Title: Bafetinib inhibits functional responses of human eosinophils in vitro

Article Type: Research Paper

Section/Category: Pulmonary, gastrointestinal and urogenital pharmacology

Keywords: bafetinib; eosinophils; Protein tyrosine kinase; inflammation

Corresponding Author: Prof. JAVIER MILARA,

Corresponding Author's Institution: Research Unit, University General Hospital, Valencia, Spain

First Author: JAVIER MILARA

Order of Authors: JAVIER MILARA; Maleles Martinez-Losa; Celia Sanz; Patricia Almudéver; Teresa Peiró; Adela Serrano; Esteban Jesus Morcillo; Cristóbal Zaragozá; Julio Cortijo

Abstract: Eosinophils play a prominent role in the process of allergic inflammation. Non-receptor associated Lyn tyrosine kinases generate key initial signals in eosinophils. Bafetinib, a specific Abl/Lyn tyrosine kinase inhibitor has shown a potent antiproliferative activity in leukemic cells, but its effects on eosinophils have not been reported. Therefore, we studied the effects of bafetinib on functional and mechanistic responses of isolated human eosinophils. Bafetinib was more potent than non-specific tyrosin kinase comparators genistein and tyrphostin inhibiting superoxide anion triggered by N-formyl-Met-Leu-Phe (fMLF; 100nM) (-log IC50= 7.25±0.04 M; 6.1±0.04 M; and 6.55±0.03 M respectively). Bafetinib, genistein and tyrphostin did not modify the [Ca2+]i responses to fMLF. Bafetinib inhibited the release of EPO induced by fMLF with higher potency than genistein and tyrphostin (-log IC50 = 7.24±0.09 M; 5.36±0.28 M; and 5.37±0.19 M respectively), and nearly suppressed LTC4, ECP and chemotaxis. Bafetinib, genistein and tyrphostin did not change constitutive apoptosis. However bafetinib inhibited the ability of granulocyte-monocyte colony-stimulating factor to prevent apoptosis. The activation of Lyn tyrosine kinase, p-ERK1/2 and p-38 induced by fMLF was suppressed by bafetinib and attenuated by genistein and tyrphostin. In conclusion, bafetinib inhibits oxidative burst and generation of inflammatory mediators, and reverses the eosinophil survival. Therefore, future anti-allergic therapies based on bafetinib, could help to suppress excessive inflammatory response of eosinophils at inflammatory sites.

Title:

Bafetinib inhibits functional responses of human eosinophils *in vitro*

Javier Milara[a, b, c], Maleles Martinez-Losa[c], Celia Sanz[d], Patricia Almudéver[c, e], Teresa Peiró[c, e], Adela Serrano[c], Esteban Jesus Morcillo[e, f], Cristóbal Zaragozá[g], Julio Cortijo[a, c, e, f].

[a]Clinical research unit (UIC), University General Hospital Consortium, Valencia, Spain. Av. tres cruces s/n. E-46014.

[b]Department of Biotechnology, Universidad Politécnica de Valencia, Spain.

[c]Research Foundation of General Hospital of Valencia, Spain. Av. tres cruces s/n. E-46014.

[d]Faculty of Biomedic Sciences, European University of Madrid; affiliated center of Valencia.

[e]Department of Pharmacology, Faculty of Medicine, University of Valencia, Spain. Blasco Ibanez 17, E-46010.

[f]CIBERES, Health Institute Carlos III, Valencia, Spain.

[g]UCMA, University General Hospital Consortium, Valencia, Spain. Av. tres cruces s/n. E-46014.

Corresponding Author: Javier Milara, PhD., Unidad de Investigación, Consorcio Hospital General Universitario, Avenida tres cruces s/n, E-46014 Valencia, Spain. Phone: +34 620231549, Fax: +34961972145, E-mail: xmilara@hotmail.com

Key words: bafetinib; eosinophils; Protein tyrosine kinase; inflammation

Abstract: 218

Eosinophils play a prominent role in the process of allergic inflammation. Non-receptor associated Lyn tyrosine kinases generate key initial signals in eosinophils. Bafetinib, a specific Abl/Lyn tyrosine kinase inhibitor has shown a potent antiproliferative activity in leukemic cells, but its effects on eosinophils have not been reported. Therefore, we studied the effects of bafetinib on functional and mechanistic responses of isolated human eosinophils. Bafetinib was more potent than non-specific tyrosin kinase comparators genistein and tyrphostin inhibiting superoxide anion triggered by *N*-formyl-Met-Leu-Phe (fMLF; 100nM) (–log IC_{50}= 7.25±0.04 M; 6.1±0.04 M; and 6.55±0.03 M respectively). Bafetinib, genistein and tyrphostin did not modify the $[Ca^{2+}]_i$ responses to fMLF. Bafetinib inhibited the release of EPO induced by fMLF with higher potency than genistein and tyrphostin (-log IC_{50} = 7.24±0.09 M; 5.36±0.28 M; and 5.37±0.19 M respectively), and nearly suppressed LTC_4, ECP and chemotaxis. Bafetinib, genistein and tyrphostin did not change constitutive apoptosis. However bafetinib inhibited the ability of granulocyte-monocyte colony-stimulating factor to prevent apoptosis. The activation of Lyn tyrosine kinase, p-ERK1/2 and p-38 induced by fMLF was suppressed by bafetinib and attenuated by genistein and tyrphostin. In conclusion, bafetinib inhibits oxidative burst and generation of inflammatory mediators, and reverses the eosinophil survival. Therefore, future anti-allergic therapies based on bafetinib, could help to suppress excessive inflammatory response of eosinophils at inflammatory sites.

1. Introduction: 500

Tissue eosinophilia is one of the hallmarks of allergic diseases and T-helper-2 (Th2)-type immune responses. Eosinophils contribute to lung injury, vascular leakage, mucus secretion, and tissue remodeling in allergic asthma by releasing cytotoxic granule proteins, reactive oxygen species (superoxide anion, eosinophil peroxidase (EPO)), and non-oxidant pro-inflammatory mediators (eosinophil cationic protein (ECP), leukotriene C4 (LTC_4)) (Hogan et al., 2008). Thus, a therapy targeting eosinophil chemotaxis, proliferation and activation should be required in allergic diseases.

Tyrosine kinases represent a class of enzymes that have been categorized into those that are receptor-linked and those that are cytosolic or non-receptor associated.

Between the receptor-linked tyrosine kinases, those associated to epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), vascular endothelial growth factor receptors (VEGFRs), stem cell factor (SCF) receptor c-kit, and fetal liver tyrosine kinase receptor 3 (FLT3) have been associated with the pathogenesis of allergic airway inflammation (Guntur and Reinero, 2012). Thus, for example, the EGFR-associated tyrosine kinase inhibitor, the anticancer drug gefitinib, alleviated allergic airway inflammation in mice (Hur et al., 2007), and the novel oral multitargeted receptor tyrosine kinase inhibitor, sunitinib, significantly inhibited airway hyperresponsiveness and airway remodeling in chronic experimental asthma (Huang et al., 2009).

The tyrosine kinases involved in signaling from G-protein-coupled receptors (GPCR) are thought to be primarily cytosolic or non-receptor associated in origin, and includes the src-related tyrosine kinase family, which comprises $p60^{src}$, $p53^{lyn}$, $p56^{lyn}$, $p56^{hck}$, $p59^{hck}$, $p62^{yes}$, $p55^{blk}$, $p55^{fgr}$, $p59^{fyn}$, $p56^{lck}$, and $p60^{yrk}$ (Corey and Anderson, 1999). GPCR agonists such as thrombin, bradykinin and bacterial peptide N-formyl-Met–Leu–

Phe (fMLF) can also invoke tyrosine kinase signaling cascades leading to MAPK pathway stimulation in different cell types (Wong, 2005). In addition, src family members including Lyn, have been suggested to initiate events leading to ERK1/2 phosphorylation in numerous GPCR systems (Bonacchi et al., 2001; El-Shazly et al., 1999; Tomkowicz et al., 2006). In this regard, the main non-receptor associated src-tyrosine kinase related with the eosinophil activation appears to be Lyn tyrosine kinase (Stafford et al., 2002). Lyn tyrosine kinase participates in eosinophil survival (Stafford et al., 2002), ERK1/2 phosphorylation and LTC_4 secretion (Zhu and Bertics, 2011) secondary to IL-5 stimulation.

Bafetinib is a novel, oral and dual Abl/Lyn tyrosine kinase inhibitor recently evaluated in hypereosinofilic syndromes, leukemia and chronic myeloid leukemia (Santos et al., 2010). Since Lyn tyrosine kinase regulates several eosinophil functions such as cell survival and activation (Adachi et al., 1999b), a specific drug directed to inhibit Lyn tyrosine kinase could be of potential value to attenuate eosinophilic allergic diseases.

Because fMLF has proven to be a valuable stimulus relevant to the activation of human eosinophils by allergens such as house dust mite or birch pollen (Svensson et al., 2007), we examined the effect of bafetinib on fMLF-induced different functional and mechanistic outputs relevant to the eosinophil activation, such as calcium signal and LTC_4, superoxide anion, ECP and EPO release. Il-5 was also used as stimulus as comparator. Furthermore, since survival of eosinophils may be relevant to allergic inflammation (Wegmann, 2011), the effects of bafetinib on the constitutive and granulocyte-monocyte colony-stimulating factor (GM-CSF) delayed apoptosis of eosinophils was examined.

2. Materials and methods

2.1. All reagents were obtained from Sigma-Aldrich unless otherwise stated

2.2. Isolation of human eosinophils and cytotoxicity assessment

Human blood from healthy donors was obtained in heparin, and PMNs were separated by standard laboratory procedures (Boyum, 1968). Eosinophils were then separated by depletion of neutrophils with anti-CD16-coated magnetic microbeads using the magnetic cell-separation system (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany) as outlined (Hansel et al., 1991; Martinez-Losa et al., 2007a). Eosinophils of >95% purity (determined by May-Grünwald–Giemsa) and viability (trypan blue exclusion) were used. For cytotoxicity assessment, the percentage of lactate dehydrogenase (LDH) release compared with values in cell lysates was taken as marker for cell damage by using a commercially available colorimetric assay as outlined (Martinez-Losa et al., 2007a). This investigation was approved by the institutional ethics committee and informed consent was obtained from all donors.

2.3. Superoxide anion generation

Release of superoxide anion from eosinophils was studied as described (Cortijo et al., 1999). In brief, generation of superoxide anion was measured as the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c with a modified microassay. With 96-well microtitre plates and a 200 µL reaction volume, 10^5 cells were added to 100 µmol/L of cytochrome c in Hanks balanced salt solution (HBSS) with 0.1% gelatin and 5 µg/mL of cytochalasin B. To initiate the reaction, cells were incubated with fMLF (100 nM) or IL-5 (20ng/ml). Immediately after the addition of activator, the reaction wells were measured for absorbance at 550 nm in a Microplate Autoreader (EL309, Bio-Tek Instruments Inc., Winooski, VT, USA) followed by repeated readings for 60 min. Each reaction was performed in duplicate and against an

identical control reaction which contained 20 µg/mL of SOD (approximately 2000 U/mg protein). Results were adjusted to represent a 1mL reaction volume, and superoxide anion generation was calculated with an extinction coefficient of $21.1x10^3$ mol/L/cm as nanomols of cytochrome c reduced per $5x10^5$ cells per time (min) minus SOD control. Treated cells were pre-incubated with bafetinib (Toronto Research Chemicals Inc. Toronto, Canada; 1nM-10µM) or the unspecific tyrosine kinase inhibitors tyrphostin (Sigma, Madrid, Spain; 10nM-100µM) or genistein (Sigma, Madrid, Spain; 10nM-100µM) for 30 min at 37 ºC before cell activation and the exposure was continued until the end of experiment. Control experiments were carried out in parallel with drug vehicle. Drug effects are expressed as percent inhibition from control values.

2.4. Intracellular Ca^{2+}-levels

Measurement of intracellular calcium concentration ($[Ca2^+]_i$) was performed as described (Cortijo et al., 1996). Cells (10^6/mL) were suspended in HBSS containing Ca^{2+} 1mM, glucose 1mM, bovine serum albumin 0.5%, and Fura-2/AM 5 µM, and incubated for 45 min at 37 ºC. After loading, eosinophils were washed in pre-warmed HBSS and resuspended ($5x10^5$ cells/mL). Then, cells were incubated at 37 ºC for 60 min with bafetinib (100 nM), tyrphostin (10 µM) or genistein (10 µM) or its vehicle before addition of fMLF (100 nM). The fluorescence intensity was monitored (excitation wavelengths 340 and 380 nm, emission wavelength 510 nm) using a spectrofluorometer (Perking Elmer LS50, Waltham, MA, USA) with thermally controlled cuvette holder and a magnetic stirrer. The peak values of $[Ca^{2+}]_i$ and the area under the curve over 60 s after activation were determined as outlined (Cortijo et al., 1996).

2.5. Quantitation of leukotriene C_4 production

LTC_4 assay was carried out as outlined (Cortijo et al., 1997). After isolation, eosinophils were resuspended in HBSS with HEPES (10mM), calcium (1mM), and magnesium (1mM), pH 7.4, supplemented with 0.1% gelatine and 20mM L-serine to a final concentration of 10^6 in 200 µL incubation volume. Eosinophils were pre-incubated with bafetinib (1nM-10µM), tyrphostin (10 µM), genistein (10 µM) or their vehicle for 30min at 37 ºC with cytochalasin B (5 µg/mL) added after the first 5 min of pre-incubation.

Thereafter, the cells were stimulated with fMLF (1 µM) or IL-5 (20ng/ml) for 30 min at 37 ºC. LTC_4 synthesis was terminated by immersion of the tubes in ice and the addition of 3 vol of ice-cold methanol. Cells were pelleted by centrifugation at 1500 g for 20min at 4 ºC. The methanolic supernatants (containing LTs released by cells) and extracts of cell pellets treated with 100% methanol for 18 h at 4 ºC (containing LTs retained intracellularly) were evaporated to dryness in a speed vacuum concentrator, and stored at -80 ºC before enzymeimmunoassay (EIA). Samples were reconstituted to original volume with ice-cold EIA buffer and LTC_4 was quantified as described by the manufacturer (Biotrak, RPN 224, Amersham Int.). Absorbance was measured at 450 nm with a microtitre plate photometer (Multiscan MKII, Labsystems, Haverhill, MA, USA). The assay uses horseradish peroxidase labelled LTC_4 and a rat anti-LTC_4. The sensitivity of the assay is 0.5 pg/well. The assay has 100% and 30% cross-reactivity for LTD_4 and LTE_4, respectively; cross-reactivity for LTB_4 was 0.3%, and cross-reactivity for other related compounds is negligible (< 0.006%).

2.6. Determination of eosinophil peroxidase release

Release of EPO was measured as outlined (Cortijo et al., 1999). Eosinophils were resuspended in HBSS-FBS, and aliquots of 10^5 cells in 100 µL were loaded onto

microplate wells. bafetinib (1nM-10μM), tyrphostin (10 μM), genistein (10 μM) or their vehicle was added to each well and the plate was incubated for 30 min at 37 ºC. Then, cells were activated with fMLF (1 μM plus 5 μg/mL of cytochalasin B) or IL-5 (20ng/ml plus 5 μg/mL of cytochalasin B). The substrate solution (0.1 mM o-phenylenediamine dihydrochloride in 0.05 M Tris-HCl containing 0.1% Triton X-100 and 1mM H_2O_2) was added to wells and the plate incubated (30 min, 37 ºC) before stopping the reaction (4 M sulphuric acid), followed by centrifugation at 350 g for 5 min. Duplicate aliquots of supernatant (100 μL) were transferred onto a new plate.

Kinetic assay for EPO was performed for supernatant of treated and untreated cells. The absorbance was determined at 492 nm using a Microplate Autoreader (EL309, Bio-Tek Instruments). The EPO release is expressed in peroxidase units/10^6 cells as determined from comparison with a standard curve. Drug effects are also expressed as per cent inhibition from control values.

2.7. Eosinophil cationic protein production

All assays were performed in duplicate at a cell concentration of $2.5x10^5$ cells/mL in supplemented Dulbecco's phosphate-buffered saline (PBS) (Hatzelmann et al., 1995). Cell suspensions were pre-incubated with bafetinib (100nM), tyrphostin (10 μM), genistein (10 μM) or its vehicle for 30 min at 37 ºC before stimulation; thereafter, the cells were activated with fMLF (100 nM) or IL-5 (20ng/ml) for 30min. Experiments were terminated by centrifugation and supernatants were prepared for storage (-20 ºC). Aliquots of the samples were taken for ECP measurements by a RIA method according to the manufacturer instructions (anti-ECP-^{125}I; Pharmacia, Uppsala, Sweden).

2.8. Eosinophil apoptosis

Human eosinophils were analysed for apoptosis after 24 h of culture by flow cytometry using annexin V-fluorescein isothiocyanate and PI following the protocol indicated by the manufacturer (Annexin-V-Fluos; Roche Applied Science, Barcelona, Spain) in a flow cytometry analyzer (CyAn TM ADP; DakoCytomation A/S DK-2600), and as previously outlined (Buenestado et al., 2006). Cells ($1x10^4$) were distinguished as viable non-apoptotic (annexin V^-/PI^-), early apoptotic (annexin V^+/ PI^-) or late apoptotic (annexin V^+/ PI^+) cells. Apoptosis was measured in the absence and presence of rhGM-CSF (1 ng/mL) in cells treated with bafetinib (100nM), tyrphostin (10 μM), genistein (10 μM) or their vehicle. The concentration of GM-CSF was selected to produce a significant eosinophil survival effect as previously reported (Martinez-Losa et al., 2007b).

2.9. Eosinophil chemotaxis

Eosinophil migration was measured with the Boyden chamber technique as previously outlined (Zigmond and Hirsch, 1973). In brief, eosinophils ($0.5x10^5$cells/ml) were placed in the upper compartment of the chamber, previously incubated for 15 minutes in the absence or presence of Bafetinib (100nM), tyrphostin (10 μM), genistein (10 μM) or their vehicle. The chemotactic factor fMLF 100 nM was placed in the lower compartment, followed by 30 minutes incubation at 37ºC. After migration, the nitrocellulose filters (8 μm pores (Sartuorious # 11301-013N) were fixed and stained with Diff Quick (Baxter Diagnostics AG) and the distance (μm) travelled into the filter was determined according to the leading front technique by microscopy examination as previously described (Buenestado et al., 2006). In each chemotactic assay, the migration distance was determined at five different filter sites.

2.10. Western blot

Western blot analysis was used to detect total phosphorylation of tyrosine residues, Lyn tyrosine kinase, p-ERK1/2 and p-38 in eosinophils. $5x10^6$ eosinophils/ mL were incubated with bafetinib (10 nM, 100 nM), tyrphostin (10 μM), genistein (10 μM) or its vehicle for 30 min at 37 ºC before stimulation; thereafter, the cells were activated with fMLF (100 nM) for 5 min. Reaction was stoped in ice, and cells were centrifuged and lysed on ice with a lysis buffer consisting of 20mM Tris, 1 mM ethylenediaminetetraacectic acid (EDTA), 0.9% NaCl, 0.1% Triton X-100, 1 mM dithiothreitol and 1 μg ml^{-1} pepstatin A supplemented by a complete protease inhibitor cocktail. The Bio-Rad assay (Bio-Rad Laboratories Ltd., Herts, UK) was used to quantify the level of protein in each sample to ensure equal protein loading. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to separate the proteins according to their molecular weight. Briefly, 20 μg of protein (denatured) mixed with 2x loading buffer (comprising 160mM Tris HCl (pH 6.8), 4% SDS, 20% glycerol, 1.4mM β-mercaptoethanol, 0.04% bromophenol blue) along with a molecular weight protein marker, Bio-Rad Kaleidoscope marker (Bio-Rad Laboratories), was loaded onto an acrylamide gel consisting of a 5% acrylamide stacking gel on top of a 12% acrylamide resolving gel and run through the gel by application of 100 V for 1 hour. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a wet blotting method. The membrane was blocked with 5% Marvel in PBS containing 0.1% Tween20 (PBS-T) and then probed with rabbit anti-human monoclonal antibody against anti-phosphotyrosine (clone PY20; Millipore, Madrid, Spain), rabbit anti-human Lyn tyrosine kinase antibody p56/p53 (cat. nº: sc-15, Santa Cruz Biotechnology/Autogen Bioclear, Wiltshire, UK), mouse anti-human phospho-ERK1/2 (cat. nº: M-9692; Sigma), rabbit anti-human monoclonal antibody phospho-p38 MAPK (Trh180/Tyr182)

(cat. nº: 28B10; Cell Signal, Boston, EEUU), and a rabbit anti-human β-actin antibody (cat. nº: A1978; Sigma) as house-keeping reference, followed by the corresponding peroxidase-conjugated secondary (1:10,000) antibody. The enhanced chemiluminescence method of protein detection using ECL-plus (GE Healthcare, Amersham Biosciences, UK) was used to detect labelled proteins.

2.11. Statistical analysis of results

Data are presented as mean±SEM of n experiments. The IC_{50} values were calculated from the concentration inhibition curves by non-linear regression analysis. Statistical analysis of data was carried out by ANOVA followed by Bonferroni's test or by Student's t test as appropriate (GraphPad Software Inc., San Diego, CA, USA). Significance was accepted when $P<0.05$.

3. Results

3.1. Bafetinib produced no cytotoxicity in human eosinophils

The cytotoxicity of bafetinib on human eosinophils has not been reported previously. Therefore, to exclude that any inhibition of eosinophil functions observed with this compound was due to cell damage, we first examined the effect of bafetinib on LDH release as a marker of cell injury. In this respect, the maximal concentrations used in the present study for bafetinib showed no cytotoxicity (values are expressed as % of total LDH) as shown by 3.14±0.81% for bafetinib 10μM (n = 4) which was not significantly different from 2.66±0.57% in control experiments (n = 5).

3.2. Effect of bafetinib on *N*-formyl-Met–Leu–Phe- and IL-5-induced superoxide anion release and $[Ca^{2+}]_i$ response.

Oxidant generation by eosinophils is relevant to inflammatory damage in allergy diseases. We tested first the ability of bafetinib to inhibit the generation of superoxide anion from human eosinophils activated by fMLF (100 nM) or IL-5 (20ng/ml) as comparator. The concentration of these stimuli was selected to produce enough levels of oxidant generation to perform inhibitory studies (7.7±1.4 nmol superoxide anion generated by $5x10^5$ cells for fMLF, n = 5; and 6.8±1.4 nmol superoxide anion generated by $5x10^5$ cells for IL-5, n = 5).

Bafetinib decreased, in a concentration-dependent way, the superoxide anion generation triggered by fMLF, with an inhibitory potency of $-\log IC_{50}$ = 7.25±0.04 M as shown in Fig. 1A. As comparators, the unspecific inhibitors of tyrosine kinases tyrphostin and genistein were also tested. Both, tyrphostin and genistein were significantly lesser potent than bafetinib inhibiting oxidative burst, reaching inhibitory potencies of $-\log IC_{50}$ = 6.55±0.03 and 6.1±0.04 M respectively (Fig 1A). Similar findings were observed for IL-5 stimuli. In this case bafetinib concentration-dependently inhibited superoxide anion generation with a potency of $-\log IC_{50}$ = 7.81±0.05 M, while inhibitory potencies for tyrphostin and genistein were significantly lower $-\log IC_{50}$ = 7.05±0.04 and 6.4±0.04 M respectively (Fig.1B)

The oxidant production by fMLF in human eosinophils requires an intracellular calcium signal (Zardini et al., 1999). Thus, addition of fMLF (100 nM) resulted in a rapid initial increase in $[Ca^{2+}]_i$ followed by a decline towards baseline levels (not shown). However, neither bafetinib nor the unspecific tyrosine kinase inhibitors tyrphostin and genistein significantly altered the peak and $AUC_{0\text{-}60s}$ responses to fMLF in human eosinophils (Table 1). Because IL-5, in human eosinophils did not enhance significant $[Ca^{2+}]_i$ mobilization (van der Bruggen et al., 1993) experiments using IL-5 as inducer of $[Ca^{2+}]_i$ were discarded.

3.3. Bafetinib inhibited eosinophil peroxidase release, leukotriene C_4 and eosinophil cationic protein production in *N*-formyl-Met–Leu–Phe- and IL-5-activated eosinophils

Activation of eosinophils with fMLF or IL-5 caused also the release of EPO and other non-oxidant inflammatory mediators such as LTC_4 and ECP. Bafetinib produced a concentration-related inhibition of EPO release (-log IC_{50} = 7.24±0.09; Fig. 2A) induced by fMLF which showed a higher potency than tyrphostin and genistein (-log IC_{50} = 5.37±0.19 and 5.36±0.28 respectively; Fig. 2A). Similar results were obtained for bafetinib (-log IC_{50} = 7.10±0.1; Fig. 2C) tyrphostin (-log IC_{50} = 5.87±0.2; Fig. 2C) and genistein (-log IC_{50} = 5.62±0.15; Fig. 2C) when IL-5 was added as stimulus.

In other experiments, bafetinib 100nM significantly reduced the increased production of LTC_4 and ECP induced by fMLF and, in a lesser extent, by IL-5. However, tyrphostin 10μM and genistein 10μM did not significantly inhibit ECP release induced by IL-5 (Fig. 3A, 3B, 3C and 3D).

3.4. Bafetinib attenuated the ability of granulocyte-monocyte colony-stimulating factor to prevent eosinophil apoptosis and reduced the *N*-formyl-Met–Leu–Phe-induced eosinophil migration

We investigated the effects of bafetinib on spontaneous apoptosis as well as in the presence of GM-CSF inflammatory cytokin which enhance eosinophil survival. Apoptosis was measured after 48 h of culture by using annexin V and PI staining of eosinophils. Bafetinib 100nM and the comparators tyrphostin 10μM and genistein 10μM produced no change in apoptosis in the absence of cytokine (Fig 4A, 4C and 4D). Bafetinib significantly attenuated the GM-CSF-induced inhibition of apoptosis in a higher proportion than tyrphostin and genistein (Fig 4B, 4E and 4F).

In experiments examining chemotaxis, eosinophil migration distance amounted 67.4 ± 5.4 μm in response to fMLF (Fig. 5). Bafetinib significantly reduced the fMLF-induced eosinophil chemotaxis in a similar way than tyrphostin and genistein (Fig. 5).

3.5. *N*-formyl-Met–Leu–Phe increased the phosphorylation of tyrosine residues and Lyn tyrosine kinase phosphorylation as well as p-ERK1/2 and p-38.

Eosinophil activation by fMLF entails the phosphorylation of multiple protein tyrosine residues of a wide range of molecular weights in order to activate different downstream signals. The Fig. 6A shows the phosphorylation of multiple tyrosine residues mainly located between 100 to 36 kD after 5 min of fMLF stimulation. Preincubation of eosinophils with tyrphostin 10μM and genistein 10μM reduced the phosphorylation of tyrosine residues induced by fMLF, mainly in proteins between 100 to 36 kD. In a similar way, bafetinib (10-100nM) inhibited the phosphorylation of tyrosine residues in proteins between 60 to 36 kD (Fig. 6A). The phosphorylation of Lyn tyrosine kinase is located between 56-53 kD and was selectively inhibited by bafetinib as shown Fig 6A and 6C. Genistein but not tyrphostin also inhibited the fMLF-induced Lyn tyrosine kinase phosphorylation (Fig. 6B). Since MAPKs p-ERK1/2 and p-38 signals mediate the activation of eosinophils (Zhu et al., 2001), and because the activation of p-ERK1/2 and p-38 are produced, in part, by the phosphorylation of tyrosine residues at 42, 44 and 43 kD respectively, we next explored the effect of bafetinib and the unspecific tyrosine kinase inhibitors tyrphostin and genistein on fMLF-induced p-ERK1/2 and p-38. In this regard, bafetinib and genistein but not tyrphostin inhibited phosphorylation of ERK1/2, and all of them suppressed p-38 (Fig. 7).

4. Discussion: 1434

The main and novel finding of the present work is that bafetinib, an oral inhibitor of the dual Abl/Lyn tyrosine kinase currently under clinical research for hypereosinofilic syndromes, is able to inhibit a wide array of functional responses of human eosinophils *in vitro* showing potential applications to allergic disorders.

Recent works have shown the value of protein tyrosine kinase inhibitors attenuating a broad range of asthmatic processes such as the activation and proliferation of inflammatory cells and resident airway cells (Wong, 2005). Thus, the unspecific protein tyrosine kinase inhibitor genistein, was the first showing a reduction of airway hyperresponsiveness, airway eosinophilia and eosinophil peroxidase activity in bronchoalveolar lavage fluid in an animal model of asthma (Duan et al., 2003). Later, a broad range of receptor protein tyrosine kinase inhibitors such as gefitinib (EGFR tyrosine kinase inhibitor), imatinib (SCF receptor c-kit, PDGFR-α and BCR-ABL protein tyrosine kinase inhibitor), masitinib (c-kit/PDGF receptor tyrosine kinase inhibitor) and sunitinib (VEGFR, SCF receptor c-kit, PDGFR-α,β and FLT3 protein tyrosine kinase inhibitor) showed favorable anti-asthmatic profile in animal asthmatic models, reducing Th2 cytokine and eosinophil number in bronchoalveolar fluid as well as bronchial remodeling (Berlin and Lukacs, 2005; Huang et al., 2009; Hur et al., 2007; Lee-Fowler et al., 2012). While receptor tyrosine kinases are critical in airway remodeling, non-receptor tyrosine kinases are one of the earliest activated signaling components in response to stimulation of immune receptors of inflammatory cells. In this regard, eosinophils, as a key effector cells in human asthma, are mainly regulated by non-receptor protein tyrosine kinase Lyn. Thus, for example, eosinophilopoiesis and eosinophil survival induced by IL-5, IL-3 and GM-CSF is controlled by Lyn tyrosine kinase (Stafford et al., 2002) because it's physical association to their βc receptors (Adachi et al., 1999a). In addition, other GPCR ligands, such as fMLF may also activate

src family members, including Lyn tyrosine kinase, although the relevance of this activation in human eosinophils has been poorly analyzed. In this regard, it has been shown previously that the activation of formyl-peptide receptors by fMLF activates Lyn tyrosine kinase, phosphorylating ERK1/2 down-stream factor which is a relevant mechanism involved in the activation of human eosinophils by allergens (Ptasznik et al., 1995; Svensson et al., 2007). Eosinophils express functional formyl peptide receptors (FPR1-FPR4) whose activation induces the release of eosinophil peroxidase (EPO), increase of eosinophil migration and intracellular calcium release (Svensson et al., 2007). In fact, inhalant allergens such as house dust mite and birch pollen activates human eosinophils through the activation of FPR1. These observations were demonstrated since FPR1 inhibitors boc-MLP and CsH inhibited house dust mite and birch eosinophil activation. Furthermore, fMLF pre-exposure desensitized the activation of eosinophils by both house dust mite and birch pollen (Svensson et al., 2007). In addition we previously used fMLF as eosinophil activator of superoxide anion generation, EPO release, LTC_4 increase and ECP release (Martinez-Losa et al., 2009) with a robust increase of these eosinophil markers of activation. Supporting these findings, the boc-MLP and CsH inhibitors of FPR1 were able to inhibit by 79 ± 10% the chemotactic movement of eosinophils toward eotaxin-1, eotaxin-2, eotaxin-3 and RANTES (cognate ligands of CCR3) (Svensson et al., 2009) confirming the relevance of FPR in eosinophil activation.

In the present work we observed that fMLF increased reactive oxygen species superoxide anion and EPO as well as ECP, which are directly involved in lung tissue remodeling of asthmatic patients (Pegorier et al., 2006). Similar findings were obtained recently by our group (Martinez-Losa et al., 2007a). Furthermore, the unspecific protein tyrosine kinase inhibitors genistein and tyrphostin dose-dependently inhibited

superoxide anion and EPO generation with similar potency, which is consistent with previous observations in human eosinophils and in guinea pig model of asthma (Dent et al., 2000; Duan et al., 2003). These observations implicate protein tyrosine kinases in fMLF-induced degranulation and reactive oxygen species formation in human eosinophils. However, the specific inhibition of Lyn kinase with bafetinib showed higher potency than genistein and tyrphostin suggesting a major role of Lyn kinase in the release of superoxide anion, EPO and ECP induced by fMLF. In contrast, previous observations indicate that Lyn kinase is not responsible of the degranulation and release of ECP in human eosinophils stimulated with IL-5 (Adachi et al., 1999b). These differences may be explained by the differences in the stimulus (fMLF *vs* IL-5) employed in this work and because the activation of the initial tyrosine kinase and subsequent signaling pathway is specific to cell type and the stimulating ligand (Wong, 2005). Supporting these results, a number of src protein tyrosine kinases have been detected to participate in the fMLF-induced neutrophil degranulation (Fumagalli et al., 2007; Yan et al., 2004). In fact, in this study IL-5 increased ECP release that was not inhibited by tyrphostin and genistein and was weakly inhibited by bafetinib, suggesting that IL-5-Lyn kinase pathway in eosinophils only induce a small amount of ECP.

LTC_4 is a member of the cysteinil-leukotrienes, which are potent mediators of airway inflammation and hypersensitivity and is released by the activated eosinophils (Munoz et al., 1999). The role of protein tyrosine kinases on the LTC_4 production in human eosinophils was established previously, since genistein and tyrphostin were able to inhibit the release of LTC_4 secondary to platelet-activating factor exposure (Dent et al., 2000). In this work, we observed that both, genistein and tyrphostin reduced the LTC_4 production following fMLF exposure and that the Lyn tyrosine kinase inhibitor bafetinib nearly abolished LTC_4.

Bafetinib, also known as NS-187, was developed to the management of imatinib-resistant leukemia since the overexpression of Lyn tyrosine kinase confers resistance to imatinib (Kimura et al., 2005). Currently, bafetinib has been assayed in a phase I clinical trial in which the non-toxic dose of 240mg twice a day reached a maximum concentration of 844 nM (Kantarjian et al., 2010), which is near of the maximum response achieved by bafetinib in this work. The application of protein tyrosine kinase inhibitors to allergic disorders is currently under debate because besides the promising inhibitory effects on nearly all allergic processes, the nonspecific protein tyrosine kinase inhibitors currently available show a wide range of side effects. Thus, a specific inhibition of a single key protein tyrosine kinase would be desirable. In this regard, a previous study showed that bafetinib only inhibited 4 of the 79 protein tyrosine kinases assayed, and that between those of the src family only Lyn tyrosine kinase was inhibited (Kimura et al., 2005). In this work, we observed that, in contrast to the non-specific inhibitors genistein and tyrphostin, bafetinib showed an inhibition of the phosphorylation of tyrosine residues in proteins between 60 to 36 kD after the stimulation with fMLF, that is in agreement with the $p53^{lyn}$ and $p56^{lyn}$ currently characterized. Furthermore the inhibitory effect of genistein and tyrphostin on Lyn protein kinase phosphorylation was lower than that observed for bafetinib (Fig. 6B), which may explain the lower potency of genistein and tyrphostin on functional responses of human eosinophils.

Thus far, bafetinib effects have been only focused on leukemic cell survival and proliferation attributed to Lyn kinase (Santos et al., 2010). Lyn tyrosine kinase is responsible of the human eosinophil survival and eosinophilopoiesis induced by IL-5, IL-3 and GM-CSF because it's physical association to the βc cytokine receptor under basal conditions (Stafford et al., 2002). Apoptosis is now considered an important mechanism in the resolution of inflammation. In this work, bafetinib, genistein and tyrphostin did not modify spontaneous apoptosis. However, bafetinib significantly

inhibited the antiapoptotic effect of GM-CSF. Since IL-5 family members are elevated in allergic diseases promoting eosinophil proliferation and survival, bafetinib could be of interest inhibiting esinophilic processes.

As part of the inflammatory cell increase in allergy, cells have to migrate to the site of allergic reaction to promote phenotypic disorders as occurs in lung tissue of asthmatics. Thus, potent chemotactic factors such as CCL5 and fMLF have been studied as eosinophil chemoatractants (Munoz et al., 1997). In this work, the non-specific inhibition of protein tyrosine kinases with genistein and tyrphostin significantly inhibited the eosinophil migration generated by fMLF in a similar extent that bafetinib. Similar results have been observed previously (Schweizer et al., 1996). However there is a lack of evidence of what type of tyrosine kinase modulates eosinophil migration. Thus, proline-rich tyrosine kinase 2, focal adhesion kinase and Src family kinases, Hck and c-Fgr have been related with eosinophil migration (Cheung et al., 2008; El-Shazly et al., 1999; Lynch et al., 2000; Zhu et al., 2008) supporting the idea that eosinophil migration may be modulated by a wide array of protein tyrosine kinases. Other mechanism implicated in eosinophil migration is the increase of intracellular Ca^{2+} which also contributes to eosinophil degranulation and activation (Ito et al., 2007; Zardini et al., 1999). In this regard, nor genistein, tyrphostin nor bafetinib reduced the $[Ca^{2+}]_i$ following fMLF exposure, which implicate alternative pathways for tyrosine kinase inhibitors attenuating eosinophil functions. Other signal pathways implicated in eosinophil survival, chemotaxis and activation are the phosphorylation of the MAPKs p-ERK1/2 and p-38 (Boehme et al., 1999; Kampen et al., 2000; Yamamura et al., 2009). Thus, Lyn tyrosine kinase and other src family kinases are able to phosphorylate ERK1/2 and p-38 (Pazdrak et al., 1995). In this work, genistein and bafetinib but not tyrphostin inhibited the phosphorylation of ERK1/2. This may be explained because

tyrphostin was unable to inhibit Lyn phosphorilation (Fig. 6B) and because Lyn tyrosin kinase phosphorylates ERK1/2. Therefore, the effects of bafetinib inhibiting functional outputs of human eosinophils may be mediated by the inhibition of Lyn tyrosin kinase and its downstream signals ERK1/2 and p38.

In conclusion, this is the first report showing the effect of bafetinib inhibiting oxidative burst and generation of inflammatory mediators, as well as reversion of the survival effect produced by inflammatory cytokine GM-CSF. Therefore, future anti-allergic therapies based on bafetinib, could help to suppress excessive inflammatory response of eosinophils at inflammatory sites, although future clinical benefit has to be proven.

Acknowledgments

This work was supported by grants SAF2011-26443 (JC), SAF2012-31042 (EM), FIS CP11/00293(JM), CIBERES (CB06/06/0027), ADE10/00020 and research grants from Regional Government (Prometeo/2008/045, 'Generalitat Valenciana'. TP received a research grant from Conselleria de Educacion, Generalitat Valenciana ACIF/2010/114. Support from the CENIT programme (Spanish Government) was obtained.

Bibliography

Adachi, T., Pazdrak, K., Stafford, S., Alam, R., 1999a. The mapping of the Lyn kinase binding site of the common beta subunit of IL-3/granulocyte-macrophage colony-stimulating factor/IL-5 receptor. J Immunol 162, 1496-1501.

Adachi, T., Stafford, S., Sur, S., Alam, R., 1999b. A novel Lyn-binding peptide inhibitor blocks eosinophil differentiation, survival, and airway eosinophilic inflammation. J Immunol 163, 939-946.

Berlin, A.A., Lukacs, N.W., 2005. Treatment of cockroach allergen asthma model with imatinib attenuates airway responses. Am J Respir Crit Care Med 171, 35-39.

Boehme, S.A., Sullivan, S.K., Crowe, P.D., Santos, M., Conlon, P.J., Sriramarao, P., Bacon, K.B., 1999. Activation of mitogen-activated protein kinase regulates eotaxin-induced eosinophil migration. J Immunol 163, 1611-1618.

Bonacchi, A., Romagnani, P., Romanelli, R.G., Efsen, E., Annunziato, F., Lasagni, L., Francalanci, M., Serio, M., Laffi, G., Pinzani, M., Gentilini, P., Marra, F., 2001. Signal transduction by the chemokine receptor CXCR3: activation of Ras/ERK, Src, and phosphatidylinositol 3-kinase/Akt controls cell migration and proliferation in human vascular pericytes. J Biol Chem 276, 9945-9954.

Boyum, A., 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest Suppl 97, 77-89.

Buenestado, A., Cortijo, J., Sanz, M.J., Naim-Abu-Nabah, Y., Martinez-Losa, M., Mata, M., Issekutz, A.C., Marti-Bonmati, E., Morcillo, E.J., 2006. Olive oil-based lipid emulsion's neutral effects on neutrophil functions and leukocyte-endothelial cell interactions. JPEN J Parenter Enteral Nutr 30, 286-296.

Corey, S.J., Anderson, S.M., 1999. Src-related protein tyrosine kinases in hematopoiesis. Blood 93, 1-14.

Cortijo, J., Pons, R., Dasi, F., Marin, N., Martinez-Losa, M., Advenier, C., Morcillo, E.J., 1997. Bronchodilator and anti-inflammatory activities of SCA40: studies in human isolated bronchus, human eosinophils, and in the guinea-pig in vivo. Naunyn Schmiedebergs Arch Pharmacol 356, 806-814.

Cortijo, J., Villagrasa, V., Navarrete, C., Sanz, C., Berto, L., Michel, A., Bonnet, P.A., Morcillo, E.J., 1996. Effects of SCA40 on human isolated bronchus and human polymorphonuclear leukocytes: comparison with rolipram, SKF94120 and levcromakalim. Br J Pharmacol 119, 99-106.

Cortijo, J., Villagrasa, V., Pons, R., Berto, L., Marti-Cabrera, M., Martinez-Losa, M., Domenech, T., Beleta, J., Morcillo, E.J., 1999. Bronchodilator and anti-inflammatory activities of glaucine: In vitro studies in human airway smooth muscle and polymorphonuclear leukocytes. Br J Pharmacol 127, 1641-1651.

Cheung, P.F., Wong, C.K., Ip, W.K., Lam, C.W., 2008. FAK-mediated activation of ERK for eosinophil migration: a novel mechanism for infection-induced allergic inflammation. Int Immunol 20, 353-363.

Dent, G., Munoz, N.M., Zhu, X., Ruhlmann, E., Magnussen, H., Leff, A.R., Rabe, K.F., 2000. Involvement of protein tyrosine kinases in activation of human eosinophils by platelet-activating factor. Immunology 100, 231-237.

Duan, W., Kuo, I.C., Selvarajan, S., Chua, K.Y., Bay, B.H., Wong, W.S., 2003. Antiinflammatory effects of genistein, a tyrosine kinase inhibitor, on a guinea pig model of asthma. Am J Respir Crit Care Med 167, 185-192.

El-Shazly, A., Yamaguchi, N., Masuyama, K., Suda, T., Ishikawa, T., 1999. Novel association of the src family kinases, hck and c-fgr, with CCR3 receptor stimulation:

A possible mechanism for eotaxin-induced human eosinophil chemotaxis. Biochem Biophys Res Commun 264, 163-170.

Fumagalli, L., Zhang, H., Baruzzi, A., Lowell, C.A., Berton, G., 2007. The Src family kinases Hck and Fgr regulate neutrophil responses to N-formyl-methionyl-leucyl-phenylalanine. J Immunol 178, 3874-3885.

Guntur, V.P., Reinero, C.R., 2012. The potential use of tyrosine kinase inhibitors in severe asthma. Curr Opin Allergy Clin Immunol 12, 68-75.

Hansel, T.T., De Vries, I.J., Iff, T., Rihs, S., Wandzilak, M., Betz, S., Blaser, K., Walker, C., 1991. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. J Immunol Methods 145, 105-110.

Hatzelmann, A., Tenor, H., Schudt, C., 1995. Differential effects of non-selective and selective phosphodiesterase inhibitors on human eosinophil functions. Br J Pharmacol 114, 821-831.

Hogan, S.P., Rosenberg, H.F., Moqbel, R., Phipps, S., Foster, P.S., Lacy, P., Kay, A.B., Rothenberg, M.E., 2008. Eosinophils: biological properties and role in health and disease. Clin Exp Allergy 38, 709-750.

Huang, M., Liu, X., Du, Q., Yao, X., Yin, K.S., 2009. Inhibitory effects of sunitinib on ovalbumin-induced chronic experimental asthma in mice. Chin Med J (Engl) 122, 1061-1066.

Hur, G.Y., Lee, S.Y., Lee, S.H., Kim, S.J., Lee, K.J., Jung, J.Y., Lee, E.J., Kang, E.H., Jung, K.H., Kim, J.H., Shin, C., Shim, J.J., In, K.H., Kang, K.H., Yoo, S.H., 2007. Potential use of an anticancer drug gefinitib, an EGFR inhibitor, on allergic airway inflammation. Exp Mol Med 39, 367-375.

Ito, W., Chiba, T., Kanehiro, A., Kato, H., Yamaguchi, K., Ueki, S., Kayaba, H., Chihara, J., 2007. Hepatocyte growth factor attenuates eotaxin and PGD2-induced chemotaxis of human eosinophils. Allergy 62, 415-422.

Kampen, G.T., Stafford, S., Adachi, T., Jinquan, T., Quan, S., Grant, J.A., Skov, P.S., Poulsen, L.K., Alam, R., 2000. Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases. Blood 95, 1911-1917.

Kantarjian, H., le Coutre, P., Cortes, J., Pinilla-Ibarz, J., Nagler, A., Hochhaus, A., Kimura, S., Ottmann, O., 2010. Phase 1 study of INNO-406, a dual Abl/Lyn kinase inhibitor, in Philadelphia chromosome-positive leukemias after imatinib resistance or intolerance. Cancer 116, 2665-2672.

Kimura, S., Naito, H., Segawa, H., Kuroda, J., Yuasa, T., Sato, K., Yokota, A., Kamitsuji, Y., Kawata, E., Ashihara, E., Nakaya, Y., Naruoka, H., Wakayama, T., Nasu, K., Asaki, T., Niwa, T., Hirabayashi, K., Maekawa, T., 2005. NS-187, a potent and selective dual Bcr-Abl/Lyn tyrosine kinase inhibitor, is a novel agent for imatinib-resistant leukemia. Blood 106, 3948-3954.

Lee-Fowler, T.M., Guntur, V., Dodam, J., Cohn, L.A., DeClue, A.E., Reinero, C.R., 2012. The tyrosine kinase inhibitor masitinib blunts airway inflammation and improves associated lung mechanics in a feline model of chronic allergic asthma. Int Arch Allergy Immunol 158, 369-374.

Lynch, O.T., Giembycz, M.A., Daniels, I., Barnes, P.J., Lindsay, M.A., 2000. Pleiotropic role of lyn kinase in leukotriene B(4)-induced eosinophil activation. Blood 95, 3541-3547.

Martinez-Losa, M., Cortijo, J., Juan, G., O'Connor, J.E., Sanz, M.J., Santangelo, F., Morcillo, E.J., 2007a. Inhibitory effects of N-acetylcysteine on the functional responses of human eosinophils in vitro. Clin Exp Allergy 37, 714-722.

Martinez-Losa, M., Cortijo, J., Juan, G., Ramon, M., Sanz, M.J., Morcillo, E.J., 2007b. Modulatory effects of N-acetyl-L-cysteine on human eosinophil apoptosis. Eur Respir J 30, 436-442.

Martinez-Losa, M., Cortijo, J., Piqueras, L., Sanz, M.J., Morcillo, E.J., 2009. Taurine chloramine inhibits functional responses of human eosinophils in vitro. Clin Exp Allergy 39, 537-546.

Munoz, N.M., Douglas, I., Mayer, D., Herrnreiter, A., Zhu, X., Leff, A.R., 1997. Eosinophil chemotaxis inhibited by 5-lipoxygenase blockade and leukotriene receptor antagonism. Am J Respir Crit Care Med 155, 1398-1403.

Munoz, N.M., Hamann, K.J., Rabe, K.F., Sano, H., Zhu, X., Leff, A.R., 1999. Augmentation of eosinophil degranulation and LTC(4) secretion by integrin-mediated endothelial cell adhesion. Am J Physiol 277, L802-810.

Pazdrak, K., Schreiber, D., Forsythe, P., Justement, L., Alam, R., 1995. The intracellular signal transduction mechanism of interleukin 5 in eosinophils: the involvement of lyn tyrosine kinase and the Ras-Raf-1-MEK-microtubule-associated protein kinase pathway. J Exp Med 181, 1827-1834.

Pegorier, S., Wagner, L.A., Gleich, G.J., Pretolani, M., 2006. Eosinophil-derived cationic proteins activate the synthesis of remodeling factors by airway epithelial cells. J Immunol 177, 4861-4869.

Ptasznik, A., Traynor-Kaplan, A., Bokoch, G.M., 1995. G protein-coupled chemoattractant receptors regulate Lyn tyrosine kinase.Shc adapter protein signaling complexes. J Biol Chem 270, 19969-19973.

Santos, F.P., Kantarjian, H., Cortes, J., Quintas-Cardama, A., 2010. Bafetinib, a dual Bcr-Abl/Lyn tyrosine kinase inhibitor for the potential treatment of leukemia. Curr Opin Investig Drugs 11, 1450-1465.

Schweizer, R.C., van Kessel-Welmers, B.A., Warringa, R.A., Maikoe, T., Raaijmakers, J.A., Lammers, J.W., Koenderman, L., 1996. Mechanisms involved in eosinophil migration. Platelet-activating factor-induced chemotaxis and interleukin-5-induced chemokinesis are mediated by different signals. J Leukoc Biol 59, 347-356.

Stafford, S., Lowell, C., Sur, S., Alam, R., 2002. Lyn tyrosine kinase is important for IL-5-stimulated eosinophil differentiation. J Immunol 168, 1978-1983.

Svensson, L., Redvall, E., Bjorn, C., Karlsson, J., Bergin, A.M., Rabiet, M.J., Dahlgren, C., Wenneras, C., 2007. House dust mite allergen activates human eosinophils via formyl peptide receptor and formyl peptide receptor-like 1. Eur J Immunol 37, 1966-1977.

Svensson, L., Redvall, E., Johnsson, M., Stenfeldt, A.L., Dahlgren, C., Wenneras, C., 2009. Interplay between signaling via the formyl peptide receptor (FPR) and chemokine receptor 3 (CCR3) in human eosinophils. J Leukoc Biol 86, 327-336.

Tomkowicz, B., Lee, C., Ravyn, V., Cheung, R., Ptasznik, A., Collman, R.G., 2006. The Src kinase Lyn is required for CCR5 signaling in response to MIP-1beta and R5 HIV-1 gp120 in human macrophages. Blood 108, 1145-1150.

van der Bruggen, T., Kok, P.T., Raaijmakers, J.A., Verhoeven, A.J., Kessels, R.G., Lammers, J.W., Koenderman, L., 1993. Cytokine priming of the respiratory burst in human eosinophils is Ca2+ independent and accompanied by induction of tyrosine kinase activity. J Leukoc Biol 53, 347-353.

Wegmann, M., 2011. Targeting eosinophil biology in asthma therapy. Am J Respir Cell Mol Biol 45, 667-674.

Wong, W.S., 2005. Inhibitors of the tyrosine kinase signaling cascade for asthma. Curr Opin Pharmacol 5, 264-271.

Yamamura, K., Adachi, T., Masuda, T., Kojima, Y., Hara, A., Toda, T., Nagase, H., Ohta, K., 2009. Intracellular protein phosphorylation in eosinophils and the functional relevance in cytokine production. Int Arch Allergy Immunol 149 Suppl 1, 45-50.

Yan, S.R., Byers, D.M., Bortolussi, R., 2004. Role of protein tyrosine kinase p53/56lyn in diminished lipopolysaccharide priming of formylmethionylleucyl- phenylalanine-induced superoxide production in human newborn neutrophils. Infect Immun 72, 6455-6462.

Zardini, D.M., Bueb, J.L., Tschirhart, E.J., 1999. Release of O2- by human umbilical cord blood-derived eosinophils: role of intra- and extracellular calcium. Cell Calcium 25, 381-389.

Zhu, X., Boetticher, E., Wang, L., Duan, Y., Learoyd, J., Leff, A.R., 2008. Proline-rich tyrosine kinase 2 regulates spreading and migration of eosinophils after beta2-integrin adhesion. Am J Respir Cell Mol Biol 39, 263-269.

Zhu, X., Sano, H., Kim, K.P., Sano, A., Boetticher, E., Munoz, N.M., Cho, W., Leff, A.R., 2001. Role of mitogen-activated protein kinase-mediated cytosolic phospholipase A2 activation in arachidonic acid metabolism in human eosinophils. J Immunol 167, 461-468.

Zhu, Y., Bertics, P.J., 2011. Chemoattractant-induced signaling via the Ras-ERK and PI3K-Akt networks, along with leukotriene C4 release, is dependent on the tyrosine kinase Lyn in IL-5- and IL-3-primed human blood eosinophils. J Immunol 186, 516-526.

Zigmond, S.H., Hirsch, J.G., 1973. Leukocyte locomotion and chemotaxis. New methods for evaluation, and demonstration of a cell-derived chemotactic factor. J Exp Med 137, 387-410.

Figure legends

Fig. 1. Inhibitory effects of bafetinib, tyrphostin and genistein on the superoxide anion generation evoked by (A) *N*-formyl-Met-Leu-Phe (fMLF; 100nM) and (B) IL-5 (20ng/ml) in isolated human eosinophils as indicated. Data are mean±SEM of five independent experiments.

Fig. 2. The effects of bafetinib, tyrphostin and genistein on eosinophil peroxidise (EPO) release triggered by *N*-formyl-Met-Leu-Phe (fMLF) and IL-5 in isolated human eosinophils. The concentration-response curve for the inhibitory effects of bafetinib, tyrphostin and genistein following fMLF (A) or IL-5 (C) stimulation is shown. Data are mean±SEM of four to five independent experiments for each drug concentration. **P*<0.05 compared with control values; #*P*<0.05 from fMLF or IL-5 alone.

Fig. 3. Effects of bafetinib, tyrphostin and genistein on the generation of leukotriene C4 (LTC_4; panel A and C) and eosinophil cationic protein (ECP; panel B and D) by isolated human eosinophils stimulated with (A, B) *N*-formyl-Met-Leu-Phe (fMLF) or (C, D) IL-5. Data are mean±SEM of four independent experiments. **P*<0.05 compared with control values; #*P*<0.05 from fMLF or IL-5 alone.

Fig. 4. (A, B) Effects of bafetinib, tyrphostin and genistein on apoptosis assessed as annexin V positive at 48 hours of culture of isolated human eosinofils. Eosinophil apoptosis was examined in the absence (A) and in the presence (B) of granulocyte-

monocyte colony-stimulating factor (GM-CSF) as indicated. Columns are mean±SEM of five independent experiments. *P<0.05 compared with GM-CSF stimulus.

(C-F) Representative flow cytometer showing annexin V staining (x-axis) and propidium iodide (PI) staining (y-axis) to assess apoptosis of human eosinophils at 48h of culture in the absence (C and D) and presence (E and F) of GM-CSF (1ng/ml), and in the absence (C and E) or presence (D and F) of bafetinib 100Nm. Viable non apoptotic eosinophils were quantified as the percentage of total population of cells that were negative for both annexin V and PI staining. Early apoptotic cells were annexin V positive and PI negative. Late apoptotic cells were annexin V and PI positive. The numbers represent the percentage of cells in each quadrant.

Fig. 5. Effects of bafetinib, tyrphostin and genistein on the migration of isolated human eosinophils exposed to a gradient of *N*-formyl-Met-Leu-Phe (fMLF; 100nM). Data are mean±SEM of five independent experiments. *P<0.05 compared with control values; #P<0.05 from fMLF alone.

Fig. 6. A) Effects of bafetinib (Baf), tyrphostin (Tyr) and genistein (Gen) on the phosphorylation of residues of global protein tyrosine kinases induced by *N*-formyl-Met-Leu-Phe (fMLF; 100nM) in isolated human eosinophils. B and D) Effects of bafetinib (Baf), tyrphostin (Tyr) and genistein (Gen) on the phosphorylation of Lyn tyrosin kinases p56/p53 induced by *N*-formyl-Met-Leu-Phe (fMLF; 100nM) in isolated human eosinophils. Pictures are representative of four different experiments.

Fig. 7. Effects of bafetinib (Baf), tyrphostin (Tyr) and genistein (Gen) on the phosphorylation of ERK1/2 and p38 proteins induced by *N*-formyl-Met-Leu-Phe (fMLF; 100nM) in isolated human eosinophils. β-actin protein staining was performed as housekeeping to ensure equal protein loading. Pictures are representative of four different experiments.

***Response to Reviewers**

The authors would like to express their gratitude for the work carried out by the referees in reviewing this manuscript. The revised manuscript has taken into account all the comments and criticisms raised by the reviewers thus improving, to our view, the quality of the revised version.

To ease the review process, in our reply, the reviewer's comments are pasted just preceding the corresponding replies, as indicated. Also, the main changes introduced in the revised manuscript are highlighted in yellow.

Figure 1
Click here to download high resolution image

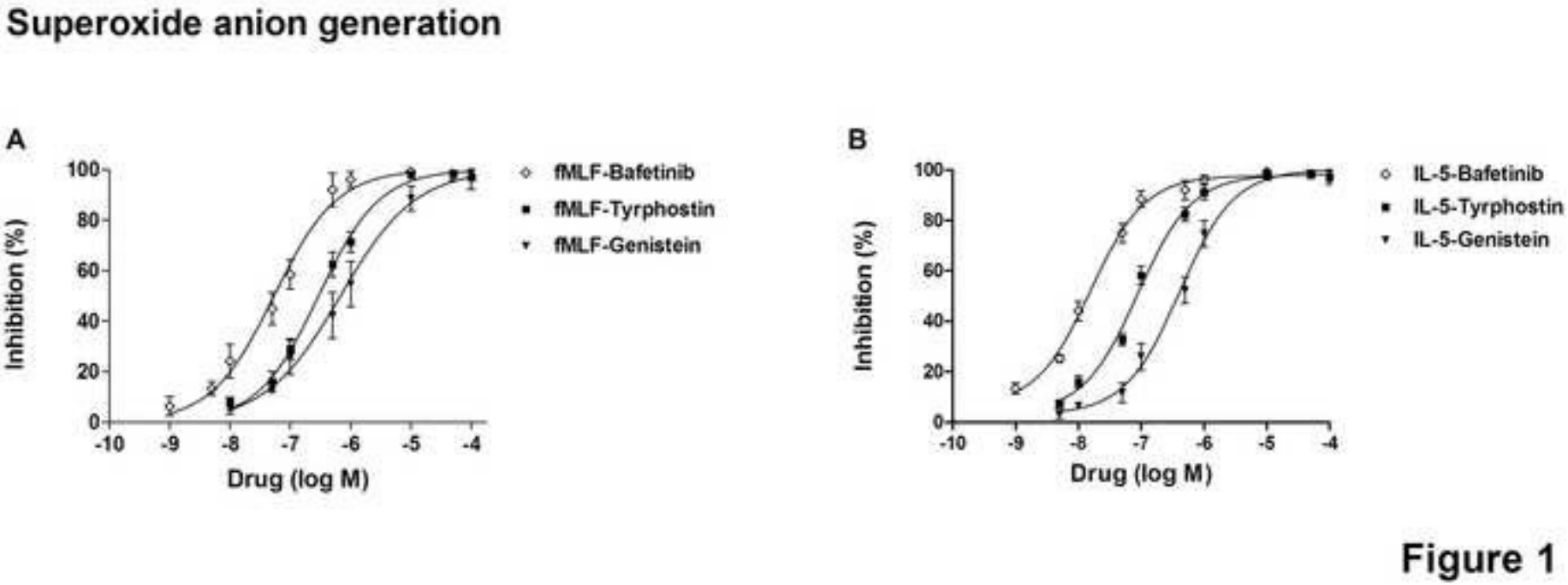

Figure 1

Figure 2

Click here to download high resolution image

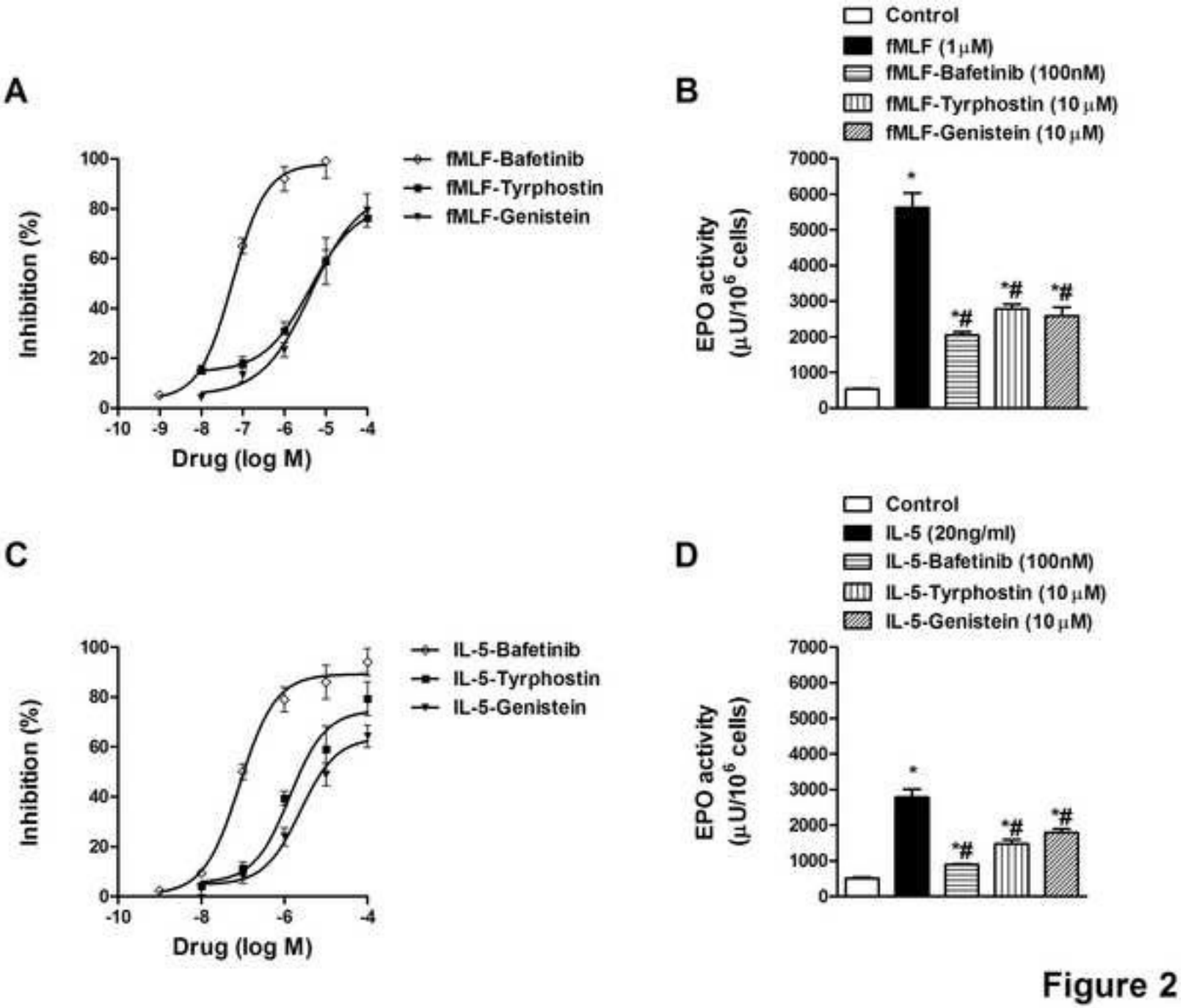

Figure 2

Figure 3

Click here to download high resolution image

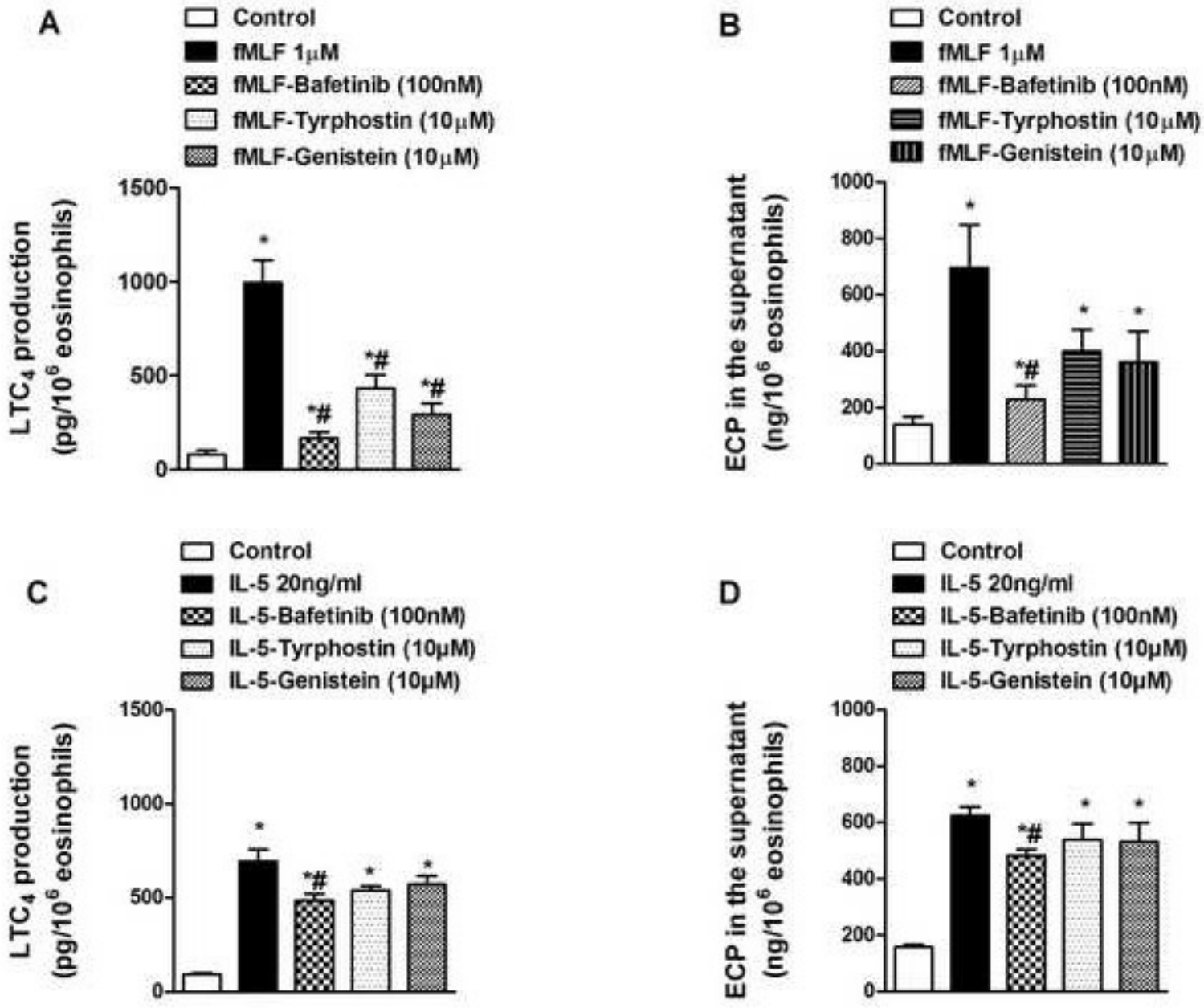

Figure 3

Figure 4
Click here to download high resolution image

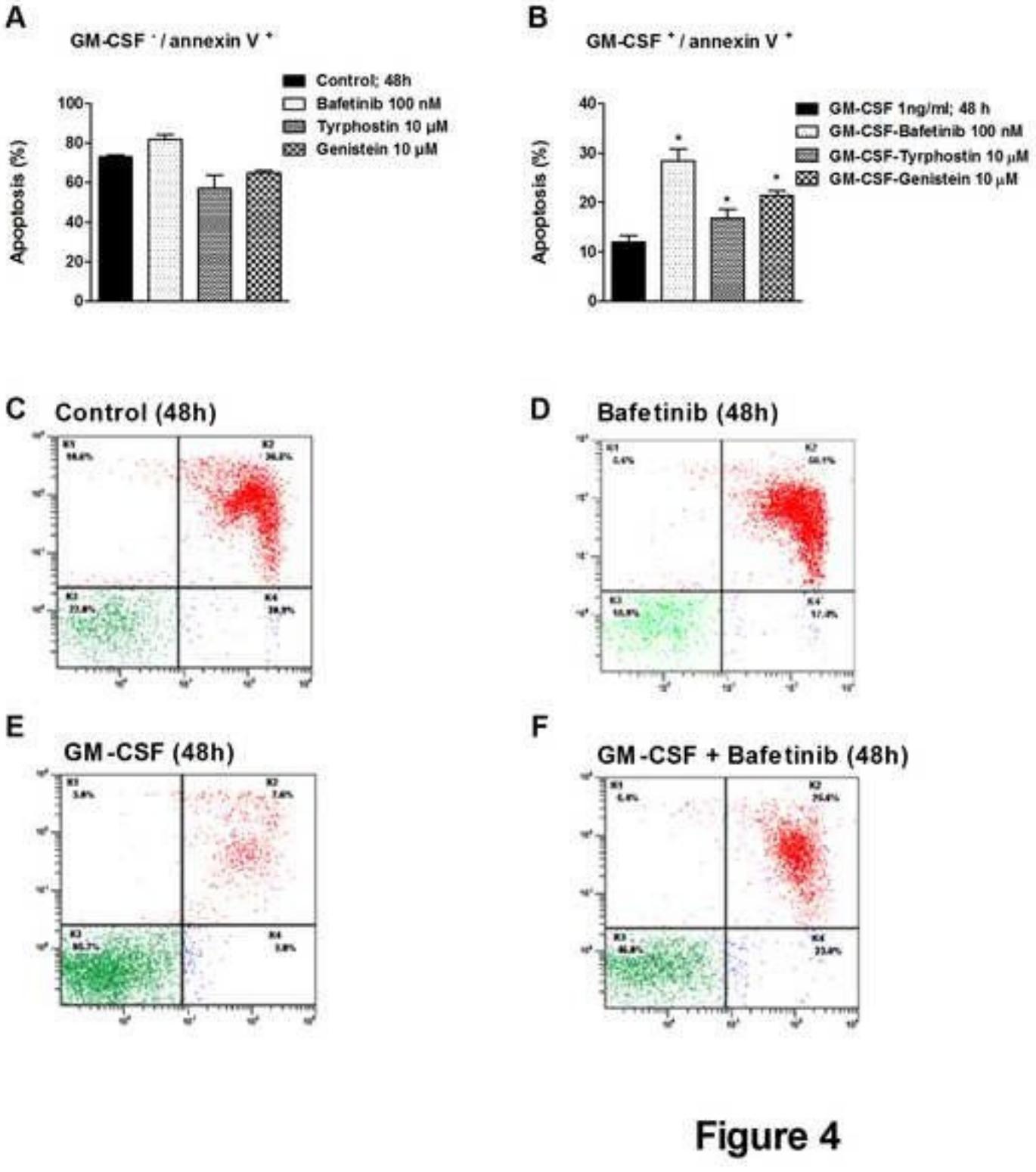

Figure 4

Figure 5
Click here to download high resolution image

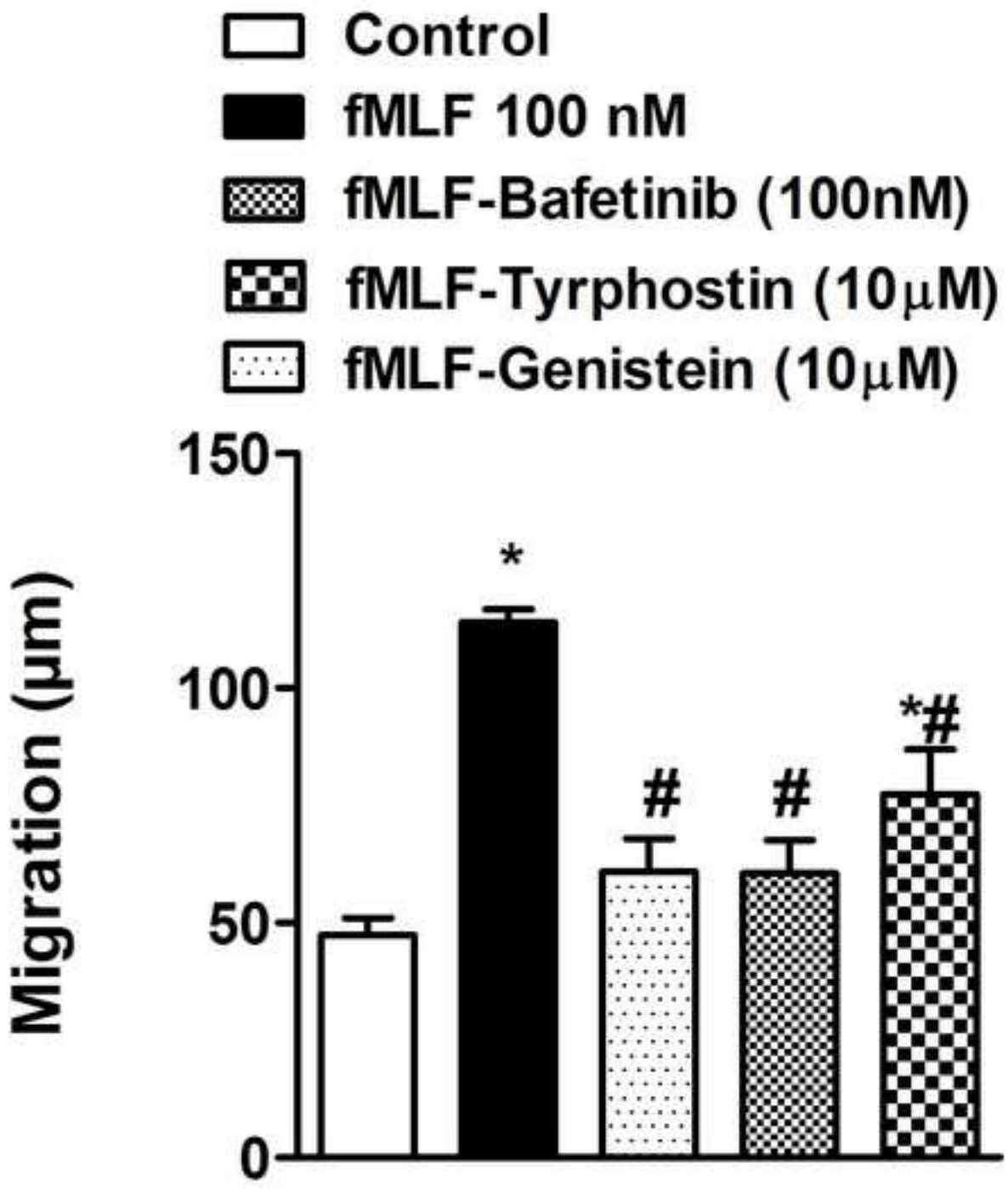

Figure 5

Figure 6
Click here to download high resolution image

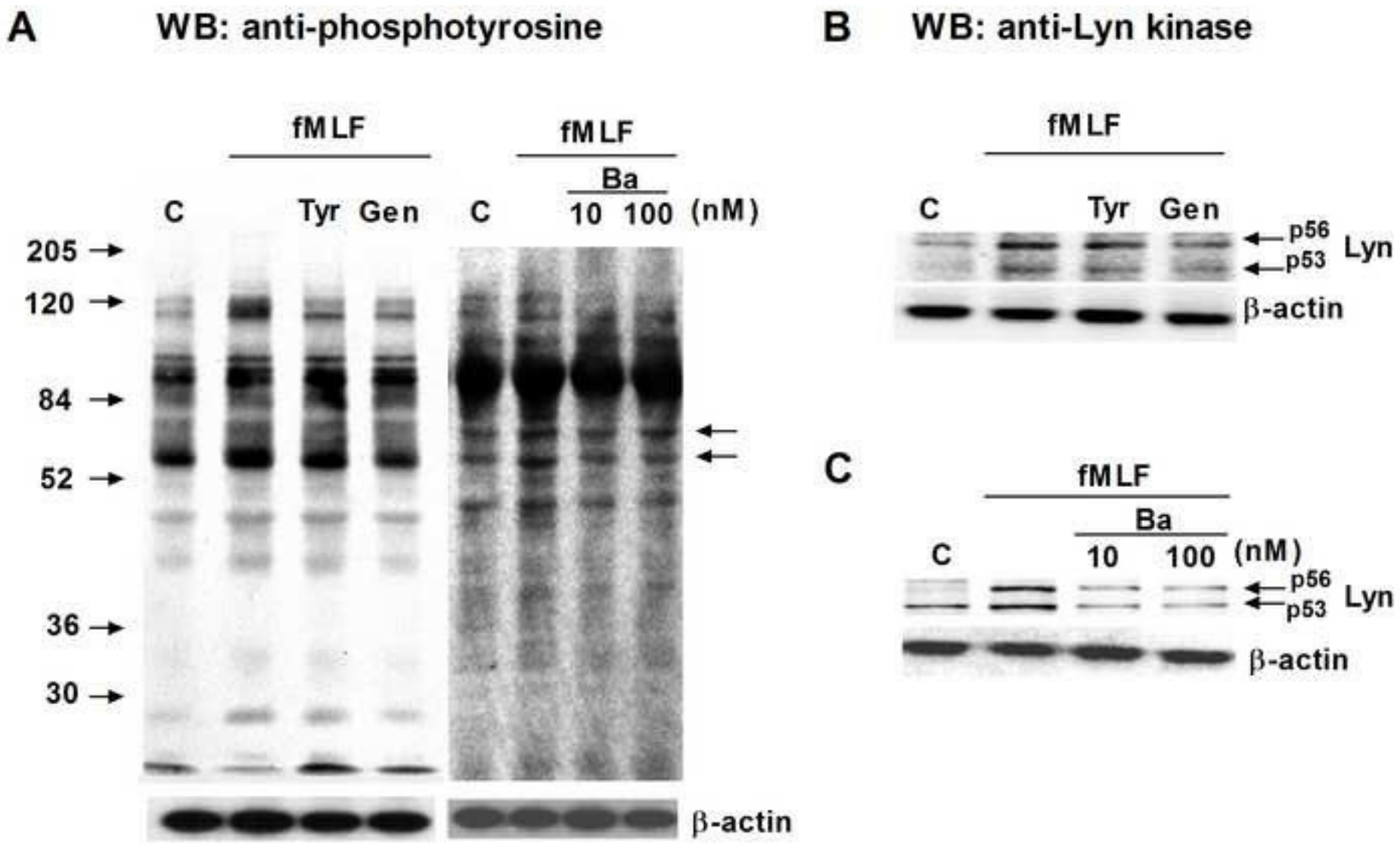

Figure 6

Figure 7
Click here to download high resolution image

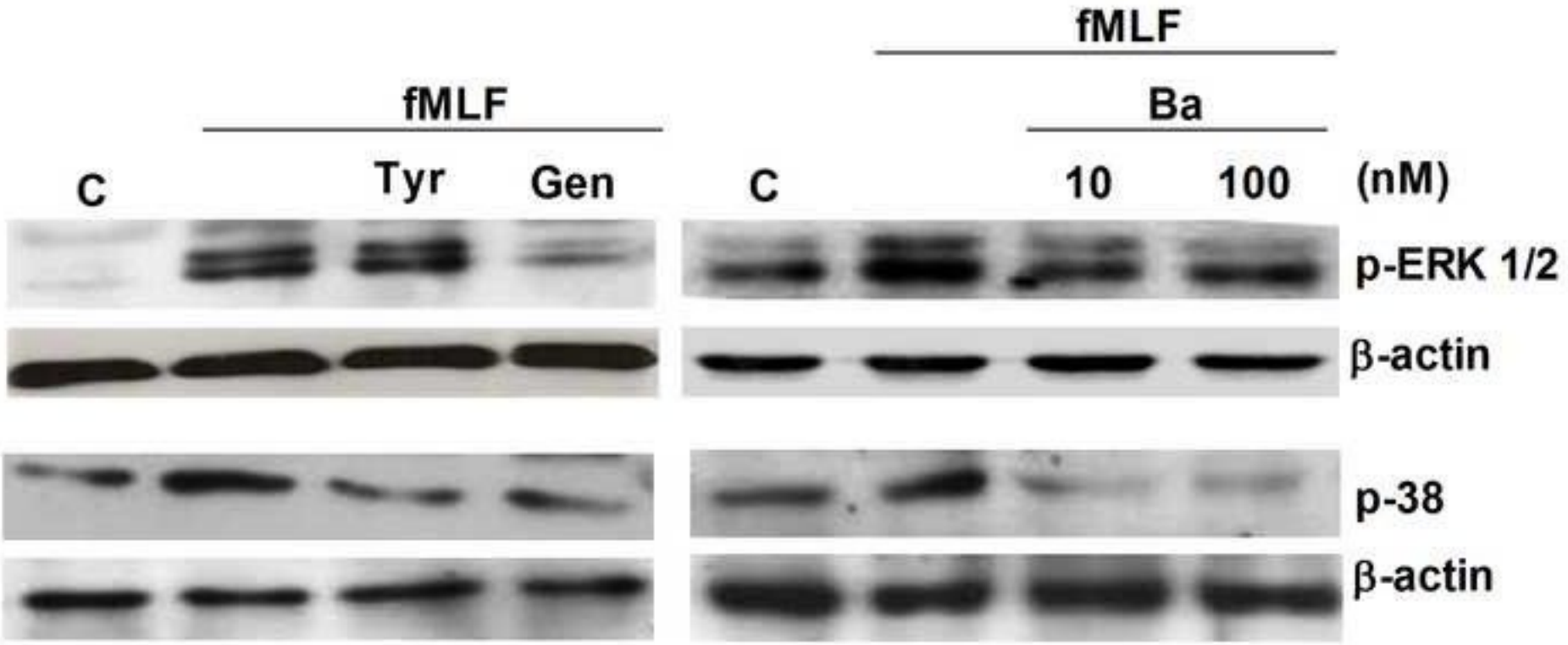

Figure 7

Tables

Table 1. Effect of bafetinib, tyrphostin and genistein on the intracellular calcium ($[Ca^{2+}]_i$) response elicited by fMLF (100 nM) in human eosinophils

	n	Baseline value (nM)	Δ (nM)	$AUC_{0\text{-}60s}$ (nM s)
Control	**5**	**136±12**	**261±34**	**7742±589**
Bafetinib (100 nM)	**5**	**153±21**	**258±46**	**6941±653**
Tyrphostin (10 µM)	**5**	**142±13**	**277±25**	**8321±921**
Genistein (10 µM)	**5**	**148±15**	**271±36**	**7932±737**

Data are mean±SEM of *n* independent experiments. Differences among groups by ANOVA were not significant (P>0.05).
The responses are assessed as the increase of $[Ca^{2+}]_i$ (Δ; peak value minus baseline), and the area under the curve in the interval 0-60s ($AUC_{0\text{-}60s}$).
fMLF, *N*-formyl-Met-Leu-Phe.