Induction of apoptosis of lung and esophageal cancer cells treated with the combination of histone deacetylase inhibitor (trichostatin A) and protein kinase C inhibitor (calphostin C)

Justin B. Maxhimer, BA,[a,b] Rishindra M. Reddy, MD,[a] Jingtong Zuo, MD,[a] George W. Cole, Jr, MD,[a] David S. Schrump, MD, FACS,[a] and Dao M. Nguyen, MD, FRCSC, FACS[a]

Objective: Histone deacetylase inhibitors mediate a potent growth-inhibitory effect in cancer cells through induction of cell-cycle arrest and apoptosis. Moreover, these agents significantly induce transcriptional activation of nuclear factor κB, as well as p21 regulated by protein kinase C, and are thought to negatively influence the ability of histone deacetylase inhibitor to effectively mediate apoptosis. This study aimed to evaluate the effect of calphostin C (a protein kinase C inhibitor) on trichostatin A (a histone deacetylase inhibitor)–mediated upregulation of nuclear factor κB and p21 promotor transcriptional activity, as well as induction of apoptosis in lung and esophageal cancer cells.

Methods: Cultured lung and esophageal cancer cells were treated with calphostin C and trichostatin A. Nuclear factor κB transcriptional activity was quantitated by using the nuclear factor κB–luciferase assay. Transcription of p21 gene and p21 protein levels was evaluated by using the p21 promoter–luciferase assay and the p21 enzyme-linked immunoassay, respectively. Apoptosis was evaluated by using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–based ApoBrdU assay. Levels of expression of nuclear factor κB–dependent antiapoptotic and proapoptotic proteins were evaluated by means of Western blotting.

Results: Exposure of lung or esophageal cancer cells to trichostatin A resulted in a dose- and cell-dependent 2-fold to greater than 20-fold increase of nuclear factor κB and p21 transcriptional activity. Treatment with trichostatin A and calphostin C led to a 50% to 90% decrease of trichostatin A– mediated upregulation of nuclear factor κB and p21 activation. Inhibition of nuclear factor κB activity resulted in significant reduction (30% to >99%) of trichostatin A– mediated activation of not only nuclear factor κB transcription but also p21 promotor activity. Importantly, 90% to 96% of thoracic cancer cells under-went apoptosis after exposure to the combination of trichostatin A plus calphostin C.

Conclusion: Inhibition of protein kinase C abrogates trichostatin A–mediated upregulation of nuclear factor κB transcriptional activity and p21 expression that is associated with profound induction of apoptosis in lung or esophageal cancer cells. Protein kinase C might be a novel target for enhancing the efficacy of histone deacetylase inhibitor in cancer therapy.

From the Section of Thoracic Oncology,[a] Surgery Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Md, and the Howard Hughes Medical Institute–National Institutes of Health Research Scholar Program,[b] Bethesda, Md.

Read at the Eighty-fourth Annual Meeting of The American Association for Thoracic Surgery, Toronto, Ontario, Canada, April 25-28, 2004.

Received for publication April 23, 2004; revisions received July 1, 2004; accepted for publication July 13, 2004.

Address for reprints: Dao M. Nguyen, MD, FRCSC, FACS, Section of Thoracic Oncology, Surgery Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Building 10, Room 2B07, 10 Center Dr, MSC 1502, Bethesda, MD 20892-1502 (E-mail: Dao_Nguyen@nih.gov).

J Thorac Cardiovasc Surg 2005;129:53-63

0022-5223/$30.00

Copyright © 2005 by The American Association for Thoracic Surgery

doi:10.1016/j.jtcvs.2004.07.051

Primary cancer of the upper aerodigestive tract remains one of the most common malignancies worldwide. Surgical resection remains the treatment of choice for early-stage cancers of the lung or esophagus. However, the majority of patients with these malignancies frequently present with locally advanced disease or with systemic metastases. They therefore require multimodality therapy consisting of cytotoxic chemotherapeutics and external beam radiation as the standard of care.[1-4] Unfortunately, metastatic lung and esophageal cancers are quite refractory to current optimal treatment regimens, with a median survival of less than 12 months. These disappointing clinical results, in combination with better understanding of the molecular basis of carcinogenesis, provide a strong impetus for the development of novel treatment methods. Molecular targeted therapeutics, aimed at oncogenic pathways operational in cancer but not normal cells, offer a novel approach to this difficult problem.

Histone deacetylase (HDAC) inhibitors are pharmacologic compounds of diverse chemical structures that induce hyperacetylation of nuclear histones and mediate potent anticancer activity. HDAC inhibitors are currently in early-phase clinical trials to treat hematologic and solid tumor malignancies.[5] It is important to note, however, that the magnitude of HDAC inhibitor–mediated induction of apoptosis is dependent on the drug concentrations, as well as the duration of drug exposure, and that the effects of HDAC inhibition are not solely related to alterations in histone acetylation.[6-8] It has been shown that treating cultured cancer cells with HDAC inhibitor in vitro results in rapid and profound transcriptional upregulation of p21 gene expression,[6,9] as well as activation of the nuclear factor κB (NF-κB) pathway.[10] Robust expression of NF-κB–dependent, antiapoptotic proteins, such as the cIAPs and Bcl-XL, have also been documented.[11] It is well described that induction of these genes impedes the ability of HDAC inhibitor to mediate apoptosis in cancer cells[12-15] and that inhibition of either p21 expression or NF-κB activation was associated with induction of apoptosis of HDAC inhibitor–treated cells.[9,16-18] Previously, Han and colleagues[19] demonstrated that HDAC inhibitor–associated upregulation of p21 gene expression is mediated by protein kinase C (PKC), and more recently, PKC is thought to be a key regulator of NF-κB transactivation.[20]

Previously, we have demonstrated that the cdk inhibitor flavopiridol potentiates cytotoxicity mediated by the HDAC inhibitor depsipeptide FK228 in a variety of thoracic malignancies in vitro. Furthermore, we have shown that flavopiridol inhibits the induction of p21 by depsipeptide and can profoundly potentiate the HDAC inhibitor–mediated apoptosis.[16] However, this study was undertaken to extend those observations by examining whether inhibition of PKC signaling by calphostin C (CC) could potentiate the cytotoxicity mediated by the HDAC inhibitor trichostatin A (TSA) in lung and esophageal cancer (EsC) cells. These experiments were predicated on the hypothesis that inhibition of PKC activity in HDAC inhibitor–treated cells would abrogate not only p21 gene activation but also NF-κB upregulation, thus leading to more pronounced HDAC inhibitor–induced apoptosis.

Materials and Methods

Cells and Reagents

The EsC cells TE2 and TE12 and the non–small cell lung cancer (NSCLC) cells H322 and H460 were maintained in RPMI-1640 culture medium supplemented with fetal calf serum (10% vol/vol), streptomycin (100 μg/mL), penicillin (100 U/mL), and glutamine (2 mmol/L). Primary human dermal fibroblasts and human umbilical vein endothelial cells were purchased from Clonetics Corp (Walkerville, Md). TSA (Sigma, St Louis, Mo), CC (Calbiochem, La Jolla, Calif), and the NF-κB inhibitor parthenolide (Alexis) were dissolved in dimethyl sulfoxide to stock solutions of 500 μmol/L or 1000 μmol/L and stored at −20°C. The adenovirus vector expressing the super repressor mutant IκB (Adv-SR-IκB) was kindly provided by Dr Albert S. Baldwin (University of North Carolina at Chapel Hill, Chapel Hill, NC).

Cell Proliferation Assay

Cultured cancer cells or primary normal cells were seeded onto 96-well microtiter plates at a density of 5×10^3/well. After an overnight incubation, cells were treated with TSA (0.10-5.0 μmol/L for 12 hours), after which they were either maintained in normal media or treated with CC (0.5-2.0 μmol/L) for 60 hours. Cell viability at the end of the assays was quantitated with (4,5-dimethylthiazo-2-yl)-2,5-diphenyl- tetrazolium bromide (MTT; Sigma).

Determination of Apoptosis

Cancer cells seeded at 2×10^5 per well in 6-well plates were treated with either TSA alone (0.5-5.0 μmol/L for 12 or 24 hours) or with the sequential combination of TSA (1.0 or 2.0 μmol/L for 12 hours) followed by CC (1.0 μmol/L for 36 hours). Treated cells were harvested at 48 hours after the onset of drug treatment, fixed in 1% paraformaldehyde and 70% ethanol, and assayed for apoptosis by using the terminal deoxynucleotidytransferase-mediated dUTP nick end labeling (TUNEL)–based ApoBrdU assay (BD Pharmingen, Torrance, Calif), as per the protocol provided by the manufacturer.

NF-κB and p21 Promotor Transcriptional Activity

The transcriptional activity of NF-κB and of the p21 promotor was evaluated by transient transfection of reporter plasmids that contain the luciferase gene under the control of a promotor with multiple NF-κB binding sites or the promotor of the wild-type p21 gene, respectively, into cells before drug treatments. Luciferase activity of cell lysates is directly correlated with the transcriptional activity of NF-κB or p21 promotor.

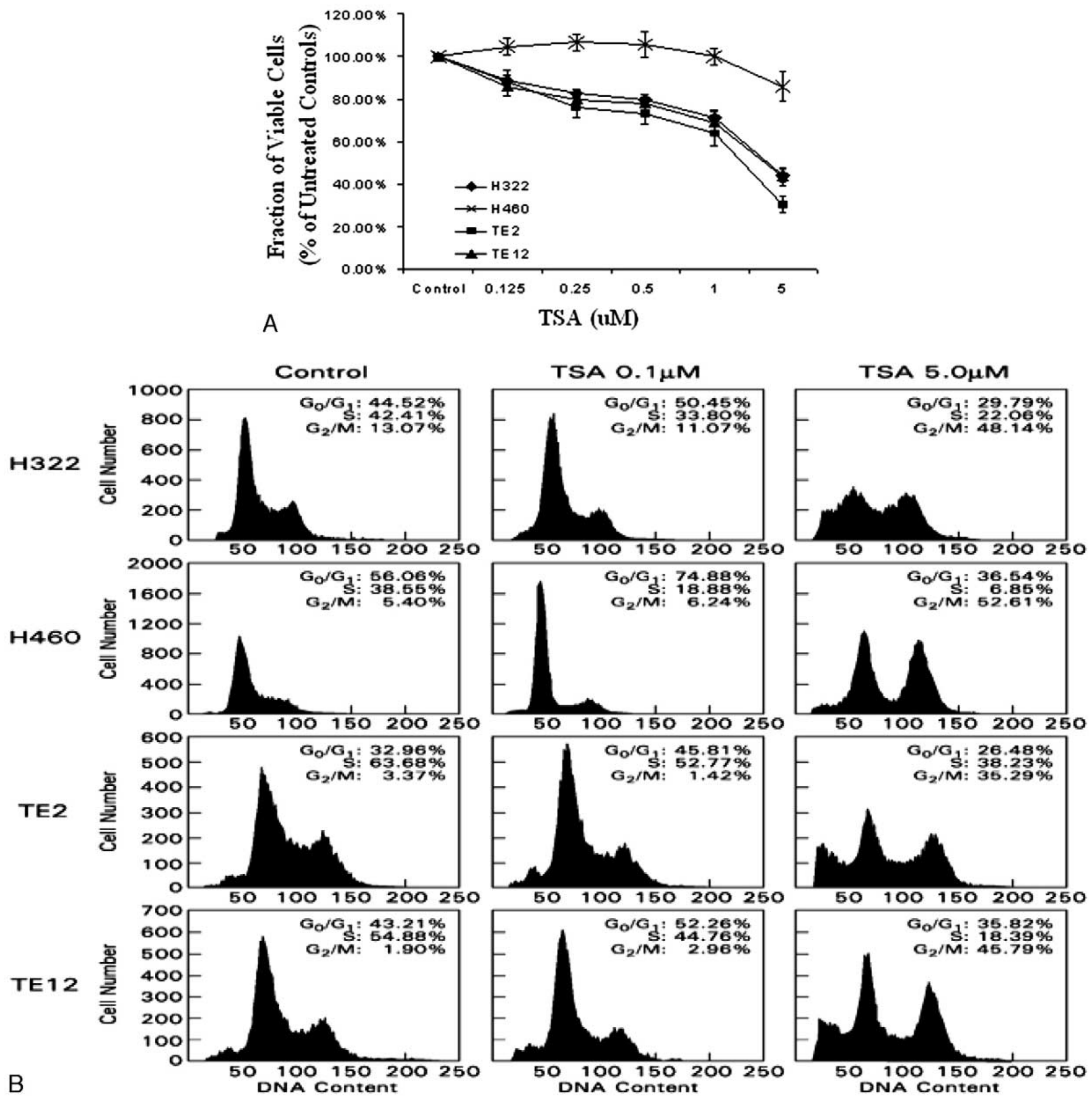


Figure 1. A, Growth-inhibitory effects of TSA on the NSCLC cells H322 and H460 and the EsC cells TE2 and TE12. Cell viability was quantitated by means of MTT assay and expressed as percentages of untreated control cells. Data are presented as means ± SEM of 4 independent experiments. B, TSA dose-dependent induction of cell growth arrest at the G0/G1 or G2/M phases of the cell cycle. Cultured cancer cells were treated with normal media and either 0.1 μmol/L or 5.0 μmol/L TSA for 12 hours and then further cultured in TSA-free complete culture media. Cells were harvested at 12 hours after completion of drug treatment, and cell-cycle analysis was performed by using propidium iodide staining and flow cytometry. Representative data of 3 independent experiments that yielded similar results are shown.

Plasmids

The human wild-type p21 promoter–luciferase fusion plasmid WWP-Luc (kindly provided by B. Vogelstein, John Hopkins University, Baltimore, Md) and the NF-κB–luciferase reporter plasmid NF-κB–Luc (Invitrogen, Carlsbad, Calif) were amplified and CsCl purified (Lofstrand Labs, Gaithersburg, Md) for transient transfection experiments.

Transfection and Luciferase Assay

Cells were plated onto 24-well plates at a density of 8×10^4/well. For transcription experiments, cells were transfected with 500 ng of DNA per well of either the WWP-Luc or NF-κB-Luc plasmid by using Fugene transfection reagent (Promega, Madison, Wis). Twenty-four hours after transfection of the reporter plasmids, cells were treated with different experimental conditions, as

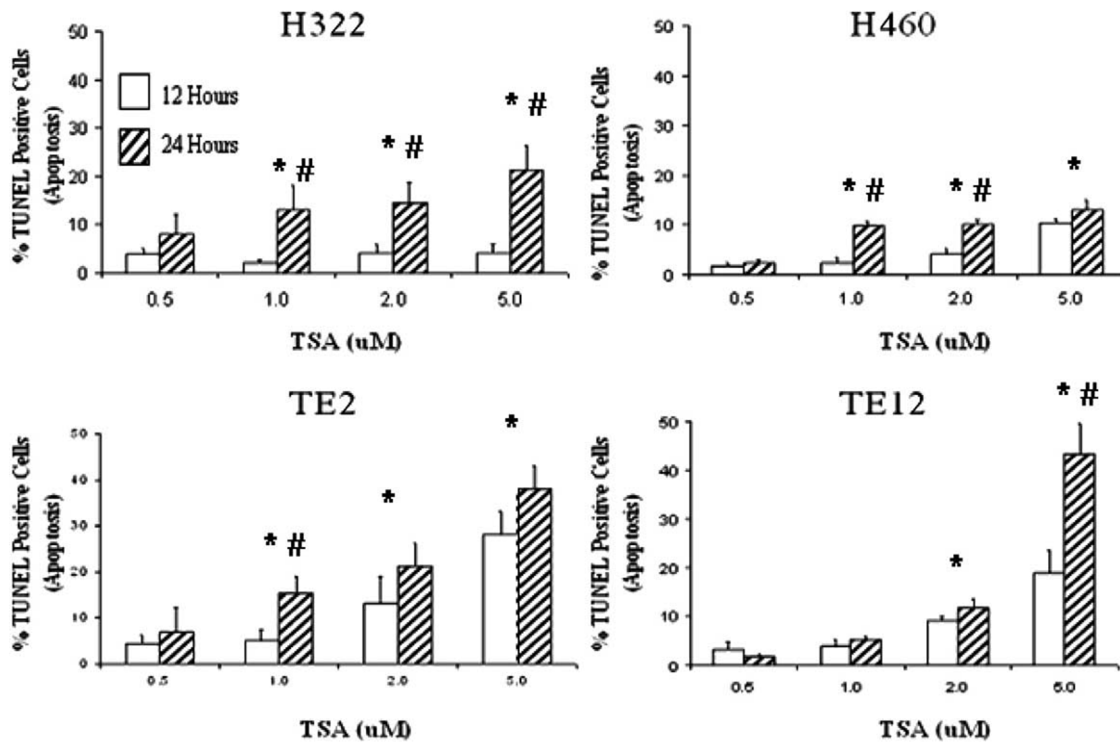


Figure 2. Induction of apoptosis by TSA treatment of the NSCLC cells H460 and H322 and the EsC cells TE2 and TE12. Cells were exposed to TSA (0.5-5.0 μmol/L) for either 12 or 24 hours and then harvested at 48 hours for determination of apoptosis with the TUNEL-based ApoBrdU assay and flow cytometry. Data are presented as means ± SEM of 4 independent experiments. #P = .004 to .046, Student t test between 12-hour and 24-hour treatment groups. *P = .0079 to .013, analysis of variance with Tukey-Kramer multiple comparison tests between TSA of 1, 2, and 5 μmol/L and TSA of 0.5 μmol/L.

outlined in the respective figure legends. The transcriptional activity of NF-κB or p21 promotor was quantitated by measuring the luciferase activity of cell lysates with the Luciferase Assay kit (Promega), according to the manufacturer's recommendation, by using the Lumat LB 9507 lucinometer (EG&G Berthold, Gaithersburg, Md).

Quantitation of p21 Protein

The levels of p21 proteins in cells treated with TSA, CC, or the TSA+CC combination were measured by using the enzyme-linked immunosorbent assay method (Oncogene Science, Boston, Mass). Cellular p21 levels were normalized for protein content of cell lysates and expressed as units per milligram of cellular protein.

Western Blotting

Cultured NSCLC or EsC cancer cells treated with TSA (0.1 and 1.0 μmol/L × 12 hours), CC (1.0 μmol/L × 12 hours), or sequential TSA (12 hours) followed by CC (12 hours) were harvested in Laemmli buffer at 24 hours after the onset of drug exposure. Cell lysates were immunoblotted by using the standard Western blot technique with monoclonal antibodies for Bcl-XL (Cell Signaling Technology, Beverly, Mass), cIAP-1 and cIAP-2 (R&D Systems, Minneapolis, Minn), Bax (Upstate Biotechnology, Lake Placid, NY), and mcl-1 (Pharmingen, San Diego, Calif). Blots were also probed for β-actin (Oncogen Research Product, Cambridge, Mass) to verify equal loadings of protein.

Data Analysis

Data are expressed as means ± SD for at least 3 independent experiments that yielded similar results. Statistical analysis was performed with the GraphPad InStat software (GraphPad, San Diego, Calif).

Results

Growth-Inhibitory Effect of TSA on NSCLC and EsC Cells

Brief exposure of cultured lung and EsC cells to TSA resulted in a dose-dependent inhibition of cell proliferation that was most pronounced in TE2, TE12, and H322 cells but not in H460 cells (Figure 1, *A*). TSA at low concentrations (0.1-1.0 μmol/L) mediated cell-cycle arrest at the G1/S checkpoint (increase of cells in the G0/G1 phase and reduction of cells in the S phase). At higher concentrations (≥2.0 μmol/L), TSA induced a profound perturbation of mitosis that was associated with significant accumulation of tetraploid (G2/M) cells, a concomitant reduction of cells in the

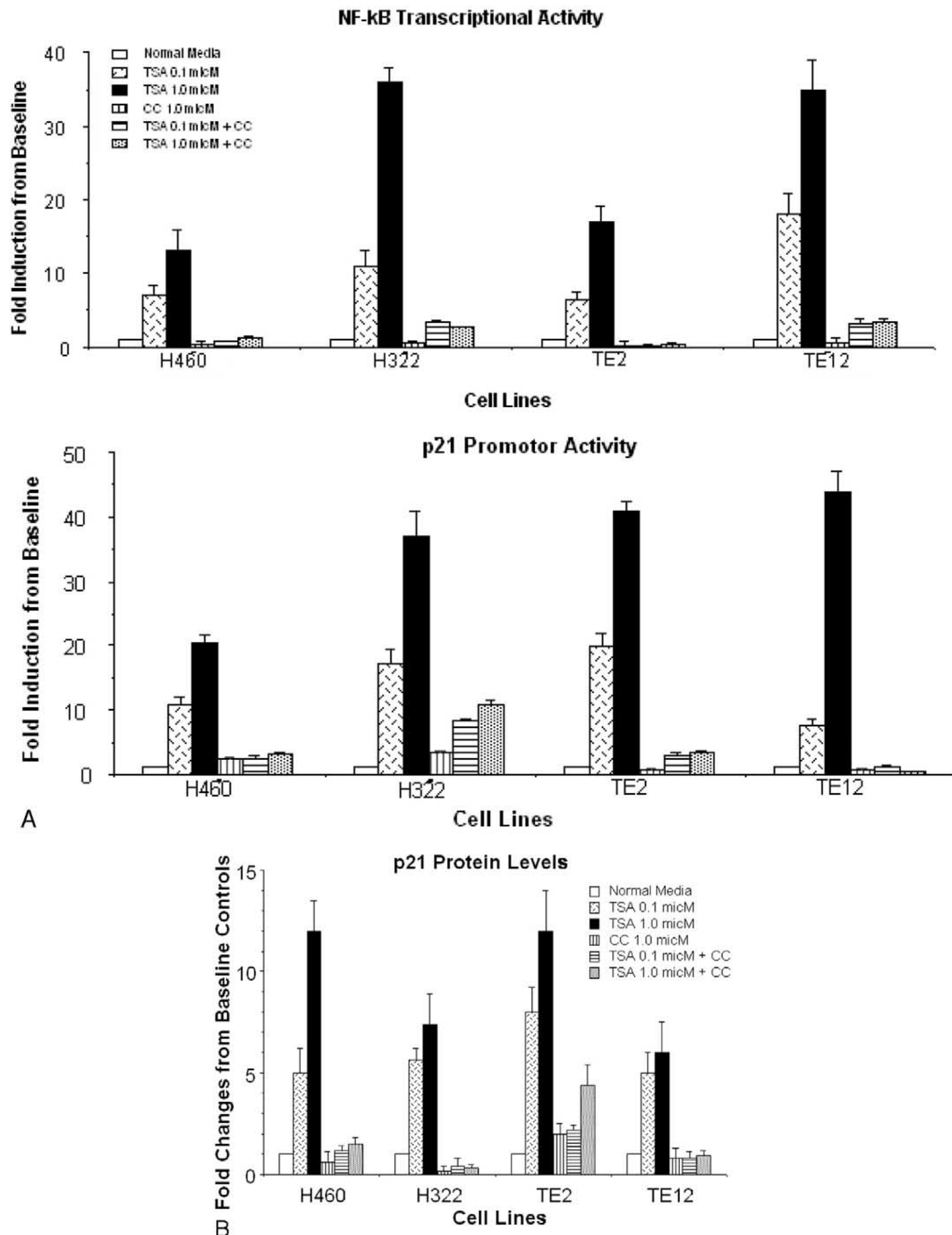


Figure 3. A, Profound inhibition of TSA-mediated upregulation of p21 promotor activity and NF-κB transcriptional activity by the PKC inhibitor CC in all NSCLC and EsC cells. Data are presented as folds of increase of luciferase activity over activity of untreated control cells (means ± SEM of 4 independent experiments). B, Inhibition of TSA-mediated upregulation of p21 levels by the PKC inhibitor CC. Protein levels of p21 in lysates of treated cells were quantitated by means of enzyme-linked immunosorbent assay. The levels were normalized for total cellular protein and then expressed as fold changes over levels of untreated control cells. Data are presented as means ± SEM of 4 independent experiments.

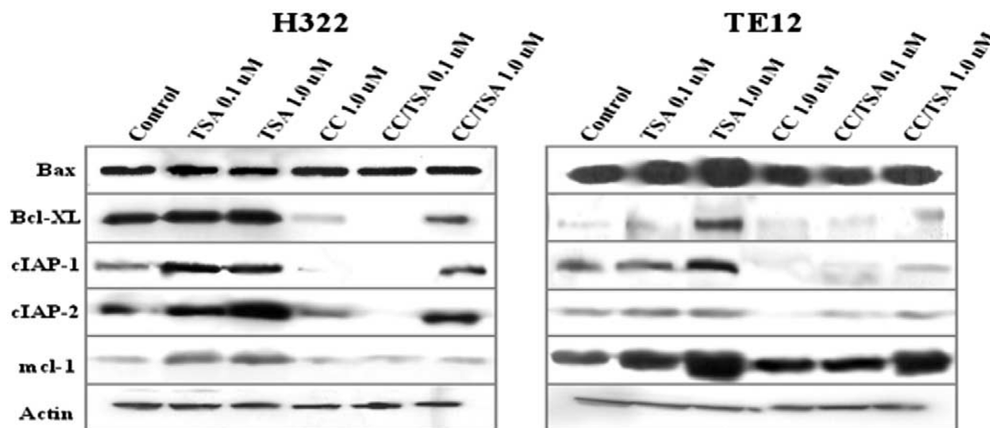


Figure 4. Western blot analysis of the effect of TSA alone and the TSA+CC combination on proapoptotic, antiapoptotic, and NF-κB–dependent proteins. Increased induction of antiapoptotic proteins is seen in treatment with TSA alone and in the proapoptotic protein, with little to no change in the combination treatment. However, abrogation of the antiapoptotic proteins is seen in the TSA+CC combination. Representative data from 2 independent experiments with comparable results are shown.

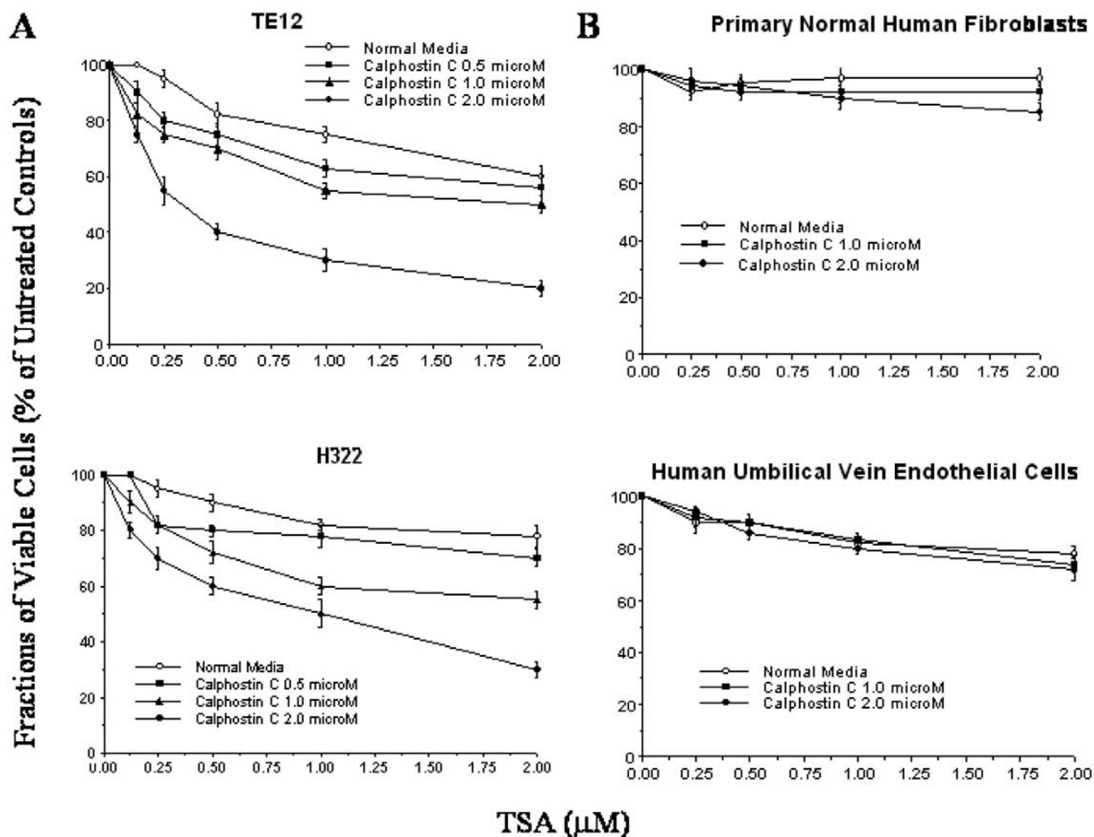


Figure 5. A, Synergistic inhibition of cell proliferation by the TSA+CC combinations in representative NSCLC H322 cells and EsC TE12 cells. Cells were treated with TSA (0.1-5.0 μmol/L) for 12 hours, followed by a replacement with drug-free culture media (TSA treatment alone) or culture media containing CC (0.25, 0.5, and 1.0 μmol/L; TSA+CC combinations). B, The TSA+CC combination effect was also examined in primary human fibroblasts and human umbilical vein endothelial cells, respectively. Cell viability was determined by using the MTT assay 60 hours after completion of TSA treatment. Dose-response curves of cells treated with the TSA+CC combinations were constructed by normalizing for the mild growth-inhibitory effect of CC treatment alone. Data are presented as means ± SEM of 4 independent experiments.

G0/G1 and S phases, and an appearance of sub-G0 cell populations (Figure 1, *B*). TSA-mediated induction of apoptosis was dependent on cell lines, the drug concentrations, and the duration of drug exposure (Figure 2). For instance, significant apoptosis was only observed in H322 and H460 cells after 24 hours of TSA exposure (P = .004 to .046, Student t test between 12-hour and 24-hour treatment groups). TSA-mediated dose-dependent induction of apoptosis was observed in all cell lines, particularly in TE2 and TE12 EsC cells, after either 12 or 24 hours of drug exposure (P = .0079 to .013, analysis of variance with the Tukey-Kramer pairwise comparison between TSA of 1, 2, and 5 μmol/L and TSA of 0.5 μmol/L).

Abrogation of TSA-Mediated Activation of NF-κB and p21 Promotor Transcriptional Activity by the PKC Inhibitor CC

Exposure of EsC and NSCLC cells to TSA (1.0 μmol/L for 6, 12, 18, or 24 hours) resulted in a treatment duration–dependent 5-fold to 30-fold upregulation of the p21 promotor and NF-κB transcriptional activity that reached a plateau at 18 and 24 hours (data not shown). Subsequent transcriptional studies were performed with a 24-hour TSA treatment schedule. The robust TSA-mediated activation of either the p21 promotor activity or the NF-κB transcriptional activity was totally abrogated by coexposure of treated cells to the PKC inhibitor CC (Figure 3, *A*). In direct correlation with the p21 promotor activity assay, the TSA-mediated increase of p21 protein expression, as determined by enzyme-linked immunosorbent assay, was significantly suppressed by CC cotreatment (Figure 3, *B*). Similarly, upregulation of NF-κB transcriptional activity by TSA was paralleled by an increased expression of the NF-κB–dependent genes, such as those of the antiapoptotic protein family cIAP-1, cIAP-2, and Bcl-XL (Figure 4). TSA-mediated upregulation of these gene expressions was significantly abrogated by the PKC inhibitor CC. Moreover, increased expression of the antiapoptotic mcl-1 protein (not currently known to be NF-κB dependent) after TSA treatment was also strongly inhibited by CC. On the other hand, levels of the proapoptotic protein Bax were not altered significantly by TSA, CC, or TSA+CC treatments. Taken together, there is a substantial increase of the proapoptotic versus antiapoptotic protein ratio, thus creating a proapoptotic milieu in cells treated with the TSA+CC combination.

Enhancement of TSA-Mediated Cytotoxicity and Apoptosis by the PKC Inhibitor CC

Subsequent exposure of TSA-treated cancer cells to different concentrations of CC resulted in a significant further reduction in cell viability compared with that seen with TSA treatment alone. This is best demonstrated by dose-response curves, where the viability of cells treated with the TSA and CC combination were normalized for the mild growth-inhibitory effect of CC (Figure 5, *A*), and of note, the

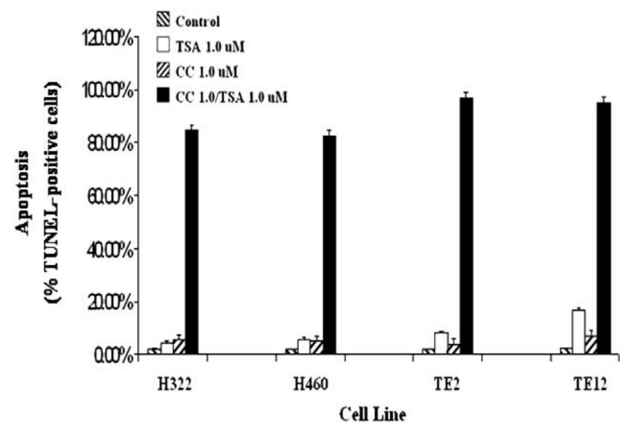


Figure 6. Profound induction of apoptosis of NSCLC H322 and H460 cells or EsC TE2 and TE12 cells by the combinations of TSA and CC. Cells were treated with TSA alone (1.0 μmol/L for 12 hours), CC alone (1.0 μmol/L continuous exposure), or TSA followed by continuous CC. Cells were harvested at 48 hours after the onset of treatment to assay for apoptosis. Data are presented as means ± SEM of 4 independent experiments.

TSA+CC drug combination was not toxic to primary normal cells (Figure 5, *B*). More importantly, although less than 15% of lung or EsC cells treated with either TSA alone or CC alone underwent apoptosis, 80% to 96% of them were TUNEL-positive within 48 hours of exposure to the TSA+CC sequential combination, creating a profound synergistic cytocidal drug combination (Figure 6).

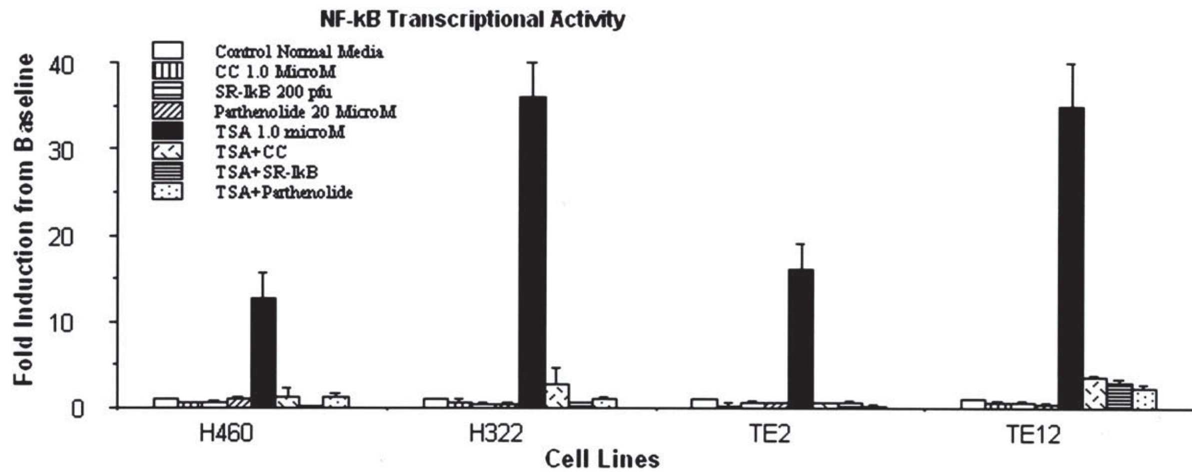
Enhancement of TSA-Mediated Apoptosis by NF-κB Inhibitor

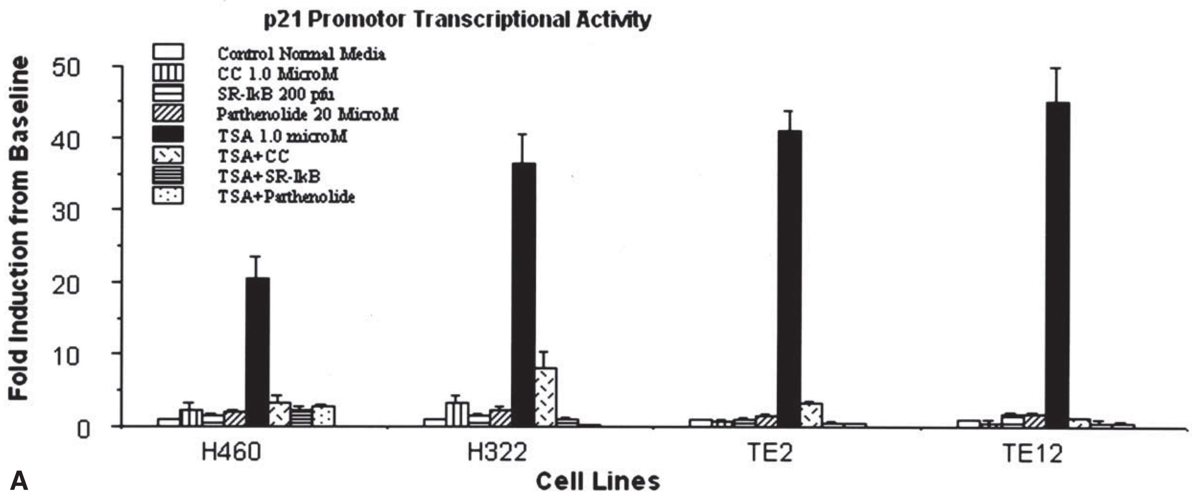
NF-κB transcriptional activity was suppressed with either a pharmacologic inhibitor, parthenolide, or by overexpression of the superrepressor IκB (SR-IκB, kinase-resistant mutant of IκB) by means of adenovirally mediated gene transfer (AdV-SR-IκB) to further elucidate the role of NF-κB in regulating p21 gene expression and apoptosis in TSA-treated cells. Parthenolide (20 μmol/L) or AdV-SR-IκB (200 pfu/cell), as predicted, completely suppressed TSA-mediated NF-κB transcriptional activation. These NF-κB inhibitors, however, also dramatically reduced TSA-induced upregulation of the p21 promotor transcriptional activity, implying that NF-κB might play a role as an upstream regulator of p21 gene expression (Figure 7, *A*). More importantly, NF-κB activation associated with TSA treatment negatively influences the ability of this HDAC inhibitor to induce cell death because profound induction of apoptosis was observed in TSA-treated H322 or TE12 cells previously infected with AdV-SR-IκB (Figure 7, *B*).

Discussion

TSA, similar to other HDAC inhibitors, induces profound dose-dependent cytoxicity in cancer cells. At lower con-

GTS





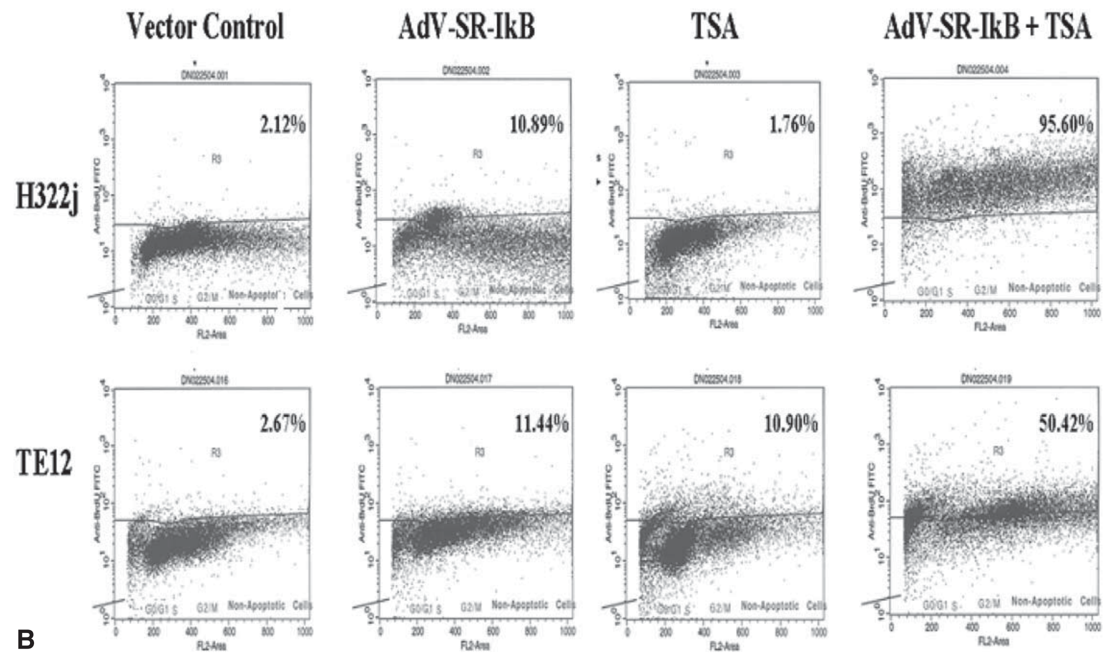


Figure 7. For legend see opposite page.

Figure 7. A, Selective inhibition of NF-κB transcriptional activity by the adenoviral vector expressing IκB (AdV-SR-IκB) or the pharmacologic NF-κB inhibitor parthenolide not only results in profound abrogation of TSA-mediated NF-κB activation but also the TSA-mediated upregulation of p21 gene transcription. Cells were transiently transfected with either the NF-κB–luciferase plasmid or the p21 promotor–luciferase plasmid before further treatment with AdV-SR-IκB (200 pfu/cell), parthenolide (20 nmol/L), and TSA (0.1 or 1.0 μmol/L). Data are presented as folds of increase of luciferase activity over activity of untreated control cells (means ± SEM of 4 independent experiments). B, Induction of apoptosis of H322 and TE12 by AdV-SR-IκB–infected cells in combination with TSA. Both AdV-SR-IκB–infected cells and vector control cells were treated with TSA (1.0 μmol/L for 12 hours) and harvested 12 hours later for quantitation of apoptosis by using the TUNEL-based ApoBrdU assay. Representative data of 3 independent experiments are shown.

centrations, TSA exerts its antiproliferative effect by arresting cells at the G0/G1 and G2/M checkpoints. Substantial apoptosis, on the other hand, is only observed after prolonged treatment of cultured lung or EsC cells with high concentrations of TSA. These conditions might not be clinically achievable without significant systemic toxicity. The phase I clinical trial of suberoylanilide hydroxamic acid (an analog of TSA) indicated that the maximally tolerated dose was 300 mg/m^2 per day on a schedule of 5 days per week for 3 weeks. Severe leukopenia and thrombocytopenia constituted the dose-limiting toxicity in this study. The plasma suberoylanilide hydroxamic acid maximum concentration was approximately 9.0 μmol/L, with a half-life of 60 minutes, resulting in an area under the curve of about 13.0 μmol/L per hour. Minor response was observed in 4 patients (4/29 [14%]).[21] The in vitro TSA treatment conditions that produced significant apoptosis in NSCLC and EsC cells in our experiments would require an estimated area under the curve of 24 or 60.0 μmol/L per hour (1.0 or 5.0 μmol/L per 24 hours of drug exposure). To enhance the clinical utility of HDAC inhibitor−based therapy for cancers, one needs to develop novel strategies to potentiate the antitumor effect of HDAC inhibitor while keeping dosing concentrations well within safe levels.

Both induction of p21 expression and increase of NF-κB transcriptional activity and expression of NF-κB–dependent genes are frequently observed in many cultured cancer cell lines treated with HDAC inhibitor. However, a common entity unifying these 2 potent antiapoptotic factors has not been elucidated. The NF-κB transcriptional factor is involved in multiple pathways and can provide protection to cancer cells against apoptosis.[14] The cdk inhibitor p21 is classically known to mediate cell arrest and, as recently described, can exert direct antiapoptotic effects,[15] and multiple lines of evidence have shown that HDAC inhibitor-mediated upregulation of p21 expression, NF-κB transcriptional activity, or both, actually inhibits drug-induced apoptosis. More importantly, studies have conclusively shown that PKC transcriptionally regulates p21 expression in HDAC inhibitor–treated cells[16,22] and in other experimental conditions; NF-κB activation is under the regulatory control of certain PKC isoforms.[20] PKC therefore appears to be a common regulator of both NF-κB and p21 transcriptional activity. Inhibition of PKC activity would suppress HDAC inhibitor–associated transcriptional activation of either p21, NF-κB, or both, and should theoretically augment drug-induced apoptosis. Our data provide clear experimental evidence to support this hypothesis. The PKC inhibitor CC effectively abrogates TSA-induced activation of both p21 promotor activity and NF-κB transcriptional activity. Substantial and synergistic induction of apoptosis was observed in all cultured thoracic cancer cells after treatment with the TSA+CC combination.

One addition to the existing body of literature is our observation that direct inhibition of NF-κB activity with SR-IκB leads to transcriptional inhibition of p21 expression. In keeping with previous observations, direct inhibition of NF-κB in TSA-treated NSCLC and EsC cells resulted in robust induction of apoptosis.[10] Knowing that SR-IκB also suppressed p21 expression in TSA-treated cells, it is not clear whether the increased cell death after Ad-SR-IκB and TSA treatment is mediated by the inhibition of NF-κB, the reduction of p21, or both. Further experiments are being performed to elucidate the molecular basis of this synergistic interaction between PKC inhibitor, NF-κB inhibitor, and HADC inhibitor combinations.

In conclusion, we describe a novel strategy developed to enhance the cytotoxic effect of HDAC inhibitor by exposing treated cells to a PKC inhibitor. The 2 drugs were brought together on the basis of the understanding of their molecular interactions and the potential for a synergistic cytotoxic effect. We identified p21 and NF-κB as exploitable factors with a common target for the development of more effective and clinically applicable HDAC inhibitor–based therapeutics for malignant disease.

References

1. Spigel DR, Greco FA. Chemotherapy in metastatic and locally advanced non-small cell lung cancer. *Semin Surg Oncol*. 2003;21:98-110.

2. Crino L, Cappuzzo F. Present and future treatment of advanced non-small cell lung cancer. *Semin Oncol*. 2002;29(suppl 9):9-16.
3. Shepherd FA. Treatment of advanced non-small cell lung cancer. *Semin Oncol*. 1994;21(suppl 7):7-18.
4. Koshy M, Esiashvilli N, Landry JC, Thomas CR Jr, Matthews RH. Multiple management modalities in esophageal cancer: epidemiology, presentation and progression, work-up, and surgical approaches. *Oncologist*. 2004;9:137-46.
5. Vigushin DM, Coombes RC. Histone deacetylase inhibitors in cancer treatment. *Anticancer Drugs*. 2002;13:1-13.
6. Yu X, Guo ZS, Marcu MG, Neckers L, Nguyen DM, Chen GA, Schrump DS. Modulation of p53, ErbB1, ErbB2, and Raf-1 expression in lung cancer cells by depsipeptide FR901228. *J Natl Cancer Inst*. 2002;94:504-13.
7. Marks PA, Rifkind RA, Richon VM, Breslow R. Inhibitors of histone deacetylase are potentially effective anticancer agents. *Clin Cancer Res*. 2001;7:759-60.
8. Elaut G, Torok G, Vinken M, Laus G, Papeleu P, Tourwe D, et al. Major phase I biotransformation pathways of Trichostatin a in rat hepatocytes and in rat and human liver microsomes. *Drug Metab Dispos*. 2002;30:1320-8.
9. Nakano K, Mizuno T, Sowa Y, Orita T, Yoshino T, Okuyama Y, Fujita T, et al. Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. *J Biol Chem*. 1997;272:22199-206.
10. Mayo MW, Denlinger CE, Broad RM, Yeung F, Reilly ET, Shi Y, Jones DR. Ineffectiveness of histone deacetylase inhibitors to induce apoptosis involves the transcriptional activation of NF-kappa B through the Akt pathway. *J Biol Chem*. 2003;278:18980-9.
11. Wang Q, Wang X, Evers BM. Induction of cIAP-2 in human colon cancer cells through PKC delta/NF-kappa B. *J Biol Chem*. 2003;278: 51091-9
12. Shin HJ, Baek KH, Jeon AH, Kim SJ, Jang KL, Sung YC, et al. Inhibition of histone deacetylase activity increases chromosomal instability by the aberrant regulation of mitotic checkpoint activation. *Oncogene*. 2003;22:3853-8.
13. Vigushin DM, Coombes RC. Histone deacetylase inhibitors in cancer treatment. *Anticancer Drugs*. 2002;13:1-13.
14. Mayo MW, Baldwin AS. The transcription factor NF-kappaB: control of oncogenesis and cancer therapy resistance. *Biochim Biophys Acta*. 2000;1470:M55-62.
15. El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, et al. WAF1, a potential mediator of p53 tumor suppression. *Cell*. 1993;75:817-25.
16. Nguyen DM, Schrump WD, Chen GA, Tsai W, Nguyen P, Trepel JB, et al. Abrogation of p21 expression by flavopiridol enhances depsipeptide-mediated apoptosis in malignant pleural mesothelioma cells. *Clin Cancer Res*. 2004;10:1813-25.
17. Nguyen DM, Schrump WD, Tsai WS, Chen A, Stewart JH 4th, Steiner F, et al. Enhancement of depsipeptide-mediated apoptosis of lung or esophageal cancer cells by flavopiridol: activation of the mitochondria-dependent death-signaling pathway. *J Thorac Cardiovasc Surg*. 2003; 125:1132-42.
18. Rosato RR, Almenara JA, Grant S. The histone deacetylase inhibitor MS-275 promotes differentiation or apoptosis in human leukemia cells through a process regulated by generation of reactive oxygen species and induction of p21CIP1/WAF1 1. *Cancer Res*. 2003;63:3637-45.
19. Han JW, Ahn SH, Kim YK, Bae GU, Yoon JW, Hong S, Lee HY, et al. Activation of p21(WAF1/Cip1) transcription through Sp1 sites by histone deacetylase inhibitor apicidin: involvement of protein kinase C. *J Biol Chem*. 2001;276:42084-90.
20. Catley MC, Cambridge LM, Nasuhara Y, Ito K, Chivers JE, Beaton A, et al. Inhibitors of protein kinase C (PKC) prevent activated transcription: role of events downstream of NF-kappaB DNA binding. *J Biol Chem.* 2004;279:18457-66.
21. Kelly WK, Richon VM, O'Connor O, Curley T, MacGregor-Curtelli B, Tong W, Klang M, et al. Phase I clinical trial of histone deacetylase inhibitor: suberoylanilide hydroxamic acid administered intravenously. *Clin Cancer Res*. 2003;9:3578-88.
22. Kim YK, Han JW, Woo YN, Chun JK, Yoo JY, Cho EJ, Hong S, et al. Activation of p21(WAF1/Cip1) transcription through Sp1 sites by histone deacetylase inhibitor apicidin: involvement of protein kinase C. *J Biol Chem*. 2001;276:42084-90.

Discussion

Dr Thomas A. D'Amico *(Durham, NC)*. Just to clarify, you did demonstrate that Bcl-XL was downregulated, correct?

Dr Maxhimer. We saw that Bcl-XL was increased with HDAC treatment, and then on addition of our PKC inhibitor, Bcl-XL was downregulated.

Dr Yolonda Colson *(Boston, Mass)*. What do you think are potential mechanisms of resistance to these different pathways that you are starting to block? Do you think that is going to be a problem?

Dr Maxhimer. As far as we have seen, our results have been consistent across cell lines. We have not looked at a meso cell line, but we have looked at a wide array and also have started to use this in an in vivo model. Our hope is that if this is going to be a consistent downregulation of these resistant pathways, that we will. We also want to use more clinically relevant drugs, HDAC inhibitors as well as PKC inhibitors, to see this effect.

Dr Raphael Bueno *(Boston, Mass)*. What happens to normal cells when you do this?

Dr Maxhimer. Nothing. With the combination therapy, there is no toxicity seen. If it gets further out, we see somewhat of a reduction in cell viability. We also had some keratinocyte data and the human umbilical vein endothelial cell lines that showed no toxicity in these cell lines.

Dr Bueno. You flipped through that slide very fast. You maintain that it is just the PKC inhibitor that downregulates the various IAPs?

Dr Maxhimer. Yes.

Dr Bueno. And that alone does not cause increased apoptosis in your cell lines?

Dr Maxhimer. I am sorry that I breezed through that too quickly. In the next graph we saw little. We saw maybe 5% to 10% with the PKC inhibitor alone that induces apoptosis, and it was in comparison with the TSA, the HDAC inhibitor, but once we put the 2 together, we saw a synergistic effect come together to see 90% to almost 100% induction of apoptosis.

Dr Bueno. Because I would wonder if you combined the PKC inhibitor with other things without the deacetylase inhibitor whether you actually need that because if you can alter the balance of the antiapoptotic genes and the proapoptotic genes, you might just be able to do with that. Have you tried to do that, the PKC with, let us say, cisplatin?

Dr Maxhimer. That is actually a great question. No, we have not looked at that, but we wanted to exploit some of the HDAC qualities of cell growth inhibition and then use that to downregulate some of its other negative effects on resistance of apoptosis. So that is kind of our scheme of why we are using these types of treatments. But, no, we have not looked at PKC inhibition with other combination therapies.

Dr Nguyen. I appreciate that question. I just want to clarify. Work has been done before, not in our laboratory but actually at

Sloan-Kettering, looking at PKC inhibition with Biostatin, and it has been shown to enhance the chemotherapeutic effect. Therefore it has been used as a target. Actually, PKC becomes a very attractive target for drug development. The other issue is, with the advent of RNA inhibitor, we can do these experiments very cleanly by targeting Bcl-2 and Bcl-XL with a construct that basically downregulates Bcl-2 or Bcl-XL, and we can hopefully see the same results.