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Estrogen Receptor β: Expression Profile and Possible Anti-Inflammatory Role in Disease

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ABSTRACT

Estrogen receptor (ER) β agonists have been demonstrated to possess anti-inflammatory properties in inflammatory disease models. The objective of this study was to determine whether ERβ agonists affect in vitro and in vivo preclinical models of asthma. mRNA expression assays were validated in human and rodent tissue panels. These assays were then used to measure expression in human cells and our characterized rat model of allergic asthma. ERB-041 [7-ethenyl-2-(3-fluoro-4-hydroxyphenyl)-1,3-benzoxazol-5-ol], an ERβ agonist, was profiled on cytokine release from interleukin-1β-stimulated human airway smooth muscle (HASM) cells and in the rodent asthma model. Although ERβ expression was demonstrated at the gene and protein level in HASM cells, the agonist failed to have an impact on the inflammatory response. Similarly, in vivo, we observed temporal modulation of ERβ expression after antigen challenge. However, the agonist failed to have an impact on the model endpoints such as airway inflammation, even though plasma levels reflected linear compound exposure and was associated with an increase in receptor activation after drug administration. In these modeling systems of airway inflammation, an ERβ agonist was ineffective. Although ERβ agonists are anti-inflammatory in certain models, this novel study would suggest that they would not be clinically useful in the treatment of asthma.

Asthma is a chronic inflammatory disease of the lung characterized by reversible airflow obstruction, airway hyperresponsiveness, and airway inflammation (Bousquet et al., 2000). Most asthmatics are effectively treated using a combination of β_2-adrenergic receptor agonists to relieve the constriction and corticosteroids to suppress inflammation (Bousquet et al., 2000). However, a small proportion of asthmatics have severe or corticosteroid insensitive asthma, which is poorly controlled by corticosteroids causing a reduced quality of life and imposing a considerable cost burden on health services (Ito et al., 2006; Moore and Peters, 2006). Moreover, there are also concerns about the systemic effects of long term corticosteroid treatment (Barnes, 1993). Thus, there is a desperate need for novel anti-inflammatory asthma therapies.

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Corticosteroids elicit their actions by binding the glucocorticoid receptor (GR), which is one of the 48 mostly ligand-activated transcription factors of the nuclear receptor super family (Gronemeyer et al., 2004). Nuclear receptors have proved amenable targets for small molecular weight pharmacological agents that modulate their activity, and there is considerable interest in the effect of modulating nuclear receptor activity in a number of diseases (Gronemeyer et al., 2004). The pharmacological modulation of estrogen receptor (ER) activity has proved therapeutically valuable for hormone therapy and the treatment of cancer (Vogelvang et al., 2004; Shang, 2006). Recent reports have also indicated that activation of ER may be beneficial in treating diseases with an inflammatory component (Harris et al., 2003, 2006), although one caveat is that asthma is more prevalent in women, which might suggest that high levels of ER activation via endogenous estrogen could be proasthmatic (Melgert et al., 2007).

In rats, ERα agonists are able to mediate most of the classical effects of estrogen, such as increased uterine wet

ABBREVIATIONS: GR, glucocorticoid receptor; ER, estrogen receptor; ERB-041, 7-ethenyl-2-(3-fluoro-4-hydroxyphenyl)-1,3-benzoxazol-5-ol; IL, interleukin; PCR, polymerase chain reaction; Ct(s), threshold cycles; HASM, human airway smooth muscle; RT, reverse transcription; GM-CSF, granulocyte macrophage-colony-stimulating factor; G-CSF, granulocyte cell-stimulating factor; Th, T-helper; WAY-202196, (3-(3-fluoro-4-hydroxyphenyl)-7-hydroxynaphthonitrile.

weight, maintenance of bone mineral density, and vasomotor stability (Harris et al., 2002; Hillisch et al., 2004). Furthermore, estrogen/estradiol has been shown to be anti-inflammatory in various models of allergic asthma through an effect mediated by activation of the ERα receptor (Degano et al., 2001; Haggerty et al., 2003; Carey et al., 2007; Matsubara et al., 2007). In contrast, ERβ-selective agonists, such as ERB-041, are incapable of producing the classical estrogen responses (Harris et al., 2003) but are able to produce benefit in models of adjuvant-induced arthritis and inflammatory bowel disease (Harris et al., 2003). The ERβ agonist WAY-202196 also increased survival in rodent models of septic shock (Cristofaro et al., 2006). Given that ERβ agonists have demonstrated utility in models of disease with an inflammatory component, we postulated that an ERβ agonist could be beneficial for the treatment of asthma through the suppression of the associated inflammation thought to drive the pathogenesis of the disease (Harris et al., 2003; Cristofaro et al., 2006). Therefore, the aim of this novel study was to determine the effects of an ERβ agonist in characterized in vitro and in vivo preclinical models of asthma-like inflammation. We initiated our investigation by first developing and validating assays to measure the mRNA expression of ERβ in our cell-based assay systems. From these data, we selected a cell type shown to express the receptor, confirmed the expression of the receptor at the protein level, and then profiled the impact of ERB-041, and a number of other selective tool compounds, on IL-1β-induced inflammation. The temporal modulation of ERβ mRNA expression in the lungs of our fully characterized rodent model of allergic asthma not only demonstrated the presence of the receptor in the target organ but also encouraged us to continue with the profiling of the selective ligand. This study is the first such study to investigate the effects of a selective ERβ agonist in human and rodent models of asthma-like inflammation.

Materials and Methods

ERβ mRNA Expression in Human and Rat Tissues: Validation of Assay. cDNA was prepared from RNA extracted from a panel of human tissues (Clontech, Mountain View, CA), and a parallel panel was collected from three male Brown Norway rats (180–200 g; this strain was chosen because it is the same as used in our asthma model) as described previously (Birrell et al., 2005). Amplification of the cDNA and detection of target PCR product were carried out by real-time PCR using predeveloped assays (Applied Biosystems, Foster City, CA). Reactions were internally controlled with the 18S rRNA internal control as described previously (Birrell et al., 2005). Results were analyzed using the Sequence Detection Software, and the relative amount of target gene transcript was normalized to the amount of 18S rRNA internal control transcript in the same cDNA sample. The data were then converted from the exponential form into linear data by using the calculation $2^{-(\text{target Ct} - \text{18S Ct})}$ and then arbitrarily multiplied by 10^6 to change the values into whole numbers. The assay was then validated according to instructions from Applied Biosystems using a tissue type (human and rat) highly expressing ERβ mRNA. Assay validations were performed to ensure that the threshold cycles (Ct) of both the target and internal control, determined in the linear exponential phase of the amplifications, had equal efficiencies.

ERβ Expression in Cells Used in Our in Vitro Inflammatory Assay Systems. ERβ mRNA expression was assessed in human cells [primary airway smooth muscle cells, lung tissue macrophages and epithelial cells plus monocyte (THP-1), and epithelial (A549) cell lines] we have previously characterized using the developed and validated assay described above. Having shown that human airway smooth muscle (HASM) cells express ERβ at the gene level, we used Western analysis (Catley et al., 2006) to show presence of the receptor at the protein level. ERβ antibody used was from Chemicon International (Temecula, CA; product no. 05-824).

ERβ mRNA Expression after Antigen Challenge in Our Characterized Rat Model of Asthma. Samples of lung tissue were collected 2, 4, 6, 8, 12, or 24 h after vehicle or antigen challenge. Level of mRNA expression was measured using the validated assay described above.

Effect of ER Agonists in Cultured Human Airway Smooth Muscle Cells. HASM cells were isolated from normal lung transplant donor tissue surplus to requirement and cultured as described previously (Belvisi et al., 1997). Consent from relatives and ethical approval were obtained. Cells were rinsed with fresh medium [Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) plus supplements as previously described] and treated with indomethacin (10^{-5} M; Sigma Chemical, Poole, Dorset, UK) for 30 min, and throughout the study, to block endogenous cyclooxygenase activity, which can affect cytokine production (Lazzeri et al., 2001). The cells were then pretreated with ERβ-selective agonists [diarylpropionitrile (Sigma Chemical) or ERB-041 (kind gift from GlaxoSmithKline PLC, Stevenage, UK)] or the ERα/β-nonselective agonist (17β-estradiol; Sigma Chemical) (10^{-12} to 10^{-5} M) for 1 h before stimulation with IL-1β (0.1 ng/ml). Dexamethasone (10^{-6} M; Sigma Chemical) was included as an intra-assay control. Twenty-4 h after stimulation, the supernatants were collected and stored at −20°C, and cells were then assayed for cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. Cytokine levels in the supernatants were determined by enzyme-linked immunosorbent assay (R&D Systems Europe Ltd, Abingdon, Oxfordshire, UK).

Impact of an ERβ Agonist in an Antigen-Driven Rat Model of Asthma. Male Brown Norway Rats (200–225 g) were obtained from Harlan UK Limited (Bicester, Oxon, UK) and housed for 1 week before initiating experiments. Food and water were supplied ad libitum. Experiments were performed in accordance with the UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986.

Ovalbumin (Sigma Chemical) sensitization and challenge in this model has previously been described (Birrell et al., 2005). For this study there were two parallel arms; the first entailed collecting plasma and lung tissue samples 6 h after the OVA challenge. The second arm of the study was designed to measure the level of airway eosinophilia in the airway lumen and lung tissue 24 h after challenge (as described in Underwood et al., 2002). Where appropriate, rats were orally dosed (5 ml/kg) with vehicle (1% dimethyl sulfoxide/66% PEG/33% H_2O) or compounds (ERB-041 at 3, 10, and 30 mg/kg) 1 h before and 1 and 6 h after challenge. Budesonide (3 mg/kg) was included as a positive control (14).

Plasma levels of ERB-041 (n.b. 5 h after last oral dose) were determined using liquid chromatography-tandem mass spectrometry. ERβ activation was demonstrated by measuring IGFBP4 mRNA expression, believed to be linked to ligand binding to this receptor (Harris et al., 2003), using the real-time RT-PCR technique outlined above.

Results

ERβ Expression. Using the validated real-time PCR assay, we demonstrated that, under these conditions, primary HASM and epithelial cells expressed ERβ mRNA to a greater extent compared with the other cell types tested (Fig. 1A). Of the two cell types shown to express ERβ, at the mRNA level, it was decided to choose HASM cells to further our work because this cell is known to be central to pathogenesis of asthma (Oliver and Black, 2006) and has been well charac-

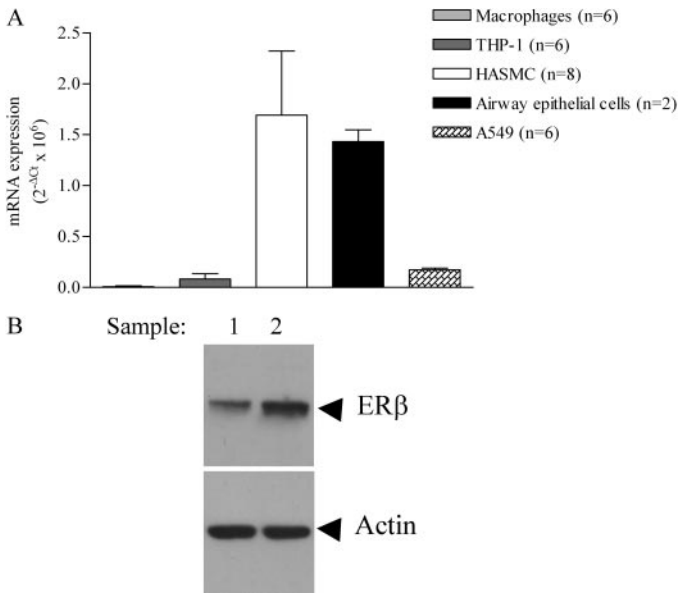

Fig. 1. Expression of ERβ in human cells. A, expression of ERβ mRNA in primary human lung cells was measured using RT-PCR from donors macrophages (n = 6; mean age, 48; age range, 34–59; four female and two male), human airway smooth muscle (n = 8; mean age, 43; age range, 34–54; three female and five male), primary airway epithelial cells (n = 2; mean age, 40; age range, 38–42; two female). ERβ mRNA expression in THP-1 cells (n = 6) and A549 (n = 6) cell lines. Data are expressed as mean ± S.E.M. ($2^{-\Delta}$Ct 10^{-6}). B, representative blot showing expression of ERβ protein in HASM cells by Western analysis.

terized in our group (Belvisi et al., 1997; Birrell et al., 2005; Catley et al., 2006). Having shown the presence of the receptor at the mRNA level, we wanted to ensure this corresponded to expression at the protein level before continuing with profiling the selective ligands. Figure 1B clearly shows a band on a Western blot that corresponds to the molecule weight for this receptor, suggesting that ERβ is indeed expressed at the protein level in these primary cells.

When we measured the level of ERβ mRNA expression in the BN allergic rat lung, we observed a temporal decrease in the level of this receptor (Fig. 2). The presence of the receptor in the primary human cell-based system (Fig. 1) and modulation of the expression levels in the preclinical asthma

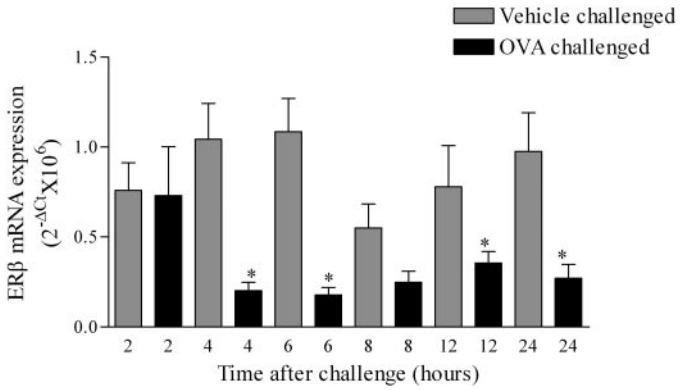

Fig. 2. Expression of ERβ mRNA in Brown Norway rat lung after ovalbumin challenge in sensitized rats. ERβ mRNA expression in the lungs taken at various time points after antigen challenge in sensitized Brown Norway rat. Data are expressed as mean ± S.E.M. ($2^{-\Delta}$Ct 10^{-6}), n = 8. *, statistical (Student t test) significance from time-matched vehicle control group.

model (Fig. 2) encouraged us to continue with studying the role of this receptor in models of asthma.

The Effect of ERβ Agonists on Cytokine Release from Human Airway Smooth Muscle. Stimulation of HASM cells with IL-1β evoked the release of a significant amount of GM-CSF, G-CSF, and IL-8 (Fig. 3). Preincubation with the ERβ agonist failed to significantly affect any of the IL-1β-induced cytokines measured (Fig. 3, A–C). The positive control dexamethasone, however, had the expected impact on these cytokines inasmuch as it blocked GM-CSF, partially blocked IL-8, and had little effect on G-CSF (Fig. 3). To confirm this lack of effect of ERB-041, we profiled a second selective ERβ agonist, diarylpropionitrile, and the dual ERα/β agonist, β-estradiol. Neither compound had any significant impact on IL-1β-induced cytokine release (data not shown).

The inflammatory process in vivo involves a diverse range of cells, and it is possible that ER agonists may be able to exert an anti-inflammatory effect on a limited range of cells that are essential to this process. Alternatively, systemic exposure to ERβ agonists could affect infiltration of inflammatory cells into the lung. Therefore, the impact of the ERβ agonist ERB-041 was examined in a clinically relevant in vivo rat model of allergic asthma.

Effect of the ERβ Agonist on Antigen-Induced Allergic Airway Inflammation in the Rat. To check for adequate compound exposure, we measured plasma levels and showed that using this dosing regimen, there was linear, dose-related compound exposure, and even 5 h after the last oral dose, there were levels in excess of 1 μM (300 ng/ml, at 30 mg/kg) in the plasma (Fig. 4A). Furthermore, the IC_{50} for ERB-041 binding to the rat ERβ ligand binding domain is 3.1 nM, and ERB-041 is able to increase the expression of the ER-responsive gene IGFBP4, with an ED_{50} of 20 nM in cultured SAOA-2 cells expressing the ERβ receptor (Harris et al., 2003). Therefore, the pharmacokinetics data show that systemic levels of ERB-041 were sufficient to activate ERβ in the lung. To confirm that there was sufficient exposure to elicit a functional effect in vivo, IGFBP4 mRNA expression in the rat lung was used as a marker of ERβ activation (Harris et al., 2003). The expression of IGFBP4 in the lung tissue was up-regulated by the top dose of ERB-041 used at this time point, indicating that there is sufficient compound exposure in the lung to activate ERβ (Fig. 4B).

The positive pharmacokinetic and pharmacodynamic data encouraged us to continue with the investigation. At the 24-h endpoint, we showed that ovalbumin challenge caused significant eosinophilia in the BALF (Fig. 5A) and lung tissue (Fig. 5B) of sensitized rats. The numbers of eosinophils in the BALF and tissue were significantly reduced by treatment with budesonide at 3 mg/kg (Fig. 5). However, treatment with ERB-041 had no significant effect on the eosinophilia in the BALF (Fig. 5A) or lung tissue (Fig. 5B).

Discussion

ERβ agonists have demonstrated utility in models of disease with an inflammatory component; therefore, we postulated that an ERβ agonist could be beneficial for the treatment of asthma through the suppression of the associated inflammation thought to drive the pathogenesis of the disease (Haggerty et al., 2003; Harris et al., 2003; Cristofaro et

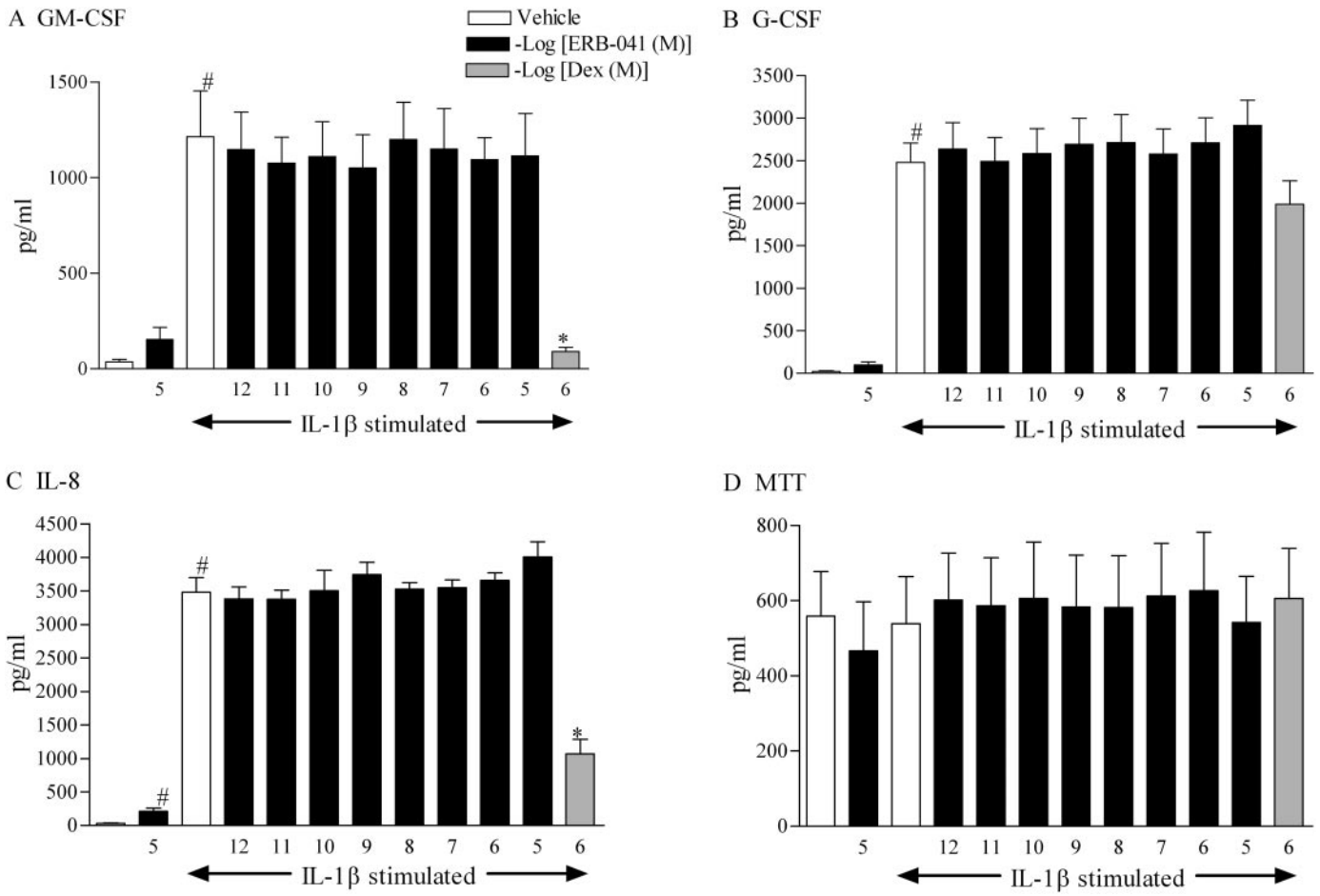

Fig. 3. Impact of ERβ agonist (ERB-041) on IL-1β-induced cytokine release from cultured human airway smooth muscle. Cells were growth arrested for 24 h and pretreated for 30 min with indomethacin before treatment with ERB-041. Twenty-four hours after stimulation with IL-1β, supernatants were collected and assayed for cytokine release (A, GM-CSF; B, G-CSF; C, IL-8). Dexamethasone was included as positive control. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (D). Data are expressed as a mean of two determinates from four patients ± S.E.M. (mean age, 43; age range, 35–50; two female and two male). #, statistical (Student's *t* test, Mann-Whitney) significance difference from nonstimulated vehicle control; ∗, statistical (one-way analysis of variance, Dunn post-test) significance difference from nonstimulated vehicle control.

al., 2006). The aim of this study was to determine the effects of an ERβ agonist in characterized in vitro and in vivo preclinical models of asthma-like inflammation. The initial results were promising inasmuch as we were able to show that HASM cells expressed the target receptor, ERβ, at the mRNA and protein level. In parallel with this, we found that ERβ expression was reduced by ovalbumin challenge in our preclinical rat model of asthma. The timing of the reduction of ERβ expression corresponds with key processes in the ovalbumin-induced inflammatory response, such as the induction of inflammatory cytokine gene expression at 6 h and the influx of immune cells at 24 h (Underwood et al., 2002). These data indicate that reduction of ERβ expression may be an important process in the development of inflammation in the rat lung after ovalbumin challenge. Thus, it seemed possible that agonist-induced activation of ERβ receptors may ameliorate effects of antigen challenge and reduce the inflammatory response. However, when we profiled the impact of various ligands in the human cell-based assay and the selective ERβ agonist in the preclinical model of asthma, we failed to observe any anti-inflammatory activity. The demonstration that ERβ agonists were effective in models of inflammatory bowel disease and arthritis raised the possibility that ERβ agonists could be used to treat a range of inflammatory diseases including asthma (Harris et al., 2003, 2006). However, the data presented here demonstrate for the first time that, although ERβ receptors are expressed in the lung, agonists have no effect on inflammation. The data presented here are on the whole negative but make an important addition to the literature regarding ERβ agonists in inflammatory disease. Our data, however, do not rule out any impact on airway smooth muscle hypertrophy/hyperplasia as suggested by others (Hughes et al., 2002). Indeed, considerable data have been published regarding the positive effects of ERβ agonists in certain models of inflammatory disease (Harris et al., 2003, 2006; Cristofaro et al., 2006; Follettie et al., 2006). However, a recent review indicated that ERβ agonists are ineffective in a range of inflammatory models including collagen-induced arthritis (Harris, 2006). Unfortunately, none of these data have been peer reviewed and published, and the experimental details are not available for critical evaluation. Thus, the data presented here are the first published accounts demonstrating the lack of effect of an ERβ agonist in an inflammatory airway disease. This is important since it demonstrates that ERβ agonists are not general anti-inflammatory agents but have some beneficial effects in certain models of inflammatory diseases and not in others.

Several studies have indicated that estrogenic compounds have a positive effect in lung disease and in animal models (Shirai et al., 1995; Cuzzocrea et al., 2001; Degano et al., 2001; Speyer et al., 2005; Carey et al., 2007; Matsubara et al., 2007). These have looked at either the effect of pregnancy on inflammatory lung disease in humans or the effect of estrogen administration to ovariectomized female mice or the effect of ovariectomy on lung inflammation. None have used selective agonists of the individual ERs (Haggerty et al., 2003). It is therefore possible that some of these positive effects were mediated through ERα, which has documented anti-inflammatory properties in some in vivo models (Harris, 2006). The fact that ERβ agonists are effective in models of IBD and arthritis but not other inflammatory disease may represent fundamental differences between the inflammatory responses present in these diseases. There are data to suggest that estrogen promotes a T-helper (Th)-2 cytokine profile, and this is why some Th-1-driven disease including arthritis show some improvement during pregnancy when estrogen levels are high (Doria et al., 2006). Conversely, there is evidence that Th-2-driven diseases such as systemic lupus erythematosus show increased flare up during pregnancy (Doria et al., 2006). Asthma is a Th-2-driven disease, and as such, it would be expected to show increased asthma pathology if ERβ receptor agonists are skewing the Th cell balance to a Th-2 profile; this was not seen in our model. However, other studies have demonstrated that estrogen has proinflammatory effects in ovalbumin-sensitized rats (Ligeiro de Oliveira et al., 2004). Since estrogen is a dual ERα/ERβ agonist, it is not clear which receptor is responsible for skewing the Th-2 cell responses. If estrogen receptor agonists are skewing the T cell response to a more Th-2 profile, then ERβ agonists may be more beneficial for the treatment of diseases where a Th-1 cell profile is thought to drive the disease such as arthritis.

This is the first report to examine the effect of specific ERβ agonists in an in vivo preclinical rat model of allergic inflammation. The ability of ERβ agonist ERB-041 to resolve inflammation in rat models of IBD and arthritis suggested that activation of ERβ may be able to resolve inflammation in a range of inflammatory disease. We were able to demonstrate the presence of the target receptor at the mRNA and protein level in the human cell type employed for in vitro assessment. In addition, antigen challenge caused temporal modulation of ERβ mRNA expression, and the dosing regimen used resulted in plasma levels in excess of 1 μM, which was associated with an increased expression of a biomarker linked to activation of ERβ. Despite these positive observations, the agonists failed to induce any measurable anti-inflammatory activity. In general, this study would suggest that ERβ agonists are not general anti-inflammatory compounds and would have questionable benefit in the treatment of asthma.

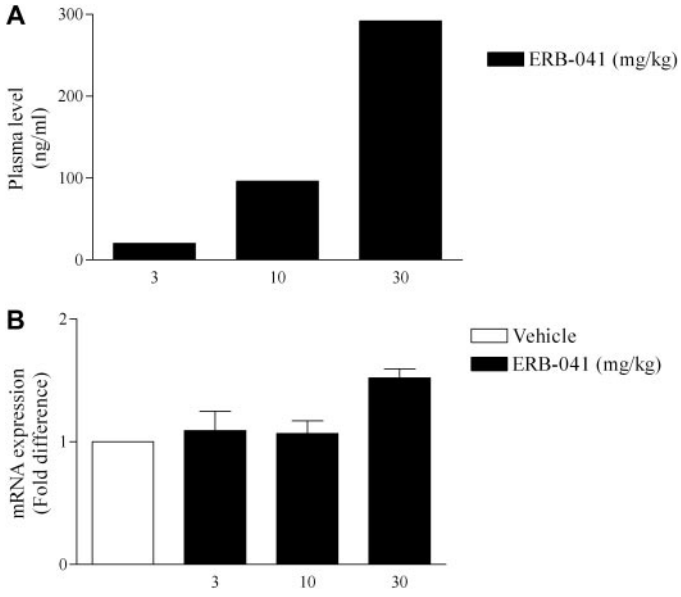

Fig. 4. ERB-041 pharmacokinetics and pharmacodynamics. Sensitized BN rats were dosed with ERB-041 and then challenged with ovalbumin. Plasma levels 5 h after the last oral dose and levels of compound were determined (A). The expression of IGFBP4 mRNA (marker of ERβ activation) in the lung tissue 5 h after the last oral dose was measured using RT-PCR (B). Data (n = 8) are expressed as means ± S.E.M.

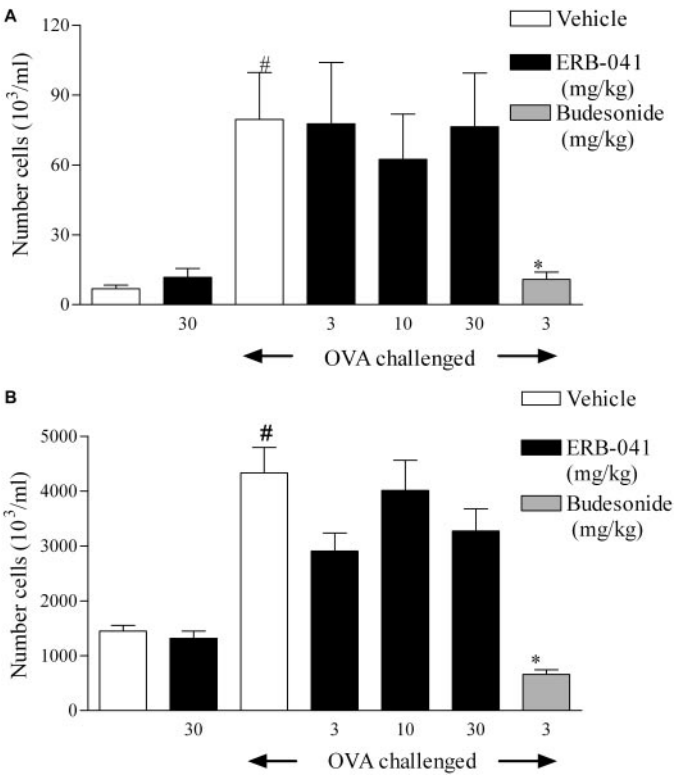

Fig. 5. Effect of ERB-041 on antigen-induced airway eosinophilia. Sensitized BN rats were dosed with ERB-041 and then challenged with ovalbumin. Twenty-fours later, the BALF and lung samples were collected. Eosinophilia was assessed in the BALF (A) and lung tissue (B). Data (n = 12) are expressed as means ± S.E.M. #, statistical (Student's t test, Mann-Whitney) significance difference from vehicle-dosed/saline-challenged group; ∗, statistical (one-way analysis of variance, Dunn post-test) significance difference from vehicle-dosed/ovalbumin-challenged group/nonstimulated vehicle control.

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