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Research Article

Modulation by polyamines of apoptotic pathways triggered by procyanidins in human metastatic SW620 cells

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Abstract. We showed previously that inhibition of polyamine catabolism with the polyamine oxidase inhibitor MDL 72527 (MDL) potentiates the apoptotic effects of apple procyanidins (Pcy) in SW620 cells. Here we report that Pcy caused an activation of the intrinsic apoptotic pathway through enhanced polyamine catabolism and mitochondrial membrane depolarization. MDL in the presence of Pcy caused a profound intracellular depletion of polyamines and exerted a protective effect on mitochondrial functions. MDL potentiation of Pcy-triggered apoptosis was reversed by addition of exogenous polyamines. In addition, MDL in combination with Pcy activated the extrinsic apoptotic pathway through enhanced TRAIL-death receptor (DR4/DR5) expression. Potentiation of Pcy-triggered apoptosis by MDL was inhibited when cells were exposed to specific inhibitors of DR4/DR5. These data indicate that the depletion of intracellular polyamines by MDL in the presence of Pcy caused a switch from intrinsic to extrinsic apoptotic pathways in human colon cancer-derived metastatic cells.

Keywords. Apoptosis, polyphenols, flavonoids, MDL 72527, mitochondria, TRAIL death receptors, polyamine, histone deacetylase.

Introduction

Apples represent an important source of polyphenolic compounds, which are divided into several classes such as flavonoid monomers and condensed tannins, the procyanidins (Pcy) [1]. Polyphenols have antioxidant properties and may prevent carcinogenesis by affecting molecular events in the initiation, promotion and progression stages [2]. We have recently reported that apple Pcy inhibit the growth of human metastatic colon carcinoma-derived SW620 cells. Pcy alter intracellular signaling pathways, polyamine metabolism and induce apoptosis [3, 4]. Polyamine metabolism in

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normal cells is regulated in a tight balance between biosynthesis and catabolism. The polyamine biosynthetic pathway involves two key enzymes: ornithine decarboxylase (ODC) necessary for the conversion of ornithine into putrescine and *S*-adenosylmethionine decarboxylase (AdoMetDC) catalyzing the decarboxylation of *S*-adenosylmethionine, the donor of propylamine necessary to the synthesis of spermidine and spermine. In the catabolic pathway (or retro-conversion pathway) acetylated polyamines are formed by spermine/spermidine acetyltransferase (SSAT), and are used as substrates by a flavin-dependent polyamine oxidase (PAO), which catalyzes their conversion back to spermidine and finally putrescine [5]. The observation that the polyamines (putrescine, spermidine and spermine) play a critical role in favoring cancer cell growth supported the development of antitumor strategies targeting the polyamine pathways [6, 7]. Neoplastic cell growth is associated with elevated polyamine biosynthetic activity [8]. We have previously reported that Pcy down-regulated polyamine biosynthesis by inhibiting ODC and AdoMetDC activities, and enhanced polyamine catabolism favoring apoptosis [3, 9]. Two main pathways control apoptotic cell death: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. These pathways may process independently or in cross-talk to activate pro-apoptotic factors and/or to inhibit anti-apoptotic mediators [10, 11].

We have recently reported that inhibition of polyamine catabolism with the specific PAO inhibitor MDL 72527 (MDL) potentiated the apoptotic effects of apple Pcy in SW620 cells [4], but MDL (50 μM) used as a single drug showed no anti-proliferative and/nor apoptotic effects. The potentiation of Pcy-triggered apoptosis by MDL seems to involve other apoptotic mechanisms than those activated by Pcy used alone. MDL, when combined with Pcy, prevented by 80% the formation of reactive oxygen species (ROS) (hydrogen peroxide and 3-acetamidopropanal) generated by PAO-catalyzed degradation of acetylated polyamines that were induced by Pcy [4]. The present report was aimed to gain more insight into the alternative apoptotic mechanisms triggered by MDL in the presence of Pcy and to determine the role of polyamines in this process.

Materials and methods

Isolation and characterization of apple Pcy. Polyphenols were purified from a cider apple (*Malus domestica*, variety Antoinette). Apples were reduced to a homogeneous powder, which was extracted by water : ethanol : acetic acid (975 : 1000 : 25). After filtration, evaporation under vacuum and freeze drying, the crude extract was dissolved in 2.5% acetic acid and separated by preparative HPLC (Lichrospher RP 18, 12 μm, Merck, Darmstadt, Germany) to remove sugars and other non phenolic polar compounds. Polyphenols were eluted with acetonitrile : water : acetic acid (300 : 700 : 25). The fractions containing polyphenols were evaporated and freeze-dried. The polyphenols were fractionated on a Fractogel column by a method adapted from Souquet et al. [12]. Pcy were characterized and quantified by thiolysis coupled with reverse phase-HPLC and diode array UV-visible detection [13]. On a weight basis, the Pcy fraction contained 78.4% Pcy, consisting of 95% (-)epicatechin and 4% (+)catechin. The mean degree of polymerization was close to four. The Pcy fraction was almost totally devoid of monomeric catechins and other phenols (< 2%). Stock solutions were prepared in dimethylsulfoxide (DMSO). MDL 72527 [N^1,N^4-bis(2,3-butanedienyl)-1,4-butaneamine dihydrochloride] was synthesized as described previously [14].

Cell culture and treatments. SW620 cells were purchased from the European Collection of Animal Cell Culture (ECACC, Salisbury, UK). They were cultured in 75-cm^2 Falcon flasks in Dulbecco's modified Eagle's medium containing 25 mM glucose and supplemented with 10% heat-inactivated (56 °C) horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% nonessential amino acids (Invitrogen Corp., Cergy Pontoise, France). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 and subcultured after trypsinization (0.5% trypsin/2.6 mM EDTA). For all experiments, cells were seeded at 0.2×10^6 cells (dishes 2.5 cm Ø) or at 1×10^6 cells (dishes 10 cm Ø). The culture medium was Dulbecco's modified Eagle's medium supplemented with 3% heat-inactivated horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml transferrin, 5 ng/ml selenium, 10 μg/ml insulin and 1% nonessential amino acids (Invitrogen Corp., France). Cells were exposed to different compounds 24 h after seeding and incubated for 48 h. Pcy was diluted in DMSO and used at a final concentration of 80 μg/ml. The final concentration of DMSO in the control and the treated group of cells did not exceed 0.1%. Stock solutions of MDL, putrescine and spermidine were dissolved in pure H_2O and tested at 50 μM for MDL and at 500 μM for putrescine and spermidine (Sigma-Aldrich, Steinheim, Germany).

Flow cytometric analysis of subG_0/G_1 cell population. Cells were harvested by trypsinization after 48-h treatment with Pcy (80 μg/ml) and/or MDL (50 μM) and washed twice with phosphate-buffered saline (PBS) 0.1 M, pH 7.2. The subG_0/G_1 cell population

(hypodiploïd apoptotic cells) distribution was analyzed after labeling cells with propidium iodide, and assays were carried out as described previously [15]. Cells were centrifuged and fixed in 1 ml methanol : PBS (9 : 1, v/v) for 30 min at 4 °C, washed twice in PBS and re-suspended in 200 µl PBS containing 0.25 mg/ml RNase A and 0.1 mg/ml propidium iodide (Sigma-Aldrich, Germany). After incubation in the dark at 37 °C for 30 min, flow cytometric analysis was assessed (FL-2: 580 nm) for 10000 cells/sample. Histograms were analyzed by the CellQuest Software (FACScan, BD Biosciences, San Jose, CA, USA). For experiments in which exogenous putrescine and spermidine were used, compounds were added in the culture medium at the final concentration of 0.5 M, 8 h before Pcy and/or MDL treatments. For experiments aimed at blocking TRAIL death receptors activation, cells were treated with antibodies against TRAIL receptors DR4 and DR5 (anti-DR4, HS10 and anti-DR5, HS201, Alexis Biochemicals Corp., Lausen, Switzerland) at 250 ng/ml 24 h before Pcy and/or MDL treatments. These antibodies used during cell culture, recognize TRAIL-receptors expressed at the cell surface and block their activation [16].

Determination of intracellular polyamine content. Cells were incubated in the presence or absence of 80 µg/ml Pcy, 50 µM MDL and combination of Pcy/MDL for 48 h. They were harvested by scraping, washed twice in PBS, homogenized by sonication in perchloric acid (200 mM), and centrifuged at 3000 *g* for 10 min after standing for 16 h at 4 °C. The acid-insoluble pellets were used for protein determination by Lowry method and the clear supernatants for the determination of polyamines and acetylated polyamines by separation of the ions pairs formed with *n*-octanesulfonic acid, and reaction of the column effluent with *o*-phthalaldehyde/2-mercaptoethanol reagent. The primary amino groups form fluorescent 1-alkylthio-2-alkylisoindole derivatives, which were determined by continuous monitoring of fluorescence intensity [17]. Results were expressed as pmol/mg protein.

cDNA array and Western blot analysis. To identify apoptosis-associated genes that are deregulated by Pcy and/or MDL, GEArray Q series Human Apoptosis Gene Array was used. This array is designed to profile the expression of 96 key genes involved in apoptosis (Superarray Bioscience Corp., Bethesda, MD, USA). The complete gene list can be found at http://www.superarray.com. The cDNA fragments are printed on a 3.8×4.8 cm nylon membrane. Total RNA was prepared by TRIzol Reagent (Invitrogen Corp., Karlsruhe, Germany). An aliquot of 4 µg of total RNA was reverse transcribed (Promega, Madison, WI, USA), and the probe was simultaneously labeled using the GEA labeling buffer mix (Biotin-16-dUTP), (Roche, Basel, Switzerland) according to the manufacturer's instruction. The membrane was prehybridized with sheared salmon sperm DNA in GEAhyb hybridization solution followed by hybridization at 68 °C overnight with continuous agitation. The membrane was washed at 68°C twice with 2× standard saline citrate, 1 % SDS, and 0.1× standard saline citrate, 0.5 % SDS, respectively. For detection of chemiluminescence, the membrane was blocked using alkaline phosphatase-conjugated streptavidin method in combination with CPD star (Roche, Switzerland). All signals were measured using the GeneGenome and GeneTools image scanning and analysis package (Syngene BIO-Imaging System; Syngene Biomaging Systems, Synoptics Ltd., Frederick, MD, USA). Signals were calculated as described by Schneider-Stock et al. [18].

For Western blot analysis, cells were harvested by trypsinization after 48-h treatment with Pcy (80 µg/ml) and/or MDL (50 µM) and washed twice with PBS. Cell pellets were lysed for 30 min at 4 °C in a buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 % Triton X-100). After an ultra-centrifugation for 30 min at 100000 *g* at 4 °C, the total protein content was determined by Lowry method. Proteins (50–100 µg) were submitted to a 12 % SDS-polyacrylamide gel electrophoresis for 2 h at 100 V. Thereafter, proteins were transferred to nitrocellulose membranes (2 h, 200 mA) (Bio-Rad Laboratories, Marnes-la-Coquette, France). Membranes were blocked with 3 % bovine serum albumin (BSA)–0.1 % Tween 20 in 10 mM Tris-HCl pH 7.5, 0.1 M NaCl overnight at 4 °C and incubated with mouse anti-human TRAIL receptor DR4 monoclonal antibody (3 µg/ml) (Clone 32A242, Stratagene, Agilent Technologies, CA, USA) or mouse anti-human beta-actin at 1 : 2000 (Chemicon Int., Hampshire, UK). Subsequently, membranes were incubated with 0.02 µg/ml horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce, Perbio Science, Brebières, France) and proteins were visualized by Super Signal West Pico Chemiluminescent Substrate System (Pierce). Intensity of bands was analyzed by Gel Doc 2000 and One 1-D Analysis Software (Bio-Rad).

Detection of cell surface expression of TRAIL-death receptors DR4 and DR5. Cells were treated and harvested as described previously for fluorescence-activated cell sorting (FACS) analysis. Cell pellets were washed twice with PBS and incubated with FITC-conjugated mouse anti-human TRAIL-death receptors DR4 and DR5 monoclonal antibodies

(1:100) (anti-DR4, HS10 and anti-DR5, HS201, Alexis Biochemicals Corp., Switzerland) or FITC-conjugated mouse IgG_1 monoclonal isotype control antibody (1:50) (BD Biosciences) for 30 min at 4 °C in the dark, as described previously [19]. After two washing steps, cells were resuspended in PBS and the green fluorescence (FL-1: 515 nm) of 10000 events/sample was analyzed by flow cytometer and CellQuest Software (FACScan, BD Biosciences).

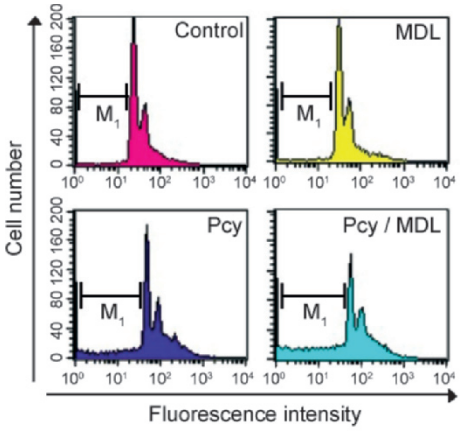
Measure of nuclear histone deacetylase activity. Cells were treated with the various drugs for 48 h and proteins contained in the nuclear compartment were extracted according to the protocol of the Nuclear Extract kit (Active Motif, Rixensart, Belgium). Protein quantity was determined by Lowry method and fluorometric detection of histone deacetylase (HDAC) activity was assessed with a HDAC Assay kit (Upstate, NY, USA). Nuclear samples were incubated with the HDAC Assay substrate allowing deacetylation of the fluorometric substrate, and fluorescence was detected by fluorescence in a Fluorolite 100, 96-well plate reader (Dynatech laboratories, Issy les Moulineaux, France) (excitation at 350–380 nm, emission at 440–460 nm).

Measure of mitochondrial membrane potential. Changes in mitochondrial membrane potential were assessed using the MitoProbe™ $DiOC_2(3)$ Assay Kit (Invitrogen Corp., France) as described previously [20]. Cells stained with $DiOC_2(3)$ can be visualized by flow cytometry with excitation at 488 nm and green (FL-1: 515 nm) or red emissions (FL-3: >600 nm) filters according to manufacturer's instructions. This method allows quantification of cells with depolarized mitochondria. Briefly, after the trypsinization step, the cells were washed once in PBS and incubated with $DiOC_2(3)$ dye at 37 °C for 30 min. For positive control, cells were incubated with a mitochondrial membrane-potential disrupter, the carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at 50 μM for 5 min at 37 °C before $DiOC_2(3)$ staining. After two washing steps, cells were re-suspended in PBS for flow cytometer analysis (10000 events/sample) and histograms were analyzed by the CellQuest Software (FACScan, BD Biosciences).

Statistical analysis. All experiments were performed at least three times. Data are reported as mean ± SE. Statistical differences between control and treated groups were evaluated using the Student's *t*-test or the Student-Neuman-Keuls multiple comparison test. Differences between groups are considered significant at $p < 0.05$.

Results

Pcy and MDL 72527 induced SW620 cell death. We have previously shown that Pcy induced apoptosis in SW620 cells [3]. Here, we compared the amount of hypodiploïd apoptotic cells ($subG_0/G_1$) 48 h after Pcy (80 μg/ml), MDL (50 μM) or Pcy/MDL combined treatments. As shown in Figure 1, MDL used as a single drug did not increase the $subG_0/G_1$ cell population when compared with control cells. However, MDL potentiated the apoptotic effects observed with Pcy by increasing significantly the hypodiploïd cell population (27% *versus* 17%).



	% of hypodiploid cells
Control	0.9 ± 0.1[a]
MDL	1.2 ± 0.3[a]
Pcy	17.0 ± 0.5[b]
Pcy / MDL	26.5 ± 0.8[c]

Figure 1. Analysis of cell hypodiploïd population after procyanidins (Pcy) and MDL 72527 (MDL) treatments. SW620 cells treated with 0.1% DMSO (control), 80 μg/ml Pcy, 50 μM MDL and Pcy/MDL for 48 h. For the measurement of hypodiploïd bodies, cells were collected and permeabilized, stained with propidium iodide and submitted to flow cytometry analysis as described in the Materials and methods section. Flow cytometry data are represented. The percent of cells in the $subG_0/G_1$ region (M_1) is indicated in the table. Data are the mean ± SE of three separate experiments and statistical differences are expressed by superscript letters ($a \neq b \neq c$, $p < 0.05$).

Effects of Pcy and MDL 72527 on polyamine metabolism. In colorectal cancer cells, polyamine metabolism is enhanced with the activation of biosynthesis and inhibition of catabolism [21]. We have previously reported that Pcy inhibited the two key enzymes of polyamine biosynthesis, ODC and AdoMetDC, and activated the polyamine catabolic pathway leading to the increased intracellular formation of ROS, amidopropanal and hydrogen peroxide, generated by the

enhanced PAO activity [4]. In the present study our aim was to compare the effects of Pcy and Pcy/MDL-combined treatments on the intracellular pool of polyamines. As shown in Figure 2A, Pcy (80 μg/ml) and MDL (50 μM) alone did not significantly alter the intracellular polyamine content. However, the combination of Pcy with MDL caused a 50% decrease in total intracellular polyamine content. As expected, the PAO inhibitor MDL blocked polyamine catabolism and provoked an important accumulation of acetylated polyamines when compared to control cells or to Pcy-treated cells. The depletion of the polyamine pool explained the reduced ($p < 0.05$) amount of acetylated polyamines in cells treated with Pcy/MDL when compared to control or to cells treated with Pcy or MDL used as single drugs (Fig. 2B).

Effect of exogenous putrescine and spermidine. To establish a possible relationship between pro-apoptotic activity and perturbation of polyamine metabolism by Pcy/MDL treatments, SW620 cells exposed to Pcy or to Pcy/MDL were incubated with exogenous putrescine and spermidine to increase the intracellular polyamine pool. An equimolar mixture of putrescine and spermidine (500 μM) was added to the culture medium 8 h before treatment for 48 h with Pcy (80 μg/ml) and/or MDL (50 μM). Addition of exogenous polyamines did not alter cell growth in non-treated SW620 cells (data not shown). As illustrated in Figure 3, the amount of apoptotic hypodiploïd cells was significantly reduced only in cells exposed to putrescine/spermidine and treated with the Pcy/MDL combination. Under these conditions, the amount of apoptotic hypodiploïd cells (27%) was reduced to the amount observed after treatment with Pcy alone (about 17%). These data show that the potentiation of apoptosis by MDL when combined with Pcy was suppressed in the presence of exogenous putrescine/spermidine. Thus, the observed enhanced apoptotic effects observed with the Pcy/MDL combination is directly related to the profound intracellular depletion of the polyamine pool.

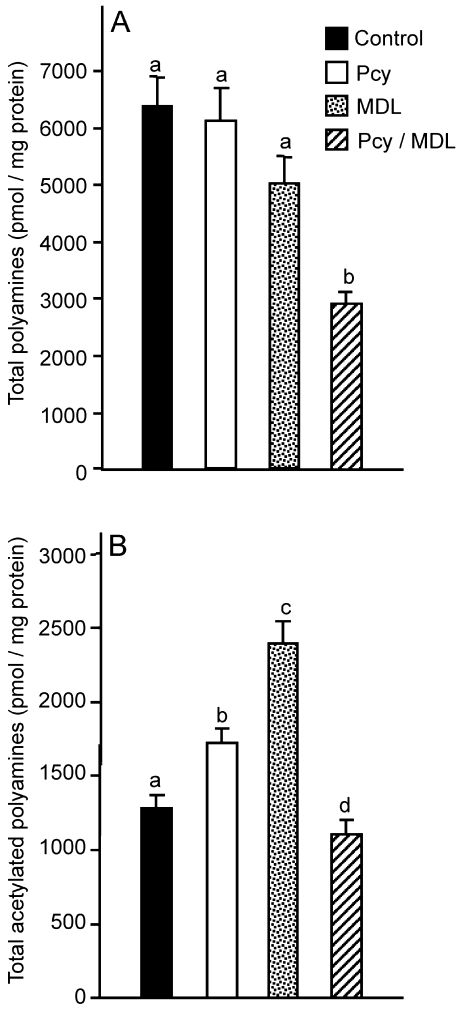


Figure 2. Effects of Pcy and MDL on intracellular polyamines. SW620 cells were exposed to 0.1% DMSO (control), 80 μg/ml Pcy, 50 μM MDL for 48 h. Cells were harvested by scraping and extracted with 0.2 N perchloric acid. Results are represented as the mean ± SE of three separate experiments and are expressed as pmol of polyamines/mg total proteins. *(A)* Concentration of total polyamines: putrescine, spermidine, spermine. (*B*) Concentration of total acetylated polyamines: N^1-acetylspermine and N^1-acetylspermidine. Columns not sharing the same superscript differ significantly ($p < 0.05$).

Effects of procyanidins and MDL 72527 on TRAIL-death receptors expression. As a first approach, to identify apoptosis-associated genes that may be deregulated by Pcy and/or MDL, we used a commercial cDNA array (GEArray Q series Human Apoptosis Gene) designed to profile the expression of 96 key genes involved in apoptosis. This study revealed that Pcy (80 μg/ml) activated the expression of the TRAIL death receptor DR4 in SW620 cells after a 24-h treatment (Fig. 4A). A 7-fold increase of DR4 mRNA was observed over the controls and MDL (50 μM) potentiated the effects of Pcy leading to a 12-fold increase over the controls. The up-regulation of DR4 mRNA was confirmed by Western blotting. Indeed, the protein expression was increased by 3-fold over the control after the combined Pcy/MDL treatment and only by 2-fold with Pcy alone; MDL used as a single drug did not significantly increase DR4 expression (Fig. 4B). We measured the cell surface expression of both TRAIL-death receptors DR4 and DR5 by flow cytometry using specific antibodies. After a 48-h treatment with Pcy and/or MDL the expression of DR4 and DR5 receptors was increased at the cell surface (Fig. 5). SW620 cells are known to be TRAIL resistant [22], and we confirmed that DR4 and DR5 receptors were not present at the cell surface

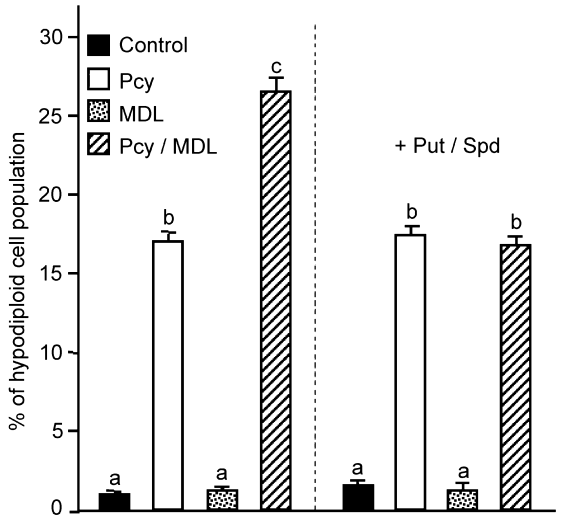


Figure 3. Effect of exogenous polyamines on SW620 cell death induced by Pcy and MDL. SW620 cells treated with 0.1 % DMSO (control), 80 μg/ml Pcy, 50 μM MDL for 48 h. Putrescine (Put) and spermidine (Spd) were added simultaneously at 500 μM in the culture medium 8 h before the treatment with various compounds. The growth of SW620 cells was not altered with exogenous polyamines under basal conditions (data not shown). Formation of hypodiploïd cells was detected by flow cytometry as described in the Materials and methods section. Histograms represent the percentage of hypodiploïd cells of 10 000 cells/sample. Data are the mean ± SE of at least three separate experiments, and columns not sharing the same superscript differ significantly ($p < 0.05$).

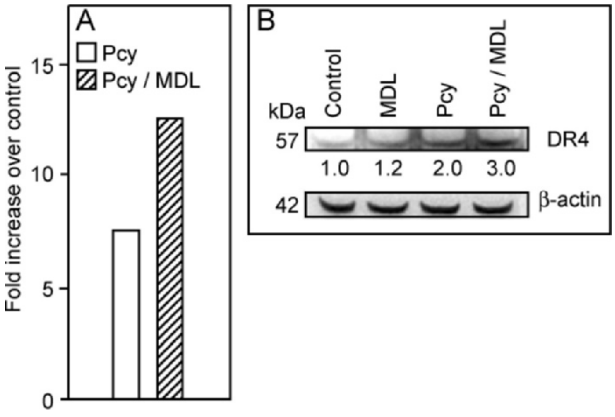


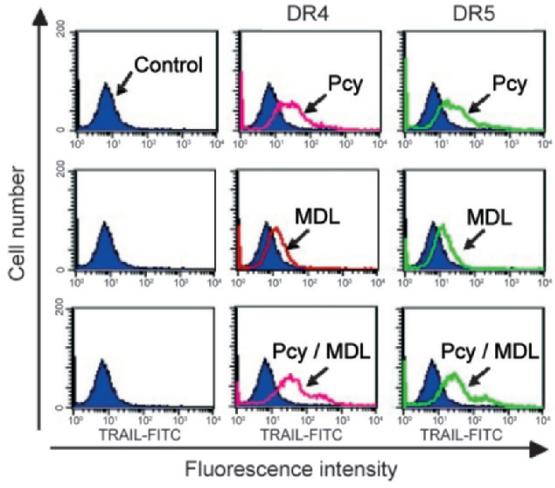
Figure 4. mRNA and protein expression levels of TRAIL-DR4 death receptor. Cells were seeded in the presence of 0.1 % DMSO (control), 80 μg/ml Pcy, 50 μM MDL and Pcy/MDL for 24 h. Human gene array was used for profiling the expression of 96 key genes involved in apoptosis after treatment with Pcy and/or MDL. Total RNA was reversed transcribed and analyzed as described in Materials and methods section. At 24 h among the 96 genes studied, death receptor DR4 gene expression was enhanced significantly after treatment with Pcy or Pcy/MDL. (*A*) Histograms represent the fold increase in expression over control cells, the level of mRNA expression was similar in control and MDL-treated cells. (*B*) Protein expression of cell death receptor DR4 was confirmed by Western blot analysis after 24-h treatment. A band of 44 kDa corresponding to DR4 protein was detected and β-actin was used as an internal control. All results are representative of three independent experiments.

of non-treated cells. After treatment with Pcy about 60 % of cells showed DR4/DR5 receptors expression at their cell surface. After combined Pcy/MDL treatment the amount of cells expressing both death receptors reached 80 %.

Effects of TRAIL death receptor inactivation on drug-induced apoptosis. To investigate the correlation between cell surface DR4/DR5 receptors expression and apoptosis, cells were pre-treated with anti-DR4 and anti-DR5 blocking antibodies for 24 h before Pcy and/or MDL treatments and the rate of apoptosis was evaluated by flow cytometry. As shown in Figure 6, the amount of apoptotic cells dropped from 25 % to 13 % with Pcy/MDL-combined treatment after addition of blocking antibodies. In contrast, in Pcy-treated cells the amount of apoptotic cells was significantly enhanced from about 17 % to 29 % by the blocking antibodies, indicating that TRAIL-death receptor pathway was not the main apoptotic pathway triggered by Pcy in these cells.

Regulation of HDAC activity. Deregulation of polyamine biosynthesis has been reported to alter histone acetyltransferase and HDAC activities [23]. Furthermore, polyamines and histone acetylation may act together to modify global chromatin structure, which in turn can be altered in polyamine-depleted cells [24]. Since a close relationship has been established between HDAC activity and the activation of promoters of the TRAIL and/or Fas pathway on cancer cells [25], we investigated the effects of MDL and Pcy on nuclear HDAC activity. As shown in Figure 7, cells treated with the Pcy/MDL combination exhibited a 50 % inhibition of HDAC activity when compared to control. A non-significant effect on HDAC activity was observed in cells treated with Pcy or MDL used as single drugs.

Mitochondrial membrane permeabilization. We have previously demonstrated that Pcy stimulated the intracellular production of ROS in SW620 cells, and that ROS formation was inhibited by MDL [4]. This indicates that the enhanced apoptotic effects observed with Pcy/MDL combination were not related to the intracellular accumulation of ROS generated by enhanced polyamine catabolism. However, the enhanced ROS formation in cells treated with Pcy, which is related to the activation of polyamine catabolism, may affect mitochondrial membrane function and favor the induction of apoptosis through an activation of the intrinsic pathway. Therefore, we examined whether Pcy and Pcy/MDL-combined treatments had an impact on mitochondrial membrane potential ($\Delta\Psi m$). Mitochondrial membrane permeabilization was assessed by flow cytometry after staining the cells with $DiOC_2(3)$. This



% of cells expressing TRAIL receptors

	DR4	DR5
Pcy	61.6 1.2 [a]	57.7 0.8 [a]
MDL	20.5 0.4 [b]	21.1 2.0 [b]
Pcy / MDL	82.7 1.1 [c]	73.8 0.5 [c]

Figure 5. Expression of TRAIL-death receptors DR4 and DR5. Cell surface expression of TRAIL-DR4 and -DR5 death receptors by flow cytometry analysis in cells treated with 0.1% DMSO (control), 80 µg/ml Pcy, 50 µM MDL and Pcy/MDL for 38 h. Cells were harvested and stained with FITC-conjugated monoclonal antibodies against the two types of TRAIL receptors (DR4 and DR5). Similar profiles were observed for control cells and isotype control with no basal DR4 or DR5 protein expression (data not shown). Data are represented on cytometry histograms and the fluorescence shifts to the right are indicative of cell death receptor expression on cell membrane (overlays). The percentage of cells with enhanced fluorescence is given in the table. Data are the mean value ± SE of at least three separate experiments and statistical differences are expressed by superscript letters ($a \neq b \neq c$, $p < 0.05$).

cationic cyanine dye accumulates in mitochondria with active membrane potentials producing red fluorescence. The intensity of fluorescence decreases in cells with impaired mitochondrial membrane potential, accompanied by a shift from red to green fluorescence. As illustrated in Figure 8, the percentage of cells showing depolarized mitochondrial membrane reached 30% after 48-h treatment with Pcy used as a single drug. In contrast, only 10% of the cells showed depolarized mitochondrial membrane when treated with the Pcy/MDL combination. Mitochondrial membrane perturbations are clearly involved in the apoptotic effects activated by Pcy, whereas MDL exhibited a protective effect on mitochondria function through its inhibitory effects on ROS formation.

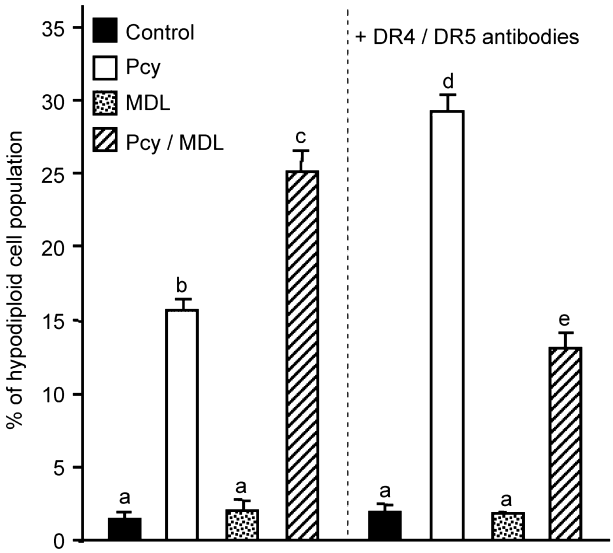


Figure 6. Analysis of hypodiploïd cells in the presence of blocking antibodies against DR4 and DR5 receptors. SW620 cells treated with 0.1% DMSO (control), 80 µg/ml Pcy, 50 µM MDL for 48 h. Antibodies anti-human cell death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) were added simultaneously in the culture medium 24 h before the treatment to various compounds. Production of hypodiploïd cells was detected by flow cytometry as described in the Materials and methods section. Histograms represent the percentage of hypodiploïd cells of 10000 cells/sample. Data are the mean ± SE of at least three separate experiments and columns not sharing the same superscript differ significantly ($p < 0.05$).

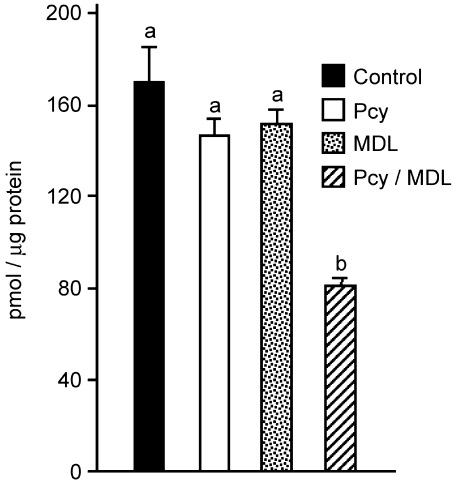


Figure 7. Measure of histone deacetylase (HDAC) activity. Cells were treated with 0.1% DMSO (control), 80 µg/ml Pcy, 50 µM MDL and Pcy/MDL for 48 h. They were harvested by scraping and nuclear extracts were prepared and analyzed as described in the Material and methods section. Histograms represent the pmol of deacetylated substrate/µg of total proteins. Data are obtained as the mean ± SE of at least three separate experiments. Columns not sharing the same superscript differ significantly ($p < 0.05$).

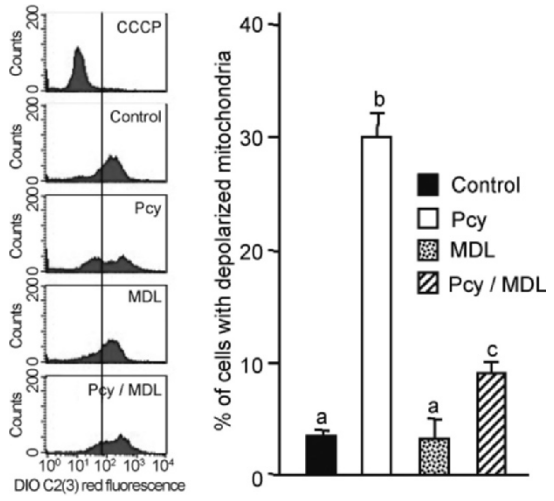


Figure 8. Flow cytometry analyses of mitochondrial membrane potential. Cells were treated with 0.1 % DMSO (control), 80 μg/ml Pcy, 50 μM MDL and Pcy/MDL for 48 h. Cells were harvested, stained with $DiOC_2(3)$ reagent and analyzed by flow cytometry. Reduction of red fluorescence corresponds to the loss of mitochondrial membrane potential ($\Delta\Psi_m$). Cytometry data and histograms are shown. Data are presented as the percentage of cells with reduced red fluorescence. Data are obtained as the mean ± SE of at least three separate experiments. Columns not sharing the same superscript differ significantly ($p < 0.05$).

Discussion

In the present study we show that MDL, the specific inhibitor of the main polyamine catabolic enzyme, the PAO [26], when used at a non-cytotoxic dose and without anti-proliferative effects [4], potentiated the apoptotic effects of apple Pcy in human colon cancer-derived metastatic SW620 cells by activating alternative apoptotic pathways, which do not operate when Pcy are used as a single drug.

The polyamine biosynthetic pathway is enhanced during carcinogenesis [27], and modulation of polyamine metabolism by drugs remains an important target for colon cancer chemoprevention [5, 9]. Apple Pcy inhibited polyamine biosynthetic enzymes and activated polyamine catabolism leading to the enhanced ROS formation, and in this context MDL inhibited ROS formation induced by Pcy [4]. This indicated that the enhanced apoptotic effects observed with MDL/Pcy combination were not related to ROS generated through a modulation of polyamine catabolism. However, Pcy-induced cell death was directly caused by enhanced ROS production since Pcy activated the intrinsic apoptotic pathway by altering mitochondrial function characterized by the ROS-triggered loss of mitochondrial membrane potential. This was confirmed by the observation that MDL, through its inhibitory effect on ROS formation generated by Pcy treatment, exerted a protective effect on mitochondrial function. Thus, the observed potentiation of Pcy-triggered apoptosis by MDL was mainly caused by the activation of another apoptotic pathway.

We used a commercially available cDNA array designed to profile the expression of 96 key genes involved in apoptosis, which might be deregulated by the Pcy/MDL combination. It was found that MDL in the presence of Pcy, up-regulated the expression (mRNA and protein) of TRAIL-death receptor DR4. This preliminary experiment led us to study the protein expression of TRAIL-DR4 and -DR5 death receptors at the cell surface. Activation of both receptors by their ligand TRAIL or by other stimuli has been reported to induce the extrinsic apoptotic pathway [28]. Using specific antibodies we found that MDL combined with Pcy enhanced the expression of both DR4 and DR5 death receptors at the cell surface. These effects were lower when Pcy were used alone. The SW620 cell line used in our study is normally resistant to TRAIL-induced apoptosis and does not express DR4 or DR5 at the cell surface [22]. The activation of TRAIL-death receptors in tumors by pharmacological agents has recently been of great interest in chemopreventive clinical trials, since this pathway can preferentially activate apoptosis in cancer cells but not in normal non-cancerous cells [29–31].

To determine whether activation of TRAIL-death receptors was involved in the potentiation of apoptosis by MDL when combined with Pcy, SW620 cells were treated with specific anti-DR4 and anti-DR5 blocking antibodies. The potentiation of apoptosis by MDL was specifically inhibited, indicating that the drug enhanced the apoptotic effects of Pcy through the activation of the extrinsic apoptotic pathway involving cell surface TRAIL-death receptors DR4 and DR5, whereas Pcy-induced apoptosis in SW620 cells occurred mainly through the activation of the mitochondrial apoptotic pathway. The observation that TRAIL DR4/DR5 blocking antibodies increased Pcy-triggered apoptosis when Pcy were used as a single drug suggests that the blocking of DR4/DR5 may cause a further imbalance leading to the increased activation of the intrinsic apoptotic pathway.

A close relationship has been established between HDAC activity and the activation of the TRAIL pathway in cancer cells since HDAC inhibitors activate apoptosis by hyperacetylation of the promoters of TRAIL-death receptors pathway in cancer cells [25]. HDAC inhibitors are promising drugs for treatment of various cancer types and several clinical trials are already in progress [32]. We investigated if there is a possible relationship between the potentiation of apoptosis by MDL through an activation of DR4/DR5-death receptors and changes in HDAC activity. It

was clearly shown that MDL in combination with Pcy caused a 50% reduction of nuclear HDAC activity in SW620 cells, whereas Pcy or MDL used as single drugs did not significantly affect the enzyme activity. These data indicate that in the presence of Pcy, MDL caused an inhibition of HDAC activity, which may favor hyperacetylation of the promoters of the TRAIL death pathway. These effects may be related to the profound depletion of the intracellular polyamine pool observed with Pcy/MDL combination. Indeed, deregulation of polyamine biosynthesis has been reported to alter histone acetyltransferase and also HDAC activities [23]. Polyamines and histone acetylation may act together to modify global chromatin structure, which in turn can be profoundly altered in polyamine-depleted cells [24]. Our data combined with those presented in the literature [9] suggest a key role for polyamines in the control of apoptosis.

Polyamine metabolism mediates the expression of various genes involved in tumor development and its alteration may affect sensibility of cells toward apoptosis [23, 24, 33, 34]. In metastatic SW620 cells, polyamine metabolism was affected differently by the single drugs (MDL or Pcy) and when MDL was combined with Pcy. When used as a single drug, MDL inhibited polyamine catabolism through an inactivation of PAO, leading to the intracellular accumulation of acetylated polyamines [35]. In this context, we showed that the total intracellular polyamine pool was not significantly altered and that MDL did not inhibit SW620 cell growth nor induce an apoptotic response. This was also related to our previous observation showing that MDL presented no inhibitory effects on biosynthetic enzymes [4]. This was in contrast to Pcy, which inhibited polyamine biosynthesis by reducing the activity of its two key enzymes, ODC and AdoMetDC, and activated also the catabolic and retro-conversion pathway, explaining why the total polyamine pool was not significantly reduced by Pcy treatment. The activation of the polyamine catabolic flux by Pcy led to the increased formation of ROS through PAO activation [4]. Our present data suggest that mitochondrial membrane perturbations by Pcy may be a consequence of ROS production by PAO, and that ROS production might be the prominent apoptotic signal. MDL, through its inhibitory effects on PAO activity, inhibited ROS formation induced by Pcy and protected the mitochondria. Thus, the potentiation of Pcy-triggered cell death by MDL is essentially related to the changes in intracellular polyamine pool caused by both the inhibition of the biosynthetic pathway (induced by Pcy) and the inhibition of the catabolic and retro-conversion pathway (induced by MDL). As shown in the present report, Pcy/MDL-combined treatment caused a depletion of the intracellular polyamine and acetylated polyamine pools. Numerous studies have shown that inhibition of ODC and/or AdoMetDc activities associated to an important reduction of the intracellular polyamine pools, initiates an activation of apoptotic processes in various cell types [36–38].

To correlate the depletion of intracellular polyamines with apoptosis, exogenous polyamines (putrescine and spermidine) were added to the culture medium a few hours before the treatment with the different compounds. We observed that the potentiation of apoptosis due to MDL was inhibited in cells treated with the Pcy/MDL combination. The amount of apoptotic cells remained similar to that observed in cells treated with Pcy alone. These data clearly indicate that the potentiation of Pcy-triggered apoptosis by MDL was a direct consequence of intracellular polyamine depletion. Thus, the potentiation of Pcy-triggered apoptosis by MDL involves a depletion of the intracellular polyamine pool, leading to the activation of an extrinsic apoptotic pathway through an up-regulation of TRAIL-death receptors expression, which might be related to an inhibition of nuclear HDAC activity.

Here, we show that MDL, a specific inhibitor of PAO, without having anti-proliferative effects or apoptotic effects on SW620 cells, potentiates Pcy-triggered apoptosis by activating an alternative apoptotic pathway that is not activated when Pcy is used alone. We found that Pcy caused an activation of the intrinsic apoptotic pathway through the alteration of mitochondrial membrane permeability. We observed that the PAO inhibitor MDL exerted a protective effect on mitochondria function, suggesting that alterations observed with Pcy were related to ROS generated by increased polyamine catabolism [4]. The potentiation by MDL of Pcy-triggered cell death seems essentially related to the depletion of the intracellular polyamine pool since MDL potentiation was reversed by exogenous polyamines. Our data suggest that MDL in the presence of Pcy activated an extrinsic apoptotic pathway involving inhibition of HDAC activity and activation of TRAIL-death receptors DR4 and DR5 in SW620 cells. This is the first report showing that abrupt changes in the intracellular polyamine pool caused by a specific drug (MDL), showing no anti-proliferative activity by itself, can initiate a switch from intrinsic to extrinsic apoptotic pathway by activating the expression of death receptors that are not active in non-treated cells. Investigations are now in progress to determine whether AP/MDL combinations are of interest for chemopreventive and/or therapeutic interventions.

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