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Histone Deacetylase Inhibitors Enhance the Apoptotic Activity of Insulin-Like Growth Factor Binding Protein-3 by Blocking PKC-Induced IGFBP-3 Degradation

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Abstract

Overexpression of insulin-like growth factor binding protein (IGFBP)-3 induces apoptosis of cancer cells. However, preexisting resistance to IGFBP-3 could limit its antitumor activities. This study characterizes the efficacy and mechanism of the combination of recombinant IGFBP-3 (rIGFBP-3) and HDAC inhibitors to overcome IGFBP-3 resistance in a subset of non-small cell lung cancer (NSCLC) and head and neck squamous cell carcinoma (HNSCC) cells. The effects of the combination of rIGFBP-3 and a number of HDAC inhibitors on cell proliferation and apoptosis were assessed *in vitro* and *in vivo* by using the MTT assay, a flow cytometry-based TUNEL assay, western blot analyses, and the NSCLC xenograft tumor model. Combined treatment with HDAC inhibitors and rIGFBP-3 had synergistic antiproliferative effects accompanied by increased apoptosis rates in a subset of NSCLC and HNSCC cell lines *in vitro*. Moreover, combined treatment with depsipeptide and rIGFBP-3 completely suppressed tumor growth and increased the apoptosis rate *in vivo* in H1299 NSCLC xenografts. Evidence suggests that HDAC inhibitors increased the half-life of rIGFBP-3 protein by blocking protein kinase C (PKC)-mediated phosphorylation and degradation of rIGFBP-3. In addition, combined treatment of IGFBP-3 with an HDAC inhibitor facilitates apoptosis through up-regulation of rIGFBP-3 stability and Akt signaling inhibition. The ability of HDAC inhibitors to decrease PKC activation may enhance apoptotic activities of rIGFBP-3 in NSCLC cells *in vitro* and *in vivo*. These results indicated that combined treatment with HDAC inhibitor and rIGFBP-3 could be an effective treatment strategy for NSCLC and HNSCC with highly activated PKC.

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Keywords

Non-small cell lung cancer; insulin-like growth factor binding protein-3; head and neck squamous cell carcinoma; Akt; histone deacetylases; protein kinase C

Introduction

Lung cancer is the leading cause of cancer deaths in the United States (28% of all cancer deaths) [1]. More than 80% of lung cancers are non-small cell lung cancer (NSCLC), and approximately 60% of patients have advanced disease at the time of diagnosis [2, 3]. Despite the use of conventional and novel therapeutic strategies, such as surgery, chemotherapy, and ionizing radiation therapy, the prognosis for NSCLC remains unsatisfactory. These data underscore the need for effective therapy for this disease. Toward this goal, we have investigated strategies for blocking the insulin-like growth factor (IGF)-mediated signaling pathway, which plays a central role in cellular growth, differentiation, and proliferation [4]. One such strategy is treatment with natural IGF-binding protein (IGFBP)-3, which binds to free IGF in the extracellular milieu with high affinity and specificity, thus reducing IGF bioavailability [5, 6].

IGFBP-3 is the most abundant IGFBP in human serum [7], where it forms a ternary complex with the acid-labile subunit of IGF [8]. IGFBP-3 sequesters IGFs away from their receptor and thereby inhibiting the mitogenic and anti-apoptotic action of IGFs [5]. IGFBP-3 also induces IGF-I-independent antitumor activities in a variety of human cancer cells [9-11]. High expression of IGFBP-3 is correlated with lower portal invasion and better prognosis in human hepatocellular carcinoma [12]. We previously found that loss of IGFBP-3 expression is a common event in patients with stage I NSCLC or HNSCC and correlates closely with poor prognosis [13, 14]. These findings provide a strong rationale for the use of IGFBP-3 in lung cancer. In addition, recombinant IGFBP-3 (rIGFBP-3) is currently under extensive preclinical investigation to determine its efficacy in cancer therapy [15].

Potential hurdles for the use of IGFBP-3 in lung cancer therapy are preexisting or acquired resistance mechanisms in NSCLC cells. For example, activation of Ras and different proteases have been involved in the nonresponsiveness of cancer cells to IGFBP-3 [16]. Indeed, co-treatment with SCH66336, a farnesyltransferase inhibitor that blocks Ras activation, enhanced therapeutic activities of IGFBP-3 in NSCLC cell lines [17] by upregulation of its stability. However, the cytotoxic actions of FTIs also implicate the modulation of other targets in addition to the inhibition of Ras proteins [18, 19]. Therefore, further clarification on the effects of Ras inhibition on apoptotic activities of IGFBP-3 is required.

Histone deacetylases (HDACs) regulate the expression of a variety of genes and proteins involved in cell cycle and apoptosis [20]. In addition to its function to decrease acetylation of histones (e.g., histone H3 and H4), HDACs also deacetylate a variety of nonhistone proteins, such as Hsp90 and RelA/p65 [21, 22]. Several HDAC inhibitors that exhibit impressive anti-tumor activity with remarkably little toxicity are now in clinical trial as mono-therapy as well as in combination with other drugs in treatment of cancers [23, 24]. Certain HDAC inhibitors suppress Ras expression and/or activity and show potent and selective antitumor activity [25, 26]. Some of these drugs, such as depsipeptide (FK228), suppress the Ras–MAP kinase signaling pathway and induce apoptosis in malignant melanoma [27]. Therefore, it is of interest to determine whether HDAC inhibitors enhance antitumor of IGFBP-3 in cancer cells.

In the present study, we sought to determine whether the therapeutic efficacy of IGFBP-3 in the treatment of NSCLC and head and neck squamous cell carcinoma (HNSCC) is enhanced by combined treatment with HDAC inhibitors *in vitro* and *in vivo*. Our data show that enhanced apoptosis induced by the drug combination in NSCLC and HNSCC cells involves blockade of protein kinase C-mediated phosphorylation, leading to increases in IGFBP-3 levels and Akt inhibition.

Materials and Methods

Cells and cell cultures, reagents, and animals

Human NSCLC cell lines with undetectable levels of IGFBP-3 expression (H1299 and H226Br) were purchased from the American Type Culture Collection (Manassas, VA). The human HNSCC cell lines UMSCC38 and SqCC35, which were originally established by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI) and Dr. Michael Reiss (Yale University, New Haven, CT), respectively, were obtained from Dr. Reuben Lotan (The University of Texas M. D. Anderson Cancer Center, Houston, TX). The NSCLC and HNSCC cell lines were maintained in RPMI 1640 and Dulbecco's modified Eagle's medium, respectively, supplemented with 10% fetal bovine serum (FBS; GIBCO-BRL, Gaithersburg, MD) in a humidified atmosphere with 5% CO_2. The HDAC inhibitors depsipeptide (FK228/FR901228) and SAHA (Vorinostat) were obtained from CTEP of National Cancer Institute and Merck and Co. Inc. (Rahway, NJ), respectively. Recombinant non-glycosylated IGFBP-3 was obtained from Insmed Inc. (Glen Allen, VA) and glycosylated recombinant IGFBP-3 was purchased from R&D Systems (Minneapolis, MN). Sodium butyrate (NaB), trichostatin A (TSA), hispidin, and the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, MO). m-Carboxycinnamic acid bis-hydroxamide (CBHA), RO31-8220, and Gö6976 were all obtained from Calbiochem-Novabiochem (Alexandria, New South Wales, Australia). Six-week-old female nude mice were purchased from Harlan Sprague Dawley (Indianapolis, IN).

Cell viability analysis, anchorage-dependent and -independent colony formation assays, and flow cytometric analysis of cell cycle distribution and apoptosis

The effects of IGFBP-3 on cell viability were analyzed by incubating cells in complete medium in the presence of with rIGFBP-3 (1 μg/ml), various concentrations of HDAC inhibitors or protein kinase inhibitors, or both. The cells were daily changed to fresh media with the same concentrations of the drug. The cell viability, cell cycle distribution, and apoptosis were analyzed after three days of treatment using the 3-4,5 dimethylthiazol-2-yl)-, 5-diphenyltetrazolium bromide (MTT) assay as described previously [28]. The anchorage-dependent and –independent colony forming abilities after twoo weeks of treatment were analyzed as described previously [28]. Data from replicate wells were presented as mean values with SD. Three independent experiments were performed with similar results; representative results of one experiment were presented.

Immunoblotting and immunoprecipitation

Immunoprecipitation was performed as described previously [29] using 1 mg of the total cell lysates using 1 μg of goat polyclonal antibodies against IGFBP-3 (Santa Cruz Biotechnology, Santa Cruz, CA) or a healthy preimmune serum as a negative control. Immunoblotting was performed using goat polyclonal antibodies against IGFBP-3 (1:4,000; Diagnostic Systems Laboratories, Webster, TX), rabbit polyclonal antibodies against pAkt (Ser473), phosphorylated PKC-α, -β, or -δ/θ, Akt (Cell Signaling, Danvers, MA Signaling), Bax, caspase-3 (Pharmingen, San Diego, CA), Bcl-2 (Roche Molecular Biochemicals,

Indianapolis, IN), pSer, or actin (Santa Cruz Biotechnology), and a mouse monoclonal antibody against poly(ADP-ribose) polymerase (PARP) (Cell Signaling).

Immune complex kinase assay

Immune complex kinase assays were performed as described previously [30] using rIGFBP-3 and MBP (myelin basic protein) as substrates.

Measurement of IGFBP-3 level by enzyme-linked immunosorbent assay (ELISA)

Supernatants were obtained from cultures of NSCLC cells treated with NaB (4 mmol/L), TSA (500 nmol/L) for 24 hours with IGFBP-3 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Each sample was assayed in triplicate.

Reverse Transcriptase-Polymerase Chain Reaction

To measure gene expression changes at the RNA level, reverse transcriptase (RT)-polymerase chain reaction (PCR) was performed. HNSCC cells were treated with (4 mmol/L), TSA (500 nmol/L) for 24 hours and total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). First, complementary DNA was synthesized from 2 μg of RNA extracted from the cells using an M-MLV RT kit (Invitrogen). Primers amplifying a region of IGFBP-3 (forward, 5'-GAAGGGCGACACTGCTTTTTC-3'; reverse 5'-CCAGCTCCAGGAAATGCTAG-3') and GAPDH (forward, 5'-GGTGAAGGTCGGTGTGAACGGATTT-3'; reverse, 5'-ATTGCCAAA GTT GTCATGGATGACC-3' were used.

Animal Experiment

The effect of the combination of IGFBP-3 and depsipeptide on the growth of H1299 tumor xenografts in athymic nude was analyzed as previously described [29]. The mice were randomly assigned to one of four treatment groups, with each group containing six mice. After tumor volume reached approximately 75 mm^3 (day 0), mice received a single subcutaneous injection of rIGFBP-3 (3 mg/kg body weight), subabdominal injection of depsipeptide (1.5 mg/kg body weight), or both. Tumor volumes were determined using caliper measurements of tumor length (L) and width (W) according to the formula $(L \times W^2)/2$. On day 24, all of the mice were killed, and tumor tissues were collected from them. The results were expressed as the mean tumor volumes (n = 6) with SD.

Statistical analysis

The data acquired using the MTT assay was analyzed using the Student *t*-test. All means and 95% CIs for eight samples were calculated using the Microsoft Excel software program (version 5.0; Microsoft Corporation, Redmond, WA). Cell-survival comparisons among groups of mice and the statistical significance of differences in tumor growth between the combination and single-agent treatment groups was analyzed using analysis of variance for a 2 × 2 factorial design. All means from triplicate to eight samples and 95% CIs were calculated using the SAS software program (version 8.02; SAS Institute, Cary, NC). In all of the statistical analyses, two-sided *P* values less than 0.05 were considered statistically significant.

Results

HDAC inhibitors and IGFBP-3 synergistically inhibit viability and anchorage-dependent and -independent growth of NSCLC and HNSCC cell lines *in vitro*

We investigated the viability of H1299 and H226Br NSCLC cells, which lack a basal level of IGFBP-3 expression probably due to the methylation in IGFBP-3 gene promoter [31], and

UMSCC38 and SqCC35 HNSCC cells after single or combined treatment of rIGFBP-3 (1 μg/ml) and the HDAC inhibitors, including NaB (5 mM), TSA (500 nM), CBHA (1 μM), and depsipeptide (10 nM). Exposure of cells to rIGFBP-3 or to HDAC inhibitors for 72 hours resulted in only a modest degree of cytotoxicity; however, combined treatment with these drugs showed synergistically enhanced effects on cell viability (Fig. 1*A*, supplementary Table 1). Similarly, treatment with rIGFBP-3 or HDAC inhibitors resulted in a modest inhibitory effect on the anchorage-dependent colony forming activity of H1299 and UMSCC38 cells but combined treatment with these drugs showed synergistic inhibition of the activity. Interestingly, IGFBP-3 increased anchorage-dependent colony formation in H226Br and SqCC35 cells by 40-50%. It has been previously shown that IGFBP-3 up-regulates IGF-I expression and induces activation of the IGF-IR pathway in certain breast cancer cells [32]. Further, IGFBP-3 can either inhibit or enhance EGF-mediated growth of breast epithelial cells [33]. Thus, IGFBP-3 appeared to have both antiproliferative and survival activities depending on the cell type and experimental conditions. Nevertheless, the combined treatment of rIGFBP-3 and HDAC inhibitor revealed significantly enhanced inhibitory effects on anchorage-dependent colony formation of these cells (Fig. 1*B*, Supplementary Table 2). Combination of rIGFBP-3 and NaB or TSA also reduced the anchorage-independent colony-forming ability of H1299 and UMSCC38 cells more significantly than did single-agent treatment (Fig. 1*C*, Supplementary Table 3). Treatment with NaB or TSA alone induced more than 60% of decrease in the anchorage-independent colony forming ability of H226Br cells and had only a modest increase in the inhibitory effects of rIGFBP3 on the ability (Fig. 1*C*). Together, these results and combined administration of these HDAC inhibitors and rIGFBP-3 appeared to have synergistic activities in suppressing viability and tumorigenic potential of NSCLC and HNSCC cells.

Apoptotic Effects of IGFBP-3 and HDAC inhibitors in NSCLC cells

Propidium Iodide (PI) staining and flow cytometry analysis was performed to determine the cell cycle distribution and apoptosis in the H1299 cells treated with rIGFBP-3 single or combination with the HDAC inhibitors. We found no obvious changes in cell cycle progression in H1299 cells treated with rIGFBP-3 for 3 days (Fig. 2*A*). In contrast, treatment with NaB, TSA, or CBHA increased the G_1-phase population of H1299 cells. Combined treatment with rIGFBP-3 and the HDAC inhibitors for the same duration led to a decrease in the G_1-, S-, and G_2/M-phase populations and an increase in the subG_0/G_1-phase population, suggesting an occurrence of apoptotic cell death after the combined treatment with rIGFBP-3 and HDAC inhibitors. Percentage of cells in specific phases of the cell cycle (Sub G_1, S, and G_2/M) by FACS analysis is shown in Supplementary Table 4. Consistent with the FACS analysis, rIGFBP-3 combined with NaB and rIGFBP-3 combined with TSA induced PARP cleavage better than single-agent treatment did (Fig. 2*B*), while these combinations did not enhance the changes in the expression of cell cycle-related molecules (p27, p21, cyclin D and cyclin E). We further tested the effects of the combined treatment on activities of Akt, $p70^{S6K}$ and Erk1/2, downstream effectors of the IGF-1R pathway, in NSCLC cells. pAkt (Ser473) and $p70^{S6K}$ levels, but not pErk1/2 level, were obviously lower in the cells with the combined treatment compared to those with single-agent treatment (Fig. 2*C*). rIGFBP-3 combined with depsipeptide also induced PARP cleavage and decreases in pAkt and $p70^{S6K}$ levels better than did single-agent treatment (Fig. 2*D*). These results suggested that a decrease in Akt activation may have contributed to the apoptotic activity of combined treatment with IGFBP-3 and the HDAC inhibitors in NSCLC cells.

Effects of combined treatment with HDAC inhibitors and rIGFBP-3 on the growth of NSCLC xenograft tumors

To determine whether combined treatment with HDAC inhibitors and rIGFBP-3 more effectively inhibits tumor growth than single-agent treatment, we assessed the effects of

HDAC inhibitors (depsipeptide, SAHA), rIGFBP-3, or a combination of the two agents on H1299 xenograft tumors established in nude mice. We observed that single-agent treatment with depsipeptide, SAHA, or rIGFBP-3 did not yield a statistically significantly reduction in H1299 xenograft tumor growth during the treatment period when compared with the control groups (Fig. 3*A*). In contrast, combination therapy resulted in an almost complete suppression of tumor growth throughout the study when compared with treatment with depsipeptide, SAHA or rIGFBP-3 alone. At day 24 or 21, the tumor volume in mice who received rIGFBP3 and depsipeptide (Supplementary Table 5) or SAHA (Supplementary Table 6) was statistically significantly smaller than the tumor volume in mice who received rIGFBP3, SAHA or depsipeptide. We did not observe unacceptable toxic effects of any of the treatment regimens as determined by physical examination and body-weight measurements (data not shown). Western blotting (Fig. 3*B*, upper) followed by densitometric analysis (Fig. 3*B*, bottom) of the H1299 xenograft tumors from the mice co-treated with rIGFBP-3 and SAHA (thrice a week for one week treatment) revealed significantly greater PARP cleavage of the 113-kD PARP compared to those from other treatment groups. These results indicated that treatment with the combination of rIGFBP-3 and depsipeptide significantly retarded tumor growth *in vivo* by inducing apoptosis.

HDAC inhibitors increase IGFBP-3 transcription and stabilize IGFBP-3 protein

We investigated the mechanisms underlying HDAC inhibitor-induced increase in apoptotic activity of IGFBP-3. Previous studies demonstrated the effects of NaB and TSA on IGFBP-3 transcription [34]. Therefore, we first tested the effects of HDAC inhibitors on mRNA levels of IGFBP-3 in UMSCC38, SqCC35, H1299, and H226Br cells. Consistent with the previous findings in MCF-7 and Hs578T breast cancer cells [35], RT-PCR revealed that HDAC inhibitors, including NaB and TSA, induced time-dependent increases in IGFBP-3 mRNA levels in UMSCC38 and SqCC35 cells (Fig. 4*A*), but not in in H1299 and H226Br cells (data not shown). Since H1299 and H226Br cells have been known to carry methylated IGFBP-3 promoter and the p53 null function [31, 36], it was not unexpected that there would be no IGFBP-3 mRNA visible in the cell lysates. We reasoned, however, that IGFBP-3 may be detectable in conditioned media from these cells if it was highly induced by HDAC inhibitors. Hence, we performed the enzyme-linked immunosorbent assay (ELISA)-based analysis of potential increase in endogenous IGFBP-3 secretion after treatment with HDAC inhibitors. We observed that TSA and NaB stimulated secretion of IGFBP-3 from 1.02 ng/ml to 1.80 ng/ml and to 2.33 ng/ml, respectively, in H1299 cells and from 0.281 ng/ml to 0.69 ng/ml and to 0.61 ng/ml, respectively, in H226Br cells (Fig. 4*B*).

Given the fact that HDAC inhibitors suppress Ras [25, 26] that is implicated in IGFBP-3 stability, we further tested the effects of NaB and TSA on exogenously added rIGFBP-3 in H1299, H226Br, and UMSCC 38 cells. Six hour after the treatment, H1299 cells treated with rIGFBP-3 and NaB or TSA showed relatively similar levels of IGFBP-3 compared to the cells treated with rIGFBP-3 alone (Fig. 4*C*). After 24 hours, H1299 cells with combined treatment showed obviously higher levels of IGFBP-3 compared to the cells treated with rIGFBP-3 alone. Similarly, H226Br cells (Fig. 4*D*) and UMSCC38 cells (supplementary Fig. 1) treated with rIGFBP-3 together with NaB, TSA or depesipeptide for 12 or 24 hours also showed obvious difference in IGFBP-3 levels compared to the cells treated with rIGFBP-3 alone. Thus, HDAC inhibitors appeared to increase IGFBP-3 expression not only through increased transcription but also through delayed protein degradation.

IGFBP-3 is phosphorylated and destabilized by PKC, and inhibitors of HDAC suppress IGFBP-3 phosphorylation by PKC

We further investigated the mechanism mediating the effects of HDAC inhibitors on IGFBP-3 stability. Because protein phosphorylation is the most common biochemical

modification that mediates protein stability, we studied whether HDAC inhibitors regulate the enzymes involved in IGFBP-3 phophorylation and subsequent degradation. IGFBP-3 has potential phosphorylation sites for protein kinase A (PKA), PKC, and DNA-dependent protein kinase (DNA-PK), p44/42 Erk1/2 [37, 38]. We assessed whether these protein kinases can phosphorylate IGFBP-3 and affect its stability using different small molecule inhibitors, including H-89 (an inhibitor of PKA), Nu7026 (an inhibitor of DNA-PK), hispidin (a selective inhibitor of PKC βI/βII), Ro 31-8220 (PKCα inhibitor), and Gö6976 (PKCα/β inhibitor). H1299 cells with rIGFBP-3 and H-89, Nu7026 (Nu), or hispidin treatment showed similar levels of IGFBP-3 levels compared to those with rIGFBP-3 treatment alone (Fig. 5*A*). In contrast, H1299 (Fig. 5*A*) and H226Br cells (data not shown) treated with rIGFBP-3 and Ro 31-8220 and Gö6976 showed increased cellular levels of IGFBP-3 compared to those treated with rIGFBP-3 alone, suggesting that suppression of PKCα activity increased the half-life of IGFBP-3.

We then assessed whether HDAC and PKC were involved in the mechanisms that induce phophorylation of IGFBP-3 in H1299 and H226Br cells. We performed immunoprecipitation using these cell lysates and an anti-IGFBP-3 antibody and analyzed serine phosphorylation of IGFBP-3 by Western blotting. The results showed that TPA had a discernible effect on IGFBP-3 phosphorylation in these cell liness, whereas treatment with Ro 31-8220, Gö6076, NaB, or TSA blocked this phosphorylation (Fig. 5*B*). We further performed an *in vitro* kinase assay to determine whether PKCα can phosphorylate IGFBP-3 *in vitro*. PKCα immunoprecipitated from H1299 and H226Br cells stimulated by TPA, an activator of PKC, rapidly raised phophorylation of rIGFBP-3 as well as MBP (a positive control) (Fig. 5*C*). PKCα-induced phophorylation of rIGFBP-3 was inhibited by RO31-8220, Gö6976, NaB, and TSA. We further assessed whether PKCα were involved in the antiproliferative activities of rIGFBP-3. We observed that treatment with Ro 31-8220 had increased the inhibitory effects of rIGFBP-3 on H1299 and H226Br cell viability (Fig. 5*D*). These observations suggest that IGFBP-3 undergoes degradation *via* PKCα-induced phosphorylation and then loses its antiproliferative activities.

Inhibitors of HDAC suppress the activity of PKC

We then examined whether treatment with HDAC inhibitors inhibited PKCα activity in these cells. As shown by western blotting using anti pPKC (pan) (βII Ser660) antibody that detects phosphorylated PKC α, βI, βII, δ, ε, η and θ homologous to pPKC βII (serine 660), more than 500 nM TSA, 1 mM NaB, and 1 μM SAHA inhibited PKC phosphorylation in H226Br (Fig. 6*A*). Further, TSA, NaB, and depsipeptide (FK228/FR901228) suppressed phosphorylation of PKCα (Fig. 6*B*) with no change in total PKC protein expression level. In contrast, the HDAC inhibitors failed to decrease pPKCδ/θ. H1299 (Fig. 6*C*) and UMSCC38 (Fig. 6*D*) cells also showed decrease in pPKCα in response to the TSA or NaB treatment. Consistent with these *in vitro* results, depsipeptide-based treatment inhibited PKCα activity *in vivo* in H1299 xenograft tumors (Fig. 6*E*) that were obtained from experiments described in Fig 3*C*. Therefore, the HDAC inhibitors appeared to preferentially affect the PKCα activity in H1299 and H226Br cells.

Discussion

In the present study, we demonstrated that combined treatment with HDAC inhibitors and IGFBP-3 enhanced the apoptotic effects of IGFBP-3 on NSCLC and HNSCC cells both *in vitro* and *in vivo*. We found that HDAC inhibitors suppressed PKC activity, which induced phophorylation and degradation of IGFBP-3. Although researchers have extensively studied the use of IGFBP-3 and HDAC inhibitors alone as proapoptotic agents, this is the first report to show that treatment with combinations of these agents significantly enhances the antitumor activities of IGFBP-3 in NSCLC and HNSCC cells.

IGFBP-3 regulates cancer cell growth in a variety of experimental systems through dual mechanisms: IGF-dependent extracellular regulation of antiproliferation and survival pathways and IGF-independent intracellular effects on proliferation and apoptosis [39]. Our laboratory has demonstrated that IGFBP-3 has antitumor activity in NSCLC cells *in vitro* and *in vivo* by inducing apoptosis and by inhibiting angiogenic and metastatic activities [9, 40]. Recombinant IGFBP-3 protein (rIGFBP-3) has also shown single-agent and combinatorial antitumor activity (additive or synergistic) with radiation, proapoptotic, and chemotherapeutic agents [41]. In a recent study, Jerome et al [41] showed that rIGFBP-3 potentiates Herceptin activity in Herceptin-resistant breast cancer cells. These findings support the rationale for the use of IGFBP-3 in the treatment of cancer, including lung cancer.

Despite the potential of IGFBP-3 to be used as a therapeutic agent for lung cancer, several NSCLC cell lines showed mild or no sensitivity to rIGFBP-3. We previously demonstrated that the apoptotic activity of IGFBP-3 is synergistically enhanced in NSCLC cells when combined with the farnesyltransferase inhibitor SCH66336, implicating Ras pathway-mediated signaling mechanisms in the development of resistance to IGFBP-3 [17]. On the basis of the effects of HDAC inhibitors on Ras activity, we assessed whether HDAC is involved in the resistance to rIGFBP-3 in NSCLC cells and found that the combined treatment with IGFBP-3 and HDAC inhibitors had greater efficacy than single-agent treatment in inducing apoptosis in NSCLC cells *in vitro* and *in vivo*; specifically, treatment with depsipeptide in combination with rIGFBP-3 completely abolished H1299 tumor xenograft growth in nude mice, whereas treatment with either agent alone had marginal effect on the growth of these xenografts. Treatment with HDAC inhibitors and rIGFBP-3 potently decreased Akt activation in NSCLC cells both *in vitro* and *in vivo*. Therefore, decreased Akt activity has orchestrated effects on the survival of NSCLC and HNSCC cells, resulting in enhanced apoptotic activity in these cells.

We also investigated the potential mechanisms responsible for the synergistic effect of HDAC inhibitors and rIGFBP-3. Investigators have shown that treatment with HDAC inhibitors, including NaB and TSA, increases IGFBP-3 transcription in hepatoma, mammary and colonic epithelial cells [34, 42]. We also observed treatment with HDAC inhibitors, including NaB and TSA, induces upregulation of IGFBP-3 expression in a subset of NSCLC and HNSCC cells. Given the reported effects of HDAC inhibitors depsipeptide and apicidin on Ras-MAPK signaling [25, 27], which induces inactivation of IGFBP-3 via induction of its destabilization [16, 17], we performed additional studies to investigate the potential role of HDAC inhibitors on the stability of IGFBP-3 protein. Indeed, treatment with HDAC inhibitors increased the half-life of IGFBP-3, indicating that increased stability of the proapoptotic IGFBP-3 protein by HDAC inhibitors contribute to the synergistic antiproliferative effect of this combination. A previous report suggested that posttranslational modifications such as glycosylation and phosphorylation can influence IGFBP activity [43]. Phosphorylation is widely accepted to be the most important general mechanism for the regulation of protein stability, enzymatic activity, localization, and interaction with other proteins [44-46]. However, the effects of phophorylation on protein stability of IGFBP-3 are still elusive. We therefore sought to determine the effects of different protein kinase inhibitors on rIGFBP-3 accumulation in H1299 and H226Br cells, which do not have endogenous expression of IGFBP-3 expression. Among the inhibitors of three kinases (PKA, DNA-PK, and PKC), PKCα inhibitor (Ro 31-8220) and PKCα/β inhibitor (Gö6076), but not inhibitors of PKA, PKCβI/βII (hispidin), and DNA-PK, could cause an increase in exogenously added IGFBP-3 protein levels. In concert with these results, stimulation of PKCα by TPA induced phosphorylation of IGFBP-3 and Ro 31-8220 and Gö6076 prevented it. Since recombinant IGFBP-3 protein (Insmed) used in our study was un-natural nonglycosylated form, which binds better to cells than the natural

glycosylated form, we compared the effect of a nonglycosylated form of recombinant IGFBP3 (from Insmed) and a commercially available glycosylated form of recombinant IGFBP3 (from R&D Systems) on the Akt phosphorylation. We observed that both forms of rhIGFBP3 similarly suppressed the phosphorylation of Akt in H1299 cells (supplementary Fig. 2*A*). We further confirmed the PKCα-induced phosphorylation and destabilization of exogenously added IGFBP-3 protein. Similar to the effects on nonglycosylated form of rhIGFBP3 (Fig. 5), treatment with HDAC inhibitors increased stability of glycosylated rhIGFBP3 (supplementary Fig. 2*B*) and suppressed TPA-induced IGFBP-3 phosphorylation on Serine residue (supplementary Fig. 2*C*). It has been previously shown that treatment with the PKCα-specific inhibitor Ro 31-8220 increases apoptosis and IGFBP-3 expression in glioblastoma cells [47]. Increased protein kinase PKCα has been associated with resistance to anticancer drug-induced apoptosis [48]. In our study, treatment with the PKCα-specific inhibitor Ro 31-8220 had enhansed the inhibitory effects of rIGFBP-3 on NSCLC cell viability. These previous and our current findings suggest that IGFBP-3 loses its apoptotic activities at least in part through PKCα-induced phosphorylation and degradation. It has been shown that IGFBP-3 contains consensus phosphorylation sites for a variety of serine/threonine kinases, including PKA, DNA-PK, and casein kinase-2 [38]. Other researchers have implicated serine phosphorylation of IGFBP-3 by DNA-PK as being functionally critical to IGFBP-3's apoptosis-inducing activity [49]. Further, phosphorylation of IGFBP-3 by casein kinase-2 decreases IGFBP-3's the acid labile subunit (ALS)-binding capability. Thus, the degree of phosphorylation of IGFBP-3 protein may be directly related to IGFBP-3 function. The possibility that IGFBP-3 is phosphorylated by more than one kinase at multiple sites is intriguing as it suggests the existence of multiple mechanisms for the regulation of IGFBP-3 function *via* complex interactions between different protein kinases and IGFBP-3. Our observations also indicated that IGFBP-3 degradation is a physiological process that may regulate IGFBP-3 expression and, consequently, IGFBP-3-dependent signaling in cancer cells.

In conclusion, we demonstrate for the first time that HDAC inhibitors have synergy with IGFBP-3 and enhance the apoptotic activity of IGFBP-3 in NSCLC and HNSCC cells. The enhanced apoptotic activity of this combination appears to result from several mechanisms, which are not limited in the context of effects of HDAC inhibitors on chromatin structure [50]. We show that HDAC inhibitors increase the stability of IGFBP-3 by suppressing PKCα activity, resulting in delayed degradation of IGFBP-3, effective inactivation of PI3K/Akt, and enhanced regulation of cell proliferation and survival. Taken together, these findings provide the rationale for combined treatment of NSCLC and HNSCC with IGFBP-3 and HDAC or PKC inhibitors. Further studies are needed to identify the optimal schedule of treatment with these combinations to increase their synergism and efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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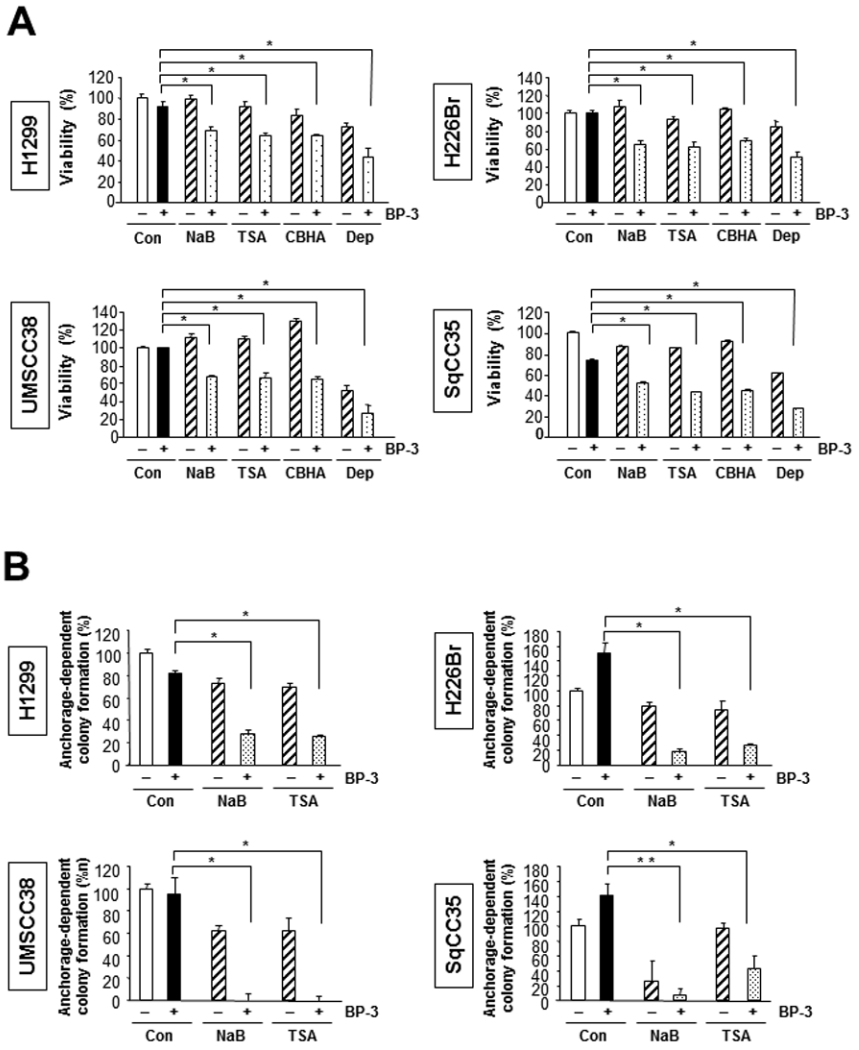
Novelty & Impact Statements

In this study, we found that treatment with HDAC inhibitors reinstated antitumor effects of IGFBP-3 by downregulating PKC-mediated degradation of IGFBP-3 and thus restoring the effects of IGFBP-3 on Akt activation and apoptosis. These results implicate PKC activation, presumably through myriad pathways, in the mechanisms of intrinsic resistance to IGFBP-3. Our findings suggest combined treatment approaches with IGFBP-3 and HDAC inhibitors as effective therapeutic strategies for patients with NSCLC and HNSCC possessing high PKC activity.



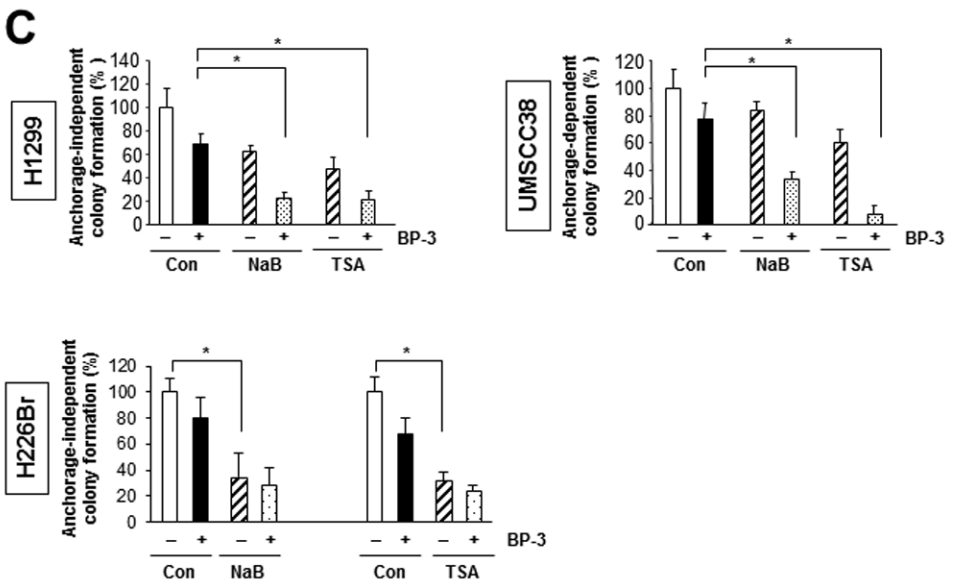
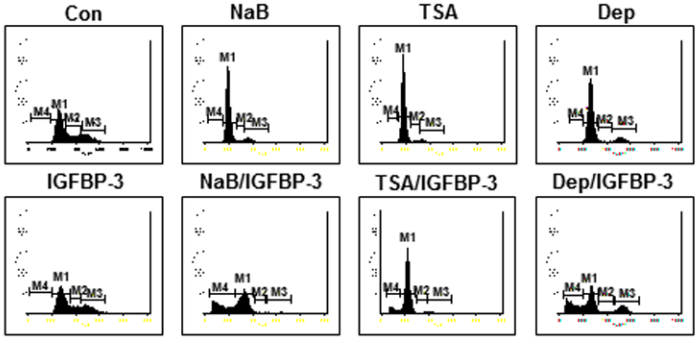


Figure 1. Growth-inhibition effect of combined treatment with rIGFBP-3 and HDAC inhibitors in NSCLC and HNSCC cells

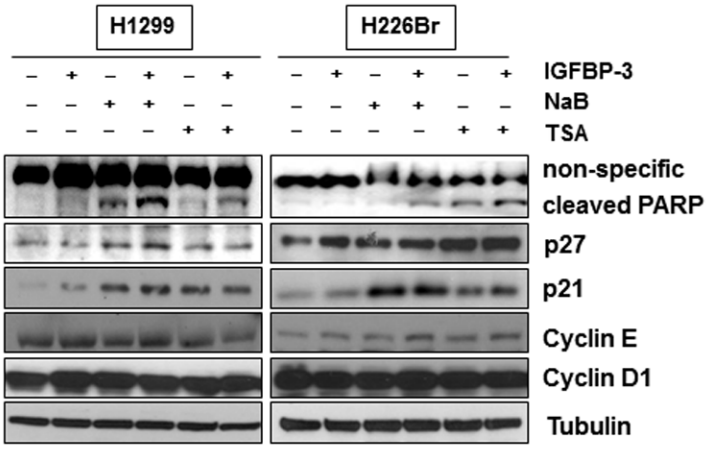
A, H1299, H226Br, UMSCC38, and SqCC35 cells were incubated with NaB (4 mM), TSA (500 nM), CBHA (1 μM), and depsipeptide (Dep, 10 nM) in the presence and absence of rIGFBP-3 (BP3, 1 μg/ml) for 72 hours. Cell viability was measured using the MTT assay. *B and C*, effects of treatment with HDAC inhibitors and rIGFBP-3 on anchorage-dependent growth (*B*) and anchorage-independent growth (*C*) of NSCLC and HNSCC cells. Relative colony formation was measured by dividing each plate density by the "untreated" plate density. For anchorage-independent formation cells were plated in RPMI 1640 medium containing 0.35 % agar on top of a base of 1 % agar in the culture medium containing NaB (4 mM) or TSA (500 nM) with or without rIGFBP-3. The bars represent the mean numbers of colonies in triplicate independent experiments. *$P < 0.05$ (Student *t*-test). Values are means + SD. The experiment is representative of three independent experiments.

A



	Con	NaB	TSA	Dep	IGFBP-3	NaB + IGFBP-3	TSA + IGFBP-3	Dep + IGFBP-3
M1(G1)	60.95	88.73	93.02	86.72	59.30	54.61	81.95	43.26
M2(S)	20.44	8.68	4.13	9.99	20.44	2.20	2.49	17.12
M3(G2/M)	18.02	2.45	2.56	1.91	18.02	3.13	1.91	5.23
M4(Sub-G1)	0.34	0.39	0.25	0.94	0.34	40.20	13.75	33.90

B



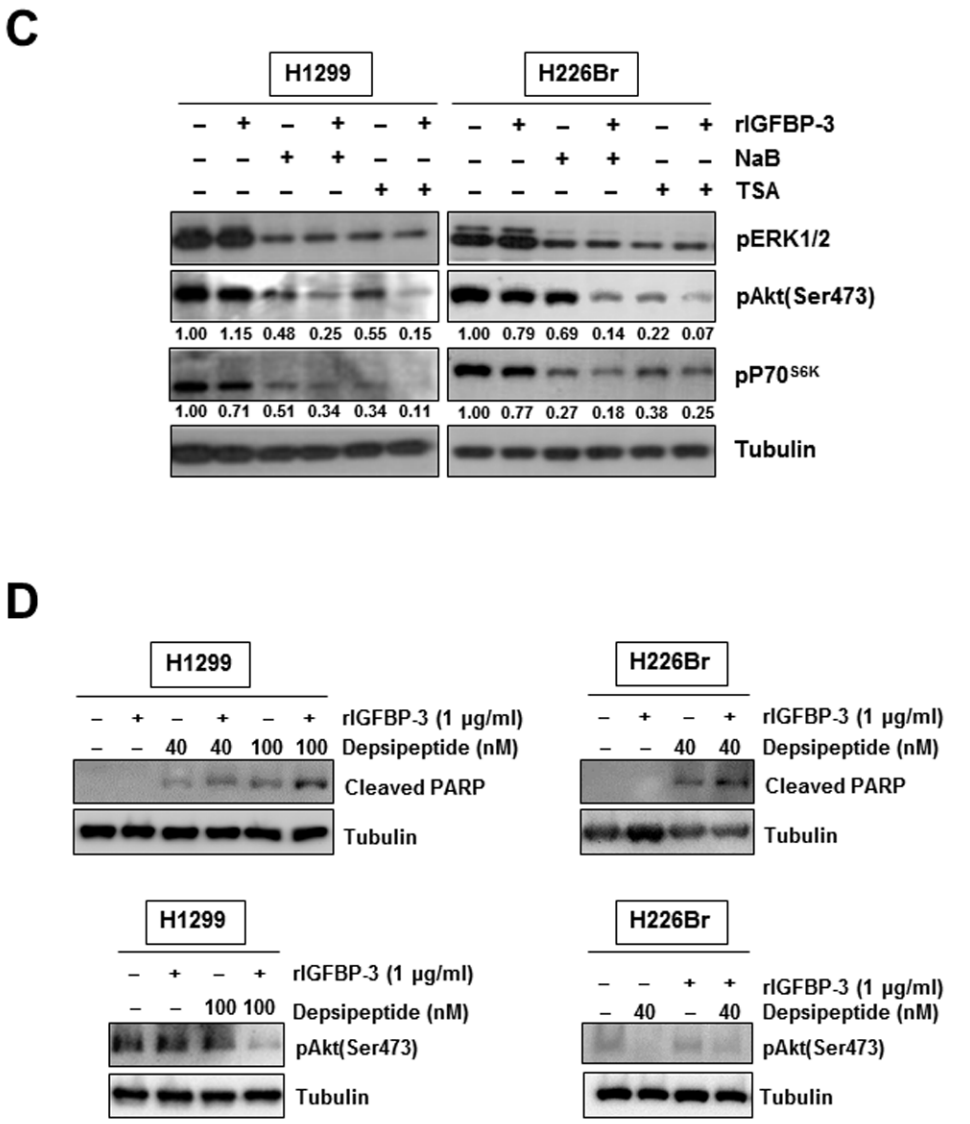
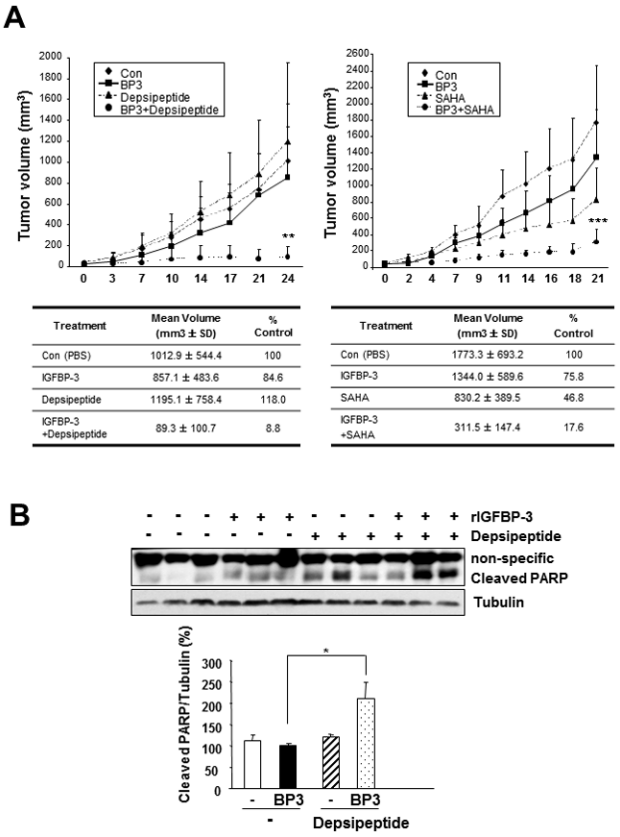


Figure 2. Effect of combined treatment with rIGFBP-3 and HDAC inhibitors on apoptosis of NSCLC cells

A, flow cytometric analyses of cell cycle distribution and apoptosis in H1299 cell. Cells treated with rIGFBP-3 (1 μg/ml) and NaB (4 mM), TSA (500 nM), or depsipeptide (10 nM) alone or in combination for 3 days. DNA content was measured by propidium iodide staining. *B*, Western blot analysis of the expression of cleaved PARP (86 kD), and cell cycle-related proteins (p21, p27, cyclin D, and cyclin E) in H1299 and H226Br cells treated with rIGFBP-3 and NaB (4 mM) and TSA (500 nM) alone or in combination. Tubulin was used as a loading comparison. A representative experiment of two experiments is shown. *C*, Western blot analysis of the expression of pAKT, pERK1/2, and pp70S6K levels in H1299 and H226Br cells treated with rIGFBP-3 and NaB (4 mM) and TSA (100 nM) alone or in combination. The expression of pAkt or pp70S6K calculated as the ratio to tubulin protein expression, which was performed by densitometric analysis. *D*, Western blot analysis of the expression of cleaved PARP and pAkt levels in H1299 and H226Br cells treated with rIGFBP-3 and depsipeptide alone or in combination. Tubulin was used as a loading comparison. A representative experiment of two experiments is shown.



Treatment	Mean Volume (mm3 ± SD)	% Control
Con (PBS)	1012.9 ± 544.4	100
IGFBP-3	857.1 ± 483.6	84.6
Depsipeptide	1195.1 ± 758.4	118.0
IGFBP-3 +Depsipeptide	89.3 ± 100.7	8.8

Treatment	Mean Volume (mm3 ± SD)	% Control
Con (PBS)	1773.3 ± 693.2	100
IGFBP-3	1344.0 ± 589.6	75.8
SAHA	830.2 ± 389.5	46.8
IGFBP-3 +SAHA	311.5 ± 147.4	17.6

Figure 3. HDAC inhibitors enhance the effect of rIGFBP-3 blockage of the growth of NSCLC in nude mice

A, treatment of mice carrying established H1299 tumor xenografts with rIGFBP-3 (3 mg/kg), depsipeptide (left, 1.5 mg/kg), SAHA (right, 50 mg/kg), or their combination was initiated on day 0. Tumors were measured twice a week and results were expressed as the mean tumor volume + SD. *** $P < 0.01$. *B*, induction of apoptosis by the combination of depsipeptide and rIGFBP-3 in H1299 tumor xenografts. The blots are representative of three independent experiments. Densitometric quantitation of PARP band intensity was performed.

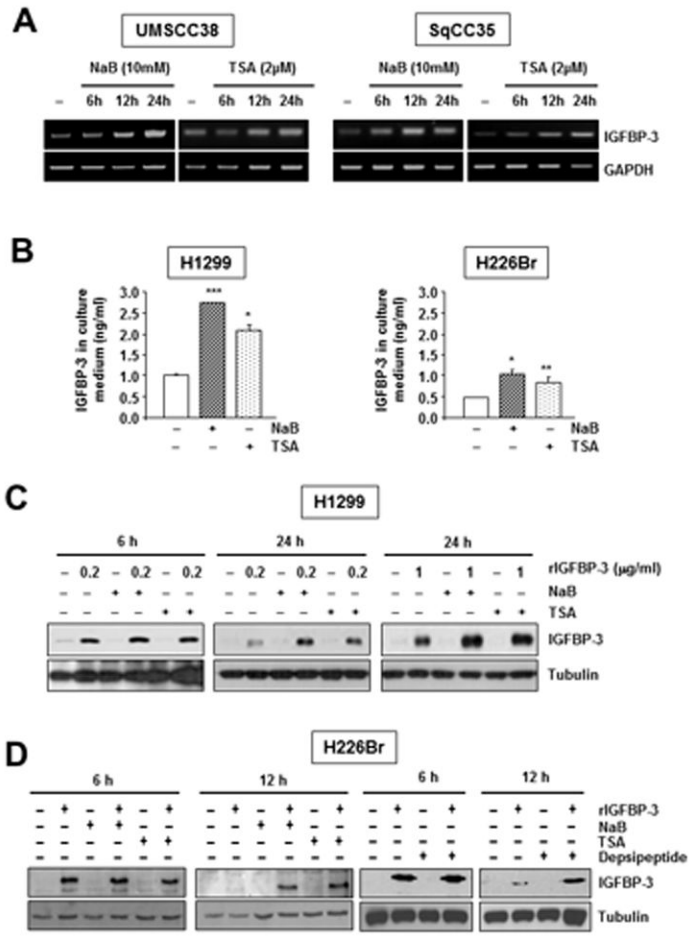


Figure 4. Increased IGFBP-3 protein expression by HDAC inhibitors in NSCLC cells

A, RT-PCR analysis of IGFBP-3 in UMSCC38 and SqCC35 cells after treatment of NaB and TSA for indicated time. *B*, ELISA assay for the IGFBP-3 level in conditioned medium from H1299 or H226Br cells treated with NaB (4 mM), TSA (500 nM) for 24 hours. The amount of IGFBP-3 was expressed as the mean+ SD. $*P < 0.05$; $**P < 0.01$; *** $P < 0.01$ (Student *t*-test). *C and D*, H1299 cells (*C*) and 226Br cells (*D*) were treated with indicated dose of rIGFBP-3 with or without NaB (4 mM), TSA (500 nM), and depsipeptide (10 nM) for 6 to 24 hours. IGFBP-3 expression was compared by Western blot analysis.

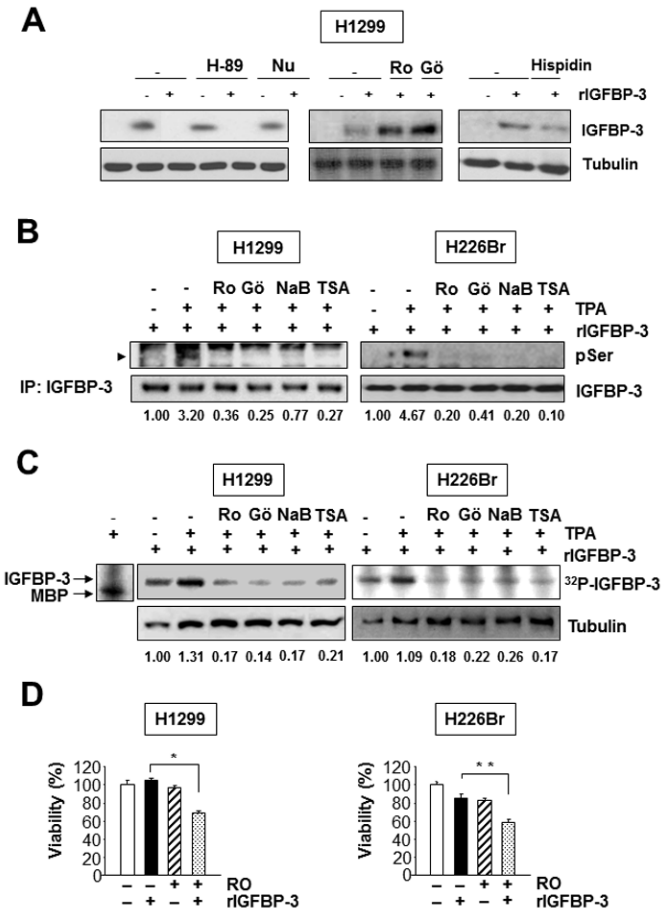


Figure 5. Increased IGFBP-3 protein expression by PKC inhibitor and effect of HDAC inhibitors on the prevention of phosphorylation of IGFBP-3 by PKC

A, H1299 cells were untreated or treated with PKA inhibitor (H-89, 10 μM), DNA-PK inhibitor (Nu7026, *Nu*, 10 μg/ml), PKCα inhibitor (Ro 31-8220, *RO*, 2.5 μM), PKCα/β inhibitor (Gö6976, *Gö*, 1 μM), and PKCβI/βII inhibitor (Hispidin, 2 μM) in combination with rhIGFBP-3 (1 μg/ml) for 1 day. *B and C*, H1299 and H226Br cells were untreated or treated with combination of rhIGFBP-3 (1 μg/ml) and Ro-318220 (2.5 μM), Gö6976 (1 μM), NaB, (4 mM), or TSA (100 nM) for 6 hours. Before harvesting, the cells were stimulated with TPA (10 nM). The phosphorylation status of IGFBP-3 was assessed using immunoprecipitation with anti-IGFBP-3 antibody and immunoblotting for phosphorylated serine (top) and IGFBP-3 (bottom) (*B*). The expression of phosphorylated IGFBP-3 was calculated as the ratio of pSer to IGFBP-3 protein expression, which was performed by densitometric analysis. Protein lysates were used for *in vitro* protein kinase activity using myelin basic protein (MBP) kinase as a substrate (*C*). The expression of IGFBP-3 was calculated as the ratio of ^{32}P-labeled-IGFBP-3 to tubulin protein expression, which was performed by densitometric analysis. *D*, H1299 and H226Br cells were treated with rIGFBP-3 (1 μg/ml), Ro 31-8220 (*RO*, 2.5 μM), or their combination for 3 days. Cell viability was measured using the MTT assay. **P* < 0.05; ***P* < 0.01. Values are means + SD.

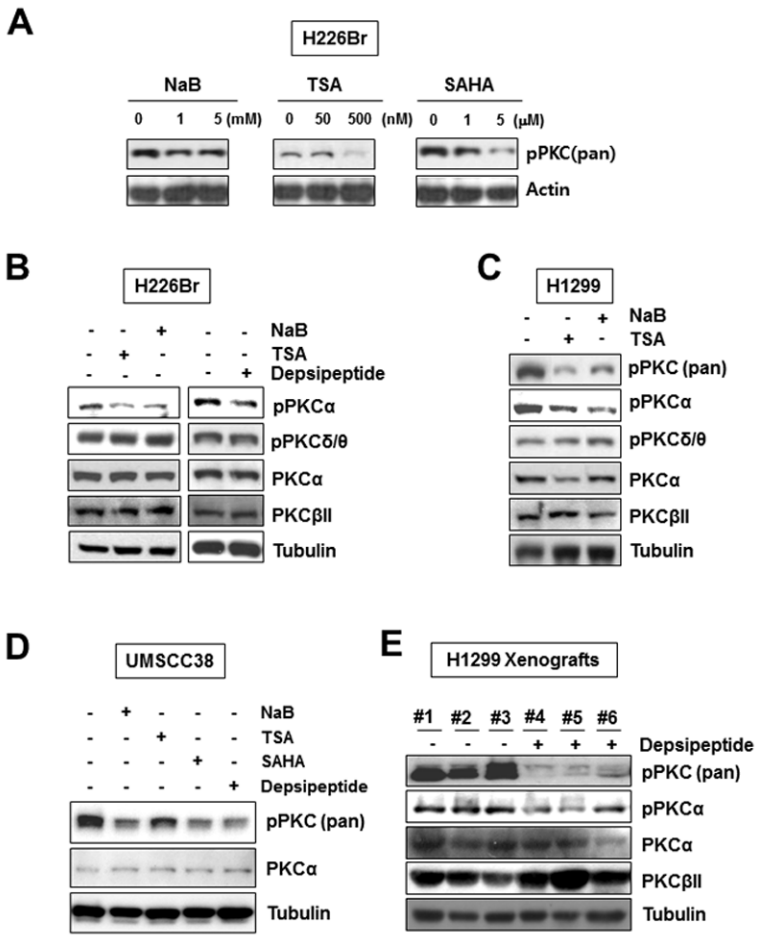


Figure 6. Effect of HDAC inhibitors on PKC activity

A, H226Br cells were treated with indicated dose of HDAC inhibitors (TSA, NaB, and SAHA) for 6 to 12 hours. *B, C*, and *D*, H226Br (*B*), and H1299 (*C*) and UMSCC38 (*D*) were treated with HDAC inhbitors (4 mM NaB, 500 nM TSA, 5 μM SAHA or 100 nM depsipeptide) for 12 hours. Western blot analysis was performed to test the effect of HDAC inhibitors on pPKC expression. *E*, Western blot analysis was performed on the tissues from H1299 xenograft tumors of mice which were treated with depsipeptide (1.5 mg/kg), rIGFBP-3 (3 mg/kg), or their combination for 24 days. Tubulin was included as a loading control.