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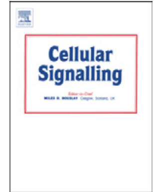

# GW5074 and PP2 kinase inhibitors implicate nontraditional c-Raf and Lyn function as drivers of retinoic acid-induced maturation ☆

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ABSTRACT

The multivariate nature of cancer necessitates multi-targeted therapy, and kinase inhibitors account for a vast majority of approved cancer therapeutics. While acute promyelocytic leukemia (APL) patients are highly responsive to retinoic acid (RA) therapy, kinase inhibitors have been gaining momentum as co-treatments with RA for non-APL acute myeloid leukemia (AML) differentiation therapies, especially as a means to treat relapsed or refractory AML patients. In this study GW5074 (a c-Raf inhibitor) and PP2 (a Src-family kinase inhibitor) enhanced RA-induced maturation of t(15;17)-negative myeloblastic leukemia cells and rescued response in RA-resistant cells. PD98059 (a MEK inhibitor) and Akti-1/2 (an Akt inhibitor) were less effective, but did tend to promote maturation-uncoupled G1/G0 arrest, while wortmannin (a PI3K inhibitor) did not enhance differentiation surface marker expression or growth arrest. PD98059 and Akti-1/2 did not enhance differentiation markers and have potential, antagonistic off-targets effects on the aryl hydrocarbon receptor (AhR), but neither could the AhR agonist 6-formylindolo(3,2-b)carbazole (FICZ) rescue differentiation events in the RA-resistant cells. GW5074 rescued early CD38 expression in RA-resistant cells exhibiting an early block in differentiation before CD38 expression, while for RA-resistant cells with differentiation blocked later, PP2 rescued the later differentiation marker CD11b; but surprisingly, the combination of the two was not synergistic. Kinases c-Raf, Src-family kinases Lyn and Fgr, and PI3K display highly correlated signaling changes during RA treatment, while activation of traditional downstream targets (Akt, MEK/ERK), and even the surface marker CD38, were poorly correlated with c-Raf or Lyn during differentiation. This suggests that an interrelated kinase module involving c-Raf, PI3K, Lyn and perhaps Fgr functions in a nontraditional way during RA-induced maturation or during rescue of RA induction therapy using inhibitor co-treatment in RA-resistant leukemia cells.

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## 1. Introduction

Two pathways that are extensively targeted via kinase inhibitors are the Raf/MEK/ERK (MAPK) and PI3K/Akt signaling cascades, which are known to be activated (often simultaneously) by growth factor, cytokine or hormone stimulation, and are frequently subject to deregulation in malignant cells [1]. The MAPK pathway, once thought to be a three-tiered chute for transducing membrane-initiated growth factor signaling, can relay complex signals that promote proliferation, mitosis, differentiation, apoptosis, motility or other cell-specific functions. Potent MAPK inhibitors cannot function as stand-alone treatments since too many processes depend on this pathway. Meanwhile PI3K/Akt activation is associated with pro-survival and anti-apoptotic signaling. There is significant interest in employing PI3K [2] and Akt [3] inhibitors to overcome resistance and re-sensitize cells to apoptosis-inducing agents.

Retinoic acid (RA) is a morphogenic compound and dietary factor that exerts pro-differentiative and anti-proliferative effects in normal and malignant contexts, including breast, lung and prostate cancers [4,5]. Yet as a cancer therapeutic, RA falls short of its initial success as a potent differentiation induction therapy for acute promyelocytic leukemia (APL, FAB M3), in which it induces remission in 80–90% of all cases [6]. There is great interest in combining RA with other chemical agents, such as other differentiation-inducing compounds, conventional chemotherapies, or kinase inhibitors, to both enhance efficacy and overcome emergent RA resistance in non-APL acute myeloid leukemia (AML). Current clinical trials assess the effect of RA combined with kinase inhibitors such as dasatinib, HDAC inhibitors, or inhibitors of lysine-specific demethylase 1 (for example: NCT00892190, NCT00867672, NCT00995332, NCT02261779). Here, we tested the effects of PD98059 (MEK inhibition), GW5074 (c-Raf inhibition), wortmannin (PI3K

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inhibition), Akti-1/2 (Akt inhibition) and PP2 (Lyn inhibition) during RA-induced maturation in the non-APL AML patient-derived myeloblastic leukemia (FAB M2) cell line HL-60 [7,8] and two RA-resistant sublines.

The patient-derived HL-60 cell line (FAB M2) lacks the PML-RARα fusion protein pathognomonic for APL, rendering HL-60 an attractive model for investigating RA-induced mechanisms in a t(15;17)-negative context [9]. RA-treated HL-60 cells undergoing differentiation display increased CD38 and CD11b expression and G1/G0 cell cycle arrest. With a doubling time of 20–24 h, RA-treated HL-60 cells complete two cell divisions and are committed to granulocytic differentiation by 48 h [10,11]. Sustained activation of the c-Raf/MEK/ERK cascade persists for 48 h and beyond after RA treatment [12]. However, inhibiting MEK after RA-induced HL-60 that has completed one division cycle does not inhibit RA-induced differentiation [13], indicating that sustained MEK or ERK activation may be necessary only during the lineage-uncommitted, priming phase and not for the second (lineage commitment) division. c-Raf phosphorylation concurrent with reduced MEK/ERK activation is known [14–18].

Despite the sustained ERK activation that occurs in RA-treated HL-60 cells, noncanonical c-Raf function has emerged as a hallmark of this system. c-Raf propels RA-induced differentiation [12,19] but induced phosphorylation at the c-Raf activating sites S338 and Y340/Y341 cannot be detected in RA-treated HL-60 [20,21]. Instead, phosphorylation occurs at the S259 putative inhibitory site [22], the S621 putative stability site [23] and the S289/296/301 c-Raf sites [24,25]. The S289/296/301c-Raf sites are targets of ERK, but whether these sites are inhibitory [26] or activating [27] remains unclear (see Discussion). pS621c-Raf undergoes nuclear translocation and interacts with transcription factors in RA-induced HL-60 cells [20,28]. Phosphorylated S259 has been shown specifically to prevent c-Raf membrane localization [29,30] and may promote Ras-independent and membrane-independent functions of c-Raf.

An RA-inducible interaction between the Src-family kinase (SFK) Lyn and pS259c-Raf was reported by Congleton et al. (2012) [24]. Lyn and Fgr are the predominant SFKs in myeloid cells [31,32] and both are upregulated by RA treatment in HL-60 cells [11,24]. Lyn displays RA-inducible phosphorylation at both its activating SFK Y416 site, which on Lyn is Y396 [24,33] and its inhibitory Y507 site [34]. Meanwhile Fgr is not phosphorylated at the SFK Y416 site (Y400 on Fgr) after RA treatment [24], implicating Lyn as the primary active SFK in RA-treated HL-60, as well as in NB4 cells [35]. We recently reported that Lyn and PI3K exhibit RA-inducible interaction and phosphorylation, and both bind to the RA-upregulated surface marker CD38, which is known to propel differentiation [36]. A Lyn/PI3K interaction in the context of differentiation has been shown [36,37]. Thus despite the attractiveness of PI3K inhibitors to diminish cell survival signaling, PI3K inhibition may have a negative effect on RA-induced maturation. Akt is the downstream effector of PI3K, and interestingly, Akt is able to phosphorylate c-Raf at S259 [38].

We previously developed two sequentially emergent RA-resistant HL-60 cells by chronic RA-exposure. Both fail to upregulate CD11b expression, G1/G0 arrest and signaling factor expression/activation after RA treatment [25]. However, one RA-resistant cell line retains RA-inducible CD38 expression (R38+ HL-60) while the other has lost this marker as well (R38- HL-60). The SFK inhibitor PP2, which enhances RA-induced differentiation in wild-type HL-60 and NB4 cells [24,39], rescues differentiation in both R38+ and R38− RA-resistant HL-60 cells [25]. Using the kinase inhibitors PD98059, GW5074, wortmannin, and Akti-1/2, we found that the c-Raf inhibitor GW5074 also emerges as an augmenter of RA-induced differentiation in wild-type and RA-resistant HL-60 cells, and had a similar effect to PP2 as reported previously. In RA-resistant cells, GW5074 was more capable in rescuing early events in induced differentiation, whereas PP2 was more capable for rescuing late events, however contrary to expectation, the two were not synergistic.

## 2. Materials and methods

### 2.1. Cell lines and treatments

HL-60 cells, derived from the original patient isolates, were a generous gift of Dr. Robert Gallagher and maintained in this laboratory. Two retinoic acid (RA)-resistant HL-60 sublines (R38+ and R38−) were isolated as described previously [25]. Cell cultures were maintained in 1640 RPMI medium (Invitrogen, Carlsbad, CA) supplemented with heat-inactivated 5% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% antibiotic/antimycotic (Invitrogen) and grown at 37 °C in a 5% $CO_2$ humidified environment. Cells were seeded at $0.2 \times 10^6$ for 48 h experiments or at $0.1 \times 10^6$ for 72 h experiments. Cell viability was monitored via Trypan blue exclusion. All-*trans*-retinoic acid (RA; Sigma, St. Louis MO) was added from a stock solution of 5 mM in ethanol to cell cultures at a final concentration of 1 μM. PD98059 (Cell signaling, Danvers MA) was added from a 10 mM stock solution in DMSO to a final concentration of 2 μM in culture. GW5074 (Sigma) was added from a 10 mM stock solution in DMSO to a final concentration of 2 μM in culture. Wortmannin (Calbiochem/EMD Chemicals, San Diego CA) was added from a 5 mM stock solution in DMSO to a final concentration of 1 μM in culture. Akti-1/2 (Calbiochem) was added from a 10 mM stock solution in DMSO to a final concentration of 1 μM in culture. PP2 (Calbiochem), obtained as a 10 mM solution in DMSO, was added to cultures at a final concentration of 10 μM. The AhR agonist 6-Formylindolo(3,2-b)carbazole (FICZ; Enzo Life Sciences, Exeter, United Kingdom), was added from a 100 μM stock solution in DMSO to a final concentration of 100 nM in culture.

### 2.2. CD38 and CD11b quantification

$0.5 \times 10^6$ cells were centrifuged at 700 rpm for 5 min, and cell pellets were resuspended in 200 μl PBS containing 2.5 μl of phycoerythrin (PE)-conjugated anti-CD38 antibody and allophycocyanin (APC)-conjugated anti-CD11b antibody (BD Pharmingen, San Jose CA). Samples were incubated at 37 °C for 1 h, then analyzed by flow cytometry on a BD LSRII flow cytometer (BD Biosciences, San Jose CA). APC fluorescence (excitation using 633 nm, red laser) was collected with a 735 nm dichroic longpass and 660/20 nm bandpass filter. PE fluorescence (excitation at 488 nm, blue laser) was collected with a 550 nm dichroic longpass and 576/26 nm bandpass filter. Gates for untreated controls were set to exclude 95% of the live cell population peak.

### 2.3. G1/G0 arrest quantification

$0.5 \times 10^6$ cells were centrifuged at 700 rpm for 5 min, and cell pellets were resuspended in 200 μl of cold (4 °C) hypotonic propidium iodide (PI) staining solution containing 50 μg/ml propidium iodide, 1 μl/ml Triton X-100, and 1 mg/ml sodium citrate in PBS. Samples were incubated overnight at 4 °C. Nuclei fluorescence was then analyzed on a BD LSRII using 488 nm excitation and collected with a 550 nm dichroic longpass and 576/26 nm bandpass filter. Doublets were excluded from the analysis.

### 2.4. Western blotting

$15 \times 10^6$ cells were washed twice with PBS via centrifugation at 700 rpm for 5 min. For total lysate collection, cell pellets were resuspended in 200–350 μl M-PER lysis buffer (Thermo Scientific, Rockford IL) supplemented with protease and phosphatase inhibitor cocktails (Sigma) at 1:100 volume ratio. After incubation on ice for 30 min, lysates were cleared via centrifugation for 30 min at 13,000 rpm and DNA pellets were discarded. For collection of cytoplasmic and nuclear fractions, cell pellets were resuspended in 200 μl CER I buffer from the NE-PER extraction kit (Thermo Scientific), followed by subsequent kit instructions. The BCA Protein Assay kit (Thermo Scientific) was used

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