Research Paper

Differential Induction of Apoptosis by Apple Procyanidins in TRAIL-Sensitive Human Colon Tumor Cells and Derived TRAIL-Resistant Metastatic Cells

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AIM: **Procyanidins (Pcy) are oligomers of catechins and epicatechins. In order to get more insight into the apoptotic effects of Pcy, we aimed to compare the death signaling pathways triggered by Pcy in TRAIL-sensitive human colon cancer cells (SW480) and in their derived TRAIL-resistant metastatic cells (SW620).**

METHODS: **Cell death, DR4/DR5, Bcl-2/Bax proteins, mitochondrial membrane potential, and reactive oxygen species (ROS) production were analyzed by flow cytometry. Bid and cytochrome *c* were studied by immunoblotting and ELISA.**

RESULTS: **Pcy (80 μg/ml) combined with exogenous TRAIL (30 ng/ml) enhanced the apoptotic effects of TRAIL in SW480 but also in SW620 cells, indicating that Pcy sensitized SW620 cells to TRAIL treatment. Pcy up-regulated the expression of death receptors DR4/DR5 of TRAIL. Activation of caspase-8 and -3 was observed in both cell lines but caspase-9 was activated only in SW620 cells. Pcy-treated SW620 cells exhibited a decrease in Bid protein level, in Bcl-2/Bax ratio, and enhanced intracellular ROS production. These events were associated with the decrease of mitochondrial membrane potential and the release of cytochrome *c* into the cytosol.**

CONCLUSION: **Our data suggest that Pcy triggers apoptosis via caspase-8 and may enhance TRAIL-induced apoptosis by activation of the extrinsic pathway in both cell lines. In the TRAIL-resistant SW620 cells, the apoptotic effects of Pcy were complex and may involve a cross-talk between the extrinsic and the mitochondrial apoptotic pathways and a Pcy-triggered intracellular ROS production favoring mitochondrial disruption. These data highlight the chemopreventive potentials of apple Pcy in colorectal cancer treatments.**

Journal of Cancer Molecules 5(1): 21-30, 2009.

Keywords:

apoptosis

colorectal cancer

flavonoids

TRAIL

mitochondria

Introduction

Colon cancer is the third most common type of cancer worldwide. The highest prevalence of colon cancer occurs in North America, Europe, Australia, and New Zealand and accounts for almost 65% of the total global incidence. The lowest incidence was seen in Central and South America, Asia and Africa, but it is now increasing in these regions [1]. Epidemiological studies suggest that the consumption of vegetables and fruits is inversely associated with colon cancer risk. Much of the protective effects of vegetables and fruits have been attributed to biologically active secondary plant metabolites which are non-nutrient constituents such as the carotenoids, the phenolic acids and flavonoids [2].

Apples represent an important source of polyphenols and especially of flavonoids in the human diet. Flavonoids present in apples are divided into different classes: flavonols like quercetin conjugates (3-galactoside, 3-glucoside, 3-rhamnoside), flavan-3-ols derivatives including monomers (catechin and epicatechin) as well as dimers, trimers and other condensed tannins named procyanidins (Pcy[2]). The Pcy are oligomeric and polymeric polyphenols formed by association of several monomeric units of flavan-3-ols [3,4]. The regular consumption of Pcy-containing foods has been associated with a reduced risk of certain cancers [2,5,6]. Flavonoids inhibit carcinogenesis by affecting molecular events in the initiation, promotion and progression stages. They are able to increase the expression of pro-apoptotic mediators and prevent or delay tumor development. In this regard, induction of apoptosis appears as one of the most important targets in a chemopreventive approach [6,7]. Over the past few years, it has been shown that Pcy trigger apoptosis through the alteration of intracellular signalling pathways in human mammary [8], prostate LNCaP [9] and

Received 10/1/08; Revised 3/18/09; Accepted 3/29/09.

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[2]**Abbreviations:** Pcy, procyanidins; TRAIL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand; DR4/DR5, death receptor 4/death receptor 5; tBid, truncated Bid; IAP, Inhibitor of Apoptosis protein; Bcl-2, B-cell lymphoma-2 protein; PI, propidium iodide; p-NA, para-nitroaniline; $DiOC_2(3)$, 3,3'-dihexyloxacarbocyanine iodide; $\Delta\Psi m$, mitochondrial membrane potential; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; ROS, reactive oxygen species; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate.

colon carcinoma-derived metastatic cells [10,11]. It has been also reported that these compounds are able to inhibit the promotion/progression phases of colon carcinogenesis in rats [10]. However, the cellular and molecular mechanisms by which Pcy induce apoptosis in cancer cells are still not well understood.

The apoptotic process may involve two major pathways: the extrinsic or death receptor-mediated pathway and the intrinsic or mitochondrial pathway. The extrinsic pathway implicates the activation of death receptors at the plasma membrane level. TRAIL (TNF-related apoptosis-inducing ligand), a member of the tumor necrosis factor superfamily, induces apoptosis selectively in tumor cells while leaving normal tissues intact [12-14]. TRAIL interacts with the death domain-containing receptors DR4 and DR5 [12,15], resulting in the formation of the active death-inducing signaling complex (DISC) that leads to the activation of caspase-8 [13]. Caspase-8 may activate two distinct death pathways: a mitochondrial-independent pathway leading directly to the activation of caspase-3 and subsequent DNA degradation (type I pathway), and another caspase-8-activated pathway involving a cross-talk with the intrinsic (mitochondrial) pathway through the cleavage of Bid protein into a truncated Bid (tBid) which translocates into the mitochondria causing mitochondrial dysfunctions and release of cytochrome *c* into the cytosol [16]. These events favor the activation of caspase-9 and downstream activation of effector caspase-3 (type II pathway)[13]. The type I pathway is regulated at the level of caspase-3 activation by IAPs (Inhibitor of Apoptosis proteins) and the type II pathway is regulated at the level of mitochondria by members of the Bcl-2 (B-cell lymphoma-2 protein) family [17].

In order to get more insight into the intimate mechanisms involved in the pro-apoptotic effects of Pcy, we aimed to compare the death-signaling pathways triggered by Pcy in TRAIL-sensitive human colon cancer SW480 cells [18,19] and in their derived TRAIL-resistant metastatic SW620 cells [18-20]. SW480 cells correspond to a primary human colon adenocarcinoma, and SW620 cells are derived from the primary tumor and isolated from a mesenteric lymph node metastasis of the same patient. The two cell lines have been validated as an *in vitro* model of colon cancer progression from a primary tumor to metastatic disease [21]. In this study, we demonstrated that Pcy was able to induce apoptosis via caspase-8 in both cell lines, and to sensitize the resistant SW620 cells to TRAIL by acting at several levels of the apoptotic signaling pathway involving the TRAIL receptors DR4/DR5, ROS production and the mitochondrial pathway.

Materials and Methods

Isolation and characterization of apple procyanidins

Polyphenols were purified from a cider apple (*Malus domestica*, variety Antoinette) as reported by Souquet *et al.* [22]. In brief, apples were reduced into 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). Fractions containing polyphenols were evaporated and freeze-dried. The polyphenols were fractionated on a Fractogel column [22,23]. Pcy were characterized and quantified by reverse-phase HPLC after thioacidolysis (Figure 1). On a weight basis, the Pcy-fraction contained 70% Pcy, consisting of 95% (-)-epicatechin and 4% (+)-catechin. The mean degree of polymerization was close to seven. The Pcy fraction was almost totally devoid of monomeric catechins and other phenols (< 2%). Pcy was diluted in DMSO and used at a final concentration of 80 µg/ml.

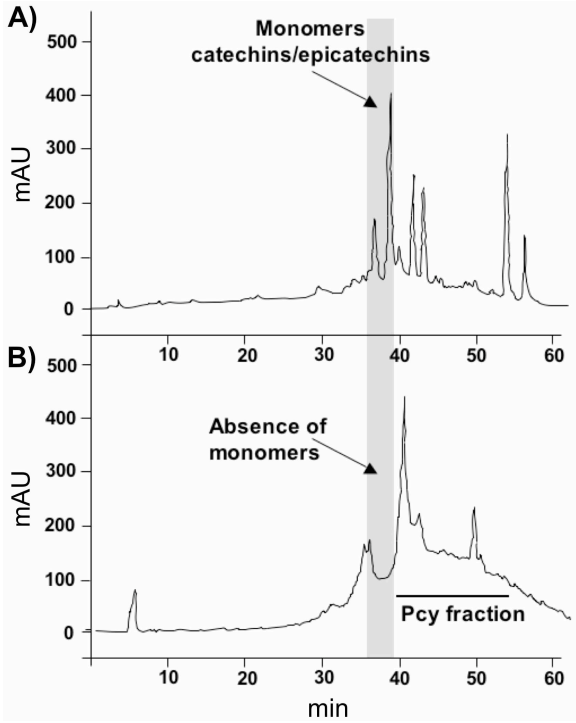

Figure 1: Representative analytical HPLC profiles (280 nm) of apple extracts. A, Total polyphenolic extracts. B, Procyanidin (Pcy) extract. Chemical structures were identified by their retention times, UV spectra and HPLC co-injections. HPLC method was according to Shoji *et al.* [42].

Cell culture and treatments

SW480 and SW620 cells were obtained from the European Collection of Animal Cell Culture (ECACC, Salisbury, UK). They were cultured in 75 cm^2 Falcon flasks with Dulbecco's modified Eagle's medium supplemented with 25 mM glucose, 2 mM L-glutamine, 10% heat (56°C)-inactivated horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% non-essential amino acids (Invitrogen, Cergy-Pontoise, France). Incubations were carried out at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was replaced every 48 h. For all experiments, horse serum was reduced to 3%, and the medium was supplemented with 10 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium (ITS-defined medium; Invitrogen). Cells were exposed to different compounds 24 h after seeding and incubated for 24 or 48 h. DMSO final concentration in culture medium was 0.1% for control and treated cells. Stock solutions of TRAIL (Alexis Biochemicals, Grünberg, Germany) were diluted in PBS and tested at 30 ng/ml. Caspase-8 inhibitor z-IETD-fmk (R&D Systems, Bad Nauheim, Germany) was diluted in PBS containing 1% BSA. Cells were pre-treated for 2.5 h with the specific caspase inhibitor (50 µM) before addition of Pcy to culture medium.

Flow cytometric analysis of sub-G_0/G_1 cell population

The amount of dying and dead cells of the cell populations was determined by flow cytometric analysis and corresponded to the percentage of hypodiploid cells present in the sub-G_0/G_1 region as shown previously [10]. Cells were seeded in culture dishes (1×10^6 cells per 10-cm internal diameter or 2×10^5 cells per 2.5-cm internal diameter) and treated with 0.1% DMSO (control), Pcy, TRAIL, or Pcy + TRAIL in the absence or presence of z-IETD-fmk. Control and treated cells were harvested by trypsinization, centri-

Print ISSN 1816-0735; Online ISSN 1817-4256

fuged and washed twice with ice-cold PBS at 1800 ×g for 5 min at 4°C, and fixed in 1 ml methanol:PBS (9:1, v/v) at -20°C for at least 30 min. The fixed cells were washed twice with ice-cold PBS and cell pellets were resuspended in 100 µl PBS containing 0.25 mg/ml RNase A and 0.1 mg/ml propidium iodide (PI; Sigma-Aldrich, Munich, Germany), incubated in darkness at 37°C for 30 min. Cell-cycle phase distribution was analysed using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). Data from 10000 events per sample were collected and analysed using the CellQuest software. Apoptosis was quantitatively measured as the percentage of hypodiploid cells in the sub-G_0/G_1 region using the Cell Fit analysis program (BD Biosciences).

Detection of cell surface expression of DR4 and DR5

Cells (1×10^6) were seeded and treated as described for cell cycle analysis. Cells were harvested by trypsinization, washed twice with ice cold PBS and incubated for 30 min at 4°C with anti-human-DR4 (mouse IgG1, clone HS101, 1:50) or anti-human-DR5 (mouse IgG1, clone HS201, 1:50) monoclonal antibody (Alexis Biochemicals Co., Stockholm, Switzerland). Cells were washed with ice-cold PBS and incubated with anti-mouse IgG1-FITC (clone STAR81F, 1:50) for 30 min at 4°C in darkness. The excess antibody was removed by two washings. Isotype control mouse IgG1-FITC antibody (BD Biosciences) was used as negative control, incubated for 30 min at 4°C in darkness and washed. Cells were resuspended in PBS, and surface expression of 10000 events per sample was analysed with a FACScan flow cytometer and CellQuest software (BD Biosciences).

Measurement of caspase activities

Caspase activities were measured by colorimetric assay kits (Sigma-Aldrich; MBL, Naka-ku Nagoya, Japan) according to the manufacturers' instructions. In brief, attached and floating cells were harvested, washed in ice-cold PBS and lysed in 100 µl of lysis buffer; protein extraction was obtained and stored at -20°C. Twenty microliter cell lysate solutions was added in the buffer containing p-nitroaniline (p-NA)-conjugated specific substrate for caspase-8 (Ac-IETD-pNA), caspase-3 (Ac-DEVD-pNA), or caspase-9 (LEHD-pNA) to a total 100 µl reaction volume and incubated at room temperature for caspase-8 and at 37°C for caspases-3 and -9 during 24 h. The concentration of p-NA released was calculated from the absorbance values at 405 nm compared to a standard curve using a spectrophotometer. Data were adjusted to the total protein content, and activity was expressed as nmol pNA/h/mg protein.

Western blot analysis of Bid protein

Cells were harvested by trypsinization after treatment and lysed for 15 min at 4°C in a lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, and 1% Triton X-100. The lysates were centrifuged at 16000 ×g for 30 min at 4°C, and the total protein content was determined by Lowry assay. Electrophoresis of cell lysate proteins was performed in 15% SDS-polyacrylamide gels for 100 min at 80 V and proteins were transferred onto nitrocellulose membranes (BioRad Laboratories, Marnes-la-Coquette, France) for 80 min at 100 V. After addition of a blocking solution (10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 0.1% Tween-20, and 3% BSA) for 1 h at room temperature, the membranes were incubated with rabbit anti-human Bid polyclonal antibody (BD Biosciences) at 1:500 or mouse anti-human β-actin at 1:2000 (Chemicon, Hampshire, UK). Subsequently, membranes were incubated with 0.02 µg/ml of HRP-conjugated secondary antibodies (Pierce, Perbio Science, Brebières, France) and proteins were visualized by Super Signal West Pico Chemiluminescent Substrate System (Pierce). Intensity of bands was analysed using BioRad Quantity One 1-D Analysis Software version 4.2.1 (BioRad).

Flow cytometric analysis of Bcl-2 and Bax expression

Cells (1×10^6) were seeded and treated as described for cell cycle analysis. Cells were harvested by trypsinization, washed twice with ice cold PBS and proceeded for the fixation and permeabilization step with the BD Cytofix/Cytoperm™ kit (BD Biosciences). According to manufacturer's instructions, pellet was resuspended in fixation/permeabilization solution for 20 min at 4°C and after washing twice in 1× BD Perm/Wash™ buffer, cells were incubated with appropriate antibodies for the detection of Bcl-2 or Bax proteins. For Bcl-2 protein detection, cells were labeled directly with 20 µl FITC-conjugated mouse anti-human Bcl-2 monoclonal antibody (BD Biosciences). For Bax level detection, cells were incubated with rabbit anti-human Bax polyclonal antibody (1:100 dilution; BD Biosciences) for 30 min at 4°C in the dark [24]. After washing twice, FITC-conjugated swine anti-rabbit antibody was added (1:10 dilution; Abcam, Cambridge, UK) for 30 min at 4°C. After washing twice in BD wash buffer, the fluorescence values of 10000 cells were analyzed using a FACScan flow cytometer (excitation 488 nm, emission pass filter FL-1: 530 nm) and CellQuest software.

Mitochondrial membrane permeability changes

Changes in mitochondrial membrane permeability were assessed by using the MitoProbe™ $DiOC_2(3)$ assay kit (Invitrogen) as described previously [25]. Cells stained with $DiOC_2(3)$ are visualized by flow cytometry with excitation at 488 nm and green filter (FL-1: 515 nm) according to manufacturer's instructions. This method allows quantifying cells with depolarized mitochondria. Briefly, cells after trypsinization 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 disrupter of mitochondrial membrane potential ($\Delta\Psi m$), carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at 50 µM for 5 min at 37°C before DiOC2(3) staining. After washing, cells were resuspended in PBS for flow cytometric analysis (10000 events/sample) and histograms were analyzed by the CellQuest software.

Measure of cytochrome c release

Cells were harvested by scrapping, washed twice in PBS and stored at -20°C. Cytochrome *c* release was detected by using the Function ELISA™ Cytochrome *c* Kit (Active Motif Europe, Rixensart, Belgium). This system is based on the sandwich ELISA method for detecting cytochrome *c* present in cellular lysates and for determining changes in mitochondrial and cytosolic fractions. Fractions were prepared using the Mitochondrial Extract Kit (Active Motif Europe). Colorimetric detection was assessed by reading absorbance at 450 nm. Data were adjusted to total protein content, and values were expressed as nmol cytochrome *c*/mg protein.

Determination of intracellular reactive oxygen species (ROS)

After treatments, cells were resuspended in pre-warmed PBS containing 8 µM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, mixed isomers; Invitrogen). The compound is deacetylated by intracellular esterases producing dichlorodihydrofluorescein that reacts with H_2O_2 to form dichlorofluorescein, which is an oxidized green fluorescent compound. Cells were incubated for 30 min at room temperature in darkness. For positive control, H_2O_2 (1.05%, v/v) was added to cell culture 15 min before trypsinization. Fluorescence was detected by flow cytometry with FL-1 filter (530 nm). Data from 10000 events per sample were collected and analysed using the CellQuest software.

Statistical analysis

All data were presented as mean ± SE from three independent experiments. Significant differences between control and treated groups were evaluated by one-way ANOVA analysis. Student's *t* test or the Student-Neuman-

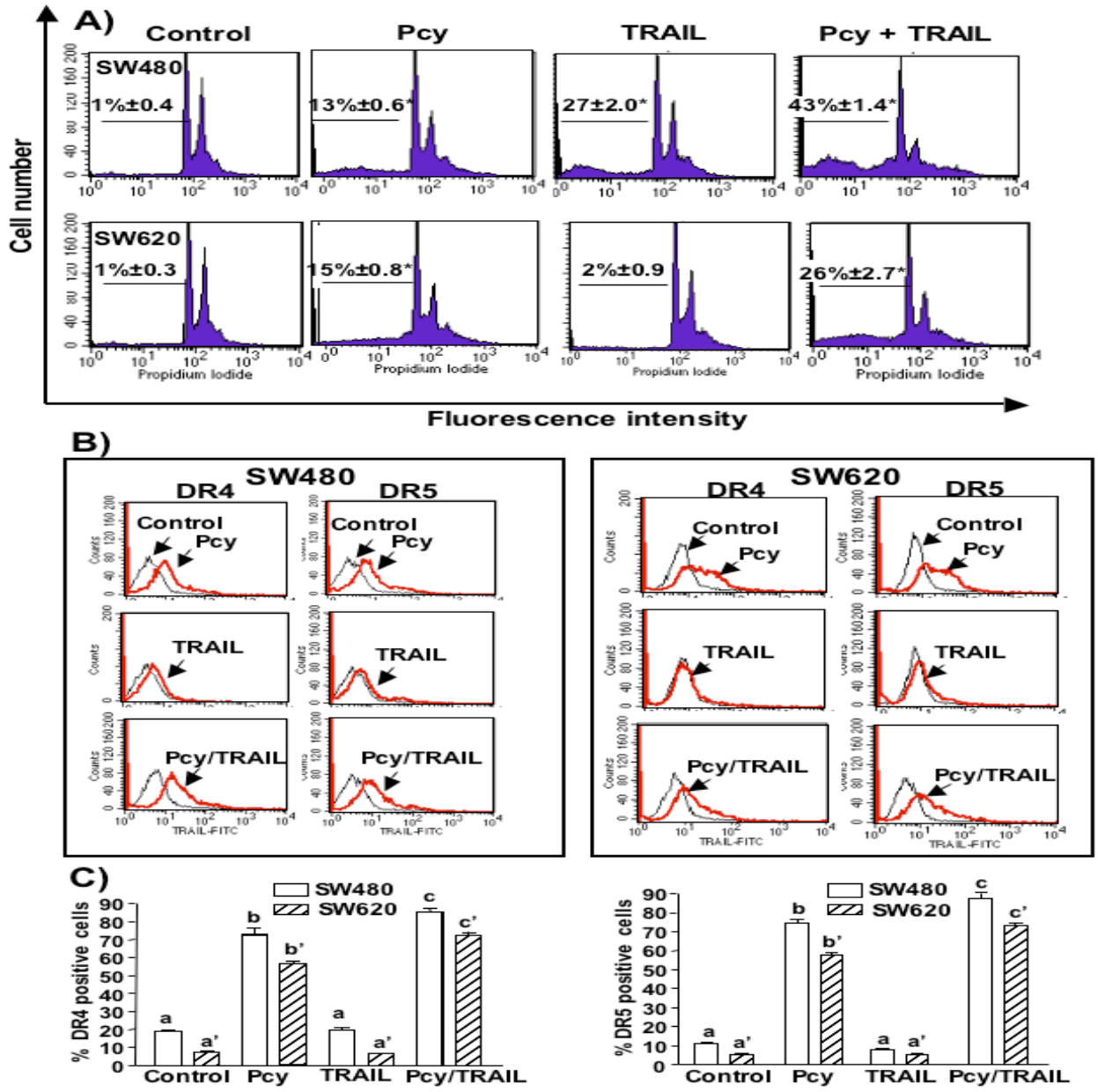

Figure 2: Effect of Pcy on TRAIL-mediated apoptosis in SW480 and SW620 cells. A, Analysis of hypodiploid cell population by flow cytometry after Pcy (80 μg/ml), TRAIL (30 ng/ml) or Pcy + TRAIL treatment of SW480 and SW620 cells for 48 h. Data are presented as mean percentage ± SE of cells in the sub-G_0/G_1 region. Data are representative of at least three different experiments. For each cell line, significant differences were found in treated *vs.* control cells and Pcy *vs.* Pcy + TRAIL, *$P < 0.05$. B, Effects of Pcy on the cell surface expression of DR4 and DR5 in SW480 and SW620 cells. Cells were exposed to 0.1% DMSO (control), Pcy (80 μg/ml), TRAIL (30 ng/ml) or Pcy + TRAIL for 48 h. Cells were incubated with FITC-conjugated anti-DR4 or -DR5 antibody and analyzed by flow cytometry. Fluorescence shift to the right is indicative of an increase of cell death receptor expression at the cell surface. C, Percentages of the cells positive for DR4 and DR5 expression. Data are presented as mean percentage ± SE of three separate experiments. For each cell line, columns not sharing the same superscript letter differ significantly: a ≠ b ≠ c and a' ≠ b' ≠ c', $P < 0.05$.

Keuls multiple comparisons test was used to determine the significance of statistical differences between data at the level of $P < 0.05$, using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Effect of Pcy on TRAIL-mediated apoptosis in SW480 and SW620 cells

TRAIL has received increased attention as a promising therapeutic agent against cancer due to its ability to induce apoptosis without harmfull effects on normal cells. However, there are tumor cells resistant to TRAIL [14,26]. Thus combined treatments of TRAIL with other therapeutic agents might enhance TRAIL-induced apoptosis. In this respect, it is important to know whether the combination of TRAIL with Pcy may represent an effective way to enhance TRAIL-induced cell death or overcome TRAIL resistance by activation of DR4/DR5 death receptors. Therefore, we compared the effects of Pcy (80 μg/ml), TRAIL (30 ng/ml) and Pcy + TRAIL on SW480 and SW620 cell death. The amount of dying and dead cells in cell populations was determined by flow cytometric analysis and corresponded to the percentage of hypodiploid cells present in the sub-G_0/G_1 region as reported previously [10]. As shown in Figure 2A, Pcy and TRAIL used as single drugs induced cell death (by 13% and

Print ISSN 1816-0735; Online ISSN 1817-4256

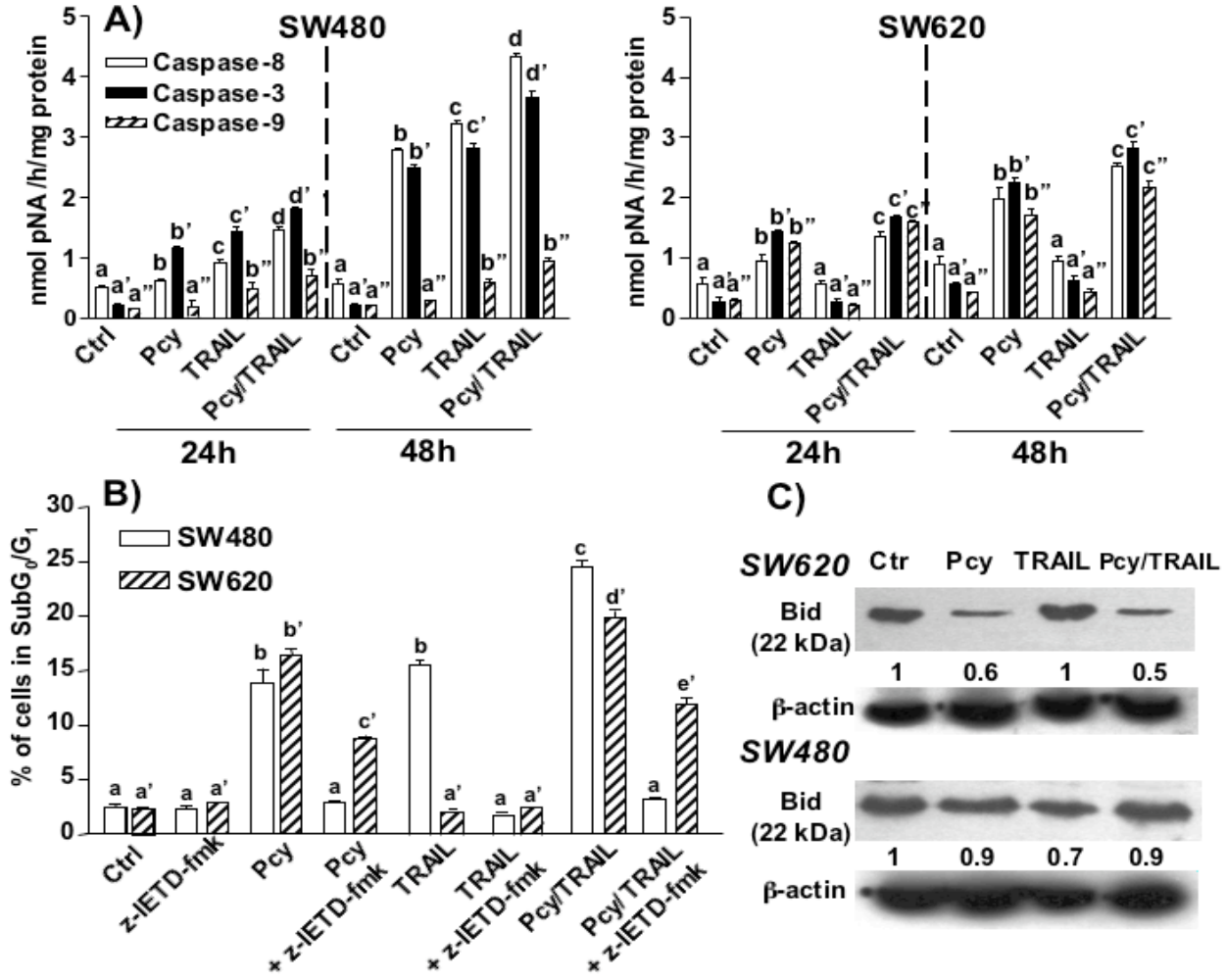

Figure 3: Effects of Pcy treatments on caspase activities. A, Activities of caspases-8, -3 and -9 in SW480 and SW620 cells. Cells were treated with 0.1% DMSO (control), Pcy, TRAIL, or Pcy + TRAIL for 24 and 48 h. At the end of the incubation period, caspase activities were measured by colorimetric assay. Data are presented as mean ± SE of three separate experiments. For each cell line at 24 or 48 h and each caspase, columns not sharing the same superscript letter differ significantly: a ≠ b ≠ c ≠ d, a' ≠ b' ≠ c' ≠ d', and a" ≠ b" ≠ c", $P < 0.05$. B, Effects of caspase-8 inhibition on Pcy-triggered apoptosis in SW480 and SW620 cells. Cells were pre-treated for 2.5 h with caspase-8 inhibitor z-IETD-fmk peptide (50 μM) before adding Pcy and/or TRAIL for 48 h. The hypodiploid cell population was analyzed by flow cytometry using PI staining. Data are presented as the mean ± SE of three independent experiments. For each cell line, columns not sharing the same superscript letter differ significantly: a ≠ b ≠ c and a' ≠ b' ≠ c' ≠ d' ≠ e', $P < 0.05$. C, Effects of Pcy and/or TRAIL on Bid protein cleavage in SW480 and SW620 cells treated for 48 h. Whole lysates were subjected to SDS-PAGE and immunoblotted with antibody specific for Bid protein. β-actin was used as a loading control. Bid processing was evidenced by a reduction of full-length protein.

27%, respectively) in SW480 cells when compared to non-treated cells (1%; $P < 0.05$). The combined treatment (Pcy + TRAIL) caused an important increase in the amount of hypodiploid cells reaching 43% of the total SW480 cell population. In SW620 cells, the addition of exogenous TRAIL did not significantly affect cell death, confirming the known TRAIL-resistance of these cells. However, Pcy increased the percentage of hypodiploid cells of the TRAIL-treated SW620 cells from 2% to 26%, with Pcy alone the percentage of hypodiploid cells reached 15% (Figure 2A).

From these data we hypothesized that the TRAIL-induced apoptosis enhanced by Pcy was associated with an increase of DR4 and DR5 death receptor expression at the cell surface of both SW480 and SW620 cells. We evaluated the effects of Pcy, TRAIL, and Pcy + TRAIL treatments on the cell surface expression of DR4 and DR5 by flow cytometry using specific antibodies. As shown in Figure 2B and 2C, DR4 and DR5 proteins were already present at the surface of SW480 cells (in 19% and 11% of cells for DR4 and DR5, respectively). Pcy treatment enhanced the amount of SW480 cells expressing DR4 and DR5 at their cell surface (72% and 77%, respectively; $P < 0.05$). No significant difference was found in the amount of DR4 and DR5 proteins in control *vs.* TRAIL-treated SW480 cells. The treatment of SW480 cells with Pcy + TRAIL enhanced significantly the expression of DR4 and DR5 at the cell surface (85% and 90%, respectively; $P < 0.05$). In SW620 cells, DR4 and DR5 were only scarcely present at the cell surface (Figure 2B and 2C), and treatment with TRAIL did not change the expression levels of DR4/DR5 receptors at the cell surface. Alternatively, Pcy used as a single drug or combined with TRAIL increased significantly the number of cells expressing DR4 (Pcy, 56%; Pcy + TRAIL, 72%) and DR5 (Pcy, 58%; Pcy + TRAIL, 73%).

Pcy induced caspase-dependent apoptosis

Activation of caspase-3 was previously demonstrated in SW620 cells treated with Pcy [10]. In order to characterize the apoptotic pathway triggered by Pcy in association with TRAIL, we examined and compared the activities of caspases-8, -3 and -9 as downstream markers of the apoptotic induction by Pcy, TRAIL and Pcy + TRAIL in both cell

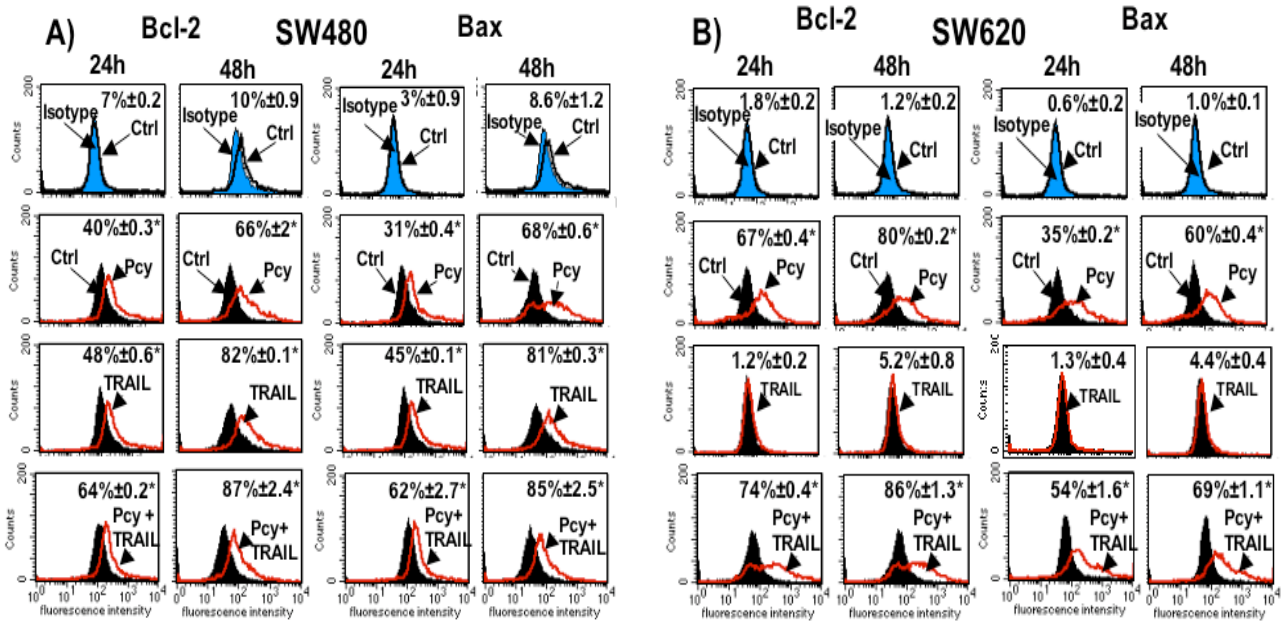

Figure 4: Effects of Pcy treatments on cytoplasmic Bcl-2 and Bax protein expression levels in SW480 (A) and SW620 (B) cells analyzed by flow cytometry. Cells were exposed to 0.1% DMSO (control), 80 μg/ml Pcy, 30 ng/ml TRAIL, or Pcy + TRAIL for 24 or 48 h. Cells were permeabilized and incubated with anti-Bcl-2 or anti-Bax antibody as described in the "Materials and Methods" section. Control cells were compared to FITC-conjugated goat anti-mouse isotype control. The overlays representing the fluorescence shifts to the right allow to measure the percentage of cells expressing Bcl-2 or Bax proteins. Data are presented as mean ± SE of three separate experiments. For each cell line at each time period, treated *vs.* control cells: $*P < 0.05$.

lines treated for 24 and 48 h. As shown in Figure 3A, Pcy used as a single drug or combined with TRAIL increased significantly caspase-8 and caspase-3 activities in SW480 and SW620 cells ($P < 0.05$). TRAIL was active only in SW480 cells and activated both caspases-8 and -3. It was noteworthy that caspase-9 activity was induced only in SW620 cells treated with Pcy or Pcy + TRAIL (Figure 3A). The involvement of caspase-8 in the Pcy, TRAIL or Pcy + TRAIL-triggered cell death was further assessed by using a specific peptide (z-IETD-fmk) inhibitor of caspase-8. As shown in Figure 3B, the ability of Pcy to induce cell death was inhibited by 80% in SW480 cells but only by 40% in SW620 cells by the caspase-8 inhibitor, suggesting that the extrinsic pathway involving the activation of DR4/DR5 death receptors may represent the main apoptotic pathway activated by Pcy in SW480 cells. These data also suggested that Pcy may activate other apoptotic pathways in SW620 cells.

Since Bid protein may represent a target for caspase-8, we analyzed Bid protein cleavage using Western blot of whole cell lysates after exposure of cells for 48 h to Pcy and/or TRAIL. As shown in Figure 3C, a decreased intensity of the native p22 form of Bid protein was detected in SW620 cells treated with Pcy alone or combined with TRAIL, but this effect was not observed with TRAIL used as a single drug. In SW480 cells, Bid was decreased after TRAIL treatment, whereas no significant effect was observed with Pcy and Pcy + TRAIL treatments (Figure 3C). These findings were not related to general protein degradation or reduced protein synthesis as evidenced by the level of β-actin protein.

Effect of Pcy on the activation of mitochondrial pathway

Activation of the apoptotic mitochondrial pathway is tightly regulated by the pro- and anti-apoptotic members of the Bcl-2 family like Bid, Bcl-2 and Bax. This event is characterized by disruption of $\Delta\Psi m$ and release of cytochrome *c* and other pro-apoptotic factors leading to activation of caspase-9 [27]. Bcl-2 is an anti-apopototic protein which promotes cell survival, whereas Bax is a pro-apoptotic member which is activated by tBid to translocate into the mitochondria where tBid can promote the insertion of Bax into the outer mitochondrial membrane [27,28]. Therefore, we investigated whether the levels of Bcl-2 and Bax proteins were affected by Pcy in SW480 and SW620 cells. As shown in Figure 4A, low levels of Bcl-2 and Bax proteins were already expressed in non-treated SW480 cells, whereas Bcl-2 and Bax proteins were not present in SW620 cells under the same conditions (Figure 4B). Treatment of both cell lines with Pcy resulted in a significant increase in the expression levels of both Bcl-2 and Bax proteins if compared with non-treated cells ($P < 0.05$; Figure 4A and 4B). No significant difference was found in the expression of Bcl-2 and Bax proteins in TRAIL-treated SW620 cells. In SW480 cells, these two proteins were increased by 41% and 42%, respectively, after TRAIL treatment for 24 h, and increased by 57% and 59%, respectively, when TRAIL was in combination with Pcy. However in SW480 cells, the Bcl-2/Bax ratio remained stable during the various treatments.

In SW620 cells, an enhanced expression of both Bcl-2 and Bax proteins was measured after Pcy treatment. However, over the 24 to 48 h period of treatment the expression of Bcl-2 was increased by 13%, whereas the expression of Bax was enhanced by 25%, and the Bcl-2/Bax ratio diminished from 2.0 to 1.3 (Figure 4B).

The mitochondrial (intrinsic) apoptotic pathway is associated with the disruption of $\Delta\Psi m$, causing a sudden increase of mitochondrial membrane permeability leading to the release of cytochrome *c* [29,30]. Thus, we examined whether Pcy and Pcy + TRAIL treatments had effects on $\Delta\Psi m$ using flow cytometry after staining cells with $DiOC_2(3)$. This cationic cyanine dye accumulates into the mitochondria with active membrane potentials, producing red fluorescence. The intensity of fluorescence decreases in the cells with impaired $\Delta\Psi m$, which is accompanied by a shift from red to green fluorescence. As shown in Figure 5A, the percentage of SW480 and SW620 cells with depolarized mitochondrial membrane increased significantly after treatment with Pcy for 48 h (21% and 46%, respectively) and

Print ISSN 1816-0735; Online ISSN 1817-4256

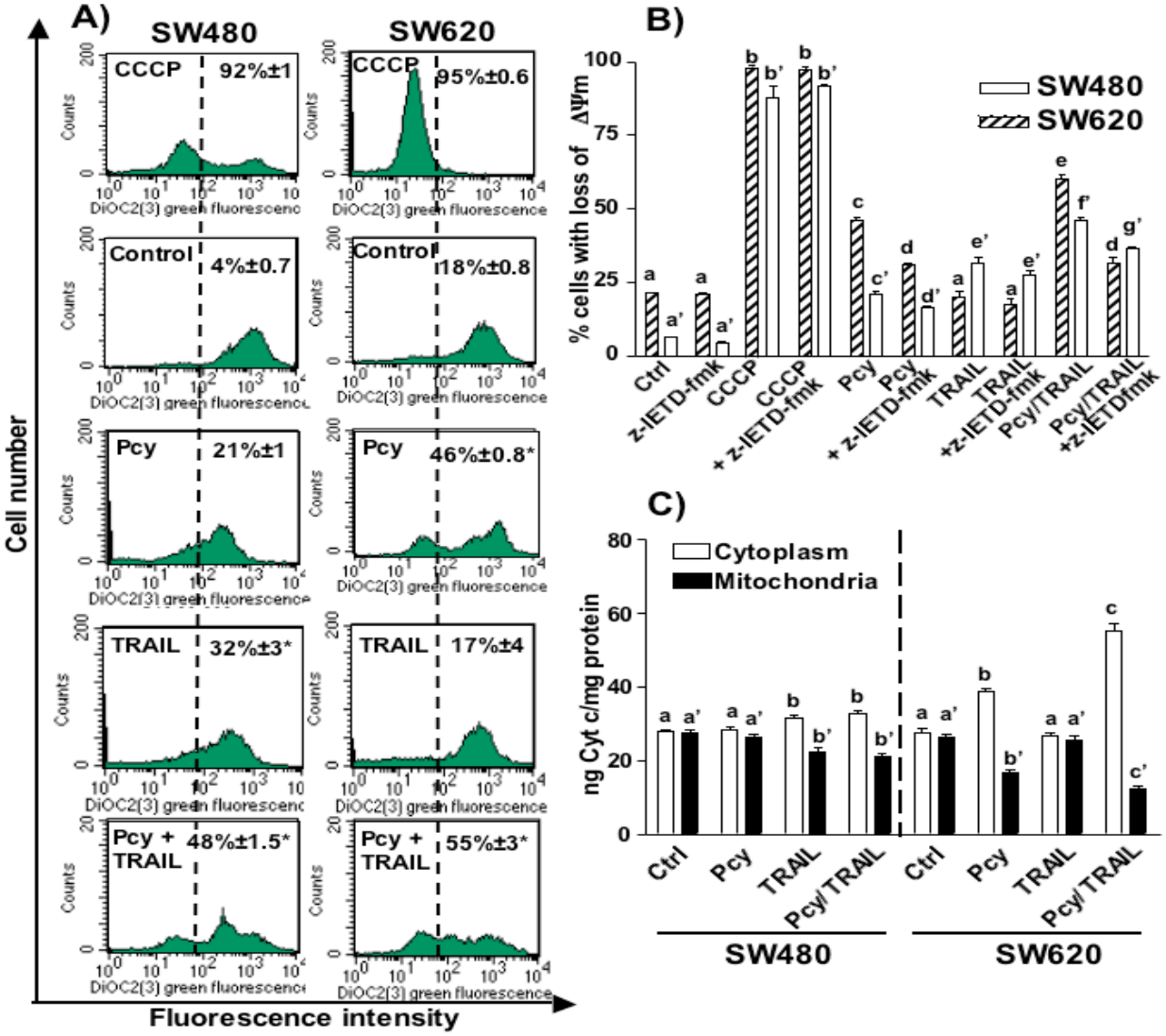

Figure 5: Effects of Pcy and/or TRAIL on mitochondrial functions in SW480 and SW620 cells. A, Flow cytometry analysis of changes in ΔΨm. Cells were treated for 48 h with Pcy (80 μg/ml) and/or TRAIL (30 ng/ml), and were harvested and stained with $DiOC_2(3)$. CCCP was used as positive control. Reduction of green fluorescence corresponds to the loss of ΔΨm. Data are representative of three experiments. For each cell line, control *vs.* treated cells: $^*P < 0.05$. B, Inhibition of Pcy-induced caspase-8 activity by z-IETD-fmk. Cells were pre-treated for 2.5 h with 50 μM z-IETD-fmk before adding 80 μg/ml Pcy and/or 30 ng/ml TRAIL for 48 h and ΔΨm was analyzed by flow cytometry. For each cell line, columns not sharing the same superscript letter differ significantly: a ≠ b ≠ c ≠ d ≠ e and a' ≠ b' ≠ c' ≠ d' ≠ e' ≠ f' ≠ g', $P < 0.05$. C, Measurement of mitochondrial and cytosolic cytochrome *c* levels. Data were presented as mean ± SE of three independent experiments. For each cell line, a ≠ b ≠ c and a' ≠ b' ≠ c', $P < 0.05$.

with Pcy + TRAIL treatment (48% and 55%, respectively). The effect was prominent in SW620 cells exposed to Pcy.

To determine whether the mitochondrial membrane depolarization observed with Pcy treatment was related to a cross-talk between the TRAIL-death receptor pathway and the mitochondrial pathway, we studied the effect of caspase-8 inhibitor on this process. The caspase-8 inhibitor (z-IETD-fmk) reduced significantly the percentage of SW620 cells with depolarized mitochondrial membrane caused by Pcy and Pcy + TRAIL treatments (Figure 5B). Interestingly, such an effect was not observed in SW480 cells treated with Pcy or TRAIL alone, and was very limited with Pcy + TRAIL. These data suggested that the mitochondrial membrane depolarization observed in Pcy-treated SW620 cells required at least partly the activation of caspase-8.

To confirm the involvement of the mitochondrial death pathway in SW620 cells, we examined the effect of Pcy treatment on mitochondrial cytochrome *c* release. As illustrated in Figure 5C, Pcy alone or combined with TRAIL induced a significant release of cytochrome *c* into the cytosol of SW620 cells, and such an effect was not observed in SW480 cells treated with Pcy, confirming the important alteration of mitochondrial membrane permeability in Pcy-treated SW620 cells.

ROS generation by Pcy

Many studies have shown that chemopreventive agents induce apoptosis in cancer cells through generation of ROS. It has been reported that apoptotic signals may disrupt the mitochondrial electron transport chain leading to ROS production such as superoxide anion, hydroxyl radical and H_2O_2 [25,28-30]. On the other hand, we have also previously reported that Pcy was able to activate intracellular polyamine catabolism in SW620 cells giving rise to ROS formation [11]. Therefore, we examined whether Pcy used as a single drug or in combination with TRAIL induced ROS formation in SW480 and SW620 cells. As shown in Figure 6, the number of ROS-producing cells was significantly en-

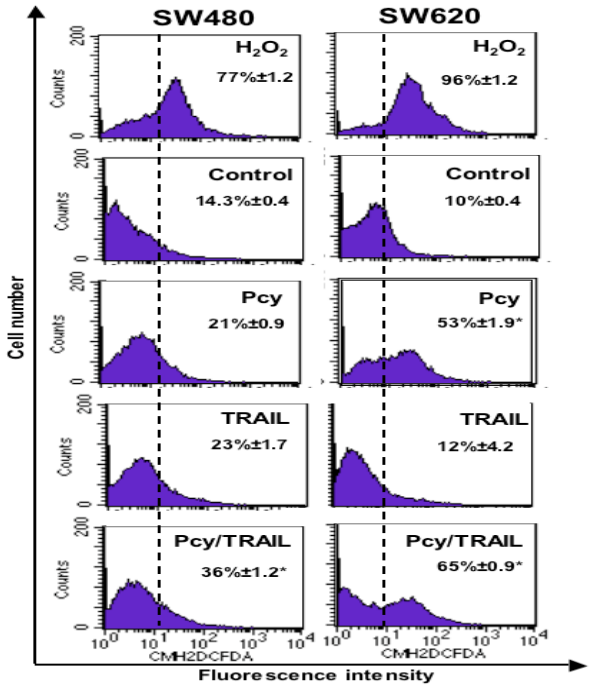

Figure 6: Effects of Pcy on ROS production in SW480 and SW620 cells. Cells were treated 48 h with Pcy (80 μg/ml), TRAIL (30 ng/ml) or Pcy + TRAIL and were collected by trypsinization. After incubation for 30 min at 37°C with CM-H_2DCFDA, oxidation of the reagent was detected by monitoring fluorescence by flow cytometry. As a positive control, H_2O_2-treated cells were included. Data are representative of three experiments. For each cell line, treated *vs.* control cells: **P* < 0.05.

hanced after 48 h of treatment with Pcy alone or in combination with TRAIL, when compared with non-treated cells (*P* < 0.05). The amount of ROS-producing cells was prominent for SW620 cells when compared with SW480 cells (53% *vs.* 22% in Pcy-treated cells, and 65% *vs.* 36% for the Pcy + TRAIL combination).

Discussion

In this study, we show that the acquired resistance to TRAIL during the progression of a primary colon adenocarcinoma to a lymph node metastasis [19,20] can be overcome by Pcy. This Pcy-induced sensitivity to TRAIL in resistant SW620 cells may occur at two levels: (i) through an up-regulation of DR4/DR5 death receptors and the activation of pro-apoptotic proteins of the Bcl-2 family like Bid and Bax, and (ii) through the cytosolic production of ROS favoring mitochondrial disruption leading to cytochrome *c* release into the cytosol and activation of caspase-9. To our knowledge, this is the first report showing that the pro-apoptotic activity of apple Pcy [10,31] may be linked to the TRAIL-receptor mediated apoptosis in colon cancer. Our present data showed that Pcy enhanced the expression of DR4 and DR5 at the surface of both SW480 and SW620 cells. Potentiation of cell death was observed with Pcy + TRAIL combination in SW480 and SW620 cells, suggesting that DR4/DR5 receptors could be actived at the cell surface, as described in a variety of tumor and transformed cells but not in most normal cells [14]. Thus, pharmacological manipulation of DR4/DR5 receptors may be crucial to bypass the resistance to TRAIL-induced apoptosis because it may preferentially kill tumor cells. Similarly, it was reported that flavonoids like quercetin [32], luteolin [33] and apigenin [34] have a critical role in TRAIL-induced apoptosis in human colon and cervical cancer cells. Addition of exogenous TRAIL combined with these flavonoids enhanced mitochondrion-dependent death pathway, and conversely, treatment with the DR5/Fc chimera protein, caspase inhibitors, or DR5 siRNA efficiently reduced apoptosis induced by co-treatments [32-35]. Our results raise the possibility that a combined treatment with Pcy and TRAIL might represent a promising approach against cancer development.

The basis for cell sensitivity and resistance to TRAIL-induced apoptosis is not fully understood and identified. It remains to be determined whether Pcy is acting similarly in both cell lines through an activation of DR4 and DR5 genes, or/and by favoring protein translocation from the cytoplasm to the cell membrane. We have previously reported that a two-fold increase in the intracellular level of DR4 mRNA was observed after Pcy treatment of SW620 cells [31], and we confirmed by Western blot an increased amount of DR4 protein, suggesting that TRAIL-death receptor axis was enhanced at the transcriptional level. In addition, it was shown that Pcy was able to interact with the cell membrane [36], resulting in fluidity changes which may also modulate the functionality of membrane-associated proteins [37] and receptors [26].

Treatments with Pcy or Pcy + TRAIL activated caspases-8 and -3 in both cell lines. The death-inducing signaling complex may have been formed, although not equally because the level of caspase-8 activation was not similar in both cell lines. The TRAIL-death receptor pathway involving directly the caspase-8/caspase-3 cascade seemed to be the prominent apoptotic pathway triggered by Pcy in SW480 cells since treatment of cells with a specific caspase-8 inhibitor reduced by 80% the apoptotic effect of Pcy. Such an effect was not observed in SW620 cells.

Activation of caspase- and mitochondrion-dependent apoptosis is not exclusive for TRAIL pathway. Another death receptor pathway, the Fas (CD95)/Fas-L system may also be involved in the activation of caspase-dependent apoptosis and the crosstalk with the intrinsic mitochondrion-dependent pathway. It has been known that SW480 cells present a Fas-sensitive phenotype whereas its derived SW620 cells are Fas-resistant [38]. It can be hypothesized that Fas-pathway might also be activated by Pcy in SW480 and/or SW620 cells. Further investigations now in progress are necessary to address these aspects.

Bax is a pro-apoptotic member activated by tBid. Our data suggest that Pcy may cause Bid cleavage and formation of tBid through the activation of caspase-8, which is directly involved in the cross-talk between the extrinsic and the mitochondrial (intrinsic) apoptotic pathways. This occurs through translocation of Bax into the mitochondria where tBid can promote the insertion of Bax into the outer mitochondrial membrane [27,28]. These events may favor mitochondrial disruption leading to cytochrome *c* release and consequently the activation of caspase-9 [16,17]. These events were only partly observed in SW480 cells since Bid and Bax proteins were not affected, cytochrome *c* was not released and caspase-9 was not activated after Pcy treatment. However, the mitochondrial membrane depolarization was observed but seemed insufficient to favor cytochrome *c* release and caspase-9 activation. It was proposed that interaction between caspase-8 and its substrate Bid might be influenced by post-translational modifications of Bid, the presence of a negative regulator in SW480 cells, and/or mutations of Bid affecting physical interaction between the molecules [27-28,39,40]. Our data suggested that, in SW480 cells, the main apoptotic pathway triggered by Pcy was the extrinsic (death receptor) pathway.

Involvement of other Bcl-2 family members as Bcl-2 and Bax may be a key step for the mitochondrion-driven apoptotic process. Likewise, Bax and Bcl-2 were increased in Pcy- and Pcy + TRAIL-treated SW480 and SW620 cells, whereas the Bcl-2/Bax ratio remained identical in SW480 cells treated with Pcy and Pcy + TRAIL, but SW620 cells exposed to Pcy displayed a progressive decrease of the Bcl-2/Bax ratio. These events paralleled with Bid truncation,

Print ISSN 1816-0735; Online ISSN 1817-4256

disruption of $\Delta\Psi m$, release of cytochrome c, and activation of caspase-9. Thus, some of the observed mitochondrial alterations may be a downstream consequence of the activation of extrinsic apoptotic pathway.

At low levels, ROS have been implicated in multiple signaling pathways and may also play an important role in apoptosis by regulating the activity of enzymes involved in cell death. Intracellular ROS levels are enhanced dramatically by environmental stress, which may cause significant damages in cellular macromolecules (such as DNA and RNA). The apoptotic process triggered by mitochondrial dysfunctions was reported to be at least partly related to ROS production [11,25,41]. ROS formation may also induce mitochondrial permeability transition, pore opening, decrease of membrane potential, cytochrome *c* release, and caspase-9 activation. Our data showed that Pcy treatment of SW620 cells led to an increased number of ROS-producing cells when compared with SW480 cells. This may also explain the higher involvement of the mitochondrial apoptotic pathway observed in SW620 cells. Indeed, we have previously shown that ROS production by Pcy was related, in great part, to a Pcy-mediated activation of polyamine catabolism which caused the intracellular formation of ROS (hydrogen peroxide and amidopropanal)[11]. This was confirmed by the observation that a specific inhibition of the polyamine catabolic pathway by a specific inhibitor (MDL 72527) caused an inhibition of ROS generated by Pcy treatment and exerted a protective effect on mitochondrial function [11,31].

In conclusion, Pcy-treatment of human colon adenocarcinoma SW480 cells enhanced TRAIL-mediated apoptosis. In the adenocarcinoma-derived metastatic SW620 cells which are TRAIL-resistant under basal conditions, an activation of the TRAIL-death receptor pathway seemed to be involved. In SW480 cells, Pcy activated mainly the extrinsic apoptotic pathway, i.e., up-regulation of DR4/DR5 expression and activation of caspases-8 and -3. In contrast, in SW620 cells, the apoptotic effect of Pcy was more complex and involved a cross-talk between the extrinsic and the mitochondrial (intrinsic) apoptotic pathway, and Pcy-triggered ROS production participated in mitochondrial disruption. The ability of Pcy to activate the TRAIL apoptotic pathway also in TRAIL-resistant cancer cells and the potentiation of Pcy-triggered cell death by TRAIL + Pcy combination highlight the chemopreventive potential of this natural agent for colorectal cancer treatments.

Acknowledgments

Maria Elena Maldonado-Celis is the recipient of a doctoral scholarship from the Francisco José Caldas Institute for the Development of Science and Technology (COLCIENCIAS), University of Antioquia, Colombia. This work was supported by a grant from the Ligue Contre le Cancer, Comité Départemental du Haut-Rhin, France.

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Print ISSN 1816-0735; Online ISSN 1817-4256