Granulocyte-Macrophage Colony-Stimulating Factor/Interleukin-3 Fusion Protein (pIXY 321) Enhances High-Dose Ara-C-Induced Programmed Cell Death or Apoptosis in Human Myeloid Leukemia Cells

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High dose Ara-C (HIDAC) induces programmed cell death (PCD) or apoptosis in vitro in human myeloid leukemia cells, which correlates with the inhibition of their clonogenic survival. Hematopoietic growth factors (HGFs) granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) have been demonstrated to enhance the metabolism and cytotoxic effects of HIDAC against leukemic progenitor cells. We examined the effect of pIXY 321 (a GM-CSF/IL-3 fusion protein) on HIDAC-induced PCD and related gene expressions as well as the PCD-mediated colony growth inhibition of human myeloid leukemia cells. Unlike the previously described effects of HGFs on normal bone marrow progenitor cells, exposure to pIXY 321 alone for up to 24 hours did not suppress PCD in HL-60 or KG-1 cells.

However, exposure to pIXY 321 for 20 hours followed by a combined treatment with Ara-C plus pIXY 321 for 4 or 24 hours versus treatment with Ara-C alone significantly enhanced the oligonucleosomal DNA fragmentation characteristic of PCD. This was temporally associated with a marked induction of c-jun expression and a significant decrease in BCL-2. In addition, the treatment with pIXY 321 plus HIDAC versus HIDAC alone produced a significantly greater inhibition of HL-60 colony growth. These findings highlight an additional mechanism of HIDAC-induced leukemic cell death that is augmented by cotreatment with pIXY 321 and may contribute toward an improved antileukemic activity of HIDAC.

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MATERIALS AND METHODS

Cells. The human myeloid leukemia cells HL-60 and KG-1 were derived from the original lines.17,18 They are maintained in suspension culture as previously described.19

Drugs. Ara-C hydrochloride was purchased from Sigma Chemicals (St Louis, MO). pIXY 321 was kindly provided by Dr Douglas E. Williams (Immunex Corp, Seattle, WA). The specific activity of pIXY 321 is 1 x 10^6 U/μg of the protein.16 pIXY 321 concentration of 5 ng/mL was used for all experiments because this was determined to maximal biologic activity as an HGF in the colony culture assays of normal and leukemic BM progenitor cells (data not shown).

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DNA Fragmentation

The internucleosomal DNA fragmentation was assayed by a modification of previously described methods.\(^3\) Cells were treated with Ara-C 1 to 100 \(\mu\text{mol} / \text{L}\) for 4 or 24 hours. Alternatively, cells (5 \(\times\) 10^6/mL) were incubated overnight for 20 hours with or without PIXY 321 (5 ng/mL). After this incubation, 1 to 100 \(\mu\text{mol} / \text{L}\) Ara-C was added for 4 hours. At the end of these treatments, cells were pelleted and washed with phosphate-buffered saline (PBS) at 4°C and disrupted by suspension for 20 minutes at 4°C in 5 mmol/L Tris-HCL buffer containing 0.5% (vol/vol) Triton-X-100 (New England Nuclear, Boston, MA) and 20 mmol/L EDTA. The cellular lysates were centrifuged at 27,000g for 20 minutes to separate low molecular weight DNA from intact chromatin. The pellet was resuspended in the lysis buffer (0.5 mL), and 50 \(\mu\text{l}\) of bovine serum albumin (BSA) (2 mg/mL) was added to this as well as to the supernatant. After adding 1 mL of 10% trichloric acid (TCA), the microfuge tubes were centrifuged for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1 mL of 100% ethanol at 4°C and incubated at –20°C. After another centrifugation, the pellet was treated with 500 \(\mu\text{l}\) of 1N sodium hydroxide to hydrolyze RNA and incubated at 37°C for 1 hour, or until it was resuspended. The reaction mixture was incubated for 15 minutes on ice, treated with 1.5 mL of 20% TCA for 30 minutes at 4°C, and then centrifuged at 4°C for 10 minutes at 1,500 rpm. The pellet was vortexed or sonicated and DNA was hydrolyzed with 1 mL of 5% perchloric acid (PCA) for 15 minutes at 90°C with frequent vortexing to break up the pellet and release DNA from the pellet. Subsequently, the reaction mixture was centrifuged at 3,000 rpm and the supernatant was treated overnight at 37°C with 2 mL of Burton’s reagent which uses a diphenylamine reaction. The absorbance at 600 nm was compared with that of 250 \(\mu\text{g}\)/mL of DNA as standard, using linear regression. The quantity of fragmented DNA in the supernatants was expressed as percent of total DNA from 2 \(\times\) 10^7 cells.

For qualitative DNA analysis, 5 \(\times\) 10^6 cells were treated with PIXY 321 and/or Ara-C, as described above. At the end of the incubations, cells were washed in PBS and resuspended in the lysis buffer, as described above. The suspension was centrifuged and DNA was extracted from the supernatant by treatment with phenol, and chloroform/isoamyl alcohol (25:24:1). To the upper aqueous layer placed in a fresh tube, 50 \(\mu\text{l}\) of 3 mol/L sodium acetate and 1 mL of 100% ethanol were added and the total cellular RNA extracted through a phenol/chloroform/isoamyl alcohol extraction and precipitation with ethanol, and electrophoresed DNA was transferred overnight to nylon membranes by standard capillary methods. Blots were UV-cross-linked and hybridized at 55°C overnight in 50% formamide/10% dextran sulfate/1% sodium dodecyl sulfate (SDS)/5.6% sodium chloride containing denatured salmon sperm DNA. Separately, fragmented DNA from drug-treated leukemic cells was used to make a cDNA probe labeled by the random hexamer priming system by Prime-a-Gene (Fisher, Norcross, GA). Blots were washed for 30 minutes at 27°C in 2X SSC/0.1% SDS, 30 minutes at 65°C in 0.1X SSC/0.1% SDS, and autoradiographed for 10 minutes to 5 hours at ~70°C.

RNA Extraction and Northern Analysis

RNA extraction. Total cellular RNA was extracted by the guanidine thiocyanate-phenol-chloroform method. After exposure to the designated concentrations and schedules of PIXY 321 and/or Ara-C (see above), cells were washed with PBS and homogenized in GIT buffer (4 mol/L guanidine isothiocyanate, 2 mol/L sodium acetate pH 4, 0.1 mol/L 2-mercaptoethanol). The homogenate was then treated with 10% Sarkosyl (Sigma, St Louis, MO), 2 mol/L sodium acetate, and the total cellular RNA extracted through a phenol/chloroform/isoamyl alcohol suspension (25:24:1) and subsequent centrifugation. The resulting RNA pellet was washed with absolute ethanol, dissolved in diethyl pyrocarbonate-treated water, and stored at –20°C until used. Total RNA extracted by this procedure has a A260/A280 ratio of 1.95. The level of BCL-2, c-jun, c-myc, and \(\beta\)-actin RNA is determined by Northern blot analysis (vide infra) using the following 32P-labeled probes: (1) the 1.8-kb BamHI/EcoRI insert of a human c-jun complementary DNA purified from a pBluescript SK (+) plasmid; (2) the human c-myc probe was a 1.3-kb EcoRI/Clal fragment from the PMC 445 plasmid; (3) the human \(\beta\)-actin probe was 600-bp EcoRI/BamHI fragment from the KSII (+) plasmid; and (4) murine BCL-2 probe was a 830-bp EcoRI/HindIII fragment from pBluescript plasmid. The probes were kindly provided as follows: Dr Donald Kufe (Dana Farber Cancer Center, Boston, MA), c-jun; Dr R. Dalla-Favera (Columbia University, New York, NY), c-myc; Dr Stanley Korsemeyer (Washington University, St Louis, MO), BCL-2; and Dr James S. Norris (Medical University of South Carolina, Charleston), \(\beta\)-actin.

Northern blot analysis. RNA hybridization analysis was performed as previously described. Total RNA (10 \(\mu\text{g}\)) obtained as described above, was mixed with electrophoresis sample buffer (100% deionized formamide, 37% formaldehyde, 10X MOPS, glyceral, 4% bromophenol blue, 4% xylene cyanol), denatured at 65°C for 15 minutes, and run on a 1.0% agarose gel in 1X MOPS buffer. The RNA in the gel was denatured with 0.05 N NaOH/0.15 mol/L NaCl for 30 minutes, and neutralized with 0.1 mol/L Tris pH 7.5/0.15 mol/L NaCl for 30 minutes. The gel was then placed in contact with nitrocellulose filter and the RNA was transferred to the solid support on a PosiBlot Pressure Blotter (Stratagene, La Jolla, CA) at 80 psi for 2 hours. The RNA was cross-linked to the filter by a UV Cross-linker (Stratagene). Prehybridization and hybridization of the RNA was performed in low stringency conditions. The nitrocellulose membrane was prehybridized overnight at 55°C in the following solution containing de-ionized formamide, 50% dextran sulfate, 10% SDS, and 100 \(\mu\text{g}/\text{mL}\) salmon sperm DNA. Nick-translated, 32P-labeled cDNA probes were heat denatured, cooled, and added to the buffer and hybridized for 18 to 72 hours at 55°C. The hybridized blots were washed twice in 2X SSC,
0.1% SDS at 65°C for 15 minutes per washing, and twice in 0.1X SSC, 0.1% SDS at 65°C, again for 15 minutes per washing. The blots were then exposed to Kodak X-Omat AR films (Eastman Kodak, Rochester, NY) with intensifying screens. The developed films were scanned by densitometry.

Colony growth of leukemic cells. The colony culture growth of the leukemic cells was determined by a minor modification of a previously described method. Briefly, cells were incubated with or without PIXY 321 (5 ng/mL) for 20 hours followed by additional treatment with or without Ara-C (10 or 100 μmol/L) for 4 hours. After these incubations, cells were washed twice and plated at a cell density of 5 × 10⁵ cells/mL of 0.3% agarose in the presence of 20% fetal bovine serum (FBS), 10% 5637 conditioned medium (CM) in 18 mm, 12 well tissue culture plates. The plates were incubated at 37°C, 5% CO₂ atmosphere and colonies consisting of 50 or more are counted at the end of 10 days.

Statistical analysis. Significant differences between values obtained in a population of leukemic cells (HL-60 or KG-1) treated with different experimental conditions were determined by paired t-test analyses.

RESULTS

The effect of the exposure to HIDAC (1, 10, or 100 μmol/L) for 4 or 24 hours on the oligonucleosomal fragmentation of DNA in HL-60 cells is depicted in Fig 1. It shows the ethidium bromide-stained agarose gels containing the electrophoresed DNA from the supernatant of lysed HL-60 cells (5 × 10⁶ cells) treated with the different doses and schedules of HIDAC. Treatment with 10 or 100 μmol/L Ara-C for 4 or 24 hours produced the characteristic ladder of oligonucleosomal DNA fragments that were 200 bp integer multiples in sizes (Fig 1, A and B, lanes 3 and 4). This effect was also seen in KG-1 cells (data not shown). A similar ladder of oligonucleosomal DNA fragmentation was observed in cells exposed to 1 μmol/L Ara-C for 24 hours (Fig 1B, lane 2). DNA in the lanes 1 in Fig 1 are from the supernatant of lysed, control cells incubated for 4 or 24 hours, respectively. Both lanes show a faint ladder of DNA fragments. This may be caused by apoptosis occurring in the small population of spontaneously differentiating HL-60 cells, as has been previously described.

Figure 2 shows the internucleosomal DNA fragmentation detected by the Southern blot method in HL-60 cells treated with different doses and exposure intervals of Ara-C. Panel A shows that a 4-hour exposure to 1.0 to 100 μmol/L (lanes 3 to 6) but not 1 μmol/L Ara-C (lane 2) produced the characteristic DNA fragmentation. It should be noted that being more sensitive, the Southern blot method could detect oligonucleosomal DNA fragmentation after treatment with 1.0 μmol/L Ara-C for 4 hours, which was not evident when the electrophoresed DNA was stained with ethidium bromide (Fig 1A, lane 2). The 50-fold greater sensitivity of the Southern blot method is further highlighted in the Fig 2B, where the DNA in the supernatant from 1 × 10⁵ lysed cells was used as opposed to the DNA from 5 × 10⁶ cells electrophoresed in the ethidium bromide-stained agarose gels. Panel B shows that a 4-hour exposure to 1 to 100 μmol/L Ara-C induced the characteristic internucleosomal DNA fragmentation that could be detected when only 1 × 10⁵ cells were used per condition. The sensitivity of the method was used to determine whether shorter than 4-hour exposure to Ara-C could result in the oligonucleosomal DNA fragmentation. Panel C shows that exposures shorter than 4 hours (0.24, 0.5, 1, and 2 hours) to 100 μmol/L Ara-C were insufficient to produce the DNA fragmentation even when 5 × 10⁶ cells were used per condition. These results suggest that the endonuclease-mediated internucleosomal DNA fragmentation produced

![Fig 1. HL-60 cells were exposed to 1.0 to 100 μmol/L Ara-C for 4 hours (A) or 24 hours (B). DNA fragments from the supernatant of lysed cells were electrophoresed in agarose gels (see text). Lane M in (A) and (B) contains a 123-bp marker DNA ladder. DNA in the four lanes of (A) (4 hours) and (B) (24 hours) was purified from cells treated as follows: lane 1, control cells; lane 2, 1.0 μmol/L Ara-C; lane 3, 10 μmol/L; lane 4, 100 μmol/L Ara-C.](image-url)
Fig 2. DNA fragmentation detected by a Southern blot method as described in the text. (A) A blot of electrophoresed DNA from the supernatant of $5 \times 10^6$ lysed HL-60 cells treated with 0.1 (lane 2), 1.0 (lane 3), 50 (lane 4), 500 (lane 5), or 1000 pmol/L Ara-C (lane 6). Lane 1 has DNA from the supernatant of untreated HL-60 cells. (B) A blot of electrophoresed DNA in the six lanes from the supernatant of $1 \times 10^6$ HL-60 cells electrophoresed in the six lanes. The lanes contain DNA from cells treated identically to the lanes in (A), but autoradiographed for 6 hours. (C) A blot of electrophoresed DNA in six lanes from the supernatant of $5 \times 10^6$ HL-60 cells treated with 100 pmol/L Ara-C for 15 minutes (lane 2), 30 minutes (lane 3), 1 hour (lane 4), 2 hours (lane 5), or 4 hours (lane 6). Lane 1 has DNA from the supernatant of untreated HL-60 cells. Autoradiography was performed for 1 hour.

by HIDAC follows treatments for intervals no shorter than 2 hours.

The effect of pIXY 321 on HIDAC induced oligonucleosomal DNA fragmentation in HL-60 and KG-1 cells is shown in Fig 3. The figure shows an ethidium bromide-stained agarose gel containing electrophoresed DNA from the supernatant of lysed 5 million cells (panel A, HL-60; panel B, KG-1 cells) in each of the lanes. It should be noted that the intensity of the ladder is only a qualitative assessment of the DNA fragmentation and the absolute differences in the quantity of fragmentation under different treatment conditions should be assessed by referring to the data in Table 1, which are discussed below. DNA in the lanes 1 of panels A and B are obtained from the supernatant of untreated, lysed HL-60 and KG-1 cells, respectively. Lane 2 in panel A and lane 3 in panel B show the characteristic ladder of DNA fragments from the cells treated with Ara-C (100 pmol/L) for 4 hours. DNA from KG-1 cells treated with pIXY 321 alone for 24 hours and then electrophoresed in lane 2, panel B did not show a significant difference from that in the lane 1, panel B. In contrast, DNA from HL-60 cells treated with pIXY 321 for 24 hours and then electrophoresed in lane 3 of panel A shows a slight increase in the intensity of the bands in the ladder. However, quantitative analysis of the fragmented DNA (Table 1) showed that pIXY 321 treatment did not

Fig 3. HL-60 (A) and KG-1 (B) cells were exposed to pIXY 321 (5 ng/mL) for 24 hours. During the last 4 hours of the incubation, Ara-C (100 pmol/L) was added to the suspension culture. After these incubations, cells were pelleted and lysed and cellular lysates were centrifuged to separate low molecular weight DNA from intact chromatin. Purified DNA fragments from the supernatant of lysed cells were electrophoresed in agarose gel (see text). DNA in lane M, 123-bp marker DNA ladder (GIBCO, BRL, Grand Island, NY). DNA in the four lanes of the two panels are from cells treated as follows. (A): Lane 1, untreated control cells; lane 2, 100 pmol/L Ara-C; lane 3, treated with pIXY 321; lane 4, treated with pIXY 321 plus Ara-C. (B): Lane 1, untreated control cells; lane 2, 100 pmol/L Ara-C; lane 3, treated with pIXY 321; lane 4, treated with pIXY 321 plus Ara-C.
with Ara-C produced a dose-dependent decrease in HL-60 colony growth. Although treatment with pIXY 321 alone significantly improved HL-60 colony growth to 124.3 ± 3.1, treatment with a combination of pIXY plus Ara-C (10 or 100 μmol/L) caused a significantly greater inhibition of colony growth as compared with Ara-C alone (Table 1). It should be noted that pIXY 321-mediated increase in HL-60 colony growth was not associated with the suppression of PCD or apoptosis, as reflected by an insignificant change in the DNA fragmentation in HL-60 cells (Table 1).

Based on the previous reports that HIDAC treatment rapidly induces c-jun and inhibits c-myc expression in human myeloid leukemia cells,10 we examined the effect of pIXY 321 on c-jun and c-myc RNA expression in HL-60 and KG-1 cells. Figure 4 shows a Northern blot of RNA from HL-60 cells hybridized to c-jun, c-myc, and β-actin probes. The first four lanes have RNA extracted from cells treated with pIXY and/or Ara-C for 4 hours in a schedule described above, while lanes 5 through 8 have RNA extracted from cells 24 hours after the 4-hour Ara-C treatment. Lanes 1 and 5 have RNA from untreated HL-60 cells, and lanes 2 and 6 have RNA from cells treated with pIXY 321 alone. In Fig 4A, exposure to pIXY 321 for up to 24 hours did not have any significant effect on c-jun expression. However, treatment with Ara-C alone markedly increased the expression of c-jun. It peaked after 4 hours of exposure to Ara-C (lane 3), but decreased significantly 24 hours after the treatment with Ara-C (lane 7).

Table 1. Effect of pIXY 321 on Ara-C-Mediated Oligonucleosomal DNA Fragmentation and Colony Growth Inhibition of HL-60 Cells

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Mean % Fragmented DNA*</th>
<th>% of Control Growth†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.5 ± 0.2</td>
<td>100.0</td>
</tr>
<tr>
<td>Ara-C (10 μmol/L)</td>
<td>8.2 ± 1.1</td>
<td>76.6 ± 3.3</td>
</tr>
<tr>
<td>Ara-C (100 μmol/L)</td>
<td>9.1 ± 1.0</td>
<td>42.8 ± 2.4</td>
</tr>
<tr>
<td>pIXY 321 (5 ng/mL)</td>
<td>0.6 ± 0.3</td>
<td>124.3 ± 3.1</td>
</tr>
<tr>
<td>pIXY 321 + Ara-C (10 μmol/L)</td>
<td>12.3 ± 1.4†</td>
<td>35.3 ± 2.8†</td>
</tr>
<tr>
<td>pIXY 321 + Ara-C (100 μmol/L)</td>
<td>13.5 ± 0.8†</td>
<td>23.6 ± 1.6†</td>
</tr>
</tbody>
</table>

Cells were incubated with or without pIXY 321 for 20 hours followed by treatment with 10 or 100 μmol/L Ara-C for 4 hours. After these treatments, cells were either washed and plated in agarose or pelleted and lysed (see text). Purified DNA in the supernatant and pellet was quantitated (see text) and DNA fragmentation was expressed as the percent of total DNA. Colony growth was counted and expressed as the percent of control colony growth.

*Mean ± SEM of three experiments.
†Mean ± SEM of five experiments.
‡Values significantly different (P < .05) from those seen in cells treated with Ara-C alone.

In lanes 4 of each panel containing DNA from cells treated with pIXY 321 plus Ara-C showed a more intense ethidium bromide staining of the ladder of DNA fragments, suggesting a greater amount of fragmented DNA in the lane, which was confirmed by the quantitative analyses of DNA fragmentation.

In Table 1, the quantitative analyses of the fragmented DNA in the supernatant released from the pellets, expressed as the mean percent of the total starting DNA, is compared with the inhibition in the colony growth due to treatment of HL-60 cells with pIXY 321 and/or Ara-C. Exposure to 10 μmol/L Ara-C increased DNA fragmentation from 0.5% ± 0.2% to 8.2% ± 1.1%, which was further enhanced to 12.3% ± 1.4% when the cells were exposed to pIXY 321 (5 ng/mL) plus Ara-C. No significant difference in the amount of DNA fragments was observed in the cells treated with 10 or 100 μmol/L Ara-C (Table 1). Cotreatment with pIXY 321 also enhanced the DNA fragmentation caused by 100 μmol/L Ara-C. Quantitation of DNA fragmentation following exposure to 1.0 μmol/L Ara-C for 24 hours showed significantly higher fragmentation as a mean percent (of two experiments performed in duplicate) of total starting DNA that was only slightly increased by cotreatment with pIXY 321, 33.2% and 34.9%, respectively. Also, not shown in Table 1, quantitation of the DNA fragmentation from untreated KG-1 cells showed a 0.2% fragmented DNA as the mean percent (of two experiments performed in duplicate) of the total starting DNA. After treatment with 100 μmol/L Ara-C for 4 hours, the DNA fragmentation increased to 10.1%. Treatment of KG-1 cells with pIXY 321 (5 ng/mL) alone for 24 hours had no effect on the DNA fragmentation, whereas cotreatment with pIXY 321 further increased Ara-C-mediated DNA fragmentation to 13.8%. Table 1 also shows that a 4-hour treatment with Ara-C produced a dose-dependent decrease in HL-60 colony growth.
was not affected by a concurrent treatment with PIXY 321. Changes in c-jun and c-myc expression caused by 10 pmol/L Ara-C.

Cells had decreased further (lane 6). Once again, PIXY 321 significantly reduced BCL-2 expression (lane 2), which this presumed housekeeping gene is also partly suppressed. Although not shown, pIXY 321 had similar effects on changes in c-jun and c-myc expression caused by 10 μmol/L Ara-C.

Figure 5 shows the effect of pIXY 321 on HIDAC-induced changes in BCL-2 expression in HL-60 cells. Exposure to (100 μmol/L) Ara-C for 4 hours produced a significant reduction in BCL-2 expression (lane 2), which was not affected by a concurrent treatment with pIXY 321 plus Ara-C (lane 4). Twenty-four hours after an exposure to HIDAC (for 4 hours), the expression of BCL-2 in HL-60 cells had decreased further (lane 6). Once again, pIXY 321 did not significantly affect the reduction in BCL-2 expression caused by Ara-C (lane 8). Lanes 7 and 8 again show a partial repression of β-actin gene caused by a 24-hour exposure to 100 μmol/L Ara-C. Similar changes in BCL-2 expression in HL-60 cells were observed after treatment with 10 μmol/L Ara-C for 4 hours and 1 μmol/L Ara-C for 24 hours. The reduction in BCL-2 expression occurred in temporal association with Ara-C ± pIXY 321-induced oligonucleosomal DNA fragmentation in HL-60 cells.

**DISCUSSION**

Recent studies have shown that a variety of DNA damaging antileukemic drugs, including cisplatin, etoposide, camptothecin, doxorubicin, and HIDAC, are also able to induce leukemic cell death by an alternative process of PCD or apoptosis,10,15,25-27 By using a Southern blot method with a 50-fold greater sensitivity to detect internucleosomal DNA fragmentation characteristic of PCD, we have been able to elaborate on previously published findings and show that a 4-hour exposure to 1.0 to 100 μmol/L Ara-C induces PCD in human myeloid leukemic cells. Also, our findings indicate that the exposure to these concentrations of Ara-C for intervals ≤2 hours does not result in the DNA fragmentation, and may indeed be the time required to activate the nuclear endonuclease-mediated internucleosomal DNA fragmentation in these cells. Because PCD is an active process of gene directed cellular self destruction, the molecular signals that govern this process are and indeed be triggered by the antileukemic agents may be modulated by a variety of growth factors or other mitogenic stimuli.11,26-28 In this report we show that the hematopoietic growth factor pIXY 321 significantly enhances HIDAC-induced PCD and colony growth inhibition of HL-60 cells. In addition, significant alterations in gene expressions described in association with PCD were also observed with treatment with pIXY 321 plus HIDAC.

The molecular signals generated by HIDAC-induced DNA damage include the activation of PKC.11 This results in a posttranslational activation of jun/AP-1 transcription factor, which then binds to the AP-1 binding target sites and causes increased c-jun transcription and expression.11,29 HIDAC-induced PCD in leukemic cells has been temporally correlated with increased c-jun transcription and expression.10,11,29 pIXY 321 is a GM-CSF/IL-3 fusion protein with improved receptor affinity and biologic activity as compared with either IL-3 and/or GM-CSF alone.16,30 Recent studies have shown that after binding to their receptors, GM-CSF and IL-3 initiate mitogenic signal transduction to the nucleus that includes serine phosphorylation and activation of Raf-1 kinase, as well as increased generation of diacylglycerol and the resultant activation of PKC.31-33 This is supported by the observation that PKC inhibitors suppress the proliferative response caused by GM-CSF and IL-3.34 PKC inhibitors have also been shown to inhibit the induction of c-jun transcription by Ara-C.29 Taken together, these reports highlight the potential interaction of pIXY 321-induced mitogenic signals with the molecular signals for PCD generated by HIDAC in leukemic cells. Parenthetically, bryostatin-1, which is a PKC stimulator, enhances Ara-C-induced DNA fragmentation and cytotoxic effects in human myeloid leukemia cells.35 A recent report has also shown that bryostatin-1 in conjunc-

**Fig 5.** HL-60 cells were exposed to pIXY 321 and/or Ara-C (100 μmol/L), as described in the text. Subsequently, cells were pelleted and total cellular RNA was extracted. The Northern blots of the electrophoresed RNA were hybridized with 32P-CTP labeled random primed cDNA probes of BCL-2 and β-actin. RNA in lanes 1 through 4 was extracted after 4 hours of Ara-C treatment and in lanes 5 through 8 from cells 24 hours after Ara-C treatment as follows: lanes 1 and 5, untreated control; lanes 2 and 6, pIXY 321 treated; lanes 3 and 7, Ara-C treated; lanes 4 and 8, pIXY 321 + Ara-C treated.
tion with GM-CSF markedly enhances Ara-C-mediated inhibition of clonogenic growth and self-renewal capacity of leukemic progenitor cells, while sparing a significant fraction of normal hematopoietic progenitor cells. If PCD is an important contributory mechanism producing substantial inhibition of the clonogenic survival of leukemic cells by the combinations of bryostatin-1, GM-CSF and Ara-C, then the data presented here suggest that the induction of PCD in different cell types may be disparately affected by the modulation of PKC activity. For example, it has been noted that phorbol esters which stimulate PKC block glucocorticoid-induced PCD in T cells, whereas the activation of PKC in sarcoma cells increases apoptosis caused by doxorubicin. The latter finding is more in line with our observations on the effects of PIXY 321 on HIDAC-induced PCD in HL-60 cells.

It is also noteworthy that unlike other PKC stimulators, treatment of HL-60 cells with PIXY 321 alone did not increase c-jun expression, nor did it further increase Ara-C-induced marked enhancement of c-jun expression. This suggests that other genes, eg, BCL-2, which is known to suppress PCD, may regulate Ara-C-induced PCD in leukemic cells. In pre-B leukemia cells, high levels of expression of BCL-2 have been demonstrated to block glucocorticoid-induced apoptosis. Recently, BCL-2 has also been shown in vitro to protect against apoptosis induced by nitrogen mustard and camptothecin. Our findings show that HL-60 cells express low levels of BCL-2, and that PIXY 321 did not affect BCL-2 expression in HL-60 cells. In addition, HIDAC-induced PCD in HL-60 cells was associated with a decrease in BCL-2 expression that was not affected by cotreatment with PIXY 321. It has been previously reported that in the cells expressing low levels of BCL-2 a continuous decrease in c-myc expression may be responsible for their growth arrest and apoptosis. Our results indicate that HIDAC induces a similar effect in HL-60 cells that is not compromised by cotreatment with PIXY 321. Of note, PIXY 321 did not further increase Ara-C-mediated inhibition of c-myc expression, although in a previous study in KG-1 cells, IL-3 was shown to increase the inhibition of c-myc caused by 1-log higher concentrations of Ara-C (1.0 mmol/L).

In addition to stimulating the proliferation and differentiation of BM progenitor cells, HGF's promote the survival of normal myeloid progenitor cells by suppressing their PCD. However, our data show that the treatment with PIXY 321 alone for up to 24 hours does not suppress PCD in HL-60 cells. It is noteworthy that HL-60 cells lack the expression of wild-type tumor suppressor gene p53. The transfection of p53 into myeloid leukemia cells has been shown to induce PCD without causing their differentiation. This suggests that the expression of the wild-type p53 is involved in apoptosis and in HL-60 cells the loss of its activity may allow their unrestricted growth. Indeed, this may be one of the mechanisms facilitating the development of myeloid leukemias. It is conceivable that a loss of p53 function in conjunction with a low expression of BCL-2 may be responsible for the inability of PIXY 321 alone to suppress PCD in HL-60 cells.

In previous studies, a combination of GM-CSF and IL-3 was shown to selectively enhance the antileukemic activity of HIDAC against leukemic but not normal BM progenitor cells. This was mostly attributed to HGF-induced increase in cycling S-phase cells and a selective enhancement in Ara-C metabolism in AML blasts. The present studies indicate that the HGF combination may also be improving the antileukemic activity of HIDAC by enhancing its ability to induce PCD in leukemic cells. Because PCD is also induced by a variety of antileukemic drugs that are not S-phase specific in their activity, HIDAC-induced PCD may also be operative in non-S phases of the cell cycle and may significantly contribute to the leukemic cell lethality. However, our data does not establish as to what extent the augmentation by PIXY 321 of Ara-C-mediated inhibition of HL-60 clonogenic survival is caused by the enhancement of PCD in the leukemic cells. This would require the use of agents or strategies that would differentially block Ara-C–induced PCD versus cell necrosis. Also, because of the inability to purify a homogenous population of normal BM progenitor cells in sufficient numbers to perform the quantitative DNA fragmentation studies presented here, we have not examined the effect of PIXY 321 on HIDAC-induced PCD in normal human BM progenitor cells. Nevertheless, if HIDAC-induced PCD plays an important role in vivo in mediating the rapid decrease of myeloid leukemia cell burden, by demonstrating that PIXY 321 augments HIDAC-induced PCD in HL-60 cells, our findings underscore an additional mechanism that can potentially be modulated to improve the antileukemic activity of HIDAC. These findings have potential implications in the setting of high-dose chemotherapy as well as ex vivo purging of AML blasts in autologous BM transplantation.

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