Transforming Growth Factor β Mimetics: Discovery of 7-[4-(4-Cyanophenyl)phenoxy]-Heptanohydroxamic Acid, a Biaryl Hydroxamate Inhibitor of Histone Deacetylase

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Abstract

Transforming growth factor β (TGF-β) is a multifunctional protein that has been shown to possess potent growth-inhibitory activity. To identify small molecular weight compounds with TGF-β-like activities, high throughput screening was performed using mink lung epithelial cells stably transfected with a TGF-β-responsive plasminogen activator inhibitor 1 promoter/luciferase construct. Biaryl hydroxamate compounds were identified that demonstrated TGF-β-like activities. 7-[4-(4-cyanophenyl)phenoxy]-heptanohydroxamic acid (A-161906) demonstrated complete TGF-β-like agonist activity in the plasminogen activator inhibitor 1/luciferase construct. A-161906 inhibited the proliferation of multiple cell lines in a concentration-dependent manner. Cells were growth arrested at the G_1-S checkpoint similar to TGF-β. Consistent with the G_1-S arrest, A-161906 induced the expression of the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$. A-161906 produced many cellular effects similar to that of TGF-β but did not displace labeled TGF-β from its receptors. Cells with mutations in either of the TGF-β receptors I or II were growth-arrested by A-161906. Therefore, the site of action of A-161906 appears to be distal to the receptors and possibly involved with the signaling events controlled by TGF-β. The TGF-β mimetic effect of A-161906 can be partially, if not entirely, explained by its activity as a histone deacetylase (HDAC) inhibitor. A-161906 demonstrated potent HDAC-inhibitory activity (IC_{50} = 9 nM). A-161906 is a novel small molecular weight compound (< 400 MW) having TGF-β mimetic activity as a result of its potent HDAC-inhibitory activity. These results and those of others demonstrate the importance of HDACs in regulation of the TGF-β signaling pathway(s).

Introduction

TGF-β[3] is a member of a pleuripotent family of growth factors that interact with receptor serine/threonine kinases. The signal transduced by these receptors produces a multitude of effects that are cell type specific. TGF-β produces profound growth inhibition on many types of cells, including normal epithelia, induces a differentiated phenotype, and has the potential to produce an apoptotic response in various cell types (1–3). Along with these profound effects of TGF-β on the growth of cells, it also affects the way cells interact with their environment through modulation of the extracellular matrix (4). These effects include increased synthesis of extracellular matrix components, *e.g.,* fibronectin and regulation of proteolytic activities, *e.g.,* collagenase production and adhesion molecule expression (5). Another component of the TGF-β response is the alteration of cell phenotype by induction of cellular differentiation that results in growth inhibition and terminal differentiation to a mature phenotype (6). These activities suggest that TGF-β is a major component of physiological processes (*e.g.,* wound healing) and that dysregulation of its normal functions are involved in pathophysiological processes such as tumorigenesis (7, 8).

The TGF-β family of growth factors, including TGF-β, activins, and bone morphogenic proteins, signal through unique transmembrane receptors TGF-βRI and TGF-βRII, which are both receptor serine/threonine kinases (9). The TGF-β dimer, the growth factor form of TGF-β, binds to a heterotetramer composed of one homodimer of each TGF-β receptor. The current model of TGF-β signaling involves TGF-β binding initially to TGF-βRII, which in itself is not sufficient to initiate TGF-β signaling. TGFR-βRII recruits, phosphorylates, and activates TGF-βRI, which then recruits specific intracellular signaling molecules that transduce the TGF-β signal. A family of Smads orchestrates TGF-β signaling through the TGF-β receptors (2, 10, 11). When TGF-βRI is phosphorylated by TGF-βRII, Smads 2 and 3 are recruited and in turn are phosphorylated by TGF-βRI. In their phosphorylated state, the Smad 2/3 heterodimer then combines with Smad 4 to enter the nucleus as a transcription factor.

Received 2/13/02; revised 5/29/02; accepted 6/26/02.

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[3] The abbreviations used are: TGF-β, transforming growth factor β; TGF-βRI or RII, transforming growth factor β receptor I or II; HDAC, histone deacetylase; Mv1Lu, mink lung epithelial cell; PAI-1, plasminogen activator inhibitor 1; HTS, high throughput screen; A-161906, 7-[4-(4-cyanophenyl)phenoxy]-heptanohydroxamic acid; IL, interleukin; TBS, Tris-buffered saline; DR26, a cell line deficient in the TGF-βRII; R1B, a cell line deficient in the TGF-βRI; TSA, trichostatin A; SAHA, suberoylanilide hydroxamic acid; TNF-α, tumor necrosis factor α; PMA, phorbol 12-myristate 13-acetate; RAR, retinoic acid receptor.

One consequence of this signaling is that cells are arrested in the G_1 phase of the cell cycle due, in part, to the induction of several specific inhibitors of the cyclin-dependent kinases p15 and p21 (1).

One phenomenon in the process of carcinogenesis is the loss of responsiveness to the normal growth-inhibitory effect of TGF-β. This is particularly observed in colon carcinomas where functional loss of one of the TGF-β receptors has been suggested to be involved in the development of the malignant phenotype and progression of this type of carcinoma (12–15). Dysfunction of TGF-β signaling, as caused by the Smad 4 mutations observed in pancreatic carcinoma, is another mechanism that results in loss of TGF-β growth-inhibitory responsiveness (16). Loss of TGF-β signaling can also be mediated by epigenetic changes rather than mutational effects, resulting in loss of TGF-β receptor expression as observed in the MiaPaCa pancreatic carcinoma cell line (16). In breast cancer cells, epigenetic repression of TGF-β signaling can be relieved by an inhibitor of HDAC, MS-275 (17). Inhibition of HDAC by MS-275 caused the accumulation of acetyl groups on the core histones associated with the TGF-β type II receptor promoter. Induction of TGF-β type II receptor expression and reactivation of the normal TGF-β signaling may be responsible for some of the antiproliferative effects seen with this HDAC inhibitor (17).

Because TGF-β is such a multifunctional protein, the identification of small molecular weight, nonpeptidic compounds that could mimic, enhance, or antagonize the effects of TGF-β may be potentially useful therapeutic agents. Compounds that demonstrated any one of these properties would have therapeutic potential because abnormalities in all of the responses to TGF-β have been associated with many different disease states (18). Through the use of HTS of a PAI-1 promoter/luciferase reporter construct in mink lung epithelial cells (Mv1Lu), we were able to identify a series of biaryl aliphatic hydroxamates with the ability to mimic the activity of TGF-β. These compounds do not interact with the TGF-β binding domain of the TGF-β receptors (*i.e.,* they are not ligands for the TGF-β receptors) but act at a point distal to the TGF-β receptors in the signaling pathway of TGF-β. Subsequently, these compounds were demonstrated to have potent HDAC-inhibitory activity that can potentially explain most, if not all, of the common properties of biaryl hydroxamates as TGF-β mimetics.

Materials and Methods

TGF-β was purchased from R&D Systems (Minneapolis, MN). All cell lines used in these studies were obtained from the American Type Culture Collection (Manassas, VA) with the exception of the Mv1Lu PAI-1/luciferase-transfected cell line. The Mv1Lu PAI-1/luciferase-transfected cell line was constructed as described by Abe *et al.* (19) and used with the permission from that investigator (D. B. Rifkin, New York University Medical Center) in Abbott Laboratories.

HTS with the Mv1Lu PAI-1/Luciferase Assay. Mv1Lu PAI-1/luciferase cells were cultured in DMEM, 10% fetal bovine serum, penicillin/streptomycin, L-glutamine, and 200 μg/ml G418. For experiments, cells were seeded into 96-well plates at 1.6×10^4 cells/well and allowed to adhere for 3 h at 37°C. Compounds (in DMSO) or TGF-β (1 ng/ml) were added, and the cells were incubated for 18 h at 37°C. Cell media were then removed, cells were washed with PBS, and then lysed with 20 μl of lysis buffer [25 mM H_3PO_4/Tris (pH 7.8), 10 mM $MgCl_2$, 1% Triton X-100, 15% glycerol, 1 mM EDTA, and 0.5% β-mercaptoethanol]. D-Luciferin (500 μM) was then added in 50 μl of reaction buffer [20 mM Tricine, 1.5 mM $MgSO_4$ (anhydrous), 0.1 mM EDTA, 33 mM DTT, 0.2 mg/ml acetyl-CoA, 0.5 mM ATP, and 6.0 mM $MgCO_3$] to each well, and the luminescence measured in a 96-well luminometer (10-s reading/well). In the presence of TGF-β, antagonism was then determined as a reduction in the amount of total luminescence and enhancement an increase in total luminescence. Individual compounds were then selected for IC_{50} determinations. Compounds that were shown to enhance the activity of TGF-β were then evaluated in the absence of TGF-β for direct agonist or mimetic activity.

Human Dermal Fibroblast Fibronectin and Collagenase Production. Human dermal fibroblasts were seeded at 50,000 cells/well in a 24-well tissue culture plate and allowed to grow for 7 days. Cells were then stimulated with TGF-β (10 ng/ml) or test compound for 15 h at 37°C. Total cellular fibronectin was extracted from each well with urea extraction buffer [10 mM Tris-HCl (pH 7.4), 10 mM EDTA, 1 mM DTT, 100 mM urea, and protease inhibitors]. Samples were centrifuged at 13,000 × *g* for 10 min, and the supernatants were analyzed for total fibronectin content using the Biomedical Technologies, Inc. (Stouchton, MA) human fibronectin ELISA kit according to the manufacturers instructions.

Cells prepared as above for the fibronectin assay were stimulated with IL-1β (10 ng/ml) for 15 h in serum-free medium containing either TGF-β or test compound (in DMSO). Cell culture medium was then removed and centrifuged at 700 × *g* for 10 min to remove any residual cells or dead cells. The supernatant was then tested for fibroblast collagenase activity using an 5-[(2-aminoethyl)amino]naphalene-1 sulfonic acid (EDANS):4-(4-dimethylaminophenylazo) benzoic acid (DABCYL) labeled peptide substrate. Fibroblast collagenolytic activity was measured in a 96-well fluorometer. Inhibition of collagenolytic activity was an indication of TGF-β-like activity. Active compounds were further evaluated for their ability to directly inhibit fibroblast collagenase by addition of the compound directly to conditioned medium containing fibroblast collagenolytic activity.

Cell Growth Inhibition Assays. Cells were seeded at 5–10 × 10^3 cells/well in a 96-well tissue culture plate for human neonatal dermal fibroblast, A375, and MCF-7 cells and at 4 × 10^4 cells/well in a 24-well tissue culture plate for Mv1Lu, murine keratinocytes, and MDA-468 cells 18 h before addition of compound or vehicle (DMSO) in complete growth medium (10% serum). After 72 h of exposure to compound, the cells were washed with PBS. Cell number was determined by AlamarBlue [modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] or [^{3}H]thymidine labeling. Treated cells were compared with vehicle-treated cells to determine growth inhibition. AlamarBlue was added in serum-free medium or PBS (10% AlamarBlue in 100 μl) and allowed to incubate for 4 h at 37°C. Cell number was measured by determining the fluorescence at 488/520

nm in an fmax fluorescence plate reader (Molecular Devices, San Diego, CA). Alternatively, after 72 h of compound exposure, [^{3}H]thymidine was added at 1 μCi/ml in complete growth medium and allowed to incorporate for 4 h at 37°C. The cells were then fixed with 5% trichloroacetic acid and washed three times with 5% trichloroacetic acid, followed by 70% ethanol. The incorporated [^{3}H]thymidine was solubilized with 0.1 N NaOH, and radioactivity was determined by liquid scintillation spectrometry. Growth inhibition was determined relative to vehicle-treated controls.

Cell Cycle Analysis. Murine keratinocytes were treated with A-161906 (3 μM) or TGF-β (10 ng/ml) for 24 h. Approximately 10^6 cells were incubated with 20 μg of propidium iodide (Sigma)/ml. DNA content was determined by fluorescence cell analysis using a FACS Caliber (Becton Dickinson) and analyzed with CellQuest software.

HDAC Inhibition. Nuclear HDACs were isolated from K562 erythroleukemia cells and partially purified by Mono-Q chromatography as described by Yoshida *et al.* (20). K562 nuclear histones were labeled with [^{3}H]-acetic acid (500 Ci/mmol, New England Nuclear) *in situ* and isolated according to Yoshida *et al.* (20). HDAC activity was determined by incubation of nuclear HDACs, with and without inhibitor (DMSO vehicle), with 10,000 cpm of [^{3}H]-labeled histone for 30 min at 37°C. The reaction was stopped by addition of 4 N HCl containing 0.16 mM acetic acid as carrier. Released [^{3}H]-acetic acid was extracted with 5 volumes of ethyl acetate, and radioactivity was determined in a liquid scintillation spectrometer. IC_{50}s were calculated by log-logit conversion of the concentration response curve.

p21 Induction and Histone Hyperacetylation. T24 bladder carcinoma cells were plated at 1×10^6 cells/100-mm tissue culture dish. After 24 h, the cells were treated with compounds in complete growth medium (McCoy's 5A modified medium with 10% FBS, penicillin and streptomycin, and glutamine), 4 h for histone hyperacetylation, and 24 h for p21 induction. Cells were washed with Dulbecco's phosphate-buffered saline once and harvested by scraping and centrifugation at 700 $\times$ *g* for 10 min. For p21 induction, whole cell lysates were prepared using 1% Tween 20 in TBS (pH 7.4) with protease inhibitors (Roche Biochemicals) and sonication. Samples were cleared by centrifugation at 20,000 $\times$ *g* at 4°C for 10 min. Supernatant protein was determined using the Bio-Rad BCA protein assay with BSA as the standard. Fifty or 100 μg of total cellular protein were loaded per lane for SDS-PAGE.

For histone hyperacetylation, nuclei were prepared by Dounce homogenization (10 strokes) in lysis buffer [10 mM Tris-HCl (pH 6.5), 50 mM $Na_2S_2O_8$, 1% Triton X-100, 10 mM $MgCl_2$, and 8.6% sucrose]. Nuclei were pelleted by centrifugation at 1000 $\times$ *g* at 4°C for 10 min, washed three times with lysis buffer, and once with wash buffer [10 mM Tris-HCl (pH 7.4), 13 mM EDTA(Na_2)]. Nuclei were disrupted by hypotonic lysis (100 μl of deionized water), and histones were extracted with 1 N H_2SO_4. After 1 h of acid extraction, the samples were centrifuged at 20,000 $\times$ *g* for 10 min, and the supernatant was added to 1 ml of ice-cold acetone and placed at −20°C overnight for histone precipitation. Histones were isolated by centrifugation, dried in a Savant speedvac and redissolved in water (50 μl). Histone protein was determined by the BCA protein reagent, and 5 μg of nuclear histone protein were loaded per lane for SDS-PAGE.

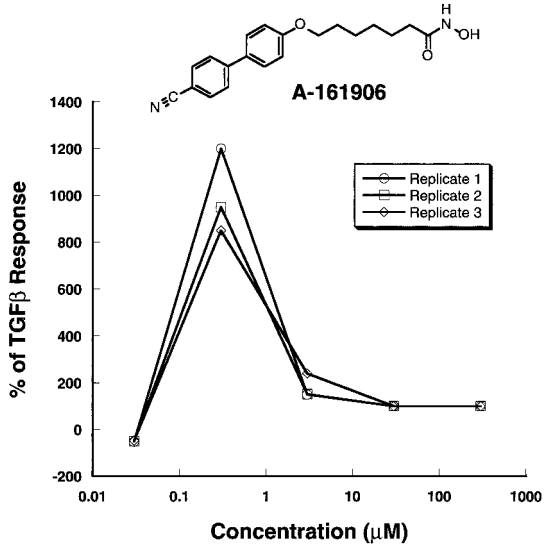


Fig. 1. Induction of PAI-1/luciferase in Mv1Lu cells in response to A-161906. Mv1Lu cells with the PAI-1/luciferase construct were treated with A-161906 for 15 h before determination of luciferase activity in cell lysates. Induction of luciferase activity was normalized relative to TGF-β (1 ng/ml) response in the same experiment. The concentration response curves from three separate experiments are shown. *Inset*, structure of A-161906.

Electrophoresis and Western Blotting. SDS-PAGE was performed using 4–12% Novex NuPAGE gels using the 4-morpholinepropanesulfonic acid buffer system (Invitrogen, San Diego, CA). Proteins were prepared at the appropriate concentrations in sample buffer (4x Novex NuPAGE sample buffer), heated at 70°C for 10 min, and loaded onto gels. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Novex) and blocked for 2 h with 5% nonfat milk in TBS. Primary antibodies were diluted in 5% nonfat milk in TBS [anti-p21 (1 μg/ml) and antihyperacetylated histone H4 (0.5 μg/ml) were both from UBI, Lake Placid, NY] and incubated with membranes overnight at 4°C. The membranes were washed three times with 0.1% Tween 20 in TBS before addition of the secondary horseradish peroxidase-conjugated antibody. Secondary antibodies were diluted in 5% nonfat milk in TBS (1:5000) and incubated for 2 h with shaking. The membranes were then washed 10 times 5 min each in 0.1% Tween 20 in TBS. Proteins were visualized by enhanced chemiluminescence with the Pierce Dura SuperSignal substrate (Pierce, Rockford, IL).

Results

HTS for Modulators of TGF-β. Mv1Lu cells stably transfected with a PAI-1 promoter-luciferase construct were used to screen the Abbott compound library for modulators of the TGF-β response (19). The design of the assay allowed for the discovery of compounds that either enhance or antagonize the effect of exogenous TGF-β. Compounds shown to have TGF-β-enhancing activity without antagonist activity were

additionally evaluated in the absence of TGF-β for their ability to mimic the TGF-β induction of PAI-1/luciferase. A compound library of 192,000 compounds was screened using the PAI-1/luciferase assay. The screen identified 236 compounds that reconfirmed as modulators of the TGF-β response, a 0.12% hit rate. One notable series of compounds identified from our compound library demonstrating TGF-β modulatory activity were the biaryl hydroxamates.

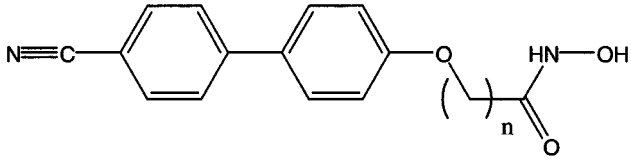
A-161906 (biaryl hydroxamate, *inset* Fig. 1) enhanced the induction of luciferase activity in the presence of TGF-β (1 ng/ml) with an EC_{50} of 0.9 μM and demonstrated no antagonist activity (≥300 μM). Therefore, A-161906 was evaluated for TGF-β agonist activity in the absence of exogenous TGF-β. Fig. 1 shows the response of three evaluations of the intrinsic TGF-β mimetic activity of A-161906 on PAI-1/luciferase induction. The bell-shaped response curve is similar to that produced by a small molecular weight mimetic of granulocyte-colony-stimulating factor (21) and other TGF-β mimetic compounds (22, 23). A-161906 produced 90% of the maximal induction of PAI-1/luciferase by TGF-β at a concentration of 3 μM. Substructure analysis of related biaryl hydroxamates demonstrated that agonist activity was dependent on the number of methylene groups between the hydroxamic acid and the ether link to the biaryl moiety (Table 1). Maximal TGF-β agonist activity was obtained with the five methylene linker, 300% of the TGF-β response at 3 μM. However, this compound demonstrated antagonistic properties in the presence of TGF-β. Therefore, A-161906 was used for additional evaluation of the intrinsic TGF-β activity of this class of compounds. Decreasing the methylene linker to 1–2 carbons resulted in compounds without TGF-β agonist or enhancing activity (Table 1). Replacing the hydroxamic moiety with a carboxylic acid resulted in inactive compounds (data not shown). Compounds with the shorter methylene linkers demonstrated TGF-β antagonist properties in the PAI-1/luciferase assay (Table 1). However, the antagonist activity could not be demonstrated in any secondary cellular assay (data not shown).

Evaluation of the TGF-β Mimetic Activity of A-161906. Table 2 lists the cellular TGF-β-like activities of A-161906. Regulation of extracellular matrix deposition and degradation is a major component of TGF-β activity (4). Like TGF-β, A-161906 increased fibronectin deposition from human dermal fibroblasts in culture by 30% at a concentration of 3 μM. Human dermal fibroblasts produce activated fibroblast collagenase when stimulated with IL-1. A-161906 inhibited the IL-1-stimulated collagenase activity with an IC_{50} of 0.3 μM. A-161906 did not directly inhibit the activity of fibroblast collagenase in a peptide-based assay (data not shown). Therefore, like TGF-β, A-161906 inhibited the induction of fibroblast collagenolytic activity by IL-1 in human dermal fibroblasts. TGF-β decreases the release of IL-8 from human lung carcinoma A549 cells when stimulated with IL-1. A-161906 inhibited the release of IL-8 from A549 cells with an IC_{50} of 0.48 μM. TGF-β is a potent regulator of immune cell function as a consequence of its potent antiproliferative effect on T cells. A-161906 inhibited the mixed lymphocyte reaction of human T cells with an IC_{50} in the 3–15 μM range.

To determine whether the effect of A-161906 was at the level of the TGF-β receptor, its ability to competitively inhibit the binding of radiolabeled TGF-β to Mv1Lu cell membranes was evaluated. At concentrations up to 300 μM, A-161906 did not competitively displace ^{125}I- TGF-β from its receptors on Mv1Lu cell membranes. Therefore, the TGF-β mimetic effects of A-161906 appear to be distal to receptor binding and possibly involved first-hand with the signal transduction mechanism of TGF-β.

Effects of A-161906 on Cell Proliferation. TGF-β is a potent antimitogenic in many different cell types, especially in epithelial cells (24). Table 3 provides a summary of antiproliferative responses with TGF-β and A-161906. In Mv1Lu cells, A-161906 inhibits proliferation as determined by inhi-

Table 1 SAR of chain length for biaryl hydroxamates



		PAI-1/luciferase induction			
		Agonist[a]	Enhancement[b]	Antagonist[b]	HDAC[c]
Compound	*n*	(% of TGF-β @ 3μM)	EC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)
2	2	na[d]	na	0.05	11.0
3	4	48	0.4	56	0.18
4	5	300	1.0	24	0.076
161906	6	90	0.9	>300	0.009
5	7	nd[d]	nd	nd	0.031

[a] Agonist activity of compounds was determined in the absence of exogenous TGF-β.
[b] Enhancement and antagonist activities were determined in the presence of a submaximal concentration of TGF-β (1 ng/ml) to allow both activities to be determined in one test.
[c] HDAC inhibitory activity was determined against K562 nuclear extracts, which contain HDACs 1, 2, and 3 by Western blot analysis as described in ''Materials and Methods.''
[d] na, not active; nd, not determined.

Table 2 TGF-β-like effects of A-161906

TGF-β effect	Response of A-161906	Magnitude
Extracellular matrix deposition[a]	Increases fibroblast fibronectin deposition	30% of TGF-β @ 3 μM
Extracellular matrix degradation[b]	Inhibits IL-1-induced fibroblast collagenase activity	IC_{50} = 0.3 μM
Inhibition of IL-1-induced IL-8 secretion[c]	Inhibits IL-1-induced IL-8 secretion	IC_{50} = 1.5 μM
Inhibition of immune responses[d]	Inhibits MLR (human)	IC_{50} = 3–15 μM
Binds extracellular TGF-β receptors[e]	No effect on ^{125}I-TGF-β binding	No effect
Reversibly inhibits normal cell proliferation[f]	Reversibly inhibits proliferation of Mv1Lu cells	IC_{50} = 0.5 μM
No effect on TGF-βR-deficient cell lines[g]	Inhibits proliferation of both TGF-βRI and II-deficient cell lines	IC_{50} = 1.5 μM

[a] Cells were stimulated with TGF-β (10 ng/ml) or test compound for 15 h. Fibronectin deposition was determined by ELISA as per "Materials and Methods".
[b] Cells were stimulated with IL-1β (10 ng/ml) for 15 h in serum-free medium containing either TGF-β (10 ng/ml) or test compound. Collagenolytic activity was determined as described in "Materials and Methods".
[c] A459 lung carcinoma cells were plated at 5.8×10^5 cells/well in a 96-well tissue culture plate. After overnight incubation, the cells were stimulated with IL-1β (1 ng/ml) in the presence of TGF-β (0.1–30 ng/ml) or test compound in DMSO (final DMSO concentration <0.1%) for 4 h. Cell-free supernatants were isolated by centrifugation, and the medium was analyzed for IL-8 by ELISA (Endogen). The 4-h incubation with IL-1β generated ~10 ng/ml IL-8.
[d] Mixed lymphocyte reaction (MLR) was performed using human lymphocytes isolated from two different donors. Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque-1077 (Sigma). Responder cells (100 μl), untreated PBMCs (1.0×10^6 cells/ml), were mixed with stimulator cells (100 μl), mitomycin C-treated PBMCs (25 μg/ml, 0.5–1.0 $\times 10^6$ cells/ml), in a 96-well tissue culture plate. Cells were allowed to incubate for 96 h, and proliferation was determined using [^{3}H]thymidine labeling.
[e] TGF-β was iodinated [^{125}I] using the Hunter-Bolton reaction. Membranes were prepared from Mv1Lu cells by lysis and centrifugation at 100,000 $\times$ *g* for 1 h. Saturable binding of [^{125}I]-TGF-β was demonstrated using Mv1Lu membranes. Binding of [^{125}I]-TGF-β was determined using Mv1Lu cell membranes with and without test compounds.
[f] Reversibility studies were carried out with Mv1Lu cells as per "Materials and Methods," except that at day 3 another dose of A-161906 was added to one group. All cells were then allowed to grow until day 8. Effects on cell proliferation were determined by manual counting of cells.
[g] Proliferation of both TGF-βR-deficient lines (DR26 and R1B) was determined as stated in "Materials and Methods" for Mv1Lu cell proliferation.

Table 3 Inhibition of cell proliferation by TGF-β and A-161906

Cell line	IC_{50} (nM)	
	TGF-β	A-161906
Mv1Lu[a]	0.028	500
Murine keratinocytes[a]	0.028	900
HDF[a]	0.05	1,500
HCT 116[b]	Nd[c]	1,200
A375[d]	Nd	2,300
MCF 7[a]	0.034	1,800
MDA-MB-468[e]	>0.4[f]	>30,000

[a] Proliferation determined by [^{3}H]thymidine incorporation.
[b] Proliferation determined in soft agar colony-forming assay.
[c] Nd, not determined for this cell line.
[d] Proliferation determined by AlamarBlue assay (modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay).
[e] MDA-MB-468 cells are defective in Smad4 signaling.
[f] >0.4 nM is > 10 ng/ml TGF-β.

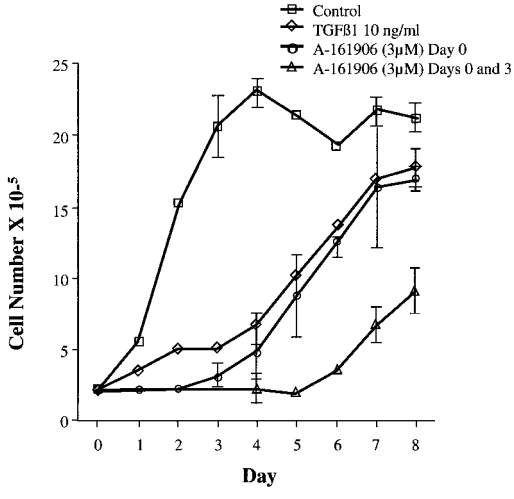


Fig. 2. Reversibility of the effects of A-161906 and TGF-β on Mv1Lu cell proliferation. Mv1Lu cells were plated in 24-well tissue culture plates and allowed to adhere overnight. Cell number was determined by manual cell count on days 1–8. On day 0, TGF-β (10 ng/ml) or A-161906 (3 μM) was added to the cells. On day 3, one set of cells was given an additional dose of A-161906 (3 μM). Proliferation was allowed to continue until day 8. Control cells were untreated.

bition of [^{3}H]thymidine incorporation with an IC_{50} of 0.5 μM. A-161906 also inhibits the proliferation of murine keratinocytes with an IC_{50} of 0.9 μM. A-161906 inhibited the proliferation of several carcinoma cell lines such as HCT-116 (colon), A375 (melanoma), and MCF-7 (breast) with IC_{50}s of 1.2, 2.3, and 1.8 μM, respectively (Table 3). A-161906 demonstrates a broad antiproliferative response in both normal and tumor cells *in vitro* similar to that of TGF-β (Table 3). To demonstrate the relative potency of A-161906 *versus* TGF-β, their effects were evaluated in human neonatal dermal fibroblasts. TGF-β is ~30,000-fold more potent than A-161906 at inhibiting the proliferation of human dermal fibroblasts (IC_{50}s of 50 pM and 1.5 μM, respectively). This difference in potency was also observed in MCF-7 breast carcinoma cells (Table 3).

To determine whether the antiproliferative effect of A-161906 is cytostatic or apoptotic, the reversibility of the antiproliferative effect was evaluated in Mv1Lu cells. As shown in Fig. 2, a single administration of TGF-β (10 ng/ml) caused an ~3-day cytostatic effect, after which the cells resumed normal growth. A-161906 (3 μM) caused a similar 3-day delay in cell growth, after which the cells resumed normal growth. If a second dose of A-161906 (3 μM) was given to the cells on day 3, an additional 3 days of growth arrest occurred, after which the cells resumed normal growth on day 6. These data suggest that A-161906, like TGF-β, causes a reversible cytostatic growth arrest in Mv1Lu cells *in vitro*.

As A-161906 did not displace TGF-β from its receptors on cell membranes, it was of interest to determine whether the growth arrest induced by A-161906 was associated with the

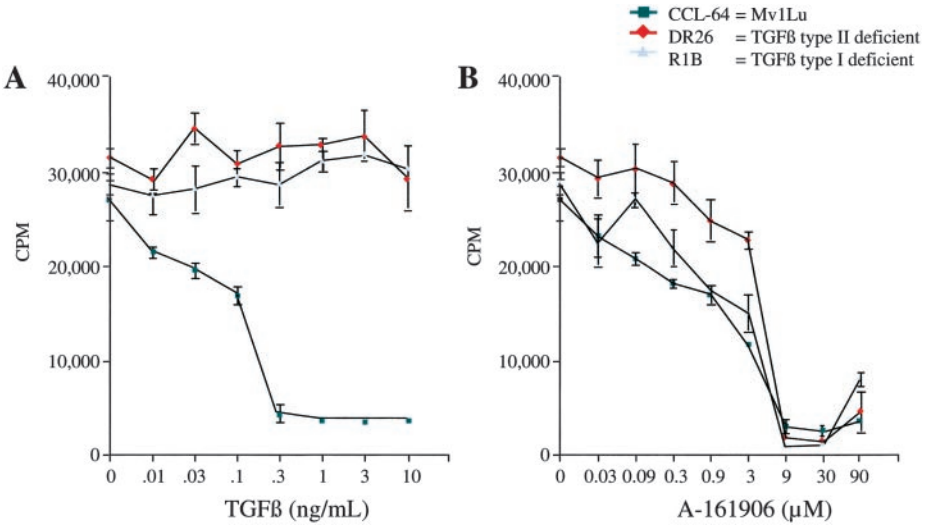
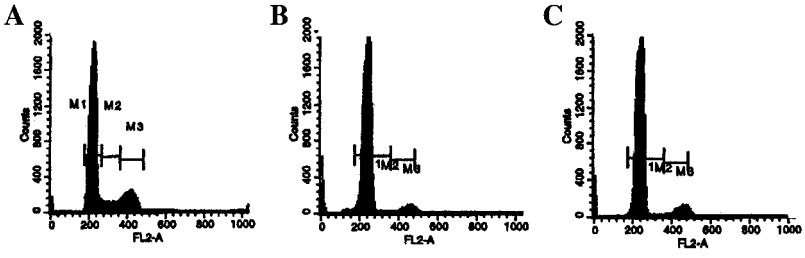


Fig. 3. Effect of A-161906 on the proliferation of TGF-β receptor-deficient cell lines. Cell lines deficient in TGF-βRII (DR26) and RI (R1B) were plated in 24-well tissue culture plates, and proliferation was analyzed as for Mv1Lu cells as described in "Materials and Methods." After 72 h of exposure to TGF-β (*A*) or A-161906 (*B*), cell proliferation was determined by [^{3}H]thymidine labeling as described in "Materials and Methods." Mv1Lu cells were used as a positive control. (n = 3).



Control		TGFβ (10 ng/ml)		A-161906 (3 μM)	
% Total		% Total		% Total	
G1	70	G1	88	G1	88
S	10	S	3	S	2
G2/M	17	G2/M	6	G2/M	8

Fig. 4. Effect of TGF-β and A-161906 on cell cycle in Mv1Lu cells. Mv1Lu cells were treated with TGF-β (*B*; 10 ng/ml) or A-161906 (*C*; 3 μM) for 24 h before cell cycle analysis using propidium iodide staining and fluorescence analysis. Control cells were untreated (*A*). n = 3, a representative histogram is shown.

receptors for TGF-β. A-161906 was evaluated in two cell lines that have inactivating mutations in one of the two TGF-β receptors DR26, which is TGF-βRII deficient, and R1B, which is TGF-βRI deficient. As shown in Fig. 3*A*, these cells were not growth arrested by TGF-β at concentrations up to 10 ng/ml, whereas the sensitive Mv1Lu cells were completely inhibited at this concentration. However, A-161906 was equipotent with IC_{50}s of ~3–5 μM against both TGF-βR-deficient cell lines (Fig. 3*B*). These results demonstrate that the effect of A-161906 is distal to the TGF-β receptors and most likely is associated with the signal transduction mechanism of TGF-β. To evaluate this further, the effects of A-161906 on the cell proliferation of a breast carcinoma that lacks Smad4 expression (MDA-468) demonstrated that these cells were quite resistant to the growth arrest induced by A-161906 (IC_{50} > 30 μM; Table 3).

Effect of A-161906 and TGF-β on Cell Cycle in Mv1Lu Cells. The effects of A-161906 were evaluated on cell cycle progression in Mv1Lu cells and compared with TGF-β. As shown in Fig. 4*A*, Mv1Lu cells in logarithmic growth have 70% of the cells in G_1 phase. Upon treatment with TGF-β (10 ng/ml), there is a prominent G_1 arrest with an increase to 88% of cells in G_1 and a concomitant decrease of cells in S and G_2-M (Fig. 4*B*). Treatment of Mv1Lu cells with A-161906 (3 μM) also resulted in a prominent G_1 arrest with an increase to 88% of cells in G_1 (Fig. 4*C*). At these concentrations, there was no observed increase in the sub-G_1 or apoptotic population of cells after 24-h treatment with either TGF-β or A-161906.

Induction of p21 Expression by A-161906. Because one of the mechanisms that leads to G_1 arrest is the induction of the cyclin-dependent kinase inhibitors, *e.g.,* $p21^{cip1/waf1}$ (25), the ability of A-161906 to induce p21 expression in T24 bladder carcinoma cells was investigated. Treatment of T24 cells with various concentrations of A-161906 led to the up-regulation of p21 expression as determined by Western blot analysis (Fig. 5). Concentrations of ≥5 μM caused the induction of p21 after 24 h of treatment. These results are consistent with the G_1 arrest and TGF-β mimetic activities of A-161906.

Histone Hyperacetylation and Inhibition of HDAC by A-161906. The responsiveness of the TGF-β signaling pathway in cells is known to be regulated by corepressors of

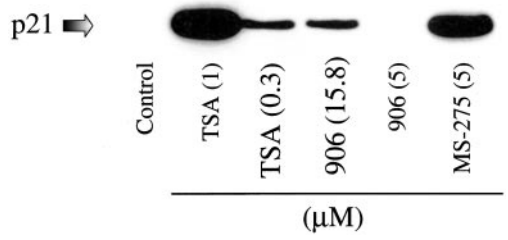


Fig. 5. Induction of p21$^{waf1/cip1}$ expression in T24 bladder carcinoma cells. T24 bladder carcinoma cells were plated at 1.0×10^6 cells/100-mm tissue culture dish and allowed to adhere overnight. The cells were then treated with A-161906 (906) at 5 and 15.8 μM or the reference HDAC inhibitors TSA at 0.3 and 1.0 μM and MS-275 (5 μM) for 24 h. p21 was analyzed by Western blot using 50 μg/lane whole cell lysate and a monoclonal antibody that is specific for p21 (Upstate Biotechnologies). A representative Western blot is shown with at least three other experiments demonstrating a similar pattern.

Fig. 6. Induction of hyperacetylation of histones H3 and H4 by A-161906. T24 bladder carcinoma cells were plated at 1.0×10^6 cells/100-mm tissue culture dish and allowed to adhere overnight. Cells were then treated for 4 h with A-161906 (15.8 μM) or the reference HDAC inhibitor TSA (100 nM). Histones were extracted by the method of Yoshida *et al.* (20) and analyzed by Western blot using specific antibodies for acetylated histone H3 **(*A*)** and H4 **(*B*;** Upstate Biotechnologies) as described in "Materials and Methods." A representative blot is shown with at least three other experiments demonstrating a similar pattern.

TGF-β-driven transcription factors that are associated with HDACs (26). Because of the structural similarity of A-161906 with HDAC inhibitors such as TSA (20) and SAHA (27), A-161906 was evaluated as an inhibitor of HDAC activity.

Inhibition of HDAC activity at the cellular level results in the accumulation of hyperacetylated histones H3 and H4 in the nucleus (20). As shown in Fig. 6, using antibodies specific for the acetylated forms of histones H3 and H4, hyperacetylation of histone H3 and H4 in nuclear extracts could be readily demonstrated in T24 cells 4 h after treatment with A-161906. This effect was also demonstrated for the known HDAC inhibitors TSA (Fig. 6) and SAHA (Refs. 20, 27; data not shown). Hyperacetylation of nuclear histones is consistent with inhibition of HDACs as a mechanism for A-161906. Other hydroxamic acid matrix metalloproteinase inhibitors or cytotoxic agents (*e.g.,* doxorubicin) do not cause the accumulation of hyperacetylated histones H3 or H4 in T24 cells (data not shown), nor does TGF-β (10 or 30 ng/ml) cause the accumulation of hyperacetylated histone H4 (Western blot analysis) in normal human dermal fibroblasts (data not shown); however, this does not preclude the acetylation of specific promoter sites in response to TGF-β.

A-161906 demonstrated potent inhibition of nuclear HDACs isolated from K562 erythroleukemia cells (28) with an IC_{50} of 9 nM (Fig. 7). Related biaryl compounds were evaluated for their ability to inhibit HDAC activity. Similar to the TGF-β mimetic activity observed on the PAI-1/luciferase construct as a function of linker (aliphatic) length in the biaryl hydroxamates, when the methylene linker is reduced from $n = 6$ (A-161906) to 1 or 2, there is a concomitant loss of HDAC inhibitory activity (Table 1). Longer methylene linkers ($n = 7$, Compound 5; Table 1) decrease the HDAC-inhibitory activity of biaryl hydroxamates. Removal of the hydroxamate moiety also results in the loss of HDAC inhibitory activity. Subsequent screening of the compound library with a HDAC preparation (HDAC 1 and 2 from K562 erythroleukemia cells) identified the biaryl hydroxamates but did not identify other

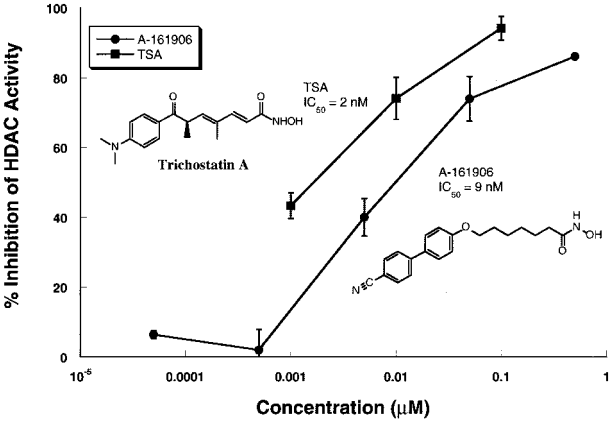


Fig. 7. Concentration response analysis of A-161906 and TSA on HDAC enzymatic activity. HDACs were partially purified from K562 erythroleukemia cells as described by Yoshida *et al.* (20) and in "Materials and Methods." HDAC activity was determined using [^{3}H]-labeled K562 core histones as described in "Materials and Methods." Approximately 10,000 cpm were used in each reaction, and the HDAC was adjusted to give a release of ~2000 cpm over a 30-min incubation. Inhibitors were preincubated with HDAC for 15 min before addition of substrate. IC_{50}s for TSA (2 nM) and A-161906 (9 nM) were determined by log-logit conversion of the concentration response curve. $n = 3$.

compounds that had demonstrated TGF-β modulatory activity as HDAC inhibitors.[4]

Effect of A-161906 on Cell Differentiation and Gelsolin Expression. TGF-β and inhibitors of HDAC cause cells, immature progenitor cells, or carcinoma cells to undergo differentiation both morphologically and at the biochemical level. T24 bladder carcinoma cells undergo a distinct morphological change when exposed to A-161906 (15.8 μM) for 24 h (compare Fig. 8, *B* and *C*). Gelsolin, an actin binding protein, has been shown to have significantly reduced pro-

[4] K. B. Glaser, unpublished data.

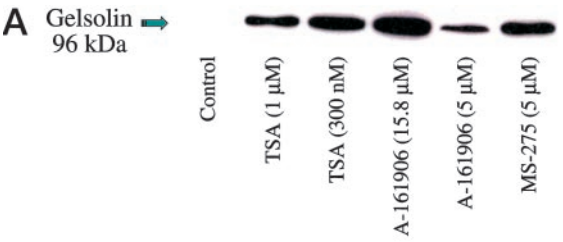
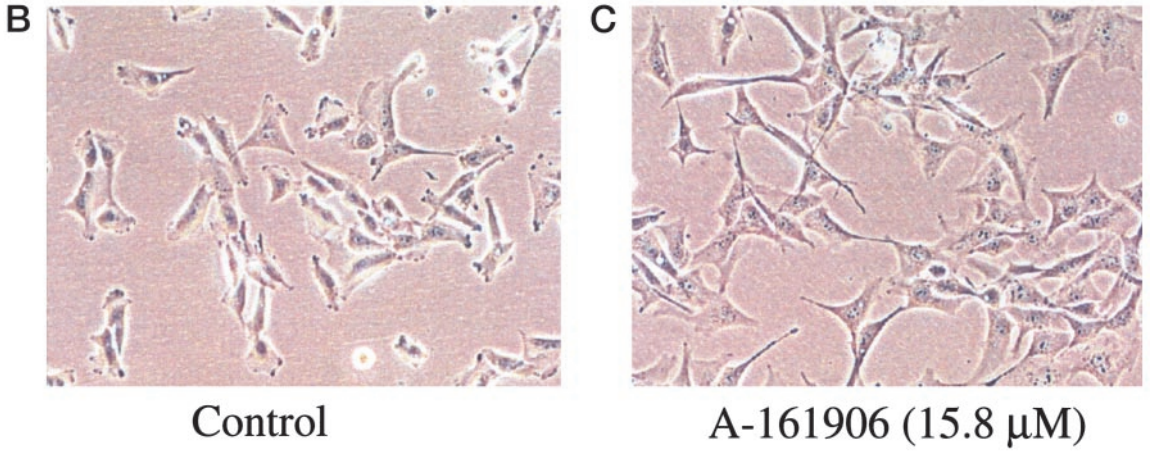


Fig. 8. Effect of A-161906 and reference HDAC inhibitors on gelsolin expression in T24 bladder carcinoma cells. T24 bladder carcinoma cells were plated at 1.0×10^6 cells/100-mm tissue culture dish and allowed to adhere overnight. Cells were treated with A-161906 and the reference HDAC inhibitors TSA and MS-275 for 24 h. Gelsolin expression (*A*) was analyzed by Western blot using 50–100 μg total cell lysate/lane as described for p21 in "Materials and Methods." Cell images were taken after 24-h compound exposure, control cells (*B*) demonstrated ruffled edges characteristic of growing T24 cells, and A-161906-treated cells (*C*) demonstrated a more spindle-like cell shape without ruffled edges.



tein amounts associated with malignant transformation (29, 30). HDAC inhibitors such as TSA have been shown to cause a differentiated phenotype and induce the expression of gelsolin (31). A-161906 induces the expression of gelsolin in T24 bladder carcinoma cells (Fig. 8A) over a 24-h incubation period, which is associated with the morphological differentiation of these cells.

Discussion

In these studies we have identified a novel small molecular weight mimetic of TGF-β, A-161906. A-161906 was originally synthesized as part of a series of metalloproteinase inhibitors at Abbott Laboratories. A-161906 is a micromolar inhibitor of TNF-α release in PMA-stimulated HL-60 cells. PMA stimulates the release of TNF-α concomitant with the differentiation of HL-60 cells. However, in a lipopolysaccharide-stimulated model in a more monocytic-differentiated THP-1 cell line, A-161906 has no effect on TNF-α release. This suggested that A-161906 had other mechanisms aside from the inhibition of TNF-α release, a metalloproteinase-dependent process, that were associated with the differentiation of HL-60 cells by PMA. HTS of a PAI-1/luciferase construct in Mv1Lu cells provided the identification of a novel activity of A-161906, modulation of transcriptional promoter activity in a cellular assay sensitive to TGF-β (32).

In cancer and other diseases where the normal responses to TGF-β have been dysregulated, compounds that modulate or mimic the activities of TGF-β would have substantial therapeutic utility (18). A-161906 was identified as an enhancer of TGF-β function and as a complete (pure) TGF-β mimetic (32). At the cellular level, this molecule possesses many of the characteristics of TGF-β. A-161906 can modulate extracellular matrix deposition and degradation independent of any effect it may have as a metalloproteinase inhibitor. A-161906 can modulate IL-1β driven processes such as IL-1β-induced IL-8 secretion from A549 cells. The ability of A-161906 to inhibit the proliferation of many different carcinoma cell lines is consistent with the activity of TGF-β. As may be expected from these types of small molecule mimetics, their ability to inhibit proliferation is substantially less potent than that of TGF-β itself (30,000-fold in human neonatal dermal fibroblasts). These results are also consistent with previous studies involving other HDAC inhibitors (*e.g.,* TSA and SAHA) that were shown to inhibit the proliferation of a wide variety of cell types (20, 27, 33).

A-161906 acts intracellularly in signal transduction pathways to block cell proliferation as evidenced by the ability of A-161906 to block the proliferation of cells that have deletions in either one of the two TGF-β receptors. This is also consistent with the involvement of HDACs in the regulation of the TGF-β signaling pathway. Recent studies have demonstrated the association of HDAC 1 with Smad 4 (10). The oncogenic forms of Ski and Sno have also been demonstrated to associate with HDACs through interactions with the corepressors N-CoR and mSin3A (26). Sno negatively regulates TGF-β signaling as to prevent the inappropriate antiproliferative activity of TGF-β (34). Upon binding of TGF-β and the subsequent binding of the Smad4 transcription factor to the TGF-β promoter, HDAC(s) dissociates from the Sno complex, and TGF-β signaling is initiated (26). This is analogous to the regulation of the differentiation of leuke-

mia cells by retinoic acids (RA). Under normal conditions, HDAC(s) is associated with the RAR-α and dissociate upon RA binding to allow cell differentiation. However, if the RAR-α is fused to the PML or even more stringently with the PZLF transcription factors, then RA at physiological concentrations cannot displace the HDAC, and differentiation does not occur. In the case of PML, pharmacological doses of RA do allow the dissociation of the HDAC; however, with PZLF:RAR-α, pharmacological doses of RA cannot dissociate the HDAC, and the system remains repressed. It has been demonstrated that in patients with the PZLF:RAR-α, inhibitors of HDAC relieve repression and allow RA-induced cytodifferentiation of leukemic cells (35). Therefore, it is not surprising that HDAC inhibitors are mimics of TGF-β because they may be alleviating the natural negative regulation exerted by the Ski/Sno:HDAC complex. In this regard, studies with A-161906 in highly Smad-dependent processes, *e.g.,* activation of the Smad binding element/CAGA-Lux reporter, suggest that there is no direct effect on Smad dimerization, translocation or binding; however, studies that directly demonstrate these effects have not been carried out.[5] These results suggest that A-161906 does not mimic the TGFβ-induced transcriptional effects directly through Smads.

Several other compounds have also been identified as modulators of TGF-β using cell lines stably expressing TGF-β driven promoters. Using the PAI-1/luciferase construct, the spiruchostatins A and B (36) and diheteropeptin (23, 37) were described as compounds possessing TGF-β-like activity. Diheteropeptin was also described as an inhibitor of HDAC (23) with an IC_{50} of 24 μM against partially purified HDACs. The spiruchostatins A and B are related to a novel series of HDAC inhibitors, FK228 (38). Using a stable construct of the palindromic Smad binding element (6SBE)/luciferase, which regulates the Smad4 tumor suppressor gene of the TGF-β signaling pathway (39), the compound scriptaid was identified as a modulator of the TGF-β signaling and also as an inhibitor of HDAC (22). Promoter constructs sensitive to TGF-β have been a fruitful resource to identify novel inhibitors of HDAC. This possibly indicates the relative importance of HDACs in the regulation of the TGF-β response in cells. The association of HDACs with multiple transcription factors in the TGF-β signaling pathway (10, 26) also demonstrate the importance of HDAC-mediated repression in the regulation of TGF-β signaling.

The structure activity relationship within the biaryl hydroxamates is consistent with the TGF-β mimetic activity of A-161906 being associated with the HDAC-inhibitory activity of these compounds. Similar relationships have been shown in the series of hydroxamic acids, including the clinical compound SAHA (27). As the number of methylenes in the linker between the hydroxamic acid and the aromatic groups is decreased, a corresponding decrease in TGF-β promoter induction and decreased HDAC inhibition is observed. Also, converting the hydroxamic acid to a carboxylic acid abolishes both activities. This phenomenon was also observed converting TSA to trichostatic acid (33). Maintaining the hydroxamic acid moiety is essential and probably relates to the ability of this moiety to chelate or bind zinc because HDACs possess a zinc atom at the catalytic site (40).

Along with the ability of A-161906 to induce the expression of the PAI-1/luciferase promoter, it is able to induce the expression of several other genes involved in cell cycle regulation and differentiation. TGF-β and A-161906 produce a profound G_1 arrest in Mv1Lu cells. A-161906 is able to induce the expression of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ that has been functionally linked to the regulation of the G_1 checkpoint in carcinoma cells (41). A-161906 also induces the expression of the differentiation marker gelsolin. Gelsolin is an actin binding protein and substrate for the caspases involved in apoptosis. In some tumor cell lines, the expression of gelsolin has been silenced presumably to provide a survival advantage (31). Addition of A-161906 or other HDAC inhibitors to these cells induces their terminal differentiation and gelsolin expression (31). In tumor cells, the combined HDAC inhibition and re-expression of p21 and gelsolin predispose these cells to apoptosis because most of these cells have lost the G_1-S checkpoint.

The identification of compounds that affect or mimic the activities of TGF-β is of considerable interest to dissect the activities of TGF-β under pathogenic conditions, most notably cancer (7). As loss of TGF-β responsiveness is a hallmark of some cancers, understanding the mechanisms that regulate TGF-β responses may aid in the development of novel antitumor therapeutics. To this end, we have undertaken the evaluation of compounds that regulate the activity of TGF-β in HTS and discovered a class of biaryl hydroxamates that mimic and/or enhance the activities of TGF-β. Their mechanism of action is most likely the disruption of the normal repressive state of the TGF-β signaling pathway by the inhibition of the HDAC that is associated with TGF-β-responsive elements in the genome. Of these compounds, A-161906 stands out as a complete TGF-β mimetic with potent inhibitory activity against nuclear HDACs. These compounds induce the expression of a stably and transiently integrated transfected gene (*e.g.,* PAI-1/luciferase Mv1Lu cell construct). In carcinoma cells, these compounds are able to induce the expression of proteins that regulate the G_1-S checkpoint $p21^{Waf1/Cip1}$ and the differentiation of cancer cells to a more mature phenotype. These aspects of the activities of A-161906 make this class of compounds a promising lead for the development of novel antitumor therapies.

Acknowledgments

We thank Professor Joan Massague for providing the cell lines (DR26 and R1B). We also thank Lori Pease for cell culture and cell proliferation support, Yung-Wu Chen and Morey Smith for technical assistance with the mixed lymphocyte reaction tests, and M. Pat Plutz for technical assistance in the submission of this manuscript. We also thank Dr. Steve Fesik for critical evaluation of this manuscript.

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