Centrosome aberrations and G_1 phase arrest after *in vitro* and *in vivo* treatment with the SRC/ABL inhibitor dasatinib

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The online version of this article contains a supplemental appendix.

ABSTRACT

Background

Dasatinib is a multitargeted inhibitor of ABL, the SRC family, and other tyrosine kinases. We sought to evaluate the effects of this drug on cell proliferation, centrosomes, mitotic spindles, and cell cycle progression *in vitro* and *in vivo*.

Design and Methods

Human dermal fibroblasts, Chinese hamster cells, human osteosarcoma cells, and blood and bone marrow mononuclear cells from 32 patients with chronic myeloid leukemia, gastrointestinal stromal tumor, and systemic mastocytosis as well as from six healthy individuals were investigated. The effects of dasatinib were compared with those of the ABL inhibitors imatinib and nilotinib, the SRC inhibitor PP2, and the ABL/LYN inhibitor INNO-406.

Results

Dasatinib induced G_1 phase arrest in all cell lines and this was associated with a decline in cyclin D1 levels. *In vitro*, centrosomal aberrations, a decrease of mitotic spindles, and G_1 phase arrest were observed. In patients, centrosome alterations were found in a median of 17% (range, 10-22%) of cells with a decrease of spindles in 8/18 patients. In comparison, imatinib, nilotinib and PP2 led to centrosome aberrations without G_1 phase arrest. INNO-406 was associated with centrosome aberrations and cell cycle arrest in G_1 phase.

Conclusions

Dasatinib blocks the G_1/S transition and inhibits cell growth. It induces centrosomal aberrations and a decrease of mitotic spindles. The effects suggest an involvement of SRC and ABL inhibition.

Key words: chronic myeloid leukemia, cytogenetics, centrosomes, spindles, tyrosine kinase inhibitors.

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Introduction

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder characterized by the Philadelphia chromosome (Ph) and the *BCR-ABL* fusion gene. This gene encodes a chimeric BCR-ABL fusion protein with constitutively enhanced tyrosine kinase activity.[1] As tyrosine kinase activity is fundamental for transformation, it is an ideal target for therapy.[2] Therefore, drugs have been developed to inhibit BCR-ABL signaling pathways associated with proliferation of the leukemic clone in BCR-ABL-positive diseases.

Imatinib (Gleevec™/Glivec®) has been introduced as first line therapy for CML and targets the tyrosine kinase activity of ABL-associated human leukemias. Apart from BCR-ABL, imatinib occupies the ATP binding sites of several other tyrosine kinases (c-KIT, PDGFRA, PDGFRB, ARG, LCK, FRK, FYN and c-FMS).[3-6] Despite good cytogenetic responses to imatinib, *BCR-ABL* point mutations associated with clinical resistance may occur in chronic and advanced stage disease.[7] In addition, clonal cytogenetic evolution, being also a major cause of resistance, plays a fundamental role in the progression of CML to advanced disease.[7,8] To overcome resistance, higher doses and combination therapy of imatinib with other drugs have been applied with moderate success.

The novel drug dasatinib (SPRYCEL®, Bristol-Myers Squibb, New York, NY, USA) is an ATP-competitive, dual SRC/ABL kinase inhibitor with antiproliferative activity against hematologic and solid tumor cell lines that is postulated to bind to the active, as well as to the inactive, conformations of the ABL kinase domains and is, therefore, effective against most imatinib-resistant forms of BCR-ABL, except the T315I BCR-ABL mutation.[9,10] SRC, a non-receptor tyrosine kinase, is a key mediator for multiple signaling pathways that regulate critical cellular functions and is often aberrantly activated in cancers. Inhibition of the SRC family members LYN and HCK, up-regulated in imatinib-resistant cells, and simultaneous inhibition of ABL are the important features of dasatinib, which also inhibits all other SRC-family members[11] and has shown activity against c-KIT and PDGFRB.[9,12,13] The efficacy and safety of dasatinib have been demonstrated in patients with Ph-positive leukemias after the failure of imatinib and the drug has been advanced into clinical trials for solid human epithelial tumors.[10,14-18]

Despite good response rates and acceptable tolerability of tyrosine kinase inhibitor treatment, little is known about long-term toxicity. Given the multitargeted function it is assumed that undesired adverse effects influencing other drug-sensitive kinases could occur. In 2000, the first cases of Ph-independent clonal evolution were observed under imatinib treatment[8,19-23] and such cases have now also been observed in a limited number of dasatinib-treated patients.[24] The question arises as to whether these alterations, presumably resulting from genetic chromosomal instability of CML patients, were uncovered by a simultaneous gradual elimination of Ph-positive clones or were induced by treatment. Although patients with aberrations in Ph-negative hematopoiesis are rare, data suggest an association with a higher risk of developing secondary myelodysplastic syndromes (MDS) or acute myeloid leukemias (AML).[25] The long-term effects of dasatinib have not been investigated as yet. Although SRC has been well studied, its exact role in the physiology of normal and malignant cells is complex and not completely understood.[26]

We recently showed that 30% of centrosomes in the chronic phase of CML are aberrant. Centrosome hypertrophy is an early event in the transformation process and precedes karyotype instability.[27] The occurrence of chromosomal aberrations in BCR-ABL-negative cells from patients being treated with imatinib raises the question of whether centrosome-associated mechanisms may be responsible. Furthermore, centrosome and chromosome aberrations were detected *in vitro* in normal human cells after treatment with imatinib or nilotinib (an imatinib derivative and second generation tyrosine kinase inhibitor; Tasigna®; Novartis Pharma, Basel, Switzerland), suggesting that both drugs may themselves play a role in the emergence of aberrations in Ph-negative cells in CML patients.[28,29]

In this study we sought to evaluate the potential of the new tyrosine kinase inhibitor dasatinib on cell proliferation, chromosomes, centrosome status, spindle formation, and cell cycle progression *in vitro* and *in vivo*. We used other compounds targeting a different spectrum of tyrosine kinases (ABL: imatinib, nilotinib; SRC: PP2; ABL/LYN: INNO-406) to pinpoint whether observed effects were of ABL or SRC-related origin.

Design and Methods

Reagents

Dasatinib was provided by Bristol-Myers Squibb, imatinib and nilotinib by Novartis. PP2 was purchased (Calbiochem-Novabiochem, San Diego, CA, USA) and INNO-406 was provided by Innovive, (New York, NY, USA). Stock 10 mM solutions were prepared in sterile water (imatinib) or dimethyl sulphoxide (DMSO) (dasatinib, nilotinib, PP2, and INNO-406).

Primary cells and cell lines

Normal human dermal fibroblasts (NHDF, Promocell, Heidelberg, Germany) and Chinese hamster embryonal fibroblasts (CHE), both with normal karyotypes (46,XY and 22,XY respectively) were grown in 100-mm tissue culture dishes in RPMI 1640 containing 10% fetal bovine serum (Invitrogen, Karlsruhe, Germany) and 1% penicillin-streptomycin (100 mM; Invitrogen) in a humidified incubator at 37°C in an atmosphere of 95% air and 5% CO_2 as described before.[28] The human osteosarcoma cell line U2OS (ATCC) was grown in McCoy's 5A (Invitrogen) with 10% fetal bovine serum, 1% penicillin-streptomycin and 0.5% neomycin (Sigma, Taufkirchen, Germany).

Inhibitory assays

Drug concentrations were chosen in accordance with clinically applied doses including C_{max} and IC_{50} values measured for NHDF cells and compounds *in vitro*. NHDF cell cultures were treated for a period of 3 weeks with: (i) 0.01, 0.05, 0.1, 0.5, 1, 10, 25, 50, 100, 250, 500 nM, 1, and

2 μM dasatinib (when administered to patients twice a day at a dose of 70 mg, the plasma half-life was 3-4 h and the C_{max} 90 nM).[30] Dasatinib inhibited cell growth of NHDF cells with an IC_{50} of 50 nM; (ii) 500 nM, 1, 2, 5 and 10 μM nilotinib (when administered to patients twice a day at a dose of 400 mg, the plasma half-life was 15-24 h and the C_{max} 3.6 μM).[30] Nilotinib inhibited NHDF cell growth with an IC_{50} of 10 μM; (iii) 5, 10, and 20 μM imatinib (when administered to patients once a day at a dose of 400 mg, the plasma half-life was 19.3 h and the C_{max} 4.6 μM).[2] Imatinib inhibited cell growth of NHDF cells with an IC_{50} of 5 μM; (iv) 500 nM, 1, and 2 μM PP2 (IC_{50} 2 μmol/L in NHDF cells; C_{max} 10 μmol/L);[31] (v) 100, 500 nM, 1, and 2 μM INNO-406 (when administered to Balb/c mice twice a day at a dose of 100 mg/kg, the C_{max} value was 4 μM).[32] INNO-406 inhibited NHDF cell growth with an IC_{50} of 1 μM. In addition, NHDF cells were treated with imatinib (5 μM) and PP2 (2 μM) simultaneously (the IC_{50} doses of imatinib and PP2 in NHDF cells) for a period of 3 weeks. For comparison of species and cell types, CHE fibroblasts and U2OS cells were treated with 500 nM, 1, and 2 μM dasatinib for a period of 3 weeks. All cell culture experiments were carried out in triplicate, accompanied by control cultures treated with corresponding concentrations of DMSO (maximum 0.2% DMSO). Controls did not show any effect of DMSO. The medium was changed and drugs were added three times a week. Confluent cell cultures were divided and transferred to fresh culture dishes once a week.

Cytogenetic analysis

At least 20 metaphases per sample (cell cultures, bone marrow/peripheral blood) were analyzed by the G-banding technique and the results presented according to the International System for Human Cytogenetic Nomenclature (ISCN, 2005), as described elsewhere.[27-29,33]

Centrosome and spindle staining

For centrosome staining, cells were immunostained with an antibody to pericentrin (Covance, Richmond, CA, USA), followed by a Cy3-conjugated secondary antibody. At least 100 cells per sample were examined.[27-29]

Spindle staining was performed in combination with centrosome staining using a polyclonal antibody to α-tubulin (#T6074, Sigma) at a dilution of 1:500 in 1 x phosphate-buffered saline containing 2% horse serum in addition to the anti-pericentrin antibody. Twenty spindles per sample were examined.[29]

Cell proliferation

NHDF cells ($3\text{-}4\times10^5$) were plated in triplicate in 100 mm tissue culture dishes in complete medium containing dasatinib (1 nM - 2 μM). For comparison cells were treated with PP2 (100 nM - 2 μM), INNO-406 (100 nM - 2 μM), imatinib (500 nM-5 μM) and nilotinib (500 nM - 2 μM) or an equal volume of DMSO (control). To assess cell proliferation, cells were harvested with trypsin, stained with trypan blue, and counted manually after 24 h, 48 h and 72 h of incubation in the presence or absence of the inhibitors. Cells that excluded trypan blue were considered viable. All experiments were performed in triplicate. The range of drug concentrations was chosen taking into account the IC_{50} values for NHDF cells (IC_{50} of dasatinib, 50 nM; range of drug concentrations, 1 nM to 2 μM). For further information (measure points; standard deviation) see also *Online Supplementary Table S1*.

Cell cycle analysis

Subconfluent NHDF cells were treated with dasatinib (0.01 nM - 2 μM; taking into the account the IC_{50} values for NHDF cells) for 1 week. To compare the observed effects of compounds targeting a different spectrum of tyrosine kinases, cells were treated with PP2 (100 nM - 2 μM), INNO-406 (100 nM - 2 μM), imatinib (2 μM - 5 μM), nilotinib (1 μM - 2 μM), and imatinib (5 μM)/PP2 (2 μM) simultaneously for 1 week. Cells were harvested, washed in 1 x phosphate-buffered saline, fixed in ice-cold methanol/acetone (1:1) and stained with propidium iodide. DNA content was measured by fluorescence-activated cell sorting using a flow cytometer (FACScan, Becton Dickinson and Company, San José, CA, USA) according to standard protocols.

Western blot analysis

Subconfluent NHDF cells were grown for 48 h with dasatinib (500 nM), imatinib (5 μM) and in the absence of drugs to confluence (5×10^5 cells in 100-mm tissue culture dishes). Cells were washed twice in ice-cold phosphate buffered saline (pH 7.4) and lysed for 10 minutes in buffer [50 mM Tris-HCl (pH 7.6; Sigma), 150 mM NaCl (Merck, Darmstadt, Germany), 1 mM EDTA (pH 8; Sigma), 1 mM EGTA (pH 8; Sigma), 1% Nonidet P-40 (USB #19628), 10% glycerol (Sigma), 50 mM NaF (Sigma) and 1 mM dithiothreitol (DTT; Invitrogen)]. Cells were scraped from plates, and cell lysates were clarified by centrifugation for 20 minutes at 13,000 rpm at 4°C. The supernatant was collected and total protein concentration was determined by the Biorad Assay. Equal protein aliquots were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to standard methods,[34] transferred to nitrocellulose membranes, immunoblotted with primary antibody (anti-actin; Calbiochem, Merck) (cyclin D1, D3 and p27; Santa Cruz Biotechnology, Heidelberg, Germany) and detected with secondary antibody (goat anti-mouse IgG horse-radish peroxidase conjugate antibody, goat anti-rabbit IgG horse-radish peroxidase conjugate antibody; Promega, Mannheim, Germany).

Patients

Seventeen CML patients in chronic phase (9 males, 8 females) with Ph and BCR-ABL-positive CML after failure of imatinib treatment and one patient with a gastrointestinal stromal tumor on dasatinib treatment (70 mg twice daily; plasma half-life 3-4 h; C_{max} of 90 nM) were investigated.[30] The duration of therapy was 3 to 16 months and all patients were under treatment at the moment of analysis. The patients had previously been treated with imatinib (n=18), hydroxyurea (n=11), interferon α (n=13), cytosine arabinoside (n=2) and nilotinib (n=1) alone or in combination. All patients on dasatinib were resistant to or intolerant of prior imatinib therapy. For comparison, five patients (three with CML and two with systemic mastocytosis) on nilotinib (400 mg per day; plasma half-life 15-

24 h; C_{max} value 3.6 μM)[30], four CML patients on imatinib (400 mg per day; plasma half-life 19.3 h; C_{max} value 4.6 μM),[2] five untreated CML patients at diagnosis and six healthy individuals were evaluated (see Table 1 for the patients' characteristics). Peripheral blood and bone marrow mononuclear cells from patients were investigated.

Statistical analysis

ANOVA and multiple regression analyses were used to compare results in the control and drug treatment groups and the effects of different drug concentrations within the same group. All statistical computations were done with the SAS software package, release 8.02 (SAS Institute Inc., Cary, NC, USA).

Results

In order to investigate the influence of dasatinib on cell proliferation, centrosome, spindle and chromosome status as well as on cell cycle, NHDF cells were analyzed after dasatinib treatment. The effects of dasatinib were compared with those of the ABL inhibitors imatinib and nilotinib, the SRC inhibitor PP2, the ABL/LYN inhibitor INNO-406, and the solvent control. Additionally, bone marrow and/or peripheral blood cells from 17 patients with CML and from one patient with a gastrointestinal stromal tumor were examined after treatment with dasatinib. Nine patients being treated with nilotinib or imatinib were also analyzed. Five untreated CML patients and six healthy donors served as controls. After 1 week of incubation, cell cycle data were assessed and levels of the key regulatory molecules cyclin D1, D3 and p27 were determined; cell proliferation studies were performed after 24 h, 48 h and 72 h; centrosome, mitotic spindle and karyotype analyses were carried out after 3 weeks of treatment.

Inhibition of NHDF cell growth after short-term exposure to dasatinib

Dasatinib suppressed the growth of NHDF cells with an IC_{50} of 50 nM (Figure 1 A and B). Even concentrations of 1 nM inhibited the growth of cells after 24 h, 48 h and 72 h. High concentrations (1 and 2 μM) of dasatinib resulted in a reduction of the total number of cells, induced cell death and appeared generally toxic to NHDF cells ($p<0.0001$ for all experiments). PP2 and INNO-406 also inhibited growth of NHDF cells (Figure 1 C and D; $p<0.0001$), but the data and IC_{50} values (2 μM and 1 μM, respectively) suggest that dasatinib is a more potent inhibitor of cell proliferation. Cells exposed to nilotinib did not show a growth disadvantage compared to untreated controls (Figure 1E; IC_{50} 10 μM; $p<0.7796$). In contrast, imatinib inhibited cell growth in a concentration-dependent fashion (Figure 1F; IC_{50} 5 μM; $p<0.0001$; *Online Supplementary Table S1*). The observed effects were reversible after removing the drugs (*data not shown*).

Dasatinib treatment in vitro results in centrosome hypertrophy and chromosome aberrations

Following a 3-week period of dasatinib treatment, centrosome and chromosome alterations were detected in NHDF cells when concentrations of 100 nM and 50 nM, respectively, were used. The highest rate of centrosome aberrations occurred at a concentration of 500 nM and chromosome defects arose in a dose-dependent manner ($p<0.0001$). CHE cells also showed centrosome and chromosome alterations after dasatinib treatment, indicating that the induced changes are species-independent. The U2OS cell line also showed centrosome alterations after dasatinib treatment, but this is a cell line with marked chromosomal alterations, with chromosome counts in the hypertriploid and hypotetraploid range. No changes in clonal behavior were observed after 3 weeks of exposure to dasatinib (Figure 2, Table 2). Cell cultures treated with imatinib, nilotinib, INNO-406 and PP2 also displayed numerical and structural centrosome as well as chromosome aberrations (compared to controls; imatinib $p<0.0001$, nilotinib $p<0.05$, INNO-406 p=n.s., and PP2 p=n.s.) (Figure 2, Table 2). After removing the drugs and culturing the cells for a further 3 weeks, the effects were reversed to some extent, but normal values were not reached (≤ 5% centrosome alterations, ≤10% chromosome aberrations; *data not shown*).[29]

Dasatinib treatment concurs with a decrease of spindles

Having observed the chromosome and centrosome defects induced by dasatinib, we assessed the spindle

Table 1. The characteristics of the patients receiving dasatinib (n=18), imatinib (n=4) and nilotinib (n=5) treatment.

Clinical and cytogenetic data	*Median (range)*	*n (%)*
Patients on dasatinib therapy (CML, n=17; GIST, n=1)		18
Age at start of dasatinib, years	57 (26-75)	
% male		50
Previous therapy		18 (100)
Imatinib		4 (22)
Imatinib, HU		1 (6)
Imatinib, IFN		4 (22)
Imatinib, HU, IFN		6 (33)
Imatinib, HU, IFN, AraC		2 (11)
Follow-up of all patients after diagnosis, months	75 (10-198)	
Follow-up of dasatinib therapy, months	11 (3-16)	
Cytogenetic data of CP CML patients		17
Complete cytogenetic responders		6 (35)
t(9;22)(q34;q11) as sole alteration		5 (29)
Additional aberrations in Ph+ cells		3 (18)
Additional aberrations in Ph- cells		3 (18)
Cytogenetic data of CML patients on imatinib		4
Complete cytogenetic responders		3
t(9;22)(q34;q11) as sole alteration		1
Cytogenetic data of patients on nilotinib		5
CML patient with complete cytogenetic response		1
CML patients with t(9;22)(q34;q11)		2
SM patients with normal karyotype		2
Controls		11
CML patients with t(9;22)(q34;q11) without prior treatment		5
Healthy controls with normal karyotype		6

n: number; AraC: cytosine arabinoside; HU: hydroxyurea; IFN: interferon α; CP: chronic phase; CML: chronic myeloid leukemia; GIST: gastrointestinal stromal tumor; SM: systemic mastocytosis; Ph+/Ph-: Philadelphia chromosome positive/negative.

status in NHDF, CHE fibroblasts and U2OS cells exposed to various drugs. After dasatinib treatment, the rate of spindle defects was low (at most 1%). However, even at low dasatinib concentrations (25 nM), a conspicuous reduction in the occurrence of mitotic spindles was observed. The regular tubulin structure was absent, the visible structure looked rather like a cap (Figure 2A, Table 2) and was located next to the nucleus (Figure 2B). At concentrations above 500 nM, no normal spindles and no characteristic tubulin structure patterns could be detected in NHDF, CHE fibroblasts and U2OS cells ($p<0.0001$). Comparable tubulin structures also occurred after treatment with INNO-406. PP2 did not induce either spindle defects or a decrease of spindles. NHDF showed aberrant spindles, but no decrease of spindles after treatment with imatinib and nilotinib. The induction of irregular tubulin patterns in dasatinib and INNO-406 treated cells points to synergistic inhibitory effects of ABL and SRC.

Combined treatment with imatinib and PP2 decreases the number of mitotic cells

To test the assumed synergistic effects of ABL and SRC inhibition, we treated NHDF with imatinib (5 μM;

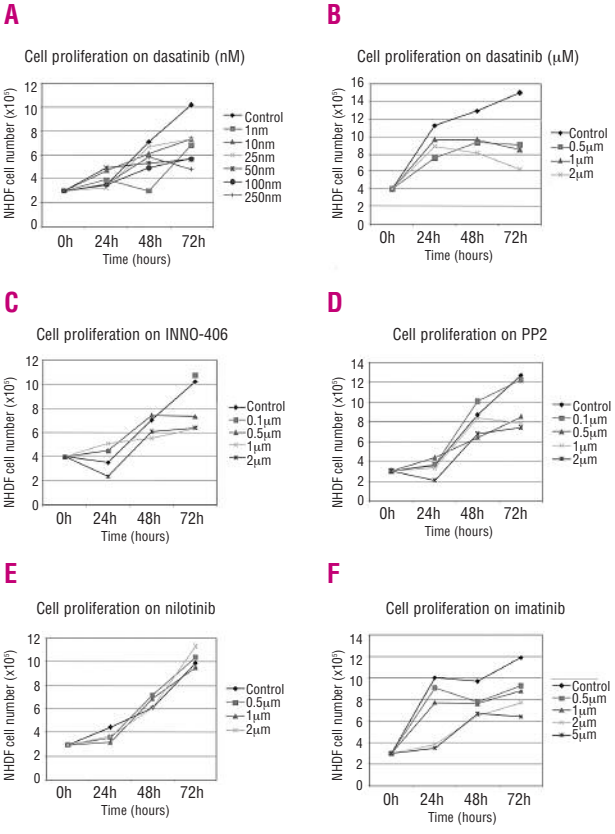

Figure 1. Effect of dasatinib, INNO-406, PP2, imatinib and nilotinib on *in vitro* growth of NHDF cells. Cells (3-4×10⁵) were plated in triplicate in 100-mm tissue culture dishes and treated with different nanomolar (A) and micromolar doses of dasatinib (B), INNO-406 (C), PP2 (D), imatinib (E) and nilotinib (F). After 24h, 48h and 72 h cell proliferation was measured by trypan blue exclusion staining and cell counting (see *Online Supplementary Table S1* for further details).

A

Species	Drugs	Normal centrosomes		Normal spindle and tubulin structure	
NHDF	Control				
		Centrosomes		Observed Phenotypes	
Species	Drugs	Numerical	Structural	Defect Spindles	Phenotypes of G_1-Arrest
NHDF	Imatinib				N. o.
	Nilotinib				N. o.
	PP2				N. o.
	Dasatinib			N. o.	
	INNO-406				
	Imatinib + PP2				
		Centrosomes		Observed Phenotypes	
Species	Drugs	Numerical	Structural	Defect Spindles	Phenotypes of G_1-Arrest
CHE	Dasatinib			N. o.	
U2OS	Dasatinib			N. o.	
CML-Patients	Dasatinib			N. o.	
GIST-Patient	Dasatinib			N. o.	
CML-Patients	Untreated			N. o.	N. o.
CML-Patients	Nilotinib				N. o.
CML-Patients	Imatinib				N. o.

B

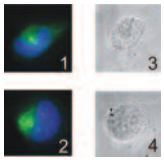

Figure 2. (A) Centrosome status and mitotic spindles in NHDF, CHE fibroblast, and U2OS cell lines and cells from chronic myeloid leukemia patients treated with different drugs. Indirect immunofluorescence staining of centrosomes was performed on untreated and on dasatinib, INNO-406, PP2, imatinib and nilotinib-treated NHDF, CHE fibroblasts, U2OS and on bone marrow and/or peripheral blood cells from patients and healthy controls. Cells were immunostained with an antibody to percentrin, followed by a Cy3-conjugated secondary antibody. Immunofluorescence staining of mitotic spindles was performed on cells and bone marrow/peripheral blood determined as above. Spindles were immunostained with an antibody directed to α-tubulin, followed by a fluoroscein isothiocynate–conjugated secondary antibody. (B) Immunostained peripheral blood cells of a patient with gastrointestinal stromal tumor visualized by immunofluorescence (1-2) and phase contrast (3-4). Abbreviation: N. o., not observed.

IC_{50} in NHDF) and PP2 (2 μM; IC_{50} in NHDF) simultaneously for 3 weeks. In treated cells, the normal tubulin cytoskeleton was absent in 25% of the analyzed cells. Instead of normal spindles, the cap-like tubulin structure similar to that seen after dasatinib and INNO-406 treatment was observed (Figure 2, Table 2). Not only was there a decrease of spindles but there were also spindle defects, as seen in imatinib and nilotinib-treated cells (2%).

Dasatinib induced G_1 cell cycle arrest in normal human fibroblasts

The inhibition of normal cell proliferation, induction of centrosome and chromosome aberrations as well as the decrease of spindles in cells exposed to dasatinib raised the question as to whether these phenomena could be related to changes of the cell cycle. We, therefore, measured the DNA content of cells treated with dasatinib using FACS analyses, and compared the results with those obtained using drugs targeting different tyrosine kinases (imatinib, nilotinib, PP2 and INNO-406; Figure 3).

Dasatinib induced a G_1 cell cycle arrest in all NHDF tested, with a shift to 2n DNA ploidy and absence of EG5 expression as a marker of G_2 phase/mitosis (*data not shown*). The dasatinib induced G_1 phase arrest was basically independent of drug concentration; only at doses of 0.5 nM and lower was dose-dependency observed (Figure 3A).

Table 2. Centrosome, chromosome and spindle status of NHDF, CHE and U2OS cells after dasatinib, imatinib, nilotinib, PP2, INNO-406 and PP2/imatinib treatment over 3 weeks.

	Concentration	*Centrosome aberrations (%)*		*Chromosome aberrations (%)*		*Spindle defects (%)*	*Phenotypes of G_1 phase arrest (%)*
		numerical	*structural*	*numerical*	*structural*		
NHDF-cultures							
+ dasatinib	control	3	2	5	5	0	0
	1 nM	1	5	10	0	0	0
	10 nM	1	5	15	0	0	0
	25 nM	2	3	15	0	3	3
IC_{50}	50 nM	2	4	20	0	0	20
C_{max} (*in vivo*)	100 nM	3	7	20	0	0	27
	250 nM	7	5	20	0	0	37
	500 nM	8	12	35	0	0	100
	1 μM	8	5	46	0	0	100
	2 μM	10	7	70	5	0	100
CHE-cultures							
+ dasatinib	control	1	4	50	0	0	0
	500 nM	10	11	70	20	0	100
	1 μM	5	10	90	10	0	100
	2 μM	7	15	90	10	0	100
U2OS-cultures							
+ dasatinib	control	3	3	c.a.	c.a.	0	0
	500 nM	6	9	c.a.	c.a.	0	100
	1 μM	9	3	c.a.	c.a.	0	100
	2 μM	8	6	c.a.	c.a.	0	100
NHDF-cultures							
+ imatinib	control	2	3	10	5	0	0
IC_{50}/C_{max} (*in vivo*)	5 μM	3	6	20	0	0	0
	10 μM	8	8	30	0	32	0
	20 μM	10	9	40	5	27	0
NHDF-cultures							
+ nilotinib	control	1	0	10	0	0	0
	1 μM	4	12	30	0	6	0
IC_{50}	2 μM	12	8	30	0	9	0
C_{max} (*in vivo*)	10 μM	6	6	25	20	53	0
	20 μM	12	6	30	5	29	0
NHDF-cultures							
+ PP2	control	1	3	10	0	0	0
	500 nM	1	5	10	0	0	0
	1 μM	1	8	10	0	0	0
IC_{50}	2 μM	7	3	20	0	0	0
NHDF-cultures							
+ INNO-406	control	3	2	10	0	0	0
	100 nM	5	5	5	0	0	7
	500 nM	16	4	35	5	0	27
IC_{50}	1 μM	5	15	43	0	0	23
	2 μM	10	8	40	0	0	17
NHDF-cultures							
+ imatinib/PP2	control	2	3	n. a.	n. a.	0	0
	5 μM/2 μM	4	12	n. a.	n. a.	2	25

NHDF: normal human dermal fibroblasts; CHE: chinese hamster embryonal cells; U2OS, osteosarcoma cell line; c. a., complex aberrant; n. a. , not analyzed; IC_{50}, 50% of NHDF cells die under treatment; C_{max}, maximal concentration in vivo.

INNO-406 also caused a G_1 arrest at doses above 100 nM (Figure 3B), while PP2 induced S phase arrest (Figure 3C). After treatment with the drug combination of imatinib and PP2, cells showed G_1 phase arrest similar to that observed after dasatinib and INNO-406 treatment (Figure 3D). Imatinib and nilotinib did not show either G_1 phase or S phase arrest (*data not shown*).

Centrosome defects and spindle decreases in dasatinib-treated patients

Since all treated cells and cell lines displayed centrosome and chromosome aberrations as well as a decrease of spindles, we were interested to determine whether patients treated with dasatinib show comparable alterations. In total, 17 CML patients and one patient with a gastrointestinal stromal tumor being treated with dasatinib (70 mg twice a day) were investigated. Five untreated CML patients in chronic phase and six healthy individuals served as controls (Table 1).

Additional chromosomal aberrations occurred in Ph-positive metaphases during dasatinib therapy. However, these aberrations were common secondary changes frequently observed in CML during disease progression. Chromosomal changes in Ph-negative hematopoiesis were observed in three CML patients. Centrosome aberrations occurred in a median of 17% (range, 10-22%) of cells. Of note, all patients were in chronic phase and had no blastic cells in the bone marrow or peripheral blood. The phenotype of G_1 phase arrest (cap-like tubulin structure), comparable to that observed *in vitro*, was detected in 8/18 patients (Figure 2A, Table 3). The patient with a gastrointestinal stromal tumor without bone marrow infiltration had centrosome aberrations in 16% of peripheral blood cells and demonstrated the characteristic phenotype of G_1 phase arrest (Figure 2B).

Five CML patients at diagnosis were analyzed as controls. All showed centrosome defects but none had the phenotype of G_1 phase arrest. Of note, these patients demonstrated high cell counts in peripheral blood and blasts in both the bone marrow and peripheral blood. Centrosome defects, already detected in 30% of cells in CML chronic phase patients at initial diagnosis by our group[27] were not, therefore, unexpected. Peripheral blood cells from six healthy controls showed normal centrosomes and karyotypes and neither spindle defects nor the characteristic phenotype of G_1 phase arrest.

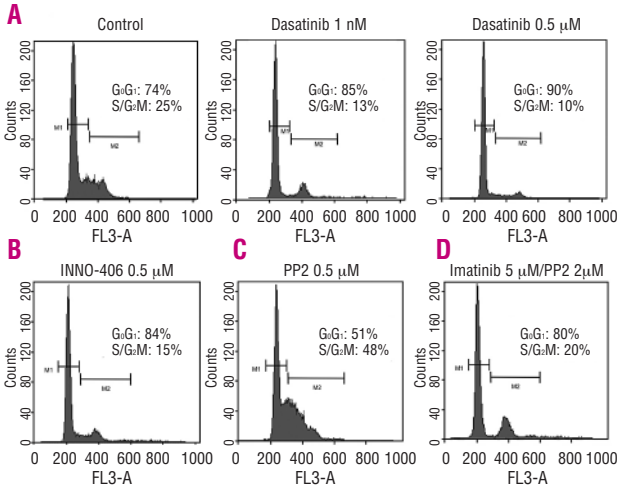

Figure 3. Cell cycle profiles of untreated, dasatinib, INNO-406 and PP2-treated NHDF. NHDF were incubated for 1 week with dasatinib (A) INNO-406 (B) PP2 (C) PP2 and imatinib (D) as indicated at the top of each graph. Controls were treated with DMSO (2 μM) instead of the drug. Subsequently, the cell cycle profile was analyzed by flow cytometry. The percentages of cells in G_1 phase and in the S, G_2 and M phases are shown in each graph.

Table 3. Centrosome and spindle status of 17 patients with chronic myeloid leukemia (CML) (#1-17) and one patient with gastrointestinal stromal tumor (#18) on dasatinib, four CML patients on imatinib (#19-22), three CML patients (#23-25) and two patients with systemic mastocytosis (#26-27) on nilotinib, five untreated CML patients at diagnosis (#28-32) and six healthy controls (#33-38).

Patient no.	*Tissue*	*Disease*	*Centrosome aberrations (%) numerical*	*Structural*	*Karyotype changes*	*Spindle defects*	*Phenotypes of G_1 phase arrest*
Dasatinib therapy							
1	BM	CML	13	9	+ (Ph$^+$ cells)	–	+
2	BM/PB	CML	6/9	10/9	–	–	+
3	BM/PB	CML	9/4	1/13	–	–	–
4	BM	CML	7	6	–	–	+
5	PB	CML	13	5	–	–	–
6	BM/PB	CML	5/12	11/4	–	–	+
7	BM/PB	CML	12/6	7/9	+ (Ph- cells)	–	–
8	BM/PB	CML	16/7	2/13	–	–	–
9	PB	CML	2	8	–	–	–
10	BM/PB	CML	7/6	13/9	+ (Ph- cells)	–	+
11	BM/PB	CML	7/12	11/9	+ (Ph$^+$ cells)	–	+
12	BM	CML	10	12	+ (Ph$^+$ cells)	–	–
13	BM/PB	CML	6/10	9/12	–	–	–
14	BM	CML	6	9	–	–	+
15	BM	CML	1	17	+ (Ph- cells)	–	–
16	BM/PB	CML	13/11	3/7	–	–	–
17	BM/PB	CML	10/13	10/6	–	–	–
18	PB	GIST	10	6	–	–	+
Imatinib therapy							
19	BM/PB	CML	5/4	20/14	–	+	–
20	BM	CML	18	10	–	+	–
21	BM/PB	CML	5/5	20/10	–	–	–
22	BM	CML	4	6	–	–	–
Nilotinib therapy							
23	BM	CML	5	5	–	–	–
24	BM/PB	CML	1/4	9/8	–	–	–
25	BM	CML	6	7	–	+	–
26	BM	SM	10	6	–	+	–
27	BM	SM	9	6	–	–	–
No therapy							
28	BM	CML	13	10	+ (Ph$^+$ cells)	–	–
29	BM	CML	10	10	–	–	–
30	BM	CML	10	10	–	–	–
31	BM	CML	11	15	–	–	–
32	BM	CML	8	9	–	–	–
33	PB	Control	2	1	–	–	–
34	PB	Control	3	0	–	–	–
35	PB	Control	0	0	–	–	–
36	PB	Control	2	0	–	–	–
37	PB	Control	2	2	–	–	–
38	PB	Control	0	2	–	–	–

CML: chronic myeloid leukemia; GIST: gastrointestinal stromal tumor; SM: systemic mastocytosis; BM: bone marrow; PB: peripheral blood; Ph+/Ph-: Philadelphia chromosome positive/negative.

Centrosome alterations and spindle defects in patients with chronic myeloid leukemia and systemic mastocytosis treated with imatinib and nilotinib without the phenotype of G_1 phase arrest

In total, we investigated three CML patients in chronic phase being treated with nilotinib after the failure of imatinib, two patients with systemic mastocytosis under nilotinib treatment and four CML patients in chronic phase being treated with imatinib (Table 1). No additional chromosomal aberrations were observed in Ph-positive metaphases in patients under nilotinib and imatinib therapy and there were no chromosomal changes in Ph-negative hematopoiesis. However, centrosome aberrations occurred in a median of 13% (range, 10-16%) of cells from patients being treated with nilotinib and in 26% (range, 25-28%) of cells from patients being treated with imatinib. Defective spindles were observed in 7% (range, 5-10%) of bone marrow cells but no characteristic phenotypes of G_1 phase arrest occurred (Table 3). This suggests that imatinib and nilotinib affect centrosomes and spindles *in vivo* (C_{max} values of 4.6 μM and 3.6 μM, respectively, are achievable in treated patients),[30] but do not influence the cell cycle in the way that dasatinib treatment does. Two patients with systemic mastocytosis without bone marrow infiltration showed elevated rates of centrosome alterations (16%) and spindle defects (5%).

Dasatinib treatment resulted in reduced cyclin D1 levels

We examined the effect of dasatinib on the key regulatory molecules cyclin D1, cyclin D3 and p27 involved in G_1-S cell cycle progression that can be regulated by SRC.[35] The G_1 arrest resulting from dasatinib was associated with a decline in cyclin D1 levels, whereas no changes were observed in imatinib-treated NHDF or in untreated controls. No changes in the protein levels of cyclin D3 and p27 were observed in either dasatinib or imatinib-treated cells (*data not shown*).

Discussion

Over the past decade, inhibitors of cellular tyrosine kinases have been developed as potential therapeutic agents for the treatment of solid tumors and leukemias. Despite the striking efficacy and good to moderate tolerability of such inhibitors, little is known about their influence on normal cells. The appearance of chromosomal abnormalities in Ph-negative cells after imatinib and dasatinib treatment and the development of secondary acute myeloid leukemia and myelodysplastic syndromes after imatinib therapy raises the questions as to whether patients have intrinsic disease-related genetic instability or whether the kinase inhibitor based therapy may be the cause of the manifestations. A combination of both is also conceivable.

In this study, we found that dasatinib had effects on NHDF *in vitro* at concentrations which are achievable *in vivo* under treatment (see Design and methods section, inhibitory assays, Table 2). These effects included induction of chromosome and centrosome alterations ($p<0.0001$) and inhibition of proliferation ($p<0.0001$). In addition, G_1 phase arrest and a decrease of spindles with a characteristic phenotype of G_1 phase arrest were observed ($p<0.0001$). Comparable results were obtained when bone marrow and peripheral blood cells of patients on dasatinib therapy were analyzed. It is, however, important to note that all patients had been treated with other drugs, including imatinib, prior to dasatinib therapy (Table 1). Because of the absence of data from patients on first line dasatinib therapy, an influence of the previous therapy on the observed chromosome and centrosome alterations cannot be excluded. Furthermore, the doses of drugs and the times of sample collection after oral administration of the drugs varied from patient to patient which could have affected the analysis and complicate interpretation of the results. Data from dasatinib-treated CHE fibroblasts and U2OS cells were comparable, suggesting that the alterations induced by dasatinib are cell and species independent.

The molecular mechanisms for the observed effects of dasatinib on karyotype stability, centrosomes and spindles may be the inhibition of one or more of its known targets. ABL tyrosine kinases interact with several proteins such as p73,[36] DNA-PK and[37] ATM,[38] involved in DNA repair mechanisms. In addition, c-ABL is an essential regulator of the RAD51 protein that plays a fundamental role in homologous recombination and in DNA repair.[39,40] Inhibition of c-ABL could enhance homologous recombination resulting in genetic instability and supernumerary functional centrosomes.[41,42] LCK and FYN (members of the SRC family), which have been identified as target molecules of dasatinib, may also be important for centrosome maintenance.[43] The association of LCK in human T cells with pericentrosomal vesicles and of FYN in Jurkat T cells and T lymphoblasts with the centrosome and mitotic spindle suggests that these kinases may be crucial for cell cycle regulation.[44] Inhibition may, therefore, affect centrosomal fidelity resulting in centrosome hypertrophy and genetic instability.

G_1 phase arrest and a decrease of spindles as indicators of a reduction of cells in mitosis after dasatinib treatment cannot be explained by inhibition of ABL or LCK and FYN alone, because these effects have never been observed *in vitro* or *in vivo* after either imatinib or nilotinib treatment. Our data appear to suggest that other members of the SRC family that play a role *in vivo* and are required for progression through the initial phase of mitosis may be important by inhibiting the cell cycle and inducing the observed G_1 phase arrest in normal human cells. The fact that INNO-406 (which also inhibits FYN)[32] produces the same results supports this concept.

In normal cells c-SRC activation is essential for PDGF-induced G_1/S transition and DNA replication. Consequently, the inhibition of SRC results in kinase-inactive SRC inducing a G_1/S block and delayed G_1/S transition.[45-48] However, SRC inhibitors induced dose-dependent anemia, edema and erythema and affected the ovaries and liver in rats.[49] Limited enzymatic selectivity, which is not correlated to the compound's core structure and which led to inhibition of other kinases in non-targeted tissues, may be possible. Since the cell cycle is important in many tissues, a common protein

kinase-dependent mechanism is conceivable. These findings concur with our results obtained in dasatinib-treated normal cells, showing deregulation of the cell cycle. In prostate cancer cells, dasatinib treatment blocked the kinase activities of LYN and SRC at low nanomolar concentrations resulting in partial inhibition of cell proliferation due to G_1 arrest.[11] Other studies have shown that *in vitro* dasatinib treatment of head and neck squamous cell carcinoma cell lines caused dose-dependent inhibition of cell growth and the cell lines showed decreased percentages of cells in G_2-M and S phases and an increased number of cells in the sub-G_0 population, consistent with cell cycle blockage in the G_1-to-S transition and induction of apoptosis.[50]

Furthermore, studies have shown that induction of the key regulator molecule p27 in dasatinib-treated cancer cell lines is associated with a decline in the levels of the proteins cyclin D1 and D3 which play important roles in regulating cell cycle progression and survival.[35,51] We found an effect of dasatinib on the regulatory molecule cyclin D1 involved in cell cycle progression which can be regulated by SRC.[26,35] SRC inhibition by dasatinib could, therefore, lead to a decrease in cyclin D1 levels resulting in G_1 cell cycle arrest and a decreased rate of spindle formation.

Unexpectedly, NHDF treated with the specific SRC inhibitor PP2 showed S phase arrest. In contrast, simultaneous PP2 and imatinib treatment resulted in less G_1 phase arrest and decreased rates of mitotic spindles. This suggests synergistic inhibitory effects of members of the tyrosine kinases (e.g., c-ABL, c-KIT) and of SRC kinases (LYN or others). Despite the previously mentioned effect, only marginal inhibition was observed and seemed to be dependent on drug potency (different IC_{50} values of drugs for NHDF).[31]

In conclusion, dasatinib blocks G_1/S transition and thereby inhibits cell growth in both normal and neoplastic cells. In addition, it induces centrosomal aberrations and decreases mitotic spindles. These effects may not be due to SRC inhibition alone but may be the result of a combination of SRC and ABL inhibition or non-specific effects on multiple kinases (c-KIT, ABL, and PDGFR) or other targets. Dasatinib is registered for the treatment of CML patients after the failure of imatinib therapy.[14,15] The adverse effects of this drug, such as neutropenia and grade 3 or 4 thrombocytopenia which occurred in 45% of patients in chronic phase and 89% of patients in accelerated phase/blast crisis and treatment-related pleural effusions,[10,30] may be caused by prolonged tissue regeneration times. Since G_1 phase arrest was observed in normal, leukemic and tumor cells, dasatinib should be considered a drug that inhibits normal and neoplastic cell growth with a strong targeted component, resulting in preferential inhibition of cells carrying a specific target, such as BCR-ABL.

Authorship and Disclosures

AF: principal investigator with primary responsibility for the publication; AF, MG, BR, AK, OF, CH, WS, PD, RH and AH: contributed to the design of the study, to the work and to the interpretation of the results. All authors checked the final version of the manuscript.

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