CORE
Metadata, citation and similar papers at core.ac
Provided by Archivio della ricerca - Università degli studi di Napoli Federico II

Journal of Cellular Physiology

Comparison of Dasatinib, Nilotinib, and Imatinib in the Treatment of Chronic Myeloid Leukemia

ROBERTO CIARCIA,[1]* SARA DAMIANO,[1] MARIA VALERIA PUZIO,[1] SERENA MONTAGNARO,[1] FRANCESCO PAGNINI,[1] CARMEN PACILIO,[2] GIUSEPPE CAPARROTTI,[3] CRISTIANA BELLAN,[4] TIZIANA GAROFANO,[5] MARIA SOLE POLITO,[4] ANTONIO GIORDANO,[4,5,6]** AND SALVATORE FLORIO[1]

[1]*Department of Veterinary Medicine and Animal Productions, University of Naples "Federico II", Naples, Italy*

[2]*Clinical Trials Unit, Istituto Nazionale per lo Studio e la Cura dei Tumori, Fondazione G. Pascale-IRCCS, Naples, Italy*

[3]*Department of Haematology, ASL Caserta (CE), Hospital Moscati, Aversa (CE), Italy*

[4]*Department of Medicine, Surgery and Neuroscience, University of Siena, Siena, Italy*

[5]*AORN Dei Colli Monaldi UOC, Oncology, Naples, Italy*

[6]*Sbarro Institute for Cancer Research and Molecular Medicine, Center of Biotechnology, College of Science and Technology, Temple University, Philadelphia, Pennsylvania*

To overcome the drug resistance phenomenon induced by Imatibib (IM), in clinical practice, are often used second generation of tyrosine kinase inhibitors as Nilotinib (NIL); a such potent inhibitor of the BCR/ABL kinase and Dasatinib (DAS), a inhibitor of BCR/ABL kinase, and inhibitor SrC family kinase. In this study we evaluated the in vivo effect of DAS, NIL, and IM on intracellular calcium concentration, oxidative stress, and apoptosis in peripheral blood leukocytes of 45 newly diagnosed patients with chronic myeloid leukaemia (CML-PBM). Our data demonstrated that treatment with DAS and NIL showed an higher modulating potential than IM on intracellular calcium concentration by inhibiting the thapsigargin, a sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA) inhibitor, and Lithium (Li) an inositol 1,4,5-triphosphate (InsP3) receptor inhibitor activities. Moreover our data demonstrated that NIL and DAS have significantly increased apoptosis more than IM by involving both intracellular calcium signaling as well as oxidative stress. The acquisition of the oxidative stress and calcium channels receptors values data could help the hematologist to modulate and improve the treatment of chronic myeloid leukaemia (CML) pathology.

J. Cell. Physiol. 231: 680–687, 2016. © 2015 Wiley Periodicals, Inc.

Chronic myeloid leukaemia (CML) is a myeloproliferative disease that originates in an haemopoietic stem cell as the result of the *t* (9;22) translocation, giving rise to the Philadelphia chromosome and bcr-abl oncoprotein p210bcr-abl. The BCR-ABL chimeric protein is thought to play a central role in the pathogenesis of Ph chromosome-positive leukaemia (Sawyers, 1999). Since several years, for the treatment of CML specific inhibitors of BRC/ABL protein kinase are employed. The first inhibitor used was imatinib mesylate (IM), a small-molecule tyrosine kinase inhibitor, which binds to an ATP-binding site on BCR-ABL, KIT, PDGF-R, and ABL protein-tyrosine kinase, thus preventing downstream signaling. In humans, this drug has been successfully used as a therapeutic agent for Philadelphia-positive chronic myeloid leukemia and gastrointestinal stromal tumor carrying a c-KIT mutation (Demetri et al., 2002). In veterinary medicine, clinical studies have reported that IM is effective against canine mast cell tumors carrying the ITD mutation on c-KIT in exon 8 or 11 and the c.1523A>T mutation in exon 9 (Kobayashi et al., 2013). However, IM is ineffective in treating cases of patients with mutations in some kinase domains (Y253F/H; E255K/V; T315I; H396P/R43,44; M244V; M351T o F359V) that confer high resistance to pharmacological treatment for which is need to increase the daily dose to be administered (Hochhaus and La Rosée, 2007). To overcome the drug resistance phenomenon, since several years, in clinical practice, second generation of tyrosine kinase inhibitors

Roberto Ciarcia and Sara Damiano contributed equally to the work.

Conflicts of interest: No potential conflict of interest was disclosed.

Contract grant sponsor: Italian Asociation Leukaemia and Lymphoma (A.I.L.) – Caserta-ONLUS.
Contract grant sponsor: Human Health Foundation - ONLUS.
Contract grant sponsor: Sbarro Health Research Organization.

*Correspondence to: Roberto Ciarcia, Department of Veterinary Medicine and Animal Productions, University of Naples "Federico II, Via Delpino 1 - 80137 Napoli, Italy.
E-mail: roberto.ciarcia@unina.it

**Correspondence to: Antonio Giordano, Sbarro Institute for Cancer Research and Molecular Medicine, Center of Biotechnology, College of Science and Technology, Temple University, Philadelphia, Pennsylvania, 12th Street, Bio Life Sciences Building Suite 431, Philadelphia, PA. E-mail: giordano@temple.edu

Manuscript Received: 9 July 2015
Manuscript Accepted: 30 July 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 3 August 2015.
DOI: 10.1002/jcp.25118

© 2015 WILEY PERIODICALS, INC.

(TKI) widely such as (NIL), a potent inhibitor of the BCR/ABL kinase structurally similar to IM, was rationally designed to improve binding affinity against BCR-ABL and improve specificity over that of IM (Weisberg et al., 2005) and (DAS), a potent inhibitor of BCR/ABL kinase (Shah et al., 2004) and inhibitor SrC family kinase, c-kit, and platelet-derived growth factor receptor (Lombardo et al., 2004). Several studies demonstrated a correlation between the activity of TKI, and alteration of intracellular calcium homeostasis. In fact the alteration in the ionized calcium concentration in the cytosol has been implicated in the initiation of secretion, contraction, and cell proliferation (Kretsinger, 1979) as well as the production of reactive oxygen species (ROS), natural by-products of aerobic metabolism, has been correlated with normal cell proliferation through activation of growth-related signalling pathways (Murrell et al., 1990). High concentration of intracellular ROS provides a direct effector mechanism for necrotic cell death and a mild increase in ROS, either H_2O_2 or O_2^- prooxidant state that could provide protection against apoptosis (Pervaiz et al., 2001). By contrast, an H_2O_2/O^{2-} ratio that favors reduction of the intracellular milieu, reduced state, sensitizes cells to apoptotic triggers that could eventually lead to spontaneous apoptosis (Clement et al., 1998). Finally the production of ROS has been correlates with normal cell proliferation through activation of growth-related signaling pathways (Murrell et al., 1990). Moreover to ROS have been ascribed a role as signalling molecules in immunity, basing inter alia on the findings that lymphocyte effector cells such as natural killer and cytotoxic T cells undergo apoptosis-like cell death after encounter with ROS-producing myeloid cells (Thoren et al., 2007). Recently, studies by Bellodi et al. (2009) showed in primary CML and K562 cells that in vitro treatment with IM induce apoptosis through inhibition of BCR/ABL protein and also through inhibition of the calcium release from the intracellular stores induced by thapsigargin. Some TKI can induce apoptosis inducing a sustained increase in concentration of intracellular Ca^{2+} resulting from depletion of the endoplasmic reticulum Ca^{2+} stores (Sergeev, 2004). In agreement with these results, recently, we demonstrated that functionality of calcium channel and oxidative stress on CML lymphomonocytes are dramatically compromised (Ciarcia et al., 2010) and that IM, in vitro, in K562 and CML cells, exerts its activity after modulation of intracellular calcium concentration (Ciarcia et al., 2013). Therefore is evident the ability of TKI to use more pathways to exert their pharmacological activity. The aim of this study was to compare the in vivo effect of NIL or DAS or IM in peripheral blood mononuclear cells of patients with (CML-PBM) on the mobilization of intracellular calcium, oxidative stress and apoptosis in order to verify some aspects of the signalling cascades.

Materials and Methods

Patients

For this study n. 45 volunteers patients with CML (21 males and 24 females; mean age 56 years; range 45–61 years), after obtaining informed consent, were selected and processed at the time of diagnosis and after oral treatment for 6 months with NIL at dose of 600 mg/die or DAS at dose of 100 mg/die or IM at dose of 400 mg/die. Eligible patients have been diagnosed with Ph + CML-CP (Cytogenetic analysis showed that Ph chromosome were 100% of positive). Chronic phase was defined by the presence of $<15\%$ blasts, $<20\%$ basophils, and $<30\%$ blasts plus promyelocytes in the peripheral blood or bone marrow.

Chemicals

(NIL) and Imatinib mesylate (IM) were kindly provided from Novartis SpA (Novartis Internation AG CH-4002 Basel Switzerland); (DAS) was provided by Bristol-Myers Squibb Srl (UK AP invoicing, Chester CH1 9LE UK); RPMI-1640 was purchased from Labtek Laboratories, Eurobio, Paris, France; Lithium chloride (Li), Thapsigargin (TG), Inositol 1,4,5-triphosphate (InsP3), phitoemoagglutinine (PHA), foetal calf serum (FCS), hystopaque, propidium iodide (PI), were obtained from SIGMA (Milan, Italy).

Leukocytes preparation

Peripheral blood mononuclear cells of healthy subjects (CS-PBM) or untreated CML patients (basal CML-PBM) or treated CML patients with DAS or IM or NIL were isolated using the modification of a method of Boyum as described by Bass et al. (1978); 15–20 ml human blood, obtained from volunteers healthy donors or CML patients, was drawn in heparin (20 U/ml), stratified on Ficoll-Hystopaque gradients (density 1.077), and centrifuged for 30 min at 600*g*. The ring of leukocytes at the interface was collected and washed two times with 5 ml of PBS. The preparation was found to be 89% pure by flow cytometric analysis (range 82–93%; red blood cells 6.3%; polymorphonucleates 2.6%; platelets 2.3%). Lymphocytes were washed by centrifugation for 5 min at 600*g* and re-suspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1% penicillin and streptomycin, 2 mM glutamine. Aliquots of lympho-monocytes were incubated for 1 hour in humidified atmosphere of 5% CO_2 at 37°C in culture plastic plates. Light microscopy and flow cytofluorimetric analysis of stained cells demonstrated that more than $96 \pm 2\%$ of the adherent cells were monocytes. Viable lymphomonocytes (as evaluated by means of the trypan blue exclusion test) were seeded at 3×10^6 cells in 6-well tissue culture plates and cultured overnight in RPMI 1640 complete medium.

CCyR assay

Complete Cytogenetic Response (CCyR) was performed by chromosome banding analysis (CBA) after short-term 24 h. Briefly, Bone marrow aspiration for cytogenetic analysis were treated with colchicine and hypotonic solution, then they were centrifuged, and the resulting pellet was fixed and washed in methanol/acetic acid (3:1). The cells were resuspended in fixative and dropped on slides. Karyotypes of samples were examined after the G banding technique and described according to International System for Human Cytogenetic Nomenclature (ISCN 1995) (Wei et al., 1995).

MMR assay

The molecular monitoring was based on peripheral blood samples collected before therapy and after treatment for 6 months. BCR-ABL transcripts level assessment was performed by real-time quantitative polymerase chain reaction (RT-Q-PCR) according to suggested procedures and recommendations (Hughes et al., 2006). The Molecular Response was defined as Major (MMR) if the BCR-ABL/ABL ratio was $\leq 0.1\%$ according to the International Scale (Branford et al., 2008).

Intracellular calcium measurement

Intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ were measured by using the radiometric fluorescent indicator dye FURA-2/AM, the membrane-permeant form of FURA-2/AM as previously described and opportunely modified (Florio et al., 2003). Briefly, CS-PBM or CML-PBM cells were washed twice in PBS in 15 ml polypropylene tubes (Falcon/Becton Dickinson Labware, Lincoln Park, NJ) resuspended in Krebs Ringer (KRH) medium (1×10^6 cells/ml) with 3.0 mM Ca^{2+}-sensitive dye FURA-2/AM for 60 min at 30°C. We also added the non ionic detergent Pluronic acid F-127 (0.02% w/v) to each incubation batch as a dispersing agent. Cell were subsequently centrifuged and resuspended in KRH calcium free or containing 1 mM $CaCl_2$. The samples were then transferred to a thermostat-equipped cuvette (37°C), maintained under

continuous stirring and analyzed in a SPEX spectrophotofluorimeter (two wavelengths excitations, 340 and 380 nm and emission intensities at 515 nm). The experiments were initiated by incubation of cell aliquots (1×10^6 cells) with several drugs for 5 min. After stimulation with thapsigargin (TG) or inositol 1,4,5-triphosphate (InsP3), the recording was continued until the end of the $[Ca^{2+}]_i$ peak. At the end of the experiment calibration was performed determining minimal fluorescence induced by 0.1% triton X – 100 in presence of 5 mM EGTA (Fmin) and maximal fluorescence induced by 3 mM $CaCl_2$ (Fmax). Intracellular calcium concentration was calculated according to the method of Grinkiewicz et al., to the equation: $[Ca^{2+}]_i = 224 \times [(R - Rmin)/Rmax - R)]$ were R is the experimentally determined fluorescence ratio (Grynkiewicz et al., 1985).

Malondialdheyde assay

Lipid peroxidation as index of the oxidative stress was determined by assaying the Malondialdheyde (MDA) production by means of the thiobarbituric acid test (TBA test) (Esterbauer and Cheeseman, 1990). Briefly: 1 mL of suspension of peripheral blood mononuclear cells (PMB cells) containing 1×10^6 cells treated or non treated for 24 h with 3 μM TG or 10 mM Li was mixed with 0.5 ml of cold 30% (w/v) trichloroacetic acid to precipitate proteins. The precipitate was pelletted by centrifugation and 1 ml of the supernatant was reacted with 1.3 mL of 0.5% (w/v) thiobarbituric acid (TBA) at 85°C for 40 min. In TBA test reaction, one molecule of MDA reacts with two molecules of TBA with the production of a pink pigment. Therefore, after cooling, the fluorescence of pigment was read at excitation of 536 nm and emission of 557 nm wavelengths in a SPEX Fluoromax spectrophotofluorimeter. The concentration of MDA was calculated respect to a calibration curve (range: 0.5–2 pmol/ml), and results were expressed as pmol of MDA/mg proteins.

SOD assay

Superoxide dismutase (SOD) activity was determined by sensitive SOD assay that utilizes a product a water-soluble formazan dye upon reduction with superoxide anion SOD Activity with absorbance at 450 nm according to the manufacturer's instructions (Assay Kit Fluka Products). Briefly, 1 ml of PBM cells containing 1×10^6 cells treated or not treated for 24 h with 3 μM TG or 10 mM Li was mixed. At the end of incubation cells were lysed and centrifuged at 14,000*g* for 5 min at 4°C and on cytosol was determined cytosolic and mitochondrial SOD activity. The starter of reaction by adding Xanthine Solution. The absorbance readings every minute for 10 min at room temperature. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. The SOD activity was expressed as % of inhibition respect to activity Control. One unit of SOD activity was defined as the amount of enzyme required for 50% inhibition of NBT reduction in the assay conditions. The SOD levels were expressed as units/mg proteins.

Apoptosis assay

Apoptosis was also evaluated with a cell death ELISA kit (Boehringer Mannheim, Indianapolis, IN) which utilizes a monoclonal antibody against histone to detect DNA fragments in the cytosolic fraction of lysed cells. Cells treated or untreated were lysed according to the manufacturer's instructions. The samples were transferred into 96-well dishes coated with a mouse monoclonal antibody against histone. After incubation and washing, anti-DNA-peroxidase was added to the wells. Cell culture supernatants removed from the cells after treatment (but before lysis) gave no signal, indicating that there were no necrotic cells during the treatment. The reaction was developed with the substrate supplied by the manufacturer and the absorbance of the wells was read at 410 nm and results expressed as absorbance of oligonucleosomes (O.D.).

Total protein assay

Total proteins were determined by the method of Lowry et al. (1951).

Statistical analysis

Data are expressed as mean ± standard deviation of four independent determinations. An error probability with $P < 0.05$ (including $P < 0.01$ and $P < 0.001$) was selected as significant. All experiments were performed in duplicate and the mean was used for analysis.

Results

Efficacy

Data related to the rate of CCyR and MMR in CML patients treated for 6 months with TKI are shown in Table 1. CCyR was achieved in 77% of patients treated with DAS, in 87% of patients treated with NIL and in 65% of patients treated with IM. The corresponding MMR rates were of 69% (DAS), 73% (NIL) and 53% (IM).

DAS, IM, and NIL inhibit calcium mobilization induced by TG or InsP3. It's known that the BCR/ABL–expressing cells show impaired ER homeostasis and are unable to activate ER calcium-mediated apoptotic pathways (Keeshan et al., 2002). Thus we investigated the in vivo effect of three different TK inhibitor on $[Ca^{2+}]_i$ in CML cells. In Figure 1 are shown the results obtained by using increasing concentrations (range 0–20 μM) of InsP3 on the mobilization intracellular calcium. PBM cells obtained from healthy patients (CS-PBM) or CML patients untreated (basal CML) or treated with DAS, IM and NIL, as described in MM, were loaded with FURA-2/AM and balanced for 10 min in a calcium-free medium for determination of InsP3 activity. InsP3 significantly reduced the mobilization of $[Ca^{2+}]_i$ in a dose dependent manner with an IC_{50} of 4.6 ± 0.52 μM (basal CML-PBM cells) respect to 3.3 ± 0.29 μM (CS-PBM cells) ($P < 0.05$). The treatment of patients with the inhibitors induced a significantly reduction ($P < 0.05$) of InsP3 activity. In fact the IC_{50} shifted from 4.6 ± 0.52 μM (basal CML-PBM cells) to 8.4 ± 0.78 (82,6%), 7.3 ± 0.71 (58.7%) or 10.1 ± 0.93 μM (119.6%) for DAS, IM, and NIL respectively.

In Panel (A) are reported the results obtained on the intracellular calcium mobilization induced by 5 μM InsP3. The treatment with DAS, IM, and NIL reduced significantly ($P < 0.05$) the intracellular calcium mobilization induced by InsP3 of 47.5 ± 5.2, 30.3 ± 2.9 and of 56.2 ± 6.7% respectively.

In Figure 2 are shown the results obtained by using increasing concentrations (range 0–30 μM) of TG, a specific inhibitor of the SERCA channels, on the intracellular calcium mobilization. PBM cells of healthy subjects (CS-PBM) or CML patients untreated (basal CML) or treated with DAS, IM, and NIL, were loaded with FURA-2/AM and balanced for 10 min in a calcium-free medium for the determination of TG activity. TG significantly reduced

TABLE 1. Cytogenetic and molecular response in treated CML patients

Drugs	Patients	CCyR (%)	MMR (%)
NIL	15	13 (87)	11 (73)
IM	17	11 (65)	9 (53)

Complete Cytogenetic Response (CCyR) and Major Molecular Response (MMR) in CML patients treated for 6 months with DAS, NIL, or IM.

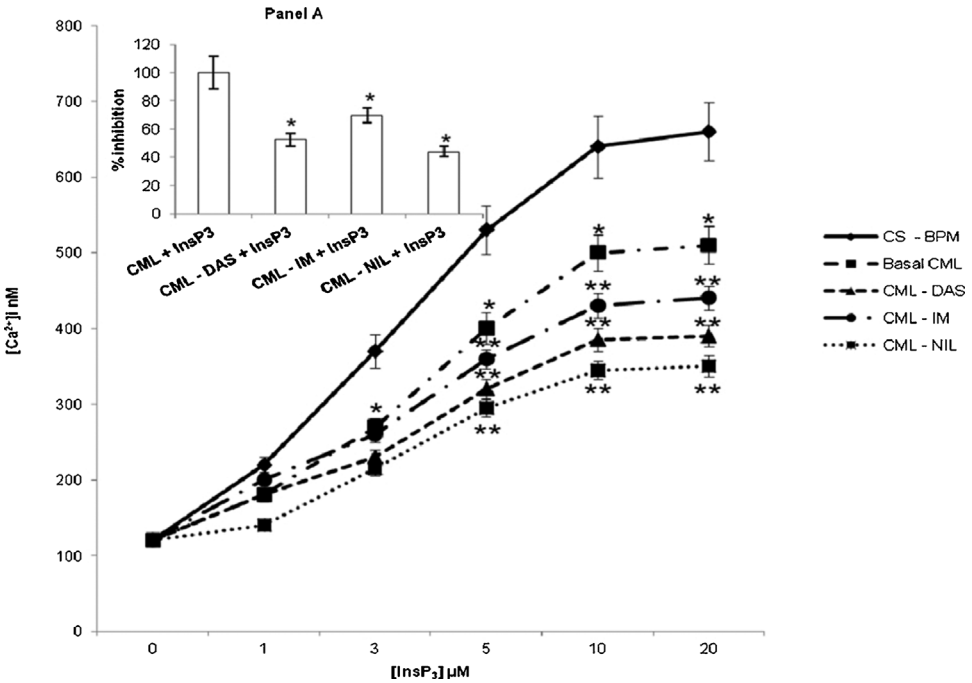

Fig. 1. In vitro activity of InsP3 on calcium levels in PBM cells obtained from healthy subjects (CS-PBM) or CML patients before (basal CML) or after treatment with DAS, IM, and NIL. 10^6 cells were incubated with increasing concentrations (0–20 μM) of InsP3 in KRH medium calcium free. Data represent the $[Ca^{2+}]_i$ values obtained after addition InsP3 (mean ± S. D.) obtained in four distinct experiments performed in duplicate. (*$P < 0.05$ vs. CS-PBM; ** $P < 0.05$ vs. basal CML). In Panel A show the percentage variation respect to basal CML of 5 μM InsP3 on calcium levels in untreated or treated CML-PBM cells. (*$P < 0.05$ vs. CML-InsP3).

the mobilization of $[Ca^{2+}]_i$ in a dose dependent manner whit on IC_{50} of 5.1 ± 0.55 (basal CML-PBM cells) respect to 3,7 ± 0.28 μM (CS-PBM cells) ($P < 0.05$). The treatment of patients with DAS, IM and NIL induced a significant reduction ($P < 0.05$) of TG activity. In fact the IC_{50} shifted from 5.1 ± 0.55 μM (basal CML-PBM cells) to 9.6 ± 0.83, 7.1 ± 0.66 or 14.4 ± 1.2 μM for DAS, IM and NIL respectively.

In Panel (A) are reported the results obtained on the intracellular calcium mobilization induced by 3 μM TG. The treatment with DAS, IM and NIL reduced significantly ($P < 0.05$) the intracellular calcium mobilization induced by TG of 44.7 ± 3.9, 26.4 ± 2.7 and of 57.9 ± 5.5% respectively.

Activity of DAS, IM, and NIL on MDA and SOD levels. We evaluated the production of MDA, a reliable marker of oxidative stress (Rossi et al., 2006) as well as the SOD activity to verify a possible correlation between induction of oxidative stress and inositol triphosphate receptor (IP3R) or SERCA signalling cascades we using the TG (specific inhibitor of the SERCA channels) and the Lithium chloride (Li) (a regulator of the levels of inositol-1,4,5-trisphosphate and inhibitor of InsP3 receptors).

The Figure 3 show the results obtained in healthy subjects (Control) and in CML-PBM cells before (basal CML) or after treatment with DAS, IM and NIL on MDA levels. 10^6 PBM cells were incubated for 24 h alone or in presence of 10 mM Li or 3 μM di TG. The levels of MDA in basal CML were significantly increased respect Control cells (209.3 ± 19.4 vs. 122.7 ± 21.1 pmol of MDA/mg of protein) ($P < 0.05$ vs. control cells). The addiction of TG induced an increase of intracellular levels of MDA in CML cells and MDA values shifted to 275.3 ± 23.5 pmol of MDA/mg of proteins ($P < 0.05$ versus basal CML). The addiction of Li induced a significant increase of MDA levels in CML cells and values shifting 259.7 ± 22.8 pmol of MDA/mg of proteins ($P < 0.05$ versus basal CML). The CML-PBM cells of patients treated with DAS, IM and NIL show higher levels of MDA respect to basal CML shifting from 209.3 ± 19.4 to 306.1 ± 28.7 or 264.5 ± 21.8 or 345.8 ± 31.4 pmol of MDA/mg of proteins respectively. The in vitro administration of TG further increased the MDA levels to 364.3 ± 29.3 or 318.9 ± 30.9 or 412.5 ± 33.8 pmol of MDA/mg of proteins for DAS, IM and NIL respectively. Finally the addiction of Li increased the MDA values to 343.1 ± 34.7 (in CML-DAS) or 307.8 ± 27.6 (in CML-IM) or 395.3 ± 34.1 (in CML-NIL) pmol of MDA/mg of proteins.

The Figure 4 show the results obtained in PBM cells from healthy subjects (Control) and CML-PBM cells before (basal CML) e after treatment with DAS, IM, and NIL on SOD activity. 10^6 CML-PBM cells were incubated for 24 h alone or in presence of 10 mM Li or 3 μM di TG. SOD activity compared to Control cells markedly decreased in the CML-PBM cells (34.6 ± 3.3 vs. 54.5 ± 6.1 U/mg proteins, $P < 0.05$). The addiction of TG to CML-PBM cells reduced the SOD activity from 34.6 ± 3.3 to 24.8 ± 2.1 ($P < 0.05$ vs. basal CML). The addiction of Li in CML-PBM cells also reduced the SOD activity decreasing from 34.6 ± 3.3 to 26.1 ± 2.3 U/mg proteins ($P < 0.05$ vs. basal CML). The in vivo treatment with DAS, IM, and NIL significantly reduced SOD activity in CML-PBM cells from 34.6 ± 3.3 to 25.3 ± 2.2 or 28.1 ± 3.1 or 20.3 ± 2.1, respectively. The in vitro administration of TG further reduced the SOD activity to 17.3 ± 1.9 or 20.4 ± 2.3 or 13.7 ± 1.4 U/mg proteins. Finally the addiction of Li reduced the SOD activity to 19.4 ± 1.6 or 22.9 ± 2.4 or 16.8 ± 1.8 for DAS, IM, and NIL, respectively.

TG and Li increase DAS, IM, and NIL-induced apoptosis. The apoptotic activity was evaluated in CML-PBM cells of patients newly diagnosed (basal CML) and after treatment with DAS, IM and NIL. 10^6 CML-PBM cells were incubated for 24 h alone or addition of 3 μM di TG. As show Figure 5 in

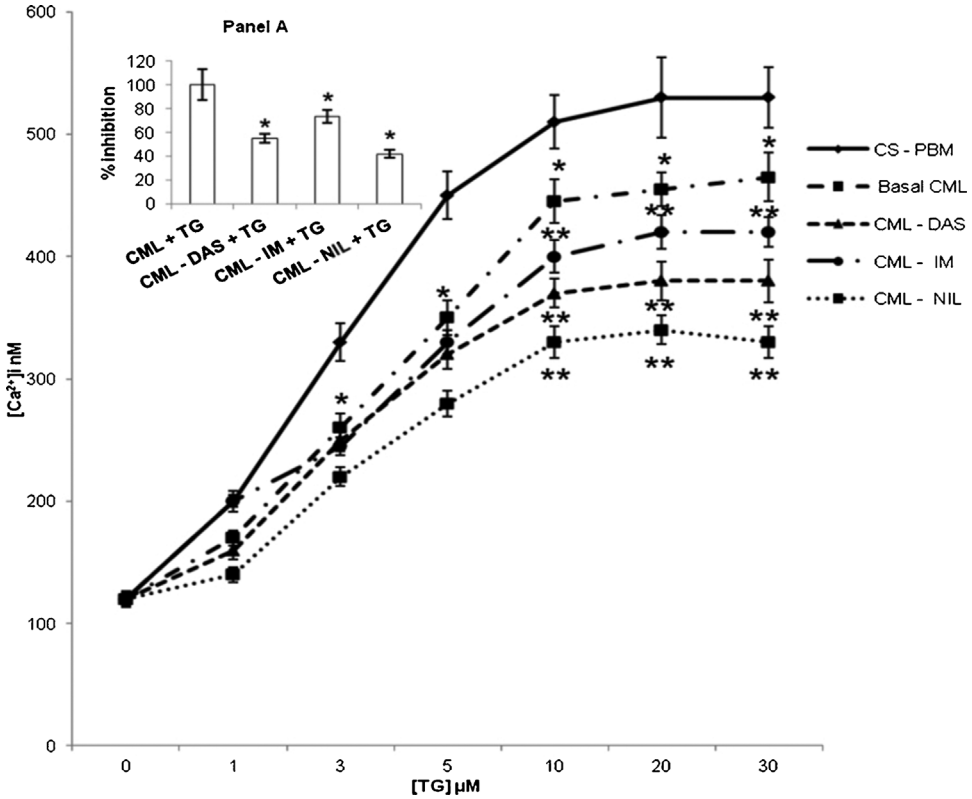

Fig. 2. In vitro activity of TG on calcium levels (CS-PBM) or CML patients before (basal CML) or after treatment with DAS, IM and NIL. 10^6 cells were incubated with increasing concentrations (0–20 µM) of TG in KRH medium calcium free. Data represent the $[Ca^{2+}]_i$ values obtained after addition TG (mean ± S. D.) obtained in four distinct experiments performed in duplicate. (* $P < 0.05$ vs. CS-PBM; ** $P < 0.05$ vs. basal CML). In Panel A show the percentage variation respect to basal CML of 3 µM TG on calcium levels in untreated or treated CML-PBM cells. (*$P < 0.05$ vs. CML-TG).

untreated CML-PBM cells the O.D. was 0.02 ± 0.0018 (without TG) and 0.11 ± 0.08 (with TG). The treatment of patients with DAS, IM, and NIL induced apoptosis in CML-PBM cells with O.D. values of 0.07 ± 0.004, 0.050 ± 0.008 and 0.080 ± 0.011 respectively. The addition in vitro of TG determined a significant increase of apoptotic activity in CML-PBM cells of patients treated with DAS, IM and NIL. In fact O.D. values shifted from 0.11 ± 0.08 (basal CML + TG) to 0.170 ± 0.02 (54.5%), 0.130 ± 0.012 (18.2%) and 0.210 ± 0.028 (90.9%) for DAS, IM, and NIL, respectively.

The Figure 6 show the results obtained using Li on apoptosis. The apoptotic activity was evaluated in CML-PBM cells before and after treatment with DAS, IM, and NIL. 10^6 CML-PBM cells were incubated for 24 h alone or after addiction 10 mM Li. The CML showed basal levels of O.D. of 0.02 ± 0.0018 (without Li) and of 0.06 ± 0.009 (with Li). The treatment with DAS, IM and NIL induced apoptosis in CML-PBM cells with O.D. values of 0.05 ± 0.004, 0.050 ± 0.008, and 0.080 ± 0.010, respectively. In vitro addition of Li determined a significant increase of apoptotic activity also in CML-PBM cells of patients treated with DAS, IM and NIL. In fact the O.D. shifted from 0.06 ± 0.009 (basal CML + Li) to 0.140 ± 0.015(133.3%), 0.120 ± 0.016 (100%), and 0.235 ± 0.021 (291.3%) for DAS, IM, and NIL, respectively.

Discussion

The IM administered at 400 mg daily, is the standard front-line therapy for patients with CML (Kantarjian et al., 2002). Unfortunately several studies have demonstrated that only the 82% of patients achieved a CCyR (Jabbour et al., 2008) and a substantial fraction of patients develops acquired resistance to IM (Hochhaus, 2006). To this end, several authors investigated the effects of new strategies in front-line treatment with the second-generation TKI, DAS, and NIL (Cortes et al., 2010). Our clinical data, reported in Table 1, confirmed that treatment with DAS, NIL was more effective respected at IM. It has been demonstrated that the endoplasmic reticulum (ER) plays an important role in the regulation of proliferation and apoptosis (Morishima et al., 2002) and high and low Bcr-Abl–expressing cells, show impaired ER homeostasis and are unable to activate ER calcium-mediated apoptotic pathways (Keeshan et al., 2002). The release of Ca^{2+} induced by $InsP_3$ from internal Ca^{2+} stores represents one of the most powerful intracellular signals within the eukaryotic cell (Camello-Almaraz et al., 2006). It has been demonstrated that TG increase cytosolic Ca^{2+} concentrations by blocking SERCA calcium pumping and produce ER stress by exerting effects on Ca^{2+}-dependent chaperones and ROS, releasing of Ca^{2+} from ER stores (Rogers et al., 1995). Our recent studies have demonstrated in CML cells a lower activity of TG and InsP3-dependent release of calcium from stores as well as a decreased function of purinergic dependent calcium channels (Ciarcia et al., 2010). The data presented herein (Figs. 1 and 2) show that treatment with DAS and NIL desensitize more the SERCA and InsP3 receptors respect to IM in CML-PBM cells. Moreover, the in vitro addition of 3 µM TG or 10 mM Li has induced, in CML cells treated in vivo with NIL and DAS, a

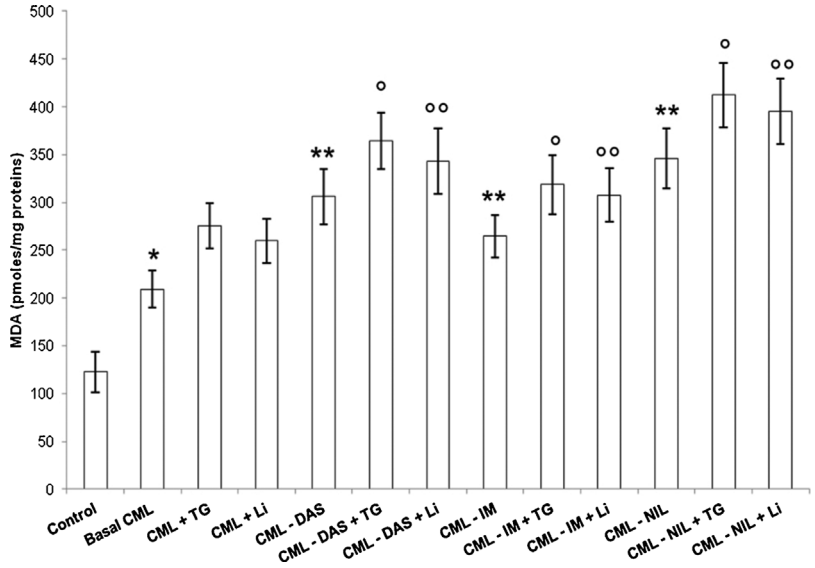

Fig. 3. In vitro effect of TG or Li on MDA levels in PBM cells obtained from healthy subjects (control) or in CML-PBM cells of patients untreated (Basal CML) or after treatment with DAS, NIL, or IM. 10^6 of CML-PBM cells incubated for 24 h alone or in association with 2 μM TG at 37°C in humidified atmosphere of 5% carbon dioxide in air. Data represents mean of MDA levels expressed in pmoles/mg proteins ± S.D. obtained in four distinct experiments performed in duplicate. (*$P < 0.05$ vs. Control; **$P < 0.05$ vs. basal CML; °$P < 0.05$ vs. CML + TG; °°$P < 0.05$ vs. CML + Li.

significantly lesser effect than in CML cells treated with IM on $[Ca^{2+}]_i$. In an in vitro experiment on primary CML cells, Bellodi et al. (2009) showed that IM induce apoptosis by inhibition of BCR/ABL protein as wells as through inhibition of the thapsigargin-induced calcium release (Hughes et al., 2006). The oscillation of cytosolic Ca^{2+} concentration is a potent activator of the intrinsic apoptotic pathway. Disruption of Ca^{2+} homeostasis induces a series of biochemical alterations leading to caspase activation and subsequent cellular apoptosis (Hajnoczky et al., 2003).

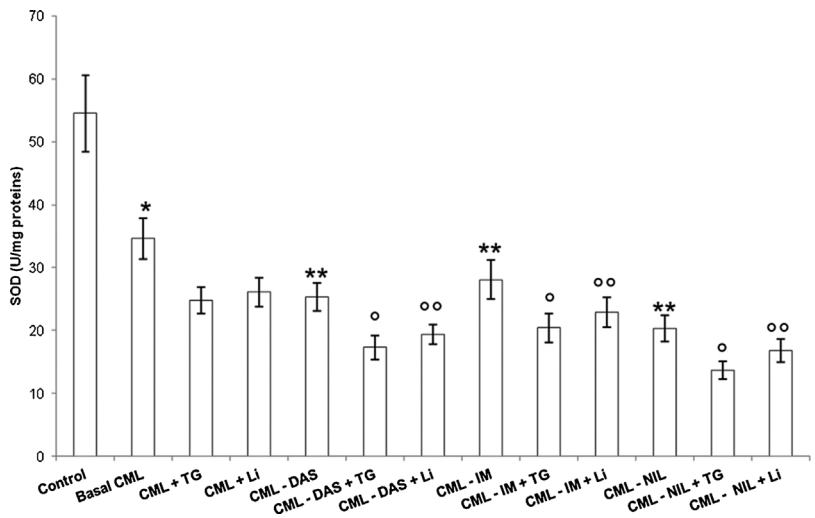

Fig. 4. In vitro effect of TG or Li on SOD activity in PBM cells obtained from healthy subjects (control) or in CML-PBM cells of patients untreated (basal CML) or after treatment with DAS, NIL or IM. 10^6 of CML-PBM cells incubated for 24 h alone or in association with 10 mM of Li at 37°C in humidified atmosphere of 5% carbon dioxide in air. Data represents mean of SOD activity expressed in U/mg proteins ± S.D. obtained in four distinct experiments performed in duplicate. (*$P < 0.05$ vs. Control; **$P < 0.05$ vs. basal CML; °$P < 0.05$ vs. CML + TG; °°$P < 0.05$ vs. CML + Li.

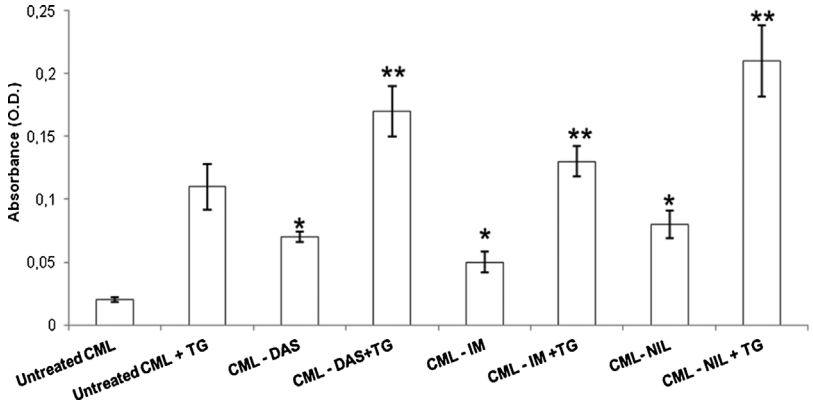

Fig. 5. In vitro effect of TG on levels of oligonucleosomes in CML-PBM cells of patients untreated or after treatment with DAS, NIL or IM. 10^6 of CML-PBM cells were incubated for 24 h alone or in association at 2 μM TG at 37°C in humidified atmosphere of 5% carbon dioxide in air. Data represents mean of Absorbance (O.D.) ± S.D. obtained in four distinct experiments performed in duplicate. (*$P < 0.05$ vs. untreated CML; **$P < 0.05$ versus Untreated CML + TG).

Recently studies by Li et al. (2014) have demonstrated, in human umbilical vein endothelial cells, that intracellular oxidative stress induced by heat leads to apoptosis triggering caspases 3 and 9 through pathways mediated by intracellular calcium and ROS. The role of ROS in carcinogenesis as well as in apoptosis has been widely documented (Jackson and Loeb, 2001; Jabs, 1999). It is known that cancer cells presented an oxidative stress higher compared to the corresponding healthy cells and that the increase of intracellular ROS is controlled by antioxidant enzymes including SOD and Glutathione (Dickinson and Forman, 2002). The manipulation of the altered state, with both oxidants that with pro-oxidants was often used by researchers to kill cancer cells (Ho et al., 2011). In fact, an inhibition of the cellular antioxidant activity leads to increased ROS levels that, in turn, are able to trigger the activation of signalling pathways, which could make cancer cells susceptible to toxic insults (Gao et al., 2012; Chen et al., 2006). Many anticancer drugs, including C2 ceramide, anti-IgM antibody and dexamethasone, induce apoptosis through the stimulation of ROS and accumulation of H2O2 while many inhibitors of apoptosis have antioxidant properties (Buttke and Sandstrom, 1994). Recently studies by Chakraborty et al. (2012) have shown that hydroxychavicol, a potent inhibitor of xanthine oxidase, purified from Piper betle leaves or prepared synthetically is able to induce apoptosis in K562 and CML cells by activation of JN-kinase ROS-dependent and revert drug resistance induced by IM. In our previous research we have demonstrated that in vitro administration an PI3K tyrosine kinase inhibitor (LY294002) and an Src kinase inhibitor (PP1) interacts in synergistic manner with IM to induce apoptosis and autophagy in CML and K562 cells (Ciarcia et al., 2013). In the present study (figs. 3, 4, 5, and 6) we showed that treatment with NIL and DAS increased oxidative stress as well as apoptosis while decreasing SOD activity more than IM. The in vivo activity of DAS, NIL, and IM on receptors SERCA and InsP3 and on ROS seems to support the hypothesis that these inhibitors induce apoptosis involved both the calcium channels pathways and oxidative stress. It, is reasonable to hypothize that the greater activity of NIL respect to IM can be correlated to its higher affinity versus BCR/ABL kinase receptors while the greater activity of DAS was due to its activity both on the BCR/ABL kinase as well as on the SrC kinase receptors. From our results it can be concluded that, in clinical practice, the data acquisition of oxidative stress and of calcium channels receptors in CML-PBM cells induced by TKI could help the hematologist to modulate therapy in CML.

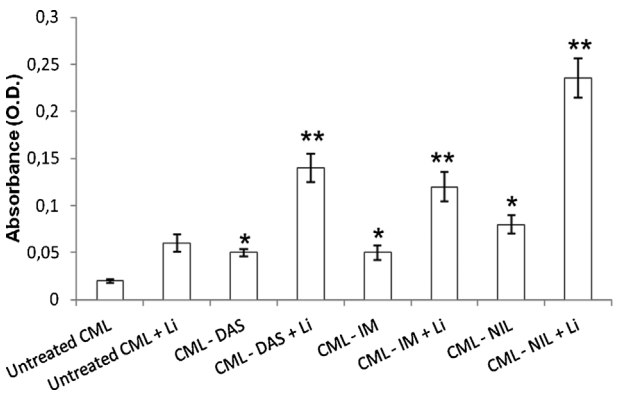

Fig. 6. In vitro effect of Li on levels of oligonucleosomes in CML-PBM cells of patients untreated or after treatment with DAS, NIL, or IM. 10^6 of CML-PBM cells were incubated for 24 h alone or in association at 10 mM of Li at 37°C in humidified atmosphere of 5% carbon dioxide in air. Data represents mean of Absorbance (O. D.) ± S.D. obtained in four distinct experiments performed in duplicate. (*$P < 0.05$ vs. untreated CML; **$P < 0.05$ vs. Untreated CML + Li).

Acknowledgments

This study was supported by grant from: Italian Asociation Leukaemia and Lymphoma (A.I.L.) – Caserta-ONLUS "ValentinaPicazio". Human Health Foundation - ONLUS (http://www.hhfonlus.org/); Sbarro Health Research Organization (http://www.shro.org/). The authors wish to thank Leonida Manco (University of Naples Federico II, Naples, Italy) for technical support.

Literature Cited

Bass DA,Dechatelet LR, McCall CE. 1978. Independent stimulation of motility and theoxidative metabolic burst of human polymorphonuclear leukocytes. J Immunol 121:172–178.

Bellodi C, Lidonnici MR, Hamilton A, Helgason GV, Soliera AR, Ronchetti M, Galavotti S, Young KW, Selmi T, Yacobi R, Van Etten RA, Donato N, Hunter A, Dinsdale D, Tirrò E, Vigneri P, Nicotera P, Dyer MJ, Holyoake T, Salomoni P, Calabretta B. 2009. Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells. J Clin Invest 119:1109–1123.

Branford S, Fletcher L, Cross NC, Müller MC, Hochhaus A, Kim DW, Radich JP, Saglio G, Pane F, Kamel-Reid S, Wang YL, Press RD, Lynch K, Rudzki Z, Goldman JM, Hughes T. 2008. Desirable performance characteristics for BCR-ABL measurement on an international reporting scale to allow consistent interpretation of individual patient response and comparison of response rates between clinical trials. Blood 112:3330–3338.

Buttke TM, Sandstrom PA. 1994. Oxidative stress as a mediator of apoptosis. Immunol Today 15:7–10.

Camello-Almaraz C, Gomez-Pinilla PJ, Pozo MJ, Camello PJ. 2006. Mitochondrial reactive oxygen species and Ca^{2+} signaling. Am J Physiol 291:C1082–C1088.

Chakraborty JB, Mahato SK, Joshi K, Shinde V, Rakshit S, Biswas N, Mukherjee I, Mandal L, Ganguly D, Chowdhury AA, Chaudhuri J, Paul K, Pal BC, Vinayagam J, Pal C, Manna A, Jaisankar P, Chaudhuri U, Konar A, Roy S, Bandyopadhyay S. 2012. Hydroxychavicol, a Piper betle leaf component, induces apoptosis of CML cells through mitochondrial reactive oxygen species-dependent JNK and endothelial nitric oxide synthase activation and overrides imatinib resistance. Cancer Sci 103:88–99.

Chen D, Chan R, Waxman S, Jing Y. 2006. Buthionine sulfoximine enhancement of arsenic trioxide-induced apoptosis in leukemia and lymphoma cells is mediated via activation of c-Jun NH2-terminal kinase and up-regulation of death receptors. Cancer Res 66:11416–11423.

Ciarcia R, Damiano S, Montagnaro S, Pagnini U, Ruocco A, Caparrotti G, d'Angelo D, Boffo S, Morales F, Rizzolio F, Florio S, Giordano A. 2013. Combined effects of PI3K and SRC kinase inhibitors with imatinib on intracellular calcium levels, autophagy, and apoptosis in CML-PBL cells. Cell Cycle 12:2839–2848.

Ciarcia R, d'Angelo D, Pacilio C, Pagnini D, Galdiero M, Fiorito F, Damiano S, Mattioli E, Lucchetti C, Florio S, Giordano A. 2010. Dysregulated calcium homeostasis and oxidative stress in chronic myeloid leukemia (CML) cells. J Cell Physiol 224:443–445.

Clement MV, Ponton A, Pervaiz S. 1998. Apoptosis induced by hydrogen peroxide is mediated by decreased superoxide anion concentration and reduction of intracellular milieu. Febs Lett 440:13–18.

Cortes JE, Jones D, O'Brien S, Jabbour E, Ravandi F, Koller C, Borthakur G, Walker B, Zhao W, Shan J, Kantarjian H. 2010. Results of dasatinib therapy in patients with early chronicphase chronic myeloid leukemia. J Clin Oncol 28:398–404.

Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, Heinrich MC, Tuveson DA, Singer S, Janicek M, Fletcher JA, Silverman SG, Silberman SL, Capdeville R, Kiese B, Peng B, Dimitrijevic S, Druker BJ, Corless C, Fletcher CD, Joensuu H. 2002. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. N Engl J Med 347:472–480.

Dickinson DA, Forman HJ. 2002. Cellular glutathione and thiols metabolism. Biochem Pharmacol 64:1019–1026.

Esterbauer H, Cheeseman KH. 1990. Determination of aldhehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. Methods enzymol 186:407–421.

Florio S, Crispino L, Ciarcia R, Vacca G, Pagnini U, de Matteis A, Pacilio C, D'Andrilli G, Kumar C, Giordano A. 2003. MPA increases idarubicin-induced apoptosis in chronic lymphatic leukaemia cells via caspase-3. J Cell Biochem 89:747–754.

Gao FH1, Liu F, Wei W, Liu LB, Xu MH, Guo ZY, Li W, Jiang B, Wu YL. 2012. Oridonin induces apoptosis and senescence by increasing hydrogen peroxide and glutathione depletion in colorectal cancer cells. Int J Mol Med 29:649–655.

Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of Ca2+ indicators with greatly improved fuorescence properties. J Biol Chem 260:3440–3450.

Hajnoczky G, Davies E, Madesh M. 2003. Calcium signaling and apoptosis. Biochem Biophys Res Commun 304:445–454.

Ho SY1, Wu WJ, Chiu HW, Chen YA, Ho YS, Guo HR, Wang YJ. 2011. Arsenic trioxide and radiation enhance apoptotic effects in HL-60 cells through increased ROS generation and regulation of JNK and p38 MAPK signaling pathways. Chem Biol Interact 193:162–171.

Hochhaus A, La Rosée Pet. 2007. Imatinib therapy in chronic myelogenous leukemia: Strategies to avoid and overcome resistance. Leukemia 18:1321–1331.

Hochhaus A. 2006. Chronic myelogenous leukemia (CML): Resistance to tyrosine kinase inhibitors. Ann Oncol 17:x274–x279.

Hughes T, Deininger M, Hochhaus A, Branford S, Radich J, Kaeda J, Baccarani M, Cortes J, Cross NC, Druker BJ, Gabert J, Grimwade D, Hehlmann R, Kamel-Reid S, Lipton JH, Longtine J, Martinelli G, Saglio G, Soverini S, Stock W, Goldman JM. 2006. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: Review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. Blood 108:28–37.

Jabbour E, Kantarjian H, Jones D, Breeden M, Garcia-Manero G, O'Brien S, Ravandi F, Borthakur G, Cortes J. 2008. Characteristics and outcomes of patients with chronic myeloid leukemia and T315I mutation following failure of imatinib mesylate therapy. Blood 112:53–55.

Jabs T. 1999. Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. Biochem Pharmacol 57:231–245.

Jackson AL, Loeb LA. 2001. The contribution of endogenous sources of DNA damage to the multiple mutations in cancer. Mutat Res 477:7–21.

Kantarjian H, Sawyers C, Hochhaus A, Guilhot F, Schiffer C, Gambacorti-Passerini C, Niederwieser D, Resta D, Capdeville R, Zoellner U, Talpaz M, Druker B, Goldman J, O'Brien SG, Russell N, Fischer T, Ottmann O, Cony-Makhoul P, Facon T, Stone R, Miller C, Tallman M, Brown R, Schuster M, Loughran T, Gratwohl A, Mandelli F, Saglio G, Lazzarino M, Russo D, Baccarani M, Morra E. International STI571 CML Study Group. 2002. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. N Engl J Med 346:645–652.

Keeshan K, Cotter TG, McKenna SL. 2002. High Bcr-Abl expression prevents the translocation of Bax and Bad to the mitochondrion. Leukemia 16:1725–1734.

Kobayashi M, Kuroki S, Ito K, Yasuda A, Sawada H, Ono K, Washizu T, Bonkobara M. 2013. Imatinib-associated tumour response in a dog with a non-resectable gastrointestinal stromal tumour harbouring a c-kit exon 11 deletion mutation. Vet J 198:271–274.

Kretsinger RH. 1979. The informational role of calcium in the cytosol. Adv Cyclic Nucleotide Res 11:1–26.

Li L, Tan H, Gu Z, Liu Z, Geng Y, Liu Y, Tong H, Tang Y, Qiu J, Su L. 2014. Heat stress induces poptosis through a Ca^{2}-mediated mitochondrial apoptotic pathway in human umbilical vein endothelial cells. PLoS One 9:e111083.

Lombardo LJ1, Lee FY, Chen P, Norris D, Barrish JC, Behnia K, Castaneda S, Cornelius LA, Das J, Doweyko AM, Fairchild C, Hunt JT, Inigo I, Johnston K, Kamath A, Kan D, Klei H, Marathe P, Pang S, Peterson R, Pitt S, Schieven GL, Schmidt RJ, Tokarski J, Wen ML, Wityak J, Borzilleri RM. 2004. Discovery of N-(2-chloro-6-methyl-phenyl)-2-(6-(4-(2-hydroxyethyl)-piperazin-1-yl)-2-methylpyrimidin-4-ylamino) thiazole-5-carboxamide BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. J Med Chem 47:6658–6661.

Lowry OH, Rosembrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275.

Morishima N, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y. 2002. An endoplasmic reticulum stress-specific caspase cascade in apoptosis: Cytochrome cindependent activation of caspase-9 by caspase-12. J Biol Chem 277:34287–34294.

Murrell GA, Francis MJ, Bromley L. 1990. Modulation of fibroblast proliferation by oxygen free radicals. Biochem J 265:659–665.

Pervaiz S, Cao J, Chao OS, Chin YY, Clement MV. 2001. Activation of the RacGTPase inhibits apoptosis in human tumor cells. Oncogene 20:6263–6268.

Rogers TB, Inesi G, Wade R, Lederer WJ. 1995. Use of thapsigargin to study Ca2+ homeostasis in cardiac cells. Biosci Rep 15:341–349.

Rossi R, Dalle-Donne I, Milzani A, Giustarini D. 2006. Oxidized forms of glutathione in peripheral blood as biomarkers of oxidative stress. Clin Chem 52:1406–1414.

Sawyers CL. 1999. Chronic myeloid leukaemia. N Engl J Med 340:1330–1340.

Sergeev IN. 2004. Genistein induces Ca2+-mediated, calpain/caspase-12-dependent apoptosis in breast cancer cells. Biochem Biophys Res Commun 321:462–467.

Shah NP1, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. 2004. Overriding imatinib resistance with a novel ABL kinase inhibitor. Science 305:399–401.

Thoren FB, Betten A, Romero AI, Hellstrand K. 2007. Cutting edge: Antioxidative properties of myeloid dendritic cells: Protection of T cells and NK cells from oxygen radical-induced inactivation and apoptosis. J Immunol 179:21–25.

Wei CH, Luh YM, Liu JH, Fan S, Hsieh RK, Chiou TJ, Tzeng CH, Chen PM. 1995. Cytogenetic studies in de novo acute nonlymphocytic leukemia. Zhonghua Yi Xue Za Zhi (Taipei) 55:296–301.

Weisberg E1, Manley PW, Breitenstein W, Brüggen J, Cowan-Jacob SW, Ray A, Huntly B, Fabbro D, Fendrich G, Hall-Meyers E, Kung AL, Mestan J, Daley GQ, Callahan L, Catley L, Cavazza C, Azam M, Neuberg D, Wright RD, Gilliland DG, Griffin JD. 2005. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. Cancer Cell 7:129–141.