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

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Received April 22, 2014; accepted March 30, 2015

ABSTRACT

Evidence is lacking on the differential effects of the two therapeutic concepts of endothelin receptor antagonists (ERAs): the blockade of only the endothelin receptor A (ETaR; selective antagonism) versus both ETaR and endothelin receptor B (ETbR; 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, enzyme-linked immunosorbent assay, and quantitative reverse transcription polymerase chain reaction. Tumor necrosis factor (TNFα) induced transcription and expression of chemokine (C-X-C motif) ligand 2 (CXCL2), chemokine (C-X-C motif) ligand 3 (CXCL3), granulocyte macrophage colony-stimulating factor (GM-CSF), and matrix metalloproteinase 12 (MMP12) in HASMCs. In concentration-response experiments, bosentan led to a significantly greater reduction of GM-CSF and MMP12 protein release than ambrisentan, whereas there was no significant difference in their effect on GM-CSF mRNA and MMP12 mRNA. Both ERAs reduced CXCL3 protein and mRNA equally but had no effect on CXCL2. Blocking mitogen-activated protein kinases revealed that both ETaR and ETbR signal through p38 mitogen-activated protein kinase, but ETbR also signals through extracellular signal-regulated kinase (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 or CXCL3 mRNA. In conclusion, blockade of each endothelin receptor subtype reduces GM-CSF transcription, but blocking ETbR additionally protects GM-CSF mRNA from degradation via ERK1/2. Accordingly, blocking both ETaR and ETbR leads to a stronger reduction of TNFα-induced GM-CSF protein expression. This mechanism might be specific to GM-CSF. Our data stress the anti-inflammatory potential of ERA and warrant 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, 2013; Barnes, 2011). They are triggered to secrete proinflammatory 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 granulocyte macrophage colony-stimulating factor (GM-CSF) and that is inhibited by endothelin receptor antagonists (ERAs) (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 the early stages of chronic inflammatory airway diseases, although it remained unclear what 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

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.

dx.doi.org/10.1124/jpet.114.215822

ABBREVIATIONS: ASMC, airway smooth muscle cell; BQ123, 2-[(3R,6R,9S,12R,15S)-6-[(H-indol-3-ylmethyl)-9-(2-methylpropyl)-2,5,8,11,14-pentaaxo-12-propan-2-yl]-1,4,7,10,13-pentazabicyclo[13.3.0]octadecan-3-yllacetic acid; BO788, sodium N-[[2R,6S]-2,6-dimethyl-1-piperidinyl carbonyl]-4-methyl-L-leucyl-N-[[1R]-1-carboxylatopentyl]-1-(methoxycarbonyl)-L-tryptophanamide; COPD, chronic obstructive pulmonary disease; CXCL2, chemokine (C-X-C motif) ligand 2; CXCL3, chemokine (C-X-C motif) ligand 3; ERA, endothelin receptor antagonist; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; ETaR, endothelin receptor A; ETbR, endothelin receptor B; GM-CSF, granulocyte macrophage colony-stimulating factor; HASMC, human airway smooth muscle cell; PD098059, 2-(2’-amino-3’-methoxyphenyl)oxanaphthalen-4-one; PH, pulmonary hypertension; SB203580, 4-[4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-1H-imidazol-5-yl]pyridine; TNFα, tumor necrosis factor α.
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 ETA/BR subtypes (Benigni and Remuzzi, 1999), which have been shown to be present on HASMCSs (Flynn et al., 1998).

ERAs 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 nonbeneficial in acute and chronic heart failure (McMurray et al., 2007; Kawanabe and Nauli, 2011), and despite encouraging results from previous clinical trials, bosentan did not show clinical efficacy in patients with histologically proven idiopathic pulmonary fibrosis in the bosentan use in interstitial lung disease-3 [BUILD-3] trial (King et al., 2011). Nonetheless, clinical efficacy was shown for the treatment of patients with pulmonary arterial hypertension 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 ET₄R (Kᵢ = 4.75 ± 1.44 nM) and ET₉R (Kᵢ = 40.9 ± 12.3 nM; each in human muscle cells) (Yuyama et al., 2004) and is, therefore, called a dual blocker (Clozow et al., 1994). A trial published in 2000 involving seven female patients 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 Food and Drug Administration in 2007 and the European Medicines Agency in 2008. Given its much higher affinity for the ET₄R (selective antagonist; ET₄R Kᵢ = 0.28 ± 0.23 nM, ET₉R Kᵢ = 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 ET₉R, clearance of ET-1, and vasodilation. Its efficacy in the treatment of pulmonary arterial hypertension 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 ERAs (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 ASM hyperplasia, bronchial vasoconstriction, and emphysema- and pulmonary fibrosis—like changes in animal models (Mukhopadhyay et al., 2006). Of note, anti-TNFα therapy proved nonbeneficial in COPD and raised concerns about the possible induction of malignancies (Bongartz et al., 2006; Rennard et al., 2007).

GM-CSF is involved in inflammatory reactions throughout the body (Hamilton and Anderson, 2004) with raised mRNA levels in bronchial biotic tissue of atopic asthmatics (Bentley et al., 1993), pulmonary inflammatory processes, and lung fibrosis (Gajewksa et al., 2003). Its expression is induced by TNFα and prevents eosinophil apoptosis in asthma, leading to an increased expression of inflammatory mediators (Esnaull 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 of an involvement of matrix metalloproteinase 12 (MMP12) in airway inflammation and remodeling. Genetic polymorphisms of MMP12 have been associated with the development of COPD and decline in lung function (Joos et al., 2002; Mukhopadhyay et al., 2010).

The chemokine (C-X-C motif) ligands 2 (CXCL2) and 3 (CXCL3) are activated in bronchialveolar 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 remodeling (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., 2008). 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 characterization were 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 Boehum (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—7 were stimulated with recombinant human TNFα (R&D Systems, Minneapolis, MN) at 20 ng/ml or with ET-1 (Sigma-Aldrich, Hamburg, Germany) at 100 nM for the indicated times. Bosentan (Actelion Pharmaceuticals, Freiburg, Germany), ambrisentan (GlaxoSmithKline, Munich, Germany), BQ123 (2-(3R,6R,8S,12R,15S)-6-(1H-indol-3-ylmethyl)-9-(2-methylpropyl)-2,5,8,11,14-pentaoxo-12-propan-2-yl-1,4,7,10,13-pentazabicyclo[13.3.0]octadecan-3-yl)acetic acid; Sigma-Aldrich), and BQ787 (sodium N'-(2,6-dimethyl-1-piperidinyl)carbonyl-4-methyl-L-leucyl-N'-(1R)-1-carboxylatopentyl-1-methoxycarbonyl)-o-tryptophanamide; Sigma-Aldrich) were added at 10⁻⁶ M or at the indicated concentrations 60 minutes before ET-1 or TNFα stimulation. Pretreatment with SB203580 (4-(4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-1H-imidazol-5-yl)pyridine; 1 μM; Calbiochem/VWR, Darmstadt, Germany) or PD088059 (2-(2-amino-3′-methoxyphenyl)-oxanaphthalene-4-one; 10 μM; Calbiochem/VWR) was carried out 30 minutes before stimulation. Gene transcription was inhibited with actinomycin D (Sigma-Aldrich) at 5 μg/ml. Cell viability was determined by trypan blue staining.

Enzyme-Linked Immunosorbent Assays. GM-CSF enzyme-linked immunosorbent assays (R&D Systems) were carried out with supernatants of subconfluent HASMCs (about 80% density) on 96-well plates as previously described (Koch et al., 2004). Correspondingly, intracellular enzyme-linked immunosorbent assays for phosphorylated p38MAPK (R&D Systems) were performed with subconfluent HASMCs on 96-well plates as described elsewhere (Knochel et al., 2009).
Quantitative Reverse-Transcription Polymerase Chain Reaction. Quantitative reverse-transcription polymerase chain reaction was performed on DNA-free total RNA extracted from HASMCs, as described previously (Knobloch et al., 2009, 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, PD098059, and/or actinomycin D on cytokine release or gene expression from HASMCs. Data sets were tested and confirmed for a Gaussian distribution by histogram analysis. The results are indicated as the mean ± S.E.M. (Gaussian distribution) or as the median with 25th to 75th interquartile range ± minimum/maximum (non-Gaussian distribution). Comparisons in time-response experiments and across more than two stimulations were performed with one-way repeated-measures analysis of variance (Gaussian distribution) or with the 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 nonlinear (sigmoidal) regression with variable parameters. All calculations were performed with GraphPad Prism (GraphPad Software, La Jolla, CA).

Results

Combined Blocking of ETAR and ETBR Leads to a Greater Reduction of TNFα-Induced GM-CSF and MMP-12 Protein Release than Blocking Only ETAR. We have previously shown in time-response experiments that TNFα induces GM-CSF protein release from cultivated HASMCs maximally after 72 hours of stimulation (Knobloch et al., 2010, 2013). Using these conditions, GM-CSF protein baseline release was 22.4 ± 5.1 pg/ml (mean ± S.E.M.), and TNFα 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 Kᵢ values (see Introduction) and the plasma half-life 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; Table 1).

TNFα induces GM-CSF mRNA levels in HASMCs at two maximums: one after 2 hours of stimulation, which is insensitive to bosentan, and a second one after 8 hours, which is sensitive to bosentan (Knobloch et al., 2009). Both bosentan and ambrisentan significantly reduced TNFα-induced GM-CSF transcription after 8 hours of stimulation—notably, without significant differences (Fig. 1, C and D; Table 1).

To check if this discrepancy between mRNA and protein sensitivity to bosentan and ambrisentan is specific for GM-CSF, we tested four additional cytokines: CXCL2, CXCL3, IL-32, and MMP12. Previously, mRNA upregulation by TNFα in HASMCs was shown for all of these cytokines by whole genome microarrays and quantitative reverse-transcription polymerase chain reaction (Knobloch et al., 2013). TNFα induced the release of CXCL2, CXCL3, and MMP12 but not IL-32 protein from HASMCs (Fig. 1, E and H; data not shown). TNFα-induced CXCL2 release was insensitive to bosentan and ambrisentan (data not shown). Therefore, IL-32 and CXCL2 were not considered for further analyses. Neither ERA modulated baseline MMP12 or CXCL3 levels (data not shown). Bosentan clearly reduced MMP12 protein release in TNFα-exposed cells; however, ambrisentan had no effect, although there was a trend for the highest concentration (Fig. 1F; Table 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); therefore, we used this concentration for the analyses of the effects of the ERAs on MMP12 and CXCL3 mRNA levels. Neither ERA modulated 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. 1, I and J; Table 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 gene, MMP12, that showed a discrepancy in sensitivity to selective and nonselective ERAs between mRNA transcription and protein expression. These data are suggestive of a post-transcriptional modification of GM-CSF and MMP12 mRNA mediated by ETBR, which was investigated further.

ETAR and ETBR Mediate TNFα-Induced GM-CSF Protein Release via p38MAPK, but Only ETBR Also Signals through ERK-1/2. ETBR 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 mitogen-activated protein kinases in our model. ERAs were used at a concentration of 10⁻⁶ M for all further experiments since this reflects the plasma concentrations reached in patients under normally dosed therapy (Treiber et al., 2007; Croxtall and Keam, 2008).

Blocking ETAR with either BQ123 or ambrisentan led to a significant reduction of TNFα-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 with blocking ERK-1/2 alone (Fig. 2A). When blocking ETBR with BQ788, there was also a significant reduction in TNFα-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, ETBR, but not ETAR, mediates TNFα-induced GM-CSF release via ERK-1/2. Blocking p38MAPK activity with SB203580 abolished TNFα-induced GM-CSF release completely.

To further elucidate the involvement of the individual endothelin receptors in p38MAPK signaling, we conducted activity experiments by measuring the phosphorylation status of p38MAPK. Our previous experiments showed that TNFα induces p38MAPK activation time dependently in waves, and that long-term, but not short-term, stimulation of p38MAPK is a consequence of a reactivation by an ET-1 feedback mechanism (Knobloch et al., 2009). Accordingly, blocking of ETAR with ambrisentan or ETAR and ETBR with bosentan had no significant influence on p38MAPK phosphorylation after stimulation with TNFα for 15 minutes (Fig. 3). ET-1 induced an increase in p38MAPK phosphorylation, which was reduced by both ERAs (Fig. 3). Importantly, bosentan, but not ambrisentan, completely abolished p38MAPK activity, and the differences in the effects between the two ERAs were statistically significant (Fig. 3). Summarized, these data show that both endothelin receptor subtypes signal through p38MAPK, but only ETBR also signals through ERK-1/2 in HASMCs.

If ETBR signals via both kinases in parallel, TNFα-induced GM-CSF release should be blocked more efficiently by combined...
use of ERK-1/2 and p38MAPK inhibitors than by use of the single inhibitors. To test this, we reduced inhibitor concentrations. Under these conditions, single use of PD098059 and SB203580 no longer had an effect; however, the combined treatment showed a reduction (Fig. 2B). ETBR 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 p38MAPK 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 with single treatments (Fig. 2C), demonstrating that endothelin-independent GM-CSF regulation by TNFα does not require ERK-1/2 activity.

**ETBR 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 compared with incubation with actinomycin D alone (Fig. 4A). In the presence of actinomycin D, bosentan, but not ambrisentan, reduced GM-CSF mRNA compared with incubation with actinomycin D alone (Fig. 4A).
We conclude that ETBR protects GM-CSF mRNA from degradation. TNFα also induced MMP12 and CXCL3 mRNA after 10 hours (Fig. 4, B and 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 ETBR does not regulate MMP12 and CXCL3 mRNA stability.

**TNFα Is Unable to Increase GM-CSF mRNA Levels in the Presence of ETBR Inhibitors in Long-Term Culture.** According to our data, ETBr-mediated GM-CSF mRNA stability explains the discrepancy between GM-CSF mRNA and protein sensitivity to bosentan and ambrisentan shown in Fig. 1, B and D, where GM-CSF mRNA was measured after 8 hours and GM-CSF protein after 72 hours of TNFα stimulation. However, if ETBr 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 ETBr, TNFα did not significantly increase GM-CSF mRNA levels. These data confirm our conclusion stated earlier.

**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 ERAs, meaning that there was no difference in sensitivity to selective and dual ERAs between mRNA and protein. However, the reduction in release of GM-CSF and MMP12 protein, although significantly decreased by both ERAs, was significantly greater after addition of the dual antagonist. This result pointed to an involvement of ETBr 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 ETBr has a strong influence on GM-CSF mRNA stability but no influence on MMP12 mRNA stability. If a discrepancy in the sensitivity to selective and dual ERAs between mRNA and protein is explained by ETBr 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 not find an indication that ETBr 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 ETBr 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, although both ETBr and ETAr signal through p38MAPK only ETAr also signals through ERK-1/2. This suggests that TNFα-dependent ET-1 signals through ETAr/p38MAPK and ETBr/p38MAPK pathways to induce GM-CSF transcription, and via the ETBr/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, whereas ambrisentan has a more favorable Ki for the ETAr, it had a higher EC50 in comparison with bosentan (see Introduction). This could mean that, to achieve the reductive effects of ERA on TNFα-induced cytokine expression, a minimum blockade of ETBr has to be achieved. As ambrisentan’s Ki for the ETBr 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 such as 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,

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**TABLE 1**

Effects of bosentan and ambrisentan on TNFα-induced cytokine release or transcription

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

—, not calculable as there was no reduction; n.d., not determined.
chemokine, and protease mRNA (Vlahos et al., 2010). Hence, therapeutic strategies to antagonize GM-CSF are worth investigating while reservations exist with regard to possible side effects such as alveolar proteinosis or an increased susceptibility to infections upon complete inhibition of GM-CSF (Vlahos et al., 2006). Since our data show that ERAs decrease TNFα-induced GM-CSF protein expression from HASMCs, they further strengthen the argument for ERAs 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 ERAs and might result in differential effects on different diseases.

Comparative data on selective and dual ERAs in humans are scarce. One paper confirmed bosentan’s blockade of ET1R when compared with 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-minute 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), 20 of whom 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 is below 50 mm Hg (Grünig et al., 2011), as was the case in this trial. The second trial was carried out by an Italian group. Forty 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 forced expiratory volume in 1 second (FEV1) at baseline was 39% in the standard care group and 37% in the bosentan group. The authors reported a significant decrease in pulmonary artery pressure, pulmonary vascular resistance, and BODE [Body-Mass Index, Airflow Obstruction, Dyspnea, and Exercise Capacity] index as well as a significant increase in the 6-minute 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 ERAs that results from our data lies in their potential to ameliorate inflammation and remodeling in early rather than late stages of chronic inflammatory 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 ERAs 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...
suggesting that there is an advantage of dual over ETAR-selective classification proved nonbeneficial. However, increased vigilance for an earlier diagnosis and more appropriate therapy (du Bois, 2012; Wenzel, 2012; Vanfleteren 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 nonbeneficial. 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 idiopathic pulmonary fibrosis expectantly allowing for an earlier diagnosis and more appropriate therapy (du Bois, 2012; Wenzel, 2012; Vanfleteren et al., 2013).

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, ETbR-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 MMP12. 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 with 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**

The authors thank Katja Urban for excellent technical assistance and Arno von dem Bussche-Ippenburg for very valuable help with the manuscript. Bosentan was provided by Actelion Pharmaceuticals. Ambrisentan was provided by GlaxoSmithKline.

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