Investigation of tyrosine kinase signaling pathways in neutrophils and in experimental autoimmune diseases

Ph.D. thesis

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INTRODUCTION

Neutrophils are short-lived, terminally differentiated innate immune cells that form the biggest population of circulating leukocytes. They are crucial elements of host defense against different microorganisms. This process is mediated by a complicated chain of events that consists of the recognition of the pathogen and the inflamed tissue, the migration to the site of inflammation, the extravasation and the elimination of the dangerous microorganisms.

Neutrophils express a wide range of cell surface receptors for exerting their physiological functions. They use their Fc receptor for binding immunoglobulin-coated particles, they get close contact with the endothelium by selectins and integrins, while their extravasation and tissue migration is also mediated by these latter molecules. Neutrophils also express G protein-coupled receptors (like those that recognize formyl peptides, other chemoattractants or chemokines), various cytokine receptors and different kinds of pattern recognition receptors (like Toll-like receptors (TLR) or C type lectins).

Signal transduction pathways downstream of these receptors generally use kinase cascades to mediate neutrophil effector responses (e.g. migration, granule content release, superoxide production or phagocytosis). Our workgroup previously published that Src family tyrosine kinases and Syk are essential in some neutrophil functions, that raised the possibility of identifying inhibitors with potential anti-inflammatory property by a high throughput screening on neutrophil functions using a validated small molecule kinase inhibitor library.

One of the most potent inhibitor in our *in vitro* screening was the anti-leukemic agent dasatinib that has a dual specificity for Abl and Src kinases. According literature data, dasatinib can not only block the growth of tumor cells, but it also has a robust inhibitory effect on the functions of various mature hematopoietic cells, like T cells, basophils or NK cells. So far, the effect of dasatinib has not been investigated on the most abundant circulating leukocyte

type neutrophils. During the second part of my Ph.D. work, I analysed the effect of dasatinib on mature human peripheral neutrophils.

While the close collaboration between innate and adaptive immunity promotes the elimination of and the defense against danger elements threatening the host, the uncontrolled activity of these sytems can result in the breakdown of immune tolerance leading to autoimmune inflammation. Autoimmune arthritides represent one of the biggest group of autoimmune diseases. Genetic and pharmacological studies have revealed that tyrosine kinases are essential in the pathogenesis of these arthritides and that neutrophils are crucial members in the effector phase of such disorders. Based on these statements, it was logical that if dasatinib have an inhibitory effect on neutrophil functions, then we should test the impact of this inhibitor in neutrophil-mediated, autoantibody-induced experimental inflammation. Next, we tried to identify the role of the main dasatinib target Src family kinases Hck, Fgr and Lyn in the immune complex-based reverse passive Arthus reaction.

Neutrophils require integrins, Src family kinases and small G proteins for some of their functions. This raised the possibility that the main Src substrate GTP-ase activating protein p190RhoGAP (in non-myeloid cells) can potentially participate in some neutrophil functions. As the fifth part of my work, I studied the role of p190RhoGAP in integrin-independent neutrophil degranulation.

As a Ph.D. student I investigated the signaling pathways of neutrophils with special emphasis on tyrosine kinase cascades. A major part of my work was based on the effect of the tyrosine kinase inhibitor dasatinib on mature human neutrophils and on the development of experimental autoimmune diseases.

A better understanding on the signaling events of neutrophils help us to improve our knowledge on the pathogenesis of inflammation and can identify potential future target molecules in the therapy of autoimmune disorders.

OBJECTIVES

During my Ph.D. work, I was working on the following projects:

1. Using a relatively high throughput screening of a focused small-molecule kinase inhibitor library on neutrophil cell functions.

2. Analyzing the effects of the tyrosine kinase inhibitor dasatinib on mature human neutrophils *in vitro*.

3. Testing the effect of dasatinib on experimental autoantibody-induced disease models (in the K/BxN serum transfer arthritis model and in the reverse passive Arthus reaction).

4. Investigating the role of the myeloid-specific Src-family tyrosine kinases (namely Hck, Fgr, Lyn) in Arthus reaction.

5. Studying the role of p190RhoGAP in integrin-independent gelatinase degranulation of mouse neutrophils.

METHODS

The kinase inhibitor library and inhibitor pretreatment

The kinase inhibitors used for the high throughput screening came from the around 30 000 molecules of the hierarchical chemical library (called the Nested Chemical Library^{TM}) of Vichem Chemie Ltd..

For the screening assay, the inhibitors were used at 10 μM concentration, while the detailed analysis with dasatinib was performed at the 10 nM-1 μM concentration range. Dimethyl sulfoxide (DMSO) treated cells served as controls. Preincubation lasted for 30 minutes at 37 °C in a magnesium-free medium. The quality of the screening assay was assessed by Z factor analysis.

Mice and bone marrow chimeras

For the investigation of the role of p190RhoGAP in integrin-independent degranulation, we used p190RhoGAP-deficient (*Grlf1*$^{tm2JSet/\ tm2JSet}$) bone marrow chimeras. As a reference point, CD18 knockout mice (*Itgb2*$^{tm2Bay/\ tm2Bay}$) were applied. In order to test the potential role of the neutrophil-expressed Src family kinase members Hck, Fgr and Lyn, animals lacking all three kinases were used (*Hck*$^{tm1Hev/tm1Hev}$/*Fgr*$^{tm1Hev/tm1Hev}$/*Lyn*$^{tm1Sor/tm1Sor}$). Wild type animals (or wild type bone marrow chimeras receiving wild type bone marrows) on the same genetic (C57BL/6) background served as controls.

Isolation of neutrophils

Human neutrophils were isolated from the peripheral blood of healthy volunteers after dextran sedimentation of red blood cells, followed by a gradient centrifugation with Ficoll. Mouse neutrophils were isolated from the bone marrow. Preparation was carried out at room temperature in an endotoxin-, calcium- and magnesium-free medium.

For the functional assays, the medium was supplemented with calcium chloride and magnesium chloride (this latter was left out in the case of the suspension assays). Functional responses were done at 37 °C.

Detection of neutrophil cell responses

Activation of neutrophils

Adhesion-dependent processes were performed by plating the cells on fibrinogen in the presence of an inflammatory mediator (e.g. Tumor necrosis factor α (TNFα), lipopolysaccharide (LPS), formyl-methionyl-leucyl-phenylalanine (fMLP). Immobilized immune complexes were formed by human serum albumin and anti-albumin antibodies or by lactoferrin and anti-lactoferrin antibodies. Adhesion-independent cell activation was assessed under megnesium-free conditions.

Detection of neutrophil cell responses

Superoxide release was measured by the reduction of cytochrome c or by a luminometric method. Gelatinase degranulation was detected by in gel zymography, lactoferrin release was measured by sandwich ELISA, cell spreading was followed by phase contrast microscopy, while cell adhesion was evaluated by the acid phosphatase assay. Migration was tested in Transwell chambers. Bacterial (*Escherischia coli* or *Staphylococcus aureus*) killing by neutrophils was measured by a bacterial survival assay after incubating neutrophils with the indicated concentration of bacteria.

Intracellular signaling events were visualized by Western blotting.

Adhesion of human and murine leukocytes in whole serum

Unfractionated dasatinib-pretreated leukocyte-rich plasma from the peripheral blood of healthy volunteers or peripheral leukocytes from mice receiving dasatinib at different doses (at 2, 5 or 10 mg/kg) were used after red

blood cell sedimentation with dextran. Adhesion was quantified by the acid phophatase assay.

Generation of autoantibody-induced inflammation in experimental mice

K/BxN serum transfer arthritis was assessed by the peritoneal injection of the serum to control or dasatinib-treated mice. The consequent inflammation was followed for 14 days; articular dysfunction was detected by a functional assay. *Reverse passive Arthus reaction* was carried out by systemic administration of ovalbumin after local anti-ovalbumin injection into the ear tissue. Local ear inflammation was evaluated compared to the control-treated other ear by a NanoSPECT/CT equipment on the basis of radioactivity. For these experiments, wild type and $Hck^{-/-}/Fgr^{-/-}/Lyn^{-/-}$ or control and dasatinib-treated mice were used.

Presentation and interpretation of data

All experiments were performed by at least three independent times. According to the experimental setup, data were subjected to Student's t test or to two-way repeated measures analysis of variance (ANOVA), followed by Tukey's post-hoc test. Statistical evaluation was carried out by the STATISTICA programme. $P < 0.05$ was considered statistically significant.

RESULTS

High throughput screening of a kinase inhibitor library on human neutrophil superoxide release

In our screening assays, we used the inhibitors at 10 μM concentration and investigated their effect on neutrophil superoxide release triggered by two physiological (Fc receptor and integrin-dependent) and one non-physiological (phorbol myristate acetate, PMA) activation systems. Inhibitors were identified as hit molecules if they could dramatically inhibit the physiological activations while leaving the PMA-triggered one intact (which correlated with cell viability and functional capacity). We found several hit molecules among the tested 2417 inhibitors. The majority of the most potent molecules with advantageous pharmacological and patent features could significantly inhibit the Src family kinases. One of the best hit molecule was found to be the clinically used Abl/Src dual specific tyrosine kinase inhibitor dasatinib.

A detailed analysis of the effects of dasatinib on mature human neutrophils *in vitro*

Effects of dasatinib on adhesion-mediated neutrophil functions

Dasatinib could block the adhesion-dependent superoxide release of neutrophils at a very low concentration in the presence of various inflammatory mediators (e.g. TNFα, the complement fragment C5a, different Toll-like receptor agonists) on an integrin-ligand (fibrinogen) surface. The inhibitory concentration 50 (IC50) was below 10 nM in the most sensitive system. Dasatinib inhibited neutrophil spreading, adhesion and lactoferrin degranulation with an IC50 below 50 nM. 100 nM dasatinib had a dramatic effect on the basal and adhesion-dependent whole tyrosine and Syk phosphorylation. While dasatinib could inhibit adhesion-dependent cell responses at a very low concentration, the molecule was relatively uneffective at the 10 nM-100 nM range on adhesion-independent TNF-triggered cell functions.

Effects of dasatinib on immune complex-mediated neutrophil cell responses

In our experiments, dasatinib effectively inhibited the immune complex-mediated spreading, superoxide release, secunder (lactoferrin) and tercier (gelatinase) degranulation processes of mature human neutrophils. The IC50 was below 50 nM at all of these cases. Similar to the adhesion-dependent phosphorylation, 100 nM dasatinib highly decreased both the basal and the Fc receptor-triggered tyrosine phosphorylation of neutrophils and had the same effect on the phosphorylation of the p38 MAP kinase and Syk.

Influence of dasatinib on neutrophil cell responses triggered by G protein-coupled receptors

Dasatinib could partially inhibit the bacterial tripeptide fMLP-triggered superoxide release and lactoferrin degranulation. In contrast with this effect, dasatinib did not influence (not in the highest applied concentration, at 1 μM) the gelatinase release and the phosphorylation of the p38 MAP kinase and ERK in neutrophils when stimulated by fMLP, interleukin 8, C5a or leukotriene B4.

Dasatinib and neutrophil migration

Even the highest dose of dasatinib did not have an influence on neutrophil cell migration in the Transwell chamber towards the chemoattractant bacterial tripeptide fMLP or interleukin 8. Migration was also not affected when the Transwell insert was coated with a complex extracellular matrix barrier (Matrigel).

Dasatinib and innate immune receptor-mediated neutrophil functions

Dasatinib inhibited the non-opsonized and heat-inactivated zymosan-mediated cell responses, but this effect was much more modest if the opsonization happened in the presence of the complement system (when

opsonization was carried out with normal human serum). Moreover, dasatinib could partially decrease Toll-like receptor-triggered neutrophil functions.

Effects of dasatinib on bacterial killing

Dasatinib-pretreated neutrophils had a slight defect in direct bacterial killing. This effect was particularly seen in the highest concentration used.

Effects on human leukocyte adhesion in the presence of whole serum

Dasatinib inhibited the adhesion capacity of human leukocytes in the presence of whole serum with an IC50 (200 nM) that fitted in the concentration range reached in dasatinib-treated patients.

Effects of dasatinib on ex vivo adhesion of murine leukocytes

Per os dasatinib-treatment could effectively decrease the *ex vivo* adhesion of unfractionated leukocytes from experimental mice with an IC50 of 4.6 mg/kg.

Dasatinib and autoantibody-induced experimental diseases

Effects on an experimental autoimmune arthritis model

Dasatinib decreased the signs of the autoantibody-induced K/BxN serum transfer arthritis in a dose-dependent manner. Preventive 50 mg/kg dasatinib-dose given twice a day significantly reduced the morphological alterations of the experimental arthritis and decreased the succeeding articular dysfunction. Furthermore, dasatinib could not only influence the development of arthritis, but it could also slow down the progression of inflammation.

Effects on the reverse passive Arthus reaction

We found that 50 mg/kg dasatinib significantly reduced the extent of inflammation in the reverse passive Arthus reaction compared to control-treated animals.

The role of Src family kinases in the reverse passive Arthus reaction

Mice with the genetic deficiency of the myeloid Src family kinases Hck, Fgr and Lyn showed a dramatically decreased reverse passive Arthus reaction compared to wild type animals.

The role of p190RhoGAP in integrin-independent degranulation of mouse neutrophils

Our results showed that the inflammatory mediator TNFα was able to trigger neutrophil gelatinase release alone, in a β_2 integrin-independent manner. Based on this observation, we performed our following experiments in cell suspensions without the presence of magnesium. The genetic deficiency of p190RhoGAP did not influence the gelatinase release of mouse neutrophils upon activation with TNFα, G protein-coupled and Toll-like receptor agonists or when stimulated with PMA. Neutrophil Fc receptor-mediated gelatinase degranulation was also found to be p190RhoGAP-independent.

CONCLUSIONS

According to the aims, we sum up our conclusions in five points:

1. During the screening of the focused, small molecule kinase inhibitor library, approximately 40 molecules were identified as potent agents which could dramatically reduce the physiological activity of neutrophils out of the 2417 tested molecules. The majority of the 40 inhibitors could significantly inhibit the Src family kinases. Our results suggest that by blocking neutrophil Src family kinases, a potential anti-inflammatory response may be achieved.

2. The Abl/Src dual specific tyrosine kinase inhibitor dasatinib had a dramatic inhibitory effect on neutrophil integrin- and Fc receptor-mediated cell responses. On the contrary, dasatinib had no or only partial effect on G protein-coupled receptor- and Pattern recognition receptor-triggered neutrophil functions.

3. Dasatinib could inhibit the signs of inflammation in neutrophil-mediated experimental animal models. This raises the possibility that dasatinib-related molecules may have therapeutic potential in autoimmune disorders characterized by a neutrophil overactivation.

4. The reverse passive Arthus reaction was significantly decreased in the genetic deficiency of the myeloid-specific Src family kinases Hck, Fgr and Lyn. With other related results of our workgroup, this observation refers to the essential role of the Src family kinases in immune complex-mediated inflammatory processes.

5. Our results showed that p190RhoGAP did not play an indispensable role in β_2 integrin-independent degranulation in mouse neutrophils.

LIST OF PUBLICATIONS

The Ph.D. thesis is based on the following publications and manuscript:

Németh T, **Futosi K**, Hably Cs, Brouns MR, Jakob SM, Kovács M, Kertész Zs, Walzog Settleman BJ, Mócsai A. Neutrophil functions and autoimmune arthritis in the absence of p190RhoGAP: Generation and analysis of a novel null mutation in mice. *J Immunol 2010;* 185:3064-3075. IF: 5,745

Futosi K, Németh T, Pick R, Vántus T, Walzog B, Mócsai A. Dasatinib inhibits proinflammatory functions of mature human neutrophils. *Blood 2012;* 119:4981-4991. IF: 9,06

Futosi K, Fodor Sz, Mócsai A. Neutrophil cell surface receptors and their intracellular signal transduction pathways. *Int Immunopharmacol 2013;* 17:638-50., Review. IF: 2,417

Kovács M, Németh T, Jakus Z, Sitaru C, Simon E, **Futosi K**, Botz B, Helyes Zs, Lowell CA, Mócsai A. Hck, Fgr and Lyn are critical for the generation of the in vivo inflammatory milieu without a direct role in leukocyte recruitment *(manuscript under review)*

Other publications:

Kenessey I, Simon E, **Futosi K**, Bereczky B, Kiss A, Erdödi F, Gallagher JT, Tímár J, Tóvári J. Antimigratory and antimetastatic effect of heparin-derived 4-18 unit oligosaccharides in a preclinical human melanoma metastasis model. *Thromb Haemost. 2009;* 102(6):1265-73. IF: 4,451

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Jani PK, Kajdácsi E, Megyeri M, Dobó J, Doleschall Z, **Futosi K**, Tímár CsI, Mócsai A, Makó V, Gál P, Cervenak L. MASP-1 induces a unique cytokine pattern in endothelial cells: a novel link between complement system and neutrophil granulocytes. *PLoS One. 2014;* 29;9(1):e87104. IF: 3,73

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