WNT and β-catenin signalling in airway smooth muscle: emerging concepts for asthma

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Selective targeting of cbp/β-catenin inhibits growth of and extracellular matrix remodelling by airway smooth muscle

Research manuscript
Characterization of smooth muscle-derived wnt-5a

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Abstract

Asthma is a heterogeneous chronic inflammatory disease, characterized by the development of structural changes (airway remodelling). β-catenin, a transcriptional co-activator is fundamentally involved in airway smooth muscle growth, and may be a potential target in the treatment of airway smooth muscle remodelling. Using small-molecule compounds that selectively target β-catenin breakdown or its interactions with transcriptional co-activators, we assessed their ability to inhibit airway smooth muscle remodelling in vitro and in vivo. ICG-001, a small-molecule compound that inhibits the β-catenin/CBP interaction, strongly and dose-dependently inhibited serum-induced smooth muscle growth and TGF-β1-induced production of extracellular matrix components in vitro. Inhibition of β-catenin/p300 interactions using IQ-1 or inhibition of tankyrase 1/2 using XAV-939 had considerably less effect. In a mouse model of allergic asthma, β-catenin expression in the smooth muscle layer was found unaltered in control versus ovalbumin-treated animals, a pattern that was found to be similar in smooth muscle within biopsies taken from asthmatic and non-asthmatic donors. However, β-catenin target gene expression was highly increased in response to ovalbumin, which was prevented by topical treatment with ICG-001. Interestingly, ICG-001 dose dependently reduced airway smooth thickness after repeated ovalbumin challenge, but had no effect on the deposition of collagen around the airways, mucus secretion or eosinophil infiltration. Together, our findings highlight the importance of β-catenin/CBP signalling in the airways and suggest ICG-001 may be a new therapeutic approach to treat airway smooth muscle remodelling in asthma.

Keywords

Asthma, airway remodelling, airway smooth muscle, extracellular matrix, β-catenin, small molecules
**Introduction**

Asthma is a chronic inflammatory disease of the large and small airways, estimated to affect 235 million people worldwide. A cardinal feature of asthma is airway hyper-responsiveness, which is defined as the exaggerated bronchoconstriction response to specific and non-specific stimuli, in which the airway smooth muscle (ASM) is fundamentally involved. Airway hyper-responsiveness has a variable and persistent component. The variable component is dependent on chronic airway inflammation, involving a wide range of inflammatory cells, whereas the persistent component is dependent on the manifestation of structural changes, or airway remodelling. Airway remodelling encompasses increased ASM mass, mucous gland hypertrophy, angiogenesis, subepithelial fibrosis and epithelial changes including cell detachment and goblet cell hyperplasia.

Chronic inflammation has long been considered the primary cause of structural tissue changes. While there is much evidence to support this, an increasing bundle of evidence suggests that airway remodelling occurs already early in the natural history of asthma, even before the onset of symptoms.

Reticular basement membrane (RBM) thickening, epithelial damage, vessel formation and increased ASM thickness have all been described in young children with asthma. Moreover, many studies have been unable to demonstrate a relationship between airway inflammation or asthma duration on one hand and severity of airway remodelling on the other, and clinical symptoms remain relatively constant with age. These findings challenge the paradigm that airway remodelling is a direct consequence of inflammation and suggest that the two may develop in parallel. Current asthma treatment is mainly focused on reducing inflammation and relaxing ASM, while no drug therapy exists that primarily targets airway remodelling.

β-catenin is a key component of adherens junctions that links family members of the transmembrane protein cadherin (N-cadherin in ASM) to the cytoskeleton and functions in cell-cell adhesion. In addition, β-catenin is a critical effector of both wingless-type and integrase 1 (WNT)-dependent and independent signalling where it functions as a transcriptional coactivator. The central event for the induction of β-catenin signalling is the accumulation of β-catenin in the cytosol.
followed by its translocation to the nucleus. In the absence of extracellular signals, levels of β-catenin are being kept low by means of an Axin-adenomatous polyposis coli (APC) complex that captures β-catenin and subjects it to phosphorylation by Glycogen synthase kinase (GSK)-3β and Casein kinase (CK)-1α, leading to its proteasomal degradation. Conversely, when WNT ligands bind FZD receptors in association with LRP-5/6 co-receptors, the destruction complex is sequestered towards the membrane, effectively rescuing β-catenin from GSK-3β-mediated phosphorylation, and allowing for its accumulation. WNT-independent mechanisms also contribute to the stabilization of β-catenin via growth factors such as Platelet-derived growth factor (PDGF) and TGF-β1 which regulate nuclear β-catenin accumulation through Akt and GSK-3β. In the nucleus, β-catenin associates with a plethora of interaction partners, consequently leading to the transcriptional regulation of genes, many of which are involved in tissue repair and remodelling. To assemble a transcriptional complex, β-catenin depends on the transcriptional co-activators CREB-binding protein (CBP) or its closely related homologue E1A-associated protein p300. In smooth muscle, β-catenin plays a critical role in pro-mitogenic signalling, which has been shown in vitro and in vivo. Moreover, TGF-β1-mediated production of extracellular matrix (ECM) proteins in ASM is dependent on β-catenin activation. The roles of CBP and p300 in these processes have not been investigated yet.

Targeting β-catenin in ASM to inhibit airway remodelling may be an interesting new approach in the context of asthmatic airway remodelling. In this study we sought to evaluate the potential of pharmacological β-catenin inhibition on ASM proliferation and ECM production, using commercially available small-molecule compounds that selectively attenuate the nuclear functions of β-catenin. To this aim, we evaluated the efficacy of ICG-001, a compound preventing β-catenin/CBP interaction, IQ-1, a compound preventing β-catenin/p300 interaction, and XAV-939, an inhibitor of tankyrase 1 and 2 which stabilizes axis inhibition protein (Axin), thereby reinforcing the β-catenin destruction complex. As ICG-001 proved most effective in attenuating ASM growth and ECM remodelling, we further explored this compound in vivo, in its ability to prevent ASM remodelling in a chronic mouse model of allergic asthma.
Results

Small-molecule compounds effectively inhibit β-catenin function in ASM cells

Three commercially available compounds were selected for this study: ICG-001 (inhibits β-catenin/CBP interaction, IC50 3 µM 37), IQ-1 (prevents β-catenin/p300 interaction, IC50 2 µM 38) and XAV-939 (a WNT-specific β-catenin antagonist that inhibits Tankyrase 1 and 2 to stabilize Axin, IC50 4/11 nM respectively 39). A TOPFlash reporter assay was used to establish their effects on β-catenin-mediated T-cell factor (TCF)-dependent gene transcription. TOPflash constructs contain copies of wild-type TCF/LEF binding sites upstream of a luciferase reporter, whereas the FOPflash contains mutated TCF/LEF binding sites. All three compounds were able to attenuate FBS-induced β-catenin/TCF target gene transcription (Figure 2A) in cultured ASM cell lines. As XAV-939 affects global levels of active β-catenin, the TOPflash results were corroborated with a Western Blot. As expected, treatment with XAV-939 inhibited protein levels of non-phospho (active) β-catenin, whereas levels of total β-catenin remained unaffected (Figure 2B). In line with this, ICG-001 and IQ-1 did not affect β-catenin expression (Figure 2C).

Inhibition of β-catenin/CBP reduces ASM cell proliferation in vitro

ASM actively utilizes β-catenin to induce cell proliferation 16,25. To further investigate this relation and the specific mechanisms involved, we studied the effects of small-molecule inhibition. Cells were treated with FBS in combination with increasing concentrations of ICG-001, IQ-1 or XAV-939. After which, the reductive capacity of the cells as an indicator for cell number was measured with an Alamar Blue conversion assay. ICG-001 and XAV-939 dose dependently inhibited FBS-induced Alamar Blue fluorescence intensity (63% and 48% reduction respectively), whereas IQ-1 had no effect (Figure 3A). To confirm whether these results were due to cellular proliferation, protein levels of phosphorylated Rb (pRb) were assessed as a marker for cell-cycle progression. ICG-001 inhibited FBS-induced expression of pRB by 68% at 3 µM compared to DMSO-treated cells (Figure 3B). IQ-1 and XAV-939 both appeared to decrease pRb expression, albeit non-significantly, and were markedly less effective in this response compared to ICG-001 (Figure 3B). To further elaborate on the underlying mecha-
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nisms involved, we followed up on these experiments by using the RTK agonist PDGF, which drives nuclear β-catenin accumulation through Akt and GSK-3β. Similar to the FBS-induced results, ICG-001, but also IQ-1 strongly inhibited PDGF-induced phosphorylation of Rb (78% and 62% reduction at 3 μM for ICG-001 and IQ-1 respectively) (Figure 3C). Again, XAV-939 was less responsive, failing to reach a statistically significant reduction (Figure 3C). Together, the data shows that inhibition of β-catenin signalling via CBP or p300 effectively attenuates ASM proliferation.

Impaired collagen 1α1 production following β-catenin inhibition in vitro

Whereas TGF-β1 has weak mitogenic effects in ASM cells, it is a potent regulator of the expression of various ECM genes in ASM, which underlies both inhibition of GSK-3β as well as de novo synthesis of β-catenin. To further explore this principle, we investigated the effects of β-catenin inhibition on ECM production by ASM cells, exposing cells to TGF-β1 co-treated with either ICG-001, IQ-1 or XAV-939. Both mRNA and protein levels were measured. Cells treated with ICG-001 exhibited a significant dose dependent reduction in TGF-β1-induced gene expression of collagen 1α1 (47% reduction at 3 μM) (Figure 4A, left upper panel). These findings were corroborated on a protein level (67% reduction at 3 μM) (Figure 4B, left panel). XAV-939 did not decrease mRNA abundance, but did reduce protein expression, although not to the same extent as ICG-001 (39% reduction at 3 μM) (Figure 4A-B, right panels). Interestingly, IQ-1 treatment resulted in increased TGF-β1-induced expression of collagen 1α1 mRNA, yet with decreased protein expression (58% reduction at 3 µM) (Figure 4A-B, middle panels). In terms of mRNA expression, ICG-001 also proved most effective for the other TGF-β1 target genes connective tissue growth factor (CTGF) and fibronectin I (Figure 4A, middle and lower panels).

Expression of target genes, but not β-catenin itself is increased in OVA-challenged mice

ICG-001 demonstrated superior effects over IQ-1 and XAV-939 in terms of inhibition of ASM cell proliferation and ECM production. For that reason, we selected ICG-001 for subsequent in vivo studies to assess the effect of CBP/β-catenin inhibition on airway remodelling using a chronic ovalbumin mouse model. Ovalbumin induces a robust pulmonary inflammatory response. The
model is widely used to reproduce the pathophysiology of clinical (allergic) asthma and displays many features of airway remodelling similar to the disease. Interestingly, repeated ovalbumin challenge did not induce differences in both total as well as non-phosphorylated (active) β-catenin levels in the ASM bundle of frozen lung tissue sections (Figure 5A-B). The fluorescence staining intensity in the ASM bundle was also low compared to surrounding tissues, such as the epithelium. However, mRNA abundance of WNT1 inducible signaling pathway protein (WISP) 1, Axin 2 and cMYC, three well-known β-catenin target genes, were increasingly expressed in whole lung homogenates of ovalbumin challenged mice (738%, 214% and 458% increase respectively vs PBS-challenged mice) (Figure 5C). Moreover, topical treatment of the airways with intranasally instilled ICG-001 30 min prior to each challenge dose dependently attenuated the ovalbumin-induced increase in WISP 1, cMYC and Axin 2 gene expression (93%, 71% and 63% reduction respectively for the 1 mM group vs OVA). These data demonstrate that β-catenin expression in ASM does not appear to be altered under conditions of allergic airway inflammation, whereas its target genes WISP 1, Axin 2 and cMYC are differentially expressed in whole lung tissue, indicating activation of β-catenin dependent gene transcription.

**β-catenin expression does not change in ASM of mild to moderate asthmatics**

The results obtained from the mouse model suggests that allergic airway inflammation does not change either the expression or the activation state of β-catenin in ASM. We wanted to know whether human asthmatic ASM would display a similar pattern. To evaluate the significance of targeting β-catenin-mediated transcription in asthma, we assessed expression levels of total and non-phosphorylated β-catenin in the ASM bundle of tissue derived from mild to moderate asthmatic and non-asthmatic donor lungs. Similar to our animal model, fluorescence emission of total β-catenin levels were no different in asthmatic ASM compared to non-asthmatic ASM (Figure 6A). We also performed immunohistochemistry, which revealed that also the active form of β-catenin was not differentially expressed between asthmatics and non-asthmatics (Figure 6B).
ICG-001 prevents OVA-induced airway smooth muscle remodelling

Because β-catenin dependent gene transcription was elevated in our animal model, we assessed the ability of ICG-001 to reduce ASM bundle thickness and ECM production, using alpha smooth muscle actin (α-SMA) and total collagen as a readout. Repeated ovalbumin challenge increased α-SMA expression measured around the airways compared to the saline-treated group (158% increase vs PBS-challenged mice). Interestingly, ICG-001 treatment fully reversed this process back to PBS-treated levels (44% reduction for the 0,1 mM group) (Figure 7A). Curiously, the smallest dose produced the strongest response, whereas the highest dose yielded no statistically significant difference in smooth muscle thickness. A Picro Sirius Red staining indicative of the total collagen deposited around the airways revealed increased production in the OVA-treated group (161% increase vs PBS-challenged mice). A declining trend towards reduced collagen levels following ICG-001 treatment can be appreciated from the data, albeit without statistical significance (Figure 7B). Together, the data indicates that in an allergic environment, ICG-001 negatively affects ASM remodelling.

Eosinophil infiltration and mucous production is not affected by ICG-001 treatment

Next to its ability to contract, the airway smooth muscle also carries an important secretory function in the airways. We were interested in whether ICG-001 could affect inflammatory events within the proximity of the ASM bundle. As expected, repeated ovalbumin challenge increased the amount of mucous covering the airway lumen (OVA vs PBS), as determined with a periodic acid-Schiff staining. However, treatment with ICG-001 did not significantly negate the increased mucus area (Figure 8A). Extending on these findings, we investigated the number of eosinophils that infiltrated around the airways, visualised by 3,3’-Diaminobenzidine. While the number of eosinophils was greatly increased in the OVA-treated group, there was no statistically significant difference between OVA and any of the ICG-001-treated groups (Figure 8B).
Figure 1: experimental procedure for the chronic allergic (ovalbumin) asthma mouse model. Mice were initially sensitised to ovalbumin (OVA) on days 1, 14 and 21 and subsequently challenged with saline or OVA aerosols for four consecutive weeks. ICG-001 or DMSO as a vehicle control was delivered intranasally via instillation 30 min prior to each challenge.
Figure 2: small-molecule compounds inhibit β-catenin in vitro. (A) TOPFlash reporter assay of immortalized human airway smooth muscle cells exposed to 10% fetal bovine serum (FBS) for 24 h with or without ICG-001, IQ-1 or XAV-939 (3 µM). Data represents six independent experiments. (B) and (C) Non-phosphorylated and total β-catenin immunoblot of lysates from cultured human airway smooth muscle cells, normalized against GAPDH. Cells were treated with XAV-939, ICG-001 or IQ-1 (10 µM) for 16 hours. Data represents four independent experiments. Data is expressed as the mean ± SEM. * vs control, $ vs FBS.
Figure 3: β-catenin inhibition reduces ASM cell proliferation in vitro. (A) Alamar Blue viability assay of immortalized human airway smooth muscle cells exposed to 10% fetal bovine serum (FBS) for 48 h with or without ICG-001, IQ-1 or XAV-939 (0.3-10 µM). Data represents six independent experiments. (B) and (C) Phosphorylated (Ser 807/811) retinoblastoma protein (Rb) immunoblot indicative of G1 cell-cycle progression, normalized against GAPDH. Cells were treated with either 10% FBS or 50 ng mL\(^{-1}\) PDGF with or without ICG-001, IQ-1 or XAV-939 (3 and 10 µM) for 16 hours. Data represents five, seven and five independent experiments for ICG-001, IQ-1 and XAV-939 treated samples respectively for (B) and five independent experiments for (C). Data is expressed as the mean ± SEM. * vs control, $ vs FBS or PDGF.
Figure 4: impaired collagen 1α1 production following β-catenin inhibition in vitro. (A) mRNA of airway smooth muscle cells pre-incubated with TGF-β1 (2 ng mL⁻¹) for 24 hours with or without ICG-001, IQ-1 or XAV-939 (3 and 10 µM) was isolated and subjected to RT-qPCR. Bars represent collagen 1α1 gene expression. Data represents five, eight and four independent experiments for ICG-001, IQ-1 and XAV-939 treated samples respectively. (B) Collagen 1α1 immunoblot, normalized against GAPDH. Cells were treated with TGF-β1 (2 ng mL⁻¹) for 48 hours with or without ICG-001, IQ-1 or XAV-939 (3 and 10 µM). Data represents five independent experiments. Data is expressed as the mean ± SEM. * vs control, $ vs TGF-β1.
Figure 5: β-catenin (target gene) expression in OVA-challenged mice. (A) Representative immunofluorescent image (left) and the quantification (right) of total β-catenin (green color), normalized to dapi from frozen lung tissue sections of mouse airways exposed to repeated allergen (ovalbumin) challenge vs PBS-treated controls. Nuclear dapi staining is represented by blue. Dashed line represents the airway smooth muscle (ASM) boundary. Alv is alveoli, Epi is epithelium. Each dot represents a single animal. (B) Immunofluorescent image of non-phosphorylated (active) β-catenin as in (A). (C) mRNA of whole lung homogenates subjected to RT-qPCR, obtained from animals exposed to ovalbumin or PBS and treated with intranasally instilled ICG-001. Group sizes are n = 9, 9, 10, 8, 7 for the respective groups (left to right). Data is expressed as the mean ± SEM. * vs PBS, $ vs OVA.
Figure 6: β-catenin expression in asthmatic airway smooth muscle. (A) Representative immunofluorescent image (left) and the quantification (right) of total β-catenin (green color) from asthmatic and non-asthmatic donor airway smooth muscle tissue, normalized to dapi (blue color). Two airways per donor were analyzed. (B) Representative immunohistochemistry image (left) and the quantification (right) of non-phosphorylated (active) β-catenin (DAB) in asthmatic and non-asthmatic donor airway smooth muscle tissue.
Figure 7: ICG-001 prevents OVA-induced airway smooth muscle remodelling. (A) Representative immunohistochemistry images (left) and the quantification (right) of an alpha smooth muscle actin staining visualized with DAB, obtained from paraffin-embedded lung tissue sections of mouse airways exposed to repeated allergen (ovalbumin) challenge vs PBS-treated controls. Data represents at least seven animals. (B) Immunohistochemistry staining as in (A) of total collagen deposition, measured with a Sirius Red staining. Group sizes are n = 10, 10, 10, 8, 10, 7 for the respective groups (left to right). Data is expressed as the mean ± SEM. * vs PBS, $ vs OVA.
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Figure 8: Effects of ICG-001 on eosinophil infiltration and mucous production. (A) Representative immunohistochemistry images (left) and the quantification (right) of a periodic acid Schiff staining, indicative of mucous, obtained from paraffin-embedded lung tissue sections of mouse airways exposed to repeated allergen (ovalbumin) challenge vs PBS-treated controls. Data represents at least seven animals. (B) Immunohistochemistry staining as in (A) of eosinophil’s endogenous peroxidase activity, measured with DAB. Group sizes are n = 10, 10, 10, 8, 10, 7 for the respective groups (left to right). Data is expressed as the mean ± SEM. * vs PBS.
Discussion

In this study we evaluated the potential of three small-molecule compounds, ICG-001, IQ-1 and XAV-939, in their ability to inhibit β-catenin function in vitro. We demonstrated that treatment with ICG-001 effectively inhibits airway smooth muscle cell cycle progression. Interaction of the co-activator CBP with β-catenin, rather than p300 or effectors that stabilize Axin appears fundamental in the regulation of this process. These results fit the previously proposed model that TCF/β-catenin/CBP interaction primarily initiates gene transcription events related to cellular proliferation, whereas TCF/β-catenin/p300 interaction is more involved in driving normal cellular differentiation. In our study there is some inhibition of cell growth following IQ-1 exposure, suggesting that in ASM cells there may be some degree of redundancy between CBP/p300 function. Alternatively, differences in endogenous expression levels could underlie the dissimilar response. IQ-1 exerts its action on p300 by inhibiting protein phosphatase 2A (PP2A). It does so by binding to its PR72 and PR130 subunits, resulting in decreased phosphorylation of p300 through an as yet undetermined mechanism. This reduces its binding affinity to β-catenin and thereby diminishing its interaction with p300 or effectors that stabilize Axin. PR72 and PR130 expression levels have been reported to be very low in smooth muscle, possibly explaining the relative mild effects of IQ-1 on ASM proliferation. Interestingly, treatment with XAV-939 was unable to produce the same degree of inhibition as ICG-001, whereas levels of non-phospho β-catenin are clearly diminished (Figure 2B). It is possible that only very low levels of β-catenin are required in the nucleus to induce cell growth and that the leftover pool after XAV-939 is sufficient to do so. In addition, XAV-939 acts upon the β-catenin destruction complex and thus primarily influences WNT-dependent signalling. The results support the notion that FBS-induced proliferation in ASM may be more driven via WNT-independent mechanisms that stabilize β-catenin independent from the destruction complex.

We further showed that β-catenin is essential in the regulation of ECM production in ASM cells. CBP appears central in this event, as exposure to ICG-001 dose dependently inhibited synthesis of both collagen 1α1 mRNA and protein following TGF-β1 stimulation. Interestingly, exposure to IQ-1 dose dependently increased mRNA abundance for collagen 1α1, but
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decreased protein yield. The discrepancy between mRNA and protein further demonstrates the complex interplay between CBP and p300 signalling or possibly off-target effects of IQ-1, and it was beyond the scope of this study to investigate the underlying principles further.

In our animal model, topical application of ICG-001 to the airways of BALB/c mice significantly reduced ASM mass induced by repeated allergen challenge. However, contrary to our in vitro findings, no changes in collagen content were found. This finding contradicts previous publications on β-catenin inhibition in murine models of fibrosis in the lung 45,46, where pharmacologic inhibition of β-catenin or overexpression of Dickkopf-1 ameliorates fibrosis. In one study, administration of ICG-001 in a Bleomycin-mouse model prevented fibrosis, and late stage application was able to reverse established fibrosis 47. Various explanations may underlie these differences. An important distinction that has to be addressed here is the model used for experimentation. In the Bleomycin model, fibrosis is initially induced by new collagen deposition, whereas the continued pathology results from the maintenance of established ECM and not due to newly synthesized collagen 48. In asthma, ECM turnover is significantly enhanced 49,50, not only by the synthesis of new proteins and other components, but also by its degradation through the activity of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) 51. Increased ECM turnover in the ovalbumin model may have obscured potential effects mediated by ICG-001. Alternatively, overlapping functions of CBP and p300 may have contributed to the observed effect, as CBP and p300 may act redundantly in some cell systems. ICG-001 competes with β-catenin to CBP, resulting in increased binding of β-catenin to additional partners like p300 52. In this case, application of ICG-001 would not be sufficient to achieve full inhibition. Moreover, next to the airway smooth muscle, other cell types like the myofibroblast also contribute to peribronchial collagen deposition 53 and it is unclear at this moment what the relative contribution of each source is. In light of this, TGF-β1-driven expression of collagen type 1 has been shown to be mediated via p300 in human fibroblasts 54,55.

Little work has been done on WNT/β-catenin or TGF-β1/β-catenin signalling in the context of asthma. Smooth muscle development in the developing mouse lung requires β-catenin signal-
ling and its activity is increased following exposure to the allergen *Aspergillus fumigatus* [27]. Furthermore, in primary bronchial epithelial cells, TGF-β1-induced epithelial to mesenchymal transition (EMT) is paralleled by activation of β-catenin signalling, which is even further enhanced when exposed to house dust mite extract [56]. In our study, we did not find any difference in either total or non-phosphorylated (active) β-catenin expression between healthy and asthmatic ASM lung tissue, which we corroborated with our ovalbumin mouse model. However, expression of the β-catenin target genes WISP 1 and Axin 2 were increased in ovalbumin challenged mice. Perhaps the limited proportion of total β-catenin translocating to the nucleus or differences between the time point at which translocation is observed (24 h after allergen challenge in our study) account for this apparent discrepancy. β-catenin is a pleiotropic gene, involved in many different cellular processes, and its activation requires tight regulation to coordinate cell behaviour. This translates into transient periods of activation, where both activation and diminution act in quick succession. Exposure to allergens could initiate a cascade of events starting with activation of β-catenin and setting the stage for the development of airway remodelling. As such, it is possible that although activation of β-catenin is a critical initiating event in the pathogenesis of asthma, it eventually becomes superfluous in the face of additional downstream targets that accumulate over time. Additionally, even when there is no change in the total β-catenin pool, there may still be changes in protein binding mediated by β-catenin, driven by the presence of additional adapter proteins that may show altered expression in asthma. This results in enhanced β-catenin target gene transcription, without any change in β-catenin abundance itself. Finally, potential changes in β-catenin expression may be limited to only a few cells. Even within one individual, smooth muscle cells exhibit different phenotypes, some synthetic in nature and others being more contractile. As these phenotypes associate with different β-catenin expression levels [25], measuring the entire ASM pool will potentially mask changes in individual cells. To answer these questions, it is important that we gather more information about the temporal behaviour of β-catenin and to assess how β-catenin target genes behave in an asthmatic environment.
Selective targeting of CBP/β-catenin

β-catenin signalling plays a crucial role in orchestrating developmental processes and maintaining tissue homeostasis in adult life. It is essential in virtually every organ system in homeostasis, including the lung. It is of no surprise that abnormal regulation is linked to such a vast array of diseases\(^5^7\). Even within the lung, β-catenin signalling performs seemingly opposing roles in cellular proliferation and differentiation\(^5^8\). This is not unexpected, given the staggering amount of binding partners that β-catenin has, modulating a plethora of downstream biological processes. The ubiquitous nature of β-catenin signalling and its pleiotropic effects make it problematic for specific targeting, particularly in a chronic setting, such as asthma. Successfully targeting β-catenin signalling in asthma requires careful balancing, where inhibition of β-catenin in off-target tissues could cause unwanted effects. Now that we begin to understand the crucial role of cofactors such as CBP and p300, and the disparate effects that they may mediate, new therapeutic avenues are beginning to emerge. ICG-001 holds much promise in this regard, as it disrupts only a very small subset of CBP interactions, initiating a transcriptional program that inhibits ASM growth. At the same time, additional genes regulated by p300 or other cofactors that are important in maintaining cellular homeostasis are not affected by the therapy, something that would not be achieved by inhibition of β-catenin alone. This shows that β-catenin/CBP inhibition may be a successful strategy for inhibition of ASM thickening in a way that is independent of inflammation, which is of clear relevance to severe asthma, in which ASM remodelling persists despite treatment with corticosteroids.

In conclusion, we show that the β-catenin/CBP interaction is essential in the regulation of airway smooth muscle proliferation and production of extracellular matrix components. In vivo, in spite of a lack of observable changes in β-catenin expression levels, asthmatic airways exhibit enhanced β-catenin target gene transcription, which can be inhibited by administration of ICG-001. Inhibition of β-catenin results in diminished airway smooth muscle thickness driven by repeated allergen challenge in a mouse model of allergic asthma, but has no effect on total collagen deposition. These findings highlight the importance of β-catenin/CBP signalling in the airways and suggest ICG-001 may be a new therapeutic approach to treat airway smooth muscle remodelling in asthma.
Materials and methods

Cell culture
Three human bronchial smooth muscle cell lines, immortalized by stable expression of human telomerase reverse transcriptase (hTERT), were used for all experiments. Generation of these cell lines has been described previously. Cells were used up to passage 30 for all experiments. Cells were grown in uncoated 100/20 mm tissue culture dishes (GBO, #664160) in DMEM (GIBCO, #42430-082) supplemented with 200 units mL⁻¹ Penicillin-Streptomycin (GIBCO, #15070-063), 2.5 µg mL⁻¹ antimycotic (GIBCO, #15290-026) and 10% vol/vol Fetal Bovine Serum (FBS) (Thermo Scientific, #SV30180.03).

TOPFlash assay
Cells grown in DMEM supplemented with antibiotics, antimycotics and 10% vol/vol FBS were seeded on uncoated 6-wells plates (Sigma Aldrich, #CLS3506) (300.000 cells/well) and were serum-starved after attachment for 12 hours in plain DMEM. Cells were transfected for 24 hours with either 1 µg TOPFlash (Addgene, #12456) or FOPFlash (Addgene, #12457) reporter plasmids using the TransIT-X2 transfection delivery system (Mirus, #MIR6000). Following transfection, cells were washed with PBS and subjected to stimulation. Luciferase activity was assayed with the Dual-Luciferase Reporter assay system (Promega, #E1910) according to the manufacturer’s instructions.

Western blot analysis
Cells were washed with PBS and incubated with RIPA lysis buffer (65 mM Tris, 155 mM NaCl, 1% Igepal CA-630, 0.25% sodium deoxycholate, 1 mM EDTA, pH 7.4, and a mixture of protease inhibitors: 1 mM Na₃VO₄, 1 mM NaF, 10 µg ml⁻¹ leupetin, 10 µg ml⁻¹ Pepstatin A, 10 µg ml⁻¹ Aprotinin). Cells were then scraped from the plate and kept on ice for 15 min. Lysates were vortexed vigorously and finally centrifuged for 10 min at 10,000 g. Lung homogenates were first pulverised with a pestle and mortar in liquid nitrogen and subsequently sonicated in RIPA lysis buffer. Following sonication, samples were processed in the same way as the cell lysates. Protein content of the supernatant fractions was determined with a BCA protein assay kit (Thermo Scientific, #23225) and subsequently subjected to SDS-PAGE, using 6% and 10% running gels (depending on protein size). Separated proteins were transferred to either nitrocellulose (Bio-rad, 0.2 µm, #162-0112) or PVDF membranes (Carl Roth, 0.45 µm,
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#T830.1), which were then blocked with ROTI®-Block blocking solution (Carl Roth, #A151.2) for 2 hours at room temperature. Membranes were incubated with primary antibodies overnight at 4 °C in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.05% (w/v) Tween-20, pH 7.4). The next day, after washing in TBST, membranes were incubated with HRP-conjugated secondary antibody for 2 hours at room temperature. Finally, blots were developed using enhanced chemiluminescence substrate (Perkin Elmer, #NEL105001EA). Digital images were quantified by densitometry using LI-COR Image Studio Lite software.

**Cell proliferation assay**

Cellular viability, or the reductive potential of the cell population, was measured with an Alamar Blue conversion assay, the conversion of which is proportional to cell number (Invitrogen, #DAL1100). Cells grown in DMEM supplemented with antibiotics, antimycotics and 10% vol/vol FBS were seeded on uncoated 24-well plates (Sigma Aldrich, #CLS3524) (30,000 cells/well) and were serum-starved after attachment for three days in serum-free DMEM supplemented with 1% ITS (insulin, transferrin, selenium) (GIBCO, #51300). Following stimulation for 48 hours, cells were washed with PBS and incubated with 10% alamarBlue® reagent together with 1x HBSS (GIBCO, #14065) at 37 °C for 30 min. Supernatant was collected and fluorescence was measured in a 96-wells plate (Sigma-Aldrich, #CLS3596) with excitation wavelength at 530 nm and emission wavelength at 590 nm.

**RT-qPCR**

Cells were washed with PBS and incubated with lysis buffer before being scraped from the plate. Lung homogenates were first pulverised with a pestle and mortar in liquid nitrogen prior to addition of lysis buffer. Isolation of mRNA was performed with a NucleoSpin® RNA isolation kit (Macherey-Nagel, # 740955.250) according to the manufacturer’s instructions. Equal amounts of cDNA were synthesized using AMV reverse transcriptase (Promega, #A3500) and diluted 15 times with RNAse-free ddH₂O. Quantitative real-time PCR was performed on an Illumina Eco Real-Time PCR system using SYBR green as the DNA binding dye (Roche, #04913914001). PCR cycling was performed with denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec for 45 cycles. Analysis of RT-qPCR data was done using LinRegPCR analysis
software \textsuperscript{31,32}. 18S ribosomal RNA, Beta-2-Microglobulin (B2M) and Ribosomal Protein L13A (RPL13A) were used as reference loci for accurate normalization of the RT-qPCR data. Primer sequences are listed in Table 1. Total RNA yield was determined with a NanoDrop ND-1000 spectrophotometer and samples were normalized accordingly.

**Animals**
All procedures described in this study were approved by the animal ethics committee (DEC) of the University of Groningen under license number DEC-6485. Inbred female BALB/c mice (n=10 per group) were obtained from Charles River (Leiden, The Netherlands). Animals were group-housed (10 per cage) in climate controlled animal quarters and provided with food and water *ad libitum*. A 12h/12h light/dark cycle was maintained. Animals were 8-12 weeks of age at the start of the experiment, weighing 16-20 g and were randomly assigned to the different experimental groups. Group sizes were calculated based on airway eosinophil infiltration. Earlier studies with the ovalbumin-challenge model within the University Medical Center Groningen has shown an average of 16 ± 5 eosinophils \textsuperscript{33}. Assuming equal variance, alpha of 0,05, 1-beta of 0,8 and an effect size of 40% a group size of 10 can be obtained. Some samples were lost either due to death of the animals or post-processing issues of the animal tissue. These samples were not replaced as the ovalbumin-challenge for this particular group of animals yielded a very robust infiltration of airway eosinophils (average of 30 ± 3 for the ovalbumin-treated animals); almost twice the size of earlier studies and therefore maintaining sufficient power.

**Allergen exposure**
To induce a chronic allergic asthmatic response, animals were sensitized to chicken-derived ovalbumin (OVA) (Sigma-Aldrich, #A5378), using Aluminum Hydroxide (AlOH\textsubscript{3}) (Imject\textsuperscript{TM}, Thermo Scientific, #77161) as an adjuvant to promote an IgE and Th2 skewed response, as previously described \textsuperscript{34} (Figure 1). In brief, animals were sensitized on days 1, 14 and 21 by means of an intraperitoneal injection of 10 µg OVA together with 1.5 mg of AlOH\textsubscript{3} dissolved in 200 µl saline. Subsequently, animals were exposed to aerosolized OVA (1% w/v in saline) or saline for 20 min/day on two consecutive days/week for four weeks. Exposures were carried out in a custom-built Perspex chamber (9 L) with a DeVilbiss Model 646 nebulizer.
Air flow was set at a rate of 40 L/min, providing aerosol with an output of 0.33 mL/min. 30 min prior to each challenge, 30 uL containing ICG-001 (0.1 mM, 0.33 mM, 1.0 mM) or DMSO as a vehicle control, suspended in PBS, was briefly vortexed and administered endotracheally via intranasal instillation immediately after brief exposure (circa 10 sec) to 5% isoflurane.

**Blood and tissue collection**

24 hours following cessation of the last inhalational exposure, animals were killed by exsanguination following a subcutaneous injection of 40 mg kg\(^{-1}\) ketamine and 0.5 mg kg\(^{-1}\) dexmedetomidine dissolved in 100 µl solvent. 0.5-1.0 mL blood was collected via cardiac puncture in 1.5 mL Eppendorf tubes and spun down to obtain serum. Lungs were harvested as follows: the post-caval, inferior lobe and left lobe were snap frozen in liquid nitrogen for mRNA and protein analysis. The leftover lungs were then inflated with 600 uL of a saline/Tissue-Tek® mixture (1:1 v/v), of which the superior lobe was subsequently fixed in formalin and later embedded in paraffin for immunohistochemistry. The middle lobe was snap frozen in liquid nitrogen and used for immunohistochemistry.

**Immunofluorescence**

Tissue-Tek®-perfused mouse lung tissue was cut with a Microm HM 525 (Thermo Scientific). Transverse cross-sections of 5 µm were used for analyses. In short, sections were washed in PBS and fixed in ice-cold Acetone for 15 min at 4 °C. The sections were then incubated with PBS containing 0.3% Triton X-100 for 5 min and then blocked in 10% normal goat serum with 1% BSA in PBS. After blocking, cells were incubated with primary antibodies diluted with 1% BSA in PBS overnight at 4°C, washed with PBS and incubated with fluorescent secondary antibody for 1 hour at room temperature. Finally, sections were incubated with Hoechst 33342 nucleic acid stain (Invitrogen, #H1399), washed in ddH\(_2\)O and mounted with ProLong® Gold antifade (Molecular Probes, #P36930).

Mean fluorescence intensity was digitally analysed with ImageJ (NIH) software. Following a 300 px background subtraction, images were thresholded and the integrated density per area from the appropriate channel was calculated, using the perimeter of the ASM relative to Hoechst 33342.
Table 1. Primer sequences used

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Species</th>
<th>Forward sequence (5'→3')</th>
<th>Reverse sequence (5'→3')</th>
</tr>
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<tbody>
<tr>
<td>Collagen 1α1</td>
<td>Homo Sapiens</td>
<td>TCAGCAAGAACCACAAAGGAC</td>
<td>CCGCCATACTCAGACTGGAAT</td>
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<tr>
<td>18S rib RNA</td>
<td>Homo Sapiens</td>
<td>CGCCGCTAGAGGTGAAAATTC</td>
<td>TTGGCAATGCTTTGCTTC</td>
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<tr>
<td>B2M</td>
<td>Homo Sapiens</td>
<td>AAGCAGCATCATGAGGTTTG</td>
<td>AAGCAAGCAAGCGAATTTGGA</td>
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<td>RPL13A</td>
<td>Homo Sapiens</td>
<td>ACCGCCCTACGCAAGAAA</td>
<td>GCTGTCACTGCTGCTACTT</td>
</tr>
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<td>WISP 1</td>
<td>Mus Musculus</td>
<td>CAATAGGAGTGTGACACAGGT</td>
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<tr>
<td>Axin 2</td>
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<td>CGGTGGGTTTCGAAATG</td>
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<tr>
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<tr>
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<td>CCTCAATGGACTCTGTTTA</td>
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<tr>
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<td>ACCGTCTACTGGGACAGA</td>
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<tr>
<td>RPL13A</td>
<td>Mus Musculus</td>
<td>AGAACACGGATTCTTACGGATTACG</td>
<td>GTTCACACCAGGAGTTAGTTA</td>
</tr>
</tbody>
</table>

nucleic acid stain. Sections were observed through a HC PL APO CS2 63x/1.4 (oil) objective on a Leica SP8 Confocal Microscope (DMI 600). Analyses were performed in a blinded fashion. This experiment has been performed at the UMCG Microscopy and Imaging Center (UMIC), which is sponsored by NWO-grants 40-00506-98-9021 (TissueFaxs) and 175-010-2009-023 (Zeiss 2p), University of Groningen.

Patient material
β-catenin staining were performed on de-identified asthmatic and non-asthmatic donor lungs not suitable for transplantation and donated for medical research, which were obtained through the International Institute for the Advancement of Medicine (Edison, NJ). Subject characteristics have been listed before 35. The study was approved by the ethics committees of the institutions involved.

Immunohistochemistry
Paraffin-embedded lung tissue was cut with a Microm HM 340E microtome (Thermo Scientific). Transverse cross-sections of 5 µm were used for analyses. In short, tissue sections were deparaffinised in xylene and rehydrated in a serial dilution of ethanol. Heat-
induced epitope retrieval was performed where necessary in a Decloaking chamber™ Nxgen pressure cooker (Biocare Medical). Sections were washed in PBS and blocked in normal serum from the species in which the secondary antibody was generated (either goat (Dako, #X0907) or rabbit (Dako, #X0902)). Primary antibody diluted in 1% BSA in PBS was incubated overnight at 4°C, then washed with PBS and incubated with 0.3% H₂O₂ in PBS for 15 min. Subsequently, sections were incubated with secondary antibody for 1 h at room temperature. Finally, sections were washed with PBS and developed with diaminobenzidine (Sigma-Aldrich, #D5637) for 5 min, followed by a hematoxylin counterstain (Sigma-Aldrich, #MHS32). Sections were then rinsed in tap water for 5 min, dehydrated in ethanol-xylene and mounted with KP-mounting medium (Klinipath, #7275).

Total collagen abundance was visualised with a Sirius red staining. In short, tissue sections were deparaffinised in xylene and rehydrated in a serial dilution of ethanol. Sections were incubated in Sirius red solution (Sirius red F33: Klinipath, #80115, picric acid: Sigma-Aldrich, #P6744) for 60 min at room temperature, followed by a 2 min wash with 0.01 N HCl. Sections were counterstained with hematoxylin (Sigma-Aldrich, #MHS32) for 5 min, rinsed with tap water for 5 min, quickly dehydrated in ethanol-xylene and mounted with KP-mounting medium (Klinipath, #7275).

Mucous was visualised with a Periodic Acid Schiff (PAS) staining. Tissue sections were deparaffinised and hydrated to deionized water. Sections were immersed in Periodic Acid solution (Sigma-Aldrich, #3951) for 15 min at room temperature, rinsed in water and immersed in Schiff’s Reagent (Sigma Aldrich, #3952016) in the dark for 30 min at room temperature. Sections were rinsed in water, counterstained with hematoxylin (Sigma-Aldrich, #MHS32) for 5 min, rinsed with tap water for 5 min, quickly dehydrated in ethanol-xylene and mounted with KP-mounting medium (Klinipath, #7275).

Digital images were quantified using ImageJ (NIH). Analyses were performed in a blinded fashion. Expression intensity was expressed as staining positive area relative to the length of the basement membrane squared (mm² / mm²).
Statistical analysis

All in vitro data represent the mean ± SEM, of at least four independent experiments, whereas for the in vivo experiments, at least seven animals were analysed per treatment group. In line with BJP guidelines, data with an n below 5 were not statistically analysed. A Shapiro-Wilk’s test (p>0.05) as well as visual inspection of the respective histograms, normal Q-Q plots and box plots was used to test whether samples were normally distributed (approximately), using IBM SPSS Statistics version 23. Comparisons between two groups were made using an unpaired Student’s t-test for normally distributed data or a Mann-Whitney U test as the non-parametric equivalent. Comparisons between three or more groups were performed using a one-way ANOVA followed by Tukey’s post hoc test for normally distributed data, or with a Kruskal-Wallis H test for non-normally distributed data. A value of p < 0.05 was considered statistically significant. Analyses were performed with GraphPad Prism (GraphPad Software, Inc.). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology 36.

Antibodies and chemicals

The following antibodies were used: GAPDH (western blot 1:3000, mouse, Santa Cruz, #sc-47724), Phospho-Rb (western blot 1:500, rabbit, Cell Signaling, #9308), α-Smooth Muscle Actin (immunohistochemistry 1:100, Abcam, #ab5694), Collagen 1α1 (western blot 1:1000, goat, Southern-Biotech, #1310-01), β-catenin (western blot 1:1000, immunofluorescence 1:300, mouse, BD Biosciences, #610154), Non-phospho (active) β-catenin (western blot 1:500, immunofluorescence 1:300, rabbit, Cell Signaling, #8814), peroxidase-conjugated anti-mouse IgG (western blot 1:3000, rabbit, Sigma-Aldrich, #A9044), peroxidase-conjugated anti-rabbit IgG (western blot 1:3000, goat, Sigma-Aldrich, #A0545), peroxidase-conjugated anti-goat IgG (western blot 1:8000, rabbit, Sigma-Aldrich, #A5420), Alexa Fluor® 633-conjugated anti-rabbit IgG (immunofluorescence 1:500, goat, Invitrogen, #A-21070).

Other reagents used include the following: DMSO (Sigma Aldrich, #472301), ICG-001 (Tocris, #4505), XAV-939 (Tocris, #3748), IQ-1 (Tocris, #4713), recombinant TGF-β1 (R&D Systems, #240-B), recombinant PDGF (Sigma-Aldrich, #P332G), ProLong® Gold Antifade Mountant (Molecular
Probes, #P36930), Hoechst 33342 (Molecular Probes, #H3570) and FBS (Thermo Scientific, #SV30180.03). All other chemicals were of analytical grade.

Acknowledgements
This study was financially supported by a NWO Vidi grant (016.126.307). The authors wish to thank Sophie I Bos and Anita I Spanjer for helping with the collection of animal tissue as well as preparation of the paraffin-embedded tissue.

Author contributions
TK conceived the study and designed the experiments, carried out the vast majority of the work, analysed the majority of the data and drafted the manuscript. SC carried out part of the in vitro work. MM carried out part of the in vivo work. TLH and DK provided and conducted work on the patient material. AJH kindly provided the human ASM cells. RG conceived the study and designed the experiments. All authors critically revised the manuscript for important intellectual content and approved its final version.

Competing financial interests
The authors declare no competing financial interests.
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Selective targeting of CBP/β-catenin


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