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The pan-ErbB receptor tyrosine kinase inhibitor canertinib induces ErbB-independent apoptosis in human leukemia (HL-60 and U-937) cells

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ABSTRACT

Epidermal growth factor (EGF) receptor tyrosine kinase inhibitors have recently been shown to display anti-neoplastic effects in human malignant myeloid cells. Our study was initiated in order to determine the effect of the pan-ErbB receptor tyrosine kinase inhibitor, canertinib (CI-1033), on growth and survival of human leukemia (HL-60 and U-937) cells. We show that treatment of HL-60 and U-937 cells with canertinib significantly inhibits growth of both cell lines in a dose-dependent manner; half maximal effective dose (IC_{50}) in HL-60 and U-937 cells was approximately 2.5 μM and 1.0 μM, respectively. Treatment with 2 μM canertinib promoted a G_1 cell cycle arrest, whereas doses of 5 μM or more induced apoptosis as determined by the Annexin V method and cleavage of poly-(ADP-ribose) polymerase (PARP). HL-60 and U-937 cells lacked EGF-receptor transcript but expressed ErbB2–4 mRNA as determined by RT-PCR. However, none of the corresponding ErbB-receptor proteins could be detected by Western blot analysis. We conclude that canertinib induces apoptosis in HL-60 and U-937 cells devoid of functional ErbB1–4 receptors. Our results suggest that canertinib could be of potential clinical interest in the treatment of acute myeloid leukemia.

Introduction

Currently, the preferred treatment of most acute leukemias is aggressive chemotherapy alone or in combination with bone marrow transplantation. However, a large proportion of the patients relapse and subsequently die from their disease [1]. Therefore, in order to improve the long-term survival, novel and preferentially less toxic therapeutic modalities are urgently needed in the treatment of patients with acute leukemia. An early example of a specific treatment of leukemia was the differentiation inducing therapy with all-*trans* retinoic acid in the case of acute promyelocytic leukemia [2]. Recently molecularly targeted treatment strategies have evolved to selectively kill malignant cells expressing specific determinants vital for growth and survival [3]. The multi-tyrosine kinase inhibitor, imatinib, was one of the first target drugs developed and is often successfully used in the treatment of chronic myeloid leukemia (CML) [4]. A wide variety of signal transduction inhibitors are being developed and are currently under clinical investigation in the treatment of different forms of hematological malignancies including acute myeloid leukemia (AML) [5]. Interestingly, recent studies have revealed that the ErbB1 tyrosine kinase inhibitors, gefitinib (ZD1839) and erlotinib, have the capacity to induce differentiation or apoptosis in ErbB1-negative AML blasts and in bone marrow cells derived from patients with myelodysplastic syndrome (MDS) [6–9]. Based on these reports, we investigated the effect of the pan-ErbB receptor tyrosine kinase inhibitor canertinib (CI-1033) on the human AML cell lines, HL-60 and U-937, with respect to cell proliferation and viability. We report that canertinib induces apoptosis in an ErbB-independent manner in both cell lines. Our results suggest that canertinib may prove useful in the treatment of patients with AML.

Materials and methods

Chemicals and growth factors. Canertinib, a generous gift from Pfizer pharmaceuticals (Ann Arbor, MI, USA), was dissolved in sterile dimethyl sulfoxide (DMSO; Sigma–Aldrich, St. Louis, MO, USA) at a stock concentration of 10 mM and stored at −20 °C. A working solution was prepared in growth medium and used within 30 min of preparation [10,11]. Gefitinib was a gift from AstraZeneca (Macclesfield, UK). Crystalline gefitinib was dissolved in DMSO at a 10 mM stock concentration. Camptothecin (Sigma–Aldrich) was dissolved in DMSO at a 1 mM stock concentration. The resulting concentration of DMSO used was less than 0.1% in the cell cultures. Recombinant human epidermal growth factor (EGF; PeproTech EC, London, UK) and recombinant human neuregulin1-β1 (NRG1-β1;

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R&D systems, UK) were dissolved in phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA: Saveen Biotech AB, Limhamn, Sweden) to a stock concentration of 20 μg/ml and 10 μg/ml, respectively.

Cell lines and culture conditions. Human promyelocytic HL-60 cells, human breast adenocarcinoma MCF-7 cells, human epidermoid carcinoma A431 cells and human lymphoblastic T cell leukemia Jurkat cells were all obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). A serum-dependent subclone (U-937-1) of the wildtype human histiocytic lymphoma U-937 cell line was used in all experiments and kindly provided by Dr. K. Nilsson, Department of Genetics and Pathology, Uppsala University, Sweden [12]. The cells were maintained as exponentially growing cultures in 75-cm^2 cell culture flasks (Costar, Cambridge, MA, USA). HL-60, U-937, Jurkat and MCF-7 cells were cultured in RPMI 1640 growth medium (GIBCO® Invitrogen, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO), 2 mM L-glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml). A431 cells were cultured in Eaglés Minimum Essential Medium (EMEM; GIBCO) supplemented as above. The cells were incubated in humidified air containing 5% CO_2 at 37 °C and subcultivated twice a week with a split ratio of 1:3. All cell cultures were routinely analyzed for mycoplasma contamination and found to be negative.

Experimental settings. For cell proliferation assay HL-60 and U-937 cells were seeded in 35 mm dishes at a concentration of 0.2×10^6 cells/ml and incubated with 0–10 μM canertinib for 72 h. For cell cycle analysis, experimentally growing HL-60 and U-937 cells ($\sim 0.4 \times 10^6$ cells/ml) were treated with or without 2 μM canertinib for 24 h. Time–response experiments were performed by incubating HL-60 and U-937 cells ($\sim 0.4 \times 10^6$ cells/ml) with 10 μM canertinib for up to 16 h before the cells were processed for Annexin V-PE analysis or protein preparation to determine PARP expression by Western blot analysis. For the dose–response experiments the cells were treated with increased concentration (0–10 μM) of canertinib for up to 16 h and subjected to Western blot analysis of PARP expression. ErbB1–4 receptor expression was analyzed in exponentially growing HL-60 and U-937 cells by Western blot. The effect of EGF and NRG1-β1 on ErbB1–4 receptor phosphorylation was determined by starving the cells in growth medium supplemented with 0.1% BSA without FCS for 24 h and then incubated in presence or absence of 50 ng/ml of the respective growth factor for 15 min.

Cell proliferation assay. HL-60 and U-937 cells were diluted in Coulter-Isoton® diluent buffer and counted using a Coulter Counter Z2 (Beckman Coulter, Krefeld, Germany). The results are presented as mean cell number of three separate experiments, each performed in duplicate.

Cell cycle analysis. The cell cycle distribution was determined using a method developed by Vindeløv, as previously described [13,14]. All chemicals were obtained from Sigma–Aldrich. The DNA content was analyzed by flow cytometry using a FACSCalibur™ (BD Biosciences, San Jose, CA, USA). The fraction of G_1-, S-, and G_2/M-phase cells was determined using ModFit Lt 3.0 (Verity Software House, Inc, Topsham, ME, USA).

Analysis of cell viability. The number of apoptotic cells was analyzed using the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences), according to the manufacturer's protocol. The detection of Annexin V-PE and 7-AAD was performed by flow cytometry analysis (FACSLSR, BD Biosciences). For each sample, data from 15,000 non-gated cells were collected and analyzed using the CellQuest Pro software (BD Biosciences). Three sets of experiments were performed for each culture condition.

Antibodies. The monoclonal ErbB1 (Ab14), ErbB2 (Ab17), and ErbB3 (Ab7) antibodies were purchased from NeoMarkers (Fremont, CA, USA). The polyclonal antibody specific to ErbB1 phosphotyrosine 1173 and the polyclonal antibody specific to ErbB2 phosphotyrosine 1248 were from Upstate biotechnology (Chicago, IL, USA). The polyclonal antibody specific to ErbB3 phosphotyrosine 1289 was from Cell Signalling Technology (Beverly, MA, USA). The monoclonal β-actin antibody was purchased from Sigma–Aldrich. The polyclonal ErbB4 (C-18) antibody and the polyclonal ErbB4 phosphotyrosine 1056 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The monoclonal poly-(ADP-ribose) polymerase (PARP) antibody was from BIOMOL International, LP (Plymouth Meeting, PA, USA). The horseradish peroxidase (HRP)-conjugated secondary polyclonal goat anti-rabbit antibody (P0448) and the polyclonal goat anti-mouse antibody (P0447) were from DakoCytomation (Glostrup, Denmark).

Western blot analysis. Protein extraction was performed as previously described [13,15]. Proteins were separated on 6% SDS–polyacrylamide gels, and then transferred to polyvinylidene fluoride (PVDF) membranes (Pall Corporation, Pensacola, FL, USA). Immunodetection was performed as previously described [13]. For PARP analysis the membranes were blocked for 1 h in TBS containing 0.1% Tween 20 (TBST) and 5% non-fat dry milk and then blocked for an additional 1 h in TBST and 5% BSA. Incubation with PARP (1:2500) was performed overnight at 4 °C. The membranes were incubated with HRP-conjugated secondary antibody (1:10,000) for 1 h at room temperature in TBST with 5% non-fat milk and the immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Bioscience, Little Chalfont Buckinghamshire, UK).

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated from exponentially growing MCF-7, HL-60 and U-937 cells using QIAamp®RNA Blood Mini Kit (Qiagen, Hilden, Germany). The RNA samples were digested with RNase-free DNase I (Qiagen) and cDNA was synthesized from 2 μg of total RNA from each sample using "First strand cDNA synthesis" kit for RT-PCR (Roche Diagnostics, Mannheim, Germany). Each PCR reaction contained 1 μl of the first strand cDNA, 1 μM of each primer, 0.2 mM of dNTP, 2 mM $MgCl_2$, 1× polymerase buffer and 2.5 U of Taq polymerase (Promega, Madison, WI, USA). The primers used were adopted from Mahtouk et al. [16]. The annealing temperature and the number of cycles are shown in Supplementary Material Table 1. PCR products were electrophoresed on 1.3% agarose gels and stained with ethidium bromide; details concerning the chosen products are also shown in Supplementary Table 1.

Statistics. The significance of differences between groups was analysed by a two-tailed Student's *t*-test. A *P* value of less than 0.05 was considered significant.

Results and discussion

In the present study, we demonstrate that the pan-ErbB receptor tyrosine kinase inhibitor, canertinib, induces G_1 cell cycle arrest followed by apoptosis in the human leukemic cell lines, HL-60 and U-937. Treatment with increasing concentrations of canertinib for 72 h resulted in a dose-dependent anti-proliferative effect in both cell lines (Fig. 1A and B). The growth inhibitory effect was significant ($p < 0.001$) already at 1 μM canertinib in exponentially growing HL-60 and U-937 cells as compared to untreated cells. The concentration of canertinib required to inhibit serum-stimulated growth by 50% (IC_{50}) was approximately 2.5 μM and 1.0 μM in HL-60 and U-937 cells, respectively. The HL-60 cells were totally growth-inhibited after treatment with 5 μM canertinib and this effect was even more pronounced in U-937 cells, where 2 μM canertinib completely inhibited cell proliferation. Also, treatment with 2 μM canertinib induced accumulation of both cell lines in the G_1 phase of the cell cycle with a concomitant decrease of cells

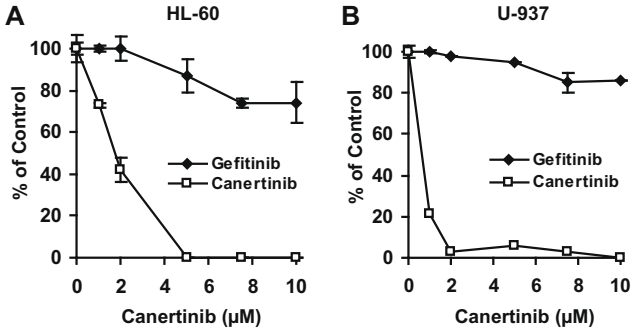

Fig. 1. Canertinib inhibits growth of HL-60 and U-937 cells. Proliferation of exponentially growing HL-60 cells (A) or U-937 cells (B) treated with increasing concentrations of canertinib or gefitinib for 72 h was determined by cell counting as described in Materials and methods. Results are given as mean value ± SD from three separate experiments performed in duplicates and plotted as a percentage of untreated cells.

Table 1
Canertinib induced cell cycle arrest. Cell cycle distribution after 24 h of treatment with 2 μM canertinib, analyzed by flow cytometry.

Cells		Cell cycle distribution (%)		
		G1	S	G2/M
HL-60	Control	57.2 ± 0.5[a]	33.9 ± 0.7	8.9 ± 0.6
	Canertinib	70.6 ± 0.9***	25.8 ± 0.7***	3.6 ± 0.5***
U-937	Control	47.4 ± 2.6	46.2 ± 1.1	6.3 ± 3.0
	Canertinib	70.1 ± 0.7***	26.5 ± 0.9***	2.8 ± 0.3***

[a] Mean ± SD.
*** $P < 0.001$.

in the S- and G_2/M cell cycle phase (Table 1). Furthermore, canertinib was also determined to be cytotoxic at a dose of 5 μM or more (Fig. 2). This effect was ascribed to induction of apoptosis since both HL-60 and U-937 cells treated with canertinib showed a significant increase of apoptotic cells as determined by Annexin V analysis. HL-60 cells incubated with 10 μM canertinib for 16 h displayed 73% apoptosis as compared to 8% in untreated cells (Fig. 2A). Analogously, treatment of U-937 cells with 10 μM canertinib resulted in a distinct time-dependent increase of apoptotic cells reaching a maximum of 100% after 16 h of incubation. Untreated exponentially growing U-937 cells displayed a basal apoptosis of 17% within the same time–period (Fig. 2B). The ability of canertinib to induce apoptosis was confirmed by Western blot analysis of PARP cleavage (Fig. 2C–F). In the time–response experiment, HL-60 cells demonstrated an increased expression of the 85 kDa PARP apoptotic fragment within 6 h of treatment with 10 μM canertinib. Complete cleavage of the 116 kDa PARP protein was achieved in both cell lines within 16 h of treatment (Fig. 2C and D). The apoptotic PARP fragment was observed after treatment with 5 μM canertinib in both cells lines, however, the pro-apoptotic effect appeared to be more potent in HL-60 than in U-937 cells. The PARP protein was completely cleaved by treatment with 10 μM canertinib in both cell lines (Fig. 2E and F).

It has recently been demonstrated that the two EGFR/ErbB1 tyrosine kinase inhibitors gefitinib and erlotinib convey cytostatic effects on HL-60 and U-937 cells as well as on myeloblasts derived from patients with AML and MDS by induction of cell differentiation [7,9]. In our study, gefitinib only marginally inhibited serum-stimulated growth of HL-60 and U-937 cells (Fig. 1A and B). This anti-proliferative effect was not assigned to apoptosis since no increased degradation of PARP could be detected by treatment

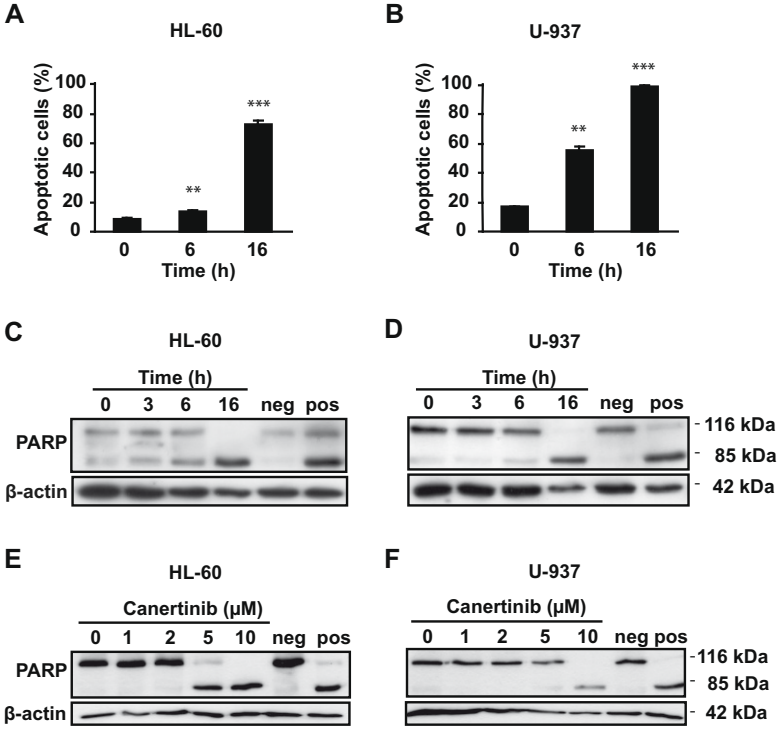

Fig. 2. Canertinib induces apoptosis in HL-60 and U-937 cells. Time course of the percentage of apoptotic cells in exponentially growing HL-60 (A) and U-937 (B) cultures treated with 10 μM canertinib for the indicated time–periods. The percentage of apoptosis was determined by staining the cells with Annexin V-PE/7-AAD followed by flow cytometry analysis. The results are given as mean value ± SD of three separate experiments. ** $P < 0.01$, *** $P < 0.001$. Time–response of PARP degradation in exponentially growing HL-60 (C) or U-937 (D) cells treated in the absence or presence of 10 μM canertinib for 3 h, 6 h or 16 h. Dose–response of PARP degradation in HL-60 (E) or U-937 (F) cells was induced with increasing concentrations of canertinib for 16 h. Both cell lines were treated with 10 μM gefitinib for 16 h served as a negative control (neg) and Jurkat cells treated with 5 μM camptothecin for 6 h were used as a positive control (pos). Western blot analysis of PARP cleavage was performed using 30 μg protein/lane of whole cell lysates. Membranes were stripped and rehybridized with an antibody specific for β-actin as an internal loading control.

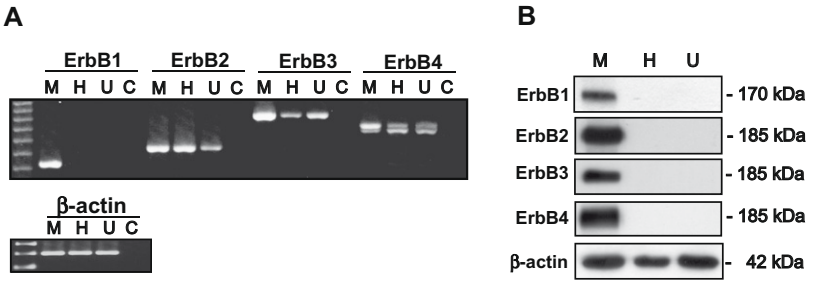

Fig. 3. Expression of ErbB1/2/3/4 receptor mRNA and protein in HL-60 and U-937 cells. (A) ErbB1/2/3/4 mRNA expression in HL-60 (H) and U-937 (U) cells. MCF-7 cells (M) and water (C) were used as positive and negative control, respectively. Total RNA, extracted from HL-60 or U-937 cells, was reverse transcribed and subsequently subjected to PCR amplification of the respective ErbB receptor cDNA. β-Actin was amplified as a control to demonstrate intact cDNA. The PCR products were separated by gel electrophoresis and visualized by ethidium bromide staining. (B) ErbB1/2/3/4 protein expressions in exponentially growing HL-60 and U-937 cells were analyzed by Western blot experiments using antibodies to ErbB1/2/3/4 receptors. MCF-7 cells were used as a positive control and β-actin detection was used as a loading control.

of both cell lines with 10 μM gefitinib (Fig. 2C–F). In contrast to a previous report, gefitinib treatment did not induce any signs of differentiation in our experimental systems (data not shown) [7]. Gefitinib has, however, been shown by others to provoke apoptosis in the U-937 cell line at a concentration of 15 μM or above, whereas erlotinib seems to lack the ability to induce apoptosis in HL-60 as well as in U-937 cells [8,9].

The expression pattern of ErbB1/2/3/4 receptor mRNA in HL-60 and U-937 cells was analyzed by RT-PCR, as depicted in Fig. 3A. The human breast cancer cell line MCF-7, known to express mRNA and protein for all four ErbB receptors, was used as a positive control [17]. In agreement with previously published data [7], no ErbB1 transcript could be identified in the two hematopoietic cell lines. In contrast, ErbB2, ErbB3, and ErbB4 mRNA was readily detectable in HL-60 and U-937 cells. Although ErbB2 transcripts have previously been reported in HL-60 cells the detection of ErbB3 and ErbB4 mRNA in HL-60 and U-937 cells is a novel finding [7]. Interestingly, two ErbB4 transcripts of 1013 bp and 965 bp were demonstrated in both cell lines (Fig. 3A), and were found to represent the cytoplasmic ErbB4 isoforms CYT-1 and CYT-2, respectively, as determined by sequencing of the two PCR products (data not shown) [18–20]. In order to determine if any of the ErbB-receptor proteins were expressed, Western blot was performed and showed that none of the ErbB receptors were expressed in either HL-60 or U-937 cells (Fig. 3B). Although the expression of the ErbB proteins could not be detected, we wanted to confirm the absence of any functional ErbB receptors in HL-60 and U-937 cells. Therefore, both cell lines were serum-starved, treated with 50 ng/ml EGF or NRG1-β1 for 15 min and subsequently analyzed for ErbB1–4-phosphorylation (Fig. 4). EGF clearly activated ErbB1 in A431 cells, but was unable to stimulate ErbB1 phosphorylation in HL-60 and U-937 cells (Fig. 4A). Moreover, neither cell line exhibited any phosphorylated ErbB2/3/4 receptors after treatment with NRG1-β1. In this case, MCF-7 cells were used as positive control and showed a clear increase of ErbB2-, ErbB3-, and ErbB4-phosphorylation after NRG1-β1 treatment (Fig. 4B).

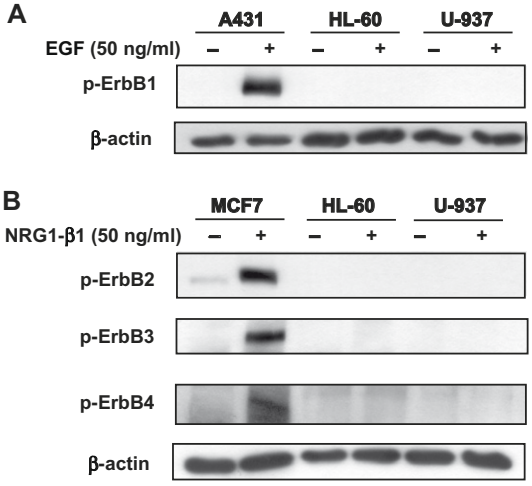

Fig. 4. Effect of EGF and NRG1-β1 on ErbB1/2/3/4 receptor phosphorylation in HL-60 cells and U-937 cells. (A) Serum starved HL-60 and U-937 cells were incubated with (+) or without (–) 50 ng/ml EGF for 15 min. Western blot analysis was performed using a pErbB1 antibody as described in Materials and methods. A431 carcinoma cells, known to overexpress ErbB1, were used as a positive control. (B) Serum starved HL-60 and U-937 cells were incubated with (+) or without (–) NRG1-β1 (50 ng/ml) for 15 min. Western blot analysis was performed using specific pErbB2/3/4 antibodies. MCF-7 breast cancer cells, known to express ErbB2/3/4, were used as a positive control.

The fact that HL-60 and U-937 cells express ErbB-2, -3, and -4 mRNA but are devoid of any of the corresponding receptor proteins suggests that these cells may indeed lack the receptor target for canertinib, that any ErbB receptor protein present is rapidly degraded or exist in a truncated form [21,22]. Others have previously demonstrated that the anti-neoplastic effect of gefitinib and erlotinib in HL-60 and U-937 cells is an off-target effect since principle targets for these drugs, the ErbB1- and ErbB2-receptor, are absent [7–9].

Conclusions

The present study demonstrates that the designated pan-ErbB inhibitor canertinib induces a G_1 cell cycle arrest and apoptosis in an ErbB receptor-independent manner in HL-60 and U-937 cells. Our results extend previous observations concerning the ability of specific ErbB tyrosine kinase inhibitors such as gefitinib and erlotinib to induce differentiation and affect viability in malignant blast cells of hematopoietic origin [6–9]. Although the specific mechanisms involved in the anti-neoplastic action of canertinib in leukemic cells remains to be elucidated, this drug might be of clinical interest as a potential therapeutic agent in the treatment of acute myeloid leukemia.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.055.

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