Analysis of Flt3 cellular signaling in leukemic cell lines by flow cytometry and phospho-specific antibodies.

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Abstract

Aberrant Flt3 signaling, often involving an internal tandem duplication or overexpression of the receptor, has been implicated in a number of acute leukemias. The effect of Flt3 inhibition on downstream signaling in leukemic cells is not well-known. Knowledge of these downstream effects will be important for the identification of mechanisms of response and resistance as well as the development of pharmacodynamic and predictive biomarkers for drugs targeting Flt3. In this study, we investigated the effect of a specific FLT3 inhibitor on signaling pathways in human myeloid and lymphoid leukemic cell lines. We used a combination of flow cytometry and phospho-specific antibodies to analyze the phosphorylation of signaling proteins in several AML and ALL cell lines following treatment with the drug or a control. Our results showed that the inhibitor decreased phosphorylation of Flt3, and of downstream kinases such as Erk, Akt, S6 ribosomal protein, Stat3, and Stat5. Further, our results indicate differences in Flt3 signaling responses in cell lines with either the Flt3 internal tandem duplication or the over-expressed Flt3 receptor. These results should further our understanding of the aberrant signaling underlying these leukemias and may lead to the discovery of specific biomarkers and more effective therapies.

Introduction

Flt3 is a receptor tyrosine kinase expressed by hematopoetic progenitor cells in the bone marrow, thymus, and lymph nodes. Amplification, mutation, or overexpression of Flt3 results in aberrant downstream signaling thus promoting cell survival, proliferation and inhibition of apoptosis. High expression of Flt3 is observed in 70% of AML cases, B-precursor cell ALL, a fraction of T-cell ALL, and CML in lymphoid blast crisis (1). Most common mutations of Flt3 kinase in cases with AML are internal tandem duplications (ITD) and activation loop mutations (AL). They result in constitutive Flt3 activity. The consequence of this activation is increased and persistent downstream signaling (2). Here we investigate the effect of a specific Flt3 inhibitor on downstream signaling in leukemic cell lines using combination of flow cytometry and phospho-specific antibodies.

Materials and Methods:

Human leukemic cell lines [MOLM14 (AML), MV4-11 (AML), MM6 (AML), and SEM (ALL)] were maintained in RPMI medium containing 10% fetal bovine serum. Cells were treated as indicated with a Flt3 inhibitor for indicated times and at indicated concentrations, harvested simultaneously, fixed with 3% formaldehyde, and permeabilized with 90% methanol. Cells were labeled with phospho-specific antibodies from Cell Signaling Technology and analyzed by flow cytometry on an FC500 flow cytometer (Beckman Coulter).

Cells	Type	Flt3 status	Other mutations
MOLM14	AML	ITD-JM-one allele	no translocation
MV411	AML	ITD-JM-two allele	4;11 translocation
MM6	AML	V592 mutation	
SEM	ALL	overexpressed	4;11 translocation

Antibodies	Site	Catalog #
Phospho-Erk, Alexa 488 conjugate	Thr202/Tyr204	#4374
Phospho-Erk, Alexa 647 conjugate	Thr202/Tyr204	#4375
Phospho-Akt, Alexa 488 conjugate	Ser473	#2336
Phospho-Tyrosine, Alexa 647 conjugate		#9415
Phospho-S6, Alexa 488 conjugate	Ser235/236	#4854
Phospho-Flt3, Alexa 488 conjugate	Tyr591	#3459
Phospho-Stat5	Tyr694	#9354
Phospho-Stat3	Tyr705	#9145

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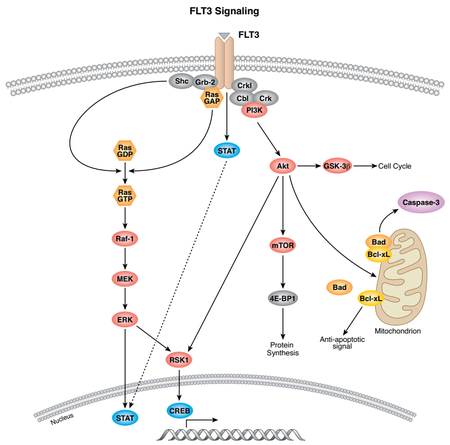
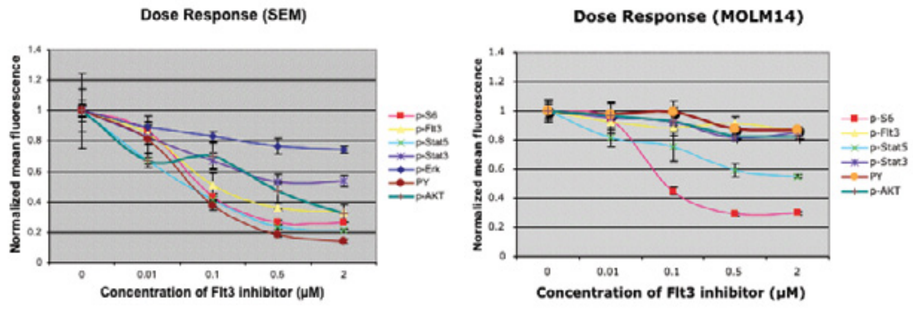
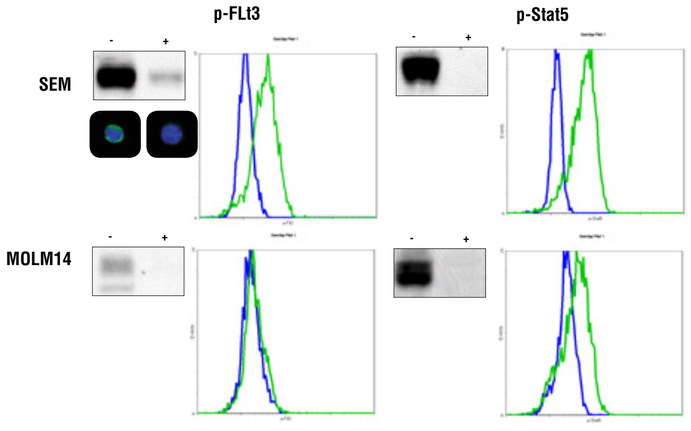


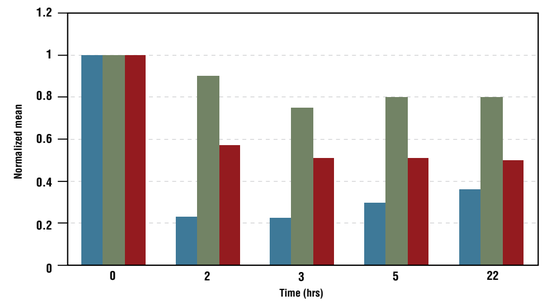


Dose response curves for SEM (top) and MOLM14 (bottom) cell lines. Cells were treated with 0 to 2 μM of Flt3 inhibitor and analyzed by flow cytometry with phospho-specific antibodies. Results are plotted as arithmetic means (±SD) of three separate experiments.



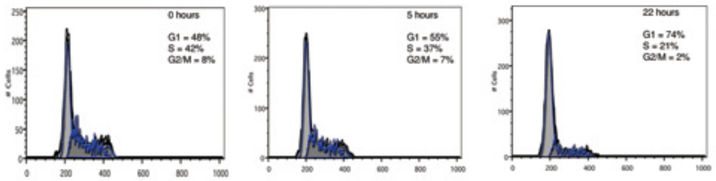
SEM and MOLM14 cells were treated with Flt3 inhibitor and analyzed by Western and flow cytometry (blue-treated, green- untreated) showing correlation between the two methods. In addition, SEM cells were labeled with p-Flt3 and examined by immunofluorescence showing complete loss of signal after treatment with the inhibitor.

Time course.



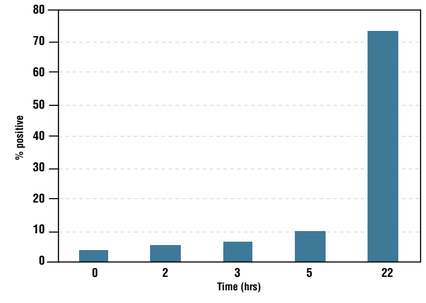
MOLM14 cells were treated with Flt3 inhibitor for 0, 1.5, 2.5, 5, and 22 hours. The cells were harvested simultaneously and analyzed for expression of p-S6 and p-Flt3.

Flt 3 inhibition induces cell cycle arrest.



MOLM14 cells were treated with Flt3 inhibitor for 0, 1.5, 2.5, 5, and 22 hours and analyzed for cell cycle phase distribution using propidium iodide and flow cytometry. DNA histograms corresponding to 0, 5, and 22 hour-time points are shown.

Percentage of Cleaved Caspase-3 positive cells.



MOLM14 cells were treated with Flt3 inhibitor for 0, 1.5, 2.5, 5, and 22 hours. The cells were harvested and analyzed for expression of Cleaved Caspase-3 by flow cytometry. Results are plotted as percentage of Cleaved Caspase positive cells at the indicated time-points.

Conclusion:

- Leukemic cell lines with different Flt3 mutations have different signaling profiles.
- The Flt3 inhibitor decreased signal from phospho-Tyrosine, phospho-Stat5, phospho-S6, phospho-Flt3, phospho-Akt, phospho-Stat3, and phospho-Erk in SEM cells (overexpress Flt3), while only phospho-S6 and phospho-Stat5 were significantly decreased in MOLM14 (Flt3 ITD), MM6 (V592), and MV411 (ITD) cells.
- Regardless of the mutation, phospho-S6 was the best indicator of Flt3 activity.
- Phospho-specific antibodies can used with flow cytometry to analyze aberrant cellular signaling in cancer, identify potential diagnostic or prognostic targets, and evaluate the efficacy of kinase inhibitors and other therapeutic agents.

References:

(1) Gilliland. Blood,1 September 2002, 100(5). (2) Jiang et al. Blood, 15 September 2004, 104 (6)

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