Biochemical Pharmacology 80 (2010) 1662–1675

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Involvement of ROS in chlorogenic acid-induced apoptosis of Bcr-Abl$^+$ CML cells

Srabanti Rakshit [a,1], Labanya Mandal [a], Bikas Chandra Pal [b], Jayashree Bagchi [a,2], Nabendu Biswas [a], Jaydeep Chaudhuri [a], Avik Acharya Chowdhury [a], Anirban Manna [a], Utpal Chaudhuri [c], Aditya Konar [d], Tulika Mukherjee [b], Parasuraman Jaisankar [b], Santu Bandyopadhyay [a,*]

[a] Department of Infectious Diseases & Immunology, Indian Institute of Chemical Biology, A Unit of Council of Scientific and Industrial Research (CSIR), 4, Raja S.C. Mullick Road, Jadavpur, Kolkata 700032, India
[b] Department of Chemistry, Indian Institute of Chemical Biology, A Unit of CSIR, Kolkata, India
[c] The Institute of Hematology and Transfusion Medicine, Medical College, Kolkata, India
[d] Department of Molecular Endocrinology, Indian Institute of Chemical Biology, A Unit of CSIR, Kolkata, India

ARTICLE INFO

Article history:
Received 26 May 2010
Accepted 23 August 2010

Keywords:
Chlorogenic acid
ROS
Apoptosis
Bcr-Abl
DR5

ABSTRACT

Chlorogenic acid (Chl) has been reported to possess a wide range of biological and pharmacological properties including induction of apoptosis of Bcr-Abl$^+$ chronic myeloid leukemia (CML) cell lines and clinical leukemia samples via inhibition of Bcr-Abl phosphorylation. Here we studied the mechanisms of action of Chl in greater detail. Chl treatment induced an early accumulation of intracellular reactive oxygen species (ROS) in Bcr-Abl$^+$ cells leading to downregulation of Bcr-Abl phosphorylation and apoptosis. Chl treatment upregulated death receptor DR5 and induced loss of mitochondrial membrane potential accompanied by release of cytochrome *c* from the mitochondria to the cytosol. Pharmacological inhibition of caspase-8 partially inhibited apoptosis, whereas caspase-9 and pan-caspase inhibitor almost completely blocked the killing. Knocking down DR5 using siRNA completely attenuated Chl-induced caspase-8 cleavage but partially inhibited apoptosis. Antioxidant NAC attenuated Chl-induced oxidative stress-mediated inhibition of Bcr-Abl phosphorylation, DR5 upregulation, caspase activation and CML cell death. Our data suggested the involvement of parallel death pathways that converged in mitochondria. The role of ROS in Chl-induced death was confirmed with primary leukemia cells from CML patients *in vitro* as well as *in vivo* in nude mice bearing K562 xenografts. Collectively, our results establish the role of ROS for Chl-mediated preferential killing of Bcr-Abl$^+$ cells.

1. Introduction

Chlorogenic acid (Chl) is one of the most abundant dietary polyphenols that has diverse biologic activities including anti-HIV activity [1], antioxidant activity [2], anti-carcinogenic activity [3], anti-allergic activity [4], modulating activity of cytochrome P450-linked enzyme [5], and apoptosis-inducing activity in human oral squamous cell carcinoma and salivary gland tumor cell lines [6]. In our earlier study, we reported that Chl-mediated inhibition of Bcr-Abl phosphorylation leads to apoptosis of Bcr-Abl$^+$ CML cells [7].

ROS play an important physiological role as secondary messengers and interfere with the expression of a number of genes and signal transduction pathways. The redox metabolism that maintains the homeostasis of ROS (ratio between production and detoxification) is critical in cell signaling and in regulation of cell death [8]. On one hand, low concentrations of ROS can promote cancer by transforming normal cells through activation of transcription factors or inhibition of tumor suppressor genes; on the other hand, elevated ROS levels inhibit cancer progression through the stimulation of pro-apoptotic-signals, leading to the death of cancer cells. Thus, ROS exert a paradoxical effect on cancer cells [9]. Tumor cells have higher levels of ROS than their normal counterparts and are therefore more sensitive to the additional oxidative stress generated by anticancer agents [10]. Emerging evidence suggests that ROS induce programmed cell death in

Abbreviations: ROS, reactive oxygen species; CML, chronic myeloid leukemia; NAC, N-acetyl-L-cysteine; JC-1, 5,5′-6,6′-tetracholoro-1,1′-3,3′-tetraethylbenzimidazolylcarbocyanine iodide; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; JNK, c-Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; FITC, fluorescein isothiocyanate; COX4, cytochrome *c* oxidase subunit 4; SMAC, second mitochondria-derived activator of caspase; PARP, poly ADP-ribose polymerase; TDT, terminal deoxy transferase; TUNEL, terminal deoxynucleotidyl-transferase enzyme-mediated dUTP end labeling; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

* Corresponding author. Tel.: +91 33 2473 3491; fax: +91 33 2472 3967.
E-mail address: santu2@iicb.res.in (S. Bandyopadhyay).
[1] Current address: Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India.
[2] Current address: Institute of Cellular Medicine, Medical School, Newcastle University, UK.

0006-2952/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2010.08.013

several cancer cells. Recently, many compounds like adaphostin [11], arsenic trioxide [12], β-phenylethylisothiocyanate (PEITC) [13] have been shown to induce apoptosis in Bcr-Abl$^+$ cells by the generation of ROS. A recent study from our laboratory demonstrated that Chl selectively induced apoptosis of Bcr-Abl$^+$ CML cell lines and primary cells from CML patients *in vitro* in a time- and dose-dependent manner and reduced xenografts of Bcr-Abl$^+$ CML cells in nude mice [7]. Chl inhibited Bcr-Abl phosphorylation and triggered p38MAPK-dependent apoptosis in these cells. Plant polyphenols are usually considered to be antioxidants, but they also exhibit prooxidant properties [14]. Most free-radical scavengers act in oxidation-reduction reactions that are reversible, and some, such as phenolic phytochemicals, depending on their structure and the conditions can act both as antioxidants and prooxidants. A recent report suggested the prooxidant property of Chl [15]. Here, we investigated whether Chl-induced downregulation of Bcr-Abl phosphorylation followed by activation of downstream signaling pathways that ultimately lead to

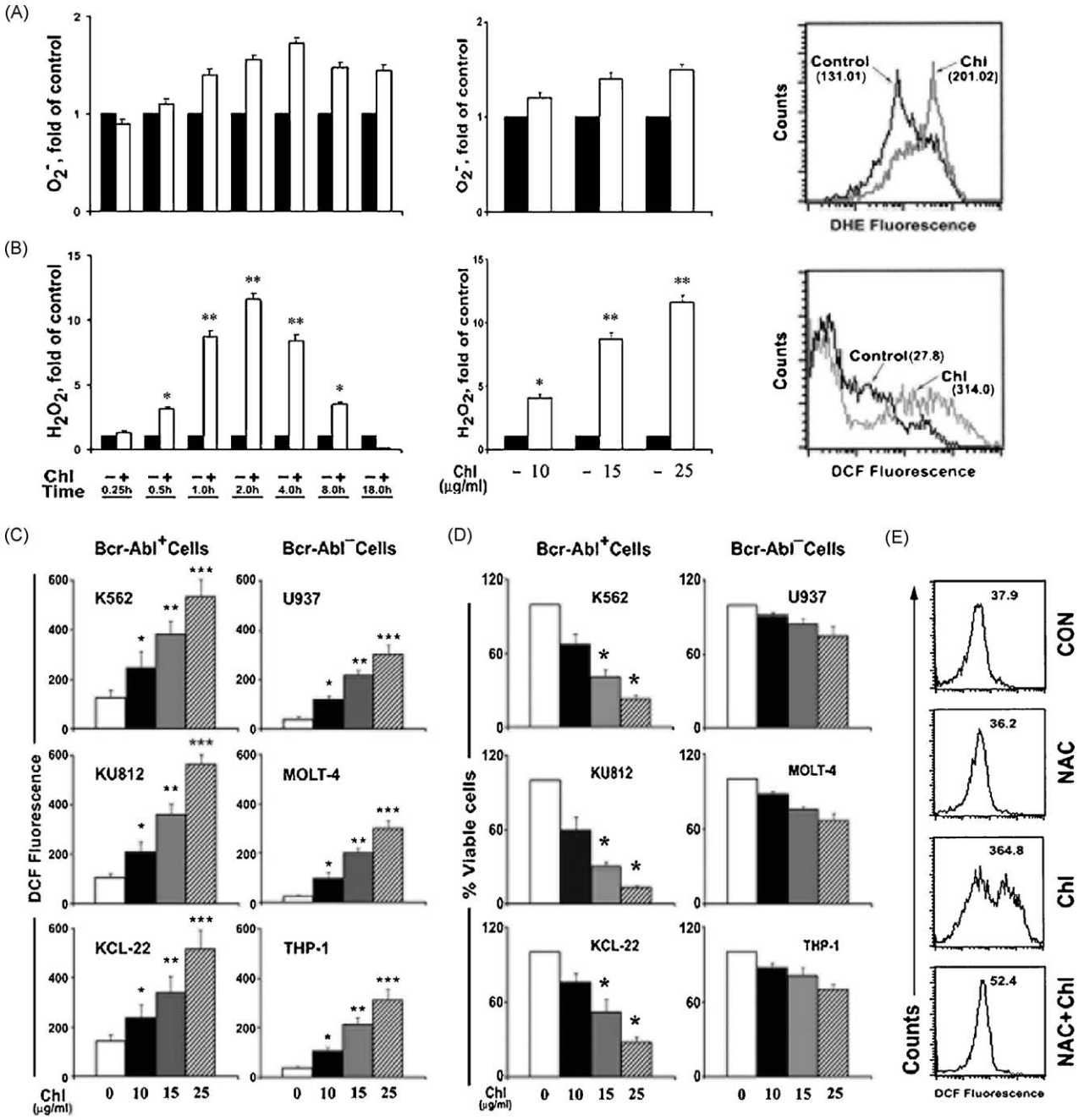

Fig. 1. Chl induces the production of intracellular O_2^- and H_2O_2 in a time and dose-dependent manner in K562 cells. (A and B) Cells were exposed to 25 μg/ml Chl for varying time periods (left panel) or to varying concentrations of Chl for 2 h (middle panel) for analysis of intracellular O_2^- (A) and H_2O_2 (B) using flow cytometry by measuring fluorescence of oxidized forms of DHE and DCFH-DA respectively (mean ± SD of three experiments; $^*p < 0.05$ compared to media treatment; $^{**}p < 0.01$ compared to media treatment). Representative histograms indicating intracellular accumulation of O_2^- and H_2O_2 upon exposure of cells to 25 μg/ml Chl for 2 h are shown in the right panel. (C) A panel of Bcr-Abl$^+$ and Bcr-Abl$^-$ cells were treated with varying concentrations of Chl for 2 h, intracellular levels of H_2O_2 were analyzed and has been represented as mean fluorescence intensity. $^*p < 0.05$ compared to media treatment; $^{**}p < 0.01$ compared to media treatment; $^{***}p < 0.001$ compared to media treatment. (D) Dose-dependent effect of Chl on the viability of Bcr-Abl$^+$ (left) and Bcr-Abl$^-$ cells (right). Cell viability was determined by Trypan blue exclusion assay after incubation for 24 h. Data represent mean ± SD of three independent experiments. $^*p < 0.05$ compared to media treatment. (E) K562 cells were preincubated with 2.5 mM NAC for 1 h followed by treatment with Chl (25 μg/ml) for 2 h for measurement of intracellular H_2O_2. Representative histograms are shown.

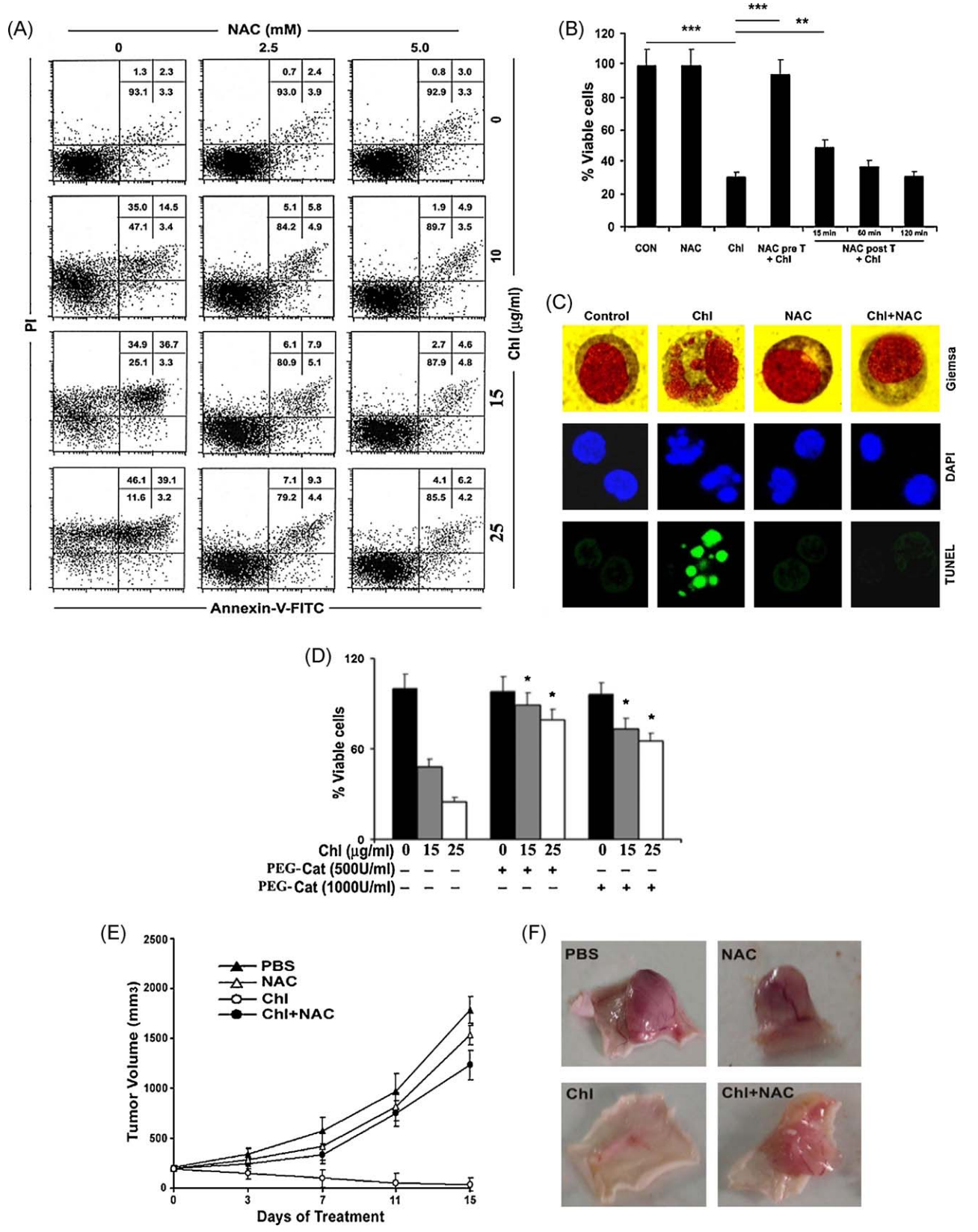

Fig. 2. Chlorogenic acid-induced apoptosis is reversed by NAC and polyethylene glycol conjugated (PEG) catalase. (A) Chl-induced apoptosis in K562 cells is inhibited by NAC. Cells were pre-treated with indicated concentrations of NAC for 1 h followed by incubation with varying concentrations of Chl for 24 h. Apoptosis was then determined by flow cytometry after staining with annexin V–FITC/PI. Data represent similar results from three independent experiments. (B) Cells were either pre-treated for 1 h with NAC (2.5 mM) before Chl treatment (25 μg/ml) or post-treated at indicated time periods with NAC (2.5 mM) after treatment with Chl (25 μg/ml). Cell viability was then assessed by Trypan blue exclusion assay. Data represent mean ± SD of three independent experiments. $^{***}p < 0.001$ compared to treatment with media alone, NAC alone or pre-treated with NAC followed by treatment with Chl; $^{**}p < 0.01$ compared to post-treatment with NAC at 15 min of Chl treatment. (C) K562 cells were pre-treated with NAC (2.5 mM) for 1 h followed by treatment with Chl (25 μg/ml) for 24 h. DNA fragmentation was then determined by staining with Giemsa (top panel), DAPI (middle panel) or by TUNEL assay (bottom panel). Data represent similar results from three independent experiments. (D) K562 cells were preincubated for 15 min with varying concentrations of PEG-catalase followed by

cell death are consequences of enhanced generation of intracellular ROS.

2. Materials and methods

2.1. Purification of chlorogenic acid

Chlorogenic acid was purified as previously described [7] from leaves of *Piper betle* which belongs to the family Piperaceae. The structure of the active compound was determined as chlorogenic acid, $C_{16}H_{18}O_9$, melting point 205–206 °C, α_D-33.25 (H_2O). Its identity was confirmed by comparing its physical data as well as its infrared (IR), nuclear magnetic resonance (^{1}H NMR), ^{13}C NMR, and mass spectral data with those of an authentic sample.

2.2. Antibodies and reagents

Antibodies were purchased from the following suppliers: Antibodies to c-Abl (rabbit polyclonal IgG), Bax (rabbit polyclonal IgG), cIAP1 (rabbit polyclonal IgG), Bcl-X_L (mouse monoclonal IgG_1), Bcl-2 (mouse monoclonal IgG_1), phospho-STAT5 (goat polyclonal IgG, Tyr-694), phospho-JNK (mouse polyclonal IgG, Thr-183/Tyr-185), phospho-p38 (rabbit polyclonal IgG, Thr-180/Tyr-182), actin (mouse monoclonal IgG_1), SMAC (goat polyclonal IgG), Bad (rabbit polyclonal IgG), Bim (rabbit polyclonal IgG), Bid (rabbit polyclonal IgG), Mcl-1 (rabbit polyclonal IgG), survivin (rabbit polyclonal IgG), XIAP (rabbit polyclonal IgG), DR4 (mouse monoclonal IgG_1), DR5 (goat polyclonal IgG), JNK2 (rabbit polyclonal IgG) and p38 (rabbit polyclonal IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody to DR5 (mouse monoclonal IgG_1) was also purchased from eBioscience (San Diego, CA, USA). Antibodies to poly ADP-ribose polymerase (PARP; mouse monoclonal IgG_1), cytochrome *c* (rat monoclonal IgG_1), caspase-3 (rabbit polyclonal IgG), caspase-9 (mouse monoclonal IgG_1), TNFR1 (mouse monoclonal IgG_{2a}) and TNFR2 (rat monoclonal IgG_{2b}) were purchased from BD Biosciences (San Jose, CA, USA). Antibodies to phospho-c-Abl (rabbit polyclonal IgG, Tyr 245), caspase-8 (mouse monoclonal IgG_1), cleaved caspase-8 (rabbit polyclonal IgG) and phospho-CrkL (rabbit polyclonal IgG, Tyr 207) were procured from Cell Signaling Technology (Danvers, MA, USA).

N-acetyl-L-cysteine (NAC), JNK specific inhibitor (SP600125), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanineiodide [JC-1], 2′,7′-dichlorodihydrofluorescein diacetate [DCFH-DA], dihydroethidium [DHE], Z-VAD-FMK (pan-caspase inhibitor), Z-IETD-FMK (caspase-8 inhibitor) and LEHD-CHO (caspase-9 inhibitor) were from Calbiochem (San Diego, CA, USA). Polyethylene glycol conjugated catalase [PEG-Cat] was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Cell lines and clinical samples

Bcr-Abl$^+$ cell lines K562, KU812 and KCL-22 and Bcr-Abl$^-$ cell lines THP-1, U937 and MOLT-4 were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 100 U/ml penicillin–streptomycin (all from Life Technologies, Carlsbad, CA, USA). Fresh peripheral blood samples from three CML patients and two healthy donors were collected and mononuclear cells were separated by HISTOPAQUE (Sigma–Aldrich) density gradient centrifugation. All experiments with human blood were conducted under an approved institutional Human Ethics Committee protocol. Informed consent was provided according to the Declaration of Helsinki.

2.4. Cell viability assay

Cells (1×10^4) in triplicate were incubated in 0.2 ml RPMI 1640 – 10% fetal bovine serum containing varying concentrations of Chl in the presence and absence of NAC or specific inhibitors of different caspases. Cell viability was determined by the Trypan blue exclusion assay [16]. Viability of primary CML cells was determined in the same way except that recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF, 100 ng/ml; R&D Systems, Minneapolis, MN, USA) was included [7].

2.5. In vivo studies on K562 xenografts

To evaluate the role of ROS in Chl-mediated killing of Bcr-Abl$^+$ cells *in vivo*, K562 xenografts were developed in nude mice as reported [7]. Chl (150 mg/kg body weight) was administered once a day for 15 days and NAC (150 mg/kg body weight) was administered on alternate days (3 days per week) via intra-peritoneal (i.p.) route. Tumor volumes were monitored and after 15 days of treatment, animals were sacrificed and photographs of the dissected tumors were taken during postmortem with Olympus CAMEDIA C-4000 Zoom digital camera. Animal studies were conducted under an approved institutional Animal Care and Use Committee protocol.

2.6. Annexin V–PI binding assay

Cells seeded at a density of 1.5×10^5 cells/ml were either pretreated with NAC or left alone for 1 h followed by incubation with Chl at different concentrations for 24 h. Apoptotic cells were quantified by Annexin V-FITC and propidium iodide [PI] binding assay using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) as described [7].

2.7. Assessment of cell morphology by Giemsa staining

To examine the apoptotic change in cell morphology, the control and Chl-treated cells were centrifuged and smears of the resultant pellet were drawn onto clean grease-free glass slides and air dried. The slides were then fixed in methanol for 10 min at 4 °C, air dried, then stained with Giemsa stain (Sigma–Aldrich) and observed under oil immersion lens of light microscope (Olympus, CH30). Microscopic photographs were taken with Olympus CAMEDIA C-4000 Zoom (4.0 megapixel) digital camera.

2.8. Confocal microscopy

Cells exposed to Chl for 24 h were collected by centrifugation, washed with ice-cold PBS and fixed with 4% paraformaldehyde (Calbiochem) for 30 min at room temperature. After permeabilization with 1% Triton X-100 (Sigma–Aldrich) for 5 min, cells were stained with 4′-6-diamidino-2-phenylindole (DAPI, Calbiochem) for 30 min [17] and were then examined with a Leica TCS SP2 confocal laser scanning microscope (DMIRB, 40× objective, Mannheim, Germany).

DNA strand breaks induced by apoptosis were identified by TdT-mediated TUNEL assay using the ApoAlert® DNA Fragmentation

treatment with indicated concentrations of Chl for 24 h. Viability was then assessed by Trypan blue dye exclusion. Data represent mean ± SD of three independent experiments. *$p < 0.05$ compared to treatment with Chl alone. (E) NAC abrogates the effect of Chl on Bcr-Abl$^+$ K562 xenografts. K562 cells embedded in Matrigel were staged in nude mice until tumors reached 200–300 mm^3. PBS or Chl (150 mg/kg) were administered i.p. once a day for fifteen days. NAC was administered i.p. three times a week at a dose of 150 mg/kg for two weeks. Error bars denote standard deviations of three different experiments. (F) Representative gross appearance of K562 xenografts after receiving indicated treatments.

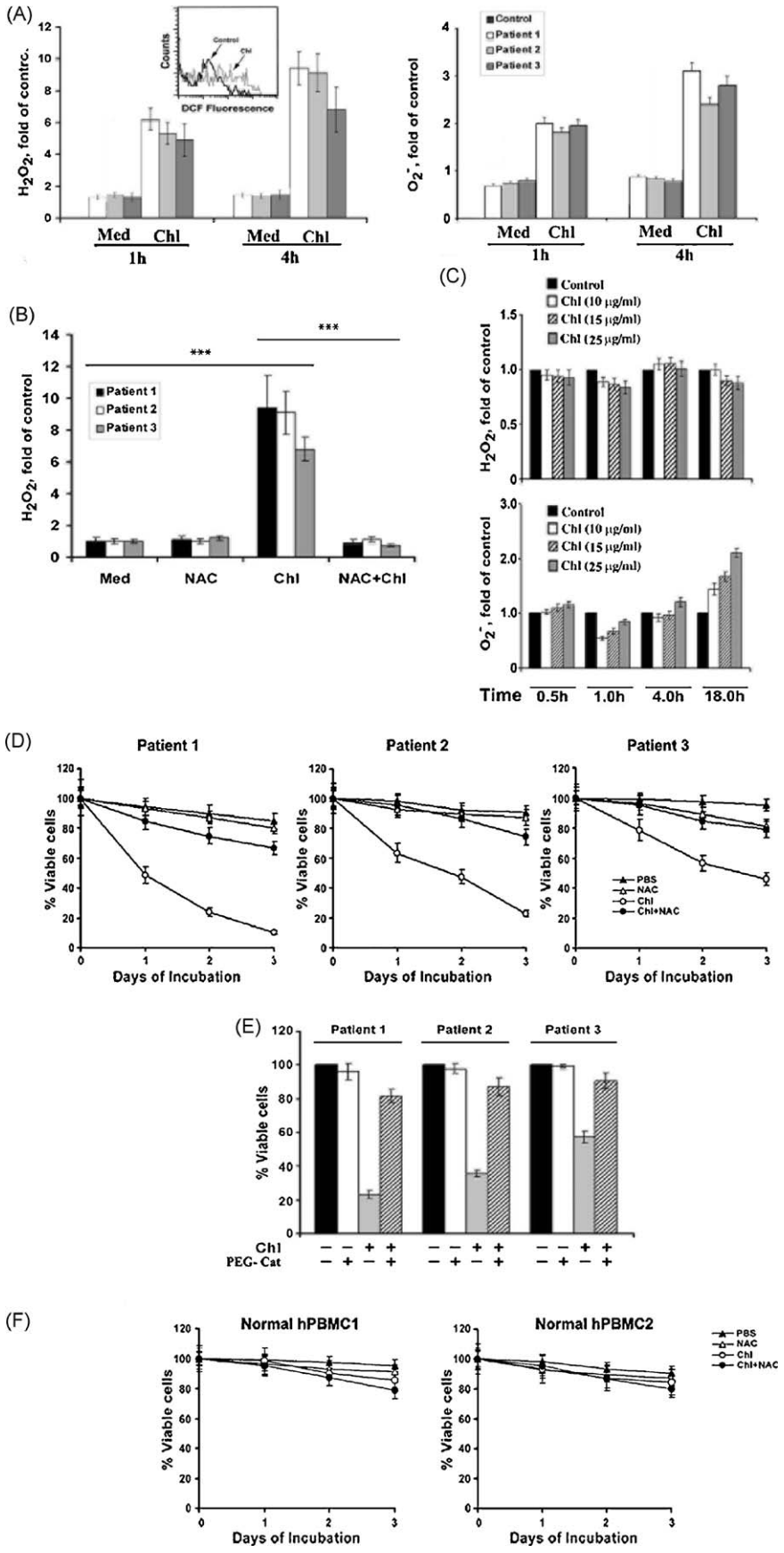

Fig. 3. Chlorogenic acid-induced ROS plays a key role in reducing viability of primary cells from Bcr-Abl$^+$ CML patients. (A) PBMC isolated from three CML patients were incubated with medium containing rhGM-CSF (100 ng/ml) or with the same medium containing 25 μg/ml Chl for 1 h and 4 h after which H_2O_2 and O_2^- were measured by flow cytometry using DCFH-DA and DHE respectively. A representative histogram of intracellular accumulation of H_2O_2 for a CML patient is shown (inset). (B) PBMC isolated from three CML patients were pre-incubated with 2.5 mM NAC for 1 h followed by treatment with 25 μg/ml Chl for 4 h after which intracellular H_2O_2 was measured by flow

Assay Kit (Clontech, Mountain view, CA, USA) following the manufacturer's protocol. TUNEL positive cells detected by confocal microscopy were considered as apoptotic cells.

For analysis of cytochrome *c* release, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100–PBS and stained with anti-cytochrome *c* antibody. After three washes with PBS–0.01% Triton X-100, samples were incubated with Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes, Carlsbad, CA, USA) for 45 min in a dark chamber. After three washes, coverslips were mounted on microscope slides in 80% glycerol in PBS. Cytochrome *c* release was imaged by a Leica TCS SP MP confocal microscope with an oil immersion objective.

2.9. Flow cytometry

Flow cytometry was performed to evaluate the surface expression of death receptors, to analyze intracellular phosphorylation status of c-Abl and MAP kinases, to measure mitochondrial membrane potential and intracellular ROS. For analysis of death receptors on the cell surface, treated and untreated cells were stained with indicated antibodies for 30 min. Isotype-matched control mouse antibodies and normal goat or rat sera were used as controls for respective antibodies. After washing, cells were incubated with multiple adsorbed FITC-conjugated secondary antibodies for 30 min, washed and analyzed in a flow cytometer (BD LSR, Becton Dickinson, San Jose, CA, USA) with Cell Quest software. Intracellular staining for different proteins was performed as reported earlier [7]. For staining of intracellular ROS, control and Chl-treated cells were incubated with 10 μM DCFH-DA and 5 μM DHE at 37 °C for 15 min in the dark for measurement of intracellular hydrogen peroxide and superoxide respectively [13]. Mitochondrial membrane potential ($\Delta\Psi_m$) was determined by flow cytometry using the lipophilic cationic probe JC-1 [16].

2.10. Immunoblotting

Immunoblotting experiments were performed on whole cell lysate, cytosolic and mitochondrial fractions of K562 cells [7,16].

2.11. Sub-cellular fractionation

The mitochondrial and cytoplasmic fractions were separated according to the ApoAlert Cell Fractionation Kit (Clontech) protocol. Anti-COX4 antibody supplied in the kit was used as the loading control to check the purity of the mitochondrial fraction.

2.12. siRNA knockdown

K562 cells were transfected with control siRNA and siRNA for DR5 (Santa Cruz Biotechnology). Transfections were carried out following the manufacturer's instructions. The transfection reagent used for siRNA transfection was purchased from Santa Cruz Biotechnology. 48 h post-transfection, the cells were treated as indicated.

2.13. HPLC analysis

Chlorogenic acid (1.0 mg) and NAC (9.0 mg) were separately dissolved in 1 ml mixed solvent [1% acetic acid in water:methanol (66:34)] .The two solutions were then mixed and the mixture was incubated for 1 h at 37 °C. The resulting solution was subjected to HPLC analysis [column: Luna 5 μC_{18} (250 mm × 4.60 mm), mobile phase: 1% acetic acid in water:methanol (66:34), flow rate: 0.5 ml/min].

2.14. Statistical analysis

Data were expressed as mean ± SD of at least three independent experiments, and statistical analysis for single comparison was performed using the Student's *t*-test. The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Chlorogenic acid treatment induces higher accumulation of intracellular ROS in Bcr-Abl$^+$ cells

In our earlier study we had shown that chlorogenic acid (Chl) induces apoptosis of Bcr-Abl$^+$ cells by inhibition of Bcr-Abl phosphorylation followed by activation of p38MAPK [7]. Since p38MAPK is also involved in oxidative stress-induced apoptosis, we wanted to test whether initial signal for Chl-induced cell death was derived from ROS generation. Intracellular levels of ROS (both O_2^- and H_2O_2) were quantified by flow cytometry using specific fluorescent probes (DHE for O_2^-; DCFH-DA for H_2O_2). Though chlorogenic acid is a well-known antioxidant [2], we investigated whether it acts as a prooxidant in CML cell lines. When Bcr-Abl$^+$ CML cell line K562 was treated with Chl, an early accumulation of H_2O_2 was observed. H_2O_2 levels had significantly increased within half an hour above the basal level and peaked by ~2 h post-treatment and reduced thereafter. However, O_2^- levels marginally increased till 4 h and later on declined to almost basal levels. The increase in DHE fluorescence was not significant (Fig. 1A). Data representing time kinetics (left panels) and dose dependency (middle panels) of O_2^- and H_2O_2 accumulation in K562 cells are shown in Fig. 1A and B respectively. Representative histograms of intracellular accumulation of O_2^- and H_2O_2 are also shown (right panels, Fig. 1A and B).

Next, a panel of Bcr-Abl$^+$ and Bcr-Abl$^-$ cell lines were selected for investigating the effect of Chl on ROS production in these cells. Since Chl-induced intracellular accumulation of H_2O_2 was significantly higher than O_2^- in K562 cells (10 fold for H_2O_2 versus 1.5 fold for O_2^-), accumulation of only H_2O_2 was examined in these panels of cell lines. Chl treatment resulted in a dose-dependent significant increase in mean DCF fluorescence in both Bcr-Abl$^+$ and Bcr-Abl$^-$ cell lines. As the basal threshold of intracellular H_2O_2 in Bcr-Abl$^+$ cells was markedly higher than the Bcr-Abl$^-$ cells, higher accumulation of intracellular H_2O_2 was observed in Bcr-Abl$^+$ cells after Chl treatment (Fig. 1C). In agreement with our earlier report [7], Chl induced more pronounced apoptotic effects on Bcr-Abl$^+$ cells compared to Bcr-Abl$^-$ leukemia cells (Fig. 1D).

To confirm our findings that Chl treatment induced ROS generation, we investigated whether NAC could neutralize intracellular ROS production by Chl. As shown in Fig. 1E, K562 cells treated with Chl exhibited a huge increase in DCF fluorescence which was reduced by ~80% on pre-treatment with 2.5 mM NAC. Experiments were performed to rule out the possibility that NAC acts directly with Chl in solution, thereby neutralizing this agent so that it cannot react with cells. Chl was incubated with NAC and

cytometry. ***$p < 0.001$ compared to treatment with media alone, NAC alone or pre-treated with NAC followed by treatment with Chl. (C) Normal human peripheral blood mononuclear cells (hPBMC) were exposed to varying concentrations of Chl for varying time periods. Intracellular H_2O_2 and O_2^- levels were then assessed by flow cytometry. (D) PBMC isolated from three CML patients were incubated with media alone (▲), NAC (2.5 mM) alone (△), Chl (25 μg/ml) alone (○) or NAC plus Chl (●) for indicated time periods for measurement of viability. (E) PBMC isolated from three CML patients were incubated as indicated for 3 days. Viability was then determined. (F) hPBMC from two donors were pre-treated with 2.5 mM NAC for 1 h followed by treatment with 25 μg/ml Chl for 3 days (medium alone (▲), NAC alone (△), Chl alone (○), NAC followed by Chl (●)). Cell viability was measured by Trypan blue exclusion assay. Error bars denote standard deviations of triplicate cultures.

then analyzed by HPLC. Results of this analysis indicated that NAC failed to react with Chl (Fig. S1).

3.2. Chl-induced ROS triggers apoptosis of K562 cells and reduces tumor burden in nude mice

To study the role of ROS accumulation in Chl-induced cytotoxicity toward K562 cells, we tested whether scavenging of ROS by NAC could attenuate the cell death mediated by Chl. As shown in Fig. 2A, not just apoptosis, necrosis also contributed to Chl-mediated cell death as manifested by significant staining with PI in absence of annexin V binding. Pre-treatment of K562 cells with NAC dose-dependently blocked cell death induced by Chl (Fig. 2A). However, post-treatment with NAC could not efficiently reverse Chl-mediated cell death (Fig. 2B). Post-treatment with NAC at 15 min of Chl treatment rescued cell death ($p < 0.01$ versus Chl treatment alone). Post-treatment with NAC at 60 min or 120 min of Chl treatment could not significantly enhance cell viability (Fig. 2B). Thus, early accumulation of ROS is critical for Chl-induced cell death.

Morphological hallmarks that are characteristic of oxidative stress include chromatin dysfunction such as single- and double-strand DNA fragmentation leading to cell death through apoptosis or necrosis [18]. DNA fragmentation is associated with the endpoint of the apoptotic process. To further support the contribution of ROS in Chl-induced cell death, we determined the effect of NAC pre-treatment on Chl-induced apoptosis by measuring DNA fragmentation. DNA fragmentation was analyzed by staining with Giemsa, DAPI and also by TUNEL assay. Chl-induced nuclear fragmentation of K562 cells, as determined by Giemsa staining, was prevented by NAC pre-treatment (Fig. 2C, top panel). This was confirmed by nuclear DAPI staining. Typical images of untreated and NAC treated cells with round intact nuclei were seen. In contrast, cells treated with 25 μg/ml Chl showed phase-bright nuclear fragmentation typical of apoptosis which was completely reversed by pre-treatment with NAC (Fig. 2C, middle panel). The protective effect of NAC on DNA fragmentation was also observed by TUNEL assay (Fig. 2C, bottom panel). Catalase, an antioxidant enzyme, is bestowed with the ability to hydrolyze H_2O_2 [19]. However, catalase is a membrane-impermeable enzyme. Covalent conjugation of catalase with polyethylene glycol (PEG) induces cell fusion and enhances cell association of this enzyme in a manner which increases cellular enzyme activity and provides prolonged protection from H_2O_2 [20]. Pre-treatment with graded concentrations of PEG-catalase resulted in significant protection, almost by 80–90%, from Chl-induced cytotoxicity indicating Chl does induce H_2O_2 and its production is critically important for Chl-induced cell death (Fig. 2D).

Similarly, *in vivo* anticancer activity of Chl was also mediated by ROS. Simultaneous administration of NAC and Chl significantly diminished the effect of Chl alone on tumor burden in nude mice transplanted with K562 cells (Fig. 2E). The representative tumor masses of K562 xenografts of nude mice receiving vehicle control or Chl with or without NAC are shown in Fig. 2F.

3.3. NAC abrogates Chl-induced intracellular ROS and apoptosis in primary CML cells

Since Chl induces significantly more intracellular ROS in Bcr-Abl$^+$ cells, we examined its effect on primary mononuclear cells isolated from CML patients. Similar results were obtained with three different CML patients confirming that Chl does induce the production of H_2O_2 and O_2^- (Fig. 3A). Representative histogram showing intracellular H_2O_2 in primary mononuclear cells of a CML patient after Chl treatment is shown in the inset of Fig. 3A. In addition, co-incubation of NAC and Chl led to a significant reduction in intracellular H_2O_2 levels in primary CML cells (Fig. 3B). Since Chl treatment increased intracellular ROS in leukemia cells, we were interested to evaluate the effect of Chl treatment on intracellular ROS in normal human peripheral blood mononuclear cells (hPBMC). Treatment of hPBMC with graded concentrations of Chl for varying time periods failed to generate H_2O_2, but induced detectable but insignificant increase in O_2^- levels (Fig. 3C).

NAC reverted Chl-induced apoptosis of K562 cells. We therefore evaluated whether NAC can exert comparable effects on primary cells isolated from CML patients. NAC pre-treatment significantly abrogated the cytotoxicity mediated by Chl in all the three CML patients (Fig. 3D). The role of ROS was further confirmed by the effect of PEG-catalase on Chl-induced apoptosis in primary mononuclear cells of CML patients (Fig. 3E). Of note, no appreciable toxicity was observed when normal hPBMC from two healthy donors were incubated with Chl (Fig. 3F).

3.4. Chl-induced inhibition of phosphorylation of Bcr-Abl and its downstream substrates is reversed by NAC

We evaluated the role of ROS on Chl-mediated inhibition of Bcr-Abl phosphorylation. K562 cells were incubated with increasing concentrations of Chl for varying time periods in the presence and absence of NAC or with graded doses of exogenous H_2O_2. Phosphorylation of Abl was evaluated by Western blot as well as by flow cytometry. Chl inhibited phosphorylation of both fused (Bcr-Abl) and unfused Abl as early as 30 min post-treatment without affecting protein expression. However, NAC pre-treatment reversed the effect on phosphorylation (Fig. 4A). Intracellular phosphorylated Abl was also demonstrated by flow cytometry. Similarly, Chl treatment abolished the phosphorylation and NAC opposed its effect (Fig. 4B). Of note, unlike Western blot, phosphorylation of Bcr-Abl and c-Abl could not be distinguished by flow cytometry. Since phosphorylation of c-Abl is negligible compared to phosphorylation of Bcr-Abl in K562 cells (Fig. 4A), reduction of phospho-Abl staining detected by flow cytometry reflected mostly the reduction of Bcr-Abl phosphorylation.

The effects of exogenously added H_2O_2 on cellular Bcr-Abl phosphorylation (demonstrated by Western blot and flow cytometry) are dose dependent; at low concentrations (upto 25 μM), H_2O_2 enhanced Bcr-Abl phosphorylation while high concentrations of H_2O_2 exerted opposite effects (Fig. 4C and D). Therefore, inhibition of Bcr-Abl phosphorylation by Chl is due to enhanced ROS production and NAC preincubation abrogates this effect.

Next we wanted to determine the effect of Chl on phosphorylation status of downstream targets of Bcr-Abl (i.e., STAT5 and CrkL) and also to evaluate whether Chl-induced ROS generation was responsible for modulation of these substrates in K562 cells. Coadministration of NAC substantially reversed Chl-induced downregulation of phospho-Stat5 and phospho-CrkL in K562 cells (Fig. 4E). These findings suggest that oxidative stress is responsible for Chl-induced disruption of Bcr-Abl mediated downstream signaling events in K562 cells.

3.5. Chl treatment abrogates mitochondrial membrane potential and leads to the release of mitochondrial proteins into the cytosol

Bcr-Abl exerts an anti-apoptotic effect by blocking the release of cytochrome *c* from mitochondria to cytosol via Bcl-2 [21]. We therefore investigated whether inhibition of Bcr-Abl phosphorylation by Chl leads to the disruption of mitochondrial membrane potential ($\Delta\Psi_m$) and the translocation of mitochondrial intermembrane space proteins into the cytoplasm. We used JC-1 staining which indicates a decrease in $\Delta\Psi_m$ by an increased fluorescence at 530 nm (FL-1) and a reduced fluorescence at 590 nm (FL-2). Exposure of K562 cells to Chl led to significant reduction in mitochondrial membrane potential which is depicted

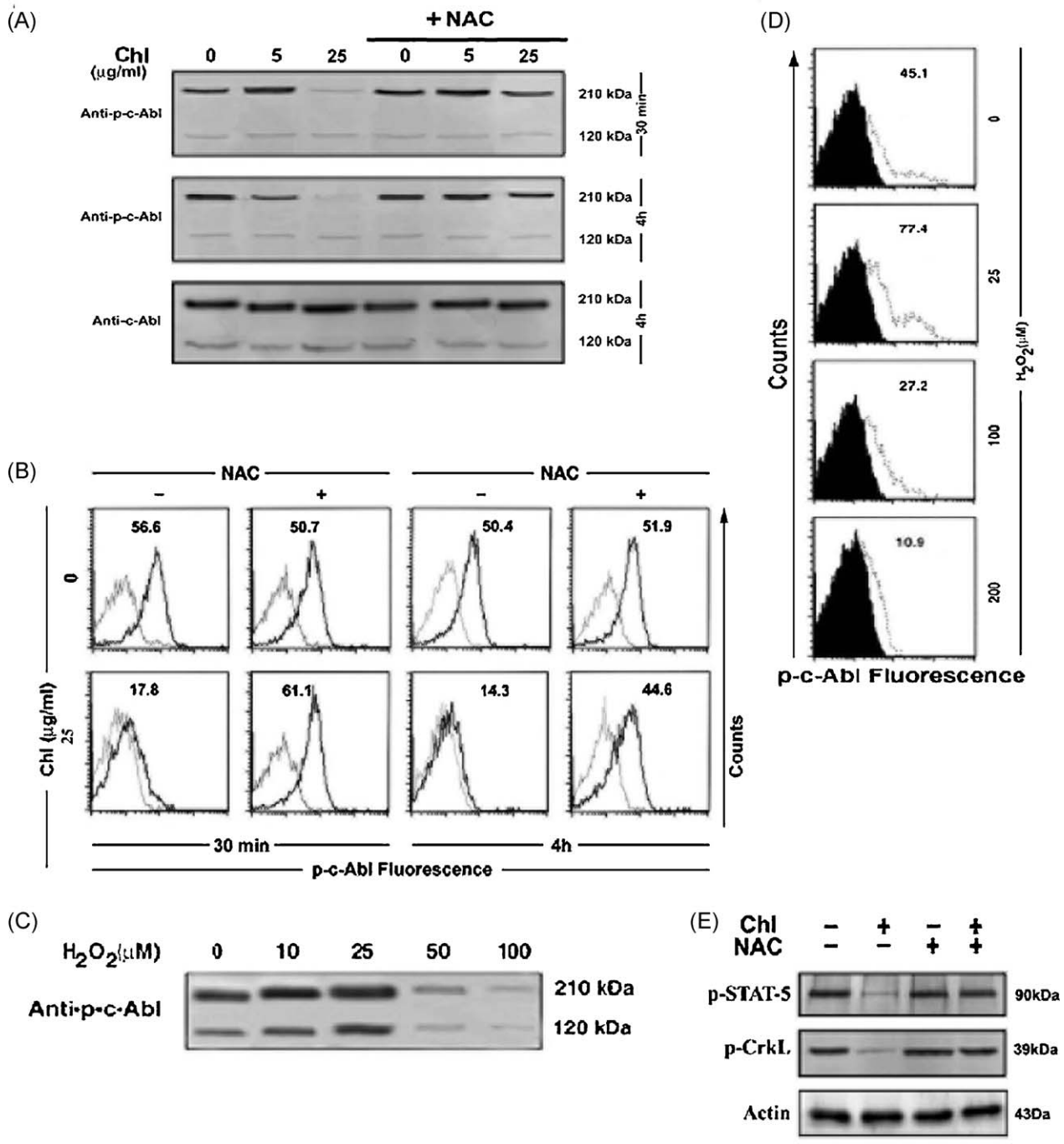

Fig. 4. NAC reverses the inhibitory effects of Chl on Bcr-Abl phosphorylation. (A) Immunoblot analysis of Bcr-Abl phosphorylation in K562 cells. Cells were pre-treated with NAC (2.5 mM) for 1 h before treatment with varying concentrations of Chl for 30 min (top panel) or 4 h (bottom panel). Whole cell lysates were subjected to Western blotting with anti-phospho-c-Abl and anti-c-Abl antibodies. (B) Flow cytometric determination of Abl phosphorylation status of K562 cells. Cells (1.5×10^5 cells/ml) were incubated in the presence or absence of NAC (2.5 mM) for 1 h before treatment with Chl (25 μg/ml) for 30 min (left panel) or 4 h (right panel). After fixing and permeabilization, cells were stained with rabbit anti-phospho-c-Abl (Tyr245) (black line) or with normal rabbit sera (gray line). Values within histograms represent the specific mean fluorescence intensity (MFI) (after subtracting the control values). (C) Effect of exogenous H_2O_2 on the phosphorylation status of Abl. K562 cells were treated with varying concentrations of exogenous H_2O_2 for 1 h, whole cell lysates were prepared and Western blotting were performed with indicated antibody. (D) K562 cells were incubated with varying concentrations of H_2O_2 for 1 h before intracellular staining of phospho-c-Abl. Filled histograms indicate staining with normal rabbit sera; dotted lines, staining with rabbit anti-phospho-c-Abl antibody. (E) Effect of Chl on the expression of downstream substrates. K562 cells were pre-incubated with 2.5 mM NAC for 1 h followed by treatment with 25 μg/ml Chl for 4 h. Expression status of p-STAT-5 and p-CrkL were analyzed by Western blot with whole cell lysates.

as progressive loss of orange-red fluorescence (upper left quadrant) and increase in green fluorescence (lower right quadrant) of JC-1 (Fig. 5A). To determine whether Chl-induced ROS generation was associated with mitochondrial membrane potential disruption, we measured JC-1 fluorescence in K562 cells treated with Chl in the presence and absence of NAC. Indeed, the Chl-mediated disruption of mitochondrial membrane potential was abolished on pre-treatment with NAC (Fig. 5B).

Western blot analysis was used to assess the effects of Chl on the expression level of cytochrome *c* and SMAC in the cytosolic and mitochondrial fractions of K562 cells. Chl treatment induced the release of cytochrome *c* and SMAC into the cytosol (Fig. 5C).

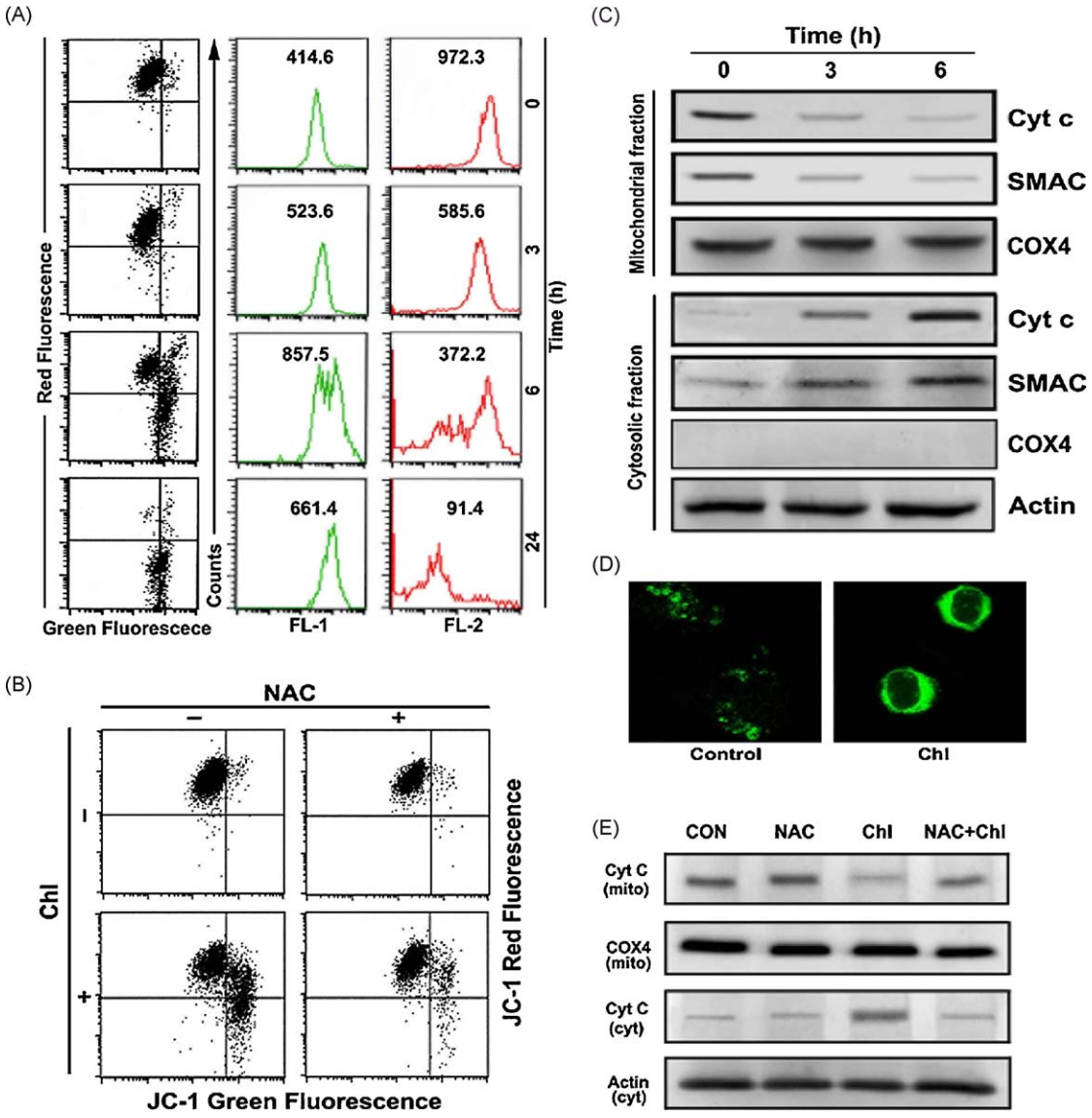

Fig. 5. Chl-induced apoptosis is mediated through mitochondrial pathway. (A) K562 cells were cultured with 25 μg/ml Chl for varying time periods and JC-1 fluorescence was analyzed by flow cytometry. Dot plots (left panel), histograms for both FL1 (middle panel) and FL2 (right panel) fluorescence are shown. Values within histograms represent the mean fluorescence intensity (MFI). (B) Cells were pre-treated with NAC (2.5 mM) for 1 h followed by treatment with Chl (25 μg/ml) for 6 h. JC-1 fluorescence was analyzed by flow cytometry. (C) Expression status of cytochrome *c* and SMAC in mitochondrial and cytosolic fractions of K562 cells after treatment with Chl (25 μg/ml). COX4 and actin were used as loading controls for mitochondrial and cytosolic fractions respectively. (D) K562 cells were treated with Chl (25 μg/ml) for 6 h. Cells were then fixed, permeabilized and stained with anti-cytochorome *c* antibody and analyzed by confocal microscopy. (E) K562 cells were pre-treated with NAC for 1 h followed by treatment with Chl for 6 h. Expression of cytochrome *c* was analyzed in the mitochondrial and cytosolic fractions by Western blot.

Cytochrome *c* release was also confirmed by confocal microscopy (Fig. 5D). NAC pre-treatment conferred significant protection against Chl-induced release of cytochrome *c* to the cytosol (Fig. 5E). Collectively, these findings indicated that Chl treatment abrogates mitochondrial membrane potential and then leads to the release of pro-apoptotic mitochondrial proteins cytochrome *c* and SMAC into the cytosolic fraction of K562 cells, and all these events are initiated by ROS.

3.6. Chl-induced caspase activation is downstream of intracellular ROS generation

Activation of apoptotic caspase cascade is an important event in cytotoxic drug-induced apoptosis. Therefore we wanted to investigate whether treatment of cells with Chl leads to caspase activation and whether or not it is a consequence of Chl-mediated ROS generation. First, we wanted to determine the effect of different caspase inhibitors on Chl-induced apoptosis. K562 cells were treated with 25 μg/ml Chl for 24 h, either alone or in combination with 25 μM Z-VAD-FMK (pan-caspase inhibitor), 25 μM Z-IETD-FMK (caspase-8 inhibitor), or 25 μM LEHD-CHO (caspase-9 inhibitor). Each caspase inhibitor alone had minimal effect on the viability of K562 cells. Z-VAD-FMK or LEHD-CHO treatment led to almost complete blockade of apoptosis, although Z-IETD-FMK partially but significantly inhibited Chl-mediated cell death in K562 cells (Fig. 6A). In addition, we found that Chl induced cleavage of caspase-3, -8, and -9 and degradation of the typical caspase-3 substrate PARP (Fig. 6B). Moreover, Chl-induced caspase-3 activation and PARP cleavage was abolished in K562 cells pre-treated with NAC (Fig. 6C). These results indicate that treatment of cells with Chl resulted in a dramatic increase in caspases-9, -3, and -8 processing, as well as PARP degradation suggesting the involvement of both extrinsic and intrinsic pathways of apoptosis. Combination of Chl and each caspase inhibitor

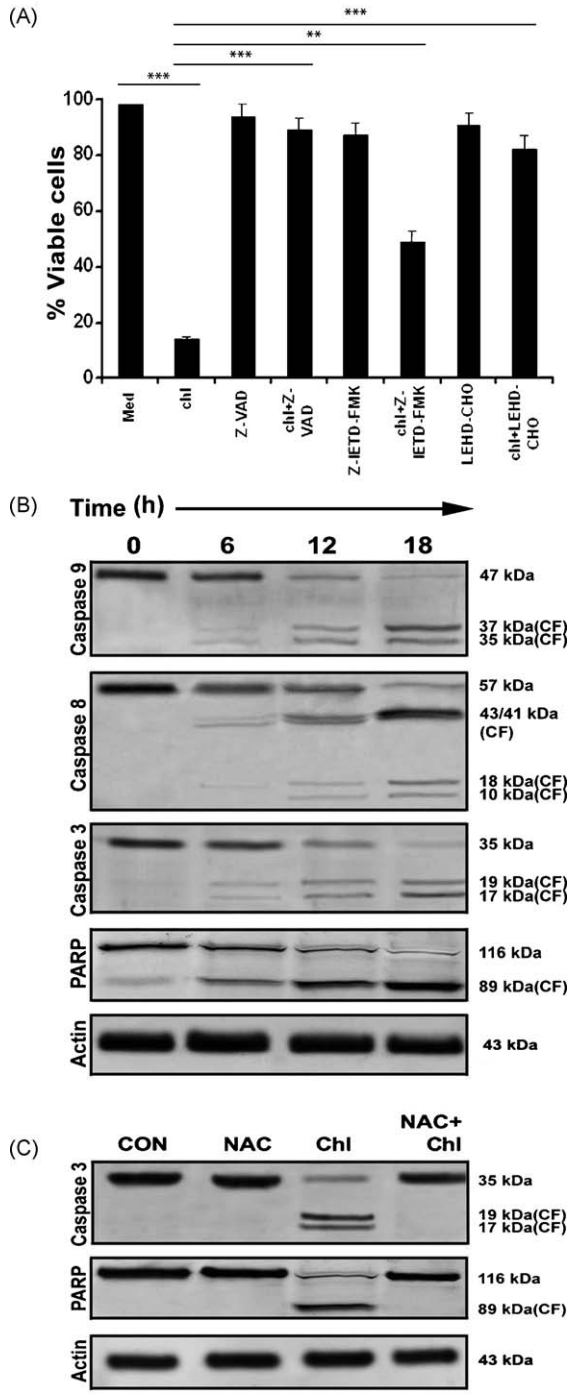

Fig. 6. Chl-induced caspase activation is reversed by NAC. (A) Effects of caspase inhibitors on Chl-treated K562 cells. Inhibition of Chl-induced death of K562 cells was estimated in a co-culture with a panel of caspase inhibitors. Cells were pre-incubated with indicated caspase inhibitors (25 μM each) for 1 h before the addition of 25 μg/ml Chl for 24 h. Cell viability was measured by Trypan blue dye exclusion. Values represent the mean ± SD for three separate experiments. $^{**}p < 0.01$ versus indicated treatment; $^{***}p < 0.001$ versus indicated treatments. (B) Time course of caspase-3, -8, -9 and PARP cleavage in Chl-treated K562 cells. Cells were cultured with 25 μg/ml Chl for the indicated times. Whole cell lysates were prepared and subjected to Western blotting with indicated antibodies. CF denotes cleaved fragment. (C) Effect of NAC on Chl-induced cleavage of caspase-3 and PARP. Cells were pre-treated with 2.5 mM NAC for 1 h before treatment with 25 μg/ml Chl for 18 h. Whole cell lysates were subjected to Western blotting.

significantly blocked Chl-induced apoptosis but NAC coadministration neither caused PARP cleavage nor reduced the level of procaspase-3. Thus, ROS generation plays a critical role in caspase activation and is an upstream event in Chl-mediated cell lethality.

3.7. Chl-induced ROS upregulates death receptors and activates extrinsic pathway

Chl activated caspase-8 and respective specific inhibitor partially blocked Chl-induced apoptosis in K562 cells. In addition, death receptor-mediated activation of caspase-8 can be induced downstream of caspase-3 by caspase-6 [22]. To establish whether caspase-8 cleavage is important or not, experiments were performed on the role of death receptor-mediated pathway in Chl-mediated apoptosis. FACS analysis demonstrated significant increase on the surface expression of DR5 after Chl treatment (Fig. 7A). In contrast, DR4 was only marginally increased and increase in the levels of TNFRs was undetectable (Fig. 7A). Next, we evaluated the role of Chl-induced ROS generation in the upregulation of death receptors. Pre-treatment with NAC attenuated Chl-induced upregulation of DR5 (Fig. 7B). Collectively, these results suggest that Chl-induced upregulation of DR5 requires the generation of ROS.

To determine whether Chl-mediated upregulation of DR5 is critical for Chl-induced apoptosis, the effect of siRNA-mediated knockdown of DR5 was evaluated for both Chl-mediated apoptosis and caspase-8 cleavage. Suppression of DR5 expression by transfection with DR5 siRNA completely attenuated Chl-induced caspase-8 cleavage but partially blocked apoptosis (Fig. 7C). These results suggest that death receptor-mediated extrinsic pathway is responsible partly but not solely for Chl-mediated apoptosis.

3.8. Modulation of pro-apoptotic and anti-apoptotic regulatory proteins is mediated by Chl-induced ROS

In CML cells, Bcr-Abl upregulates Bcl-2 and Bcl-xL through activation of STAT5, inhibits release of cytochrome *c* and prevents caspase activation. All these events confer resistance to apoptosis [23,24]. We therefore investigated whether Chl treatment modified the expression of Bcl-2 family members. Treatment with Chl resulted in the translocation of Bax from cytosol to the mitochondria indicating Bax activation alongwith an increase in the expression of Bad, Bim and cleavage of Bid and also reduction in Bcl-xL and Bcl-2 levels. There was no significant alteration in Mcl-1 expression by Chl (Fig. S2A). NAC pre-treatment prevented Bid cleavage and reduction in Bcl-xL and Bcl-2 expression confirming that all these events are mediated by Chl-induced ROS (Fig. S2B).

Because of the importance of inhibitor of apoptosis (IAP) proteins particularly survivin in conferring CML cells with a growth and survival advantage by inhibition of pro-apoptotic caspases [25], we evaluated the status of their expression in K562 cells upon Chl exposure. Chl induced a time-dependent reduction in the expression of survivin, cIAP1 and XIAP (Fig. S2C). Interestingly, NAC pre-treatment significantly reversed the effect of Chl on IAP proteins indicating the involvement of ROS (Fig. S2D). Thus, downregulation of Bcl-xL, Bcl-2, survivin, XIAP and cIAP1 might be contributing to Chl-induced cell death. Alternatively, these downregulations may reflect caspase-mediated cleavage of the indicated proteins [26,27]. Experiments in the presence of pan-caspase inhibitor support the later possibility (Fig. S2E).

3.9. Chl-induced ROS leads to the activation of JNK and p38 MAPK kinases

Chl induced the activation of JNK and p38 MAPK which was neutralized by pre-treatment with NAC. These findings were validated by Western blot (Fig. S3A) and flow cytometry (Fig. S3B). Thus, Chl-induced activation of these MAP kinases is mediated via Chl-induced ROS generation. The functional significance of Chl-induced activation p38 MAP kinase has been assessed earlier [7].

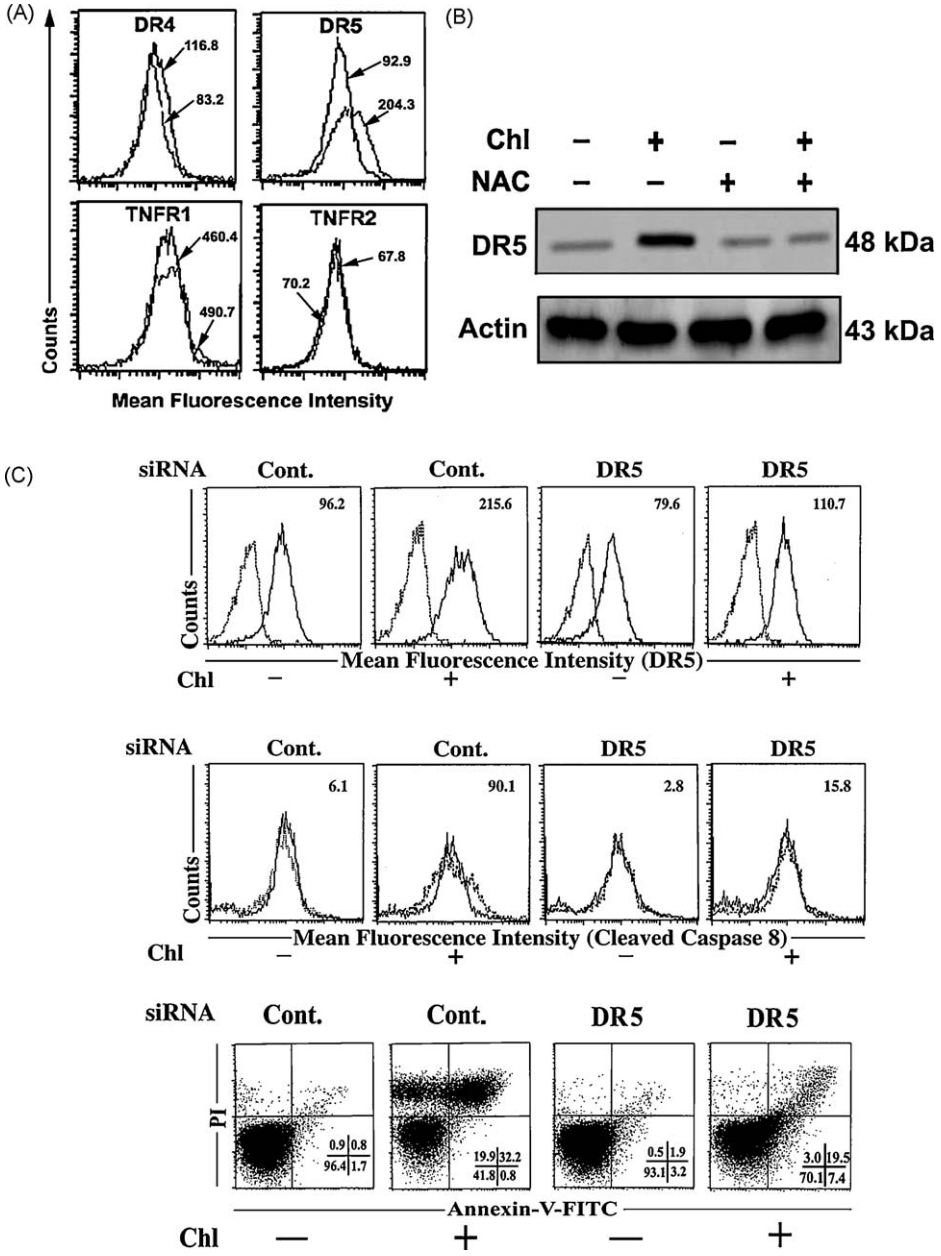

Fig. 7. Chl treatment upregulates death receptors (DRs). (A) Flow cytometric analysis of death receptor expression on the plasma membrane of K562 cells. Cells were treated with 25 μg/ml Chl for 8 h, washed and stained with indicated antibodies or isotype-matched control antibodies. Medium treated cells are shown in black solid line and Chl-treated cells are shown in grey solid line. Mean fluorescence intensity (MFI) of isotype-matched control antibodies was less than 20 under all treatment conditions and for all death receptors (not shown). Results are representative of three separate experiments. (B) Cells were pre-treated with 2.5 mM NAC for 1 h followed by treatment with Chl for 8 h and the expression of death receptors were analyzed by Western blot on whole cell lysates. (C) Suppression of DR5 expression by siRNA attenuates Chl-induced cleavage of caspase-8, and apoptosis. K562 cells were transfected with control siRNA or DR5 siRNA. 48 h after transfection, cells were treated with 25 μg/ml Chl for 24 h for analysis of cleaved caspase-8 and apoptosis. Upper panel, flow cytometry analysis to confirm the downregulation of DR5 by siRNA transfection (dotted line, staining with control antibody; solid line, staining with anti-DR5 antibody; values in the histograms represent specific mean fluorescence intensity (MFI) after subtracting the respective control values. Middle panel, analysis of caspase-8 cleavage by flow cytometry. Solid line, staining with control antibody; dotted line, staining with anti-cleaved caspase-8 antibody; values in the histograms represent specific MFI. Lower panel, analysis of apoptosis by flow cytometry.

To assess the role of pronounced JNK activation on Chl-mediated apoptosis, K562 cells were exposed to 25 μg/ml Chl for 24 h in the presence or absence of 20 μM SP600125, a pharmacologic inhibitor of JNK [28]. Coadministration of SP600125 attenuated Chl-induced cell death (Fig. S3C) and reversed Chl-mediated loss of mitochondrial membrane potential (Fig. S3D). Collectively, these findings implicate that JNK activation, a downstream event of ROS generation, plays an important role in mediating Chl-induced mitochondrial dysfunction and apoptosis of K562 cells.

4. Discussion

We have shown previously that chlorogenic acid, an ester of caffeic and quinic acid, isolated from *P. betle* leaf extract, suppresses growth of Bcr-Abl$^+$ cells including Bcr-Abl$^+$ primary leukemic cells of CML patients in culture as well as K562 xenografts *in vivo* [7]. However, the sequence of events leading to inhibition of Bcr-Abl phosphorylation and cell death was not clearly defined. Using K562 cells, originally isolated from a CML patient with blast crisis, we now demonstrate that the initial signal for Chl-induced apoptosis is

derived from Chl-induced ROS. Mounting evidence suggests that ROS play an important role in apoptosis induction under both physiological and pathological conditions and are also recognized for playing a dual role by displaying both deleterious and beneficial effects [29]. The "two-faced" character of ROS is substantiated by growing body of evidence that ROS within cells act as secondary messengers in intracellular signaling cascades, which induce and maintain the oncogenic phenotype of cancer cells [30]. However, ROS can also induce cellular senescence and apoptosis. Here we demonstrate that modulation of intracellular ROS alters the cytotoxic activity of Chl.

Exposure of a panel of Bcr-Abl$^+$ and Bcr-Abl$^-$ cells to graded concentrations of Chl led to preferential enhancement of ROS generation in Bcr-Abl$^+$ cells as indicated by an increase in oxidation of DCFH-DA. Consistent with this finding, we also noticed that Chl exhibits preferential toxicity towards Bcr-Abl$^+$ cells at the doses tested. Bcr-Abl$^+$ cells are more sensitive than Bcr-Abl$^-$ cells to ROS inducing agents [10,13]. Previous reports have demonstrated that primary leukemia cells isolated from various types of leukemia exhibit a significant increase in ROS in their malignant cells compared to their normal counterparts [10]. Leukemic cells with higher basal ROS contents are more sensitive to ROS inducing agents than those with lower ROS contents [31]. However, our data suggest that not only the threshold of ROS but also intrinsic differential sensitivity to ROS may be responsible for the observed differential cytotoxicity of Bcr-Abl$^+$ and Bcr-Abl$^-$ cells to Chl.

We evaluated the role of ROS in mediating Chl-induced cell death. For this purpose, we used the thiol specific antioxidant, N-acetyl-L-cysteine (NAC) which protects cells by increasing intracellular GSH levels and scavenging ROS by behaving like catalase [32]. NAC pre-treatment scavenged intracellular ROS and almost completely blocked Chl-induced apoptosis of Bcr-Abl$^+$ CML cell line, primary cells of CML patients *in vitro* and K562 xenografts *in vivo*. Importantly, protective effect of NAC was time-dependent: pre-treatment was effective and post-treatment was marginally effective only at earlier time point, emphasizing the role of early production of ROS in Chl-induced cytotoxicity. Thus, oxidative damage plays a key role in the apoptosis process induced by Chl. This result is in agreement with previous reports that NAC decreases the activity of ROS-dependent anticancer agents such as arsenic trioxide and sulforaphane [33,34]. PEG-catalase pre-treatment also attenuated the apoptosis confirming the role of ROS in Chl-induced cell death.

Next we evaluated the role of ROS in Chl-mediated inhibition of Bcr-Abl phosphorylation. Recently, it was reported that NAC attenuated the PEITC-induced oxidative stress in CML cells and prevented the degradation of BCR-ABL and cell death [35]. Our data indicate that NAC pre-treatment reversed the effect of Chl on Bcr-Abl phosphorylation. Additionally, previous studies reported that H_2O_2 activates c-Abl [36]. Our data suggest that the effects of exogenously added H_2O_2 on cellular Bcr-Abl phosphorylation is dose dependent; at low concentrations (upto 25 μM), H_2O_2 enhances Bcr-Abl phosphorylation while high concentrations of H_2O_2 exert opposite effects. Bcr-Abl phosphorylates several substrates and activates a myriad of signal transduction pathways such as Ras, ERK, STAT, NFκB and PI3K/Akt all of which can stimulate cell proliferation and mediate resistance to apoptosis [37]. The transcription factors Stat3 and Stat5a/b have been implicated in Bcr-Abl induced initial transformation [38]. CrkL, a prominent substrate of the Bcr-Abl oncoprotein in chronic myelogenous leukemia binds to both Bcr-Abl and c-Abl [39]. Chl-induced ROS prevented the phosphorylation of both Bcr-Abl substrates, STAT5 and CrkL which was reverted by NAC.

Interestingly, mitochondria are regarded both as the source and target of ROS. In fact it has been postulated that ROS may play a dual role in apoptosis, either as activators of permeability transition or a consequence of this transition, depending on the death stimulus [40]. ROS generation leads to the free-radical attack of membrane phospholipids followed by depletion of mitochondrial membrane potential with the opening of the permeability transition pore (PTP) resulting in the release of intermembrane proteins, such as cytochrome *c* to the cytosol. Chl-induced ROS generation in K562 cells was accompanied by disruption of the mitochondrial membrane potential and release of cytochrome *c* and SMAC from mitochondria to the cytosol. Chl-induced ROS generation was evident as early as 30 min after treatment. However, the considerable loss of mitochondrial membrane potential and cytosolic release of mitochondrial pro-apoptotic proteins was observed only after 6 h post-treatment with Chl. Thus, ROS act as upstream signaling molecules to initiate Chl-mediated cell death. This is consistent with the finding that pre-treatment of K562 cells with NAC not only prevents ROS generation but also confers near-complete protection against Chl-induced mitochondrial membrane potential disruption and cytochrome *c* release.

Anticancer drug-induced apoptosis is generally mediated via extrinsic (death receptor) or intrinsic (mitochondrial) pathway but in some cases both pathways may be involved in inducing cell death. Chl treatment resulted in an increase in caspases-9, -3, and -8 processing as well as PARP degradation. Combination of Chl and pan-caspase or caspase-9 inhibitor significantly blocked Chl-induced cell death and NAC coadministration considerably attenuated both caspase-3 and PARP cleavage. Since Chl-induced caspase-8 cleavage and cell death was partially blocked with the caspase-8 inhibitor, the role of different death receptors in Chl-induced cell death was evaluated. Death receptors exert many different biological functions, including the regulation of cell death and survival, differentiation and immune regulation. Death receptors are part of the tumor necrosis factor (TNF) receptor gene superfamily, which comprises more than 20 proteins, for example, CD95, TRAIL receptors, and TNF receptors (TNFR) [41]. Chl treatment preferentially enhanced DR5 expression and knocking down DR5 by siRNA transfection completely attenuated caspase-8 cleavage but partially reversed apoptosis. Various chemopreventive agents like sulforaphane, curcumin and rosiglitazone upregulate DR5 expression through ROS-mediated pathway [42]. Therefore, we examined whether ROS generation could also be involved in Chl-induced DR5 upregulation. Pretreatement with NAC significantly reduced Chl-induced DR5 upregulation. Taken together, our data suggest that Chl-induced apoptosis is orchestrated by the cooperative effects of both extrinsic and intrinsic pathways and that early generation of ROS plays a key role in both the pathways.

The Bcl-2 family proteins have emerged as critical regulators of the mitochondria mediated apoptosis by functioning as either promoters or inhibitors of the cell death process [43]. Bcl-2 inhibits the mitochondria depolarization and ROS production, while Bax induces mitochondria depolarization and ROS production [44]. Treatment of K562 cells with Chl led to a decrease in anti-apoptotic and an increase in pro-apoptotic members of the Bcl-2 family, and NAC pre-treatment significantly reversed the effect of Chl.

Bcr-Abl has a much stronger anti-apoptotic effect than Bcl-xL, suggesting that additional/alternative survival pathways are involved [45]. Survivin, an inhibitor of apoptosis protein is involved in the blockade of mitochondrial injury and caspase activation conferred by Bcr-Abl, therefore, represents a therapeutic target downstream of Bcr-Abl [46]. Moreover, the pro-survival actions of the Bcr-Abl kinase have also been associated with altered expression of another anti-apoptotic protein XIAP. Survivin is overexpressed in Bcr-Abl$^+$ CML patients in all phases of the disease whereas its expression is very low in samples from healthy individuals and in Bcr-Abl$^-$ CML patients [47]. Recently, it has been reported that disruption of survivin sensitizes Bcr-Abl$^+$ cells to imatinib-induced

apoptosis and was further enhanced by inhibition of catalase [48]. We therefore investigated the effect of Chl-induced ROS on members of the IAP family proteins. A time-dependent reduction in the expression of survivin as well as XIAP and cIAP1 was observed. NAC markedly attenuated this effect of Chl indicating that the ROS mediates Chl-induced downregulation of IAP family proteins. Moreover, survivin and Bcl-2 underwent caspase-mediated cleavage because Chl-induced downregulation of these two proteins was reversed in the presence of pan-caspase inhibitor.

JNK and p38 MAPK are involved in stress responses and cell death [49,50]. It is known that JNK signaling is necessary for the stress-induced release of cytochrome *c* and programmed cell death. In our earlier study it was documented that Chl treatment resulted in the activation of stress activated kinase p38 in Bcr-Abl$^+$ cells. Activation of p38 MAPK was thought to be a consequence of inhibition of Bcr-Abl phosphorylation [7]. Furthermore, other related studies have shown that treatment of Bcr-Abl$^+$ cells with various agents that suppress their growth, such as IFNα [51], imatinib mesylate [52] and dasatinib [53] also result in activation of the p38 MAPK pathway. Notably, in all these studies, pharmacological inhibition of p38 MAPK significantly abrogated the induction of pro-apoptotic or growth inhibitory effects in response to these drugs, implicating a key role for p38 MAPK in the initiation of antileukemic responses in Bcr-Abl$^+$ cells. Here we demonstrate that Chl-induced activation of p38 MAPK and JNK was mediated by ROS.

In conclusion, our study suggests that Chl-induced disruption of mitochondrial membrane potential, release of cytochrome *c*, activation of caspases, upregulation of death receptors and pro-apoptotic regulatory proteins and activation of JNK and p38 MAP kinases may or may not be mediated by the inhibition of Bcr-Abl phosphorylation. Chl-induced ROS can directly induce apoptosis by disrupting mitochondrial membrane potential, activating caspases and other apoptotic pathways.

Acknowledgements

We thank Dr. Carlo Gambacorti Passerini, Instituto Nazionale Tumori, Milan, Italy for providing KU812 and KCL-22 cell lines. The present study was supported by the Council of Scientific and Industrial Research (CSIR) and the Department of Biotechnology (DBT), New Delhi.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.08.013.

References

[1] McDougall B, King PJ, Wu BW, Hostomsky Z, Reinecke MG, Robinson Jr WE. Dicaffeoylquinic and dicaffeoyltartaric acids are selective inhibitors of human immunodeficiency virus type 1 integrase. Antimicrob Agents Chemother 1998;42:140–6.
[2] Kono Y, Kashine S, Yoneyama T, Sakamoto Y, Matsui Y, Shibata H. Iron chelation by chlorogenic acid as a natural antioxidant. Biosci Biotechnol Biochem 1998;62:22–7.
[3] Tanaka T, Nishikawa A, Shima H, Sugie S, Shinoda T, Yoshimi N, et al. Inhibitory effects of chlorogenic acid, reserpine, polyprenoic acid (E-5166), or coffee on hepatocarcinogenesis in rats and hamsters. Basic Life Sci 1990;52:429–40.
[4] Ito H, Miyazaki T, Ono M, Sakurai H. Antiallergic activities of rabdosiin and its related compounds: chemical and biochemical evaluations. Bioorg Med Chem 1998;6:1051–6.
[5] Teel RW, Huynh H. Modulation by phytochemicals of cytochrome P450-linked enzyme activity. Cancer Lett 1998;133:135–41.
[6] Jiang Y, Sakagami H, Satoh K, Takayama F, Watanabe S, Kusama K. Induction of cell death by chlorogenic acid in human oral tumor cell lines. Phytomedicine 2000;7:483–91.
[7] Bandyopadhyay G, Biswas T, Roy KC, Mandal S, Mandal C, Pal BC, et al. Chlorogenic acid inhibits Bcr-Abl tyrosine kinase and triggers p38 mitogen-activated protein kinase-dependent apoptosis in chronic myelogenous leukemic cells. Blood 2004;104:2514–22.
[8] Jabs T. Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. Biochem Pharmacol 1999;57:231–45.
[9] Laurent A, Nicco C, Chereau C, Goulvestre C, Alexandre J, Alves A, et al. Controlling tumor growth by modulating endogenous production of reactive oxygen species. Cancer Res 2005;65:948–56.
[10] Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov 2009;8:579–91.
[11] Chandra J, Hackbarth J, Le S, Loegering D, Bone N, Bruzek LM, et al. Involvement of reactive oxygen species in adaphostin-induced cytotoxicity in human leukemia cells. Blood 2003;102:4512–9.
[12] Yi J, Gao F, Shi G, Li H, Wang Z, Shi X, et al. The inherent cellular level of reactive oxygen species: one of the mechanisms determining apoptotic susceptibility of leukemic cells to arsenic trioxide. Apoptosis 2002;7:209–15.
[13] Trachootham D, Zhou Y, Zhang H, Demizu Y, Chen Z, Pelicano H, et al. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by β-phenylethyl isothiocyanate. Cancer Cell 2006;10:241–52.
[14] Lee KW, Lee HJ. The roles of polyphenols in cancer chemoprevention. Biofactors 2006;26:105–21.
[15] Fan GJ, Jin XL, Qian YP, Wang Q, Yang RT, Dai F, et al. Hydroxycinnamic acids as DNA-cleaving agents in the presence of Cu(II) ions: mechanism, structure–activity relationship, and biological implications. Chemistry 2009;15:12889–9.
[16] Rakshit S, Bagchi J, Mandal L, Paul K, Ganguly D, Bhattacharjee S, et al. N-acetyl cysteine enhances imatinib-induced apoptosis of Bcr-Abl$^+$ cells by endothelial nitric oxide synthase-mediated production of nitric oxide. Apoptosis 2009;14:298–308.
[17] Kapuscinski J. DAPI: a DNA-specific fluorescent probe. Biotech Histochem 1995;70:220–33.
[18] Higuchi Y. Chromosomal DNA fragmentation in apoptosis and necrosis induced by oxidative stress. Biochem Pharmacol 2003;66:1527–35.
[19] Deisseroth A, Dounce AL. Catalase: physical and chemical properties, mechanism of catalysis, and physiological role. Physiol Rev 1970;50:319–75.
[20] Beckman JS, Minor Jr RL, White CW, Repine JE, Rosen GM, Freeman BA. Superoxide dismutase and catalase conjugated to polyethylene glycol increases endothelial enzyme activity and oxidant resistance. J Biol Chem 1988;263:6884–92.
[21] Amarante-Mendes GP, Naekyung Kim C, Liu L, Huang Y, Perkins CL, Green DR, et al. Bcr-Abl exerts its antiapoptotic effect against diverse apoptotic stimuli through blockage of mitochondrial release of cytochrome *c* and activation of caspase-3. Blood 1998;91:1700–5.
[22] Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, et al. Ordering the cytochrome *c*-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J Cell Biol 1999;144:281–92.
[23] Horita M, Andreu EJ, Benito A, Arbona C, Sanz C, Benet I, et al. Blockade of the Bcr-Abl kinase activity induces apoptosis of chronic myelogenous leukemia cells by suppressing signal transducer and activator of transcription 5-dependent expression of Bcl-xL. J Exp Med 2000;191:977–84.
[24] Deming PB, Schafer ZT, Tashker JS, Potts MB, Deshmukh M, Kornbluth S. Bcr-Abl-mediated protection from apoptosis downstream of mitochondrial cytochrome *c* release. Mol Cell Biol 2004;24:10289–9.
[25] Fang ZH, Dong CL, Chen Z, Zhou B, Liu N, Lan HF, et al. Transcriptional regulation of survivin by c-Myc in BCR/ABL-transformed cells: implications in anti-leukaemic strategy. J Cell Mol Med 2009;13:2039–52.
[26] Cheng EH, Kirsch DG, Clem RJ, Ravi R, Kastan MB, Bedi A, et al. Conversion of Bcl-2 to a Bax-like death effector by caspases. Science 1997;278:1966–8.
[27] Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS, Reed JC. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. EMBO J 1999;18:5242–51.
[28] Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, et al. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A 2001;98:13681–6.
[29] Pelicano H, Carney D, Huang P. ROS stress in cancer cells and therapeutic implications. Drug Resist Updat 2004;7:97–110.
[30] Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med 2010;48:749–62.
[31] Zhou Y, Hileman EO, Plunkett W, Keating MJ, Huang P. Free radical stress in chronic lymphocytic leukemia cells and its role in cellular sensitivity to ROS-generating anticancer agents. Blood 2003;101:4098–104.
[32] Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radic Biol Med 1989;6:593–7.
[33] Park MJ, Lee JY, Kwak HJ, Park CM, Lee HC, Woo SH, et al. Arsenic trioxide (As_2O_3) inhibits invasion of HT1080 human fibrosarcoma cells: role of nuclear factor-kappaB and reactive oxygen species. J Cell Biochem 2005;95:955–69.
[34] Singh SV, Srivastava SK, Choi S, Lew KL, Antosiewicz J, Xiao D, et al. Sulforaphane-induced cell death in human prostate cancer cells is initiated by reactive oxygen species. J Biol Chem 2005;280:19911–24.
[35] Zhang H, Trachootham D, Lu W, Carew J, Giles FJ, Keating MJ, et al. Effective killing of Gleevec-resistant CML cells with T315I mutation by a natural compound PEITC through redox-mediated mechanism. Leukemia 2008;22:1191–9.
[36] Sun X, Majumder P, Shioya H, Wu F, Kumar S, Weichselbaum R, et al. Activation of the cytoplasmic c-Abl tyrosine kinase by reactive oxygen species. J Biol Chem 2000;275:17237–40.

[37] Van Etten RA. Mechanisms of transformation by the BCR-ABL oncogene: new perspectives in the post-imatinib era. Leuk Res 2004;28(Suppl. 1): S21–8.
[38] Hoelbl A, Schuster C, Kovacic B, Zhu B, Wickre M, Hoelzl MA, et al. Stat5 is indispensable for the maintenance of bcr/abl-positive leukaemia. EMBO Mol Med 2010;2:98–110.
[39] Johnson KJ, Griswold IJ, O'Hare T, Corbin AS, Loriaux M, Deininger MW, et al. A BCR-ABL mutant lacking direct binding sites for the GRB2, CBL and CRKL adapter proteins fails to induce leukemia in mice. PLoS One 2009;4:e7439.
[40] Kroemer G, Reed JC. Mitochondrial control of cell death. Nat Med 2000;6:513–9.
[41] Mahmood Z, Shukla Y. Death receptors: targets for cancer therapy. Exp Cell Res 2010;316:887–99.
[42] Kim YH, Jung EM, Lee TJ, Kim SH, Choi YH, Park JW, et al. Rosiglitazone promotes tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by reactive oxygen species-mediated up-regulation of death receptor 5 and down-regulation of c-FLIP. Free Radic Biol Med 2008;44:1055–68.
[43] Kuwana T, Newmeyer DD. Bcl-2-family proteins and the role of mitochondria in apoptosis. Curr Opin Cell Biol 2003;15:691–9.
[44] Kirkland RA, Franklin JL. Bax, reactive oxygen, and cytochrome *c* release in neuronal apoptosis. Antioxid Redox Signal 2003;5:589–96.
[45] Amarante-Mendes GP, McGahon AJ, Nishioka WK, Afar DE, Witte ON, Green DR. Bcl-2-independent Bcr-Abl mediated resistance to apoptosis: protection is correlated with up regulation of Bcl-xL. Oncogene 1998;16:1383–90.
[46] Wang Z, Sampath J, Fukuda S, Pelus LM. Disruption of the inhibitor of apoptosis protein survivin sensitizes Bcr-Abl–positive cells to STI571-induced apoptosis. Cancer Res 2005;65:8224–32.
[47] Conte E, Stagno F, Guglielmo P, Scuto A, Consoli C, Messina A. Survivin expression in chronic myeloid leukemia. Cancer Lett 2005;225:105–10.
[48] Kwee JK, Luque DG, Ferreira AC, Vasconcelos FC, Silva KL, Klumb CE, et al. Modulation of reactive oxygen species by antioxidants in chronic myeloid leukemia cells enhances imatinib sensitivity through survivin downregulation. Anticancer Drugs 2008;19:975–81.
[49] Shen HM, Liu ZG. JNK signaling pathway is a key modulator in cell death mediated by reactive oxygen and nitrogen species. Free Radic Biol Med 2006;40:928–39.
[50] Obata T, Brown GE, Yaffe MB. MAP kinase pathways activated by stress: the p38 MAPK pathway. Crit Care Med 2000;28:N67–77.
[51] Mayer IA, Verma A, Grumbach IM, Uddin S, Lekmine F, Ravandi F, et al. The p38 MAPK pathway mediates the growth inhibitory effects of interferon-alpha in BCR-ABL-expressing cells. J Biol Chem 2001;276:28570–7.
[52] Parmar S, Katsoulidis E, Verma A, Li Y, Sassano A, Lal L, et al. Role of the p38 mitogen-activated protein kinase pathway in the generation of the effects of imatinib mesylate (STI571) in BCR-ABL-expressing cells. J Biol Chem 2004;279:25345–52.
[53] Dumka D, Puri P, Carayol N, Lumby C, Balachandran H, Schuster K, et al. Activation of the p38 Map kinase pathway is essential for the antileukemic effects of dasatinib. Leuk Lymphoma 2009;50:2017–29.