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Oestrogen receptor beta: expression profile and possible anti-inflammatory role in disease

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Running Title: Effect of ERβ agonist in asthma models

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Abbreviations: ER - Oestrogen Receptor

GR - Glucocorticoid Receptor

HASM – Human Smooth Muscle Airway

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Abstract

Background: Oestrogen receptor (ER) β agonists have been demonstrated to possess anti-inflammatory properties in inflammatory disease models. **Objectives:** To determine if ERβ agonists impact on *in vitro* and *in vivo* pre-clinical models of asthma. **Methods:** mRNA expression assays were validated in human and rodent tissue panels. These assays were then used to measure expression in human cells and our characterised rat model of allergic asthma. ERB-041, an ERβ agonist, was profiled on cytokine release from IL-1β-stimulated human airway smooth muscle (HASM) cells and in the rodent asthma model. **Results:** Although ERβ expression was demonstrated at the gene and protein level in HASM cells, the agonist failed to impact on the inflammatory response. Similarly, *in vivo*, we observed temporal modulation of ERβ expression after antigen-challenge. However, the agonist failed to impact on the model end-points such as airway inflammation even though plasma levels reflected linear compound exposure and was associated with an increase in receptor activation after drug administration. **Conclusions:** In these modelling systems of airway inflammation an ERβ agonist was ineffective. **Capsule Summary:** 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.

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Introduction

Asthma is a chronic inflammatory disease of the lung characterised by reversible airflow obstruction, airway hyper-responsiveness 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 & 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.

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 oestrogen receptor (ER) activity has proved therapeutically valuable for hormone therapy and the treatment of cancer (Shang, 2006; Vogelvang et al, 2004). Recent reports have also indicated that activation of ER may be beneficial in treating diseases with an inflammatory component (Harris et al, 2003; Harris, 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 pro-asthmatic (Melgert et al, 2007).

In rats ERα agonists are able to mediate most of the classical effects of oestrogen such as increased uterine wet 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 (Haggerty et al, 2003; Dgeano et al, 2001; Carey et al, 2007; Matsubara et al, 2007). In contrast, ERβ selective agonists, such as ERB-041, are incapable of producing the classical oestrogen 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). Since, 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 (Cristofaro et al, 2006; Harris et al, 2003). Therefore, the aim of this novel study was to determine the effects of an ERβ agonist in characterised *in vitro* and *in vivo* pre-clinical 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 this 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 (7-ethenyl-2-(3-fluoro-4-hydroxyphenyl)-1,3-benzoxazol-5-ol), 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 characterised 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.

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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 Laboratories) and a parallel panel collected from 3 male Brown Norway rats (180-200 g, this strain was chosen because it is the same as used in our asthma model) as previously described (Birrell et al, 2005). Amplification of the cDNA and detection of target PCR product was carried out by Real-Time PCR using pre-developed assays (Applied Biosystems, Warrington, UK). Reactions were internally controlled with the 18S rRNA internal control as previously described (Birrell et al, 2005). Results were analysed using the Sequence Detection Software and the relative amount of target gene transcript was normalised 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 characterised using the developed and validated assay described above.

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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 Upstate/Chemicon (product number #05-824).

ERβ mRNA expression after antigen challenge in our characterised rat model of asthma.

Samples of lung tissue were collected 2, 4, 6, 8, 12 or 24 hours 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 previously described (Belvisi et al, 1997). Consent from relatives and ethical approval were obtained. Cells were rinsed with fresh medium (Dulbecco's Modified Eagle's Medium (DMEM, Gibco, UK) plus supplements as previously described) and treated with indomethacin (10^{-5}M, Sigma UK) for 30 minutes, and throughout the study, to block endogenous cyclo-oxygenase activity which can impact on cytokine production (Lazzeri et al, 2001). The cells were then pre-treated with ERβ selective agonists (Diarylpropionitrile (DPN, Sigma, UK) or (ERB-041, kind gift from GlaxoSmithKline, UK) or the ERα/β non-selective agonist (17β-Estradiol, Sigma, UK) (10^{-12}-10^{-5}M) for 1 hour prior to stimulation with IL-1β (0.1 ng/ml). Dexamethasone (10^{-6}M, Sigma, UK) was included as an intra-assay control. Twenty-four hours after stimulation, the supernatants were collected and stored at -20°C, cells were then assayed for cell viability by MTT assay. Cytokine levels in the supernatants were determined by ELISA (R&D systems, UK).

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Impact of an ERβ agonist in an antigen driven rat model of asthma

Male Brown Norway Rats (200-225g) were obtained from Harlan UK Limited (Bicester, 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, UK) sensitisation 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 six hours 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 hours after challenge (as described in 14). Where appropriate rats were orally dosed (5 ml/kg) with vehicle (1% DMSO:66% PEG:33% H_20) or compounds (ERB-041 at 3, 10 and 30 mg/kg) 1 hour before and 1 and 6 hours after challenge. Budesonide (3 mg/kg) was included as a positive control (14).

Plasma levels of ERB-041 (n.b. 5 hours 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 to the other cell types tested (Figure 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 as this cell is known to be central to pathogenesis of asthma (Oliver & Black, 2006) and has been well characterised in our group (Birrell et al, 2005;Catley et al, 2006;Belvisi et al, 1997). 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 which 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 (Figure 2). The presence of the receptor in the primary human cell based system (Figure 1) and modulation of the expression levels in the pre-clinical asthma model (Figure 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 (Figure 3). Pre-incubation with the ERβ agonist failed to significantly impact on any of the IL-1β-induced cytokines measured (Figure 3A, B and C). The positive control dexamethasone, however, had the expected impact on

these cytokines insomuch as it blocked GM-CSF, partially blocked IL-8 and had little effect on G-CSF (Figure 3). To confirm this lack of effect of ERB-041 we profiled a second selective ERβ agonist DPN 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 which 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 hours after the last oral dose there were levels in excess of 1 μM (= 300 ng/ml, at 30 mg/kg) in the plasma (Figure 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 20nM in cultured SAOA-2 cells expressing the ERβ receptor (Harris et al, 2003). Therefore, the PK data shows that systemic levels of ERB-041 were sufficient to activate ERβ in the lung. To confirm 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 (Figure 4B).

The positive pharmacokinetic and pharmacodynamic data encouraged us to continue with the investigation. At the 24hr end point we showed that ovalbumin challenge caused significant eosinophilia in the BALF (Figure 5A) and lung tissue (Figure 5B) of sensitised rats. The numbers of eosinophils in the BALF and tissue were significantly reduced by treatment with budesonide at 3 mg/kg (Figure 5). However, treatment with ERB-041 had no significant effect on the eosinophilia in the BALF (Figure 5A) or lung tissue (Figure 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; Cristofaro et al, 2006; Harris et al, 2003). The aim of this study was to determine the effects of an ERβ agonist in characterised *in vitro* and *in vivo* pre-clinical models of asthma-like inflammation. The initial results were promising insomuch 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 pre-clinical 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 hours and the influx of immune cells at 24 hours (Underwood et al, 2002). This data indicates 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. When, however, we profiled the impact of various ligands in the human cell based assay and the selective ERβ agonist in the pre-clinical 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; Harris, 2006). However, the data presented here demonstrates for the first time that, although ERβ receptors are expressed in the lung, agonists have no

effect on inflammation. The data presented here is on the whole negative but makes an important addition to the literature regarding ERβ agonists in inflammatory disease. Our data, however does not rule out any impact on airway smooth muscle hypertrophy/hyperplasia as suggested by others (Hughes et al 2002). Indeed, **c**onsiderable data has been published regarding the positive effects of ERβ agonists in certain models of inflammatory disease (Cristofaro et al, 2006; Harris et al, 2003; Cristofaro et al, 2006; Follettie et al, 2006; Harris, 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 this data has been peer reviewed and published and the experimental details are not available for critical evaluation. Thus the data presented here is one of 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 (Speyer et al, 2005; Cuzzocrea et al, 2001; Shirai et al, 1995; Degano et al, 2001; 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 oestrogen administration to ovariectomised 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 is data to suggest that oestrogen 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 oestrogen 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 oestrogen has pro-inflammatory effects in ovalbumin sensitised rats (Ligeiro de Oliveira et al, 2004). Since oestrogen is a dual ERα/ERβ agonist it is not clear which receptor is responsible for skewing the Th-2 cell responses. If oestrogen 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* pre-clinical 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 1μM 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.

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Legends for Figures

Figure 1: Expression of ERβ in human cells.

A: The 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 to 59, 4 female and 2 male), human airway smooth muscle (n=8 mean age 43, age range 34 to 54, 3 female and 5 male), primary airway epithelial cells (n=2, mean age 40, age range 38 to 42, 2 female). ERβ mRNA expression in THP-1 cells (n=6) and A549 (n=6) cell lines. Data is expressed as mean ± s.e.mean ($2^{-\Delta}Ct\ 10^{-6}$)

B: Representative blot showing expression of ERβ protein in HASM cells by Western analysis.

Figure 2: Expression of ERβ mRNA in Brown Norway rat lung after ovalbumin challenge in sensitised rats.

ERβ mRNA expression in the lungs taken at various time points after antigen challenge in sensitised Brown Norway rat. Data is expressed as mean ± s.e.mean ($2^{-\Delta}Ct\ 10^{-6}$), n = 8. * indicates statistical (Student T test) significance from time-matched vehicle control group.

Figure 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 hours and pre-treated for 30 min with indomethacin prior to 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 MTT (D). Data is expressed as a mean of two determinates from 4 patients ±

s.e.mean (mean age 43, age range 35 to 50, 2 female and 2 male). # indicates statistical (Student T test, Mann-Whitney) significance difference from non-stimulated vehicle control, * indicates statistical (One-way ANOVA, Dunn post-test) significance difference from non-stimulated vehicle control,

Figure 4: ERB-041 pharmacokinetics and pharmacodynamics.

Sensitised BN rats were dosed with ERB-041 and then challenged with ovalbumin. Plasma levels 5 hours after the last oral dose and levels of compound determined (A) The expression of IGFBP4 mRNA (marker of ERβ activation) in the lung tissue 5 hours after the last oral dose measured using RT-PCR (B). Data (n=8) is expressed as means ± s.e.mean.

Figure 5: Effect of ERB-041 on antigen-induced airway eosinophilia.

Sensitised 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) is expressed as means ± s.e.mean. # indicates statistical (Student T test, Mann-Whitney) significance difference from vehicle dosed / saline challenged group, * indicates statistical (One-way ANOVA, Dunn post-test) significance difference from vehicle dosed / OVA challenged group/non-stimulated vehicle control.

Figure 1A:

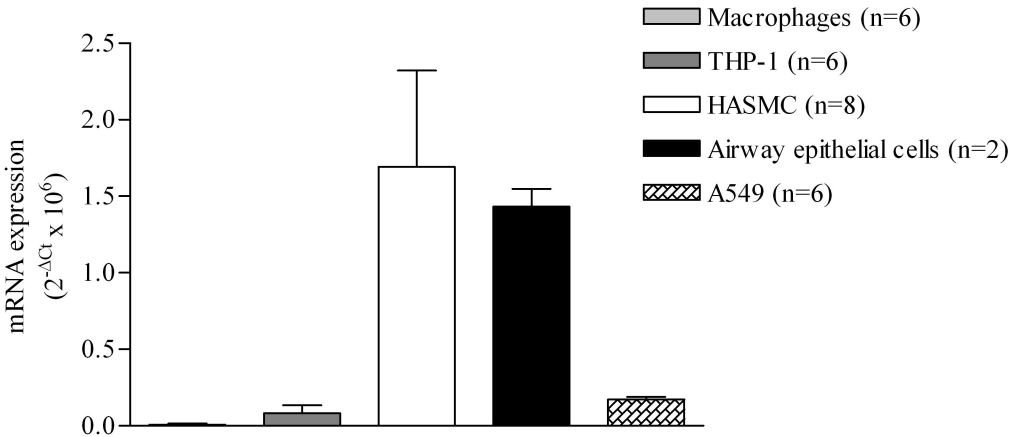

Figure 1B:

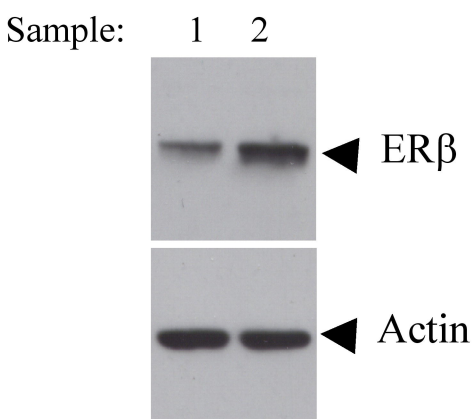

Figure 2:

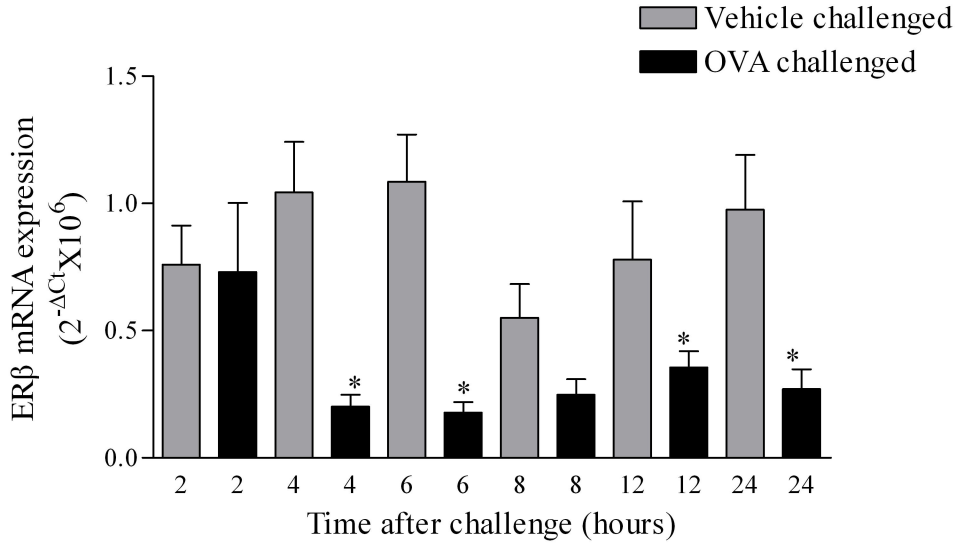
Vehicle challenged
OVA challenged
ERβ mRNA expression
($2^{-\Delta Ct}X10^6$)
1.5
1.0
0.5
0.0
*
2 2 4 4 6 6 8 8 12 12 24 24
Time after challenge (hours)

Figure 3:

Figure 3A: GM-CSF

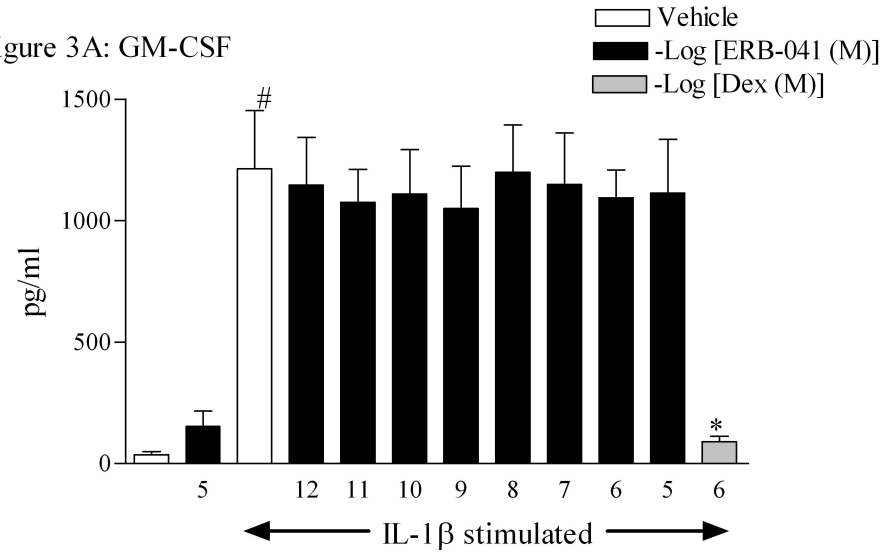

Figure 3B: G-CSF

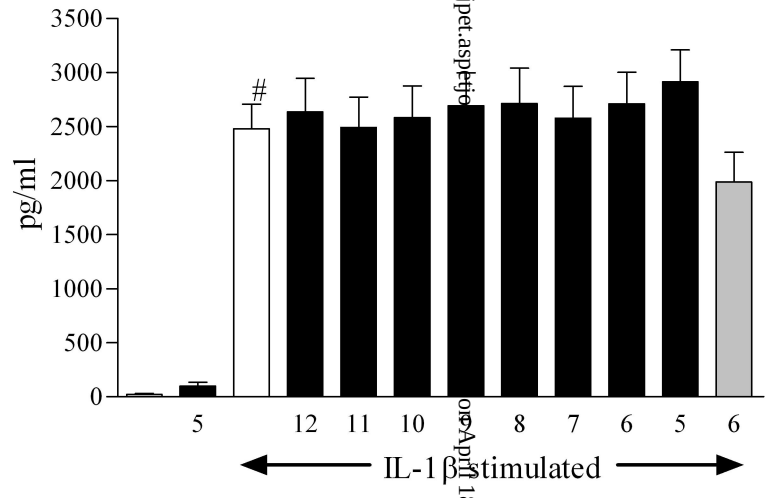

Figure 3C: IL-8

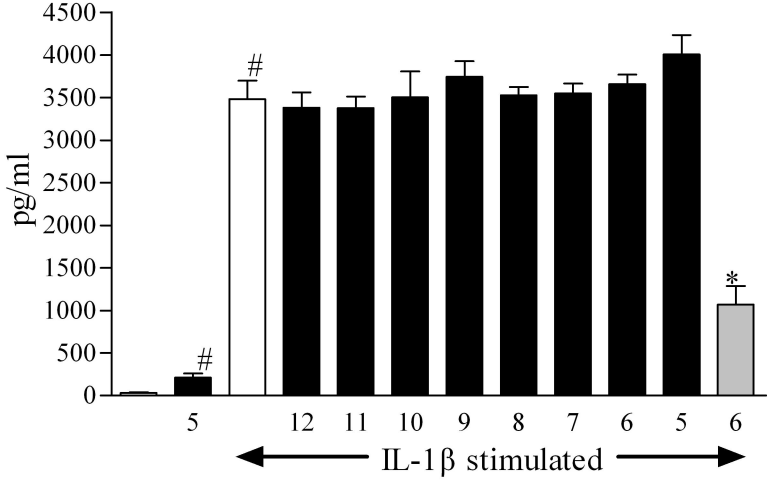

Figure 3D: MTT

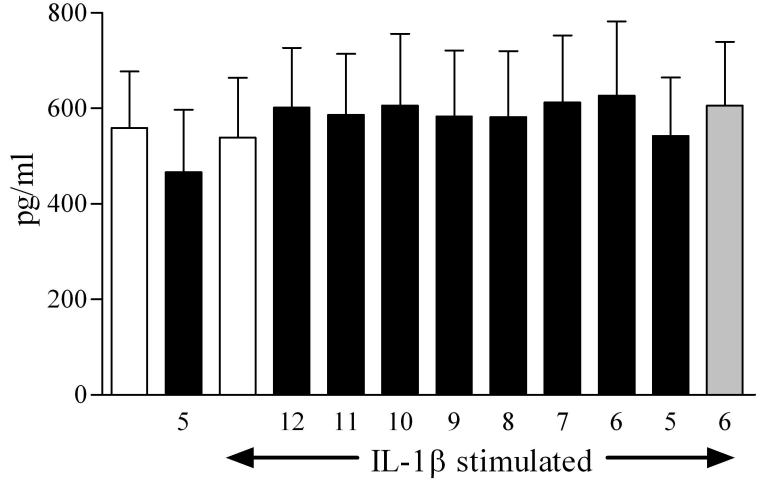

Figure 4A

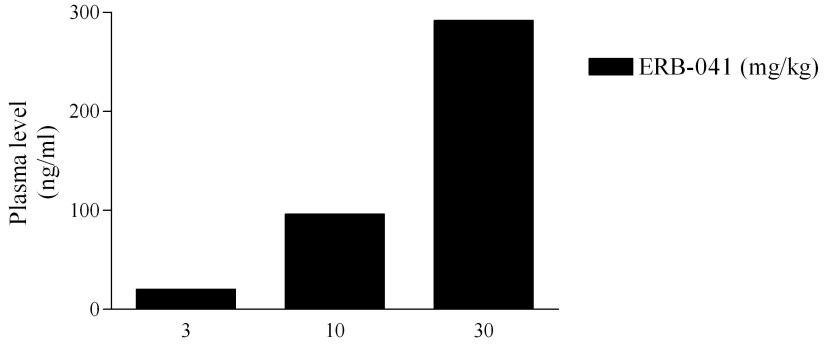

Figure 4B

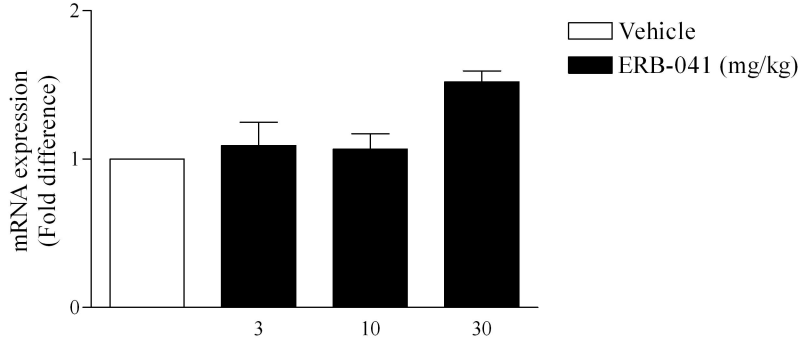

Figure 5A:

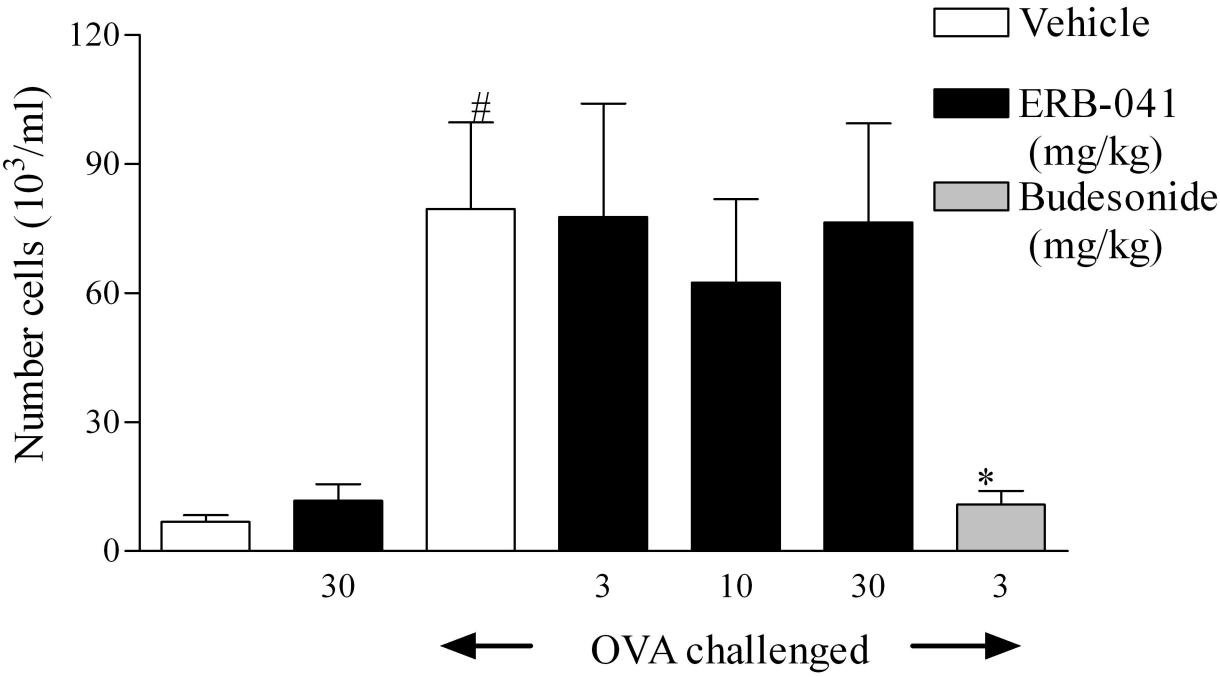

Figure 5B: