Cyanidin-3-rutinoside, a Natural Polyphenol Antioxidant, Selectively Kills Leukemic Cells by Induction of Oxidative Stress*

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Anthocyanins are a group of naturally occurring phenolic compounds widely available in fruits and vegetables in human diets. They have broad biological activities including antimutagenesis and anticarcinogenesis, which are generally attributed to their antioxidant activities. We studied the effects and the mechanisms of the most common type of anthocyanins, cyanidin-3-rutinoside, in several leukemia and lymphoma cell lines. We found that cyanidin-3-rutinoside extracted and purified from the black raspberry cultivar Jewel induced apoptosis in HL-60 cells in a dose- and time-dependent manner. Paradoxically, this compound induced the accumulation of peroxides, which are involved in the induction of apoptosis in HL-60 cells. In addition, cyanidin-3-rutinoside treatment resulted in reactive oxygen species (ROS)-dependent activation of p38 MAPK and JNK, which contributed to cell death by activating the mitochondrial pathway mediated by Bim. Down-regulation of Bim or overexpression of Bcl-2 or Bcl-xL considerably blocked apoptosis. Notably, cyanidin-3-rutinoside treatment did not lead to increased ROS accumulation in normal human peripheral blood mononuclear cells and had no cytotoxic effects on these cells. These results indicate that cyanidin-3-rutinoside has the potential to be used in leukemia therapy with the advantages of being widely available and selective against tumors.

Natural products derived from plants have recently received much attention as potential chemopreventive and chemotherapeutic agents. Among them great attention has been given to natural products with established antioxidant activities and less toxicity in normal cells, such as tea polyphenols and resveratrol (1–3). These substances appear very promising for cancer prevention and treatment in preclinical models and clinical trials (1–4).

Perhaps among the most common type of plant polyphenols is flavonoids, which provide much of the flavor and color to fruits and vegetables. More than 5000 different flavonoids have been described (5). There are six major subclasses of flavonoids (5); anthocyanins, flavones (e.g. apigenin), flavonoids (e.g. quercetin), flavonones (e.g. naringenin), catechins of flavanols (e.g. epicatechin), and isoflavones (e.g. genistein). Anthocyanins probably deserve the most attention, as the daily uptake of anthocyanins in the human diet is remarkable, estimated at 180–215 mg/day in the United States (6), which is much higher than the total intake estimated for other flavonoids (23 mg/day).

Anthocyanins are the glycosylated form of anthocyanidins, which are polyhydroxyl and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts (7). Structurally, there are more than a dozen different anthocyanidins in plants, but cyanidin is the most frequently found, naturally occurring anthocyanidin. Cyanidin is widely available in the human diet through crops such as beans, fruits, vegetables, and red wine (7), and cyanidin-3-rutinoside (C-3-R)2 is a common glycosylated form (8).

Anthocyanins demonstrate strong antioxidant activities in a variety of in vitro assays (7–10). These natural compounds can react with reactive oxygen species (ROS) and thus interrupt the propagation of new free radical species. The double bonds present in the phenolic ring, the hydroxyl side chains, and even the glycosylation contribute to the scavenging activity. Anthocyanins, particularly cyanidin glycosides, have been found to possess a broad spectrum of biological activities, including scavenging effects on activated carcinogens and mutagens and effects on cell cycle regulation (7, 8, 11–13). Previous work has indicated that cyanidin-rich berry or pomegranate extracts or purified cyanidins possess remarkable chemopreventive activities in cell culture and animal models (11, 14–17). Furthermore, cyanidin glycosides purified from berry extracts possess proapoptotic properties in human cancer cells including leukemia cells (13, 18–20). These studies suggest that the antitumor activities of cyanidin glycosides could be related to their ability to induce apoptosis and that these products may have potential.

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‡The abbreviations used are: C-3-R, cyanidin-3-rutinoside; DCF, 2′,7′-dichlorofluorescein; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; NAC, N-acetyl-cysteine; PBMC, peripheral blood mononuclear cells; PEITC, β-phenylethyl isothiocyanate; PI, propidium iodide; shRNA, short hairpin RNA; JNK, c-Jun NH2-terminal kinase; MAP, mitogen-activated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AFC, 7-amino-4-trifluoromethyl coumarin; Z, benzoyloxy carbonyl.
as agents for cancer treatment as well. All of these activities are thought to be related to the antioxidant properties of the cyanidins (8, 21–23). However, this notion has not been carefully examined, particularly for the apoptotic activity. Furthermore, the molecular mechanisms and signaling pathways initiated by cyanidin glycosides in cell death are also poorly understood.

In the present study, we have found that cyanidin-3-rutinoside paradoxically increases the level of peroxides in the human leukemia cells, and this increase is responsible for its apoptotic effects. Cyanidin-3-rutinoside subsequently activates the p38 MAP kinase and the pro-death Bcl-2 family proteins, leading to mitochondrial release of apoptogenic factors and cell death. In contrast, cyanidin-3-rutinoside caused little ROS generation in the normal human peripheral blood mononuclear cells (PBMC) and had very low toxicity toward these cells. Our study thus indicates that natural antioxidative products such as cyanidin 3-rutinoside may exhibit pro-oxidant activities selectively in leukemic cells, an effect that could be exploited in the development of antitumor agents having a low toxicity toward normal cells.

**EXPERIMENTAL PROCEDURES**

Reagents—C-3-R (purity >99%) was purified from black raspberry cultivar Jewel extract as described previously (10). Purified C-3-R was stored at −80 °C, and the stock solution (150 mM) was prepared fresh before use by dissolving the powder in Me2SO.

The following primary or secondary antibodies were used: anti-Bak (NT) (Upstate Biotechnology, Lake Placid, NY); anti-Bak (Ab-1) (Oncogene, Cambridge, MA); anti-Bax (N-20) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Bax (clone 6A7), anti-Bcl-2, anti-JNK (clone 666), anti-cytochrome c, and anti-Smac (all from BD Biosciences, San Jose, CA); anti-Bcl-xL, anti-Bim, antiphosphorylated JNK and p38 MAP kinase (all from Cell Signaling, Beverly, MA); anti-β-actin (Sigma); anti-COX IV subunit II (Invitrogen-Molecular Probes); and horse-radish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA).

Cell Lines and Cell Cultures—The human leukemia and lymphoma cell lines HL-60 (myeloblastic), MOLT-4 (lymphoblastic), and Daudi (lymphoblastic) were obtained from American Type Culture Collection (Manassas, VA). Human leukemia cells HL-60/neo, HL-60/Bcl-2, and HL-60/Bcl-xL were derived from the laboratory of Dr. Kapil N. Bhalla (Medical College of Georgia) and cultured as described previously (24). The T cell acute lymphoblastic leukemia cell line, CCRF-CEM, and its transfectants were used in this study. Human PBMC were prepared from healthy donors (Pittsburgh Blood Bank) using Ficoll-Hypaque centrifugation. The leukemia/lymphoma cell lines and PBMC were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Analysis of Cell Death—Apoptotic and necrotic cell death were determined as described previously, with modifications (26). Briefly, cells were double-stained with 10 μmol/liter bisbenzimide Hoechst 33258 and propidium iodide (PI, 1 μg/ml) (Invitrogen-Molecular Probes) for 30 min and analyzed by fluorescence microscopy. Approximately 300–500 cells/condition were randomly selected and assessed. Hoechst 33258 positive cells with apoptotic (condensed and/or fragmented) nuclei were considered as apoptotic cells regardless of whether they were PI positive or not, whereas PI-stained cells without apoptotic nuclear changes were considered as necrotic cells.

Caspase activities were measured using 15 μg (caspase-3) or 20 μg (caspase-8 and -9) of proteins and 20 μM fluorescent substrates (Ac-DEVD-AFC, Ac-IETD-AFC, and Ac-LEHD-AFC for caspase-3, -8, and -9, respectively) (Biomol, Plymouth Meeting, PA) (26). The fluorescence signals were detected using a fluorometer (Tecan GENios, Durham, NC) at excitation and emission wavelengths of 400 and 510 nm, respectively.

Cytochrome c release and Bax translocation to the mitochondria were analyzed by immunoblot assay using the cytosolic and membrane fractions prepared from treated cells. Subcellular fractionation was conducted using limited plasma membrane permeabilization with 0.05% digitonin as described previously (27).

Measurement of ROS—For the in vitro study, superoxide radicals were quantified spectrophotometrically in a xanthine/xanthine oxidase system as described previously (28). The final results were expressed as percent inhibition of superoxide radical production in the presence of an antioxidant (C-3-R, ascorbic acid, or Trolox). The assay for hydrogen peroxide (H2O2) levels was carried out following procedures described previously (29). The final results were expressed as percent inhibition of H2O2 levels in a Ti(IV)-H2O2 complex system in the presence of antioxidants.

For determination of the cellular ROS level, two different methods were used. First, at various times (15 min to 4 h) after C-3-R treatment, HL-60 cells were incubated for another 15 min with either 2 μM dihydroethidium bromide or 2 μM 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA). These ROS-dependent fluorescent probes were converted to ethidium or 2′,7′-dichlorofluorescein (DCF) in the presence of superoxide radicals or H2O2, respectively, which can be detected by flow cytometry (26). To measure the intracellular accumulation of C-3-R-induced H2O2 production, cells were treated simultaneously with C-3-R and H2DCFDA for 1.5–3 h and then harvested for flow cytometry analysis. In another set of experiments, intracellular H2O2 levels were determined by a method based on horseradish peroxidase-dependent oxidation of phenol red, which is assessed by the increased absorbance at 610 nm (30).

Immunofluorescence and Immunoblot Analysis—Immunofluorescence staining was carried out as described previously (26). Treated cells were harvested and pelleted to glass slides by Cytospin® (Thermo Shandon Inc., Pittsburgh, PA). Cells were fixed in 4% paraformaldehyde/phosphate-buffered saline (pH 7.4) for 1 h at 4 °C and permeabilized in 0.5% Triton X-100 for another 1 h. Following incubation of the primary antibodies, the molecules of interest were detected using Alexa Fluor-conjugated secondary antibodies and fluorescence microscopy.

For the immunoblot assay, treated cells were lysed with radioimmune precipitation assay buffer supplemented with 1 μM EDTA and a mixture of protease inhibitors. Proteins in the lysates were quantified and separated by SDS-PAGE, trans-
ferred to a polyvinylidene difluoride membrane, and probed with the relevant antibodies.

RESULTS

C-3-R Selectively Induces Apoptosis in Human Leukemic Cells—Treatment of the human leukemia cell line HL-60 with C-3-R induced a significant amount of apoptosis in a time- and dose-dependent manner (Fig. 1A) based on the morphology of the nucleus. About 50% of the cells became apoptotic within 18 h when 50 μM C-3-R was applied, and virtually all cells were apoptotic in the presence of 120 μM or greater C-3-R. There was very little necrosis as measured by PI staining. Consistently, a significant amount of caspase-3 and caspase-9 activity could be detected in a dose-dependent manner, which peaked around 16 h (Fig. 1, B and C). The detected activity was reduced thereafter, likely because of the increased cell death. Consequently, C-3-R-induced apoptosis and caspase activation in HL-60 cells could be suppressed by the effector caspase inhibitor DEVD-
CHO (data not shown) and the pan-caspase inhibitor Z-VAD-fmk (data not shown) and the pan-caspase inhibitor Z-VAD-fluoromethyl ketone (Fig. 1D). The mitochondrial pathway could be an important mechanism contributing to C-3-R-induced apoptosis, as we found that cytochrome c (Fig. 1E) and Smac (see below) was released upon C-3-R treatment.

C-3-R also induced apoptosis in other human leukemia/lymphoma cell lines including MOLT-4, Daudi, and CCRF-CEM (Fig. 1F). It had little toxicity against the normal human PBMC as measured by nuclear staining with Hoechst 33258 and by MTT assay (Fig. 2, A and B). This probably is not a surprising finding, as C-3-R is a common constituent in human diets (5, 6). This property is similar to resveratrol, a natural product widely studied for its chemopreventive benefits (4). C-3-R had some toxicity against proliferating PBMC stimulated with phytohemagglutinin based on Hoechst staining (Fig. 2A), although this was not as obvious in the MTT assay. This toxicity, however, was much lower than that found in proliferating HL-60 cells (Fig. 1A). We also observed a similar level of toxicity of resveratrol in the proliferating PBMC using the Hoechst assay (data not shown). These observations suggest that proliferating PBMC may be, in general, more susceptible to these chemicals.

C-3-R Selectively Alters the Intracellular Level of Peroxides in HL-60 Cells—Structurally C-3-R possesses the phenolic rings typical of the anthocyanidins (Fig. 3A), suggesting that it has antioxidant activity similar to that of other members of the family. Indeed, when using the standard in vitro assays, we found that C-3-R demonstrated concentration-dependent inhibition of H$_2$O$_2$ production in the Ti(IV)-H$_2$O$_2$ complex system (Fig. 3B). C-3-R could also effectively inhibit superoxide and peroxyl radicals (ROO$^*$) production (data not shown). This activity might be related to its chemopreventive activity in the normal cells (8, 21, 22). Consistently, we have found that treatment of normal human PBMC with C-3-R led to reduced intracellular peroxide levels in the time frame of 15 min to 4 h based on the use of the cell-permeable nonfluorescent H$_2$DCFDA, which is oxidized to generate the fluorescent product DCF by the peroxides (Fig. 2C). In addition, the increased reduction of MTT in the C-3-R-treated cells could also suggest an overall less oxidative environment in these cells (Fig. 2B).

In contrast, when the leukemic HL-60 cells were treated with C-3-R, there was an increased level of H$_2$O$_2$ accumulated in the cells. In the first assay, we added H$_2$DCFDA only for the last 15 min of the culture, at each given time point, to detect the intracellular level of H$_2$O$_2$ at that particular moment. We found that although similar to what was observed in the normal PBMC, C-3-R treatment in HL-60 cells led to the reduction of intracellular peroxides in the first 15 min of the treatment. The peroxide level began to reverse within 30 min and was significantly above the control level by 1 h of culture (Fig. 3C). The accumulated level of peroxide was gradually reduced thereafter to the control level by 4 h after treatment. To examine the overall impact of C-3-R treatment on the intracellular accumulation of H$_2$O$_2$, we added the H$_2$DCFDA at the beginning of the culture through the first 1.5 and 3 h. This allowed the level of DCF to accumulate as the production of H$_2$O$_2$ was increased. The result indicated that despite the initial reduction of H$_2$O$_2$ in the first 15 min after treatment, the net level of H$_2$O$_2$ was increased during the first 3 h and peaked at the first 1.5 h (Fig. 3D). We confirmed this result using an enzyme-based assay that measures the concentration of H$_2$O$_2$ based on the horseradish peroxidase-mediated oxidation of H$_2$O$_2$.
phenol red (30) (Fig. 3E). In contrast to the observed change in H₂O₂ levels, there were no significant changes in the intracellular superoxide (O₂⁻) levels using dihydroethidium staining (data not shown). These results suggest that C-3-R led to a biphasic change in the intracellular redox status, with the net impact being an increase in the intracellular H₂O₂ level.

C-3-R-induced Apoptosis Could Be Mediated by ROS, p38 MAP Kinase, and JNK—Based on the above observations, it was possible that C-3-R-induced apoptosis could be related to its activity in enhancing cellular peroxide level. To investigate this possibility, we first determined whether a preincubation of a nontoxic low dose of H₂O₂ would inhibit C-3-R-induced apoptosis. Such treatment is well known to induce an adaptive response from the cells, which leads to cellular resistance to the subsequent challenge of a larger dose of H₂O₂ due to the induction of antioxidative gene expression (31). Indeed, this pretreatment with 0.5–4 µM H₂O₂ for a period of 3 h dramatically suppressed C-3-R-induced apoptosis in HL-60 cells (Fig. 4A). Consistently, C-3-R-induced apoptosis, caspase activity, and mitochondrial release of apoptogenic factors also could be suppressed by the general antioxidant N-acetyl-cysteine (NAC) or the H₂O₂ scavenger catalase (Fig. 4, B and C). These observations suggest that C-3-R-induced oxidative stress can activate the mitochondrial apoptosis pathway in HL-60 cells.

To explore the signaling events upstream the mitochondria, we examined the redox-sensitive stress kinase pathways, which are involved in apoptosis (32). In untreated HL-60 cells, neither p38 MAP kinase nor JNK was phosphorylated (Fig. 4D). Upon C-3-R treatment, there was a rapid increase in the phosphorylation of both p38 MAP kinase and JNK, correlating with the increase of intracellular peroxides levels (Fig. 3, C–E). Indeed, treatment of cells with the antioxidant NAC and the H₂O₂ scavenger catalase suppressed the activation of p38 MAP kinase and JNK at the early time point (Fig. 4E).
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Although JNK activation seemed to be relatively transient, p38 MAP kinase phosphorylation was sustained throughout the time course. However, both the p38 MAP kinase inhibitor SB203580 and the JNK inhibitor SP600125 could significantly inhibit mitochondrial release of cytochrome c and Smac, caspase activation, and apoptosis induced by C-3-R (Fig. 4, B and C), indicating that both stress kinases were involved in the C-3-R-activated mitochondrial apoptosis pathway following C-3-R-induced oxidative stress.

Activation of the Mitochondrial Pathway by C-3-R—To understand how C-3-R-mediated oxidative stress and stress kinase activated the mitochondrial apoptosis pathway, we examined activation of the pro-death Bcl-2 family proteins, which are perhaps the most important molecules promoting this pathway (33). The multidomain pro-death molecules, Bax and Bak, are responsible for the mitochondrial release of apoptogenic factors. When activated, they become oligomerized, which can be detected by conformation-sensitive antibodies.

Indeed, the mitochondria localized Bak could be activated by C-3-R treatment as shown by immunostaining with a conformation-sensitive antibody (Ab-1) (Fig. 5A). Immunostaining with a conformation-sensitive anti-Bax antibody (clone 6A7) also detected Bax in a distinctive punctated pattern following C-3-R treatment, suggesting that the normally cytosol-located Bax was translocated to the mitochondria and was in an activated status (Fig. 5A). Fractionation studies confirmed Bax translocation upon C-3-R treatment (Fig. 5B). Interestingly, catalase, SB203580, or SP600125 had a much stronger effect on Bax conformational change (Fig. 5C) than on Bax translocation (Fig. 5B). These data suggest that ROS and stress kinases mainly mediate the second step of Bax activation, the oligomerization.

This activation step of Bax and Bak (oligomerization and conformation change) was most likely caused by the BH3-only Bcl-2 family proteins (33). Among the possible BH3-only molecules that could be activated under the current experimental system, we focused on Bim, as it is reported to be activated by stress kinases, mostly via direct phosphorylation of protein (34–37). We found that although the general level of BimEL did not change significantly following C-3-R treatment, the migration pattern of BimEL was noticeably altered (Fig. 5D). There were about three species of BimEL that could be detected in the nontreated HL-60 cells, which represent different phosphorylation statuses of BimEL as shown previously (34–36, 38). Treatment of C-3-R rapidly induced an increase in the slowest migrating species that remained for at least several hours (Fig. 5D). When the inhibitors of the stress kinases were added to the culture, this up-shift was significantly suppressed (Fig. 5E). These results thus indicate that increased BimEL phosphorylation following C-3-R treatment is correlated with the activation of p38 MAP kinase and JNK, which could contribute to its proapoptotic activity as shown in earlier studies (34–37).

Because suppression of Bim expression using the RNAi-based approach in HL60 cells was not successful, we used another leukemic cell line, CCRF-CEM, for which a subline stably expressing a shRNA against Bim has been established and characterized (25). This cell line was susceptible to C-3-R-mediated apoptosis in a dose-dependent manner (Fig. 1F), which could be further suppressed by the p38 MAP kinase
inhibitor SB203580 and the antioxidants NAC and catalase to the same extent as observed in HL-60 cells (Fig. 5F), suggesting that a conserved pathway was activated. Bim expression was considerably reduced in the CCRF-CEM subline stably expressing a shRNA against Bim, and indeed this subline was significantly resistant to C-3-R-induced apoptosis as compared with the subline expressing the shRNA vector only (Fig. 5F). Furthermore, no further reduction of apoptosis was seen in the Bim knocked-down cells upon treatment with SB203580, NAC, or catalase, supporting the notion that Bim is the downstream target of ROS and p38 MAP kinase.

Conversely, HL-60 cells that overexpressed anti-death Bcl-2 or Bcl-xL (Fig. 6A) were significantly resistant to C-3-R-induced apoptosis and caspase activation at all tested doses (Fig. 6, B and C). These results indicate that C-3-R-induced HL-60 apoptosis is mediated mainly by the mitochondrial pathway, which is regulated by the Bcl-2 family proteins.

**DISCUSSION**

Anthocyanins such as C-3-R have broad biological effects in antimutagenesis and anticarcinogenesis and have been hailed as useful natural agents for chemoprevention (8, 18, 21, 22). These activities are generally attributed to their antioxidant properties, as determined in a variety of in vitro assays. Although we have confirmed that C-3-R, purified from black raspberries, does possess the antioxidant activity in these in vitro assays, we find that it actually causes ROS accumulation in leukemic cells and that the increased oxidative stress is responsible for its proapoptotic activity.

It is not yet clear how C-3-R promotes oxidative stress in leukemic cells. We observed the increase of peroxides, but not superoxides, in the treated cells. The surge of ROS can be cumulative, and is sufficient to activate the downstream apoptotic events. It is likely that treatment with C-3-R could inactivate the glutathione antioxidant system, which is involved in the scavenging of the peroxides. This assumption is supported by the protective effects of NAC observed in this study, which could regain the glutathione levels in C-3-R-treated cells.

C-3-R, on the other hand, did not induce increased levels of peroxide in the normal PBMC, where the intracellular H$_2$O$_2$ levels actually remained lower following treatment, suggesting...
that C-3-R could have a radical scavenging activity in these cells. It is not quite clear why C-3-R has a different impact on the redox status in the transformed leukemic cells versus the normal PBMC. This could, however, be related to the notion that transformed cells and normal cells may have different resting levels of ROS and antioxidant capacity (39, 40).

It is interesting to note that several other natural products, such as β-phenylethyl isothiocyanate (PEITC), a natural compound found in consumable cruciferous vegetables (41), known to be antioxidants in in vitro assays, demonstrate pro-oxidant activities in in vivo studies, which are responsible for their apoptotic effects in tumor cells. Notably, PEITC also shows a relatively higher selection toward the tumor cells for its killing effects and ROS generation (41). These observations indicate that the dual effects on the redox status could be a shared feature in some biologically active natural products that are being evaluated for their chemoprevention and/or chemotherapeutic activities.

The intracellular redox environment could be determined by the relative rate of production and removal of ROS. Changes in the ROS level or the intracellular redox environment can significantly modulate physiologic and pathologic processes. It has been generally postulated that tumor cells produce more ROS than normal cells, and this is connected with increased proliferation and tumorigenesis (39, 40). Oxygen radicals such as superoxide and hydrogen peroxide can act directly as signaling molecules. Alternatively, a change in redox environment can affect signaling cascades and redox-sensitive transcription factors involved in proliferation, survival, and hormonal response.

However, excessive ROS production can lead to cellular damage and induce cell death (40). Because of the pro-oxidant status of the cancer cells, they seem to be more susceptible than normal cells to treatment with agents that cause oxidative stress, as epitomized by the study using PEITC (41). Our findings that C-3-R, also a natural product commonly found in fruits and vegetables, selectively induces the accumulation of peroxides in the leukemic cell line HL-60, but not in normal PBMC, point in the same direction and suggest that this approach is valid in seeking new generation of therapeutic agents with lower side effects, considering that many anticancer drugs currently used in the clinic have strong cytotoxicity toward normal cells.

It may be that not all tumor cells are subjected to such selective killing. Although we have observed that several leukemia/lymphoma cell lines (HL-60, MOLT4, Daudi, and CCRF-CEM) are sensitive to C-3-R, we have also found that a lung adenocarcinoma cell line, A549, and a hepatocellular carcinoma cell line, SMMC-7721, are resistant to treatment with C-3-R (data not shown). It is possible that the redox status of these cells is different from that of leukemic cells such as HL-60. Alternatively C-3-R may not be sufficiently potent in changing the redox balance to allow the development of oxidative stress in some cancer cells. These hypotheses could be tested in future experiments.

ROS can cause cell death via a variety of mechanisms, among which the activation of stress kinases plays an important role (32). Our studies indicate the activation of p38 MAP kinase and JNK by C-3-R in a ROS-dependent manner in HL-60 and CCRF-CEM cells. The exact mechanisms by which ROS activate the MAP kinase pathways are not fully understood in many cases and could depend on the types of cells and stimuli. ROS could activate the upstream ASK1 (32) or inactivate the MAP kinase phosphatase (42) leading to sustained phosphorylation and activation of p38 MAP kinase or JNK. Future work should be directed toward exploring these possibilities.

Both ROS and JNK/p38 MAP Kinase-initiated apoptosis have been closely linked to the intrinsic pathway characterized by the mitochondrial release of apoptogenic factors such as cytochrome c and Smac (32). Mitochondrial activation is most critically regulated by the Bcl-2 family proteins. Although anti-death molecules such as Bcl-2 and Bcl-xL inhibit mitochondria activation, the pro-death molecules promote it (33). Our findings indicate that C-3-R-induced p38 MAP kinase and JNK activation promotes the activation of the BH3-only molecule, BimEL, and Bax and Bak conformation change. Earlier studies showed that Bim could be sequestered in the cytosol by its binding with the dynein light chain-1 and is released from the microtubules following phosphorylation in response to apoptotic stimuli (35, 38). Bim binds and antagonizes the anti-death

FIGURE 6. Bcl-2 or Bcl-xL can protect HL-60 cells from apoptosis induced by C-3-R. A, HL-60 cell lines stably expressing the mammalian expression vector (Neo) or human Bcl-xL, or Bcl-2 were analyzed by immunoblot assay for Bcl-xL (left panel) or Bcl-2 (right panel). B and C, HL-60 stable cell lines expressing the vector, Bcl-xL, or Bcl-2 were treated with C-3-R (0–80 μM) for 18 h. The percentage of cells with apoptotic nuclei as determined by Hoechst 33258 staining was quantified (B). Alternatively, cell lysates were prepared and analyzed for the activities of caspase-3 (C, upper panel) and caspase-9 activities (C, lower panel).
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Bcl-2 or Bcl-xL, thus activating Bax or Bak (43). BimEL can be phosphorylated at Ser65 by JNK (36) or p38 MAP kinase (37) in response to growth factor deprivation, UV stimulation, or sodium arsenate, all agents that can readily induce oxidative stress. p38 MAP kinase could also transcriptionally up-regulate Bim in leukemic cells following glucocorticoids treatment (25). Our finding that C-3-R could promote p38 MAP kinase and JNK-mediated BimEL phosphorylation is consistent with these studies. Furthermore, inhibition of Bim expression led to the suppression of C-3-R-induced apoptosis to the same extent as the p38 MAP kinase inhibitor and the antioxidant, supporting the role of Bim in C-3-R-induced apoptosis. It has to be noted, however, that this pathway is likely not the only pro-death mechanism activated by C-3-R, as suppressing ROS, stress kinases, or Bim could block C-3-R-induced apoptosis significantly but not completely.

In conclusion, the selective toxicity of C-3-R in leukemic cells is due significantly to its paradoxical pro-oxidant effects in cancer cells, which activate the stress kinases and subsequently the pro-death Bcl-2 family proteins and the mitochondrial apoptotic pathway. This property of C-3-R could be further explored for the development of antitumor agents with a higher therapeutical index.

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