Inhibition of Transforming Growth Factor-β Signaling by Low Molecular Weight Compounds Interfering with ATP- or Substrate-Binding Sites of the TGFβ Type I Receptor Kinase†

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ABSTRACT: Transforming growth factor-β (TGFβ) is a potent regulator of cell proliferation, differentiation, apoptosis, and migration. TGF-β type I receptor (TβR-I), which has intrinsic serine/threonine kinase activity, is a key component in activation of intracellular TGFβ signaling. We studied two different classes of TβR-I inhibitors, i.e., compounds interfering with the ATP-binding site of the kinase and substrate-mimicking peptides. We found that pyridinylimidazole compounds inhibited TβR-I kinase at micromolar concentration. A representative compound, SB203580, inhibited in vivo Smad2 phosphorylation by TβR-I and affected TGFβ-dependent transcriptional activation. Peptides mimicking the TβR-I phosphorylation sites at the C-terminus of Smad2 also inhibited the autophosphorylation of TβR-I and phosphorylation of Smad2 by TβR-I in vitro and in vivo, whereas a similar peptide from Smad5 was without effect. The substrate-mimicking peptide, fused to penetratin, inhibited a TGFβ1-dependent transcriptional response in a luciferase reporter assay and ligand-dependent growth inhibition of Mv1Lu cells. Thus, the substrate-mimetic peptide is a new type of specific inhibitor of the TGFβ signaling in vivo.

TGFβ[1] is a polypeptide growth factor involved in regulation of cell proliferation, differentiation, apoptosis, and migration (*1*, *2*). Two types of TGFβ-specific serine/threonine kinase receptors, type I and type II, are essential for the TGFβ signaling (*3*). Upon interaction with the ligand, a homodimer of TβR-II recruits two TβR-I molecules and activates their kinases by phosphorylation of serine residues in the GS region of TβR-I (*4*, *5*). In this heterotetrameric complex, activated TβR-I phosphorylates Smad proteins, which are major components of the TGFβ intracellular signaling pathway (*6*, *7*).

TGFβ is involved in pathogenesis of many diseases, including cancer, as well as fibrotic and immunological disorders. TGFβ signaling has a tumor promoting effect at late stages of tumorigenesis, when malignant cells have lost responsiveness to the growth inhibitory action of TGFβ, via action on nonmalignant cells surrounding the tumor cells, such as immune cells, endothelial cells, and connective tissue cells (*8*, *9*). An excessive activation of TGFβ signaling can also cause fibrotic disorders, as TGFβ is a potent stimulator of extracellular matrix formation (*10*). *TGFβ1* null mice die due to a severe wasting syndrome, which is in agreement with the potent immunosuppressor action of TGFβ (*11*). Tools to regulate TGFβ signaling would be warranted to selectively modulate TGFβ-dependent processes in various medical conditions.

TβR-I kinase can be regulated by interaction with other proteins or by phosphorylation (*12*). Phosphorylation of TβR-I in the juxtamembrane GS region by TβR-II is crucial for its activation, whereas TβR-I-interacting proteins have a modulatory effect on TGFβ signaling (*3*−*5*, *12*). Thus, an efficient way to affect TGFβ signaling is by affecting the kinase activity of TβR-I.

Low molecular weight compounds have been successfully used as potent inhibitors of tyrosine kinases as well as serine/threonine kinases (*13*). Most of these inhibitors block the ATP-binding sites of the respective enzymes. Despite significant similarity of the ATP-binding sites in kinases, it has been possible to develop inhibitors with a high degree of selectivity. However, absolute specificities have not been achieved, which complicates their use in treatment of diseases (*13*, *14*). Another way to achieve specificity is to use inhibitors affecting the substrate-binding site. These inhibitors may have high specificities, e.g., inhibitory peptides for protein kinase C (PKC), p60c-src protein tyrosine kinase, cAMP-dependent protein kinase A (PKA), and myosin light chain kinase (*15*−*17*). In this paper, we have explored two approaches to inhibit the TβR-I kinase, i.e., by known serine/threonine kinase inhibitors and by substrate-mimicking peptides.

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[1] Abbreviations: antp, antennapedia peptide penetratin; PKA, cAMP-dependent protein kinase A; PKC, protein kinase C; pS2, specific anti-phosphorylated Smad2 polyclonal antiserum; TGFβ, transforming growth factor-β; TβR-I, TGF-β type I receptor; TβR-II, TGF-β type II receptor.

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MATERIALS AND METHODS

Constructs and Reagents. To express a GST-TβR-I fusion protein, the complete cytoplasmic part (amino acid residues 148−503) of constitutively active TβR-I (T204D) FLAG-tagged at the C-terminus was inserted into the pGEX4T-1 vector (Pharmacia). The protein was produced in *Escherichia coli* strain B21 and purified using glutathione−Sepharose essentially as described (*18*). Purified protein was checked by SDS−PAGE and subsequent Coomassie brilliant blue R-250 staining and by Western blot using anti-FLAG antibodies (M2; Eastman Kodak). GST-Smad3deltaMH1, GST-Smad2, and GST-Smad1 were purified as described elsewhere (*18*). SB203580, SB202190, SB202474, SC68376, PD169316, roscovitine, and H7 were obtained from Calbiochem; PD98059 and staurosporine were obtained from Sigma.

Cells. Mv1Lu and COS-7 cells were obtained from ATCC (LGC, Teddington) and cultured in DMEM with 10% FBS.

Peptide Synthesis. The antennapedia peptide penetratin RQIKIWFQNRRMKWKK (*19*) was used as a control (antp) or was linked with peptides derived from the C-terminus of wild-type Smad2 or from Smad2 and Smad5 in which the phosphorylatable two serine residues were changed to alanine residues: RQIKIWFQNRRMKWKKTQMGSPSVRCSSMS-COOH (antp-Sm2S), RQIKIWFQNRRMKWKKTQMG-SPSVRCSAMA-COOH (antp-Sm2A), and RQIKIWFQN-RRMKWKKTQMGSPLNPISAVA-COOH (antp-Sm5A). Peptides were synthesized using the Fmoc chemistry as described (*20*). Peptides were purified by reverse-phase HPLC using a C_{18} column, and the quality and purity of the peptides were confirmed by MALDI-TOF-MS analysis.

In Vitro Kinase Assay. GST-TβR-I activity was assayed in vitro using the known TβR-I substrates GST-Smad3deltaMH1 and GST-Smad2; GST-Smad1 was used as a control for specificity. The reaction mixture (20 μL) contained 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol (DTT), 5 μM ATP plus 0.5 μCi of [γ-^{32}P]ATP (Redivue), and inhibitors at different concentrations. Reaction was initiated by an addition of 0.02 μg of purified GST-TβR-I, followed by incubation at 22 °C for 20 min. Reaction was terminated by addition of 5 μL of five times concentrated SDS-containing sample buffer. Samples were subjected to SDS−PAGE followed by analysis in a Fuji X2000 phosphorimager. For all in vitro enzyme assays, the percentage of inhibition was calculated as $100(1 - A_i/A_0)$, where A_i and A_0 are levels of GST-Smad phosphorylation in the presence and absence of inhibitors, respectively. The IC_{50} concentration for each compound is defined as a concentration required to inhibit GST-TβR-I activity by 50%. Phosphorylated GST-Smad3deltaMH1 and autophosphorylated GST-TβR-I were analyzed by tryptic phosphopeptide mapping as described previously (*5*).

For in vitro kinase assay of type I and type II TGFβ family receptors, p38α and SAPKα2 kinases, COS-7 cells were transfected with the kinase expression vectors or the control empty pcDNA3 vector using the DEAE-dextran method (*25*). Proteins were extracted in a lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1% NP-40, 10 μg/mL aprotinin, 0.1 mM PMSF). HA-tagged proteins (ALK1 to ALK7, p38α, SAPKα2) were immunoprecipitated with anti-HA antibody (12CA5; Roche); His_6-tagged proteins (type II receptors) were precipitated with Ni-NTA agarose (Qiagen). The precipitates were washed in extraction buffer, with 20 mM imidazole for Ni-NTA agarose-precipitated proteins, and two times with kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT). The kinase reaction was initiated by addition of 20 μL of kinase buffer containing 0.5 μCi of [γ-^{32}P]ATP, 5.0 μM ATP, and inhibitory peptides at a final concentration of 10 μM. The reaction was performed at 22 °C for 20 min and terminated by addition of SDS-containing sample buffer and boiling for 5 min. The reaction products were analyzed by SDS−PAGE and exposure in a Fuji X2000 phosphorimager. Equal loading was controlled by immunoblotting aliquots of the samples with a HA probe (Y-11; Santa Cruz) or anti-His antibody (Qiagen).

Growth Inhibition Assay. Growth inhibition assay with Mv1Lu cells, using evaluation of [^{3}H]thymidine incorporation, was performed as described earlier (*5*, *20*). Briefly, cells were preincubated with peptides for 30 min and then treated or not with TGFβ1 (0.1 ng/mL) for 24 h. During the last 2 h of incubation, [^{3}H]thymidine was added. Radioactivity incorporated into DNA was measured in a scintillation counter. No cytotoxic effects of peptides were observed after 24 h incubation with cells, as evaluated by observation of cells in a microscope.

Luciferase Assays. Luciferase assays with CAGA(12)-luc and ARE-luc reporters were performed as described earlier (*21*, *22*).

Activation of Smad2. The effect of inhibitors on the TβR-I-mediated phosphorylation of substrates in vivo was determined by immunoblot analysis using specific anti-phosphorylated Smad2 polyclonal antiserum (pS2) (*23*). Mv1Lu cells were stimulated with TGFβ1 in the absence or presence of inhibitors or antennapedia penetratin-fused peptides. Phosphorylation of Smad2 was visualized by immunoblotting of the whole cell extract with pS2 antiserum.

RESULTS

Inhibition of TGFβ Signaling by Compounds Interfering with the ATP-Binding Pocket of the TβR-I Kinase. To construct an assay for inhibition of the TβR-I kinase, we produced in bacteria the intracellular part of the TβR-I receptor (amino acid residues 148−503) fused to GST through its N-terminus (GST-TβR-I). In GST-TβR-I, Thr204 was replaced by an aspartic acid residue, which leads to a constitutive activation of the TβR-I kinase (*24*). Purified GST-TβR-I kinase efficiently phosphorylated itself, as well as the C-terminal part of Smad3 and full-length GST-Smad2 as substrates. Consistent with the kinase specificity of TβR-I, no phosphorylation of GST-Smad1 was observed (Figure 1A). Two-dimensional phosphopeptide maps showed similar patterns of autophosphorylation of GST-TβR-I and wild-type full-length TβR-I (Figure 1B) (*5*). Moreover, the phosphopeptide map of GST-Smad3 phosphorylated by GST-TβR-I contained the C-terminal phosphopeptides, which are the sites of wild-type Smad3 activating phosphorylation in vivo by TβR-I (Figure 1C) (*25*). Thus, the produced GST-TβR-I preserved the specificity in phosphorylation of Smad substrates.

To assess the efficiency of inhibition of TGFβ signaling by compounds interfering with ATP binding, we studied the

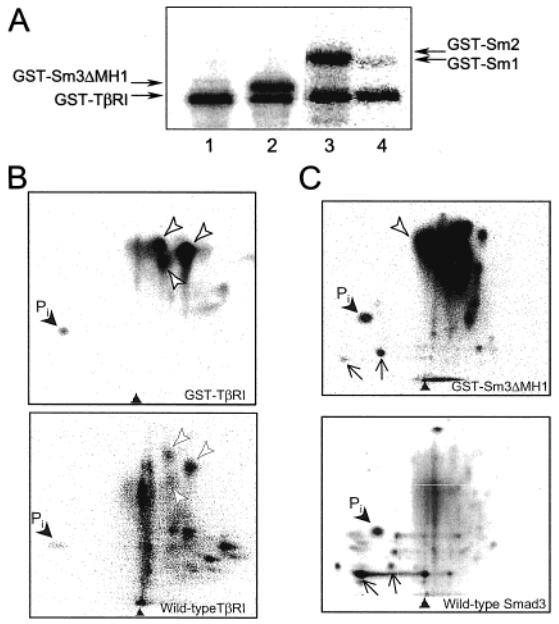


FIGURE 1: GST-TβR-I kinase preserves specificity for substrate phosphorylation. (A) Purified GST-TβR-I was subjected to the in vitro kinase assay alone (1) or with purified GST-Smad3deltaMH1 (2), GST-Smad2 (3), or GST-Smad1 (4). Samples were resolved by SDS−PAGE, and labeled proteins were visualized by using a Fuji X2000 phosphorimager. Migration positions of GST-TβR-I, GST-Smad3deltaMH1, GST-Smad2, and GST-Smad1 are shown. A representative experiment out of three performed is shown. (B) Autophosphorylated GST-TβR-I, shown in lane 1 of panel A, and wild-type TβR-I activated by addition of TGFβ1 were subjected to tryptic digestion and two-dimensional phosphopeptide mapping. Arrowheads indicate the position of phosphopeptides observed in maps of GST-TβR-I (upper panel) and wild-type TβR-I (lower panel) (*5*). (C) GST-Smad3deltaMH1, shown in lane 2 of panel A, and wild-type Smad3 activated by treatment of Mv1Lu cells with TGFβ1 were subjected to two-dimensional phosphopeptide mapping. Arrows show migration positions of the C-terminal peptide with single or double (left spot) phosphorylation on maps of GST-Smad3 (upper panel) and wild-type Smad3 (lower panel) (*25*). The arrowhead shows the migration of the linker-derived peptide with multiple phosphorylations. Sample application points in panels B and C are shown by triangles. P_i indicates the migration position of inorganic phosphate.

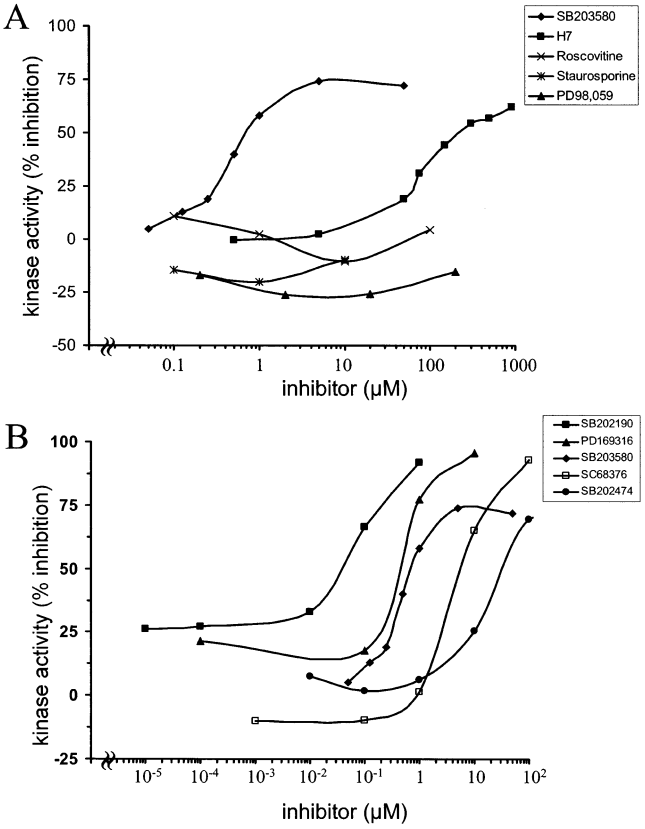


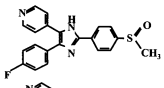
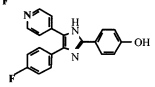
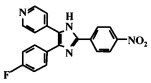
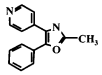
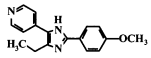
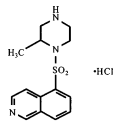
FIGURE 2: Inhibition of GST-TβR-I kinase by inhibitors interfering with ATP binding. Kinase activity of GST-TβR-I was evaluated by phosphorylation of the GST-Smad3deltaMH1 in the in vitro kinase assay. The percent of inhibition was calculated as $100(1 - A_i/A_0)$, where A_i and A_0 are the levels of phosphorylation in the presence or absence of the inhibitor, respectively. Kinase inhibitors representing different structural groups (A) and inhibitors of the pyridinylimidazole group (B) were prepared according to manufacturer's recommendations. Inhibitors were added in final concentrations as indicated. Samples were subjected to SDS−PAGE, and radioactivity incorporated in GST-Smad3deltaMH1 was evaluated by using a Fuji X2000 phosphorimager. A representative experiment out of three performed is shown.

effect of known inhibitors of serine/threonine kinases on phosphorylation of substrates by GST-TβR-I. We selected inhibitors which represent five different structural classes, i.e., H7 (isoquinoline group), roscovitine (isopropylpurine group), PD98059 (flavone group), SB203580 (pyridinylimidazole group), and staurosporine. Among them, SB203580 and H7 inhibited TβR-I-dependent phosphorylation of GST-Smad3deltaMH1 in an in vitro kinase assay. The IC_{50} of SB203580 for inhibition of GST-TβR-I was 700 nM compared to 34 nM for inhibition of the p38 kinase (Figure 2A). The IC_{50} value for H7 was 200 μM for inhibition of GST-TβR-I, compared to 97 μM for inhibition of myosin light chain kinase. The other inhibitors, PD98058, roscovitine, and staurosporine, did not have an effect on the kinase activity of GST-TβR-I (Figure 2A). A similarity in the ATP-binding sites of p38 and TβR-I kinases has been reported earlier, and the observed inhibitory effect of SB203580 is in agreement with previous findings (*26*).

To explore molecular features which can be of importance for the inhibition of TβR-I by pyridinylimidazole compounds, we tested a panel of inhibitors with substitutions in both phenyl rings, as well as in the imidazole ring (Figure 2B and Table 1). We found that SB202190 has a similar IC_{50} toward GST-TβR-I and p38 kinase, 20 nM compared to 16 nM, respectively. SB202190 has a hydroxyl group at position 4 of the 2-phenyl ring, compared to methylsulfinyl (SB203580) or nitro groups (PD169316). SC68376, which lacks fluor in the 4-fluorophenyl ring, has the imidazole replaced by oxazole and the 2-phenyl ring replaced by a methyl group, compared to SB202190, SB 203580, and PD169316, respectively, and has increased IC_{50} for both p38 kinase and GST-TβR-I to 2−5 μM and 5 μM, respectively. Interestingly, SB202474, which does not affect p38 kinase, inhibited the kinase activity of GST-TβR-I; however, the IC_{50} was rather high, 30 μM (Table 1). This suggests that, in contrast to p38 kinase, the 4-fluorophenyl ring is not essential for inhibition of the TβR-I kinase in the in vitro assay and that the size of the 2-imidazole substituent affects the efficiency of the inhibition.

To explore whether SB203580 affects the kinase activity of TβR-I in vivo, we studied phosphorylation of endogenous Smad2 in Mv1Lu cells treated with TGFβ1 and SB203580. Cells were pretreated or not with SB203580 before addition of TGFβ1, and the phosphorylation status of Smad2 was evaluated by immunoblotting with antibodies against the two

Table 1: Inhibition of GST-TβR-I Kinase by Low Molecular Weight Compounds Interfering with the ATP-Binding Site

Substance	Structure	IC50 (μM)			
		GST-TßR-I		p38 MAP	
		in vitro	*in vivo*	*in vitro*	*in vivo*
SB203580		0.7	10	0.034 [a]	0.6 [a]
SB202190		0.02	ND	0.016 [a]	0.35 [a]
PD169316		0.35	ND	0.089 [a]	ND
SC68376		5.0	ND	2-5 [a]	ND
SB202474		30	ND	ND [a]	ND
H7		200	ND	ND [b]	ND

[a] For references see Calbiochem product data sheets. ND, not determined. [b] K_i for myosin light chain kinase, 97 μM; protein kinase A, 3.0 μM; protein kinase C, 6.0 μM; and protein kinase G, 5.8 μM.

phosphorylated C-terminal serine residues of Smad2 (pS2), which are direct targets of TβR-I kinase (*20*, *27*). We found that pretreatment of cells with SB203580 at 25 μM significantly reduced TGFβ1-dependent phosphorylation of Smad2 (Figure 3A). Quantification of the signals showed that SB203580 inhibited TGFβ1-induced Smad2 phosphorylation by 57% (data not shown). H7 also inhibited Smad2 phosphorylation, but its effect was weaker than that of SB203580 (Figure 3A). This is in agreement with the lower efficiency of H7 in inhibition of kinase activity of GST-TβR-I in the in vitro assay (Figure 2A). SB203580 at similar concentrations inhibited TGFβ-dependent activation of the luciferase reporter, CAGA(12)-luc, transfected in Mv1Lu cells (Figure 3B). The inhibitory effect of SB203580 was observed already at a concentration of 10 μM and was most pronounced after 6 h of TGFβ1 stimulation. Our results show for the first time that in vivo SB203580 inhibits phosphorylation of endogenous Smad2 by TβR-I and affects a TGFβ-specific transcriptional response.

Inhibition of TGFβ Signaling by Substrate-Mimicking Peptides. Inhibitors interfering with the ATP-binding site of kinases often have a limited specificity, as ATP-binding pockets of different kinases have significant similarities. In contrast, protein substrate-recognizing surfaces have less similarities and, therefore, can provide specificity for targeting of kinases. To explore the possibility of inhibition of TβR-I kinase by interfering with the binding of substrate to the kinase, we analyzed peptides corresponding to the C-terminus of Smad2 (antp-Sm2S), since the two serine residues at the C-terminus of Smad2 and Smad3 are known as efficient substrates of TβR-I kinase (*20*, *25*, *27*). As phosphorylation of serine residues by the kinase could lead to a quick dissociation of the peptide from the kinase, we also synthesized C-terminal peptides with a substitution of the phosphorylatable serine to alanine residues (antp-Sm2A)

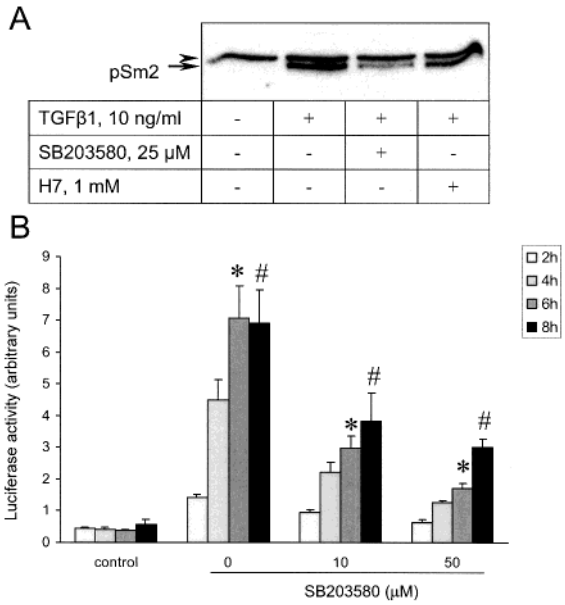


FIGURE 3: SB203580 is a potent inhibitor of TβR-I signaling. (A) Mv1Lu cells were stimulated with 10 ng/mL TGFβ1 for 30 min in the presence or absence of SB203580 and H7, as indicated. Smad2 phosphorylated at the C-terminus was detected by immunoblotting of the whole cell extract with pS2 antibody. The migration position of phosphorylated Smad2 (pSm2) is shown by an arrow, and the arrowhead shows the migration of a nonspecific band. (B) Mv1Lu cells were transiently transfected with CAGA(12)-luc and Lac-Z (β-gal) reporter plasmids. Cells were stimulated with TGFβ1 (10 ng/mL) for 2, 4, 6, and 8 h in the presence of SB203580 at the indicated concentrations, and luciferase activity was measured. The luciferase activity was normalized to expression of Lac-Z. *, #, $p < 0.05$; cells pretreated with SB203580 were compared to non-pretreated cells.

as a possible pseudosubstrate. A pseudosubstrate occupies the substrate-binding site but cannot be phosphorylated and therefore does not dissociate from the kinase and inhibit substrate phosphorylation. Smad2 peptides inhibited autophosphorylation of GST-TβR-I in the in vitro kinase assay (Figure 4A); the antp-Sm2A peptide had a stronger effect than the antp-Sm2S peptide. No or only weak inhibition of autophosphorylation was observed with a peptide mimicking the C-terminus of Smad5, which is not a substrate of TβR-I. Antp-Sm2A peptide also inhibited the phosphorylation at the C-terminus of GST-Smad2, while the antp-Sm2S effect was less pronounced (Figure 4B). The effect of antp-Sm2A and antp-Sm2S peptides was observed at 10 μM, and maximal inhibition was found at a concentration 200 μM (data not shown).

To evaluate whether the substrate-mimicking peptides affect kinases of type I and type II receptors of the TGFβ family, we studied the influence of peptides on autophosphorylation of all known mammalian type I receptors [ALK-1, ActR-I (ALK-2), BMPR-IA (ALK-3), ActR-IB (ALK-4), TβR-I (ALK-5), BMPR-IB (ALK-6), and ALK-7], TβR-II, ActR-II, BMPR-II, p38α, and SAPKα2 (Figure 5). The kinases were expressed in COS-7 cells and purified by using specific anti-tag antibodies or Ni-NTA agarose, and kinase activity was tested in the presence of peptides. We found that antp-Sm2A and antp-Sm2S peptides inhibited the TβR-I kinase, without any effect on the other kinases (Figure 5), indicating that these peptides are recognized more efficiently by TβR-I than by the other tested kinases.

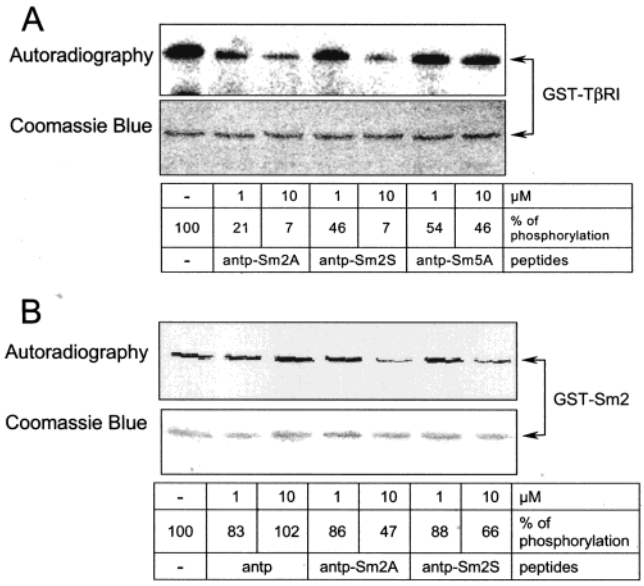


FIGURE 4: Inhibition of GST-TβR-I kinase by substrate-mimicking peptides. (A) A GST-TβR-I autophosphorylation assay was performed in the absence or presence of antp-Sm2S, antp-Sm2A, and antp-Sm5A peptides at concentrations of 1 and 10 μM, as indicated. Arrows show the migration of GST-TβR-I. (B) Phosphorylation of GST-Smad2 by GST-TβR-I was performed in an in vitro kinase assay. The peptides were added as indicated. Arrows show the migration position of GST-Smad2 (GST-Sm2). Phosphorylated GST-TβR-I and GST-Smad2 were visualized after SDS−PAGE and exposure in a Fuji X2000 phosphorimager. The gels stained with Coomassie blue are shown to control equal loading. Representative experiments out of three (A) or two (B) performed are shown.

To evaluate whether the peptides also affect TβR-I kinase in vivo, we tested their influence on TGFβ-dependent stimulation of Smad2 phosphorylation. For this assay, we used peptides which contain in their N-termini a sequence (penetratin) corresponding to the third helix of the homeodomain of antennapedia, a *Drosophila* transcription factor (*19*, *28*); penetratin or peptides fused to penetratin are efficiently taken up by cells. The molecular mechanism of penetratin-mediated cellular uptake is nonendocytotic and transporter/receptor-independent (*19*, *28*). Penetratin-fused peptides corresponding to the C-terminus of Smad2 with intact C-terminal serine residues (antp-Sm2S) or the same residues replaced by alanine residues (antp-Sm2A), as well as a peptide corresponding to the C-terminus of Smad5 with the serine residues replaced by alanine residues (antp-Sm5A), and a control penetratin peptide (antp), were analyzed. Direct monitoring in a fluorescence microscope of uptake by cells of these peptides conjugated with FITC confirmed efficient uptake within the first 10 min of incubation, which was sustained for at least 2 h (data not shown). We found that pretreatment of Mv1Lu cells with the antp-Sm2A peptide led to an inhibition of the TGFβ1-dependent phosphorylation of endogenous Smad2, with 67% of inhibition compared to nontreated cells (Figure 6A). The antp-Sm2S peptide had a weaker effect, and the antp-Sm5A peptide did not have any effect. Effective concentrations of the Smad2 peptides were higher in these in vivo analyses, compared to the in vitro tests, 50−100 μM compared to 10 μM, respectively (Figures 4 and 6A); this probably reflects a shorter half-life of the peptides in cells. The effect of antp-Sm2A and antp-Sm2S peptides occurs on the level of receptor kinase activation, since in an in vitro kinase assay with immunoprecipitated constitutively active TβR-I expressed in Mv1Lu cells, antp-Sm2A and antp-Sm2S peptides inhibited autophosphorylation of TβR-I, while antp-Sm5A peptide had only a marginal effect (data not shown). These data are similar to the results of the in vitro kinase assay (Figures 4 and 5), strongly suggesting that antp-S2A peptide is an inhibitor of TβR-I both in vivo and in vitro.

To explore whether the substrate-mimicking peptides can affect TGFβ-dependent signaling, we performed an assay with a luciferase reporter under control of a Smad2-responsive element. The ARE-luc reporter was transfected in Mv1Lu cells together with xFAST-1, and cells were pretreated with peptides or not, followed by stimulation with TGFβ1. Pretreatment of cells with antp-Sm2A inhibited TGFβ1-dependent stimulation up to 31%, while antp-Sm2S had no effect (Figure 6B).

To explore whether the antp-Sm2A peptide can interfere with the TGFβ-dependent effects on cells, we performed an assay with Mv1Lu cells, which are widely used in studies of TGFβ effects and are potently growth-inhibited by TGFβ (*29*). We found that antp-Sm2S and antp-Sm2A peptides at a concentration of 50 μM reverted to a significant extent the inhibitory action of TGFβ1, while the antp-Sm5A peptide did not have any effect (Figure 7); for cells treated with antp-Sm2A peptide inhibition in response to TGFβ1 was only 26%, compared to 66% of inhibition for the cells treated with antp peptide only or cells not treated with peptides. In this assay, antp-Sm2A peptide was more efficient than antp-Sm2S, suggesting that the peptide has characteristics of a pseudosubstrate. Thus, our data show that substrate-mimicking peptides specifically inhibit TGFβ-dependent signaling.

DISCUSSION

Search for specific inhibitors of kinases involved in intracellular signaling is an important task in the development of drugs, since it may target selected regulatory pathways. Most of the known kinase inhibitors act through interaction with the ATP-binding site of kinases. In this study, we showed that, among the tested serine/threonine kinase inhibitors, compounds of the pyridinylimidazole class inhibited TβR-I kinase in vitro and TGFβ signaling in vivo (Figures 2 and 3). These compounds are also known as potent inhibitors of the p38 MAP kinase. The similarity between the ATP-binding sites of p38 and TβR-I kinases has suggested that TβR-I kinase can be sensitive to pyridinylimidazole compounds, and Eyers et al. have shown that SB230580 inhibits autophosphorylation of TβR-I and TβR-II in vitro (*26*). Our results provide additional insight into the molecular mechanism whereby TβR-I is inhibited by the pyridinylimidazole compounds. We found that IC_{50}'s of different SB203580-related compounds differ in regard to inhibition of TβR-I compared to the p38 kinase (Table 1). The sensitivity of the TβR-I kinase to SB202474, which does not inhibit the p38 kinase, suggests that the 4-fluorophenyl ring is not essential for interaction of the inhibitors with residues in the ATP-binding site of TβR-I kinase. This can be explained by differences in the residues which form bonds with the 4-fluorophenyl ring of the inhibitor in p38β vs TβR-I kinases, i.e., Leu75 vs Arg255, Leu86 vs Phe262, and Thr106 vs Ser280, respectively (*29*, *30*) (Figure 8). A similar

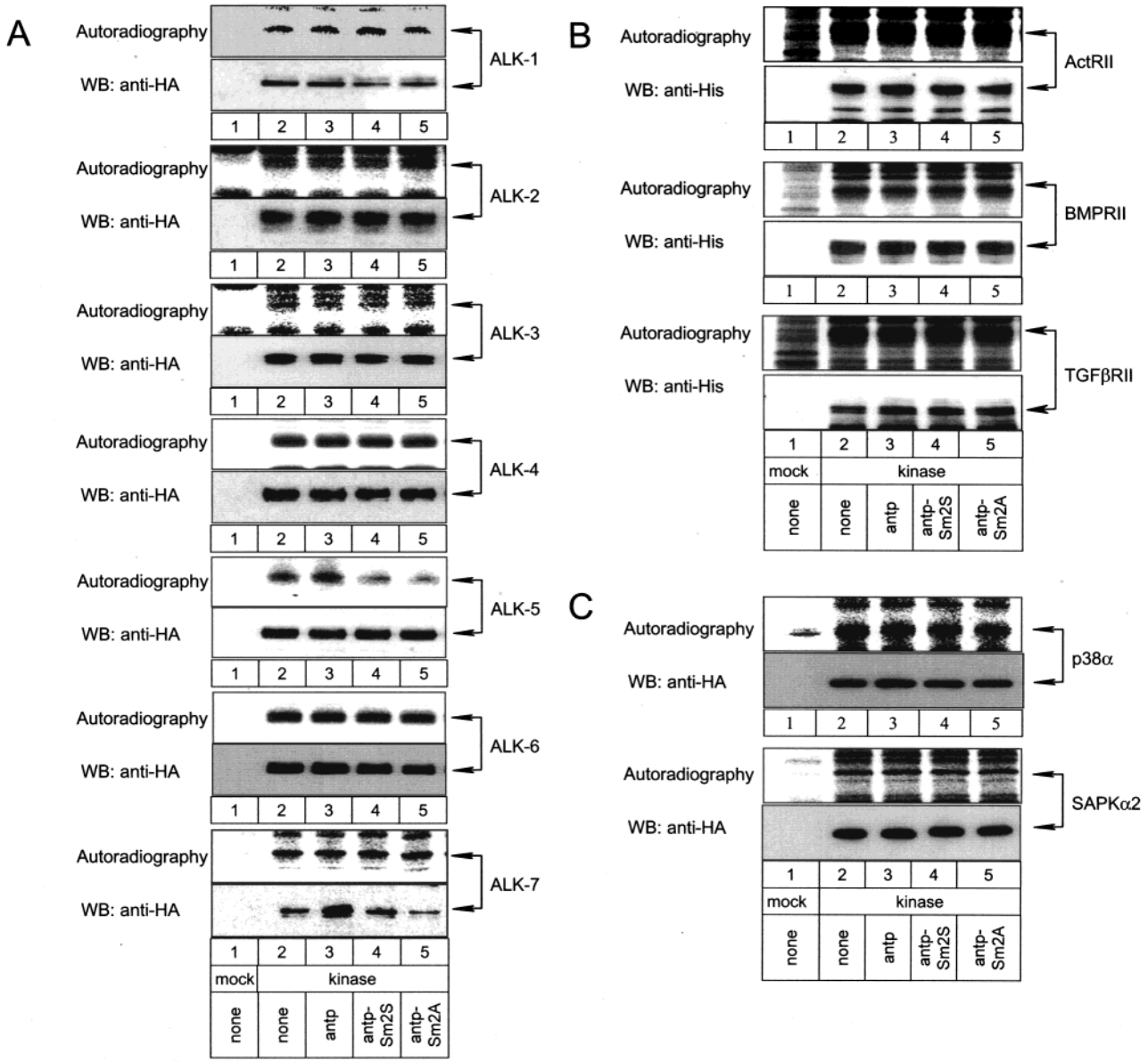


FIGURE 5: Substrate-mimicking peptides inhibit TβR-I receptor kinase but have no effect on other type I and type II receptors of the TGFβ family and do not affect p38α and SAPKα2 kinase activities. The receptors and kinases were expressed in COS-7 cells and purified by precipitation with anti-tag antibodies or Ni-NTA agarose. Type I receptors [ALK-1, ActR-I (ALK-2), BMPR-IA (ALK-3), ActR-IB (ALK-4), TβR-I (ALK-5), BMPR-IB (ALK-6), and ALK-7] were HA-tagged (A). TβR-II, ActR-II, and BMPR-II were His_6-tagged (B), and p38α and SAPKα2 were HA-tagged (C). In vitro kinase assays were performed in the presence of 10 μM peptides or not, as indicated. Equal loading and expression of kinases were controlled by immunoblotting with anti-tag antibodies (lower panels). Migration positions of kinases on autoradiographs and immunoblots are indicated. A representative experiment out of two performed is shown.

observation has been made by Callahan and colleagues (*31*). The presence of nitro or methylsulfinyl groups in the 2-phenyl ring increased the IC_{50} for TβR-I more than 10 times, compared to the presence of a hydroxyl group, while for p38 kinase the differences were only 2−4 times (Table 1). This suggests that the presence of a bulky group in the 2-phenyl ring creates a hindrance, which can lead to a weaker inhibition of the TβR-I kinase. The requirement of a nonbulky group as a 2-imidazole substituent and the dispensability of 4-fluorophenyl suggest that the mechanism of inhibition of TβR-I kinase by SB203580 differs from the mechanisms of p38 inhibition.

The ability of SB203580 to inhibit direct phosphorylation of a substrate, Smad2, by TβR-I in vivo and the potent inhibition of transcriptional activation (Figure 3) suggest that pyridinylimidazole compounds can provide a basis for development of highly specific TβR-I inhibitors. Recently, Laping et al. (*33*) and Inman et al. (*34*) have shown that another pyridinylimidazole analogue, SB431542, can inhibit TGFβ signaling. However, SB431542 lacks absolute specificity as it inhibits also other kinases, i.e., p38α, ALK4, and ALK6. Results by us and others provide information of specificity and potency of various pyridinylimidazole analogues, which will be valuable for further development of highly specific inhibitors.

Inhibitors acting through binding to the ATP-binding site often suffer from low specificity, since ATP-binding sites in all studied kinases share significant similarity. Another potential problem with these inhibitors is that their effects have been determined only for a part of the kinases predicted from the human genome. Therefore, additional kinases can also be affected by these inhibitors. Our data about the

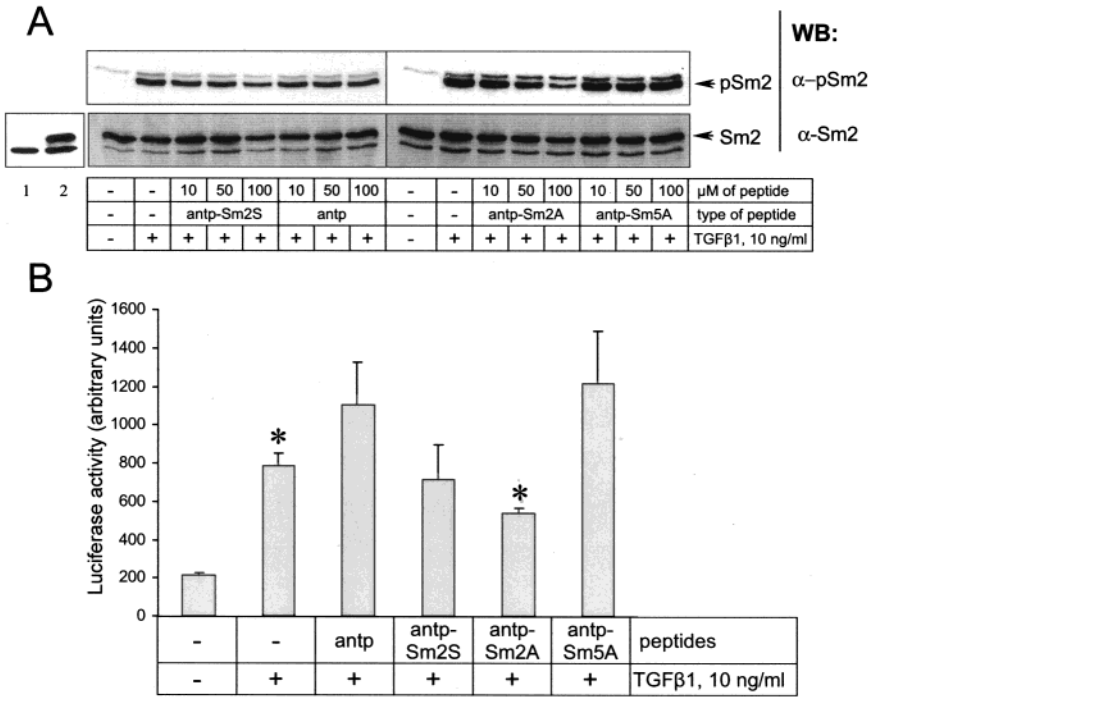


FIGURE 6: Substrate-mimicking peptides inhibit TGFβ signaling. Phosphorylation of endogenous Smad2 was inhibited by pretreatment of cells with peptides. Mv1Lu cells were incubated with TGFβ1 alone or with antp-Sm2S, antp-Sm2A, antp-Sm5A, and control antp peptides, as indicated. After 15 min of incubation with TGFβ1, phosphorylated Smad2 was detected by immunobloting of the whole cell extract with pS2 antibody (upper panel). To control equal loading, the same membrane was reprobed with anti-Smad2 antibody (lower panel). The specificity of anti-Smad2 antibody is shown on the left lower panel using the total lysates of Smad2 null mouse embryonal fibroblasts (1) and wild-type Mv1Lu cells (2). Arrows show migration positions of phosphorylated Smad2 (pSm2, upper panel) and Smad2 (Sm2, lower panel). (B) Mv1Lu cells were transfected with ARE-luc, xFAST-1, and Lac-Z (β-gal) plasmids. Twenty hours after transfection, cells were incubated with TGFβ1 (10 ng/mL) and peptides, as indicated. The final concentration for all peptides was 50 μM. After 18 h luciferase activity was measured. For normalization, β-gal activity was measured. Representative experiments out of three (A) or two (B) performed are shown. *, $p < 0.05$; cells pretreated with antp-Sm2A peptide before addition of TGFβ1 were compared to cells treated with TGFβ1 only.

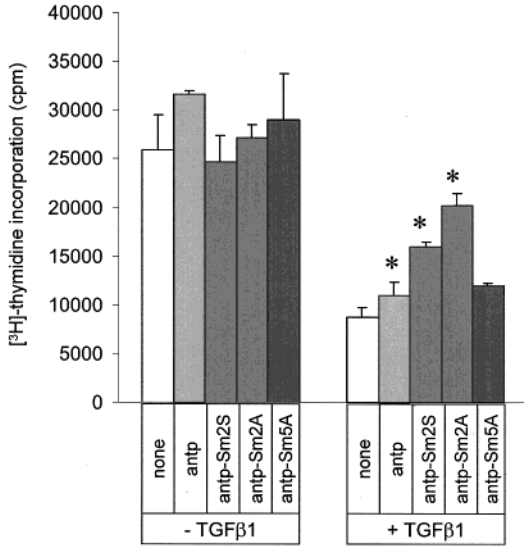


FIGURE 7: Substrate-mimicking peptides revert TGFβ-dependent inhibition of DNA synthesis. Mv1Lu cells were pretreated with peptides at a final concentration of 50 μM and incubated with TGFβ1 (0.1 ng/mL) or not for 24 h, as indicated. [^{3}H]Thymidine was added to cells for the last 2 h of incubation, and radioactivity incorporated into DNA was measured. A representative experiment out of four performed is shown. *, $p < 0.05$; cells pretreated with antp-Sm2A or antp-Sm2S peptides were compared to cells pretreated with antp peptide.

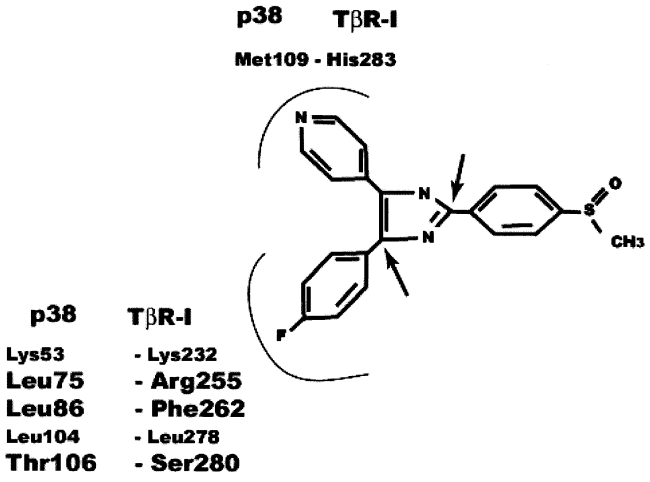


FIGURE 8: Schematic presentation of residues which may interact with SB203580 in p38 and TβR-I kinases. Residues which differ are shown in bold. Arrows show the position of substituents affecting the affinity of the inhibitor to TβR-I kinase.

inhibition of TβR-I by pyridinylimidazole inhibitors at a concentration range similar to that of p38 kinases is an example of this challenge.

To find specific inhibitors, we explored the possibility of affecting the TβR-I kinase by competition with its substrate-(s). Sequential phosphorylation of the two C-terminal serine residues in Smad2 and Smad3 by TβR-I is known to trigger intracellular TGFβ signaling (*20*). We found that peptides corresponding to the 14 most C-terminal amino acids of Smad2 inhibited efficiently autophosphorylation of TβR-I in vitro and phosphorylation of Smad2 in vitro and in vivo (Figures 4 and 6A; data not shown). These peptides did not

affect kinase activity of other type I and type II receptors, p38α and SAPKα2 (Figure 5). The absence of an effect on type II receptors, p38α, SAPKα2, and bone morphogenetic protein signaling-specific type I receptors was expected, since these kinases show a difference in substrate specificity compared to TβR-I. However, the lack of a significant effect on autophosphorylation of ActR-IB and ALK-7, which also phosphorylate Smad2 and Smad3, was unexpected (Figure 5A). This suggests that type I receptors with similar substrate specificity can have different affinity for the substrate, which may depend on the variability of substrate−kinase interacting surfaces. This issue is currently under investigation.

Similar approaches were used for the generation of specific inhibitors of other kinases, e.g., $p60^{c-src}$, PKC, Erk, and PKA (*15*−*17*, *32*). Substrate-mimetic peptides compete at the substrate-binding site, potentially providing higher specificity, compared to competition at the ATP-binding pocket. Moreover, substitution of phosphorylatable residues to nonphosphorylatable, e.g., serine or threonine to alanine residue, significantly increases the affinity of an inhibitory peptide to a kinase. This is due to the inability of the γ-phosphoryl transfer from ATP to an acceptor residue and, therefore, an absence of repulsing force, which is the major contributing factor to the dissociation of phosphorylated substrate from a kinase. Our observation of a higher inhibitory effect of a peptide with phosphorylatable serine residues replaced with alanine residues, compared to the serine-containing peptide, is consistent with the notion that the antp-Sm2A is a pseudosubstrate inhibitor for TβR-I.

Our results show that substrate-mimicking peptides represent a promising approach for the development of specific inhibitors of TGFβ signaling in vivo (Figures 6 and 7). Efficient delivery of the peptides into cells in vivo was ensured by the use of the antennapedia peptide penetratin. Penetratin provides a basis for new generation of peptide-delivery vectors, and it has allowed specific modulation of signaling processes in different studies (*19*, *28*). The molecular mechanism of the inhibition of TGFβ signaling comprises blocking of TβR-I kinase activity, as was shown in in vitro and in vivo assays (Figures 4, 5, and 6A). The inhibitory effect of the peptides was observed both in a short-term (30 min) and in a long-term (18−24 h) assay, i.e., Smad2 phosphorylation and growth inhibition, respectively. This suggests that the peptides were stable in cells and that they did not have a cytotoxic effect during the time of the assays. Thus, the described peptides represent a new class of inhibitors of TGFβ signaling. In combination with the recent developments of methods to introduce peptides into living cells, these peptides will be important tools for regulation of TGFβ signaling.

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