by time-lapse video microscopy demonstrated that even at the IC_{90} value (0.7 μM) of BNP1350, only about 12% of cells disattached and died during the first 48 h (data not shown). The peak value of the second wave (7.9%) was much higher than that of the first wave of DNA fragmentation (3.7% of DNA released from well). PFGE analysis showed that the first wave of DNA damage was associated with megabase DNA fragmentation, whereas the secondary wave of DNA damage was associated with 50- to 300-kb DNA fragmentation in addition to the megabase DNA damage (Fig. 3B). The DNA fragmentation may be induced as a consequence of collisions between replication forks and the cleavable complexes. The appearance of 50- to 300-kb DNA fragmentation from 16 h after 2-h exposure may result from increased cyclin E and cdk2 kinase activities (Yin et al., 1999). These results suggest that the second wave of DNA fragmentation induced by BNP1350 was associated with the drug effect on cell cycle regulation.

Cell cycle arrest is a universal response to DNA damage (Rao, 1980; Konopa, 1988). Extensive DNA damage leads to prolonged cell cycle arrest and cell death (Zhang et al., 1990; Tsao et al., 1992). Meanwhile, the cell cycle arrest enables cells to repair damaged DNA, complete DNA replication, or both. An analysis of cell cycle checkpoint response after DNA damage induced by BNP1350 revealed a complex series of events, with two major patterns of cell cycle perturbations observed. After exposure to a low concentration of BNP1350 (IC_{50}), A253 cells accumulated primarily in G_2 phase at 24 h and persisted at 48 h after 2-h exposure. In contrast, treatment with a high concentration of BNP1350 (IC_{90}) resulted in S phase arrest. Similar observations were found in the cells treated with SN-38. These results suggest that the observed changes in cell cycle distribution after DNA damage may result from the effects seen on cell cycle checkpoint regulatory proteins.

The transition from G_2 to M phase in all eukaryotic cells is tightly linked to the kinase activity of cyclin B/cdc2 complexes. In CPT-treated cells, G_2 arrest is associated with down-regulation of cyclin B/cdc2 kinase activity. The importance of the G_2 checkpoint for CPT cytotoxicity is suggested by the correlation between G_2 breakthrough and CPT cytotoxicity (Tsao et al., 1992; Goldwasser et al., 1996). It seems likely that both cell cytotoxicity and G_2 arrest are triggered by the same event: the interaction between replication machinery and the reversible cleavable complex (Liu, 1989). The G_2 checkpoint is probably critical at low levels of DNA damage because higher concentrations of CPT arrest cells in S phase. The inverse correlation between replicon elongation and degree of G_2 breakthrough suggests that the S and G_2 checkpoints may be interrelated and share common cellular pathways (Zakian, 1995). This notion is in agreement with previous publications linking the S and G_2 checkpoint control systems (Hartwell and Kastan, 1994; Paulovich and Hartwell, 1995).

DNA damage can induce cell cycle arrest via other pathways. The DNA damage checkpoint kinase, chk1, is important for the G_2 checkpoint that delays entry into mitosis in the presence of damaged DNA. Cells that lack chk1 are hypersensitive to agents that induce DNA damage, because they enter mitosis with damaged DNA and subsequently die (al-Khodairy et al., 1994; Wan et al., 1999). Thus, the integrity of the chk1-dependent DNA damage checkpoint pathway

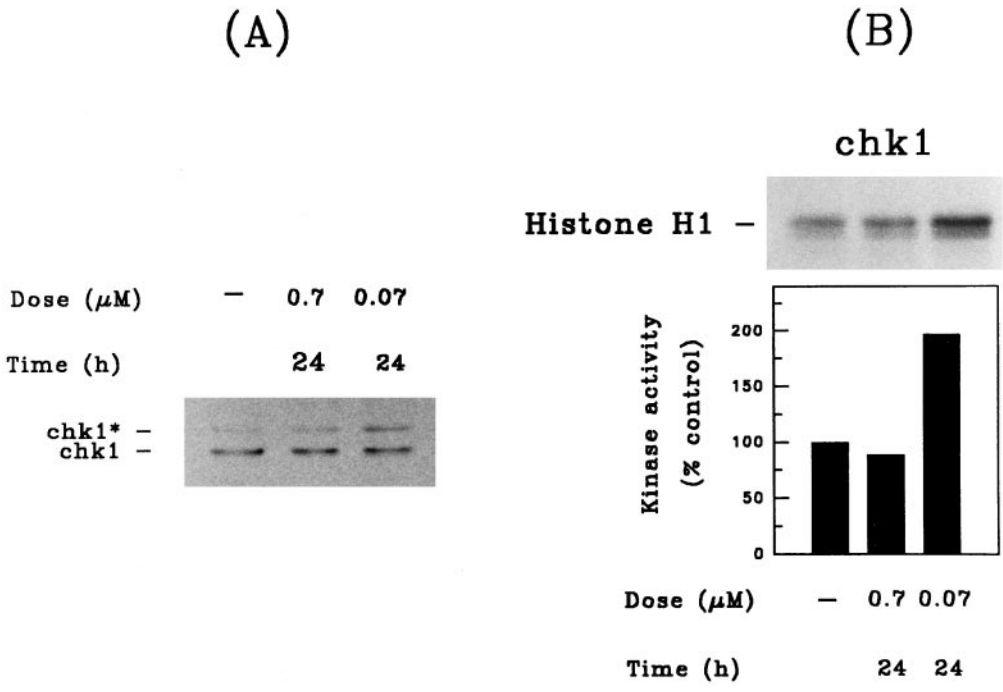

Fig. 7. Effect of BNP1350 on chk1 phosphorylation. Exponentially growing cells were treated with 0.07 or 0.7 μM BNP1350 for 2 h, incubated in drug-free medium for an additional 24 h, and then harvested. Cell lysates for Western blot analysis (A) were prepared in RIPA buffer, and cell lysates for histone H1 assay (B) were prepared in lysis buffer as described in *Materials and Methods*. chk1* indicates phosphorylated chk1.

may be an important determinant of cellular sensitivity to DNA-damaging agents. Although chk1 action after DNA damage depends on the activity of several other components of the DNA damage checkpoint pathway, including rad3, cdc25, and 14-3-3 proteins (Chen et al., 1999; Lopez-Girona et al., 1999), there is interest in the alteration of chk1 phosphorylation in response to DNA damage induced by BNP1350. This observation shows that the signaling pathway to chk1 may be prominent during the DNA damage-associated G_2 phase arrest. A low concentration of BNP1350 arrested A253 cells in G_2 phase of the cell cycle, with a significant increase in chk1 phosphorylation observed. Slightly decreased chk1 phosphorylation was found in the cells treated with a higher concentration of BNP1350. This is consistent with other reports that the inhibition of the human checkpoint kinase chk1 abrogates G_2/M arrest in response to DNA damage (Roshak et al., 1999). These results indicate that chk1 may be a molecular mediator of DNA damage checkpoint response by BNP1350 in A253 cells. The activation of chk1 might not only delay mitotic entry but also increase the capacity of A253 cells to survive.

Another DNA damage responsive factor is nuclear factor-κB (NF-κB). It is activated by various stress situations, including topoisomerase poisons. Likewise, ataxia telangiectasia mutated (ATM) kinase along with other checkpoint kinases are important components in response to DNA damage. Ectopic expression of the ATM protein increases the activation of NF-κB in response to CPT, indicating that the ATM protein is required for activation of NF-κB after DNA damage (Piret et al., 1999). The role of ATM or NF-κB pathways as part of the G_2 response to BNP1350-induced DNA damage is therefore possible, but further investigation is required to prove this association.

In conclusion, this study demonstrated that BNP1350 induced S phase (high concentration) and G_2 phase (low concentration) arrest in A253 cells. This is correlated with the changes in cell cycle checkpoint kinase phosphorylation, which is involved in cell cycle arrest in response to DNA damage. Therefore, a better prediction of sensitivity to BNP1350 may be obtained by the characterization of checkpoint pathways, thereby providing a new approach to improving the cytotoxicity of DNA-damaging agents.

Acknowledgments

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