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Flavopiridol inhibits TGF-β-stimulated biglycan synthesis by blocking linker region phosphorylation and nuclear translocation of Smad2

Muhamad A. Rostam, Aravindra Shajimoon, Danielle Kamato, Partha Mitra, Terrence J. Piva, Robel Getachew, Yingnan Cao, Wenhua Zheng, Narin Osman, and Peter J. Little.

Kulliyyah of Allied Health Sciences, International Islamic University Malaysia, Kuantan, Pahang, Malaysia - MAR

School of Health and Biomedical Sciences, RMIT University, Bundoora, Victoria, Australia – AS, TJP, RG, NO, PJL

School of Pharmacy, Pharmacy Australia Centre of Excellence, The University of Queensland, Woolloongabba, Queensland, Australia – DK, PM, PJL

Department of Pharmacy, Xinhua College of Sun Yat-sen University, Tianhe District, Guangzhou, 510520, China University- YC, PJL

Faculty of Health Sciences, University of Macau, Taipa, Macau, China – WZ

Monash University, Departments of Medicine and Immunology, Central and Eastern Clinical School, Alfred Health, Melbourne, Victoria, Australia.- NO

Running title: Flavopiridol inhibits Smad linker phosphorylation

Corresponding author :

Prof Peter J. Little,

School of Pharmacy, University of Queensland,

20 Cornwall Street, Woolloongabba, Queensland, Australia

T: +61733461991

F: +61733461999

E: P.little@uq.edu.au

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List of Abbreviations

Alk-5: Activin-like Kinase

C4ST-1: chondroitin 4-sulfotransferase

CAMK: calcium calmodulin dependent kinase

CDK: cyclin-dependent kinases

ChGn-2: chondroitin N-acetylgalactosaminyltransferase-2

ChIP: chromatin immunoprecipitation

ChSy-1: chondroitin sulfate synthase-1

CPC: Cetylpyridinium chloride

CS/DS: chondroitin sulfate/dermatan sulfate

ERK: extracellular-signal regulated kinase

GAG: glycosaminoglycan

GSK: glycogen synthase kinase

JNK: Jun N-terminal kinase

LDL: low-density lipoproteins

MAPK: mitogen activated protein kinase

PI3K: phosphatidylinositol 3′-kinase

ROCK: rho-associated protein kinase

TGF-β: Transforming Growth Factor Beta

TGFβRI: TGF-β receptor

VSMCs: vascular smooth muscle cells

XT-1: xylosyltransferase-1

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ABSTRACT

Transforming Growth Factor (TGF)-β is a pleiotropic growth factor implicated in the development of atherosclerosis for its role in mediating glycosaminoglycan (GAG) chain hyperelongation on the proteoglycan biglycan, a phenomenon that increases the binding of atherogenic lipoproteins in the vessel wall. Phosphorylation of the transcription factor Smad has emerged as a critical step in the signalling pathways that control the synthesis of biglycan, both the core protein and the GAG chains. We have used flavopiridol, a well-known cyclin-dependent kinase (CDK) inhibitor, to study the role of linker region phosphorylation in the TGF-β-stimulated synthesis of biglycan. We used radiosulfate incorporation and SDS-PAGE to assess proteoglycan synthesis; RT-PCR to assess gene expression and chromatin immunoprecipitation (ChIP) to assess the binding of Smads to the promoter region of GAG synthesis genes. Flavopiridol blocked TGF-β-stimulated synthesis of mRNA for the GAG synthesizing enzymes, chondroitin 4-sulfotransferase (C4ST-1), chondroitin sulfate synthase-1 (ChSy-1), TGF-β mediated proteoglycans synthesis as well as GAG hyperelongation were also blocked by flavopiridol. Flavopiridol blocked TGF-β-stimulated Smad2 phosphorylation at both the serine triplet and the isolated threonine residue in the linker region. The binding of Smad to the promoter region of the C4ST-1 and ChSy-1 genes was stimulated by TGF-β and this response was blocked by flavopiridol demonstrating that linker region phosphorylated Smad can pass to the nucleus and positively regulate transcription. These results demonstrate the validity of the kinases, which phosphorylate the Smad linker region as potential therapeutic target(s) for the development of an agent to prevent atherosclerosis.

INTRODUCTION

Cardiovascular disease is the leading cause of death among adults worldwide and atherosclerosis is the major underlying pathology (Nigro et al., 2006; Deaton et al., 2011). The early stages of the development of atherosclerotic lesions in human pathology occur due to the accumulation of atherogenic lipids on proteoglycans, mostly the chondroitin sulfate/dermatan sulfate (CS/DS) proteoglycan, biglycan (Nakashima et al., 2007; Nakashima et al., 2008). Modification of the synthesis and structure of proteoglycans, predominantly glycosaminoglycan (GAG) chain hyperelongation, results in increased binding to apolipoproteins on lipids *in vitro* (Little et al., 2002; Ballinger et al., 2004; Little et al., 2008) leading to the trapping of atherogenic low-density lipoproteins (LDL) in the blood vessel wall. GAG chain hyperelongation occurs by growth factor stimulation of the expression of the GAG chain synthesizing enzymes in vascular smooth muscle cells (VSMCs) (Little et al., 2002; Ivey and Little, 2008; Yang et al., 2009; Burch et al., 2010; Cardoso et al., 2010; Yang et al., 2010; Osman et al., 2014). Prevention of this change in proteoglycan structure by targeting the hormone and growth factor signalling pathways has been proposed and demonstrated as a therapeutic target to prevent atherosclerosis (Ballinger et al., 2004; Little et al., 2007; Osman et al., 2008; Little et al., 2011); the signalling pathways are the preferred target because these pathways are specific for VSMCs which is not the case for the action of the elongation enzymes themselves which are ubiquitously expressed and functional in most tissues of the body. Vasoactive growth factors mediate proteoglycan core protein expression and independently modify the structure of the GAG chains on proteoglycans by stimulating the expression of the genes for the GAG chain elongation enzymes (Osman et al., 2011; Kamato et al., 2016; Rostam et al., 2016).

Transforming growth factor-β (TGF-β) is a pleiotropic growth factor linked to vascular disease (Bobik et al., 1999) which acts via serine/threonine kinase cell surface receptors (Derynck

and Zhang, 2003; Massagué et al., 2005). TGF-β stimulates the expression of biglycan in VSMCs and also stimulates the elongation of its GAG chains which results in hyperelongated GAG chains that show increased binding of the modified biglycan to LDL (Little et al., 2002; Burch et al., 2010; Rostam et al., 2016). TGF-β signalling involves the regulation of gene expression by Smad transcription factors (Massagué et al., 2005). This signalling pathway is responsible for the transcription and translation of enzymes which can regulate GAG chain synthesis and structure (Yang et al., 2009). Anggraeni *et al* 2011 showed a correlation between increased mRNA expression of the synthesizing enzymes chondroitin 4-sulfotransferase (C4ST-1) and chondroitin N-acetylgalactosaminyltransferase-2 (ChGn-2) involved in chondroitin sulfate (CS) and GAG elongation with lipid deposition and the development of atherosclerosis in a mouse model (Anggraeni et al., 2011).

To date, most studies on TGF-β signalling pathways have focused on the response of TGF-β receptors (TGFβRI) also known as Activin-like kinase (Alk-5), directly activate Smad transcription factors (Smad2 or Smad3) in the carboxy terminus (Derynck and Zhang, 2003; Massagué et al., 2005). However, specific residues of the Smad linker region phosphorylation can regulate a wide range of cellular events (Kamato et al., 2013; Yumoto et al., 2013). The Smad linker region pathway signals through activation of serine/threonine kinases including the mitogen activated protein kinase (MAPK), extracellular-signal regulated kinase (ERK), Jun N-terminal kinase (JNK) and p38 kinase, Akt, cyclin-dependent kinases (CDK), rho-associated protein kinase (ROCK), calcium calmodulin dependent (CAM) kinase and glycogen synthase kinase (GSK)-3 as well as via activation of the tyrosine kinase Src and phosphatidylinositol 3′-kinase (PI3K) (Kamato et al., 2013). MAPK and CDK show a preference for specific serine/threonine residues in the linker region essential for the regulation, stability, activity and nuclear transport of R-Smads

(Matsuzaki et al., 2009; Burch et al., 2011).

In the original characterization of the cell biology of linker region phosphorylation of Smad transcription factors, Ras-dependent linker region phosphorylation inhibited the nuclear translocation and hence the gene regulatory action of phosphorylated Smad (Kretzschmar et al., 1997). However, and in contrast, our data has demonstrated that Smad linker region phosphorylation is essential to the process of GAG chain elongation and hyperelongation on biglycan (Burch et al., 2010). Thus, Smad linker region phosphorylation and the question of the role of linker region phosphorylation on the nuclear translocation of these transcription factors has not been answered.

In an earlier study of the role of various kinase inhibitors in blocking Smad phosphorylation and GAG hyperelongation, we identified flavopiridol as a potent inhibitor of GAG elongation (Rostam et al., 2016). Flavopiridol (Alvocidib) was the first cyclin-dependent kinase inhibitor to be tested in human clinical trials (Senderowicz and Sausville, 2000a). It is a flavonoid alkaloid and CDK9 kinase inhibitor previously under clinical development for the treatment of acute myeloid leukaemia (Mariaule and Belmont, 2014). Flavopiridol thus presents as a useful tool to study the role of linker region phosphorylation in TGF-β-stimulated biglycan synthesis. In a study of kinase inhibitors and Smad linker phosphorylation and TGF-β stimulation of GAG gene expression, GAG elongation and core protein (biglycan) expression, flavopiridol had very potent and substantial inhibitory effects (Rostam et al., 2016). We have reported on the role of Smad linker region phosphorylation on driving the expression of the genes for the enzymes which are rate limiting for the elongation of GAG chains on biglycan and also the resultant size of the biglycan molecules related to changes in the size of the chondroitin sulfate/dermatan sulfate (CS/DS) GAG chains (Rostam et al., 2016). So, based on the potency and efficacy of flavopiridol

in this *in vitro* model of atherogenesis, we have used this compound to further explore the role of Smad linker region phosphorylation in driving the expression of GAG synthesizing genes.

We report that flavopiridol inhibits GAG elongation on biglycan, inhibits Smad linker region phosphorylation and Smad binding to consensus sites on the promoter regions of C4ST-1 and ChSy-1 demonstrating a pathway which convincingly shows the involvement of Smads in GAG hyperelongation in VSMCs. The data points to very specific pathways that may represent therapeutic targets for the prevention of the changes in biglycan structure which mediate lipid binding in the vessel wall as the earliest stage of human atherosclerosis.

EXPERIMENTAL PROCEDURES

Materials- Human recombinant TGF-β was purchased from R&D systems (Minneapolis, USA). Foetal bovine serum (FBS) was purchased from CSL (Parkville, Australia). Cell culture materials were from GIBCO BRL (Grand Island, USA). Trypsin-versene, antibiotics (penicillin, streptomycin), flavopiridol and SB431542, Dulbecco's phosphate-buffered saline (PBS) (10X), sodium dodecyl sulfate (SDS), and 2-Mercaptoethanol and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, USA). The 18S primer, RNeasy® Mini Kit, QuantiTect® Reverse Transcription Kit, QuantiFast™ SYBR® Green PCR kit and the Rotor-Gene® Q Series software were from Qiagen (Chadstone, Australia). Anti-rabbit immunoglobulin-G (IgG) horseradish peroxidase (HRP), anti-mouse IgG HRP, anti-phospho-Smad2 (Ser245/255/250), anti-α tubulin and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were from Cell Signaling Technology (Danvers, USA). Primers for GAG synthesizing genes and biglycan (table 1) were purchased from GeneWorks Pty LTD (Thebarton, Australia) Amersham ECL Prime chemiluminescent detection reagent was from GE Healthcare (Paramatta, Australia). Bovine Serum Albumin (BSA) was from Bovogen Biologicals Pty. Ltd. (Keilor, Australia). Bicinchoninic acid protein assay kit was from Thermo Scientific (Rockford, USA). Chemiluminescent molecular weight marker (MagicMark XP) and chromogenic molecular weight marker (BenchMark) were from Invitrogen (Auckland, New Zealand). TEMED, Tris Base and Glycine were from Amresco (Solon, USA). Tween-20, 30% acrylamide/Bis solution, ammonium persulfate, Polyvinylidene fluoride (PVDF) membrane and Image Lab V5.0 imaging software were from BioRad Laboratories (Hercules, USA). Phospho-Smad2/3L (Thr220/Thr179) rabbit IgG polyclonal antibody was a gift from Professor Koichi Matsuzaki (Kansai Medical University, Osaka, Japan). Carrier-free [^{35}S]-SO_4 was from ICN Biomedicals (Irvine, USA). Cetylpyridinium

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chloride (CPC) was from Uni-lab Chemicals and Pharmaceuticals (Mumbai, India).

Tissue Culture-Human VSMCs were isolated using the explant technique from discarded segments of the saphenous veins from patient donors undergoing surgery at the Alfred Hospital (Melbourne, Australia) under ethics approval from The Alfred Hospital Ethics Committee (Melbourne, Australia). Cells were seeded into 6-well plates at $8x10^5$ cells/well in low-glucose (5 mM) DMEM with 10% (v/v) FBS and antibiotics and maintained until confluent. Cells were then serum deprived by culturing in low glucose (5 mM) DMEM with 0.1% (v/v) FBS for 48 h prior to experimentation. Experiments were conducted using cells from passage 14–19.

Quantitative RT-PCR analysis-Measurement of the mRNA levels of GAG synthesizing enzymes was conducted using quantitative real-time polymerase chain reaction (RT-PCR). Total RNA was extracted using the Qiagen RNeasy® Mini Kit. cDNA (1000 ng/µl) was synthesized using the Qiagen QuantiTect® Reverse Transcription kit. Quantitative RT- PCR was performed using the QuantiFast™ SYBR® Green PCR kit. Data was normalized to the ribosomal 18S housekeeping gene. All experiments were performed at least three times.

Western Blotting- Whole cell lysates were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. Membranes were blocked with 5% (w/v) BSA and incubated with primary antibody (1:1000) as indicated in figure legends followed by species-specific secondary antibody (anti-rabbit IgG HRP, anti-mouse IgG HRP). Enhanced chemiluminescence (ECL) was used to detect protein of interest. Blots were imaged using the BioRad gel documentation system and densitometry analysis was performed with the BioRad Image Lab V5.0 imaging software. Each experiment was conducted at least three times.

Quantification of radiolabel incorporation into proteoglycans- Quiescent cells in 24-well plates were changed to fresh medium containing [^{35}S]-sulfate (50 µCi/ml) in the presence or absence of TGF-β (2 ng/ml) and inhibitors for 24 h. Secreted proteoglycans were harvested from the media with added protease inhibitors (5 mM benzamidine in 0.1 M 6-aminocaproic acid). Incorporation of the radiolabel into proteoglycans was measured by the CPC precipitation assay described previously (Nigro et al., 2002; Tannock et al., 2002).

Determination of proteoglycan size- Proteoglycans labelled with [^{35}S]-sulfate were isolated through DEAE-Sephacel anionic exchange mini columns. Samples were washed with low-salt buffer (8 M urea, 0.25 M NaCl, 2 mM disodium EDTA, 0.5% Triton X-100). Proteoglycans were eluted using high-salt buffer (8 M urea, 3 M NaCl, 2 mM disodium EDTA, 0.5% Triton X-100). Aliquots (25,000 cpm) were precipitated (1.3% potassium acetate, 95% ethanol) and chondroitin sulfate was added as a "cold carrier". Samples were resuspended in buffer (8 M urea, 2 mM disodium EDTA, pH 7.5), to which an equal volume of sample buffer was added. Radiolabelled proteoglycans were separated on 4–13% acrylamide gels with a 3% stacking gel. Processed and dried gels were scanned on a Cyclone Plus Phosphor Imager (Perkin Elmer, USA).

ChIP Assay- Approximately, 6 x 10^6 cells were cultured in a serum-free medium for 48 h before they were treated with TGF-β (2 ng/ml) for 6 h. For both flavopiridol and TGF-β treatment, cells were pre-incubated for 30 min with 500 nM flavopiridol before they were treated with TGF-β for 6 h. After TGF-β incubation, the cells were treated with 1.0% formaldehyde for 10 min at room temperature before they were harvested, washed twice with cold PBS and resuspended in 1X lysis buffer (50 mM Tris-HCl, pH8.1, 10 mM EDTA, and 1.0% SDS) supplemented with protease inhibitors (Roche, USA). Cell suspensions were sonicated for 10 cycles (10 sec for each cycle followed by 1 min interval) using sonicator with half of its maximum capacity and

centrifuged at 20,000xg for 10 min. Extracts were diluted at 1:10 ratio with immunoprecipitation (IP) dilution buffer (50 mM Tris-HCl, pH8.1, 150 mM NaCl, 2 mM EDTA and 1.0% TritonX-100) and incubated for 2 h with 25 µl of protein A/G beads (Santa Cruz Biotech, USA) at 40°C. Extracts were centrifuged and the supernatants were re-incubated with anti-SMAD antibody (Cell Signaling, USA) or corresponding control IgG for overnight at 4°C. DNA bound protein-antibody complexes were captured after further incubation with 50 µl of protein A/G beads for 60 min. Complexes were washed once with TSE-I (20 mM Tris-HCl, pH8.1, 150 mM NaCl, 2 mM EDTA, 1.0% TritonX-100 and 0.1% SDS), four times with TSE-II (20 mM Tris-HCl, pH8.1, 500 mM NaCl, 2 mM EDTA, 1.0% TritonX-100 and 0.1% SDS), once with buffer Buffer-III (10 mM Tris-HCl, pH8.1, 1 mM EDTA, 1.0% Deoxycholate, 1.0% NP-40 and 0.25 M LiCl) and finally three times with TE (10 mM Tris-HCl and 1 mM EDTA). DNA-protein complexes were extracted by two time incubations (5 min each time) with the freshly prepared Extraction buffer (1.0% SDS and 100 mM $NaHCO_3$) and incubated another 6 h at 65°C to uncouple the protein-DNA complex. The DNA fragments were precipitated by adding twice the volume of 100% ethanol, 150 mM of sodium acetate and 10 µg of glycogen and finally fragments were purified through PCR purification kit (Invitrogen, USA). Elutes were analysed through RT-PCR to determine the enrichments. PCR condition for ChSy-1 was 95°C 2 min/95°C 10 sec, 56°C 15 sec and 72°C 30 sec/ for 40 cycles whilst the PCR condition for C4ST-1 and XT-1 was 95°C 2 min/95°C 10 sec, 55°C 15 sec and 72°C 30 sec/ for 40 cycles.

Images- The experiments in this paper were conducted with multiple CDK inhibitors however the lack of specificity of these inhibitors makes interpretation of the data uncertain; with the known actions of flavopiridol from our earlier paper (Rostam et al., 2016) on Smad phosphorylation, we have presented the data only for flavopiridol and thus the gel images been

prepared to only show the flavopiridol and relevant other control agents being the agonist (TGF-β) and control antagonist (SB431542).

Statistical Analysis- Data are presented as mean ± Standard Error of the Mean (S.E.M.) and analysed for statistical significance using a one-way analysis of variance (ANOVA) followed by the least significant difference post hoc analysis. Results were considered statistically significant at $P < 0.05$ or $P < 0.01$, as indicated.

RESULTS

*Flavopiridol concentration-dependently inhibits the TGF-β-stimulated mRNA expression of GAG synthesizing enzymes in human VSMC*s. C4ST-1 and ChSy-1 are leading candidates to be rate limiting enzymes for the elongation of GAG chains while XT-1 catalyzes the addition of a xylose residue to the serine in the biglycan core protein as the initial step in the formation of a GAG chain (Götting et al., 2000; Izumikawa et al., 2011). We investigated the effects of flavopiridol on TGF-β-stimulated expression of these three GAG synthesizing enzymes (Fig. 1). VSMCs were treated with TGF-β (2 ng/ml) for 6 h and the mRNA expression of C4ST-1, ChSy-1 and XT-1 was upregulated (1.5–3.0), (3.0–4.9), and (1.5–2.1)-fold, respectively (P<0.01) (Fig. 1) compared to untreated controls. Treatment of VSMCs with flavopiridol (5– 500 nM) caused a concentration-dependent decrease in TGF-β stimulated mRNA expression of C4ST-1, ChSy-1, and XT-1 (Fig. 1). The TGFβRI/Alk-5 inhibitor, SB431542 (Burch et al., 2010) abolished TGF-β mediated C4ST-1, ChSy-1, and XT-1 mRNA expression (Fig. 1A, 1B & 1C, lane 9).

Effect of flavopiridol on TGFβRI/Alk-5-stimulated biglycan mRNA expression in VSMC. Biglycan is one of the major lipid binding CS/DS proteoglycan produced by VSMCs. Biglycan consists of a core protein to which two CS/DS GAG chains are covalently attached. Growth factors including TGF-β regulate the core protein and GAG chain synthesis (Ballinger et al., 2004; Osman et al., 2011). As a prelude to studies on the expression of GAG initiation and elongation genes, we also investigated the effects of flavopiridol on the TGF-β-stimulated synthesis of biglycan mRNA (Fig. 2) (Ballinger et al., 2004). TGF-β increases the expression of biglycan in VSMCs via the Akt pathway but this pathway is not involved in GAG hyperelongation (Burch et al., 2010). TGF-β treatment increased biglycan mRNA expression 1.4-fold (P<0.05) (Fig. 2). Both

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flavopiridol (1 μM) and SB431542 (3 μM) treatment totally inhibited TGF-β-stimulated biglycan mRNA expression (Fig. 2).

Flavopiridol effects on TGF-β-stimulated biglycan synthesis and GAG hyperelongation.

Although the elongation enzymes, C4ST-1 and ChSy-1 are purported or teleologically considered to be rate limiting in the synthesis and elongation of GAG chains, this has not been definitively demonstrated. Therefore, to confirm that the action of flavopiridol on the expression of these genes (C4ST-1 and ChSy-1) is functionally relevant, we assessed its effects at two concentrations on the size of biglycan molecules synthesized and secreted by TGF-β-stimulated human VSMCs. Thus, as an assessment of total proteoglycan synthesis being a combination of core protein expression and incorporation of sulfate into GAG chains (Ballinger et al., 2004), we used a CPC precipitation assay and measured the incorporation of radioactive sulfate [^{35}S]-SO_4 into secreted proteoglycans (mostly biglycan) over 24 h. Treatment with TGF-β increased [^{35}S]-SO_4 incorporation into secreted proteoglycans by two fold as compared to untreated controls (Fig. 3A & 3C). When VSMCs were treated with flavopiridol (1 μM) for 24 h, there was a complete inhibition of [^{35}S]-SO_4 incorporation into proteoglycans (Fig. 3A). Flavopiridol (10 μM) also elicited a similar effect (Fig. 3C). The TGFβRI/Alk-5 inhibitor, SB431542 completely inhibited TGF-β-stimulated [^{35}S]-SO_4 incorporation into proteoglycans in these cells (Fig. 3A & 3C).

Assessment of the apparent size of biglycan molecules provides evidence of changes in the size of the GAG chains because the size of the core protein is fixed and only the CS/DS chain size can vary (Little et al., 2002; Ballinger et al., 2004). Biglycan synthesized and secreted by TGF-β treated cells showed a marked decrease in electrophoretic mobility (corresponding to an increase in the apparent size of the molecules) compared to biglycan synthesized and secreted by untreated

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cells (Fig. 3B & 3D). In the presence of flavopiridol (1 & 10 μM), the effect of TGF-β treatment was blocked such that there was an increase in biglycan electrophoretic mobility (Fig. 3B & 3D). VSMCs treated with TGF-β in the presence of SB431542 had a similar biglycan size to control cells (Fig. 3B & 3D). These results demonstrate that the increase in the size of biglycan molecules in TGF-β-stimulated VSMCs is blocked by flavopiridol. It can also be noted in these experiments that TGF-β stimulation increases the expression of biglycan core proteins consistent with the mRNA expression data shown (Fig. 2). In the SDS-PAGE shown in Fig. 3 lane 3 vs lane 1, shows strong darkening of the band consistent with higher levels of biglycan and this effect is blocked by flavopiridol (Fig. 3 lane 4 vs lane 3). The data shows that the effects of flavopiridol on the expression of C4ST- 1 and ChSy-1 correlates with the effects on the size of biglycan molecules which is consistent with the proposition that the activity of these two enzymes is rate limiting for the synthesis and elongation of GAG chains.

Effect of flavopiridol on TGF-β-stimulated Smad linker region phosphorylation of specific serine and threonine sites. As a starting point for the investigation of the role of Smad linker region phosphorylation in TGF-β-stimulated GAG gene expression and GAG hyperelongation, we investigated its effect on the phosphorylation of the cluster of serine residues (Ser 245, 250 and 255) as well as the threonine site (Thr 220) in the linker region of Smad2. Western blotting was utilized to determine the time-course of TGFβRI mediated Smad2 linker region phosphorylation. The level of phospho-Smad2L (Ser 245, 250 and 255) reached a peak 1 h post-TGF-β treatment (Fig. 4A). Treatment with TGF-β for 1 h, increased the phosphorylation of Smad2L (Ser 245/Ser 250/Ser 255) by 2.7-fold (Fig. 4B) while flavopiridol (1 μM) treatment

inhibited this TGF-β mediated phosphorylation. SB431542 (3 μM) inhibited the TGF-β-mediated phosphorylation of Smad2L (Fig. 4B).

Site-specific antibody for phospho-Thr220 was used to investigate the involvement of the Thr220 residue (Matsuzaki et al., 2009; Kamato et al., 2014). We have previously reported that TGF-β treatment stimulates the phosphorylation of Thr220 with a peak response at 1 h (Rostam et al., 2016). In the current experiments, TGF-β (2 ng/ml) stimulation of VSMCs resulted in a 2.3-fold increase in phospho-Smad2L (Thr220) (Fig. 5). Flavopiridol completely prevented the TGF-β-induced phosphorylation of Smad2L (Thr220). These results indicate that the phosphorylation of multiple Smad linker region phosphorylation sites which can be blocked by flavopiridol are most likely responsible for the effects of TGF-β on the expression of GAG synthesizing genes and of GAG hyperelongation. The temporal aspects of the phosphorylation of the Ser and Thr sites were sufficiently different to suggest different signalling pathways however the total inhibition of both pathways by flavopiridol highlights its broad inhibitory effects.

Smads bind to promoter regions of GAG synthesizing genes in a flavopiridol-dependent manner. The question of the role of linker region phosphorylation of Smads in determining phosphoSmad distribution and specifically in modulating Smad nuclear translocation is a major unresolved question in TGF-β signalling and cell biology. We earlier observed a dose dependent inhibition of TGF-β-stimulated C4ST-1, ChSy-1 and XT-1 mRNA expression in the presence of flavopiridol (Fig. 1). Considered with the Smad linker region phosphorylation data in Fig. 4 and Fig. 5, these data suggest that flavopiridol-dependent linker region Smad phosphorylation is critical for the TGF-β driven expression of GAG synthesizing genes. Analysis of the promoter region sequences of GAG genes investigated in this study revealed single consensus Smad

binding sites were present 600 bp upstream of the C4ST-1 and ChSy-1 initiation sites but no equivalent Smad binding site was present in the promoter region of the XT-1 gene. XT-1 expression could thus serve as a negative control for studies of Smad binding to C4ST-1 and ChSy-1 genes. We performed ChIP assays to determine the enrichment of Smad binding in that region after TGF-β treatment. We observed a marked increase of Smad binding in the promoter of C4ST-1 (>20 fold) and ChSy-1 (>5 fold) genes and the increased binding was absent for the XT-1 gene (Fig. 6). In VSMCs pretreated with flavopiridol (500 nM), TGF-β-stimulated Smad enrichment was markedly reduced for both the C4ST-1 and ChSy-1 genes. In relation to XT-1 expression as there was no stimulation with TGF-β there was no inhibition by flavopiridol. This data demonstrates that TGF-β-stimulated GAG gene expression is mediated directly by Smad transcription factors which are linker region polyphosphorylated by a flavopiridol-sensitive kinase, most likely a CDK.

DISCUSSION

Proteoglycans with structural modifications are implicated in the early stages of the atherosclerotic process and the pathways regulating their synthesis and properties are potential targets for the development of therapeutic agents. Such a therapeutic agent would block GAG hyperelongation and reduce the binding of the proteoglycan to LDL and such a therapeutic approach would be used in tandem with a HMG-CoA reductase inhibitor (statin) which reduces blood cholesterol levels (Little et al., 2007; Little et al., 2008; Little et al., 2011). TGF-β plays a role in atherosclerosis and in this context it is a potent and highly effective agent to mediate hyperelongation of GAG chains on the proteoglycan, biglycan. TGF-β signals via its Type I and Type II cell surface serine/threonine kinase receptors and on to regulation of the phosphorylation

of regulatory Smads (Smad2/3), which pass to the nucleus and regulate gene transcription (Derynck and Zhang, 2003; Massagué et al., 2005). There are multiple pathways for the regulation of Smads via phosphorylation (Burch et al., 2011). Phosphorylation of Smad2 in the linker region has emerged as a pathway regulating the expression of the genes for the enzymes, which mediate GAG elongation. In this study, we investigated the role of Smad linker region phosphorylation in mediating the expression of GAG elongation genes in human VSMCs using flavopiridol, a well-known CDK inhibitor that inhibited the increased expression of these genes (Rostam et al., 2016).

We show that flavopiridol inhibits (a) TGF-β stimulation of the expression of the genes which are considered to be rate limiting for the elongation of GAG chains on biglycan and (b) TGF-β-stimulated increase in the size of the biglycan molecules which reflects the increased size of the CS/DS GAG chains (Little et al., 2002; Little et al., 2010; Little et al., 2013). TGF-β treatment increased the phosphorylation of the serine triplet as well as the isolated threonine residue in the linker region of Smad2 (Rostam et al., 2016). We also found that the linker region and the carboxy-terminal phosphorylated Smad bound to the critical regions of the genes for C4ST-1 and ChSy-1 but not XT-1 exactly as predicted from the structural analysis of these regions. The increased binding of the linker region of phosphorylated Smad2 to the promoter region of C4ST-1 and ChSy-1 shows that this transcription factor can pass to the nucleus and regulate, in our case upregulate, gene transcription. Our data thus strongly supports the notion that the Smad linker region is a site of integration of TGF-β signalling as well as mediating transcriptional regulation of GAG synthesizing genes in the cell nucleus.

Linker region phosphorylation was originally demonstrated to be inhibitory for TGF-β signalling by blocking the translocation of this phosphorylated entity to the cell nucleus (Kretzschmar et al., 1999). Our data clearly shows that for the TGF-β mediated regulation of GAG

gene expression the Smad linker region facilitates and not inhibits this response. These discrepancies in gene expression and cellular responses require further analysis of TGF-β signalling and the role of Smad linker region phosphorylation as a master regulator and integrator. Indeed, in view of the clear role of linker region phosphorylation in mediating the up-regulation of gene expression, we also suggest that it is worth re-addressing the role of carboxy terminal phosphorylation in TGF-β signaling.

In VSMCs, GAG hyperelongation by TGF-β stimulation is dependent upon both transcription and translation (Yang et al., 2009). GAG synthesizing enzymes (C4ST-1, ChSy-1 and XT-1) are responsible for sulfation and GAG chain (hyper) elongation; multiple other hormones and growth factors stimulate GAG elongation (Ballinger et al., 2009; Getachew et al., 2010). In many studies, Vascular Endothelial Growth Factor (VEGF) stimulates GAG elongation but not in retinal endothelial cells (Al Gwairi et al., 2016). As to the *in vivo* relevance of these findings, Emoto and colleagues have shown that the mRNA expression of some GAG synthesizing genes increases over 8 weeks in an atherosclerotic mouse model and this increase correlates with increased atherosclerosis in the mice (Anggraeni et al., 2011).

TGF-β mediated proteoglycan synthesis in VSMCs involves Smad2 linker region phosphorylation (Burch et al., 2010). The Smad3 linker region is a target for CDK2 and CDK4 phosphorylation (Matsuura et al., 2004). In epithelial cells, TGFβRI and Ras-associated kinases, including Erk, c-Jun NH_2-terminal kinase (JNK), and CDK4 (Matsuura et al., 2004) differentially phosphorylate Smad2/3 in the carboxy-terminus, linker region or both (Matsuzaki et al., 2009). Smad2/3 phospho isoforms can differentially interact with Smad4 to either translocate or be blocked from entering the nucleus to initiate gene transcription (Derynck and Zhang, 2003). Kinase specific phosphorylation of Smad2/3 isoforms creates a complex signalling cascade that

regulates the switching of TGF-β mediated tumor suppressive effects in early stages of cancer to advanced carcinomas (Matsuzaki, 2011). In VSMCs, the specificity of these signalling pathways is not well understood however it is important and further analyses will facilitate a better understanding of the cell biology of TGF-β signalling and in identification of new therapeutic targets - perhaps the kinases which are in the cascades leading to Smad linker region phosphorylation - for the treatment of atherosclerosis (Little et al., 2007).

In vascular endothelial cells, Smad2 linker region residues are phosphorylated by different serine/threonine kinases that alter plasminogen-activator inhibitor 1 mRNA expression (Kamato et al., 2014). Here we used two antibodies, with one detecting the cluster of serine residues (Ser245/250/255) and the second, the phosphorylated threonine residue (Thr220). Flavopiridol blocked all of these phosphorylations so we are not able to describe a relationship between individual residues that are phosphorylated and the expression of individual GAG synthesizing enzymes but this work is currently under way in our laboratory.

Although there have been many studies on the role played by Smads in the expression of genes (Matsuzaki, 2013; Morikawa et al., 2013), there are none for GAG genes showing the definitive involvement of these transcription factors. Indeed the current favoured hypothesis is that linker region phosphorylation of Smad2/3 prevents the translocation of the Smad entity to the nucleus whereas our data indicates a correlation between the phosphorylation status of the linker region and increased expression of GAG elongation genes. This is an important point in the understanding of the cell biology of TGF-β signalling. To investigate this point we used ChIP assays to assess the direct binding of Smads to promoter regions of three GAG synthesizing genes. We determined bioinformatically that the two GAG genes, C4ST-1 and ChSy-1 possessed Smad binding sites whereas the GAG chain initiation enzyme XT-1 did not possess this Smad binding

site. TGF-β treatment of VSMC led to a marked increase in Smad enrichment on the C4ST-1 and ChSy-1 genes but not on the XT-1 gene. Both of these responses were attenuated in cells treated with flavopiridol. This data strongly suggests that there is direct activation of Smad mediated GAG gene expression by TGF-β and that this is dependent upon Smad linker region phosphorylation (as described above). It is noted that studies of XT-1 expression after TGF-β treatment showed small but statistically significant increases (see Fig. 6). This indicates that there are Smad independent pathways of TGF-β-stimulated GAG gene expression which is not inconsistent with our earlier findings of the existence of multiple pathways mediating the effects of TGF-β in VSMCs.

The therapeutic rational for these studies is that there is the potential for a specific kinase inhibitor or an inhibitor of multiple kinases (Bernard et al., 2016) to be a therapeutic agent for the reduction in atherosclerosis and the prevention of cardiovascular disease (Little et al., 2007). It can also be mentioned that there is an alternative approach of targeting GAG chain biosynthesis which is to use GAG-directed antibodies which block the ionic interaction between GAG chains and ApoB100; the veracity of this approach has recently been demonstrated in a mouse model of intimal hyperplasia where the accelerated atherosclerosis was shown to be due to lipoprotein binding to modified proteoglycans and the interaction and hence the atherosclerosis could be blocked by GAG directed antibodies (Kijani et al., 2017). It follows in this area that an "anti-proteoglycan" or more specially an "anti-GAG" agent would work in therapeutic tandem with a HMG- CoA reductase inhibitor such as a statin. Here, the statin would reduce the blood cholesterol level (Gotto, 2002) and a proteoglycan synthesis inhibitor capable blocking the hyperelongation of GAG chains on biglycan or the interaction between GAG chains and LDL in the vessel wall would render vessel wall less sticky for atherogenic lipoproteins (Ballinger et al., 2010). It needs

to be considered what would be the impact of a CDK inhibitor on normal physiology. In clinical practice CDK inhibitors are associated with a range of adverse effects which include secretory diarrhoea and a pro-inflammatory syndrome (Senderowicz and Sausville, 2000b). Thus, the potential exists for CDK inhibitors, such as flavopiridol and related agents, to have a variety of effects beyond the identified therapeutic target which in this case is the role of hyperelongated GAG chains in the aetiology of the early stages of atherosclerosis. The need will be for studies in animal models and later in translational studies to establish if a highly specific inhibitor of the signalling pathways mediating GAG hyperelongation can successfully target proteoglycan changes in the vessel wall associated with the early stages of atherosclerosis at doses that do not generate unwanted side effects. We have already provided proof of concept data for this hypothesis (Ballinger et al., 2010; Getachew et al., 2010) and now need to determine the optimum target in the signalling pathways that mediate growth factor effects of GAG synthesizing enzyme gene expression and GAG hyperelongation in human VSMCs.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: PJL and NO jointly designed and coordinated the study**.** MAR performed, analysed the experiments, and wrote the manuscript. AS assisted in the experiments performed. RG performed and analysed the experiments. YC contributed to the interpretation of the results and the finalisation of the manuscript. PM performed the experiments descried in Fig. 6 and assisted with the writing of the associated section. WZ contributed to the analysis of the data and signalling pathways. All authors reviewed the interpretation of results, and approved final version of the manuscript.

REFERENCES

Al Gwairi O, Osman N, Getachew R, Zheng W, Liang XL, Kamato D, Thach L and Little PJ (2016) Multiple Growth Factors, But Not VEGF, Stimulate Glycosaminoglycan Hyperelongation in Retinal Choroidal Endothelial Cells. *Int J Biol Sci* **12**:1041-1051.

Anggraeni VY, Emoto N, Yagi K, Mayasari DS, Nakayama K, Izumikawa T, Kitagawa H and Hirata K (2011) Correlation of C4ST-1 and ChGn-2 expression with chondroitin sulfate chain elongation in atherosclerosis. *Biochem Biophys Res Commun* **406**:36-41.

Ballinger ML, Ivey ME, Osman N, Thomas WG and Little PJ (2009) Endothelin-1 activates ETA receptors on human vascular smooth muscle cells to yield proteoglycans with increased binding to LDL. *Atherosclerosis* **205**:451-457.

Ballinger ML, Nigro J, Frontanilla KV, Dart AM and Little PJ (2004) Regulation of glycosaminoglycan structure and atherogenesis. *Cell Mol Life Sci* **61**:1296-1306.

Ballinger ML, Osman N, Hashimura K, Haan JBD, Jandeleit-Dahm K, Allen T, Tannock LR, Rutledge JC and Little PJ (2010) Imatinib inhibits vascular smooth muscle proteoglycan synthesis and reduces LDL binding in vitro and aortic lipid deposition in vivo. *J Cell Mol Med* **14**:1408-1418.

Bernard R, Getachew R, Kamato D, Thach L, Osman N, Chan V, Zheng W and Little PJ (2016) Evaluation of the potential synergism of imatinib-related poly kinase inhibitors using growth factor stimulated proteoglycan synthesis as a model response. *J Pharm Pharmacol* **68**:368-378.

Bobik A, Agrotis A, Kanellakis P, Dilley R, Krushinsky A, Smirnov V, Tararak E, Condron M and Kostolias G (1999) Distinct Patterns of Transforming Growth Factor-β Isoform and Receptor Expression in Human Atherosclerotic Lesions: Colocalization Implicates TGF-β in Fibrofatty Lesion Development. *Circulation* **99**:2883-2891.

Burch M, Zheng W and Little P (2011) Smad linker region phosphorylation in the regulation of extracellular matrix synthesis. *Cell Mol Life Sci* **68**:97-107.

Burch ML, Yang SN, Ballinger ML, Getachew R, Osman N and Little PJ (2010) TGF-beta stimulates biglycan synthesis via p38 and ERK phosphorylation of the linker region of Smad2. *Cell Mol Life Sci* **67**:2077-2090.

Cardoso LE, Little PJ, Ballinger ML, Chan CK, Braun KR, Potter-Perigo S, Bornfeldt KE, Kinsella MG and Wight TN (2010) Platelet-derived growth factor differentially regulates the expression and post-translational modification of versican by arterial smooth muscle cells through distinct protein kinase C and extracellular signal-regulated kinase pathways. *J Biol Chem* **285**:6987-6995.

Deaton C, Froelicher ES, Wu LH, Ho C, Shishani K and Jaarsma T (2011) The Global Burden of Cardiovascular Disease. *J Cardiovasc Nurs* **26**:S5-S14 10.1097/JCN.1090b1013e318213efcf.

Derynck R and Zhang YE (2003) Smad-dependent and Smad-independent pathways in TGF-β family signalling. *Nature* **425**:577-584.

Getachew R, Ballinger ML, Burch ML, Reid JJ, Khachigian LM, Wight TN, Little PJ and Osman N (2010) PDGF β-Receptor Kinase Activity and ERK1/2 Mediate Glycosaminoglycan Elongation on Biglycan and Increases Binding to LDL. *Endocrinology* **151**:4356-4367.

Götting C, Kuhn J, Zahn R, Brinkmann T and Kleesiek K (2000) Molecular Cloning and Expression of Human UDP-d-Xylose:Proteoglycan Core Protein β-d-Xylosyltransferase and its First Isoform XT-II. *J Mol Biol* **304**:517-528.

Gotto AM (2002) Statins: Powerful Drugs for Lowering Cholesterol: Advice for Patients. *Circulation* **105**:1514-1516.

Ivey ME and Little PJ (2008) Thrombin regulates vascular smooth muscle cell proteoglycan synthesis via PAR-1 and multiple downstream signalling pathways. *Thromb Res* **123**:288-297.

Izumikawa T, Okuura Y, Koike T, Sakoda N and Kitagawa H (2011) Chondroitin 4-O-sulfotransferase-1 regulates the chain length of chondroitin sulfate in co-operation with chondroitin N-acetylgalactosaminyltransferase-2. *Biochem J* **434**:321-331.

Kamato D, Burch ML, Piva TJ, Rezaei HB, Rostam MA, Xu S, Zheng W, Little PJ and Osman N (2013) Transforming growth factor-β signalling: Role and consequences of Smad linker region phosphorylation. *Cell Signal* **25**:2017-2024.

Kamato D, Rostam MA, Piva TJ, Babaahmadi Rezaei H, Getachew R, Thach L, Bernard R, Zheng W, Little PJ and Osman N (2014) Transforming growth factor β-mediated site-specific Smad linker region phosphorylation in vascular endothelial cells. *J Pharm Pharmacol* **66**:1722-1733.

Kamato D, Thach L, Getachew R, Burch M, Hollenberg MD, Zheng W, Little PJ and Osman N (2016) Protease activated receptor-1 mediated dual kinase receptor transactivation stimulates the expression of glycosaminoglycan synthesizing genes. *Cell Signal* **28**:110-119.

Kijani S, Vazquez AM, Levin M, Boren J and Fogelstrand P (2017) Intimal hyperplasia induced by vascular intervention causes lipoprotein retention and accelerated atherosclerosis. *Physiological reports* **5**.

Kretzschmar M, Doody J and Massague J (1997) Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. *Nature* **389**:618-622.

Kretzschmar M, Doody J, Timokhina I and Massague J (1999) A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. *Genes Dev* **13**:804-816.

Little PJ, Ballinger ML and Osman N (2007) Vascular wall proteoglycan synthesis and structure as a target for the prevention of atherosclerosis. *Vasc Health Risk Manag* **3**:117.

Little PJ, Burch ML, Getachew R, Al-aryahi S and Osman N (2010) Endothelin-1 stimulation of proteoglycan synthesis in vascular smooth muscle is mediated by endothelin receptor transactivation of the transforming growth factor-[beta] type I receptor. *J Cardiovasc Pharmacol* **56**:360-368.

Little PJ, Chait A and Bobik A (2011) Cellular and cytokine-based inflammatory processes as novel therapeutic targets for the prevention and treatment of atherosclerosis. *Pharmacol Ther* **131**:255-268.

Little PJ, Osman N and O'Brien KD (2008) Hyperelongated biglycan: the surreptitious initiator of atherosclerosis. *Current Opinion in Lipidology* **19**:448-454.

Little PJ, Rostam MA, Piva TJ, Getachew R, Kamato D, Guidone D, Ballinger ML, Zheng W and Osman N (2013) Suramin inhibits PDGF-stimulated receptor phosphorylation, proteoglycan synthesis and glycosaminoglycan hyperelongation in human vascular smooth muscle cells. *J Pharm Pharmacol* **65**:1055-1063.

Little PJ, Tannock L, Olin KL, Chait A and Wight TN (2002) Proteoglycans Synthesized by Arterial Smooth Muscle Cells in the Presence of Transforming Growth Factor-β1 Exhibit Increased Binding to LDLs. *Arterioscler Thromb Vasc Biol* **22**:55-60.

Mariaule G and Belmont P (2014) Cyclin-Dependent Kinase Inhibitors as Marketed Anticancer Drugs: Where Are We Now? A Short Survey. *Molecules* **19**:14366.

Massagué J, Seoane J and Wotton D (2005) Smad transcription factors. *Genes Dev* **19**:2783-2810.

Matsuura I, Denissova NG, Wang G, He D, Long J and Liu F (2004) Cyclin-dependent kinases regulate the antiproliferative function of Smads. *Nature* **430**:226-231.

Matsuzaki K (2011) Smad phosphoisoform signaling specificity: the right place at the right time. *Carcinogenesis* **32**:1578-1588.

Matsuzaki K (2013) Smad phospho-isoforms direct context-dependent TGF-beta signaling. *Cytokine Growth Factor Rev* **24**:385-399.

Matsuzaki K, Kitano C, Murata M, Sekimoto G, Yoshida K, Uemura Y, Seki T, Taketani S, Fujisawa J and Okazaki K (2009) Smad2 and Smad3 phosphorylated at both linker and COOH-terminal

regions transmit malignant TGF-beta signal in later stages of human colorectal cancer. *Cancer Res* **69**:5321-5330.

Morikawa M, Koinuma D, Miyazono K and Heldin CH (2013) Genome-wide mechanisms of Smad binding. *Oncogene* **32**:1609-1615.

Nakashima Y, Fujii H, Sumiyoshi S, Wight TN and Sueishi K (2007) Early human atherosclerosis: accumulation of lipid and proteoglycans in intimal thickenings followed by macrophage infiltration. *Arterioscler Thromb Vasc Biol* **27**:1159-1165.

Nakashima Y, Wight TN and Sueishi K (2008) Early atherosclerosis in humans: role of diffuse intimal thickening and extracellular matrix proteoglycans. *Cardiovasc Res* **79**:14-23.

Nigro J, Dilley RJ and Little PJ (2002) Differential effects of gemfibrozil on migration, proliferation and proteoglycan production in human vascular smooth muscle cells. *Atherosclerosis* **162**:119-129.

Nigro J, Osman N, Dart AM and Little PJ (2006) Insulin Resistance and Atherosclerosis. *Endocr Rev* **27**:242-259.

Osman N, Ballinger ML, Dadlani HM, Getachew R, Burch ML and Little PJ (2008) p38 MAP kinase mediated proteoglycan synthesis as a target for the prevention of atherosclerosis. *Cardiovasc Hematol Disord Drug Targets* **8**:287-292.

Osman N, Getachew R, Burch M, Lancaster G, Wang R, Wang H, Zheng W and Little PJ (2011) TGF-β stimulates biglycan core protein synthesis but not glycosaminoglycan chain elongation via Akt phosphorylation in vascular smooth muscle. *Growth Factors* **29**:203-210.

Osman N, Getachew R, Thach L, Wang H, Su X, Zheng W and Little PJ (2014) Platelet-derived growth factor-stimulated versican synthesis but not glycosaminoglycan elongation in vascular smooth muscle is mediated via Akt phosphorylation. *Cell Signal* **26**:912-916.

Rostam MA, Kamato D, Piva TJ, Zheng W, Little PJ and Osman N (2016) The role of specific Smad linker region phosphorylation in TGF-beta mediated expression of glycosaminoglycan synthesizing enzymes in vascular smooth muscle. *Cell Signal* **28**:956-966.

Senderowicz AM and Sausville EA (2000a) Preclinical and Clinical Development of Cyclin-Dependent Kinase Modulators. *J Natl Cancer Inst* **92**:376-387.

Senderowicz AM and Sausville EA (2000b) Preclinical and clinical development of cyclin-dependent kinase modulators. *J Natl Cancer Inst* **92**:376-387.

Tannock LR, Little PJ, Wight TN and Chait A (2002) Arterial smooth muscle cell proteoglycans synthesized in the presence of glucosamine demonstrate reduced binding to LDL. *J Lipid Res* **43**:149-157.

Yang SNY, Burch ML, Getachew R, Ballinger ML, Osman N and Little PJ (2009) Growth factor-mediated hyper-elongation of glycosaminoglycan chains on biglycan requires transcription and translation. *Arch Physiol Biochem* **115**:147-154.

Yang SNY, Burch ML, Tannock LR, Evanko S, Osman N and Little PJ (2010) Transforming growth factor-β regulation of proteoglycan synthesis in vascular smooth muscle: Contribution to lipid binding and accelerated atherosclerosis in diabetes. *J Diabetes* **2**:233-242.

Yumoto K, Thomas PS, Lane J, Matsuzaki K, Inagaki M, Ninomiya-Tsuji J, Scott GJ, Ray MK, Ishii M, Maxson R, Mishina Y and Kaartinen V (2013) TGF-β-activated Kinase 1 (Tak1) Mediates Agonist-induced Smad Activation and Linker Region Phosphorylation in Embryonic Craniofacial Neural Crest-derived Cells. *J Biol Chem* **288**:13467-13480.

Author contributions:

Participated in research design: Little, Osman, Cao, Piva, Zheng,

Conducted experiments: Rostam, Shajimoon, Getachew, Mitra

Performed data analysis: Rostam, Getachew, Mitra, Kamato

Wrote or contributed to the writing of the manuscript: Rostam, Little, Osman, Cao, Zheng, Kamato, Piva

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FIGURE LEGENDS

FIGURE 1: Effect of flavopiridol on TGF-β-stimulated mRNA expression of GAG synthesizing enzymes (C4ST-1, ChSy-1, and XT-1). VSMCs were pre-incubated with flavopiridol (5–500 nM) or SB431542 (3 μM) for 30 min before being treated with TGF-β (2 ng/ml) for 6 h. The expression of (A) C4ST-1, (B) ChSy-1, and (C) XT-1 was assessed by RT-PCR. Histograms represent the mean + S.E.M. of three individual experiments which indicate the fold-change of mRNA expression compared to untreated control. ## $P < 0.01$ basal versus TGF-β (2 ng/ml), **$P < 0.01$ inhibitor versus TGF-β (2 ng/ml). Experiments were analysed using one-way analysis of variance.

FIGURE 2: Flavopiridol and biglycan mRNA. VSMCs were pre-incubated with the presence and absence of flavopiridol (1 μM), and an Alk-5 inhibitor SB431542 (3 μM) for 30 min followed by treatment with TGF-β (2 ng/ml) for 4 h. The histogram shows the mean fold-change + S.E.M. from three individual experiments, compared to untreated control. ##$P < 0.01$ basal versus TGF-β (2 ng/ml), **$P < 0.01$ and *$P < 0.05$ inhibitor versus TGF-β (2 ng/ml), using one-way analysis of variance.

FIGURE 3: Effect of flavopiridol on TGF-β-stimulated radiosulfate incorporation into proteoglcyans. (A) VSMCs were treated with or without flavopiridol (1 μM) and SB431542 (3 μM) for 24 h with presence of TGF-β (2 ng/ml) and [^{35}S]-SO_4 (50 μCi/ml). Medium containing secreted proteoglycans was harvested and spotted onto 3MM paper and were quantitated by CPC precipitation to measure radiolabel incorporation. **(B)** Secreted proteoglycans were isolated using

ion exchange chromatography (DEAE-Sephacel) and concentrated by ethanol/potassium acetate precipitation and electrophoresed on a 4–13% SDS-PAGE. The histogram shows the mean + S.E.M. of the fold-change compared to untreated control. ##P < 0.01 basal versus TGF-β (2 ng/ml), **P < 0.01 and *P < 0.05 inhibitor versus TGF-β (2 ng/ml), n = 3 experiments using one-way analysis of variance. **(C)** Similar treatment as indicated in (A) with flavopiridol 10 μM. **(D)** Similar method as detailed in (B) with flavopiridol at 10 μM.

FIGURE 4: TGF-β-mediated phosphorylation of Smad2 linker region (Ser245/250/255) in human VSMCs. (A) Time course (0–2 h) of TGF-β (2 ng/ml) on phospho-Smad2L levels in VSMCs. Western blot of cell lysates were probed with primary antibody phospho-Smad2L (Ser245/250/255) rabbit polyclonal or α-tubulin rabbit polyclonal antibody followed by peroxidase labeled anti-Rabbit IgG secondary antibody. **(B)** Effects of flavopiridol on TGF-β/Alk-5-mediated Smad2L phosphorylation in VSMCs. Cells were treated with TGF-β (2 ng/ml) for 1 h in the presence and absence of flavopiridol (1 μM) as well as the Alk-5 inhibitor SB431542 (3 μM). The membrane was probed with phospho-Smad2L (Ser245/250/255) as detailed in (A). The Western blot is a representative of three independent experiments. Histogram is a densitometric quantitation of the three independent experiments and indicates the mean ± S.E.M. of the fold change over that observed in untreated control. ## P < 0.01 basal versus TGF-β (2 ng/ml), **P < 0.01 and *P < 0.05 inhibitor versus TGF-β (2 ng/ml), using one-way analysis of variance.

FIGURE 5: The effects of flavopiridol on TGF-β/Alk-5 mediated Smad2L (Thr220) phosphorylation in VSMCs. VSMCs were pre-incubated for 30 min in the absence or presence

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of flavopiridol (1 μM) or the Alk-5 inhibitor SB431542 (3 μM) with TGF-β (2 ng/ml) stimulation for 1 h. Western blot of cell lysates were probed with an anti-phospho-Smad2L (Thr220) rabbit polyclonal primary antibody followed by peroxidase labeled anti-Rabbit IgG secondary antibody together with anti-GAPDH HRP-conjugated monoclonal antibody for equal loading. Histograms are a densitometric quantitation of three independent experiments and show the mean + S.E.M. of the fold change of expression relative to untreated controls. ## $P < 0.01$ basal versus TGF-β (2 ng/ml), **$P < 0.01$ and *$P < 0.05$ inhibitor versus TGF-β (2 ng/ml), using one-way analysis of variance.

FIGURE 6. TGF-β induced GAG gene expression is associated with increased Smad binding in the promoter region of C4ST-1 and ChSy-1 but not in XT-1. VSMCs were treated with TGF-β (2 ng/ml) for 6 h to stimulate gene activation. In order to determine the effect of flavopiridol on Smad binding, cells were pre-incubated with flavopiridol before they were treated with TGF-β for 6 h. Extracts were used to perform ChIP assay by using anti-Smad antibody at 1:50 dilution or normal rabbit IgG (1.0 μg) as control. The amount of Smad binding was estimated by RT-PCR by using gene specific primers described in the materials and methods. For each gene, Smad enrichment was represented as fold-change in comparison to the corresponding control IgG. Data presented here is the mean + SD of two independent experiments and in each case RT-PCR was performed at least twice to confirm the reproducibility of the results.

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Table 1: Target gene and primer sequence

Target Gene	Real-time PCR primer Sequence (5'-3')
C4ST-1	Forward 5' AGG GTC GTG CAG TTT CCT TA 3' Reverse 5' AGG GTT AGG GGG TTT CTG GT 3'
ChSy-1	Forward 5' GAA GAG GAC GGG GAC AGC 3' Reverse 5' CCT TTA AGA GGG GAC CAT GC 3'
XT-1	Forward 5' AAG GTC CGC ATA CCA AAC AC 3' Reverse 5' TAT CCT CAT GGG GTG GT 3'
Biglycan	Forward 5'-CTC AAC TAC CTG CGC ATC TCA G-3' Reverse 5'-GAT GGC CTG GAT TTT GTT GTG-3'

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Figure 1.

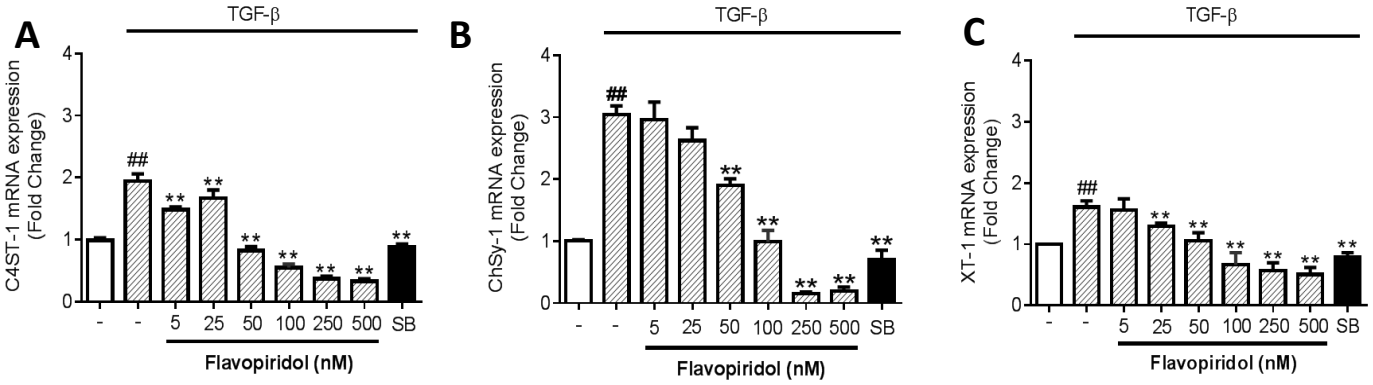

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Figure 2.

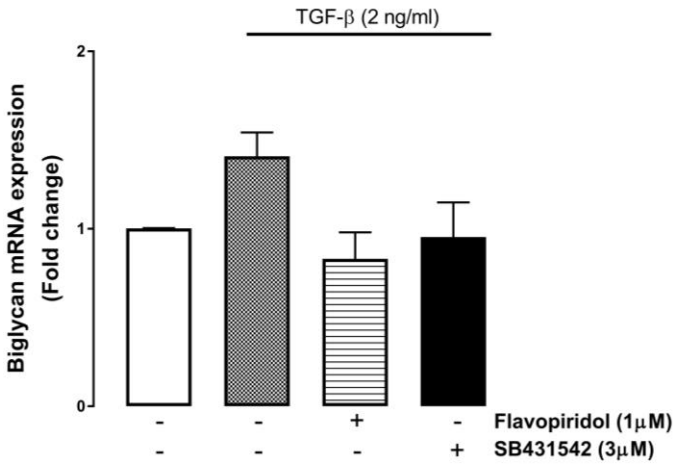

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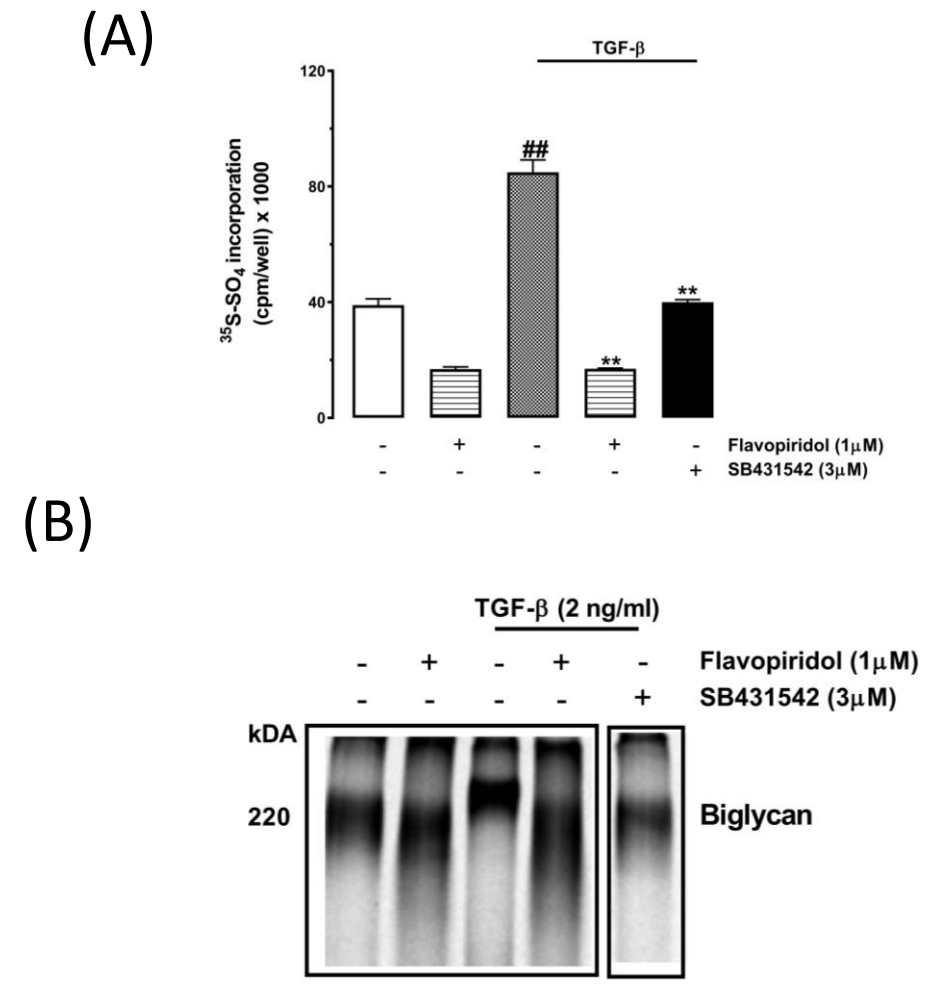
Figure 3.



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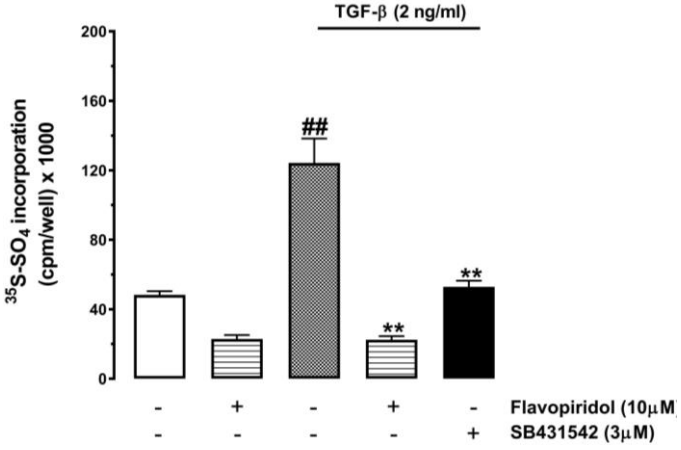
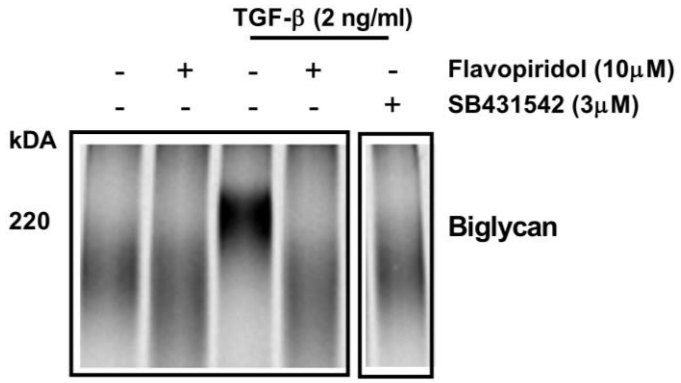
(C)



(D)



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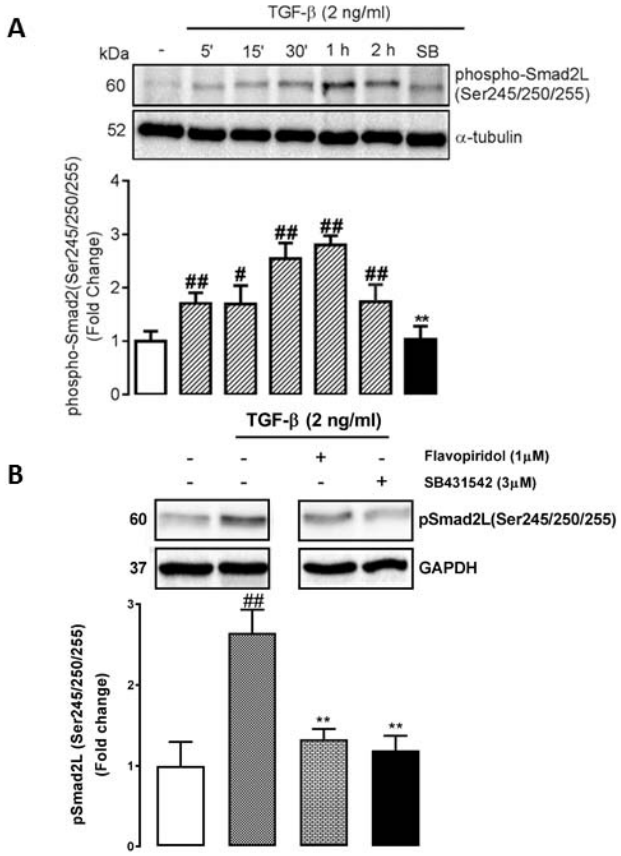

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Figure 5.

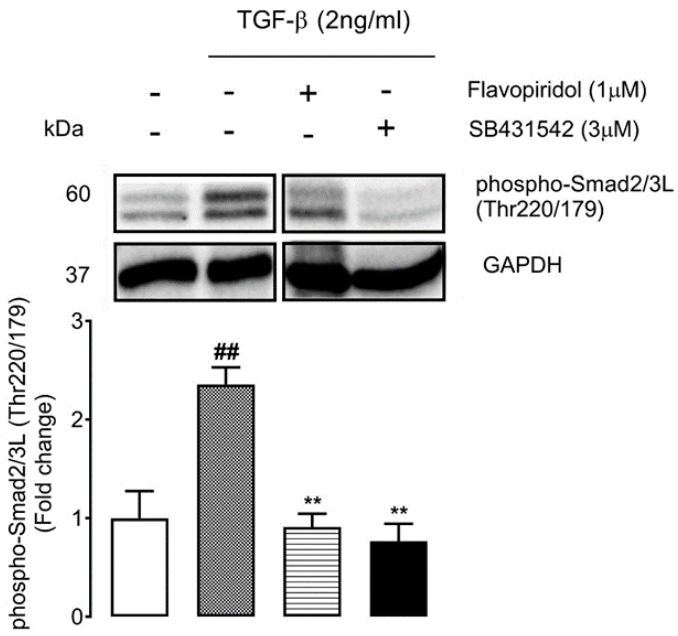

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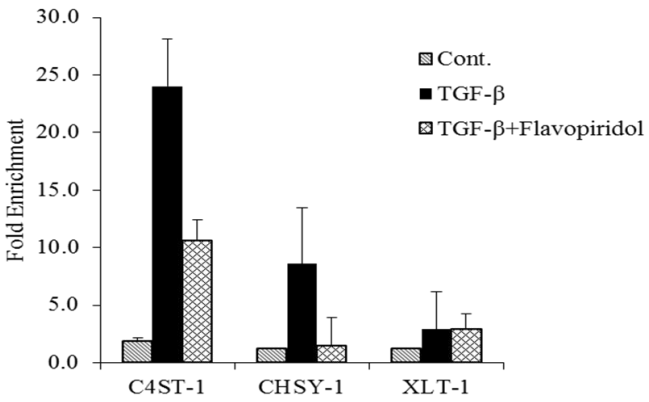
Figure 6.



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