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Erlotinib and gefitinib for the treatment of myelodysplastic syndrome and acute myeloid leukemia: A preclinical comparison

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ABSTRACT

Erlotinib and gefitinib, two inhibitors of the epidermal growth factor receptor (EGFR), can stimulate apoptosis and differentiation of myeloid cell lines that lack EGFR, unveiling a novel, therapeutically exploitable off-target effect of tyrosine kinase inhibitors. Here, we performed a side-by-side comparison of erlotinib and gefitinib effects on a broad spectrum of malignant myeloid cell lines, as well as on primary myeloblasts freshly purified from the bone marrow of patients with myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). Both erlotinib and gefitinib induce apoptosis of a cell line (KG-1) that represents AML, and differentiation in another cell line (P39) derived from a patient with high-risk MDS. In this setting, erlotinib was more efficient than gefitinib. Erlotinib and gefitinib were equipotent in inducing apoptosis of primary $CD34^+$ myeloblasts from MDS and AML patients, yet had no toxic effect on $CD34^+$ progenitor cells from healthy donors. Although the response of individual MDS and AML patients in vitro was highly heterogeneous, the pro-apoptotic effects of erlotinib and gefitinib correlated significantly. These results suggest that erlotinib and gefitinib share a mechanistically related off-target effect that may be taken advantage of for the therapy of MDS and AML.

1. Introduction

Myelodysplastic syndromes (MDS) are clonal hematopoietic stem cell disorders, which are characterized by ineffective hematopoiesis leading to peripheral cytopenias and a frequent progression to acute myeloid leukemia (AML). MDS are classified based on morphology and blast cell percentage in blood and bone marrow [1,2]. Main prognostic factors of MDS, for progression to AML and survival, include the number of cytopenias, percentage of marrow blasts and cytogenetic abnormalities. Those factors are combined in an International Prognostic Scoring System (IPSS) that distinguishes four subgroups with significantly different risk of progression to AML and survival: low, intermediate 1 (int-1), intermediate 2

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(int-2), and high. While low and intermediate 1 subgroups are often grouped together as low-risk MDS, intermediate 2 and high subgroups are considered as high-risk MDS [3].

Low-risk MDS are characterized by increased apoptosis of marrow progenitors, that explains to a large extent the cytopenias. On the contrary, at later disease stages (high-risk MDS), a progressive increase in marrow blasts, coupled to a decrease in apoptosis, is seen, and this progressive infiltration leads to cytopenias through marrow failure [4,5]. In these high-risk MDS patients, allogeneic stem cell transplantation remains the only option for curative treatment, yet is only applicable in 15–20% of all patients.

AML is highly heterogeneous in morphology, cytogenetics, and prognosis [6]. The single most important prognostic indicator in AML with respect to treatment efficacy, long term remission and overall survival is the karyotype [7–11]. Accordingly, patients can be classified into three risk groups: (a) favorable, (b) intermediate, or (c) unfavorable. Whereas core-binding factor AML (that is AML with t [8,21] or inv [16]) and AML with t [15,17] constitute the favorable risk-group, patients exhibiting a normal karyotype in the malignant clone are in the intermediate risk group. A complex karyotype, with three or more chromosome abnormalities, defines the unfavorable risk group [12,13]. As true in MDS, aggressive treatment regimens are associated with severe toxicity in AML and are thus frequently not applicable in an elderly patient population that often suffers from considerable co-morbidity.

Both high-risk MDS and AML require new therapeutic approaches that rely on targeted therapies rather than on the use of cytotoxic components such as anthracyclins that might exert unacceptable side effects. We and others have reported that drugs originally designed to inhibit the receptor tyrosine kinase epidermal growth factor receptor (EGFR) may exert therapeutic off-target effects on MDS and AML cells (which do not express EGFR) [14–16]. Two small inhibitors of EGFR, gefitinib (Iressa®, AstraZeneca) and erlotinib (Tarceva®, Roche) were initially designed to antagonize the deregulated tyrosine kinase activity of EGFR in solid tumors [17–20]. Gefitinib induces differentiation and arrests proliferation in AML cells lines (U937, HL-60, Kasumi-1) and in primary blast cells from patients with AML [14]. Erlotinib causes differentiation, G1 phase arrest and/or apoptosis of MDS/AML cell lines (P39, HL-60, KG-1) and induces similar effects in myeloblasts from high-risk MDS and AML patients [16]. Two case reports on patients with non-small cell lung cancer (NSCLC) and MDS or AML demonstrated that monotherapy with erlotinib can exert beneficial effects on high-risk MDS and AML in vivo, causing hematological improvement (MDS) or complete remission (AML) [15,16].

Based on these results, we decided to perform a side-by-side comparison of the effects of erlotinib and gefitinib on MDS and AML cell lines and primary myeloblasts of MDS and AML.

2. Patients, materials and methods

2.1. Patients

Samples of patients or healthy volunteers were assessed after obtaining informed consent according to the Declaration of Helsinki. The diagnosis of AML and MDS was determined by cytology of peripheral blood and bone marrow myeloblasts, according to the WHO and FAB classification, as well as by conventional cytogenetic analysis. In patients with MDS, risk groups were determined using the International Prognostic Scoring System [3].

2.2. Cell lines and selection of $CD34^+$ cells

Mononuclear cells (MNC) of peripheral blood or bone marrow were isolated using a Ficoll-Paque PLUS density gradient (Amersham Biosciences, Sunnyvale, CA, USA). To obtain $CD34^+$ cells from MNC, a positive selection using the MiniMacs system (Miltenyi Biotec, Bergisch Gladbach, Germany) was carried out. Subsequently, $CD34^+$ cells were cultured in Iscove modified Dulbecco medium (IMDM, Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco) [21]. The high-risk MDS cell line P39/Tsugane was kindly provided by Dr. Yoshida Takeda, Japan, the KG-1 and U937 cell lines by Dr. Martin Ruthardt, Germany, and HL-60 cells by Dr. Bruno Cassinat, France, respectively. MOLM-13 and MV4-11 cells were purchased from the Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Note that the P39 cell line was morphologically and cytogenetically clearly different from HL-60 cells [22]. All cell lines were cultured in FCS-supplemented RPMI 1640 (Gibco). Unless specified differently, cells were seeded at a concentration of 1×10^5 cells/ml.

2.3. Assessment of apoptosis and differentiation

10^5 cells derived from patients or the indicated cell lines were resuspended in 1 ml of culture medium and incubated with the indicated dosages of erlotinib (Roche, Basel, Switzerland) or gefitinib (AstraZeneca, London, United Kingdom) dissolved in DMSO. Control cells were incubated with the corresponding amount of DMSO (0.02%) (Gibco). At the applied concentrations and at the depicted time points, DMSO did not affect apoptosis nor differentiation, as compared to untreated cells (data not shown). Therefore results depict only the controls incubated with 0.02% DMSO (''control''). Apoptotic cells were quantified by cytofluorometric analysis using a FACScan (Becton Dickinson, Mountain View, CA) as described previously [23–25]. Thus, cells were stained with the vital dye propidium iodide (PI; 5 μg/ml; Sigma, Steinheim, Germany) and concomitantly with either $DiOC_6(3)$ (3,3 dihexyloxacarbocyanine iodide; 40 nM; Molecular Probes, Eugene, OR, USA) for 15 min at 37 °C, or with Annexin-V-FITC (Becton Dickinson, Heidelberg, Germany) to determine the mitochondrial transmembrane potential or phosphatidyl serine exposure, respectively [21,25].

To assess differentiation by cytofluorometry, cells were harvested, washed and stained with a PE-conjugated anti-CD11b antibody (clone ICRF44, Becton Dickinson) indicating myelocytic differentiation, as previously described [16]. Analysis was carried out on live cells, which were gated based on, forward and side scatter patterns. Isotypic mouse IgG1 (Becton Dickinson) was used to determine threshold parameters.

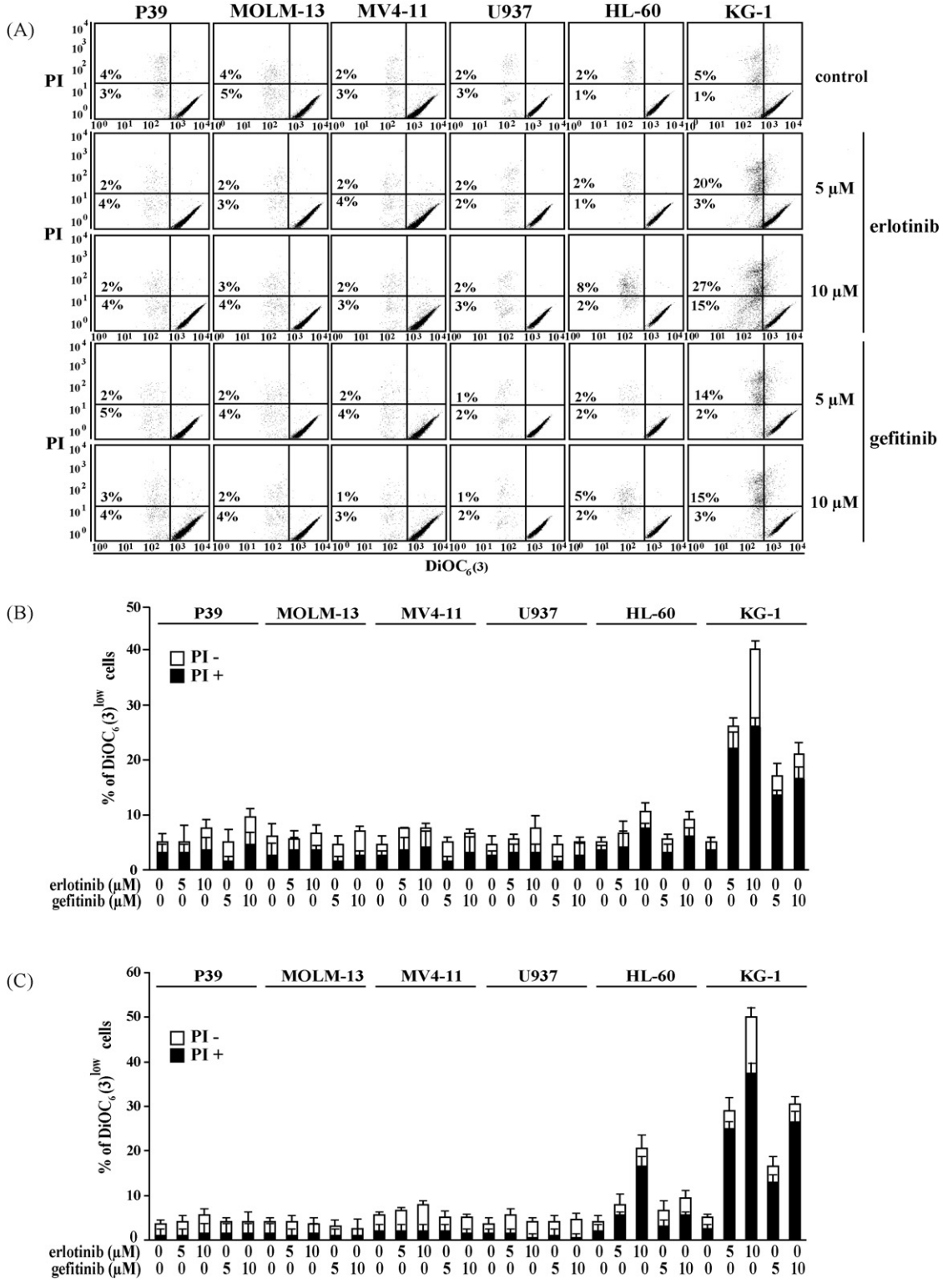

Fig. 1 – Erlotinib and gefitinib decrease the viability of selected myeloid cell lines. The myeloid cell lines P39, MOLM-13, MV4-11, U937, HL-60 and KG-1 were cultured for up to 6 days in the presence of 0.02% DMSO (corresponding to the amount of DMSO used to dissolve 10 μM of erlotinib or gefitinib, "control") or the indicated doses of erlotinib and gefitinib. (A) Representative FACS diagrams assessing dissipation of the mitochondrial transmembrane potential by $DiOC_6(3)$ staining, and loss of viability by staining with PI after an incubation period of 3 days with the indicated doses of erlotinib or gefitinib, respectively. (B) Quantitation of the data depicted in (A). (C) Quantitation of the loss of viability in the myeloid cell lines after 6 days of incubation with the indicated concentrations of erlotinib and gefitinib. Results depicted in (B) and (C) are means ± S.D. of triplicates. This experiment was repeated at least three times, yielding comparable results.

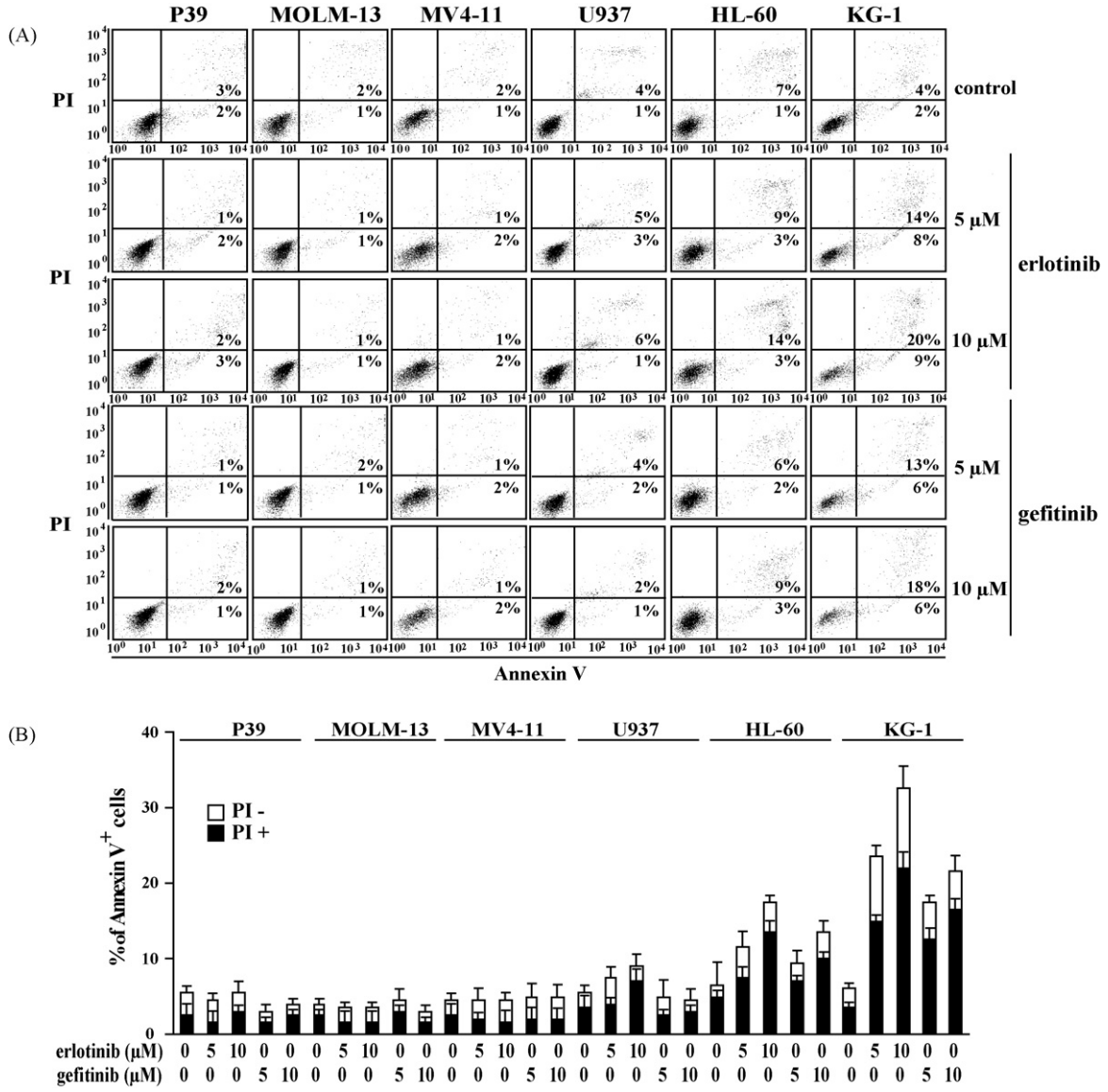

Fig. 2 – Pro-apoptotic off-target effects of erlotinib and gefitinib on MDS/AML cell lines. Myeloid cell lines were treated with the indicated doses of erlotinib and gefitinib (and DMSO-only-treated cells served as controls) and stained after 3 days of incubation with Annexin V-FITC/PI. (A) Representative FACS diagrams depicting the percentages of dying (Annexin V^+/PI^-) or dead (Annexin V^+/PI^+) myeloid cells in the respective cell lines. (B) Quantitation of the data depicted in (A). Results are means ± S.D. of triplicates. This experiment was repeated at least three times, yielding comparable results.

2.4. *Statistics*

Statistics were calculated with the help of Excel Software (Microsoft, Redmond, WA), SPSS software (SAS Institute, Cary, NC), and Scion Image 4.0 (Scion Corporation, Frederick, MD). Apoptosis induced by erlotinib and gefitinib in freshly isolated cells from MDS and AML patients was depicted using a boxplot graph, where the horizontal represents the median, the box the 25th percentile, and the whiskers the extreme. Statistical significance was assessed using the Student's *t*-test.

3. Results and discussion

3.1. *Pro-apoptotic off-target effects of erlotinib and gefitinib on MDS/AML cell lines*

To evaluate the potential therapeutic effects of erlotinib and gefitinib, both agents were added to a panel of myeloid cell lines that represent distinct subtypes of myeloid malignancy: P39 (which is derived from a patient with high-risk MDS), MOLM-13 (established from a patient with acute myelomonocytic leukemia after initial MDS), MV4-11 (acute myelo-

monocytic leukemia), U937 (histiocytic lymphoma with monocytic properties), HL-60 and KG-1 (both AML). Erlotinib or gefitinib were used at concentrations that can be therapeutically achieved in patients, that is in the order of 1–10 μM [20,26]. In a first series of experiments, we evaluated the effect of both agents, side-by-side, on the viability of cells (determined with the vital dye propidium iodine, PI) and the mitochondrial transmembrane potential ($\Delta\Psi_m$, measured with the $\Delta\Psi_m$-sensitive probe $DiOC_6(3)$) whose dissipation is indicative of incipient cell death [25].

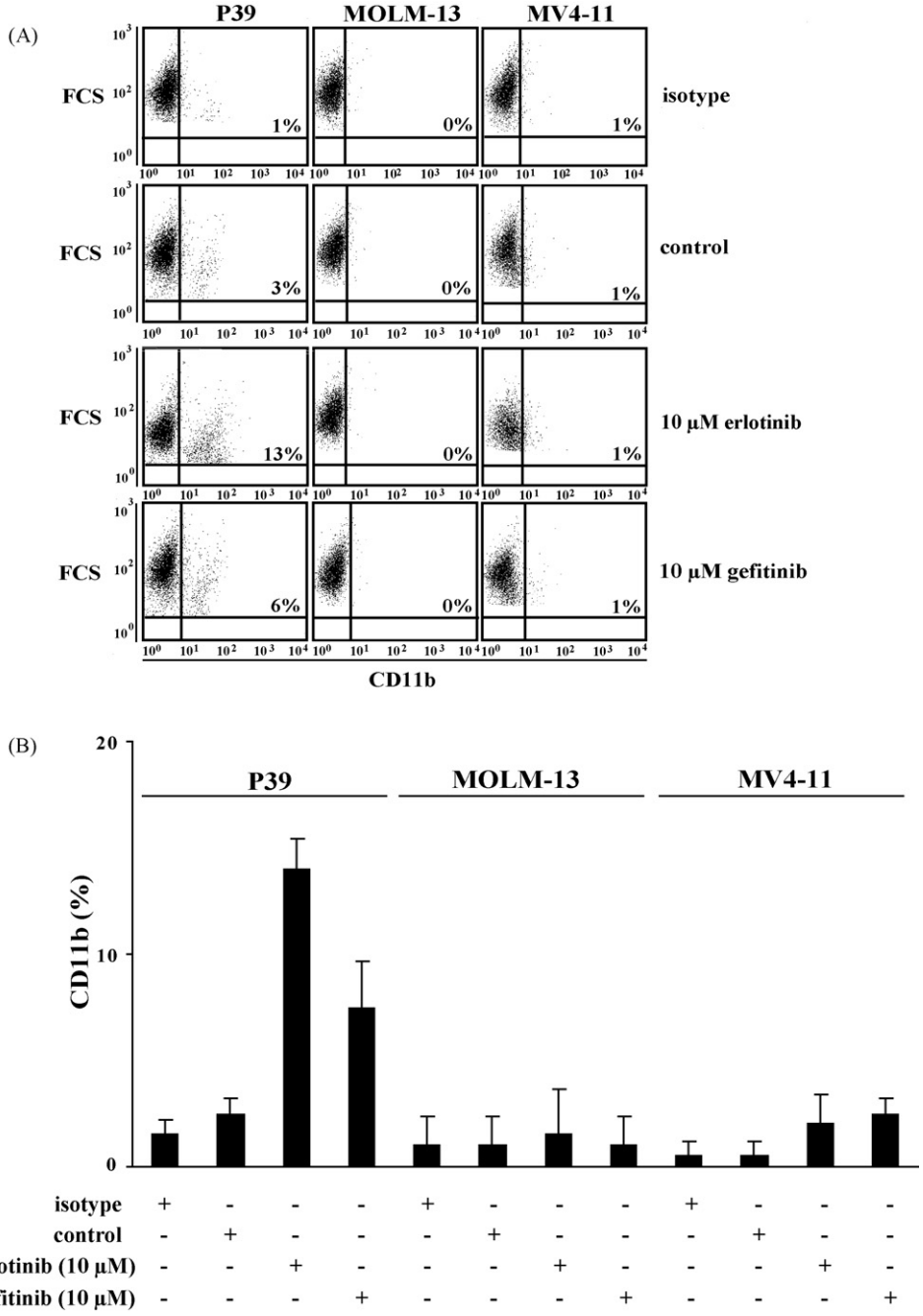

Fig. 3 – Capacity of erlotinib and gefitinib to induce surface expression of the myelocytic marker CD11b in myeloid cell lines. The myeloid cell lines P39, MOLM-13 and MV4-11 were incubated for 3 days with 0.02% DMSO ("control"), or 10 μM erlotinib or gefitinib, respectively. Cell surface expression of the myelocytic marker CD11b was assessed after staining with a PE-conjugated anti-CD11b antibody. Isotype controls served to determine threshold parameters. Analysis was carried out on live cells, which were gated based on, forward and side scatter patterns. FSC denotes forward scatter. (A) Representative FACS diagrams depicting CD11b expression as a marker of myelocytic differentiation. (B) Quantitation of the data depicted in (A). Results are means ± S.D. of triplicates. This experiment was repeated at least three times, yielding comparable results.

The only cell line in the tested panel that consistently died in response to both EGFR inhibitors was KG-1, which manifested $\Delta\Psi_m$ loss (reduced $DiOC_6(3)$ staining) and plasma membrane permeabilization (PI positivity), 3 days (Fig. 1A and B) and 6 days (Fig. 1C) after incubation with 5–10 μM of the drugs. The effect of erlotinib was consistently more pronounced than that of gefitinib (Fig. 1A–C). HL-60 cells manifested a minor apoptotic response at 6 (but not at 3) days after addition of 10 μM erlotinib (but not gefitinib), while P39, MOLM-13, MV4-11 and U937 failed to manifest signs of imminent or completed cell death (Fig. 1A–C). Similar results were obtained when in addition to viability, a bona-fide marker of apoptosis (namely exposure of phosphatidylserine) was determined by staining with Annexin V. KG-1 (and to a much lower degree HL-60) cells exhibited signs of apoptosis after incubation with the EGFR antagonists, while the other tested cell lines remained unaffected (Fig. 2A and B). Once again, erlotinib was more effective than gefitinib in killing KG-1 and HL-60 cells.

These results imply that both erlotinib and gefitinib can exert pro-apoptotic effects on EGFR-negative myeloid cells [14–16], depending on the cellular context.

3.2. *Differentiation-inducing effects of erlotinib and gefitinib on MDS/AML cell lines*

The first report on EGFR antagonists on AML cells insisted on the differentiation-inducing effect of gefitinib [14]. Thus both gefitinib and erlotinib demonstrated their capacity to induce differentiation in *ex vivo* patients' cells [14,16]. As a result, we determined the capacity of erlotinib or gefitinib to overcome the differentiation block characteristic of myeloid malignancies and to induce the acquisition of CD11b, a marker of myelocytic differentiation [21]. A significant fraction of P39 cells cultured in the presence of 10 μM erlotinib became positive for CD11b expression. No such effect was observed when erlotinib was replaced by gefitinib (Fig. 3A and B). In contrast, none of these agents-induced CD11b exposure in

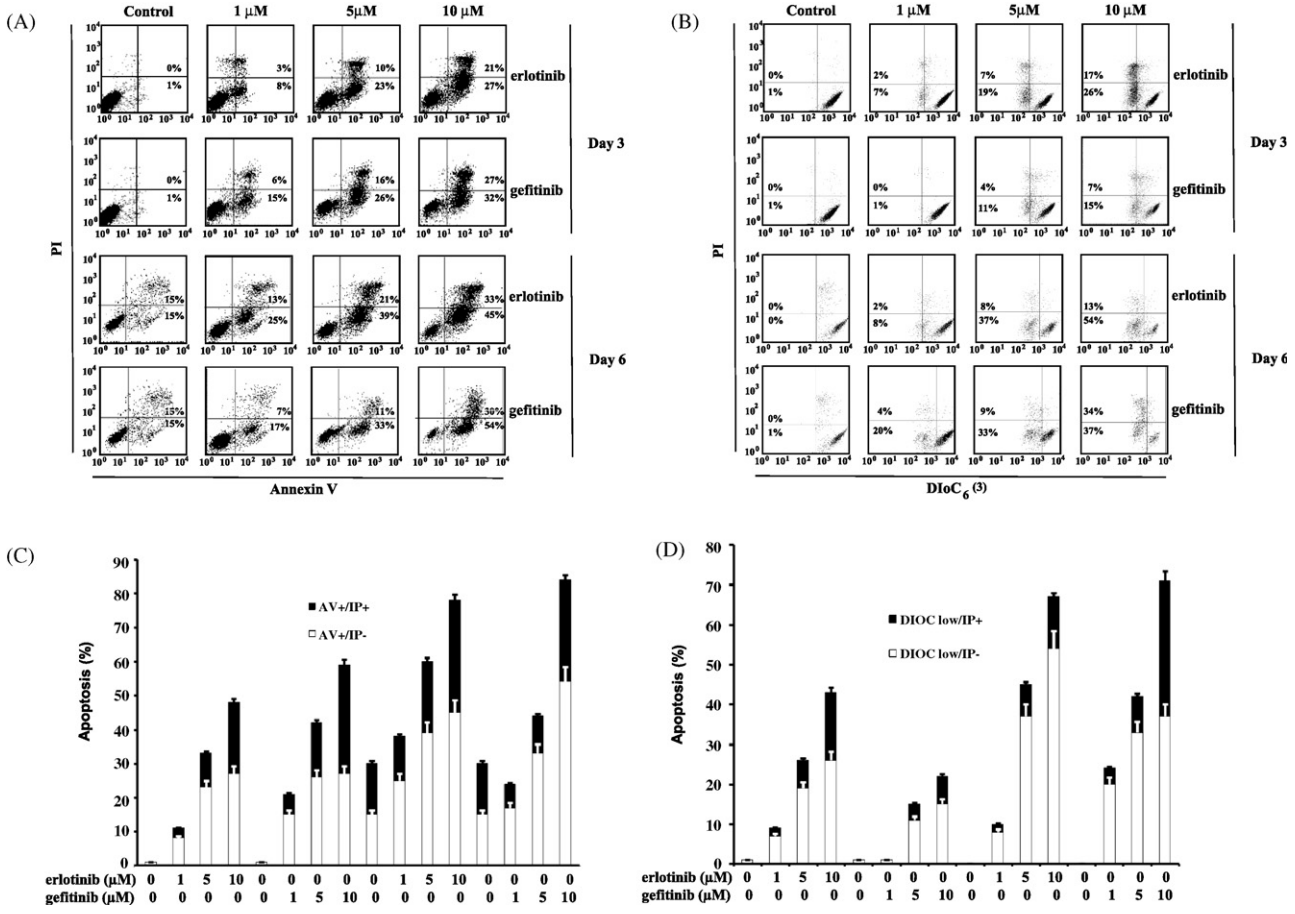

Fig. 4 – Therapeutic effects of erlotinib and gefitinib on myeloblasts *ex vivo*. $CD34^+$ bone marrow cells from a patient diagnosed with an AML were incubated with increasing dosages (1, 5, and 10 μM) of erlotinib [16] and gefitinib. $CD34^+$ cells incubated with 0.02% DMSO served as solvent control. (A) Representative FACS diagrams depicting the percentages of dying (Annexin V^+/PI^-) or dead (Annexin V^+/PI^+) myeloid cells after 3 (upper panels) and 6 days (lower panels) of the incubation period. (B) Representative FACS diagrams assessing dissipation of mitochondrial transmembrane potential by $DiOC_6(3)$ staining and loss of viability by staining with PI after an incubation period of 3 (upper panels) and 6 days (lower panels) of the incubation period. (C) and (D) Quantitation of the data depicted in (A) and (B), respectively.

Table 1 – Characteristics of the subjects included in this study

N	Sex	Age (years)	Diagnosis (WHO)	% bone marrow blast	Karyotype	IPSS	% of living cells/control			
							Day 3		Day 6	
							Gefi	Erlo	Gefi	Erlo
1	Female	38	Control	0	Normal	n.a.	–	–	96	88
2	Male	57	Control	0	Normal	n.a.	–	–	100	100
3	Female	76	Del 5q Sd	0	Del (5q)	LOW	68	58	80	100
4	Male	76	RAEB-1	7	Normal	INT-1	71	70	–	–
5	Male	68	RAEB-2	14	Del 7	HIGH	–	–	37	23
6	Female	72	RAEB-2	19	−7	HIGH	25	15	25	15
7	Female	77	RAEB-1	7	Del (11q)	INT-2	67	58	80	90
8	Male	59	AML	36	Complex	n.a.	84	79	–	–
9	Male	56	AML	70	Normal	n.a.	64	29	64	70
10	Female	80	sAML	30	Del (9p)	n.a.	49	48	21	23
11	Female	73	AML	34	Normal	n.a.	67	78	13	31
12	Female	23	AML	96	Inv (16)	n.a.	44	68	14	31
13	Female	66	AML	40	Normal	n.a.	62	93	–	–
14	Female	46	AML	66	Normal	n.a.	78	72	–	–

RAEB, refractory anemia with excess of blasts; sAML, secondary AML (after MDS); Del, deletion; complex, complex karyotype as defined by the detection of 3 or more aberrations; Inv, inversion; IPSS, International Prognostic Scoring System; n.a., not applicable; the percentage of living cells is depicted after incubation with 10 μM erlotinib (erlo) or gefitinib (gefi) after 3 and 6 days (as compared to the percentage of living cells incubated with 0.02% DMSO) as determined by $DiOC_6(3)$/PI and Annexin V/PI staining.

MOLM-13 or MV4-1. Noteworthy, P39 cells that undergo differentiation do not enter the apoptotic program (at least within 6 days) (compare Figs. 1–3).

3.3. *Pro-apoptotic off-target effects of erlotinib and gefitinib on primary MDS/AML cells*

$CD34^+$ myeloblasts were purified from the bone marrow of patients with AML or MDS on a column that specifically retains $CD34^+$ cells. Then, these blasts were cultured for 3–6 days in the presence of increasing concentrations (1–10 μM) of erlotinib or gefitinib and the frequency of dead (PI^+) or dying ($DiOC_6(3)^{low}$ or Annexin V^+) cells was determined by cytofluorometry. As exemplified for one patient diagnosed with AML, a significant fraction of cells died in response to both molecules, in a time- and dose-dependent fashion (Fig. 4A–D). Of note, erlotinib and gefitinib acted with a similar degree of efficacy. This side-by-side comparison of erlotinib and gefitinib was extended to a total of 14 clinical samples (Table 1) including 2 normal controls that exhibited no

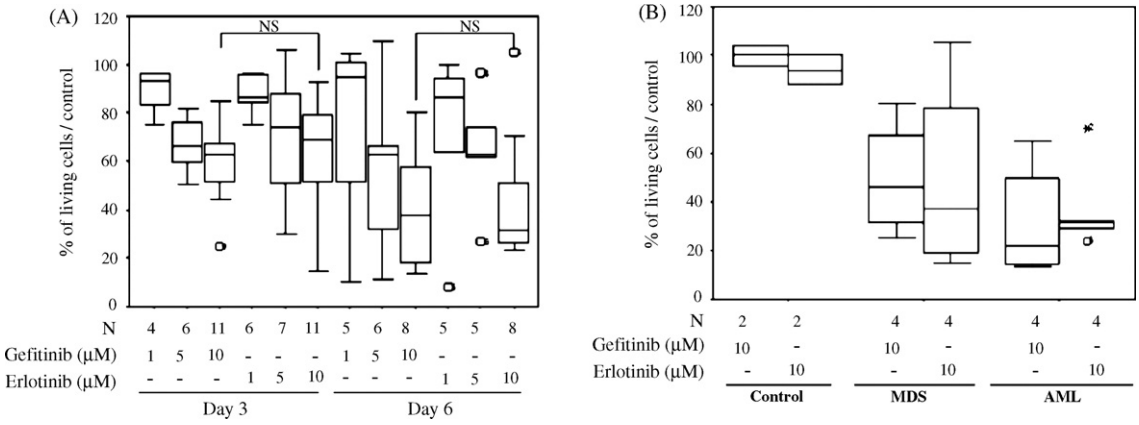

Fig. 5 – Comparison of the apoptosis-inducing effect of erlotinib and gefitinib in primary MDS/AML cells. $CD34^+$ cells from healthy volunteers and patients with MDS or AML (patient characteristics are detailed in Table 1) were incubated with the indicated doses of erlotinib or gefitinib. Results are depicted using a boxplot graph, where the horizontal represents the median, the box the 25th percentile, and the whiskers the extreme. Statistical significance was assessed using the Student's *t*-test. (A) Comparison of the cytotoxic effect (assessed by staining with Annexin V/PI and $DiOC_6(3)$/PI) of increasing dosages (1, 5, and 10 μM) of erlotinib or gefitinib. The box plot depicts the decrease in living cells as compared to DMSO-treated (0.02%) control cells after 3 and 6 days of incubation. N, number of samples assessed under the respective medication and dosage. NS, not significant. (B) Comparison of the cytotoxic effect (assessed by staining with Annexin V/PI and $DiOC_6(3)$/PI) of 10 μM erlotinib or gefitinib in $CD34^+$ cells from healthy volunteers (control) and patients with MDS and AML. The box plot depicts the decrease in living $CD34^+$ cells as compared to DMSO-treated (0.02%) cells after 6 days of incubation. N, number of samples assessed in the respective subgroup.

significant toxic effects of either EGFR antagonist. The response of individual patients to erlotinib and gefitinib was highly heterogeneous. Comparison of the efficacy of both agents on the entire population of patients with MDS or AML yielded no significant differences between erlotinib and gefitinib (Fig. 5A). Similarly, there was no trend in favor of a higher potency of either of the two agents when the patients were subdivided into groups with MDS or AML (Fig. 5B). However, there was a strong correlation between the apoptotic response to erlotinib and the one to gefinitib, meaning that erlotinib responders were also sensitive to gefitinib and vice versa (Table 1, Fig. 5).

Altogether, these preclinical results indicate that myeloblast from most but not all patients with MDS or AML concordantly respond to erlotinib or gefinitib.

4. Concluding remarks

The results shown in this study provide some insights on the mode of action and on the potential clinical use of erlotinib versus gefitinib for the treatment of high-risk MDS and AML. One important insight comes from the systematic comparison of distinct myeloid cell lines that respond to erlotinib or gefitinib by activating two distinct molecular programs, differentiation or apoptosis. The capacity of these drugs to induce apoptosis and differentiation renders them particularly interesting for a potential treatment of myeloid malignancies, since – comparable to already established treatment regimen – they are able to induce cell death and to overcome the block of differentiation characteristic for these malignancies. Of note, both processes, apoptosis and differentiation, were induced more efficiently by erlotinib than by gefitinib, yet did not correlate among each other. Differentiation (as assessed by induction of the myelo-monocytic marker CD11b) could occur in cells that did not undergo apoptosis. It is also important to note that only a minority of cell lines (including those representing post-MDS AML or primary AML) underwent apoptosis in response to erlotinib or gefitinib (and this side-by-side comparison of resistant and sensitive myeloid cell lines constitutes a prerequisite for the identification of the kinase(s) targeted by these agents), while in a majority of MDS and AML patients, ex vivo purified myeloblasts activated the death program in response to the two EGFR antagonists. The reason for this discrepancy is elusive, since the molecular target of the pro-apoptotic action of EGFR has not been characterized yet. However, it is more than plausible that cell lines do not necessarily recapitulate the cellular context that applies to primary myeloblasts from patients.

When added to primary blasts from high-risk MDS or AML patients, both erlotinib and gefitinib were equipotent in inducing apoptosis (which is another difference with the KG-1 and HL-60 cells, in which erlotinib was more efficient than gefitinib). A majority of patients' samples exhibited a therapeutic response to both agents, but those samples who failed to respond to one of the agents exhibited a cross-resistance to the other agent, suggesting that erlotinib and gefitinib act on the same target. The nature of this target is unknown. However, it must be distinct from the EGFR (because MDS and AML cells do not express EGFR) and is likewise another tyrosine kinase. We are currently in the process of identifying this kinase, hoping that this information would resolve the enigma on which patients would benefit from treatment with erlotinib or gefitinib. The identification of such a biomarker may have a profound impact on the clinical management of high-risk MDS and AML.

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