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Selective inhibitors of type I receptor kinase block cellular transforming growth factor-β signaling☆

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Abstract

Transforming growth factor (TGFβ) is a 25-kDa dimeric polypeptide that plays a key role in a variety of physiological processes and disease states. Blocking TGFβ signaling represents a potentially powerful and conceptually novel approach to the treatment of disorders in which the signaling pathway is constitutively activated, such as cancer, chronic inflammation with fibrosis and select immune disorders. In this paper, we describe the biological properties of a novel series of quinazoline-derived inhibitors of the type I transforming growth factor receptor kinase (TβKIs) that bind to the ATP-binding site and keep the kinase in its inactive conformation. These compounds effectively inhibited TGFβ-induced Smad2 phosphorylation in cultured cells in vitro with an IC_{50} between 20 and 300 nM. Moreover, TβKIs were able to broadly block TGFβ-induced reporter gene activation. Finally, TβKIs inhibited TGFβ-mediated growth inhibition of normal murine mammary epithelial cells (NMuMG) and mink lung epithelial cells (Mv1Lu), and TGFβ-induced epithelial-mesenchymal transdifferentiation (EMT) of NMuMG cells. Thus, these chemical TβKIs have the potential to be further developed as anti-cancer and -fibrosis agents. In addition, they represent valuable new tools for dissecting the biochemical mechanisms of TGFβ signal transduction and understanding the role of TGFβ signaling pathways in different physiological and disease processes.

Keywords: Transforming growth factor (TGFβ); Receptor kinase; Smad; Cancer; Fibrosis

1. Introduction

The transforming growth factor (TGFβ) family of polypeptides comprises a group of highly conserved dimeric proteins with a molecular weight of approximately 25 kDa. TGFβ is a multifunctional growth factor that plays a key role in a variety of physiological processes including embryonic development, wound healing, hematopoiesis and immune function, as well as disease states, including cancer, chronic inflammation with fibrosis, and immune disorders [1]. Mammalian cells express three highly homologous isoforms of TGFβ: TGFβ1, TGFβ2, and TGFβ3. TGFβ1 is the most abundant isoform in most cells and tissues. The TGFβ signal is transduced by a pair of transmembrane serine–threonine kinase receptors [2]. Binding of biologically activated, mature TGFβ to type II receptor (TβR-II) homodimers results in recruitment of two type I receptor (TβR-I) molecules into heterotetrameric complexes, which in turn, allows the activation of the TβR-I kinase by TβR-II. In response to receptor activation, two cytosolic proteins, Smad2 and Smad3, become transiently

Abbreviations: TGFβ, transforming growth factor-β; TβR-I, TGFβ type I receptor; TβR-II, TGFβ type II receptor; MAPK, mitogen-activated protein kinase; TβKI, TβR kinase inhibitor; FBS, fetal bovine serum; DMSO, dimethylsulfoxide; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TBS, tris-buffered saline; pSmad2, phosphorylated Smad2; EMT, epithelial-to-mesenchymal transdifferentiation

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associated with and phosphorylated by the TβR-I kinase, allowing them to form heteromeric complexes with a third homologue, Smad4. These complexes are translocated into the nucleus, where they bind to DNA in a sequence-specific manner, which results in the transcriptional regulation of wide range target genes involved in cell differentiation, proliferation, apoptosis, migration, and extracellular matrix production [3]. In addition, recent evidence suggests that Smad-independent activation of the p38/MAPK pathway may play an important role in TGFβ signaling as well [4].

TGFβ has been implicated as a critical cytokine in the development and progression of many epithelial malignancies [5]. In normal epithelial cells, TGFβ acts as a strong inhibitor of cell growth [5,6]. However, in later stages of tumorigenesis, carcinoma cells frequently escape from TGFβ-mediated cell cycle control [5,6]. Moreover, TGFβ is secreted and activated by many carcinoma cells and is thought to promote tumor progression by inducing angiogenesis, facilitating tumor cell invasion and/or metastasis, and inhibiting anti-tumor immunity [5].

Activation of TGFβ stimulates the production of various extracellular matrix proteins and inhibits the degradation of these matrix proteins. Persistent activation of TGFβ appears to play a key role in causing fibrosis associated with chronic inflammation in disease states such as pulmonary fibrosis, chronic glomerulonephritis, and primary biliary cirrhosis [7]. Moreover, TGFβ regulates almost every facet of the immune response, including the growth and differentiation of precursors for multiple hematopoietic lineages, and the proliferation and migration of mature immune cells into sites of injury or response. Thus, TGFβ appears to be a key mediator of chronic inflammation and fibrosis by virtue of its effects on both inflammatory and immune cells as well as fibroblasts [8].

Blocking TGFβ signaling represents a potentially powerful and conceptually novel approach to the treatment of metastatic cancer, fibrogenic conditions and select immune disorders [9–11]. As TβR-I is the key signaling molecule within the TGFβ pathway, and is a transmembrane serine–threonine receptor kinase, it represents an excellent drug target. Using high-throughput screening of chemical libraries against purified kinases in vitro, we have identified a series of novel potent and selective quinazoline-derived inhibitors of the TβR-I kinase.

Using NPC-30345 as the representative of this new class of TβR-I kinase antagonists, Huse et al. [12] solved the crystal structure of the TβKI in complex with the TβR-I kinase. The TβR-I kinase domain displays a canonical protein kinase fold, consisting of an N-terminal lobe (N-lobe) composed of a five-strand beta sheet and a regulatory alpha helix as well as a larger, primarily helical C-terminal lobe (C-lobe). The ATP-binding site lies in the deep cleft between the two lobes and it is this structure that is occupied by NPC-30345. NPC-30345 selectively recognizes TβR-I in a catalytically inactive conformation and appears to stabilize the inhibited form [12].

In the current study, we have begun to characterize the biochemical and biological effects of these compounds in mammalian cells. TβKIs block TβR-I kinase activity in cultured cells, TGFβ-mediated regulation of target genes, and TGFβ's effects on cell growth and epithelial-to-mesenchymal transdifferentiation (EMT). Besides their potential for drug development, these small molecule inhibitors represent invaluable new tools for dissecting the molecular mechanisms of TGFβ signal transduction and the role of TGFβ signaling in physiological and pathological processes.

2. Material and methods

2.1. Cell culture

NMuMG normal murine mammary epithelial cells (obtained from Dr Daniel DiMaio, Yale University) were maintained in high glucose Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) FBS (GIBCO-BRL). Human breast carcinoma cell lines (MDA-MB-435 and MDA-MB-231, derived from malignant pleural effusions; and ZR-75-1 derived from malignant ascites) and Mv1Lu mink lung epithelial cells (obtained from Dr J. Massagué, Memorial-Sloan Kettering Cancer Center) were maintained in RPMI 1640 (GIBCO-BRL) supplemented with 10% (v/v) FBS, epidermal growth factor (10 ng/ml; Upstate Biotechnology, Inc.,), insulin (5 μg/ml; Collaborative Research), transferrin (10 μg/ml; Collaborative Research), and gentamicin (10 μg/ml) or in DMEM/F12 (GIBCO-BRL) supplemented with pyruvate (10 mM; GIBCO-BRL), and non-essential amino acids (1 mM; GIBCO-BRL).

2.2. Reagents

Human recombinant TGFβ1 was obtained from Austral Biologicals and dissolved in 4 mM HCl, with 1 mg/ml bovine serum albumin. TβKIs (US patent no. 6031) were provided by Scios, Inc. (Fremont, CA): NPC-30488 and the inactive control compound, NPC-31181, were dissolved in DMSO (50 mM stock solution); NPC-30345 and SD-093 were dissolved in DMSO (10 mM stock solution); NPC-34016 was dissolved in 0.05% (w/v) acetic acid (4 mM stock solution). All stock solutions were stored at −70 °C and diluted to the working concentration immediately prior to use.

2.3. Expression and purification of soluble intracellular kinase domain of TβRI and other protein kinases

In order to have access to a stable source of purified catalytically active enzyme, the baculovirus system was used to produce large quantities of soluble recombinant proteins in insect cells (Sf9 lepidopterin cells). The soluble

intracellular kinase domain of human TβR-I receptor was cloned by PCR. Briefly, cDNA encoding the complete cytoplasmic domain of TβR-I (residues 151–503) was amplified and inserted into the baculovirus expression vector, pFastBac HT (Life Technologies). The resulting fusion protein contains an N-terminal polyhistidine (6xHis) sequence for rapid purification and a rTEV protease cleavage site. Recombinant baculovirus was produced and used to infect Sf9 lepidopterin cells at a multiplicity of infection of 1. Cells were harvested 3 days post-infection and protein expression was verified by SDS–PAGE. Other human recombinant serine–threonine and tyrosine kinases (protein kinase A (PKA); protein kinase C (PKC); protein kinase D (PKD); cell division cycle 2 (cdc2); epidermal growth factor receptor (EGFR); p38α MAP kinase; p38β MAP kinase; p38γ MAP kinase; Jun-terminal kinase 1 (JNK1); and extracellular signal-regulated protein kinase 2 (ERK2)) were produced in a similar manner.

2.4. Purification of GST–Smad2 and in vitro kinase assay

The cytoplasmic protein, Smad2, contains the characteristic C-terminal Ser-Ser-X-Ser (SSXS) motif in which the two most terminal serine residues become phosphorylated in response to TGFβ or activin signaling. To express GST–Smad2, a 91 kDa fusion protein of GST and almost full-length Smad2, the construct was inserted into the pGEX-4F-1 vector (Pharmacia). The protein was produced in *Escherichia coli* strain B21 and purified using glutathione Sepharose 4B (Pharmacia). Purified protein was checked by SDS–PAGE and Coomassie brilliant blue R-250 staining, as well as by Western blot using a polyclonal goat anti-Smad2 (S-20) antibody (1:100). Co-incubation of purified GST–Smad2 (3.5 μM) with 20 μM cold ATP and purified TβR-I kinase in kinase buffer (50 mM Tris (pH 7.5), 100 mM NaCl, 10 mM $MnCl_2$, 10 mM DTT, 0.05% Triton X-100; total reaction volume 50 μl) for 60 min at 25 °C yielded two radiolabeled bands which represented phosphorylated GST–Smad2 and TβR-I, respectively.

2.5. Detection of Smad expression by Western blotting

For detection of Smad proteins, semi-confluent cell cultures were lysed in situ in buffer composed of 150 mM NaCl, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100 in the presence of protease inhibitors for 30 min at 4 °C. Cell lysates were then subjected to SDS–PAGE electrophoresis and transferred to nitrocellulose membranes. Activated Smad2 (pSmad2) was detected using our own rabbit anti-pSmad2 at 1:1000 dilution [13]. Smad2 was detected using rabbit anti-Smad2 (Zymed Laboratories) at 1:500 dilution. Blots were developed using a 1:2000 dilution of horseradish peroxidase-tagged goat anti-rabbit IgG (Calbiochem) and the bands visualized using DuPont NEN Chemiluminescence Reagent or Amersham ECL reagent using the protocols recommended by the manufacturers. Radiographs were scanned and the digitized images subjected to densitometry using NIH Image or ImageJ software (NIH, Bethesda, MD).

2.6. Effects of TβKIs on TGFβ-regulated genes

In order to determine the effects of TβKIs on TGFβ-regulated gene expression, we carried out transient transfection assays using a number of different reporter gene assays in Mv1Lu mink lung epithelial cells. Three different firefly luciferase reporter gene constructs were used: pSBE4, in which four tandem repeats of a Smad4-specific DNA-binding element (SBE) drive luciferase expression; p3TP-Lux, which contains TGFβ-response elements from the collagenase and PAI-1 gene promoters as well as three tetradecanoyl phorbol acetate-response elements [14]; and p15P751-luc (Dr X.F. Wang, Duke University), which contains the INK4B gene promoter. For transfections, cells were plated at 1.4×10^5 cells per well in six-well cluster dishes and allowed to adhere overnight at 37 °C. Transfections of 1 μg of reporter plasmid DNA were carried out using Lipofectin (GIBCO-BRL) as previously described [13]. Four hours later, cells were treated with TβKIs or vehicle only, followed by the addition of 100 pM TGFβ 15 min later. Forty-eight hours post-transfection, cells were harvested by scraping them with a rubber policeman into reporter lysis buffer (Promega Corporation). To control for variations in transfection efficiency, we used the Dual-Luciferase Reporter Assay System (Promega). This involves co-transfecting a small amount (0.1 μg) of pRL-TK, a plasmid expressing a Renilla luciferase reporter gene under control of a thymidine kinase promoter (Promega). Twenty microliters of cell lysate was mixed with 100 μl of the appropriate luciferase assay reagent and photon emission was measured for 10 s using a Series 20 Barthold Luminometer (Turner Designs).

2.7. Cell proliferation assays

Mv1Lu or NMuMG cells were plated in duplicate wells in 24-well plates at 5×10^5 or 1×10^4 cells/well, respectively, and allowed to adhere overnight. Cells were treated with TGFβ1, TβKI, or the combination. Cell numbers were determined using a Model 0039 Coulter Counter (Beckman Coulter) during mid-exponential growth (72 or 96 h for Mv1Lu and NMuMG cells, respectively).

2.8. Epithelial-to-mesenchymal transdifferentiation

NMuMG cells were plated at 4×10^3 cells/well in eight-well culture slides (Becton-Dickinson Falcon), and allowed to adhere overnight, cells were then treated with TGFβ1, TβKI, or the combination for 24 h incubation, washed with phosphate-buffered saline (PBS) twice and fixed with ice-cold methanol for 5 min (for hematoxylin–eosin staining)

or with 10% Millonigs formalin fixative (Surgipath Medical Industries, Inc.; for E-cadherin and F-actin staining). Morphological changes were assessed by viewing hematoxylin and eosin-stained slides. For assessment of subcellular F-actin fiber distribution, slides were incubated with 0.8 ml acetone/well for 5 min at −20 °C. Following two washes with PBS, slides were incubated with 0.165 μM Alexa-Fluor® 488-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR) with 1% (w/v) BSA in PBS for 20 min at 20 °C in the dark. For E-cadherin immunostaining, air-dried slides were incubated with 5% (v/v) goat serum for 20 min at room temperature, followed by incubation with 2 μg/ml mouse monoclonal anti-E-cadherin antibody (Transduction Laboratories) in 2.5% (v/v) goat serum for 1 h at room temperature. The cells were then washed 3 × 5 min with PBS, followed by incubation with 2 μg/ml fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 45 min in the dark. In both cases, stained slides were then washed 3 × 5 min with PBS, mounted using Permount® and viewed using a Zeiss epifluorescence microscope (model no. 090477; Carl Zeiss) equipped with a MTI CCD camera (model DC 330E, DAGE-MTI Inc.).

3. Results

3.1. Primary screen of Scios, Inc., proprietary chemical compound libraries

Scios, Inc., owns a proprietary, diverse chemical library consisting of 60,000 compounds, which includes a 6000 compound set directed toward another serine–threonine protein kinase, p38α. Given the reported similarities between the ATP-binding pocket of the p38α- and TβR-I and -II kinases [15,16], these compounds were tested against both TGFβ receptor kinases at 15 μM by high-throughput screening. For any compound exhibiting greater than or equal to 50% inhibition of receptor autophosphorylation at the screening concentration, a six-point IC_{50} determination was performed. Using this high-throughput screen, a series of compounds were identified, which inhibited the activity of purified TβR-I kinase at submicromolar concentrations.

3.1.1. Selectivity and potency of TβKIs in vitro

Selectivity of TβR-I inhibitors was examined by testing their effects in in vitro kinase assays against a broad panel of different kinases, which included both serine–threonine and tyrosine kinases (Fig. 1). The early compounds, NPC-30345 and -30488, potently inhibited both the TβR-I and -II kinases, while the later compounds, NPC-34016 and SD-093, are selective inhibitors of the TβR-I kinase. Moreover, as shown in Table 1, the most recently developed selective compound, SD-093, also has the greatest potency against the TβR-I kinase in vitro. Even though these compounds can also inhibit several members of the MAP kinase family and the EGF receptor kinase when used at concentrations of 10–50 μM (Fig. 1), they are much more selective inhibitors of the TβR-I kinase at submicromolar concentrations. Thus, SD-093 inhibits TβR-I approximately 10-fold more strongly than p38α, 25-fold more strongly than EGFR and TβR-II, and more than 50-fold more strongly than p38γ, JNK1, PKA, PKC, PKD, and ERK2 (data not shown).

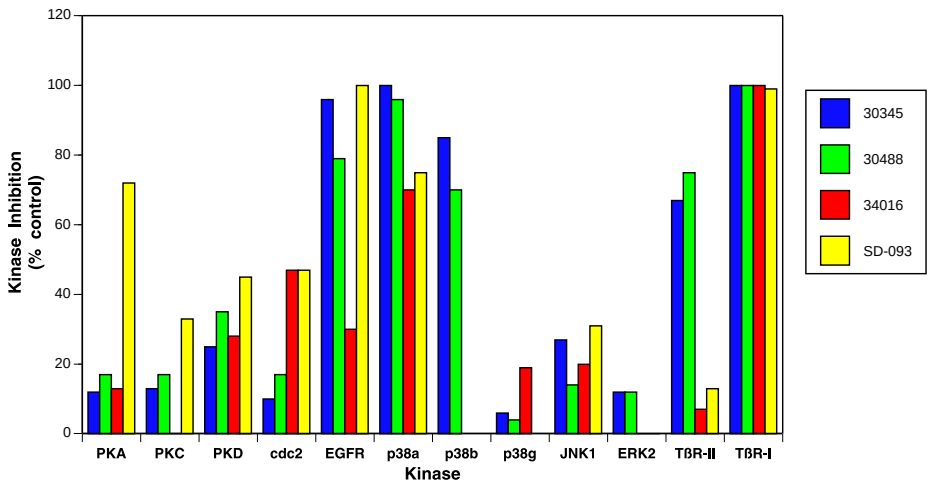

Fig. 1. Selectivity of TβR-I kinase inhibitors. Initial high-throughput screening identified a number of lipophilic small compounds that exhibited significant activity against both the TβR-I and -II kinases. Selectivity was further examined by testing the effects on a broad panel of different additional serine–threonine and tyrosine kinases. These included protein kinase A (PKA); protein kinase C (PKC); protein kinase D (PKD); cell division cycle 2 (cdc2); epidermal growth factor receptor (EGFR); p38α MAP kinase; p38β MAP kinase; p38γ MAP kinase; Jun-terminal kinase 1 (JNK1); and extracellular signal-regulated protein kinase 2 (ERK2). NPC-30345, -30488, and -34016 were tested at 10 μM, while SD-093 was tested at 50 μM. The early compounds, NPC-30345 and -30488, potently inhibited both the TβR-I and -II kinases, while the later compounds, NPC-34016 and SD-093, are selective inhibitors of the TβR-I kinase. At 10–50 μM, this series of compounds can also potently inhibited several members of the MAP kinase family, as well as the EGFR kinase. However, at submicromolar concentrations, SD-093 is particularly selective. Thus, SD-093 inhibits TβR-I approximately 10-fold more strongly than p38α, 25-fold more strongly than EGFR and TβR-II, and more than 50-fold more strongly than p38γ, JNK1, PKA, PKC, PKD, and ERK2 (data not shown).

Table 1
Potency of TRKIs in cell-free and cellular systems

Compound	IC_{50} against TβR-I kinase in vitro (nM)	IC_{50} against TβR-I kinase in cultured cells (nM)
NPC-31181	>10000	>10000
NPC-30345	140	200–250
NPC-30488	470	300–550
NPC-34016	192	625
SD-093	33.2	40

Quinazoline-derived serine–threonine kinase inhibitors were tested against purified TβR-I kinase using in vitro kinase assays, as well as for their ability to inhibit TGFβ-induced phosphorylation of Smad2 in cultured cells (see Fig. 2) as described in Section 2. The relative and absolute potencies of the different compounds as determined by in vitro kinase assay was highly predictive of their efficacy in cultured cells, indicating the excellent cellular uptake and activity of this class of compounds.

3.2. TβKIs inhibit TGFβ-induced Smad2 phosphorylation in whole cells

The TβR-I kinase primarily catalyzes the phosphorylation of Smad2 (pSmad2), which can be detected using a specific rabbit polyclonal antibody that we recently generated [13]. In in vitro kinase assays using purified recombinant constitutively active TβR-I kinase and recombinant GST–Smad2 fusion protein, the pSmad2 antibody detects a band of approximately 83 kDa, the density of which is proportional to the amount of active enzyme (Fig. 2A). Moreover, treatment of cultured human keratinocytes with TGFβ induces pSmad2 (as recognized by our antibody) in a dose- and time-dependent manner [13,17]. Thus, we can utilize the density of the signal detected by the pSmad2 antibody as a semi-quantitative marker of cellular TβR-I kinase activity. As shown in Fig. 2B, pretreatment of MDA-MB-435 breast cancer cells with TβKIs inhibited TGFβ-induced Smad2 phosphorylation in a dose-dependent manner, with IC_{50} values ranging from >300 nM for NPC-30488 to 40 nM for SD-093 (Table 1, Fig. 2B). Similar results were obtained using two other breast cancer cell lines, MDA-MB-231 and ZR-75-1 (data not shown). Thus, TβKIs effectively enter into cells and are capable of inhibiting the target enzyme in vivo. Moreover, the potency of the different TβKIs in vitro was highly predictive of their efficacy in whole cells (Table 1).

3.3. Effects of TβKIs on TGFβ-regulated genes

TGFβ regulates a broad range of target genes [18]. In order to determine whether blocking TβR-I kinase activity by TβKIs inhibited TGFβ-regulated gene expression, we carried out transient transfection assays using a number of different reporter gene assays in Mv1Lu mink lung epithelial cells, which are exquisitely sensitive to TGFβ. As shown in Fig. 3A, NPC-30345, -30488, and SD-093 inhibited TGFβ-induced activation of p3TP-Lux in a dose-dependent manner. The differences in activity between the three

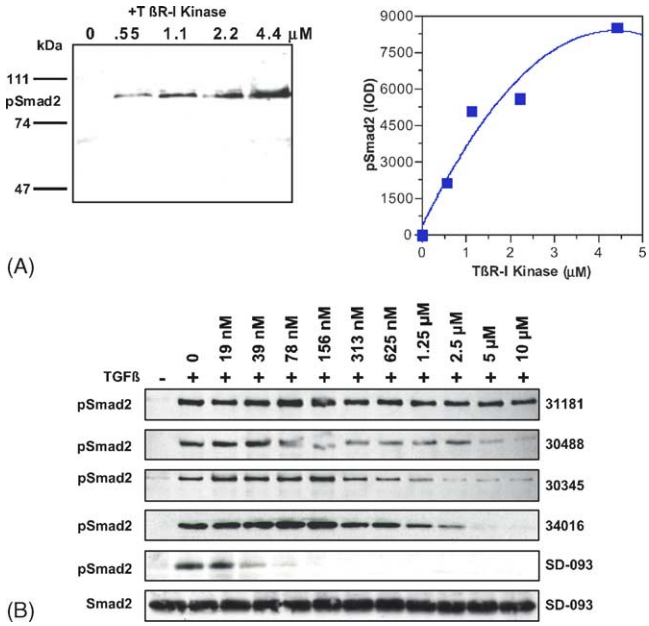

Fig. 2. Effects of TβR-I kinase inhibitors on TGFβ-induced Smad2 phosphorylation in vitro. (A) Purified GST–Smad2 (3.5 µM) was incubated with 20 µM cold ATP and purified TβR-I kinase at the concentrations indicated in kinase buffer (50 mM Tris (pH 7.5), 100 mM NaCl, 10 mM $MnCl_2$, 10 mM DTT, 0.05% Triton X-100; total reaction volume 50 µl) for 60 min at 25 °C. The reaction products were resolved by SDS–PAGE and then subjected to Western blotting using the pSmad2-specific rabbit antibody as described in Section 2. The amount of detectable phosphorylated GST–Smad2 product, as measured by the integrated optical density (IOD) of the pSmad2-specific band, increased as a function of the TβR-I kinase concentration in the reaction. (B) Semi-confluent MDA-MB-435 breast cancer cells were treated with varying concentrations of TβKIs or vehicle only followed15 min later by TGFβ1 (100 pM). Two hours later, cells were lysed and extracts immunoblotted with anti-phospho-Smad2 antibody. The antibody detects a band of approximately 58 kDa, which represents pSmad2, but does not react with unphosphorylated Smad2 in untreated cells. Integrated optical densities of the bands were plotted to calculate an estimated IC_{50} for each TβKI. NPC-31181 was included as inactive control compound.

compounds parallels the difference in potency as inhibitors of Smad2 phosphorylation by the TβR-I kinase (Fig. 2B). As shown in Fig. 3B, inhibition of TβR-I signaling with NPC-30345 blocked the activation of all three reporter gene constructs by TGFβ in a dose-dependent manner. The quantitatively weaker effect on the INK4B promoter construct may be due to partial inhibition of mitogenic kinases, resulting in derepression of the INK4B gene. Thus, TβKIs broadly block the effects of TGFβ on target genes, and this activity is proportional to their potency as inhibitors of TβR-I kinase.

3.4. Effects of TβKIs on TGFβ-mediated growth inhibition

TGFβ exerts two major effects on epithelial cells. It is the most potent known inhibitor of cell cycle progression of non-malignant epithelial cells [19]. Second, it induces EMT, a phenotype characterized by a decrease in cell–cell

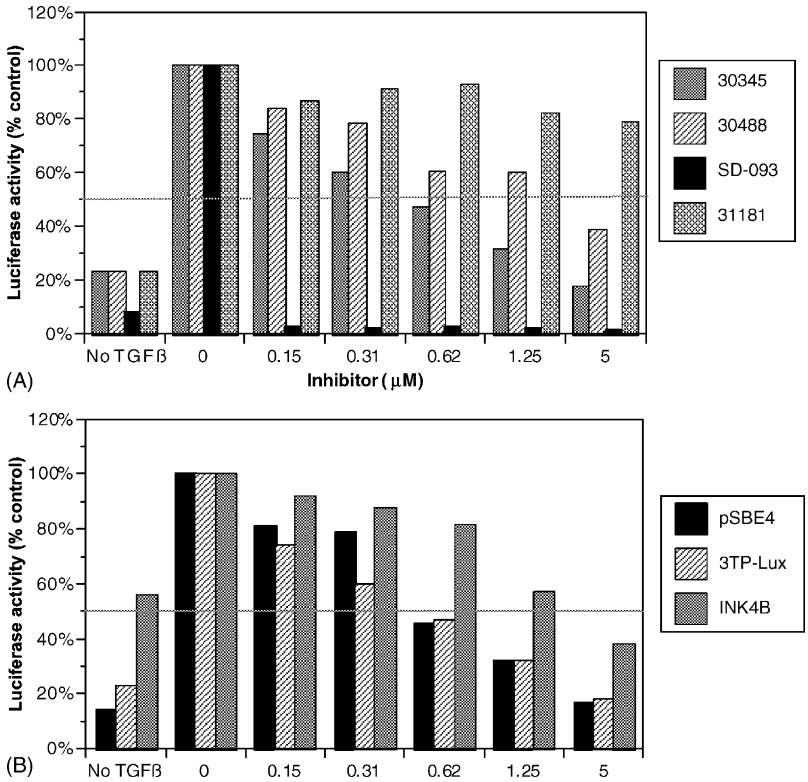

Fig. 3. Effects of TβR-I kinase inhibitors on TGFβ-regulated reporter genes. (A) Mv1Lu mink lung epithelial cells were transiently transfected with 3TP-Lux. Four hours later, cells were treated with TβKI or vehicle only, followed by the addition of TGFβ (100 pM) 15 min later. Luciferase activity in cell extracts was measured 24 h later. Results were normalized for *Renilla* luciferase activity to correct for differences in transfection efficiency between experiments. Both compounds (NPC-30345 and -30488) blocked 3TP-Lux activation by TGFβ with IC_{50} of 0.31–0.62 μM and 1.25–5 μM, respectively, while control compound, NPC-31181 was inactive. (B) NPC-30345 inhibited all three TGFβ-regulated reporter gene constructs pSBE4, p3TP-Lux, and p15P751-luc.

cohesion, the acquisition of a spindle-shaped morphology, and increased motility [20,21]. To determine whether or not the TβKIs were able to block TGFβ-mediated growth inhibition, we treated TGFβ-sensitive Mv1Lu cells and NMuMG cells with TβKIs. At a concentration of 100 pM, TGFβ inhibited Mv1Lu cells growth by 75% (Fig. 4A). Pretreatment with NPC-30345 partly reversed TGFβ-mediated growth inhibition in a dose-dependent manner. Interestingly, NPC-30488 had no detectable effect in this assay, suggesting that its inhibitory effects on mitogenic kinases might override its activity against TβR-I (Fig. 4A). As shown in Fig. 4B, TGFβ inhibits growth of both Mv1Lu and NMuMG cells in a dose-dependent manner, with an IC_{50} of <5 and <50 pM, respectively. In this case, pretreatment of cells with 1 μM SD-093 was able to completely block TGFβ-mediated growth inhibition.

3.5. Effects of TβKIs on TGFβ-induced EMT

As shown in Fig. 5, treatment of NMuMG cells with TGFβ for 24 h induced a spindle-shaped morphology with decreased cell–cell cohesion, conversion of the F-actin cytoskeleton from submembranous fibers to a transcellular stress fibers, and redistribution of membrane-associated E-cadherin to the cytoplasm. This TGFβ-induced EMT could be completely blocked by pretreatment of cells with 1 μM SD-093.

4. Discussion

Excessive production of bioactive TGFβ has been implicated in chronic inflammatory conditions associated with fibrosis, immuno-suppression, successful parasite infection, and cancer metastasis [9–11]. Blocking the effect of TGFβ represents a potentially powerful approach to the treatment of these diverse disorders. Proof of concept has already been provided by studies showing that neutralizing antibodies to TGFβ, the TGFβ-binding proteoglycan, decorin, anti-sense TGFβ oligonucleotides or dominant-negative TβR-II receptor mutants are all capable of inducing tumor regression in mouse models [22–30]. Recently, two groups of investigators showed that in vivo treatment with a soluble TβR-II receptor:Fc fusion protein protects mice against cancer metastasis without adverse side effects [28,29]. Furthermore, administration of decorin, TGFβ neutralizing antiserum, or soluble TβR-II receptor:Fc fusion protein successfully reduced matrix protein accumulation in rats with glomerulonephritis [23,25]. Finally, Smad7, a natural antagonist of TβR-I/Smad signaling, was

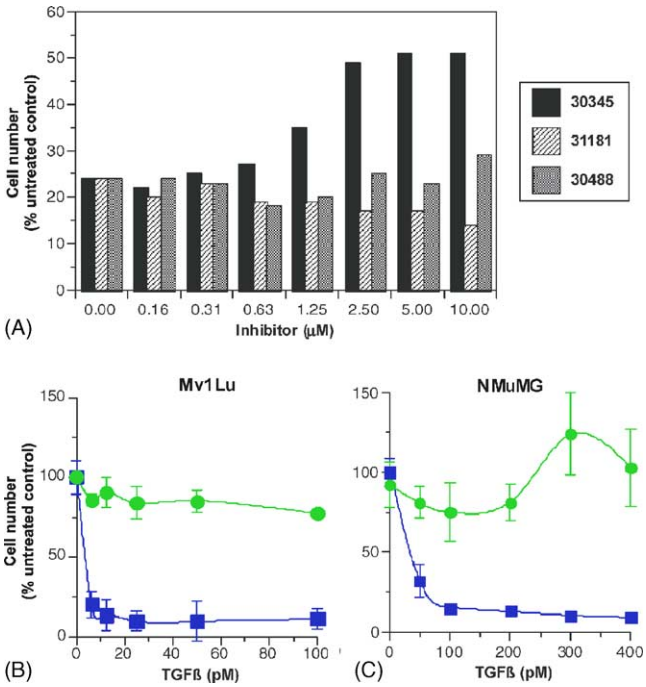

Fig. 4. Effects of TβR-I kinase inhibitors on TGFβ-mediated growth inhibition of epithelial cells. (A) Mv1Lu cells were plated at 2×10^{-4} cells/well in 24-well plates. After 24 h, TβKIs or vehicle only were added at the indicated concentrations, followed by TGFβ1 (100 pM) 15 min later. Cells were detached and counted after 72 h. Cell growth was inhibited by 75% in the absence of compound. Treatment with NPC-30345 partly reversed TGFβ-mediated growth inhibition, while the control compound, NPC-31181, had no effect. Interestingly, NPC-30488 had no detectable effect in this assay. (B) Mv1Lu or NMuMG cells were plated at 1×10^4 cells/well in 24-well plates. After 24 h, SD-093 or vehicle only was added followed by TGFβ1 at indicated concentrations 15 min later. Cells were detached and counted after 72 h. TGFβ1 (■) inhibited cell growth of both cell lines by 90%, with an IC_{50} of <50 pM. Pretreatment of cells with SD-093 (1 μM) (●) completely blocked TGFβ-mediated growth inhibition.

able to prevent bleomycin-induced lung fibrosis in mice when introduced into their bronchi using an adenoviral vector [31,32].

As TβR-I is the key signaling molecule within the TGFβ pathway, and is a transmembrane serine–threonine receptor kinase, it represents an excellent target for the development of novel anti-cancer and anti-fibrotic agents. Recently, a number of low molecular weight compounds that potently and selectively inhibit key tyrosine kinases have been developed into clinically effective anti-cancer agents [33,34]. Most existing protein kinase inhibitors are competitive inhibitors of ATP binding to the enzyme. The design of such inhibitors faces two major challenges: First, because the intracellular [ATP] is between 5 and 10 mM, and the K_m is in the order of 5 μM, competitive inhibitors need to have an extremely high affinity for the ATP binding site. Because the structure of ATP binding sites is highly conserved among protein kinases, the second major challenge is to design truly selective competitive inhibitors of ATP. To identify highly selective small molecular TβR-I antagonists, we used in vitro kinase assays for high-throughput screening of large numbers of compounds against TβR-I and a panel of 12 structurally related kinases. Using this approach, we were able to identify a series of progressively more potent and selective agents. NPC-30488, -30345, -34016, and SD-093 showed progressively greater potency and better selectivity against TβR-I over TβR-II, EGFR, and mitogen-activated protein kinases.

In order to evaluate effects of small molecules on TβR-I kinase activity, we developed an activation state-specific anti-phospho-Smad2 antibody that can be used both in Western blots and immunohistochemistry [35]. Using recombinant constitutively active TβR-I kinase, we found that in vitro phosphorylation of Smad2 can be quantified using the anti-phospho-Smad2 (pSmad2) antibody. TβKI blocked TGFβ-induced phosphorylation of Smad2 in a dose-dependent manner in whole cells, while a compound that was inactive in in vitro assays was also inactive in this cell-based assay. Among the TβKIs, SD-093 showed the highest potency with an IC_{50} of 20–40 nM.

We also validated TβKIs' efficacy in blocking TGFβ signaling on downstream target genes. NPC-30345, -30488, and SD-093 successfully inhibited TGFβ-dependent activation of p3TP-Lux, which contains response elements from the collagenase and PAI-1 gene promoters. Moreover, as exemplified by NPC-30345, this effect extended to other TGFβ-regulated gene promoters, including pSBE4 in which four tandem repeats of a Smad4-specific DNA-binding element (SBE) drive luciferase and p15P751-luc, which contains the INK4B gene. The INK4B promoter was relatively less affected, probably because of a confounding effect of NPC-30345 on mitogenic signaling pathway kinases. This also explains why NPC-30345 only partially reversed the effect of TGFβ on Mv1Lu cell growth, particularly as Mv1Lu are highly dependent on EGF for growth in culture. In contrast, the more selective TβKI, SD-093, completely blocked TGFβ-induced growth inhibition.

TGFβ induces EMT in epithelial and endothelial cells [20,21,36,37]. EMT is a complex process associated with alterations in epithelial cell junctions, changes in cell morphology, reorganization of the cell cytoskeleton, expression of fibroblastic markers, and enhancement of cell migration [20,21,36,37]. A number of studies have provided evidence that TGFβ contributes to tumor cell invasion and metastasis by inducing EMT [38]. The TβKI, SD-093, completely blocked TGFβ-induced epithelial-EMT of NMuMG cells. Whether these agents are capable of blocking TGFβ-driven EMT of carcinoma cells is currently being explored.

Besides the TβKIs described in this study, several series of imidazole-based compounds have been developed that inhibit TβR-I and related serine/threonine protein kinases [39–44]. The prototype compound in one series, SB-203580, is a non-selective inhibitor of TβR-I, which is an order of magnitude less potent in cell-free assays, and two orders of magnitude less potent in cell-based assays

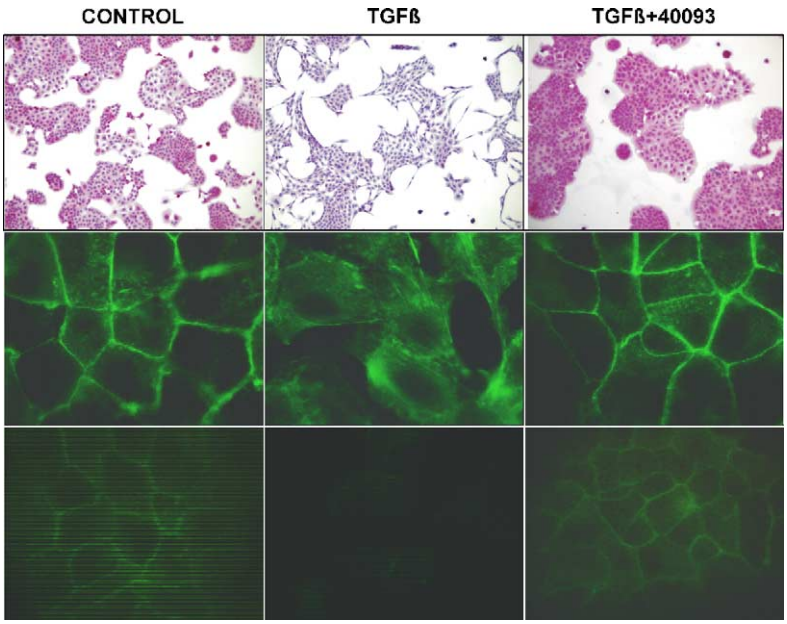

Fig. 5. Effects of TβR-I kinase inhibitors on TGFβ-mediated EMT. NMuMG cells (4000 cells/well) were seeded on 8-well culture slides and allowed to adhere overnight. Cells were then treated with TGFβ (100 pM) in the absence or presence of SD-093 (1 μM), or with vehicle only (control). TGFβ-induced EMT was clearly detectable 24 h later, as evidenced by fibroblastoid morphology (upper row), and redistribution of actin filaments (middle row) and of E-cadherin (bottom row). Pretreatment of cells with SD-093 (1 μM) completely prevented TGFβ-induced EMT.

than our best compound, SD-093 [39]. The derivative compound, SB-202190, is somewhat more potent than SB-203580 but similarly non-selective [39]. More recently, Callahan et al. [40] described SB-431542, a second generation compound with a 100-fold selectivity for the TβR-I, Alk4, and Alk7 kinases as compared to p38α kinase in cell-free assays [41]. SB-431542 completely inhibits TGFβ-induced Smad2 phosphorylation in whole cells at concentrations ranging from 1 to 5 μM [41,42]. Thus, in this respect, SB-431542 seems to be significantly less potent than SD-093. Similarly, SB-431542 blocks TGFβ-induced reporter gene activation with an IC_{50} of approximately 0.5 μM [41,42]. This is similar to our first generation compound, NPC-30345, but inferior to SD-093. Finally, Laping et al. [42] reported that SB-431542 inhibited TGFβ-mediated induction of collagen and fibronectin mRNA as well as protein at low nanomolar concentrations, with complete inhibition at 1 μM. The somewhat surprisingly high potency of the compound in this regard may be due to its cross-reactivity with other kinases besides TβR-I that may mediate these cellular responses. More recently, DaCosta Byfield et al. [44] described a third generation compound, SB-505124, that inhibits TβR-I kinase activity with an IC_{50} of approximately 50 nM, and blocks TGFβ-mediated R-Smad phosphorylation, reporter gene activation and apoptosis at submicromolar concentrations. Thus, the potency of this compound appears to be very similar to that of SD-093. Interestingly, besides TβR-I (Alk5), SB-505124 also effectively inhibits activin- but not BMP-induced R-Smad phosphorylation, reflecting its activity against Alk4 and Alk7 [41,44]. Even though we have not tested the effects of our TβKIs on Alk4 and 7, they are likely to inhibit these activin receptors as well, given their high degree of protein sequence homology with TβR-I (Alk5). Moreover, SD-093 fails to block BMP2-induced Smad1 and 5 phosphorylation, suggesting that it is not capable of inhibiting BMP type I receptors (Alk3 and 6), at least in whole cells (G. Subramanian and M. Reiss, unpublished observations).

In summary, we have demonstrated that quinazoline-based small molecular inhibitors of the TβR-I kinase selectively and effectively block TGFβ signaling in vitro. These TβKIs are readily taken up by cultured cells, block TGFβ-induced phosphorylation of Smad2 and regulation of target genes, and effectively prevent TGFβ-mediated growth inhibition and EMT. These compounds appear to be at least as selective and more potent than the recently described pyridinylimidazole, SB-431542 [41,42]. Thus, these compounds have great potential to be further developed as novel anti-cancer and anti-fibrotic agents. In addition, these compounds represent invaluable new tools for dissecting the mechanisms of TGFβ signal transduction and helping us understand the role of TGFβ signaling pathways in physiological processes and disease states.

References

[1] Roberts AB, Flanders KC, Heine UI, Jakowlew S, Kondaiah P, Kim SJ, et al. Transforming growth factor-beta: multifunctional regulator of differentiation and development. Philos Trans Royal Soc Lon B Biol Sci 1990;327(1239):145–54.
[2] Massagué J. TGFβ signal transduction. Annu Rev Biochem 1998;67:753–91.
[3] Hill CS. The Smads. Int J Biochem Cell Biol 1999;31(11):1249–54.

[4] Yu L, Hebert MC, Zhang YE. TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J 2002;21(14):3749–59.
[5] Reiss M. TGFβ and cancer. Microbes Infect 1999;1(15):1327–47.
[6] Fynan TM, Reiss M. Resistance to inhibition of cell growth by transforming growth factor-β and its role in oncogenesis. Crit Rev Oncog 1993;4:493–540.
[7] Branton MH, Kopp JB. TGF-beta and fibrosis. Microbes Infect 1999;1(15):1349–65.
[8] Sime PJ, O'Reilly KM. Fibrosis of the lung and other tissues: new concepts in pathogenesis and treatment. Clin Immunol 2001;99(3): 308–19.
[9] Chen W, Wahl SM. Manipulation of TGF-beta to control autoimmune and chronic inflammatory diseases. Microbes Infect 1999;1(15): 1367–80.
[10] Omer FM, Kurtzhals JA, Riley EM. Maintaining the immunological balance in parasitic infections: a role for TGF-beta? Parasitol Today 2000;16(1):18–23.
[11] Dumont N, Arteaga CL. Targeting the TGF beta signaling network in human neoplasia. Cancer Cell 2003;3(6):531–6.
[12] Huse M, Muir TW, Xu L, Chen YG, Kuriyan J, Massague J. The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. Mol Cell 2001;8(3):671–82.
[13] Yan W, Vellucci VF, Reiss M. Smad protein expression and activation in transforming growth factor-beta refractory human squamous cell carcinoma cells. Oncol Res 2000;12(3):157–67.
[14] Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, et al. TGF beta signals through a heteromeric protein kinase receptor complex. Cell 1992;71(6):1003–14.
[15] Eyers PA, Craxton M, Morrice N, Cohen P, Goedert M. Conversion of SB 203580-insensitive MAP kinase family members to drug-sensitive forms by a single amino-acid substitution. Chem Biol 1998;5(6): 321–8.
[16] Cohen P, Goedert M. Engineering protein kinases with distinct nucleotide specificities and inhibitor sensitivities by mutation of a single amino acid. Chem Biol 1998;5(7):R161–4.
[17] Xie W, Kim D, Haffty BG, Rimm DL, Reiss M. Frequent alterations of Smad signaling in human head-&-neck squamous cell carcinomas—a tissue microarray analysis. Oncol Res 2003;14:61–73.
[18] Yang YC, Piek E, Zavadil J, Liang D, Xie D, Heyer J, et al. Hierarchical model of gene regulation by transforming growth factor beta. Proc Natl Acad Sci USA 2003;100(18):10269–74.
[19] Reiss M, Dibble CL. Reinitiation of DNA synthesis in quiescent mouse keratinocytes; regulation by polypeptide hormones. In Vitro 1988;24:537–44.
[20] Miettinen PJ, Ebner R, Lopez AR, Derynck R. TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. J Cell Biol 1994;127(6 Pt 2): 2021–36.
[21] Piek E, Moustakas A, Kurisaki A, Heldin CH, ten Dijke P. TGF-(beta) type I receptor/ALK-5 and Smad proteins mediate epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells. J Cell Sci 1999;112(Pt 24):4557–6458.
[22] Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT. Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. J Clin Invest 1993;92:2569–76.
[23] Isaka Y, Brees DK, Ikegaya K, Kaneda Y, Imai E, Noble NA, et al. Gene therapy by skeletal muscle expression of decorin prevents fibrotic disease in rat kidney. Nat Med 1996;2(4):418–23.
[24] Bandyopadhyay A, Zhu Y, Cibull ML, Bao L, Chen C, Sun L. A soluble transforming growth factor beta type III receptor suppresses tumorigenicity and metastasis of human breast cancer MDA-MB-231 cells. Cancer Res 1999;59(19):5041–6.
[25] Isaka Y, Tsujie M, Ando Y, Nakamura H, Kaneda Y, Imai E, et al. Transforming growth factor-beta1 antisense oligodeoxynucleotides block interstitial fibrosis in unilateral ureteral obstruction. Kidney Int 2000;58(5):1885–92.
[26] Bandyopadhyay A, Zhu Y, Malik SN, Kreisberg J, Brattain MG, Sprague EA, et al. Extracellular domain of TGFbeta type III receptor inhibits angiogenesis and tumor growth in human cancer cells. Oncogene 2002;21(22):3541–51.
[27] McEarchern JA, Kobie JJ, Mack V, Wu RS, Meade-Tollin L, Arteaga CL, et al. Invasion and metastasis of a mammary tumor involves TGF-beta signaling. Int J Cancer 2001;91(1):76–82.
[28] Yang YA, Dukhanina O, Tang B, Mamura M, Letterio J, MacGregor J, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. J Clin Invest 2002;109(12):1607–15.
[29] Muraoka RS, Dumont N, Ritter CA, Dugger TC, Brantley DM, Chen J, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. J Clin Invest 2002;109(12):1551–9.
[30] Kobie JJ, Wu RS, Kurt RA, Lou S, Adelman MK, Whitesell LJ, et al. Transforming growth factor beta inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. Cancer Res 2003;63(8):1860–4.
[31] Nakao A, Fujii M, Matsumura R, Kumano K, Saito Y, Miyazono K, et al. Transient gene transfer and expression of Smad7 prevents bleomycin-induced lung fibrosis in mice. J Clin Invest 1999;104(1):5–11.
[32] Nakao A, Miike S, Hatano M, Okumura K, Tokuhisa T, Ra C, et al. Blockade of transforming growth factor beta/Smad signaling in T cells by overexpression of Smad7 enhances antigen-induced airway inflammation and airway reactivity. J Exp Med 2000;192(2):151–8.
[33] Ottmann OG, Druker BJ, Sawyers CL, Goldman JM, Reiffers J, Silver RT, et al. A phase 2 study of imatinib in patients with relapsed or refractory Philadelphia chromosome-positive acute lymphoid leukemias. Blood 2002;100(6):1965–71.
[34] Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, et al. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. N Engl J Med 2002;347(7):472–80.
[35] Xie W, Mertens JC, Reiss DJ, Rimm DL, Camp RL, Haffty BG, et al. Alterations of Smad signaling in human breast carcinoma are associated with poor outcome–a tissue microarray study. Cancer Res 2002; 62:497–505.
[36] Boyer AS, Erickson CP, Runyan RB. Epithelial-mesenchymal transformation in the embryonic heart is mediated through distinct pertussis toxin-sensitive and TGFbeta signal transduction mechanisms. Dev Dyn 1999;214(1):81–91.
[37] Brown CB, Boyer AS, Runyan RB, Barnett JV. Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. Science 1999;283(5410):2080–2.
[38] Wakefield LM, Roberts AB. TGF-beta signaling: positive and negative effects on tumorigenesis. Curr Opin Genet Dev 2002;12(1):22–9.
[39] Yakymovych I, Engstrom U, Grimsby S, Heldin CH, Souchelnytskyi S. Inhibition of transforming growth factor-beta signaling by low molecular weight compounds interfering with ATP- or substrate-binding sites of the TGF beta type I receptor kinase. Biochemistry 2002;41(36):11000–7.
[40] Callahan JF, Burgess JL, Fornwald JA, Gaster LM, Harling JD, Harrington FP, et al. Identification of novel inhibitors of the transforming growth factor beta1 (TGF-beta1) type 1 receptor (ALK5). J Med Chem 2002;45(5):999–1001.
[41] Inman GJ, Nicolas FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, et al. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol 2002; 62(1):65–74.
[42] Laping NJ, Grygielko E, Mathur A, Butter S, Bomberger J, Tweed C, et al. Inhibition of transforming growth factor (TGF)-beta1-induced

extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542. Mol Pharmacol 2002;62(1): 58–64.

[43] Sawyer JS, Anderson BD, Beight DW, Campbell RM, Jones ML, Herron DK, et al. Synthesis and activity of new aryl- and heteroaryl-substituted pyrazole inhibitors of the transforming growth factor-beta type I receptor kinase domain. J Med Chem 2003;46(19):3953–6.

[44] DaCosta Byfield S, Major C, Laping NJ, Roberts AB. SB-505124 is a selective inhibitor of transforming growth factor-{beta} type I receptors ALK4, ALK5, and ALK7. Mol Pharmacol 2004;65(3):744–52.