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**Investigation into the potential anti-inflammatory effects of endothelin antagonists  
in a murine model of experimental monosodium urate peritonitis.**

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## ABSTRACT

Endothelin (ET)-1 has been detected in many inflammatory pathologies including rheumatoid arthritic patients, asthma and ischaemic-reperfusion injury. In this study we have investigated the effect of a panel of different ET-1 antagonists displaying different selectivity's for the receptors in a murine model of experimental inflammatory peritonitis. Systemic treatment of mice with the ETA antagonist FR139317 inhibited neutrophil accumulation. However, a greater degree of inhibition was observed with the ETB antagonist BQ-788 and the ET(A&B) antagonist PD145065, all these effects occurred without altering peripheral blood cell counts. Release of the CXC chemokine KC was significantly reduced by the FR139317 and PD145065 but not by BQ-788. Evaluation of the therapeutic potential of these antagonists showed that PD145065 inhibited neutrophil migration and KC release, whilst the others caused a non-significant reduction in these parameters. Parameters of endothelial cell activation showed that urate stimulated IL-1 $\beta$  release was inhibited by BQ-788 and PD145065 but not by FR139317, whilst ET-1 was only inhibited by the mixed antagonist. A different scenario was observed with respect to release of the CXC chemokine KC with FR139317 and PD145065 being effective, whilst with a marker of PMN activation the ETA and mixed antagonist inhibited adhesion molecule expression. This data shows that ET-1 antagonists elicit different mechanisms of actions in the way they display their anti-migratory effects in a murine model of monosodium urate crystal peritonitis.

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Monosodium urate (MSU) crystal deposition into the joint articular space is responsible for the inflammatory condition known as gouty arthritis (McCarty *et al.*, 1962). Oedema and erythema of the joints with severe pain and infiltration of polymorphonuclear (PMN) leukocytes are associated with this pathology (Terkeltaub *et al.*, 1992, Dieppe *et al.*, 1979). MSU crystals cause the synthesis of CXC chemokines selective for PMN, such as murine KC (Getting *et al.*, 1997) but not that of a CC chemokine selective for monocytes, such as monocyte chemoattractant protein-1 (Hachicha *et al.*, 1995) a scenario that will lead to PMN but not monocyte migration. In addition, MSU crystal activation of intra-articular mononuclear phagocytes, also induces secretion of interleukin-8 (Terkeltaub *et al.*, 1991). All these studies provide further evidence that gouty arthritis is mainly a PMN driven pathology. Murine KC shows a 60% homology to human GRO- $\alpha$  and this chemokine has been shown to be selective for the human CXCR2. In mice only the CXCR2 exists, whereas in humans both CXCR2 and CXCR1 are present. KC binds to the murine CXCR2 and this has been shown to play a major role in neutrophil chemotaxis. Studies have shown that mice lacking the CXCR2 have fewer neutrophils infiltrating into an artificial air-pouch during MSU crystal-induced gouty synovitis (Terkeltaub *et al.*, 1998).

ET-1, was first identified in 1988, is a 21 amino acid peptide produced by endothelial cells and is a potent vasoconstrictor being implicated in hypertension, stroke and ischaemic-reperfusion injury (Hofman *et al.*, 1998). The involvement of ET-1 in inflammatory pathologies has been documented including arthritic pathologies (Yoshida *et al.*, 1998), with detection in human synovial tissue (Wharton *et al.*, 1992), whilst

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increased production of ET-1 occurs in the synovial fluid compared to plasma in patients with inflammatory arthritides (Miyasaka *et al.*, 1992), a fact confirmed more recently in rheumatoid arthritic patients (Haq *et al.*, 1999) and arthritic joints (Gutierrez *et al.*, 1996). ET-1 release has long been known to lead to neutrophil migration in rabbits (Elferink and de Koster, 1994) and cause neutrophil driven ischaemia in hearts (Andrasi *et al.*, 2002). An efficacy with antagonists directed at the ET-1 receptors at inhibiting inflammation has also been demonstrated in models of asthma with FR139317 inhibiting airway hyperresponsiveness (D'Agostino *et al.*, 1999), lung eosinophilia (Fujitani *et al.*, 1997) and paw oedema (Sampaio *et al.*, 1995).

To date no studies have investigated the role of ET-1 antagonists in gouty arthritis, a neutrophil driven pathology in which MSU crystals cause the influx of neutrophils into the joint articular space.

In this study we have investigated the effects of the ETA, FR139317 (Sogabe *et al.*, 1993, Filep *et al.*, 1995), ETB, BQ-788 (Karaki *et al.*, 1994, Hayasaki *et al.*, 1996) and ET(A&B), PD145065 (McMurdo *et al.*, 1993, Battistini *et al.* 1994) antagonists on neutrophil migration in a murine model of experimental gouty peritonitis when administered either prophylactically or therapeutically. We have also evaluated their effect on adhesion molecule expression and release of pro-inflammatory chemokines and cytokines from cultured macrophages (MØ) and endothelial cells.

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## METHODS AND MATERIALS

### **Animals**

Male TO (Tuck Original, white out bred strain) mice (20-22 g body weight) were purchased from Tuck (Battlesbridge, Essex, UK). Mice were maintained on a standard chow pellet diet with tap water *ad libitum* using a 12 h light/dark cycle. Animals were used 3-4 days after arrival. Animal work was performed according to Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986).

### **Peripheral blood leukocyte counts.**

Mice received i.v. injection of 1000 pmol FR139317, BQ-788 and PD145065, 2 h before blood collection by cardiac puncture following terminal anaesthesia. Differential cell counts were determined by light microscopy following staining in Turk's solution as previously described (Getting *et al.*, 1999). Plasma was collected and stored at  $-20^{\circ}\text{C}$  prior to analysis for KC levels by commercially available ELISA.

### ***In vivo* model of PMN accumulation**

MSU crystal peritonitis was induced by injection of 3 mg MSU crystals in 0.5 ml phosphate buffered saline (PBS) as reported (Getting *et al.*, 1997, 1999, 2001, 2003). Mice were sacrificed at different time-points between 2 and 48 h post crystal injection, animals were killed by  $\text{CO}_2$  exposure, peritoneal cavities washed with 3 ml of PBS containing 3mM ethylenediaminetetraacetate (EDTA) and  $25\text{u/ml}^{-1}$  heparin. Aliquots of lavage fluid were then stained with Turk's solution and differential cell counts performed using a Neubauer haemocytometer and a light microscope (Olympus B061). Lavage

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fluids were then centrifuged at 400 g x 10 min and supernatants stored at -20°C prior to several biochemical determinations.

### **Cytokine and Chemokine quantification by ELISA**

Murine IL-1 $\beta$  and KC levels in the lavage fluids and plasma were quantified with a commercially available Quantikine™ ELISA purchased from R&D Systems (Oxfordshire, UK). In brief, lavage fluids (50  $\mu$ l) were assayed for IL-1 $\beta$  and compared with a standard curve ranging from 0 to 0.5 ng/ml of standard cytokine or for KC and compared to a standard curve ranging from 0 to 1 ng/ml of standard chemokine. The ELISAs showed negligible (<1%) cross-reactivity with several murine cytokines and chemokines (data as furnished by the manufacturer).

### **ET-1 quantification by enzyme immunoassay (EIA)**

ET-1 levels in the cell culture supernatants were quantified with a commercially available EIA purchased from Cayman chemical (Ann Arbor, MI, USA). In brief, cell culture supernatants (100  $\mu$ l) were assayed for ET-1 and compared with a standard curve ranging from 0 to 1 ng/ml of standard ET-1.

### **Drug treatment**

The ETA antagonist FR139317 (100-1000 pmol) (Sogabe *et al.*, 1993, Filep *et al.*, 1995), ETB antagonist BQ-788 (100-1000 pmol) (Karaki *et al.*, 1994, Hayasaki *et al.*, 1996), mixed ET(A&B) antagonist PD145065 (100-1000 pmol) (McMurdo *et al.*, 1993, Battistini *et al.* 1994) or PBS (100  $\mu$ l) were administered i.v. either 30 min prior or 2 h

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after MSU crystals (3 mg in 0.5 ml sterile PBS i.p.). FR139317 was purchased from TOCRIS (Avonmouth, Bristol, UK), BQ-788 was purchased from Bachem Ltd. (Saffron Walden, Essex, UK) and PD145065 was purchased from Sigma (Poole, Dorset, UK). All peptides were stored according to the manufacturers instructions.

### **Assays of cell activation**

Endothelial cell culture: Murine endothelial cells ( $300 \times 10^5$ /well) were obtained as previously described (Lim *et al.*, 2003) and maintained and cultured in DMEM with glutamax, sodium pyruvate, glucose and pyridoxine, 10% FCS, 100  $\mu$ M HEPES, 1% nonessential amino acids, 0.05 mg/ml gentamycin and grown on gelatin coated flasks (0.5%) at 37°C under humidified conditions with 5% CO<sub>2</sub> / 95% air. Non-adherent cells were then washed off and adherent cells were incubated with the ET-1 antagonists FR139317, BQ-788, PD145065 (100-1000 nM) for 30 min in DMEM medium. Cells were then stimulated with 1mg/ml MSU crystals (a concentration chosen from preliminary experiments) (Getting *et al.*, 1999) and the cell-free supernatants collected 30 min later. KC and IL-1 $\beta$  levels were measured by ELISA as described above.

Primary culture of M $\phi$ : An enriched population of peritoneal M $\phi$  (>95% pure) was prepared by 2 h adherence at 37°C under humidified conditions with 5% CO<sub>2</sub> / 95% air in RPMI-1640 supplemented with 10% FCS, by culturing  $1 \times 10^6$  M $\phi$  in 24-well plates. Non-adherent cells were washed off using warm media, and adherent cells (>95% M $\phi$ ) were then incubated with either PBS or 1000 nM of FR139317, BQ-788 and PD145065 for 30 min in RPMI-1640 medium. Cells were then stimulated with 1 mg/ml MSU



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crystals (a concentration chosen from previous experiments) (Getting *et al.*, 1999) and cell-free supernatants collected 2 h later. KC levels were measured by ELISA as described above.

*PMN CD11b up-regulation:* a whole blood protocol was used to quantify CD11b expression in basal conditions and after cell stimulation (Tailor *et al.*, 1997). Briefly, 1 ml of mouse blood was incubated with the different antagonists (1000 nM) for 15 min at 37°C in a shaker water bath prior to addition of 0.1 µM platelet-activating factor (PAF) and a further 15 min incubation. Control tubes were incubated without PAF. At the end, blood aliquots (200 µl) were incubated with rat IgG (10 µg/ml) or rat anti-mouse CD11b (clone 5C6; Serotec, Oxford, UK). After 1h at 4°C, FITC-conjugated rabbit anti-mouse IgG was added for a further 45 min, and FACS analysis performed using a FACScan II analyser (Becton Dickinson, Mountain View, CA) with air-cooled 100 mV argon ion laser tuned to 448 nm and a Consort 32 computer running Lysis II software. CD11b expression was measured in the FL1 (green) channel and quantified as computed units (channel numbers) and expressed as median fluorescence intensity (MFI).

### **Statistics**

Data are reported as mean  $\pm$  SE of *n* distinct observations. Statistical differences were calculated on original data by ANOVA followed by Bonferroni test for intergroup comparisons (Berry and Lindgren, 1990), or by unpaired Student's *t* test (two-tailed) when only two groups were compared. A threshold value of  $P < 0.05$  was taken as significant.

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## RESULTS

### **Effect of ET-1 antagonists on PMN accumulation and KC levels in a model of MSU crystal peritonitis.**

As shown in Table 1, the *in vivo* administration of FR139317, BQ-788 and PD145065 did not alter circulating peripheral blood leukocyte counts or plasma levels of the CXC chemokine KC as measured at 2 h following collection of blood by cardiac puncture.

We then evaluated the effect of selective and mixed antagonists on PMN migration and as a marker of inflammation, the CXC chemokine KC in cell-free lavage fluids. The selective ETA antagonist FR139317 caused a dose dependent attenuation of PMN migration into the peritoneal cavity. The highest dose tested (1000 pmol) here caused a 32 % reduction, 300 pmol caused a more modest 15% reduction, whilst the lowest dose was essentially inactive (Figure 1A). At the anti-migratory dose of 1000 pmol, FR139317 caused a significant attenuation of KC release into the peritoneal cavity, with a 28% reduction being observed. Surprisingly the lower dose of 300 pmol caused a 40% reduction in KC release (Figure 1B). The selective ETB antagonist BQ-788 showed a similar degree of inhibition at 300 and 1000 pmol with a 25% and 35% reduction being observed respectively (Figure 2A). However, this antagonist was unable to inhibit the release of the CXC chemokine KC into peritoneal cavity (Figure 2B). To ascertain if inhibition of both receptors led to an enhanced anti-inflammatory effect, the mixed ET(A&B) antagonist PD145065 was evaluated. At 1000 pmol we were able to inhibit PMN migration by 54% whilst lower doses were not effective (Figure 3A). KC release into the peritoneal cavity was inhibited at 1000 pmol by 31% (Figure 3B). In naïve mice

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the number of PMN and level of KC was below the detection limits of these assays (data not shown).

### **Therapeutic administration of the selective ET-1 antagonists on PMN accumulation in experimental gout.**

In these experiments the therapeutic potential of the antagonists was evaluated at the dose of 1000 pmol per mouse since this was found to be the most active when given prophylactic. The selective ETA antagonist FR139317 caused a non-significant 25% reduction in the neutrophil migration into the peritoneal cavity when given 2 h after the initiation of the inflammatory response (Figure 4A) and this inhibition was associated with a 32% reduction in the CXC chemokine KC (Figure 4B) and 23% reduction in IL-1 $\beta$  release (Figure 4C). A different scenario was observed for the selective ETB antagonist BQ-788 this failed to significantly attenuate the neutrophil migration (Fig. 4A), KC or IL-1 $\beta$  levels (Figure 4B and C). To ascertain if inhibition of both receptors led to an enhanced therapeutic anti-inflammatory effect the mixed ET(A&B) antagonist PD145065 was evaluated. PMN migration was inhibited by 51% (Figure 4A), whilst KC (Figure 4B) and IL-1 $\beta$  (Figure. 4C) were both inhibited a significant 32%.

### **Effect of anti-migratory dose of endothelin antagonists over a time course**

We next decided to see if the antagonists were able to modulate the inflammatory response elicited by MSU crystals over a time course. Mice were treated with 1000 pmol of PD145065, FR139317 or BQ-788 and their effects on PMN migration and release of KC were investigated at different time points. MSU crystal injection caused a peak

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release of the chemokine at 2 h ( $3272 \pm 190$  pg/ml) it then fell dramatically to  $882 \pm 125$  pg/ml at 6 h and back to a near basal level between 24 and 48 h. The maximal detection of PMN migration was observed 6 h following MSU crystal injection and remained high up to 24 h. Investigation of the effect of the mixed antagonist PD145065 showed a significant reduction of 38% at 2h reducing PMN migration from  $1.3 \pm 0.3$  to  $0.8 \pm 0.1 \times 10^6$ /mouse (\* $P < 0.05$  vs. PBS, n=4) and a 33% reduction in the release of the CXC chemokine KC reducing it from  $3272 \pm 190$  to  $2192 \pm 75$  pg/ml (\* $P < 0.05$  vs. PBS, n=4). At the later time point of 24 h an anti-migratory effect was observed, although no effect was seen at 48 h. With respect to the chemokine an inhibitory effect was seen at 6 h although at later time-points no effect was observed. We next evaluated the ETA antagonist FR139317 that was inactive at 2, 24 and 48 h post MSU crystal-injection and caused a modest but significant inhibition at 6 h. A strange observation was noted with respect to KC release with a significant reduction of 23% from  $3272 \pm 190$  to  $2515 \pm 176$  pg/ml at 2 h ( $P < 0.05$  vs. PBS, n=4) and 28% at 6 h, whilst no effect was observed at any other time-point. Finally the ETB antagonist BQ-788 was evaluated and found to be anti-migratory at 6 and 24 h post MSU crystal stimulation although no effect was observed on KC release (Table 2).

### ***In vitro* effect of ET-1 antagonists on IL-1 $\beta$ and ET-1 release from cultured endothelial cells.**

The effect of ET antagonists on IL-1 $\beta$  and ET-1 release *in vitro* was evaluated as a marker of endothelial cell activation following stimulation with urate crystals utilising the most effective dose observed *in vivo*. FR139317 at 100-1000 nM was inactive and failed

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to inhibit IL-1 $\beta$  release, whilst BQ-788 caused a non-significant inhibition of 36% at 300 nM and a significant 60% inhibition at 1000 nM respectively. PD145065 caused a 28% reduction at 300 nM similar to that observed for BQ-788, whilst at 1000 nM there was a significant inhibition of 52% (Figure 5A). A different scenario was observed with respect to the effect of the antagonists on ET-1 release. FR139317 and BQ-788 were inactive and failed to inhibit ET-1 release. However, antagonism of both receptors using PD145065 caused a near complete abrogation of ET-1 release at 1000 nM with an approximate 90% reduction being observed (Figure 5B).

#### **Effect of ET-1 antagonists on KC release *in vitro* from cultured peritoneal M $\phi$ .**

The effect of ET-1 antagonists on release of the CXC chemokine KC *in vitro* was evaluated as a marker of M $\phi$  activation. The ETA antagonist FR139317 at 1000 nM caused a 57% reduction in KC levels from  $456 \pm 103$  pg/ml to  $198 \pm 14$  pg/ml (\*P<0.05, n=4). PD145065 caused a 45% reduction whilst BQ-788 was inactive (Figure 6A).

#### **Effect of ET-1 antagonists on PAF induced CD11b expression on murine PMN.**

The effect of ET-1 antagonists on PAF induced CD11b expression *in vitro* was evaluated as a marker of PMN activation. PAF (0.1  $\mu$ M) caused a significant increase in CD11b expression of  $210 \pm 16$  MFI units compared to PBS  $104 \pm 20$  (\*P<0.05, n=3). Treatment of whole blood with FR139317 and PD145065 at 1000 nM caused a 28% and 31% reduction respectively in CD11b expression (\*P<0.05 vs. PBS, n=3) whilst no effect was observed with BQ-788 (Figure 6B).

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## DISCUSSION

In this study we report the novel discoveries that ET-1 antagonists are anti-inflammatory in a model of experimental MSU peritonitis. They exert their effects in part by suppressing the release of chemotactic agents and reducing adhesion molecule expression, ultimately leading to inhibition of neutrophil accumulation. This study has been prompted by the observation that ET-1 led to the migration of rabbit neutrophils (Elferink and de Koster, 1994). However, we now report here for the first time that ET-1 antagonists are efficacious in a murine model of gouty peritonitis. These data suggest that antagonism of ET receptors could be of benefit in neutrophil driven pathologies.

MSU crystals injected into the peritoneal cavity have been shown to produce an intense and long-lasting accumulation of PMN into the mouse peritoneal cavity, recently characterized in terms of role of resident cells and requirements for adhesion molecules (Getting *et al.*, 1997). Prior to the accumulation of PMN there is a release of chemotactic cytokines and chemokines during this inflammatory reaction (Getting *et al.*, 1999). In this study we decided to monitor the CXC chemokine KC and the pro-inflammatory cytokine IL-1 $\beta$ , since these have been shown to be produced in other models of experimental (Terkeltaub *et al.*, 1998, Matsukawa *et al.*, 1998) and human (Hachicha *et al.*, 1995, di Giovine *et al.*, 1991) gouty arthritis.

Systemic administration of the ETA antagonist FR139317 inhibited MSU crystal-induced PMN accumulation into the mouse peritoneal cavity and this was associated with a reduction in KC release in the inflammatory exudates. These findings suggest that ETA

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antagonists can inhibit neutrophil migration, a finding previously observed with BQ-123 and migration of rabbit neutrophils (Elferink and de Koster, 1994) and in neutrophil driven ischaemia in hearts with the selective antagonist LU135252 attenuating the neutrophil mediated injury (Andrasi *et al.*, 2002). The importance of ETA antagonists in inhibiting inflammation has also been demonstrated in models of asthma with FR139317 inhibiting airway hyperresponsiveness (D'Agostino *et al.*, 1999), lung eosinophilia (Fujitani *et al.*, 1997) and paw oedema (Sampaio *et al.*, 1995). The ETB antagonist BQ-788 also inhibited PMN migration but failed to modulate KC levels, whilst the ET(A&B) antagonist PD145065 inhibited both parameters. A potential reason for the lack of efficacy on KC release with BQ-788 is that both FR139317 and PD145065 are inhibiting KC release from mast cells and MØ. MSU crystals have long been known to release KC from MØ and mast cells (Getting and Perretti, 2000) and cause PMN migration (Schramm *et al.*, 2002). Since the ETA antagonist BQ-610 but not the ETB antagonist IRL-1038 can inhibit mast cell degranulation, it is possible that mast cells express ETA receptors and antagonism of the receptor will prevent the release of this chemokine (Boros *et al.*, 2002). This anti-migratory effect is in agreement with other studies, which have shown a protective effect in other neutrophil driven pathologies such as ischaemic reperfusion injury (Pernow and Wang, 1997) and in coronary vasoconstriction with the non-selective antagonist SB209670 (Klemm *et al.*, 1995). We believe this is the first time that an anti-inflammatory activity has been described in a model of MSU crystal peritonitis.

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We then investigated if these antagonists possessed any therapeutic potential, so they were administered after the onset of inflammation. Antagonists were administered 2 h after MSU crystal injection, the time-point chosen due to the fact that this is where the peak release of the CXC chemokine KC was observed (Getting *et al.*, 1999). Both FR139317 and BQ-788 caused a modest inhibition although this was not significant and little effect on the levels of detectable KC and IL-1 $\beta$  were observed. However, a significant inhibition was observed for the mixed antagonist PD145065 and its anti-migratory effect appeared to be due to inhibition of KC and IL-1 $\beta$ . These findings are not surprising given the fact that this antagonist when dosed prophylactically inhibited the release of this chemokine. Therefore antagonism of both receptors appears to be required once the inflammatory response has been initiated for an anti-migratory effect to occur. The requirement of switching off both receptors for an enhanced anti-inflammatory profile is not altogether surprising given the fact that ETA receptors are expressed on neutrophils and FR139317 can inhibit ET-1 induced up regulation of CD11b/CD18 expression, whilst BQ-788 had no effect. However, BQ-788 but not FR139317 can inhibit ICAM-1 expression on endothelial cells (Zouki *et al.*, 1999).

Given the anti-migratory effects of these antagonists their effect over a time course was evaluated as to allow determination at which time point they exerted their effects. MSU crystal injection caused a peak of release of the chemokine KC at 2 h and this was followed by a maximal accumulation of PMN at 6 h and is in agreement with previous studies (Getting *et al.*, 1997). At the 2 h time-point only PD145065 inhibited PMN migration whilst at 6 h all antagonists were anti-migratory. At 24 h only the mixed and



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ETB antagonist displayed any anti-migratory effect. This data could suggest that antagonism of either A or B receptors at early time-points will lead to an inhibition in PMN migration whilst at later time-points antagonism of the B receptor will prevent cell migration.

We then evaluated the effect of the ET-1 antagonists on MSU crystal stimulated endothelial cells by measuring IL-1 $\beta$  and ET-1 since urate crystals have previously been shown to release IL-1 $\beta$  in a murine model of peritonitis (Getting *et al.*, 2001). We found that BQ-788 and PD145065 exhibited a dose-dependent inhibition in IL-1 $\beta$  release, whilst FR139317 was essentially inactive compared to the control. A different profile was observed on ET-1 release with antagonism of both receptors being required to see an inhibitory effect. Therefore antagonism of ETB receptors leads to a decrease in the production of IL-1 $\beta$  and ET-1 from endothelial cells and ETA receptors appear not to play a role. An observation that is in agreement with the fact that endothelial cells do not express ETA receptors (Zouki *et al.*, 1999) and that inhibition of IL-1 $\beta$  will prevent the induction of ET-1 (Yoshizumi *et al.*, 1990).

Previous studies have highlighted that MSU crystals can stimulate resident peritoneal M $\emptyset$  to release the pro-inflammatory chemokine KC (Getting *et al.*, 1999) FR139317 and PD145065 inhibited KC release from cultured M $\emptyset$  at 1000 nM whilst BQ-788 was inactive. This inhibition in KC from cultured M $\emptyset$  could explain the anti-inflammatory effects observed *in vivo*, as FR139317 and PD145065 inhibited KC as detected in lavage fluids from the peritoneal cavity. An effect of an ETA receptor antagonist on mouse

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peritoneal MØ function is in agreement with a previous study showing that BQ-123 could inhibit ET-1 induced production of prostaglandin E<sub>2</sub> and the expression of cyclooxygenase 2 protein. However in this study they also found an effect with BQ-788 something that we were unable to do this could be due to the different strain of mice used (C57 compared to Swiss albino).

Finally given the fact that ET-1 receptors can be activated by PAF (Yoshizumi *et al.*, 1990), we investigated the effect of the antagonists on PAF induced up regulation of CD11b expression on murine PMN. FR139317 and PD145065 inhibited CD11b expression whilst BQ-788 was inactive. Given that ET-1 has been shown to down regulate the expression of L-selectin and increases CD11b expression (Zouki *et al.*, 1999), a scenario that will lead to an increase in leukocyte migration. Our findings showing an effect of the ETA and ET(A&B) antagonist but no effect with an ETB antagonist are in agreement with this study.

In conclusion the ET antagonists display novel anti-migratory effects in a murine model of MSU crystal-induced inflammation. Different profiles were observed for each antagonist and their effect was altered depending on whether given prophylactically or therapeutically. The data would also suggest that inhibition of both receptors lead to an enhanced anti-inflammatory effect and is partially due to the fact that the antagonists would target the release and action of cytokines and chemokines, and adhesion molecule expression either on the surface of the emigrating PMN or on the endothelium. These findings suggest that ET antagonists could be beneficial in treating certain inflammatory conditions. In the future further studies could be utilised in the rabbit as both CXCR1 and CXCR2 are expressed as seen in humans. Previously an anti-IL-8 antibody has been

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shown to attenuate neutrophil infiltration and joint swelling associated with MSU crystal injection (Nishimura *et al.*, 1997). Figure 7 shows a schematic model for the involvement of ET-1 antagonists in modulating the host inflammatory response elicited by MSU crystals.

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### Footnotes

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Compound structures are as follows: BQ-788 (C<sub>34</sub>H<sub>51</sub>N<sub>5</sub>O<sub>7</sub>, N-cis-2,6-Dimethylpiperidinocarbonyl-b-tBu-Ala-D-Trp(1-methoxycarbonyl)-D-Nle-OH).

FR139317 (C<sub>33</sub>H<sub>44</sub>N<sub>6</sub>O<sub>5</sub>, N-[N-[-N(Hexahydro-1H-azepin-1-yl)carbonyl]-L-leucyl]-1-methyl-D-tryptophyl]-3-(2-pyridinyl)-D-alanine). PD145065 (C<sub>52</sub>N<sub>65</sub>N<sub>7</sub>O<sub>10</sub>, N-acetyl- $\alpha$ -[10,11-Dihydro-5H-dibenzo[a,d]cycloheptadien-5-yl]-D-Gly-Leu-Asp-Ile-Ile-Trp).

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Legends to figures:

**Figure 1: Anti-Inflammatory effects of the ETA antagonist FR139317 in MSU crystal induced peritonitis.** Mice were treated i.v. with FR139317 (100-1000 pmol) or PBS (100  $\mu$ l) 30 min prior to an i.p. injection of MSU crystals (3 mg in 0.5 ml sterile PBS) on PMN migration (Panel A) and KC release (Panel B) as assessed at 6 h time-point. Data are mean  $\pm$  SE of n=8 mice per group. \*P<0.05 vs. control group.

**Figure 2: Anti-Inflammatory effects of ETB antagonist BQ-788 in MSU crystal induced peritonitis.** Mice were treated i.v. with BQ-788 (100-1000 pmol) or PBS (100  $\mu$ l) 30 min prior to an i.p. injection of MSU crystals (3 mg in 0.5 ml sterile PBS) on PMN migration (Panel A) and KC release (Panel B) as assessed at 6 h time-point. Data are mean  $\pm$  SE of n=8 mice per group. \*P<0.05 vs. control group.

**Figure 3: Anti-Inflammatory effects of the mixed ET(A&B) antagonist PD145065 in MSU crystal induced peritonitis.** Mice were treated i.v. with PD145065 (100-1000 pmol) or PBS (100  $\mu$ l) 30 min prior to an i.p. injections of MSU crystals (3 mg in 0.5 ml sterile PBS) on PMN migration (Panel A) and KC release (Panel B) as assessed at 6 h time-point. Data are mean  $\pm$  SE of n=8 mice per group. \*P<0.05 vs. control group.

**Figure 4: Effect of ET-1 antagonists given therapeutically in MSU crystal induced peritonitis.** Mice were treated i.v. with 1000 pmol of FR139317, BQ-788, PD145065 or PBS (100  $\mu$ l) 2 h after i.p. injections of MSU crystals (3 mg in 0.5 ml sterile PBS) on

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PMN migration (Panel A), KC release (Panel B) and IL-1 $\beta$  (Panel C) as assessed at 6 h time-point. Data are mean  $\pm$  SE of n=8 mice per group. \*P<0.05 vs. control group.

**Figure 5: Effect of ET-1 antagonists on IL-1 $\beta$  and ET-1 release from cultured murine endothelial cells.** FR139317 (100-1000 nM, open squares), BQ-788 (100-1000 nM, open circles), PD145065 (100-1000 nM, filled squares) or PBS (dotted line) were added to adherent cultured murine endothelial cells ( $300 \times 10^5$ ), 30 min prior to stimulation with 1 mg/ml MSU crystals. Supernatants were removed 30 min later and cell-free aliquots analysed for IL-1 $\beta$  (Panel A) and ET-1 (panel B) content using specific ELISA and EIA. Data are mean  $\pm$  SE of n=4 determinations in triplicate. \*P<0.05 vs. relevant PBS control.

**Figure 6: Effect of ET-1 antagonists on KC release from cultured M $\phi$  and CD11b expression on PMN.** Panel A: FR139317, BQ-788, PD145065 (1000 nM) or PBS (dotted line) were added to adherent cultured M $\phi$  ( $1 \times 10^6$ ), 30 min prior to stimulation with 1 mg/ml MSU crystals. Supernatants were removed 30min later and cell-free aliquots analysed for KC content (Panel A) using a specific ELISA. Panel B: Mouse whole blood were incubated with or without FR139317, BQ-788, PD145065 (1000 nM) or PBS prior to stimulation with PAF and quantification of CD11b antigen on PMN by flow cytometry. Controls samples received either a non-immune IgG or were not stimulated with PAF. Data are mean  $\pm$  SE of n=4 determinations in triplicate. \*P<0.05 vs. relevant PBS control.

**Figure 7: Schematic model for the involvement of ET-1 antagonists in modulating the host inflammatory response elicited by MSU crystals.** Antagonists that block the ETA receptor (filled square) lead to an inhibition in PAF induced CD11b expression in

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the PMN. MSU crystal stimulation of MØ leads to release of KC release which is inhibited by antagonists that block the ETA receptor, whilst activation of ETB (filled circles) receptors on endothelial cells (EC) leads to IL-1 $\beta$  release and ET-1 which is abrogated by antagonists of this receptor. Therefore inhibition of adhesion molecule expression and pro-inflammatory chemokine/cytokines ultimately leads to a reduction in PMN migration to the inflamed site.



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**Table 1: Effect of FR139317, BQ-788 and PD145065 on peripheral blood count and plasma KC levels.**

<b>Treatment</b>	<b>Blood Cells (x10<sup>5</sup> per ml)</b>			<b>Plasma</b>
	<b>PMN</b>	<b>Monocytes</b>	<b>Lymphocytes</b>	<b>KC (pg/ml)</b>
<b>PBS</b>	6.3 ± 1.1	7.2 ± 0.9	22.7 ± 1.2	185 ± 27
<b>FR139317</b>	5.8 ± 1.6	6.8 ± 1.3	22.3 ± 1.2	184 ± 16
<b>BQ-788</b>	6.4 ± 0.9	7.5 ± 1.5	19.7 ± 0.7	179 ± 33
<b>PD145065</b>	6.0 ± 0.8	7.0 ± 0.5	20.7 ± 0.3	181 ± 23

Mice (n=6 per group) were treated i.v. with the different antagonists at 1000 pmol/mouse and sacrificed 2 h later for blood collection. Peripheral blood leukocyte counts were obtained following staining in Turk's solution and differential counting with a light microscope. Plasma KC levels were determined using a commercially available ELISA.

Data are mean ± SEM.

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**Table 2: Effect of FR139317, BQ-788 and PD145065 on MSU crystal induced PMN migration and stimulated KC release over a time course.**

Treatment	Time (h)			
	2	6	24	48
<b>PMN (10<sup>6</sup>/mouse)</b>				
PBS	1.3 ± 0.3	7.1 ± 0.4	6.5 ± 0.4	1.9 ± 0.2
FR139317	1.3 ± 0.4	5.6 ± 0.4*	6.2 ± 0.3	2.0 ± 0.4
		(-21%)		
BQ-788	1.0 ± 0.3	4.8 ± 0.5*	4.8 ± 0.2*	2.0 ± 0.4
		(-32%)	(-26%)	
PD145065	0.8 ± 0.1*	3.7 ± 0.2*	4.8 ± 0.2*	1.9 ± 0.1
	(-38%)	(-48%)	(-26%)	
<b>KC (pg/ml)</b>				
PBS	3272 ± 190	882 ± 125	105 ± 10	34 ± 5
FR139317	2515 ± 176*	635 ± 33*	88 ± 7	35 ± 11
	(-23%)	(-28%)		
BQ-788	2942 ± 102	743 ± 72	106 ± 5	31 ± 8
PD145065	2192 ± 75 *	603 ± 75*	94 ± 16	23 ± 6
	(-33%)	(-28%)		

Mice (n=4 per group) were treated i.v. with ET-1 antagonists (1000 pmol/mouse) and sacrificed at different time-points between 2 and 48 h later. Peritoneal cavities were lavaged with 3 ml of PBS supplemented with 3 mM EDTA and 25 u/ml heparin.

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Differential cell counts were performed with a light microscope following staining in Turks solution and cell free supernatants analysed for KC by ELISA. Data are mean  $\pm$  SEM.

Figure 1

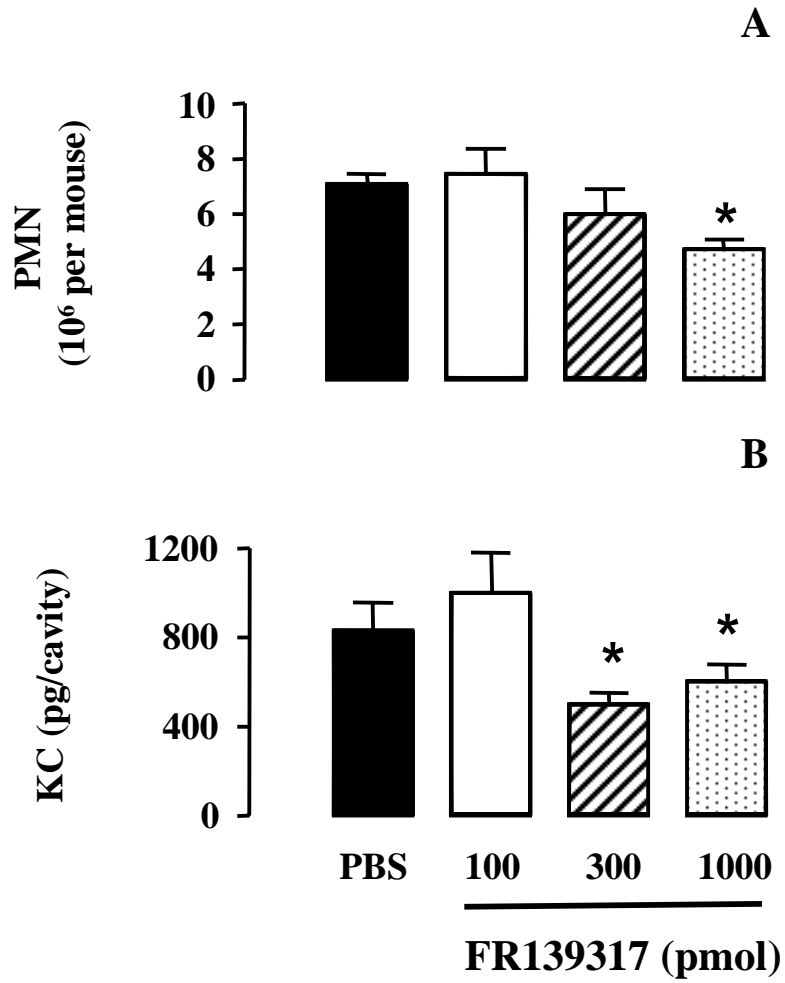
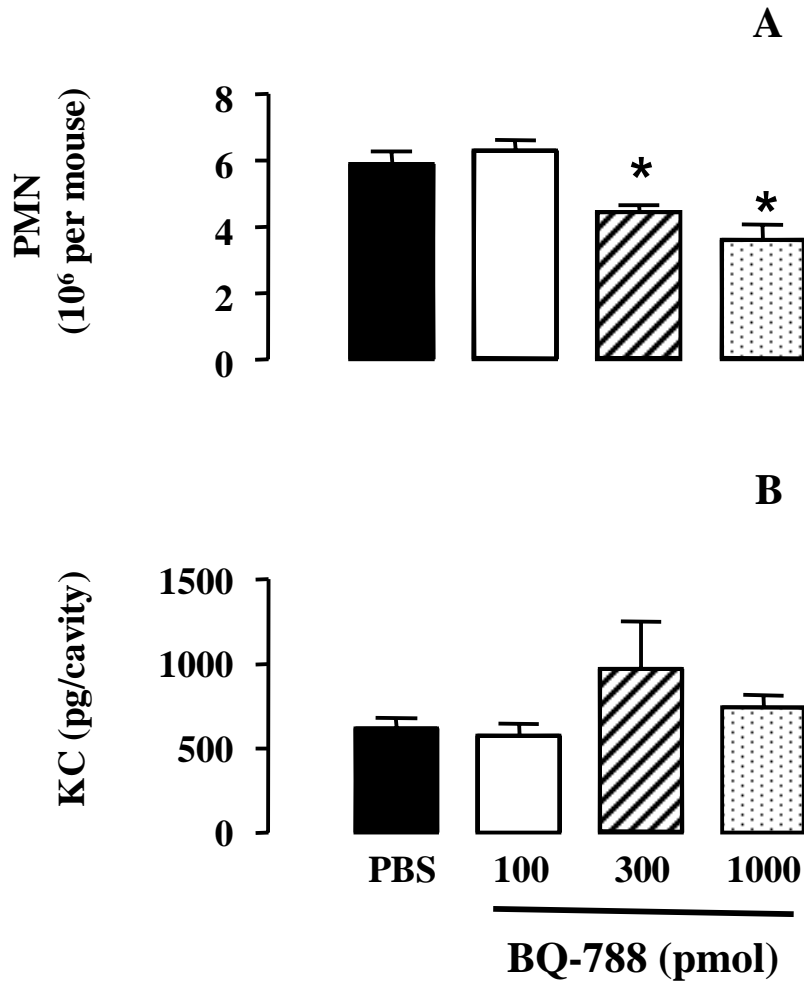


Figure 2



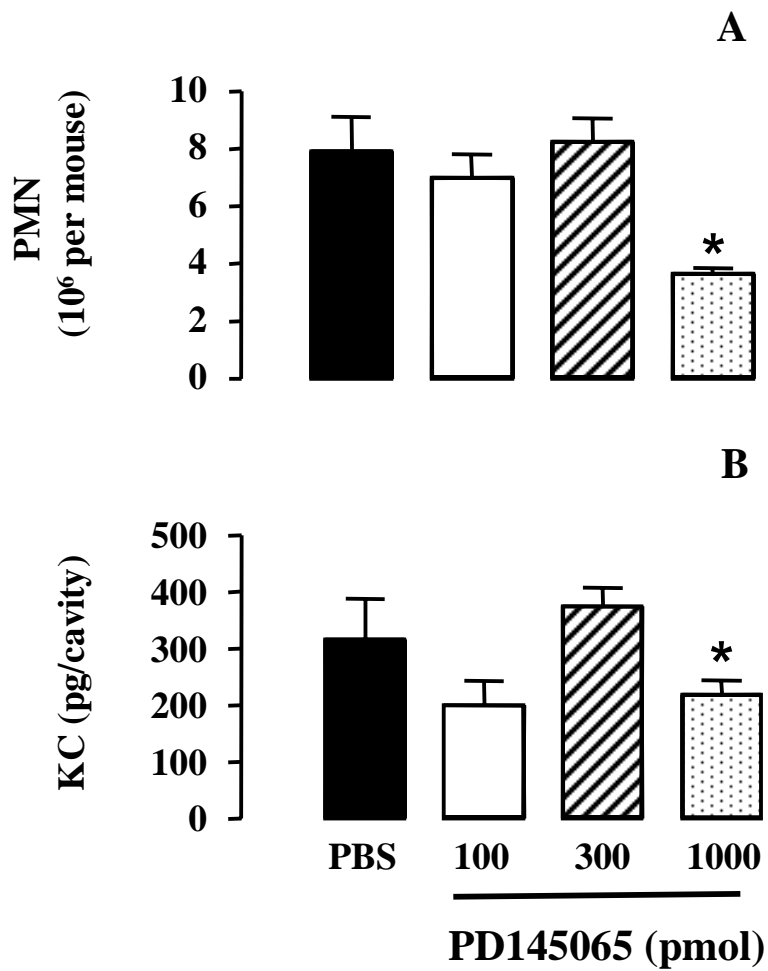


Figure 4

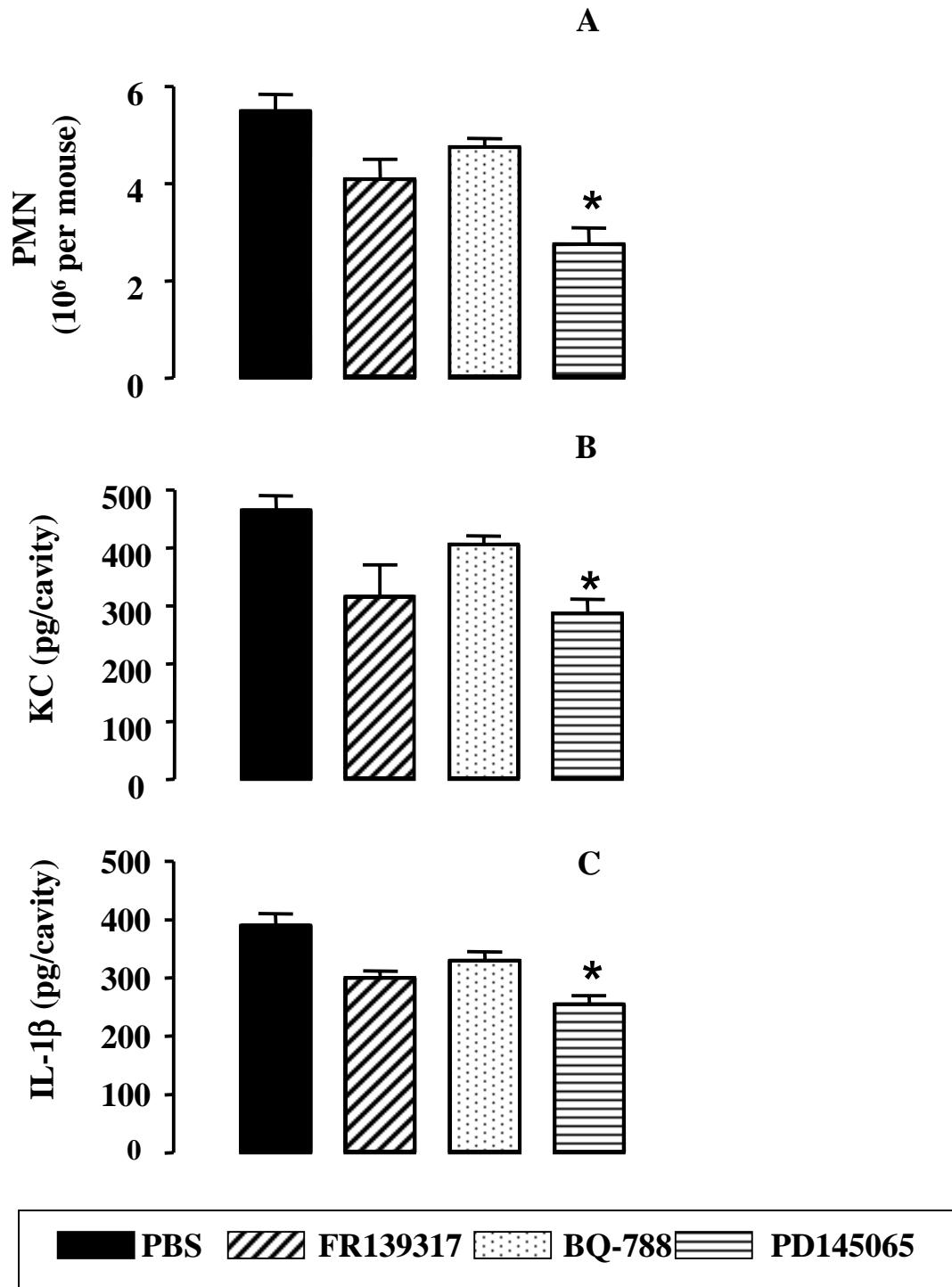


Figure 5

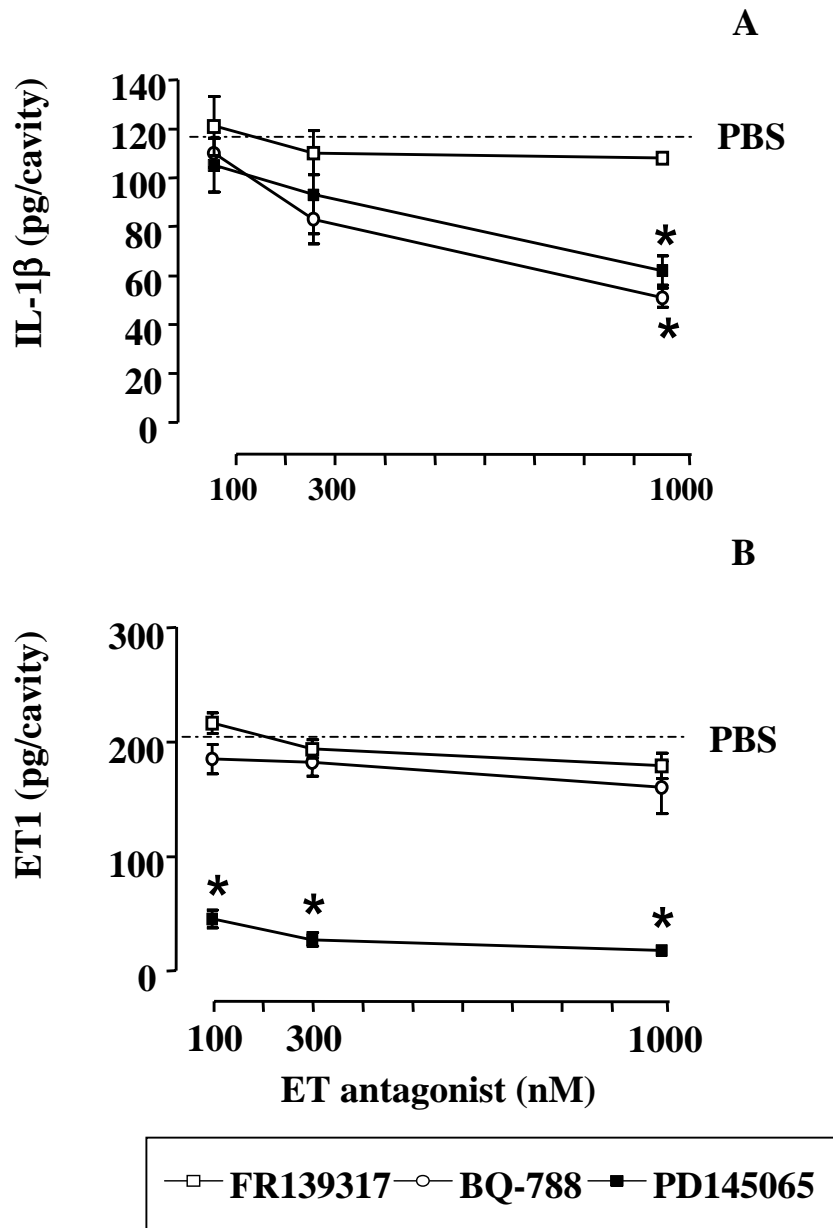




Figure 6

