Synergistic induction of apoptosis by simultaneous disruption of the Bcl-2 and MEK/MAPK pathways in acute myelogenous leukemia

Michele Milella, Zeev Estrov, Steven M. Kornblau, Bing Z. Carter, Marina Konopleva, Ana Tari, Wendy D. Schober, David Harris, Clinton E. Leysath, Gabriel Lopez-Berestein, Ziwei Huang, and Michael Andreeff

Recent studies suggest that the Bcl-2 and mitogen-activated protein kinase (MAPK) pathways together confer an aggressive, apoptosis-resistant phenotype on acute myelogenous leukemia (AML) cells. In this study, we analyzed the effects of simultaneous inhibition of these 2 pathways. In AML cell lines with constitutively activated MAPK, MAPK kinase (MEK) blockade by PD184352 strikingly potenti-ated the apoptosis induced by the small-molecule Bcl-2 inhibitor HA14-1 or by Bcl-2 antisense oligonucleotides. Isobologram analysis confirmed the synergistic nature of this interaction. Moreover, MEK blockade overcame Bcl-2 overexpression-mediated resistance to the proapoptotic effects of HA14-1. Most importantly, simultaneous exposure to PD184352 significantly (P = .01) potentiated HA14-1-mediated inhibition of clonogenic growth in all primary AML samples tested. These findings show that the Bcl-2 and MAPK pathways are relevant molecular targets in AML and that their concurrent inhibition could be developed into a new therapeutic strategy for this disease. (Blood. 2002; 99:3461-3464)

Study design

Cell cultures

AML cell lines (OCI-AML3, HL-60, and KG1) were cultured under standard conditions14 and harvested in log-phase growth for every experiment. Bone marrow samples were obtained during routine diagnostic assessment after informed consent, in accordance with regulations and protocols sanctioned by the Human Subjects Committee of the University of Texas M. D. Anderson Cancer Center. Cell viability was evaluated by triplcic counting of trypan blue dye-excluding cells under a light microscope. AML blast colony assays were performed as previously described.15 HL-60 cells stably transfected with either Bcl-2 (HL-60/Bcl-2) or empty vector control (HL-60/neo) were kindly provided by Dr K. Bhalla (Moffitt Cancer Center, University of South Florida, Tampa, FL).16 PD184352 (2-[2-chloro-4-ido-phenilamino]-N-cyclopropylmethoxy-3,4-difluoro-benzamide), a highly selective inhibitor of MEK activation,13,17 was kindly provided by Dr J. S. Sebolt-Leopold (Cancer Molecular Sciences, Pfizer Global Research & Development, Ann Arbor, MI). HA14-1 (ethyl 2-amino-6-bromo-4-[1-cyano-2-ethoxy-2-oxoethyl]-4H-chromene-3-carboxylate), a small organic molecule selected for its ability to bind the surface pocket of Bcl-2, thereby disrupting its heterodimerization with Bax,12 (M.A., manuscript in preparation, December 2001), was obtained from Maybridge (Cornwall, United Kingdom); a structurally related
compound lacking Bcl-2 binding activity (ethyl 4-[cyano(ethoxy carbonyl)methyl]-4H-chromene-3-carboxylate) was used as negative control in all the experiments involving HA14-1. P-ethoxy Bcl-2 AS, complementary to the Bcl-2 translation initiation site (5'-CAGCGTGCCATCTCTTTCCC-3'), previously shown to efficiently decrease Bcl-2 expression in myeloid leukemic cells, or a scrambled sequence (5'-TCGACCTGGATCCTGCCC-3', nonsense, [NS]) were incorporated into liposomes as previously described.5

**Western blotting and apoptosis assays**

MAPK phosphorylation was detected by Western blot analysis as previously described.7 To measure mitochondrial membrane potential (ΔΨm), cells were loaded with CMXRos (300 nM) and MitoTracker Green (100 μM, both from Molecular Probes, Eugene, OR) for 1 hour at 37°C. The ΔΨm was then assessed by measuring CMXRos retention (red fluorescence) while simultaneously adjusting for mitochondrial mass (green fluorescence).16 Caspase activation was detected by flow cytometry using a fluorescein-conjugated cell-permeable peptide (FAM-VAD-FMK) that irreversibly and selectively binds to activated caspases (caspase-1 through -9) (CaspaTag, Intergen, Purchase, NY). Annexin V binding of externalized phosphatidylserine, cell permeability, and staining of nuclear DNA were analyzed as previously published.7

**Statistical analysis**

Synergism, additive effects, and antagonism were assessed using the Chou-Talalay method19 and CalcuSyn software (Biosoft, Ferguson, MO).

Briefly, the dose-effect curve for each drug alone was determined based on the experimental observations using the median-effect principle; the combination index (CI) for each experimental combination was then calculated according to the following equation:

$$CI = \frac{(D_1)}{(D_{1x})} + \frac{(D_2)}{(D_{2x})} + \frac{(D_1)(D_2)}{(D_{1x})(D_{2x})}$$

where (D1) and (D2) are the doses of drug 1 and drug 2 that have x effect when used in combination and (D1x) and (D2x) are the doses of drug 1 and drug 2 that have the same x effect when used alone. When CI = 1, this equation represents the conservation isobologram and indicates additive effects. CI values less than 1.0 indicate a more than expected additive effect (synergism).

**Results and discussion**

Three AML cell lines with different degrees of MAPK activation were used in this study. In OCI-AML3 (Figure 1A) and HL-60 cells, which show high constitutive MAPK activity, PD184352 (10 μM) rapidly (1 hour) abrogated MAPK phosphorylation by inhibiting the upstream kinase MEK. Conversely, KG1 cells showed little, if any, constitutive MAPK phosphorylation. However, PD184352 was able to abrogate phorbol myristate acetate-induced MAPK activation in these cells (Figure 1A). In OCI-AML3 and HL-60 cell lines, the simultaneous disruption of

![Figure 1. MEK inhibition potentiates HA14-1 cytotoxicity and apoptosis in AML cell lines with constitutive MAPK activation.](image-url)
Bcl-2/Bax heterodimerization by HA14-1 and interruption of MEK-to-MAPK signaling by PD184352 resulted in a striking decrease in cell viability (48 hours; Figure 1B). Conversely, PD184352 did not significantly modify the response of KG1 cells to HA14-1 (Figure 1B).

We then analyzed the apoptotic response of OCI-AML3 cells to HA14-1 alone or in combination with PD184352. Consistent with the disruption of Bcl-2 function,20 HA14-1 (12.5 µM) induced rapid (2 hours) but transient mitochondrial depolarization, whereas caspase activation was detected only after 24 hours and in a smaller fraction of the cells (Figure 1C-D). Simultaneous treatment with PD184352 (1.25 µM) did not affect the early (2-6 hours) phase of HA14-1–induced mitochondrial depolarization but strikingly potentiated both loss of ΔΨm and caspase activation at later times (24-48 hours; Figure 1C-D). The apoptotic nature of the cell death triggered by simultaneous disruption of the Bcl-2 and MAPK pathways was confirmed by the exposure of phosphatidylserine on the outer leaflet of the plasma membrane and a decrease in the DNA content to sub-G1 levels (data not shown).

The kinetics of apoptotic events observed in response to HA14-1 alone is consistent with previous results showing that mitochondrial depolarization alone, in the absence of caspase activation, cannot trigger a full apoptotic response.21 Preliminary data from our group suggest that, in fact, AML cells that only lose ΔΨm in response to HA14-1 retain substantial in vitro clonogenic ability (M.M., unpublished results, December 2001). The late potentiation observed in response to simultaneous MEK inhibition is compatible with recent evidence that the MAPK pathway protects against apoptosis at the level of cytosolic caspase activation, strongly implicating an inhibitor of apoptosis protein-like molecule.22 In this regard, we have recently demonstrated that MAPK inhibition specifically inhibits the expression of survivin in AML cells with constitutive MAPK activation within a compatible time frame.7,14 Alternatively, these 2 pathways may interact via MAPK-mediated phosphorylation of Bcl-2 itself and/or other Bcl-2 family members, such as BAD.9,23-25

We further analyzed the pharmacologic interactions between HA14-1 and PD184352 using a fixed-ratio experimental design and found that the simultaneous disruption of both pathways resulted in the synergistic (CI < 1.0) induction of apoptosis in cell lines showing constitutive MAPK activation (OCI-AML3 and HL-60; Figure 2A). Moreover, the exploration of a wider range of HA14-1 and PD184352 doses using different drug ratios (2:1 to 20:1) further confirmed the synergistic nature of this interaction (Table 2).
Table 1. Proapoptotic synergism of different HA14-1:PD184352 ratios in AML cell lines

<table>
<thead>
<tr>
<th>HA14-1:PD184352 ratio</th>
<th>OCI-AML3</th>
<th>HL-60</th>
<th>KG1</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:1</td>
<td>0.34 ± 0.03</td>
<td>0.41 ± 0.1</td>
<td>N/A</td>
</tr>
<tr>
<td>10:1</td>
<td>0.46 ± 0.01</td>
<td>0.41 ± 0.1</td>
<td>1.31 ± 0.1</td>
</tr>
<tr>
<td>20:1</td>
<td>0.47 ± 0.01</td>
<td>0.41 ± 0.1</td>
<td>N/A</td>
</tr>
</tbody>
</table>

N/A indicates not available.

*Average ± SD of the CI values obtained at the ED_{50}, ED_{75}, and ED_{90}.

1). Conversely, the combination of HA14-1 and PD184352 at a 10:1 ratio had a slightly antagonistic effect in KG1 cells (CI > 1.2; Figure 2A and Table 1).

The specificity of the interaction between Bcl-2 and the MEK/MAPK module was further studied using liposome-delivered Bcl-2 AS. As shown in Figure 2B, the combination of Bcl-2 AS and PD184352 at a 8:1 ratio caused substantially more apoptosis than either agent alone. Isobologram analysis confirmed that the proapoptotic interaction between Bcl-2 AS and PD184352 was indeed synergistic (CI = 0.36 ± 0.03; Figure 2B inset). Moreover, while Bcl-2 overexpression produced by stable gene transfer increased the resistance of HL-60–to-HA14-1–induced apoptosis (Figure 2C, left panels), this effect was overcome by simultaneous treatment with PD184352 (Figure 2C, right panels), which shifted the 90% effective dose (ED_{90}) of HA14-1 from 32 μM to 22 μM (ED_{90} in parental HL-60 cells, 23 μM).

Although widely used and undoubtedly useful as a model to conduct mechanistic studies, culture-adapted cell lines may not accurately reflect the behavior of patient-derived cancer cells. We therefore sought to confirm our cell line findings in primary AML samples. In all bone marrow samples tested in the AML blast clonogenic assay (n = 5), simultaneous exposure to a fixed concentration of PD184352 (1.25 μM) significantly potentiated the colony inhibitory effect of escalating doses of HA14-1 (6-12 μM; Figure 2D).

With rare exceptions, neoplastic cell growth is the result of multiple genetic alterations; therefore, any new clinically successful therapeutic strategies will most likely draw on the mechanism-based manipulation of multiple, cross-talking pathways involved in growth and survival control. From the standpoint of AML, our findings indicate that the simultaneous disruption of both the Bcl-2 and MAPK pathways synergistically induces apoptosis in cells showing constitutive MAPK activation. Because we have recently demonstrated that patients with AML with antiapoptotic Bax/Bcl-2 ratios and activated MAPK have a uniformly poor prognosis (S.M.K., manuscript submitted, September 2001), the disruption of the antiapoptotic crosstalk between the Bcl-2 and MAPK pathways could be useful exploited for therapeutic purposes in a population of patients with AML that is currently the least responsive to conventional treatment strategies.

Acknowledgments

The authors thank Judith S. Sebolt-Leopold (Pfizer Global Research and Development) for kindly providing PD184352, Jennifer Jones and Matthew Womble for their help with primary AML samples, and Rosemarie Lauzon for her assistance with the manuscript.

References


and chemotherapy of tumor cells resulting in the induction of apoptosis and loss of clonogene-


13. Carter BZ, Millela M, Altieri DC, Andreuff M, Cyto-


17. Erhardt P, Schreemser EJ, Cooper GM. B-Raf in-
hibits programmed cell death downstream of cyto-
tochrome C release from mitochondria by activat-

18. Breitschopf K, Haendeler J, Malcho P, Zeher AM, Dimmeter S. Posttranslational modification of Bcl-2 facilitates its proteasome-dependent degra-


20. Domin AM, Smith JH, Craig RW. Myeloid cell leukemia 1 is phosphorylated through two distinct pathways, one associated with extracellular sig-


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