Published in final edited form as:

Cell Signal. 2012 February ; 24(2): 476–483. doi:10.1016/j.cellsig.2011.09.027.

Structural Basis for Specificity of TGF β Family Receptor Small Molecule Inhibitors

Abiodun A. Ogunjimi¹, Elton Zeqiraj¹, Derek F. Ceccarelli¹, Frank Sicheri^{1,2,*}, Jeffrey L. Wrana^{1,2,*}, and Laurent David^{1,*}

¹Center for Systems Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, M5G 1X5, Ontario, Canada

²Dept. of Molecular Genetics, University of Toronto, 1 King's College Circle, Toronto, M5S 1A8, Ontario, Canada

Abstract

Transforming growth factor- β (TGF β) receptor kinase inhibitors have a great therapeutic potential. SB431542 is one of the mainly used kinase inhibitors of the TGF β /Activin pathway receptors, but needs improvement of its EC₅₀ (EC₅₀ = 1 μ M) to be translated to clinical use. A key feature of SB431542 is that it specifically targets receptors from the TGF β /Activin pathway but not the closely related receptors from the bone morphogenic proteins (BMP) pathway. To understand the mechanisms of this selectivity, we solved the crystal structure of the TGF β type I receptor (T β RI) kinase domain in complex with SB431542. We mutated T β RI residues coordinating SB431542 to their counterparts in activin-receptor like kinase 2 (ALK2), a BMP receptor kinase, and tested the kinase activity of mutated T β RI. We discovered that a Ser280Thr mutation yielded a T β RI variant that was resistant to SB431542 inhibition. Furthermore, the corresponding Thr283Ser mutation in ALK2 yielded a BMP receptor sensitive to SB431542. This demonstrated that Ser280 is the key determinant of selectivity for SB431542. This work provides a framework for optimizing the SB431542 scaffold to more potent and selective inhibitors of the TGF β /Activin pathway.

Keywords

ALK5; SB431542; ALK2; dorsomorphin; A8301

1. Introduction

TGF β family proteins (TGF β , BMPs, Activin/Nodal, GDFs and AMH) are secreted morphogens that signal through heteromeric complexes of transmembrane type I and type II

*Correspondence to: Dr. Laurent David, Post-Doc, SLRI, ldavid@lunenfeld.ca, Tel: 1-416-586-4800 x2363, Fax: 1-416-586-8869; Dr. Jeff Wrana, Sr. Investigator, SLRI, wrana@lunenfeld.ca, Tel: 1-416-586-4800 x2791, Fax: 1-416-586-8869; Dr. Frank Sicheri, Sr. Investigator, SLRI, sicheri@lunenfeld.ca, Tel: 1-416-586-4800 x2336, Fax: 1-416-586-8869.

Accession codes

Coordinates and structure factors have been deposited in the Protein Data Bank under accession code PDBID: 3TZM.

Supplementary Information contains 5 figures.

serine/threonine kinase receptors to orchestrate a wide array of cellular signals in development and disease.

TGF β family ligand-receptor interaction triggers the formation of specific type I and type II heterotetrameric receptor complexes. The type II receptors then phosphorylate the type I receptors, which in turn phosphorylate down-stream effectors, the receptor-activated Smads (R-Smads). Phosphorylated R-Smads assemble with Smad4, accumulate in the nucleus and regulate transcription via interaction with transcription factor partners [1]. The seven TGF β family type I receptors cluster in two groups depending on the R-Smad they target. ALK4, T β RI (also referred to as ALK5) and ALK7 induce the phosphorylation of Smad2 and 3; this is called the “TGF β /Activin pathway”. ALK1, 2, 3 and 6 induce the phosphorylation of Smad1, 5 and 8, and this is referred to as the “BMP pathway” (Fig S1A). In these pathways it is the identity of the activated type I receptor that is critical in defining transcriptional outputs, as the two groups of R-Smads trigger different, and often opposite, cellular responses.

Enhanced signalling output mediated via the intracellular kinase domains of the TGF β family receptors is important for a variety of pathological processes such as cancer progression, fibrosis, as well as parasitic infection by *Trypanosoma cruzi*. Therefore, small molecule inhibitors of TGF β signalling that specifically target the kinase domains have great therapeutic potentials.

Multiple inhibitors are commonly used to dissect the myriad biological and pathological functions of TGF β family cytokines. During the first attempt to find inhibitors for T β RI, by using a T β RI kinase assay coupled to compound screening, Callahan et al. found that 2,4,5-substituted imidazoles could be potent inhibitors [2]. This resulted in a series of 2,4,5-substituted imidazoles [2-4]. Later on, virtual small molecules screening aimed at finding new molecules targeting the T β RI ATP-binding pocket yielded a series of 2,4,5-substituted pyrazole inhibitors [5, 6]. 2,4,5-substituted imidazoles and pyrazoles are the two main inhibitor families used to inhibit the TGF β /Activin pathway (Fig S1).

Recently, multiple inhibitors belonging to the substituted-imidazole group (SB431542 and its derivatives SB505124 or SB525334) or substituted-pyrazole group (LY364947 and its derivative A8301) have been benchmarked in a panel of 123 kinases [7]. This confirmed that these kinase inhibitors are specifically targeting the TGF β - but not the BMP-pathway receptors, which makes them invaluable. However, the molecular determinants conferring inhibitor specificity of those inhibitors toward the TGF β /Activin but not the BMP pathway are unknown. Understanding how this specificity is regulated is needed to enhance the usefulness of these pathway specific inhibitors, through chemical engineering.

We have solved the structure of SB431542 bound to the T β RI protein kinase domain, with the aim of understanding the molecular basis for SB431542 inhibitor specificity. The structure enabled a systematic mutational analysis of TGF β - and BMP-receptors (T β RI and ALK2), coupled with specific TGF β - or BMP-receptor activity readouts to discern the basis for inhibitor specificity. We have discovered that the gatekeeper residue S280 in T β RI is the key determinant for the selectivity of both SB431542 and A8301.

2. Materials and Methods

2.1. Protein expression and purification

The T β RI kinase domain (residues 200-503) was cloned into a pFastBac-HTa vector, expressed in Sf9 cells and purified using nickel affinity chromatography. The 6 $\times$ His purification tag was cleaved overnight by incubation with TEV protease at 4 °C. T β RI was then purified using an S75 Superdex gel filtration column pre-equilibrated in 25 mM Tris-HCl pH 7.5, 150 mM NaCl and 1 mM DTT.

2.2. Crystallization, data collection and structure determination

T β RI was mixed with SB431542 at final concentrations of 0.25 mM and 1 mM respectively and incubated on ice for 60 min prior to crystallisation. Crystals were grown using the hanging drop vapour diffusion method by mixing 1.5 μ l of T β RI-SB431542 complex with 1.5 μ l of mother liquor containing 100 mM imidazole pH 8.0 and 10% PEG 8000. Full-sized crystals of the T β RI-SB431542 complex grew after 5 days. Crystals were cryoprotected with mother liquor supplemented with 25% glycerol (v/v) before flash freezing in liquid nitrogen. Diffraction data were collected at NE CAT beamline 24-ID-C. The structure was solved by molecular replacement using MOLREP [8] and the T β RI structure (PDBID 1PY5, [9]) as a search model. The structure was refined by iterative rounds of refinement with REFMAC [10] and manual model building with the program COOT [11].

2.3. Alignment and Mutagenesis

Sequences alignments were performed using MUSCLE [12] and edited using ALINE [13]. Mutants of ALK2ca and T β RIca were generated using conventional site directed mutagenesis technique. Each mutant was sequenced-verified.

2.4. Reporter gene constructs, expression plasmids and inhibitors

Reporter plasmids pGL3 (BRE)-luc was kindly provided by Dr P. ten Dijke (Leiden University Medical Center, Netherlands). 3TP-luc, T β RI and ALK2 were previously generated in the lab. SB431542 and dorsomorphin were purchased from Sigma-Aldrich; A8301 from Tocris.

2.5. Luciferase activity assay

NIH-3T3 cells were seeded in 24 well plates for 24h, then transfected in Opti-MEM using lipofectamine and PLUS reagent (Invitrogen) with 0.1 μ g pGL3 (BRE²)-luc or 0.2 μ g p3TP-luc, 0.05 μ g of pCMV5 β -gal and 0.05 μ g of either pCMV-GFP or a vector expressing ALK2 or T β RI. Four hours after transfection, cells were treated with inhibitors (SB431542, A8301 or dorsomorphin) at the indicated concentrations for 15h. Cells were lysed, then Firefly luciferase and β -galactosidase activities were measured, using a luminometer (Berthold) or a plate reader (Perkin-Elmer), respectively [14]. Values of fold activation were calculated as relative luciferase activity compared with untreated cells. Data are representative of at least three independent experiments. Error bars represent standard deviation calculated from data derived from 3 individually transfected wells of NIH-3T3 cells.

2.6. Protein expression and Western blotting

293T cells were transfected with plasmids expressing ALK5ca and ALK5ca S280T, by calcium phosphate. 48h after transfection, cells were lysed in 0.1% (v/v) TNTE and separated on SDS-PAGE. After transfer on nitrocellulose membrane, the membranes were probed with anti-Phospho-Smad2 (Cell Signalling, 1/2500), anti-Smad2 (Cell Signalling, 1/2500) anti-HA (Roche, 1/5000) and anti-Actin (Sigma, 1/10,000) antibodies.

3. Results

3.1. Structure of the T β RI-SB431542 complex

To investigate the basis for selectivity of SB431542 towards TGF β pathway receptors (EC_{50} = 1 μ M) versus BMP pathway receptors (no inhibition at 20 μ M), we co-crystallized SB431542 bound to T β RI kinase domain (residues 200-503). Diffraction data collection to 1.7 Å resolution, structure solution by molecular replacement and refinement, resulted in a final model with good statistics (Table 1). Residues 370-371 (within the activation segment) and 497-503 (corresponding to the C-terminus) were disordered and are absent from the final model. T β RI displays the classical kinase domain organisation comprising a small N-terminal lobe and a larger C-terminal lobe (Fig 1A). The T β RI activation loop (region between DFG and APE motifs) attains the extended conformation found in active protein kinases [15]. The Leu residue of the DLG motif (equivalent to the DFG motif in most kinases) assumes the so-called “DFG in” conformation [16]. T β RI in complex with SB431542 resembles the T β RI kinase conformation reported in the FKBP12-T β RI complex (RMSD 0.3 Å over 207 C α atoms) [17]. Clear electron density was observed for the SB431542 compound (see unbiased $|F_o| - |F_c|$ electron density maps in Fig 1B). SB431542 has a heterocyclic ring structure (Fig S1) and acts as a type I kinase inhibitor [18] by occupying the purine (ATP) binding region and adjacent hydrophobic sites near the hinge region; the latter defined as the linker between the N- and C-terminal kinase lobes (Fig 1A). The first residue of the hinge region is referred to as the gatekeeper residue, which often fills a hydrophobic pocket adjacent to the ATP purine-binding site [19]. T β RI however contains a small residue at the gatekeeping position (S280), thus allowing for the hydrophobic pyridinyl ring of SB431542 to be comfortably accommodated within this pocket (Fig 1B). The position of the pyridinyl ring adjacent to the gatekeeper residue is further stabilised through hydrophobic interactions via residues A230 (β 3 sheet), L260 (α C- β 5 loop) and L278 (β 4 sheet). L260 and A230 contact both the pyridinyl and benzodioxol rings, which adopt a non-co-planar conformation (Fig 1B).

The benzodioxol ring of SB431542 is sheltered by hydrophobic residues A230, L260, Y282 (hinge region) and L340 (β 7 sheet) (Fig 1B). A hydrogen bond links the benzodioxol oxygen of SB431542 and the amide nitrogen of H283 from the hinge region of the T β RI kinase domain (Fig 1B), a feature common to many ATP competitive kinase inhibitors. The classical ion pair between K232 (VAIK motif) and E245 (helix α C), reflective of an active-like kinase conformation, is maintained and places K232 in hydrogen bonding distance to the imidazole ring of SB431542, which is also stabilised by V219 (β 7 strand) through hydrophobic interactions, as well as by coordination by an ordered water molecule (Fig 1B). The SB431542 benzamide ring, sandwiched between the glycine-rich (Gly-rich, GXGXXG)

loop and the activation segment, is coordinated in part through hydrogen bonding to D351 (Fig 1B). Compared to the other cyclic moieties of SB431542, the benzamide ring appears loosely coordinated. The main SB431542 contacts contributing to binding involve the pyridinyl, benzodioxol and imidazole moieties interacting with residues from the hinge region and N- and C-lobes of T β RI.

3.2. The gatekeeper residue is responsible for TGF β selective inhibition by SB431542

To understand why SB431542 selectively inhibits T β RI but not its closely related counterpart ALK2, we superimposed the structure of ALK2 kinase domain (PDBID 3H9R) onto the structure of T β RI bound to SB431542, and looked for differences in residues contributing to SB431542 binding. Inspection of the SB431542-kinase contacts revealed that of the 12 residues directly contacting the bound inhibitor, only the gatekeeper residue S280 differs between ALK2 and T β RI. S280 is conserved in all the TGF β /Activin pathway receptors, while it is replaced by T283 in all BMP pathway receptors (Fig S2).

To assess if S280 indeed contributes to selective inhibitor binding we mutated this position to threonine (its counterpart in ALK2), and tested for the ability of SB431542 to inhibit T β RI. We used a constitutively active form of T β RI (T β RIca, T204D), as our goal was to analyse the effect of the mutation on the kinase activity, and not on ligand binding or on type I/II transactivation. We co-transfected T β RIca with a reporter gene (3TP-Lux) that is specifically activated by Smad2 and 3, the downstream targets of T β RI [20]. T β RIca strongly activated 3TP-lux when compared to the wild type T β RI (>15 fold) (Fig 2A). This activation was potently suppressed by SB431542 in a dose dependent manner and T β RIca activity was diminished to basal levels at 20 μ M SB431542 (Fig 2A). We then tested a T β RIca S280T mutant in the presence of SB431542. We found that T β RIca (S280T) strongly activated 3TP-lux, but unlike the WT receptor, was completely resistant to SB431542 inhibition when tested up to 20 μ M (Fig 2A). These results indicate that S280 confers selectivity in SB431542-mediated inhibition of T β RI-like kinases. We note that both T β RIca and T β RIca (S280T) have similar expression levels as shown by Western blotting (Fig S3).

We next explored the possibility that additional residues could also be important for SB431542 selective inhibition. Sequence alignment of TGF β and BMP pathway receptor kinases revealed non-conserved residues as possible contributors to T β RI-SB431542 specificity (Fig S2). Namely Q208, F216, E228, V279, A368 and H371 corresponding to L211, Y219, N231, I282, G371 and P374 in ALK2 (Fig 2B and S2). Although not in direct contact with SB431542 (Fig 2B), we questioned if these differing residues contributed indirectly to the selective inhibition of T β RI by SB431542. Additionally, the orientation of F216 is flipped $\approx 180^\circ$ toward the outside of the binding pocket in the presence of SB431542 (compared to ALK5 apo structure PDBID 11AS - Fig S4A). Assuming the same side chain flip will occur in ALK2, the corresponding residue Y219 would be predicted to directly contact SB431542 (Fig S4A). However, substitution of T β RIca residues to their ALK2 counterparts, F216Y, E228N, V279I and A368G-H371P, all yielded mutant receptors that were equally sensitive to inhibition by 5 μ M of SB431542 (Fig 2C). This suggests that these differing residues do not contribute to T β RI selective inhibition by SB431542.

Analysis of combinational mutations further revealed no additional amino acids or group of amino acids that regulated SB431542 specificity (Fig S4B).

3.3. T β RIca (S280T) is resistant to A8301 inhibition

Since the gatekeeper residue defines sensitivity to SB431542, we next investigated if the same residue (S280) also regulates specificity to substituted-pyrazole compounds. Superimposition of T β RI-SB431542 complex with the structure of T β RI bound to LY364947 (precursor of A8301 - PDBID 1PY5) [9], revealed similarities of T β RI - inhibitor binding (Fig 3A). The two T β RI structures show little overall difference (RMSD of 0.3 Å over 293 C α atoms) with the exception of a shift in the orientation of the side chain of D351 (D351 interacts with the benzamide ring of SB431542 and the imidazole ring of LY364947 - Fig 3). Comparison of the two structures led us to hypothesize that the S280T mutation would also render T β RIca resistant to A8301 inhibition. Indeed, A8301 inhibited T β RIca in a dose dependent manner with complete inhibition at 50 nM A8301 (Fig 3B), while T β RIca (S280T) was resistant to A8301 inhibition under the same conditions (Fig 3B). These findings demonstrate the importance of S280 in conferring selectivity of substituted-imidazole and -pyrazole small molecule inhibitors towards T β RI-like kinases.

3.4. T283S mutation renders ALK2 sensitive to SB431542

ALK2 has a threonine at the gatekeeper position and is resistant to SB431542, therefore, we mutated the ALK2 gatekeeper residue T283 to the Ser residue found in T β RI and assessed sensitivity to SB431542. For this we used a constitutively active ALK2 mutant (ALK2ca, Q207D) and a BMP reporter gene (BRE²-luc) (Fig S1A) [21]. We found that the single T283S substitution of the ALK2 gatekeeper residue resulted in ALK2ca inhibition by SB431542 (Fig 4A). Notably, the EC₅₀ of SB431542 for ALK2ca (T283S) was similar to the EC₅₀ of SB431542 on T β RI activity (EC₅₀ ALK2ca T283S = 2.5 μM, EC₅₀ T β RIca = 1 μM). These results confirm that the gatekeeper residue in TGF β family receptors is critical for inhibitor specificity among the compounds we tested.

To further delineate the molecular basis for specific TGF β versus BMP receptor inhibition, we compared the binding mode of SB431542 to T β RI with that of the BMP inhibitor dorsomorphin to ALK2 (PDBID 3H9R) (Fig 4B). We examined the co-crystal structure of ALK2 in complex with dorsomorphin and observed that in contrast to T β RI-SB431542 structure, dorsomorphin does not contact the gatekeeper position but instead is mainly coordinated by hydrogen bonds with H286 in the hinge region of the ALK2 kinase domain (Fig 4B). Thus, we hypothesized that inhibition by dorsomorphin would not be affected by the T283S mutation. Consistent with this notion, dorsomorphin comparably inhibited both ALK2ca T283S and ALK2ca in the BMP pathway reporter assay (complete inhibition at 5 μM) (Fig 4C). Moreover, none of the T β RIca mutants generated (see Fig 2C and Fig S4B) was sensitive to dorsomorphin (data not shown).

4. Discussion

In this study we show that the selective inhibitory function of the substituted-imidazole SB431542 on TGF β - versus BMP-signalling depends on serine 280 at the gatekeeping

position within the ATP binding cleft of the kinase domain. Mutation of this serine to threonine (S280T) conferred complete resistance of T β RI to inhibition by SB431542. Furthermore, a reciprocal mutation of threonine to serine at this position in ALK2 (T283S) rendered ALK2 sensitive to SB431542. Moreover, we showed that the gatekeeper residue was also important for the substituted-pyrazole A8301 inhibition of T β RI. A number of co-crystal structures of T β RI kinase domain-inhibitors with several types of core moieties are available. To our knowledge, all co-crystallized inhibitors appear to occupy the available space in the ATP-binding back-pocket of T β RI (Fig S5). This suggests that S280 could be a key determinant of most T β RI inhibitors.

Altogether, our structural and functional studies point out the importance of having a small gatekeeper residue in T β RI that allows for SB431542 binding. Interestingly, 11 out of the 518 protein kinases have residues with small or no side chains (e.g. Ser, Ala and Gly) at the gatekeeper position [Kinase Sequence Database (<http://sequoia.ucsf.edu/ksd/>)]. Six protein kinases have serine as a gatekeeper, three of which are TGF β family kinases sensitive to SB431542 inhibition (ALK4, T β RI and ALK7) and the other three are c-Abl, RETGC-1 and STRAD β . Of these, c-Abl is not inhibited by SB431542 suggesting that other binding pocket determinants, in addition to the small gatekeeper residue, contribute to the selectivity of SB431542 inhibition [7, 22].

Substituted-imidazole TGF β pathway inhibitors (SB4321542 and its close analogue SM16 - Fig S1) have been successfully used in animal disease models for fibrosis, Chagas disease and cancer. Usage of SM16 resulted in decreased fibrosis and decreased myofibroblast induction in the lung [23], kidney [24], liver [25] or blood vessels [4]. SB431542 was successfully used in a mouse model of Chagas disease, which results from the infection of cardiomyocytes by *Trypanozoma cruzi* parasites and led to decreased parasitaemia and prevented heart damage [26]. Finally, SM16 inhibited primary tumour progression and metastasis in mouse breast cancer and mesothelioma tumour models [27, 28]. Moreover, a substituted-pyrazole, LY2157299 [29], is being tested in phase II on patients with hepatocellular carcinomas (<http://clinicaltrials.gov>).

These studies bear great promises for therapeutical use of TGF β /Activin pathway inhibitors based on substituted-imidazole or -pyrazole scaffolds. Our results pave the way forward for structural-based drug design aimed at improving the selectivity and potency of TGF β /Activin pathway inhibitors, which will help their translation to the clinic.

5. Conclusions

- Mutation of the gatekeeper residue of T β RI (Ser280) to the equivalent residue in ALK2 (Thr283) renders T β RI insensitive to SB431542, a substituted imidazole selective T β RI inhibitor.
- The Ser280Thr mutant of T β RI is also insensitive to A8301, a substituted pyrazole selective T β RI inhibitor.

- Analysis of the deposited structures of T β RI in complex with small molecules inhibitors suggests that Ser280 is a key determinant of most of the selective T β RI inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Andrew Hinck and Gerald Gish for critically reviewing this manuscript.

Financial disclosure

Supported by funds from CIHR to JLW (MOP14339) and FS (MOP36399). LD is recipient of a CIHR Fellowship and EZ is a Sir Henry Wellcome Postdoctoral Fellow. JLW is an HHMI International Scholar and CRC Chair in Systems Biology and FS is a CRC Chair in Structural Biology of Signal Transduction. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

- [1]. Attisano L, Wrana JL. *Science*. 2002; 296:1646–1647. [PubMed: 12040180]
- [2]. Callahan JF, Burgess JL, Fornwald JA, Gaster LM, Harling JD, Harrington FP, Heer J, Kwon C, Lehr R, Mathur A, Olson BA, Weinstock J, Laping NJ. *Journal of medicinal chemistry*. 2002; 45:999–1001. [PubMed: 11855979]
- [3]. Inman GJ, Nicolas FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, Laping NJ, Hill CS. *Mol Pharmacol*. 2002; 62:65–74. [PubMed: 12065756]
- [4]. Fu K, Corbley MJ, Sun L, Friedman JE, Shan F, Papadatos JL, Costa D, Lutterodt F, Sweigard H, Bowes S, Choi M, Boriack-Sjodin PA, Arduini RM, Sun D, Newman MN, Zhang X, Mead JN, Chuaqui CE, Cheung HK, Cornebise M, Carter MB, Josiah S, Singh J, Lee WC, Gill A, Ling LE. *Arteriosclerosis, thrombosis, and vascular biology*. 2008; 28:665–671.
- [5]. Singh J, Chuaqui CE, Boriack-Sjodin PA, Lee WC, Pontz T, Corbley MJ, Cheung HK, Arduini RM, Mead JN, Newman MN, Papadatos JL, Bowes S, Josiah S, Ling LE. *Bioorganic & medicinal chemistry letters*. 2003; 13:4355–4359. [PubMed: 14643325]
- [6]. Tojo M, Hamashima Y, Hanyu A, Kajimoto T, Saitoh M, Miyazono K, Node M, Imamura T. *Cancer Sci*. 2005; 96:791–800. [PubMed: 16271073]
- [7]. Vogt J, Traynor R, Sapkota GP. *Cellular signalling*. 2011; 23:1831–1842. [PubMed: 21740966]
- [8]. Vagin A, Teplyakov A. *Acta crystallographica. Section D, Biological crystallography*. 2010; 66:22–25.
- [9]. Sawyer JS, Anderson BD, Beight DW, Campbell RM, Jones ML, Herron DK, Lampe JW, McCowan JR, McMillen WT, Mort N, Parsons S, Smith EC, Vieth M, Weir LC, Yan L, Zhang F, Yingling JM. *Journal of medicinal chemistry*. 2003; 46:3953–3956. [PubMed: 12954047]
- [10]. Murshudov GN, Vagin AA, Dodson EJ. *Acta Crystallogr D Biol Crystallogr*. 1997; 53:240–255. [PubMed: 15299926]
- [11]. Emsley P, Cowtan K. *Acta Crystallogr D Biol Crystallogr*. 2004; 60:2126–2132. [PubMed: 15572765]
- [12]. Edgar RC. *Nucleic Acids Res*. 2004; 32:1792–1797. [PubMed: 15034147]
- [13]. Bond CS, Schuttelkopf AW. *Acta Crystallogr D Biol Crystallogr*. 2009; 65:510–512. [PubMed: 19390156]
- [14]. Labbe E, Silvestri C, Hoodless PA, Wrana JL, Attisano L. *Mol Cell*. 1998; 2:109–120. [PubMed: 9702197]
- [15]. Nolen B, Taylor S, Ghosh G. *Molecular cell*. 2004; 15:661–675. [PubMed: 15350212]
- [16]. Zuccotto F, Ardini E, Casale E, Angiolini M. *Journal of medicinal chemistry*. 2010; 53:2681–2694. [PubMed: 20000735]

- [17]. Huse M, Chen YG, Massague J, Kuriyan J. *Cell*. 1999; 96:425–436. [PubMed: 10025408]
- [18]. Zhang J, Yang PL, Gray NS. *Nat Rev Cancer*. 2009; 9:28–39. [PubMed: 19104514]
- [19]. Liu Y, Shah K, Yang F, Witucki L, Shokat KM. *Bioorganic & medicinal chemistry*. 1998; 6:1219–1226. [PubMed: 9784863]
- [20]. Wrana JL, Carcamo J, Attisano L, Cheifetz S, Zentella A, Lopez-Casillas F, Massague J. *Cold Spring Harb Symp Quant Biol*. 1992; 57:81–86. [PubMed: 1339707]
- [21]. Korchynskyi O, ten Dijke P. *The Journal of biological chemistry*. 2002; 277:4883–4891. [PubMed: 11729207]
- [22]. Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, Chan KW, Ciceri P, Davis MI, Edeen PT, Faraoni R, Floyd M, Hunt JP, Lockhart DJ, Milanov ZV, Morrison MJ, Pallares G, Patel HK, Pritchard S, Wodicka LM, Zarrinkar PP. *Nature biotechnology*. 2008; 26:127–132.
- [23]. Bonniaud P, Margetts PJ, Kolb M, Schroeder JA, Kapoun AM, Damm D, Murphy A, Chakravarty S, Dugar S, Higgins L, Protter AA, Gauldie J. *American journal of respiratory and critical care medicine*. 2005; 171:889–898. [PubMed: 15563636]
- [24]. Grygielko ET, Martin WM, Tweed C, Thornton P, Harling J, Brooks DP, Laping NJ. *The Journal of pharmacology and experimental therapeutics*. 2005; 313:943–951.
- [25]. de Gouville AC, Boullay V, Krysa G, Pilot J, Brusq JM, Loriolle F, Gauthier JM, Papworth SA, Laroze A, Gellibert F, Huet S. *British journal of pharmacology*. 2005; 145:166–177. [PubMed: 15723089]
- [26]. Waghbi MC, de Souza EM, de Oliveira GM, Keramidas M, Feige JJ, Araujo-Jorge TC, Bailly S. *Antimicrobial agents and chemotherapy*. 2009; 53:4694–4701. [PubMed: 19738024]
- [27]. Rausch MP, Hahn T, Ramanathapuram L, Bradley-Dunlop D, Mahadevan D, Mercado-Pimentel ME, Runyan RB, Besselsen DG, Zhang X, Cheung HK, Lee WC, Ling LE, Akporiaye ET. *Anticancer Res*. 2009; 29:2099–2109. [PubMed: 19528470]
- [28]. Suzuki E, Kim S, Cheung HK, Corbley MJ, Zhang X, Sun L, Shan F, Singh J, Lee WC, Albelda SM, Ling LE. *Cancer research*. 2007; 67:2351–2359. [PubMed: 17332368]
- [29]. Bueno L, de Alwis DP, Pitou C, Yingling J, Lahn M, Glatt S, Troconiz IF. *European journal of cancer*. 2008; 44:142–150. [PubMed: 18039567]

Figure 1. Crystal structure of TβRI-SB43154 complex

(A) Structure of TβRI Kinase domain (blue) in complex with SB431542. The structure of SB431542 (yellow) is shown as sticks occupying the ATP binding cleft between the kinase N- and C-lobes. (B) Magnified stereo view of SB431542 compound coordination in the binding cleft showing residues contacting SB431542 and the conservation of the ATP binding cleft between TGFβ and ALK2. Hydrogen bonds are represented by dashed lines and a water molecule is depicted as a red sphere.

Figure 2. TβRIca S280T mutant is resistant to SB431542

(A) NIH-3T3 cells were transfected with 3TP-lux, β-gal and constructs expressing TβRI wt, or TβRIca with the indicated ATP binding cleft mutations. After treatment with various doses of SB431542, Luciferase activity was measured and results were normalized to β-gal activity. (B) Worm representation of the N-lobe of TβRI showing residues selected for mutagenesis based on lack of conservation among TGFβ type I receptors (see also sequence alignment in Fig S2). (C) The experiment was conducted as in (A), with the indicated constructs.

Figure 3. Similarities between A8301 and SB431542

(A) Magnified ribbon representation of superimposed co-crystal structures of TβRI- A8301 and TβRI-SB431542 complexes. Dashed lines: Hydrogen bonds; blue sphere: amide nitrogen of His283. (B) NIH-3T3 cells were transfected with 3TP-lux, β-gal and indicated constructs. Dose response was determined by treating cells with varying concentrations of A8301 compound.

Figure 4. ALK2 gatekeeper residue mutant T283S is sensitive to SB431542

(A) NIH-3T3 cells were transfected with BRE-Luc, β -Gal and plasmid expressing ALK2ca or ALK2ca T283S. Dose response was determined by treating cells with varying concentrations of SB431542 compound (A). (B) Superimposition of the ATP binding cleft of T β RI-SB431542 and ALK2-dorsomorphin complexes. (C) The experiment was performed as in (A), but with dorsomorphin.

Table 1
Data collection, structure determination and refinement statistics for T β RI-SB431542 complex

Space group	P2 ₁
Unit cell	a = 41.8 Å, b = 77.7 Å, c = 90.1 Å $\alpha = \beta = \gamma = 90^\circ$
Number of molecules/asu	1
Resolution range (Å)	30 – 1.7 (1.76-1.70)
Observed reflections	183820
Unique Reflections	31955 (3236)
Redundancy	5.8 (5.6)
<i>I</i> / σ <i>I</i>	39.8 (5.4)
Completeness (%)	96.8 (99.8)
<i>R</i> _{sym}	0.085 (0.0383)
<i>R</i> _{work} / <i>R</i> _{free}	0.172/0.205
Rms deviations	
Bonds (Å)	0.009
Angles (°)	1.23
<i>B</i> -factor rmsd (Å ²) Backbone bonds	0.96
Average <i>B</i> -factor (Å ²)	
Protein	19.85
Ligand (SB431542)	15.05
Water	29.28
Ramachandran plot statistics (%)	
Most favored region	97.9%
Additional allowed region	2.1%
Disallowed region	0.0%

Values for the highest resolution shell are given in parentheses

asu, = asymmetric unit