Novel triiodophenol derivatives induce caspase-independent mitochondrial cell death in leukemia cells inhibited by Myc

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Abstract

2,4,6-Triiodophenol (Bobel-24, AM-24) was originally described as a nonsteroid antiinflammatory molecule. We have synthesized three derivatives of Bobel-24 (Bobel-4, Bobel-16, and Bobel-30) and tested their activities as putative antileukemic agents. We have found that Bobel-24 and Bobel-16 were dual inhibitors of cyclooxygenase and 5-lipoxygenase, whereas Bobel-4 and Bobel-30 were selective against 5-lipoxygenase. We have tested the antiproliferative activity of these compounds on a panel of cell lines derived from myeloid and lymphoid leukemias (K562, Raji, HL-60, and Molt4). The cytotoxic IC₅₀ in these cell lines ranged between 14 and 50 μmol/L, but it was higher for nontransformed cells such as 32D, NIH3T3, or human leukocytes. All compounds showed cytotoxic activity on all tested cell lines, accompanied by DNA synthesis inhibition and arrest in the G₀/G₁ phase. Bobel-16, Bobel-4, and Bobel-24 induced a caspase-independent cell death in K562 and Raji cells, accompanied by chromatin condensation, cytochrome c release, and dissipation of mitochondrial membrane potential in a concentration-dependent manner and production of reactive oxygen species. As the proto-oncogene MYC is involved in mitochondrial biogenesis and survival of leukemia cells, we tested its effect on bobel activity. Bobel-24 induced down-regulation of MYC in K562 and, consistently, ectopic expression of MYC resulted in partial protection towards the cytotoxic effect of Bobel-24. In conclusion, Bobel derivatives induce a caspase- and Bcl-2-independent cell death in which mitochondrial permeabilization and MYC down-regulation are involved. Bobels may serve as prototypes for the development of new agents for the therapy of leukemia. [Mol Cancer Ther 2006;5(5):1166–75]

Introduction

Prostaglandins and leukotrienes are signaling mediators generated from arachidonic acid by the action of cyclooxygenases (COX-1 and COX-2) and 5-lipoxygenases (5-LOX), respectively. COX is expressed in most cells, whereas 5-LOX is mainly expressed in inflammatory cells such as polymorphonuclear leukocytes (1). Leukotrienes modulate the growth of several cell types and it has also been shown that various 5-LOX metabolites from arachidonic acid regulate murine and human hematopoiesis in vitro (2). COX and 5-LOX inhibitors are used as anti-inflammatory drugs (3). Some 5-LOX inhibitors inhibit the proliferation of malignant human hematopoietic cell lines although they do not seem to affect the growth of normal hematopoietic cells (4). Several reports have described that activation of 5-LOX and increase of its intermediate by-products (hydroperoxides), induce programmed cell death (PCD) in human T cells, neutrophils (5), and Jurkat cells (6). Despite the ability of LOX or COX inhibitors to cause growth inhibition and/or cell death (7–9), the mechanisms by which these inhibitors act and whether their cytotoxic effects could be characterized as apoptosis, necrosis, or other forms of PCD, is unclear. Nevertheless, the fact that the concentrations of 5-LOX or COX inhibitors required to induce cell death are usually higher than those required to inhibit LOX or COX activity, and that these compounds remain active in cells deficient in LOX (9, 10) or COX activity (11), suggest that mechanisms other than LOX or COX inhibition must also be involved in their antiproliferative effects.

MYC oncogene encodes the transcription factor c-Myc (herein termed Myc) that plays a pivotal role in the control of cell proliferation, cell differentiation, cell size, and apoptosis (12–14).

Myc activates genes involved in cell cycle control, energetic metabolism, protein and ribosome synthesis,
and other functions. Also, an important number of Myc target genes are repressed by Myc (15). In some models, enforced expression of Myc in the absence of growth factors results in apoptosis, whereas in others, Myc acts as a survival factor and is down-regulated in response to treatments causing cell death or growth arrest (16, 17). Recently, it has been shown that Myc is also involved in mitochondrial biogenesis (18).

Consistent with these *ex vivo* effects, deregulated expression of MYC is found in a wide array of human cancers (13, 19). Leukemia and lymphoma are clear examples of cancers in which MYC deregulation has been described and associated with tumor progression (19–21). Also, transgenic models with Myc deregulated expression in lymphoid tissue or erythroid precursors have shown that MYC induces lymphomagenesis or erythroleukemia, respectively (21, 22). Our previous work also showed a survival effect of Myc on K562, a CML-derived cell line (23).

The present study examines the mechanism by which a new family of synthetic dual 5-LOX and COX inhibitors, named bobels, with different specificities and selectivities toward 5-LOX and COX, induce cell death in leukemia. Our results show that these compounds produce mainly caspase-independent PCD (24). Moreover, cell death is usually accompanied by reactive oxygen species (ROS) production, mitochondrial membrane depolarization, S phase depletion, and Myc down-regulation. These inhibitors are potential drugs for the treatment of leukemia.

**Materials and Methods**

**Cell Culture and Reagents**

Molt4, HL60, K562, Raji, 32D, and NIH3T3 cell lines were obtained from American Type Culture Collection. NIH3T3 were grown in DMEM and the rest of the cell lines were cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum, t-glutamine (2 mmol/L), and penicillin/streptomycin (50 units/mL). The medium for 32D cells was also supplemented with a 10% supernatant of WEHI-3B (as an interleukin-3 source). K562 cell lines carrying Karyotyping (KmcyB) or overexpressing Bcl-2 (KBcl2, K562 transduced with Bcl-2 retrovirus) have been described elsewhere (25, 26). Cyclosporin A (CsA) and A23187 were purchased from Sigma (St. Louis, MO). Benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethyl ketone was from Calbiochem (La Jolla, CA). Bobel-24 (2,4,6-triiodophenol, AM-24) and Bobel-30 was described in Results. Bobels were purified (Santander, Spain). The synthesis of Bobel-4, Bobel-16, and Bobel-24 (2,4,6-triiodophenol, AM-24) was provided by Industrial Farmaceutica Cantabria (La Jolla, CA). Bobel-24 (2,4,6-triiodophenol, AM-24) was provided by Industrial Farmaceutica Cantabria (La Jolla, CA). The synthesis of Bobel-4, Bobel-16, and Bobel-30 was described in Results. Bobels were purified by recrystallization from acetone/water (Bobel-16 and Bobel-30) and by passage through a preparative chromatographic column on SiO2 eluting with a mixture of ethyl acetate and n-hexane (1:1; Bobel-4). The purity of the compounds was tested by TLC. Stock solutions were prepared in DMSO. For the treatments, cells growing at a density of 80,000 cells/mL were treated with the indicated concentrations of bobels for 24 hours. Control cells were treated with the corresponding volumes of the bobel vehicle.

**5-LOX and COX IC50 Determination Assays**

5-LOX inhibition assay was done in suspensions of human polymorphonuclear leukocytes prepared as described previously (27). Drugs dissolved in DMSO were added to 350 μL of leukocytes suspensions, adjusted to a density of 18 × 10⁶ cells/mL, containing 2 mmol/L CaCl₂ and 1.5 mmol/L MgCl₂ and kept at 37°C for 5 minutes prior to the addition of substrate and calcium ionophore. The reaction was started by adding 8.33 μmol/L (final concentration) of [¹⁴C]-arachidonic acid (56 mCi/mmol, American Biosciences, Buckinghamshire, United Kingdom) and 5 μmol/L of calcium ionophore A23187. After 5 minutes at 37°C, the reaction was stopped by adding 300 μL of cold stop solution (2% acetic acid in methanol), and the samples were kept at −80°C until eicosanoid analysis. Analysis of 5-LOX-derived compounds was done in a reverse-phase high-pressure liquid chromatography column as described previously (27). 5-LOX activity was evaluated as the sum of peak radioactivity counts associated with all eicosanoids formed through the 5-LOX pathway: leukotriene B₄, 6-trans-leukotriene B₄ epimers, 5,6-dihydroxy-eicosatetraenoic acid epimers, 5-hydroxy-12-methoxy-leukotriene B₄, 20-hydroxy-leukotriene B₄, and 5-hydroxyeicosatetraenoic acid.

COX inhibition assays were done in human platelet suspensions prepared as described previously (28). Drugs were added to 175 μL of platelet suspensions, adjusted to a density of 170 × 10⁶ platelets/mL and incubated at 37°C for 5 minutes prior to the addition of substrate. The reaction was started by adding 15 μmol/L of [¹⁴C]-arachidonic acid (final concentration). After 5 minutes at 37°C, the reaction was stopped as described above and the prostanooids produced were analyzed by reverse phase high-pressure liquid chromatography (27). The COX activity was evaluated as the sum of peak radioactivity counts associated with all eicosanoids formed through the COX pathway: thromboxane B₂, prostaglandin F₂α, prostaglandin E₂, prostaglandin D₂, and 12-hydroxy-eptadecatrienoic acid.

**Arachidonic Acid Metabolism of K562 and Raji Leukemic Cell Lines**

A 0.5 mL aliquot of a cell suspension at a density of 20 × 10⁶ cells/mL in RPMI (without serum) was incubated at 37°C in the presence of 25 μmol/L [¹⁴C]-arachidonic acid for 15 minutes. To test the 5-LOX activity, cells were preincubated with 0.2 mmol/L of acetyl salicylic acid (Sigma) for 30 minutes in RPMI at 37°C to irreversibly inhibit COX activity. Cells (10⁷ cells in 0.5 mL) were washed and incubated with 25 μmol/L [¹⁴C]-arachidonic acid and 5 μmol/L calcium ionophore A23187 at 37°C for 15 minutes. The reaction was stopped and the samples were analyzed as described above (27). The drugs at the final concentrations indicated in Results were added to the cell suspensions 20 minutes before the addition of the radioactive substrate, and samples were processed as indicated above.

**Cell Viability Assays**

Viability assays were done by using the “Cell Proliferation Kit II” (XTT assay, Roche, Germany). Cells were plated at a density of 5,000 cells per well on 96-well plates in 100 μL of culture medium. After 12 hours, 0.8 μL of propidium iodide solution (5 μg/mL) was added to each well and incubated for 48 hours. After 48 hours of incubation, 50 μL of XTT labeling mixture was added to each well and incubated for an additional 4 hours. The absorbance was determined at 490 nm.

**Evaluation of the Cell and Nuclear Morphology by Phase Contrast and Fluorescent Microscopy**

Cells were pretreated with 1 ng of Hoechst 33342/mL medium for 24 hours, and later plated in 96-wells plate at 8,000 cells/well in 100 μL of medium for 12 hours. Then, the cells were incubated with different concentrations of compound for 16, 24, or 48 hours. The cell and chromatin morphology was recorded under 20× or 40× magnification in a contrast (bright-field) microscopy and fluorescence microscopy with Axiovert-200M microscope (Zeiss, Jena, Germany).

**Cell Cycle Analysis**

Cell lines (1.5 × 10⁶ cells in 10 mL of medium) were treated for 24 hours with bobel compounds and then supplied with 1 μmol/L bromodeoxyuridine in complete medium for 1 hour at 37°C. The cells were then fixed in 70% ethanol overnight at 4°C and stained with FITC labeled anti-bromodeoxyuridine antibody (DAKO, Carpinteria, CA) for 30 minutes at room temperature followed by the addition of propidium iodide solution (5 μg/mL). Cells were then analyzed on a Becton Dickinson FACScan instrument using CellQuest software (BD Biosciences, San Jose, CA).

**Mitochondrial Membrane Potential Detection**

Mitochondrial membrane potential (ΔΨ) was determined with 5,5′,6,6′-tetrachloro-1,1′-tetraethyl-benzimidazolylcarbocyanine iodide reagent (BD MitoScreen Kit, BD Biosciences). Cell lines (0.8 × 10⁶ cells in 10 mL) were harvested after 16 to 20 hours treatment with Bobel compounds or vehicle, centrifuged at 400 × g for 10 minutes, and the cell pellet was resuspended in 0.5 mL of 5,5′,6,6′-tetrachloro-1,1′-tetraethyl-benzimidazolylcarbocyanine iodide solution and incubated at 37°C for 10 to 15 minutes and analyzed by flow cytometry (Becton Dickinson FACScan instrument). A dot-plot of red fluorescence (FL2) versus green fluorescence (FL1) was drawn. Red fluorescence corresponds to living cells with intact mitochondrial ΔΨ and green fluorescence to cells with lost ΔΨ.

**ROS Detection**

ROS generation was assayed using dihydroethidium probes (Molecular Probes, Invitrogen, San Diego, CA). The reaction is relatively specific for superoxide anion (O₂⁻) production. Cells were harvested after treatment, and incubated with 4 μmol/L dihydroethidium in RPMI medium without fetal bovine serum and without phenol red for 45 minutes. After that, cells were rinsed with medium, the cell pellet was resuspended in 0.5 mL of medium, and immediately analyzed by flow cytometry. The number of counts versus green fluorescence (FL1-Height) is shown.

**Preparation of Cytosolic and Mitochondria-Enriched Fractions**

To determine mitochondrial cytochrome c release, 0.8 × 10⁶ cells (in 10 mL) treated with bobels were harvested and washed with 150 μL of extraction buffer [20 mmol/L Hepes-KOH (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 1 mmol/L DTT, 250 mmol/L sucrose, 100 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL pepstatin A, 10 μg/mL leupeptin, and 2 μg/mL aprotinin]. The cell pellet was resuspended in 100 μL of buffer and kept on ice for 30 minutes. Then, cells were homogenized using a glass dounce homogenizer and centrifuged twice at 20,000 × g for 30 minutes at 4°C. The pellet obtained represented the nuclear mitochondria–enriched fraction; supernatant represented the cytoplasmic fraction in which cytochrome c is released. Western blot was done from the cytoplasmic fraction.

**Western Blot Analysis**

Whole protein lysates were obtained as described previously. Fifty micrograms of protein cell extracts were resolved by 12% or 15% SDS-PAGE and then transferred to a nitrocellulose membrane. Blots were incubated with the following primary antibodies anti-PARP (Roche Diagnostics, Mannheim, Germany), anti-caspase-3, anti-caspase-7, anti-caspase-8, anti-Myc, anti-Bcl-XL, anti-Bid, anti-cytochrome c (BD PharMingen, San Diego, CA), anti-caspase-9 (Cell Signaling Technology, Beverly, MA), anti-cyclin B1, anti-p21, anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PCNA, anti-cyclin D3, anti-cyclin A, anti-Bax, anti-Bad, and anti-Bcl-2 (BD Transduction, San Diego, CA), anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon International, Temecula, CA). Secondary antibodies were from Jackson ImmunoResearch Laboratories (Bar Harbor, ME) and the working dilutions were 1:20,000 to 1:10,000. The levels of proteins were visualized by an enhanced chemiluminescence system (SuperSignal; Pierce, Rockford, IL) and autoradiographed.

**Northern Blot Analysis**

RNAs were prepared with the Trizol reagent according to the manufacturer’s instructions (Invitrogen), separated by electrophoresis through an agarose-formaldehyde gel and transferred to nitrocellulose (15 μg of total RNA per lane). The probe for human Myc was a 1.4-kb fragment of human Myc cDNA (30). Probe labeling, with [α-³²P]dCTP, and filter hybridization were carried out according to standard procedures. The amount and integrity of the RNAs were assessed by staining of the filter with ethidium bromide, which reveals 28S and 18S rRNA.

**Results**

**Synthesis and Cytotoxic Activities of Bobel Derivatives**

Bobel-4, Bobel-16, and Bobel-30 (Fig. 1) were synthesized by iodination at positions 2, 4, or 6 of the corresponding noniodinated phenols carrying the adequate carbon residues or chlorine atoms at positions 2, 3, 4, or 6. Iodination was done by means of an aromatic electrophilic substitution.
Bobel-24, Bobel-16, and Bobel-30 are white crystalline solids with quite high fusion points, Bobel-24 (157°C), Bobel-16 (86°C–89°C), and Bobel-30 (137°C–139°C), whereas Bobel-4 is an oil (liquid at room temperature), thus, with a low fusion point (31). Bobel-4 also differs from the other three in that it is easily oxidizable. The isopropyl groups, specific to Bobel-4, have two carbon residues that make this compound more lipophilic.

We analyzed the in vitro inhibitory activity of bobels on 5-LOX and COX. The obtained IC50s are shown in Table 1. Bobel-4 has the highest selectivity and inhibitory potency on 5-LOX (2.25 μmol/L) although it is less potent as a COX inhibitor. Bobel-16 has the higher inhibitory activity on COX, but also effectively inhibited 5-LOX. Thus, Bobel-16 is a potent dual inhibitor. Bobel-24 (leader compound) was less potent on COX than the other compounds. In summary, Bobel-4 shows 23-fold higher potency toward 5-LOX than to COX, whereas Bobel-30 shows 6.5-fold higher potency and Bobel-24 shows a 2.5-fold higher potency towards 5-LOX than to COX. In this work, we focused on the cytotoxic activities of Bobel-24 (the series leader), Bobel-4, and Bobel-16.

Bobel-16 and Bobel-4 Cause the Lowest Survival on Leukemia Cell Lines

We tested the antiproliferative activity of the bobels on four human leukemia cell lines: K562 (derived from chronic myeloid leukemia), HL60 (derived from acute myeloid leukemia), Raji (derived from B-lymphoblastic leukemia), and Mol4 (derived from T lymphoblastic leukemia). The XTT cell viability assay revealed that bobels were cytotoxic in the micromolar range on all tested cell lines (Table 2). However, the antiproliferative IC50 of Bobel-16 was the lowest for the four cell lines (IC50 ~ 20 μmol/L). This result correlates with the fact that Bobel-16 behaves as a dual COX/5-LOX inhibitor with the highest inhibitor potency on both COX and 5-LOX enzymes. HL60 and Raji cells were the most sensitive towards Bobel-16 treatment (IC50 ~ 17 μmol/L). The leader compound, Bobel-24, had less antiproliferative activity than the other three derivatives. All these concentrations are clinically relevant because human plasmatic values (Cmax) of 212 μmol/L Bobel-24 (the series leader) can be obtained and are well tolerated (32). We also tested the activity of Bobel-4, Bobel-16, and Bobel-24 on three non-transformed cell types: 32D (murine myeloid cells), NIH3T3 (murine fibroblasts), and human leukocytes from blood donors. In these three cases, the cells were less sensitive to the cytotoxic effect of the Bobel-24 and Bobel-16, whereas the sensitivity to Bobel-4 was similar to K562 and Raji (Table 2). The growth curves for the hematologically derived cells (K562, Raji, 32D, and leukocytes) are shown in Fig. 2A. The curves show the relative resistance of 32D and human leukocytes to the cytotoxic effects of Bobel-16 and Bobel-24.

As Bobel-24 was originally introduced and described as a COX and LOX inhibitor (31, 33), we asked whether these inhibitions were responsible for the cytotoxic activity. We analyzed whether Bobels interfered with the arachidonic acid metabolism by analyzing metabolites of arachidonic acid in K562 (myeloid cell line) and Raji (lymphoid cell line). Cells were labeled in vitro with [14C]-arachidonic acid and its metabolites analyzed by chromatography. We did not detect 5-LOX activity in K562 cells, nor did we find COX activity in Raji cells. In K562 cells, COX activity was inhibited by 72%, 47%, and 50% when cells were treated for 20 minutes with Bobel-24, Bobel-16, and Bobel-4, respectively, at concentrations corresponding to their IC50s (as stated in Table 2). In Raji cells, 5-LOX activity was inhibited by 96%, 2%, and 100% when cells were treated for 20 minutes with Bobel-24, Bobel-16, and Bobel-4, respectively, at concentrations corresponding to their IC50s (as stated in Table 2). In both cell lines, but more dramatic for Raji cells, bobel concentrations that allow half-maximal growth rate (Table 2) resulted in >50% inhibition of COX and 5-LOX activities. Thus, the antiproliferative or cytotoxic effect of the bobels did not correlate with their inhibition of COX or 5-LOX enzymatic activity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μmol/L)</th>
<th>5-LOX</th>
<th>COX</th>
</tr>
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<tbody>
<tr>
<td>Bobel-4</td>
<td>2.25 ± 1.08</td>
<td>51.45 ± 2.15</td>
<td></td>
</tr>
<tr>
<td>Bobel-16</td>
<td>10.24 ± 0.89</td>
<td>6.34 ± 0.94</td>
<td></td>
</tr>
<tr>
<td>Bobel-24</td>
<td>10.46 ± 1.67</td>
<td>26.82 ± 7.51</td>
<td></td>
</tr>
<tr>
<td>Bobel-30</td>
<td>2.76 ± 0.62</td>
<td>17.89 ± 5.40</td>
<td></td>
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</tbody>
</table>

NOTE: The activities were determined in vitro as detailed in Materials and Methods. IC50 is the concentration that results in half enzymatic activity with respect to untreated controls.
Membrane and Chromatin Modification during Induction of Cell Death by Bobel Compounds

To explore the mechanism by which these compounds decrease cell viability, we first assessed annexin V binding, which detects the degree of phosphatidylserine externalization on the plasmatic membrane, an early marker of apoptosis and late marker of other PCD. The binding of annexin V was small for K562 and Raji after shorter treatment times (<4% after 24 hours; data not shown).

The previous result suggests the induction of a nonclassical apoptotic pathway for the Bobel-induced cell death, either programmed or accidental necrosis. To explore the cytotoxic mechanism of bobels, we investigated whether bobels provoked nuclear condensation and chromatin fragmentation. For this, we stained with the DNA-specific dye, Hoechst. Raji cells were treated with Bobel-4 and K562 cells were treated with Bobel-16 (Fig. 2B). The results showed that both drugs induced chromatin condensation after 16 or 24 hours of treatment. We also observed that bobels induced membrane blebbing at high concentrations (bright-field micrographs in Fig. 2B). We conclude that the mechanism by which these compounds act is not by accidental necrosis because all of them produce chromatin condensation and fragmentation, different levels of annexin exposure and nuclear condensation and membrane blebbing, and all these features are absent in accidental necrosis (34, 35).

The cell death is not a consequence of p53 activation by bobels, as K562 and Raji cells carry inactivated p53 alleles (36).

Effect of Bobel Compounds on the Cell Cycle

We next analyzed the apoptosis and cell cycle distribution of K562 and Raji cells in response to bobels through cytometric analysis of propidium iodide–stained cells. These compounds induced an accumulation of cells in the sub-G₁ fraction (i.e., with partially degraded chromatin, a cell death indicator) in K562 and, to a lesser extent, in Raji cells, after 24 hours of treatment (Fig. 2C). However, bobels did not provoke chromatin degradation, as assessed by the sub-G₁ fraction in 32D cells (Fig. 2C), consistently with its higher resistance to bobel-mediated cell death (Table 2).

On the other hand, flow cytometry revealed that the bobels induced, as expected, a profound repression of DNA synthesis (S phase fraction detected by anti-bromodeoxyuridine–FITC) in K562 and Raji cells (Fig. 2C). We also

Table 2. Effect of Bobel-4, Bobel-16, Bobel-30, and Bobel-24 in cell survival of leukemic cell lines (K562, HL60, Raji, and Molt4) and nontransformed cells (32D, human leukocytes, and NIH3T3)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>IC₅₀ (µmol/L)</th>
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<tbody>
<tr>
<td></td>
<td>Bobel-4</td>
</tr>
<tr>
<td>K562</td>
<td>28.0 ± 4.3</td>
</tr>
<tr>
<td>HL-60</td>
<td>54.7 ± 8.0</td>
</tr>
<tr>
<td>Raji</td>
<td>32.1 ± 1.3</td>
</tr>
<tr>
<td>Molt4</td>
<td>33.2 ± 1.4</td>
</tr>
<tr>
<td>32D</td>
<td>37.0 ± 8.5</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>24.2 ± 2.1</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>30.5 ± 1.4</td>
</tr>
</tbody>
</table>

NOTE: IC₅₀ is the concentration that results in half viability with respect to untreated cells, expressed as µmol/L. Values are averages of at least three independent determinations ± SE.

Abbreviation: ND, not determined.

Figure 2. Effect of Bobel-4, Bobel-16, and Bobel-24 in cell survival of leukemic cell lines (K562, HL60, Raji, and Molt4). A, cytotoxicity in leukemic cell lines (K562 and Raji), nontransformed myeloid cells (32D), and human leukocytes were determined by the XTT test after 48 h of incubation with Bobel-4, Bobel-16, and Bobel-24 at the indicated concentrations.

B, characterization of membrane modification, cell morphology, and chromatin during induction of cell death by Bobel-4 (2-fold, IC₅₀) for 16 h in Raji cells and 40 µmol/L of Bobel-16 (2-fold, IC₅₀) for 24 h in K562 cells. The cell morphology was displayed by contrast microscopy (phase contrast images, top) and nuclear staining by Hoechst staining (fluorescent images, bottom). Original magnification, ×20 (Raji) and ×40 (K562). Control cells treated with vehicle. C, bobels induce depletion of S phase in leukemic cells. Cell cycle fluorescence-activated cell sorting analysis on cells incubated in the presence of vehicle or 2-fold IC₅₀ of bobel compounds (as stated in Table 2) for 24 h.
determined whether some regulatory components of the G1 and M phases of the cell cycle, such as cyclin D3 (the cyclin D with highest expression in K562), cyclin A, and cyclin B, which were down-regulated by bobels in a dose-dependent manner (data not shown).

**Bobel-24 and Bobel-16 Induce Caspase-Independent Cell Death in Leukemia Cells**

To further investigate the mechanism of cell death induced by the bobels, we analyzed the activation of pro-caspase-3 or caspase-7 by Bobel-24, Bobel-16, or Bobel-4 by Western blot. We did not observe the activation of caspase-3 and caspase-7 at bobel concentrations equivalent to 2-fold IC50 (Fig. 3A). Also, we did not detect any significant proteolysis of the PARP protein, a known caspase substrate in K562 and Raji (Fig. 3A). Furthermore, we did not detect changes in the levels of the antiapoptotic protein Bcl-2 and Bcl-xL and of the proapoptotic Bax and Bid in both cell lines (data not shown).

Thus, these compounds induce a mostly caspase-independent cell death in leukemic cells. We next asked whether the cell death mechanism elicited by bobels could involve the mitochondrial pathway. Thus, we first asked whether the mitochondrial integrity could be affected by the bobel compounds by determining cytochrome c release. The results show that Bobel-24 and Bobel-16 induced cytochrome c release in K562 and Raji cells after 16 hours of treatment (Fig. 3B). This suggests that these bobels affected the mitochondrial permeability.

**Effect of Bobels on Mitochondrial Membrane Permeabilization and ROS Production**

The finding that bobels alter the mitochondrial membrane permeability of leukemia cells suggests that they could also induce the production of ROS, which is a common mediator of cell death (37). To study whether this was also operative in bobel-mediated leukemic cell death, we analyzed whether ROS production was increased. For this, we stained cells treated with the bobel compounds with dihydroethidium, a cell-permeable fluorescence dye that reacts with a broad spectrum of ROS. ROS production (e.g., oxidized dihydroethidium) was quantified by flow cytometry. As a positive control for ROS production, we determined the relative fluorescent shift in H2O2-treated cells. The viable cells were gated by forward- and side-scatter values. The results showed that treatment of Raji and K562 cells with Bobel-16 and Bobel-24 results in ROS production similar to H2O2-treated cells (Fig. 3C). The results are consistent with a cell death mechanism involving ROS production, at least for Bobel-24 and Bobel-16.

We also analyzed whether bobels induced mitochondrial membrane permeabilization and subsequent membrane depolarization (ΔΨ), which is associated with most forms of cell death involving the mitochondria. To that purpose, we stained the cells with a fluorescent cationic dye, 5,5′,6,6′-tetrachloro-1,1′-tetraethyl-benzimidazolylcarbocyanine iodide, that exhibits potential-dependent accumulation in
mitochondria which can be detected by flow cytometry. Bobel-16 in K562 and Raji cell lines induced loss of ΔΨ, and to a lesser degree, Bobel-24, in K562 cells (Fig. 4A). In contrast, Bobel-4 hardly induces loss of ΔΨ in K562 cells, whereas it readily induces loss of ΔΨ in Raji cells. However, higher levels of Bobel-4, such as 3-fold IC50, in the K562 line, induces mitochondrial depolarization after 16 hours (data not shown).

The former results suggest that the bobels could impair the mitochondrial membrane functionality and hence generate ROS. We analyzed whether loss of ΔΨ is essential for bobel-induced cell death by pretreating the cells with CsA, an inhibitor of the mitochondrial permeability transition pore. For that, we preincubated K562 cells with 2 μmol/L of CsA for 30 min followed by their incubation with bobel for 16 hours (Fig. 4B). CsA inhibits the membrane depolarization induced by 2-fold IC50 Bobel-16 (B-16) for 16 h. Black and gray columns, similar to (A). Left, pretreatment of cells with 2 μmol/L CsA for 30 min rescues the depolarization of mitochondrial membrane induced by 2-fold IC50 Bobel-16 (B-16) for 16 h. Black and gray columns, similar to (A). Right, pretreatment of cell with 2 μmol/L CsA partially suppresses cell death induced by Bobel-16, as determined by the XTT viability assay. V, control cells treated with vehicle alone. B, CsA reduces bobel-induced depolarization and cell death. Left, pretreatment of cells with 2 μmol/L CsA for 30 min rescues the depolarization of mitochondrial membrane induced by 2-fold IC50 Bobel-16 (B-16) for 16 h. Black and gray columns, similar to (A). Right, pretreatment of cell with 2 μmol/L CsA partially suppresses cell death induced by Bobel-16, as determined by the XTT viability assay. V, control cells treated with vehicle alone. C, z-VAD, a pan-caspase inhibitor, does not increase cell viability in Bobel-24-treated K562 cells. K562 or Jurkat cells were preincubated with or without 25 μmol/L benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethyl ketone for 1 h previous to treatment with Bobel-24 (2 × IC50 concentration) for 48 h. D, Bcl-2 does not confer resistance to bobel-induced cell death in K562 cells. Immunoblot showing the expression of Bcl-2 of parental K562 and KbcI2 cells treated for 24 h with Bobel-24 (used at a concentration of 2 × IC50). The expression of glyceraldehyde-3-phosphate dehydrogenase is also shown to assess protein loading. E, parental K562 and KbcI2 cells were treated with the indicated concentrations of Bobel-4, Bobel-16, and Bobel-24 for 24 h, and cell viability was assayed by the XTT assay. Columns, mean values from four independent experiments; bars, SE.
leukemia and plays a role in the survival in many leukemia cell lines, including K562 and Raji. Also, Myc stimulates mitochondrial biogenesis. Therefore, we asked whether Myc could modify the cell death mediated by bobels. We first analyzed Myc expression in cells treated with the bobel compounds. K562 and Raji cells were treated for 24 hours with Bobel-24, Bobel-16, and Bobel-4 at different concentrations. We found that, in both cell lines, MYC mRNA expression is down-regulated by Bobel-24 and, to a lesser extent, by Bobel-16, as assessed by Northern blot (Fig. 5A). The same result was observed at the protein level, as assessed by Western blot (Fig. 5B). MYC down-regulation was more prominent after treating cells longer than 24 hours in all conditions (data not shown). However, Bobel-4, at 2× IC50 concentration did not significantly affect the levels of Myc protein in K562 cells (Fig. 5B), consistent with the divergent chemical and cytotoxic properties of Bobel-4.

In view of the dramatic down-regulation of Myc elicited by Bobel-24 and also because this is the leader compound, we asked whether Myc overexpression could rescue the cytotoxic effect of Bobel-24. We used the cell line, KmycB, as a tool to address this question. KmycB cells are a K562 subline stably transfected with a MYC expression vector in which the oncogene is under the control of the metallothionein promoter and is inducible by the addition of Zn2+ to the culture medium (25). The results showed that KmycB cells expressed significant levels of exogenous Myc mRNA and protein (Fig. 5C) when treated with Zn2+ or Zn2+ plus Bobel-24. In contrast, Bobel-24 completely abolished endogenous MYC mRNA (Fig. 5C, left) and protein (Fig. 5C, right) expression as expected. We next tested the cytotoxic effect of Bobel-24 on the KmycB cell line in the presence or absence of Myc induction. The results (Fig. 5D, left) showed that the ectopic expression of Myc partially rescued KmycB cells from the cytotoxic effect of Bobel-24 (16 hours). As a control, we also tested the effect of Bobel-4 (which hardly decreased the Myc levels in K562, Fig. 5A and B) on the viability of KmycB cells. Consistently, the ectopic expression of Myc did not rescue the viability loss provoked by Bobel-4 in KmycB cells (Fig. 5D, right). Thus, under conditions in which Myc is down-regulated (i.e., with Bobel-24 in K562), Myc ectopic expression antagonized the cytotoxic effect of Bobel-24, whereas in the conditions in which Myc expression is unmodified (i.e., with Bobel-4) ectopic Myc does not modify the cytotoxic response to the compound.

Discussion

Bobel-24 (2,4,6-triiodophenol, AM-24) was originally designed as a potential antiinflammatory drug with inhibitory activities against COX and LOX. Here, we have found that it exerts a potent cytotoxic activity against several leukemia cell lines. We have designed and described three new derivatives of Bobel-24: Bobel-4, Bobel-16, and Bobel-30. These molecules show different inhibitory selectivities toward 5-LOX and COX, and induce cell death in leukemia cell lines.

Bobel-16 is a dual inhibitor of COX and 5-LOX and shows the highest cytotoxic potency over the studied cell lines. In contrast, Bobel-4 is the most selective against 5-LOX. In many cases, there is no good correlation between the extent of COX or 5-LOX inhibition and the cytotoxic effect on the tested cell lines. Therefore, their cytotoxic mechanism does not seem to be directly related to the inhibition of these enzymatic activities. However, it is important to note that the IC50 of these compounds for all the tested cell lines are within the concentration range clinically attainable (32). Interestingly, Bobel-24 and Bobel-16 show lower cytotoxic activity on nontransformed cells (32D, NIH3T3, leukocytes) than in leukemia cell lines, either from myeloid origin (K562, HL60) or lymphoid origin (Raji, MOLT). This effect is not found for Bobel-4, which presents physicochemical properties divergent from the other bobel compounds.

In the Raji and K562 cell lines, bobels provoke chromatin condensation (pyknosis, nuclear shrinkage), chromatin fragmentation, different degrees of phosphatidylserine

Figure 5. Effects of Bobel-24, Bobel-16, and Bobel-4 on MYC expression. K562 and Raji cells were incubated for 24 h with the indicated concentrations of Bobel-24 and Bobel-4, expressed as fold of the corresponding IC50 for each cell line (see Fig. 2A). A, MYC mRNA expression as determined by Northern analysis. The rRNAs on the filter stained with ethidium bromide are shown to assess the loading of RNA. B, Myc protein expression as determined by Western analysis. Expression of β-actin was used as a loading control. Myc rescues the viability of K562 cells treated with bobel-24. C, MYC expression in KmycB cells. Cells were pretreated for 6 h in the presence of 75 μmol/L of ZnSO4 to induce ectopic Myc expression, then Bobel-24 (used at 75 μmol/L) was added and Myc expression was determined by Northern blot (left) after 36 h and Western blot (right) after 16 h. Left, bigger endogenous and smaller exogenous MYC transcripts in KmycB cells (arrows). D, Myc partially rescues KmycB cells from Bobel-24-induced cell death. KmycB cells were seeded at 1.2 × 105 cells/mL in the presence or absence of 75 μmol/L ZnSO4 for 6 h. Then, cells were plated and incubated in 96-well culture plates with the indicated concentrations of Bobel-24 (left) and Bobel-4 (right) for 16 h and cell viability as determined by the XTT assay. Columns, mean values from four independent experiments; bars, SE.
exposures, and membrane blebbing. However, we did not detect the activation of pro-caspase-3 or pro-caspase-7. Moreover, a pan-caspase inhibitor or Bcl-2 overexpression does not rescue cytotoxicity by bobels. These features are indicative of PCD (apoptosis-like PCD and necrosis-like PCD) that work mainly through caspase-independent pathways, but it is different from so-called accidental necrosis (34, 38).

Thus, we observed that several cell death mediators are involved in the bobel-induced cell death. They include generation of ROS, dissipation of mitochondrial transmembrane potential (ΔΨm), and cytochrome c release, which suggest a redox-linked mechanism. Caspase-independent mitochondrial death associated with mitochondrial dysfunction has been previously reported (39). Thus, we have shown that Bobel-24 and Bobel-16 induce total or partial depolarization of the mitochondrial membrane in all studied cell lines, and Bobel-4 only in Raji cells or at a concentration >2-fold IC50 on K562 cells, again, consistently with its divergent physico-chemical properties. Pretreatment with CsA, an inhibitor of permeability transition pore and hence of the membrane depolarization, was able to partially prevent cell death induced by bobels. Although 5-LOX and COX activity inhibition are not directly responsible for the bobel-induced cell death through the mitochondria, their inhibition could amplify some of the cytotoxic effects produced by these compounds by increasing free arachidonic acid or other lipid second messengers (such as the GL3 ganglioside and ceramide), which are involved in mitochondrial outer membrane permeabilization (38, 40). On the other hand, ROS, the concentrations of which are also increased by cPLA2 and arachidonic acid (38, 41), could also lead to severe mitochondrial dysfunction and produce necrotic PCD or apoptotic PCD. The cytochrome c release and production of arachidonic acid are also involved in the cell death pathways induced by the bobel compounds in pancreatic carcinoma cell lines, suggesting that this might be a general mechanism of action of bobels. The reduction in S phase provoked by bobel treatment may be related to the dramatic Myc down-regulation, as Myc is a major regulator of the G1-S transition (14, 15). In agreement with this idea, we observed that the bobel compound provoking a less potent DNA synthesis inhibitory effect on K562 (Bobel-4, Fig. 2C), also provokes a smaller decrease in Myc expression (Fig. 5B).

In many tumor cells, particularly hematologic neoplasias, Myc down-regulation can lead to apoptosis (17, 42). Also, in some models, the cell death mediated by MYC down-regulation is associated with an increase in the production of ROS and cytochrome c release (43–45). Moreover, it has recently been shown that Myc increases mitochondrial mass and function (18).

Our results are in line with these data and suggest that the down-regulation of MYC by bobel compounds induces mitochondrial dysfunction, leading to induction of the downstream events of PCD, such as ROS generation and cytochrome c release. This hypothesis was confirmed by using a K562 cell line with conditional expression of MYC, in which Myc expression remains unabated in the presence of bobel. Under these conditions, these cells show a resistance to the cytotoxic effect of Bobel-24, suggesting that bobels induce cytotoxicity, in part, through the down-regulation of MYC.

In conclusion, this work describes the cytotoxic effect of a new series of molecules on leukemia cells by inducing a caspase-independent cell death. This effect is exerted through the production of ROS, mitochondrial membrane permeability alteration, and MYC down-regulation.

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