Erlotinib exhibits anti-neoplastic off-target effects in AML and MDS: a preclinical study

Simone Boehrer¹²³⁴*, Lionel Adès¹²³⁴*, Thorsten Braun¹²⁴, Lorenzo Galluzzi¹²³, Jennifer Grosjean¹², Claire Fabre¹², Génèviève Le Roux⁴, Claude Gardin⁴, Antoine Martin⁴, Stéphane de Botton⁵, Pierre Fenaux¹²³⁴, and Guido Kroemer¹²³

¹INSERM, U848, 94805 Villejuif, France; ²Institut Gustave Roussy, 94805 Villejuif, France; ³Université Paris-Sud/Paris XI; ⁴Service d'Hématologie Clinique, Hôpital Avicenne, AP-HP, Université Paris XIII, Bobigny, France;

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Correspondence to:
Dr. Guido Kroemer
INSERM, U848
Institut Gustave Roussy, PR1
39, rue Camille Desmoulins
F-94805 Villejuif
France
Tel. 33-1-42 11 60 46
Fax 33-1-42 11 60 47
e-mail: kroemer@igr.fr

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Off-target effect of erlotinib in MDS and AML

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Abstract

Erlotinib, an inhibitor of the epidermal growth factor receptor (EGFR), induces differentiation, cell cycle arrest and apoptosis of EGFR-negative myeloblasts of patients with myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML), as well as in EGFR-negative cell lines representing these diseases (P39, KG-1 and HL-60). This off-target effect can be explained by inhibitory effects on JAK2. Apoptosis induction coupled to mitochondrial membrane permeabilization occurred independently from phenotypic differentiation. In apoptosis-sensitive AML cells, erlotinib caused a rapid (within less than 1 hour) nucleocytoplasmic translocation of nucleophosmin-1 (NPM-1) and p14ARF. Apoptosis-insensitive myeloblasts failed to manifest this translocation, yet became sensitive to apoptosis induction by erlotinib when NPM-1 was depleted by RNA interference. Moreover, erlotinib reduced the growth of xenografted human AML cells in vivo. Erlotinib also killed CD34+ bone marrow blasts from MDS and AML patients while sparing normal CD34+ progenitors. This ex vivo therapeutic effect was once more associated with the nucleocytoplasmic translocation of NPM-1 and p14ARF. One patient afflicted with both MDS and non-small cell lung cancer manifested hematological improvement in response to erlotinib. In summary, we here provide novel evidence in vitro, ex vivo and in vivo for the potential therapeutic efficacy of erlotinib in the treatment of high-risk MDS and AML.
Introduction

Both high-risk MDS and AML require new therapeutic approaches. On theoretical grounds, such approaches should target transformed cells and cause them to initiate at least one of three distinct, yet interwoven biological processes: (i) cell death by apoptosis, (ii) preferentially irreversible cell cycle blockade or (iii) terminal differentiation.

A recent overall trend in oncology is the conception of so-called targeted therapies in which oncogenic events or surface markers are taken advantage of to disrupt the essential transforming event and to selectively destroy tumor cells with a maximum of specificity and a minimum of side effect on non-transformed cells \(^1\). Examples of such targeted therapies applied to hematological malignancies include selective tyrosine kinase inhibitors (with imatinib as a ground-breaking paradigm for the treatment of chronic myeloid leukemia). For the treatment of solid tumors, quintessential examples are the small inhibitory compounds targeting the epidermal growth factor receptor (EGFR) in non-small cell lung cancer (NSCLC) \(^2\). Such small molecules directed against the EGFR include gefitinib and erlotinib \(^3\)-\(^5\).

Surprisingly, a recent study revealed the capacity of gefitinib to induce differentiation in three AML cell lines (U937, HL60, and Kasumi-1) which all lack expression of the EGFR \(^6\), thus unraveling an interesting off-target effect of a compound that was believed to specifically act on EGFR-expressing cells. Based on this report, we studied the effects of the EGFR inhibitor erlotinib on EGFR-negative AML and MDS cells. Here, we report that erlotinib has an anti-neoplastic activity on MDS and AML cells that includes a pro-apoptotic effect. The therapeutic efficacy of erlotinib is demonstrated \(in \text{ vitro, ex vivo}\) on MDS/AML-derived
malignant myeloblasts, as well as in vivo. Based on these data, we propose the therapeutic off-target use of erlotinib for the treatment of high-risk MDS and AML.

**Patients, Materials and Methods**

**Patients**

Samples from 31 patients or healthy volunteers were assessed in our study (Table 1). Informed consent of all patients and healthy subjects was provided according to the Declaration of Helsinki. The diagnosis of AML and MDS was based on cytology of peripheral blood and bone marrow according to the WHO classification, as well as on conventional cytogenetic analysis.

**Cell lines and selection of CD34\(^+\) cells**

Mononuclear cells (MNC) from the bone marrow (BM) were isolated by Ficoll-Paque PLUS density gradient (Amersham Biosciences, Sunnyvale, CA, USA). To isolate CD34\(^+\) cells from MNC a positive selection using the MiniMacs system (Miltenyi Biotec, Bergisch Gladbach, Germany) was carried out. CD34\(^+\) cells were maintained in Iscove modified Dulbecco medium (IMDM, Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS). The high-risk MDS cell line P39/Tsugane (kindly provided by Dr. Yoshida Takeda, Japan), and the KG-1 and HL60 cell lines (kindly provided by Dr. Martin Ruthardt, Germany, and Dr. Bruno Cassinat, France, respectively) were cultured in FCS-supplemented RPMI 1640 (Gibco). Unless otherwise indicated, cells were seeded at a concentration of 1 x 10\(^5\) cells/ml.

**Assessment of cell death, cell cycle distribution, proliferation, and differentiation**

10\(^5\) cells derived from patients or cell lines were resuspended in 1 ml of culture medium and incubated with the indicated dosages of erlotinib (Roche, Basel, Switzerland). Control cells were incubated with the corresponding amount of DMSO. In the applied concentrations, DMSO neither affected proliferation, apoptosis, nor cell cycle distribution as compared to cells left untreated (data not shown). But since DMSO induced minor signs of differentiation,
all assays assessing the differentiation-inducing capacity of erlotinib were carried out in the presence ("DMSO") as well as in absence of DMSO ("control"). To inhibit activation of caspases cells were incubated with 100µM z-VAD-fmk. Apoptotic cells were detected by cyttofluorometric analysis using a FACScan (Becton Dickinson, Mountain View, CA) as described previously 7,8. Cells were stained with propidium iodide (PI; 5µg/mL; Sigma, Steinheim, Germany) and concomitantly with either 40nM fluorochrome DiOC6(3) (3,3 dihexyloxacarbocyanine iodide; Molecular Probes, Eugene, OR, USA) for 15 minutes at 37°C, or Annexin-V–FITC (Becton Dickinson) following the manufacturer’s instructions to determine the mitochondrial transmembrane potential or phosphatidyl serine exposure, respectively 9-11.

For conventional cell cycle analysis, cells were harvested, washed in PBS and stained with propidium iodide (PI, 25 µg/ml, Sigma) followed by an incubation period of 30 min at 37°C. Subsequently, cell cycle distribution was determined by cyttofluorometric analysis using a FACS Vantage (Becton Dickinson).

Changes in proliferation were assessed by adding a predefined amount of immunoflorescent beads (AlignFlow, Invitrogen, Karlsruhe, Germany), emitting a signal at 488nm to 300µl of the cells cultured in suspension following the manufacturer’s instructions. In the subsequent flourocytometric analysis 3000 events elicited by the beads were counted with the concomitantly acquired number of cultured cells, allowing quantification of cell numbers in relation to the number of beads.

To flourocytometrically assess differentiation, cells were harvested, washed and stained with a PE-conjugated anti-CD11b antibody (clone ICRF44, Becton Dickinson) indicating myelocytic differentiation as described previously12. Isotypic mouse IgG1 (Becton Dickinson) was used to set threshold parameters.

Morphological assessment of differentiation and apoptosis was carried out following Wright-Giemsa staining of cytospins. To quantify differentiation and apoptosis at least 100 cells of each condition were assessed for signs of differentiation (decrease of cytoplasmic basophilia, appearance of granulation, lobulation of the nucleus) or apoptosis (chromatin condensation and nuclear fragmentation).

**Analysis of the phosphoproteome**

Differential expression of phosphoproteins was assessed in KG-1 cells cultured 8h in the presence of 10 µM erlotinib or 0.02% DMSO. Analysis was carried out using the Kinex
antibody microarray (Kinex Bioinformatics Corporation, Vancouver, BC, Canada). Samples were prepared following the manufacturer’s instructions.

Fluorocytometric assessment of STAT-5 activation

Cells were harvested, washed and subsequently fixed and permeabilized using the BD Cytofix/Cytoperm kit (Becton Dickinson) following the manufacturer’s instructions. Cells were first stained with the primary antibody detecting activated STAT-5 (P-Tyr694, Cell Signaling), followed by staining with a secondary FITC-conjugated antibody. Isotypic controls were used to set threshold parameters.

Knock-down of proteins by small interfering RNA (siRNA)

Cells were transfected with the Nucleofactor system (Amaxa, Cologne, Germany) as described previously 12 using two different siRNAs specific for NPM-1 (sense strain: 5-CAC CAC CAG UGU CUU AAG TT-3, Sigma) or JAK-2 (sense strain: 5-GAA GAG CAC CUA AGA GAC UTT-3; Eurogentec, Seraing, Belgium). Two different scramble siRNAs were used as controls. For knock-down of Src (pp60Src), PDGFRα and PDGFRβ SMARTpool siRNA together with the control siRNA and the respective antibodies were purchased from Dharmacon (Lafayette, CO, USA).

Immunoblot analysis

Lysates from 5x10^6 cells were separated on sodium dodecyl sulfate (SDS)–polyacrylamide gels and electroblotted onto PVDF membranes following standard procedures. The membrane was blocked with 5% nonfat, dry milk and incubated with the respective primary antibody: Actin (mouse monoclonal Ab; Chemicon, Temecula, CA), EGFR (rabbit polyclonal Ab; Santa Cruz, Santa Cruz, CA, USA); Cyclin E (mouse monoclonal Ab; Santa Cruz), Cyclin D1 (mouse monoclonal Ab; Santa Cruz), Retinoblastoma (mouse monoclonal Ab; Cell Signaling, Danvers, MA, USA), Retinoblastoma (P-Ser780, P-Ser795, P-Ser807/811, rabbit monoclonal Abs; Cell Signaling), STAT-5 (rabbit monoclonal Ab; Cell Signaling), activated STAT-5 (P-Tyr694; rabbit monoclonal Ab; Cell Signaling), NPM-1 (rabbit monoclonal Ab; Cell Signaling), JAK-2 (rabbit polyclonal Ab; Santa Cruz), activated JAK-2 (P-Tyr1007/1008;
rabbit monoclonal Ab; Cell Signaling). Antibodies against Src (pp60Src), PDGFRα and PDGFRβ were purchased (together with the respective SMARTpool siRNA) from Dharmacon. Blots were stained with either goat anti–rabbit peroxidase-labeled or goat anti–mouse peroxidase-labeled secondary antibody (Amersham, Arlington Heights, IL, USA) and were revealed using an enhanced chemiluminescence detection system (Amersham).

**Immunofluorescence**

Cells of patients or cell lines were allowed to adhere on polylysine-L coverslips (Sigma) and were fixed in 4% paraformaldehyde at room temperature. Cells were then permeabilized with SDS 0.1% for 10 minutes, washed in PBS, and stained with the indicated primary antibodies: cytochrome c (mouse monoclonal Ab; BD Pharmingen, Heidelberg, Germany), endonuclease G (rabbit polyclonal Ab; Pro-Science, Woburn, MA), Hsp60 (mouse polyclonal Ab; Sigma), activated caspase-3 (rabbit polyclonal Ab, Cell Signaling), NPM-1 (rabbit or mouse monoclonal Ab; Santa Cruz), p14ARF (mouse monoclonal Ab; Sigma), NF-κB p65 (rabbit polyclonal Ab; Santa Cruz) and revealed with the adequate secondary antibody coupled with Alexa 568 (red) or Alexa 488 (green) fluorochromes (Molecular Probes/Invitrogen). DNA of cells was counterstained with DAPI (Molecular Probes/Invitrogen). At least one hundred cells for each slide were examined independently with a LSM 510 confocal microscope (Zeiss, Thornwood, NY) at 63x magnification. Background correction of fluorescence was performed with the LSM 5 image browser (Zeiss).

**Evaluation of the therapeutic efficacy of erlotinib in a murine leukemia model**

Antileukemic activity of erlotinib was evaluated in female, severe combined immunodeficient (SCID) mice (Charles River, Wilmington, MA). Animals were housed in suitable cages under specified pathogen-free conditions in rooms maintained at 23°C and 50% humidity, with a 12-hour light/12-hour dark cycle. The mice were quarantined during the acclimatization period of at least a week. Food (Standard 1320 and 1430; Altromin, Lange, Germany) and acidified water (pH 2.5-3.0) were available *ad libitum*. Mice (in groups of 4) were injected intraperitoneally with 1×10^6 KG-1 cells on day 0 as described previously. Mice were then randomly assigned to the treatment or control group. Treatment was initiated on day 7 following intraperitoneal injection of tumor cells. The treatment group received a daily dose
of 100mg/kg erlotinib (diluted in sterile water and 10% DMSO) orally on five consecutive days, followed by a treatment pause of two days. Mice in the control group received water/DMSO accordingly. Mice were controlled daily for signs of toxicity and tumor/aszites development. Xenografts were measured daily in two dimensions with a caliper. At the end of the studies, all animals were euthanized by cervical dislocation under isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethylether; CuraMED Pharma, Karlsruhe, Germany) anesthesia.

**Explanted tumor and specimen investigations**

Explanted tumors were fixed in 3.8% buffered formaldehyde, embedded in Paraplast (Sherwood Medical, Norfolk, NE), and 5 µm sections prepared. For histopathologic examination, sections were stained with hematoxylin/eosin (HE) and examined by light microscopy at x200 magnification by a certified histopathologist. To assess the presence of malignant cells in the ascites, peritoneal exudate was collected by peritoneal lavage and cells were visualized on cytopsins by Wright-Giemsa staining.

**Statistics**

Statistics were analyzed using Excel Software (Microsoft, Redmond, WA), SPSS software (SAS Institute, Cary, NC), and Scion Image 4.0 (Scion Corporation, Frederick, MD). Tumor-free survival in mice was analyzed by using the Kaplan-Meier method. Statistical significance was determined employing the log-rank test. Apoptosis induced by erlotinib in ex vivo cells of the MDS and AML subgroups is depicted using a boxplot graph, statistical significance was assessed using the Student’s t-test.

**Results**

**Off-target effects of erlotinib on MDS/AML cell lines**

In contrast to the non-small cell lung cancer cell line A549, the AML cell lines KG-1 and HL60 as well as the MDS-derived cell line P39 lack EGFR expression (Figure 1A), meaning that any effect of the *bona fide* EGFR antagonist erlotinib on these cells must be considered as "off-target". Erlotinib efficiently induced the apoptosis-associated phosphatidylserine exposure (detected with Annexin V-FITC conjugates) followed by the loss of viability (detected with the vital dye propidium iodide, PI) in KG-1 cells (Figure 1, B and C), yet failed
to do so in P39 (Figure 1D) and HL60 cells (Figure 1E). Erlotinib also reduced the absolute number of viable KG-1 (and to a lesser extent the viability of P39 or HL60 cells) cells (Figure 1F and G). Phosphoproteome analysis of KG-1 cells revealed that erlotinib influenced the phosphorylation status of numerous proteins (data not shown), including the retinoblastoma (Rb) protein, which is critical for G1/S transition of the cell cycle, as well as the platelet-derived growth factor receptors PDGFRα and PDGFRβ, which are involved in hematopoietic differentiation. We therefore assessed the effect of erlotinib on cell cycle progression and differentiation of malignant myeloblasts.

Erlotinib had no effect on the total levels or Rb protein, yet reduced the phosphorylation of Rb on serines 807/811, 780 and 795, coupled to a reduction in the G1/S cyclins E and D1 (Figure 2A). Accordingly, erlotinib led to a blockade in G1 with a consequent diminution of S and G2/M phases (Figure 2, B and C). This cell cycle blockade manifested before erlotinib-treated KG-1 cells became apoptotic and accumulated in the subG1 phase. Erlotinib induced cell cycle arrest in KG-1 cells, and - although to a lesser extent - in P39 and HL60 cells (Figure 2, D and E).

When exposed to erlotinib or all-trans retinoic acid (ATRA), KG-1 cells manifested comparable signs of differentiation, namely a reduction in cytoplasmic basophilia and an increased nuclear lobulation that occurred in non-apoptotic cells (Figure 3, A and B). Similar signs of differentiation could be observed in P39 and HL60 cells, which also upregulated the myelocytic differentiation antigen CD11b (Figure 3, C-H). Since these cells did not (or only marginally) undergo apoptosis in response to erlotinib, the pro-apoptotic and differentiation-inducing effects of erlotinib can be dissociated (Figure 3, E and H). Altogether, these results indicate that erlotinib can exert anti-neoplastic off-target effects on a range of malignant myeloid cell lines.
Mechanisms of erlotinib-mediated cell death

The phenotype of cell death induced by erlotinib in KG-1 was clearly apoptotic, with early phosphatidylserine exposure (Figure 1, B and C), loss of nuclear DNA (Figure 2), and nuclear pyknosis and karyorrhexis (Figure 3A). Moreover, KG-1 cells incubated with erlotinib manifested biochemical hallmarks of apoptosis, namely the release of cytochrome \(c\) from mitochondria, coupled to the activation of caspase-3, as determined by immunofluorescence staining. In addition, a significant fraction of erlotinib-treated KG-1 cells exhibited the mitochondrial release of the caspase-independent death effector endonuclease G (Figure 4, A and B). No such effect could be detected for P39 (Figure 4, C and D) cells, which did not die in response to erlotinib (Figure 1-3). Inhibition of caspases by Z-VAD-fmk suppressed the activation of caspase-3 in KG-1 cells treated with erlotinib, yet had no effect on the mitochondrial release of cytochrome \(c\) or endonuclease G (Figure 4B), indicating that mitochondrial outer membrane permeabilization occurred independently from (and presumably upstream of) caspase activation in erlotinib-induced cell death. Z-VAD-fmk fully blocked the (caspase-dependent) pyknosis/karyorrhexis of KG-1 cells treated by erlotinib, yet failed to inhibit the (caspase-independent) exposure of phosphatidylserine and cell death induced by erlotinib (Figure 4E and data not shown). Hence, caspase activation accompanies erlotinib-induced cell death, yet is not required for cellular demise.

Erlotinib reduced the (auto)phosphorylation of the oncogenic JAK2 kinase on tyrosine 1007/1008, when added to KG-1 cells (Figure 4F), in accord with the previously reported capacity of erlotinib to directly inhibit JAK2.\(^{17}\) The transcription factor STAT-5, a direct target for phosphorylation by JAK2 on tyrosine 694\(^{18}\), was hypophosphorylated after erlotinib treatment, as determined by immunoblot (Figure 4F) and confirmed by cytofluorometric analysis (Figure 4G; note the left shift of the staining after erlotinib as compared to the vehicle control). Thus both assays demonstrate that erlotinib alone is
sufficient to reduce the constitutive STAT-5 activation in KG-1 cells. In order to assess the functional relevance of the JAK2-STAT-5 signaling in erlotinib-induced apoptosis, expression of JAK2 was down-regulated by RNA interference. As depicted in Figure 4H and I, abrogation of JAK2 expression alone was sufficient to diminish activation of STAT-5 and to concomitantly induce apoptosis in KG-1 cells. The combination of JAK2 knock-down and erlotinib did not induce more apoptosis than erlotinib alone. This result is compatible with the hypothesis that erlotinib causes apoptosis at least in part by inhibiting JAK2. In contrast, erlotinib was still able to increase the apoptosis induced by knock-down of the PDGFR and Src, suggesting that these kinases are not (or less) relevant to the pro-apoptotic effect of erlotinib (Supplemental Figure 1).

As a further tentative to explore the anti-neoplastic action of erlotinib, we studied its effects on the myelomonocytic master regulator nucleophosmin-1 (NPM-1). In KG-1 cells, erlotinib stimulated the rapid (within 30 min) redistribution of NPM-1 from the nucleus to the cytoplasm (Figure 5, A and B). This translocation was always accompanied by that of the tumor suppressor protein p14ARF (Figure 5, A and B), which is know to interact with NPM-1.\(^{19-22}\) Apoptosis-resistant P39 cells failed to release NPM-1, p14ARF and NF-κB p65 – which is constitutively activated in P39 cells and imparts an anti-apoptotic signal\(^{12}\) - from their nuclei (Figure 5, C-F). Depletion of NPM-1 with two distinct siRNAs was not sufficient to induce apoptosis, nor did it affect the apoptogenic action of erlotinib on KG-1 cells (Figure 5, G-I). However, downregulation of NPM-1 established the sensitivity of P39 cells (which normally did not undergo apoptosis, Figures 1-3) in response to erlotinib (Figure 5, G and H). These results suggest that inactivation of NPM-1 would be essential for the lethal action of erlotinib.

*In vivo and ex vivo* effects of erlotinib on malignant cells of MDS and AML
To verify that a therapeutic effect of erlotinib in EGFR-negative myeloblasts can be obtained in vivo, we inoculated KG-1 cells into the peritoneal cavity of severe combined immunodeficient (SCID) mice, a manipulation that lead to the formation of malignant ascites (Figure 6A), peritoneal carcinosis, and the development of palpable tumors in the lower abdomen (Figure 6, B and C). In this setting, oral treatment with erlotinib (5 days per week, 100 mg/kg/day) significantly suppressed the development of clinically detectable tumors (Figure 6, C and D). Thus, erlotinib can exert anti-neoplastic effects in vivo, at an acceptable level of toxicity.

Next, we determined whether erlotinib acts on freshly purified CD34+ bone marrow blasts from AML patients, comparing them to CD34+ bone marrow progenitors from healthy volunteers. While normal CD34+ cells were not harmed by erlotinib (Figure 7A), AML-derived CD34+ bone marrow blasts exhibited a dose- and time-dependent increase in apoptosis, as determined by two distinct methods: first by assessing the frequency of cells that exposed phosphatidylserine on their surface (and hence stained positive with Annexin V-FITC conjugates), and secondly by measuring the portion of cells that lost the mitochondrial transmembrane potential (ΔΨm, and hence manifest reduced incorporation of the ΔΨm-sensitive cyanine dye DiOC6(3)), before they lost viability and incorporated PI (Figure 7B).

As to be expected from the results obtained with the erlotinib-sensitive AML cell line KG-1 (Figure 5, A and B), primary myeloblasts from patients that responded to erlotinib ex vivo, manifested a rapid nucleocytoplasmic translocalization of NPM-1 and p14ARF (Figure 7, D and F), which was not seen in normal CD34+ bone marrow cells (Figure 7, C and E). Moreover, the ex vivo treatment of primary myeloblasts responding to erlotinib recapitulated two further effects that we observed on KG-1 cells: an increase in the surface expression level of the myelocytic marker CD11b and morphological signs of differentiation (Figure 7, G and H). Ex vivo treatment of CD34+ bone marrow cells from a cohort of control subjects and
patients with MDS and AML confirmed that an increasing fraction of patients - but not the healthy volunteers - manifested a strong (>20%) apoptosis induction upon erlotinib administration (Figure 7I). We could not correlate the therapeutic response with the mutational status of NPM-1 (because only one AML patient in our cohort carried a NPM-1 mutation).

During the course of our study, we had the opportunity to evaluate the in vivo effects of erlotinib in a patient diagnosed with two malignancies: a metastatic EGFR-positive non-small cell lung cancer (NSCLC) and a high-risk MDS (RAEB-2) with a normal karyotype. Noteworthy, the patient’s CD34+ bone marrow myeloblasts responded to erlotinib ex vivo (Figure 7J). Since the NSCLC progressed after chemotherapeutic treatment (and because the patient’s considerable co-morbidity presented a contraindication for further chemotherapy), a single agent treatment with 150 mg/day erlotinib for the NSCLC (a disease where this drug is approved by European Union Health Authorities) was initiated, while high-risk MDS was treated with best supportive care. Routine blood examinations carried out before and during erlotinib monotherapy revealed that the patient manifested a transient, objective hematopoietic improvement (according to the IWG-2006 criteria) on platelet and neutrophil counts (without achieving a complete remission since the initial percentage of bone marrow blasts remained stable at around 15% during the treatment). This hematopoietic improvement was maintained throughout the duration of erlotinib treatment (34 days). However, after discontinuation of erlotinib platelet and neutrophil counts declined (Figure 7K).

Discussion

A previous article by Golub’s group reported two possible anti-neoplastic effects of the EGFR antagonist gefinitib on AML cells: differentiation and proliferative arrest. Based on the assumption that it would be preferable to induce cell death, we investigated whether the
EGFR antagonist erlotinib might induce apoptosis in myeloid malignancies. Erlotinib was unable to induce apoptosis in HL60 cells (Figure 1-3) and U937 cells (not shown), two cell lines investigated also by Golub's group 6, although erlotinib efficiently induced the differentiation of HL60, U937 and P39 cells (Figure 3 and data not shown). In contrast, one particular AML cell line, KG-1, was potently killed by erlotinib, suggesting that the lethal response to this compound is dictated by a poorly understood cellular context. Importantly, these results reveal that differentiation and cell cycle blockade induced by EGFR antagonists exerting off-target effects on AML cells are not automatically linked to an apoptotic response. This idea is also corroborated by the fact that some experimental interventions that block erlotinib-induced differentiation (such as the knock-down of the PDGFR or Src) do not suppress the apoptotic response (Supplemental Figure 1).

Erlotinib kills KG-1 cells by triggering apoptosis, based on morphological signs (chromatin condensation and nuclear fragmentation that define apoptosis) and biochemical criteria (early phosphatidylserine exposure before loss of viability, nuclear DNA degradation giving rise to a peak of subdiploid cells). Apoptosis can either be induced following the extrinsic pathway (in which ligation of death receptors causes apical caspase activation in the death inducing signaling complex) or the intrinsic pathway (in which mitochondrial outer membrane permeabilization [MOMP] causes the liberation of the caspase activator cytochrome \(c\)). It appears clear that erlotinib stimulates the intrinsic pathway, based on the observation that mitochondria released cytochrome \(c\) (and other death effectors such as endonuclease G) before caspase-3 was activated and that caspase inhibition was unable to prevent mitochondrial outer membrane permeabilization and cell death (Figure 4). While the particular apoptotic execution pathway that is triggered by erlotinib appears clear, it remains an ongoing conundrum through which molecular events erlotinib finally causes MOMP.
As a possibility, direct inhibition of JAK2 may account for the apoptogenic activity of erlotinib on KG-1 AML cells (Figure 4). The evidence that erlotinib functions by disrupting signaling of JAK2/STAT-5 is of particular importance if one takes into account that these molecules are considered to be crucial during leukemogenesis and deregulated in a broad spectrum of hematological malignancies. Another molecular event that could be associated with the lethal effect of erlotinib was the nucleocytoplasmic translocation of the NPM-1/p14ARF complex. Thus, cell lines that were killed by erlotinib manifested this translocation whereas others that were resistant against erlotinib failed to do so. Resistant cell lines became susceptible to erlotinib-mediated killing when NPM-1 was knocked down by specific siRNAs (Figure 5). Although this association is interesting, we lack mechanistic insights into the way how erlotinib inactivates NPM-1 and how the inactivation of NPM-1 makes cells permissive to the lethal action of erlotinib. These issues require further clarification in future studies.

Our study unravels a remarkable internal coherence between the in vitro, ex vivo and in vivo results that were obtained on AML cell lines and primary, patient-derived malignant myeloblasts. Thus, normal CD34+ bone marrow cells did not respond to erlotinib, while blasts from a fraction of MDS and AML patients were killed by erlotinib while manifesting the nucleocytoplasmic translocation of the NPM-1/p14ARF complex, as well as an apoptosis-independent differentiation. The anti-neoplastic effect of erlotinib could be confirmed in vivo, in a mouse model of xenografted human AML cells, as well as in one patient who presented both a high-risk MDS as well as a NSCLC (which constituted the indication for the therapeutic administration or erlotinib). Noteworthy, a recently published case report of a patient suffering concomitantly from AML and NSCLC provides further clinical evidence for the therapeutic efficacy of erlotinib in EGFR-negative myeloid malignancies. Thus this
patient, who initially received a monotherapy with erlotinib, achieved a complete remission of his AML that was maintained for several months \(^29\).

From a more general point of view, the results presented here confirm an emerging concept in the field of targeted anti-cancer therapy: targeted therapies do not only work through an action on the molecular target that they were originally designed for. As an example, imatinib was rationally designed to antagonize the deregulated tyrosine kinase activity of Bcr-Abl (and hence to treat leukemias bearing the Philadelphia chromosome creating the Bcr-Abl fusion oncogene)\(^30\). However, later imatinib turned out to be useful for the treatment of malignancies relying on the activating mutation of c-kit (in gastrointestinal stroma cell tumors) or the PDGFR (in hypereosinophilic syndrome)\(^31,32\). In experimental models, imatinib can even cause the regression of tumors \textit{in vivo} that do not respond to the drug \textit{in vitro}, presumably though the inhibition of non-mutated tyrosine kinases expressed by the host immune system\(^33,34\). From this viewpoint, the present report adds another example of a therapeutically warranted off-target effect of tyrosine kinase inhibitors. Through a hitherto unexplained molecular pathway, the EGFR antagonist erlotinib kills MDS and AML cells that lack the EGFR. Needless to say, that this discovery may constitute a first step towards a novel therapeutic application of this tyrosine kinase inhibitor.

\textbf{Author Contribution Statement:}\nS.B., L.A. designed, performed the research and analyzed the data. T.B., L.Z., J.G., C.F., G.L. A.M. performed some of the research. C.G., S.B., P.F. Provided bone marrow samples, discussed the results and provided helpful suggestions. G.K. designed the experiments and supervised their conduct, wrote the paper, and analyzed the data.

\textbf{Conflict of Interest Disclosure:}\nThe authors declare no competing financial interests.
Table 1: Characteristics of healthy volunteers, MDS and AML patients.

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<sup>a</sup>healthy volunteer; <sup>b</sup>refractory anemia ; <sup>c</sup>refractory anemia with ringsideroblasts; <sup>d</sup>refractory anemia with excess of blasts (5%-9%: RAEB-1, 10%-19%: RAEB-2); <sup>e</sup>therapy-related AML; <sup>f</sup>secondary AML; <sup>g</sup>complex karyotype was defined as 3 or more cytogenetic abnormalities; <sup>h</sup>wildtype NPM-1; <sup>i</sup>internal tandem duplication of NPM-1.
References

2. Toschi L, Cappuzzo F. Understanding the new genetics of responsiveness to epidermal growth factor receptor tyrosine kinase inhibitors. Oncologist. 2007;12:211-220.
References

Figure 1. Proapoptotic effect of erlotinib on EGFR-negative myeloid cell lines. (A) Absence of EGFR expression on KG-1, P39 and HL60 cells, as determined by immunoblot. A549 cells were included as positive control. (B) Representative Annexin V-FITC/PI stainings of erlotinib-treated KG-1 cells, 3 days after treatment, as compared to DMSO-only (0.02%) treated controls. (C-E) Quantitation of dying (Annexin V+/PI-) or dead (Annexin V+/PI+) KG-1 (C), P39 (D) or HL60 (E) cells, 3 or 6 days after culture with the indicated concentrations of erlotinib. (F, G) Influence of erlotinib on the number of viable KG-1 (F), P39 and HL60 (G) cells. Results are means ± SD of triplicates. These experiments were repeated at least three times, yielding comparable results.

Figure 2. Cell cycle effects of erlotinib on EGFR-negative myeloid cell lines. (A) Immunoblot detection of Rb phosphorylation and G1/S cyclins. KG-1 cells were treated for the indicated period with 10 µM erlotinib, followed by lysis, SDS-PAGE and immunochemical detection of the indicated antigens. (B) Cell cycle analysis of KG-1 cells, 24 h after treatment with 10 µM erlotinib as compared to cells treated with DMSO (0.02%) only. (C-E) Quantitation of erlotinib-mediated cell cycle effects on KG-1 (C), P39 (D) or HL60 (E) cells. Results are means ± SD of triplicates of one experiment representative for four.

Figure 3. Erlotinib-induced differentiation of EGFR-negative myeloid cell lines. (A) Representative Wright-Giemsa staining of KG-1 cells cultured 6 days in the absence (control) or presence of DMSO (0.02%), erlotinib (10 µM) or all-trans retinoic acid (ATRA, 1 µM), revealing signs of differentiation, that is reduced cytoplasmic basophilia and nuclear lobulation, or apoptosis, that is chromatin condensation (pyknosis) and nuclear fragmentation (karyorrhexis). (B) Quantitation of results, as obtained in A on ≥100 cells. (C-H) Erlotinib-
driven differentiation in P39 (C-E) or HL60 (F-H) cells. Differentiation was quantified (X±SD of triplicates) by immunofluorocytometric measurement of surface CD11b on day 3 (quantified in D, G) and day 6 (C, D and F, G) or by Wright-Giemsa staining of cytospins on day 6 of the incubation period (C, E and F, H). Results are representative of at least three independent experiments.

Figure 4. Erlotinib induces caspase-independent mitochondrial outer membrane permeabilization (MOMP) and disruption of the JAK2/STAT-5 pathway. (A) Representative fluorescence microphotographs of KG-1 cells treated with 10 µM erlotinib or DMSO as a control, after staining for the visualization of nuclear chromatin, cytochrome c, endonuclease G and activated caspase-3 (caspase 3a). Note the mitochondrial distribution of cytochrome c and endonuclease G as cytoplasmic (non-nuclear) dots at the same location as the mitochondrial matrix marker Hsp60 or their diffuse distribution throughout the cell. (B) Quantitative assessment of the data obtained as in A, for KG-1 cells cultured for three days in the absence or presence of erlotinib and/or the pan-caspase inhibitor Z-VAD-fmk. (C, D) Immunofluorescence assessment of MOMP and caspase-3 activation in erlotinib-treated P39 cells. The same technology as in A and B was used on P39 cells. Results are means ± SD of triplicates of one experiment representative of three. (E) The pan-caspase inhibitor Z-VAD-fmk blocks pyknosis and karyorrhexis in erlotinib-treated KG-1 cells. Cells were treated as described in Figure 4A and B. (F-I) Erlotinib disrupts signaling of the JAK2/STAT-5 pathway. (F) Erlotinib decreases phosphorylation of JAK2 and STAT-5. KG-1 cells treated for 30 min or 3 h with erlotinib were subjected for immunoblot detection of JAK2 and STAT-5 phosphorylation. (G, H) Impact of erlotinib and JAK2 on STAT-5 phosphorylation. After depletion of JAK2 with two distinct siRNAs (see insert in H) and 24h of erlotinib treatment (10 µM), cells were permeabilized and subjected to the immunofluorometric quantitation of
STAT-5 phosphorylation. Representative FACS histograms obtained for the first of two control and JAK2-specific siRNAs are shown in G (grey curves indicate isotype controls) and quantitative data (expressed as % of positive cells, X±SD, n=3) are depicted in H. Note the decrease in STAT-5 activation (shift towards the isotype) upon erlotinib treatment alone and upon down-regulation of JAK2. (I) Impact of erlotinib and JAK2 on cell death. KG-1 cells were transfected with control siRNAs or JAK2-specific siRNAs (day 0) and then cultured in the absence or presence of 10 µM erlotinib (from day 1-2 of transfection), followed by determination of the frequency (X±SD, n=3) of dying and dead cells using the Annexin V/PI staining method employed in Figure 1B. This experiment has been repeated twice, yielding similar results.

**Figure 5: Influence of nucleophosmin-1 (NPM-1) on erlotinib-mediated cytolysis.** (A) Typical immunofluorescence pictures obtained after staining of KG-1 cells with antibodies specific for NPM-1 and p14ARF. Chromatin was counterstained using DAPI. Note the nuclear localization of NPM-1 and p14ARF that is lost after erlotinib treatment (10 µM, 30 min) in favor of a cytoplasmic staining. (B) Quantitation of the data obtained as in A, 30 min or 3 h after addition of erlotinib. (C-F) Failure of erlotinib to affect the subcellular localization of NPM-1 (C-F), p14ARF (C, D) and NF-κB p65 (E, F) in erlotinib-resistant P39 cells. Representative immunofluorescence microphotographs are shown in C and E and quantitative results are reported in D and F. (G-I) Effect of NPM-1 knockdown on the apoptosis-inducing capacity of erlotinib in P39 and KG-1 cells. NPM-1 was knocked down with two distinct siRNA heteroduplexes (inserts in H and I). 24 h after transfection erlotinib (or DMSO as a solvent control) was added for 48 h, and the frequency of dying and dead cells was measured by means of the AnnexinV/PI method. Representative FACS pictograms are shown for P39
cells in G, and quantitative results (X±SD, n=3) are depicted for both P39 and KG-1 cells in H and I.

Figure 6. Therapeutic efficacy of erlotinib on human AML xenografts in SCID mice. (A, B) Cytological and histopathological features of KG-1 cells injected into SCID mice. Forty days after intraperitoneal inoculation of 10⁶ KG-1 cells, ascites was subjected to Wright-Giemsa staining (A) and abdominal tumors were analyzed by HE staining (B) to demonstrate the presence of AML cells in the tissues. (C) Representative mice that were vehicle-only or erlotinib-treated with the presence or absence of abdominal tumor masses are shown. (D) Kaplan-Meier plot showing tumor-free survival. Mice were inoculated with KG-1 cells on day 0 and oral erlotinib administration was started on day 7 (5 days per week, 100 mg/kg/day). This experiment has been repeated once, yielding similar results.

Figure 7. Therapeutic effects of erlotinib ex vivo and in vivo. (A, B) Representative FACS diagrams of CD34⁺ bone marrow cells from a healthy volunteer and an AML patient with a normal karyotype. Cells were cultured with medium plus DMSO or erlotinib for 3-6 days, followed by the assessment of cell death with two distinct methods, the DiOC₆(3)/PI and Annexin V/PI staining of dying (DiOC₆(3)low PI⁻ or AnnV⁺PI⁻) and dead cells (PI⁺). (C-F) NPM-1 and p14ARF translocation induced by erlotinib in AML cells (D, F), but not in normal CD34⁺ bone marrow cells (C, E). Cells were treated and assessed as described in Figure 6 A-D. (G, H) Induction of myeloid differentiation by erlotinib in malignant myeloblasts. CD34⁺ bone marrow cells from a high-risk MDS (G, H) and an AML patient (H) were cultured for 6 days in the presence of 0.02% DMSO or 10 μM erlotinib. CD11b surface exposure was measured by FACS analysis while excluding apoptotic cells. (G) The morphology and percentage of cells exhibiting signs of erlotinib-induced differentiation were determined by
Wright-Giemsa staining as described in Figure 3 (G, H). (I) Quantitative comparison of apoptosis induction in a cohort of normal controls and different subgroups of MDS/AML patients. Results are depicted as a boxplot, giving the increase in apoptosis induced by erlotinib (10 µM, 3 days) in vitro/ex vivo as compared to DMSO-only treated controls in the indicated subgroups. K: Karyotype; NS: not significant; *p<0.01. (J, K) Comparison of the *ex vivo* and *in vivo* effects of erlotinib on a patient with high-risk MDS. A patient diagnosed with metastatic EGFR-positive NSCLC and MDS RAEB-2 was treated with a monotherapy of erlotinib for the NSCLC. (J) Apoptosis-inducing effect of erlotinib on the patient’s CD34+ bone marrow cells *ex vivo* (3 days, 10 µM) as assessed by staining with DiOC6(3)/PI. (K) Routine blood examinations before and during monotherapy with erlotinib demonstrated the *in vivo* effect of erlotinib on platelet and neutrophil counts resulting in a hematological improvement. D-32: day 32 before erlotinib treatment; D0: start of erlotinib therapy; D+34: day 34 under erlotinib treatment.
Fig. 1
Fig. 2
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Fig. 5
**Fig. 6**
Fig. 7
Erlotinib exhibits anti-neoplastic off-target effects in AML and MDS: a preclinical study

Simone Boehrer, Lionel Ades, Thorsten Braun, Lorenzo Galluzzi, Jennifer Grosjean, Claire Fabre, Genevieve Le Roux, Claude Gardin, Antoine Martin, Stephane de Botton, Pierre Fenaux and Guido Kroemer