Retinoic Acid/Alpha-Interferon Combination Inhibits Growth and Promotes Apoptosis in Mantle Cell Lymphoma through Akt-Dependent Modulation of Critical Targets

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Abstract
Mantle cell lymphoma (MCL) is characterized by a profound deregulation of the mechanisms controlling cell-cycle progression and survival. We herein show that the combination of 9-cis-retinoic acid (RA) and IFN-α induces marked antiproliferative and proapoptotic effects in MCL cells through the modulation of critical targets. Particularly, IFN-α enhances RA-mediated G0–G1 cell accumulation by downregulating cyclin D1 and increasing p27Kip1 and p21WAF1/Cip1 protein levels. Furthermore, RA/IFN-α combination also induces apoptosis by triggering both caspases-8 and -9 resulting in Bak and Bak activation. In particular, RA/IFN-α treatment downregulates the antiapoptotic Bcl-xL and Bfl-1 proteins and upregulates the proapoptotic BH3-only Noxa protein. Sequestration of Mcl-1 and Bfl-1 by upregulated Noxa results in the activation of Bid, and the consequent induction of apoptosis is inhibited by Noxa silencing. Noxa upregulation is associated with nuclear translocation of the FOXO3a transcription factor as consequence of RA/IFN-α-induced Akt inhibition. Pharmacologic suppression of Akt, but not of TORC1, increases Noxa protein levels and downregulates Bfl-1 protein supporting the conclusion that the inhibition of the Akt pathway, the resulting FOXO3a activation and Noxa upregulation are critical molecular mechanisms underlying RA/IFN-α-dependent MCL cell apoptosis. These results support the potential therapeutic value of RA/IFN-α combination in MCL management.

Introduction
Mantle cell lymphoma (MCL) is a distinct CD5⁺ B-cell non-Hodgkin lymphoma (NHL) characterized by the t(11;14)(q13;q32) translocation that leads to overexpression of cyclin D1 and subsequent cyclin D/Rb pathway deregulation (1, 2). Nevertheless, other genetic changes, such as c-myc gene, low levels of the cyclin-dependent kinase (CDK) inhibitor p27Kip1, and p53 deregulation (3–5), are probably required for MCL development. Gene expression profiling of MCL cells has recently shown the dysregulation of several genes/proteins involved in NF-κB, PI3K/Akt, and mTOR signaling pathways, which are all constitutively activated in MCL cells (6–9). These findings suggest that a profound alteration of pathways regulating cell survival is likely responsible for the aggressive clinical behavior of MCL, which usually show poor response to conventional therapeutic regimens and a very unfavorable prognosis, even when the disease is treated with high-dose therapy and hematopoietic stem cell transplantation (1). Therefore, new therapeutic options need to be explored in patients affected by these lymphomas.

We have recently shown that 9-cis-retinoic acid (RA) induces marked antiproliferative responses in MCL cells by interfering with G1–S transition through the inhibition of ubiquitination and proteasome-dependent degradation of p27Kip1. Retinoic acid–upregulated p27Kip1 binds to cyclin D1/CDK4 complexes, resulting in decreased CDK4 kinase activity and pRb hypophosphorylation. Notably, retinoic acid also inhibited the growth-promoting effect induced in primary MCL cells by microenvironmental factors such as CD40 triggering and interleukin (IL)-4 (10). These findings make these compounds highly attractive in terms of potential clinical efficacy in this setting. Because retinoic acid alone does not induce relevant apoptotic responses in MCLs, we investigated the ability of retinoic acid to cooperate with IFNs in inducing apoptosis in MCL cells. Indeed, RA/IFN combination was previously shown to exert synergistic antiproliferative and proapoptotic effects in different cancer cell systems (11, 12). With particular regard to the proapoptotic activity, these compounds can variably promote both the downregulation of antiapoptotic proteins such as Bcl-2, Bcl-xL, and Mcl-1 (12, 13), often overexpressed in B-NHLs, including MCL (14–18) and/or the induction of proapoptotic proteins, including Bax, Noxa, and XAF1 (12, 19–23).
Intriguingly, recent studies showed a critical role of Noxa/Mcl-1 interaction in regulating MCL cell survival. In fact, both Noxa-upregulating and BH3-mimetic drugs were shown to induce significant apoptotic responses in MCL (24–26). Noxa belongs to the BH3-only subfamily of proteins with proapoptotic activity (27), which may directly or indirectly promote Bax and Bak activation. A crucial event in this process is the selective interaction with and the consequent inactivation of the prosurvival members of the Bcl-2 family, which, specifically for Noxa, are represented by Mcl-1 and A1/Bfl-1 (28).

On these grounds, we have investigated the ability of RA/IFN-α treatment to induce apoptotic responses in MCL cells and, particularly, to shift the critical balance between anti- and proapoptotic regulators in favor of apoptotic machinery activation. Moreover, taking into account our previous findings indicating that the PI3K/Akt pathway is critical for MCL cell survival and that Akt, but not mTOR, inhibition induces apoptotic responses in MCL (9), we also investigated the effects of RA/IFN-α treatment on the inherent PI3K/Akt activation.

Materials and Methods

Patient samples

Four patients with MCL were identified on the basis of morphologic, immunophenotypic, and molecular criteria according to World Health Organization (WHO) lymphoma classification (Table 1). The study was conducted in accordance with protocols approved by the local Institutional Review Board, and all patients gave their informed consent. Mononuclear cells were isolated from unicellular suspension and immunoblotting extracts preparation, immunoprecipitation, and immunoblotting

Proliferation assay, apoptosis, and caspase activity detection

Cell proliferation was evaluated by ³H-thymidine uptake (10) and apoptosis by Annexin-V/propidium iodide (PI) stains and/or by active/cleaved caspase-3. Activation of caspases-8, -9, and -3 was evaluated using a fluorimetric commercial kit (Immunochemistry Technologies). All flow cytometric analyses were conducted on a FC500 flow cytometer (Beckman Coulter).

Antibodies and reagents

Bcl-2 antibody was from Calbiochem; Bcl-xL, A1/Bfl-1, Mcl-1, Bax, Bid (rabbit), Puma, phospho-Akt (Ser473), phospho-S6rp (Ser235/236), phospho-GSK-3α/β (S21/9), phospho-FOXO3a (S318/321), Akt, and active caspase-3 (D175) antibodies were from Cell Signaling Technology; Noxa, Mcl-1 (Y37), Bcl-2A1 (EP517Y) antibodies were from Abcam; p27Kip1 and p21Cip1/Waf1 from BD Transduction Laboratories; PARP (F2), cyclin D1 (DCS-6), p57Kip2, p45Skp2, and Cks1 from Santa Cruz Biotechnology; Bid (mouse) antibody from SouthernBiotech; and Bak (NT) and FOXO3a antibodies from Millipore. Vital nuclear dye DRAQ5, SH5 (Akt inhibitor), and LY294002 were purchased from Alexis Biochemicals; rapamycin, G418, 9-cis-RA (Sigma); and IFN-α (IntronA, SP Europe) were used at 1 μmol/L and 1,000 U/mL, respectively. RO 40-6655 (RARγ agonist) was kindly provided by Dr. W. Bollag (Hoffmann-LaRoche, Inc.) and SR11237 (RXR agonist) was a kind gift of Dr. H. Gronemeyer (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France).

Extracts preparation, immunoprecipitation, and immunoblotting

Whole-cell lysate extracts and immunoprecipitated samples were prepared as previously described (10). Proteins were fractionated using SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblotting was conducted using the Enhanced Chemiluminescence plus Detection System (PerkinElmer).

Intracellular flow cytometry

Cells (10⁶) were fixed with 2% of paraformaldehyde, permeabilized with 500 μL of cold methanol, and incubated with the primary antibodies for 1 hour at room temperature. After 2 washes with PBS/0.5% bovine serum albumin, cells were incubated for 30 minutes in ice with phycoerythrin

Table 1. MCL cases

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Sex/age, y</th>
<th>Malignant cells (%)</th>
<th>Type</th>
<th>Cyclin D1</th>
<th>p27Kip1</th>
<th>Sample analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCL4</td>
<td>F/72</td>
<td>95</td>
<td>Classical</td>
<td>+</td>
<td>NA</td>
<td>Lymph node biopsy</td>
</tr>
<tr>
<td>MCL5</td>
<td>M/50</td>
<td>86</td>
<td>Classical</td>
<td>+</td>
<td>Low</td>
<td>Lymph node biopsy</td>
</tr>
<tr>
<td>MCL6</td>
<td>M/64</td>
<td>95</td>
<td>Classical</td>
<td>+</td>
<td>Low</td>
<td>Lymph node biopsy</td>
</tr>
<tr>
<td>MCL7</td>
<td>M/72</td>
<td>96</td>
<td>Classical</td>
<td>+</td>
<td>Low</td>
<td>Spleen biopsy</td>
</tr>
</tbody>
</table>

NOTE: The expression of cyclin D1 and p27Kip1 proteins was detected by immunohistochemistry. Abbreviation: NA, not available.
(PE)-anti-rabbit secondary antibody and analyzed by flow cytometry.

**Bid–Mcl-1 and Bid–A1/Bfl-1 colocalization and FOXO3a nuclear internalization**

Cells (10⁶ per sample) were fixed, permeabilized, and labeled as described above. Samples were acquired with the ImageStream X (Amnis) using the INSPIRE software. For colocalization experiments, samples were labeled with Mcl-1 or A1/Bfl-1 antibodies and Bid antibody and DRAQ5. Then, cells double-positive for Mcl-1 and Bid or A1/Bfl-1 and Bid were selected and compared using an algorithm of the IDEAS software which calculates the specificity and the degree of the fluorescence signals colocalization through the Similarity Bright Details Score (SBDS; see Supplementary Methods; ref. 31). For the analysis of FOXO3a nuclear internalization, samples were labeled with an antibody against FOXO3a (1:100) at 4°C overnight. Then, cells were stained with the PE-anti-rabbit secondary antibody and DRAQ5. Using an algorithm of the IDEAS software, the similarity score (SS; ref. 31) between FOXO3a and DRAQ5 staining was calculated for each sample.

**Noxa silencing**

Two different short hairpin RNAs (shRNA) PMAIP1 (phorbol-12-myristate-13-acetate–induced protein 1) constructs were obtained by subcloning the double-stranded 64-mer oligonucleotide containing the PMAIP1 target sequences (A: 5’-AACTGAACTTCCGCGCAG-3’; B: 5’-TCTGATATCCAACTCT-3’) into the pSUPERretro-GFP/neo vector (pSUPER; OligoEngine). Infectious supernatant from pSUPER, and pSUPER-retro-shPMAIP1 retrovirotransfected Phoenix cells were collected after 48 hours and used for 3 cycles of infections (32). Upon infection, cells were selected with G418 (1 mg/mL) and the infection efficiency was checked by flow cytometry (97% GFP-positive cells). Different clones of infected cells were then obtained after seeding cells in 96-well plate at an initial density of 25 cells per well in 200 μL of medium supplemented with G418.

**Results**

**IFN-α significantly enhances the antiproliferative activity exerted by retinoic acid in MCL cells**

The 9-cis-RA is the isomer with the strongest antiproliferative activity against MCL cells (10). Treatment of SP53 cells with 9-cis-RA (1 μmol/L) in combination with IFN-α (1,000 U/mL) for 2, 4, and 7 days resulted in a more pronounced inhibition of MCL cell proliferation as compared with cells treated with retinoic acid alone, showing an additive effect (Fig. 1A). Consistently, 9-cis-RA/IFN-α combination increased the number of MCL cells in G0/G1 (not shown), suggesting a likely involvement of regulators of G1 to S-phase transition. Immunoblotting analysis showed that IFN-α enhances the upregulation of the p27<sup>Kip1</sup> protein induced by 9-cis-RA as a result of a more pronounced inhibition of p45<sup>Skp2</sup> and Cks1, 2 SCF<sup>Skp2</sup>

![Figure 1. A. IFN-α enhances the antiproliferative activity exerted by 9-cis-RA in MCL cells. DNA synthesis was assessed in SP53 cells by ³H-thymidine incorporation after 6 hours. Points, mean from triplicate wells; bars, SD. The results are representative of 1 of 3 experiments. B, RA/IFN-α combination upregulates p27<sup>Kip1</sup> and p21<sup>WAF1/Cip1</sup> in SP53 cells (3 days). C, RA/IFN-α-induced p21<sup>WAF1/Cip1</sup> upregulation is not a p53-only–dependent event, being detected in MCL cell lines with wild-type (SP53, Granta-519) and mutated p53 (Mino, Jeko-1; 3 days). Ml, mutated; wt, wild-type. D, RA/IFN-α combination downregulates cyclin D1 expression in SP53 and Mino cells (5 days).](image-url)
complex components that are required for proteasome-dependent p27Kip1 degradation (Fig. 1B). Furthermore, 9-cis-RA/IFN-α combination also induces p21WAF1/Cip1 upregulation, whereas the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B). Notably, p21WAF1/Cip1 upregulation was detected in MCL cell lines with the levels of p57Kip2 were unaffected (Fig. 1B).

9-cis-RA sensitizes MCL cells to the proapoptotic effect of IFN-α through both RARs and RXRs

We previously showed that retinoic acid alone does not induce relevant apoptotic responses in MCL (10). Given the ability of IFN-α to cooperate with retinoic acid in inhibiting MCL cell growth, we also explored the possible induction of proapoptotic effects. To this end, SP53 and Mino cells were treated with each drug alone or in combination for 3 and 5 days, and apoptosis was evaluated using Annexin-V/PI staining. RA/IFN-α combination induced more pronounced apoptotic effects in both MCL cell lines than single treatments (Fig. 2A). In particular, sequential treatment experiments indicated that a 24-hour pretreatment with 9-cis-RA sensitizes MCL cells to the proapoptotic effect of IFN-α, whereas the reverse induced only modest effects (Fig. 2B). Considering that the pleiotropic effects of retinoids are mainly mediated by 2 classes of nuclear receptors, the RARs and RXRs (33, 34), and that 9-cis-RA is a pan-RAR and -RXR agonist, the relative contribution of distinct retinoic receptors was assessed using synthetic selective agonists for RARα (RO 40-6055) and RXR (SR11237). As shown in Fig. 2C, both RARα and RXR agonists sensitize MCL cells to IFN-α—induced apoptosis, although the relative contribution of RXRα is markedly higher. These findings are clinically relevant, as RARα agonists are associated with less pronounced side effects than the pan-RAR/RXR agonist 9-cis-RA when used in vivo.

9-cis-RA/IFN-α–dependent MCL cell apoptosis involves multiple caspase activation and modulation of anti- and proapoptotic proteins

The involvement of initiator and effector caspases in 9-cis-RA/IFN-α–induced apoptosis was investigated in SP53 and Mino cells using specific fluorimetric caspase assays. Time course experiments showed that both caspases-8 and -9 are activated, almost simultaneously, after 36 hours of 9-cis-RA/IFN-α treatment; nevertheless, at later time points, their activity differs (Fig. 3A). In fact, caspase-8 activation increases progressively, whereas the caspase-9 activity, after the initial triggering, increases more slowly, showing a downmodulation at 60 hours and a subsequent increase at 72 hours (Fig. 3A). Given the involvement of the mitochondrial pathway, the role of Bak and Bax proteins was analyzed by flow cytometry using antibodies specific for the N-terminal domains of these proteins that are exposed only upon Bak and Bax activation. As shown in Fig. 3B, the conformational changes of Bak and Bax were detectable only in cells exposed to 9-cis-RA/IFN-α (3 and 5 days).
concomitantly with the presence of cleaved caspase-3, as an indicator of ongoing apoptosis.

Given that the interactions between pro- and antiapoptotic members of the Bcl-2 superfamily are essential for mitochondrial integrity, the expression levels of several proteins of the Bcl-2 and BH3-only families were analyzed by immunoblotting in SP53 cells treated with 9-cis-RA, IFN-α, or their combination. While no change in Bcl-2 and Mcl-1 expression levels was observed in the different conditions, a marked downregulation of the Bcl-xL and A1/Bfl-1 antiapoptotic proteins was induced by 9-cis-RA/IFN-α (Fig. 3C). Although this treatment induced conformational changes indicating Bax and Bak activation, the expression levels of these proteins in untreated and treated cells were comparable. Analysis of BH3-only proteins disclosed a marked increase of Noxa protein levels, especially in 9-cis-RA/IFN-α–treated cells, whereas the expression of Puma did not appreciably change. The levels of the full-length Bid protein decreased significantly as likely consequence of its activation by caspase-dependent cleavage (Fig. 3C). Notably, 9-cis-RA/IFN-α treatment was also able to significantly upregulate Noxa concomitantly with enhanced caspase-3 activation in all 4 primary MCL cultures investigated (Fig. 3D). This effect was apparently specific for lymphoma cells, as 9-cis-RA/IFN-α did not upregulate Noxa, nor exerted any proapoptotic activity in normal B lymphocytes obtained from 2 different donors (not shown). Taken together, these results indicate that 9-cis-RA/IFN-α combination triggers both mitochondrial/intrinsic and death receptor/extrinsic apoptotic pathways and promotes the shift of the critical balance between anti- and proapoptotic proteins in favor of apoptotic machinery activation.

Noxa is a critical mediator of 9-cis-RA/IFN-α–induced apoptosis

The BH3-only protein Noxa was shown to be one of the critical players of the apoptotic responses induced in MCL cells by different drugs, such as bortezomib and the MDM2 inhibitor Nutlin-3 (24, 25). Interestingly, immunoblotting experiments showed that Noxa upregulation was induced by 9-cis-RA and at
higher levels by 9-cis-RA/IFN-α cotreatment. These effects are probably mediated by transcriptional regulation, as shown by microarray-based expression profiling carried out in SP53 cells (not shown). To assess the role of Noxa in 9-cis-RA/IFN-α–dependent apoptosis, we knocked down Noxa expression by retrovirally infecting Mino cells with a pSUPER.retro-GFP/neo vector (pSUPER) carrying 2 different shRNAs, corresponding to nucleotides 106 to 124 and 132 to 148 of PMAIP1 consensus coding sequence and GFP (pSUPER.shPMAIP1). Silencing of Noxa prevents the 9-cis-RA/IFN-α–induced upregulation of the protein and consequently reduces the extent of treatment-dependent apoptosis in Mino cells assessed by cleaved caspase-3 and PARP (Fig. 4A). Considering the ability of Noxa to specifically bind and consequently inactivate the antiapoptotic Mcl-1 and A1/Bfl-1 proteins, the interactions between Noxa and these 2 Bcl-2 family

Figure 4. A, Noxa knockdown reduces RA/IFN-α–dependent apoptosis. Mino cells infected with empty vector (pSUPER) and 2 different clones (CL.a and CL.b) of cells infected with vector containing shPMAIP1 sequence A and 1 clone (CL.c) with the sequence B (see Materials and Methods) were treated with 9-cis-RA/IFN-α for 3 days. Total cell lysates (50 μg) were analyzed by immunoblotting for Noxa, cleaved caspase-3, and PARP. B, RA/IFN-α combination promotes the formation of Noxa-Mcl-1 and Noxa-A1/Bfl-1 complexes. SP53 cells were cultured in absence or presence of 9-cis-RA/IFN-α for 3 days. Mcl-1 (left) and A1/Bfl-1 (right) were immunoprecipitated from 500 μg of total proteins. Immunoprecipitated (IP; bound) and nonimmunoprecipitated (unbound) fractions were analyzed by immunoblotting for Noxa, cleaved caspase-3, and PARP. C, RA/IFN-α–induced Noxa favors Bid displacement from Bid-Mcl-1 and Bid-Bfl-1 interactions in vivo. Mino cells were cultured in the absence (untreated) or presence of 9-cis-RA/IFN-α for 3 days, then 10^6 cells per sample were labeled with primary antibodies against Noxa, Mcl-1, and A1/Bfl-1 proteins. C, RA/IFN-α–induced Noxa favors Bid displacement from Bid-Mcl-1 and Bid-Bfl-1 interactions in vivo. Mino cells infected with empty vector (pSUPER) and 2 different clones (CL.a and CL.b) of cells infected with vector containing shPMAIP1 sequence A and 1 clone (CL.c) with the sequence B (see Materials and Methods) were treated with 9-cis-RA/IFN-α for 3 days. Total cell lysates (50 μg) were analyzed by immunoblotting for Noxa, cleaved caspase-3, and PARP. B, RA/IFN-α combination promotes the formation of Noxa-Mcl-1 and Noxa-A1/Bfl-1 complexes. SP53 cells were cultured in absence or presence of 9-cis-RA/IFN-α for 3 days. Mcl-1 (left) and A1/Bfl-1 (right) were immunoprecipitated from 500 μg of total proteins. Immunoprecipitated (IP; bound) and nonimmunoprecipitated (unbound) fractions were analyzed by immunoblotting for Noxa, cleaved caspase-3, and PARP. C, RA/IFN-α–induced Noxa favors Bid displacement from Bid-Mcl-1 and Bid-Bfl-1 interactions in vivo. Mino cells were cultured in the absence (untreated) or presence of 9-cis-RA/IFN-α for 3 days, then 10^6 cells per sample were labeled with primary antibodies against Bid (1:50) and Mcl-1 (1:100) or (E) A1/Bfl-1 (1:75). The vital nuclear dye DRAQ5 was added to each sample. A total of 20 × 10^3 cells were acquired with the ImageStream X and analyzed with a specific algorithm for the SBDS calculation. Data are representative of 1 of 3 independent experiments.
members were investigated. Most of 9-cis-RA/IFN-α–induced Noxa co-immunoprecipitated with Mcl-1, the remaining amount being associated to A1/Bi-1 (Fig. 4B). Furthermore, the sequestration of Mcl-1 by upregulated Noxa results in the displacement of the full-length Bid protein from Bid–Mcl-1 complexes (Fig. 4C), allowing thus the consequent Bid activation through enzyme cleavage. The resulting truncated Bid may thus directly contribute to the activation of the Bak and Bax apoptotic effectors. Taking advantage from multispectral imaging flow cytometry, we analyzed the colocalization between Bid and Mcl-1 and A1/Bi-1 also in vivo. To this end, we set up a protocol in which the cells were stained with specific antibodies to Mcl-1 or A1/Bi-1 and Bid proteins and then the Bid–Mcl-1 and Bid–A1/Bi-1 colocalization was analyzed only in double-positive live cells. As shown in Fig. 4D and E, the SBDS detected in untreated samples was 2.48 ± 0.42 for Bid–Mcl-1 and 2.45 ± 0.49 for Bid–A1/Bi-1, and in both cases, the score significantly decreased when the cells were treated for 3 days with RA/IFN-α. Moreover, in treated samples, the percentage of cells showing a significant colocalization of the 2 proteins (with SBDS ≥ 2.25) was reduced from 72% to 19.2% for Bid–Mcl-1 and from 66.4% to 17.2% for Bid–A1/Bi-1. These results indicate that the treatment induces the displacement of Bid from Bid–Mcl-1 and Bid–A1/Bi-1 complexes through Noxa upregulation and this event precedes and promotes the apoptotic process.

9-cis-RA/IFN-α–dependent MCL cell apoptosis is mediated by inhibition of the PI3K/Akt pathway

We and others recently showed that the PI3K/Akt pathway is constitutively activated in MCL, resulting in a strong deregulation of both cell proliferation and survival (8, 9). Particularly, pharmacologic inhibition of phosphoinositide 3-kinase (PI3K) or Akt induced significant levels of apoptosis in both established cell lines and primary MCL cultures, whereas the TORC1 inhibitor rapamycin induced no or only modest effects on cell survival (9). On these grounds, we investigated the ability of 9-cis-RA/IFN-α to interfere with the constitutive activation of these kinases. In particular, SP53 cells were treated for 3 days with 9-cis-RA, IFN-α, or their combination and analyzed for the presence of the phosphorylated form of Akt and of its substrates GSK-3β and FOXO3a. In addition, TORC1 activation was investigated by analyzing the phosphorylation status of one of its main substrates, the S6 ribosomal protein. Interestingly, 9-cis-RA inhibited Akt and mTOR activation, as shown by the downregulation of phospho-(S473)-Akt, of its substrates phospho-(Ser21)-GSK-3β and phospho-(Ser318/321)-FOXO3a, and of phospho-(S235/236)-S6RP, and more importantly, IFN-α significantly enhanced the inhibitory effects exerted by retinoic acid on these kinases (Fig. 5A). In particular, 9-cis-RA/IFN-α–induced Akt inhibition was associated with Noxa upregulation and the decrease of A1/Bi-1 protein levels (Fig. 5B). Furthermore, the PI3K/Akt inhibitor LY294002 induced a marked upregulation of Noxa and a complete depletion of A1/Bi-1, whereas rapamycin did not affect the levels of these proteins (Fig. 5C), consistent with our previous demonstration that rapamycin is unable to induce apoptosis in MCL cells (9).

Notably, Noxa upregulation induced by the treatment was observed in MCL cell lines carrying either wild-type (SP53) or mutant p53 (Jeko-1; Fig. 5B), supporting the hypothesis that a different transcription factor is involved in this phenomenon. Interestingly, FOXO3a activates the transcription of several genes, including PMAIP1, which encodes for the Noxa protein (35). FOXO3a transcriptional activity is regulated by the control of its intracellular localization through the phosphorylation/dephosphorylation of different serine/threonine residues. In particular, Akt-dependent phosphorylation on Thr32, Ser318/321, and Ser253 abolishes its nuclear translocation. Given the ability of 9-cis-RA/IFN-α to inhibit Akt-dependent FOXO3a phosphorylation, using multispectral imaging flow cytometry, we evaluated FOXO3a intracellular localization after 48 hours of exposure to 9-cis-RA/IFN-α, SH5 (10 μmol/L), or rapamycin (0.1 μmol/L). As shown in Fig. 5D, FOXO3a protein is clearly retained in the cytoplasm of untreated cells, whereas in 9-cis-RA/IFN-α– and SH5-treated cells, the protein is also detectable within the nucleus in 58.7% and 64.1% of cells, respectively. In contrast, rapamycin did not affect FOXO3a intracellular localization, confirming that this transcription factor is Akt- but not TORC1-dependent. Notably, the analyses were conducted excluding apoptotic cells, given that FOXO3a nuclear internalization and consequent Noxa upregulation are 2 events occurring in the first steps of the apoptotic process. These results are consistent with a role of FOXO3a as a molecular mediator of the 9-cis-RA/IFN-α–induced Noxa upregulation. Moreover, these findings indicate that the 9-cis-RA/IFN-α combination induces MCL cell apoptosis through inhibition of the inherent Akt activation, and the consequent increment of FOXO3a activity and, in turn, of Noxa expression (Fig. 6A and B).

Discussion

As our understanding of the biology of MCL advances, novel agents rationally designed to target the key pathogenic mechanisms of MCL, such as cyclin D1, cyclin/CDK inhibitors, proapoptotic proteins, mTOR, and proteasome, continue to emerge. Previously, we showed that the constitutive PI3K/Akt/mTOR activation contributes to the stability of cyclin D1 and p27Kip1 in MCL cells (9, 36), suggesting that this signaling pathway may be a crucial therapeutic target. While the therapeutic potential of TORC1 inhibitors is being extensively studied in patients with relapsed or refractory MCL (37), specific inhibitors of the upstream kinase Akt are still under evaluation in phase I clinical trials (38). Nevertheless, the Akt kinase may constitute a more effective target in MCL than TORC1, as Akt inhibition not only reduces proliferation but also induces significant apoptotic responses (9).

Herein, we show that RA/IFN-α cotreatment has significant effects on both proliferation and cell survival by affecting key molecular targets of MCL, such as cyclin D1, p27Kip1, Akt, and Noxa (Fig. 6A and B). In particular, IFN-α enhances the anti-proliferative activity exerted by 9-cis-RA by inducing a down-regulation of cyclin D1, which is strongly overexpressed in most MCLs. Nevertheless, the observation that cyclin D1 overexpression alone is not sufficient for MCL development and that its downregulation has only limited effects on MCL cell proliferation and survival (39) indicates that additional targets should...
Furthermore, p21WAF1/Cip1 upregulation is induced irrespective of the p53 mutational status of the cells, thus excluding a deregulated p53 (40, 41), a characteristic that could promote resistance to novel drugs targeting the HDM2/p53 pathway, such as the MDM2 antagonist Nutlin-3 (25) and MI-63 (42).

Intriguingly, the antiproliferative activity of RA/IFN-α combination triggers both the death receptor/extrinsic and the intrinsic apoptotic pathway, such as the MDM2 antagonist Nutlin-3 (25) and MI-63 (42).

The combination inhibits the inherent PI3K/Akt pathway activation in SPS3 and Jeko-1 cells (72 hours). Total cell lysates (100 μg) were subjected to immunoblotting using phosphospecific antibodies. C, inhibition of Akt, but not of TORC1, is associated with Noxa upregulation and A1/Bfl-1 depletion. SPS3 cells were untreated (C) or treated with 50 μmol/L LY294002 (LY) or 0.1 μmol/L rapamycin (Rapa) for 48 hours and total cell lysates were analyzed for the expression of phospho-Akt, Noxa, and A1/Bfl-1 proteins. D, RA/IFN-α treatment promotes FOXO3a nuclear localization. Mino cells were untreated or treated with 9-cis-RA/IFN-α, SH5 (10 μmol/L), or rapamycin (0.1 μmol/L) for 48 hours and labeled with antibody against FOXO3a (1:100) and the vital nuclear dye DRAQ5. Cells (20 × 10⁶) were acquired with ImageStream X and analyzed with the IDEAS software. FOXO3a nuclear localization was calculated as similarity score between FOXO3a and DRAQ5 intensities. Data are representative of 1 of 3 independent experiments.

More relevant in a therapeutic perspective is the demonstration that, unlike retinoic acid alone (10), the RA/IFN-α combination induces significant levels of apoptosis in both established cell lines and primary MCL cultures. The exposure of MCL cells to 9-cis-RA for 24 hours and the following addition of IFN-α indicate this sequential treatment as the most effective combination, providing thus the rationale for the design of appropriate treatment schedules. Moreover, we also show that 9-cis-RA ability to sensitize MCL cells to IFN-α-dependent proapoptotic effect involves both RARs and RXRs. These findings are of potential clinical relevance, as RARα agonists are associated with less pronounced side effects than the pan-RAR/RXR agonist 9-cis-RA when used in vivo. RA/IFN-α combination triggers both the death receptor/extrinsic and the mitochondrial/intrinsic apoptotic pathways and promotes the activation of the proapoptotic effectors Bak and Bax.
Moreover, RA/IFN-α treatment induced upregulation of Noxa and the concomitant Mcl-1 and A1/Bfl-1 inactivation as a result of protein–protein interactions. (24–26). In contrast to other compounds inducing Noxa-dependent MCL cell apoptosis, RA/IFN-α combination does not increase Mcl-1 protein levels and even downregulates A1/Bfl-1. Intriguingly, our results show that, under normal conditions, both Mcl-1 and A1/Bfl-1 can be bound to the full-length form of Bid, thus preventing its cleavage and repressing its activation. MCL cell exposure to RA/IFN-α combination relieves this repression through a competitive inhibition exerted by Noxa, which favors Bid displacement from pro/antiapoptotic protein complexes and its subsequent activation.

Notably, we took advantage of multispectral imaging flow cytometry to selectively analyze Bid–Mcl-1 and Bid–A1/Bfl-1 colocalization in cells with morphometric features of early apoptosis, a distinction that is not usually feasible in co-immunoprecipitation experiments. This methodologic approach is particularly relevant if we consider that the binding of Mcl-1 or A1/Bfl-1 to Bid abolishes its proapoptotic activity and that RA/IFN-α–induced Bid displacement from these complexes is an early event in the activation of apoptotic machinery.

Noxa was initially identified as transcriptional target of p53 (43), but now it is well known that it could be induced also by other factors, as it occurs in neuronal cells where it is induced by the activation of the FOXO3a transcription factor, a down-stream target of the Akt kinase (35). We previously showed that the P38/Akt pathway is crucial for MCL cell survival and that the inhibition of Akt, but not of TORC1, induced apoptosis in MCL cells, suggesting that an Akt substrate different from TORC1 is likely involved in mediating these effects. The results presented herein support the conclusion that the inhibition of
the Akt pathway by RA/IFN-α, resulting in FOXO3a dephosphorylation/activation and its subsequent nuclear internalization followed by Noxa upregulation, is one of the main molecular mechanisms underlying RA/IFN-α-dependent MCL cell death. Our findings are also consistent with a relevant role of RA/IFN-α-induced Akt inhibition in mediating MCL cell growth inhibition (9, 36). In particular, this drug combination mimics the effects induced in MCL cells by pharmacologic inhibition of Akt, which results in cyclin D1 downregulation and p27^Kip1 overexpression. Overall, the results of the present study show that how the combination 9-cis-RA/IFN-α affects critical molecular targets of MCL, thus providing a strong rationale for the clinical application of known and relatively cheap drugs in the management of this aggressive and poorly responsive lymphoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
25. Tatey Y, Sesagari D, Jin L, Rudelius M, Davies-Hill T, Miyake K, et al. MDM2 antagonist nutlin-3 displays antiproliferative and...


Retinoic Acid/Alpha-Interferon Combination Inhibits Growth and Promotes Apoptosis in Mantle Cell Lymphoma through Akt-Dependent Modulation of Critical Targets

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