The BH3-only protein Noxa is stimulated during apoptosis of chronic lymphocytic leukemia cells triggered by M2YN, a new plant-derived extract

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Abstract. Deficiency of apoptosis is a hallmark of chronic lymphocytic leukemia (CLL) cells. M2Yn is a natural extract from plants of central Asia, identified for its antiangiogenic properties and its ability to block the migration of malignant cells. Here, we report that *in vitro* treatment of cells derived from CLL patients with M2Yn results in internucleosomal DNA fragmentation, phosphatidylserine externalization, mitochondrial membrane depolarization, caspase-3 activation and cleavage of the caspase substrate PARP-1. The extents of these effects depend on the patients and are mostly comparable to those of flavopiridol or hyperforin, two known plant-derived apoptosis inducers of CLL cells. M2Yn does not modulate Mcl-1 expression, while downregulation of this antiapoptotic protein is involved in the action of flavopiridol. By contrast, M2Yn, like hyperforin, upregulates the Noxa protein, possibly by inhibiting proteasomal activity. This BH3-only protein is known to trigger the activation of the pro-apoptotic protein Bak through displacement of the Mcl-1/Bak complex at the mitochondrial membrane, as actually observed here in M2Yn-treated cells. Our data, therefore, show that M2Yn can induce the caspase-dependent mitochondrial pathway of apoptosis in CLL cells via a mechanism resembling that of hyperforin. Our data also confirm that the BH3-only protein Noxa is a relevant target for CLL therapy.

Introduction

Chronic lymphocytic leukemia (CLL), the most frequent leukemia in Western countries (1), still remains an incurable

disease despite recent therapeutic advances with the combination of purine analogs, alkylating agents and monoclonal antibodies (2,3). CLL is characterized by clonal expansion of CD5⁺ B lymphocytes which accumulate in the blood and are deficient in their apoptotic program (4). Therefore, new combination treatment strategies based on the re-establishment of apoptosis and on newly identified molecular targets have to be developed (2,4).

A number of agents are known for their ability to induce apoptosis of CLL cells *in vitro*, such as proteasome inhibitors, histone deacetylase (HDAC) inhibitors or various types of plant-derived compounds. Among them, flavopiridol, a semi-synthetic flavonoid, was the first to be discovered (5). Afterwards, we and others have shown the proapoptotic properties of a number of natural products such as roscovitine, silvestrol, gossypol, resveratrol and other polyphenols, combretastatin analogues, some xanthenes as well as hyperforin, a phloroglucinol purified from *Hypericum perforatum* or St John's wort (6-11). Most of these agents, which often display antitumor and antiangiogenic properties, activate the caspase-dependent mitochondrial pathway of apoptosis, but through different mechanisms. By its capacity to inhibit short-lived protein mRNA transcription, flavopiridol (and other cyclin-dependent kinase inhibitors) downregulates antiapoptotic proteins which are overexpressed in leukemic cells, including Mcl-1 (12,13), a Bcl-2 family member thought to be crucial for apoptosis deficiency in CLL (14). In contrast to flavopiridol, hyperforin does not inhibit Mcl-1 but induces the expression of the proapoptotic protein Noxa (15) belonging to the BH3-only subclass of the Bcl-2 family (16). Proteasome and HDAC inhibitors also upregulate Noxa (17,18). However, clinical trials performed in CLL with apoptosis inducers turned out to be disappointing. This is the case for flavopiridol (19), bortezomib, a proteasome inhibitor (20) or HDAC inhibitors (21), while no clinical trial with hyperforin has been reported so far. It therefore appears necessary to identify new therapeutic agents capable of specifically targeting certain Bcl-2 family proteins.

M2Yn is a natural extract isolated from plants in high altitude areas of central Asia which has been used in traditional

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medicine for centuries to alleviate symptoms associated with viral infections, particularly hepatitis-like diseases. M2Yn was characterized in our laboratory for its potential antiangiogenic properties (Mirshahi *et al*, unpublished data). Actually, this compound inhibits the formation of vascular tubules by human bone marrow and microvascular endothelial cells *in vitro*. Moreover, it blocks the migration of invasive mammary carcinoma cells, as well as filopode and lamellipode formation by corneal fibroblasts via Rho protein modifications, which are critical events in cell migration and tumor invasiveness.

In the present work, we have investigated the effects of M2Yn on nuclear, membrane and mitochondrial events of apoptosis on CLL patients' cells *ex vivo* in comparison with flavopiridol and hyperforin, two apoptosis inducers of vegetal origin. We show evidence that M2Yn can trigger the caspase-dependent mitochondrial pathway of apoptosis in the leukemic cells, involving upregulation of the proapoptotic Noxa without affecting the expression of the antiapoptotic Mcl-1. Our data also confirm that the BH3-only protein Noxa is an attractive target for new therapeutic strategies in CLL.

Patients and methods

Patients, cells and cell culture. Blood samples from CLL patients were obtained from the Hematology Department of Saint-Antoine Hospital (Paris, France) directed by one of us (Jean-Pierre Marie), after written informed consent in accordance with the revised Helsinki protocol. Diagnosis was established according to standard clinical criteria and to the International Workshop on CLL (IWCLL), including lymphocyte morphology and co-expression of CD5, CD20 and CD23 antigens (22). A total of 7 untreated patients (4 men and 3 women), all classified as Binet stage A, with a mean age of 63±10 years were selected. The patients were randomly chosen inasmuch as CD38 and ZAP-70 expression, cytogenetic data and mutational VH status were available only for some of them, thus hampering a risk-group analysis. Leukemic B cells were isolated from peripheral blood of CLL patients with a purity ≥95% as previously described (23) and were immediately cultured. Cell cultures were performed at 37°C in a humidified atmosphere containing 5% CO₂ in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics and 10% FCS (PAA Laboratories, Pasching, Austria) at seeding densities of 2-4x10⁶ cells/ml. Cell concentrations were measured with a Z2 Coulter counter (Beckman-Coulter, Les Ulis, France) and cell viability was evaluated using a colorimetric assay based on WST1 tetrazolium salt cleavage by mitochondrial dehydrogenases (Roche Diagnostic, Meylan, France).

Reagents. M2Yn was isolated from medicinal plants harvested in high altitude areas of Tajikistan. The plants including their propolis were dried and the resulting powder (100 g) was subjected to alcoholic extraction (10% w/v in 70% ethanol). After filtration, the extract was dry-concentrated and samples were dissolved at the concentration of 3 mg/ml in 70% ethanol. The M2Yn extract thus obtained endows with antiangiogenic and antimigratory properties *in vitro*: it inhibits the formation of pseudo-tubules by endothelial cells grown on matrigel,

decreases the motility of corneal fibroblasts and blocks the migration of invasive mammary carcinoma cells (Mirshahi *et al*, unpublished data). These biological activities are detectable with a concentration of 0.5 µg/ml, reaching optimal values with 1-2 µg/ml. M2Yn is much more active than different propolis of european origin. The partial chemical analysis of M2Yn extract revealed that it contains several flavonoids, tocopherols, polyunsaturated fatty acids and chlorophyll, and mass spectrometry showed the presence of several polypeptides unknown in protein data base. However, M2Yn retained its biological properties upon protein depletion by saturated ammonium sulfate. Flavopiridol was kindly provided by Aventis Pharmaceuticals (Bridgewater, NJ, USA). Hyperforin was purified by one of us (Isabelle Bombarda) in the laboratory which described the procedure (24). Most other reagents and chemicals were from Sigma (Sigma Aldrich, Saint Quentin Fallavier, France), except when otherwise stated.

Phosphatidylserine (PS) externalization. This membrane marker of apoptosis was quantified by specific binding of FITC-conjugated annexin V (Bender Medsystems, Vienna, Austria) with or without simultaneous labeling with propidium iodide (PI), as previously detailed (23). The percentages of cells positive for annexin V-FITC and PI negative (early apoptosis) and of double positive cells (late apoptosis) were determined with a flow cytometer (Beckman Coulter).

Internucleosomal DNA fragmentation. The cleavage of chromatin and DNA into nucleosomes, a typical nuclear event of apoptosis, was evaluated by detection of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) in cell lysates from aliquots of 20,000 cells, using an ELISA with anti-histones and anti-DNA fragments mAbs (Cell Death Detection ELISAPLUS, Roche Diagnostics) according to a technique used previously (23). Nucleosomes levels were measured using streptavidin/biotin/peroxidase system and reading of the absorbance at 405 nm. Values obtained from triplicate samples never differed more than 10%.

Mitochondrial membrane depolarization. Activation of the mitochondrial pathway of apoptosis leads to mitochondrial membrane permeabilization characterized by a dissipation of the membrane potential. This depolarization can be quantified by a shift of the red to the green fluorescence of the specific probe JC-1, and therefore by a decrease of red/green fluorescence ratio, according to a technique described previously (23). Briefly, 2x10⁶ cells/ml were incubated in an assay buffer containing 2 µM JC-1 for 15 min at 37°C, then washed and distributed in a microtitration plate. Green and red fluorescence (respectively: excitation wavelength 485 nm/emission wavelength 525 nm, and excitation wavelength 485 nm/emission wavelength 595 nm) were simultaneously recorded with a microplate reader cytofluorometer (Wallac Victor-2, Perkin-Elmer, Norwalk, MT, USA).

Caspase-3 activity assay. Enzymatic activity of caspase-3 was determined by the capacity of cell lysates to cleave a specific substrate according to techniques previously used (23). Briefly, aliquots of cell lysates (10-20 µg) were incubated with the fluorogenic caspase-3 substrate DEVD-aminocoumarin

(DEVD-AMC) and the release of AMC was then recorded every 10 min for 2 h with a Victor-2 thermostated at 37°C (excitation wavelength: 380 nm/emission wavelength: 460 nm). The specificity of the reaction was assessed by the addition of unlabeled Ac-DEVD-CHO caspase-3 inhibitor (Sigma-Aldrich).

Western blotting. Protein expression was studied by Western blotting, as detailed previously (23). Equal amounts of the cell lysates (30-50 µg of proteins) were electrophoresed by SDS-PAGE. Separated proteins were electrotransferred on PVDF membranes which were further incubated with primary antibodies recognizing the molecules of interest. The immunoblotted proteins were revealed with secondary HRP-specific antibodies (DakoCymation, Glostrup, Denmark) and a system of enhanced chemiluminescence (Amersham ECL Western Blotting Analysis System, GE Healthcare, Orsay, France). The primary antibodies used were: the rabbit polyclonal S-19 anti-Mcl-1 and the mouse mAb anti-Bcl-2 (both from Santa Cruz Biotechnology, Tebu, Le Perray en Yvelines, France); two mouse mAb anti-Noxa, clone 6D619 (US Biologicals, Euromedex, Mundolsheim, France) and IMG-249 (Imgenex, Clinisciences, Montrouge, France); the mouse mAb anti-Bak Ab-1 specific for the active form of Bak (Calbiochem, VWR International SAS, Fontenay, France); the mouse mAb anti-PARP-1 [Poly(ADP-ribose) Polymerase-1] recognizing both the native form of 116 kDa and the cleaved fragment 85 kDa (Santa Cruz Biotechnology); the rabbit polyclonal anti-p27 (N20) recognizing both the native form (27 kDa) and the cleaved fragment p23 (Santa Cruz Biotechnology); the anti-beta actin (clone C4; MP Biomedicals, Illkirch, France) used for protein content monitoring and standardization.

Immunoprecipitation experiments. Cells were lysed for 30 min at 4°C with 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), 20 mM Tris-HCl/pH 8.0, 150 mM NaCl and a cocktail of protease inhibitors. Cell lysates were pre-cleared for 30 min at 4°C with Protein G-agarose beads (Santa Cruz Biotechnology) equilibrated with lysis buffer in the presence of the isotype antibody. Pre-cleared lysates were then incubated overnight at 4°C with pre-equilibrated Protein G beads and the mouse mAb anti-Mcl-1 (MCL-1801) from Santa Cruz Biotechnology. Immunoprecipitates were washed and mixed with non-reducing electrophoresis sample buffer, and beads were discarded by boiling for 3 min. Immunoprecipitates were finally subjected to Western blot analysis using the following primary antibodies: either anti-Mcl-1 (S-19) as a control of IP, anti-Noxa (6D619) or the rabbit polyclonal anti-Bak NT (Upstate, Millipore, Saint Quentin en Yvelines, France).

Immunofluorescence and flow cytometry analysis. The expression of Noxa protein was also analyzed by indirect immunofluorescence on cells permeabilized at 4°C with the Cytotfix/Cytoperm kit (BD Biosciences, Le Pont de Claix, France), as previously described (23). Cells were incubated with for 30 min with primary antibodies: either goat polyclonal anti-Noxa (N15, Santa Cruz Biotechnology) or isotype control (goat IgG). Cells were further incubated with a secondary FITC-conjugated antibody against goat Ig before

to be analyzed by flow cytometry using an EPICS Altra flow cytometer (Beckman-Coulter) in order to record the profiles of fluorescence and the percentages of fluorescent cells.

RT-PCR analysis of mRNA expression. Total RNA was extracted from CLL cells with the Trizol method and reverse transcription was performed as previously detailed (25). The primers used for Noxa and Mcl-1 (synthesized Eurobio, Les Ulis, France) and for β 2-microglobulin (Sigma Proligo France) were as follows: Noxa (forward) 5'-GTG CCC TTG GAA ACG GAA GA-3', and (reverse) 5'-CCA GCC GCC CAG TCT AAT CA-3'; Mcl-1 (forward) 5'-ATC TCT CGG TAC CTT CGG GAG C-3', and (reverse) 5'-CCT GAT GCC ACC TTC TAG GTC C-3'; β 2-microglobulin (forward) 5'-CAT CCA GCG TAC TCC AAA GA-3', and (reverse) 5'-GAC AAG TCT GAA TGC TCC A-3'. The annealing temperature of primers was 57°C. PCR products were visualized by electrophoresis in 2% agarose gel containing 0.2 mg/ml ethidium bromide. The sizes of amplified cDNAs were 165 bp for β 2-microglobulin, 446 bp for Mcl-1, and 260 bp for Noxa.

Proteasome activity assay. Proteasome activity was determined with a luminescent assay, the Proteasome-Glo™ Chymotrypsin-like cell-based assay (Promega France, Charbonnières-les-Bains, France) that measures chymotrypsin-like protease activity associated with the proteasome complex directly in intact cells. This assay has been used to evaluate the activity of bortezomib in a variety of cancer cell lines (26). It has three components: a buffer containing the proteasome complex, a luciferin detection reagent and the luminogenic substrate, succinyl-LLVY-aminoluciferin. The proteasome cleavage generates a luminescent signal produced by the luciferase reaction. Briefly, 100 µl of the reagent mixture was added to 2x10⁵ cells/100 µl in 96-well microtitration plates for 15 min at 37°C, and the luminescence was immediately recorded with a spectrophotometer Wallac Victor-2 microplate reader.

Results

M2Yn induces apoptosis of primary CLL cells. To investigate the effects of M2Yn on internucleosomal DNA fragmentation in CLL cells from different patients, cells were treated in culture with various M2Yn concentrations ranging from 0.5 to 12 µg/ml for different periods of time not exceeding 48 h in order to prevent spontaneous apoptosis in the untreated controls. It turned out that M2Yn treatment resulted in a time- and dose-dependent stimulation of DNA fragmentation, and Fig. 1 shows the maximal effect observed after 18 h with 6 µg/ml in comparison with 1 µM flavopiridol and 10 µg/ml hyperforin. The amplitude of this effect of M2Yn differed depending on the patients but was comparable to the effects of flavopiridol and hyperforin: a 3.9-fold mean increase over untreated controls was found with M2Yn versus 4.1- and 5.1-fold with respectively flavopiridol and hyperforin. M2Yn treatment also stimulated PS externalization as illustrated in Table I: the percentages of cells positive for PS externalization markedly augmented when compared to untreated controls. These data indicate that M2Yn is capable of inducing the apoptosis of CLL cells *in vitro*.

Figure 1. Effect of M2Yn on internucleosomal DNA fragmentation in CLL patient cells and comparison with flavopiridol and hyperforin. Leukemic cells from 4 CLL patients were cultured for 18 h in the absence (controls) or the presence of either 6 $\mu\text{g/ml}$ M2Yn, 1 μM flavopiridol, or 10 $\mu\text{g/ml}$ (18 μM) hyperforin, then cytoplasmic histone-associated DNA fragments (nucleosomes) were measured as described in Materials and methods.

M2Yn-induced apoptosis involves the caspase-dependent mitochondrial pathway. To examine whether the apoptosis elicited by M2Yn was caspase-dependent, the enzymatic activity of the effector caspase-3 was analyzed by measuring the capacity of cell lysates to cleave a specific substrate. Results showed that caspase-3 activity was stimulated after exposure of CLL patient cells to M2Yn. The examples of two patients are presented in Fig. 2A where caspase-3 activity was enhanced by M2Yn to the same extent as flavopiridol or hyperforin in one patient, but to a lesser extent in another one. Furthermore, the release of apoptogenic factors responsible for the cascade of caspase activation is allowed by pore opening at the mitochondrial membrane. This is associated with a dissipation of the transmembrane potential. It clearly appears that M2Yn treatment of CLL cells resulted in the diminution of the mitochondrial membrane potential, as shown in Fig. 2B. The amplitude of this effect depended on individual patient and was comparable to that obtained with flavopiridol or hyperforin. These results indicate that M2Yn triggers the caspase-dependent mitochondrial pathway of apoptosis in CLL cells.

M2Yn treatment stimulates Noxa expression in CLL cells. We then analyzed the effects of M2Yn on the expression of three Bcl-2 family proteins regulating the mitochondrial pathway of apoptosis: the proapoptotic BH3-only Noxa known to be upregulated during hyperforin-induced apoptosis (12), and two antiapoptotic proteins which are overexpressed by CLL cells and downregulated by flavopiridol, Bcl-2 and the crucial Mcl-1 (12,23). Apoptosis induction was monitored in parallel by the cleavages of PARP-1, a substrate of caspases, and of p27^{kip1} which is overexpressed by CLL cells and downregulated during apoptosis triggered by flavopiridol or hyperforin through caspase-dependent cleavage (23). Western blotting obtained from four patients are presented in Fig. 3A. As expected (15), flavopiridol-induced PARP-1 and p27 cleavages were associated with a strong Mcl-1 inhibition, a slight Bcl-2 cleavage and no stimulatory effect on Noxa levels. As also expected (15), hyperforin elicited PARP-1, p27^{kip1} and Bcl-2 cleavages to a lesser extent than flavopiridol, did not inhibit

Table I. Effects of M2Yn on PS externalization in CLL cells in comparison with flavopiridol and hyperforin.

	Percentages of cells				
	A ⁻ /PI ⁻	PI ⁺	A ⁺	A ⁺ /PI ⁺	Total A ⁺
Controls	62	4	11	22	33
M2Yn	36	2	35	26	61
Flavopiridol	11	1	72	15	87
Hyperforin	18	9	23	49	72

CLL cells from patient no. 5 were treated for 42 h with 6 $\mu\text{g/ml}$ M2Yn, 1 μM flavopiridol, or 20 $\mu\text{g/ml}$ hyperforin, or not treated (controls). PS externalization was evaluated by labeling with FITC-annexinV/PI and flow cytometry analysis, as described in Materials and methods. Data are from one experiment representative of three. A⁻/PI⁻, double negative viable cells; PI⁺, PI positive necrotic cells; A⁺, annexin V positive cells (early apoptosis); A⁺/PI⁺, double positive cells (late apoptosis); Total A⁺, apoptotic cells.

Mcl-1 but strongly enhanced Noxa levels. It turned out that treatment with M2Yn resulted in the stimulation of Noxa expression versus untreated controls, with hardly detectable effects on Mcl-1 levels or Bcl-2 cleavage. Mcl-1 expression was nearly not affected even with high concentrations of M2Yn (data not shown). M2Yn-promoted Noxa stimulation was dose-dependent and was accompanied with PARP-1 and p27 cleavages to extent similar to those observed with hyperforin. The effect of M2Yn on Noxa expression was not as strong as that of the phloroglucinol, but was observed with all the patients in the study (7/7). Moreover, it was obtained with two different anti-Noxa antibodies and was also detected by immunofluorescence (with a third antibody) and flow cytometry analysis: indeed both fluorescence intensity and the percentage of positive cells increased after treatment with M2Yn (Fig. 3B). These results show that unlike flavopiridol, but similarly to hyperforin, M2Yn induces Noxa upregulation without detectable effect on Mcl-1 expression and that this effect is associated with apoptosis.

M2Yn does not increase Noxa mRNA expression but inhibits proteasome activity. To evaluate whether the effect of M2Yn on Noxa protein resulted from transcription activation, RT-PCR experiments were performed. Fig. 4A shows that Noxa mRNA levels remained unchanged after treatment of CLL cells with M2Yn. No effect on Mcl-1 mRNA levels was observed while these levels decreased after treatment with flavopiridol, in agreement with previous data (13). These results indicate that the stimulatory effect of M2Yn on Noxa protein does not seem to result from a transcriptional regulation.

Because Noxa is known to be upregulated by inhibition of proteasomal degradation (16,17,27) and because a number of plant-derived compounds are natural proteasome inhibitors (28), we decided to study the effect of M2Yn on proteasomal activity in CLL cells. As seen in Fig. 4B, M2Yn treatment induced a dose-dependent reduction in proteasomal activity

Figure 2. Effects of M2Yn on caspase-3 activity and on the mitochondrial membrane potential in comparison with flavopiridol and hyperforin. Cells from different CLL patients were treated with either 6 μ g/ml M2Yn, 1 μ M flavopiridol, or 10 μ g/ml hyperforin, or left untreated (controls), then analyzed for caspase-3 activity and variation of mitochondrial membrane potential. (A) M2Yn stimulates caspase-3 activity, as measured by the capacity of cell lysates to cleave the fluorogenic specific substrate DEVD-AMC every 10 min during 2 h; the specificity of the enzymatic reaction was monitored by simultaneous incubation of the cell lysates with an inhibitor of caspase-3 activity (DEVD-CHO), as exemplified with M2Yn-treated cells (M2Yn/inhibitor). (B) M2Yn induces a dissipation of the mitochondrial membrane potential, as revealed by a decrease in the red/green fluorescence ratio using the specific probe JC-1. Data are means of triplicate values with SD never exceeding 10%. See Materials and methods for experimental procedures.

even more efficiently than hyperforin, although to a lower extent than bortezomib, a proteasome inhibitor used as a reference. It is thus possible that such a post-translational regulation might contribute to M2Yn-promoted Noxa upregulation.

M2Yn stimulates Mcl-1/Noxa association, Mcl-1/Bak dissociation and the active form of Bak. Noxa is known to insert its BH3 domain into the hydrophobic groove of Mcl-1 resulting in the displacement of the complex that Mcl-1 forms with the proapoptotic Bcl-2 family protein Bak, and thus in Bak release and activation (16). We therefore investigated whether Noxa, when upregulated by M2Yn, can interact with Mcl-1 and displace Mcl-1/Bak complex. To this end, co-immunoprecipitation experiments were performed on CLL cell lysates immunoprecipitated by an anti-Mcl-1 antibody. Results show clearly that treatment with M2Yn stimulated

the co-precipitation of Noxa with Mcl-1 as compared to low basal levels detected in untreated cells (Fig. 5A). This effect was also observed when cells were treated with hyperforin but not with flavopiridol, as expected because the latter does not induce Noxa expression. Simultaneously, the high levels of Bak co-precipitating with Mcl-1 in untreated controls were lowered upon exposure to M2Yn or hyperforin (Fig. 5A). These data provide evidence that M2Yn promoted both the interaction of Noxa with Mcl-1 and the dissociation of Mcl-1/Bak complex in CLL cells, suggesting a displacement of this complex. To verify that this effect was actually accompanied with Bak activation, the expression of the active form of Bak was studied. As seen in Fig. 5B, treatment of CLL cells with M2Yn resulted in a concentration-dependent stimulation of activated Bak levels, with a maximal extent closely resembling the effect of hyperforin. It can be noted that a similar result was observed upon exposure to flavopiridol that does not induce Noxa upregulation but Mcl-1 downregulation.

Discussion

The identification of new agents having highly specific targets is necessary to improve CLL therapy. Proteins of the Bcl-2 family appear as potential targets because they regulate survival/apoptosis balance via the control of pore opening at the mitochondrial membrane, allowing the release of apoptogenic factors (16). Moreover, this delicate balance influences CLL cell fate (29). Several reports have recently revealed the crucial role of Mcl-1 in the apoptotic defect of CLL cells (14,30). Besides, loss of Mcl-1 is sufficient by itself to trigger programmed cell death (31). Due to its specific interaction with Mcl-1, the BH3-only Noxa seems also to be an interesting target for CLL therapy (15,16,32). Actually, Noxa antagonizes Mcl-1, thus facilitating the activation of Bak, responsible for the mitochondrial membrane permeabilization and the subsequent caspase cascade.

In the present study, we show that the natural extract M2Yn, like a number of antitumor/antiangiogenic compounds of vegetal origin (flavopiridol, resveratrol and other polyphenols, hyperforin), is capable of inducing the apoptosis of primary CLL cells. As most apoptosis inducers, M2Yn activates the caspase-dependent mitochondrial pathway. This is attested by stimulation of DNA fragmentation, PS external-

A **B**

Figure 3. M2Yn stimulates Noxa protein expression in CLL cells. CLL cells of patients were treated for 18 h with either 3 or 6 $\mu\text{g/ml}$ M2Yn, 1 μM flavopiridol (F) or 10 $\mu\text{g/ml}$ hyperforin (H) and in untreated controls (C). (A) Western blot analysis of the expression and/or cleavage of different proteins showing that M2Yn stimulates both Noxa expression and PARP-1 cleavage without modulating Mcl-1 levels. (B) Immunofluorescence and flow cytometry analysis of Noxa expression in CLL cells from patient no. 4 after 18-h treatment with 6 $\mu\text{g/ml}$ M2Yn and in untreated controls (C); the data represent the fluorescence profiles obtained with anti-Noxa antibody (pale grey) in comparison with those obtained with isotype control Ig (dark grey) and the percentages of positive cells are indicated.

A **B**

Figure 4. M2Yn treatment does not modulate Noxa mRNA expression but inhibits proteasomal activity in CLL cells. Cells from patients 3, 6 and 7 were cultured for 18 h in the presence of 1.5 or 3 $\mu\text{g/ml}$ M2Yn, 1 μM flavopiridol (F), 10 $\mu\text{g/ml}$ hyperforin (H), or 5 nM bortezomib (B) or in their absence (controls, C). (A) Representative RT-PCR analysis of Noxa and Mcl-1 mRNA levels with $\beta 2\text{-m}$ as a control of RNA content; (-): buffer without cDNA. (B) Dose-dependent inhibition of proteasomal activity by M2Yn (mean of duplicates in a representative experiment).

ization, mitochondrial membrane permeabilization, caspase-3 activation as well as cleavage of the caspase substrate PARP-1. M2Yn has no direct effect on the expression of Mcl-1 but neutralizes its antiapoptotic activity through Noxa upregulation. Indeed, by interacting with Mcl-1, Noxa displaces Mcl-1/Bak complex leading to Bak activation. Consequently, the mechanism of action of M2Yn is different from that of flavopiridol and other plant-derived compounds acting through downregulation of antiapoptotic proteins including Mcl-1 (12,13). By upregulating the proapoptotic Noxa, M2Yn employs a mechanism shared by hyperforin, proteasome and HDAC inhibitors (15,17,18,32). This effect appears to result from a regulation at the protein level, as suggested by the ability of M2Yn to inhibit proteasomal activity in CLL cells, as do proteasome inhibitors (17) and hyperforin (Zaher *et al*, unpublished). With the exception of hyperforin which has not been so far tested therapeutically, apoptosis inducers of CLL cells such as flavopiridol, HDAC and proteasome inhibitors turned out to be disappointing in clinical trials, showing toxicity, adverse effects and/or no significant therapeutic activity (19-21). This is not surprising because their targets are not really specific: flavopiridol inhibits the transcription of a large number of proteins (33); HDAC and proteasome inhibitors enhance the expression of not only Noxa but also antiapoptotic proteins such as Mcl-1 (34,35). The observation that M2Yn does not modulate Mcl-1 expression seems therefore of great interest. The characterization of the activity of

Figure 5. M2Yn stimulates Noxa/Mcl-1 association, Mcl-1/Bak dissociation and activated Bak expression. CLL cells from patients 3, 6 and 7 were treated for 18 h with either 1 μ M flavopiridol (F), 10 μ g/ml hyperforin (H) or 6 μ g/ml M2Yn, or left untreated (controls, C). (A) Representative co-immunoprecipitations experiments showing that M2Yn treatment increases the co-precipitation of Noxa in Mcl-1 immunoprecipitates (IP) and that it decreases the co-precipitation of Bak in Mcl-1 IP (right panel). (B) Representative Western blot analysis showing that the levels of the active form of Bak are dose-dependently stimulated by M2Yn after 18-h treatment.

M2Yn and of its mechanism of action suggests that it belongs to a possible class of plant-derived apoptosis inducers capable of specifically targeting the BH3-only protein Noxa through upregulation. This family of compounds includes hyperforin, a phloroglucinol, and the natural proteasome inhibitor celastrol, a triterpen which was reported to induce apoptosis by activating Noxa (27). It has to be noted that triterpenoids can induce apoptosis of CLL cells (36). It is possible that a number of flavonoids (quercetin, apigenin, myricetin) known for their capacity to inhibit proteasome activity (28) would be able to induce apoptosis by enhancing Noxa expression. In addition, Noxa upregulation was reported to be associated with the proapoptotic effects of several polyphenols such as resveratrol, phenoxodiol or tetramethoxystilbene as well as the flavonoid isoliquiritigenin (37-40). Therefore, such a family of natural specific Noxa upregulators might be useful for the development of novel combining therapeutic protocols in CLL. As regards hyperforin, a derivative called aristoforin has been described with improved stability and solubility as well as better biological activity (41). By contrast, the biochemical structure of M2Yn is not yet fully defined. The natural extract consists of a mixture of several components retaining its biological activity upon protein depletion. Several flavonoids were found among these components, and experiments are in progress in order to identify which of them is (are) responsible for the proapoptotic activity of M2Yn. The complete chemical structure of M2Yn will allow to determine whether this activity may also be attributed to other types of compounds such as phloroglucinols or terpenoids.

Data from the present study confirm that the BH3-only protein Noxa is an attractive target for CLL therapy. The

concept of BH3 mimetics has recently emerged (29,30), and several BH3 mimetics displaying the capacity to antagonize the antiapoptotic Bcl-2/Bcl-xL/Bcl-w proteins have been described such as ABT-737 (42). As regards the crucial role of Mcl-1 in CLL, the use of Noxa mimetics should be of great interest. In connection with this, the clinical developments of pan-BH3 mimetics which efficiently mimic Noxa and/or target Mcl-1 (2), such as Obatoclax/GX15-070, Gossypol/AT-101 or the peptide 072RB, seem promising (8,43-45).

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