ORIGINAL PAPERS

The Chk1-Cdc25C regulation is involved in sensitizing A253 cells to a novel topoisomerase I inhibitor BNP1350 by bax gene transfer

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Promotion of apoptosis may potentiate the sensitivity of tumor cells to chemotherapeutic agents, thus improving the efficacy of cancer treatment. The transfection of the proapoptotic *bax* gene, which results in the overexpression of bax protein, augments the growth inhibition of A253 cells by BNP1350. Increased drug response was associated with the induction of DNA fragmentation in the size of 30–200 Kb, generating a cleaved fragment of 18 kDa from full-length 21 kDa bax and the cleavage of PARP. A253/vec cells treated with 0.07 μM(IC_{50}) of BNP1350 accumulated in G_2 phase at 24 h after drug removal. In contrast, A253/Bax cells treated with an equimolar concentration of BNP1350 primarily displayed a G_1 phase accumulation with a concurrent decrease in G_2 phase. Certain cell cycle regulatory protein expression and activities were altered following drug exposure in both cell lines under similar conditions. Cdk2- and cdc2-associated H1 kinase activities were markedly increased in the A253/Bax cell line with marginal increased activity in the A253/vec cell line. A chk1 activity assay was performed with GST-cdc25C (200–256) or GST-cdc25C^{S216A} (200–256) fusion proteins as the substrate. Increased chk1 activity was observed in the A253/vec cell line, with little change in the A253/Bax cell line, when exposed to equimolar concentrations of BNP1350 (0.07 μM). A Western blot of immunoprecipitated chk1 indicated that increased chk1 phosphorylation following DNA damage induced by BNP1350 was accompanied by the observed G_2 accumulation in the A253/vec cell line, while only a slight increase in chk1 phosphorylation was seen in the A253/Bax cell line. A decreased expression of cdc25C was observed in the BNP1350-treated A253/Bax cells, but not in the A253/vec cell line. Following exposure to BNP1350, increased binding of 14-3-3 proteins to chk1 occurred in both cell lines, with more being observed in the A253/vec cell line. The data have shown that inhibition of the chk1 pathway accompanied by the abrogation of G_2 arrest is involved in sensitizing A253 cells to BNP1350 by *bax* gene transfer. These findings suggest that *bax* gene transfer sensitizes A253 cells to BNP1350 through apoptosis promoting and G_2/M DNA damage checkpoint regulatory pathways. *Oncogene* (2001) **20,** 5249–5257.

Introduction

The mechanisms to kill tumor cells by inducing apoptosis have become a focus in the study of tumor treatment strategies. Among numerous genes involved in the regulation of apoptosis, the bcl-2 family gene products are the key regulators of apoptosis. The family is characterized by proteins that inhibit (e.g. bcl-2, bcl-X_L) or promote apoptosis (e.g. bax). The widely expressed *bax* gene is one of the well-characterized pro-apoptotic genes and its overexpression leads to apoptosis in a wide variety of cells and tissues, with or without other additional stimuli (Xiang *et al.*, 1996; Yin *et al.*, 1999). The importance of *bax* gene expression in the clinical outcome of cancer patients has been recognized. Reduced expression of *bax* is associated with poor response rates to combination chemotherapy and shorter survival in woman with metastatic breast cancer (Krajewski *et al.*, 1995). On the other hand, *bax* overexpression enhances the activity of chemotherapeutic agents against cancer and improves the clinical outcome (Tai *et al.*, 1998). The data from our laboratory have shown that the observed increase of antitumor activity in A253/Bax xenografts was associated with an enhanced induction of apoptosis *in vivo* (Guo *et al.*, 2000). Additional evidence has shown that *bax* enhances intracellular drug accumulation of chemotherapeutics (Strobel *et al.*, 1998). It has been proposed that the *bax* gene acts as a tumor suppressor and serves as a good candidate for cancer gene therapy.

Gene therapy using the transfected *bax* gene has proven to be useful in killing cancer cells (Arafat *et al.*, 2000; Xiang *et al.*, 2000), because it may not only kill cancer cells directly, but also increase the sensitivity of radiation and chemotherapeutic agents. Published papers reported that transfection of the *bax* gene into cancer cells shows a more potent antitumor response by enhancing drug-induced apoptosis (Coll *et al.*, 1998;

McPake *et al.*, 1998; Strobel *et al.*, 1996; Sugimoto *et al.*, 1999; Vogelbaum *et al.*, 1998). The cytotoxicity of *bax* overexpression is caused primarily by mitochondrial dysfunction which is involved in the apoptosis triggered by caspase-8 (Shinoura *et al.*, 2000). We have previously shown that *bax* overexpression and dimerization of mitochondrial *bax* in *Bax*-transfected A253 cells are associated with the increased drug response by enhancing drug-induced apoptosis (Guo *et al.*, 1999). This suggests that the induction of *bax* overexpression with mitochondrial dysfunction results in an enhanced cytotoxic effect in cancer cells.

Recent work indicates that the apoptosis regulatory proteins not only induce or inhibit apoptosis, but also can directly impinge on the cell cycle machinery (Brady *et al.*, 1996; Gil-Gomez *et al.*, 1998; Lind *et al.*, 1999; Linette *et al.*, 1996). Several reports have shown that activation of various cell cycle regulatory proteins, such as the cyclins and cyclin-dependent kinases (cdks), correlates with apoptosis (Hsu *et al.*, 1999; Hakem *et al.*, 1999; Jacotot *et al.*, 2000). Furthermore, several oncoproteins and suppressor proteins may simultaneously influence the cell cycle and the propensity to undergo apoptosis (Sard *et al.*, 1999; Jacotot *et al.*, 2000). It has been shown that cell cycle regulation and apoptosis may be interconnected. However, the molecular mechanisms linking the various effects of pro-apoptotic or antiapoptotic regulatory proteins in the cell cycle machinery are still largely obscure. One major conundrum concerns the mechanisms by which pro-apoptotic regulatory proteins can influence cell cycle checkpoint regulatory pathways.

Progression through the cell cycle can be transiently delayed by the generation of DNA damage (Elledge, 1996). The induction of a G_2 delay after DNA damage depends, at least in part, on inhibition of cyclin B1/cdc2 activity through phosphorylation of the cdc2 subunit at Thr[14] and Tyr[15] (Ohi and Gould, 1999). The G_2 delay also requires the activation of the other components of the DNA damage checkpoint pathway, including the protein kinase chk1 (Walworth *et al.*, 1993; al-Khodairy *et al.*, 1994; Walworth and Bernards, 1996). Activated chk1 phosphorylates cdc25C on Ser[216], which is kept inactive through its interaction with 14-3-3 proteins (Peng *et al.*, 1997, 1998). According to the model, phosphorylated cdc25C bound to 14-3-3 proteins would be prevented from dephosphorylating cdc2 and driving cells into mitosis (Chan *et al.*, 2000). However, Chen *et al.* (1999) reported that association of chk1 with 14-3-3 proteins is stimulated in response to DNA damage. DNA damage results in phosphorylation of chk1 and the 14-3-3 proteins bind preferentially to the phosphorylated form. The authors suggested that the interaction between the 14-3-3 proteins and cdc25C does not require chk1 function and is unaffected by DNA damage, in sharp contrast to the interaction between the 14-3-3 proteins and chk1 (Chen *et al.*, 1999). Moreover, there is evidence that the chk1 inhibitor indolocarbazole abrogates G_2 phase arrest by DNA damage (Jackson *et al.*, 2000). Thus, the regulation of chk1 pathway might be an important marker in sensitizing tumor cells by *bax* gene transfer.

We have recently found that altered chk1 phosphorylation following DNA damage appears to be associated with specific phases of cell cycle arrest induced by a novel topo I inhibitor BNP1350 (Yin *et al.*, 2000) and acquired resistance of the A253 cells to BNP1350 may involve the chk1 pathway (Yin *et al.*, submitted). In this report, we investigated the effects of *bax* gene transfer on the sensitivity of A253 cells to BNP1350 and whether overexpression of *bax* by gene transfer technology can enhance drug sensitivity through promotion of apoptosis and the inhibition of the chk1 pathway.

Results

Increased in vitro *drug sensitivity to BNP1350 in A253/Bax transfectant*

The growth inhibiting effect of BNP1350 was examined in A253, A253/vec and A253/Bax cells by SRB assay. As depicted in Figure 1, the IC_{50} of BNP1350 against A253, A253/vec and A253/Bax cells were 0.07, 0.07 and 0.007 μM, respectively. The cell growth inhibition of A253/Bax cells by BNP1350 was significantly higher than that of the parental and A253/vec cell lines. There was no difference in the IC_{50} of BNP1350 between the parental and A253/vec cells. Since A253/Bax cells stably express approximately 50 times higher bax protein than the parental cells, increased *in vitro* sensitivity to BNP1350 is associated with overexpression of bax in stable A253/Bax transfectant.

Measurement of BNP1350-induced DNA fragmentation by PFGE

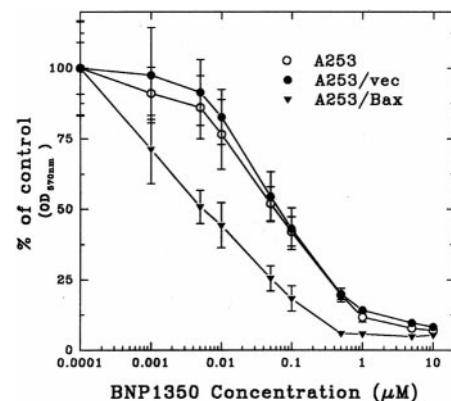To explore whether *bax* gene transfer is associated with pro-apoptotic DNA fragmentation, the patterns of

Figure 1 Growth inhibition of A253, A253/vec and A253/Bax cells treated with BNP1350. Exponentially growing cells were treated with various concentrations of BNP1350 for 2 h and then incubated in drug-free medium for four doubling times after drug exposure. 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 8 wells of culture

DNA fragmentation induced by BNP1350 in A253, A253/vec and A253/Bax cells were analysed by pulse-field gel electrophoresis. At 24 h after 2 h exposure to 0.07 μM BNP1350, significantly increased DNA fragmentation in the size of 30–200 Kb was observed in A253/Bax, when compared to the parental or A253/vec cells (Figure 2). Since the appearance of pro-apoptotic DNA fragmentation ranging between 50–300 Kb was associated with apoptotic cell death (Oberhammer *et al*., 1993; Collins *et al*., 1997), treatment with BNP1350 markedly increased the apoptotic cell death in the A253/Bax transfectant cell line.

Expression studies of sets of apoptosis-regulating gene products

Because the bcl-2 family proteins are the key regulators of apoptotic cell death, we examined the expression of bcl-X_L and bax gene products in both the A253/vec and A253/Bax cell lines. The results as shown in Figure 3 indicated that increased expression of bax protein was observed in A253/Bax cells. There is also a cleaved fragment at 18 kDa of *bax* seen only in the A253/Bax cells. To determine whether the cleavage of PARP, a nuclear substrate of caspase-3, was involved in increased *in vitro* sensitivity to BNP1350, we evaluated the extent of the cleavage of PARP after drug treatment. The results in Figure 3 have shown that PARP was cleaved to the expected 89 kDa fragment from 113 kDa only in the A253/Bax cell line.
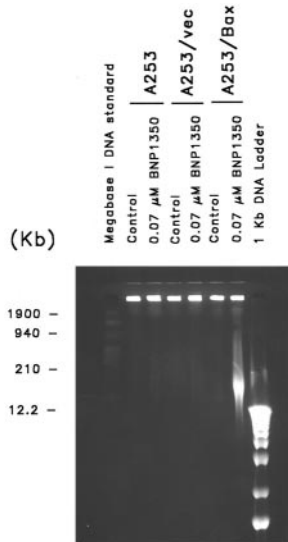
G_2 phase abrogation observed in sensitizing A253/Bax transfectant to BNP1350

The three cell lines were exposed to equimolar concentrations of BNP1350 (0.07 μM) for 2 h and further incubated without the drug for up to 48 h. The cells were harvested at the indicated time points in Figure 4 and analysed for cell cycle distribution. As shown in Figure 4, A253 and A253/vec cells accumulated in the G_2 phase at 24 and 48 h after drug removal, but decreased G_2 phase accumulation and increased numbers of cells in G_1 phase were observed in the A253/Bax cells treated at this concentration. The patterns of cell cycle distribution
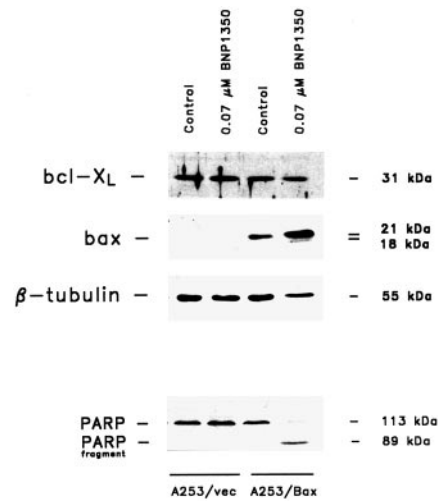
Figure 3 Western blot analysis of the expression of the apoptosis-regulatory proteins. Cells were treated with 0.07 μM BNP1350 for 2 h, washed twice in fresh medium and then incubated in drug-free medium for an additional 24 h. Fifty μg of cell lysates were loaded onto each lane, separated in SDS–PAGE and were examined using anti-bcl-X_L and anti-bax antibodies, followed by incubation with secondary antibody conjugated to peroxidase. Caspase-3 cleavage products, 113 kDa PARP and 89 kDa fragment, were examined using anti-PARP antibodies. ß-tubulin signals were also obtained to ensure equivalency of protein loading
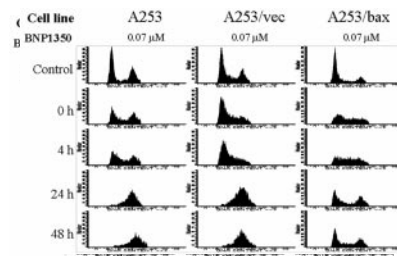
Figure 2 PFGE analysis for BNP1350-induced DNA fragmentation. Exponentially growing cells were treated with 0.07 μM BNP1350 for 2 h and then incubated in drug-free medium for 24 h. Cells were harvested for preparation of agarose plugs containing DNA as described in Materials and methods. Determination of DNA damage by PFGE was carried out in one phase: 90 s pulse time, 16 h running time at 170 V at 14°C in 0.8% agarose. DNA ladder of 1 Kb and megabase I DNA standards are suitable for sizing double strand DNA from 0.001 to 1.9 Mb

Figure 4 Effect of BNP1350 on cell cycle distribution of A253, A253/vec and A253/Bax cells. Cell lines were exposed to identical concentrations (0.07 μM) of BNP1350 for 2 h and then incubated in drug-free medium for the indicated times. The cells were stained with propidium iodide and analysed by flow cytometry. Abscissa values are proportional stained DNA content and ordinate values indicate the relative numbers of cell cycle distribution

induced by BNP1350 in A253/Bax cells were quite different from that of the parent and A253/vec cells. These results indicate that G_2 phase abrogation plays an important role in sensitizing A253/Bax cells to BNP1350.

Expression studies of sets of cell cycle regulatory proteins

Under our experimental conditions used previously, the expression of cyclin A/cdk2 and cyclin B/cdc2 proteins were examined by Western blot in the three cell lines. The results as shown in Figure 5 indicate that increased expression of cyclin A/cdk2 and cyclin B/cdc2 proteins was observed in the parental and A253/vec cell lines, but no significant increase in cyclin A, cyclin B or cdc2 protein expression in the A253/Bax cell line. In general, after treatment with equimolar concentrations of BNP1350, there was no momentous difference of cyclin A/cdk2 and cyclin B/cdc2 protein expression patterns between the parental, A253/vec or A253/Bax cell lines.

Cdk2 and cdc2-associated H1 kinase activities after BNP1350 treatment in A253/Bax transfectant

Since cyclins act by binding to and activating a series of cdks, *in vitro* cdk kinase activities of the appropriate immunoprecipitates prepared from BNP1350-treated cell lines were evaluated under previously mentioned experimental conditions. Cdk2 and cdc2-associated kinase activities were assayed using histone H1 as the *in vitro* substrate. As shown in Figure 6, cdk2-associated H1 kinase activities increased in both A253/vec and A253/Bax cell lines, but significantly more in the *bax*-transfected cell line. Cdc2-associated H1 kinase activity was markedly increased in the A253/
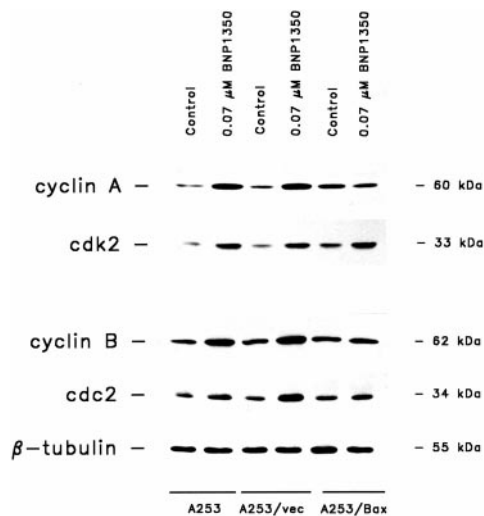
Bax cell line and only a slight increase in activity was seen in the A253/vec cell line. *Bax* gene transfer was shown to be associated with increased cdk2 and cdc2 kinase activity after BNP1350 treatment.

Effect of BNP1350 on chk1 phosphorylation and activity

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 abrogation is associated with lowered chk1 phosphorylation and inhibited chk1 activities. A Western blot of immuno-precipitated chk1 indicated that increased chk1 phosphorylation following DNA damage induced by 0.07 μM BNP1350 was accompanied by the observed G_2 accumulation in the A253/vec cell line, while only a slight increase in the A253/Bax cell line (Figure 7a). The data as shown in Figure 7b indicate that chk1 activity increased in A253/vec cells, but little change was observed in the A253/Bax cell line under the same experimental conditions.

Decreased cdc25C protein expression and binding of 14-3-3 proteins to chk1

To confirm interaction between chk1, cdc25C and the 14-3-3 proteins, 14-3-3 and cdc25C protein expressions were determined. As shown in Figure 8a, decreased expression of cdc25C protein occurred in the A253/Bax cells but not in the A253/vec cell line after drug treatment. Moreover, to confirm whether 14-3-3 proteins bind to chk1, chk1 immunoprecipitation assays were performed by Western blot analysis. As
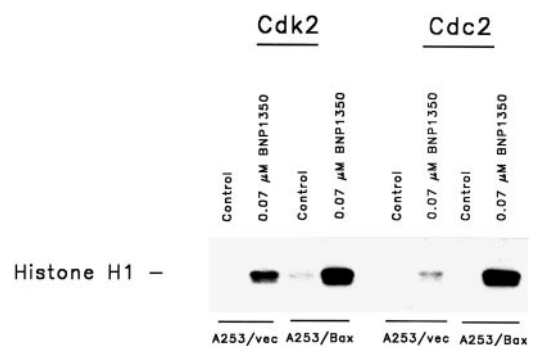

Figure 5 Western blot analysis of the expression of cell cycle regulatory proteins. A253 cell lines were exposed to 0.07 μM BNP1350 for 2 h and then incubated in drug-free medium for an additional 24 h. Cell lysates were prepared and 50 μg of total cell extracts were separated on 10% SDS–PAGE. Protein bands were detected with antibodies to cyclin A, B, cdk2 and cdc2. ß-tubulin signals are shown for equal loading

Figure 6 *In vitro* assay for cdk activities. A253 cell lines were exposed BNP1350 as described in the legend to Figure 5 and kinase activities were detected 24 h after 2 h exposure. The preparation of cell lysates is described in Materials and methods. Two hundred μg of total cell extract were immunoprecipitated with the indicated antibodies. Two μg of histone H1 as the substrate were incubated with each immunoprecipitate plus 5 μCi [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
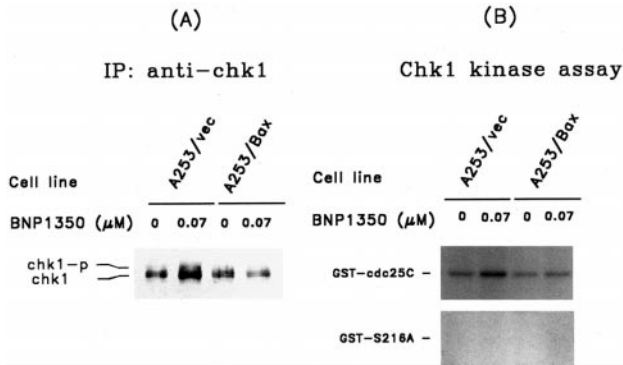
Figure 7 Effect of BNP1350 on chk1 phosphorylation and activity. Exponentially growing cells were treated with BNP1350 as described in the legend to Figure 5, then incubated in drug-free medium for an additional 24 h and harvested. Preparation of cell lysates is described in Materials and methods. Cell lysates were immunoprecipitated with an anti-chk1 monoclonal, followed by Western blot with anti-chk1 polyclonal antibody (**a**). The immunoprecipitates were assayed for kinase activity using GST-cdc25C or GST-S216A as the substrate (**b**)
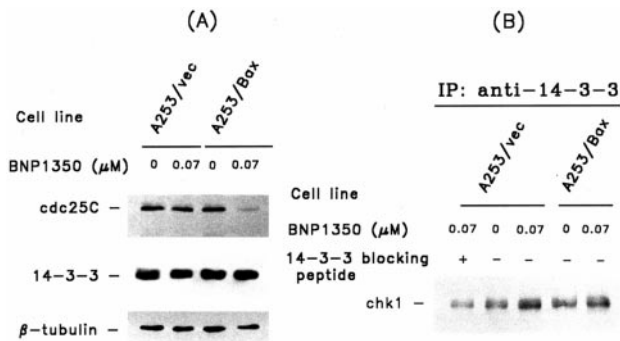

Figure 8 Effect of BNP1350 on 14-3-3 and cdc25C protein expressions (**a**) and binding of 14-3-3 proteins to chk1 or cdc25C (**b**). Both cell lines were treated and harvested as described in Figure 5. Cell lysates for Western blot analysis were prepared in RIPA buffer (**a**) and cell lysates for immunoprecipitation assay were prepared in lysis buffer (**b**) as described in Materials and methods. Lysates were immunoprecipitated with anti-14-3-3 antibodies. Immune complexes were washed and resolved by SDS–PAGE. The binding of 14-3-3 proteins to chk1 was visualized by immunoblotting. Neutralization with blocking peptides is available as control

shown in Figure 8b, increased binding of 14-3-3 proteins to chk1 occurred in both cell lines, with more being observed in the A253/vec cell line following exposure to BNP1350.

Discussion

In this study, we have found that the cell growth inhibition of A253/Bax cells by BNP1350 was significantly higher than that of the parental and A253/vec cell lines (Figure 1). A253/Bax cells stably express approximately 50 times higher bax protein than the parental cells, indicating that increased *in vitro* sensitivity to BNP1350 is associated with overexpression of bax in stable A253/Bax transfectant cells (Guo

et al., 1999). Moreover, our previous report also indicates that A253/Bax cells exhibited a significant increase in *in vitro* sensitivity to various anticancer drugs, including tomudex (9.5-fold), SN-38 (13.8-fold), doxorubicin (7.9), taxol (3.1-fold), 5-FU (2.7-fold) and 5-FU/LV (4.5-fold), compared to the parental and A253/vec cell lines. These results demonstrated a correlation between the levels of bax expression and *in vitro* drug sensitivity.

Potentiated *in vitro* sensitivity to BNP1350 is related to an increase in BNP1350-induced DNA fragmentation in the size of $30-200$ Kb and the cleavage of PARP (113 kDa) into the 89 kDa fragment observed in the A253/Bax cell line (Figures 2 and 3). Additionally, the effects of bax on apoptotic DNA fragmentation and apoptosis-associated protein expression appears to be tightly linked to cell cycle regulation, since A253 and A253/vec cell lines treated with 0.07 μM of BNP1350 accumulated in G_2 phase at 24 h after drug removal, but abrogated G_2 phase arrest was observed in the A253/Bax cell line treated with an equimolar concentration of BNP1350 (Figure 4). These results indicate that *bax* overexpression might be coupled with the abrogation of G_2 phase arrest and the enhanced sensitivity of A253 cells to BNP1350, in addition to the promotion of apoptosis. Analysis of several cell cycle regulatory protein expressions showed no momentous difference between these cell lines. Cdk2- and cdc2-associated H1 kinase activity, however, was markedly increased in the A253/Bax cell line with negligibly increased activity in the A253/vec cell line under the same experimental conditions. This shows that activation of cyclin B-dependent kinase is associated with the abrogation of G_2 phase arrest induced by BNP1350 in the A253/Bax cell line.

DNA damage will lead to arrest of the cell cycle progression at specific phases of the cell cycle, the so-called 'cell cycle checkpoints'. Since the checkpoints help to prevent further damage and give the cell time to repair the lesions that already occurred, G_2 arrest is usually associated with increased viability following drug treatment (Dubrez *et al*., 1995; Goldwasser *et al*., 1996). This delay of cell cycle progression requires the activation of the DNA damage checkpoint kinase chk1 (Walworth *et al*., 1993; al-Khodairy *et al*., 1994). 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). Roshak *et al*. (1999) reported that tumor cells can take advantage of the G_2 checkpoint to arrest following DNA damage and avoid immediate cell death. In the presence of the chk1 inhibitor, indolocarbazole, the cells did not arrest in the G_2 phase of the cell cycle following γ-irradiation or treatment with topotecan, but continued into mitosis. It may be possible to synergistically augment tumor cell death induced by DNA damage and circumvent resistance by abrogating the G_2 checkpoint arrest. These studies suggest that chk1 activity is required for G_2 arrest following DNA damage (Jackson *et al*., 2000). It was also reported that the chk1 protein kinase

inhibitor, UCN-01, can potentiate camptothecin-induced cytotoxicity (Graves *et al*., 2000). Our data indicated that A253/Bax cells exhibited a significant inhibition of chk1 activity and potent enhancement of BNP1350-induced cell growth inhibition. These results demonstrate a relationship between inhibition of the chk1 pathway and the abrogation of the G_2 arrest accompanied by the potentiation of drug-induced cytotoxicity.

Our previous studies show that the signaling pathway to chk1 may be prominent during the DNA damage-associated G_2 phase arrest in the A253 cell line. A low concentration of BNP1350 arrested this cell line in the G_2 phase of the cell cycle, with a significant increase in chk1 activity. No obvious alteration of chk1 activity was found in the cells treated with a higher concentration of BNP1350 (Yin *et al*., 2000). The data indicated that altered chk1 activity might predict for drug sensitivity. We also found that after exposure to equimolar concentrations of BNP1350 (0.7 μM), A253 cells accumulated primarily in S phase, but G_2 phase accumulation was observed in the drug-resistant A253/BNPR cell line at 48 h after drug removal. The results indicate that increased chk1 activity was accompanied by G_2 phase accumulation observed in the A253/BNPR cells (Yin *et al*., submitted). These results suggest a potential role of the activation of chk1 pathway in the cellular resistance to BNP1350 in the A253/BNPR cell line.

The protein kinase chk1 is thought to be capable of phosphorylating the Ser[216] site of cdc25C in mammalian cells. The maintenance of cdc25C phosphorylation on Ser[216] after DNA damage is thought to prevent premature progression from G_2 into mitosis (Peng *et al*., 1997, 1998). Since the phosphorylation of cdc25C on Ser[216] is an important upstream event during the induction of G_2 arrest in cells exposed to DNA-damaging agents, GST-cdc25C (200–256) or GST-cdc25C[S216A] (200–256) is used as the substrate for the chk1 activity assay. In this study, A253/vec cells which displayed an increase in chk1 activity were accompanied by G_2 phase accumulation after treatment with 0.07 μM BNP1350. No significant increase in chk1 activity coupled with the abrogation of G_2 phase arrest was observed in the A253/Bax cells under similar experimental conditions. Thus, it has been shown that the cell cycle effects of *bax* gene transfer may function at the level of the G_2 DNA damage checkpoint regulatory pathways. Additionally, our data indicated that the inhibition of chk1 activity is coupled with a decrease in cdc25C expression, which activates cdc2 resulting in the abrogation of G_2 phase arrest following BNP1350 treatment in the bax-overexpressing A253/Bax cells.

The signal transduction cascade leading to the alterations of chk1 activity is also thought to play a role in the G_2 DNA damage checkpoint regulation. Walworth *et al*. (1996) proposed that the association of chk1 with 14-3-3 proteins is stimulated in response to DNA damage. DNA damage results in phosphorylation of chk1 and the 14-3-3 proteins bind preferentially to the phosphorylated form (Chen *et al*., 1999). Our

data were consistent with the Walworth's proposition and indicated that increased binding of 14-3-3 proteins to chk1 occurred in both cell lines, with more being observed in the A253/vec cell line following exposure to BNP1350. Therefore, chk1 action with regard to the potentiation of BNP1350 cytotoxicity in the A253/Bax cell line is associated with the abrogation of G_2 phase arrest which may depend on the regulation of 14-3-3 protein binding to chk1 by phosphorylation/dephosphorylation of cdc25C on Ser[216] (Lopez-Girana *et al*., 1999). Furthermore, it has recently been reported that the onset of mitosis is controlled by the cyclin dependent kinase cdc2p. Cdc2p activity is controlled through the balance of phosphorylation and dephosphorylation of tyrosine-15 (Y15) by the Wee1p kinase and cdc25p phosphatase (Raleigh and O'Connell, 2000). These results provided a formal link between the chk1 pathway and Wee1p kinase.

In this report, our results have demonstrated for the first time the link between *bax* gene transfer and the chk1 regulatory pathway. These observations also identify the chk1 pathway as a potential target of both apoptosis and the G_2 phase abrogation by *bax* gene transfer. These results are consistent with reports that the apoptosis regulatory proteins themselves can directly impinge on the cell cycle machinery (Brady *et al*., 1996; Linette *et al*., 1996).

One obvious argument against a major overlap between cell cycle regulation and apoptosis control is the fact that the cell cycle inhibitory effect by bcl-2 is mechanistically different from the apoptosis-inhibitory effect because a specific mutation of the bcl-2 gene in the BH4 domain abolishes its anti-proliferative effect yet leaves its anti-apoptotic potential (Huang *et al*., 1997). Thus, although *bax* gene transfer may simultaneously influence the cell cycle regulation and the propensity to undergo apoptosis, it is not clear whether the putative apoptosis checkpoints are different from those determining the advancement of the cell cycle.

In summary, we demonstrated that *bax* gene transfer highly and efficiently increases sensitivity of human head and neck carcinoma A253 cells to BNP1350. Increased sensitivity of A253 cells to BNP1350 is associated not only with apoptosis promotion, but also with the G_2 phase abrogation which requires the function of G_2 DNA damage checkpoint kinase chk1. Thus, we suggest that *bax* gene transfer sensitizes A253 cells to BNP1350 through apoptosis promoting and the chk1 pathways. This suggestion will be useful not only for furthering our understanding of the role of the G_2 DNA damage checkpoints and the putative apoptosis checkpoints in the modulation of drug sensitivity, but also for innovative chemotherapeutic strategies.

Materials and methods

Drugs and chemicals

BNP1350 was kindly supplied by BioNumerik Pharmaceuticals, Inc. (San Antonio, TX, USA). The compound was

dissolved in DMSO with final dilutions made in the growth medium. Proteinase K and RNase A were obtained from Roche Diagnostics, Corp. (Indianapolis, IN, USA). [^{32}P]ATP (specific activity 3000 Ci/mmole) was obtained from NEN Life Science Products (Boston, MA, USA). SRB and sarkosyl were obtained from Sigma Chemical Co. (St. Louis, MO, USA). G418 (Geneticin) was purchased from GIBCO BRL, Life Technologies (Grand Island, NY, USA).

Cell line

The human HNSCC cell line A253 was purchased from American Type Culture Collection (Rockville, MD, USA) and grown as a monolayer culture in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO-BRL, Life Technologies). All treatments were carried out using exponentially growing cell cultures. The cell lines were free from mycoplasma as tested with the GEN-PROBE mycoplasma TC rapid detection system every 2 months (GEN-PROBE Inc, San Diego, CA, USA).

Transfection

Human HNSCC A253 cells were transfected with a full-length bax cDNA, which was constructed into the *EcoR*I site of pcDNA3 expression vector (Invitrogen, Carlsbad, CA, USA). The pcDNA3/Bax or pcDNA3 empty vectors were transfected into A253 cells using LIPOFECT-AMINE™ Reagent (GIBCO-BRL, Life Technologies), according to the manufacturer's protocol. After transfection, cells were selected for neomycin resistance by treating with G418 (GIBCO-BRL, Life Technologies). The bax-transfected cell line was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and G418. Individual G418 resistant clones were picked up, expanded and analysed for bax expression by immunoblotting of total cellular protein. A253/Bax cells stably express about 50 times higher bax protein than the parental or A253/vec cells.

Drug sensitivity test *in vitro*

Cell growth inhibition of A253, A253/vec and A253/Bax cells by BNP1350 was estimated using the total protein SRB assay as described elsewhere (Skehan *et al.*, 1990). Approximately 600 cells/well were seeded in each well of a 96-well plate and cultured for 24 h at 37°C. These cells were treated with BNP1350, which was diluted in culture medium, for 2 h, and then incubated in drug-free medium for four doubling times post drug exposure. The cells were fixed with 10% trichloroacetic acid (TCA) 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).

PFGE analysis for DNA fragmentation

The procedure for 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 HBSS. An equal volume of 2% low melting point agarose prepared in HBSS was added at 50°C. The mixture was poured into the molds immediately. 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 of 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. Agarose gels were prepared in $0.5 \times$ BRL TBE buffer (75 mM Tris, 25 mM boric acid and 0.1 mM EDTA, pH 8.9) as described previously (Panadero *et al.*, 1995). Electrophoresis was carried out using Hex-A-Field horizontal gel electrophoresis apparatus (GIBCO-BRL), which contains a hexagonal array of electrodes having a reorientation angle of 120°. The electrophoresis was performed in TBE buffer at 14°C with buffer circulation. Following electrophoresis, gels were stained with ethidium bromide and then photographed on a UV-transilluminator.

Cell cycle analysis

Cell cycle distribution was analysed using flow cytometry. Exponentially growing cells were exposed to BNP1350 for 2 h, washed twice, and then maintained in drug-free medium. At the indicated time intervals, the cells were harvested. Approximately 10^6 cells were resuspended in 1 ml of modified Krishan Buffer with propidium iodide (PI, Molecular Probes, Eugene, OR, USA) (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 protected from light for 30–60 min. The cells were pelleted, resuspended in fresh modified Krishan Buffer with PI and filtered. Measurements of DNA content were obtained using a FACScan flow cytometer (Becton Dickson, San Jose, CA, USA). Analysis of the data was performed using the WinMDI Version 2.7 Software program.

Immunoblot analysis

Cells were harvested at selected time points after exposure to BNP1350 and lysed in RIPA buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl, 0.5% SDS, 0.5% sodium deoxycholate, 0.5% Nonidet P40, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 0.4 TIU aprotinin). The protein content was determined by the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts (50 μg/lane) of the lysate were loaded and separated on SDS–PAGE, and then transferred onto a nitrocellulose membrane (Bio-Rad Laboratories). Western blotting was performed with the following antibodies: anti-bcl-X_L monoclonal antibody (Trevigen, Inc., Gaithersburg, MD, USA), polyclonal rabbit anti-human bax (DAKO Corporation, Carpinteria, CA, USA), anti-PARP polyclonal antibody (Roche Diagnostics Corp.), anti-cyclin A monoclonal antibody IgG$_1$ (BF683), anti-cdk2 polyclonal antibody IgG (M2), anti-cdc2 polyclonal antibody IgG (H-297) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and anti-cyclin B monoclonal antibody (Transduction Laboratories, Inc., Lexington, KY, USA), and visualized using the Renaissance chemiluminescence reagent kit (NEN Life Science Products). Anti-ß-tubulin mAb (clone TUB 2.1, Sigma) was used to demonstrate equal protein loading.

Immunoprecipitation and kinase activity assay

Immunoprecipitation and *in vitro* kinase activity assays were performed by the procedure described previously with minor modification (Tsai *et al.*, 1993). 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% Nonidet P-40, 5 mM DTT, 10 mM NaF, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 0.1 mM sodium orthovanadate) and sonicated twice using

SONIC dismembrator (ARTEK Systems Corp., Farmingdale, NY, USA). After centrifugation, clarified materials were incubated with protein A agarose (PIERCE, Rockford, IL, USA) 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). Cell lysates were immunoprecipitated with 2.5 μg of anti-chk1 polyclonal antibody (FL-476) (Santa Cruz Biotechnology Inc.), and immunocomplexes were recovered with protein A agarose. 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 (ATP). The kinase activity was determined in 20 μl of reaction mixture containing GST-cdc25c and 5 μCi of [^{32}P]γATP in 20 μl kinase buffer at 30°C for 20 min. The GST-cdc25C or GST-cdc25C^{S216A} fragment were used as a substrate. After incubation, 20 μl of 2 × Laemmli sample buffer (Laemmli, 1970) was added to each sample, the reaction products were analysed by SDS–PAGE and phosphorylated proteins were detected by autoradiography.

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