

Univerzita Karlova v Praze

Přírodovědecká Fakulta

Studijní program: Vývojová a buněčná biologie



Mgr. Adam Obr

Adhesion structures of leukemic cells and their regulation by Src family kinases

Adhezní struktury leukemických buněk a jejich regulace kinázami rodiny Src

Dizertační práce

Školitelka: RNDr. Kateřina Kuželová, PhD

Ústav hematologie a krevní transfuze

Praha, 2018

Prohlašuji, že jsem dizertační práci vypracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla použita k získání jiného nebo stejného akademického titulu.

V Praze, 6. 3. 2018

Acknowledgement

I would like to thank to all collaborators and colleagues who have contributed to the publications presented in this thesis. Special thanks belong to my supervisor, Dr. Kateřina Kuželová, for her professional guidance, patience, and friendly approach.

I wish also to thank to my wife, Bára, for her help in both professional and personal way.

OBSAH

Abstract	1
Abstrakt	2
Introduction	3
Cell adhesion to the extracellular matrix	3
Adhesion signaling and the adhesion assembly	4
Mechanotransduction and maturation of focal adhesion	6
Hematopoietic cell adhesion	7
Mature hematopoietic cells	7
Immature hematopoietic cells	8
Src family kinases	9
Structure of Src family kinases	10
Src kinases in hematopoietic cells	11
Src kinases in hematopoietic cell adhesion	11
Src kinases in leukemias	12
Electric Cell-substrate Impedance Sensing	14
The aims of the study	16
Results	17
Introduction of the Real-time impedance measurement of cell interactions with extracellular matrix	17
The effect of tyrosine kinase inhibitors, imatinib and dasatinib, on the leukemic cell adhesion	18
Adhesion structures in leukemia cells and their regulation by the Lyn kinase	19
Discussion	22
Conclusions	24
Publication list & contribution	25
References	26
Reprint of publications	37

ABSTRACT

Adhesion signaling is a field of cell biology studied mostly on adherent cell types. However, hematopoietic cells grow in suspension, and use adhesion to the extracellular matrix (ECM) only in their early development, or – in case of differentiated cells – to perform the tasks they are specialized for. Peripheral leukemic cells are derived from more or less immature hematopoietic precursors that have, among other alterations, defects in adhesion to the bone marrow microenvironment. On the other hand, leukemic stem cells (LSC) use adhesion to the bone marrow ECM as a mean to evade chemotherapy, and are a source of the minimal residual disease, and of the disease relapses. Kinases of the Src family (SFK) are known regulators of adhesion signaling in adherent cell types, and their overexpression and/or hyperactivation is often seen in malignant diseases. They are also involved in hematological disease progression and resistance to therapy, particularly in several types of leukemias. In the present work, we used a variety of methods including microimpedance measurement, fluorimetric measurement of adhered cell fraction, immunoblotting, confocal microscopy, and interference reflection microscopy. Our results indicate that active Lyn kinase, a hematopoietic SFK, is present in adhesion structures of leukemic cells as well as in those of adherent cells. In leukemic cells, elevated Lyn activity correlates with loosening the cell-fibronectin interactions. However, Lyn is not necessary for the structure formation, and unlike in adherent cells, the effect of its kinase activity on the structure stability is limited. Given the established role of Lyn in many cellular processes of hematopoietic cells, we assume that its function in adhesion structures is to regulate cell proliferation and survival. Lyn may also affect the adhesion structures in kinase-independent manner.

ABSTRAKT

Adhezní signalizace je v buněčné biologii studována především na adherentních buněčných typech. Hematopoetické buňky se ale většinou vyskytují volně, netvoří mezibuněčné spoje a k extracelulární matici (ECM) adherují pouze v raných stádiích svého vývoje, či – v případě diferenciovaných buněk – při výkonu speciální funkce. Leukemické buňky v periferní krvi (tj. v krevním oběhu) jsou odvozeny od nezralých hematopoetických prekurzorů, u nichž došlo mimo jiné k poruše adheze k mikroprostředí kostní dřeně. Oproti tomu leukemické kmenové buňky (LSC) díky adhezi k ECM kostní dřeně unikají účinkům chemoterapie a jsou zdrojem minimální reziduální nemoci a relapsů. Kinázy rodiny Src (SFK) jsou známými regulátory adhezních drah u adherentních buněk a jejich exprese a/nebo aktivita je často zvýšená u maligních onemocnění. U pevných nádorů zvýšená aktivita SFK koreluje se zvýšenou invazivností nádorů a tvorbou metastáz. SFK hrají roli i při progresi hematologických onemocnění a jejich rezistenci k terapii, především u různých typů leukémií. Z našich výsledků dosažených mimo jiné pomocí měření mikroimpedance, fluorimetrického měření frakce adherovaných buněk, imunoblottingu, konfokální mikroskopie a interferenční reflexní mikroskopie vyplývá, že aktivní kináza Lyn, která patří mezi hematopoetické SFK, je přítomna v adhezních spojkách leukemických i adherentních buněk. U leukemických buněk koreluje zvýšení aktivity Lyn se zeslabením interakcí s fibronectinem. Lyn ani jiné SFK se nepodílejí na tvorbě adhezních struktur leukemických buněk a na rozdíl od adherentních buněk je jejich vliv na stabilitu adheze omezený. Vzhledem k prokázané roli Lyn v buněčných procesech u hematopoetických buněk předpokládáme, že z adhezních struktur přenáší signály vedoucí k regulaci proliferace a přežití buněk, případně se na regulaci adhezních struktur podílí nezávisle na kinázové aktivitě.

INTRODUCTION

CELL ADHESION TO THE EXTRACELLULAR MATRIX

Fundamental cellular processes such as adhesion and migration require precise communication between cells and their environment (Geiger et al., 2001). To reach their site of action, cells in multicellular organisms move not only through the extracellular matrix (ECM), but also on top of each other, between each other, and even through each other. Our knowledge about cell adhesion and migration is mainly based on studies of migration of the adherent cell types. In migrating cells, new adhesions form during the cellular movement when the moving cell extends diverse protrusions towards the desired way (described as the leading edge). The protrusions are controlled by the physical properties of ECM, for example its rigidity, as well as by the cell's reaction to the ECM. The ECM influences directly the signaling pathways leading to the rearrangement of the actin cytoskeleton. The leading edge then engages in new adhesions with the ECM, pulling the cell forward (Ridley, 2011). Several mechanisms of extending the leading edge are described in the literature. Lamellipodia are thin, sheet-like structures at the leading edge of the migrating cell, are capable of extending to long distances and pulling the cell through the tissues (Friedl and Gilmour, 2009). Filopodia are exploratory extensions from the plasma membrane that contain parallel bundles of actin filaments. They are important for neural growth cone guidance and angiogenic blood vessels outgrowth (Gupton and Gertler, 2007; Eilken and Adams, 2010). Both lamellipodia and filopodia are driven by actin polymerization directly pushing the plasma membrane forward. Invadosome is a collective term for an invadopodium (found in invasive cancer cells) and a podosome (the primary type of adhesion structures found in mature hematopoietic cells). Invadosomes are defined as actin-dependent adhesive and/or protrusive structures formed specifically on the ventral surface of highly migratory and invasive cells, like leukocytes, endothelial cells, and transformed tumor cells (Linder, 2009). These protrusions allow for the local degradation of the matrix by secretion of metalloproteases to clear a path for the migrating cell (Buccione, Caldieri and Ayala, 2009). The last type of protrusions, membrane blebbing, has been described to drive directional cell migration during development (Charras and Paluch, 2008). All these types of migrative protrusions can coexist at the leading edge (Diz-Muñoz et al., 2010). All those protrusion types are in some way influenced or created by the actin cytoskeleton, polymerization of which is regulated by a balance of Rho-family GTPase activation and inhibition leading to subsequent specific protein phosphorylation patterns. Even the mRNA localization affects

formation of new adhesion structures, through localized translation of regulatory proteins (Besse and Ephrussi, 2008; Wang et al., 2017).

Adhesion receptor-mediated mechanosensing also regulates the cell behavior. The interplay between the ECM and the cells affects cell spreading (Qiu et al., 2014), contraction (Cariaga-Martínez et al., 2014), migration (Pathak and Kumar, 2012; Paluch, Aspalter and Sixt, 2016), and differentiation (Moore et al., 2011; Wen et al., 2014), as those processes are sensitive to substrate stiffness. This balance also changes in disease (Jaalouk and Lammerding, 2009) or ageing (Snedeker and Gautieri, 2014). On systemic level, the stiffness of the microenvironment is important to the cells of the adaptive immune system, when sensing a matrix-associated antigen (Shaheen et al., 2017). The degree of stiffness of the substrates presenting the antigens efficiently regulates the activation of both B and T lymphocytes (Saitakis et al., 2017). Adhesion of the cells to the ECM was also shown to contribute to tumor cell resistance to the radiation therapy and chemotherapy, as well as to targeted therapies (Eke et al., 2012; Eke and Cordes, 2015). The formation of cell-matrix adhesions and integrin signaling is regulated by mechanical tension mediated by myosin II-generated contractile forces exerted on cell-matrix adhesions. There is no correlation between the adhesion size and the force amplitude being transmitted (Schiller and Fässler, 2013; Oakes and Gardel, 2014).

ADHESION SIGNALING AND THE ADHESION ASSEMBLY

Cells attach to the ECM primarily through a range of integrin-containing adhesions, like focal complexes, focal adhesions, and fibrillar adhesions, and recently identified reticular adhesions (Balaban et al., 2001; Hynes, 2002; Lock, Wehrle-Haller and Stromblad, 2008; Lock et al., 2017). Though structurally and functionally varied, they share a substance of their molecular composition, termed adhesome (Zaidel-Bar and Geiger, 2010; Hytönen and Wehrle-Haller, 2014), suggesting that those adhesions are merely a "continuum" of the same process. Therefore, for the sake of simplicity, the only type of adhesion described in details here will be the focal adhesion.

The formation of a new cell-ECM adhesion starts by activation of the cellular receptors, integrins, and their binding to the ECM proteins. Integrins, transmembrane heterodimers of α and β subunits, can sense chemical and mechanical properties of the ECM (Campbell and Humphries, 2011). Dimerization of those subunits leads to the formation of 24 known unique $\alpha\beta$ combinations with distinct and often overlapping ligand-binding specificities. When

activated by a mechanical load, they undergo a conformation change leading to an increase in their affinity to various intracellular proteins (Geiger and Yamada, 2011; Su et al., 2016; Rahikainen et al., 2017) and cluster together, which probably thermodynamically facilitates the binding of other integrins to the ECM (Reister-Gottfried et al., 2008).

One of the first intracellular events following the integrin clustering is an increase in phosphorylation of Src family kinases (SFKs; Bershadsky, Balaban and Geiger, 2003), either by recruiting the phosphatases capable of dephosphorylating the inhibitory Y⁵²⁷ (Zou, Kitaura, Reeve, Long, Victor L.J. Tybulewicz, et al., 2007) or by direct interaction of the integrin intracellular domain and SFKs (Arias-Salgado et al., 2003). SFKs can also be activated by integrins indirectly, via the focal adhesion kinase (FAK) that upon interaction with the integrins undergoes autophosphorylation on Y³⁹⁷, which creates an SH2-binding site, resulting in SFK recruitment to the site of the nascent adhesion. Upon binding, Src enhances the phosphorylation on Y³⁹⁷ and on several other tyrosine residues on FAK molecule (Cary et al., 2002). This creates a fully active FAK-SFK complex that phosphorylates other proteins, including paxillin and p130^{Cas}. That subsequently leads to modulation of Rac-specific guanosine-exchange factors (GEFs) DOCK180 and β -Pix that are recruited to the emerging focal adhesion (ten Klooster et al., 2006). This activates Rac and promotes membrane ruffling and lamellipodia formation as well as induction of actin polymerization via the WASP/WAVE factors (Welch and Mullins, 2002). The FAK-SFK complex also inhibits the activity of the RhoA GTPase via the GTPase activating protein p190RhoGAP (Bass et al., 2008). Inhibition of the RhoA activity leads to upregulation of the Rac1 activity, which in turn promotes formation of lamellipodia and focal adhesion dynamics (Huveneers and Danen, 2009).

The events following FAK and SFK activation are largely dependent on the cellular context, membrane localization, and compartmentalization of the signaling events (de Diesbach et al., 2008); however it seems that modulation of FAK-SFK signaling does not affect the molecular composition of the assembled adhesion complex (Horton et al., 2016). The FAK-SFK dependent phosphorylation also drives other molecules to the FAs.

Several studies identified that integrin signaling, possibly through FAK, promotes tumorigenesis by maintaining the pool of slow-cycling stem-like cells that are primary targets for oncogenic transformation (Sachs et al., 2012). Integrin signaling has also been identified as the cause or one of the causes of several cancer type resistance to radiotherapy or

chemotherapy (Dickreuter et al., 2017). In some types of cancer, however, agonists of integrin signaling seem to improve the response to therapy (Tolomelli et al., 2017).

Deregulation of c-Src tyrosine kinase activity in tumor cells is associated predominantly with deregulation of cell adhesion and cytoskeletal changes, leading to a motile, more invasive cell phenotype. In solid tumors, c-Src activation leads to a disruption of the cell-cell contacts mediated by E-cadherins that can be restored by the c-Src inhibition (Nam et al., 2002). Elevated c-Src activity has been associated with invasive properties and poor prognosis in several types of cancer, including breast (Reissig et al., 2001), ovarian (Wiener et al., 2003), and colon cancer (Allgayer et al., 2002).

SFK activity is also essential for a focal adhesion turnover (Fincham and Frame, 1998). Elevated c-Src activity is associated with a disruption of the actin stress fibers followed by disassembly of focal adhesions (Fincham, Chudleigh and Frame, 1999; Webb et al., 2004).

MECHANOTRANSDUCTION AND MATURATION OF FOCAL ADHESION

Focal adhesions (FA) are the sites connecting the ECM to the cytoskeleton. The connection provides the physical tension necessary for the adhesion maturation. The cell adhesion to the ECM modulates many cellular responses, like proliferation, differentiation, survival, migration, and organization of the cytoskeleton (Legate, Wickström and Fässler, 2009).

Mechanotransduction is the cellular process of sensing a mechanical stimulus and converting it to a biochemical response. Mechanical stimulus is in this sense represented by mechanical cues of the microenvironment; including not only all components of force, stress, and strain, but also substrate rigidity, topology, and adhesiveness. The signal is transferred by the extracellular receptors through the cytoskeleton to the force-sensing apparatus, subsequently converted into a chemical signal, and finally into a cellular response (Liu, Chen and Zhu, 2015). In our tissues, cells experience numerous mechanical stimuli; for example the shear stress from the blood flow, or stretching and compression forces in a moving muscle. When in extracellular environment, cells are surrounded by the extracellular matrix that is composed of many proteins. The exact composition of the ECM is tissue-specific, the usual components being collagens, vitronectin, laminin, and fibronectin.

Talin, vinculin, and tensin are three structural proteins providing a physical link between integrins and the actin cytoskeleton (Atherton et al., 2015). They are also the most conserved parts of the integrin adhesome (Horton et al., 2015). Their activation depends on

conformational changes caused by mechanical forces (Atherton et al., 2015). When active, talin and vinculin bind each other and the cytoskeleton, leading to the maturation and stabilization of FAs (Humphries et al., 2007). Other molecules contributing to the maturation of FAs are FAK, kindlins, and paxillin. FAK phosphorylation is required for cells to respond to the substrate stiffness during cell spreading (Swaminathan, Alushin and Waterman, 2017). That also means that SFKs are involved in mechanosensing, since they mediate the FAK phosphorylation.

HEMATOPOIETIC CELL ADHESION

Mature hematopoietic cells

Unlike adherent cells, mature cells of hematopoietic origin usually move freely through the organism and use the adhesion to the ECM only in cases of performing specialized functions. For example, leukocytes in the process of diapedesis attach to and migrate on top of endothelial cells lining the blood vessels, before crossing the endothelium between two endothelial cells (paracellular pathway), or transcellularly, by inducing a membrane channel formation through a single endothelial cell. The transcellular pathway has been demonstrated to be used by leukocytes in vivo in the bone marrow, thymus, secondary lymphoid organs and structures, and during pathological conditions, across the blood-brain barrier and the blood-retinal barrier. Several types of integrins are involved in determination of the preference for distinct migration types of leukocytes (Phillipson et al., 2006). During the transcellular migration, leukocytes are using invadosome-like dynamic protrusions (ILPs) (Carman, 2009). Although these leukocyte structures share many features of classical invadosomes, other properties are unique. For example, ILPs has been observed only in cell-cell contact, not in cell-matrix environment. Additionally, it is not yet clear if ILPs are capable of degrading the extracellular matrix (a hallmark of the invadosomes). ILPs can also act as a dynamic mechanosensor, as the lifespan of ILPs seems to be dependent on substrate rigidity and the forces affecting the cells (Collin et al., 2008). Transcellular diapedesis was also documented in certain metastatic tumor cells (Azzali, 2006) and even in stem cells (Laird, von Andrian and Wagers, 2008). Integrin signaling in immune cells is dependent on a SFK-mediated phosphorylation of ITAM (Palacios and Weiss, 2004; Mócsai et al., 2006). Similar observation was made in osteoclasts, where SFKs are necessary for the signaling from $\alpha_v\beta_3$ integrins (Zou, Kitaura, Reeve, Long, Victor L J Tybulewicz, et al., 2007).

In addition to responding to externally applied forces, cells can internally generate and exert forces on their surface receptors, for example by regulation of actin polymerization, retrograde flow, and myosin II contraction. Those mechanisms are recently being shown as an important part of T-cell receptor (TCR) and B-cell receptor (BCR) signaling in immune cells (Bashour et al., 2014; Wan et al., 2015; Hu and Butte, 2016). Forces applied to these receptors has been shown to regulate many aspects of TCR and BCR signaling, including intracellular calcium flux (Pryshchep et al., 2014; Feng et al., 2017), regulation of major histocompatibility complex (MHC) dissociation from the TCR and pre-TCR (Das et al., 2015, 2016; Mallis et al., 2015), and potentiation of cytotoxic killing effect of target cells (Basu et al., 2016). Both T and B cells use mechanical forces to amplify antigen discrimination (Axmann et al., 2012; Natkanski et al., 2013; Das et al., 2016). Unlike receptors for soluble agonists, TCR and BCR bind immobilized ligands and as such are subjected to mechanical forces, and signaling from those receptors can be triggered by force (Upadhyaya, 2017).

Immature hematopoietic cells

Immature hematopoietic cells use adhesion to the ECM during their development in the process of hematopoiesis. The primary locus for hematopoiesis is bone marrow, where the microenvironment facilitates the survival, differentiation and proliferation of hematopoietic stem cells (HSCs). HSCs are a rare subpopulation of hematopoietic cells at the top of the hematopoietic hierarchy. They represent a functionally heterogeneous cell population, the variability being e.g. in their degree of self-renewal (Ema et al., 2005), lifespan (Yang et al., 2005), and differentiation capabilities (Muller-Sieburg et al., 2012). There are probably several subpopulations of HSCs, each one characterized by expression of a specific marker or markers (Kiel et al., 2005; Kent et al., 2009; Benveniste et al., 2010).

The specific bone microenvironment regulating HSCs, commonly referred to as a niche, comprises of several cell types. Exact contribution of those to HSC differentiation is not clear. Each niche has distinct properties inducing the HSC homing, mobilization, self-renewal, or lineage commitment. The properties include the autocrine, paracrine, and endocrine signals as well as direct cell-cell interactions needed for the HSC to self-renew and differentiate into all blood cell lineages.

HSCs in the specific niches are supported by fibroblast-like stromal cells from mesenchymal origin, osteoblasts, and osteoclasts, that secrete soluble factors and extracellular matrix

proteins that are responsible for homing and survival of hematopoietic cells. These molecules, like IL-6 and SCF-1, also serve as homing factors and support for malignant hematopoietic cells as well as for tumor cells metastasized to the bone (typically epithelial tumors). This environment can also provide protection against chemotherapy. The resistance against chemotherapy could be mediated by soluble factors, or by the cell adhesion (Meads, Gatenby and Dalton, 2009). Evidence suggests that there are separate niches for quiescent and proliferating HSCs in the BM (Kunisaki et al., 2013; Acar et al., 2015).

The endosteal niche. In adult hematopoiesis, HSCs reside in a region close to the endosteal bone surface (Gong, 1978). Upon transplantation, HSCs preferentially migrate to this endosteal region in close association with bone-lining cells, at least in mice (Lo Celso et al., 2009). Cells isolated from this region show higher proliferative potential as well as long-term hematopoietic reconstitution potential (Haylock et al., 2007; Grassinger et al., 2010). The main cellular contributors of the endosteal niche are osteoblasts.

The perivascular niche. The perivascular niche is situated near the blood vessels. This is essential for a gas exchange, delivery of nutrients, and waste removal in the BM (Kiel et al., 2005). The perivascular niche is quite heterogenous. It comprises of endothelial cells, leptin receptor perivascular-expressing cells, nestin-expressing mesenchymal cells, and CXCL12-abundant reticular cells. Endothelial cells line the blood vessels that bring oxygen and nutrients to the bone marrow. They regulate HSCs via cell to cell contact and via secreting angiocrine factors (Butler et al., 2010; Kobayashi et al., 2010). Leptine receptor is a well-established marker for MSCs. LepR⁺ perivascular stromal cells are responsible for the trilineage MSCs differentiation into adipocytes, osteoblasts, or chondrocytes in the adult BM (Zhou et al., 2014). Quiescent (dormant) HSCs have been reported to reside in the perivascular niche (Kunisaki et al., 2013).

SRC FAMILY KINASES

Src family kinases (SFKs) are a group of eight related non-receptor tyrosine kinases involved in regulation of many cellular processes, like cell cycle progression, apoptosis, adhesion, and cell migration (Frame, 2004). Five of the kinases (Lyn, Fgr, Hck, Lck, and Blk) are expressed almost exclusively in cells with hematopoietic origin, and the three remaining kinases (c-Src, Yes, Fyn) are expressed ubiquitiously. Additionally, three other kinases – Brk, Frn, and Srm – are often classified as SFKs. However, their sequence similarity to c-Src is not as high as in

the other SFKs. They also lack myristoylation, a post-translational modification that is indispensable in the other eight SFKs.

The founding member of the group, c-Src, was described in 1976 as a cellular variant of a v-SRC oncogene from a Rous sarcoma virus (Stehelin et al., 1976). Overexpression and/or overactivation of the c-Src kinase is commonly observed in tumors. In many of them, c-SRC has been established as a therapeutical target (Rösel et al., 2013).

Structure of Src family kinases

SFKs are modular proteins, consisting of four main domains. The domains, named SH (SRC homology), are shared in all SFKs and are present in many other signaling components in the cell. The N-terminal domain, also called SH4, is unique for each SFK. The SH4 domain contains acylation sites that can be myristoylated and/or palmitoylated. This is important for targeting of the kinase to specific membrane compartments, such as lipid rafts or lysosomal membrane (Jakus et al., 2004; Poincloux et al., 2009). The following part contains the SH3 domain binding polyproline helices (PxxP motif), and an SH2 domain binding phosphorylated tyrosine. These domains are necessary for the kinase autoinhibition, where intramolecular interactions of the SH3 and SH2 domains keep the kinase in its closed, inactive state. At the C-terminus of the SFK sequence, the kinase domain (SH1) and a regulatory tail are localized. In the activation loop of the kinase domain, Y⁴¹⁶ (c-Src) phosphorylation marks an active kinase. Inhibitory tyrosine residue (Y⁵²⁷ in c-SRC) is present at the very end of the C-terminus. Y⁵²⁷, if phosphorylated, is bound by the SH2 domain of the same kinase molecule, and supports its closed conformation. This is further consolidated by binding of the SH3 domain to the linker between SH2 and SH1 domains (Thomas and Brugge, 1997).

Interactions between SH2 and phosphorylated Y⁵²⁷, and between SH3 and the SH2-SH1 linker are rather weak. That allows for an elegant system of the kinase activation, where the bond between the activator proteins and SH2/SH3 domains of the kinase is stronger than the intramolecular bonds in the closed kinase. This results in release of the intramolecular bonds. The kinase is now allowed to switch to an open conformation and exposes the activation loop with Y⁴¹⁶ now available to a phosphorylation. That subsequently results in the stabilization of the open conformation, and the kinase is considered fully active.

Some SFKs are known to be expressed in cells in multiple isoforms. The variants are generated either by the alternative splicing (Lyn), or by two different start codons used for the

translation (Hck). For both Lyn and Hck, differences in a subcellular localization between the isoforms (Möhn et al., 1995; Carréno et al., 2000) and even functional differences (Poincloux et al., 2009) have been proposed.

Src kinases in hematopoietic cells

While functions of ubiquitous SFKs largely overlap (Abram and Lowell, 2007), the expression of the remaining SFKs is restricted to hematopoietic cell types. The more advanced the stage of the hematopoietic cell development, the less the ubiquitous SFKs Src, Yes, and Fyn are expressed and the more hematopoietic cell-specific SFK expression takes over in the cell. It also seems that it is the induction of the hematopoietic SFKs expression what reduces ubiquitous SFKs levels (Amy R. Dwyer et al., 2016).

In macrophages, the SFKs play an important role in outside-in signaling, implicating their role in an environment-mediated regulation of the cell proliferation, survival, apoptosis, phagocytosis, adhesion and migration. All macrophage types express Lyn, Hck, and Fgr at roughly equivalent levels (Abram and Lowell, 2008). Upon activation, macrophages can upregulate their c-Src expression (Boulet et al., 1992; Leu et al., 2006). As the SFKs can act as both positive and negative regulators of certain pathways and partially compensating for each other, dissecting the roles of individual SFKs is rather challenging. Also, SFK expression in macrophages appears to be dependent on their stimulation level (Amy R Dwyer et al., 2016).

Src kinases in hematopoietic cell adhesion

Integrin activation and subsequent SFK activation is important for migration, proliferation, but also for differentiation and survival of leukocytes (Suen et al., 1999; Zamir et al., 1999; Mayadas and Cullere, 2005; Shi and Simon, 2006). SFK activity is also necessary for macrophage migration (Digiacomio et al., 2017). Macrophages are the first responders of the innate immune system, both as tissue-resident cells and as monocytes recruited from the blood flow into the sites of inflammation, where they subsequently differentiate into macrophages. Motile macrophages also promote disease progression in a wide range of conditions, including cancer (Murray and Wynn, 2011). In contrast with Hck and Fgr deficient macrophages, Lyn^{-/-} cells show hyperadhesive phenotype that correlates with reduced migratory properties in vitro. Similarly, neutrophils from Lyn^{-/-} mice show evidence of increased integrin-mediated responses following adhesion (Pereira and Lowell, 2003; Pereira et al., 2004). Hyperactivation of both Hck and Lyn caused by knockout of

phosphatase CD45 leads to the inability of the cell to maintain stable adhesion (Roach et al., 1997). Fgr can also act as a negative regulator of integrin-mediated adhesion in a kinase-independent manner (Vines et al., 2001).

Src kinases in leukemias

In general, leukemias are heterogenous disorders of the hematopoietic progenitor cells. Abnormalities have been identified that affect the balance between cell proliferation, survival, and differentiation. Over the last few years, several studies have concluded that leukemogenesis is a process in which multiple events involving independent genetic alterations in protooncogene or tumor-suppressor genes, together with epigenetic or environmental factors, contribute to the development of the full malignant phenotype. The cooperation of several mutations leads to the emergence of leukemic cells capable of proliferation, but not of differentiation (Gilliland, 2002).

Chronic myeloid leukemia

Philadelphia chromosome (Ph)—positive chronic myeloid leukemia (CML) is characterized by an expression of p210^{Bcr-Abl} fusion kinase (further referred to here as Bcr-Abl) that is a sole oncogenic driver of the disease. Bcr-Abl induced oncogenicity requires SFK activity (Hayette et al., 2011). The Bcr-Abl oncogene phosphorylates and activates c-Src, Lyn, and Hck, and confers hyperproliferative and antiapoptotic advantages (Wilson et al., 2002; Hu et al., 2004; Gamas et al., 2009). Once activated by Bcr-Abl, SFKs appear to carry out a positive feedback to the oncogenic kinase (Pene-Dumitrescu et al., 2008). This results in overactivation of both the SFKs and Bcr-Abl. SFKs can also serve as a signaling intermediate between Bcr-Abl and other factors important in the CML pathology, like STAT5 (Klejman et al., 2002). Rubbi et al. (2011) showed that in Ph⁺ CML cells, most of the hyperphosphorylation is dependent on the SFK activity, and that treatment of the cells with imatinib induces a decrease in the SFK activation. SFK hyperactivation is observed even in CML leukemic stem cells (LSCs; (Palacios and Weiss, 2004; Breccia et al., 2013). Additionally, the overactivation of the SFKs, particularly Lyn and Hck, correlates with the disease progression (Hayette et al., 2011). SFKs connect the Bcr-Abl to a PI3K signaling (Lyn) and STAT5 signaling (Hck), two signaling cascades deregulated in the CML. Knockdown of Fyn restores the CML cell sensitivity to imatinib (Gao et al., 2009).

Acute myeloid leukemia

Acute myeloid leukemia (AML) is the most common myeloid leukemia in adults. Multiple genetic abnormalities are frequently detected in AML, and they usually lead to a deregulation of transcription factors or growth factor receptors (Bourri  et al., 2013). Overexpression of the SFKs, mostly Lyn and Hck, is often observed in AML.

Lyn is the predominant SFK expressed in AML (Roginskaya et al., 1999). Overexpression of Lyn was proposed to be an important factor in the disease development (Saito et al., 2013). Associated with FLT3-internal tandem duplications (ITD) leading to STAT5 activation, it may promote the FLT3-ITD oncogenic signaling found in many AML cases (Okamoto et al., 2007). Lyn also shows an aberrant localization in AML blasts, possibly contributing to its deregulated function (Dos Santos et al., 2008). The transforming potential of Lyn is furthermore illustrated by its fusion to Tel. Tel-Lyn fusion product activates STAT5 independently of JAK2 and promotes leukemogenesis (Ingley, 2012). Recently, Lyn kinase was acknowledged among the targets with the most therapeutic potential in AML (Edwards, Eryildiz and Tyner, 2018).

Fyn interaction with FLT3 was also identified as a factor in progression of AML (Chougule, Kazi and R nnstrand, 2016). More recently, FLT3 was also associated with the cellular transformation induced by the Lck kinase (Marh ll, Kazi and R nnstrand, 2017).

Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults, characterized by the accumulation of malignant B-cells in the blood. Lyn is the most relevant SFK in B-lymphocytes, often hyperactivated and aberrantly expressed (Contri et al., 2005; Hussein et al., 2009). Its substrates including Syk, PI3K, HS1, cortactin, and PP2A are highly activated in CLL (Gattazzo et al., 2014; Zonta et al., 2014, 2015; Martini et al., 2017). Lyn also aberrantly activates STAT3 (Silva, 2004). The presence of Lyn in CLL cells is not necessary for the development of the disease, but Lyn expression in the associated macrophages is needed for the CLL progression in vivo (Nguyen et al., 2016). Recently, a role of Lyn in the CLL apoptotic signaling was proposed, leading to CLL progression (Tibaldi et al., 2017). Inhibition of Lyn in CLL cells leads to their death (Veldurthy et al., 2008). Lyn overexpression is also one of the proposed mechanisms of CLL resistance to the treatment. Overexpression of Lck, normally expressed in T-cells, contributes to B-cell CLL (Talab et al., 2013).

Acute lymphocytic leukemia

Acute lymphocytic leukemia (ALL) is another type of hematological malignancy characteristic by the overexpression of Lyn (Guo et al., 2012; Vázquez-Franco et al., 2012). The role of SFKs seems to be more or less restricted to the Ph⁺ ALL, where p190^{Bcr-Abl} oncogene is expressed. In B-cells, p190^{Bcr-Abl} induces expression and activates Lyn, Fgr, and Hck, and they are all involved in B-ALL transformation (Hu et al., 2004; Fei et al., 2010). Efficient transformation of the B-cells requires at least two of the SFKs. Finally, as in myeloid leukemias, SFKs are involved in ALL LSC signaling (Yamazaki et al., 2011).

ELECTRIC CELL-SUBSTRATE IMPEDANCE SENSING

The electrical cell-substrate impedance sensing, sometimes referred to as electric cell-substrate impedance spectroscopy (ECIS), is a measuring system for quantification of the cell behavior within adherent cell layers. Cells are grown in special culture chambers on top of golden electrodes, where a weak altering current is applied between the electrodes, and the potential is measured. When the cells attach, their membrane restricts the current flow, and changes in the impedance are detected (Giaever and Keese, 1984; Szulcek, Bogaard and van Nieuw Amerongen, 2014).

The advantage of using impedance-measuring devices as opposed to traditional dye- or microscopy- based analysis of cell adhesion, is that the devices measure in a continuous readout, rather than at a single time-point. The applied voltage is small enough so the cells are not affected in any way (Giaever and Keese, 1993).

The impedance changes are dependent on the number, morphology, and size of the cells adhering onto the electrodes, and on the strength of their attachment to the bottom of the well. Measurements are based on the whole cell population rather than on individually selected cells, which is usually an upside, but in some cases the population analysis is flawed, for example when examining transfected cells with low efficiency of transfection.

The device for impedance measurement has been conceived for adherent cell monitoring. Cells growing in suspension usually do not attach to the wells and the impedance signal is very low. We have developed a new application of this method consisting in precoating the wells with an ECM protein which does not represent a negative effect to the measurement (Giaever and Keese, 1993). We have shown that in this case, the impedance signal specifically reflects cell interaction with the coated surface. The assay optimization is

described in the first article this thesis is based on. Subsequently, we used this method for real-time monitoring of the effect of selected inhibitors on leukemia cell adhesion to fibronectin.

THE AIMS OF THE STUDY

The aim of the thesis was to contribute to the clarification of the adhesion processes in leukemic cells, in particular the role of Src family kinases in leukemic cell adhesion signaling. The aims were dissected using specific inhibitors and many methods like confocal microscopy, interference reflection microscopy (IRM), immunoblotting, and real-time monitoring of cell interactions with a coated surface (ECIS).

The individual goals of the thesis were:

- (1) to introduce a non-invasive method for monitoring leukemic cell interaction with fibronectin in real time, and to verify its relevance by comparing the data with well-established methods
- (2) to explore the effect of SFK inhibitors to the cell ability to interact with fibronectin
- (3) to dissect the roles of individual SFKs in regulation of the leukemic cell adhesion

RESULTS

INTRODUCTION OF THE REAL-TIME IMPEDANCE MEASUREMENT OF CELL INTERACTIONS WITH EXTRACELLULAR MATRIX.

In previous years, we discovered that the CML-derived cells adhere almost exclusively to fibronectin (Kuzelova et al., 2010), one of the major proteins in the bone marrow extracellular matrix. Instruments allowing for non-invasive, real-time monitoring of cell-surface interactions using measurement of impedance between interdigitated electrodes embedded into the bottom of special plates (Real-Time Cell Analysis, RTCA) were previously developed as a tool for monitoring of the proliferation and survival of adherent cells. During the adaptation of the system for suspension cell use, we used a 120 kD fibronectin fragment (FNF) to coat the wells of the respective plates. We showed that in FNF-coated wells, the signal rises proportionally to the cell number inserted into the experiment; in BSA-coated wells, the signal remained low even with high cell number in the well. By evaluation of the impedance signal specificity in combination with microscopy, we determined the optimal cell number for our experiments. The adherent cell fraction (ACF) is different for each leukemic cell line in its fundamental state and RTCA signal intensity for several leukemic cell lines correlated with ACF. The ability of RTCA device to detect the changes in cell interaction with fibronectin was verified by using 100 nM 12-O-tetradecanoylphorbol-13-acetate (TPA). The cells treated with this cell adhesion activator showed a steep increase in impedance signal immediately after its addition.

The usefulness of the system in regard of tracking the cell reaction to therapeutics was shown using histone deacetylase (HDAC) inhibitors, suberoylanilid hydroxamic acid and tubastatin A. Our previous work with those compounds showed that they increase the leukemic cell ACF to fibronectin (**citace**), and the use of microimpedance measurement allowed for tracking the dynamics of this process in real time. We also showed that simultaneously with the HDAC inhibitor-induced increase in adhesivity, phosphorylation of Lyn kinase on the autophosphorylation site decreases, indicating lowering of its kinase activity.

THE EFFECT OF TYROSINE KINASE INHIBITORS, IMATINIB AND DASATINIB, ON THE LEUKEMIC CELL ADHESION

Imatinib is a specific inhibitor of the fusion kinase Bcr-Abl, the product of an oncogene responsible for the development of chronic myelogenous leukemia (CML). Bcr-Abl affects several cellular processes, including adhesion signaling. It is also presumed that Bcr-Abl is the factor responsible for the release of immature cells from the bone marrow to the peripheral blood. Imatinib is currently used as a first-line treatment in CML therapy. Dasatinib, a dual inhibitor of Abl and Src kinases, is utilized in cases of CML relapses, or in cases of suboptimal or no response to the imatinib treatment (e.g. due to Bcr-Abl kinase domain mutations). To evaluate the effect of both inhibitors on the adhesion of leukemic cells, we used two CML-derived cell lines, JURL-MK1 and MOLM-7, and two Bcr-Abl negative cell lines, HEL and Jurkat.

Both inhibitors slowed down the cell growth and induced cell death with much greater effectivity in Bcr-Abl positive cells than in cell lines lacking the oncogene. Caspase activation and first signs of apoptosis were not present until at least 6 hours post treatment. The kinetics of the cell-FN interactions was measured using the RTCA iCELLigence system (ACEA Biosciences). Imatinib addition induced an increase in microimpedance levels only in cells expressing Bcr-Abl; the amplitude of the effect was dose-dependent. In Bcr-Abl negative cell lines, no significant changes were observed. The effects of dasatinib on cell adhesion were fairly complex. Low concentrations of the inhibitor (0,5 – 10 nM) induced similar changes in microimpedance as imatinib. With increasing dose, the amplitude of the signal was lowered, and at the concentration of about 100 nM, changes in the signal were not detectable any more. Very high dasatinib concentrations (more than 1 μ M) induced impedance changes with dynamical characteristics distinct from the low-dose dasatinib. The changes induced at very high dasatinib concentrations, unlike those at lower concentrations, were observed even in Bcr-Abl negative cells and in primary patient samples.

Two independent phenomena can influence changes in the microimpedance, the character of the cell-surface interaction (cell-covered area of the surface, adhesion tightness), or changes in the number of the cells in the culture that interact with the coated surface. This was reflected by comparing the data from impedance analysis with the effect of imatinib and dasatinib on the fraction of cells adhered to fibronectin (ACF). In agreement with the RTCA analysis, dasatinib at low concentrations increased the ACF in Bcr-Abl positive cells, but

failed to do so at 100 nM concentration. However, the changes in ACF were not as pronounced as in the microimpedance measurement. That stood true for both the cells treated with an inhibitor prior to seeding onto FNF and the cells treated only after adhering onto a fibronectin layer.

Given the dual potency of dasatinib, it was necessary to dissect the effect via the inhibition of Bcr-Abl and SFK. The type of expressed SFKs vary in the individual cell lines, as well as activation levels of the respective SFKs. The EC50 values for dasatinib-induced SFK inhibition (assessed by the phosphorylation level of the autophosphorylation site of respective kinase) were by an order of magnitude lower for the Bcr-Abl expressing cell lines (~2 nM) than for other cells (~20 nM). Given that Bcr-Abl can activate the SFKs, we determined the EC50 values for SFK inhibition with imatinib, as imatinib should not directly interact with SFKs. Since imatinib induced dephosphorylation of both Bcr-Abl and SFKs at the same concentration, we concluded that the kinases transactivate each other.

To track the intracellular localization of SFKs we used a pan-specific antibody against the phosphorylation site that marks the active kinase (P-SFK). The signal of phosphorylated SFK accumulated in the cell adhesion spots which were visualized using IRM. In MOLM-7 cells, which express both Lyn and Lck kinases, we showed that Lyn is situated in adhesion spots, whereas Lck is present mainly in the nuclear envelope. The signal from P-SFK antibody was suppressed in both Bcr-Abl positive cell lines with 100 nM dasatinib, and other SFK-specific inhibitor, PP2.

ADHESION STRUCTURES IN LEUKEMIA CELLS AND THEIR REGULATION BY THE LYN KINASE

To differentiate between the roles of individual SFKs in leukemic cell adhesivity, we utilized dasatinib in 100 nM concentration, which suppresses virtually all SFK kinase activity. For comparison, we used another SFK-specific inhibitor, SKI-1 (Src kinase inhibitor-1). To explore the dependence of leukemic cell adhesion on the cytoskeletal tension, we utilized cytochalasin D, an inhibitor of actin polymerization. Only Bcr-Abl negative cell lines were used to perform the experiments to rule out the effect of the fusion kinase.

Leukemic cell lines as well as primary cells from patients are substantially heterogenous from the point of view of the cell morphology, adhesion structures, and fraction of cells with ability to adhere to fibronectin. On the other hand, common characteristics of leukemic cells

is the adhesion structure dynamics and the formation of exploratory adhesions and membrane protrusions. In adhesion structures detected with IRM we were not able to observe/identify none of the typical markers of adhesion structures (focal adhesions, podosomes). The canonical markers of focal adhesions, i.e. paxillin and vinculin, were exceptionally present in adhesion points for some cell lines, but none of the markers was usable in all leukemic cells. Phosphorylated Src kinases, on the other hand, frequently colocalized with adhesion structures.

Each of the cell lines has a unique expression profile in terms of Src kinase expression and activation level. Leukemic cells with lower fundamental adhesiveness have in general more activated SFKs.

Given the preceding results, we have chosen for subsequent analyzes Bcr-Abl negative cell lines predominantly expressing the Lyn kinase, OCI-AML3 and HEL. We verified the functionality of the used SFK inhibitors, dasatinib and SKI-1, both when we treated the cells in suspension and for the cells incubated on fibronectin. SKI-1, when added to the cells incubated on FNF, surprisingly increased phosphorylation of the Lyn kinase. Increase in phosphorylation levels was observed also in Lyn substrates, HS-1 and Pyk2, which indicated Lyn hyperactivation.

Microimpedance analyses were in this case performed using the ECIS Z Θ , which allows for the impedance measurement at a wide range of frequencies of the electrical field and decomposes the microimpedance signal into capacitance and resistance. That facilitated the interpretation of the results, since the capacitance and resistance reflect different phenomena in the cell behavior.

In adherent cell lines HeLa and HEK-293T, addition of dasatinib as well as of cytochalasin D induced a steep decrease in microimpedance. In the majority of leukemia cells, dasatinib had no marked effect on the microimpedance signal and did not affect the cell ability to interact with fibronectin when the cells were pretreated before seeding onto fibronectin-coated surface and creation of the focal contacts. Dasatinib also did not affect the leukemic cell morphology, nor their capability of creating the protrusions and new adhesion contacts. The same results were observed with SKI-1, despite the SFK activation.

The behavior of Lyn in adherent cells was inspected using HeLa cells transfected with Lyn-eGFP coding plasmid. The signal of Lyn-eGFP was enriched in focal adhesions. The Lyn-eGFP expression did not cause any changes in the cell morphology compared with the

controls. When treated with 100 nM dasatinib, the cells appeared to be more tensioned in comparison with the untreated controls, independently of Lyn-eGFP presence. In transfected cells, dasatinib treatment caused relatively fast accumulation of Lyn-eGFP signal in focal adhesions. Similar changes, only slower and less apparent, were observed using SKI-1.

DISCUSSION

Abnormalities in cell adhesion signaling contribute to the aberrant behavior of leukemia blasts in several types of hematological malignancies. The typical example is the chronic myeloid leukemia (CML), where the adhesion processes are deregulated by the aberrant signaling of Bcr-Abl and altered integrin $\beta 1$ -mediated adhesion and adhesion-mediated growth regulation (Prosper and Verfaillie, 2001). Adhesion to the extracellular matrix affects the progenitor proliferation and resistance to apoptosis. Tyrosine kinase inhibitors used in clinical practice may restore the interactions of the cells with the bone marrow, but that in turn leads to the persistence of minimal residual disease and the disease relapses (Weisberg et al., 2007; Konig et al., 2008). The progression of several hematological malignancies (CML, AML, ALL, B-CLL, multiple myeloma) is correlated with higher expression of hematopoietic kinases of the Src family. SFK targeting is considered a part of anti-leukemia therapy (Dos Santos et al., 2013). Lyn kinase is also involved in the adhesion and migration of differentiated hematopoietic cells, like macrophages and neutrophils.

According to the widely-accepted model of adherent cell adhesion, binding of the cell to the extracellular matrix is mediated mainly by integrins and integrin-associated protein complexes. The composition and architecture of these complexes change in time during maturation of the adhesion. Mature structures—focal adhesions—are physically connected to the actinomyosin cytoskeleton, or to the intermediate filaments. In a recent work of Horton and colleagues, a consensus “adhesome” has been defined based on molecular composition of integrin-associated complexes in 7 different cell types, including K562, a leukemia cell line (Horton et al., 2015). It was shown that leukemic cells lack proteins with force-recognition domains responsible for transmitting signal represented by mechanical force. The authors suggested that the K562 dataset likely represented a higher number of immature adhesions resembling those formed in adherent cells before the mechanical tension is applied. In agreement with those findings, we found that adhesion structures of leukemic cells are highly dynamic, and that the cells do not form stress fibers. Additionally, the contact points seen in IRM do not colocalize with markers used to label the focal adhesions. Indeed, paxillin was not identified by Horton et al. as a part of the adhesome of K562 cells (Horton et al., 2015), even though it was present in all data sets obtained from adherent cells. On the other hand, Lyn kinase was present exclusively in the adhesome of K562 cells, and indeed our results show its colocalization with cell–ECM contact points in several leukemia cell lines as well as in primary samples.

Dasatinib, as an Abl/SFK inhibitor, is used as a second-line CML treatment. In our models, dasatinib at 100 nM concentration was able to completely inhibit SFK activity. As 100 nM dasatinib was not able to interfere with the cells' capability of adhering to fibronectin, we concluded that SFKs are not necessary for the first steps in adhesion formation. A very similar effect was observed using other SFK inhibitor, saracatinib.

Surprisingly, Lyn was activated following cell treatment with another SFK inhibitor, SKI-1, when the cells were incubated on a fibronectin layer prior to the treatment. The increase of Lyn activity correlated with a decrease in adhered cell fraction as well as with cell-type dependent decreases in microimpedance levels. This adds to our previous observation of the HDAC effects on cell adhesion to the ECM, where strengthening of the interactions with FN was associated with Lyn dephosphorylation. Additionally, we noted that in basal state of the cells, higher ACF correlates with lower SFK activity; in adherent cell lines, active SFKs are almost not present. Taken together, SFK activity negatively correlates with the tightness of leukemic cell interactions with fibronectin.

Treatment of the cells with dasatinib did not prevent cytochalasin D- or SKI-1-induced detachment of the cells. That indicates the kinase activity of SFKs is not necessary for the adhesion disassembly, either. Dasatinib also has no observable effect on leukemic cell morphology.

The absence of the effect of SFK activity on leukemic cell adhesion structure tightness contrasted with the well-established function of SFKs in adherent cell adhesion regulation. In adherent cells, SFK signaling works against the signaling changes induced by mechanical tension (Fincham and Frame, 1998); however, there is no such tension in leukemic cells, and that is probably the reason that the SFK kinase activity does not seem important in leukemic cell adhesion regulation.

Given the well-established function of SFKs in the development and progress in leukemias (O'Laughlin-Bunner, 2001; Orschell et al., 2008), we assume that SFKs transmit the signal from the ECM-binding structures to other cellular processes, like cell proliferation and survival. It is also possible that SFKs affect the adhesion signaling in other ways than through their kinase activity.

CONCLUSIONS

The main conclusions of this work are the following:

- (1) We have successfully implemented the method of real-time monitoring of cell interactions with extracellular matrix proteins using impedance measurement. The ECIS Z0 device (Applied Biosciences) is more convenient for this purpose than RTCA xCELLigence (ACEA Biosciences), given its higher sensitivity and the signal stability during mechanical perturbations, and the possibility of the signal decomposition to resistance and capacitance.
- (2) Tyrosine kinase Lyn, a member of the Src family of kinases, is in its active (autophosphorylated) form present in adhesion structures of leukemic cells and in adherent HeLa cells. In leukemic cells, amplification of Lyn activity correlates with weaker cell interaction with fibronectin.
- (3) Lyn (and other Src family kinases present in leukemic cells) is not involved in adhesion structure assembly. Unlike in adherent cells, the impact of SFK kinase activity to the adhesion complex stability is minimal.
- (4) Given the well-established role of Lyn in many processes in hematopoietic cells and leukemias, we assume that Lyn in adhesion structures transfers the signal leading to the regulation of proliferation and cell survival, or can participate in adhesion signaling in kinase-independent manner.

PUBLICATION LIST & CONTRIBUTION

(1) **Real-time monitoring of hematopoietic cell interaction with fibronectin**

fragment: The effect of histone deacetylase inhibitors

Obr A, Röselová P, Grebeňová D, Kuželová K; Cell Adhesion & Migration, 2013

IF = 2,336

Experiment design and performance, data analysis and interpretation, contribution to the writing of the manuscript.

(2) **Real-time analysis of Imatinib- and Dasatinib- induced effects on chronic myelogenous leukemia cell interactions with fibronectin**

Obr A, Röselová P, Grebeňová D, Kuželová K; PLOS ONE, 2014

IF = 3,403

Experiment design and performance, data analysis and interpretation, contribution to the writing of the manuscript.

(3) **Group I PAK inhibitor IPA-3 induces cell death and affects cell adhesivity to fibronectin in human hematopoietic cells**

Kuželová K, Grebeňová D, Holoubek A, Röselová P, Obr A; PLOS ONE, 2014

IF = 3,403

Microimpedance measurement and data analysis. The publication was not used as a foundation for this thesis, albeit the topic is closely related. An abstract of the publication is provided at the end of the thesis.

(4) **Adhesion structures in leukemia cells and their regulation by Src family kinases**

Röselová P, Obr A, Holoubek A, Grebeňová D, Kuželová K; Cell Adhesion & Migration, 2017

IF = 3,872

Experiment design and performance, data analysis and interpretation, contribution to the writing of the manuscript.

REFERENCES

- Abram, C. L. and Lowell, C. A. (2007) 'Convergence of immunoreceptor and integrin signaling', *Immunological Reviews*. Blackwell Publishing Ltd, 218(1), pp. 29–44. doi: 10.1111/j.1600-065X.2007.00531.x.
- Abram, C. L. and Lowell, C. A. (2008) 'The diverse functions of Src family kinases in macrophages.', *Frontiers in bioscience : a journal and virtual library*. NIH Public Access, 13, pp. 4426–50. doi: 10.2741/3015.
- Acar, M. et al. (2015) 'Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal', *Nature*. Nature Publishing Group, 526(7571), pp. 126–130. doi: 10.1038/nature15250.
- Allgayer, H. et al. (2002) 'Activation of Src kinase in primary colorectal carcinoma', *Cancer*. John Wiley & Sons, Inc., 94(2), pp. 344–351. doi: 10.1002/cncr.10221.
- Arias-Salgado, E. G. et al. (2003) 'Src kinase activation by direct interaction with the integrin beta cytoplasmic domain', *Proceedings of the National Academy of Sciences of the United States of America*, 100, p. 13298–13302 ST–Src kinase activation by direct. doi: 10.1073/pnas.2336149100.
- Atherton, P. et al. (2015) 'Vinculin controls talin engagement with the actomyosin machinery', *Nature communications*. Nature Publishing Group, 6, pp. 1–12. doi: 10.1038/ncomms10038.
- Axmann, M. et al. (2012) 'Determination of Interaction Kinetics between the T Cell Receptor and Peptide-Loaded MHC Class II via Single-Molecule Diffusion Measurements', *Biophysical Journal*, 103(2), pp. L17–L19. doi: 10.1016/j.bpj.2012.06.019.
- Azzali, G. (2006) 'On the transendothelial passage of tumor cell from extravasal matrix into the lumen of absorbing lymphatic vessel', *Microvascular Research*, 72(1–2), pp. 74–85. doi: 10.1016/j.mvr.2006.03.005.
- Balaban, N. Q. et al. (2001) 'Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates', *Nature Cell Biology*. Nature Publishing Group, 3(5), pp. 466–472. doi: 10.1038/35074532.
- Bashour, K. T. et al. (2014) 'CD28 and CD3 have complementary roles in T-cell traction forces.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 111(6), pp. 2241–6. doi: 10.1073/pnas.1315606111.
- Bass, M. D. et al. (2008) 'p190RhoGAP is the convergence point of adhesion signals from alpha 5 beta 1 integrin and syndecan-4.', *The Journal of cell biology*. Rockefeller University Press, 181(6), pp. 1013–26. doi: 10.1083/jcb.200711129.
- Basu, R. et al. (2016) 'Cytotoxic T Cells Use Mechanical Force to Potentiate Target Cell Killing', *Cell*, 165(1), pp. 100–110. doi: 10.1016/j.cell.2016.01.021.
- Benveniste, P. et al. (2010) 'Intermediate-Term Hematopoietic Stem Cells with Extended but Time-Limited Reconstitution Potential', *Cell Stem Cell*. Cell Press, 6(1), pp. 48–58. doi: 10.1016/J.STEM.2009.11.014.
- Bershadsky, A. D., Balaban, N. Q. and Geiger, B. (2003) 'Adhesion-Dependent Cell Mechanosensitivity', *Annual Review of Cell and Developmental Biology*. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA , 19(1), pp. 677–695. doi: 10.1146/annurev.cellbio.19.111301.153011.
- Besse, F. and Ephrussi, A. (2008) 'Translational control of localized mRNAs: restricting protein synthesis in space and time', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 9(12), pp. 971–980. doi: 10.1038/nrm2548.
- Boulet, I. et al. (1992) 'Lipopolysaccharide- and interferon-gamma-induced expression of hck and lyn

tyrosine kinases in murine bone marrow-derived macrophages', *Oncogene*, 7(4), p. 703—710. Available at: <http://europepmc.org/abstract/MED/1373483>.

Bourri , B. et al. (2013) 'SAR103168: a tyrosine kinase inhibitor with therapeutic potential in myeloid leukemias', *Leukemia & Lymphoma*. Taylor & Francis, 54(7), pp. 1488–1499. doi: 10.3109/10428194.2012.745071.

Breccia, M. et al. (2013) 'Systematic review of dasatinib in chronic myeloid leukemia.', *OncoTargets and therapy*, 6, pp. 257–65. doi: 10.2147/OTT.S35360.

Buccione, R., Caldieri, G. and Ayala, I. (2009) 'Invadopodia: specialized tumor cell structures for the focal degradation of the extracellular matrix', *Cancer and Metastasis Reviews*. Springer US, 28(1–2), pp. 137–149. doi: 10.1007/s10555-008-9176-1.

Butler, J. M. et al. (2010) 'Endothelial Cells Are Essential for the Self-Renewal and Repopulation of Notch-Dependent Hematopoietic Stem Cells', *Cell Stem Cell*. Cell Press, 6(3), pp. 251–264. doi: 10.1016/J.STEM.2010.02.001.

Campbell, I. D. and Humphries, M. J. (2011) 'Integrin structure, activation, and interactions.', *Cold Spring Harbor perspectives in biology*. Cold Spring Harbor Laboratory Press, 3(3), p. a004994. doi: 10.1101/cshperspect.a004994.

Cariaga-Mart nez, A. E. et al. (2014) 'Phosphoinositide 3-kinase p85beta regulates invadopodium formation.', *Biology open*, pp. 1–13. doi: 10.1242/bio.20148185.

Carman, C. V (2009) 'Mechanisms for transcellular diapedesis: probing and pathfinding by "invadosome-like protrusions".', *Journal of cell science*. The Company of Biologists Ltd, 122(Pt 17), pp. 3025–35. doi: 10.1242/jcs.047522.

Carr no, S. et al. (2000) 'Lack of palmitoylation redirects p59Hck from the plasma membrane to p61Hck-positive lysosomes.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 275(46), pp. 36223–9. doi: 10.1074/jbc.M003901200.

Cary, L. A. et al. (2002) 'Src Catalytic but Not Scaffolding Function Is Needed for Integrin-Regulated Tyrosine Phosphorylation , Cell Migration , and Cell Spreading Src Catalytic but Not Scaffolding Function Is Needed for Integrin-Regulated Tyrosine Phosphorylation , Cell Migratio', *Society*, 22(8), pp. 2427–2440. doi: 10.1128/MCB.22.8.2427.

Lo Celso, C. et al. (2009) 'Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche', *Nature*. Nature Publishing Group, 457(7225), pp. 92–96. doi: 10.1038/nature07434.

Collin, O. et al. (2008) 'Self-Organized Podosomes Are Dynamic Mechanosensors', *Current Biology*, 18(17), pp. 1288–1294. doi: 10.1016/j.cub.2008.07.046.

Contri, A. et al. (2005) 'Chronic lymphocytic leukemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to defective apoptosis.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 115(2), pp. 369–78. doi: 10.1172/JCI22094.

Das, D. K. et al. (2015) 'Force-dependent transition in the T-cell receptor β -subunit allosterically regulates peptide discrimination and pMHC bond lifetime', *Proceedings of the National Academy of Sciences*, 112(5), pp. 1517–1522. doi: 10.1073/pnas.1424829112.

Das, D. K. et al. (2016) 'Pre-T Cell Receptors (Pre-TCRs) Leverage V β Complementarity Determining Regions (CDRs) and Hydrophobic Patch in Mechanosensing Thymic Self-ligands', *Journal of Biological Chemistry*, 291(49), pp. 25292–25305. doi: 10.1074/jbc.M116.752865.

Dickreuter, E. et al. (2017) 'The cancer cell adhesion resistome: mechanisms, targeting and translational approaches.', *Biological chemistry*, 398(7), pp. 721–735. doi: 10.1515/hsz-2016-0326.

de Diesbach, P. et al. (2008) 'Differential subcellular membrane recruitment of Src may specify its downstream signalling', *Experimental Cell Research*. Academic Press, 314(7), pp. 1465–1479. doi:

10.1016/J.YEXCR.2008.01.015.

Digiacomio, G. et al. (2017) 'Fibronectin induces macrophage migration through a SFK-FAK/CSF-1R pathway', *Cell Adhesion & Migration*. Taylor & Francis, 11(4), pp. 327–337. doi: 10.1080/19336918.2016.1221566.

Diz-Muñoz, A. et al. (2010) 'Control of Directed Cell Migration In Vivo by Membrane-to-Cortex Attachment', *PLoS Biology*. Edited by W. A. Harris. Public Library of Science, 8(11), p. e1000544. doi: 10.1371/journal.pbio.1000544.

Dwyer, A. R. et al. (2016) 'Src family kinase expression and subcellular localization in macrophages: implications for their role in CSF-1-induced macrophage migration', *Journal of Leukocyte Biology*, 100(1), pp. 163–175. doi: 10.1189/jlb.2A0815-344RR.

Dwyer, A. R. et al. (2016) 'Src family kinase expression and subcellular localization in macrophages: implications for their role in CSF-1-induced macrophage migration.', *Journal of leukocyte biology*, 100(July), pp. 163–175. doi: 10.1189/jlb.2A0815-344RR.

Edwards, D., Eryildiz, F. and Tyner, J. W. (2018) 'RNAi screening of the tyrosine kinome in primary patient samples of acute myeloid leukemia', *bioRxiv*. Available at: <http://biorxiv.org/content/early/2018/01/30/256040.abstract>.

Eilken, H. M. and Adams, R. H. (2010) 'Turning on the angiogenic microswitch', *Nature Medicine*. Nature Publishing Group, 16(8), pp. 853–854. doi: 10.1038/nm0810-853.

Eke, I. et al. (2012) ' β_1 Integrin/FAK/cortactin signaling is essential for human head and neck cancer resistance to radiotherapy.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 122(4), pp. 1529–40. doi: 10.1172/JCI61350.

Eke, I. and Cordes, N. (2015) 'Focal adhesion signaling and therapy resistance in cancer', *Seminars in Cancer Biology*. Academic Press, 31, pp. 65–75. doi: 10.1016/J.SEMCANCER.2014.07.009.

Ema, H. et al. (2005) 'Quantification of Self-Renewal Capacity in Single Hematopoietic Stem Cells from Normal and Lnk-Deficient Mice', *Developmental Cell*. Cell Press, 8(6), pp. 907–914. doi: 10.1016/J.DEVCEL.2005.03.019.

Fei, F. et al. (2010) 'Development of resistance to dasatinib in Bcr/Abl-positive acute lymphoblastic leukemia', *Leukemia*, 24(4), pp. 813–820. doi: 10.1038/leu.2009.302.

Feng, Y. et al. (2017) 'Mechanosensing drives acuity of $\alpha\beta$ T-cell recognition', *Proceedings of the National Academy of Sciences*, 114(39), pp. E8204–E8213. doi: 10.1073/pnas.1703559114.

Fincham, V. J. and Frame, M. C. (1998) 'The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility', *EMBO J*, 17, p. 81–92 ST–The catalytic activity of Src is dispe. Available at: <http://dx.doi.org/10.1093/emboj/17.1.81>.

Fincham, V. J., Chudleigh, A. and Frame, M. C. (1999) 'Regulation of p190 Rho-GAP by v-Src is linked to cytoskeletal disruption during transformation', *Journal of Cell Science*, 112(6), p. 947 LP-956. Available at: <http://jcs.biologists.org/content/112/6/947.abstract>.

Frame, M. C. (2004) 'Newest findings on the oldest oncogene; how activated src does it.', *Journal of cell science*, 117(Pt 7), pp. 989–998. doi: 10.1242/jcs.01111.

Friedl, P. and Gilmour, D. (2009) 'Collective cell migration in morphogenesis, regeneration and cancer', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 10(7), pp. 445–457. doi: 10.1038/nrm2720.

Gamas, P. et al. (2009) 'Inhibition of imatinib-mediated apoptosis by the caspase-cleaved form of the tyrosine kinase Lyn in chronic myelogenous leukemia cells', *Leukemia*, 23(8), pp. 1500–1506. doi: 10.1038/leu.2009.60.

- Gao, Y. et al. (2009) 'Oxidative Stress Promotes Transcriptional Up-regulation of Fyn in BCR-ABL1-expressing Cells', *Journal of Biological Chemistry*, 284(11), pp. 7114–7125. doi: 10.1074/jbc.M804801200.
- Gattazzo, C. et al. (2014) 'Cortactin, another player in the Lyn signaling pathway, is over-expressed and alternatively spliced in leukemic cells from patients with B-cell chronic lymphocytic leukemia.', *Haematologica*. *Haematologica*, 99(6), pp. 1069–77. doi: 10.3324/haematol.2013.090183.
- Geiger, B. et al. (2001) 'Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk.', *Nature reviews. Molecular cell biology*, 2(11), pp. 793–805. doi: 10.1038/35099066.
- Geiger, B. and Yamada, K. M. (2011) 'Molecular architecture and function of matrix adhesions.', *Cold Spring Harbor perspectives in biology*. Cold Spring Harbor Laboratory Press, 3(5), p. a005033. doi: 10.1101/cshperspect.a005033.
- Giaever, I. and Keese, C. R. (1984) 'Monitoring fibroblast behavior in tissue culture with an applied electric field', *Proceedings of the National Academy of Sciences of the United States of America*, 81(12), pp. 3761–3764. doi: 10.1073/pnas.81.12.3761.
- Giaever, I. and Keese, C. R. (1993) 'A morphological biosensor for mammalian cells.', *Nature*, 366(6455), pp. 591–592. doi: 10.1038/366591a0.
- Gilliland, D. G. (2002) 'Molecular genetics of human leukemias: new insights into therapy.', *Seminars in hematology*, 39(4 Suppl 3), pp. 6–11. doi: 10.1053/shem.2002.36921.
- Gong, J. K. (1978) 'Endosteal marrow: a rich source of hematopoietic stem cells.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 199(4336), pp. 1443–5. doi: 10.1126/SCIENCE.75570.
- Grassinger, J. et al. (2010) 'Phenotypically identical hemopoietic stem cells isolated from different regions of bone marrow have different biologic potential.', *Blood*. American Society of Hematology, 116(17), pp. 3185–96. doi: 10.1182/blood-2009-12-260703.
- Guo, Y. et al. (2012) 'Oridonin in combination with imatinib exerts synergetic anti-leukemia effect in Ph+ acute lymphoblastic leukemia cells in vitro by inhibiting activation of LYN/mTOR signaling pathway', *Cancer Biology & Therapy*. Taylor & Francis, 13(13), pp. 1244–1254. doi: 10.4161/cbt.21460.
- Gupton, S. L. and Gertler, F. B. (2007) 'Filopodia: the fingers that do the walking.', *Science's STKE : signal transduction knowledge environment*. American Association for the Advancement of Science, 2007(400), p. re5. doi: 10.1126/stke.4002007re5.
- Hayette, S. et al. (2011) 'Longitudinal studies of SRC family kinases in imatinib- and dasatinib-resistant chronic myelogenous leukemia patients', *Leukemia Research*, 35(1), pp. 38–43. doi: 10.1016/j.leukres.2010.06.030.
- Haylock, D. N. et al. (2007) 'Hemopoietic Stem Cells with Higher Hemopoietic Potential Reside at the Bone Marrow Endosteum', *Stem Cells*. John Wiley & Sons, Ltd., 25(4), pp. 1062–1069. doi: 10.1634/stemcells.2006-0528.
- Horton, E. R. et al. (2015) 'Definition of a consensus integrin adhesome and its dynamics during adhesion complex assembly and disassembly', *Nature Cell Biology*, 17(12), pp. 1577–1587. doi: 10.1038/ncb3257.
- Horton, E. R. et al. (2016) 'Modulation of FAK and Src adhesion signaling occurs independently of adhesion complex composition', *Journal of Cell Biology*, 212(3), pp. 349–364. doi: 10.1083/jcb.201508080.
- Hu, K. H. and Butte, M. J. (2016) 'T cell activation requires force generation.', *The Journal of cell biology*. Rockefeller University Press, 213(5), pp. 535–42. doi: 10.1083/jcb.201511053.

- Hu, Y. G. et al. (2004) 'Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia', *Nature Genetics*, 36, p. 453–461 ST–Requirement of Src kinases Lyn, Hck. doi: 10.1038/ng1343.
- Humphries, J. D. et al. (2007) 'Vinculin controls focal adhesion formation by direct interactions with talin and actin', *Journal of Cell Biology*, 179, p. 1043–1057 ST–Vinculin controls focal adhesion f. doi: 10.1083/jcb.200703036.
- Hussein, K. et al. (2009) 'Opposite expression pattern of Src kinase Lyn in acute and chronic haematological malignancies', *Annals of Hematology*. Springer-Verlag, 88(11), pp. 1059–1067. doi: 10.1007/s00277-009-0727-5.
- Huveneers, S. and Danen, E. H. J. (2009) 'Adhesion signaling - crosstalk between integrins, Src and Rho.', *Journal of cell science*, 122(Pt 8), pp. 1059–69. doi: 10.1242/jcs.039446.
- Hynes, R. O. (2002) 'Integrins: Bidirectional, allosteric signaling machines', *Cell*, 110, p. 673–687 ST–Integrins: Bidirectional, allosteric. doi: 10.1016/s0092-8674(02)00971-6.
- Hytönen, V. P. and Wehrle-Haller, B. (2014) 'Protein conformation as a regulator of cell-matrix adhesion.', *Physical chemistry chemical physics : PCCP*, 16(14), pp. 6342–57. doi: 10.1039/c3cp54884h.
- Charras, G. and Paluch, E. (2008) 'Blebs lead the way: how to migrate without lamellipodia', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 9(9), pp. 730–736. doi: 10.1038/nrm2453.
- Chougule, R. A., Kazi, J. U. and Rönstrand, L. (2016) 'FYN expression potentiates FLT3-ITD induced STAT5 signaling in acute myeloid leukemia', *Oncotarget*, 7(9), pp. 9964–74. doi: 10.18632/oncotarget.7128.
- Ingle, E. (2012) 'Functions of the Lyn tyrosine kinase in health and disease.', *Cell communication and signaling : CCS*, 10(1), p. 21. doi: 10.1186/1478-811X-10-21.
- Jaalouk, D. E. and Lammerding, J. (2009) 'Mechanotransduction gone awry', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 10(1), pp. 63–73. doi: 10.1038/nrm2597.
- Jakus, Z. et al. (2004) 'Responses of neutrophils to anti-integrin antibodies depends on costimulation through low affinity Fc gamma Rs: full activation requires both integrin and nonintegrin signals.', *Journal of immunology (Baltimore, Md. : 1950)*, 173(3), pp. 2068–77. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15265942> (Accessed: 18 October 2017).
- Kent, D. G. et al. (2009) 'Prospective isolation and molecular characterization of hematopoietic stem cells with durable self-renewal potential.', *Blood*. American Society of Hematology, 113(25), pp. 6342–50. doi: 10.1182/blood-2008-12-192054.
- Kiel, M. J. et al. (2005) 'SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells', *Cell*. Cell Press, 121(7), pp. 1109–1121. doi: 10.1016/J.CELL.2005.05.026.
- Klejman, A. et al. (2002) 'The Src family kinase Hck couples BCR/ABL to STAT5 activation in myeloid leukemia cells', *Embo Journal*, 21, p. 5766–5774 ST–The Src family kinase Hck couples. doi: 10.1093/emboj/cdf562.
- ten Klooster, J. P. et al. (2006) 'Targeting and activation of Rac1 are mediated by the exchange factor beta-Pix', *Journal of Cell Biology*, 172, p. 759–769 ST–Targeting and activation of Rac1 are. doi: 10.1083/jcb.200509096.
- Kobayashi, H. et al. (2010) 'Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells', *Nature Cell Biology*. Nature Publishing Group, 12(11), pp. 1046–1056. doi: 10.1038/ncb2108.
- König, H. et al. (2008) 'Enhanced BCR-ABL kinase inhibition does not result in increased inhibition

of downstream signaling pathways or increased growth suppression in CML progenitors', *Leukemia*, 22, p. 748–755 ST–Enhanced BCR–ABL kinase inhibition d. Available at: <http://dx.doi.org/10.1038/sj.leu.2405086>.

Kunisaki, Y. et al. (2013) 'Arteriolar niches maintain haematopoietic stem cell quiescence', *Nature*. Nature Publishing Group, 502(7473), pp. 637–643. doi: 10.1038/nature12612.

Kuzelova, K. et al. (2010) 'Suberoylanilide Hydroxamic Acid (SAHA) at Subtoxic Concentrations Increases the Adhesivity of Human Leukemic Cells to Fibronectin', *Journal of Cellular Biochemistry*, 109(1), p. 184–195 ST–Suberoylanilide Hydroxamic Acid (SAH. doi: 10.1002/jcb.22397.

Laird, D. J., von Andrian, U. H. and Wagers, A. J. (2008) 'Stem Cell Trafficking in Tissue Development, Growth, and Disease', *Cell*. Cell Press, 132(4), pp. 612–630. doi: 10.1016/J.CELL.2008.01.041.

Legate, K. R., Wickström, S. A. and Fässler, R. (2009) 'Genetic and cell biological analysis of integrin outside-in signaling.', *Genes & development*. Cold Spring Harbor Laboratory Press, 23(4), pp. 397–418. doi: 10.1101/gad.1758709.

Leu, T.-H. et al. (2006) 'Lipopolysaccharide-induced c-Src expression plays a role in nitric oxide and TNF α secretion in macrophages', *Molecular Immunology*. Pergamon, 43(4), pp. 308–316. doi: 10.1016/J.MOLIMM.2005.03.015.

Linder, S. (2009) 'Invadosomes at a glance', *Journal of Cell Science*, 122(17), pp. 3009–3013. doi: 10.1242/jcs.032631.

Liu, B., Chen, W. and Zhu, C. (2015) 'Molecular Force Spectroscopy on Cells', *Annual Review of Physical Chemistry*. Annual Reviews , 66(1), pp. 427–451. doi: 10.1146/annurev-physchem-040214-121742.

Lock, J. G. et al. (2017) 'Reticular adhesions: A new class of adhesion complex that mediates cell-matrix attachment during mitosis', *bioRxiv*. Cold Spring Harbor Laboratory, p. 234237. doi: 10.1101/234237.

Lock, J. G., Wehrle-Haller, B. and Stromblad, S. (2008) 'Cell-matrix adhesion complexes: Master control machinery of cell migration', *Seminars in Cancer Biology*, 18, p. 65–76 ST–Cell–matrix adhesion complexes: Master. doi: 10.1016/j.semcancer.2007.10.001.

Mallis, R. J. et al. (2015) 'Pre-TCR ligand binding impacts thymocyte development before $\alpha\beta$ TCR expression', *Proceedings of the National Academy of Sciences*, 112(27), pp. 8373–8378. doi: 10.1073/pnas.1504971112.

Marhäll, A., Kazi, J. U. and Rönnstrand, L. (2017) 'The Src family kinase LCK cooperates with oncogenic FLT3/ITD in cellular transformation', *Scientific Reports*, 7(1), p. 13734. doi: 10.1038/s41598-017-14033-4.

Martini, V. et al. (2017) 'Cortactin, a Lyn substrate, is a checkpoint molecule at the intersection of BCR and CXCR4 signalling pathway in chronic lymphocytic leukaemia cells.', *British journal of haematology*, 178(1), pp. 81–93. doi: 10.1111/bjh.14642.

Mayadas, T. N. and Cullere, X. (2005) 'Neutrophil β 2 integrins: moderators of life or death decisions', *Trends in Immunology*. Elsevier Current Trends, 26(7), pp. 388–395. doi: 10.1016/J.IT.2005.05.002.

Meads, M. B., Gatenby, R. A. and Dalton, W. S. (2009) 'Environment-mediated drug resistance: a major contributor to minimal residual disease', *Nature Reviews Cancer*. Nature Publishing Group, 9(9), pp. 665–674. doi: 10.1038/nrc2714.

Mócsai, A. et al. (2006) 'Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs', *Nature Immunology*. Nature Publishing Group,

7(12), pp. 1326–1333. doi: 10.1038/ni1407.

Möhn, H. et al. (1995) 'The src-family protein-tyrosine kinase p59hck is located on the secretory granules in human neutrophils and translocates towards the phagosome during cell activation.', *The Biochemical journal*. Portland Press Ltd, 309 (Pt 2)(Pt 2), pp. 657–65. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/7626033> (Accessed: 6 March 2018).

Moore, N. M. et al. (2011) 'Synergistic enhancement of human bone marrow stromal cell proliferation and osteogenic differentiation on BMP-2-derived and RGD peptide concentration gradients', *Acta Biomaterialia*. Elsevier, 7(5), pp. 2091–2100. doi: 10.1016/J.ACTBIO.2011.01.019.

Muller-Sieburg, C. E. et al. (2012) 'Stem cell heterogeneity: implications for aging and regenerative medicine.', *Blood*. American Society of Hematology, 119(17), pp. 3900–7. doi: 10.1182/blood-2011-12-376749.

Murray, P. J. and Wynn, T. A. (2011) 'Obstacles and opportunities for understanding macrophage polarization', *Journal of Leukocyte Biology*, 89(4), pp. 557–563. doi: 10.1189/jlb.0710409.

Nam, J.-S. et al. (2002) 'Src Family Kinase Inhibitor PP2 Restores the E-Cadherin/Catenin Cell Adhesion System in Human Cancer Cells and Reduces Cancer Metastasis', *Clinical Cancer Research*, 8(7), p. 2430 LP-2436. Available at: <http://clincancerres.aacrjournals.org/content/8/7/2430.abstract>.

Natkanski, E. et al. (2013) 'B cells use mechanical energy to discriminate antigen affinities.', *Science* (New York, N.Y.), 340(6140), pp. 1587–90. doi: 10.1126/science.1237572.

Nguyen, P.-H. et al. (2016) 'LYN Kinase in the Tumor Microenvironment Is Essential for the Progression of Chronic Lymphocytic Leukemia.', *Cancer cell*, 30(4), pp. 610–622. doi: 10.1016/j.ccell.2016.09.007.

O'Laughlin-Bunner, B. (2001) 'Lyn is required for normal stem cell factor-induced proliferation and chemotaxis of primary hematopoietic cells', *Blood*, 98(2), pp. 343–350. doi: 10.1182/blood.V98.2.343.

Oakes, P. W. and Gardel, M. L. (2014) 'Stressing the limits of focal adhesion mechanosensitivity.', *Current opinion in cell biology*. Elsevier Ltd, 30C, pp. 68–73. doi: 10.1016/j.ceb.2014.06.003.

Okamoto, M. et al. (2007) 'Lyn is an important component of the signal transduction pathway specific to FLT3/ITD and can be a therapeutic target in the treatment of AML with FLT3/ITD', *Leukemia*, 21(3), pp. 403–410. doi: 10.1038/sj.leu.2404547.

Orschell, C. M. et al. (2008) 'Deficiency of Src family kinases compromises the repopulating ability of hematopoietic stem cells.', *Experimental hematology*. Elsevier, 36(5), pp. 655–66. doi: 10.1016/j.exphem.2008.01.002.

Palacios, E. H. and Weiss, A. (2004) 'Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation', *Oncogene*, 23, p. 7990–8000 ST-Function of the Src-family kinases. doi: 10.1038/sj.onc.1208074.

Paluch, E. K., Aspalter, I. M. and Sixt, M. (2016) 'Focal Adhesion–Independent Cell Migration', *Annual Review of Cell and Developmental Biology*, 32(1), p. annurev-cellbio-111315-125341. doi: 10.1146/annurev-cellbio-111315-125341.

Pathak, A. and Kumar, S. (2012) 'Independent regulation of tumor cell migration by matrix stiffness and confinement.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 109(26), pp. 10334–9. doi: 10.1073/pnas.1118073109.

Pene-Dumitrescu, T. et al. (2008) 'An inhibitor-resistant mutant of Hck protects CML cells against the antiproliferative and apoptotic effects of the broad-spectrum Src family kinase inhibitor A-419259', *Oncogene*, 27(56), pp. 7055–7069. doi: 10.1038/onc.2008.330.

Pereira, S. et al. (2004) 'The inhibitory receptor PIR-B negatively regulates neutrophil and

- macrophage integrin signaling.’, *Journal of immunology* (Baltimore, Md. : 1950). American Association of Immunologists, 173(9), pp. 5757–65. doi: 10.4049/JIMMUNOL.173.9.5757.
- Pereira, S. and Lowell, C. (2003) ‘The Lyn tyrosine kinase negatively regulates neutrophil integrin signaling.’, *Journal of immunology* (Baltimore, Md. : 1950), 171(3), pp. 1319–27. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12874221>.
- Phillipson, M. et al. (2006) ‘Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade.’, *The Journal of experimental medicine*. The Rockefeller University Press, 203(12), pp. 2569–75. doi: 10.1084/jem.20060925.
- Poincloux, R. et al. (2009) ‘The oncogenic activity of the Src family kinase Hck requires the cooperative action of the plasma membrane- and lysosome-associated isoforms.’, *European journal of cancer* (Oxford, England : 1990), 45(3), pp. 321–7. doi: 10.1016/j.ejca.2008.11.020.
- Prosper, F. and Verfaillie, C. M. (2001) ‘Regulation of hematopoiesis through adhesion receptors’, *Journal of Leukocyte Biology*, 69(March), p. 307–316 ST–Regulation of hematopoiesis through. Available at: <http://www.jleukbio.org/content/69/3/307.short> (Accessed: 9 December 2013).
- Pryshchep, S. et al. (2014) ‘Accumulation of serial forces on TCR and CD8 frequently applied by agonist antigenic peptides embedded in MHC molecules triggers calcium in T cells.’, *Journal of immunology* (Baltimore, Md. : 1950). American Association of Immunologists, 193(1), pp. 68–76. doi: 10.4049/jimmunol.1303436.
- Qiu, Y. et al. (2014) ‘Platelet mechanosensing of substrate stiffness during clot formation mediates adhesion, spreading, and activation’, *Proceedings of the National Academy of Sciences*, 111(40), p. 14430 LP-14435. Available at: <http://www.pnas.org/content/111/40/14430.abstract>.
- Rahikainen, R. et al. (2017) ‘Mechanical stability of talin rod controls cell migration and substrate sensing’, *Scientific Reports*. Nature Publishing Group, 7(1), p. 3571. doi: 10.1038/s41598-017-03335-2.
- Reissig, D. et al. (2001) ‘Elevated activity and expression of Src-family kinases in human breast carcinoma tissue versus matched non-tumor tissue’, *Journal of Cancer Research and Clinical Oncology*. Springer-Verlag, 127(4), pp. 226–230. doi: 10.1007/s004320000197.
- Reister-Gottfried, E. et al. (2008) ‘Dynamics of Specific Vesicle-Substrate Adhesion: From Local Events to Global Dynamics’, *Physical Review Letters*. American Physical Society, 101(20), p. 208103. doi: 10.1103/PhysRevLett.101.208103.
- Ridley, A. J. (2011) ‘Life at the Leading Edge’, *Cell*. Cell Press, 145(7), pp. 1012–1022. doi: 10.1016/J.CELL.2011.06.010.
- Roach, T. et al. (1997) ‘CD45 regulates Src family member kinase activity associated with macrophage integrin-mediated adhesion’, *Current Biology*. Cell Press, 7(6), pp. 408–417. doi: 10.1016/S0960-9822(06)00188-6.
- Roginskaya, V. et al. (1999) ‘Therapeutic targeting of Src-kinase Lyn in myeloid leukemic cell growth.’, *Leukemia*, 13(6), pp. 855–61. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10360372> (Accessed: 5 March 2018).
- Rösel, D. et al. (2013) ‘Drugs for solid cancer: the productivity crisis prompts a rethink.’, *OncoTargets and therapy*. Dove Press, 6, pp. 767–77. doi: 10.2147/OTT.S45177.
- Rubbi, L. et al. (2011) ‘Global Phosphoproteomics Reveals Crosstalk Between Bcr-Abl and Negative Feedback Mechanisms Controlling Src Signaling’, *Science Signaling*, 4(166), p. ra18-ra18. doi: 10.1126/scisignal.2001314.
- Sachs, N. et al. (2012) ‘Loss of integrin $\alpha 3$ prevents skin tumor formation by promoting epidermal turnover and depletion of slow-cycling cells.’, *Proceedings of the National Academy of Sciences of*

the United States of America, 109(52), pp. 21468–73. doi: 10.1073/pnas.1204614110.

Saitakis, M. et al. (2017) ‘Different TCR-induced T lymphocyte responses are potentiated by stiffness with variable sensitivity.’, *eLife*. eLife Sciences Publications, Ltd, 6. doi: 10.7554/eLife.23190.

Saito, Y. et al. (2013) ‘A Pyrrolo-Pyrimidine Derivative Targets Human Primary AML Stem Cells in Vivo’, *Science Translational Medicine*, 5(181), p. 181ra52-181ra52. doi: 10.1126/scitranslmed.3004387.

Dos Santos, C. et al. (2008) ‘A critical role for Lyn in acute myeloid leukemia.’, *Blood*, 111(4), pp. 2269–79. doi: 10.1182/blood-2007-04-082099.

Dos Santos, C. et al. (2013) ‘The Src and c-Kit kinase inhibitor dasatinib enhances p53-mediated targeting of human acute myeloid leukemia stem cells by chemotherapeutic agents.’, *Blood*. American Society of Hematology, 122(11), pp. 1900–13. doi: 10.1182/blood-2012-11-466425.

Shaheen, S. et al. (2017) ‘Substrate stiffness governs the initiation of B cell activation by the concerted signaling of PKC β and focal adhesion kinase.’, *eLife*. eLife Sciences Publications, Ltd, 6. doi: 10.7554/eLife.23060.

Shi, C. and Simon, D. I. (2006) ‘Integrin signals, transcription factors, and monocyte differentiation.’, *Trends in cardiovascular medicine*. Elsevier, 16(5), pp. 146–52. doi: 10.1016/j.tcm.2006.03.002.

Schiller, H. B. and Fässler, R. (2013) ‘Mechanosensitivity and compositional dynamics of cell–matrix adhesions’, *EMBO reports*, 14(6), p. 509 LP-519. Available at: <http://embor.embopress.org/content/14/6/509.abstract>.

Silva, C. M. (2004) ‘Role of STATs as downstream signal transducers in Src family kinase-mediated tumorigenesis’, *Oncogene*, 23(48), pp. 8017–8023. doi: 10.1038/sj.onc.1208159.

Snedeker, J. G. and Gautieri, A. (2014) ‘The role of collagen crosslinks in ageing and diabetes - the good, the bad, and the ugly.’, *Muscles, ligaments and tendons journal*. CIC Edizioni Internazionali, 4(3), pp. 303–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25489547> (Accessed: 5 March 2018).

STEHENLIN, D. et al. (1976) ‘DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA’, *Nature*. Nature Publishing Group, 260(5547), pp. 170–173. doi: 10.1038/260170a0.

Su, Y. et al. (2016) ‘Relating conformation to function in integrin $\alpha_5\beta_1$ ’, *Proceedings of the National Academy of Sciences*, 113(27), pp. E3872–E3881. doi: 10.1073/pnas.1605074113.

Suen, P. W. et al. (1999) ‘Impaired integrin-mediated signal transduction, altered cytoskeletal structure and reduced motility in Hck/Fgr deficient macrophages’, *Journal of Cell Science*, 112, p. 4067–4078 ST–Impaired integrin–mediated signal.

Swaminathan, V., Alushin, G. M. and Waterman, C. M. (2017) ‘Mechanosensation: A Catch Bond That Only Hooks One Way’, *Current Biology*. Elsevier Ltd., 27(21), pp. R1158–R1160. doi: 10.1016/j.cub.2017.09.023.

Szulcek, R., Bogaard, H. J. and van Nieuw Amerongen, G. P. (2014) ‘Electric cell-substrate impedance sensing for the quantification of endothelial proliferation, barrier function, and motility.’, *Journal of visualized experiments : JoVE*. MyJoVE Corporation, (85). doi: 10.3791/51300.

Talab, F. et al. (2013) ‘LCK Is an Important Mediator of B-Cell Receptor Signaling in Chronic Lymphocytic Leukemia Cells’, *Molecular Cancer Research*, 11(5), pp. 541–554. doi: 10.1158/1541-7786.MCR-12-0415-T.

Thomas, S. M. and Brugge, J. S. (1997) ‘Cellular functions regulated by Src family kinases’, *Annual Review of Cell and Developmental Biology*, 13, p. 513–609 ST–Cellular functions regulated by Src. doi: 10.1146/annurev.cellbio.13.1.513.

- Tibaldi, E. et al. (2017) 'Targeted activation of the SHP-1/PP2A signaling axis elicits apoptosis of chronic lymphocytic leukemia cells.', *Haematologica*. Ferrata Storti Foundation, 102(8), pp. 1401–1412. doi: 10.3324/haematol.2016.155747.
- Tolomelli, A. et al. (2017) 'Can Integrin Agonists Have Cards to Play against Cancer? A Literature Survey of Small Molecules Integrin Activators', *Cancers*. Multidisciplinary Digital Publishing Institute, 9(12), p. 78. doi: 10.3390/cancers9070078.
- Upadhyaya, A. (2017) 'Mechanosensing in the immune response', *Seminars in Cell & Developmental Biology*. Academic Press, 71, pp. 137–145. doi: 10.1016/J.SEMCDB.2017.08.031.
- Vázquez-Franco, J. E. et al. (2012) 'Src, Akt, NF- κ B, BCL-2 and c-IAP1 may be involved in an anti-apoptotic effect in patients with BCR-ABL positive and BCR-ABL negative acute lymphoblastic leukemia', *Leukemia Research*, 36(7), pp. 862–867. doi: 10.1016/j.leukres.2012.03.020.
- Veldurthy, A. et al. (2008) 'The kinase inhibitor dasatinib induces apoptosis in chronic lymphocytic leukemia cells in vitro with preference for a subgroup of patients with unmutated IgVH genes', *Blood*, 112(4), pp. 1443–1452. doi: 10.1182/blood-2007-11-123984.
- Vines, C. M. et al. (2001) 'Inhibition of β 2 Integrin Receptor and Syk Kinase Signaling in Monocytes by the Src Family Kinase Fgr', *Immunity*. Cell Press, 15(4), pp. 507–519. doi: 10.1016/S1074-7613(01)00221-7.
- Wan, Z. et al. (2015) 'The activation of IgM- or isotype-switched IgG- and IgE-BCR exhibits distinct mechanical force sensitivity and threshold.', *eLife*. eLife Sciences Publications, Ltd, 4. doi: 10.7554/eLife.06925.
- Wang, T. et al. (2017) 'Extracellular matrix stiffness and cell contractility control RNA localization to promote cell migration', *Nature Communications*. Nature Publishing Group, 8(1), p. 896. doi: 10.1038/s41467-017-00884-y.
- Webb, D. J. et al. (2004) 'FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly', *Nat Cell Biol*, 6, p. 154–161 ST-FAK-Src signalling through paxillin,. doi: http://www.nature.com/ncb/journal/v6/n2/supinfo/ncb1094_S1.html.
- Weisberg, E. et al. (2007) 'Second generation inhibitors of BCR-ABL for the treatment of imatinib-resistant chronic myeloid leukaemia', *Nat Rev Cancer*, 7, p. 345-356 ST-Second generation inhibitors of BCR-. Available at: <http://dx.doi.org/10.1038/nrc2126>.
- Welch, M. D. and Mullins, R. D. (2002) 'Cellular Control of Actin Nucleation', *Annual Review of Cell and Developmental Biology*. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA , 18(1), pp. 247–288. doi: 10.1146/annurev.cellbio.18.040202.112133.
- Wen, J. H. et al. (2014) 'Interplay of matrix stiffness and protein tethering in stem cell differentiation', *Nature Materials*. Nature Publishing Group, 13(10), pp. 979–987. doi: 10.1038/nmat4051.
- Wiener, J. R. et al. (2003) 'Activated SRC protein tyrosine kinase is overexpressed in late-stage human ovarian cancers.', *Gynecologic oncology*. Elsevier, 88(1), pp. 73–9. doi: 10.1006/GYNO.2002.6851.
- Wilson, M. B. et al. (2002) 'Selective pyrrolo-pyrimidine inhibitors reveal a necessary role for Src family kinases in Bcr-Abl signal transduction and oncogenesis', *Oncogene*, 21, p. 8075–8088 ST-Selective pyrrolo-pyrimidine inhib. doi: 10.1038/sj.onc.1206008.
- Yamazaki, H. et al. (2011) 'Regulation of cancer stem cell properties by CD9 in human B-acute lymphoblastic leukemia', *Biochemical and Biophysical Research Communications*, 409(1), pp. 14–21. doi: 10.1016/j.bbrc.2011.04.098.
- Yang, L. et al. (2005) 'Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term hematopoietic

- stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients.’, *Blood*. American Society of Hematology, 105(7), pp. 2717–23. doi: 10.1182/blood-2004-06-2159.
- Zaidel-Bar, R. and Geiger, B. (2010) ‘The switchable integrin adhesome.’, *Journal of cell science*, 123(Pt 9), pp. 1385–1388. doi: 10.1242/jcs.066183.
- Zamir, E. et al. (1999) ‘Molecular diversity of cell-matrix adhesions’, *Journal of Cell Science*, 112, p. 1655–1669 ST–Molecular diversity of cell–matrix.
- Zhou, B. O. et al. (2014) ‘Leptin-Receptor-Expressing Mesenchymal Stromal Cells Represent the Main Source of Bone Formed by Adult Bone Marrow’, *Cell Stem Cell*. Cell Press, 15(2), pp. 154–168. doi: 10.1016/J.STEM.2014.06.008.
- Zonta, F. et al. (2014) ‘Lyn-mediated procaspase 8 dimerization blocks apoptotic signaling in B-cell chronic lymphocytic leukemia.’, *Blood*. American Society of Hematology, 123(6), pp. 875–83. doi: 10.1182/blood-2013-02-485540.
- Zonta, F. et al. (2015) ‘Lyn sustains oncogenic signaling in chronic lymphocytic leukemia by strengthening SET-mediated inhibition of PP2A.’, *Blood*, 125(24), pp. 3747–55. doi: 10.1182/blood-2014-12-619155.
- Zou, W., Kitaura, H., Reeve, J., Long, F., Tybulewicz, V. L. J., et al. (2007) ‘Syk, c-Src, the $\alpha\text{v}\beta 3$ integrin, and ITAM immunoreceptors, in concert, regulate osteoclastic bone resorption.’, *The Journal of cell biology*. Rockefeller University Press, 176(6), pp. 877–88. doi: 10.1083/jcb.200611083.
- Zou, W., Kitaura, H., Reeve, J., Long, F., Tybulewicz, V. L. J., et al. (2007) ‘Syk, c-Src, the $\alpha\text{v}\beta 3$ integrin, and ITAM immunoreceptors, in concert, regulate osteoclastic bone resorption’, *The Journal of Cell Biology*, 176(6), pp. 877–888. doi: 10.1083/jcb.200611083.

REPRINT OF PUBLICATIONS

- (1) **Real-time monitoring of hematopoietic cell interaction with fibronectin fragment: The effect of histone deacetylase inhibitors**
Obr A, Röselová P, Grebeňová D, Kuželová K; Cell Adhesion & Migration, 2013
- (2) **Real-time analysis of Imatinib- and Dasatinib- induced effects on chronic myelogenous leukemia cell interactions with fibronectin**
Obr A, Röselová P, Grebeňová D, Kuželová K; PLOS ONE, 2014
- (3) **Adhesion structures in leukemia cells and their regulation by Src family kinases**
Röselová P, Obr A, Holoubek A, Grebeňová D, Kuželová K; Cell Adhesion & Migration, 2017

Real-time monitoring of hematopoietic cell interaction with fibronectin fragment

The effect of histone deacetylase inhibitors

Adam Obr, Pavla Röslová, Dana Grebenová and Kateřina Kuželová*

Department of Cellular Biochemistry; Institute of Hematology and Blood Transfusion; Prague, Czech Republic

Keywords: cellular adhesion, microimpedance, fibronectin, SAHA, tubastatin A, Lyn

Abbreviations: RTCA, real-time cell analysis; FNF, fibronectin fragment (cell attachment region); SAHA, suberoylanilide hydroxamic acid; tubA, tubastatin A; ECM, extracellular matrix; DMSO, dimethylsulfoxide; TPA, 12-O-tetradecanoylphorbol-13-acetate

Real-time cell analysis (RTCA) system based on measurement of electrical microimpedance has been introduced to monitor adherent cell cultures. We describe its use for real-time analysis of hematopoietic cell adhesion to bone marrow stroma proteins. Cells growing in suspension do not generate any significant change in the microimpedance signal until the surface with embedded microelectrodes is coated with a cell-binding protein. We show that in this case, the microimpedance signal specifically reflects cell binding to the coated surface. The optimized method was used to monitor the effect of two histone deacetylase inhibitors, suberoylanilide hydroxamic acid (SAHA) and tubastatin A, on JURL-MK1 cell adhesion to cell-binding fragment of fibronectin (FNF). Both compounds were used in non-toxic concentrations and induced an increase in the cell adhesivity. The kinetics of this increase was markedly slower for SAHA although tubulin hyperacetylation occurred rapidly for any of the two drugs. The strengthening of cell binding to FNF was paralleled with a decrease of Lyn kinase activity monitored using an anti-phospho-Src family antibody. The inhibition of Src kinase activity with PP2 accordingly enhanced JURL-MK1 cell interaction with FNF. Actin filaments were present at the proximity of the plasma membrane and in numerous membrane protrusions. In some cells, F-actin formed clusters at membrane regions interacting with the coated surface and these clusters colocalized with active Lyn kinase. Our results indicate that the role of Src kinases in the regulation of hematopoietic cell adhesion signaling is similar to that of c-Src in adherent cells.

Introduction

The interactions of hematopoietic cells with their environment are important for their proper development and function. Stem and progenitor hematopoietic cells reside in the bone marrow and their ability to survive, proliferate and differentiate depends from contacts with the bone marrow extracellular matrix (ECM) as well as with stromal cells.¹ Hematopoietic cell interaction with the extracellular matrix is also involved in stem cell homing to the bone marrow which is required for a successful hematopoietic recovery after transplantation.² The mature forms of hematopoietic cells are released into the peripheral blood and the lymph where they circulate without stable attachment to a matrix. Nevertheless, they still form transient contacts with endothelial cells and matrix proteins, e.g., during lymphocyte migration to inflammatory sites.³ Several hematological malignant diseases, such as chronic myeloid leukemia or multiple myeloma, are characterized by alteration of cell adhesivity to ECM proteins.^{4,5} However, the molecular mechanisms of these defects are

poorly understood. Leukemic cell adhesion to the bone marrow microenvironment also confers chemotherapy drug resistance, promotes leukemia cell survival and contributes to the residual disease.^{6,7}

The basic method to assess the cellular adhesivity to a matrix protein consists in cell incubation in wells coated with the protein of interest, washing and determination of the number of the attached cells (cell counting or cell staining followed by photometric detection). To our experience, this method allows for reliable detection of long-lasting changes in the cellular adhesivity to the coated surface when the difference between samples represents at least 20% of the adhered cell fraction. Our previous screening experiments have shown that cells derived from chronic myelogenous leukemia (JURL-MK1 and K562 cell lines) specifically attach to fibronectin, but not to vitronectin, laminin or collagens.⁸ We thus use fibronectin as a simple model of the bone marrow extracellular matrix proteins.

The real-time cell analysis (RTCA) system (Roche) has been introduced for continuous monitoring of adherent cell cultures. This label-free and non-invasive method is based on the

*Correspondence to: Kateřina Kuželová; Email: Katerina.Kuzelova@uhkt.cz
Submitted: 01/18/13; Revised: 03/11/13; Accepted: 04/03/13
<http://dx.doi.org/10.4161/cam.24531>

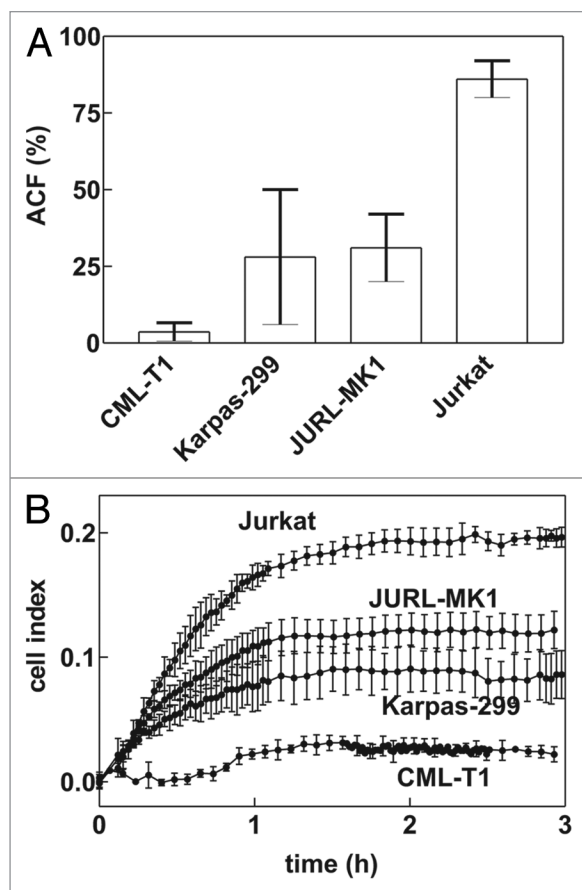


Figure 1. Adhesivity of different cell lines to fibronectin fragment (FNF). (A) Fraction of adhered cells after 1 h incubation in FNF-coated wells as determined by the end-point method for the indicated leukemic cell lines. The bars show means and standard deviations from multiple experiments (7 to 20). (B) Microimpedance signal from RTCA apparatus. E-plate was coated with FNF, equilibrated in RPMI and 60,000 cells from the indicated cell line was added to wells in quadruplicates. Means and standard deviations of quadruplicates are plotted. The results are representative from several experiments (3 to 15).

measurement of electrical impedance between interdigitated microelectrodes which are integrated on the bottom of tissue culture plates.⁹ The impedance measurement provides quantitative information about the biological status of the cells, including cell number, viability, and morphology of adherent cells. In this work, we describe its use for real-time monitoring of hematopoietic cell adhesion to fibronectin fragment-coated surfaces.

We have previously shown that suberoylanilide hydroxamic acid (SAHA) and tubastatin A (tubA) increase leukemic cell adhesivity to fibronectin.^{8,10} We now describe the use of RTCA system for monitoring the kinetics of changes in cell interaction with surface coated with cell-binding fragment of fibronectin (FNF) upon treatment with these histone deacetylase inhibitors.

Results

Tissue culture plates (200 μ L wells) with embedded electrodes were coated with fibronectin fragment (FNF) or bovine serum

albumin (BSA) as a control. In the first series of experiments, different amount of JURL-MK1 cells (in 100 μ L of cell suspension) were applied to the wells, which were pre-equilibrated with RPMI medium (100 μ L). In non-coated or BSA-coated wells, the cell addition induced no or modest change in the measured microimpedance. We observed no significant increase in microimpedance even when the cells were seated to the bottom of the wells by short centrifugation. In FNF-coated wells, the signal increased in time reaching a plateau after about 2 h. Thereafter, the signal usually exhibited a slow decrease. The signal intensity increased with the cell number and 60,000 cells per well were sufficient to obtain clearly specific response. Higher cell numbers were considered not suitable as microscopic examination showed that about 100,000 cells almost entirely cover the area of the well bottom and additional cells would not have free access to the FNF-coated surface.

We have first studied the cell adhesivity to FNF using the end-point protocol: the cells were incubated for 1 h in FNF-coated wells, then the non-adhered cells were washed out and the attached cells were quantified using fluorometric measurement. We found that the adherent cell fraction (ACF) largely differed among various cell lines (Fig. 1A). Very similar results were obtained from the real-time monitoring of cellular adhesivity using RTCA device (Fig. 1B).

The ability of RTCA system to detect changes in cell binding to FNF was tested using the known activator of adhesion signaling pathways, 12-O-tetradecanoylphorbol-13-acetate (TPA). JURL-MK1 cells were allowed to adhere for about 2 h to FNF-coated wells and 1 μ L TPA (100 nM final concentration in the well) was added thereafter. The microimpedance signal started to increase immediately after TPA addition (Fig. 2A). Similar marked effect of TPA on the microimpedance signal was observed for other cell lines, e.g., for Jurkat cells (Fig. 2B), as well as for primary leukemic cells (data not shown). In the control wells (no addition), we observed only a small decrease of the signal which was probably due to slight mechanical perturbation of the cells during the manipulation (see also Fig. 4). In fact, transient signal fluctuations were regularly observed after each manipulation with the wells or with the entire RTCA device, even after gentle opening/closing the door of the incubator where the device was placed.

We also noted that the addition of compounds which were dissolved in dimethyl sulfoxide (DMSO) produced a sharp transient increase in the microimpedance signal. We hypothesized that this effect was an artifact being due to low DMSO conductivity. We thus tested the effect of DMSO addition to wells containing solely 200 μ L RPMI medium (without cells). As it is shown in Figure 3, the addition of 0.2–2 μ L DMSO (resulting in 0.1–1% DMSO in the well) was followed by transient abrupt increase of the microimpedance. For up to 0.4% DMSO, the signal returned to the basal level within 30 min. However, more durable signal change was observed at higher DMSO concentrations. In the subsequent experiments, DMSO amount was always kept at less than 0.1%.

The optimized RTCA system was then used to monitor the kinetics of drug effects on the cellular adhesivity to FNF. We have previously reported that suberoylanilide hydroxamic acid

(SAHA) treatment of leukemic cells induced an increase in the adherent cell fraction which was measured using the end-point protocol after 24 or 48 h incubation with the effector.⁸ Also, we found that another histone deacetylase inhibitor, tubastatin A (tubA), increased JURL-MK1 cell adhesivity to FNF as well.¹⁰ The effects of SAHA and tubastatin A on JURL-MK1 cell interaction with FNF was studied using RTCA device (Fig. 4). The cells were allowed to adhere for about 2 h and 0.5–1 μ M SAHA or 10 μ M tubA has been added thereafter. In agreement with the data obtained from the end-point method, both agents induced an increase in the observed microimpedance in comparison with the untreated controls. However, while the effect of tub A was nearly immediate, SAHA-induced increase occurred after a delay (5 to 9 h, range from 8 independent experiments). The effect of another histone deacetylase inhibitor, sodium butyrate, on JURL-MK1 cell interaction with FNF was closely similar to that of SAHA (data not shown).

Tubulin belongs to the known substrates of HDAC6 and SAHA or tub A treatment thus should result in tubulin acetylation. The kinetics of the increase in α tubulin acetylation was analyzed using western blotting and acetylation-specific anti-tubulin antibody. As shown in Figure 5, both drugs induced tubulin hyperacetylation within 1 h following the drug addition (the half-life was 20 to 30 min).

In our previous work, we analyzed in detail the kinetics of histone acetylation after SAHA treatment of JURL-MK1 cells and found that the extent of acetylation at multiple different acetylation sites progressively increased for 4 to 10 h, reaching up to 30-fold of the control value.¹⁰ We thus searched for changes in the acetylation level of histones upon tubA treatment, both by western blotting and immunofluorescence microscopy, for up to 24 h incubation with the drug. However, the increase in H2AK5, H4K8 and H4K16 levels was only mild, if any (2-fold at the maximum) and at any time point lower than that induced by SAHA (data not shown).

Src-family kinases (SFKs) are known to be essential for signal transduction from cell surface receptors including integrins. We thus explored the effect of HDAC inhibitors on SFK activity using a phospho-specific antibody recognizing pTyr416 in c-Src or the corresponding tyrosine residues in other SFKs (Lyn, Hck, Fyn, Lck and Yes). We analyzed mRNA levels for different members of SFK in JURL-MK1 cells and found that the dominant Src kinases expressed in these cells were Lyn A and Lyn B (data not shown). In agreement with this finding, the anti-phospho-SFK antibody reacted with two major bands of 56 and 53 kDa, probably corresponding to A and B isoforms of Lyn kinase (Fig. 6A). The intensity of p-SFK staining was reduced after treatment with SAHA, tubA or TPA. The effect was more pronounced and faster for tubA in comparison with SAHA (Fig. 6B). These results suggested that Lyn kinase activity may be related to JURL-MK1 cell adhesivity to FNF. We thus tested the effect of PP2, an inhibitor of Src family kinases and found that Lyn kinase inhibition with PP2 indeed resulted in an increase of the microimpedance signal in RTCA analyzer (Fig. 7).

In the attempt to reveal possible Lyn interaction with cell adhesion structures, JURL-MK1 cells interacting with

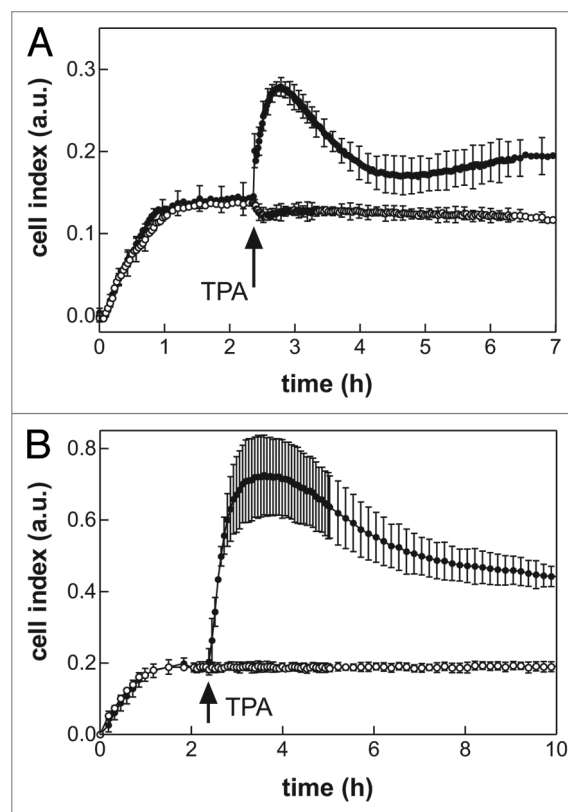


Figure 2. Effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on cell interaction with fibronectin (FNF). JURL-MK1 (A) or Jurkat (B) cells (60,000 per well), were allowed to adhere to FNF-coated wells and TPA (100 nM final concentration) was added after about 2 h. Open symbols: control wells without TPA, closed symbols: TPA-treated. Means and standard deviations of triplicates are shown. The time of TPA addition is marked with an arrow.

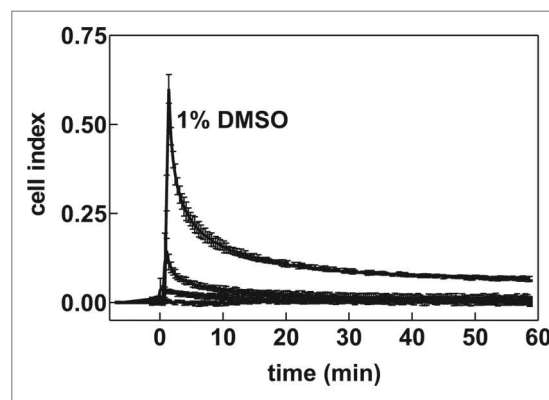


Figure 3. Transient effect of DMSO addition on the microimpedance signal. Non-coated wells were filled with RPMI medium and increasing volumes of DMSO were added at the time 0 to wells in triplicates. Means and standard deviations of triplicates are shown for control (no DMSO) and DMSO 0.1%, 0.4% and 1%. The experiment was repeated with closely similar results.

FNF-coated surface were analyzed using confocal microscopy. Filamentous actin (F-actin) was stained with FITC-phalloidin

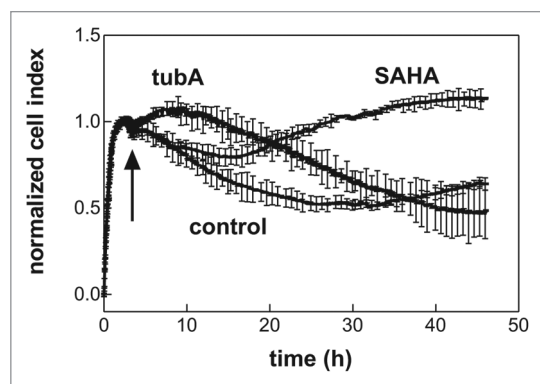


Figure 4. Effect of SAHA and tubastatin A on JURL-MK1 cell interaction with fibronectin fragment (FNF). The cells (60,000/well) were allowed to adhere to FNF and SAHA (1 μ M) or tubastatin A (10 μ M) were added at the time point indicated with the arrow. The lines are averages and standard deviations from triplicates. Similar time courses of the microimpedance signal were obtained in repeated experiments.

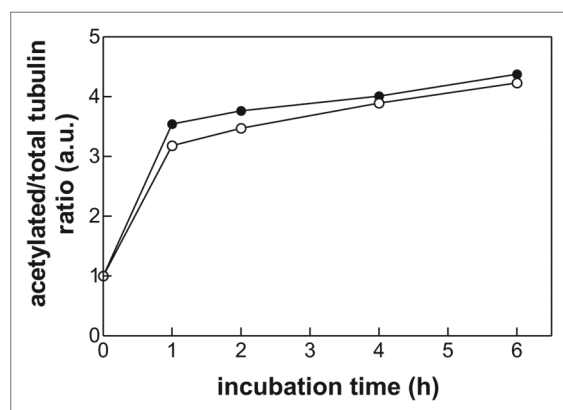


Figure 5. Kinetics of α tubulin acetylation after treatment of JURL-MK1 cells with 0.5 μ M SAHA (open circles) or 10 μ M tubastatin A (closed circles). Cell aliquots were taken at different time intervals and the relative amount of acetylated tubulin and total tubulin was determined using specific antibodies and western blotting. The ratio of acetylated tubulin/tubulin was expressed in relative units with regard to the control sample (without treatment).

while the localization of paxillin, vinculin, active (autophosphorylated) Lyn and inactive Lyn was visualized using immunofluorescence labeling. F-actin was present mainly at the proximity of the plasma membrane and in multiple fine membrane protrusions (Fig. 8A and B). After about 10–20 min incubation on FNF-coated surface, the cells started to spread, increasing their area in optical slices adjacent to the coated surface (Fig. 8B). The fraction of spread cells progressively increased with time reaching a maximum (20–30% of total cell number) after about 1 h. No cell spreading was observed on non-coated coverslips. In some spread cells, F-actin signal was concentrated into bright spots (up to 1–2 μ m in diameter) where it usually co-localized with the active form of Lyn kinase (Fig. 8C and D). On the other hand, no co-localization with F-actin has been detected for the inactive Lyn, vinculin or paxillin (data not shown). Both vinculin and paxillin displayed diffuse, homogeneous staining without any

discernible structure, with the exception of paxillin concentration in an area near the cell nucleus, presumably the centrosome (not shown).

Discussion

The real-time cell analysis system was optimized for hematopoietic cell monitoring. When the system is used for adherent cell types, 10,000 cells per well is usually recommended. In the case of hematopoietic cells, higher cell number had to be applied, probably because of the smaller cell size and also due to a weaker cell attachment. The diameter of JURL-MK1 cells is of 10–12 μ m (as measured using BioRad cell counter) while adherent cells typically cover the area of 200–1,000 μ m².¹¹ Furthermore, even minor mechanical perturbation of the RTCA system regularly produced transient decrease of the observed signal indicating that the attachment force is low and/or that the binding is highly dynamic.

The amplitude of the signal from RTCA measurement correlated well with the cellular adhesivity to FNF for different cell lines as it was determined by the end-point measurement (Fig. 1). These results further confirm that the observed signal mainly reflects the cell binding to FNF.

Using a known activator of adhesion pathways, TPA, we documented the ability of RTCA system to follow fast changes in cell interactions with the coated surface (Fig. 2). TPA treatment results in activation of intracellular substrates such as the protein kinase C, in cytoskeleton reorganization and in strengthening of integrin binding to fibronectin.^{12–14} A large increase in the microimpedance signal upon TPA addition was obtained not only for JURL-MK1 cells (Fig. 2A), but also for Jurkat cells (Fig. 2B), which are much more adherent than JURL-MK1. We determined by the end-point measurement that more than 85% of Jurkat cells remain attached to FNF after well washing in the absence of TPA (Fig. 2A) and only a small increase of this number (of about 5%) could be detected by this method after TPA treatment (data not shown). Thus, the increase in RTCA signal observed upon TPA addition reflects strengthening of the interaction rather than an increase in the number of the attached Jurkat cells.

Dimethyl sulfoxide is often used to solubilize compounds with biological activity. It is thus important to take into account that DMSO addition to water solution induces transient increase of microimpedance at the cell bottom (Fig. 3). It is likely that a drop of DMSO, which has a higher density than water, forms a layer with higher impedance at the well bottom. The layer is then progressively dissipated by diffusion and the impedance returns nearly to the original value if the final DMSO concentration is not higher than 0.4%.

After the series of proof-of-concept measurements, the system was used to monitor the effect of two histone deacetylase inhibitors—SAHA and tubA—on the interaction of leukemic cells with fibronectin fragment. Inhibition of histone deacetylases (HDACs) represents a new strategy in human cancer therapy since these enzymes play a fundamental role in regulating gene expression.^{15,16} SAHA is the first HDAC inhibitor that has

been approved for clinical use (treatment of cutaneous T-cell lymphoma). The mechanism of SAHA action is complex, cell type-dependent and can involve growth arrest, differentiation, senescence and various forms of cell death, such as the apoptosis or the mitotic cell death.¹⁷ We have recently reported that SAHA at subtoxic doses, which are achievable in clinical applications, also increases cell adhesivity to fibronectin in a variety of leukemic cell lines.⁸ TubA is a selective HDAC inhibitor that is highly efficient in inhibiting HDAC6 but it inhibits other HDACs as well when it is used in micromolar range. We expected that tubA could affect the cellular adhesivity to matrix proteins through acetylation of tubulin, the major structural protein of microtubules, which belongs to substrates of HDAC6. It was reported that decreased tubulin acetylation is related to increased cell migration and, on the other hand, tubulin hyperacetylation slows down the turnover of focal adhesions.^{18–20} RTCA analysis of the effects of SAHA and tubastatin A confirmed that both these drugs intensified JURL-MK1 cell interaction with FNF, although with different kinetics (Fig. 4). The effect of tubA treatment was nearly immediate while at least 5 h incubation with SAHA was necessary to induce a change in the microimpedance signal. The delay could be due to slower penetration of SAHA into the cells and we thus analyzed the acetylation of α tubulin as a marker of the drug presence in the cell. As shown in Figure 5, tubulin acetylation occurs rapidly after addition of either tubA or SAHA. This indicates that the drug penetration into the cells is fast and, also, that tubulin hyperacetylation is probably not the direct cause of the increase in JURL-MK1 cell adhesivity to FNF, at least in the case of SAHA.

The kinetics of SAHA-induced increase in JURL-MK1 cell adhesion to FNF is similar to that of histone acetylation described in our previous study.¹⁰ We thus hypothesized that the kinetics of histone acetylation might be faster in the case of tubA and this difference could explain the observed difference in the kinetics of cell interaction with FNF. However, we found only modest increase in histone acetylation when JURL-MK1 cells were treated with 10 μ M tubA.

The involvement of SFKs in the cell adhesion and migration is the most extensively described for the prominent member of the family, c-Src.²¹ This kinase binds to the cytoplasmic tails of integrins and assures among others maximal activation of the focal adhesion kinase (FAK), which is required both for focal adhesion assembly and disassembly.^{22,23} Sustained Src signaling promotes rapid focal adhesion turnover and cell migration while the stable cell attachment to the extracellular matrix is associated with an attenuation of Src activity.²¹ Hematopoietic cells predominantly express SFKs different from c-Src (such as Lyn or Hck). Also, the cytoskeleton structure of these cells is different from that of adherent cell types. While adherent cells form actin fibers and attach to the extracellular matrix through focal adhesions, mature leukocytes form specialized small adhesion structures, podosomes, which differ from focal adhesions, e.g., by dynamic turnover and the capacity to degrade the extracellular matrix.²⁴ Interestingly, it has been reported that podosomes are generated from focal adhesions due to c-Src overexpression in adherent cells.²⁵ The nature of adhesion structures in hematopoietic progenitors and

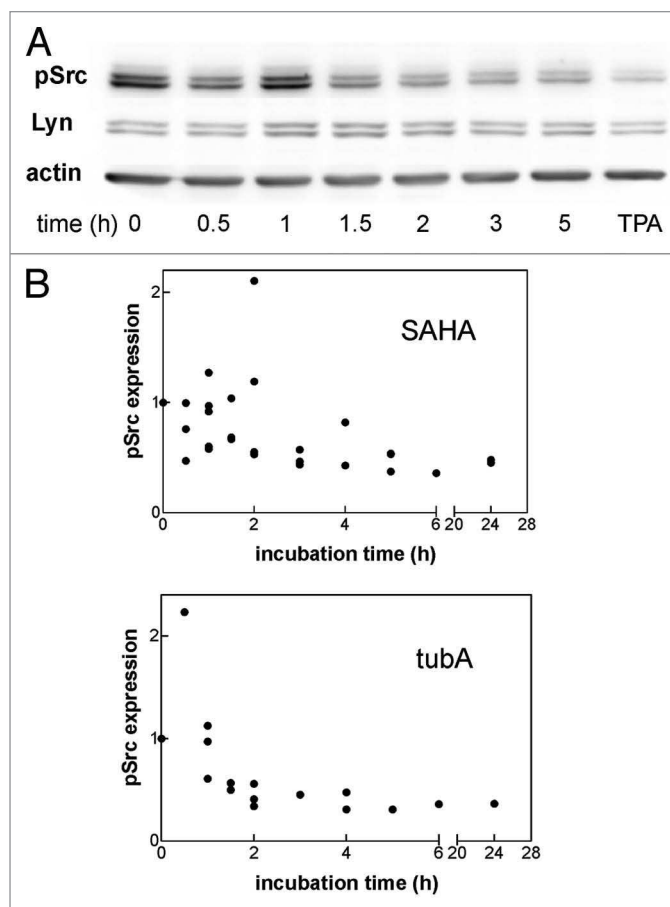


Figure 6. Effect of SAHA and tubastatin A on the activity of Src family kinases. JURL-MK1 cells were treated with 0.5 μ M SAHA or 10 μ M tubastatin A for up to 24 h and the level of autophosphorylated (active) Src kinases as well as that of Lyn kinase were assessed by western-blotting. (A) Representative blots from SAHA-treated cells at different incubation times. The effect of 30 min treatment with 100 nM TPA is shown in the last lane for comparison. (B) Summary results of anti-pSFK western-blot from repeated experiments ($n = 4$ for SAHA, $n = 3$ for tubA). The sum of the two main pSFK bands from each lane was expressed as relative to the sum in the corresponding control lane.

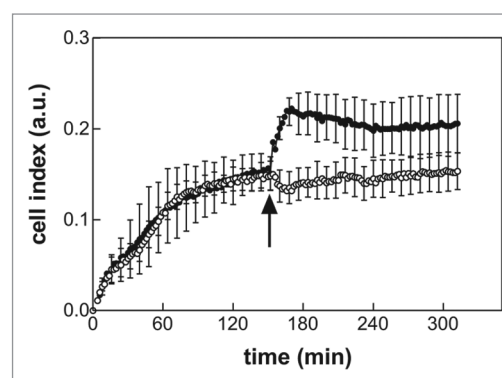


Figure 7. Effect of PP2 on JURL-MK1 cell interaction with FNF. The cells (60,000 per well), were allowed to adhere to FNF-coated wells and PP2 (10 μ M final concentration) was added thereafter. Open symbols, control wells without treatment; closed symbols, PP2. Means and standard deviations of triplicates are shown. The time of PP2 addition is marked with an arrow. Similar results were obtained in repeated experiments.

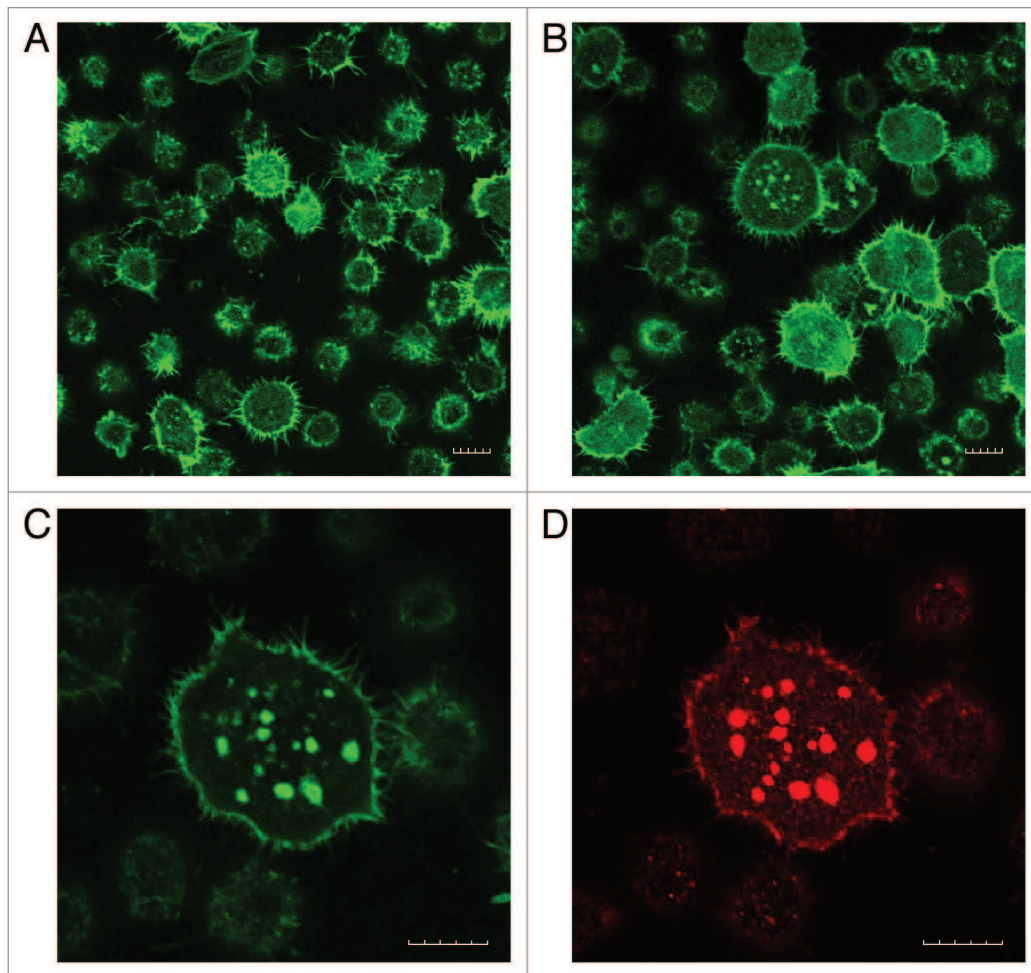


Figure 8. F-actin and p-SFK distribution in JURL-MK1 cells on FNF. The cells were seeded to FNF-coated slide, incubated for 15 min (**A**) or 1 h (**B–D**) at 37°C, then fixed and stained with FITC-phalloidin (**A–C**) to visualize actin fibers and anti-p-SFK antibody (**D**) to detect the active form of Lyn kinase. The microscope was focused to the plane adjacent to the coated slide. (**A and B**) show representative images from repeated experiments. (**C and D**) show a cell with prominent F-actin clusters. Scale bars: 10 μ m.

unmature cells like leukemic cell lines has not been well described to date. However, the involvement of hematopoietic SFK members in the regulation of these structures may be similar to that of c-Src. The multi-specific antibody we used detects two dominant bands of active SFKs in JURL-MK1 cells (Fig. 6A), which are likely to correspond to two splicing isoforms of Lyn kinase.²⁶ Indeed, two bands at the same molecular weight are detected in JURL-MK1 cells using a specific anti-Lyn antibody (Fig. 6A). Using the phospho-specific antibody, we showed that the level of active SFKs was reduced by both HDAC inhibitors (Fig. 6B) and also by TPA (last lane in Fig. 6A). On the other hand, although it has been reported that HDAC inhibitors can induce downregulation of c-Src level,^{27,28} neither SAHA nor tubA reduced total Lyn expression level (Fig. 6A for SAHA, not shown for tubA). Src family kinase inhibition with PP2 resulted in an immediate rise of the microimpedance signal (Fig. 7). It is thus possible that HDAC inhibition reduces SFK activity and thereby leads to stabilization of adhesion structures.

It has been shown that adhesion of hematopoietic CD34⁺ progenitors to fibronectin is mediated by $\alpha 5 \beta 1$ integrins (VLA-5).²⁹

JURL-MK1 cells are CD34 positive and express $\beta 1$ and $\beta 2$ integrins, including $\alpha 5 \beta 1$.⁸ We suppose that the adhesion structures are formed at the cytoplasmic parts of integrins as it occurs in adherent cells and lymphocytes. However, adhesion structures in leukocytes are small and dynamic and their visualization is challenging.³⁰ All known forms of adhesion structures associate with polymerized actin. In JURL-MK1 cells, F-actin was found in numerous membrane protrusions (filopodia) and, in addition, seemed to aggregate into clusters of different size at the contact of the cell membrane with fibronectin (Fig. 8). However, we were unable to correlate the localization of F-actin with common markers of adhesion structures, such as paxillin and vinculin. Although the nature and the function of the clusters is not clear, they may be regulated by Lyn kinase, as the active form of Lyn was found to localize to them. Previously, Lyn has been shown to localize in putative adhesion structures (F-actin rich spots) formed in another chronic myelogenous leukemia-derived cell line, K562.³¹ Identification of molecular composition and function of adhesion structures in hematopoietic cells will require further investigation.

Materials and Methods

Materials. Fibronectin fragment (120 kDa cell attachment region) was purchased from Chemicon International. Suberoylanilide hydroxamic acid (SAHA) was obtained from Cayman Chemical (distributed by the Axxora platform) and 2 mM stock solution was prepared in dimethyl sulfoxide (DMSO). Tubastatin A was supplied by BioVision and 10 to 20 mM stock solutions were made in DMSO. Further dilutions of both effectors were made in sterile water. 12-O-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma, stock solution (2 mM) was prepared in ethanol. The mouse monoclonal antibody to acetylated α tubulin was purchased from Abcam, the mouse monoclonal antibody against α tubulin conjugated with a fluorescence dye (Alexa488) from Invitrogen. The anti-phospho-Src family (Tyr416) and anti-non-phospho-Src family antibody was from Cell Signaling, mouse anti-Lyn antibody and mouse anti-paxillin antibody from BD Biosciences. The antibodies against acetylated histones H2AK5, H4K16 and H4K8, anti-histone H2A and H4 antibodies and anti-vimentin were from Abcam.

Cell culture. JURL-MK1, CML-T1 and Karpas-299 cell lines were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures), Jurkat cells from the European Collection of Animal Cell Cultures. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 4 mM L-alanyl-L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C in 5% CO₂ humidified atmosphere.

Plate coating with fibronectin fragment (FNF). To prepare the coated plate, 50 μ L of fibronectin fragment solution (20 μ g/mL in distilled water) was added to each well of a Nunc Maxisorp 96-well microtitration plate or of 16-well E-plate for RTCA and the plates were subsequently incubated overnight in the cold (10°C). Then, the wells were washed three times in PBS and the remaining protein adherence sites were blocked by 200 μ L 1% bovine serum albumin (BSA) in PBS for at least 30 min at room temperature. The plate was washed once again in PBS immediately before use. Control wells (BSA-coated) were incubated with 4% BSA instead of FNF overnight in the cold.

Adhesivity measurement—end point protocol. The end-point method for assessment of cellular adhesivity to FNF has been described previously.⁸ Briefly, the cells (1×10^4) were seeded into FNF-coated wells of a microtitration plate and incubated for 1 h at 37°C. Then, the wells were washed with PBS/Ca²⁺/Mg²⁺ using a multichannel adaptor to the suction-pump and the remaining cells were quantified by means of fluorescent labeling (Cy-Quant Cell Proliferation Assay Kit; Molecular Probes). The adherent cell fraction (ACF) was calculated using the fluorescence signal from FNF-coated plate and that from reference plate, which contained the total cell number.

Real-time cell analysis. The real-time analysis was performed using RTCA XCelligence DP system from Roche, which was placed in an incubator (at 37°C) with regulated CO₂ content (5%). FNF-coated E-plates containing 100 μ L RPMI 1640 culture medium per well were equilibrated at 37°C and the electrical microimpedance signal (cell index) was set to zero in these conditions. The cells (60,000 cells if not specified otherwise) were added in 100 μ L of suspension in RPMI, usually in quadruplicates. After the cell attachment (saturation of the microimpedance signal), effectors were added in a small volume of solvent (max. 2 μ L). Special care was taken to limit cell perturbation during the manipulation as even mild shaking resulted in signal fluctuations.

Electrophoresis and western blotting. The amount of selected proteins was analyzed using electrophoretic separation of cell lysates on 10% polyacrylamide gels and western blotting using specific antibodies. The method was described in detail previously.³² The chemiluminescence or fluorescence signal from membranes was detected and evaluated using G-box iChemi XT4 digital imaging device (Syngene Europe). The ratio of signals from acetylated tubulin and from total tubulin was calculated for each sample and the relative increase in tubulin acetylation after different incubation times was expressed as fold increase of the ratio value from the control sample (prior to drug addition). Membranes which were assessed for phosphorylated Src kinases were reprobed using anti-actin antibody and the level of p-SFK was expressed as relative to the actin level.

Confocal microscopy. The morphology of JURL-MK1 cells on fibronectin-coated surface was analyzed using Olympus Fluoview FV1000 microscope. The cells were seeded on FNF-coated slides (Superfrost Plus, Thermo Scientific) and incubated for the specified time interval at 37°C in a humidified chamber. Cells were fixed in 2% PFA, permeabilized in 0.3% Triton X-100 and treated with Image-iT FX signal enhancer before staining with FITC-conjugated phalloidin to visualize polymerized actin or with primary antibodies against pSFK, non-pSFK, paxillin or vinculin followed by the appropriate fluorescent secondary antibody. The samples were mounted in mowiol mounting media and the confocal fluorescence signals were recorded along with the differential interference contrast image.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The work was supported by the Ministry of Health of the Czech Republic (Project for conceptual development of research organization, 00023736) and by European Union (ERDF OPKK CZ.2.16/3.1.00/24/001). We wish to thank M. Voráčová for the expert technical assistance in western blotting.

References

- Prosper F, Verfaillie CM. Regulation of hematopoiesis through adhesion receptors. *J Leukoc Biol* 2001; 69:307-16; PMID:11261776.
- Sahin AO, Buitenhuis M. Molecular mechanisms underlying adhesion and migration of hematopoietic stem cells. *Cell Adh Migr* 2012; 6:39-48; PMID:22647939; <http://dx.doi.org/10.4161/cam.18975>
- Long EO. ICAM-1: getting a grip on leukocyte adhesion. *J Immunol* 2011; 186:5021-3; PMID:21505213; <http://dx.doi.org/10.4049/jimmunol.1100646>
- Salesse S, Verfaillie CM. Mechanisms underlying abnormal trafficking and expansion of malignant progenitors in CML: BCR/ABL-induced defects in integrin function in CML. *Oncogene* 2002; 21:8605-11; PMID:12476307; <http://dx.doi.org/10.1038/sj.onc.1206088>
- Kuzelová K, Hrkál Z. Rho-signaling pathways in chronic myelogenous leukemia. *Cardiovasc Hematol Disord Drug Targets* 2008; 8:261-7; PMID:19075636; <http://dx.doi.org/10.2174/187152908786786241>
- Maffei R, Fiorcari S, Bulgarelli J, Martinelli S, Castelli I, Deaglio S, et al. Physical contact with endothelial cells through β 1- and β 2-integrins rescues chronic lymphocytic leukemia cells from spontaneous and drug-induced apoptosis and induces a peculiar gene expression profile in leukemic cells. *Haematologica* 2012; 97:952-60; PMID:22207686; <http://dx.doi.org/10.3324/haematol.2011.054924>
- Becker PS. Dependence of acute myeloid leukemia on adhesion within the bone marrow microenvironment. *Scientific World Journal* 2012; 2012:856467; PMID:22346731; <http://dx.doi.org/10.1100/2012/856467>
- Kuzelová K, Pluskalová M, Brodská B, Otevřelová P, Elkněrová K, Grebenová D, et al. Suberoylanilide hydroxamic acid (SAHA) at subtoxic concentrations increases the adhesivity of human leukemic cells to fibronectin. *J Cell Biochem* 2010; 109:184-95; PMID:19911379; <http://dx.doi.org/10.1002/jcb.22397>
- Xi B, Yu N, Wang X, Xu X, Abassi YA. The application of cell-based label-free technology in drug discovery. *Biotechnol J* 2008; 3:484-95; PMID:18412175; <http://dx.doi.org/10.1002/biot.200800020>
- Grebenová D, Röslová P, Pluskalová M, Halada P, Rösel D, Suttar J, et al. Proteins implicated in the increase of adhesivity induced by suberoylanilide hydroxamic acid in leukemic cells. *J Proteomics* 2012; 77:406-22; PMID:23022583; <http://dx.doi.org/10.1016/j.jprot.2012.09.014>
- Park K, Millet LJ, Kim N, Li H, Jin X, Popescu G, et al. Measurement of adherent cell mass and growth. *Proc Natl Acad Sci U S A* 2010; 107:20691-6; PMID:21068372; <http://dx.doi.org/10.1073/pnas.1011365107>
- Davis GE, Camarillo CW. Regulation of integrin-mediated myeloid cell adhesion to fibronectin: influence of disulfide reducing agents, divalent cations and phorbol ester. *J Immunol* 1993; 151:7138-50; PMID:7505022.
- Imamura H, Takaishi K, Nakano K, Kodama A, Oishi H, Shiozaki H, et al. Rho and Rab small G proteins coordinately reorganize stress fibers and focal adhesions in MDCK cells. *Mol Biol Cell* 1998; 9:2561-75; PMID:9725912.
- Tulla M, Helenius J, Jokinen J, Taubenberger A, Müller DJ, Heino J. TPA primes α 2 β 1 integrins for cell adhesion. *FEBS Lett* 2008; 582:3520-4; PMID:18804470; <http://dx.doi.org/10.1016/j.febslet.2008.09.022>
- Monneret C. Histone deacetylase inhibitors. *Eur J Med Chem* 2005; 40:1-13; PMID:15642405; <http://dx.doi.org/10.1016/j.ejmech.2004.10.001>
- Marks PA, Xu WS. Histone deacetylase inhibitors: Potential in cancer therapy. *J Cell Biochem* 2009; 107:600-8; PMID:19459166; <http://dx.doi.org/10.1002/jcb.22185>
- Xu WS, Parmigiani RB, Marks PA. Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene* 2007; 26:5541-52; PMID:17694093; <http://dx.doi.org/10.1038/sj.onc.1210620>
- Tran AD, Marmo TP, Salam AA, Che S, Finkelstein E, Kabarriti R, et al. HDAC6 deacetylation of tubulin modulates dynamics of cellular adhesions. *J Cell Sci* 2007; 120:1469-79; PMID:17389687; <http://dx.doi.org/10.1242/jcs.03431>
- Wang YH, Yan ZQ, Qi YX, Cheng BB, Wang XD, Zhao D, et al. Normal shear stress and vascular smooth muscle cells modulate migration of endothelial cells through histone deacetylase 6 activation and tubulin acetylation. *Ann Biomed Eng* 2010; 38:729-37; PMID:20069369; <http://dx.doi.org/10.1007/s10439-009-9896-6>
- Wu Y, Song SW, Sun J, Bruner JM, Fuller GN, Zhang W. Ilp45 inhibits cell migration through inhibition of HDAC6. *J Biol Chem* 2010; 285:3554-60; PMID:20008322; <http://dx.doi.org/10.1074/jbc.M109.063354>
- Guarino M. Src signaling in cancer invasion. *J Cell Physiol* 2010; 223:14-26; PMID:20049846; <http://dx.doi.org/10.1002/jcp.22011>
- Huveneers S, Danen EH. Adhesion signaling - crosstalk between integrins, Src and Rho. *J Cell Sci* 2009; 122:1059-69; PMID:19339545; <http://dx.doi.org/10.1242/jcs.039446>
- Ruest PJ, Roy S, Shi E, Mernaugh RL, Hanks SK. Phosphospecific antibodies reveal focal adhesion kinase activation loop phosphorylation in nascent and mature focal adhesions and requirement for the autophosphorylation site. *Cell Growth Differ* 2000; 11:41-8; PMID:10672902.
- Dovas A, Cox D. Signaling networks regulating leukocyte podosome dynamics and function. *Cell Signal* 2011; 23:1225-34; PMID:21342664; <http://dx.doi.org/10.1016/j.cellsig.2011.02.004>
- Oikawa T, Takenawa T. PtdIns(3,4)P2 instigates focal adhesions to generate podosomes. *Cell Adh Migr* 2009; 3:195-7; PMID:19262173; <http://dx.doi.org/10.4161/cam.3.2.7510>
- Dos Santos C, Demur C, Bardet V, Prade-Houdellier N, Payrastre B, Récher C. A critical role for Lyn in acute myeloid leukemia. *Blood* 2008; 111:2269-79; PMID:18056483; <http://dx.doi.org/10.1182/blood-2007-04-082099>
- Maa MC, Chang MY, Hsieh MY, Chen YJ, Yang CJ, Chen ZC, et al. Butyrate reduced lipopolysaccharide-mediated macrophage migration by suppression of Src enhancement and focal adhesion kinase activity. *J Nutr Biochem* 2010; 21:1186-92; PMID:20149623; <http://dx.doi.org/10.1016/j.jnutbio.2009.10.004>
- Mologni L, Cleris L, Magistroni V, Piazza R, Boschelli F, Formelli F, et al. Valproic acid enhances bosutinib cytotoxicity in colon cancer cells. *Int J Cancer* 2009; 124:1990-6; PMID:19123474; <http://dx.doi.org/10.1002/ijc.24158>
- Peled A, Kollet O, Ponomarev T, Petit I, Franitza S, Grabovsky V, et al. The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. *Blood* 2000; 95:3289-96; PMID:10828007.
- Vicente-Manzanares M, Horwitz AR. Adhesion dynamics at a glance. *J Cell Sci* 2011; 124:3923-7; PMID:22194302; <http://dx.doi.org/10.1242/jcs.095653>
- Poincloux R, Cougoule C, Daubon T, Maridonneau-Parini I, Le Cabec V. Tyrosine-phosphorylated STAT5 accumulates on podosomes in Hck-transformed fibroblasts and chronic myeloid leukemia cells. *J Cell Physiol* 2007; 213:212-20; PMID:17503465; <http://dx.doi.org/10.1002/jcp.21112>
- Kuzelová K, Grebenová D, Brodská B. Dose-dependent effects of the caspase inhibitor Q-VD-OPh on different apoptosis-related processes. *J Cell Biochem* 2011; 112:3334-42; PMID:21751237; <http://dx.doi.org/10.1002/jcb.23263>



Real-Time Analysis of Imatinib- and Dasatinib-Induced Effects on Chronic Myelogenous Leukemia Cell Interaction with Fibronectin

Adam Obr^{1,2}, Pavla Röslová¹, Dana Grebeňová¹, Kateřina Kuželová^{1*}

¹ Department of Cellular Biochemistry, Institute of Hematology and Blood Transfusion, Prague, Czech Republic, ² Department of Cell Biology, Faculty of Science, Charles University in Prague, Prague, Czech Republic

Abstract

Attachment of stem leukemic cells to the bone marrow extracellular matrix increases their resistance to chemotherapy and contributes to the disease persistence. In chronic myelogenous leukemia (CML), the activity of the fusion BCR-ABL kinase affects adhesion signaling. Using real-time monitoring of microimpedance, we studied in detail the kinetics of interaction of human CML cells (JURL-MK1, MOLM-7) and of control BCR-ABL-negative leukemia cells (HEL, JURKAT) with fibronectin-coated surface. The effect of two clinically used kinase inhibitors, imatinib (a relatively specific c-ABL inhibitor) and dasatinib (dual ABL/SRC family kinase inhibitor), on cell binding to fibronectin is described. Both imatinib and low-dose (several nM) dasatinib reinforced CML cell interaction with fibronectin while no significant change was induced in BCR-ABL-negative cells. On the other hand, clinically relevant doses of dasatinib (100 nM) had almost no effect in CML cells. The efficiency of the inhibitors in blocking the activity of BCR-ABL and SRC-family kinases was assessed from the extent of phosphorylation at autophosphorylation sites. In both CML cell lines, SRC kinases were found to be transactivated by BCR-ABL. In the intracellular context, EC50 for BCR-ABL inhibition was in subnanomolar range for dasatinib and in submicromolar one for imatinib. EC50 for direct inhibition of LYN kinase was found to be about 20 nM for dasatinib and more than 10 μ M for imatinib. Cells pretreated with 100 nM dasatinib were still able to bind to fibronectin and SRC kinases are thus not necessary for the formation of cell-matrix contacts. However, a minimal activity of SRC kinases might be required to mediate the increase in cell adhesivity induced by BCR-ABL inhibition. Indeed, active (autophosphorylated) LYN was found to localize in cell adhesive structures which were visualized using interference reflection microscopy.

Citation: Obr A, Röslová P, Grebeňová D, Kuželová K (2014) Real-Time Analysis of Imatinib- and Dasatinib-Induced Effects on Chronic Myelogenous Leukemia Cell Interaction with Fibronectin. PLoS ONE 9(9): e107367. doi:10.1371/journal.pone.0107367

Editor: Giovanni Maga, Institute of Molecular Genetics IMG-CNR, Italy

Received: May 23, 2014; **Accepted:** August 13, 2014; **Published:** September 8, 2014

Copyright: © 2014 Obr et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: The work was supported by the Ministry of Health of the Czech Republic (Project for conceptual development of research organization No. 00023736) and by European Union (grants ERDF OPK CZ.2.16/3.1.00/24001 and CZ.2.16/3.1.00/28007). AO acknowledges the support of the Grant Agency of Charles University (grant No. 498214). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: Katerina.Kuzelova@uhkt.cz

Introduction

Hematopoietic cell interaction with the extracellular matrix of the bone marrow influences the cell behaviour and development. The microenvironment regulates e.g. cell division rate, resistance to apoptosis and cell differentiation [1]. Chronic myelogenous leukemia (CML), which is characterized by the presence of the fusion tyrosine kinase BCR-ABL, is associated with altered cell interaction with extracellular matrix proteins [2,3]. In the advanced phases of CML, leukemic progenitors are prematurely released into the blood stream, probably due to decreased cell adhesivity to the bone marrow and to an altered response to chemokines, such as SDF-1. Since the introduction of targeted therapy using tyrosine kinase inhibitors, the majority of patients remain in long-term complete remission. However, the discontinuation of the therapy usually leads to disease relapse which indicates that the residual disease remains to be a major issue in CML management [4]. Intense research in this field indicates that the leukemic burden arises from quiescent, non-dividing cells

which are resistant to treatment [5,6]. Understanding the mechanisms regulating cell quiescence, such as cell adhesion to the bone marrow matrix, is thus important for further progress in CML therapy.

We have recently described a novel approach to monitor hematopoietic cell interaction with selected extracellular matrix proteins. Real-time measurement of microimpedance allows for monitoring changes in cell adhesion to surfaces coated with the protein of interest. In this work, we applied this method to study the interaction of CML-derived cell lines with fibronectin and the effects of the most commonly used tyrosine kinase inhibitors, imatinib (mainly targeting BCR-ABL) and dasatinib (a dual ABL/SRC family kinase inhibitor).

Materials and Methods

Chemicals

Dasatinib was purchased from Selleckchem, 50 mM and 1 mM stock solutions were made in sterile dimethylsulfoxide. Imatinib

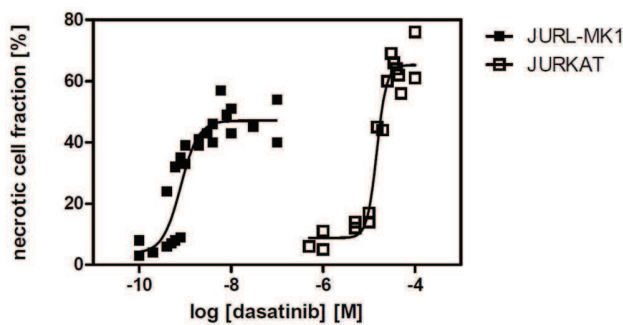


Figure 1. Dasatinib-induced cell death in BCR-ABL-positive and -negative cell lines. JURL-MK1 (full squares) and JURKAT (empty squares) cells were treated with dasatinib at different concentrations as indicated. After 45–48 h incubation, the fraction of non-viable cells was determined by counting of trypan blue-stained samples. Summary of 3 independent experiments for each cell line is shown.
doi:10.1371/journal.pone.0107367.g001

was obtained from Novartis (Basel, Switzerland), 2 mM stock solution was prepared in sterile water. Fibronectin fragment (120 kDa cell attachment region) was purchased from Chemicon International (CA, U.S.A.). To prepare a fibronectin-coated plate, 50 μ l of fibronectin fragment solution (20 μ g/ml in sterile water) was added to each well of a Nunc Maxisorp 96-well microtitration plate or of a 16-well E-plate used for real-time cell adhesion monitoring. The plates were subsequently incubated overnight at 10°C. After incubation, the plates were washed three times in PBS and blocked in 1% bovine serum albumin (BSA) in PBS (200 μ l/well, 30 min at room temperature). The plate was washed in PBS once again immediately before use.

Antibodies against phospho-SRC (Y417) family (#2101) and phospho-c-ABL (Y245, #2861) were purchased from Cell Signaling. Antibodies against HCK (#610278) and LYN (#610003) were purchased from BD Biosciences, antibody against LCK (Y123, #ab32149) was purchased from Abcam.

Cell isolation and culture

JURL-MK1 cell line was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), JURKAT cell line was from European Collection of Animal Cell Cultures (Salisbury, UK). MOLM-7 and HEL cells were provided by J. Minowada [7] and P. Martin [8], respectively.

Cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in 5% CO₂ humidified atmosphere.

Primary blood cells were obtained from leukapheresis product from patients with chronic myelogenous leukemia. Peripheral blood mononuclear cells (PBMC) were separated by standard density gradient centrifugation using Histopaque-1077 (Sigma) and maintained in RPMI 1640 medium described above.

Ethics statement

Primary leukemic blood cells were isolated from leukapheresis products, following written informed consent of the patient as to the use of biological material for research purposes. The research was approved by the Ethics Committee of the Institute of Hematology and Blood Transfusion.

Measurement of cell adhesivity to fibronectin

The end-point method for assessment of cellular adhesivity to fibronectin-coated surface has been described previously [9]. Briefly, the cells (1×10^4) were seeded into fibronectin-coated wells on a microtitration plate and incubated for 1 h at 37°C. Then, the cells were washed with PBS/Ca²⁺/Mg²⁺ and the remaining cells were quantified by means of fluorescent labelling (CyQuant Cell Proliferation Assay Kit; Molecular Probes). The adherent cell fraction (ACF) was calculated using the fluorescence signal from fibronectin-coated plate and the signal obtained from a reference plate that contained the total cell number.

Real-time microimpedance measurement

The real-time cell analysis was performed as described previously [10]. RTCA xCELLigence DP system (Acea Biosciences, San Diego, CA, U.S.A.) was placed in an incubator (at 37°C) with 5% CO₂ content. Fibronectin-coated E-plates were filled with 100 μ l of RPMI 1640 and equilibrated at 37°C. The microimpedance signal (cell index) was set to zero in these conditions. The cells (60000/well) were added in 100 μ l of suspension in RPMI 1640. Imatinib was pre-diluted in RPMI 1640 and added in total volume of 2 μ l/well in triplicates or quadruplicates, respectively. Dasatinib was pre-diluted in sterile DMSO and added in total volume of 0.2 μ l/well in triplicates or quadruplicates. The final DMSO concentration in wells did not exceed 0.1%. DMSO (0.1%) was also added to control wells.

Table 1. EC50 values for effects induced by imatinib and dasatinib.

cell line:	imatinib EC50	dasatinib EC50
JURL-MK1	proliferation (0.12 $\pm$ 0.05) μ M	proliferation (0.7 $\pm$ 0.1) nM
	cell death (0.35 $\pm$ 0.12) μ M	cell death (0.8 $\pm$ 0.3) nM
MOLM-7	proliferation (0.34 $\pm$ 0.04) μ M	proliferation (1.0 $\pm$ 0.4) nM
	cell death (1.8 $\pm$ 0.8) μ M	cell death (2.1 $\pm$ 1.0) nM
HEL	>10 μ M	proliferation (15 $\pm$ 1) μ M
		cell death (39 $\pm$ 6) μ M
JURKAT	>10 μ M	proliferation (11 $\pm$ 3) μ M
		cell death (21 $\pm$ 4) μ M

Cells were treated with the inhibitors at different concentrations for 48 h and counted. Cell viability was assessed using Trypan blue exclusion test. Measured values were fitted by sigmoidal curves using GraphPad Prism 5.0 software. Given EC50 values for inhibition of cell growth and for cell death induction are means and standard deviations from at least 3 independent experiments.

doi:10.1371/journal.pone.0107367.t001

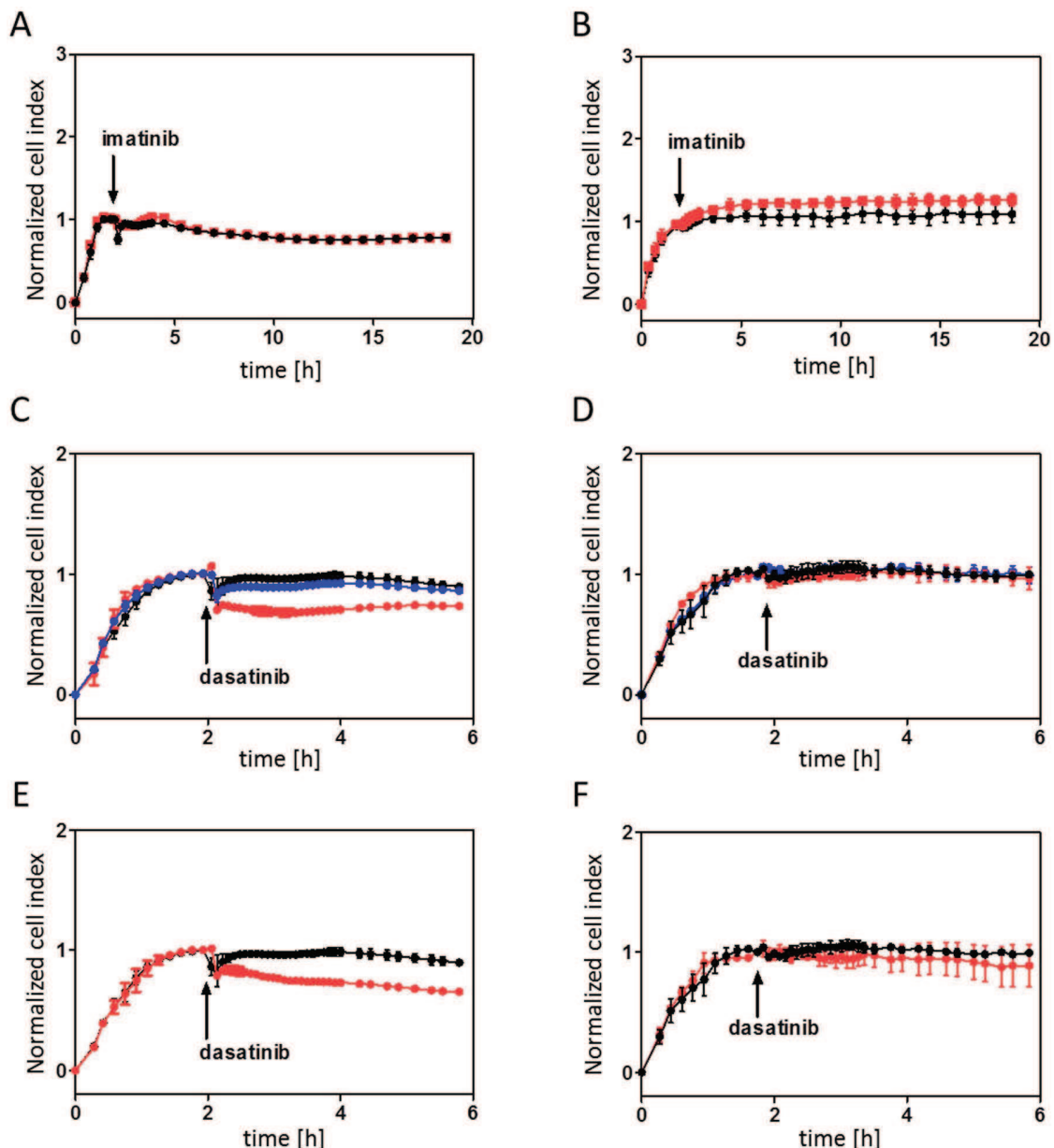


Figure 2. Changes in cell interaction with fibronectin after inhibitor treatment. The cells (6×10^4 per well) were seeded into fibronectin-coated E-plates. After the microimpedance signal stabilization, the appropriate inhibitor was added in triplets. Black circles: control cells. Time of inhibitor addition is indicated by an arrow. Microimpedance signal (cell index) was normalized to 1 at the time of inhibitor addition. The graphs show mean and standard deviation of well triplets. A,C,E: JURK-MK1 cells, B,D,F: MOLM-7 cells. A,B: imatinib was added at 1 μ M (blue circles) or 10 μ M (red squares) final concentration. C,D: dasatinib was added at 2 nM (blue circles) or 10 nM (red squares) final concentration. E,F: dasatinib was added at 100 nM final concentration (red circles). doi:10.1371/journal.pone.0107367.g002

Immunoblotting

SDS-polyacrylamide gel electrophoresis was performed as described before [11]. Briefly, the cells (5×10^6) were lysed in modified RIPA buffer and 15 μ g of total protein (30 μ g for BCR-ABL detection) was resolved with SDS-PAGE and transferred for 1 h (3 h for BCR-ABL) on a nitrocellulose membrane. Proteins of interest were detected using the appropriate antibodies and the chemiluminescent signal was visualised and evaluated using G-BOX iChemi XT4. To determine EC₅₀ values, relative band

intensities at different inhibitor concentrations were fitted with sigmoidal curves using GraphPad Prism 5 software.

Flow cytometry

The cells (1×10^6) were washed once in PBS, fixed and permeabilized with FIX&PERM Cell Permeabilization Kit (An Der Grub, Kaumberg, Austria), and incubated with anti-phospho-SRC family antibody overnight at 4°C and then with anti-rabbit PE-conjugated secondary antibody for 1 h at room temperature.

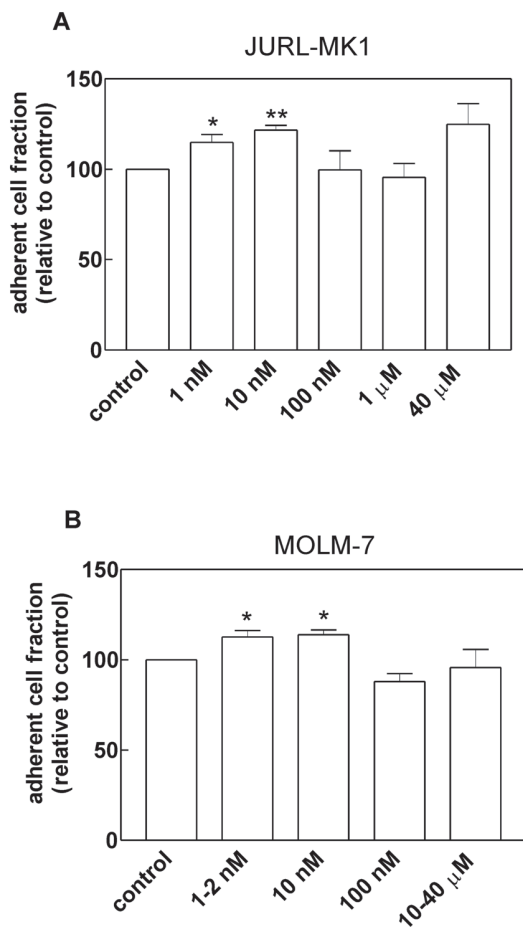


Figure 3. Changes in the adhered cell fraction induced by dasatinib pretreatment. JURL-MK1 (A) or MOLM-7 (B) cells were pretreated for 30 min with dasatinib at the indicated concentrations, seeded into FN-coated wells and incubated for 1 h at 37°C. Thereafter, the plate was washed and the fraction of attached cells was determined fluorimetrically and normalized using the value found in the control sample. The graphs show the means and standard deviations from at least 4 experiments for each condition, the statistical significance of the observed differences was evaluated using Student's paired t-test. Results significantly differing from untreated controls are denoted by asterisks (* $p < 0.05$, ** $p < 0.01$). doi:10.1371/journal.pone.0107367.g003

Detection of PE signal was performed using LSRT Fortessa flow cytometer (BD Biosciences).

Immunofluorescence microscopy

The cells were plated on fibronectin coated coverslips or glass bottom dishes, incubated for 60 minutes in CO₂ incubator, fixed with 2% paraformaldehyde, permeabilized in 0.3% Triton/PBS and incubated with anti-phospho-SRC family primary antibody and a secondary antibody (Alexa Fluor 488 Goat Anti-Rabbit). F-actin was labeled with rhodamine phalloidin (Molecular Probes). Samples were analyzed using an Olympus Fluoview FV1000 confocal laser scanning microscope.

Interference reflection microscopy

Cells were incubated for 1 h on fibronectin-coated coverslip and fixed with 2% paraformaldehyde. The interference in reflected light was observed by means of FV-1000 confocal microscope

(Olympus), using 405 nm laser beam and focusing to the coated glass surface.

Results

We analyzed the effects of imatinib mesylate and dasatinib on two CML-derived cell lines (JURL-MK1 and MOLM-7), and two cell lines not expressing BCR-ABL protein (HEL and JURKAT). As expected, both tyrosine kinase inhibitors (TKIs) reduced cell growth and induced cell death in BCR-ABL-positive cells while they were considerably less toxic in BCR-ABL-negative cells. Fig. 1 illustrates the difference in dasatinib toxicity between JURL-MK1 and JURKAT cells. EC50 values for cell growth inhibition and for cell death induction in all four cell lines are given in Table 1.

We have previously shown that imatinib mesylate affected JURL-MK1 cell adhesivity to fibronectin (FN), one of the major components of the extracellular matrix [12]. During first several hours of imatinib treatment, the cell fraction adhering to FN increased. On the contrary, longer imatinib treatment resulted in a reduction of cell binding to FN, probably due to the onset of apoptosis. These results suggested that BCR-ABL inhibition strengthens JURL-MK1 cell interaction with FN. Similar results were also obtained using dasatinib (Figure S1). After about 6 h of dasatinib treatment, but not before, caspases became activated and apoptotic cells started to appear (Figure S1, panels A–C). Following 24 h treatment, the apoptosis was triggered in a large cell fraction and, accordingly, cell adhesivity to fibronectin lowered. This decrease was prevented by simultaneous addition of the caspase inhibitor Q-VD-OPh (Figure S1, panel D). We also performed standard Annexin V staining following 6 h treatment of JURL-MK1 and MOLM-7 cells with 10 nM dasatinib and found no significant increase in the number of early apoptotic cells at this time point.

In this work, we studied the kinetics of cell-FN interaction after TKI treatment in detail using a recently introduced method based on monitoring of microimpedance between two interdigitated microelectrode systems embedded in the bottom of the wells of a microtitration plate [10]. We showed that, in case of hematopoietic cells, the microimpedance signal (denoted as “cell index”) reflects cell interaction with FN-coated surface. Figure 2 shows representative examples of the observed kinetics for the two CML cell lines, JURL-MK1 and MOLM-7. Cells were seeded into E-plates and incubated for 1–2 h to allow for cell attachment to the coated cell bottom. After signal stabilization, the drug was added in a small drop while the corresponding solvent was added to control wells as any mechanical perturbation usually produced minor changes, too. Imatinib mesylate addition to BCR-ABL-positive cells resulted in nearly immediate increase in the microimpedance signal, the effect being enhanced at higher imatinib concentration (Figure 2A,B). On the other hand, no appreciable change occurred in the control BCR-ABL-negative cell lines (Figure S2). More complex behaviour was observed after dasatinib treatment: at low (about 0.5 to 10 nM) concentration, dasatinib effect was similar to that of imatinib (Figure 2C,D). With increasing dasatinib dose, the effect was gradually suppressed and disappeared at several tens of nM (Figure 2E,F). Furthermore, at very high dasatinib concentration (up from 1 μM), an increase in the signal was observed in CML-derived cell lines as well as in BCR-ABL-negative cell lines (Figure S3) and even in freshly isolated primary mononuclear cells.

The increase in the microimpedance signal may be due to an increase in the fraction of interacting cells, but also to an increase in the individual cell area in contact with the coated surface (cell

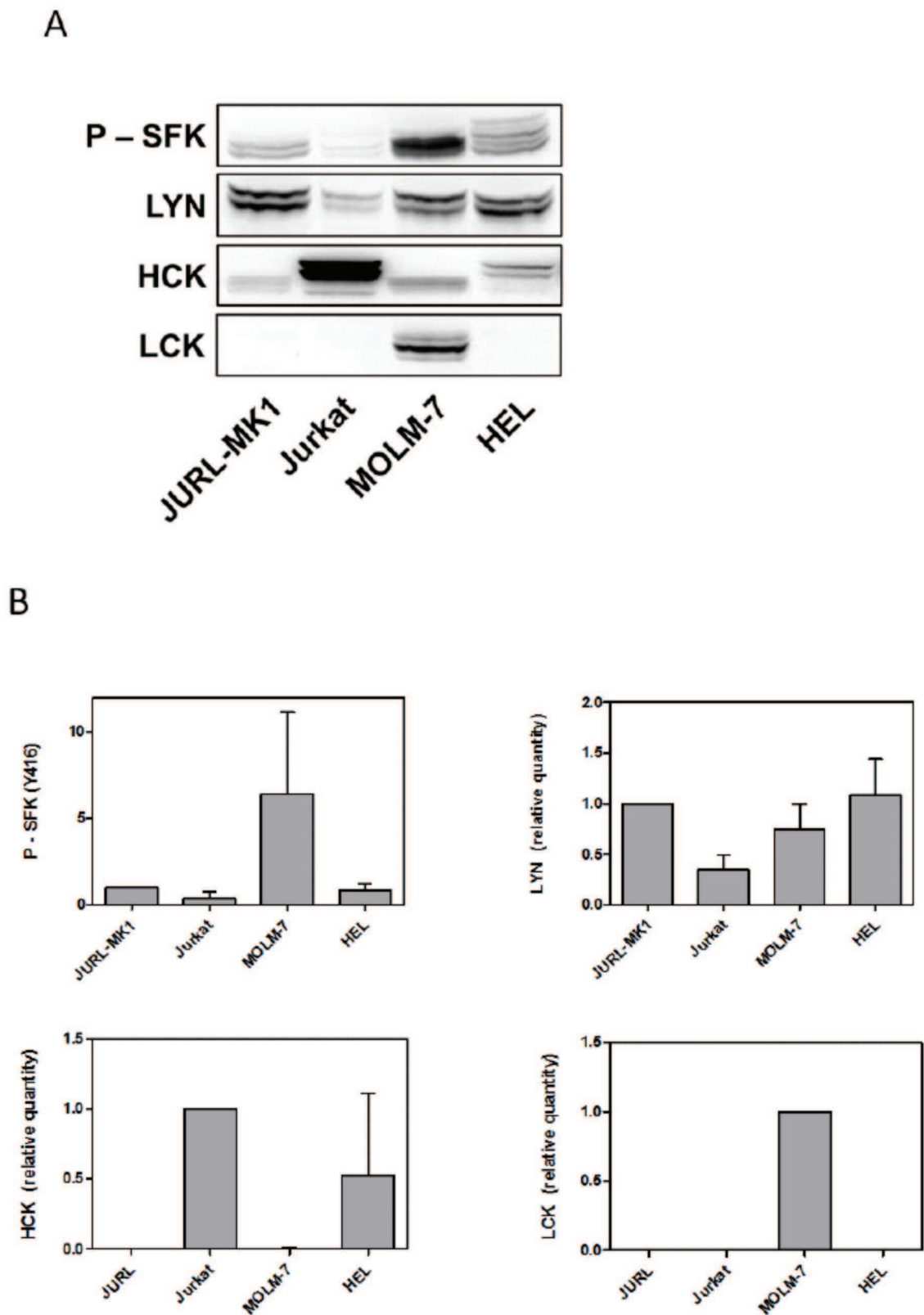


Figure 4. SRC family kinase profiles of leukemic cell lines. A: p-Y416 SFK, LYN, HCK and LCK expression pattern in the used cell lines. B: Summary of relative expression levels of p-Y416 SFK, LYN, HCK and LCK kinases in cell lines. Several (2–5) western-blot were quantified and normalized to the signal of JURL-MK1 cell line (p-SFK, LYN), JURKAT cell line (HCK) or MOLM-7 cell line (LCK).
doi:10.1371/journal.pone.0107367.g004

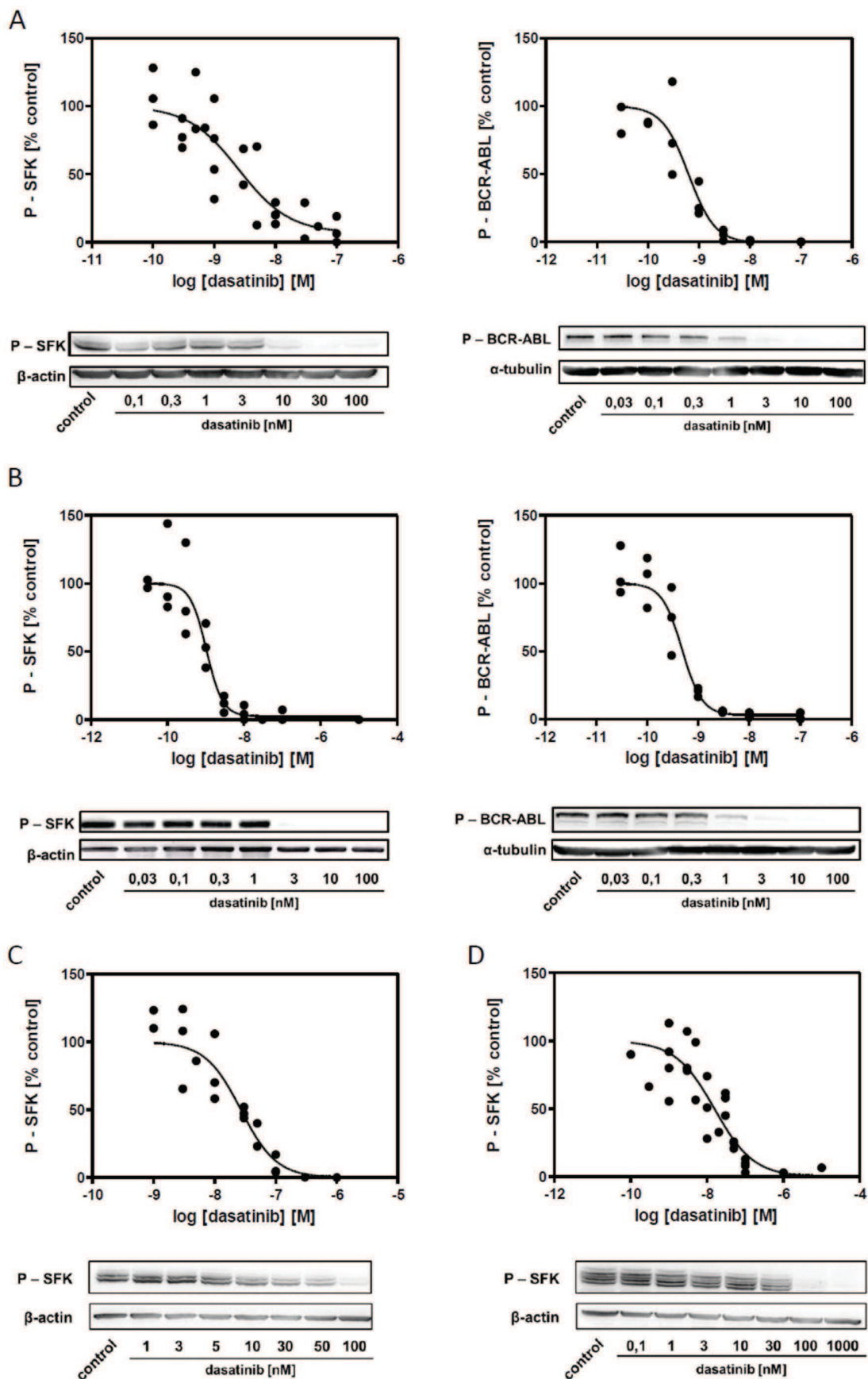


Figure 5. Dasatinib-induced dephosphorylation of SFK and BCR-ABL. Phosphorylation status of SFK and BCR-ABL after 2 h incubation with dasatinib. Quantification summary from 3 independent experiments and representative western-blots are shown for each cell line. A: JURL-MK1, B: MOLM-7, C: HEL, D: JURKAT.
doi:10.1371/journal.pone.0107367.g005

spreading) or to closer cell nestling against the surface. We thus compared the fraction of cells attached to FN-coated wells for control and dasatinib-treated cells. The cells were either seeded into the coated plate and subsequently treated with dasatinib or pretreated for 30 min with the drug and seeded thereafter. In both experimental settings, the results were similar. Both in JURL-MK1 and MOLM-7 cells, low dasatinib concentrations (1 to 10 nM), but not the intermediate ones (100 nM–1 μ M), induced a mild increase in the number of attached cells (Figure 3). However, the changes were less pronounced in comparison with those seen in microimpedance measurements (Figure 2C,D).

To account for possible roles of BCR-ABL and SRC-family kinases (SFks) in the observed adhesivity changes, we analyzed the extent and the kinetics of inhibition of these kinases due to TKI treatment. First, we screened the studied cell lines for mRNA transcripts of the individual members of SRC kinase family that are known to be expressed in hematopoietic cells (LYN A, LYN B, HCK, LCK, FGR). We found that all the four cell lines express mRNA for two Lyn splicing variants, LynA and LynB. In addition, Hck and Lck mRNA, but not Fgr, were expressed in some of them. The protein products of the relevant SFK genes were subsequently quantified by means of western-blotting (Figure 4). SFKs contain several autophosphorylation sites which may be used to assess the activation status of these kinases. The phospho-specific antibody we used recognizes Tyr 416 in c-SRC and the corresponding residues in HCK, LYN and LCK. LYN A and B are detected at 56 and 53 kDa, respectively, LCK at 54–55 kDa and two HCK isoforms (produced using different sites of initiation of translation) at 59 and 61 kDa.

To detect the activated form of BCR-ABL protein, phospho-specific antibody against phosphorylated Tyr245 of c-ABL was used and the band at 210 kDa corresponding to BCR-ABL was quantified.

The decrease in levels of phosphorylated BCR-ABL and SFKs after 2 h incubation with dasatinib at different concentration is documented in Figure 5 and EC50 values for BCR-ABL and SFK inhibition obtained from repeated experiments are given in Table 2. Note that similar results were obtained when the levels of phosphorylated SFKs were analyzed using flow-cytometry instead of western-blotting (Figure S4). We have also performed similar experiments with primary mononuclear cells from CML

patients in the chronic phase of the disease (N = 3) and EC50 values for SFK dephosphorylation after 2 h dasatinib treatment ranged between 20 and 29 nM.

When comparing EC50 values for dasatinib-mediated SFK inhibition in BCR-ABL-positive vs BCR-ABL-negative cells, we noted a clear difference indicating that SFK dephosphorylation in BCR-ABL-positive cells might be due to an indirect effect. SFKs are known to be targets of BCR-ABL and inhibition of BCR-ABL can thus result in a decrease in SFK activity. We thus assessed SFK phosphorylation status after treatment with imatinib which is considered to be relatively specific for BCR-ABL inhibition. Indeed, 1 μ M imatinib treatment markedly reduced SFK phosphorylation in BCR-ABL-positive cells while it had no or mild effect in HEL cells (Figure 6 and Figure S4).

The time course of kinase dephosphorylation in JURL-MK1 cells is shown in Figure 7. The effect of imatinib addition was very rapid: BCR-ABL was dephosphorylated in 5 minutes and the activity of SFKs was reduced at the same time. The kinetics of dephosphorylation was somewhat slower for 2 nM dasatinib. However, the decrease of BCR-ABL and SFK phosphorylation also occurred simultaneously. As expected, 100 nM dasatinib was sufficient to rapidly inhibit virtually all SFK molecules.

To support our hypothesis that SFKs may be involved in regulation of cell interaction with fibronectin, we studied the intracellular localization of active SFKs using the phospho-specific anti-SFK antibody. MOLM-7 cells on fibronectin had a spread morphology with numerous marked membrane protrusions while JURL-MK1 cells had a more compact form. Figure 8 A,B (right panels) shows images obtained using the interference reflection microscopy which allows for visualization of cell areas that are near to the coated surface. Immunofluorescence staining clearly shows an accumulation of active SFKs at the ends of membrane protrusions and in other areas of cell-surface contacts in MOLM-7 cells (Figure 8A, left panel). In JURL-MK1 cells, the active SFKs were found mainly in the contours of cell-surface contact areas and their localization to the sites of the closest contact was less evident due to different morphology (Figure 8B). As expected, 100 nM dasatinib treatment resulted in complete disappearance of the green fluorescent signal from adhesion sites (Figure S5). The same effect was also reached using the most commonly used SFK inhibitor PP2 (Figure S5, right panels). As the main SFKs in

Table 2. EC50 values for dasatinib-induced dephosphorylation of BCR-ABL and SFKs.

cell line:	BCR-ABL (nM)	SFK (nM)
JURL-MK1	0.6 (0.4–1.0)	2.5 (1.0–6.2)
MOLM-7	0.5 (0.3–0.7)	1.0 (0.7–1.6)
HEL	-	27 (15–48)
JURKAT	-	15 (6–41)
		HCK 5.2 (1.8–15)
		LYN 22 (9–53)

Cells were incubated for 2 h with dasatinib at different concentrations and the levels of phosphorylated kinases were then determined by western-blotting using phospho-specific antibodies. Relative values (% of controls) from at least 3 independent experiments were merged and the summary results were fitted with sigmoidal dose-response curves using GraphPad Prism 5.0 software. The table shows the obtained EC50 values along with 95% confidence interval. In case of JURKAT cells, bands corresponding to p-HCK and p-LYN were also evaluated separately.
doi:10.1371/journal.pone.0107367.t002

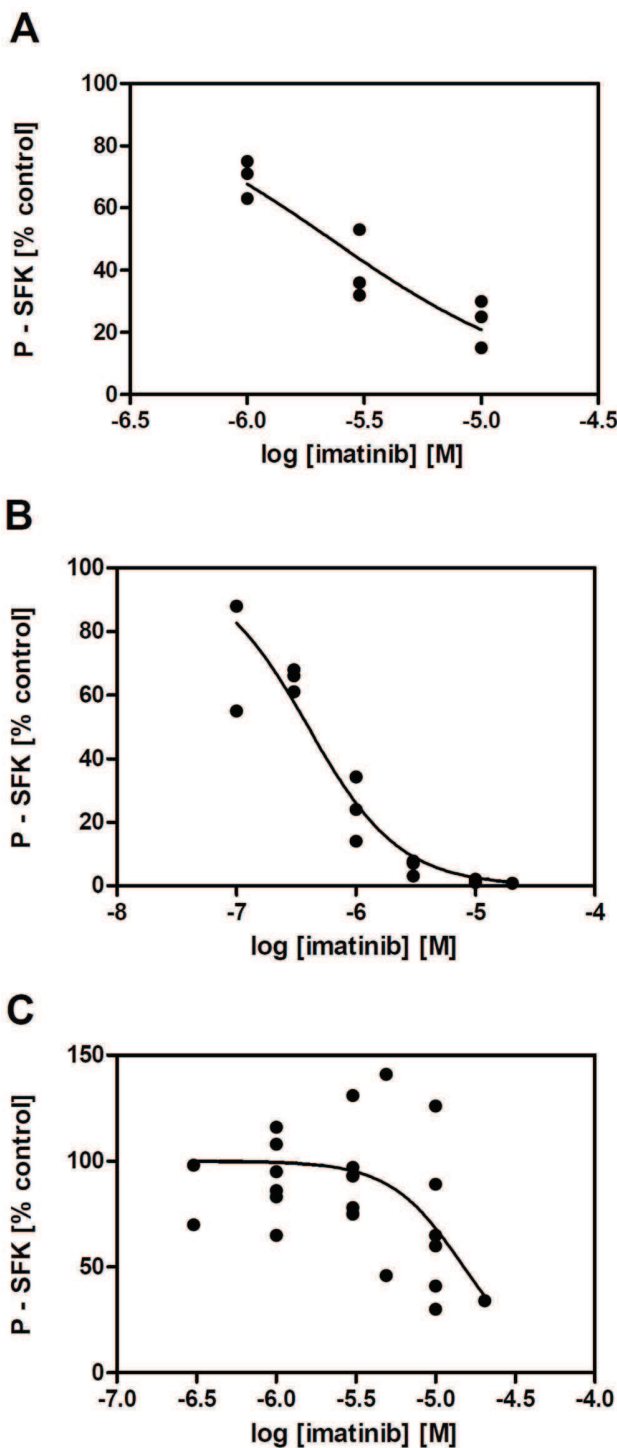


Figure 6. Imatinib-induced dephosphorylation of SFK in BCR-ABL-positive and -negative cell lines. Phosphorylation status of SFK after 2 h incubation with imatinib was assessed by western blotting. Summary of independent experiments for JURL-MK1 (A), MOLM-7 (B) and HEL cells (C). doi:10.1371/journal.pone.0107367.g006

JURL-MK1 and MOLM-7 were LYN and LYN+LCK, respectively, we also analyzed the intracellular localization of these individual kinases. LCK was mainly present in the nuclear

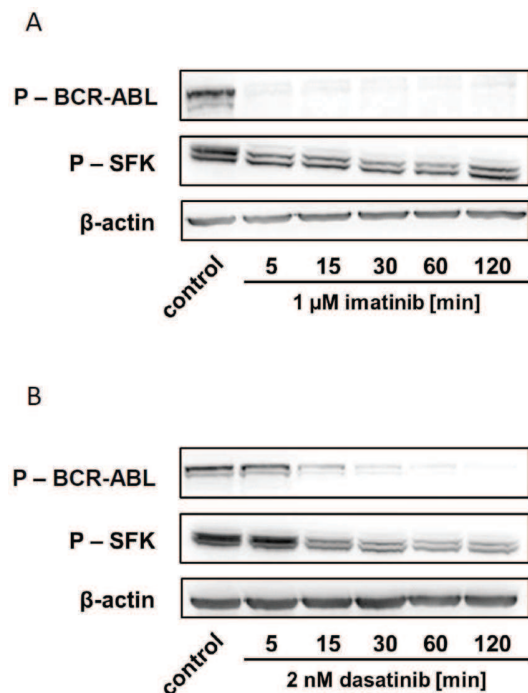


Figure 7. Time-course of BCR-ABL and SFK dephosphorylation after treatment with inhibitors. Time-resolved decrease in the phosphorylation status of BCR-ABL and SFK in JURL-MK1 cells after treatment with 1 μM imatinib (A) or 2 nM dasatinib (B). The experiment was repeated with closely similar results. doi:10.1371/journal.pone.0107367.g007

membrane (green signal in Figure 8C) while LYN was predominantly found in the cytoplasmic membrane and in the cytoplasm (green signal in Figure 8D for MOLM-7 cells, similar staining pattern was observed in JURL-MK1 cells).

Discussion

The analysis of cytotoxic effects of both tyrosine kinase inhibitors (Table 1) confirmed that these effects are related to the inhibition of BCR-ABL protein. EC₅₀ values for JURL-MK1 and MOLM-7 cell death induction by imatinib are in agreement with results previously described for other cell lines [13–15] as well as with reported IC₅₀ values for purified BCR-ABL protein (0.2–0.3 μM, [16,17]). Similarly, toxic effects of dasatinib on BCR-ABL-positive cells occur in the concentration range corresponding to IC₅₀ value of dasatinib towards the isolated BCR-ABL (0.8–1.3 nM, [16,18,19]) and to EC₅₀ values for BCR-ABL dephosphorylation in the intracellular context (Table 2, [14,15,19–21]). On the other hand, inhibition of SRC kinases does not have toxic effects in BCR-ABL-negative cells. The induction of cell death at high dasatinib concentrations (more than 1 μM) is undoubtedly due to the inhibition of unspecific dasatinib targets.

Using real-time monitoring of microimpedance, we performed kinetic analysis of the effects of imatinib and dasatinib on hematopoietic cell interaction with fibronectin fragment as a simple model of the bone marrow extracellular matrix. It is known that c-ABL signaling affects the adhesion pathways at many points [22], and that BCR-ABL-transformed cells show abnormalities in adhesion processes [3,23–26]. However, the resulting effect of BCR-ABL transformation on cell adhesion remains unclear, and seems to depend on cell type and/or used methodic approach. In a

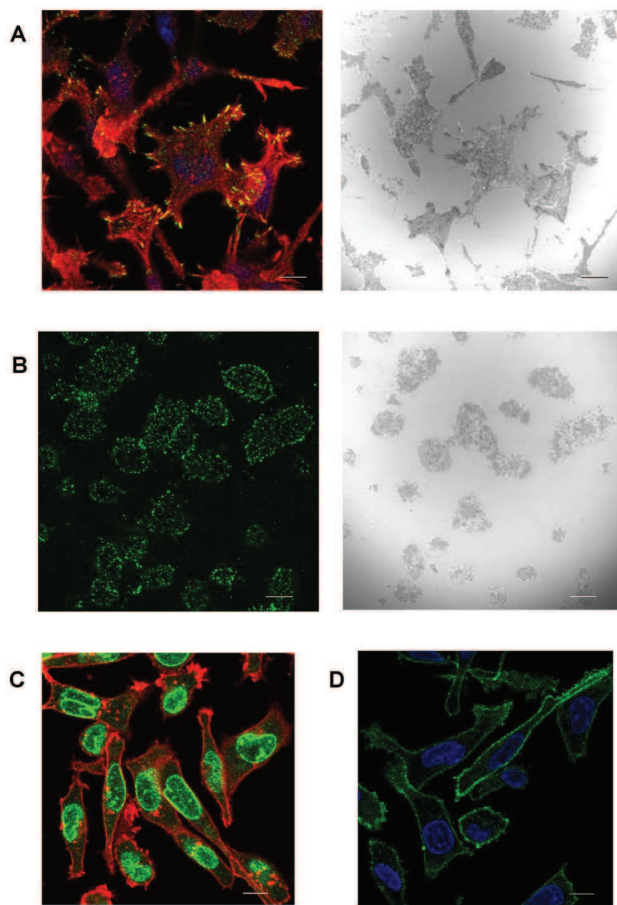


Figure 8. Microscopic analysis of SRC kinases localization in cells on fibronectin. MOLM-7 (A, C, D) or JURL-MK1 (B) cells were plated on fibronectin-coated slide, incubated for 1 h at 37°C, fixed and stained with antibodies against phospho-SFK (A, B), LCK (C) or LYN (D). A, B: Immunofluorescence images (left) were taken from the slice immediately adjacent to the FN-coated surface. Right images in A and B were captured in interference reflection mode. Green signal: SFK; red: actin (stained with phalloidin), blue: cell nuclei (DAPI). Scale bars: 10 μ m.

doi:10.1371/journal.pone.0107367.g008

similar way, SFKs are involved in cell adhesion to the extracellular matrix in adherent cell types [27–29] and in mature hematopoietic cells, but the role of SFKs in hematopoietic progenitor cell adhesion signaling is still rather unclear.

Imatinib mesylate and low-dose dasatinib strengthened the interaction of JURL-MK1 and MOLM-7 cells with fibronectin (Figure 2 A–D). The effect occurred shortly after inhibitor addition and lasted for many hours. The subsequent decrease is likely to be related to the onset of apoptosis [12]. We have also detected drug-induced increase in the number of cells which are firmly bound to FN, although this effect was less pronounced (Figure 3). This indicates that an increase in cell spreading or closer cell contact with the coated surface contributes to the observed changes in microimpedance signal.

Both imatinib and low-dose dasatinib were found to reduce the phosphorylation (activity) of SFKs in BCR-ABL-positive cells. This effect is probably indirect, resulting from the loss of SFK stimulation by BCR-ABL [30,31], as 1 μ M imatinib does not significantly reduce SFK phosphorylation in BCR-ABL-negative HEL cells (Figure 6, Figure S4) although IC₅₀ values for isolated

LYN were reported to be 0.35–2.2 μ M [17,32,33]. In addition, in our experiments, EC₅₀ values for SFK inhibition by dasatinib in BCR-ABL negative cells are by one order of magnitude higher than those in BCR-ABL-positive cells (Table 2). EC₅₀ of dasatinib for LYN inhibition in the intracellular context is of about 20 nM (Table 2, values for JURKAT and HEL cells), while reported IC₅₀ values for recombinant enzyme inhibition range from 1.7 to 8.5 nM [16,34,35]. We have also recently reported that PP2, an inhibitor of SFKs, increases JURL-MK1 cell adhesivity to FN. However, we found later that PP2 reduces the activity of BCR-ABL as well, although it is not clear if the decrease in BCR-ABL phosphorylation is due to a loss of SFK-mediated stimulation or to direct inhibition (Figure S6). No really specific small molecule inhibitor is currently available and it is thus difficult to dissect the individual roles of BCR-ABL and SFKs in dynamic cell response to imatinib and dasatinib. Nevertheless, as the inhibitors induce no significant increase of the microimpedance signal from BCR-ABL-negative cells, it is likely that the effects of TKIs on CML cell adhesion are prevalently due to BCR-ABL inhibition.

The increase in CML cell adhesivity to FN was suppressed at higher dasatinib doses (e.g. 100 nM) corresponding to concentrations which are achieved in clinical settings [36]. This indicates that dasatinib at these concentrations targets a protein which is required for dynamic cell interaction with fibronectin. We speculate that such target could be represented by SFKs as suggested by EC₅₀ values for their inhibition by dasatinib (Table 2). Indeed, SFKs are important regulators of adhesion signaling in adherent cells. The prototypic member of the family, c-SRC, belongs to well-known oncogenes. In physiological conditions, c-SRC is maintained inactive and is activated only transiently. Sustained increased activity of c-SRC shifts the dominance in adhesion signalization towards RAC1/CDC42 axis instead of RHOA and cells transformed with c-SRC acquire migratory phenotype including anchorage-independent growth and loss of contact inhibition. The other members of SRC family are much less explored, especially in relation to the cell adhesivity. Some of them (LYN, HCK, FGR, LCK, BLK) are more or less specifically expressed in hematopoietic cells where they are required for normal hematopoiesis. The current model describes SFKs as rheostats in immune cell signaling, with positive or negative net response being dependent on a number of stimulatory conditions [37]. Major SFKs expressed in myeloid cells, LYN and HCK, seem to have mutually opposed functions in integrin-mediated signaling in mature myeloid cells. In neutrophils and macrophages, LYN acts as a negative regulator of integrin-mediated adhesion, as LYN $-/-$ cells show hyper-adhesive phenotype [38]. On the other hand, HCK and FGR $-/-$ macrophages and neutrophils show disruption of integrin-mediated signaling, suggesting a positive role for HCK and FGR kinases in integrin-mediated adhesion [39,40]. Neutrophils deficient in HCK and FGR also display defects in migration processes [41]. In immature hematopoietic precursors, downregulation of LYN expression using siRNA resulted in higher adhesion to stroma and reduced CXCR4-dependent chemotaxis [42], which is needed for hematopoietic cell homing to the bone marrow [6].

In our cell lines, LYN was omnipresent while HCK and LCK were found in some of them only (Figure 4). MOLM-7 cells express LYN and LCK, the latter being localized mainly to the nuclear membrane (Figure 8C). In keeping with previously reported results obtained in other cells [43], LYN was predominantly present in the cytoplasmic membrane and, to a lesser extent, in the cytoplasm (Figure 8D). Importantly, immunofluorescence staining showed that active SFKs accumulate in adhesion structures formed at MOLM-7 cell contacts with FN-coated

surface, especially at the extremities of prominent membrane protrusions (Figure 8A). This would support the hypothesis that hematopoietic SRC kinases, and especially LYN, are involved in rearrangement of adhesion structures and that a minimal LYN activity is required for BCR-ABL-induced changes in cell interaction with fibronectin. On the other hand, SFKs are clearly not required to the establishment of cell contacts with FN as the cells pretreated with high-dose dasatinib were still able to attach to FN-coated surface (Figure 3).

In any case, it appears from our experiments that dasatinib at therapeutic concentrations does not enhance CML cell binding to fibronectin (Figures 2 E,F and 3). It is not known if this is also true for primary leukemic blasts and their interaction with relevant extracellular matrix proteins. However, if so, dasatinib-based therapy might lead to more efficient eradication of the residual disease in comparison with imatinib treatment which could enhance the protective effect provided by the bone marrow microenvironment to leukemic blasts.

The changes observed at very high dasatinib concentrations (more than 1 μ M, Figure S3) are not relevant for the clinical practise. Nevertheless, these results suggest that dasatinib at these concentrations targets a protein which is important for hematopoietic cell adhesion signaling in general, as these effects were observed in all the studied cell lines as well as in primary mononuclear cells. Therefore, uncovering of this target will be the subject of a subsequent proteomic study.

Supporting Information

Figure S1 Kinetics of dasatinib-induced cell death and changes in cell adhesivity to fibronectin. A: Apoptosis induction in JURL-MK1 and MOLM-7 cells during treatment with 10 nM dasatinib was assessed by flow-cytometry. Cell fraction in sub-G1 region of cell cycle phase distribution in samples stained with propidium iodide. B: Caspase activation during cell incubation with 10 nM dasatinib was monitored using fluorogenic substrate Ac-DEVD-AFC following the same protocol as in the reference 12. C: Caspase activation in cells treated with 1 to 100 nM dasatinib for 2 or 6 h (same method as in B). D: Kinetics of changes in JURL-MK1 cell adhesivity to fibronectin following treatment with 2 nM dasatinib alone or in combination with 10 μ M Q-VD-OPh (method as in Fig. 3). (PDF)

Figure S2 Changes in cell interaction with fibronectin after imatinib treatment. The cells (6×10^4 per well) were seeded into fibronectin-coated E-plates. After the microimpedance signal stabilization, the appropriate inhibitor was added in triplets. Black circles: control cells. Time of inhibitor addition is indicated by an arrow. Microimpedance signal (cell index) was normalized to 1 at the time of inhibitor addition. The graphs show means and standard deviations of well triplets. A,C,E: HEL cells, B,D,F: JURKAT cells. A,B: imatinib was added at 10 μ M (red squares) final concentration. C,D: dasatinib was added at 2 nM (blue

circles) or 10 nM (red squares) final concentration. E,F: dasatinib was added at 100 nM final concentration (red circles). (PPTX)

Figure S3 Changes in cell interaction with fibronectin after treatment with dasatinib at high concentrations. JURL-MK1 (A) and HEL (B) cells (6×10^4 per well) were seeded into fibronectin-coated E-plates. After stabilization of the microimpedance signal, 10 μ M dasatinib (red circles) was added in triplets. Time of addition is indicated by an arrow. Black circles: control cells treated with 0.1% DMSO. The graphs show means and standard deviations of the triplets. Microimpedance signal (cell index) was normalized to 1 at the time of inhibitor addition. (PPTX)

Figure S4 Flow-cytometric analysis of SFK phosphorylation. Cells were incubated for 2 h with imatinib or dasatinib at different concentrations, fixed and stained with anti-pSFK(Tyr416) antibody and secondary PE-anti-rabbit antibody. Mean fluorescence intensity (MFI) was measured using BD LSR Fortessa flow-cytometer and normalized to the value from the corresponding untreated control. The graphs show summary values from all independent experiments. (PPTX)

Figure S5 Effect of dasatinib on phospho-SFK signal in microscopic preparations. MOLM-7 cells were plated on fibronectin-coated slide, incubated for 30 min at 37°C and treated for additional 30 min with 2 nM or 100 nM dasatinib or with 10 μ M PP2. (PDF)

Figure S6 Western blot analysis of BCR-ABL dephosphorylation after treatment of JURL-MK1 cells with PP2. JURL-MK1 cells were treated with PP2 at the indicated concentrations for 2 h, lysed and the level of phosphorylated BCR-ABL was assessed using anti-phospho-ABL antibody. Then the cells were fixed and stained with anti-phospho-SFK antibody (top images). Green: SFK, red: actin (stained with phalloidin), blue: nuclei (DAPI). Bottom images represent the same visual field in differential interference contrast mode (DIC). Representative images are shown for each condition. (PDF)

Acknowledgments

We wish to thank H. Pilcová and M. Voráčová for skilled technical assistance. Thanks are due to P. Jendelová and K. Turnovcová (Institute of Experimental Medicine, ASCR, Prague) for providing access to RTCA device.

Author Contributions

Conceived and designed the experiments: AO KK. Performed the experiments: AO PR DG KK. Analyzed the data: AO PR DG KK. Contributed reagents/materials/analysis tools: KK. Contributed to the writing of the manuscript: AO KK.

References

1. Arai F, Hirao A, Suda T (2005) Regulation of hematopoiesis and its interaction with stem cell niches. *Int J Hematol* 82: 371–376.
2. Verfaillie CM, Hurley R, Lundell BI, Zhao C, Bhatia R (1997) Integrin-mediated regulation of hematopoiesis: Do BCR/ABL-induced defects in integrin function underlie the abnormal circulation and proliferation of CML progenitors? *Acta Haematol* 97: 40–52.
3. Wertheim JA, Forsythe K, Druker BJ, Hammer D, Boettiger D, et al. (2002) BCR-ABL-induced adhesion defects are tyrosine kinase-independent. *Blood* 99: 4122–4130.
4. Mauro MJ (2013) Striving to achieve safe, permanent treatment discontinuation in chronic myeloid leukemia. *Leuk Res* 37: 1395–1403.
5. Barnes DJ, Melo JV (2006) Primitive, quiescent and difficult to kill: The role of non-proliferating stem cells in chronic myeloid leukemia. *Cell Cycle* 5: 2862–2866.
6. Weisberg E, Azab AK, Manley PW, Kung AL, Christie AL, et al. (2012) Inhibition of CXCR4 in CML cells disrupts their interaction with the bone marrow microenvironment and sensitizes them to nilotinib. *Leukemia* 26: 985–990.
7. Tsuji-Takayama K, Kamiya T, Nakamura S, Matsuo Y, Adachi T, et al. (1994) Establishment of multiple leukemia cell lines with diverse myeloid and/or megakaryoblastoid characteristics from a single Ph1 positive chronic myelogenous leukemia blood sample. *Hum Cell* 7: 167–171.

8. Martin P, Papayannopoulou T (1982) HEL cells: A new human erythroleukemia cell line with spontaneous and induced globin expression. *Science* 216: 1233–1235.
9. Kuželová K, Pluskalová M, Brodská B, Otevřelová P, Elknerová K, et al. (2010) Suberoylanilide hydroxamic acid (SAHA) at subtoxic concentrations increases the adhesivity of human leukemic cells to fibronectin. *J Cell Biochem* 109: 184–195.
10. Obr A, Röslová P, Grebeňová D, Kuželová K (2013) Real-time monitoring of hematopoietic cell interaction with fibronectin fragment: The effect of histone deacetylase inhibitors. *Cell Adh Migr* 7: 275–282.
11. Grebeňová D, Röslová P, Pluskalová M, Halada P, Rösel D, et al. (2012) Proteins implicated in the increase of adhesivity induced by suberoylanilide hydroxamic acid in leukemic cells. *J Proteomics* 77: 406–422.
12. Kuželová K, Pluskalová M, Grebeňová D, Pavlásková K, Halada P, et al. (2010) Changes in cell adhesivity and cytoskeleton-related proteins during imatinib-induced apoptosis of leukemic JURL-MK1 cells. *J Cell Biochem* 111: 1413–1425.
13. Burgess MR, Skaggs BJ, Shah NP, Lee FY, Sawyers CL (2005) Comparative analysis of two clinically active BCR-ABL kinase inhibitors reveals the role of conformation-specific binding in resistance. *Proc Natl Acad Sci U S A* 102: 3395–400.
14. Manley PW, Cowan-Jacob S, Mestan J (2005) Advances in the structural biology, design and clinical development of bcr-abl kinase inhibitors for the treatment of chronic myeloid leukaemia. *Biochim Biophys Acta* 1754: 3–13.
15. Redaelli S, Piazza R, Rostagno R, Magistrini V, Perini P, et al. (2009) Activity of bosutinib, dasatinib, and nilotinib against 18 imatinib-resistant BCR/ABL mutants. *Journal of Clinical Oncology* 27: 469–471.
16. O'Hare T (2005) Combined abl inhibitor therapy for minimizing drug resistance in chronic myeloid leukemia: Src/Abl inhibitors are compatible with imatinib. *Clinical Cancer Research* 11: 6987–6993.
17. Niwa T, Asaki T, Kimura S (2007) NS-187 (INNO-406), a bcr-Abl/Lyn dual tyrosine kinase inhibitor. *Analytical chemistry insights* 2: 93–106.
18. Chen H, Adams E, Van Schepdael A (2013) Study of Abl1 tyrosine kinase inhibitors by liquid chromatography-electrospray ionization-mass spectrometry. *Talanta* 107: 88–94.
19. Martinelli G, Soverini S, Rosti G, Baccarani M (2005) Dual tyrosine kinase inhibitors in chronic myeloid leukemia. *Leukemia* 19: 1872–9.
20. Vainstein V, Eide CA, O'Hare T, Shukron O, Druker BJ (2013) Integrating in vitro sensitivity and dose-response slope is predictive of clinical response to ABL kinase inhibitors in chronic myeloid leukemia. *Blood* 122: 3331–4.
21. Cassuto O, Dufies M, Jacquel A, Robert G (2012) All tyrosine kinase inhibitor-resistant chronic myelogenous cells are highly sensitive to ponatinib. *Oncotarget* 3: 1557–1565.
22. Bradley WD, Koleske AJ (2009) Regulation of cell migration and morphogenesis by abl-family kinases: Emerging mechanisms and physiological contexts. *J Cell Sci* 122: 3441–3454.
23. Bazzoni G, Carlesso N, Griffin JD, Hemler ME (1996) Bcr/Abl expression stimulates integrin function in hematopoietic cell lines. *J Clin Invest* 98: 521–528.
24. Maia V, Ortiz-Rivero S, Sanz M, Gutierrez-Berzal J, Alvarez-Fernandez I, et al. (2013) C3G forms complexes with bcr-abl and p38alpha MAPK at the focal adhesions in chronic myeloid leukemia cells: Implication in the regulation of leukemic cell adhesion. *Cell Commun Signal* 11: 9.
25. Salgia R, Pisick E, Sattler M, Li JL, Uemura N, et al. (1996) p130CAS forms a signaling complex with the adapter protein CRKL in hematopoietic cells transformed by the BCR/ABL oncogene. *J Biol Chem* 271: 25198–25203.
26. Salgia R, Li JL, Ewaniuk DS, Pear W, Pisick E, et al. (1997) BCR/ABL induces multiple abnormalities of cytoskeletal function. *J Clin Invest* 100: 46–57.
27. Huvencers S, Danen EH (2009) Adhesion signaling - crosstalk between integrins, src and rho. *J Cell Sci* 122: 1059–1069.
28. Mitra SK, Schlaepfer DD (2006) Integrin-regulated FAK-src signaling in normal and cancer cells. *Curr Opin Cell Biol* 18: 516–523.
29. Playford MP, Schaller MD (2004) The interplay between src and integrins in normal and tumor biology. *Oncogene* 23: 7928–7946.
30. Meyn MA, Wilson MB, Abdi FA, Fahey N, Schiavone AP, et al. (2006) Src family kinases phosphorylate the bcr-abl SH3-SH2 region and modulate bcr-abl transforming activity. *The Journal of biological chemistry* 281: 30907–16.
31. Wu J, Meng F, Lu H, Kong L, Bornmann W, et al. (2008) Lyn regulates BCR-ABL and Gab2 tyrosine phosphorylation and c-bbl protein stability in imatinib-resistant chronic myelogenous leukemia cells. *Blood* 111: 3821–3829.
32. Kimura S, Ashihara E, Maekawa T (2006) New tyrosine kinase inhibitors in the treatment of chronic myeloid leukemia. *Curr Pharm Biotechnol* 7: 371–379.
33. Dubreuil P, Letard S, Ciufolini M, Gros L, Humbert M, et al. Masitinib (AB1010), a potent and selective tyrosine kinase inhibitor targeting KIT. *PLoS ONE*: e7258.
34. Deguchi Y, Kimura S, Ashihara E, Niwa T, Hodohara K, et al. (2008) Comparison of imatinib, dasatinib, nilotinib and INNO-406 in imatinib-resistant cell lines. *Leuk Res* 32: 980–983.
35. Nam S, Kim D, Cheng JQ, Zhang S, Lee JH, et al. (2005) Action of the src family kinase inhibitor, dasatinib (BMS-354825), on human prostate cancer cells. *Cancer Res* 65: 9185–9189.
36. Imagawa J, Tanaka H, Matsumoto K, Morita K, Harada Y, et al. (2012) A sharp fluctuation in peripheral blood cells shortly after dasatinib administration. *Int J Hematol* 96: 194–199.
37. Scapini P, Pereira S, Zhang H, Lowell CA (2009) Multiple roles of lyn kinase in myeloid cell signaling and function. *Immunol Rev* 228: 23–40.
38. Pereira S, Lowell C (2003) The lyn tyrosine kinase negatively regulates neutrophil integrin signaling. *J Immunol* 171: 1319–1327.
39. Suen PW, Ilic D, Cavegion E, Berton G, Damsky CH, et al. (1999) Impaired integrin-mediated signal transduction, altered cytoskeletal structure and reduced motility in Hck/Fgr deficient macrophages. *J Cell Sci* 112 (Pt 22): 4067–4078.
40. Mocsai A, Ligeti E, Lowell CA, Berton G (1999) Adhesion-dependent degranulation of neutrophils requires the src family kinases fgr and hck. *J Immunol* 162: 1120–1126.
41. Fumagalli L, Zhang H, Baruzzi A, Lowell CA, Berton G (2007) The src family kinases hck and fgr regulate neutrophil responses to N-formyl-methionyl-leucyl-phenylalanine. *J Immunol* 178: 3874–3885.
42. Nakata Y, Tomkowicz B, Gewirtz AM, Ptasznik A (2006) Integrin inhibition through lyn-dependent cross talk from CXCR4 chemokine receptors in normal human CD34+ marrow cells. *Blood* 107: 4234–4239.
43. Ingley E (2012) Functions of the lyn tyrosine kinase in health and disease. *Cell Commun Signal* 10: 21.

Correction

Correction: Real-Time Analysis of Imatinib- and Dasatinib-Induced Effects on Chronic Myelogenous Leukemia Cell Interaction with Fibronectin

The *PLOS ONE* Staff

Figure 2 was incorrectly published as a duplicate of Figure S2.
Please see the correct Figure 2 here.



Citation: The *PLOS ONE* Staff (2014) Correction: Real-Time Analysis of Imatinib- and Dasatinib-Induced Effects on Chronic Myelogenous Leukemia Cell Interaction with Fibronectin. *PLoS ONE* 9(9): e110070. doi:10.1371/journal.pone.0110070

Published: September 30, 2014

Copyright: © 2014 The *PLOS ONE* Staff. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

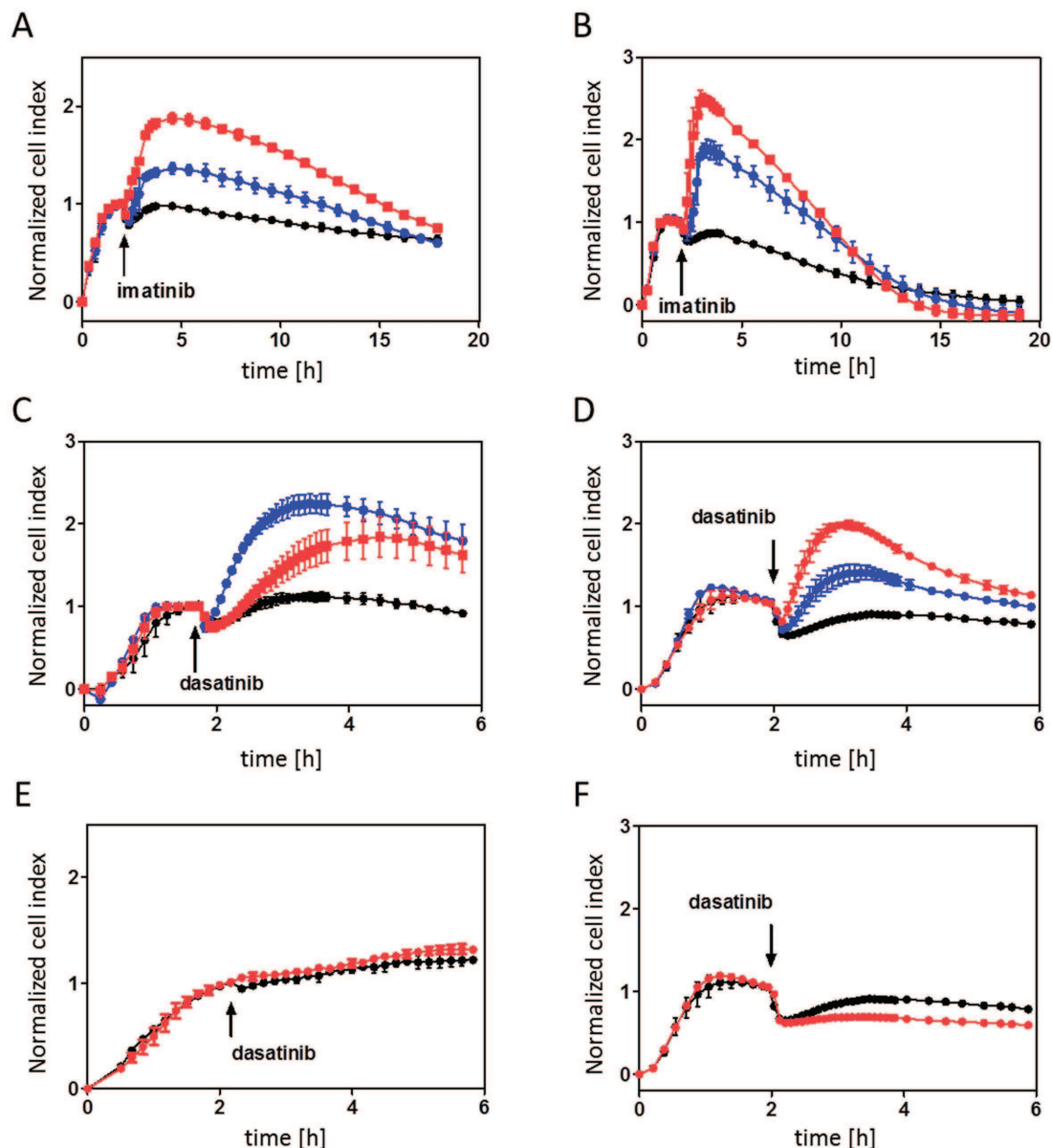


Figure 2. Changes in cell interaction with fibronectin after inhibitor treatment. The cells (6×10^4 per well) were seeded into fibronectin-coated E-plates. After the microimpedance signal stabilization, the appropriate inhibitor was added in triplets. Black circles: control cells. Time of inhibitor addition is indicated by an arrow. Microimpedance signal (cell index) was normalized to 1 at the time of inhibitor addition. The graphs show mean and standard deviation of well triplets. A,C,E: JURL-MK1 cells, B,D,F: MOLM-7 cells. A,B: imatinib was added at 1 μ M (blue circles) or 10 μ M (red squares) final concentration. C,D: dasatinib was added at 2 nM (blue circles) or 10 nM (red squares) final concentration. E,F: dasatinib was added at 100 nM final concentration (red circles). doi:10.1371/journal.pone.0107367.g002

Reference

1. Obr A, Röslová P, Grebeňová D, Kuželová K (2014) Real-Time Analysis of Imatinib- and Dasatinib-Induced Effects on Chronic Myelogenous Leukemia Cell Interaction with Fibronectin. PLoS ONE 9(9): e107367. doi:10.1371/journal.pone.0107367

RESEARCH PAPER



Adhesion structures in leukemia cells and their regulation by Src family kinases

Pavla Röslová, Adam Obr, Aleš Holoubek, Dana Grebeňová, and Kateřina Kuželová 

Department of Proteomics, Institute of Hematology and Blood Transfusion, U Nemocnice 1, Prague, Czech Republic

ABSTRACT

Interaction of leukemia blasts with the bone marrow extracellular matrix often results in protection of leukemia cells from chemotherapy and in persistence of the residual disease which is on the basis of subsequent relapses. The adhesion signaling pathways have been extensively studied in adherent cells as well as in mature haematopoietic cells, but the adhesion structures and signaling in haematopoietic stem and progenitor cells, either normal or malignant, are much less explored. We analyzed the interaction of leukemia cells with fibronectin (FN) using interference reflection microscopy, immunofluorescence, measurement of adherent cell fraction, real-time microimpedance measurement and live cell imaging. We found that leukemia cells form very dynamic adhesion structures similar to early stages of focal adhesions. In contrast to adherent cells, where Src family kinases (SFK) belong to important regulators of focal adhesion dynamics, we observed only minor effects of SFK inhibitor dasatinib on leukemia cell binding to FN. The relatively weak involvement of SFK in adhesion structure regulation might be associated with the lack of cytoskeletal mechanical tension in leukemia cells. On the other hand, active Lyn kinase was found to specifically localize to leukemia cell adhesion structures and a less firm cell attachment to FN was often associated with higher Lyn activity (this unexpectedly occurred also after cell treatment with the inhibitor SKI-1). Lyn thus may be important for signaling from integrin-associated complexes to other processes in leukemia cells.

ARTICLE HISTORY

Received 21 November 2016
Revised 13 April 2017
Accepted 16 June 2017

KEYWORDS

adhesion; ECIS;
hematopoietic cell; leukemia;
Lyn; Src family kinases

Introduction

Ten enzymes with homology to c-Src have been identified up to date and are collectively referred to as Src family kinases (SFK). The expression of 5 members of this family tends to be restricted to haematopoietic cells and increased activity of these haematopoietic cell-related SFK is often associated with worse prognosis in leukemias.

Involvement of the founding member of the family, c-Src, in the regulation of adhesion and migration of adherent cells is well documented. The expression and the activity of c-Src are correlated with advanced malignancy, higher invasiveness and poor prognosis in a variety of human cancers. Historically, it was the transformation of fibroblasts with the constitutively active form of Src from Rous sarcoma virus, which induced formation of invadosomes.¹ It is known that c-Src interacts with focal adhesion kinase (FAK), binds to adhesion complexes and phosphorylates a large number of their components. The amount of phosphorylated tyrosine at adhesion sites decreases with maturation and stabilization of focal adhesions. The signaling from c-Src promotes the activity of Rac1/Cdc42 which are required

e.g. for the formation of membrane protrusions, and reduces that of RhoA which is responsible for the mechanical tension of cytoskeletal fibers and thereby for adhesion complex maturation.² It is not clear, however, if the kinase activity of c-Src is necessary for the assembly or disassembly of adhesion complexes. A recent proteomic analysis of integrin adhesion complexes has shown that the composition of these structures is largely insensitive to SFK or FAK inhibition.³ The architecture of adhesion complexes seems to be modulated mainly by mechanical force which alters the composition of protein complexes through mechanosensors like talin or Cas.⁴⁻⁶

Although SFK have partially overlapping functions, some of their activities are highly specific. Both transactivation and mutual inhibition between individual members of the family have been described. SFK can be divided to 2 categories according to their sequence homology: Lyn-related (Lyn, Hck, Lck, Blk), which are expressed mainly in haematopoietic cells, and Src-related (Src, Yes, Fyn, Fgr), which are expressed ubiquitously.⁷ The sequence homology between c-Src on one hand and Lyn, Hck or Lck on the other hand is relatively low⁸ and

CONTACT Kateřina Kuželová  kuzel@uhkt.cz  Department of Proteomics, Institute of Hematology and Blood Transfusion, U Nemocnice 1, 12820 Prague, Czech Republic.

 Supplemental data for this article can be accessed on the [publisher's website](#).

© 2017 Taylor & Francis

the role of these haematopoietic cell-related SFK in adhesion signaling is much less explored than that of c-Src. It is known, however, that Lyn kinase influences adhesion and migration in different cell types. Lyn depletion leads to decreased migration rate in neutrophils⁹ and macrophages.⁸ Lyn is also required for long-term engraftment of transplanted haematopoietic stem and progenitor cells¹⁰ as well as for platelet activation and thrombus formation.¹¹ Several papers suggested a role for Lyn in migration and adhesion of adherent cells, too.^{12–14} Although Lyn function in haematopoietic cell adhesion and migration often resembles that of c-Src in adherent cells, opposing effects of these 2 kinases have been described in endothelial cells where Lyn strengthens cell-cell junctions and inhibits vascular leakage.¹⁵ Lyn also acts in opposition to Hck in mastocyte activation.¹⁶

Interaction of immature haematopoietic cells with the bone marrow microenvironment is important for many processes, including proliferation and differentiation of haematopoietic progenitor cells, progenitor mobilization in blood cell donors, homing of transplanted progenitors in recipients, or persistence of residual disease in leukemias which is associated with protective effects of the bone marrow on leukemia blasts during chemotherapy. Kinases of the Src family represent possible therapeutic targets in many types of cancer and their inhibition may have impact on several physiologic and pathophysiological processes. In this work, we focused on the role of SFK, and more specifically Lyn, in leukemia cell interaction with a canonical bone marrow protein, fibronectin.

Results

In screening experiments involving different proteins of the extracellular matrix (ECM), we identified fibronectin (FN) as the most general interacting partner for leukemia cells (see e.g., Supplementary Fig. S1) and we thus focused to this component of the bone marrow ECM when characterizing adhesion structures of leukemia cells. The use of a specific fibronectin fragment (120 K cell-binding fragment) further increased cell binding to FN-coated surfaces. To evaluate the firmness of cell attachment to the surface, we determined the adherent cell fraction (ACF), i.e. the percentage of cells that remain on FN layer after PBS $\text{Ca}^{2+}/\text{Mg}^{2+}$ wash. To analyze in detail cell interaction with FN-coated surfaces, we used immunofluorescence (IF), live cell imaging, interference reflection measurement (IRM), and real-time monitoring of impedance (Electric Cell-substrate Impedance Sensing, ECIS). To analyze the role of kinases of the Src family (SFK) we used dasatinib, a potent clinically used SFK inhibitor. We have previously found that EC50 value for SFK inhibition in Bcr-Abl-negative leukemia cells is about 20 nM.¹⁷ In the present study, we used 100 nM dasatinib

which provides nearly complete inhibition of SFK. For comparison, we also tested the effects of an alternative SFK inhibitor, SKI-1. Potential cytotoxicity of the inhibitors used was assessed by counting cells stained with Trypan blue and by flow-cytometry analysis of propidium iodide-stained samples during 48h incubation with 100 nM dasatinib or 20 μM SKI-1. We detected no significant effect of the inhibitors on the cell viability in this time frame. We only noted a decreased proliferation rate of HEL and OCI-AML3 cells treated with SKI-1.

Cell morphology on FN-coated surface

The morphology of leukemia cells and of their adhesion structures on a FN layer was found to be variable in leukemia cell lines as well as in primary cells from patients with acute myeloid leukemia (AML). Examples of IRM images shown in Fig. 1 illustrate different extent of cell spreading, various cell shapes as well as different size and arrangement of adhesion points. The percentage of adhered cells (ACF) ranged from 2–4% in CML-T1 cells to 60–90% in the most adherent cell types (OCI-AML3, HEL) and was also variable in primary cells (range from 7 to 67%, samples from 14 AML patients were analyzed). Cell interaction with the coated surface was very dynamic and although the cells did not extensively migrate on the surface, they were continuously forming „exploratory“ protrusions. Multiple transient small contact points were visible in IRM imaging of living cells. We have tested several canonical markers of adhesion structures known from adherent cell types (focal adhesions) or from normal mature haematopoietic cells (podosomes), but none of them specifically localized to the adhesion points (dark areas seen in IRM) although talin was usually present. Paxillin and vinculin were found in some of them only, depending on the cell type and conditions. Frequently, a nice correlation between immunofluorescence and IRM images was observed for a phospho-specific antibody detecting the autophosphorylated (active) form of Src family kinases (pTyr416 in c-Src and the corresponding residues in Lyn, Hck, Lck, Fyn and Yes). Examples of immunofluorescence images are given in Supplementary Fig. S2. Although no actin stress fibers are usually found in leukemia cells, we detected such structures in cells treated with phorbol 12-myristate 13-acetate (Supplementary Fig. S3). In this case, the adhesion points were similar to those of adherent cells and colocalized very well with vinculin and paxillin.

Expression of Src family members in leukemia cells

The amount of c-Src, and of other Src family members which are known to be expressed in haematopoietic cells, was assessed by western-blotting (Fig. 2). As the different members of the family slightly differ in their molecular

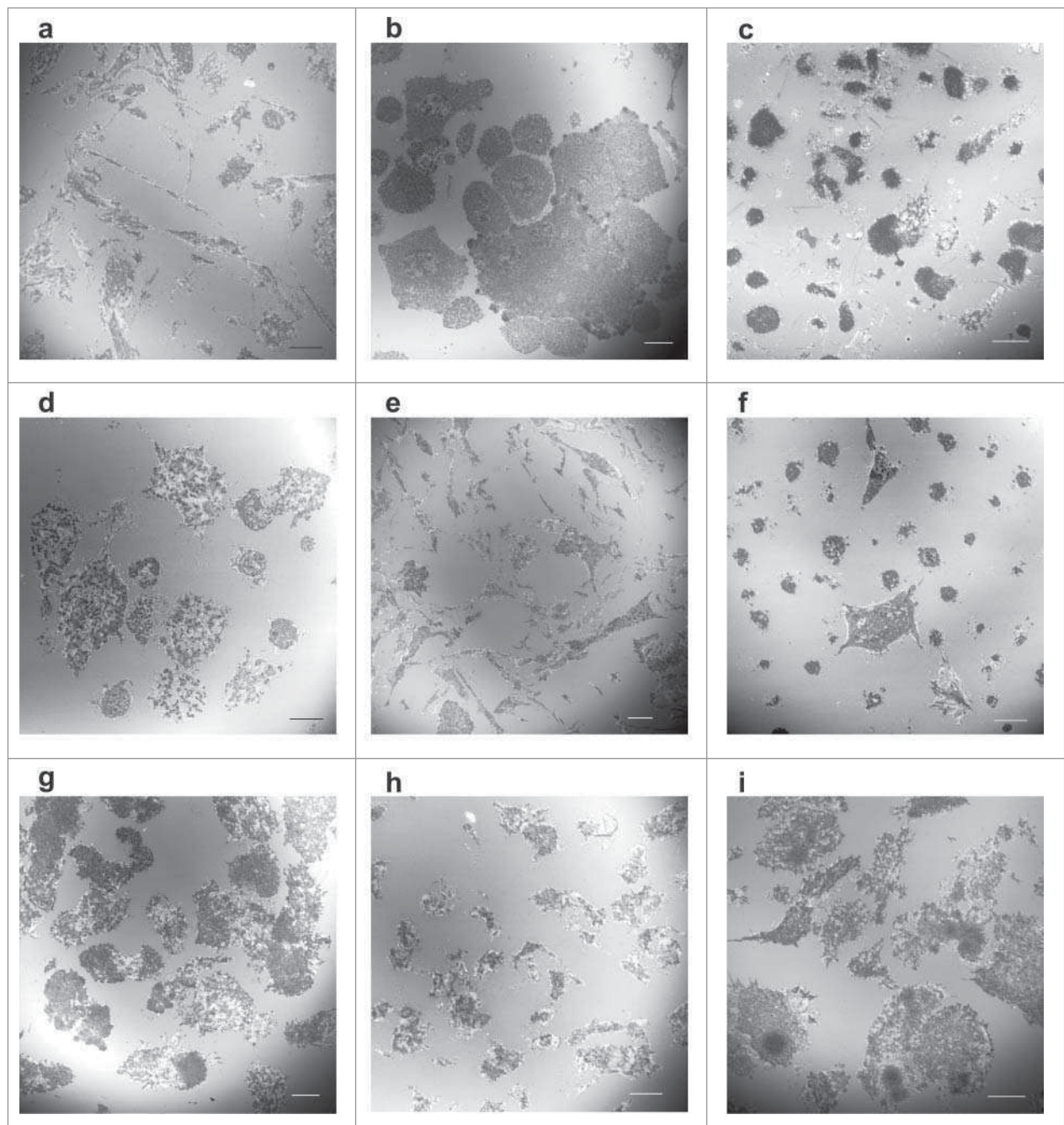


Figure 1. Morphology of contact areas between leukemia cells and fibronectin-coated surface. Leukemia cell lines (a-f) or primary cells from leukemia patients (g-i) were seeded on FN-coated slides and incubated for 1 h at 37°C. The cell parts which are very close to the surface were visualized by interference reflection microscopy. The mean diameter of cells in suspension is 12–16 μm for cell lines and 6–9 μm for primary cells. Scale bars: 10 μm . Characteristic IRM images for leukemia/lymphoma cell lines OCI-AML3 (a), HEL (b), HL-60 (c), K562 (d), MOLM-7 (e), Karpas-299 (f). Primary cells from 3 different patients with AML are shown in (g-i).

weights, it was possible to assign the bands detected by the phospho-specific antibody (denoted as pSFK in Fig. 2) to the corresponding kinases (see Supplementary Fig. S4). With the exception of CML-T1 cells which express mainly Lck, the predominant active Src family kinase detected by pSFK antibody in haematopoietic cell lines was Lyn (double band at 53/56 kDa). Although c-Src (60 kDa) was also expressed in haematopoietic cells,

its activity seems to be rather low judged from pSFK blots. The overall SFK activity in adherent cells (HeLa, HEK293T) was low in comparison with leukemia cells.

Based on our previous findings¹⁷ and on the literature data, we focused on Lyn as possible regulator of adhesion structures in haematopoietic cells and we selected cell lines with predominant Lyn activity (OCI-AML3, HEL, Karpas-299 and MOLM-7) for further analyses.

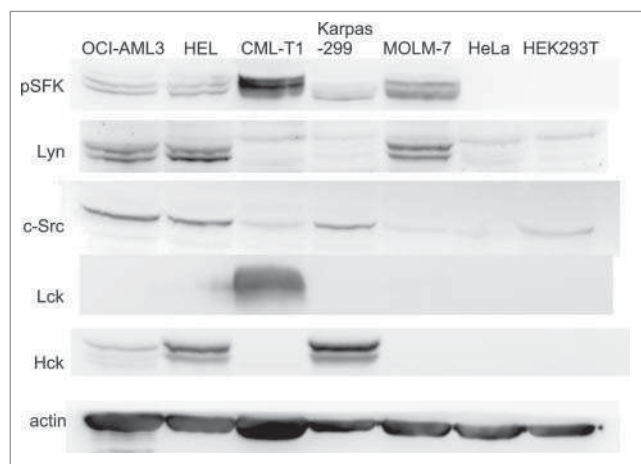


Figure 2. Expression levels of Src family kinases and of their active forms. Expression of c-Src, Lyn, Hck and Lck was assessed in human leukemia/lymphoma cell lines as well as in 2 human adherent cell lines (HeLa, HEK293T). The phospho-specific antibody against pSFK (Tyr416) recognizes an autophosphorylation site shared by all these kinases which serves as a marker of kinase activity. The same number of cells was harvested for all samples, actin was used as a control for total protein load.

Effect of kinase inhibitors on adherent cell fraction

Effect of 2 well-known inhibitors of Src family kinase activity, i.e., dasatinib and SKI-1 (Src kinase inhibitor 1), was analyzed in 2 settings: (i) cells were pretreated with the inhibitors for 1 h, then seeded on FN and left adhere for 1 h before PBS/ Ca^{2+} / Mg^{2+} wash, or (ii) cells were seeded on FN, left adhere and only then treated with the inhibitors, incubated and washed. Cell pretreatment with dasatinib or SKI-1 had no or small impact on cell ability to bind to FN (Fig. 3A). Dasatinib had only limited effect also when it was added to cells on FN (Fig. 3B). On the other hand, a larger decrease in ACF occurred after 30 min treatment of pre-adhered HEL and OCI-AML3 cells with SKI-1 (Fig. 3B). Although dasatinib had no significant effect on HEL cells after 30 min (Fig. 3B), it strengthened HEL cell adhesion after longer incubation time (90 min, Fig. 3C). This indicated that SFK inhibition may result in firmer cell attachment to the coated surface. However, no such effect was observed in the other leukemia cell lines. As SFK are supposed to be involved in the disassembly of adhesion structures, we tested if the inhibition of SFK activity by dasatinib affected cell detachment induced by cytochalasin D, an inhibitor of actin polymerization which is known to reduce the adhesivity of adherent cells through disruption of mechanical tension.¹⁸ Cytochalasin D (5 μM , 1 h) lowered the fraction of adhered HEL cells by 27% (average from 4 experiments) and this decrease in ACF remained almost unaffected by cell pretreatment with

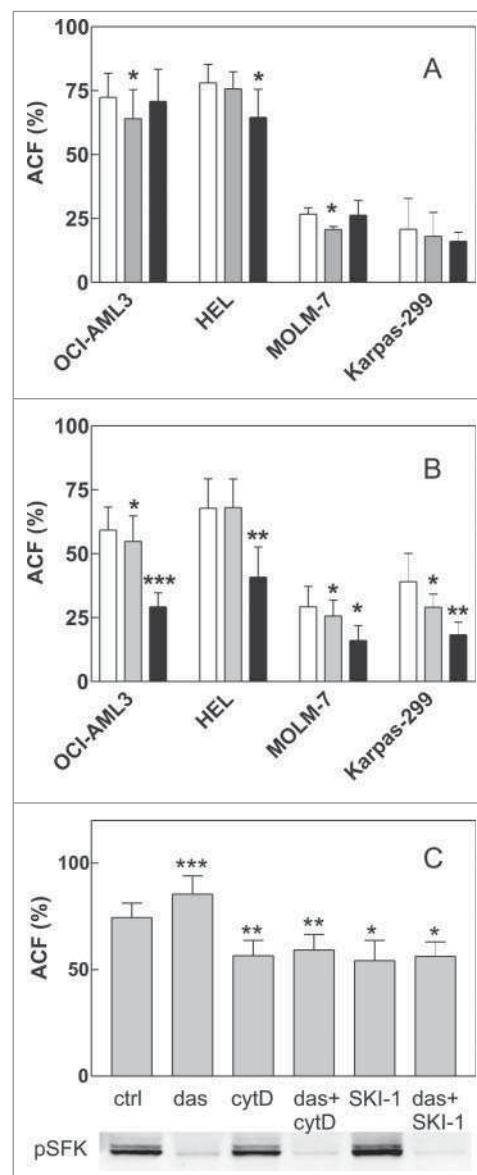


Figure 3. Effect of SFK inhibitors and cytochalasin D on cell attachment to fibronectin. A: Cells were pretreated with 100 nM dasatinib or 20 μM SKI-1 for 1 h and then seeded to FN-coated wells. The fraction of adhered cells was determined after 1 h incubation of the plate at 37°C. White bars: controls, gray bars: dasatinib, black bars: SKI-1. B: Cells were seeded on FN and left adhere for 1.5 h. Thereafter, inhibitors were added for additional 30 min at the same concentrations as in A. White bars: controls, gray bars: dasatinib, black bars: SKI-1. C: HEL cells were seeded into FN-coated wells. Dasatinib was added after 1.5 h to the corresponding wells, then 5 μM cytochalasin D or 20 μM SKI-1 was added after additional 30 min. The plate was further incubated for 1 h and the adhered cell fraction was determined thereafter. The level of phosphorylated SFK was assessed in parallel by western-blotting. All graphs show means and standard deviations from 3 to 7 independent experiments for each condition, each of them performed in quadruplets. The treated samples were compared with untreated controls using paired t-test: *p less than 0.05, **p less than 0.01, ***p less than 0.001.

100 nM dasatinib (Fig. 3C). No effect of cytD on cell adhesivity to FN was observed in the other cell lines (data not shown). Dasatinib pretreatment also failed to prevent the decrease in ACF induced by SKI-1, both in HEL (Fig. 3C) and OCI-AML3 cells (data not shown), although dasatinib efficiently inhibited SFK activity (western-blot in Fig. 3C). The results presented in Fig. 3C were analyzed using paired t-test. The increase of ACF after 90 min dasatinib treatment as well as the decrease of ACF due to SKI-1 or cytD treatment was statistically significant. On the other hand, no significant effect of dasatinib pretreatment was found for cytD or SKI-1-treated samples. Similar results were obtained using ANOVA test: a significant difference of variation was found for SKI-1 or cytD treatment (p less than 0.001) whereas no significant interaction was detected between dasatinib and SKI-1 or cytD ($p = 0.131$).

Effect of kinase inhibitors on Lyn activity

To verify the extent of kinase inhibition in the different conditions used in Fig. 3, OCI-AML3 cells in suspension or adhered to FN were treated with dasatinib or SKI-1 and Lyn phosphorylation at the autophosphorylation site was monitored by western-blotting (Fig. 4). When the cells were treated in suspension, both inhibitors reduced Lyn phosphorylation to negligible values. The effect of dasatinib was comparable on cells in suspension and on pre-adhered cells (Fig. 4A), but SKI-1 induced an unexpected increase in Lyn autophosphorylation when added to cells on FN (Fig. 4B, C). To confirm that the increased phosphorylation was actually associated with an increase in the kinase activity, we also analyzed the phosphorylation status of 2 known Lyn substrates, HS-1 (haematopoietic -specific analog of cortactin¹⁹) and Pyk2 (haematopoietic analog of focal adhesion kinase). Indeed, the phosphorylation of Lyn targets (Tyr 397 in HS-1 and Tyr402 in Pyk2) increased upon treatment with SKI-1. Similar results were obtained for HEL cell line (Supplementary Fig. S5).

Microimpedance analysis

Changes in cell interaction with FN induced by treatment with Src family kinase inhibitors were further studied using real-time measurement of microimpedance. This technique allows for monitoring of changes in the cell spreading or in the distance of the cell ventral membrane from the coated surface. The instrument enables measurement at multiple frequencies of the electric field and calculates the components of impedance, i.e., resistance and capacitance. The capacitance at high field frequencies (40 kHz or more) involves transcellular

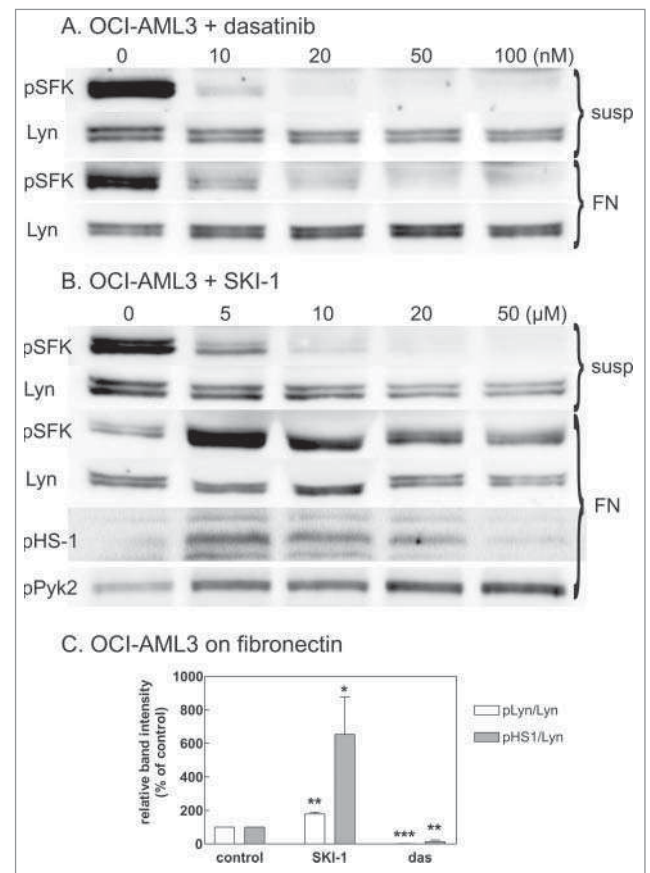


Figure 4. Effect of SFK inhibitors on Lyn activity. OCI-AML3 cells were treated with dasatinib or SKI-1, either in suspension (susp) or after adhesion to FN-coated surface (FN). Lyn activity was assessed from the extent of phosphorylation at Lyn autophosphorylation site (Tyr397) and on 2 known Lyn substrates, HS-1 and Pyk2. Lyn autophosphorylation was analyzed from bands at 53 and 56 kDa on western-blotting incubated with pan anti-pSFK antibody. A-B: Representative western-blotting from cells treated for 1 h with increasing concentrations of dasatinib (A) or SKI-1 (B). C: Summary results from 4 experiments performed with 20 μ M SKI-1 or 100 nM dasatinib treatment of pre-adhered OCI-AML3 cells (cell binding to FN for 1.5 h, then 30 min inhibitor treatment). Note that the exposition time for different membranes was not the same and was optimized to show the changes in pSFK level under different conditions.

currents and mirrors essentially the surface coverage (i.e., cell spreading in our experiments).^{18,20} The resistance at lower frequencies (400 Hz to 5 kHz) involves paracellular currents which are influenced both by the intercellular area and by cell closeness to the coated surface. We reported previously that the microimpedance signal progressively increases for 1 to 1.5 h following leukemia cell addition to FN-coated wells, reflecting the cell attachment and spreading.²¹

Figure 5A shows representative examples of extensive changes induced in adherent cells upon treatment with dasatinib or with cytochalasin D (cytD), an inhibitor of actin polymerization which releases the cytoskeletal

tension and promotes disassembly of focal adhesions. Panels B-D show the effect of dasatinib, cytD and SKI-1 on OCI-AML3 cells, as a representative example of leukemia cell lines, and on HEL cells, an erythroleukemia

cell line which is to some extent similar to adherent cells. Pretreatment with dasatinib did not significantly affect the ability of leukemia cells to attach to FN (Fig. 5B, left). The amplitude of changes in the resistance was reduced

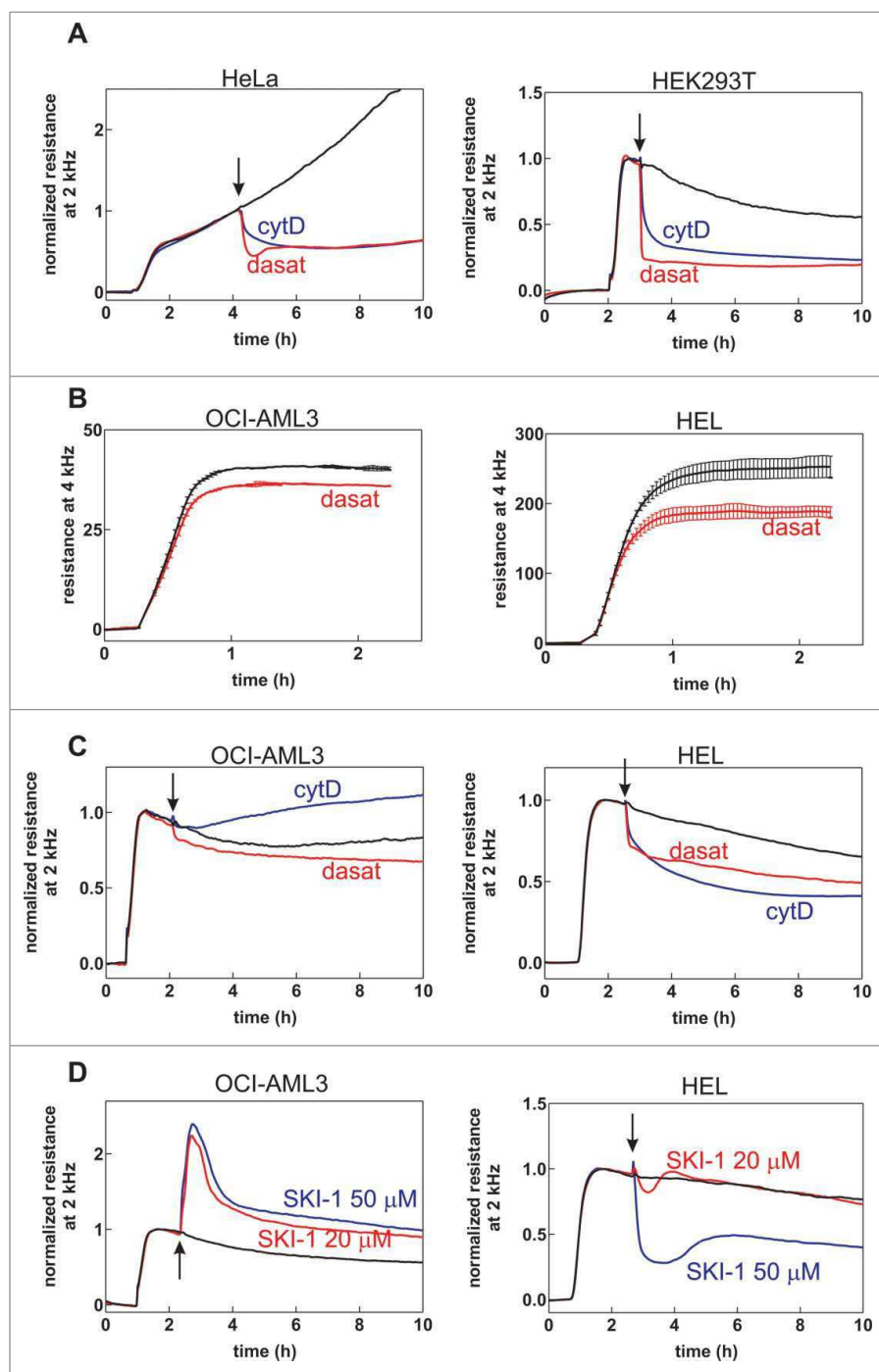


Figure 5. Monitoring of cell-FN interaction through microimpedance measurement. The effect of dasatinib (100 nM), cytochalasin D (5 μ M) and SKI-1 (20 or 50 μ M) is shown for adherent (HeLa, HEK293T) and leukemic (OCI-AML3, HEL) cells. The arrows indicate the time of inhibitor addition to cells on FN. The curves are means from well duplicates. The baseline values (before cell addition) were subtracted. In A, C and D, the resistance was normalized to 1 at a time point just before addition of inhibitors. In B, non-normalized values are shown. A: Effect of dasatinib or cytochalasin D addition on the microimpedance signal from adherent cells (left – HeLa, right – HEK293T). B: Effect of 1h dasatinib pretreatment on the microimpedance signal from attaching leukemia cells (left – OCI-AML3, right – HEL). C: Effect of dasatinib or cytochalasin D addition on the signal from leukemic cells (left – OCI-AML3, right – HEL). D: Effect of SKI-1 on the signal from leukemic cells (left – OCI-AML3, right – HEL).

by dasatinib treatment in HEL cells (Fig. 5B, right), probably due to reduced cell spreading. However, the rate of the attachment was unchanged indicating that the cell capacity to form adhesion structures is retained in the absence of SFK activity. Unlike the adherent cells, leukemia cells were also largely insensitive to dasatinib addition (cf Fig. 5A with Fig. 5C, left) whereas the response of HEL cells was closer to the adherent cells (cf Fig. 5A with Fig. 5C, right). CytD treatment resulted in a slow increase in the microimpedance signal in leukemia cells, in contrast to adherent cells and HEL cells. Finally, SKI-1 produced a transient marked increase in the resistance in OCI-AML3 cells and concentration-dependent changes different from those induced by dasatinib in HEL cells (Fig. 5D). SKI-1 induced changes in OCI-AML3 cells (Fig. 5D, left) were not markedly influenced by the frequency of the electric field and thus probably reflect a transient increase in the mean cell area.

Effect of kinase inhibitors on leukemia cell morphology and dynamics

We failed to detect any effect of 100 nM dasatinib treatment on cell morphology and dynamics, regardless of leukemia cell type and of experimental settings (cell pre-treatment or addition to adhered cells). As expected, the signal of the phospho-specific (Tyr416) antibody against SFK disappeared from immunofluorescence images in the presence of dasatinib. However, the cells still formed membrane protrusions and short-lived contacts with the surface as monitored using IRM, with no apparent change in their morphology and assembly/disassembly rate.

Similarly, SKI-1 did not seem to induce marked changes in the morphology and dynamics of the treated cells and of their adhesion structures. In agreement with the results of western-blot, this inhibitor enhanced the signal of the phospho-specific antibody against SFKs in cells plated on FN, specifically in adhesion sites. The anti-pSFK (Tyr416) antibody was nearly undetectable in control OCI-AML3 cells and SKI-1 treatment resulted in a marked increase of the fluorescence signal which was clearly localized to adhesion sites detected in IRM (Fig. 6A). In MOLM-7 cells, which have higher level of active Lyn even without treatment, the signal was distributed both in adhesion sites and in multiple small foci in the cytoplasm. SKI-1 treatment highlighted the staining in the adhesion sites while attenuating the signal from the other cell parts (Fig. 6B). Lyn activation by SKI-1 is thus limited to the proximity of adhesion complexes. Additional examples of changes in pSFK signal after cell treatment with SKI-1 are included in Supplementary Fig. S6.

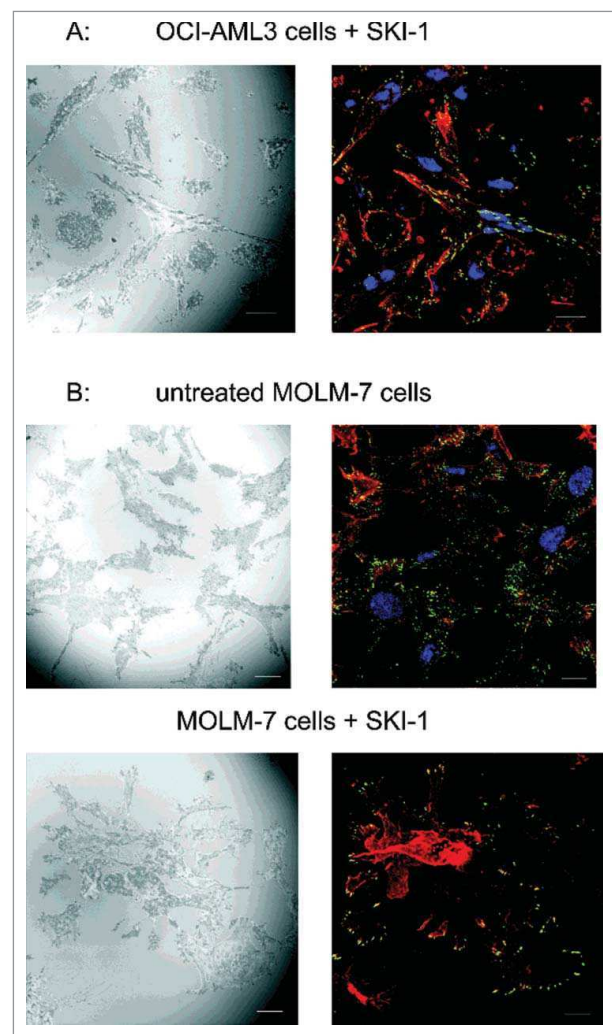


Figure 6. Localization of autophosphorylated Lyn kinase in cells treated with SKI-1. The cells were incubated for 1 h on fibronectin, then treated with 20 μ M SKI-1 for 30 to 60 min. The sample was fixed and the active Lyn was detected using anti-phospho-SFK (Tyr416) and Alexa-488-conjugated secondary antibody. Red: actin polymers visualized by phalloidin, blue: nuclei (DAPI). IRM and IF from the same visual field are shown in each pair of images. A: OCI-AML3 cells, B: MOLM-7 cells. Scale bars: 10 μ m.

Effect of kinase inhibitors on HeLa cells

To investigate Lyn behavior in adherent cells, we transfected HeLa cells with plasmids coding for Lyn kinase fused with the green fluorescent protein (Lyn-eGFP). Although the fluorescence signal was spread over the whole cell, focal adhesions were usually enriched (Fig. 7). We detected no difference in the morphology of focal adhesions between control and transfected cells as well as between successfully transfected cells (with eGFP signal, 8–15% of cells) and cells without Lyn-eGFP expression. Cells treated with 100 nM dasatinib had larger focal adhesions and appeared to be more tensioned in comparison with the untreated controls, independently of the presence of Lyn-eGFP. In parallel, dasatinib

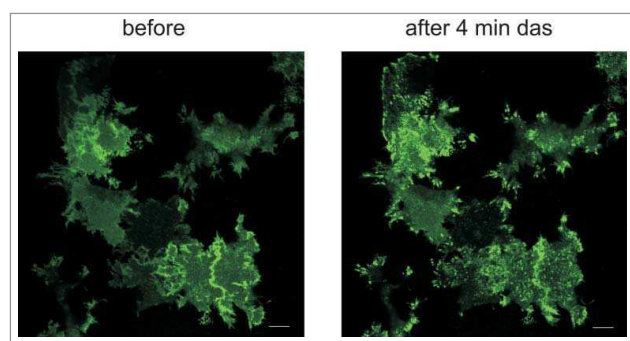


Figure 7. Changes in the distribution of Lyn-eGFP signal upon treatment of HeLa cells with dasatinib. HeLa cells were transfected with plasmid coding for Lyn-eGFP. The figure shows the fluorescence intensity from eGFP before and 4 min after treatment of cells with 100 nM dasatinib. Scale bars: 10 μ m.

treatment resulted in a fast (in several minutes) accumulation of Lyn-eGFP in focal adhesions (Fig. 7, right panel, Supplementary Fig. S7). Similar effects were observed after cell treatment with SKI-1, but the changes were slower and less apparent.

Discussion

Abnormal function of cell adhesion signaling contributes to aberrant behavior of leukemia blast cells. A typical example is the chronic myelogenous leukemia (CML) which is characterized by expression of the fusion protein BCR-ABL with tyrosine kinase activity, SH3 domain and actin-binding domain. CML cells display altered β_1 -integrin-dependent adhesion and adhesion-mediated growth regulation²² as well as increased ability to survive in the absence of interactions with the bone marrow. A number of other adhesion receptors, including CD44 and L-selectin have also been implicated in abnormal trafficking and growth of CML blasts.²³ In addition, BCR-ABL downregulates the expression of the chemokine receptor CXCR4 and thereby alters CML cell response to SDF-1.^{24,25} It has also been reported that p190 form of Bcr-Abl (expressed in acute lymphocytic leukemia) but not p210 form (expressed in CML) induces invadopodia formation upon transfection into BaF3 cells.²⁶ Treatment with tyrosine kinase inhibitors used in CML therapy may restore the interaction of CML cells with the bone marrow niches which in turn results in acquisition of stroma-mediated chemoresistance and may aggravate the residual disease.^{17,27,28} Similarly, multiple reports have already described altered expression of adhesion receptors (e.g., CXCR4, CD44, VLA-4, CD166) in AML cells^{29,30} as well as differences in interaction with bone marrow components between primary AML blasts and normal progenitor cells.³¹ Targeting of cell-matrix interactions is considered to be a promising tool

for eradication of the residual disease in AML. In B-cell chronic lymphocytic leukemia (CLL), increased levels of cortactin, which is involved in regulation of cell motility, was found to correlate with negative prognostic factors.³²

Increased expression or activity of haematopoietic cell-related SFK is often associated with leukemia progression and resistance to treatment. For example, SFK have been described to play a role in CML,³³ acute lymphoblastic leukemia,³⁴ AML,³⁵ multiple myeloma³⁶ and B-cell CLL.³⁷ Increased levels of Lyn kinase were associated with enhanced cytoskeletal activity in B-cell CLL.³⁸ Dasatinib, a dual inhibitor of BCR-ABL and SFK, is used in CML treatment, especially in cases of resistance to imatinib mesylate, a specific BCR-ABL inhibitor. Targeting of SFK is also considered as a part of combined anti-leukemia therapy.³⁵

Cell binding to fibronectin is mediated by integrins and associated protein complexes. In adherent cells, the composition and the architecture of these complexes evolve during cell attachment and spreading and the mature adhesion structures are connected to actin-myosin fibers as well as to intermediate filaments. A large set of integrin-associated proteins (so-called consensus integrin adhesome) has been defined on the basis of the composition of adhesion complexes from 7 different cell types, including one leukemia cell line (K562).³⁹ In comparison with adherent cells, the integrin adhesome of K562 cells lacked in particular proteins with force-recognition domains and the authors suggested that K562 data set likely represented a higher proportion of immature structures that form before applied myosin-II-generated cytoskeletal forces. In agreement with this result, we found that adhesion structures of leukemia cells were very dynamic and the cells usually did not contain actin stress fibers. Also, the contact points seen in IRM did not colocalize with markers which are generally used to visualize focal adhesions. Indeed, paxillin did not form part of K562 integrin adhesome although it was present in all data sets from adherent cells.³⁹ On the other hand, Lyn was found exclusively in the adhesome of K562 cells. In our experiments, SFK localization in contact points was clearly evident mainly for the active (autophosphorylated) form, in several leukemia cell lines as well as in primary cells from AML patients (Supplementary Fig. S6).

Dasatinib is a potent SFK inhibitor used in CML treatment and we confirmed nearly complete Lyn inhibition by 100 nM dasatinib in our cell models (Fig. 3C and Fig. 4A). Our results show that SFK are not required for leukemic cell attachment to FN as the adherent cell fraction was not very different in the presence of dasatinib (Fig. 3A) and the kinetics of cell binding monitored through the microimpedance signal were also nearly the same (Fig. 5B, left). A minor effect of dasatinib on the impedance signal could be attributed to a small decrease

in the mean cell area. It is known that c-Src attenuates RhoA activity, limits the actinomyosin contractility and allows for cell spreading. Cell shrinking due to SFK inhibition is thus not surprising and suggests that Lyn could have similar role in HEL cells where dasatinib induced a more marked reduction of the resistance signal (Fig. 5B and C, right).

Suprisingly, Lyn kinase was activated following cell treatment with another SFK inhibitor SKI-1 when the cells were on FN layer (Fig. 4B, Supplementary Fig. S5). The increase in Lyn activity correlated with a significant decrease of adherent cell fraction (Fig. 3B) as well as with changes in microimpedance signal which were cell type-dependent (Fig. 5D). In HEL cells, lower resistance could reflect looser cell-surface contact corresponding to lower ACF, but the effect was already apparent at 20 μ M SKI-1 concentration on ACF (Fig. 3B) whereas 50 μ M was needed to produce a significant change in the microimpedance signal (Fig. 5D). In OCI-AML3 cells, Lyn activity may stimulate membrane blebbing and transient cell spreading on the surface, which would mask the decrease in resistance due to weakening of the cell attachment to the surface. The increase in Lyn activity in adhesion points after treatment with SKI-1 is probably due to the inhibition of an unspecific target in addition to SFK. To further confirm this assumption, we also tested a third SFK inhibitor, saracatinib. In our cell systems, the effect of saracatinib on Lyn activity was similar to that of dasatinib, i.e., it gradually reduced the bands seen on anti-pSFKs western-blot, both in suspension and in cells on FN, with EC50 of about 1 μ M. No increase in Lyn activity was noted in the range from 30 nM to 20 μ M (data not shown).

Lyn kinase has been repeatedly reported to affect adhesion and migration in mature haematopoietic cells. Lyn knockout resulted in hyperadhesivity and impaired migration in macrophages,⁸ migration defects were also observed in neutrophils after Lyn knockdown using siRNA.⁹ Our results indicate that in leukemia cells, the kinase activity of SFK including Lyn is dispensable for cell attachment to fibronectin. On the other hand, active Lyn localizes to adhesion structures and increased Lyn activity is associated with looser cell binding: cell treatment with SKI-1 induced simultaneously an increase in Lyn activity (Fig. 4, Supplementary Fig. S5) and a decrease in adherent cell fraction (Fig. 3B). We have previously reported that leukemic cell treatment with histone deacetylase inhibitors resulted in an increase in adherent cell fraction in parallel with a decrease of Lyn activity.²¹ We have also noted that cell lines with lower SFK activity (OCI-AML3, HEL and adherent cell lines) are more adherent than cells with higher SFK activity (MOLM-7, CML-T1).

Transfection of fluorescently labeled Lyn into adherent HeLa cells showed that Lyn localizes in focal adhesions of these cells, too (Fig. 7). In agreement with previous studies,^{6,40,41} SFK inhibition by dasatinib induced an increase in focal adhesion size suggesting that focal adhesions are rapidly stabilized in the absence of SFK signaling. On the contrary, we failed to detect any effect of dasatinib on adhesion structure morphology and dynamics in leukemia cells and an increase in ACF was observed only in HEL cells treated with dasatinib for a longer time (90 min, Fig. 3C). In addition, dasatinib pretreatment did not prevent the decrease in cell adhesivity induced by cytochalasin D or SKI-1 (Fig. 3C) which means that cells can detach from FN in the absence of SFK signaling. This indicates that Lyn does not directly participate in the adhesion structure disassembly and that the decrease in cell adhesivity following SKI-1 treatment is not causally related to Lyn activation.

Interestingly, one leukemia cell line (HEL) resembles to some extent to adherent cells both by more firm attachment to FN in the presence of dasatinib and by partial sensitivity to cytochalasin D (Fig. 3C, Fig. 5C). Formation of actin stress fibers and of integrin-containing focal adhesions in HEL cells stimulated with PMA and seeded on FN has already been described previously.^{42,43} However, according to our results, HEL cell line is not a representative example of leukemia cells as to the adhesion structures and signaling. This infers the hypothesis that the main reason for difference between adherent and leukemia cells is the absence of cytoskeletal fibers which precludes focal adhesion maturation and stabilization. In adherent cells, SFK would act in opposition to mechanical force⁴¹ whereas no space for SFK activity would be provided in the absence of this force in leukemia cells.

In conclusion, Lyn kinase localizes in adhesion structures of leukemia cells as well as of adherent HeLa cells. In leukemia cells, its increased activity correlates with lower cell adhesivity to fibronectin. However, SFK activity is not required for adhesion structure assembly or function and, in contrast to adherent cells, its impact on the stability of the attachment is limited. With regard to documented involvement of Lyn in many important processes in immature haematopoietic cells,^{10,44} we suppose that the role of Lyn in adhesion structures of these cells is to link the cell-matrix interaction to signaling regulating cell growth and survival.

Material and methods

Chemicals and antibodies

Fibronectin fragment (120 kDa cell attachment region) was purchased from Merck (#F1904) and used at 20

$\mu\text{g/ml}$ concentration for surface coating. Dasatinib was obtained from Selleckchem (#S1021), 200 μM stock solution was made in sterile dimethylsulfoxide (DMSO). Src Kinase Inhibitor I (SKI-1) was from Abcam (#ab120839), 50 mM stock solution was prepared in sterile DMSO. The stock solution of cytochalasin D (Sigma, #C8273) was 10 mM in DMSO. Further dilutions of all compounds were made with RPMI-1640 medium.

Antibodies against the following targets were used: phospho-Src Family (Tyr416) (Cell Signaling, #2101), c-Src (Calbiochem, #OP07), Lyn (BD Biosciences, #610003), Hck (BD Biosciences, #610278), Lck (Abcam, #ab 32149), vinculin (Abcam, #ab129002), paxillin (BD Biosciences, #610051), talin 1 (Abcam, #ab157808), phospho-HS-1 (Tyr397) (Cell Signaling, #8714S), phospho-Pyk2 (Tyr402) (Life Technologies, #44-618G), β -actin (Sigma, #A5441).

Cell isolation and culture

Cell lines were purchased from DSMZ (Braunschweig, Germany – OCI-AML3, JURL-MK1, Karpas-299, CML-T1) or ECACC (Salisbury, UK – HL-60, K562). HEL, HeLa and HEK293T cells were obtained as a gift and authenticated using analysis of short tandem repeats (May 2016), the results were compared with ATCC database. The cell line MOLM-7 which is not commercially available was obtained from Dr J. Minowada.⁴⁵ All the purchased cell lines were shortly expanded after receipt and stored in frozen aliquots in liquid nitrogen. Freshly resuscitated aliquots were used for experiments and the cells were not passaged for more than 6 months. The majority of cell lines were cultured in RPMI-1640 medium with 10% fetal calf serum, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin at 37°C in 5% CO₂ humidified atmosphere, except for OCI-AML3 (α -MEM medium, 20% FCS) and HEK293T (DMEM medium).

Primary cells from patients with acute myeloid leukemia (AML) were obtained from leukapheresis at diagnosis. All patients provided their written informed consent as to the use of their biologic material for research purposes, in accordance with the Helsinki Declaration. The leukapheretic products were diluted 20-fold in PBS and the mononuclear cell fraction was then separated using the standard protocol for Histopaque-1077 (Sigma, #H8889). Analysis by flow cytometry (CD45/SSC dot-plots) confirmed high prevalence of leukemia blasts in mononuclear cell samples.

Measurement of adherent cell fraction (ACF)

The end-point method for assessment of cellular adhesivity to fibronectin-coated surface has been described previously.⁴⁶ Briefly, the cells (1×10^4) were seeded into fibronectin-

coated wells on a microtitration plate and incubated for 1 h at 37°C. Then, the cells were washed with PBS/Ca²⁺/Mg²⁺ and the remaining cells were quantified by means of fluorescent labeling (CyQuant Cell Proliferation Assay Kit; Molecular Probes, #C7026). The adherent cell fraction (ACF) was calculated using the fluorescence signal from fibronectin-coated plate and the signal obtained from a reference plate that contained the total cell number.

Immunofluorescence microscopy (IF)

The cells were plated on fibronectin-coated glass bottom dishes, incubated for 60 minutes in CO₂ incubator, fixed with 2% paraformaldehyde, permeabilized in 0.3% Triton/PBS and incubated with primary and secondary antibodies. F-actin was labeled with Alexa Fluor[®] 647 Phalloidin (Molecular Probes, #A22287). Samples were analyzed using an Olympus FluoView FV1000 confocal laser scanning microscope, focusing on the sample layer adjoining to the FN-coated substrate.

Interference reflection measurement (IRM) and live cell imaging

Cells were incubated for 1 h on fibronectin-coated coverslip and used for live cell imaging and time-lapse microscopy or fixed with 2% paraformaldehyde. The interference in reflected light was observed by means of FV-1000 confocal microscope (Olympus), using 405 nm laser beam and focusing to the coated glass surface.

Real-time monitoring of electric cell-substrate impedance (ECIS)

Impedance measurements were performed using the ECIS Ztheta apparatus (Applied Biophysics). The wells of a 8W10E+ plate were filled with 150 μl FN solution (20 $\mu\text{g/ml}$ in 0.15 M NaCl), incubated for 30 min at the room temperature, washed with RPMI medium without calf serum and filled with 200 μl medium with serum. The baseline was monitored for about 1 h before addition of cells (150 to 200 thousands per well in 200 μl). The subsequent progressive impedance increase lasted for 1 to 1.5 h until the cell attachment to the well bottom reached an equilibrium state. The instrument automatically decomposes the impedance signal into resistance and capacitance.

Immunoblotting

The cells (5×10^6) were pelleted by centrifugation and lysed for 15 min / 4°C in modified RIPA lysis buffer (50 mM HEPES; 0.15 M NaCl; 2 mM EDTA; 0.1% NP-40; 0.05% sodium deoxycholate) with freshly added protease

and phosphatase inhibitors. Cellular debris was disposed by centrifugation (16.000 g / 4°C / 15 min), and the lysate was mixed 1:1 (v/v) with 2x Laemmli sample buffer.

In the experiments involving cell harvest from FN, the cells (1×10^6) were seeded into FN-coated wells of a 12-well microtitration plate. After incubation with inhibitors, the fraction of cells in suspension was collected by aspirating the media from the wells and pelleted by centrifugation. The remaining adhered cells were simultaneously scrapped into lysis buffer (500 μ l of modified RIPA lysis buffer with phosphatase and protease inhibitors), on ice. The 2 cell fractions were combined and incubated in RIPA for 30 min at 4°C. The lysate was then concentrated using Amicon Ultra centrifugal filters (Sigma, cat. no. Z677108, 10 kDa cutoff) to the final volume of 50 μ l. The concentrate was mixed 1:1 (v/v) with 2x Laemmli sample buffer.

An equivalent of 20 μ g of total protein was resolved on polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with primary antibodies overnight at 4°C in 3% BSA in PBS with 0,1% Tween-20 (PBST), washed in PBST 6 times, and incubated with corresponding HRP-conjugated secondary antibodies for 1 h. Chemiluminescence signal was developed by Pierce SuperSignal® Substrate (Thermo Scientific, #34080F), and detected and assessed by G:BOX iChemi XT-4 (Syngene).

Cell transfection with Lyn-eGFP plasmid

Lyn kinase-coding cDNA was amplified from cDNA library (Jurkat cells, Origene) by PCR using merged primers containing appropriate restriction sites (Fw: AAAAACTCGAGCATGGGATGTATAAAATCAAAAGGG, Rv: AAAAAAGGATCCCAGGCTGCTGCTGGTATTG). By standard methods of molecular cloning, the cDNA fragment was inserted into vector peGFP-N2 (originally Clontech) designed for expression of target protein tagged with the fluorescent protein at its C-terminus. The construct was amplified in *E. coli* and purified with the PureYield Plasmid Miniprep System (Promega). This purified plasmid was then transfected into HeLa cells using the jetPRIME transfection reagent (Polyplus Transfection) following the manufacturer's instructions.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors wish to thank M.Voráčková and H.Pilcová for expert technical assistance.

Funding

This work was supported by the Grant Agency of the Czech Republic under grant No 16-16169S and the Ministry of Health of the Czech Republic under project for conceptual development of the research organization No 00023736.

ORCID

Kateřina Kuželová  <http://orcid.org/0000-0002-9628-6255>

References

- [1] Guarino M. Src signaling in cancer invasion. *J Cell Physiol* 2010; 223:14-26; PMID:20049846
- [2] Huveneers S, Danen EH. Adhesion signaling - crosstalk between integrins, Src and Rho. *J Cell Sci* 2009; 122:1059-69; PMID:19339545; <https://doi.org/10.1242/jcs.039446>
- [3] Horton ER, Humphries JD, Stutchbury B, Jacquemet G, Ballestrem C, Barry ST, Humphries MJ. Modulation of FAK and Src adhesion signaling occurs independently of adhesion complex composition. *J Cell Biol* 2016; 212:349-64; PMID:26833789; <https://doi.org/10.1083/jcb.201508080>
- [4] del Rio A, Perez-Jimenez R, Liu R, Roca-Cusachs P, Fernandez JM, Sheetz MP. Stretching single talin rod molecules activates vinculin binding. *Science* 2009; 323:638-41; PMID:19179532; <https://doi.org/10.1126/science.1162912>
- [5] Yao M, Goult BT, Klapholz B, Hu X, Toseland CP, Guo Y, Cong P, Sheetz MP, Yan J. The mechanical response of talin. *Nat Commun* 2016; 7:11966; PMID:27384267; <https://doi.org/10.1038/ncomms11966>
- [6] Teckchandani A, Cooper JA. The ubiquitin-proteasome system regulates focal adhesions at the leading edge of migrating cells. *Elife* 2016; 5:pii: e17440; PMID:27656905; <https://doi.org/10.7554/eLife.17440>
- [7] Ingley E. Functions of the Lyn tyrosine kinase in health and disease. *Cell Commun Signal* 2012; 10:21; PMID:22805580; <https://doi.org/10.1186/1478-811X-10-21>
- [8] Abram CL, Lowell CA. The diverse functions of Src family kinases in macrophages. *Front Biosci* 2008; 13:4426-50 [pii]; PMID:18508521; <https://doi.org/10.2741/3015>
- [9] He Y, Kapoor A, Cook S, Liu S, Xiang Y, Rao CV, Kenis PJ, Wang F. The non-receptor tyrosine kinase Lyn controls neutrophil adhesion by recruiting the CrkL-C3G complex and activating Rap1 at the leading edge. *J Cell Sci* 2011; 124:2153-64; PMID:21628423; <https://doi.org/10.1242/jcs.078535>
- [10] Orschell CM, Borneo J, Munugalavada V, Ma P, Sims E, Ramdas B, Yoder MC, Kapur R. Deficiency of Src family kinases compromises the repopulating ability of hematopoietic stem cells. *Exp Hematol* 2008; 36:655-66; PMID:18346837; <https://doi.org/10.1016/j.exphem.2008.01.002>
- [11] Zhi H, Dai J, Liu J, Zhu J, Newman DK, Gao C, Newman PJ. Platelet Activation and Thrombus Formation over IgG Immune Complexes Requires Integrin α IIb β 3 and Lyn Kinase. *PLoS One* 2015; 10:e0135738; PMID:26291522; <https://doi.org/10.1371/journal.pone.0135738>
- [12] Penzes K, Baumann C, Szabadkai I, Orfi L, Keri G, Ullrich A, Torka R. Combined inhibition of AXL, Lyn and p130Cas

- kinases block migration of triple negative breast cancer cells. *Cancer Biol Ther* 2014; 15:1571-82; PMID:25482942; <https://doi.org/10.4161/15384047.2014.956634>
- [13] Roseweir AK, Qayyum T, Lim Z, Hammond R, MacDonald AI, Fraser S, Oades GM, Aitchison M, Jones RJ, Edwards J. Nuclear expression of Lyn, a Src family kinase member, is associated with poor prognosis in renal cancer patients. *BMC Cancer* 2016; 16:229, 016-2254-9; PMID:26984511; <https://doi.org/10.1186/s12885-016-2254-9>
- [14] Pham H, Birtolo C, Chheda C, Yang W, Rodriguez MD, Liu ST, Gugliotta G, Lewis MS, Cirulli V, Pandol SJ, et al. Essential role of lyn in Fibrosis. *Front Physiol* 2016; 7:387; PMID:27630579; <https://doi.org/10.3389/fphys.2016.00387>
- [15] Han J, Zhang G, Welch EJ, Liang Y, Fu J, Vogel SM, Lowell CA, Du X, Cheresch DA, Malik AB, et al. A critical role for Lyn kinase in strengthening endothelial integrity and barrier function. *Blood* 2013; 122:4140-9; PMID:24108461; <https://doi.org/10.1182/blood-2013-03-491423>
- [16] Hong H, Kitaura J, Xiao W, Horejsi V, Ra C, Lowell CA, Kawakami Y, Kawakami T. The Src family kinase Hck regulates mast cell activation by suppressing an inhibitory Src family kinase Lyn. *Blood* 2007; 110:2511-9; PMID:17513616; <https://doi.org/10.1182/blood-2007-01-066092>
- [17] Obr A, Roselova P, Grebenova D, Kuzelova K. Real-time analysis of imatinib- and dasatinib-induced effects on chronic myelogenous leukemia cell interaction with fibronectin. *PLoS One* 2014; 9:e107367; PMID:25198091; <https://doi.org/10.1371/journal.pone.0107367>
- [18] Michaelis S, Wegener J, Robelek R. Label-free monitoring of cell-based assays: combining impedance analysis with SPR for multiparametric cell profiling. *Biosens Bioelectron* 2013; 49:63-70; PMID:23711901; <https://doi.org/10.1016/j.bios.2013.04.042>
- [19] Scielzo C, Bertilaccio MT, Simonetti G, Dagklis A, ten Hacken E, Fazi C, Muzzio M, Caiola V, Kitamura D, Restuccia U, et al. Hs1 has a central role in the trafficking and homing of leukemic B cells. *Blood* 2010; 116:3537-46; PMID:20530793; <https://doi.org/10.1182/blood-2009-12-258814>
- [20] Wegener J, Keese CR, Giaever I. Electric cell-substrate impedance sensing (ECIS) as a noninvasive means to monitor the kinetics of cell spreading to artificial surfaces. *Exp Cell Res* 2000; 259:158-66; PMID:10942588; <https://doi.org/10.1006/excr.2000.4919>
- [21] Obr A, Roselova P, Grebenova D, Kuzelova K. Real-time monitoring of hematopoietic cell interaction with fibronectin fragment: The effect of histone deacetylase inhibitors. *Cell Adh Migr* 2013; 7:275-82; PMID:23567296; <https://doi.org/10.4161/cam.24531>
- [22] Prosper F, Verfaillie CM. Regulation of hematopoiesis through adhesion receptors. *J Leukoc Biol* 2001; 69:307-16; PMID:11261776
- [23] Martin-Henao GA, Quiroga R, Sureda A, Gonzalez JR, Moreno V, Garcia J. L-selectin expression is low on CD34+ cells from patients with chronic myeloid leukemia and interferon- α up-regulates this expression. *Haematologica* 2000; 85:139-46
- [24] Jin L, Tabe Y, Konoplev S, Xu Y, Leysath CE, Lu H, Kimura S, Ohsaka A, Rios MB, Calvert L, et al. CXCR4 up-regulation by imatinib induces chronic myelogenous leukemia (CML) cell migration to bone marrow stroma and promotes survival of quiescent CML cells. *Molecular Cancer Therapeutics* 2008; 7:48-58; PMID:18202009; <https://doi.org/10.1158/1535-7163.MCT-07-0042>
- [25] Chen YY, Malik M, Tomkowicz BE, Collman RG, Ptasznik A. BCR-ABL1 alters SDF-1 α -mediated adhesive responses through the β 2 integrin LFA-1 in leukemia cells. *Blood* 2008; 111:5182-6; PMID:18339898; <https://doi.org/10.1182/blood-2007-10-117705>
- [26] Daubon T, Rochelle T, Bourmeyster N, Genot E. Invadopodia and rolling-type motility are specific features of highly invasive p190(bcr-abl) leukemic cells. *Eur J Cell Biol* 2012; 91:978-87; PMID:22717125; <https://doi.org/10.1016/j.jecb.2012.04.006>
- [27] König H, Holtz M, Modi H, Manley P, Holyoake TL, Forman SJ, Bhatia R. Enhanced BCR-ABL kinase inhibition does not result in increased inhibition of downstream signaling pathways or increased growth suppression in CML progenitors. *Leukemia* 2008; 22:748-55; PMID:18273048; <https://doi.org/10.1038/sj.leu.2405086>
- [28] Weisberg E, Wright RD, McMillin DW, Mitsiades C, Ray A, Barrett R, Adamia S, Stone R, Galinsky I, Kung AL, et al. Stromal-mediated protection of tyrosine kinase inhibitor-treated BCR-ABL-expressing leukemia cells. *Mol Cancer Ther* 2008; 7:1121-9; PMID:18445657; <https://doi.org/10.1158/1535-7163.MCT-07-2331>
- [29] Becker PS. Dependence of acute myeloid leukemia on adhesion within the bone marrow microenvironment. *ScientificWorldJournal* 2012; 2012:856467; PMID:22346731; <https://doi.org/10.1100/2012/856467>
- [30] Brault L, Roivo A, Decker S, Dierks C, Tzankov A, Schwaller J. CXCR4-SERINE339 regulates cellular adhesion, retention and mobilization, and is a marker for poor prognosis in acute myeloid leukemia. *Leukemia* 2014; 28:566-76; PMID:23817178; <https://doi.org/10.1038/leu.2013.201>
- [31] Hanke M, Hoffmann I, Christophis C, Schubert M, Hoang VT, Zepeda-Moreno A, Baran N, Eckstein V, Wuchter P, Rosenhahn A, et al. Differences between healthy hematopoietic progenitors and leukemia cells with respect to CD44 mediated rolling versus adherence behavior on hyaluronic acid coated surfaces. *Biomaterials* 2014; 35:1411-9; <https://doi.org/10.1016/j.biomaterials.2013.11.011>
- [32] Gattazzo C, Martini V, Frezzato F, Trimarco V, Tibaldi E, Castelli M, Facco M, Zonta F, Brunati AM, Zambello R, et al. Cortactin, another player in the Lyn signaling pathway, is overexpressed and alternatively spliced in leukemic cells from patients with B-cell Chronic Lymphocytic Leukemia. *Haematologica* 2014; 99(6):1069-77; <https://doi.org/10.3324/haematol.2013.090183>
- [33] Li S. Src-family kinases in the development and therapy of Philadelphia chromosome-positive chronic myeloid leukemia and acute lymphoblastic leukemia. *Leuk Lymphoma* 2008; 49:19-26; PMID:18203007; <https://doi.org/10.1080/10428190701713689>
- [34] Hu Y, Liu Y, Pelletier S, Buchdunger E, Warmuth M, Fabbro D, Hallek M, Van Etten RA, Li S. Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia. *Nat Genet* 2004; 36:453-61; PMID:15098032; <https://doi.org/10.1038/ng1343>
- [35] Dos Santos C, McDonald T, Ho YW, Liu H, Lin A, Forman SJ, Kuo YH, Bhatia R. The Src and c-Kit kinase

- inhibitor dasatinib enhances p53-mediated targeting of human acute myeloid leukemia stem cells by chemotherapeutic agents. *Blood* 2013; 122:1900-13; PMID:23896410; <https://doi.org/10.1182/blood-2012-11-466425>
- [36] Gertz MA. New targets and treatments in multiple myeloma: Src family kinases as central regulators of disease progression. *Leuk Lymphoma* 2008; 49:2240-5; PMID:19052970; <https://doi.org/10.1080/10428190802475311>
- [37] Veldurthy A, Patz M, Hagist S, Pallasch CP, Wendtner CM, Hallek M, Krause G. The kinase inhibitor dasatinib induces apoptosis in chronic lymphocytic leukemia cells in vitro with preference for a subgroup of patients with unmutated IgVH genes. *Blood* 2008; 112:1443-52; PMID:18550857; <https://doi.org/10.1182/blood-2007-11-123984>
- [38] ten Hacken E, Scielzo C, Bertilaccio MT, Scarfò L, Apollonio B, Barboglio F, Stamatopoulos K, Ponzoni M, Ghia P, Caligaris-Cappio F. Targeting the LYN/HS1 signaling axis in chronic lymphocytic leukemia. *Blood* 2013; 121:2264-73; PMID:23325840; <https://doi.org/10.1182/blood-2012-09-457119>
- [39] Horton ER, Byron A, Askari JA, Ng DH, Millon-Fremillon A, Robertson J, Koper EJ, Paul NR, Warwood S, Knight D, et al. Definition of a consensus integrin adhesion complex and its dynamics during adhesion complex assembly and disassembly. *Nat Cell Biol* 2015; 17:1577-87; PMID:26479319; <https://doi.org/10.1038/ncb3257>
- [40] Kaplan KB, Bibbins KB, Swedlow JR, Arnaud M, Morgan DO, Varmus HE. Association of the amino-terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527. *EMBO J* 1994; 13:4745-56; PMID:7525268
- [41] Fincham VJ, Frame MC. The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. *EMBO J* 1998; 17:81-92; PMID:9427743; <https://doi.org/10.1093/emboj/17.1.81>
- [42] Jarvinen M, Ylanne J, Vartio T, Virtanen I. Tumor promoter and fibronectin induce actin stress fibers and focal adhesion sites in spreading human erythroleukemia (HEL) cells. *Eur J Cell Biol* 1987; 44:238-46; PMID:3319626
- [43] Ylanne J, Cheresch DA, Virtanen I. Localization of beta 1, beta 3, alpha 5, alpha v, and alpha IIb subunits of the integrin family in spreading human erythroleukemia cells. *Blood* 1990; 76:570-7; PMID:1696147
- [44] O'Laughlin-Bunner B, Radosevic N, Taylor ML, Shivakrupa DeBerry C, Metcalfe DD, Zhou M, Lowell C, Linnekin D. Lyn is required for normal stem cell factor-induced proliferation and chemotaxis of primary hematopoietic cells. *Blood* 2001; 98:343-50; PMID:11435302; <https://doi.org/10.1182/blood.V98.2.343>
- [45] Tsuji-Takayama K, Kamiya T, Nakamura S, Matsuo Y, Adachi T, Tsubota T, Imanishi J, Minowada J. Establishment of multiple leukemia cell lines with diverse myeloid and/or megakaryoblastoid characteristics from a single Ph1 positive chronic myelogenous leukemia blood sample. *Hum Cell* 1994; 7:167-71; PMID:7873501
- [46] Kuzelova K, Pluskalova M, Brodska B, Otevrelova P, Elknerova K, Grebenova D, Hrkál Z. Suberoylanilide hydroxamic acid (SAHA) at subtoxic concentrations increases the adhesivity of human leukemic cells to fibronectin. *J Cell Biochem* 2010; 109:184-95; PMID:19911379; <https://doi.org/10.1002/jcb.22397>

Group I PAK Inhibitor IPA-3 Induces Cell Death and Affects Cell Adhesivity to Fibronectin in Human Hematopoietic Cells

Kateřina Kuželová*, Dana Grebeňová, Aleš Holoubek, Pavla Röselová, Adam Obr

Department of Cellular Biochemistry, Institute of Hematology and Blood Transfusion, Prague, Czech Republic

Abstract

P21-activated kinases (PAKs) are involved in the regulation of multiple processes including cell proliferation, adhesion and migration. However, the current knowledge about their function is mainly based on results obtained in adherent cell types. We investigated the effect of group I PAK inhibition using the compound IPA-3 in a variety of human leukemic cell lines (JURL-MK1, MOLM-7, K562, CML-T1, HL-60, Karpas-299, Jurkat, HEL) as well as in primary blood cells. IPA-3 induced cell death with EC50 ranging from 5 to more than 20 μ M. Similar range was found for IPA-3-mediated dephosphorylation of a known PAK downstream effector, cofilin. The cell death was associated with caspase-3 activation, PARP cleavage and apoptotic DNA fragmentation. In parallel, 20 μ M IPA-3 treatment induced rapid and marked decrease of the cell adhesivity to fibronectin. Per contra, partial reduction of PAK activity using lower dose IPA-3 or siRNA resulted in a slight increase in the cell adhesivity. The changes in the cell adhesivity were also studied using real-time microimpedance measurement and by interference reflection microscopy. Significant differences in the intracellular IPA-3 level among various cell lines were observed indicating that an active mechanism is involved in IPA-3 transport.

Citation: Kuželová K, Grebeňová D, Holoubek A, Röselová P, Obr A (2014) Group I PAK Inhibitor IPA-3 Induces Cell Death and Affects Cell Adhesivity to Fibronectin in Human Hematopoietic Cells. PLoS ONE 9(3): e92560. doi:10.1371/journal.pone.0092560

Editor: Mirjam M. Zegers, NCMLS, Radboud University Nijmegen Medical Center, Netherlands

Received September 26, 2013; Accepted February 25, 2014; Published March 24, 2014

Copyright: 2014 Kuželová et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The work was supported by the Ministry of Health of the Czech Republic (Project for conceptual development of research organization No 00023736) and by European Union (grants ERDF OPK CZ.2.16/3.1.00/24001 and CZ.2.16/3.1.00/28007). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: kuzel@uhkt.cz