Title Page

Endothelin Receptor B Protects Granulocyte Macrophage Colony Stimulating Factor mRNA from Degradation

David Jungck, Jürgen Knobloch, Sandra Körber, Yingfeng Lin, Jürgen Konradi, Sarah Yanik, Erich Stoelben, Andrea Koch

Author affiliations
Medical Clinic III for Pneumology, Allergology, Sleep and Respiratory Medicine, Bergmannsheil University Hospital, Bochum, Germany (D. J., J. K., S. K., S. Y., A. K.).
Department of Pneumology, Clinic III for Internal Medicine, University of Cologne, Cologne, Germany (Y. L., J. K.).
Department of Thoracic Surgery, Lungenklinik Merheim, Kliniken der Stadt Köln, Cologne, Germany (E. S.).
Running Title: Endothelin Receptor B Protects GM-CSF mRNA from Degradation

Corresponding author: Prof. Dr. Andrea Koch, Medical Clinic III, Bergmannsheil University Hospital, Bürkle-de-la-Camp-Platz 1, 44789 Bochum, Germany. Tel.: +49-234-302-3530; Fax: -6440. andrea.koch@bergmannsheil.de

Number of Text Pages: 37
Number of Tables: 1
Number of Figures: 6
Number of References: 53
Number of Words in the Abstract: 250
Number of Words in the Introduction: 980
Number of Words in the Discussion: 1335
Abbreviations

ASMCs; airway smooth muscle cells
CXCL2; chemokine (C-X-C motif) ligand 2
CXCL3; chemokine (C-X-C motif) ligand 3
COPD; chronic obstructive pulmonary disease
ET-1; endothelin-1
ET\textsubscript{A}R; endothelin receptor A
ET\textsubscript{B}R; endothelin receptor B
ERA; endothelin receptor antagonists
ERK; extracellular signal-regulated kinase
GM-CSF; granulocyte macrophage colony stimulating factor
HASMCs; human airway smooth muscle cells
IL-32; interleukin-32
MMP12; matrix metalloproteinase 12
PAH; pulmonary arterial hypertension
PH; pulmonary hypertension
p38\textsuperscript{MAPK}; p38 mitogen-activated protein kinase
TNF\textalpha; tumor necrosis factor \textalpha

Recommended section assignment: Inflammation, Immunopharmacology, and Asthma
Abstract

Evidence lacks on the differential effects of the two therapeutic concepts of endothelin receptor antagonists (ERA): the blockade of only the endothelin-receptor-A (ET<sub>A</sub>R) (selective antagonism) versus both ET<sub>A</sub>R and ET<sub>B</sub>R (dual blockade). Ambrisentan, a selective ERA, and bosentan, a dual blocker, are both available for therapy. We hypothesized that there are differences in the potential of ERAs to ameliorate inflammatory processes in human airway smooth muscle cells (HASMCs) and aimed to unravel underlying mechanisms. We used HASMC culture, ELISA and qRT-PCR. TNFα induced transcription and expression of CXCL2, CXCL3, GM-CSF and MMP12 in HASMCs. In concentration-response experiments, bosentan led to a significantly greater reduction of GM-CSF and MMP12 protein release than ambrisentan while there was no significant difference in their effect on GM-CSF and MMP12 mRNA. Both ERA reduced CXCL3 protein and mRNA equally but had no effect on CXCL2. Blocking mitogen-activated protein kinases revealed that both ET<sub>A</sub>R and ET<sub>B</sub>R signal through p38<sup>MAPK</sup>, but ET<sub>B</sub>R also signals through ERK-1/-2 to induce GM-CSF expression. In the presence of the transcription inhibitor actinomycin D, bosentan but not ambrisentan reduced GM-CSF but not MMP12 and CXCL3 mRNA. In conclusion, blockade of each endothelin receptor subtype reduces GM-CSF transcription but blocking ET<sub>B</sub>R additionally protects GM-CSF mRNA from degradation via ERK-1/-2. Accordingly, blocking both ET<sub>A</sub>R and ET<sub>B</sub>R leads to a stronger reduction of TNFα induced GM-CSF protein expression. This mechanism might be specific for GM-CSF. Our data stress the anti-inflammatory potential of ERA and warrants further investigation of their utility in chronic inflammatory airway diseases.
Introduction

It is well established that Airway Smooth Muscle Cells (ASMCs) take part in the inflammatory processes of chronic airway diseases (Clarke et al., 2009; Knobloch et al., 2010; Barnes, 2011; Knobloch et al., 2013). They are being triggered to secrete pro-inflammatory cytokines and alter the expression of cell adhesion molecules (Damera et al., 2009). We have previously shown in human ASMCs (HASMCs) that tumor necrosis factor α (TNFα) activates an endothelin-1 (ET-1) autoregulatory positive feedback mechanism that sustains the expression of ET-1 and GM-CSF and that is inhibited by endothelin receptor antagonists (ERA) (Knobloch et al., 2009). TNFα induced GM-CSF expression and ET-1 transcription by signaling through p38 mitogen-activated protein kinase (p38MAPK), ET-1 induced its own transcription by signaling through endothelin receptor subtype A (ETAR) and p38MAPK, and ET-1 induced GM-CSF transcription through both Endothelin receptor subtypes A and B (ETBR), p38MAPK and extracellular signal-regulated kinase (ERK)-1/-2. These results suggested further investigation of the therapeutic potential of ERA in early stages of chronic inflammatory airway diseases while it remained unclear which impact the individual Endothelin receptor subtypes have on GM-CSF expression and which kinases they signal through.

ET-1 is produced as preproendothelin, released in its precursor form, big-ET-1, from the synthesizing cells and is subsequently cleaved by endothelin converting enzyme into the active 21 amino acid peptide. Discovered as a vasoconstrictor (Yanagisawa et al., 1988), it is now known to also have an important role in inflammatory processes. Together with TNFα, ET-1 was described as a cytokine initiating inflammatory processes in the airways (Hamilton and Anderson, 2004). It acts mainly locally and signals through ETAR/BR subtypes (Benigni and Remuzzi, 1999) which have been shown to be present on HASMCs (Flynn et al., 1998).
ERA were researched extensively after the discovery of ET-1 and were soon discovered to hold anti-inflammatory potential (Finsnes et al., 1997). However, many setbacks occurred during these efforts: Tezosentan proved non-beneficial in acute and chronic heart failure (Kawanabe and Nauli, 2011; McMurray et al., 2007), and despite encouraging results from previous clinical trials, bosentan did not show clinical efficacy in patients with histologically proven idiopathic pulmonary fibrosis in the BUILD-3 trial (King et al., 2011). Nonetheless, clinical efficacy was shown for the treatment of patients with pulmonary arterial hypertension (PAH) and scleroderma-related digital ulcers, and recent results and flaws in previous trials justify research in abandoned areas (Kohan et al., 2012).

Bosentan is an endothelin receptor antagonist with almost equal affinity to both ET\textsubscript{A}R (K\textsubscript{i} 4.75 ± 1.44 nM) and ET\textsubscript{B}R (K\textsubscript{i} 40.9 ± 12.3 nM; each in human muscle cells; Yuyama et al., 2004) and is, therefore, called a dual blocker (Clozel et al., 1994). A trial in 7 female patients published in 2000 first showed a positive influence on pulmonary hypertension (PH) (Williamson et al., 2000). It was subsequently approved for therapy in the European Union in 2002 as an orphan drug after two further clinical trials demonstrated efficacy in PH (Channick et al., 2001; Rubin et al., 2002).

Ambrisentan was approved for therapy by the FDA in 2007 and the EMEA in 2008. Given its much higher affinity for the ET\textsubscript{A}R (selective antagonist; ET\textsubscript{A}R K\textsubscript{i} 0.28 ± 0.23 nM, ET\textsubscript{B}R K\textsubscript{i} 250 ± 50 nM in human muscle cells; Maguire et al., 2012) the theoretical concept arose that a better clinical outcome was achieved by conserving the potentially beneficial effects of stimulation of the ET\textsubscript{B}R, clearance of ET-1 and vasodilation. Its efficacy in the treatment of PAH was first shown in a trial with 64 patients (Galié et al., 2005). However, there is no clinical evidence to support an advantage of selective over dual ERA (Opitz et al., 2008).
TNFα is an important cytokine in inflammatory airway diseases. It is implied in inflammatory and remodeling processes in chronic bronchitis, chronic obstructive pulmonary disease (COPD) and asthma, and leads to ASMC hyperplasia, bronchial vasoconstriction and emphysema- and pulmonary fibrosis-like changes in animal models (Mukhopadhyay et al., 2006). Of note, anti-TNFα therapy proved non-beneficial in COPD and raised concerns about the possible induction of malignancies (Rennard et al., 2007; Bongartz et al., 2006).

GM-CSF is involved in inflammatory reactions throughout the body (Hamilton and Anderson, 2004) with raised mRNA levels in bronchial bioptic tissue of atopic asthmatics (Bentley et al., 1993), pulmonary inflammatory processes and lung fibrosis (Gajewska et al., 2003). Its expression is induced by TNFα and prevents eosinophil apoptosis in asthma leading to an increased expression of inflammatory mediators (Esnault and Malter, 2001). GM-CSF levels are raised in induced sputum of COPD patients (Vlahos et al., 2006). Its mRNA is rapidly degraded due to an adenosine-uridine-rich sequence in its 3’ untranslated region (Shaw and Kamen, 1986) but is stabilized by receptor signaling (Tebo et al., 2003).

There is strong evidence for an involvement of matrix metalloproteinase 12 (MMP12) in airway inflammation and remodelling. Genetic polymorphisms of MMP12 have been associated with the development of COPD and decline in lung function (Mukhopadhyay et al., 2010; Joos et al., 2002).

The chemokine (C-X-C motif) ligands 2 (CXCL2) and 3 (CXCL3) are activated in bronchoalveolar lavage cells of asthmatics resistant to corticosteroids (Goleva et al., 2008) and induce an increased migration of HASMCs from asthmatic patients suggesting a contribution to airway remodelling (Al-Alwan et al., 2013).

An increased expression of Interleukin-32 (IL-32) has been shown in macrophages, alveolar walls and bronchiolar epithelium of patients with COPD (Calabrese et al.,
Moreover, IL-32 levels are increased in the serum of asthmatic patients (Meyer et al., 2012).

The aim of this study was to assess whether differences between selective versus dual ERA exist in their potential to ameliorate inflammatory processes in HASMCs, specifically the expression of GM-CSF, MMP12, CXCL2, CXCL3 and IL-32 in response to TNFα, and to achieve mechanistic insight into the associated pathways. To this end, we compared mRNA and protein expression in response to TNFα stimulation after blocking endothelin receptors with bosentan or ambrisentan in HASMCs.
Materials and Methods

Acquisition, isolation and cultivation of HASMCs

HASMCs were dissected from resected lung tissue of patients undergoing surgery for pulmonary tumors as previously established (Oltmanns et al., 2003). Cultivation and characterisation was carried out as stated elsewhere (Raidl et al., 2007). This study was approved by the ethics committees of the Universities of Cologne (02-004) and Bochum (4257-12), Germany, and all patients gave written informed consent.

Stimulation of HASMCs

Cells were cultivated until they reached subconfluence and then deprived of serum for 24 hours before stimulation as described elsewhere (Raidl et al., 2007), HASMCs at passages 2 to 7 were stimulated with recombinant human TNFα (R&D systems, Minneapolis, Minnesota, USA; catalogue no. 210-TA) at 20 ng/ml or with ET-1 (Sigma, Hamburg, Germany; catalogue no. E7764) at 100 nM for the indicated times. Bosentan (Actelion Pharmaceuticals, Freiburg, Germany), ambrisentan (GSK, Munich, Germany), BQ123 (Sigma; catalogue no. B150) and BQ788 (Sigma; catalogue no. B157) were added at 10⁻⁶ M or at the indicated concentrations 60 min before ET-1 or TNFα stimulation. Pretreatment with SB203580 (1 µM, Calbiochem/VWR, Darmstadt, Germany; catalogue no. 559389) or PD098059 (10 µM Calbiochem/VWR; catalogue no. 513000) was carried out 30 min before stimulation. Gene transcription was inhibited with actinomycin D (Sigma-Aldrich catalogue no. A9415) at 5 µg/ml. Cell viability was determined by trypan blue staining.

Enzyme-linked immunosorbent assays

GM-CSF ELISAs (R&D systems; catalogue no. DY215) were carried out with supernatants of subconfluent HASMCs (about 80% density) on 96-well-plates as previous-
ly described (Koch et al., 2004). Correspondingly, intracellular ELISAs for phosphory-
lated p38^{MAPK} (R&D systems; catalogue no. KBC869) were performed with subconfluent HASMCs on 96-well-plates as described elsewhere (Knobloch et al., 2009).

**Quantitative reverse transcription-PCR (RT-PCR)**

Quantitative RT-PCR was performed on DNA-free total RNA extracted from HASMCs as described previously (Knobloch et al., 2009; Knobloch et al., 2013).

**Statistical analysis**

Statistical analyses were performed to examine the effects of TNFα or ET-1 alone or in combination with bosentan, ambrisentan, BQ123, BQ788, SB203580, PD098050 and/or actinomycin D on cytokine release or gene expression from HASMCs. Datasets were tested and confirmed for a Gaussian distribution by histogramm analysis. The results are indicated as mean ± SEM (gaussian distribution) or as median with 25th to 75th interquatile range ± minimum/maximum (non-Gaussian distribution). Comparisons in time response experiments and across more than two stimulations were done with one-way repeated measures ANOVA (Gaussian distribution) or with Friedman test (non-Gaussian distribution) with 95% confidence intervals. For separate comparisons of each stimulation post-hoc Bonferroni Holm or post hoc Dunn tests were performed. Comparisons between two parameters were analyzed by paired, two-tailed student's t-test. Concentration response curves were calculated by non-linear (sigmoidal) regression with variable parameters. All calculations were done with GraphPad Prism.
Results

Combined blocking of ET$_A$R and ET$_B$R leads to a greater reduction of TNF$\alpha$-induced GM-CSF and MMP-12 protein release than blocking only ET$_A$R

We have shown previously in time-response experiments that TNF$\alpha$ induces GM-CSF protein release from cultivated HASMCs maximally after 72 hours of stimulation (Knobloch et al., 2010; Knobloch et al., 2013). Using these conditions, GM-CSF protein baseline release was 22.4 ± 5.1 pg/ml (mean ± SEM) and TNF$\alpha$ clearly induced GM-CSF release (Fig. 1A). To investigate the effects of bosentan and ambrisentan in this context, we performed concentration response experiments and used the drugs according to their K$_i$ values (see introduction) and to the plasma half-life time of bosentan (Treiber et al., 2007; Croxtall and Keam, 2008) at a range of 10$^{-12}$ to 10$^{-4}$ M. Both drugs significantly reduced GM-CSF protein, but bosentan led to a significantly stronger reduction than ambrisentan (Fig. 1B; Tab. 1).

TNF$\alpha$ induces GM-CSF mRNA levels in HASMCs at two maximums: one after two hours of stimulation, which is insensitive to bosentan, and a second one after eight hours, which is sensitive to bosentan (Knobloch et al., 2009). Both bosentan and ambrisentan significantly reduced TNF$\alpha$ induced GM-CSF transcription after 8 hours of stimulation - notably, without significant differences (Fig. 1C, D; Tab. 1).

To check if this discrepancy between mRNA and protein sensitivity to bosentan and ambrisentan is specific for GM-CSF we tested four further cytokines: CXCL2, CXCL3, IL-32 and MMP12. Previously, mRNA upregulation by TNF$\alpha$ in HASMCs was shown for all of these cytokines by whole genome microarrays and qRT-PCR (Knobloch et al., 2013). TNF$\alpha$ induced the release of CXCL2, CXCL3 and MMP12 but not of IL-32 protein from HASMCs (Fig. 1E, H; data not shown). TNF$\alpha$-induced CXCL2 release was insensitive to bosentan and ambrisentan (data not shown).
Therefore, IL-32 and CXCL2 were not considered for further analyses. Both ERAs did not modulate baseline MMP12 and CXCL3 levels (data not shown). Bosentan clearly reduced MMP12 protein release in TNFα-exposed cells; however, ambrisentan had no effect, though there was a trend for the highest concentration (Fig. 1F; Tab. 1). The difference in the effects between the two ERAs was statistically significant. Both drugs reached maximal effects on GM-CSF mRNA at 1 µM (Fig. 1D) which is why we used this concentration for the analyses of ERA effects on further cytokine mRNA levels. Both ERAs did not modulate TNFα-induced MMP12 mRNA levels (Fig. 1G).

Both bosentan and ambrisentan partially reduced CXCL3 protein release and mRNA levels in TNFα-exposed cells but in both cases without differences between the two ERAs (Fig. 1I, J; Tab. 1). Notably, the effect of bosentan on CXCL3 protein had a clear trend but did not reach statistical significance.

In summary, we found one further protein, MMP12, that showed a discrepancy in sensitivity between mRNA and protein to selective and non-selective ERAs. This data is suggestive of a posttranscriptional modification of GM-CSF and MMP12 mRNA mediated by ET₄R, which was investigated further.

**ET₄R and ET₅R mediate TNFα induced GM-CSF protein release via p38MAPK, but only ET₅R also signals through ERK-1/-2**

ET₅R has previously been shown to regulate ET-1 mRNA stability via p38MAPK and ERK in vascular endothelial cells (Farhat et al., 2008). ERK has also been shown to mediate GM-CSF mRNA stabilization in peripheral blood eosinophils after stimulation with TNFα and fibronectin (Esnault and Malter, 2002). Therefore, we next investigated the role of MAPKs in our model. ERA were used at a concentration of 10⁻⁶ M for all further experiments since it reflects the plasma concentrations reached in patients under normally dosed therapy (Treibet et al., 2007; Croxtall and Keam, 2008).
Blocking ET\textsubscript{A}R with either BQ123 or ambrisentan led to a significant reduction of TNF\textsubscript{α} induced GM-CSF release. The additional blockade of ERK-1/-2 activity with PD098059 led to a further significant reduction in GM-CSF release; this was also significantly different in comparison to blocking ERK-1/-2 alone (Fig. 2A). When blocking ET\textsubscript{B}R with BQ788 there was also a significant reduction in TNF\textsubscript{α} induced GM-CSF release, but the additional blockade of ERK-1/-2 activity with PD098059 led to no further significant reduction in GM-CSF levels (Fig. 2A). Hence, ET\textsubscript{B}R but not ET\textsubscript{A}R mediates TNF\textsubscript{α} induced GM-CSF release via ERK-1/-2. Blocking p38\textsuperscript{MAPK} activity with SB203580 abolished TNF\textsubscript{α} induced GM-CSF release completely.

In order to further elucidate the involvement of the individual endothelin receptors in p38\textsuperscript{MAPK} signaling we conducted activity experiments by measuring the phosphorylation status of p38\textsuperscript{MAPK}. Our previous experiments showed that TNF\textsubscript{α} induces p38\textsuperscript{MAPK} activation time-dependently in waves and that long- but not short-term stimulation of p38\textsuperscript{MAPK} is a consequence of a reactivation by an ET-1 feedback mechanism (Knobloch et al., 2009). Accordingly, blocking of ET\textsubscript{A}R with ambrisentan or ET\textsubscript{A}R and ET\textsubscript{B}R with bosentan had no significant influence on p38\textsuperscript{MAPK} phosphorylation after stimulation with TNF\textsubscript{α} for 15 minutes (Fig. 3). ET-1 induced an increase in p38\textsuperscript{MAPK} phosphorylation, which was reduced by both ERA (Fig. 3). Importantly, bosentan but not ambrisentan completely abolished p38\textsuperscript{MAPK} activity, and the differences in the effects between the two ERA were statistically significant (Fig. 3). Summarized, these data show that both endothelin receptor subtypes signal through p38\textsuperscript{MAPK} but only ET\textsubscript{B}R also signals through ERK-1/-2 in HASMCs.

If ET\textsubscript{B}R signals via both kinases in parallel, TNF\textsubscript{α}-induced GM-CSF release should be blocked more efficiently by combined use of ERK-1/-2 and p38\textsuperscript{MAPK} inhibitors than by use of the single inhibitors. To test this, we reduced inhibitor concentrations. Under these conditions, single use of PD098059 and SB203580 did not have effects
anymore; however, the combined treatment showed a reduction (Fig. 2B). ERAs reduced TNFα-induced GM-CSF release only partially and from the data obtained so far it could not be ruled out that endothelin receptor-independent GM-CSF expression in response to TNFα is mediated by ERK-1/-2, which could also explain the additive effects of ERK-1/-2 and p38\textsuperscript{MAPK} inhibition on GM-CSF shown in Fig. 2B. However, the combined treatment with PD098059 and bosentan did not show additional effects on GM-CSF compared to single treatments (Fig. 2C) demonstrating that endothelin-independent GM-CSF regulation by TNFα does not require ERK-1/-2 activity.

**\textit{ET}β\textit{R protects GM-CSF but not MMP12 mRNA from degradation}\**

TNFα led to a strong induction of GM-CSF transcription after 10 hours of stimulation (Fig. 4A). To elucidate post-transcriptional effects of the endothelin receptor subtypes, we added actinomycin D after 8 hours of stimulation with TNFα to stop transcription. Then, we added bosentan or ambrisentan and measured GM-CSF mRNA. Actinomycin D alone did not significantly reduce GM-CSF mRNA in TNFα-exposed HASMCs (Fig. 4A). In the presence of actinomycin D both drugs significantly reduced GM-CSF mRNA compared to stimulation with TNFα alone (Fig. 4A) indicating constitutive mRNA turnover. Importantly, in the presence of actinomycin D, bosentan but not ambrisentan reduced GM-CSF mRNA compared to incubation with actinomycin D alone (Fig. 4A).

We conclude that \textit{ET}β\textit{R} protects GM-CSF mRNA from degradation. TNFα also induced MMP12 and CXCL3 mRNA after ten hours (Fig. 4B, C). Actinomycin D reduced MMP12 mRNA but there was no additional effect when it was combined with bosentan or ambrisentan (Fig.4B). CXCL3 mRNA levels were not modulated by Actinomycin D alone or in combination with ERAs (Fig. 4C). This indicates that \textit{ET}β\textit{R} does not regulate MMP12 and CXCL3 mRNA stability.
TNFα is unable to increase GM-CSF mRNA levels in the presence of ETβR inhibitors in long-term culture

According to our data, ETβR-mediated GM-CSF mRNA stability explains the discrepancy between GM-CSF mRNA and protein sensitivity to bosentan and ambrisentan shown in Figs. 1B and D, where GM-CSF mRNA was measured after 8 hours and GM-CSF protein after 72 hours of TNFα stimulation. However, if ETβR-blockade leads to accelerated GM-CSF mRNA degradation, differences in the effects of bosentan and ambrisentan on GM-CSF mRNA should be seen in long-term culture. To test this, we stimulated HASMCs for 24 hours with TNFα. TNFα significantly induced GM-CSF mRNA levels and this was not significantly different in the presence or absence of ambrisentan (Fig. 5). However, in the presence of bosentan or BQ788, a specific inhibitor for ETβR, TNFα did not significantly increase GM-CSF mRNA levels. These data confirm our conclusion stated above.
Discussion

We showed that both dual and selective endothelin receptor antagonism with bosentan or ambrisentan led to a significant and equal reduction of TNFα induced GM-CSF and CXCL3, but not MMP12 transcription in HASMCs after 8 hours of stimulation. CXCL3 protein was reduced equally by both ERA meaning that there was no difference in sensitivity to selective and dual ERA between mRNA and protein. However, the reduction in release of GM-CSF and MMP12 protein, although significantly decreased by both ERA, was significantly greater after addition of the dual antagonist. This result pointed to an involvement of ET₄R in a post-transcriptional modification of GM-CSF and MMP12 mRNA or protein. Measuring the amount of TNFα induced GM-CSF and MMP12 mRNA after having stopped transcription and blocked the respective receptors proved that ET₄R has a strong influence on GM-CSF mRNA stability but had no influence on MMP12 mRNA stability. If a discrepancy in the sensitivity to selective and dual ERAs between mRNA and protein is explained by ET₄R regulation of mRNA stability, this mechanism should not play a role for cytokines that do not have this discrepancy in sensitivity. In accordance with this reasoning, we could indeed not find an indication that ET₄R regulates CXCL3 mRNA stability. In further consideration of the MMP12 results, our data point to a mechanism that is specific to GM-CSF. This raises the question of a role for selective ET₄R antagonism in certain chronic airway diseases.

TNFα activates an ET-1 autoregulatory positive feedback mechanism in HASMCs (Knobloch et al., 2009), and we have shown here that while both ET₄R and ET₄R signal through p38MAPK only ET₄R also signals through ERK-1/-2. This suggests that TNFα-dependent ET-1 signals through ET₄R/p38MAPK and ET₄R/p38MAPK pathways to induce GM-CSF transcription, and via the ET₄R/ERK pathway to protect GM-CSF mRNA from degradation (Fig. 6). In support of this, ERK has been shown to regulate
GM-CSF mRNA stability in other cell types exposed to TNFα (Esnault and Malter, 2002).

Interestingly, while ambrisentan has a more favorable $K_i$ for the ET$_A$R it had a higher EC$_{50}$ in comparison to bosentan (see introduction). This could mean that in order to achieve the reductive effects of ERA on TNFα induced cytokine expression a minimum blockade of ET$_B$R has to be achieved. As ambrisentan's $K_i$ for the ET$_B$R is comparatively high this would explain the higher concentration needed to achieve the shown effects.

GM-CSF has been shown to be elevated in chronic inflammatory lung diseases like COPD and is thought to be an early driver of lung remodeling. Data from a subchronic smoking mouse model proved that neutralization of GM-CSF at an early stage of intense cigarette smoke exposure reduced the number of macrophages and neutrophils in bronchoalveolar lavage fluid, suppressed macrophage proliferation and reduced cytokine, chemokine and protease mRNA (Vlahos et al., 2010). Hence, therapeutic strategies to antagonize GM-CSF are worthwhile investigating while reservations with regard to possible side effects like alveolar proteinosis or an increased susceptibility to infections upon complete inhibition of GM-CSF exist (Vlahos et al., 2006). Since our data show that ERA decrease TNFα induced GM-CSF protein expression from HASMCs they further strengthen the argument for ERA as anti-inflammatory drugs. Considering that they only reduce but do not abolish GM-CSF protein expression and that small amounts of GM-CSF are sufficient for lung homeostasis (Vlahos et al., 2006), the induction of side effects becomes less probable.

The ERA mediated reduction of TNFα induced CXCL3 and MMP12 expression further broadens the anti-inflammatory potential of ERA and might result in differential effects on different diseases.
Comparative data on selective and dual ERA in humans are scarce. One paper confirmed bosentan's blockade of ETₐR when compared to sitaxentan in healthy men (MacIntyre et al., 2010). There are no published clinical trials on the use of ambrisentan in COPD, but two trials on the efficacy of bosentan in patients with COPD have been published (Stolz et al., 2008). The results of the first trial from a Swiss group were discouraging as there was no improvement in the primary endpoint, the 6-min walking test, in the bosentan group after 12 weeks of treatment. The chosen population comprised 30 patients (20 of whom received bosentan) with severe or very severe COPD (Global Initiative for Chronic Obstructive Lung Disease stages III and IV) of whom 20 were diagnosed with PH detected by echocardiography. It should be noted that this is not the recommended diagnostic tool for PH and may deliver incorrect results, especially when the systolic pulmonary artery pressure (PAP) is below 50 mmHg (Grüning et al., 2011) as was the case in this trial. The second trial was carried out by an Italian group. 40 patients with COPD and PH confirmed by right heart catheterization were enrolled and randomized to receive either standard care or standard care plus bosentan for a period of 18 months. The average FEV₁ at baseline was 39% in the standard care group and 37% in the bosentan group. The authors reported a significant decrease in PAP, pulmonary vascular resistance and BODE index as well as a significant increase in the 6-min walking distance for the bosentan group. However, the trial was not double blinded and it seems no primary endpoint had been defined (Valerio et al., 2009).

While these results are interesting they concern patients suffering from advanced stages of COPD and PH. In these patients extensive lung remodeling with loss of functional tissue will have already taken place. It is important to stress, though, that the possible utility of ERA that results from our data lies in their potential to ameliorate inflammation and remodeling in early rather than late stages of chronic inflam-
matory airway diseases. Accordingly, the disappointing results from the BUILD-3 trial also stem from a patient population with advanced idiopathic pulmonary fibrosis. In support of this, we have previously shown that ERA regulate a broad spectrum of inflammatory and remodeling genes in HASMCs after stimulation with TNFα. In these experiments the addition of bosentan led to the reduction of a larger number of these genes in comparison to ambrisentan. Also, bosentan caused a stronger reduction of MMP13 mRNA levels independently from gene transcription. We concluded that ETβR was responsible for ameliorating mRNA degradation (Knobloch et al., 2013).

Since symptoms of chronic inflammatory airway diseases frequently only occur in advanced stages the question of how to identify groups of patients that might profit from the administration of ERA arises. At least in COPD - where current therapeutic strategies can at best only slow disease progression - there is the advantage of knowing that cigarette smoke is the main risk factor for developing the disease. But merely treating patients at risk could do more harm than good, especially since an 'at risk' group is no longer part of the guidelines as this classification proved non-beneficial. However, increased vigilance will help identify smoking patients who have developed an early stage of COPD through the use of lung function screening tests. Moreover, many efforts are under way to identify new diagnostic methods and subgroups of patients with Asthma, COPD and IPF expectantly allowing for an earlier diagnosis and more appropriate therapy (Vanfleteren et al., 2013; Wenzel, 2012; du Bois, 2012).

We have shown here that the anti-inflammatory potential of dual receptor antagonism with bosentan is greater in HASMCs than that of selective ETAR antagonism with ambrisentan suggesting that there is an advantage of dual over ETAR selective antagonism concerning inflammation. We have demonstrated a mechanism, ETβR-dependent mRNA stability, that explains the superiority of dual blockers for GM-CSF.
However, this mechanism cannot be generalized for all cytokines with greater sensitivity to dual than selective blockers as it does not apply to MMP-12. Moreover, we have only examined the effect on four cytokines in this study and have shown previously that ambrisentan is more effective in reducing transcription and release of CXCL10, IL-23A/IL-23, and WISP1 when compared to bosentan. Given the good safety profile of bosentan and ambrisentan their anti-inflammatory properties should be further elucidated in clinical trials examining early stages of chronic inflammatory airway diseases.

**Acknowledgments**

We thank Katja Urban for excellent technical assistance and Arno von dem Bussche-Ippenburg for very valuable help with the manuscript. Bosentan was kindly provided by Actelion Pharmaceuticals, Freiburg, Germany. Ambrisentan was kindly provided by GlaxoSmithKline, Munich, Germany.
Authorship Contributions

Participated in research design: Jungck, Knobloch, Koch.

Conducted experiments: Jungck, Knobloch, Körber, Lin, Konradi, Yanik.

Contributed new reagents or analytic tools (primary HASMCs): Stoelben.

Performed data analysis: Jungck, Knobloch.

Wrote or contributed to the writing of the manuscript: Jungck, Knobloch, Koch.
References


Treiber A, Schneiter R, Häusler S and Stieger B (2007) Bosentan is a substrate of human OATP1B1 and OATP1B3: inhibition of hepatic uptake as the common mech-


Footnotes

Funding

This work was funded by Actelion Pharmaceuticals, Germany. Actelion Pharmaceuticals was not involved in study design; in the collection, analysis, and interpretation of data; in the writing of the manuscript; or in the decision to submit the paper for publication.
Table 1  Effects of bosentan and ambrisentan on TNFα induced cytokine release or transcription. EC₅₀ and E₉₅ values were determined from concentration response curves in figure 1. n. d. = not determined.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>ERA</th>
<th>EC₅₀ (M)</th>
<th>E₉₅ ± S.E.M. (%) reduction</th>
<th>% reduction ± S.E.M. at 10⁻⁶ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF protein</td>
<td>Bosentan</td>
<td>4.5 x 10⁻⁸</td>
<td>63.71 ± 3.026</td>
<td>60.66 ± 14.39</td>
</tr>
<tr>
<td></td>
<td>Ambrisentan</td>
<td>1.1 x 10⁻⁷</td>
<td>54.83 ± 2.478</td>
<td>46.66 ± 9.635</td>
</tr>
<tr>
<td>GM-CSF mRNA</td>
<td>Bosentan</td>
<td>2.2 x 10⁻⁸</td>
<td>49.04 ± 5.432</td>
<td>52.22 ± 19.51</td>
</tr>
<tr>
<td></td>
<td>Ambrisentan</td>
<td>4.8 x 10⁻⁸</td>
<td>51.73 ± 7.115</td>
<td>48.70 ± 29.13</td>
</tr>
<tr>
<td>MMP12 protein</td>
<td>Bosentan</td>
<td>9.7 x 10⁻⁸</td>
<td>45.38 ± 5.404</td>
<td>37.47 ± 22.55</td>
</tr>
<tr>
<td></td>
<td>Ambrisentan</td>
<td>-</td>
<td>-</td>
<td>9.83 ± 25.92</td>
</tr>
<tr>
<td>MMP12 mRNA</td>
<td>Bosentan</td>
<td>n. d.</td>
<td>n. d.</td>
<td>-2.70 ± 14.39</td>
</tr>
<tr>
<td></td>
<td>Ambrisentan</td>
<td>n. d.</td>
<td>n. d.</td>
<td>0.25 ± 37.83</td>
</tr>
<tr>
<td>CXCL3 protein</td>
<td>Bosentan</td>
<td>5.1 x 10⁻⁷</td>
<td>44.48 ± 5.862</td>
<td>29.84 ± 18.18</td>
</tr>
<tr>
<td></td>
<td>Ambrisentan</td>
<td>2.7 x 10⁻⁶</td>
<td>50.96 ± 13.85</td>
<td>19.36 ± 20.92</td>
</tr>
<tr>
<td>CXCL3 mRNA</td>
<td>Bosentan</td>
<td>n. d.</td>
<td>n. d.</td>
<td>45.77 ± 35.87</td>
</tr>
<tr>
<td></td>
<td>Ambrisentan</td>
<td>n. d.</td>
<td>n. d.</td>
<td>37.92 ± 13.06</td>
</tr>
</tbody>
</table>
Figure Legends

**Figure 1** Effect of ERAs on cytokine expression in TNFα-exposed HASMCs. HASMCs were stimulated with TNFα at 20 ng/ml for 72 hours. Bosentan (BOS) or ambrisentan (AMB) were added to the medium at the indicated concentrations one hour before stimulation with TNFα. After incubation, RNA was extracted and subjected to semi-quantitative reverse transcription PCR (RT-PCR) with subsequent gel electrophoresis and densitometric analyses of the PCR signals (C, D, G, J) or cytokine concentrations in the supernatant were measured by ELISA (A, B, E, F, H, I). (A, E, H) Values were normalised to solvent controls. A value of 1 corresponds to an average concentration of 22.4 ± 15.37 (GM-CSF), 135.8 ± 8.5 (MMP12) or 480.9 ± 199.9 (CXCL3) pg/ml. (C, D, G, J) Values for target genes were normalized to GAPDH references and to the unstimulated control, which was set to 1. GM-CSF levels are expressed as fold induction versus the unstimulated control. Induction data for MMP12 and CXCL3 were already shown in Knobloch et al. (2013), ERA effects shown in G and J were investigated in these experimental series. (B, D, F, I, G, H) Data were calculated as % reduction (negative values) versus stimulation with TNFα alone (TNFα<sub>max</sub>). Data are presented as mean ± SEM (A, B, E, F, I; each n = 9); (C, D; each n=11); (G; n=8); (J; n=5) or as median (bar within box) with 25th to 75th interquartile range (box) ± minimum/maximum (error bars) (H; n=9). Curves were created by non-linear regression analyses with sigmoidal curve fit using variable slopes. r² (goodness of fit): (B) bosentan 0.788; ambrisentan 0.8605; (D) bosentan 0.1958; ambrisentan 0.4605; (F) bosentan 0.4932; (I) bosentan 0.4932; ambrisentan 0.4589. (A, C, E) TNFα effects were analyzed by paired t-tests versus control. In (H) Wilcoxon signed rank test was used. (B, D, F, G, I, J) Whole curves or bars were compared.
using a paired t-test and a one-sample t-test to a hypothetical value of 0 (= no reduction): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

**Figure 2** Endothelin receptors A and B mediate TNFα induced GM-CSF expression through p38MAPK, but ERK-1/-2 only responds to ETβR. HASMCs were stimulated with TNFα at 20 ng/ml for 72 hours. SB203580 at 1 µM and PD098059 at 10 µM (A, C) or at indicated concentrations (B) were added 30 minutes before stimulation with TNFα. BQ123, BQ788, ambrisentan (A) and bosentan (C), all at $10^{-6}$ M were added 1 hour before stimulation with TNFα. After incubation, GM-CSF concentrations in the supernatant were measured by ELISA and values were normalised to solvent controls in A and C. A value of 1 corresponds to an average GM-CSF concentration of $34.71 \pm 26.34$ pg/ml. Data are presented as mean ± SEM of n = 5 individual experiments (A, C) or as median (bar within box) with 25th to 75th interquartile range (box) ± minimum/maximum (errors bars) (B; n=6) (A, C) One way repeated measures ANOVA ($P < 0.0001$) with post hoc bonferroni-holm test or (B) paired t-test: $$ P < 0.01$$, $$$$ P < 0.001$ related to solvent controls; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ related to the indicated values or to TNFα stimulation.

**Figure 3** Bosentan abolishes p38MAPK phosphorylation after stimulation with Endothelin-1. HASMCs were incubated with ET-1 at 100 nM or TNFα at 20 ng/ml for 15 minutes. Bosentan at $10^{-6}$ M or ambrisentan at $10^{-6}$ M were added 1 hour before stimulation with ET-1 or TNFα. After incubation, the activity of p38MAPK was measured by an intracellular ELISA for phosphorylated p38MAPK (p-p38MAPK) with total p38MAPK (t-p38MAPK) as a reference. Values for p-p38MAPK were normalised to t-p38MAPK and
related to solvent controls. Each graph represents the mean ± SEM of n = 5 individual experiments. One way repeated measures ANOVA ($P < 0.0001$) with post hoc bonferroni-holm tests: $P$ values are indicated in the graph (related to the indicated values or to solvent controls if placed on top of a bar); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

**Figure 4** Endothelin receptor subtype B is involved in stabilising GM-CSF mRNA. HASMCs were stimulated with TNFα at 20 ng/ml for 10 hours. After 8 hours of stimulation with TNFα Actinomycin D at 5 µg/ml was added to the medium. After 9 hours of stimulation with TNFα bosentan or ambrisentan, each at $10^{-6}$ M, were added to the medium. After incubation, the RNA was extracted and subjected to semi-quantitative RT-PCR with subsequent gel electrophoresis and densitometric analyses of the PCR signals. Values for target genes were normalized to GAPDH references and to the unstimulated control, which was set to 1. GM-CSF, MMP12 and CXCL3 levels are expressed as fold induction versus the unstimulated control. Each graph represents the mean ± SEM (A, B) or the median (bar within box) with 25th to 75th interquartile range (box) ± minimum/maximum (error bars) (C) of n = 4 individual experiments. One way repeated measures ANOVA ($P < 0.0001$) with post hoc bonferroni-holm tests (A, B) or Friedman test ($P = 0.0224$) with post hoc Dunn test (C): $P$ values are indicated in the graph (related to the indicated values or to solvent controls if placed on top of a bar); ** $P < 0.01$, *** $P < 0.001$.

**Figure 5** After 24 hours of stimulation with TNFα a significant difference between differentially blocking the individual endothelin receptor subtypes becomes apparent.
HASMCs were stimulated with TNFα at 20 ng/ml for 24 hours. 1 hour before stimulation with TNFα BQ788 at 10⁻⁶ M, bosentan at 10⁻⁶ M or ambrisentan at 10⁻⁶ M were added to the medium, respectively. After incubation, the RNA was extracted and subjected to semi-quantitative RT-PCR with subsequent gel electrophoresis and densitometric analyses of the PCR signals. Values for target genes were normalized to GAPDH references and to the unstimulated control, which was set to 1. GM-CSF levels are expressed as fold induction versus the unstimulated control. Each graph represents the mean ± SEM of n = 3 individual experiments. One way repeated measures ANOVA (P = 0.0054) with post hoc bonferroni-holm tests: P values are indicated in the graph (related to solvent controls); ** P < 0.01.

Figure 6  Model to explain the effects of pharmacologic modulation of the activities of endothelin receptor subtypes on granulocyte-macrophage colony stimulating factor (GM-CSF) transcription and expression after stimulation of HASMCs with tumor necrosis factor α (TNFα). As shown elsewhere (Knobloch et al., 2009), TNFα stimulation activates an autoregulatory positive feedback loop of endothelin-1 (ET-1) expression that leads to transcription and expression of GM-CSF. In this context, endothelin receptor subtype A (ET_A_R) exclusively signals through p38MAPK while subtype B (ET_B_R) also signals through ERK-1/-2. Both, ET_A_R and ET_B_R signal through p38MAPK to induce GM-CSF transcription. Additionally, GM-CSF mRNA is protected from degradation by ET_B_R via ERK-1/-2. Consequently, blocking the individual receptor subtypes leads to differential effects: blocking both ET_A_R and ET_B_R with bosentan (BOS) leads to a greater reduction in GM-CSF expression than blocking ET_A_R alone with ambrisentan (AMB) because the protective effect on GM-CSF mRNA mediated by ET_B_R is abolished.
**Figure 1**

A. GM-CSF [fold induction]

B. % reduction of TNFα effect

C. GM-CSF/GAPDH mRNA level ratio

D. % reduction vs. TNFα max

E. MMP12 [fold induction]

F. % reduction of MMP-12 effect

G. MMP12/GAPDH

H. CXCL3 [fold induction]

I. % reduction of CXCL3 effect

J. CXCL3/GAPDH

K. % reduction vs. TNFα max
Figure 2

A

B

C

GM-CSF [fold-induction]

GM-CSF [pg/ml]

Figure 2
Figure 3
Figure 4

A

B

C

GM-CSF/GAPDH mRNA level ratio

MMP12/GAPDH mRNA level ratio

CXCL3/GAPDH mRNA level ratio

TNFα - + + + + +
Actinomycin D - - + + + +
Bosentan - - - - + +
Ambrisentan - - - - - +

TNFα - + + + + +
Actinomycin D - - + + + +
Bosentan - - - - + +
Ambrisentan - - - - - +

TNFα - + + + + +
Actinomycin D - - + + + +
Bosentan - - - - + +
Ambrisentan - - - - - +

** P < 0.01
*** P < 0.001
Figure 6