Simultaneous inhibition of c-Abl and Src kinases abrogates the exaggerated expression of profibrotic genes in cultured systemic sclerosis dermal fibroblasts

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Abbreviations

c-Abl:	c-Abelson kinase
Src:	Src kinase
ECM:	Extra cellular matrix
EndoMT:	Endothelial-to-mesenchymal transition
MMPs:	Matrix Metalloproteinases
PDGF:	Platelet-derived growth factor
PKC-δ:	Protein kinase C delta
TGF-β:	Transforming growth factor-β
α-SMA:	α-smooth muscle actin

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ABSTRACT

Objective. *To examine the effects of simultaneous inhibition of c-Abl and Src kinases on the gene expression and in vitro production of profibrotic molecules by dermal fibroblasts from patients with diffuse systemic sclerosis (SSc) of recent onset.*

Methods. *Dermal fibroblasts from normal individuals or from patients with diffuse cutaneous SSc fulfilling the American College of Rheumatology/EULAR SSc classification criteria were cultured in media containing increasing concentrations of the dual c-Abl and Src kinase inhibitor Bosutinib for 24 h. Total soluble collagen in cell culture supernatants was quantified. Western blots were performed for quantitative assessment of type I collagen, fibronectin, and α-smooth muscle actin (α-SMA) production. Quantitative PCR was performed to examine the effects of Bosutinib on the expression of profibrotic and TGF-β-responsive genes in cultured SSc dermal fibroblasts.*

Results. *Simultaneous inhibition of c-Abl and Src kinases with Bosutinib reduced the expression of numerous fibrosis-associated genes including COL1A1, COL1A3, FN, and TGFβ and the production of the corresponding proteins by SSc dermal fibroblasts. Bosutinib also decreased the transition of normal dermal fibroblasts into activated myofibroblasts induced by TGF-β as evidenced by reduction of α-SMA in cell extracts from normal and SSc dermal fibroblasts.*

Conclusion. *Simultaneous inhibition of c-Abl and Src kinases with Bosutinib abrogates the exaggerated expression of genes encoding fibrillar collagens, fibronectin, and TGF-β-responsive genes and reduces type I collagen, fibronectin and α-SMA production by SSc dermal fibroblasts in vitro. Bosutinib also abrogates TGF- β-induced transition of normal fibroblasts to activated myofibroblasts. These results indicate that inhibition of c-Abl and Src kinases activity may be an effective disease modifying antifibrotic therapeutic intervention for SSc.*

Introduction

Systemic sclerosis (SSc) is a systemic autoimmune disorder characterised by severe fibroproliferative vasculopathy mostly affecting the microvasculature, progressive fibrosis of skin and internal organs, and numerous abnormalities in innate, cellular, and humoral immunity (1-7). Cumulative survival for SSc has improved during the past two decades, however, there is still a very high mortality rate with a 30–40% disease-related mortality at ten years (8). Although organ-specific therapies have substantially decreased SSc morbidity and mortality, approved disease modifying therapy for the fibrotic process in SSc does not currently exist. Numerous therapeutic agents have been utilised for SSc disease modification, all with disappointing results leading to the absence of effective treatment for this disease (1-3, 9, 10). Thus, the lack of effective therapy for SSc-associated tissue fibrosis is one of the most challenging unmet needs in SSc management.

The pathogenesis of SSc is complex (4, 9, 11-13). A currently accepted hypothesis posits that SSc is a vascular disease initiated by an unknown injury to endothelial cells of small arterioles in a genetically predisposed host (14-16). The endothelial injury leads to endothelial cell activation, upregulation of adhesion molecules and release of various cytokines, chemokines, and other mediators which in turn are responsible for the recruitment and perivascular cellular infiltration by macrophages and

activated oligoclonal T cells. The cells recruited to the perivascular milieu produce elevated levels of growth factors, most notably TGF-β and CTGF, which are responsible for the initiation and progression of the fibrotic process (17-19). TGF-β is also the most potent inducer of the phenotypic conversion of tissue resident quiescent fibroblasts into activated myofibroblasts (20). The unrestrained myofibroblast activation in affected SSc tissues results in increased synthesis and structural reorganisation of the extracellular matrix (ECM), including the exaggerated deposition of Type I and Type III collagens, fibronectin, COMP and other non-fibrillar ECM macromolecules. Myofibroblast activation also causes inhibition of relevant matrix degrading metalloproteinases (MMPs), further increasing tissue fibrosis. TGF-β also induces endothelial to mesenchymal transition, a complex profibrotic pathway resulting in the phenotypic conversion of endothelial cells into activated myofibroblasts that appears to contribute to SSc fibrotic and vascular alterations as reviewed recently (21-23).

Extensive studies on SSc pathogenesis have demonstrated the crucial role of TGF-β signalling and TGF-β downstream targets in the development of SSc tissue and vascular fibrosis (11-13, 17-19). The signalling pathways involve receptor-activated kinases, as well as non-receptor associated tyrosine kinases such as c-Abl, Src kinases, PI3 kinase, protein kinase Cδ (PKC-δ), and very likely, other not yet identified kinases (24-30). Numerous studies have shown that TGF-β-induced c-Abl activation contributes to SSc fibrosis and vasculopathy (31-34), and that c-Abl is involved in TGF-β-induced endothelial to mesenchymal transition (EndoMT), an effect mediated by a synergistic interaction with PKC-δ (34). The role of c-Abl as an important mediator of tissue fibrosis in SSc has been further supported by studies *in vitro* in cultured fibroblasts as well as *in vivo* in animal models of SSc (31, 32). Furthermore, uncontrolled clinical studies have described improvement in cutaneous and SSc-associated interstitial lung disease in response to c-Abl inhibition by imatinib mesylate therapy (35-38). Another kinase that plays an important role in the pathogenesis of tissue fibrosis in SSc is Src (27, 28, 39), as it has been shown that TGF-β stimulation of normal and SSc dermal fibroblasts strongly activated Src kinase signaling and that Src kinase inhibition with either specific small molecule-inhibitors or by induced expression of a mutant Src protein caused potent antifibrotic effects (39). Several additional studies such as the demonstration of Src kinase Smad 2/3 activation (40), have provided strong evidence of Src kinase participation in SSc-associated tissue fibrosis.

Several *in vitro* and *in vivo* experimental animal models of fibrosis have suggested that inhibition of both c-Abl and Src tyrosine kinases represents an extremely attractive antifibrotic therapeutic approach for SSc and other fibrotic disorders (25, 28). Bosutinib is a second generation BCRc-Abl inhibitor approved by the FDA for treatment of Philadelphia chromosome positive chronic myelogenous leukaemia (41-43). Bosutinib is a potent inhibitor of both c-Abl and Src kinases and it is likely that their inhibition may cause profound alterations in numerous cellular functions and molecular events including those involved in canonical and non-canonical TGF-β signalling (44,45). Furthermore, inhibition of Src activation by Bosutinib is highly specific since it does not affect the PDGF initiated pathways. This point is important because the lack of PDGF pathway inhibition eliminates the serious side effects encountered with kinase inhibitors that inhibit PDGF signalling. Of further importance, the safety profile of Bosutinib is substantially more favourable than that of other kinase inhibitors such as Imatinib and Nilotinib, particularly regarding fluid retention and cardiac conduction problems, and therefore, Bosutinib can be used in patients intolerant to other kinase inhibitors (46).

Here we examined the effects of the simultaneous inhibition of c-Abl and Src kinases with the dual kinase inhibitor Bosutinib on the molecular events mediating the exaggerated fibrotic process in SSc employing dermal fibroblasts isolated from patients with early diffuse SSc.

Methods

Isolation and culture of normal and SSc dermal fibroblasts

Dermal fibroblast cell lines were established from skin biopsies from normal individuals or from patients with SSc followed at the Scleroderma Center of Thomas Jefferson University. The SSc cell lines were established from patients fulfilling the American College of Rheumatology/EULAR 2013 criteria for the classification of SSc (47). All SSc cell lines were obtained from skin biopsies surgically excised from the leading edge of clinically apparent SSc lesions from patients with diffuse SSc of recent onset as described previously (29). Normal fibroblasts were obtained from age- and gender-matched normal volunteers or from individuals without SSc undergoing orthopedic surgical procedures. All studies were approved by the Thomas Jefferson University IRB in accordance with the Declaration of Helsinki for the study of human subjects. Fibroblast cultures were established from the biopsies employing procedures described previously (29). For all studies, only early passage fibroblasts (<8) were employed. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Gibco), 1% vitamins, 2 mM glutamine, antibiotics and fungizone, and incubated at 37°C in a 5% CO_2 humidified atmosphere. The cell cultures were supplemented with 50 μg/ml ascorbic acid for 24h prior to initiation of the experiments to optimise their collagen production levels.

Bosutinib treatment of normal and SSc dermal fibroblasts

Eight different strains of SSc fibroblasts and four different strains of normal fibroblasts were studied. The cells were plated in 60 mm plastic culture dishes and cultured until they reached confluency. For assessment of Bosutinib cytotoxicity, two SSc and two normal cell lines were studied. The cells were cultured in media containing 0, 3, 6, 15, 30, and 60 nM of Bosutinib for 24h followed by determination of cell numbers and cytotoxicity assessment employing the colorimetric WST-1 assay (Roche). To examine Bosutinib

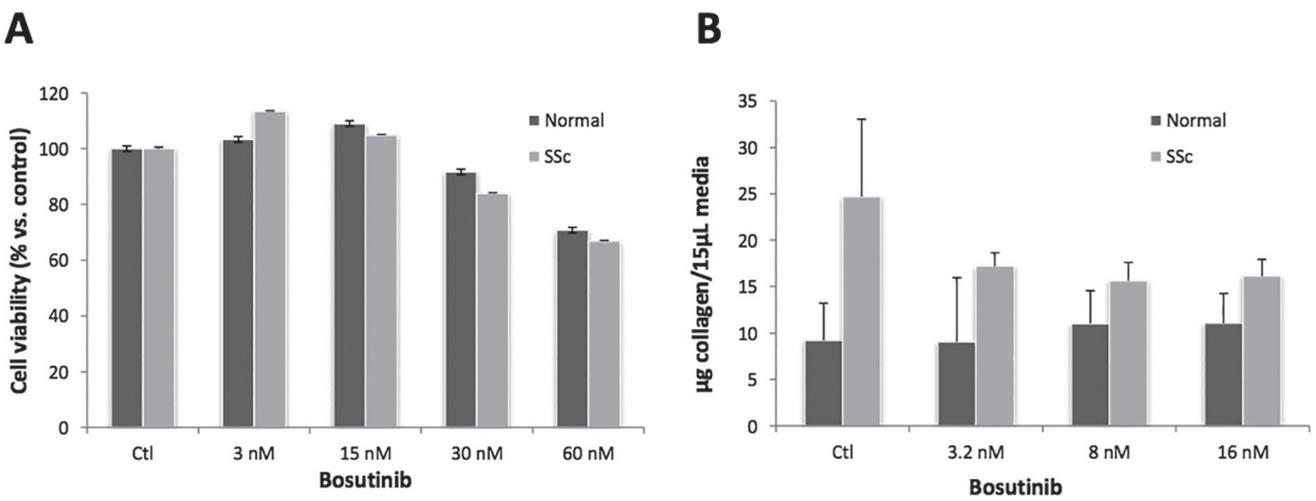

Fig. 1. Effects of Bosutinib on fibroblast viability and collagen production. **A**. Effect of Bosutinib treatment on cellular viability of cultured normal and SSc dermal fibroblasts. Cellular cytotoxicity was assessed with the colorimetric WST-1 assay. **B**. Effect of Bosutinib treatment on the amounts of collagen secreted by cultured normal and SSc dermal fibroblasts. The total amount of collagen in the culture media of normal and SSc fibroblasts during a 24 h culture period was measured using the Sircol assay. The data shown are the average of the Bosutinib effects on collagen secreted by two normal and two SSc cell lines.

effects on TGF-β-stimulated collagen production and α-SMA protein levels, two normal dermal fibroblast cell lines were treated with TGF-β1 (10 ng/mL) alone or with TGF-β1 plus 10nM Bosutinib for 24h.

Bosutinib effect on collagen production
Total soluble collagen in cell culture supernatants was quantified using the Sircol collagen assay (Biocolor). One mL of Sirius red dye was added to 15 µL aliquots of each supernatant, followed by incubation under gentle rotation for 30 min at room temperature. After centrifugation at 12,000g for 10 min, the collagen-bound dye was redissolved with 1 mL of 0.5 M NaOH and the absorbance was measured at 540nm.

Assessment of fibroblast macromolecule production employing Western blots
Western blots of cell culture media and of cell extracts were performed for quantitative assessment of type I collagen production, fibronectin, and α-SMA employing specific human anti-collagen type I (SouthernBiotech), fibronectin (Santa Cruz Biotechnology) and α-SMA (Abcam) antibodies. Samples of culture media or cell extracts were heated to 95°C for 5 min and then resolved on a NUPAGE 4-12% Bis-Tris gradient gel (Novex Life Technologies). The proteins were transferred to nitrocellulose iBlot gel transfer stacks, and then the stacks were blocked for 1 h at room temperature in blocking buffer (Li-Cor Biosciences). The antibodies were added to the buffer and incubated with the blots overnight at 4 ºC. Western blots were analysed with the Odyssey infrared image system (LI-COR). The protein bands were visualised with anti-mouse or anti-rabbit antibodies (LI-COR) and the intensity of the bands was measured by the Odissey IR reader. The values obtained were normalised to tubulin levels and are presented in bar graphs as arbitrary fluorescence units.

Assessment of the effects of simultaneous c-Abl/Src kinase inhibition on ECM macromolecule transcript levels by semi-quantitative PCR
To examine the effects of simultaneous c-Abl/Src kinase inhibition on the profibrotic gene expression profile of SSc dermal fibroblasts and myofibroblast activation, normal and SSc fibroblasts were grown in culture under control conditions (untreated) or treated with various Bosutinib concentrations. The cells were harvested following 24 h of treatment and total RNA extracted using the TRIzol reagent. RT-PCR amplification was performed with 1 µg of RNA to generate first strand cDNA employing SuperScript II reverse transcriptase (Invitrogen). PCR was performed using specific primers for COL1A2 and COL3A1, FN1, ACTA2, CTGF and TWIST1. The transcripts levels of TGF-β1, TGF-β2, and TGF-β3 were also assessed. Quantitative real time PCR was performed utilising the QuantiTect SYBR green PCR kit on an ABI PRISM 7700 real time PCR machine. The values obtained were normalised to those of 18S RNA. Relative quantitation was employed to determine gene expression levels in Bosutinib treated normal and SSc cultured dermal fibroblasts as described previously (48).

Results

Bosutinib effects on phosphorylation levels of Src and Abl in normal and SSc dermal fibroblasts
To confirm Bosutinib inhibition of Src and Abl phosphorylation in cultured SSc dermal fibroblasts we used the Kinex™ antibody microarray (Kinexus), which allows the identification and semiquantitative assessment of phosphorylation events induced by essentially all known human kinases employing pan-specific antibodies and phospho-site-specific antibodies (49). As a control we also examined the phosphorylation of PKC-δ, a kinase that has not been shown to be affected by Bosutinib. The Kinex™ KAM-1.2 chip compares two samples at a time utilising 540 pan-specific antibodies and 270 phosphosite-specific antibodies to detect specific

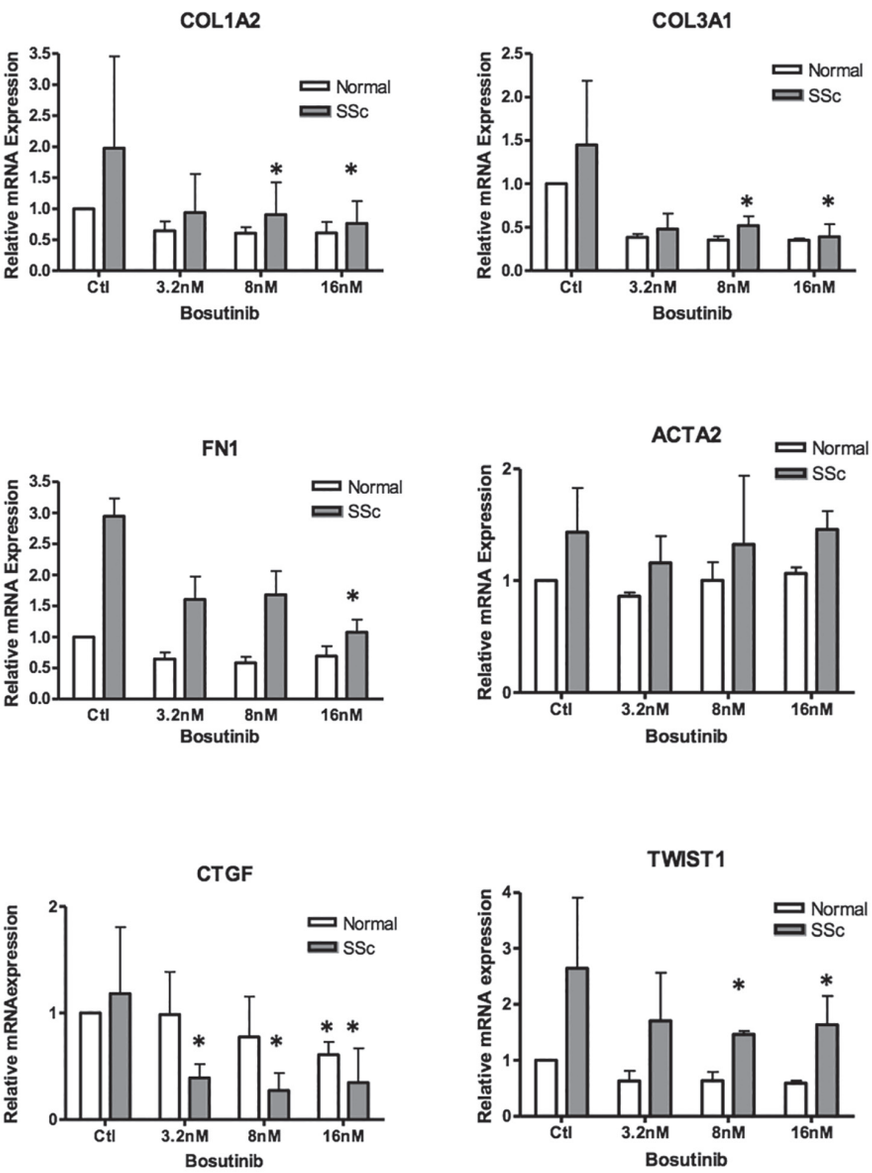

Fig. 2. Effect of Bosutinib on fibroblast expression of genes encoding extracellular matrix proteins, α-SMA, CTGF and TWIST1. Relative mRNA expression levels of COL1A2, COL3A1, FN1, ACTA2, CTGF and TWIST1 assessed by Real Time PCR analysis in normal and SSc dermal fibroblasts under treatment with Bosutinib. The level of 18S expression was used as endogenous control. The results are the average of three separate experiments.
*Statistically significant difference from gene expression in untreated (Ctl) cells (*p*-value <0.05).

differences in the phosphorylation of at least 248 phosphosites between the two samples. These antibody microarrays are highly specific and sensitive owing to the utilisation of fluorescent labeling of all proteins in the cell lysates before their capture by the antibodies bound to the microchips (49). Two SSc cell lines were treated with 16 nM Bosutinib for 24 h and following removal of the culture media and extensive washing, the cell layers were utilised for Kinexus kinomic arrays comparing Bosutinib treated with untreated cells. The results showed that Bosutinib inhibited Src phosphorylation by 25% in one cell line and by 28% in the other cell line whereas, Abl phosphorylation was reduced by 16% in one cell line and by 41% in the other cell line. In contrast, no changes were found in PKC-δ phosphorylation in either cell line following treatment of Bosutinib (Data not shown). Therefore, the results indicated that Bosutinib caused substantial reduction of Src and Abl phosphorylation but did not affect the phosphorylation of an unrelated kinase.

Bosutinib effects on normal and SSc dermal fibroblast viability

Normal and SSc dermal fibroblast cell lines were assayed for Bosutinib cytotoxicity. The results shown in Figure 1A represent the mean ± SD of three independent experiments of the proportion of viable cells remaining following 24 h treatment with Bosutinib (3 nM to 60 nM) compared with untreated cells (Ctl). The results indicated that there were grossly dose dependent cytotoxic effects of Bosutinib on cellular viability at concentrations of 30 nM and 60 nM but there were no significant cytotoxic effects at the lower concentrations examined. Therefore, all subsequent studies were performed employing Bosutinib concentrations lower than 30 nM. The results also showed differences in the cytotoxic effects dependent on whether the cell lines studied were normal or SSc cells. At the 30 nM concentration of Bosutinib the maximal observed cytotoxicity was 8% for normal cells whereas it was 18% for SSc cells.

Bosutinib effects on normal and SSc dermal fibroblast total collagen production

Total soluble collagen was quantified in cell culture supernatants of two normal and two SSc cell lines treated with 0, 3.2, 8, and 16 nM of Bosutinib for 24 h. Data representing the mean ± SD of collagen content (μg) of three samples of each cell line assayed in triplicate are shown in Figure 1B. The untreated SSc fibroblasts displayed a greater than two-fold higher collagen production compared to untreated normal fibroblasts. The results showed a dose-related reduction of SSc fibroblast collagen production by Bosutinib. Quantitative assessment indicated that in the SSc fibroblasts Bosutinib at the 3.2 nM concentration caused an average of 35% reduction in collagen production and at 8 nM concentration the inhibition reached 40 to 42%. There was no additional inhibitory effect at the higher concentration (16 nM) studied. In contrast, collagen production was not significantly affected by Bosutinib in the normal fibroblasts even at the higher concentration examined (16 nM).

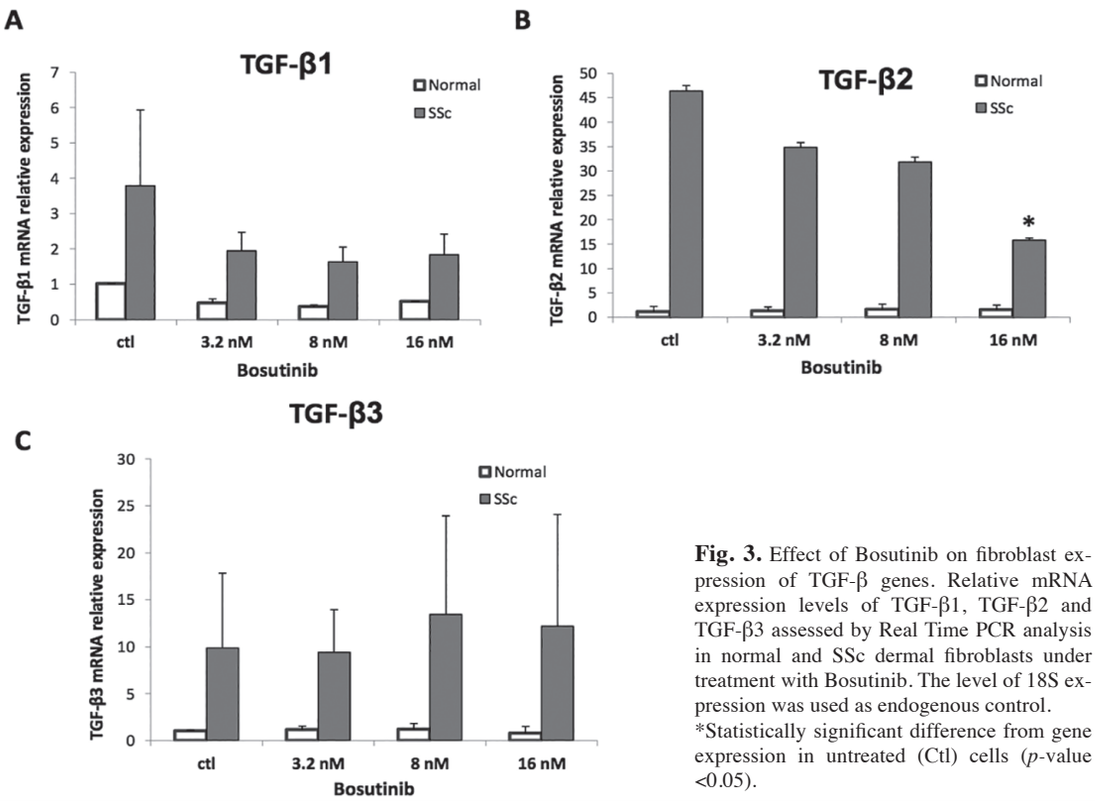

Fig. 3. Effect of Bosutinib on fibroblast expression of TGF-β genes. Relative mRNA expression levels of TGF-β1, TGF-β2 and TGF-β3 assessed by Real Time PCR analysis in normal and SSc dermal fibroblasts under treatment with Bosutinib. The level of 18S expression was used as endogenous control.
*Statistically significant difference from gene expression in untreated (Ctl) cells (*p*-value <0.05).

Bosutinib effects on normal and SSc dermal fibroblast gene expression

The expression levels of several relevant genes assessed by RTPCR showed that SSc fibroblasts displayed remarkable increases in the expression of profibrotic genes compared with normal fibroblasts. For example, the expression of COL1A2 was approximately two-fold, the expression of COL3A1 and ACTA2 was approximately one and a half-fold, and, quite remarkably, the expression of FN1 and TWIST1 was about three-fold greater in the SSc cells compared to the normal cells. Bosutinib caused a dose related decrease of mRNA transcripts for COL1A2, COL3A1 and FN1 in both normal and SSc dermal fibroblasts although these effects were only statistically significant for the SSc cells (Fig. 2). However, Bosutinib did not have a significant effect on ACTA2 expression levels in either cell type. Bosutinib also caused a dose-related reduction in the expression of genes encoding CTGF and TWIST1 (Fig. 2) and, TGF-β1 and TGF-β2, reaching statistical significance at the higher concentration tested (Fig. 3). Bosutinib, however, did not have any detectable effects on TGF-β3 expression levels.

Bosutinib effects on fibronectin and type I collagen protein production by normal and SSc dermal fibroblasts

To validate at the protein level the gene expression results we quantified the amounts of type I collagen and FN produced and secreted by control and Bosutinib-treated normal and SSc dermal fibroblasts employing Western blots. The results showed a marked reduction of fibronectin production of about 40% at a concentration of 16 nM of Bosutinib in both normal and SSc dermal fibroblasts. Remarkably, Bosutinib at the same concentration caused a greater than 50% reduction in the markedly increased levels (greater than two-fold) of collagen type I produced by the SSc cells, whereas it only caused a 25% reduction in collagen production in normal fibroblasts (Fig. 4).

Bosutinib effects on α-SMA protein and TGF-β1 protein levels in normal and SSc dermal fibroblasts

Western blots of cell extracts were performed to examine the effects of Bosutinib on α- SMA, a marker of myofibroblast activation. The baseline concentration of α-SMA protein was significantly elevated in SSc dermal fibroblasts compared to normal cells (Fig. 5A). We then examined the effects of Bosutinib on α-SMA produced by TGF-β stimulated normal fibroblasts. For this purpose, two cell lines of normal fibroblasts were treated with 10 ng/mL of TGF-β1 alone or with 10 ng/mL of TGF-β1 plus 10nM Bosutinib for 24h. Western blots of cell extracts showed that TGF-β treatment increased α-SMA production by greater than 10-fold and that Bosutinib treatment resulted in a reduction of approximately 50% of this effect (Fig. 5B). Subsequent studies were performed to examine whether Bosutinib was also capable of reversing the myofibroblast phenotype of SSc fibroblasts during a short term culture and to determine whether Bosutinib exerted any effects on production of the potent profibrotic growth factor TGF-β. The results showed that at the highest concentration used (16nM), Bosutinib caused a significant reduction of α-SMA protein levels in the SSc fibroblasts compared with untreated SSc fibroblasts (Fig. 5C). Analysis of the effects of Bosutinib on TGF-β1 production by the SSc cells are shown in Figure 5D. Western blot of cell extracts from two SSc dermal fibroblast cell lines showed a dose dependent de-

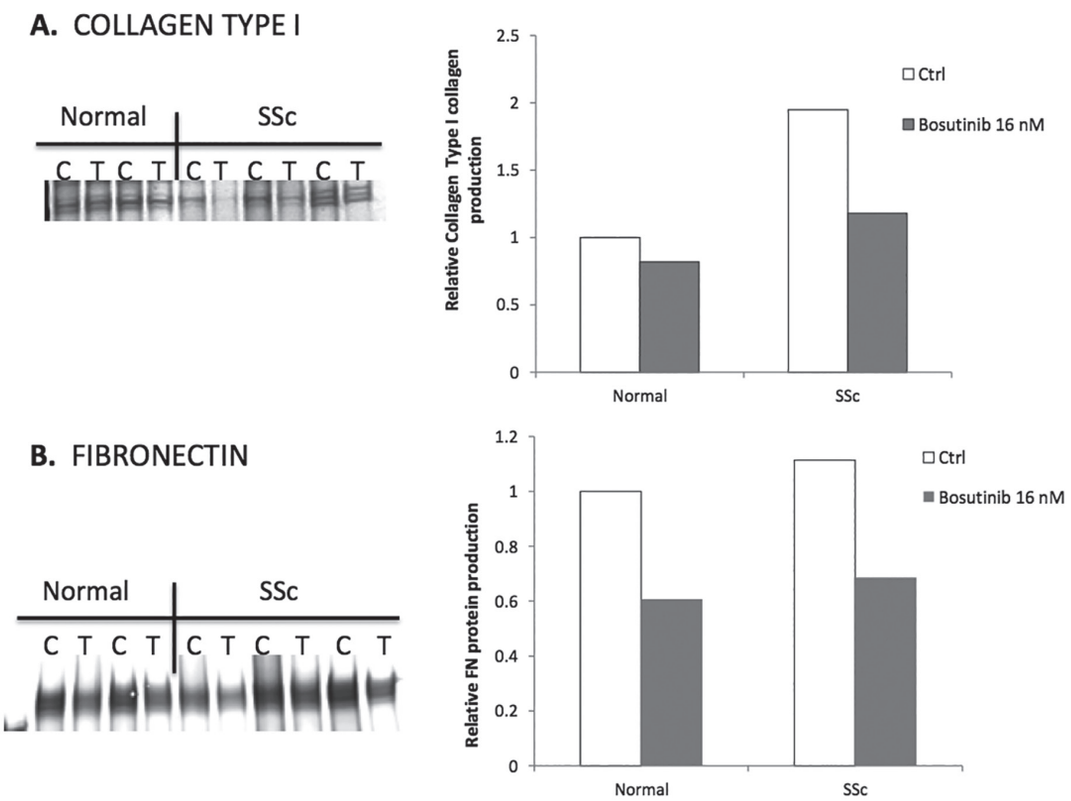

Fig. 4. Effect of Bosutinib on fibroblast collagen type I and fibronectin protein production. Western blot analysis of Type I collagen and fibronectin secreted into the culture media by untreated (control) and Bosutinib treated (16nM) normal and SSc dermal fibroblasts.
Bar graphs represent the means ± SD from three separate experiments.
C: Control;
T: Bosutinib treated samples.

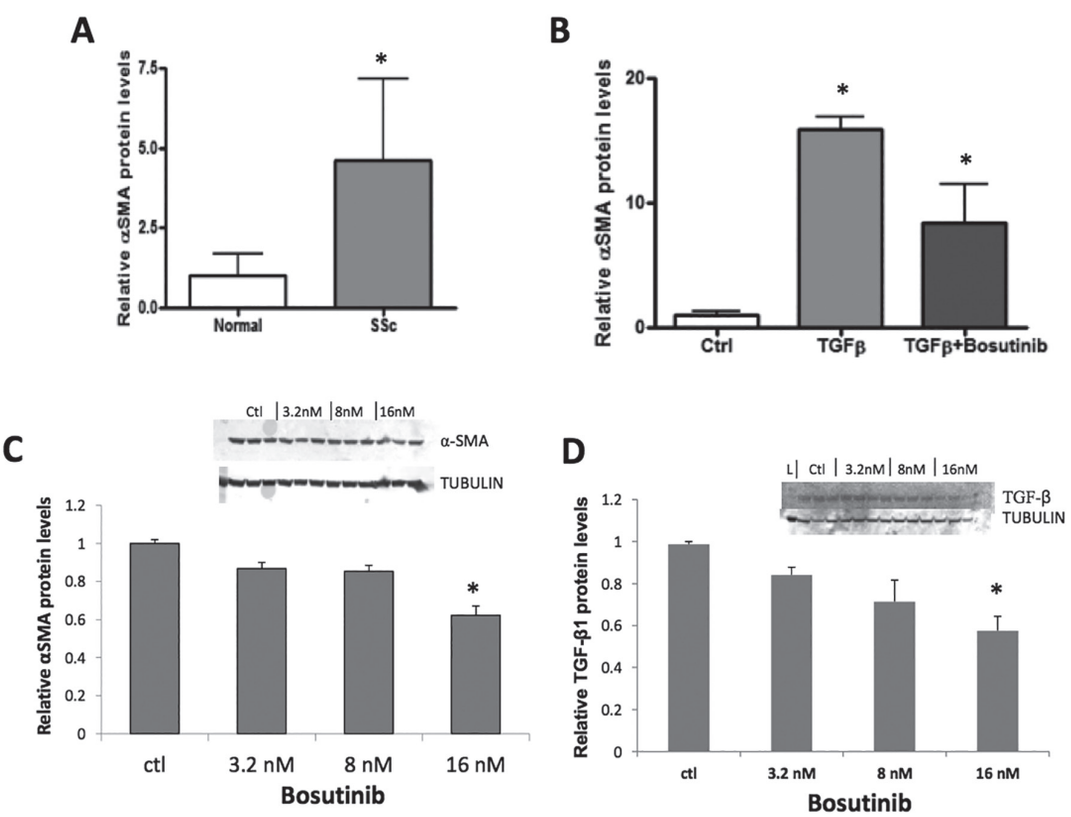

Fig. 5. Effect of Bosutinib on fibroblast α-SMA and TGF-β1 protein levels in normal and SSc cells. **A.** Western blot analysis of α-SMA produced by normal and SSc dermal fibroblasts.
B. Western blot analysis of α-SMA produced by normal dermal fibroblasts treated with TGF-β1 (10 ng/mL) alone or with TGF-β1 plus Bosutinib (10nM).
C-D. Western blot analysis of α-SMA and TGF-β1 protein levels present in the SSc dermal fibroblast cells extracts from control and Bosutinib (10nM) treated cells. Bar graphs represent protein levels following normalisation with the intensity of the tubulin band. Two normal (B) and 4 SSc cultured dermal fibroblast cell lines were examined (C, D).
*Statistically significant difference from protein production in untreated (Ctl) cells (p-value <0.05).

crease in TGF-β1 levels following Bosutinib treatment reaching approximately a 40% reduction at 16nM, a highly statistically significant effect.

Discussion

There is a strong rationale for studying the effects of tyrosine kinase inhibition as an antifibrotic approach for SSc (24, 25, 28, 33). Although the pathogenesis of skin and tissue fibrosis in SSc is not completely understood, the hallmark of SSc tissue involvement is a progres-

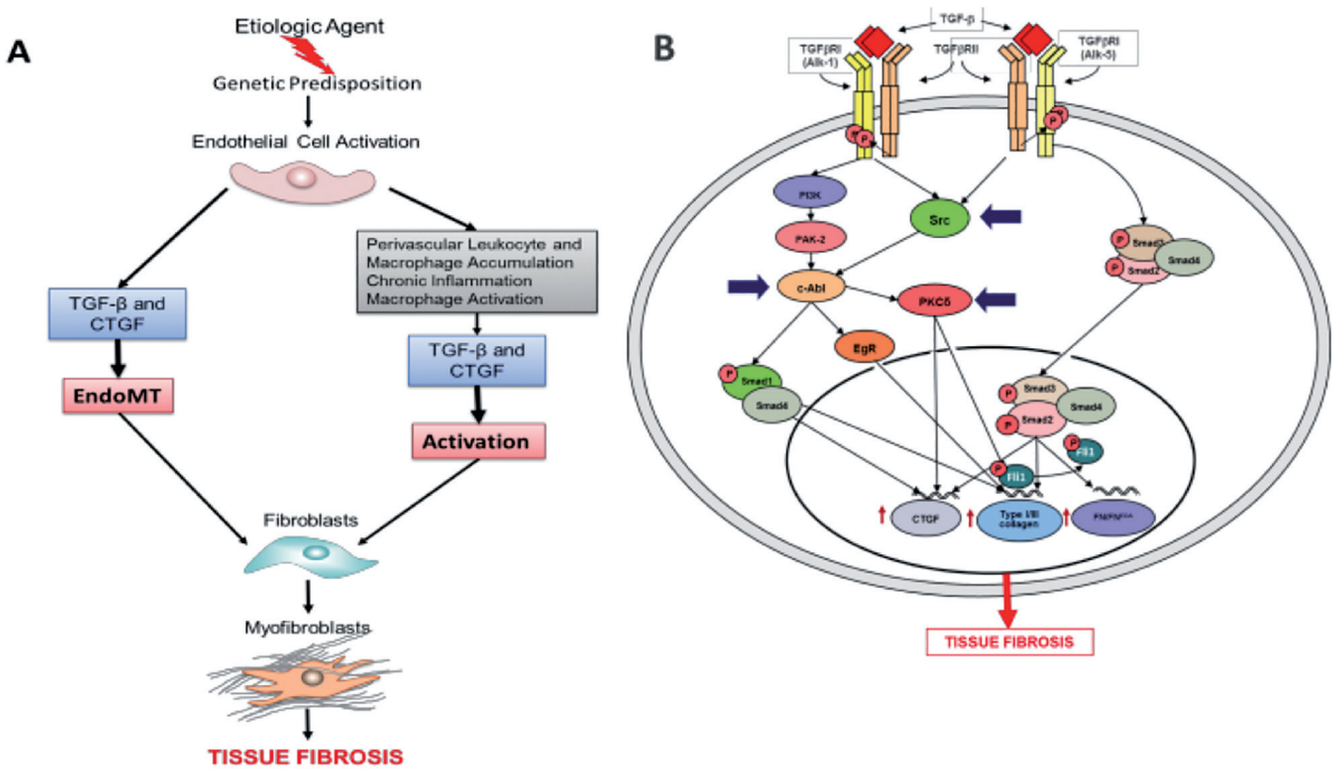

Fig. 6. Diagrammatic representation of the role of endothelial cell injury in the pathogenesis of SSc tissue fibrosis and the antifibrotic effects of Bosutinib. **A.** Diagram illustrating the role of endothelial cell alterations in SSc pathogenesis. Endothelial injury may be initiated by numerous mechanisms in a genetically predisposed host including the actions of free radicals or chemical and microbial agents that injure endothelium. Endothelial injury is associated with the release of potent profibrotic cytokines, growth factors and other endothelial cell products that in association with the effects of activated profibrotic macrophages cause activation of resident fibroblasts. Endothelial cell injury may also induce the phenotypic conversion of endothelial cells into activated myofibroblasts through the EndoMT process. Fibroblast activation in turn results in fibrosis in affected tissues and perpetuates the vasculopathy and vascular wall fibrosis. **B.** Diagram showing the TGF-β canonical and non-canonical signalling pathways leading to tissue fibrosis. The thick arrows indicate the sites of potential inhibition induced by Bosutinib [Adapted from refs. 21, 22].

sive fibrotic and sclerotic process initiated by activation of microvascular endothelial cells, caused by currently unidentified mechanisms, in a genetically predisposed host. The endothelial cell injury triggers a complex cascade of molecular alterations including release of cytokines, chemokines and growth factors, initiation of integrin-mediated pathways, and marked gene expression changes and it has been suggested that vascular alterations are a primary event in SSc pathogenesis (14-16). Numerous studies (14-16, 21-23) have shown that in SSc the endothelial cells induce profibrotic activation of quiescent resident tissue fibroblasts. It has also been shown that endothelial cells in SSc undergo a phenotypic transition into activated myofibroblasts, a process known as endothelial to mesenchymal transition or EndoMT (21-23) causing progressively increasing tissue fibrosis in the dermis and various internal organs. This postulated sequence of events is diagrammatically illustrated in Figure 6A and is supported by extensive experimental evidence (14-16, 21-23).

Although the detailed molecular mechanisms involved in the fibroblast to myofibroblast transition have not been fully identified, it has become apparent that numerous transcription factors such as SNAI1, SNAI2 and TWIST have been implicated in the final step of this process. The increased expression of TWIST1 in SSc fibroblasts described here also very likely plays an important role in myofibroblast activation in SSc. Thus, TWIST1 expression inhibition by Bosutinib may also be considered an additional molecular mechanism supporting its use as a therapeutic intervention to abrogate the exaggerated ECM overproduction of SSc and other fibrotic diseases.

We examined here the hypothesis that crucial phosphorylation reactions in the activation of fibroblasts involve the c-Abl and Src tyrosine kinases and that activation of these kinases is ultimately responsible for the profibrotic activation of myofibroblasts resulting in the progressive tissue fibrosis in SSc. We further posited that Bosutinib disruption of fibroblast c- Abl and Src tyrosine kinase signalling pathways should abrogate the profibrotic activation and gene expression of SSc fibroblasts as illustrated diagrammatically in Figure 6B. It should also be emphasised that c-Abl and Src kinases may induce the activation of other intracellular kinases through phosphorylation of crucial tyrosine residues in their folding regions and/or in the active sites. These events may further aggravate the SSc fibrotic process. One relevant example of this highly important circuitry is PKC-δ, a kinase recently identified as a potent stimulator of the profibrotic phenotype of mesenchymal cells in SSc (29). Numerous studies have shown that c-Abl and Src kinases phosphorylate various PKC-δ tyrosine residues including Y-64

(phosphorylated by c-Abl) and Y-311 (phosphorylated by either c-Abl or c-Src) resulting in the activation of numerous PKC-δ effects and of its nuclear translocation where it can induce potent and varied effects on gene expression (50-52). Indeed, a recent study demonstrated that inhibition of PKC-δ activity protected salivary glands from the fibrotic effects of ionising radiation (53). The purpose of the study described here was to examine the effects of Bosutinib, an improved second generation tyrosine kinase inhibitor that simultaneously targets the c-Abl and Src kinases (41, 42), two kinases that we postulate are crucial components of the pathogenesis of TGF-β-associated tissue fibrosis, on gene expression and production of pro-fibrotic molecules by dermal fibroblasts from patients with SSc *in vitro*. The results demonstrate that the simultaneous inhibition of c-Abl and Src kinases with Bosutinib abrogates the exaggerated expression of numerous profibrotic genes and the production of fibrosis-related ECM macromolecular components by SSc fibroblasts *in vitro* including collagens, fibronectin, CTGF, and quite remarkably, also TGF-β1 and TWIST1. These potent effects were observed at non-cytotoxic Bosutinib concentrations and were substantially more pronounced in the SSc dermal fibroblasts than in the normal cells. These observations provide strong evidence supporting the concept that c-Abl/Src kinase inhibition may interfere favourably with the early events in SSc fibrosis pathogenesis, and therefore, may result in clinically significant improvement in the progressive fibrotic process characteristic of the disease. These results also provide a strong rationale for the subsequent performance of clinical trials to examine the potential antifibrotic effects of Bosutinib and to determine whether this drug may represent an effective SSc disease modifying therapeutic agent as discussed recently (54).

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