Parthenolide Cooperates with NS398 to Inhibit Growth of Human Hepatocellular Carcinoma Cells through Effects on Apoptosis and G0-G1 Cell Cycle Arrest

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Abstract

Chemotherapy to date has not been effective in the treatment of human hepatocellular carcinoma. More effective treatment strategies may involve combinations of agents with activity against hepatocellular carcinoma. Parthenolide, a nuclear factor-κB (NF-κB) inhibitor, and NS398, a cyclooxygenase (COX)-2 inhibitor, have been shown to individually suppress the growth of hepatocellular carcinoma cells in vitro. To investigate their effects in combination, three human hepatocellular carcinoma lines (Hep3B, HepG2, and PLC) were treated with parthenolide and/or NS398. Parthenolide (0.1-10 μM/L) and NS398 (1-100 μM/L) each caused concentration-dependent growth inhibition in all cell lines. The addition of parthenolide to NS398 reduced the concentration of NS398 required to inhibit hepatocellular carcinoma growth. Because parthenolide and COX-2 inhibitors have been reported to influence NF-κB activity, the effects on this pathway were investigated. The combination of parthenolide/NS398 inhibited phosphorylation of the NF-κB-inhibitory protein IκBα and increased total IκBα levels. NF-κB DNA-binding and transcriptional activities were inhibited more by the combination than the single agents in Hep3B and HepG2 cells but not in PLC cells. The response of PLC cells to NS398 was augmented by p65 small interfering RNA to inhibit NF-κB p65 protein expression. The combination of parthenolide/NS398 increased apoptosis only in PLC cells, suggesting that the combination may decrease the apoptotic threshold in these cells. In Hep3B and HepG2 cells, combination treatment with NS398 and parthenolide altered the cell cycle distribution resulting in more G0-G1 accumulation. Cyclin D1 levels were further decreased by combination treatment in all cell lines, correlating with the cell cycle alterations. Our results suggest that parthenolide may be effective in combination with COX-2 inhibitors for the treatment of hepatocellular carcinoma. (Mol Cancer Res 2006;4(6):387–400)

Introduction

Hepatocellular carcinoma is one of the most common causes of solid organ malignancy in the world (1). The incidence of hepatocellular carcinoma in the United States is increasing, doubling in the last 20 years (1). Definitive treatment of hepatocellular carcinoma through surgical resection and/or transplantation can provide long-term survival; however, the vast majority of hepatocellular carcinoma is identified at an advanced stage, so the number of individuals who are candidates for surgical treatment are few (2, 3). Chemotherapy and other systemic treatments for patients with hepatocellular carcinoma have been largely ineffective (2, 4). More effective therapies need to be developed to treat hepatocellular carcinoma in patients who are not surgical candidates.

Targeting novel cellular growth and survival signaling pathways may hold promise for the treatment of hepatocellular carcinoma. One such growth/survival signaling pathway involves the transcription factor nuclear factor-κB (NF-κB) that was first identified as a regulator of genes involved in the immune and inflammatory responses (5). More recently, NF-κB activation has been discovered to play a role in oncogenesis and has been linked to the regulation of apoptosis, the cell cycle, and metastasis (6-12). Constitutively active NF-κB may also contribute to the chemoresistance of cancer cells (13, 14). Activation of the NF-κB pathway in several different solid tumors provides protection from the antiproliferative effects of a variety of agents (13, 15-19). In hepatocellular carcinoma, NF-κB is constitutively active (20, 21); therefore, the NF-κB pathway may be a promising therapeutic target and inhibitors of this pathway should be evaluated.

Regulation of the NF-κB pathway can occur at several levels. NF-κB is usually sequestered in the cytoplasm as an inactive complex with the inhibitory IκB proteins. In response to a signal activating the NF-κB pathway, the IκB proteins are phosphorylated by the IKK complex, targeting them for degradation. NF-κB is then liberated and can translocate into the nucleus to activate the expression of specific genes (8). Because of its role in NF-κB regulation, the inhibitory protein IκBα has been identified as tumor suppressor gene (22).
Sesquiterpene lactones, isolated from Mexican Indian medicinal plants, have been shown to inhibit NF-κB (23). Parthenolide, an abundant sesquiterpene lactone found in the medicinal herb feverfew (Tanacetum parthenium), is currently used for the treatment of migraine headaches (24, 25). In prostate cancer cells and breast cancer cells with constitutive NF-κB activity, parthenolide inhibits the DNA-binding activity of NF-κB (26, 27). In the present study, we evaluated the effect of parthenolide on NF-κB activity, growth, apoptosis, and cell cycle control in hepatocellular carcinoma cell lines in vitro.

The cyclooxygenase (COX) pathway has also been implicated in hepatic tumorigenesis. Expression of the isoform COX-2 is elevated in hepatocellular carcinoma and may correlate with the degree of tumor differentiation (28-30). Early studies showed that COX-2 inhibitors, such as NS398, increase the rates of apoptosis in hepatocellular carcinoma cells in vitro (28, 31). Although these results suggest COX-dependent mechanisms (28, 32), the doses of NS398 required to induce apoptosis were much higher than those necessary for COX-2 inhibition. Therefore, COX-independent mechanisms may also be involved in the growth-inhibitory effects of COX-2 inhibitors (33, 34). Moreover, we have shown previously that the COX-2 inhibitor NS398 can exert substantial antiproliferative effects in the HepG2 and Hep3B hepatocellular carcinoma cell lines in vitro despite the absence of COX expression (35). Similarly, inhibition of tumor growth has also been shown in other COX-deficient neoplastic cell lines (36-38). Taken together, these findings suggest that COX-2 inhibitors may mediate their growth-inhibitory effects at least in part through COX-independent mechanisms.

Because we and others have shown previously that COX-2 inhibitors suppress the growth of hepatocellular carcinoma cells in vitro, COX-2 inhibitors may be effective treatment for hepatocellular carcinoma. However, for resistant cancers, such as hepatocellular carcinoma, single-agent chemotherapy will likely be unsuccessful; therefore, combination chemotherapy targeting pathways critical for growth and survival may be more effective. To investigate whether inhibition of the NF-κB pathway can sensitize hepatocellular carcinoma cells to the antiproliferative effects of COX-2 inhibitors in vitro, we evaluated the effects of parthenolide in combination with the COX-2 inhibitor NS398 on growth, apoptosis, and cell cycle.

Results

Growth Effects of Parthenolide on Hepatocellular Carcinoma

To determine the effects of the NF-κB inhibitor parthenolide on growth, three human hepatocellular carcinoma cell lines (Hep3B, HepG2, and PLC) were treated with increasing concentrations of parthenolide (0-10 μmol/L) for 72 hours. Growth was assessed by cell counts and expressed relative to control-treated cells (Fig. 1A). Parthenolide inhibited hepatocellular carcinoma growth in a dose-dependent manner in all three cell lines. Significant growth inhibition was observed between 2.5 and 10 μmol/L parthenolide in HepG2 and PLC cells (P < 0.001). In Hep3B cells, significant inhibition was observed between 2.5 and 10 μmol/L (P < 0.05 for 2.5 μmol/L and P < 0.001 for 5 and 10 μmol/L). Thus, Hep3B and HepG2 cells were the most sensitive to parthenolide (IC₅₀, ~6-7 μmol/L) and the PLC cells were least sensitive to parthenolide (IC₅₀, >10 μmol/L). The differential response to parthenolide correlated with the level of basal NF-κB DNA-binding activity normalized to equal cell number as measured by electrophoretic mobility shift assay (EMSA) and an ELISA-based assay (Fig. 1B and C). The amount of protein in each of the EMSA extracts prepared from an equal number of cells ranged from 5 to 10 μg; binding was linear within this range. In both EMSA and ELISA assays, the more resistant PLC cells exhibited higher levels of active NF-κB per cell than the more responsive Hep3B and HepG2 cells.

Growth Effects of Parthenolide in Combination with the COX-2 Inhibitor NS398

We have reported previously that the COX-2 inhibitor NS398 inhibits growth of hepatocellular carcinoma cells in vitro despite the absence of COX-2 protein (35). To determine if the growth-inhibitory effects of NS398 could be augmented by coadministration with a NF-κB inhibitor, hepatocellular carcinoma cells were treated with NS398 and parthenolide, alone or in combination, for 72 hours. Growth was assessed by cell counts and expressed relative to control-treated cells (Fig. 2A). The lower-dose combination of 5 μmol/L parthenolide and 50 μmol/L NS398 significantly inhibited growth more than the respective single agents in all cell lines. Parthenolide had additive effects in combination with NS398 and increased the sensitivity of hepatocellular carcinoma cells to growth inhibition at lower doses of NS398. In Hep3B and HepG2 cells, respectively, the low-dose combination of 5 μmol/L parthenolide and 50 μmol/L NS398 was either significantly more or equally effective at inhibiting growth compared with 100 μmol/L NS398 alone; however, in PLC cells, 100 μmol/L NS398 alone was more effective than the low-dose combination. Transfection with p65 RelA subunit small interfering RNA (siRNA) to inhibit NF-κB p65 protein expression significantly augmented the response of PLC cells to 50 μmol/L NS398, showing that the relative resistance of these cells to the combination is due to the high level of NF-κB in these cells (Fig. 2B).

Effect of Parthenolide and/or NS398 on the Level of Phosphorylated and Total IκB Proteins

To identify the possible mechanism(s) for the complementary growth inhibition mediated by the combination of parthenolide and NS398, we investigated the NF-κB pathway. NF-κB is usually sequestered in the cytoplasm as an inactive complex with the inhibitory IκB proteins. Inhibition of NF-κB can be accomplished by increasing the relative amount of IκB proteins or by decreasing IκB phosphorylation and degradation. The most abundant IκB protein is IκBα. To study the effects of parthenolide and NS398 on the phosphorylation and expression of IκBα, cells were treated with parthenolide (5 μmol/L) and NS398 (50-100 μmol/L) for 1 to 24 hours followed by stimulation with tumor necrosis factor-α (TNF-α), an endogenous factor that activates NF-κB. TNF-α treatment facilitated the detection of changes in levels of phosphorylated and total IκBα by Western blot. Combination treatment with parthenolide and NS398 decreased the amount of phosphorylated IκBα in all cell lines by 6 and 24 hours (Fig. 3). In Hep3B cells, parthenolide
alone and NS398 alone decreased phosphorylated IκBα at 6 and 24 hours although not as much as combination treatment. Single-agent treatment did not cause noticeable decreases in phosphorylation of IκBα in the other cell lines. Taken together, these results show that the combination of parthenolide and NS398 may be targeting the NF-κB pathway by inhibiting the phosphorylation of IκBα.

Treatment with parthenolide alone increased the amount of total IκBα in each of the cell lines at all time points that were evaluated. Combination treatment caused a greater increase in total IκBα than single-agent treatment in HepG2 cells. In Hep3B and PLC cells, the effects of the combination were similar to that observed with parthenolide alone. These results suggest that parthenolide alone and in combination with NS398 can cause a reduction in IκBα phosphorylation and an accumulation of the inhibitory IκBα proteins, which may lead to inhibition of the NF-κB pathway in hepatocellular carcinoma cells.

**Effects of Parthenolide and NS398 on NF-κB-Binding and Transcriptional Activities**

To determine whether the accumulation of IκBα protein correlated with changes in NF-κB DNA-binding activity, cells were treated with parthenolide (5 μmol/L) and NS398
(50 μmol/L), alone and in combination, for 3 hours. An EMSA was done using radiolabeled probe for NF-κB as well as SP-1 as an internal control (Fig. 4A). Parthenolide inhibited DNA binding by NF-κB in all cell lines. In PLC and HepG2 cells, NS398 also inhibited the ability of NF-κB to bind DNA; in contrast, NS398 did not noticeably decrease NF-κB-binding activity in Hep3B cells. In all cell lines, low-dose combination treatment inhibited binding to the same degree as parthenolide alone. Similar trends were observed when NF-κB binding was measured by ELISA after 24 hours of treatment, except that the higher-dose combination inhibited binding more effectively than the single agents in HepG2 and Hep3B cells (Fig. 4B). In contrast, neither combination was more effective than the single agents in PLC cells.

To assess the effects of parthenolide and NS398 on NF-κB-directed transcriptional activity, hepatocellular carcinoma

![Graph A: Hep3B Growth](image)

![Graph B: HepG2 Growth](image)

![Graph C: PLC Growth](image)

![Graph D: p65 siRNA Response](image)

**FIGURE 2.** Effect of parthenolide and NS398 on hepatocellular carcinoma growth. A. Hep3B, HepG2, and PLC cells were treated with parthenolide (5 μmol/L) and/or NS398 (50-100 μmol/L) for 72 hours. After treatment, trypan blue–excluded cell counts were done. Percentage of vehicle control values from at least three independent experiments done in duplicate. Columns, mean; bars, SE. Black columns, single agents; hatched columns, combinations. *, P < 0.05 versus single agents. B. p65 siRNA and NS398 response. PLC cells were either mock transfected or transfected with negative control siRNA or p65 RelA siRNA. After 72 hours, cells were harvested for Western blot analysis (NF-κB and actin). For growth studies, transfected cells were treated with 50 μmol/L NS398 for 72 hours and cell counts were done. Columns, mean of three independent siRNA experiments; bars, SE. *, P < 0.05 versus negative control siRNA-transfected cells treated with NS398.
FIGURE 3. Effect of parthenolide and NS398 on the phosphorylation status and total levels of IκBα. Hep3B (A), HepG2 (B), and PLC (C) cells were treated with parthenolide (5 μmol/L) and/or NS398 (50-100 μmol/L) for 1 to 24 hours followed by stimulation with TNF-α (5 ng/mL) to aid in the detection of changes in IκBα. Lysates were prepared and analyzed by Western blot. Actin levels confirm similar protein loading. Representative blots.
cells were transfected with the NF-κB-luciferase reporter and treated with parthenolide (5 μmol/L) and/or NS398 (50 μmol/L) for 24 hours. Cells were treated with parthenolide/NS398 and then treated with TNF-α for 10 minutes before harvesting. Nuclear extracts were prepared and analyzed with an ELISA specific for p65 NF-κB. Columns, mean of two independent experiments; bars, SE. Black columns, single agents; hatched columns, combinations. *, P < 0.05 versus single agents.

C. NF-κB-directed transcription. Cells were transfected with the NF-κB luciferase reporter and treated with parthenolide and/or NS398 for 24 hours. Cells were harvested and luciferase activity was measured and normalized to total protein. Columns, mean of two independent experiments; bars, SE. Black columns, single agents; hatched columns, combinations. *, P < 0.05 versus single agents.

Effects of Parthenolide and NS398 on Apoptosis

To determine if the growth inhibition following treatment with parthenolide and NS398 was caused by an increase in apoptosis, cells were treated with parthenolide (5 μmol/L) and
NS398 (50 μmol/L), alone and in combination, for 48 hours. Apoptosis was measured using a DNA fragmentation ELISA. NS398 alone did not significantly affect apoptosis in any of the cell lines (Fig. 5). In Hep3B and HepG2 cells, parthenolide alone caused a significant increase in apoptosis; in PLC cells, the increase in apoptosis induced by parthenolide was not significant. The combination of parthenolide and NS398 significantly induced apoptosis in PLC cells but not in Hep3B and HepG2 cells. Similar trends were observed at 24 hours (data not shown). Thus, decreases in growth following combination therapy may be mediated by increased apoptosis in PLC cells but not in Hep3B and HepG2 cells.

**Effects of Parthenolide and NS398 on Cell Cycle Phase Distribution**

Because changes in apoptosis alone could not completely account for the observed growth-inhibitory effects of parthenolide and NS398 in two of the cell lines, cell cycle phase distribution analysis was done after drug treatment. Following synchronization, cells were treated with parthenolide (5 μmol/L) and NS398 (50-100 μmol/L), alone and in combination, for 24 hours. To determine cell cycle distribution, DNA content was quantified using flow cytometry. Parthenolide and NS398 both tended to cause an increase in the percentage of cells in G0-G1 in Hep3B and HepG2 cells (Fig. 6). However, this increase was statistically significant only in HepG2 cells at the 100 μmol/L concentration of NS398. Neither drug caused significant changes in the percentage of cells in G2-M in any of the cell lines (data not shown). The combination of parthenolide and NS398 (50 and 100 μmol/L) caused significant G0-G1 cell cycle arrest in Hep3B and HepG2 cells. Increases in G0-G1 resulted from decreases in S phase. In PLC cells, little change was observed in G0-G1 after combination treatment. Thus, the combination of parthenolide and NS398 causes G0-G1 cell cycle accumulation in Hep3B and HepG2 cells.

**Effects of Parthenolide and NS398 on G1-S-Phase Cell Cycle Regulatory Proteins**

To identify potential contributors to the observed G0-G1 cell cycle arrest, cells were treated with parthenolide (5 μmol/L) and NS398 (50-100 μmol/L), alone and in combination, for 24 hours and the levels of several G1-S-phase regulatory proteins were determined by Western blot (Fig. 7). No appreciable changes were observed in the levels of the small-molecule cell cycle inhibitors, p21WAF1 and p27Kip1, after drug treatment in any of the cell lines. In contrast, the levels of cyclin E, which promotes G1-S-phase transition late in G1 by activating cyclin-dependent kinase 2, were affected by drug treatment. NS398 treatment alone decreased cyclin E levels in a concentration-dependent manner in all cell lines. The combination of parthenolide and NS398 decreased the levels of cyclin E in all cell lines, but the decrease was similar to that observed with NS398 alone. The levels of cyclin D1, which promotes G1-S-phase transition early in G1 by activating cyclin-dependent kinase 4 and 6, also changed after drug treatment. Parthenolide alone decreased the levels of cyclin D1 in PLC and Hep3B cells but not in HepG2 cells. NS398 alone caused a decrease in the levels of cyclin D1 in a concentration-dependent manner in Hep3B cells and a slight decrease in HepG2 and PLC cells.

Importantly, the combination of parthenolide and NS398 further decreased cyclin D1 levels relative to single-agent treatment in all cell lines. Thus, the combination of parthenolide and NS398 effectively decreases the level of cyclin D1 in hepatocellular carcinoma, correlating with effects on cell cycle and growth.

![FIGURE 5. Effect of parthenolide and NS398 on apoptosis in hepatocellular carcinoma. Hep3B, HepG2, and PLC cells were treated with parthenolide (5 μmol/L) and/or NS398 (50 μmol/L) for 48 hours. An ELISA was used to quantify DNA fragmentation as a measure of apoptosis. Apoptosis is expressed relative to vehicle control-treated cells (set equal to 1). Columns, mean of at least three different experiments; bars, SE. Black columns, single agents; hatched columns, combination. *, P < 0.05 versus control.](image-url)
FIGURE 6. Effect of parthenolide and NS398 on cell cycle distribution in hepatocellular carcinoma. Hep3B, HepG2, and PLC cells were placed in serum-free medium to synchronize the cell cycle for 24 hours. After synchronization, cells were treated with parthenolide (5 μmol/L) and/or NS398 (50-100 μmol/L) for 24 hours. Cell cycle distribution was determined by flow cytometry. A, Representative set of histograms for HepG2-treated cells. B, Percentage of cells in G0-G1. Columns, mean of at least three independent experiments; bars, SE. Black columns, single agents; hatched columns, combinations. *, P < 0.05 versus control.
Discussion

COX-2 inhibitors have been shown to have anticancer potential in a variety of cancer types both in vitro and in vivo (39). Although COX-2 may play an important role in tumorigenesis, we have shown that the COX-2 inhibitor NS398 can effectively inhibit hepatocellular carcinoma growth in vitro even in the absence of COX-2 expression (35). Furthermore, we and others have shown that the concentration of NS398 required for growth inhibition is often higher than that required for inhibition of COX-2 activity (28, 33, 35, 40, 41). These data suggest that COX-2 inhibitors may mediate their growth-inhibitory effects in part by COX-2-independent mechanisms and raise concern that high doses of these drugs may be required to achieve desired antiproliferative effects.

Recent reports indicate that long-term administration of high doses of COX-2 inhibitors may increase the risk of adverse cardiovascular events (42-44). Although less important for short courses of chemotherapy, these findings could have significant implications for the long-term use of COX-2 inhibitors as chemotherapy or chemoprevention. Analyses of COX-2 inhibitors indicate that the risk of adverse cardiovascular events is reduced at lower doses (45, 46). This underscores the importance of minimizing drug dose when administering COX-2 inhibitors.

In the present study, we showed that the NF-κB inhibitor parthenolide and the COX-2 inhibitor NS398 each inhibited hepatocellular carcinoma growth in a concentration-dependent manner. In addition, the combination of parthenolide and NS398 reduced the concentration of NS398 required to inhibit hepatocellular carcinoma growth. The combination of parthenolide and 50 μmol/L NS398 was at least as effective at inhibiting growth as 100 μmol/L NS398 alone in Hep3B and HepG2 cells. Although less effective than 100 μmol/L NS398 alone, the combination also inhibited growth to a greater degree than the single agents in PLC cells. The relative resistance of PLC cells to parthenolide alone as well as the combination may be due to the presence of more active NF-κB per cell compared with Hep3B and HepG2 cells. The latter was confirmed by showing that PLC cells could be sensitized to low-dose NS398 by transfection with p65 RelA siRNA to inhibit NF-κB p65 protein expression, showing that the relative resistance of these cells to the combination is due to the high level of NF-κB in these cells.

Parthenolide has been shown to inhibit the transcription factor NF-κB by targeting the IκB kinase (47). Additionally, COX-2 inhibitors have also been reported to inhibit the NF-κB pathway, although the mechanisms are not as well understood (48, 49). Therefore, we investigated the effects of parthenolide and NS398 on the NF-κB pathway in Hep3B cells. We found that parthenolide by itself caused an accumulation of total IκB levels in hepatocellular carcinoma cells, an effect observed previously in HeLa cells (47). Although COX-2 inhibitors have been reported to prevent degradation of IκB in non–small cell lung carcinoma (50), we did not observe noticeable accumulation of total IκB by NS398 alone. However, when used in combination with parthenolide, phosphorylation of IκBα was prevented more effectively than

![FIGURE 7. Effect of parthenolide and NS398 on the levels of several G1-S-phase regulatory proteins. Hep3B (A), HepG2 (B), and PLC (C) cells were treated with parthenolide (5 μmol/L) and/or NS398 (50-100 μmol/L) for 24 hours. Lysates were prepared and analyzed by Western blot. Actin levels confirm equivalent protein loading of lysates. Fold relative expression was determined by densitometry (control set equal to 1). Representative blots.](mcr.aacrjournals.org)
by either agent alone uniformly in each cell line examined. In HepG2 cells treated with the combination, this resulted in increased total I-Bα protein levels. These findings suggest that in some hepatocellular carcinoma types these agents may complement one another in targeting the I-B kinase complex. Furthermore, the combination inhibited NF-κB DNA-binding and transcriptional activities more than the single agents in two of the hepatocellular carcinoma cell lines, Hep3B and HepG2, but not in PLC cells. Taken together, our results show that in Hep3B and HepG2 cells parthenolide and NS398 individually target the NF-κB pathway and that the combination cooperatively targets the NF-κB pathway. The combination may be mediating its effects in part via NF-κB independent mechanism(s) as well, especially in PLC cells. Our laboratory is currently investigating the effect of these agents on other cellular signaling pathways.

The NF-κB pathway provides protection from apoptosis in several human cancers (6, 51, 52). We found that parthenolide caused an increase in apoptosis in two of the hepatocellular carcinoma cell lines, correlating with the inhibitory effects on the NF-κB pathway. This finding is consistent with a report that parthenolide can induce apoptosis in hepatocellular carcinoma cells by causing oxidative stress (53). In contrast, NS398 did not induce substantial apoptosis despite the ability of this compound to inhibit hepatocellular carcinoma growth effectively. Combination therapy was effective at increasing apoptosis only in PLC cells. Although the induction of apoptosis could explain the growth inhibition observed after combination treatment in PLC cells, it was insufficient to account for the growth suppression observed in the other hepatocellular carcinoma cell lines examined.

Although the relationship between NF-κB and apoptosis has been thoroughly investigated, less information is available regarding the role of NF-κB in cell cycle regulation (54). Overexpression of the p65 subunit of NF-κB causes G1 arrest in pro-B cells (55); however, this effect may be dependent on the developmental stage of the cell because p65 overexpression does not cause G1 arrest in mature B cells. In contrast to immature B cells, NF-κB activity is elevated during the G0-G1 cell cycle transition in mouse fibroblasts (56). Furthermore, NF-κB activation is required for cell cycling in estrogen receptor-negative breast cancer cells and regenerating hepatocytes (57, 58). NF-κB inhibition has been reported to be associated with G1 arrest in hepatocellular carcinoma (59). We report a trend toward G0-G1 arrest following treatment with parthenolide alone. Similarly, NS398 caused G0-G1 cell cycle arrest in a subset of the hepatocellular carcinoma cell lines examined. This effect of NS398 in hepatocellular carcinoma has been reported previously by our group and others, although the mechanism has not been determined (32, 35, 60). Importantly, when parthenolide was combined with NS398, the observed G1 arrest was greater than that caused by either agent alone, correlating with the enhanced growth inhibition that was observed with combination therapy. Thus, the drug combination may be inhibiting hepatocellular carcinoma growth via combined effects on the cell cycle. Interestingly, the cell line that showed the most impressive apoptotic response to combination therapy did not undergo G1 arrest, suggesting that the combination mediates its effects differently according to hepatocellular carcinoma cell type. The combination modulates the apoptotic threshold in some cases and the cell cycle to achieve complementary growth inhibition in others.

A physical interaction between NF-κB and the cell cycle regulatory protein cyclin E has been described (61); however, the consequences of this association have not been elucidated. We found that treatment with NS398 alone decreased cyclin E expression in a concentration-dependent manner. This effect of NS398 may not be common to all COX-2 inhibitors, as Cheng et al. reported that treatment with the selective COX-2 inhibitor etodolac did not alter cyclin E expression (62). We also observed that the effects of the combination on cyclin E levels were similar to the effects of the single agents. Thus, changes in cyclin E expression do not seem to be contributing to effects of the combination on cell cycle or hepatocellular carcinoma proliferation. Furthermore, drug treatment did not consistently affect the expression of the G1-S-phase inhibitors, p21WAF1 and p27Kip1.

Cyclin D1 is closely related to the family of D-type cyclins that control cell cycle progression by activating cyclin-dependent kinase 4 and 6, which then advance through the G1 phase of the cell cycle (63). NF-κB controls cyclin D1 expression through direct transcriptional regulation (64-66). We found that single-agent treatment with parthenolide or NS398 decreased endogenous cyclin D1 expression in some hepatocellular carcinoma cells; however, the effect of the combination of parthenolide and NS398 was uniform. The combination further decreased cyclin D1 levels, correlating with the effects of the combination on the cell cycle and growth.

We provide evidence that coadministration of parthenolide decreases the effective dose of NS398 that is required for growth inhibition of hepatocellular carcinoma. Furthermore, the combination of these agents induces apoptosis in one cell line and G1 cell cycle arrest in two others, in association with a decrease in cyclin D1 levels. Although the response to parthenolide as well as to the combination correlates with effective targeting of the NF-κB pathway, other NF-κB/COX-2-independent mechanisms may be involved as well. For therapeutic applications, our findings suggest that the use of NF-κB inhibitors may decrease the effective dose of COX-2 inhibitors required for treatment, thus reducing toxicity. Therefore, the addition of a NF-κB inhibitor, such as parthenolide, to chemotherapeutic or chemopreventive regimens that employ COX-2 inhibitors should be considered.

**Materials and Methods**

**Cell Culture**

Human hepatocellular carcinoma cell lines (HepG2, Hep3B, and PLC) were obtained from American Type Culture Collection (Manassas, VA). These cells were cultured in MEM-α (Life Technologies Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C (5% CO2, 95% O2; BioWhittaker, Walkersville, MD). For experiments, cells were plated and after 24 hours treated with NS398 (Biomol, Plymouth Meeting, PA) and/or parthenolide (Sigma-Aldrich, St. Louis, MO). Doses employed were based on logarithmic concentration-response
curves. Parthenolide was dissolved in ethanol (40 mmol/L stock) and stored at −20°C. NS398 was dissolved in DMSO and stored at −20°C. Recombinant human TNF-α (R&D Systems, Minneapolis, MN) was dissolved in PBS containing 0.1% bovine serum albumin (10 μg/mL) and stored at −20°C.

**Cell Counts**

Cells were plated in six-well plates (1 × 10⁵ per well for HepG2, 1 × 10⁵ per well for Hep3B, and 3.0 × 10⁵ per well for PLC). After 24 hours, the cells were treated with parthenolide and/or NS398 for 72 hours. Trypan blue–excluded cell counts were determined by light microscopy using a hemacytometer. Exposure to the drugs for 72 hours was determined to be the optimal length of time for observing maximum effects on growth. IC₅₀ values were determined from the parthenolide dose-response curve.

**Western Blotting**

Where indicated, cells were treated with TNF-α (5 ng/mL) for 5 minutes before harvesting. Cells were lysed in radioimmunoprecipitation assay buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 1 mmol/L Na₃VO₄), and the supernatants were obtained. Cell lysates (10 μg total protein, determined previously to be within linear range) were resolved by SDS-PAGE on 4% to 20% gradient gels (Novex, Carlsbad, CA) and transferred to Immobilon-P membranes (Millipore, Billerica, MA). The blots were incubated for 1 hour in blocking solution (TBS-Tween 20 with 5% nonfat dry milk) followed by primary antibody either overnight at 4°C (total IκBα and phosphorylated IκBα) or for 2 hours at room temperature (all other antibodies). Membranes were incubated with the appropriate secondary antibody (horseradish peroxidase–conjugated IgG) for 1 hour followed by detection using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). The primary antibodies included phosphospecific (Ser32) IκBα and total IκBα antibodies (Cell Signaling Technology, Beverly, MA), cyclin D1, cyclin E, p21 (Neomarkers, Inc., Fremont, CA), p27 (BD PharMingen, San Diego, CA), NF-κB p65 (Upstate, Lake Placid, NY), and actin (Santa Cruz Biotechnology, Santa Cruz, CA). Fold relative expression was quantitated by densitometry and calculated relative to the control (set equal to 1).

**Electrophoretic Mobility Shift Assay**

Cells were plated in six-well plates and grown to 50% to 70% confluency. Cells were incubated with parthenolide and/or NS398 for 3 hours. A treatment time of 3 hours was determined to be optimal for observing effects on DNA binding by EMSA. For drug treatment experiments, cells were treated with TNF-α (5 ng/mL) for 10 minutes before harvesting. Whole-cell lysates were prepared and incubated with radiolabeled probes specific for NF-κB or the internal control SP-1 (Promega, Madison, WI) as described previously (67). Unless stated, extracts containing equivalent amounts of total protein (6 μg) were assayed. DNA-protein complexes were separated by electrophoresis and visualized by autoradiography. Fold relative binding was quantitated by densitometry (control set equal to 1) and normalized to the SP-1 internal control.

**NF-κB ELISA**

Cells were plated in six-well plates and grown to at least 50% confluence. Nuclear extracts were prepared from either exponentially growing cells or cells treated with NS398/parthenolide for 24 hours using the NucBuster extraction kit (Novagen/EMD Biosciences, San Diego, CA). For the NS398/parthenolide experiments, cells were treated with TNF-α (5 ng/mL) for 10 minutes before harvesting. Unless stated, nuclear extracts containing equivalent amounts of total protein were assayed. NF-κB DNA-binding activity was measured using the NoShift Transcription Factor Assay kit specific for p65 NF-κB (Novagen/EMD Biosciences).

**Transfection**

Cells were plated in six-well plates and 24 hours later transfected with the NF-κB luciferase reporter construct (3XκB-B-luc) using FuGENE 6 (Roche Diagnostics, Indianapolis, IN). After 24 hours, cells were treated with NS398/parthenolide for an additional 24 hours. Cells were harvested using PLB lysis buffer as suggested by the manufacturer (Promega). After the addition of luciferin, luciferase activity was detected by a luminometer and normalized to total protein.

**Small Interfering RNA**

Cells grown in 12-well plates overnight (~30% confluent) were transfected with 50 mmol/L p65 RelA (accession no. NM_021975) siRNA (SMARTpool, Dharmacon, Lafayette, CO) or negative control siRNA (nontargeting siRNA SMARTpool, Dharmacon) and 3 μL Mirus Trans-IT TKO transfection reagent (Mirus Bio, Madison, WI) in 600 μL total volume/well as recommended by the manufacturer. Six hours post-transfection, the media volume was increased to 1 mL and 50 μmol/L NS398 was added for 72 hours before harvesting for protein or cell counts.

**Apoptosis ELISA**

The Cell Death Detection ELISA (Roche Diagnostics) quantifies the amount of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) produced by cells undergoing apoptosis. Cells were plated in 96-well plates (1.0 × 10⁴ per well for HepG2, 5.0 × 10³ per well for Hep3B, and 1.0 × 10³ per well for PLC) and after 24 hours treated with parthenolide, NS398, or the combination. After 48 hours, cell lysates were prepared and assayed as recommended by the manufacturer. Relative apoptosis was determined by a ratio of the average absorbance of the treatment wells to the average absorbance of the control wells.

**Cell Cycle Analysis**

To determine the cell cycle phase distribution, flow cytometric analysis was employed. Cells were plated in six-well plates (2 × 10⁵ per well for HepG2, 7.5 × 10⁴ per well

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Unpublished observations.
for Hep3B, and $3 \times 10^5$ per well for PLC). The following day, the cells were incubated in serum-free medium for 24 hours to synchronize the cells. After synchronization, medium containing serum and parthenolide, NS398, or the combination was added for 24 hours. The cells were then harvested, treated with RNase A (1 μg/μL), and stained with propidium iodide (50 ng/μL) on ice for at least 30 minutes. Samples were analyzed by flow cytometry and cell cycle phase distribution was determined using Modfit software (Verity Software House, Inc., Topsham, ME) to analyze DNA content histograms.

**Statistics**

Differences in growth between control cells and parthenolide-treated cells were compared by one-way ANOVA and Dunnett’s t test. To determine whether growth effects were additive or synergistic, an interaction statistic was obtained by analyzing the combination experiments by factorial ANOVA and differences between means were then compared using Tukey’s Honestly Significant Difference. Post hoc tests were only done if $P < 0.05$ by ANOVA. Differences in growth between the combination and single agents were determined by Student’s t test. Apoptosis and cell cycle distribution experiments were analyzed in the same manner as the parthenolide growth experiments.

**References**

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Molecular Cancer Research

Parthenolide Cooperates with NS398 to Inhibit Growth of Human Hepatocellular Carcinoma Cells through Effects on Apoptosis and G0-G1 Cell Cycle Arrest

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