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EGFR inhibitors exacerbate differentiation and cell cycle arrest induced by retinoic acid and vitamin D_3 in acute myeloid leukemia cells

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Abbreviations: ABC, ATP-binding cassette; AML, acute myeloid leukemia; APC, allophycocyanin; APL, acute promyelocytic leukemia; ATRA, all-*trans* retinoic acid; CFSE, 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester; DAPI, 4',6-diamidino-2-phenylindole; $DiOC_6(3)$, 3,3′-dihexiloxalocarbocyanine iodide; FAB, French-American-British; FITC, fluorescein isothiocyanate; EGFR, epidermal growth factor receptor; JNK1, c-JUN N-terminal kinase 1; MAPK, mitogen-activated protein kinase; MAPK2K, MAPK kinase; mTOR, mammalian target of rapamycin; NBT, nitroblue tetrazolium chloride; PDGFR, platelet-derived growth factor receptor; PI, propidium iodide; RT, room temperature; SFK, SRC family kinase; TKI, tyrosine kinase inhibitor; VD, 1α,25-hydroxycholecalciferol

By means of an unbiased, automated fluorescence microscopy-based screen, we identified the epidermal growth factor receptor (EGFR) inhibitors erlotinib and gefitinib as potent enhancers of the differentiation of HL-60 acute myeloid leukemia (AML) cells exposed to suboptimal concentrations of vitamin A (all-*trans* retinoic acid, ATRA) or vitamin D (1α,25-hydroxycholecalciferol, VD). Erlotinib and gefitinib alone did not promote differentiation, yet stimulated the acquisition of morphological and biochemical maturation markers (including the expression of CD11b and CD14 as well as increased NADPH oxidase activity) when combined with either ATRA or VD. Moreover, the combination of erlotinib and ATRA or VD synergistically induced all the processes that are normally linked to terminal hematopoietic differentiation, namely, a delayed proliferation arrest in the G_0/G_1 phase of the cell cycle, cellular senescence, and apoptosis. Erlotinib potently inhibited the (auto)phosphorylation of mitogen-activated protein kinase 14 (MAPK14, best known as $p38^{MAPK}$) and SRC family kinases (SFKs). If combined with the administration of ATRA or VD, the inhibition of $p38^{MAPK}$ or SFKs with specific pharmacological agents mimicked the pro-differentiation activity of erlotinib. These data were obtained with 2 distinct AML cell lines (HL-60 and MOLM-13 cells) and could be confirmed on primary leukemic blasts isolated from the circulation of AML patients. Altogether, these findings point to a new regimen for the treatment of AML, in which naturally occurring pro-differentiation agents (ATRA or VD) may be combined with EGFR inhibitors.

Introduction

Leukemia constitutes a type of hematological malignancy characterized by a prominent increase in circulating immature white blood cells named "blasts." Leukemogenesis involves a compendium of cell-autonomous genetic and epigenetic alterations that interfere with the homeostatic regulation of the hematopoietic (stem cell) compartment. Generally, this results in the accumulation of leukemic (stem) cells that gradually take over normal hematopoiesis as they acquire all hallmarks of cancer.[1,2] Thus, curative antileukemic therapies may be conceived to achieve at least 1 out of 3 distinct purposes. First, cytotoxic agents can be designed to selectively kill leukemic (stem) cells, mostly by stimulating mitochondrial apoptosis. Second, cytostatic agents may be used to block the unrestrained proliferation of leukemic cells, thereby reducing their relative occupancy of the bone marrow. Third, differentiation-stimulating agents should stimulate the transition of leukemic (stem) cells to a mature phenotype, thereby abolishing their proliferative potential and allowing them to undergo terminal differentiation-associated cell

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death.[3-5] A prominent example of such a differentiation therapy is all-*trans* retinoic acid (ATRA), the biologically active variant of vitamin A, which has been successfully employed for decades in the treatment of acute promyelocytic leukemia (APL).[6] Similarly, 1α,25-hydroxycholecalciferol, the active form of vitamin D_3 (VD) also known as calcifediol, and many of its analogs can stimulate the terminal differentiation of leukemic cell lines as well as primary myeloid precursors, and their therapeutic value has been tested in different clinical trials.[7,8] However, the clinical development of VD as an antileukemic agent appears to stand at an impasse, for 2 reasons. First, the high doses of VD that are required to stimulate myeloid differentiation can cause moderate to severe adverse effects related to Ca^{2+} metabolism. Second, the administration of VD has been associated (at least in specific settings) with the rapid development of resistance.[9,10] Thus, no differentiation therapies are currently approved for the clinical management of leukemias other than APL (French-American-British subtype M3).

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of hematopoietic progenitors and represents one of the most common forms of acute leukemia affecting adults.[11] Although AML is a relatively rare disease, accounting for slightly over 1% of cancer-related deaths in the western world, its incidence is expected to augment as the population ages.[12] AML develops along a complex, multistep course characterized by the progressive accumulation of a variety of genetic defects that either confer a proliferative/survival advantage to myeloid progenitors (e.g., *FLT3* or *KIT* mutations) or contribute to the failure of these cells to differentiate into mature granulocytes or monocytes (e.g., *CEBPA* or *NPM1* mutations).[13,14] The clinical management of AML patients younger than 60 y is based on high-dose chemotherapy and, upon relapse, bone marrow transplantation.[15] However, the use of cytotoxic chemotherapy in the elderly is associated with high rates of morbidity and mortality.[16,17] Novel antileukemic drugs have brought about a few improvements in disease outcome among elderly patients.[18]

Because the incidence of AML affecting old patients augments (along with the progressive increase in life expectancy of the general population), novel therapeutic paradigms for the clinical management of leukemia in this patient subset are urgently awaited. Differentiation therapies may represent a valuable alternative to cytotoxic agents in this setting, as they are generally associated with comparatively less severe side effects. However, most chemicals agents with a pro-differentiation activity described in the last 2 decades do not target a disease-specific lesion such as ATRA, which selectively modulates the activity of PML-RARα (the etiological determinant of APL),[19] and generally are not potent enough to promote terminal differentiation. Recently, several groups, including ours, have proposed epidermal growth factor receptor (EGFR) inhibitors, such as gefitinib[20,21] and erlotinib,[22-24] as potential candidates for the treatment of AML, although the expression of EGFR by AML cells is a subject of controversy.[24,25] Both gefitinib and erlotinib have been reported to exert a mild differentiation-inducing effect in vitro,[24,26,27] which, however, has not been confirmed in vivo.

In the present study, we addressed the question as to whether the maturation of AML cells exposed to suboptimal doses of ATRA and VD may be exacerbated by the concomitant administration of other therapeutically relevant agents. We report that erlotinib and gefitinib synergistically interact with ATRA and VD to stimulate the terminal differentiation of AML cells.

Results and Discussion

EGFR inhibitors stimulate the differentiation of AML cells in synergy with ATRA and VD

To identify novel agents that may induce or favor the differentiation of AML cells, we developed an automated screening system involving the immunofluorescence microscopy-based detection of one particular marker of myeloid maturation, CD11b,[28] in human HL-60 (**Fig. 1**) and MOLM-13 (data not shown) cells. We then employed this system to screen the US Drug Collection (which encompasses most FDA-approved drugs, plus a large amount of compounds that have reached clinical development), finding ATRA (employed at a final concentration of 1 μM) as the most effective differentiation-inducing agent of the library (**Fig. 1A and B**). To identify possible synergistic interactions between anticancer agents that are currently employed in the clinic and known differentiation-inducing chemicals, we repeated such screen on compounds from the Oncology Drug Set (which comprises most FDA-approved chemotherapeutics; final concentration = 10 μM), alone or in the presence of a suboptimal dose of ATRA (100 nM) or VD (50 nM). These screens revealed that 2 EGFR inhibitors, erlotinib and gefitinib, are capable of stimulating the differentiation of HL-60 cells when combined with ATRA or VD, yet have limited, if any, differentiation-inducing activity when employed as single agents (**Fig. 1C and D**). To validate these findings, we performed cytofluorometric assays for the detection of the differentiation markers CD11b and CD14 on HL-60 cells exposed to suboptimal doses of ATRA or VD, alone or combined with optimal concentrations of several FDA-approved tyrosine kinase inhibitors (TKIs) (**Fig. 2A and B**). A number of TKIs stimulated the expression of CD11b on the surface of HL-60 cells, if co-administered with ATRA or VD (**Fig. 2C**). Moreover, several TKIs, notably erlotinib, favored the acquisition of the monocyte marker CD14 by HL-60 cells exposed to VD, but not to ATRA (**Fig. 2D**).

The capacity of erlotinib to promote the ATRA- or VD-induced differentiation of AML cells was confirmed by 3 additional techniques, namely (1) the morphological evaluation of cells upon May–Grünwald–Giemsa staining, revealing the differentiation-associated decrease in cytoplasmic basophilia accompanied with increased cytoplasmic granularity and reduced nucleus/cytoplasm ratio[29] (**Fig. 3A and B**), (2) the measurement of the respiratory burst that characterizes mature myeloid cells, based on the conversion of nitroblue tetrazolium chloride (NBT) to a blue insoluble product (formazan) by NADPH oxidases[30] (**Fig. 3C and D**), and (3) the cytochemical assessment of monocyte-specific esterase activity, based on the conversion of

1-naphthyl acetate into 1-naphthol (which forms an insoluble red-brown dye in the presence of a diazonium salt)[31] (**Fig. 3E and F**). All these methods confirmed that erlotinib and gefitinib fail to induce the differentiation of HL-60 cells on their own, yet exacerbate the pro-differentiation activity of suboptimal doses of ATRA or VD. Similar findings were obtained when the ability of erlotinib and gefitinib to accentuate differentiation as induced by ATRA and VD was tested on MOLM-13 cells (**Fig. S1**).

We conclude that TKIs, and in particular erlotinib, can synergize with ATRA or VD in promoting the differentiation of AML cells.

Antiproliferative and pro-apoptotic effects of EGFR inhibitors combined with ATRA or VD

Physiologically, the terminal differentiation of myeloid precursors toward the granulocytic or monocytic lineage is coupled to a progressive reduction in cell proliferation followed by apoptosis.[32,33] We therefore sought to determine the potential antiproliferative and pro-apoptotic effects of EGFR inhibitors combined with ATRA or VD. For the quantitative assessment of cell number, a fixed amount of fluorescent beads was added to differentiating cell cultures, followed by the cytofluorometric determination of the cell/bead ratio (**Fig. 4A and B**). In addition, we quantified cell proliferation using 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE), a plasma membrane-permeant fluorescent dye that, upon stable incorporation into the cytoplasm, dilutes by a factor of 2 with each successive round of mitosis (**Fig. 4C and D**).[34] Both these experimental settings revealed the capacity of erlotinib and gefitinib to exacerbate the antiproliferative effects of ATRA and VD (**Fig. 4A–D**). Such a cytostatic activity was particularly pronounced when erlotinib and gefitinib were combined with VD rather than with ATRA, which per se exerted cytostatic effects (**Fig. 4A–D**). In line with previous reports,[21,35] erlotinib also had an antiproliferative activity, yet the combination of erlotinib plus ATRA or VD was more efficient at inhibiting the proliferation of HL-60 cells than any of these agents employed alone (**Fig. 4A–D**).

Cytofluorometric analyses confirmed the capacity of EGFR inhibitors to block HL-60 cells in the G_0/G_1 phase of the cell cycle when combined with ATRA or VD (**Fig. 4E and F**). HL-60 cell cultures exposed to ATRA displayed multiple hallmarks of apoptosis, including the accumulation of cell corpses with a subdiploid DNA content (**Fig. 4E and F**), the dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$) (**Fig. 5A and B**), the exposure of phosphatidylserine on the cell surface (**Fig. 5C and D**), as well the permeabilization of plasma membranes[36,37] (**Fig. 5A–D**). Conversely, erlotinib and VD alone had negligible pro-apoptotic effects in this setting, yet caused consistent levels of apoptotic cell death when combined (**Figs. 4E and F and 5D**). Clonogenic assays confirmed these findings. The combination of erlotinib plus ATRA or VD near-to-completely abolished the clonogenic potential of HL-60 cells, while neither of these agents alone achieved such dramatic effects (**Fig. 5E and F**).

These results indicate that erlotinib synergizes with ATRA and VD in inducing the terminal differentiation of HL-60 cells, a process that is coupled to a proliferative arrest and to the induction of apoptosis.

Mechanisms of action underlying the pro-differentiation activity of erlotinib

Several protein kinases have been involved in myeloid cell differentiation, including mitogen-activated protein kinase 1 (MAPK1, best known as extracellular signal-regulated kinase, ERK), which positively regulates the process,[38,39] as well as

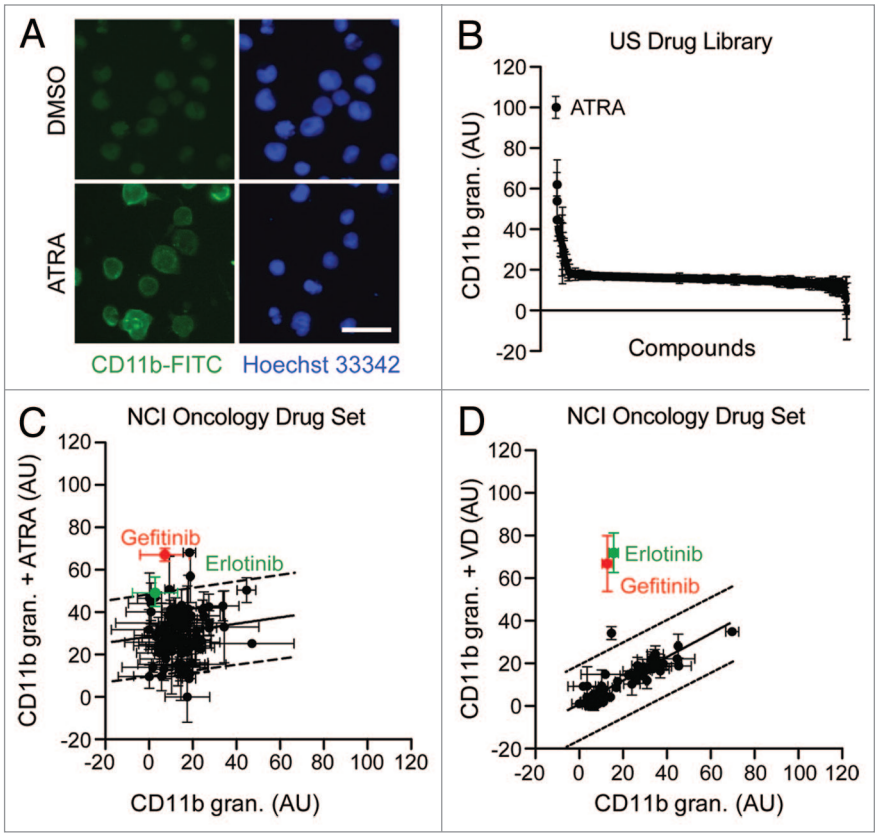

Figure 1. Automated screening platform for the identification of differentiation-inducing agents. (**A–D**). Human acute myeloid leukemia HL-60 cells were exposed to DMSO (control conditions) (**A–D**), compounds from the US Drug Library (final concentration = 1 μM) (**A and B**), or compounds from the NCI Oncology Drug Set (final concentration = 10 μM) (**C and D**), alone or in combination with 100 nM all-*trans* retinoic acid (ATRA) or 50 nM 1α,25-hydroxycholecalciferol (VD), for 3 d, and the processed for the automated fluorescence microscopy-assisted detection of CD11b. Representative images (scale bar = 20 μm) and quantitative data on CD11b granularity (AU, means ± SD, n = 3 parallel replicates) upon intra- and inter-plate normalization are reported in (**A**) and (**B–D**), respectively. In panels (**C and D**), dashed lines delimit the zone of statistical non-significance (90% prediction interval).

MAPK14 (best known as p38^{MAPK}) and multiple SRC family kinases (SFKs), which inhibit it.[40,41] Immunoblotting revealed that erlotinib and gefitinib inhibit the activating phosphorylation of p38^{MAPK} and SFKs, yet fail to suppress that of ERK and MAPK8 (best known as c-JUN N-terminal kinase 1, JNK1) (**Fig. 6A**). Rather erlotinib (but less so gefitinib) appeared to stimulate ERK phosphorylation (**Fig. 6A**).

Of note, the pharmacological inhibition of p38^{MAPK} with SB203580 and SB202190 (data not shown) or that of SFKs with PP2—but neither that of MAPK kinase 1 and 2 (MAP2K1 and MAP2K2) with U01326, nor that of JNK1 with SP600125, nor that of the mammalian target of rapamycin (mTOR) with rapamycin—favored the maturation of HL-60 cells in the presence of ATRA or VD (but not in their absence), hence mimicking the effect of erlotinib (**Fig. 6B**). Thus, the inhibition of p38^{MAPK} and SFKs may account for the differentiation-inducing activity of erlotinib. Next, we wondered whether SB203580 and PP2 might also mimic the cytostatic and pro-apoptotic effects of erlotinib. Indeed, SB203580 and (less so) PP2 were able to enhance the cell cycle-arresting effect of ATRA and VD (**Fig. 6C**) and to synergize with the latter (but not with the former, as ATRA induced per se consistent degrees of cell death) in the killing of HL-60 cells (**Fig. 6D**). When combined with VD, SB203580 and PP2 also mimicked erlotinib in its

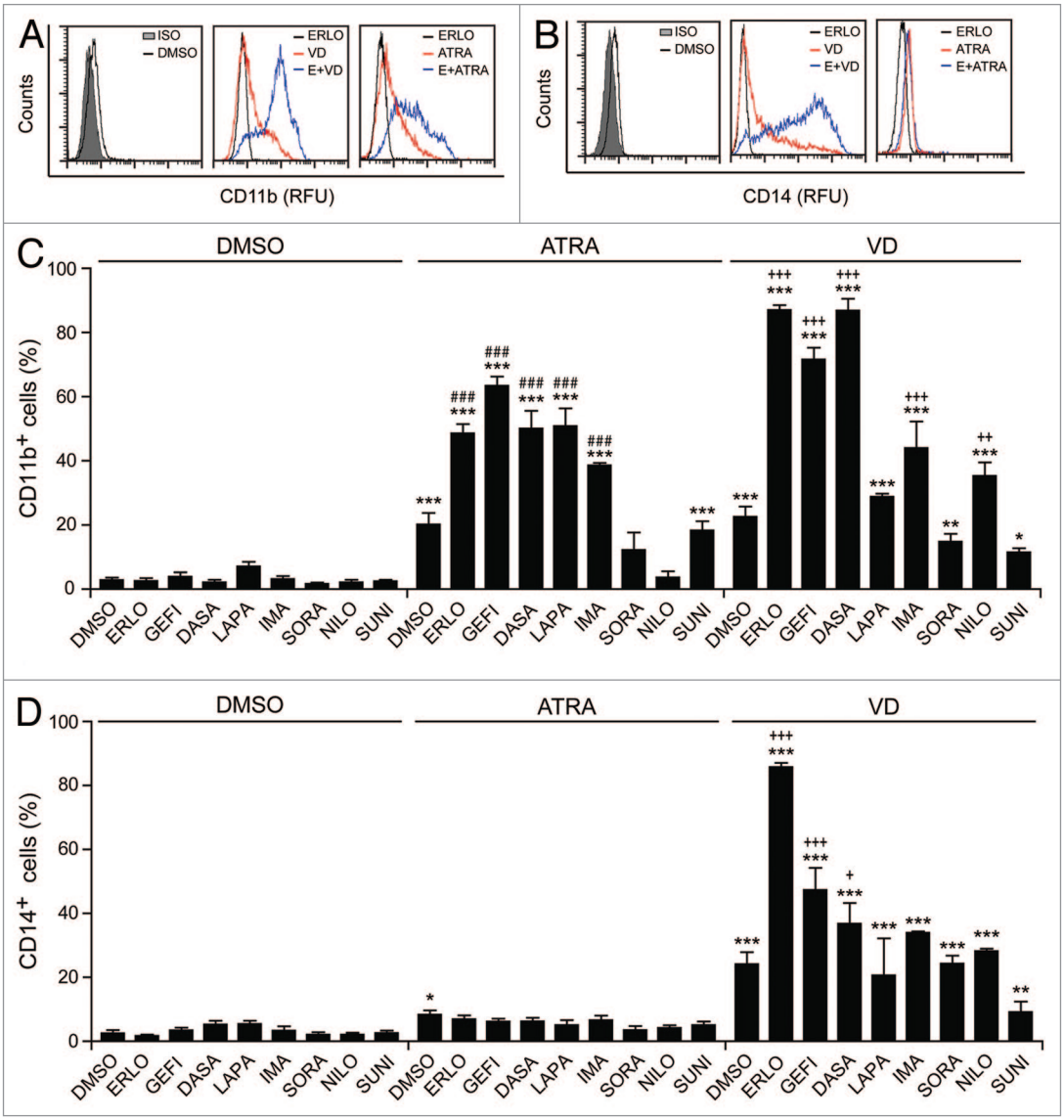

Figure 2. Effects of tyrosine kinase inhibitors on myeloid differentiation. (**A–D**). Human acute myeloid leukemia HL-60 cells were treated with 10 μM erlotinib (ERLO, E), 10 μM gefitinib (GEFI), 100 nM dasatinib (DASA), 5 μM lapatinib (LAPA), 5 μM imatinib (IMA), 5 μM sorafenib (SORA), 500 nM nilotinib (NILO), 500 nM sunitinib (SUNI), or an equal volume of DMSO, alone or combined with 100 nM all-*trans* retinoic acid (ATRA) or 50 nM 1α,25-hydroxycholecalciferol (VD), for 3 d, and then processed for the cytofluorometry-assisted detection of CD11b (**A–C**) or CD14 (**B–D**) expression. Panels (**A and B**) report representative CD11b and CD14 expression profiles, respectively. ISO, isotype control. Panels (**C and D**) illustrate quantitative data on the percentage of CD11b- and CD14 -expressing cells, respectively (means ± SEM; n = 2–3). *P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA plus Dunnett post-hoc test) as compared with DMSO-treated cells; ###P < 0.001 (ANOVA plus Bonferroni post-hoc test), as compared with ATRA-treated cells; +P < 0.05, ++P < 0.01 +++P < 0.001 (ANOVA plus Bonferroni post-hoc test), as compared with VD-treated cells.

ability to elicit 2 hallmarks of cellular senescence, namely (1) an arrest in the G_0/G_1 phase of the cell cycle (defined by a diploid DNA content and quantified by cytofluorometry upon Hoechst 33342 staining) coupled to reduced RNA synthesis (assessed by cytofluorometry upon pyronin Y staining)[42] (**Fig. 7A and B**), and (2) the acquisition of senescence-associated β-galactosidase activity[43] (**Fig. 7C and D**).

Altogether, these results are compatible with the hypothesis that erlotinib stimulates the differentiation of myeloid cells by inhibit $p38^{MAPK}$ and SFKs.

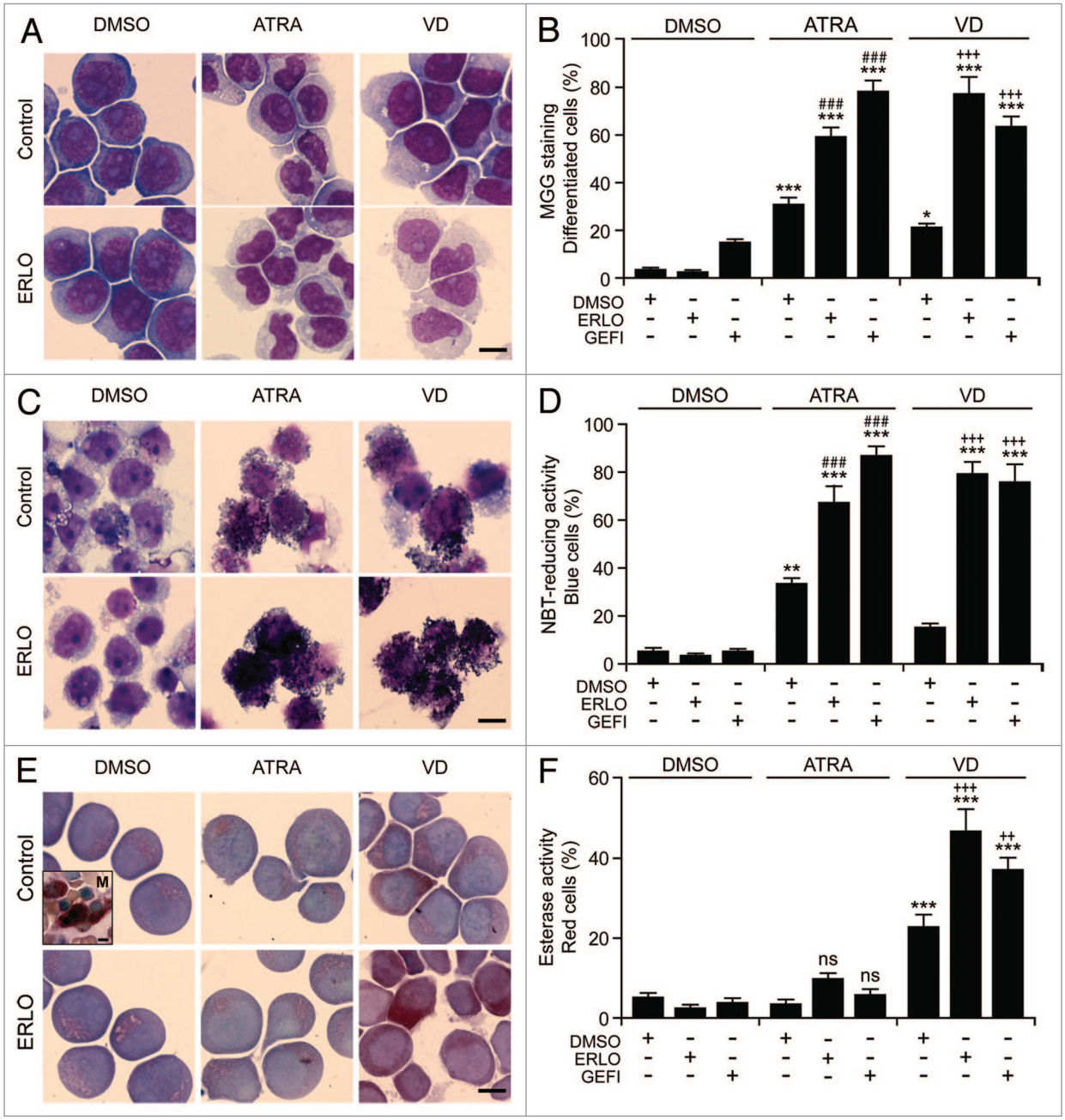

Figure 3. Morphological and functional assessment of the differentiation-inducing activity of EGFR inhibitors. (**A–F**). Human acute myeloid leukemia HL-60 cells were treated with 10 μM erlotinib (ERLO), 10 μM gefitinib (GEFI), or an equal volume of DMSO, alone or in combination with 100 nM all-*trans* retinoic acid (ATRA), or 50 nM 1α,25-hydroxycholecalciferol (VD), for 3 d, then cytospun and processed for the cytochemical assessment of myeloid differentiation upon May–Grünwald–Giemsa staining (**A and B**) or the colorimetric detection of NADPH oxidase (**C and D**) or α-naphtyl acetate esterase (**E and F**) enzymatic activities. Panels (**A, C, and E**) depict representative images (scale bars = 10 μm), whereas quantitative data on the percentage of cells exhibiting morphological signs of differentiation (decreased cytoplasmic basophilia, nuclear lobulation and cytoplasmic granulation), nitroblue tetrazolium chloride (NBT)-reducing activity and α-naphtyl acetate esterase activity are reported in (**B, D, and F**), respectively (means ± SEM; n = 3 with at least 100 cells/condition). The inset in (**E**) depicts a megakaryocyte (M) exhibiting intense α-naphtyl acetate esterase activity (positive control). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (ANOVA plus Dunnett post-hoc test), as compared with DMSO-treated cells; n.s., not significant, ###*P* < 0.001 (ANOVA plus Bonferroni post-hoc test), as compared with ATRA-treated cells; ++*P* < 0.01, +++*P* < 0.001 (ANOVA plus Bonferroni post-hoc test), as compared with VD-treated cells.

Differentiation-inducing effects of erlotinib on primary AML blasts

The results presented above underscore the capacity of erlotinib and gefitinib combined with either ATRA or VD to drive the differentiation of HL-60 (**Figs. 1–7**) and MOLM-13 cells (**Fig. S1**). We next investigated whether a similar effect might be observed on primary leukemic cells isolated from the peripheral blood of AML patients (**Table S1**). Indeed, a fraction

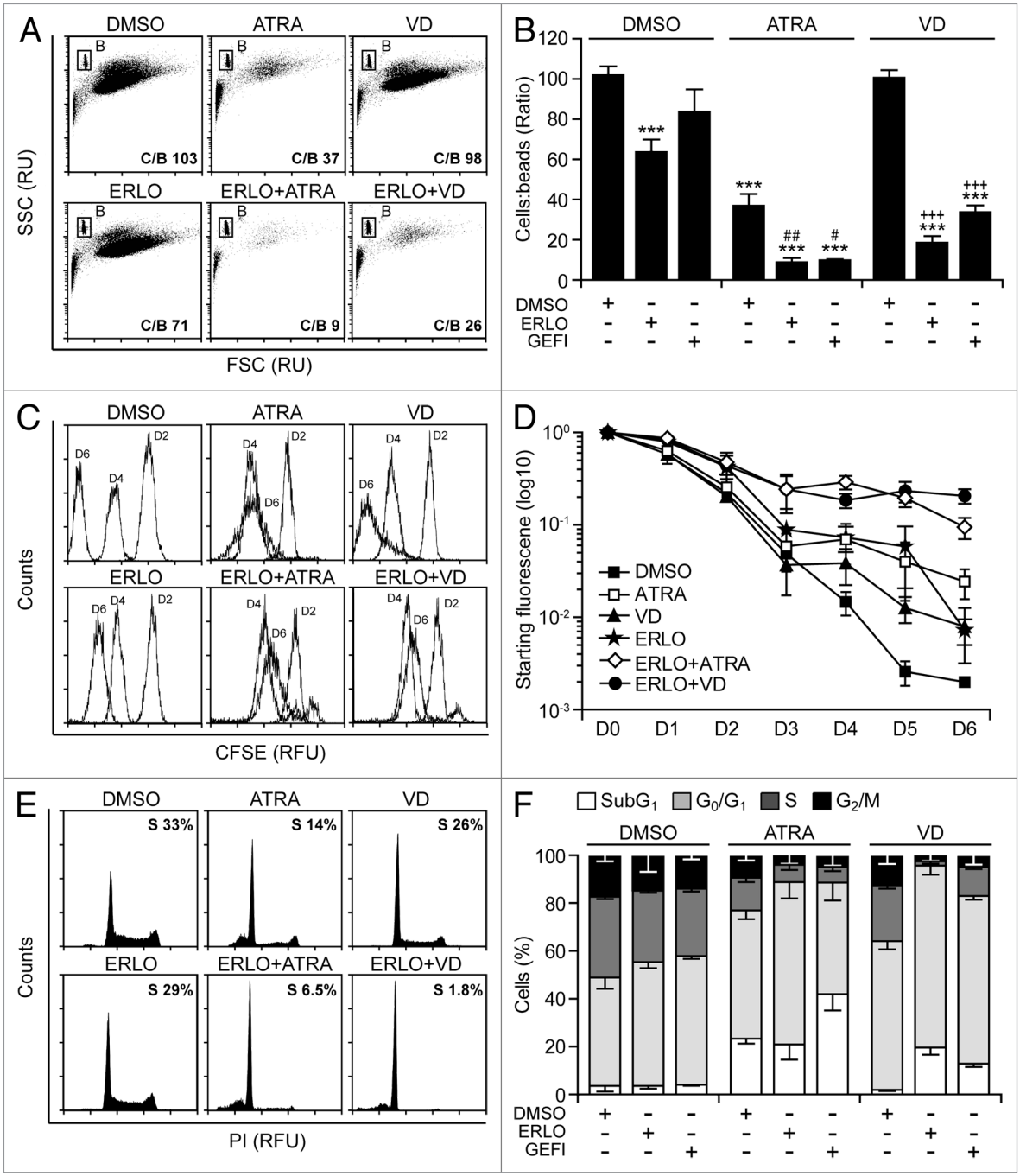

Figure 4. Antiproliferative response associated with myeloid differentiation. (**A–F**). Human acute myeloid leukemia HL-60 cells were treated with 10 μM erlotinib (ERLO), 10 μM gefitinib (GEFI), or an equal volume of DMSO, alone or in combination with 100 nM all-*trans* retinoic acid (ATRA) or 50 nM 1α,25-hydroxycholecalciferol (VD), for the indicated number of days (7, where not specified), followed by (**A, B, E, and F**) or alongside with (**C and D**) the assessment of proliferation (**A–D**) or cell cycle distribution (**E and F**) based on the bead-assisted cytofluorometric quantification of cell number (**A and B**), the CFSE-dependent assessment of replication (**C and D**), or the analysis of DNA content upon propidium iodide staining (**E and F**). Representative dot plots, CFSE emission profiles and cell cycle distributions are reported in (**A, C, and E**), respectively. In (**A**), the cell:bead (C/B) ratio is reported. In (**E**), numbers refer to the percentage of cells with a DNA content >2n and <4n (compatible with the S phase of the cell cycle). Quantitative data on the C/B ratio, CFSE fluorescence and distribution in different phases of the cell cycle are reported in (**B, D, and F**), respectively (means ± SEM; n = 3). ***$P < 0.001$ (ANOVA plus Dunnett post-hoc test), as compared with DMSO-treated cells; #$P < 0.05$, ##$P < 0.01$ (ANOVA plus Bonferroni post-hoc test), as compared with ATRA-treated cells; +++$P < 0.001$ (ANOVA plus Bonferroni post-hoc test), as compared with VD-treated cells.

of AML samples acquired the differentiation marker CD11b more robustly (difference in the percentage of CD11b$^+$ cells > 10%) upon treatment with erlotinib plus ATRA or VD than upon exposure to any of these agents alone (**Fig. 8**). This applied to 4 out of 24 AML samples treated with ATRA plus erlotinib and 4 out of 9 AML samples treated with VD plus erlotinib.

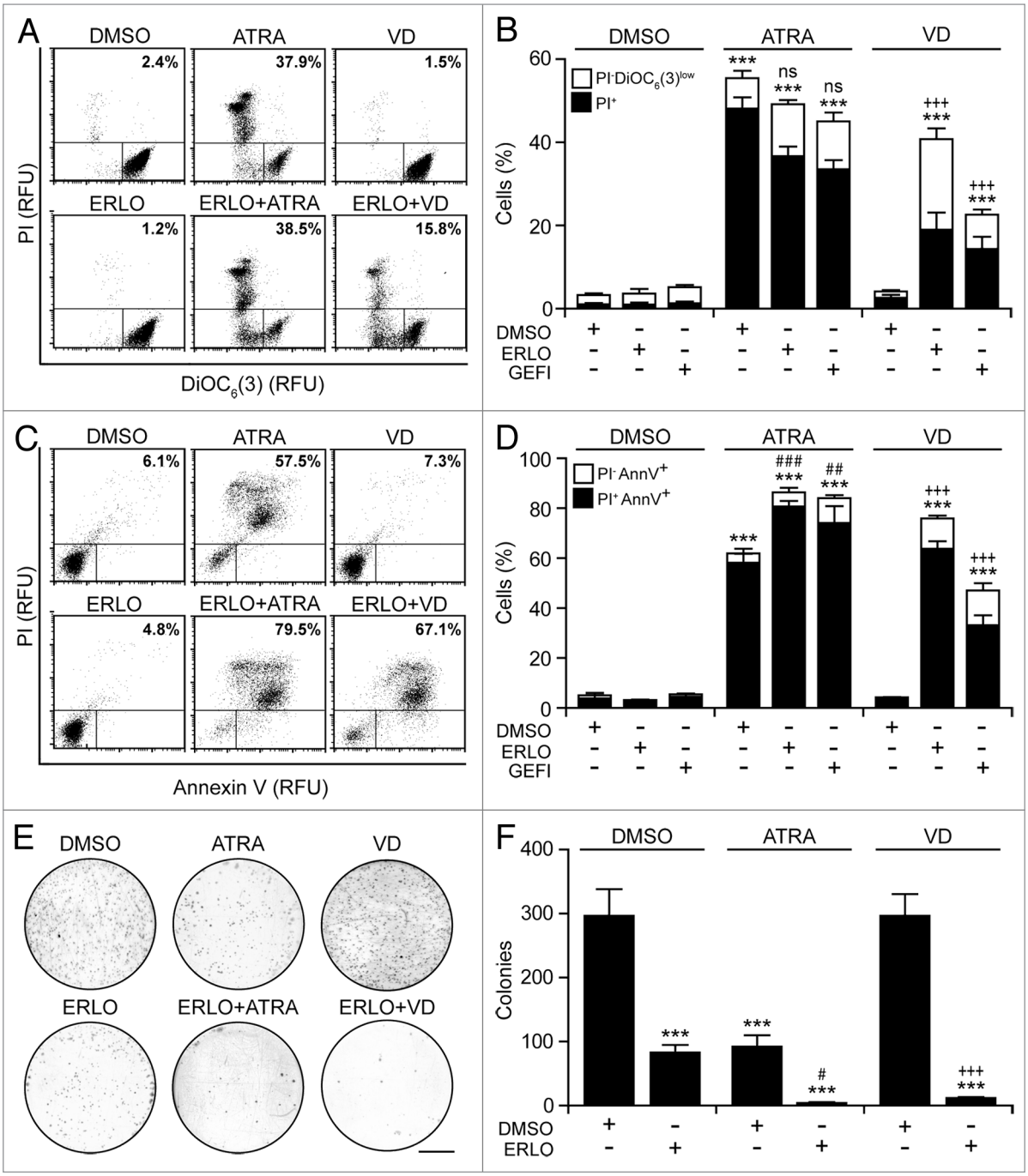

Figure 5. Apoptotic response of AML cells undergoing terminal differentiation. (**A–D**). Human acute myeloid leukemia (AML) HL-60 cells were treated with 10 μM erlotinib (ERLO), 10 μM gefitinib (GEFI), or an equal volume of DMSO, alone or in combination with 100 nM all-*trans* retinoic acid (ATRA), or 50 nM 1α,25-hydroxycholecalciferol (VD), for 7 d (**A and B**) or 14 d (**C and D**), followed by the determination of apoptosis-related parameters upon $DiOC_6(3)$/propidium iodide (PI) (**A and B**) or AnnexinV/PI (**C and D**) co-staining. Representative dot plots are depicted in (**A and C**) (numbers refer to the percentage of PI$^+$ cells). In (**B and D**), black and white column illustrate the percentage of dead (PI$^+$) or dying ($PI^-DiOC_6(3)^{low}$ or PI^-AnnV^+) cells, respectively (means ± SEM; n = 3). (**E and F**) Alternatively, HL60 cells were cultured in solid media in the presence of 10 μM ERLO or an equivalent volume of DMSO, alone Oncology Drug Set (which comprises most FDA-approved chemotherapeutics) or combined with 100 nM ATRA or 50 nM VD for 14 d, followed by the assessment of clonogenicity. Representative images (scale bar = 1 cm) and quantitative data (means ± SEM; n = 3) are reported in (**E and F**), respectively. In (**B, D, and F**), ***P < 0.001 (ANOVA plus Dunnett post-hoc test), as compared with DMSO-treated cells; #P < 0.05, ##P < 0.01, ###P < 0.001 (ANOVA plus Bonferroni post-hoc test), as compared with ATRA-treated cells; +++P < 0.001 (ANOVA plus Bonferroni post-hoc test), as compared with VD-treated cells.

Thus, erlotinib appears to exacerbate the capacity of ATRA and VD to promote the differentiation not only of AML cell lines, but also of primary leukemic cells from AML patients, at least in a fraction of cases.

Concluding Remarks

Our results reveal the capacity of erlotinib to favor the differentiation of primary or cultured AML cells as triggered by ATRA or VD. In particular, erlotinib stimulated the acquisition of multiple differentiation markers by HL-60 cells exposed to suboptimal doses of ATRA (CD11b expression on the cell surface and NADPH oxidase activity) or VD (CD14 expression on the cell surface and monocyte-specific esterase activity). Moreover, in the presence of ATRA or VD, the pharmacological inhibition of EGFR with erlotinib promoted several processes that normally accompany terminal differentiation, including a proliferation arrest in the G_0/G_1 phases of the cell cycle, cellular senescence, and apoptosis. Of note, erlotinib (and gefitinib) alone exerted limited pro-differentiation activity, yet strongly synergized with VD (and less so with ATRA) in inducing the maturation of AML cells, confirming previous results by Miranda and colleagues.[44] Along similar lines, gefitinib alone reportedly fails to induce the differentiation of APL NB4 cells, yet synergizes with arsenic trioxide (As_2O_3) in doing so.[45] Moreover, the SFK inhibitor PP2 has recently been shown to rescue inducible differentiation in emergent ATRA-resistant myeloblastic leukemia cells, confirming the important role of SFKs in this process.[46]

The detailed molecular mechanisms whereby erlotinib exerts differentiation-inducing effects remain largely elusive. However, it appears plausible that this activity involves the inhibition of $p38^{MAPK}$ and/or SFKs, as (1) several distinct EGFR-targeting agents are known to interfere with the enzymatic functions of these kinases,[47,48] and (2) the pharmacological inhibition of $p38^{MAPK}$ or SFKs mimicked the differentiation-inducting effects of EGFR inhibitors combined with ATRA of VD.[40,44,49] $p38^{MAPK}$

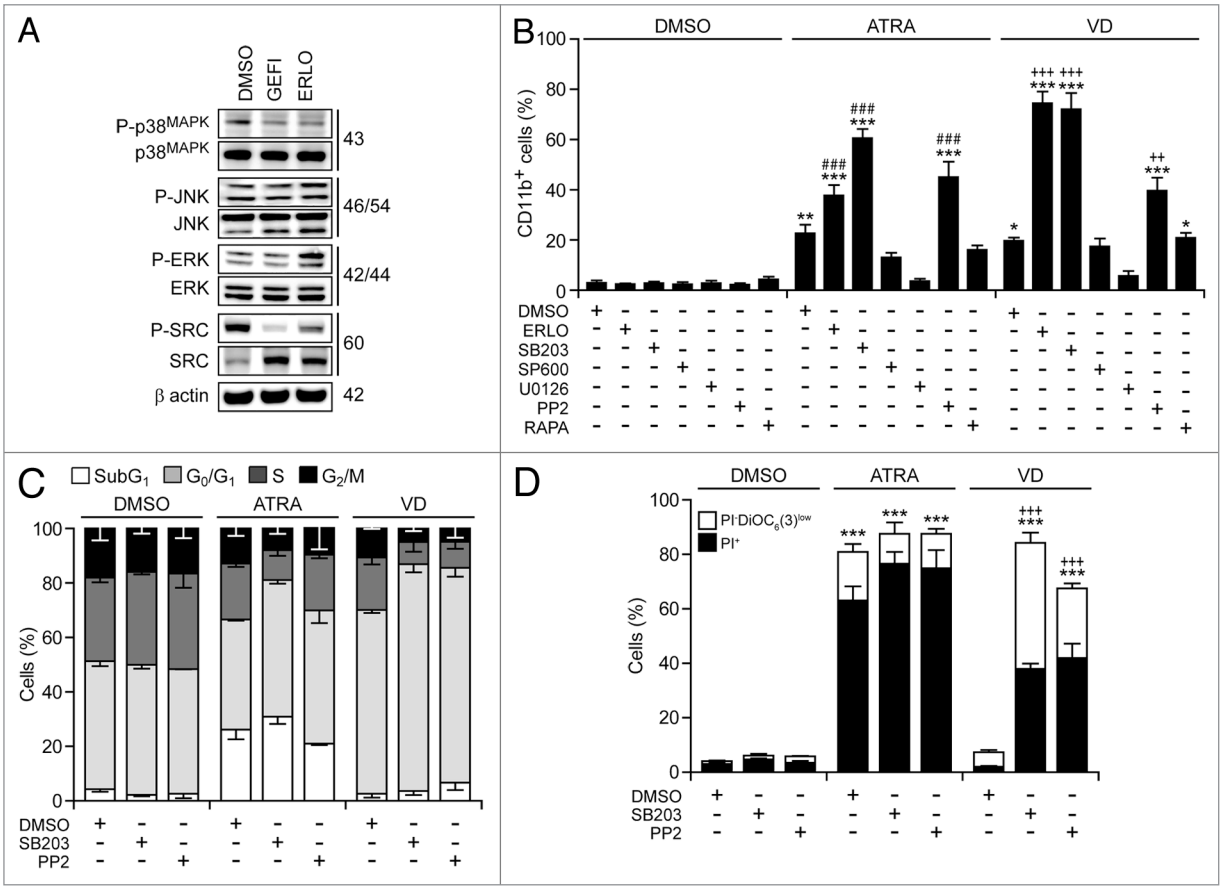

Figure 6. Molecular mechanisms underlying the differentiation-inducing effects of EGFR inhibitors. (**A**). Serum-starved human acute myeloid leukemia HL-60 cells were treated with 10 μM erlotinib (ERLO), 10 μM gefitinib (GEFI) or an equal volume of DMSO for 1 h, and then processed for the immunoblotting-assisted assessment of the phosphorylation status of the indicated proteins. Representative immunoblots are reported. β actin levels were monitored to ensure equal lane loading. (**B–D**). HL60 cells were exposed to 10 μM ERLO, 10 μM SB203580 (SB203), 10 μM SP600165 (SP600), 5 μM U0126, 10 μM PP2, 100 nM rapamycin (RAPA), or an equivalent volume of DMSO, alone or in combination with 100 nM all-*trans* retinoic acid (ATRA) or 50 nM 1α,25-hydroxycholecalciferol (VD), for 72 h, then processed for the cytofluorometric assessment of CD11b expression, upon immunostaining with a CD11b-specific antibody (**B**), cell cycle distribution, upon propidium iodide (PI) staining (**C**), or apoptosis-related parameters, upon $DiOC_6(3)$/PI co-staining (**D**). Quantitative data on the percentage of cells expressing CD11b and distribution in different phases of the cell cycle are reported in (**B and C**), respectively (means ± SEM; n = 3). In (**D**), black and white column illustrate the percentage of dead (PI^+) or dying ($PI^-DiOC_6(3)^{low}$) cells (means ± SEM; n = 3). In (**B and D**), $^*P < 0.05$,$^{**}P < 0.01$,$^{***}P < 0.001$ (ANOVA plus Dunnett post-hoc test), as compared with DMSO-treated cells; $^{###}P < 0.001$ (ANOVA plus Bonferroni post-hoc test), as compared with ATRA-treated cells; $^{++}P < 0.01$, $^{+++}P < 0.001$ (ANOVA plus Bonferroni post-hoc test), as compared with VD-treated cells.

phosphorylates serine and threonine (rather than tyrosine) residues and hence is unlikely to be directly inhibited by erlotinib or gefitinib. A recent report suggests that SRC is not a direct target of erlotinib either.[50] Thus, the erlotinib/gefitinib-mediated inhibition of $p38^{MAPK}$ and SFKs must involve an indirect signal transduction cascade that deserves further scrutiny. Interestingly, a large panel of TKIs (some of which exert a biological activity overlapping with that of erlotinib) also favored the differentiation of AML cells in the presence of ATRA and/or VD. These encompass alternative EGFR inhibitors (such as lapatinib),[51] as well as imatinib (targeting BCR-ABL, KIT and platelet-derived growth factor receptor, PDGFR)[52] and dasatinib,[53,54] which has a quite distinct inhibitory spectrum[55] including the SFK LYN.[56] The molecular effects of these TKIs remain to be precisely elucidated.

Of note, erlotinib has previously been shown to synergize with other anticancer agents, namely azacytidine and etoposide, in reducing the proliferation and inducing the apoptotic demise of AML cells.[57,58] These synergistic effects mainly reflect the ability of erlotinib to exacerbate the intracellular accumulation of azacytidine and etoposide as it inhibits multiple transmembrane transporters of the ATP-binding cassette (ABC) family.[57,58] However, at least under some circumstances, PP2 and SB203580 exacerbate the antiproliferative and cytotoxic response of HL-60 cells exposed to azacytidine. Moreover, the pharmacological inhibition of ABC transporters also promotes to some extent the differentiation-inducing effects of VD (Lainey et al., unpublished observations). Thus, there may be a link between the pharmacokinetic effects of erlotinib and its ability to inhibit $p38^{MAPK}$ and SFKs that warrants further investigation.

In synthesis, our results reveal an antileukemic cooperation between 2 groups of therapeutic agents, (1) relatively non-toxic natural compounds like ATRA and VD, which are well known for their differentiation-inducing activity, and (2) TKIs such as erlotinib, which can amplify the biological effects of the former. Erlotinib and gefitinib may normalize multiple signal transduction pathways that are deregulated in leukemic cells, including those centered around MAPKs and SFKs, and hence

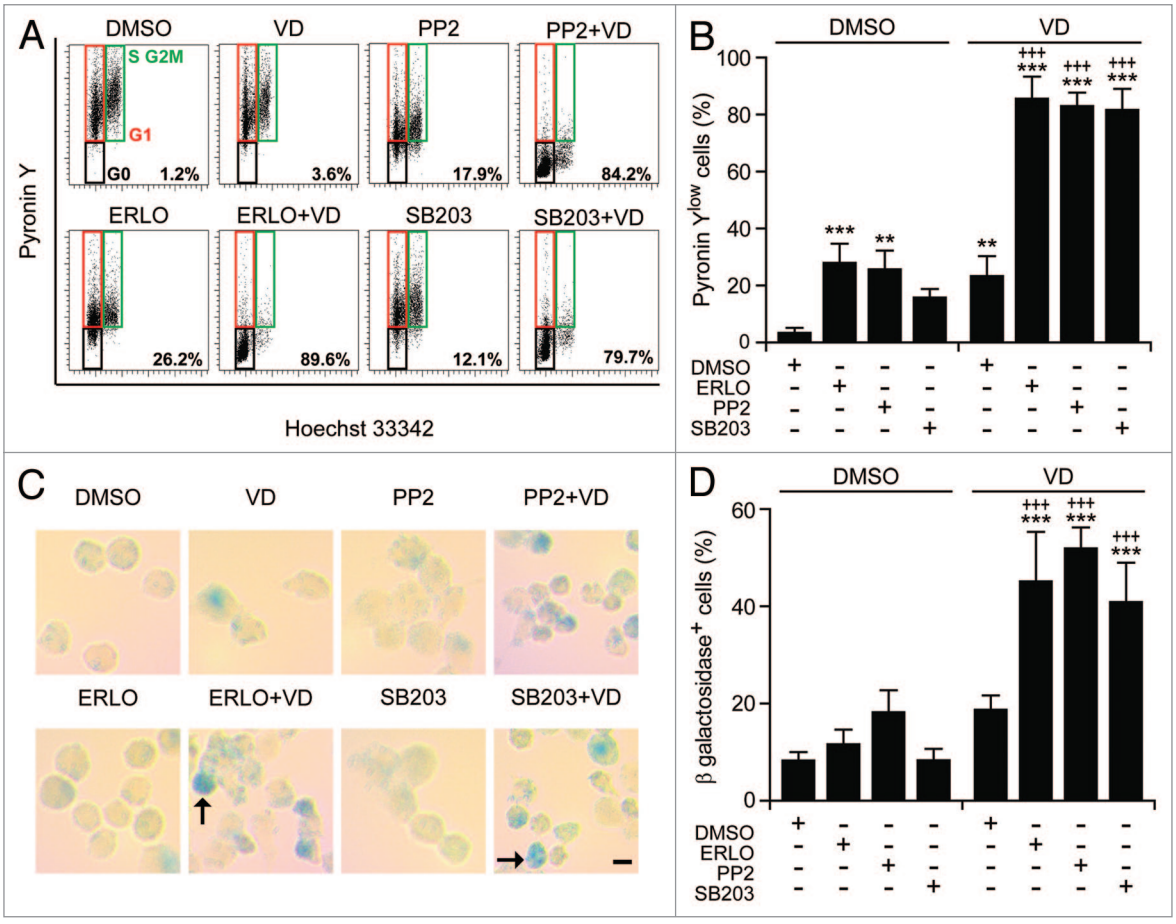

Figure 7. Metabolic aspects of myeloid differentiation. (**A–D**). Human acute myeloid leukemia HL-60 cells were treated with 10 μM erlotinib (ERLO), 10 μM PP2, 10 μM SB203580 (SB203), or an equivalent volume of DMSO, alone or in combination with 50 nM 1α,25-hydroxycholecalciferol (VD), for 7 d, then processed for the cytofluorometric detection of DNA and RNA content, upon Hoechst 33342/Pyronin Y co-staining (**A and B**) or the colorimetric detection of senescence-associated β-galactosidase activity (**C and D**). Representative dot plots and images are reported in (**A**) (numbers refer to the percentage of cells exhibiting a 2n DNA content and reduced RNA levels) and (**C**) (scale bar = 5 μM; arrows indicate senescent cells), respectively. Panels (**B and D**) report quantitative data on the percentage of Pyronin Y^{low} cells in the G_0/G_1 phase of the cell cycle or cells expressing senescence-associated β-galactosidase, respectively (means ± SEM; n = 3). $^{**}P < 0.01$, $^{***}P < 0.001$ (ANOVA plus Dunnett post-hoc test), as compared with DMSO-treated cells; $^{+++}P < 0.001$ (ANOVA plus Bonferroni post-hoc test), as compared with VD-treated cells.

may be useful as part of combinatorial therapeutic regimens. Further clinical studies are needed to fully elucidate the antineoplastic potential of these EGFR inhibitors and determine which specific subsets of AML patients may obtain therapeutic benefits from the administration of erlotinib and gefitinib.

Materials and Methods

Chemical, cell lines, and culture conditions

Unless otherwise indicated, media and supplements for cell culture were obtained by Gibco®-Life Technologies™ and plasticware from Corning. Erlotinib hydrochloride, gefitinib, imatinib mesylate, sorafenib, and sunitinib were purchased from LC Laboratories; dasatinib, lapatinib ditosylate, and nilotinib from Selleck Chemicals LLC; 1α,25-hydroxycholecalciferol (VD), all-*trans* retinoic acid (ATRA) and rapamycin from Sigma-Aldrich; 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl) pyrazolo[3,4-α]pyrimidine (PP2), SB203580, SB202190, and U0126 from Calbiochem; and SP600125 from InvivoGen. All chemicals were dissolved in DMSO and stored at –20 °C. Human AML HL-60 and MOLM-13 cells were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and cultured in RPMI 1640 medium supplemented with 20% fetal calf serum (FCS). Cells in the logarithmic phase of growth seeded at a density of 5×10^4 cells/mL were used for most experiments. For the long-term evaluation of cell death or senescence (at day 14), cells were seeded at the concentration of 1×10^4 cell/mL.

Patient samples and CD34$^+$ cell selection

Patient samples were studied after obtaining informed consent, as per the Declaration of Helsinki. AML was diagnosed by the morphological examination of peripheral blood and bone marrow biopsies, according to the World Health Organization (WHO) 2008 and French–American–British (FAB) classifications. Relevant clinicopathological features of the patients included in this study are reported in **Table S1**. To obtain CD34$^+$ cells, peripheral blood mononuclear cells (PBMCs) were isolated on a Ficoll–Paque PLUS density gradient (Amersham Biosciences) and the subjected to positive selection with the MiniMacs system (Miltenyi Biotec), according to the manufacturer's instructions. Patient-derived CD34$^+$ cells were cultured in standard conditions (37 °C, 5% CO_2) in Iscove modified Dulbecco medium (IMDM) supplemented with 1% *L*-glutamine, 100 units/mL penicillin sodium, 100 μg/mL streptomycin sulfate, 10 ng/mL interleukin-3 (Peprotech), 10 ng/mL interleukin-6 (Peprotech), 50 ng/mL thrombopoietin (Peprotech), 100 ng/mL FLT3-ligand (Miltenyi Biotec), 50 ng/mL stem cell factor (SCF) (Miltenyi Biotec), and 20% BIT 9500 serum substitute (200 μg/mL transferrin, 10 μg/mL insulin, 2% bovine serum albumin; StemCell Technologies).

High-throughput screening for myeloid cell differentiation

Ten thousand HL-60 or MOLM-13 cells were seeded in V-shaped 96-well plates in 100 μL complete medium and treated with compounds from the US Drug Collection (Microsource Discovery Systems; final concentration = 1 μM) or the Oncology Drug Set (NCI/DTP Open Chemical Repository Collection; final concentration = 10 μM), alone or combined with 100 nM ATRA or 50 nM VD, for 3 d. Thereafter, cells were washed twice with PBS and stained with PBS supplemented with 10 μg/mL Hoechst 33342 (Molecular Probes®-Life Technologies™), to visualize nuclear morphology; 1:2000 Far Red Live/Dead® Fixable Dead Cell Stain (Molecular Probes®-Life Technologies™), a vital dye that only incorporates into dead cells owing to permeabilized plasma membranes; 1:200 Human FcR Blocking Reagent (Miltenyi Biotec), to minimize unspecific antibody binding; and 1:25 fluorescein isothiocyanate (FITC)-conjugated anti-CD11b antibodies (clone Bear1, Beckman Coulter), for assessing granulo-monocytic differentiation, for 20 min at 4 °C. Cells were then washed, transferred into poly-*L*-lysine pre-treated Black/Clear 96-well Imaging Plates (Becton Dickinson), spun for 5 min at 350 g and fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature (RT). Four view fields per well were acquired by means of a BD Pathway 855 automated imaging station (Becton Dickinson) equipped with a 20× objective (Olympus)

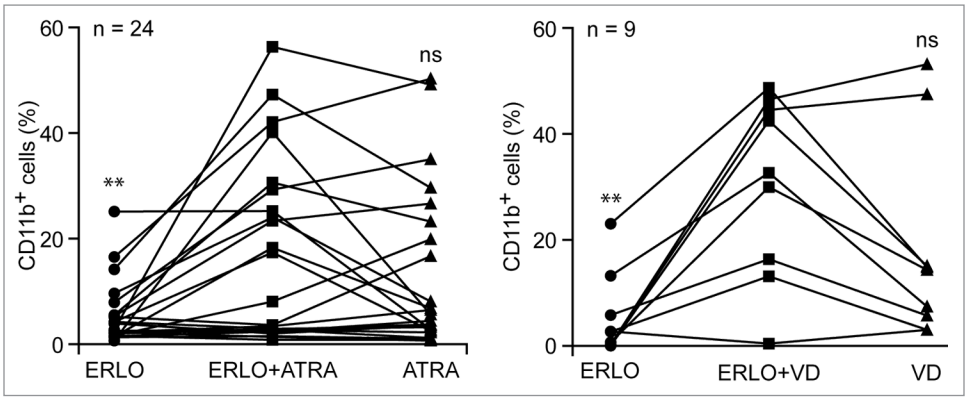

Figure 8. Effects of differentiation-inducing agents on primary leukemic cells from AML patients. CD34$^+$ cells isolated from the peripheral blood of acute myeloid leukemia (AML) patients were treated with 10 μM erlotinib (ERLO), 100 nM all-*trans* retinoic acid (ATRA), 50 nM 1α,25-hydroxycholecalciferol (VD) or an equivalent volume of DMSO, alone or in combination, for 3 d, then processed for the cytofluorometric assessment of CD11b expression upon immunostaining with a CD11b-specific antibody. Results are expressed as differences between the percentage of CD11b$^+$ cells upon treatment and that recorded in control conditions (i.e., among DMSO-treated cells). n.s., not significant, $^*P < 0.05$, $^{**}P < 0.01$ (ANOVA plus Student post-hoc *t* test), as compared with cells treated with ERLO plus ATRA or ERLO plus VD.

and coupled with a robotized Twister II plate handler (Caliper Life Sciences). Finally, image were segmented and analyzed for nuclear area, Live/Dead® Fixable Dead Cell Stain uptake, FITC intensity and FITC granularity by means of the BD AttoVision™ software (Becton Dickinson). Data were invariably normalized to take into account inter- and intra-plate signal variations.

Assessment of cellular differentiation by conventional approaches

For the morphological/cytochemical analysis of differentiation, AML cells were cytospun onto slides, fixed with absolute methanol for 15 min at RT, and then sequentially stained with the May–Grünwald and Giemsa reagents, as previously described.[29] Morphological and cytochemical hallmarks of differentiation (i.e., decreased cytoplasmic basophilia, decreased nucleus/cytoplasm ratio, appearance of granulation, nuclear lobulation) were then quantified among at least 100 cells for experimental condition on a Primo Star microscope (Zeiss) equipped with a Plan-Achromat 100×/1.25 oil immersion objective and an AxioCam EEc 5s CCD camera (both from Zeiss).

For the cytofluorometric assessment of differentiation, AML cells were harvested, washed with PBS, incubated with the Human FcR Blocking Reagent for 15 min at RT, and then stained with allophycocyanin (APC)-conjugated anti-CD11b (5 µL/sample; clone D12, Becton Dickinson) and phycoerythrin (PE)-conjugated anti-CD14 antibodies (10 µL/sample; clone RMO52, Beckman Coulter) for 20 min at 4 °C (under protection from light) in the presence of 10 µg/mL 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes®-Life Technologies™). Isotypic APC-conjugated mouse IgG2a and PE-conjugated IgG1 antibodies (both from Beckman Coulter) were used to determine threshold levels. Samples were run on a FACSCalibur™ (BD Biosciences) or a Gallios™ cytometer (Beckman Coulter) and first-line statistical evaluations were performed on live ($DAPI^-$) cells by means of the CellQuest™ (BD Biosciences) or Kaluza™ (Beckman Coulter) software.

The capacity of mature myeloid cells to undergo respiratory bursts was assessed by measuring their nitroblue tetrazolium chloride (NBT)-reducing activity, as previously described.[59] Briefly, AML cells were harvested and incubated for 30 min at 37 °C with Hanks buffer freshly supplemented with 1 µg/mL phorbol-12-myristate-13-acetate and 2 mg/mL NBT (both from Sigma-Aldrich). Samples were then cytospun on slides for 5 min at 350 g and the percentage of blue cells (containing insoluble formazan) was quantified on a Primo Star microscope (Zeiss) equipped with a Plan-Achromat 100× oil immersion objective. At least 100 cells per experimental condition were counted. Monocyte-specific α-naphtyl acetate esterase activity was evaluated with the LEUCOGNOST® EST kit (Merck Millipore), according to the manufacturer's instructions.

Cytofluorometric assessments of cell proliferation

Cell number was quantified by adding a predefined amount of fluorescent AlignFlow™ Plus Alignment Beads (Molecular Probes®-Life Technologies™), emitting at 488 nm, to 500 µL of cell suspensions. Thereafter, samples were evaluated on a Gallios™ cytometer (Beckman Coulter) for the number of cells acquired together with 3000 beads (both identified based on forward and side scatter parameters), allowing for the estimation of cell:bead ratios. Alternatively, cell proliferation was assessed by means of the CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes®-Life Technologies™), as previously described.[34,60]

Clonogenic assays

For the assessment of clonogenicity, 2×10^3 HL-60 cells were seeded in MethoCult® H4434 classic methylcellulose-based medium (StemCell Technologies) and treated with 2.5 µM erlotinib, 0.1 µM ATRA, 50 nM VD, or an equivalent volume of DMSO, alone or in combination. Fourteen days later, dishes were imaged by means of a G:BOX imaging system (Syngene) and colonies were quantified with the GeneTools software (Syngene).

Cytofluorometric assessment of apoptosis and cell cycle distribution

For apoptosis determinations, cells were co-stained with 1 µg/mL propidium iodide (PI) (Sigma-Aldrich), which identifies cells with ruptured plasma membranes, and either 20 nM 3,3′dihexiloxalocarbocyanine iodide ($DiOC_6(3)$; Molecular Probes®-Life Technologies™), which measures mitochondrial transmembrane potential ($\Delta\psi_m$), or FITC-conjugated AnnexinV (Beckton Dickinson), which evaluate phosphatidylserine exposure, as previously reported.[61,62] For the assessment of cell cycle distribution, 5×10^5 cells were collected, washed once with ice-cold PBS and permeabilized with 100 µL 0.5% Triton X-100. Thereafter, cells were stained with 50 µg/mL PI in 200 µL PBS supplemented with 20 µg/mL (w/v) RNase A (Molecular Probes®-Life Technologies™) for 1 h at 4 °C. Cytofluorometric acquisitions were performed on a FACSCalibur™ or a Gallios™ cytometer at a low flow rate mode. First-line analysis was performed with the CellQuest™, DIVA 6.1 (BD Biosciences) or Kaluza™ software, upon gating on the events characterized by normal forward and side scatter parameters and discrimination of doublets in a FSC-A vs. FSC-H bivariate plot.

Assessment of quiescence and senescence

To precisely distinguish the G_0 and G_1 phases of the cell cycle, cells were harvested and incubated with 8 µM Hoechst 33342 in complete culture medium for 30 min at 37 °C, followed by staining with and 100 µg/mL Pyronin Y (Sigma-Aldrich), which specifically labels RNA, in the presence of 10 µM Hoechst 33342, for additional 15 min at 37 °C. Tubes were then kept on ice until the acquisition on a FACSVantage flow cytometer (BD Biosciences) equipped with blue (488 nM) and UV (350 nM) lasers. The percentage of resting cells (in the G_0 phase of the cell cycle) was estimated based on events having a diploid DNA content and low RNA levels on Hoechst 33342 vs. Pyronin Y dot plots. Cell senescence was evaluated using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology), according to the manufacturer's instructions.

Immunoblotting

Immunoblotting was performed according to conventional procedures.[63,64] Briefly, cells were lysed on ice in a buffer containing 1% NP40, 20 mM HEPES pH 7.9, 10 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM orthovanadate, 1 mM PMSF, 1 mM dithiothreitol, and 10 µg/mL aprotinin, leupeptin, pepstatin. Total protein extracts were then separated on pre-cast NuPAGE® Novex® 4–12% Bis-Tris Gels (Invitrogen®-Life Technologies™)

and subjected to standard immunoblotting procedures based on primary antibodies specific for phospho-p38^{MAPK} (Thr180/Tyr182) (rabbit monoclonal, #9215; Cell Signaling Technology); p38^{MAPK} (rabbit monoclonal, #2371; Cell Signaling Technology); phospho-p44/42^{MAPK} (Thr202/Tyr204) (rabbit monoclonal, #4377; Cell Signaling Technology); p44/42^{MAPK} (rabbit monoclonal, #4695; Cell Signaling Technology); phospho-SAPK/JNK (Thr183/Tyr185) (mouse monoclonal, #9255; Cell Signaling Technology); SAPK/JNK (rabbit antiserum, #9252; Cell Signaling Technology); phospho-SRC (Tyr416) (rabbit antiserum, #2101; Cell Signaling Technology); total SRC (mouse monoclonal, #05–184; Upstate Biotechnology-Millipore); and β actin (mouse monoclonal, #MAB1501; Chemicon-Millipore). Primary antibodies were revealed with appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham) combined with an enhanced chemiluminescence detection system (Amersham) on an ImageQuant LAS 4000 camera (GE Healthcare).

Data treatment and statistical analyses

Unless otherwise specified, all experiments were performed in triplicate parallel instances and independently repeated at least 2 times. Data were analyzed with Excel (Microsoft) and Prismv.5 (GraphPad Software Inc). Statistical significance was invariably assessed by means of ANOVA followed by Dunnett, Bonferroni, or Student *t* post-hoc tests, as appropriate. P values < 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

The authors declare no conflicts of interest.

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Supplementary Materials

Supplementary materials may be found here:
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