Enhanced Growth Inhibition by Combination Differentiation Therapy with Ligands of Peroxisome Proliferator-activated Receptor-γ and Inhibitors of Histone Deacetylase in Adenocarcinoma of the Lung

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ABSTRACT

Purpose: **Histone deacetylase (HDAC) inhibitors and ligands of the peroxisome proliferator-activated receptor γ (PPARγ) have been shown previously to induce growth arrest and differentiation in a variety of cancer cell lines. The purpose of this study was to determine whether HDAC inhibitors function similarly in non-small cell lung cancer (NSCLC) and whether combination treatment with HDAC inhibitors and PPARγ ligands is more efficacious than either agent alone.**

Experimental Design and Results: **Nanomolar concentrations of trichostatin A induced growth arrest in five of seven NSCLC cell lines, whereas sodium phenylbutyrate (PB) was markedly less potent. In adenocarcinomas, trichostatin A up-regulated general differentiation markers (gelsolin, Mad, and p21/WAF1) and down-regulated markers of the type II pneumocyte progenitor cell lineage (MUC1 and SP-A), indicative of a more mature phenotype. PB had a similar effect. Simultaneous treatment with a PPARγ ligand and PB enhanced the growth inhibition in adenocarcinomas but not in nonadenocarcinomas. Growth arrest was accompanied by markedly decreased cyclin D1 expression but not enhanced differentiation.**

Conclusions: **The present study demonstrates potent growth-inhibitory and differentiation-inducing activity of HDAC inhibitors in NSCLC and suggests that combination differentiation therapy should be explored further for the treatment of lung adenocarcinomas.**

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INTRODUCTION

The induction of terminal differentiation, with its obligatory growth cessation, is an intriguing approach to the treatment of solid tumors. Although cancer has long been described as a disorder of cellular differentiation, the reinstitution of tissue-specific terminal differentiation programs in malignant epithelial cells has not been feasible clinically thus far. Recent data show, however, that ligands of the PPARγ,[2] a member of the steroid receptor superfamily that is a key regulator of adipogenesis (reviewed in Ref. 1), induce differentiation in liposarcoma, breast, colon, and lung cancer cell lines (2–5). Similarly, data are accruing that the acetylation state of histones has an important role in the regulation of expression of genes involved in proliferation and differentiation, and that HDAC inhibitors are potent differentiating agents *in vitro* (6). Thus, differentiation-based strategies deserve additional scrutiny.

Reversible acetylation of NH_2-terminal tails of the core histone proteins is important in the modulation of chromatin structure and in determining the accessibility of gene promoter regions to transcriptional regulators. The acetylation state of histones is governed by the combined activities of specific cellular enzymes, histone acetyltransferases, and HDACs (reviewed in Ref. 7). Histone acetylation is thought to have a crucial role in gene expression because transcriptionally activated genes have been found to be associated with highly acetylated loci, whereas transcriptionally inactive genes are associated with hypoacetylation (8). The role of histone acetylation in transcriptional activation is further supported by the findings that multiple transcription factors possess intrinsic histone acetylase activity (9), mutants lacking this activity fail to activate their target genes (10), and transcriptional repression correlates with histone deacetylation (11). CBP, p300, and p300/CBP-associated factor are transcriptional coactivators with intrinsic histone acetylase activity (12). These coactivators have been shown to have a key role in myogenic differentiation (13), whereas, conversely, mutations involving the p300/CBP family have been documented in colorectal and gastric cancers (14). Furthermore, HDACs contribute to acute promyelocytic leukemogenesis when recruited by the abnormal fusion protein PML-RARα to retinoic acid receptor target genes, repressing transcription and preventing differentiation (15).

Although the precise mechanisms underlying cell cycle

[2] The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor γ; CBP, cAMP-responsive element-binding protein; NSCLC, non-small cell lung cancer; HDAC, histone deacetylase; TSA, trichostatin A; PB, phenylbutyrate.

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arrest or differentiation mediated through histone acetylation have not been elucidated, HDAC inhibitors have been shown to induce cell cycle arrest and a differentiated phenotype in a variety of tumor types including leukemia, colon cancer, and breast cancer (reviewed in Ref. 6). Our study addressed the potential of pharmacological inhibitors of HDAC to modulate the differentiation status in NSCLC cell lines. HDAC inhibitors were found to induce growth arrest and differentiation in multiple NSCLC cell lines. Of particular relevance to future potential clinical studies, combination differentiation treatment with a clinically available weak HDAC inhibitor and low doses of a PPARγ ligand (a class of agents currently in use for the treatment of diabetes mellitus) resulted in enhanced growth arrest in adenocarcinoma cell lines. Additional studies are warranted to examine differentiation-based therapies using HDAC inhibitors and PPARγ ligands in NSCLC, particularly adenocarcinoma, treatment.

MATERIALS AND METHODS

Cell Culture. The NSCLC cell lines (H157, H322, H358, H441, H520, and H1299) were obtained from the National Cancer Institute-Navy Medical Oncology Branch (Bethesda, MD). The NSCLC cell line A549 was obtained from the American Type Culture Collection (Rockville, MD). All cell lines were maintained in continuous culture in RPMI 1640 supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS (Life Technologies, Inc., Gaithersburg, MD). The PPARγ ligand ciglitizone was purchased from Biomol (Plymouth Meeting, PA), and the HDAC inhibitors TSA and PB were purchased from Biomol and Triple Crown America (Perkasie, PA), respectively.

RNA Isolation and Northern Blot Analysis. Total cellular RNA isolation from cultured cells, Northern blot transfer, and hybridization with [^{32}P]dCTP-labeled probes were performed as described previously (5). The following cDNA probes, described previously (5), were used: (*a*) the *Eco*RI/*Xho*I digestion fragment of human *MUC1* cDNA (American Type Culture Collection); and (*b*) the *Eco*RI/*Hin*dIII digestion fragment of human SP-A (kind gift of J. Whitsett, University of Ohio, Cincinnati, OH).

Western Analysis. Western analysis was performed as described previously (5). Briefly, cell extracts were prepared in lysis buffer [60 mM Tris (pH 6.8), 2% SDS, 100 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.1 trypsin inhibitory unit/ml aprotinin, and 10 μM leupeptin], boiled for 5 min, and clarified. Fifty μg of each protein lysate were then electrophoresed in 6, 8, or 12% polyacrylamide minigels (Novex, San Diego, CA). Proteins were transferred to nitrocellulose filters, and detection was performed using various primary antibodies and enhanced chemiluminescence (ECL; Amersham Life Science, Arlington Hills, IL). Antibodies to the following proteins were used: anti-gelsolin (1:2500, G37820; Transduction Laboratories, Lexington, KY), anti-PPARγ (1:2000, PA3-821; Affinity BioReagents, Golden, CO), anti-p21 (1:1000, SC-397; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-Mad1 (1:1000, SC-222; Santa Cruz Biotechnology). Experiments were performed a minimum of three times with different cell lysates.

Cell Proliferation Assays. Anchorage-dependent growth was measured by CellTiter96 nonradioactive cell proliferation assay (Promega Corp., Madison, WI). Anchorage-independent growth was assessed by soft agarose clonogenic assays as described previously (5). Briefly, viable cells, as judged by trypan blue dye exclusion, were seeded at a density of 4×10^4 cells/ml in 60-mm dishes in RPMI 1640 with 10% fetal bovine serum and 0.35% agarose on a base layer of 0.7% agarose. DMSO, TSA, or PB was added to both bottom and top agarose layers. Assays were performed in triplicate on at least three separate occasions, and colonies were counted at 10–14 days.

RESULTS

HDAC Inhibitors Induce Growth Arrest in NSCLC Cell Lines. The effect of two structurally unrelated HDAC inhibitors on the growth of NSCLC cell lines was examined. As shown in Fig. 1*A*, treatment of two adenocarcinoma cell lines with 2 mM PB, a concentration achieved in humans in Phase I clinical trials (16), resulted in marked slowing of cell growth. TSA was substantially more potent, with 250–500 nM concentrations leading to growth cessation followed by cell death. Growth inhibition was concentration dependent. Examination of the effect of both HDAC inhibitors on a panel of NSCLC cell lines revealed that the growth inhibition mediated by TSA was not limited to the adenocarcinoma subtype (Fig. 1*B*), whereas PB induced growth arrest only in adenocarcinomas (H358, H441, and A549). Sensitivity to TSA was not predictive of sensitivity to PB.

The effect of HDAC inhibitors on anchorage-independent cell growth was assessed by soft agarose clonogenic assay in two cell lines (Table 1). In contrast to anchorage-dependent growth, anchorage-independent growth was abolished completely by low doses of TSA and by PB in both cell lines. This included the squamous cell carcinoma cell line H157, which was relatively resistant to PB and low doses of TSA in monolayer growth assays.

HDAC Inhibitors Induce Differentiation in NSCLC. To determine whether the growth inhibition mediated by HDAC inhibitors was accompanied by differentiation, analysis of multiple markers of the differentiated state was performed. The normal lung is composed of multiple epithelial cell lineages with differing proliferative potentials and characterized by distinct differentiation markers. To date, no single marker pathognomonic for terminal differentiation of all lung epithelial lineages has been described. Therefore, we examined multiple markers associated with differentiation in general (*i.e.,* "general" differentiation markers: gelsolin, Mad, and p21) as well as markers associated with specific lung cell types (*i.e.,* lineage-specific markers: MUC1 and SP-A), as we have done previously in assessing the differentiating potential of PPARγ agonists in NSCLC (5). To control for the effects of cell confluence, marker expression in TSA-treated cells was compared with expression in logarithmically growing vehicle-treated cells. We have shown previously that expression of these differentiation markers does not change during 72 h of treatment with the vehicle control DMSO (5).

Both 250 and 500 nM TSA up-regulated the expression of all three general differentiation markers, gelsolin, Mad, and the

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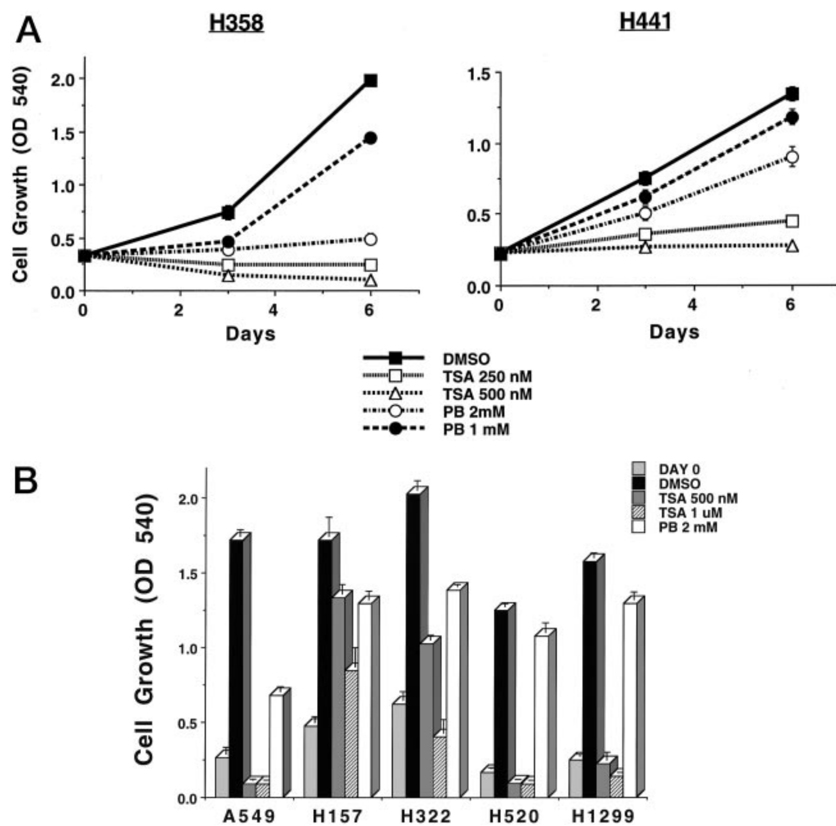

Fig. 1 Effect of HDAC inhibitors on cell growth. Anchorage-dependent cell growth in the presence of HDAC inhibitors or the vehicle control DMSO was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay. *A*, growth kinetics of H358 and H441 cells treated with TSA or PB. *B*, effect of TSA and PB on the growth of multiple NSCLC cell lines. Cells were plated at low density, and growth was assessed after 6 days of continuous culture. *Bars*, SD.

Table 1 Inhibition of anchorage-independent growth by HDAC inhibitors

Cells were cloned in soft agarose in the continuous presence of the vehicle DMSO, TSA, or PB, and colonies were counted after 10 or 14 days (A549 and H157, respectively).

Cell line	No. of colonies (% of control)			
	DMSO	TSA 250 nM	TSA 100 nM	PB 2 mM
A549	387 ± 64 (100%)	1.7 ± 1.7 (0%)	0 ± 0 (0%)	5.3 ± 1.2 (0%)
H157	162 ± 4 (100%)	1 ± 0.8 (0%)	1.3 ± 0.9 (0%)	0 ± 0 (0%)

cyclin-dependent kinase inhibitor p21 (Fig. 2, *A* and *B*) in a manner consistent with differentiation. Gelsolin is an actin-regulatory protein that is expressed at low levels in most cancer cell lines as well as in primary lung cancers, although it is highly expressed in the surrounding histologically normal lung (17). Overexpression of gelsolin in a bladder cancer cell line resulted in reversion of the malignant phenotype (18). Mad is a member of the myc family of interacting proteins that is closely linked to differentiation in leukemic as well as lung cancer cell line model systems (5, 19). Similarly, p21/Waf1 expression has been closely correlated with induced differentiation in multiple cell culture model systems (5). The changes in all three of these markers are consistent with the induction of a more mature, slower-growing phenotype. PB had a similar effect on the general differentiation markers (Figs. 2*B* and 3*D*).

Examination of lung lineage-specific markers revealed that *MUC1* and *SP-A*, both specific for the type II pneumocyte in the alveolar epithelium (20), were markedly down-regulated by TSA treatment (Fig. 2*C*). The type II pneumocyte is a peripheral lung progenitor cell with capacity to repopulate the epithelial surface after injury or during carcinogenesis. The down-regulation of *MUC1* and *SP-A* by TSA treatment suggests that differentiation away from the progenitor type II pneumocyte lineage has been induced (5, 20). Taken in context with up-regulation of general differentiation markers and growth arrest, HDAC inhibitors modulate the differentiation status of NSCLC cell lines.

Growth Arrest and Induction of Differentiation by Combination Treatment with PPARγ Ligand and HDAC Inhibitor. To address whether combination treatment with lower concentrations of unrelated differentiation inducers would be more efficacious than higher doses of either agent alone, we examined the effects of the PPARγ ligand ciglitizone and the weak HDAC inhibitor PB on multiple NSCLC cell lines. Treatment with ciglitizone and PB resulted in enhanced growth arrest (Fig. 3, *A* and *B*) and morphological changes with more abundant, flattened cytoplasm and an increased cytoplasmic:nuclear

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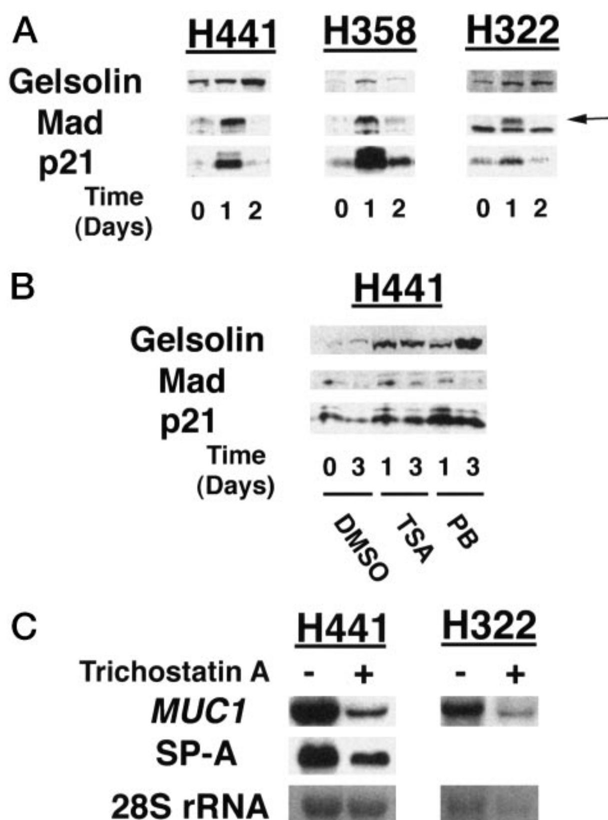

Fig. 2 Effects of HDAC inhibitors on differentiation markers. Total cellular protein or RNA was isolated as indicated. Protein analysis was performed by Western blotting using antibodies against gelsolin, Mad, and p21, whereas RNA analysis was performed by Northern blotting using cDNA probes for *MUC1* and SP-A. *A*, induction of "general" differentiation markers by 250 nM TSA. Cells were treated for the indicated time with 250 nM TSA, and total protein was isolated. *B*, induction of "general" differentiation markers by 500 nM TSA or 2 mM PB. The adenocarcinoma cell line H441 was treated for the indicated time with either agent or the vehicle control DMSO. *C*, expression of lineage-specific differentiation markers. Total cellular RNA was prepared after 24 h of treatment with 500 nM TSA. Ethidium bromide shadowing of 28S rRNA was used to assess RNA loading in different lanes.

ratio (Fig. 3*C*). This enhanced growth arrest was seen in three of three adenocarcinoma cell lines (H358, H441, and A549) but not in two squamous cell carcinomas, one large cell carcinoma, and one adenocarcinoma with squamous differentiation potential (results not shown).

To determine whether the growth arrest and morphological changes induced by the combination treatment with PPARγ agonist and HDAC inhibitor were accompanied by enhanced differentiation in NSCLC cells, the expression of differentiation markers and cell cycle regulatory proteins was examined. As shown in Fig. 3*D*, the general differentiation marker gelsolin was induced by the combination, but not more than with PB alone. The cyclin-dependent kinase inhibitor p21 was transiently induced by the combination (not more than with PB alone) and then was down-regulated below baseline, as is frequently seen during differentiation. This suggests that the combination treatment did not lead to a greater degree of differentiation than PB alone. The combination treatment did, however, result in more complete hypophosphorylation of Rb and greater inhibition of cyclin D1 than either ciglitizone or PB alone. These changes in Rb and cyclin D1 may be responsible for or may simply be a reflection of the greater growth inhibition occurring with combination treatment.

DISCUSSION

Despite new advances in our understanding of the molecular biology of lung cancer and the introduction of new treatment strategies, lung cancer remains the leading cause of cancer death in the United States. Novel approaches toward the treatment and prevention of lung cancer are therefore urgently needed. Although epithelial carcinogenesis is characterized by inappropriate cell proliferation and/or altered patterns of cell death, the ability to undergo terminal differentiation may be retained after neoplastic transformation. Multiple *in vitro* studies of breast, colon, and lung cancer cell lines support this hypothesis (3–5). The recent demonstration that administration of the PPARγ agonist troglitazone to patients with liposarcoma resulted in evidence of histological and biochemical differentiation in biopsied tissues suggests that terminal differentiation can also be induced pharmacologically *in vivo* (21).

Results from our study show for the first time that HDAC inhibitors are, indeed, potent inhibitors of NSCLC cell growth and modulate differentiation markers in a manner consistent with a more mature phenotype. HDAC inhibitors have been shown previously to arrest growth and induce differentiation *in vitro* in a variety of transformed cell types, including erythroleukemia, neuroblastoma, and colon and breast carcinomas (6, 7). TSA has also been shown to have potent antitumor activity *in vivo* in a *N*-methyl-*N*-nitrosourea-induced rat mammary carcinoma model, in the absence of any appreciable toxicity (22). Curiously, a number of the tumors from the rats treated with TSA, but not the control DMSO, were either benign fibroadenomas or tubular adenomas, indicating that differentiation was also occurring *in vivo*. The data from these studies as well as from our study indicate that HDAC inhibitors have the capacity to modulate the maturation of a variety of different cell types, including epithelial cells with complex genetic abnormalities, *in vitro*, and potentially *in vivo* as well.

We have shown previously that PPARγ ligands, which also induce differentiation in a variety of epithelial cell types (2–4), induce differentiation in NSCLC cell lines (5). In the current study, we extend these observations to show that low doses of a PPARγ ligand (which alone do not substantially inhibit growth or induce differentiation) in combination with the weak HDAC inhibitor PB resulted in more profound growth arrest than treatment with either drug alone. This occurred in the absence of enhanced differentiation. Curiously, the enhanced growth arrest was seen only in adenocarcinoma cell lines and not in other histological subtypes. This may reflect the inherently greater sensitivity of the adenocarcinomas to PB, because the non-adenocarcinoma cell lines showed minimal growth arrest after PB treatment. However, when the more potent HDAC inhibitor TSA was used at concentrations that inhibited growth by >50% in combination with low doses of ciglitizone, no enhancement of

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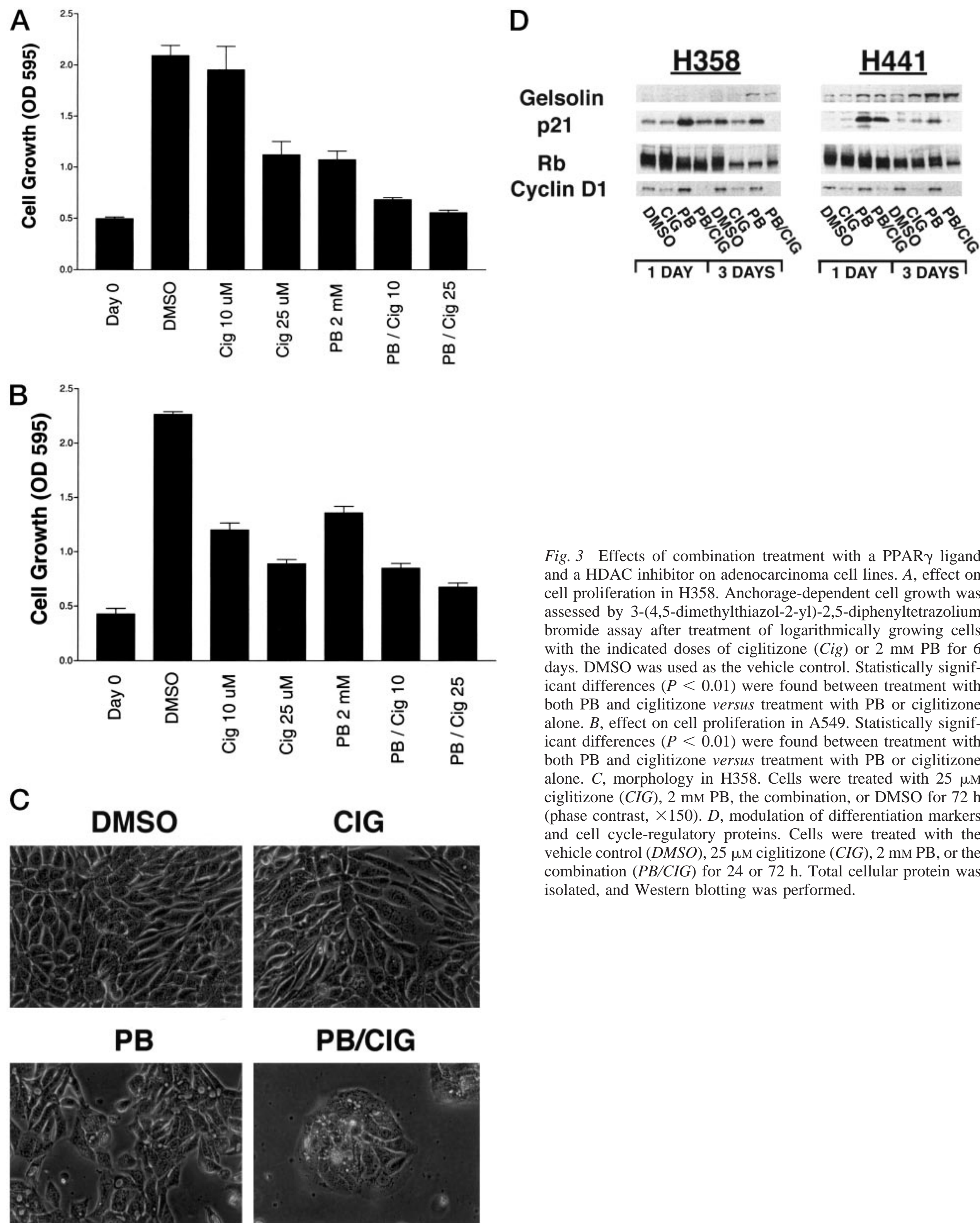

Fig. 3 Effects of combination treatment with a PPARγ ligand and a HDAC inhibitor on adenocarcinoma cell lines. *A*, effect on cell proliferation in H358. Anchorage-dependent cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after treatment of logarithmically growing cells with the indicated doses of ciglitizone (*Cig*) or 2 mM PB for 6 days. DMSO was used as the vehicle control. Statistically significant differences ($P < 0.01$) were found between treatment with both PB and ciglitizone *versus* treatment with PB or ciglitizone alone. *B*, effect on cell proliferation in A549. Statistically significant differences ($P < 0.01$) were found between treatment with both PB and ciglitizone *versus* treatment with PB or ciglitizone alone. *C*, morphology in H358. Cells were treated with 25 μM ciglitizone (*CIG*), 2 mM PB, the combination, or DMSO for 72 h (phase contrast, ×150). *D*, modulation of differentiation markers and cell cycle-regulatory proteins. Cells were treated with the vehicle control (*DMSO*), 25 μM ciglitizone (*CIG*), 2 mM PB, or the combination (*PB/CIG*) for 24 or 72 h. Total cellular protein was isolated, and Western blotting was performed.

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growth arrest compared with TSA alone occurred (results not shown). These data suggest that combination differentiation therapy potentiates the efficacy of relatively weak agents or agents given at subtherapeutic concentrations. Currently, PB is in Phase I/II clinical trials (16), and PPARγ agonists are part of standard clinical care for type II diabetes mellitus, although they are being used at concentrations that may be somewhat lower than required for differentiation induction *in vitro* (5). Therefore, our results suggest a strategy for maximizing the efficacy of these agents within the confines of current clinical usage and have clear implications for the design of potential clinical trials.

Combination differentiation therapy also resulted in greater and more rapid inhibition of cyclin D1, raising the question as to whether the enhanced growth inhibition occurred through this mechanism. Accumulating data suggest that cyclin D1 overexpression occurs frequently and early during lung carcinogenesis and may therefore be an important therapeutic target (23, 24). Ablation of cyclin D1 affords protection against mammary carcinogenesis driven by *neu* and *ras*, but not *myc* or *Wnt-1*, in transgenic mice (25). *neu* and *ras* are known to act directly on the cyclin D1 promoter (25). Although results of comparable lung carcinogenesis transgenic studies are not available, it is notable that mutations of *ras* and *Her2/neu* are primarily found in the adenocarcinoma histological subtype of NSCLC. Additional studies will be necessary to determine whether combination differentiation therapy selectively targets *ras*- and/or *neu*-driven lung carcinogenesis by interfering with cell cycle progression through cyclin D1 down-regulation.

Similarly, combination differentiation therapy led to a transient increase in p21, followed by a more profound p21 down-regulation, than treatment with either agent alone. This pattern of transient p21 induction followed by down-regulation below baseline has been shown in several epithelial differentiation model systems, including PPARγ ligand-induced differentiation of NSCLC cell lines (5, 26).[3] In addition to mediating growth arrest during differentiation, p21 may have other functions as well. Wang and Walsh (27) showed that p21 protects C2C12 myoblasts from apoptosis during myocyte differentiation, suggesting that p21 shunts cells into a differentiation pathway and away from apoptosis. Di Cunto *et al.* (26), on the other hand, showed that forced expression of p21 prevents the expression of late markers of terminal differentiation in keratinocytes, suggesting that p21 functions early during differentiation and must be eliminated for the full differentiation program to be executed. Although the role of p21 in NSCLC differentiation remains to be clarified, the p21 expression pattern observed during treatment with HDAC inhibitors or the combination used in this study is consistent with the induction of differentiation.

Differentiation induction by all-*trans* retinoic acid already plays a role in the treatment of a nonepithelial malignancy, acute promyelocytic leukemia. Warrell *et al.* (28) reported recently that treatment with all-*trans*-retinoic acid and PB resulted in a complete cytogenetic remission in a patient with refractory acute promyelocytic leukemia, providing the first clinical demonstration that combination differentiation therapy may represent an important therapeutic advance. These data, together with the accumulating preclinical evidence linking HDAC inhibitors and PPARγ ligands to solid-tumor differentiation, suggest that the induction of differentiation using a combination of agents may be an attainable goal in the treatment of multiple malignancies and warrants additional evaluation.

[3] E. Szabo and T-H. Chang, personal communication.

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