Potentiation of apple procyanidin-triggered apoptosis by the polyamine oxidase inactivator MDL 72527 in human colon cancer-derived metastatic cells

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Abstract. Apple procyanidins have chemopreventive properties in a model of colon cancer, they affect intracellular signalling pathways, and trigger apoptosis in a human adenocarcinoma-derived metastatic cell line (SW620). In the present study we investigated relationships between procyanidin-induced alterations in polyamine metabolism and apoptotic effects. Apple procyanidins diminish the activities of ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase, key enzymes of polyamine biosynthesis, and they induce spermidine/spermine N^1-acetyltransferase, which initiates retroconversion of polyamines. As a consequence of the enzymatic changes polyamine concentrations are diminished, and N^1-acetyl-polyamines accumulate in SW620 cells. In contrast with expectations MDL 72527, an inactivator of polyamine oxidase (PAO), improved the anti-proliferative effect of procyanidins, and caused an increase of the proportion of apoptotic cells, although it prevented the formation of hydrogen peroxide and 3-acetamidopropanal, the cytotoxic products of PAO-catalysed degradation of N^1-acetylspermidine and N^1-acetylspermine. Addition of 500 μM N^1-acetylspermidine to the culture medium in the presence of procyanidins mimicked the effect of MDL 72527. Therefore we presume that the enhanced procyanidin-triggered apoptosis by MDL 72527 is mediated by the accumulation of N^1-acetyl-polyamines. The observation that apple procyanidins enhance polyamine catabolism and reduce polyamine biosynthesis activity similar to known inducers of SSAT, without sharing their toxicity, and the potentiation of these effects by low concentrations of MDL 72527 suggests apple procyanidins for chemopreventive and therapeutic interventions.

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Introduction

Apples contain several classes of polyphenols such as flavonoid monomers and condensed tannins, the procyanidins (1). The polyphenol content of apples varies from one variety to another, but procyanidins are predominant (2). They consist mainly of condensed (-)-epicatechin units with a small proportion of (+)-catechin. Polyphenolic compounds are known to affect processes that are important for cancer development. Thus, they have antioxidant and radical scavenging properties, and they may prevent carcinogenesis by affecting molecular events in the initiation, promotion and progression stages (3). We have recently reported (4) that apple procyanidins inhibit the growth of human metastatic colon carcinoma-derived SW620 cells. They alter intracellular signalling pathways and induce apoptosis. The apoptotic effects were associated with a 50% reduction of protein kinase C (PKC) activity (4). Interrelations between PKC activity and polyamine metabolism have repeatedly been found. It was reported for instance that PKC inactivation inhibits the induction of ornithine decarboxylase (ODC), a key enzyme of polyamine biosynthesis (5). Transgenic mice overexpressing PKC show a 3- to 4-fold higher expression of phorbol ester-induced epidermal ODC, when compared with normal littermates (6).

The effect of procyanidins on polyamine metabolism has so far not been studied. The intracellular polyamine content and the activities of key enzymes of polyamine biosynthesis, ODC and S-adenosyl-L-methionine decarboxylase (AdoMetDC), are increased in colonic tumours in comparison with the surrounding tissue (7), and have been considered as logical targets in cancer chemoprevention and therapy (8,9). More recently polyamines have been implicated in cell death programs (10).

Biosynthetic and catabolic reactions of polyamine interconversion are illustrated in Fig. 1. It appears that polyamines are ambivalent regulators of cell functions, promoting cell proliferation or cell death, depending on the cell type, their concentration, and the environmental signals (10-12). Since procyanidins perturbate signal transduction pathways, it could be expected that they affect also polyamine metabolism. In

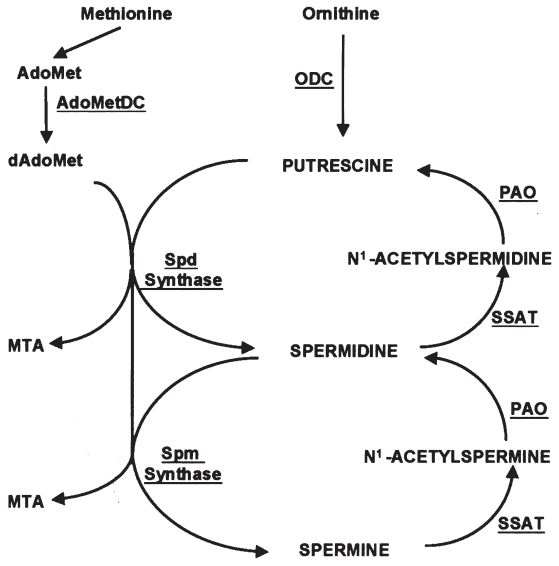

Figure 1. Polyamine interconversion pathway. Rate-limiting of the biosynthetic branch is the formation of dAdoMet and acetylation of spermine and spermidine by SSAT in the catabolic branch. In addition to the general interconversion reactions, oxidative deaminations are involved in the terminal elimination of polyamines (not shown). Acetylated polyamines are also excreted by cells. AdoMetDC, S-adenosylmethionine decarboxylase; AdoMet, S-adenosylmethionine; dAdoMet, decarboxylation product of AdoMet; MTA, methylthioadenosine; ODC, ornithine decarboxylase; PAO, polyamine oxidase; Spd, spermidine; Spm, spermine; SSAT, spermine/spermidine N^1-acetyltransferase.

the present study, we investigated, therefore, interrelations between procyanidins and polyamine metabolism of SW620 colon adenocarcinoma-derived metastatic cells. Our data show a previously unknown ability of procyanidins to activate polyamine acetylation and to down-regulate simultaneously polyamine biosynthesis. By using the PAO inactivator MDL 72527 at 50 μM, a dose which inactivates PAO, but does not inhibit cell growth (13), we demonstrate that the apoptotic effect of procyanidins is unrelated to the formation of reactive oxygen species (ROS), which are generated by enhanced oxidation of polyamines, and that MDL 72527 potentiates the procyanidin-triggered apoptosis. These effects seem to be correlated with the increased formation of N^1-acetyl derivatives of spermidine and spermine.

Materials and methods

Isolation and characterization of apple procyanidins. 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* (14). Procyanidins were characterized and quantified by thiolysis coupled with reverse-phase HPLC and diode array UV-visible detection (15). On a weight basis, the procyanidin fraction contained 78.4% procyanidins, consisting of 95% (-)epicatechin and 4% (+)catechin. The mean degree of polymerization was close to four. The procyanidin fraction was almost totally devoid of monomeric catechins and other phenols (<2%).

Cell culture and treatments. SW620 cells are rapidly growing metastatic cells. They were chosen for this work because we have extensive experience on the polyamine metabolism and effects of the PAO inactivator MDL 72527 on this cell line (16,17). They were obtained from the European Collection of Animal Cell Culture (Salisbury, UK). Cells were seeded at $3x10^3$ cells per well in 96-well culture clusters or at $1x10^6$ cells in culture dishes (100-mm diameter). They were cultured in modified Eagle's medium (containing 25 mM glucose, glutamax) and supplemented with 3% heat-inactivated (56˚C) horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids, 5 μg/ml transferrin, 5 ng/ml selenium and 10 μg/ml insulin (Gibco, Invitrogen Corp., Cergy-Pontoise, France). The use of horse serum avoids oxidative deamination of spermidine and spermine by serum amine oxidase. MDL 72527 [N^1,N^4-bis(2,3-butanedienyl)-1,4-butaneamine dihydrochloride] was synthesized as described previously (18). N^1-acetylspermidine was synthesized as described by Dredar *et al* (19). Stock solutions of the procyanidins (50 mg/ml) were prepared in dimethylsulfoxide (DMSO). Owing to the limited solubility of procyanidins in aqueous media, the highest concentration used was 50 μg/ml culture medium. The final concentration of DMSO in the culture medium was 0.1%. Cells were exposed for different times to procyanidins and/or MDL 72527, starting 24 h after seeding. Culture media were replaced every 48 h. Cell growth was stopped by addition of 50 μl/well trichloroacetic acid (50% v/v), and cell proteins were determined by staining with Sulforhodamine B (20). The relationship between cell number (protein content/well) and absorbance at 490 nm is linear from 0 to 200,000 cells per well.

Cell cycle analysis. Cell cycle distribution was analysed by labeling cells with propidium iodide. Assays were carried out as described by Nicoletti *et al* (21). Briefly, $1x10^6$ cells were seeded in culture dishes and harvested by trypsinisation (0.5% trypsin in 2.6 mM EDTA). For cell sorting SW620 cells were collected by centrifugation, and fixed in 1 ml methanol:PBS (9:1 v/v). After washing twice in PBS, cells were re-suspended in 200 μl PBS containing 0.25 mg/ml RNAse A and 0.1 mg/ml propidium iodide (Sigma-Aldrich, St. Quentin Fallavier, France). After incubation in the dark at 37˚C for 30 min, the fluorescence of 10,000 cells was analyzed using a FACScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA, USA).

Hydrogen peroxide assay. Cells were treated for 24 h with procyanidins in the absence or presence of MDL 72527. Intracellular hydrogen peroxide was determined by a method analogous to that developed by Bass *et al* (22) but using incubations of 30 min at 37˚C with 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (Invitrogen, Eugene, OR,

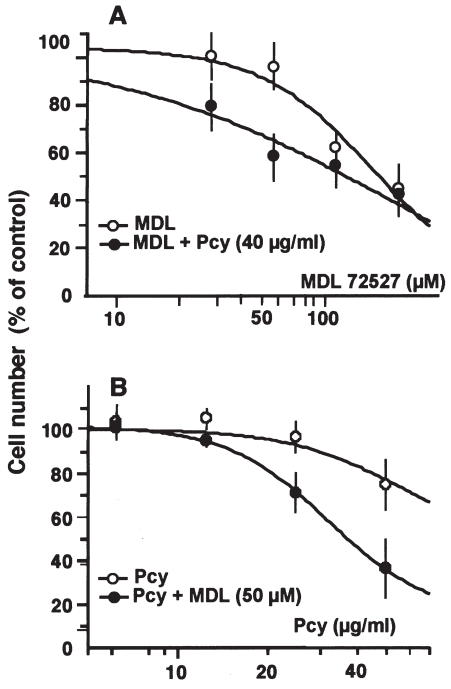

Figure 2. Inhibition of the growth of SW620 cells by MDL 72527 and procyanidins (Pcy). Dose response curves. Starting 24 h after seeding cells were exposed to MDL 72527, Pcy or both drugs for 48 h, and cell protein in wells was determined. The error bars indicate ± SE.

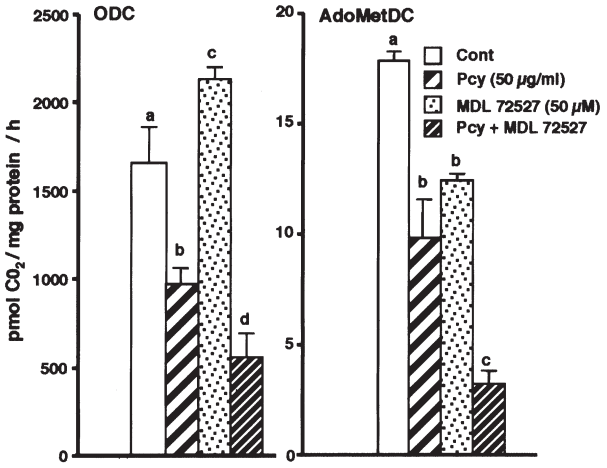

Figure 3. Effect of apple procyanidins (Pcy 50 μg/ml) and MDL 72527 (50 μM) on ODC and AdoMetDC cells after 24-h exposure to drugs. Results are means ± SE. of three separate experiments. Columns not sharing the same superscript differ significantly ($p<0.01$).

USA) as detector reagent. Cells were harvested by trypsinisation, and washed with PBS. Fluorescence resulting from the oxidation of the reagent was detected in 10,000 cells by flow cytometry. Samples incubated with hydrogen peroxide before trypsinisation served as positive controls.

ODC and AdoMetDC assays. Cells were homogenized in 100 mM Tris-HCl buffer, pH 7.4 (1 mM EDTA, 1 mM dithiothreitol, 0.5 μM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride). After centrifugation at 33,000 x g for 25 min at 4˚C, the supernatants were collected and ODC and AdoMetDC assays were performed. ODC activity was evaluated by measuring $^{14}CO_2$ formation from [1-^{14}C]L-ornithine (55 mCi/mmol, Amersham Pharmacia Biotech, Orsay, France) (23) and AdoMetDC by measuring $^{14}CO_2$ formed from [1-^{14}C]S-adenosylmethionine (60 mCi/mmol, Amersham Pharmacia Biotech) (24).

Polyamine determinations. Cells were homogenized in perchloric acid (200 mM), and the homogenates were centrifuged at 1500 x g for 10 min after standing for 16 h at 2˚C. The clear supernatants were diluted with perchloric acid (200 mM) and 200 μl aliquots were applied on a reversed-phase column for separation. The polyamines and their N^1-acetyl derivatives were determined by separation of the ion pairs formed with n-octanesulfonic acid, reaction of the column effluent with o-phthalaldehyde/2-mercaptoethanol reagent, and monitoring of fluorescence intensity (25).

Statistical analysis. All experiments were performed at least three times. If not stated otherwise, data are means ± SE. Statistical differences between groups were evaluated by one-way ANOVA and specific differences were identified using the Sudent's t-test or the Student-Neuman-Keuls multiple comparison test. Differences with $p \leq 0.01$ are considered statistically significant.

Results

Cell growth inhibition by procyanidins and MDL 72527. Fig. 2A shows the dose-effect relationship of MDL 72527 on cell growth after 48-h exposure to the drug, at concentrations ranging from 30 to 125 μM. MDL 72527 reduced the growth rate only at doses exceeding 50 μM. Procyanidins (40 μg/ml) caused approximately 20% growth inhibition. At this concentration they enhanced significantly the antiproliferative effect of the PAO inactivator. As is shown in Fig. 2B, 50 μM MDL 72527 potentiated the antiproliferative effect of the procyanidins; 60% growth inhibition was observed.

Alterations of polyamine metabolism. When SW620 cells were treated with procyanidins for 24 h, the activities of ODC and AdoMetDC were reduced by 50% (Fig. 3). MDL 72527 (50 μM) did not affect ODC activity, but caused a significant decrease of AdoMetDC activity, and it enhanced the effect of procyanidins on ODC and AdoMetDC activities by ~75%.

Figs. 4 and 5 illustrate the effect of various treatments on the intracellular polyamine content, and on the formation of N^1-acetyl polyamines. Cells treated with procyanidins alone exhibited a significant ($p<0.01$) decrease of spermine. This was accompanied by an increase of N^1-acetylspermidine concentration, indicating the enhancement of polyamine catabolism. Obviously apple procyanidins exert a dual effect on polyamine metabolism by down-regulating polyamine biosynthesis and by stimulating polyamine catabolism.

Disregarding diamine oxidase-catalysed terminal catabolism of polyamines to amino acids two enzymes are mainly involved in polyamine catabolism: spermidine/spermine N^1-acetyltransferase (SSAT) and PAO (26) (Fig. 1). Induction of SSAT was not accompanied by corresponding changes of PAO, which is usually expressed in excess. MDL 72527

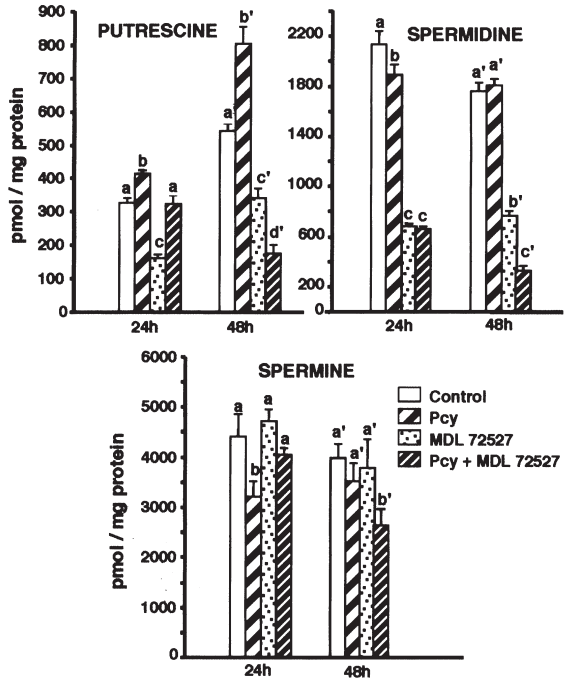

Figure 4. Effects of apple procyanidins (Pcy 50 μg/ml) and MDL 72527 (50 μM) on the polyamine content of SW620 cells. Cells were treated for 24 or 48 h with the compounds, harvested by scraping and extracted with 0.2 N perchloric acid. Results are means ± SE of three separate experiments. Columns not sharing the same superscript differ significantly ($p<0.01$).

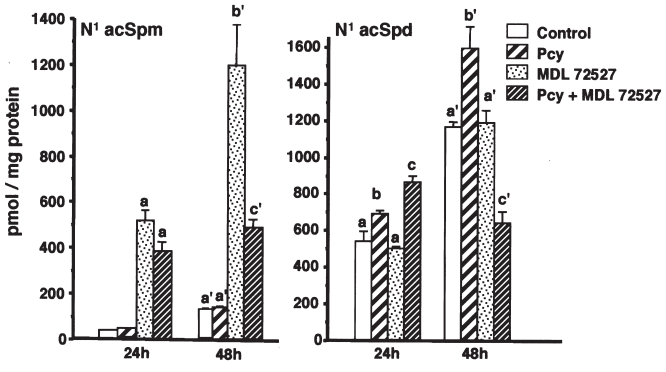

Figure 5. Effect of apple procyanidins (Pcy 50 μg/ml) and MDL 72527 (50 μM) on the formation of N^1-acetylpolyamines. Cells were treated for 24 or 48 h, harvested by scraping and extracted with 0.2 N perchloric acid. Results are the mean ± SE of three separate experiments. Columns not sharing the same superscript differ significantly ($p<0.01$).

at 50 μM, a concentration that is sufficient to inactivate PAO completely (13) but, as was shown above, had no effect on SW620 cell growth, caused the accumulation of N^1-acetylspermine and N^1-acetylspermidine (Fig. 5). This increase in N^1-acetyl derivatives is mainly due to prevention of PAO catalysed degradation, but the induction of SSAT can at present not be completely excluded, since MDL 72527 inhibits at concentrations ≥60 μM the growth of SW620 cells (13). In combination with procyanidins, MDL 72527 diminished within 48 h the intracellular content of putrescine and spermidine pools, and reduced the amount of spermine more efficiently than procyanidins alone.

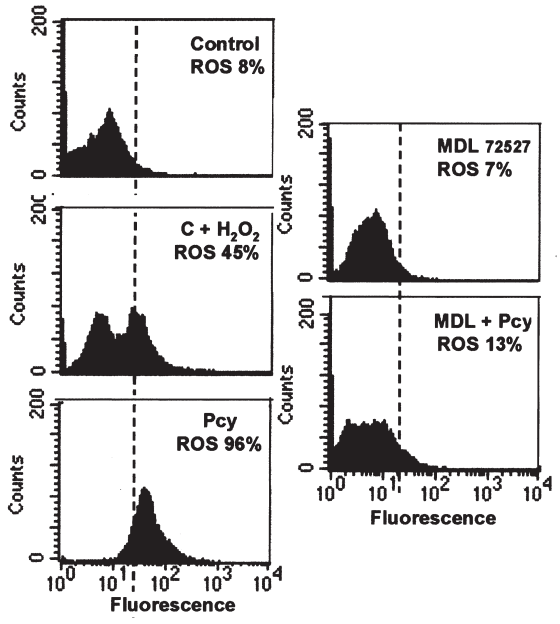

Figure 6. Generation of hydrogen peroxide in SW620 cells after treatment with apple procyanidins (Pcy 50 μg/ml) and MDL 72527 (50 μM). Cells were exposed to the drugs for 24 h and were collected by trypsinisation. After incubation for 30 min at 37°C with 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, oxidation of the reagent was detected by monitoring fluorescence using a FACScan flow cytometer. As a positive control hydrogen peroxide-treated samples were included.

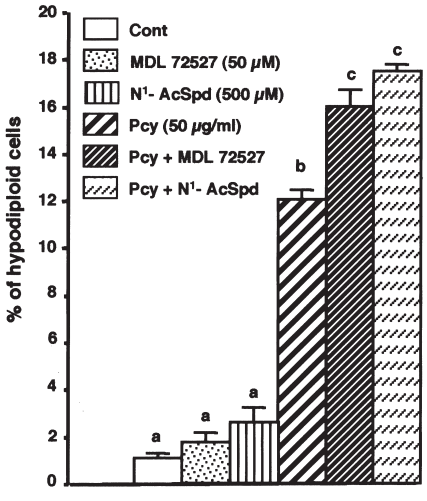

Figure 7. Percentage of hypodiploid cells after treatment with apple procyanidins (Pcy 50 μg/ml) MDL 72527 (50 μM) and N^1-acetylspermidine (500 μM). Cells were treated for 24 h with the various compounds. Hypodiploid cells were determined by flow cytometry. Results are means ± SE of three separate experiments. Columns not sharing the same superscript differ significantly ($p<0.01$).

Procyanidins and generation of reactive oxygen species (ROS). N^1-acetyl-polyamines serve as substrates of PAO, and generate hydrogen peroxide and 3-acetamidopropanal (27). Determination of intracellular hydrogen peroxide showed that procyanidins increase hydrogen peroxide accumulation (Fig. 6). When cells were incubated with the PAO inactivator and procyanidins, the formation of hydrogen peroxide was diminished by 80%, indicating that ROS formation by

procyanidins is at least to a considerable proportion directly due to the procyanidin-mediated activation of the polyamine catabolism.

Effect of MDL 72527 and N^1-acetylspermidine on procyanidin-mediated cell death. Since ROS formation is known to be implicated in cell death (10,28,29), we examined whether inhibition of ROS formation by MDL 72527 reduces cytotoxic effects triggered by procyanidins. MDL 72527 (50 μM) alone had no significant effects on cell death. However, in combination with procyanidins (50 μg/ml), the PAO inactivator increased significantly ($p<0.01$) the proportion of hypodiploid (apoptotic) cells (Fig. 7). Since the enhancement of apoptosis by procyanidins and MDL 72527 was unrelated to the accumulation of hydrogen peroxide, we hypothesised that the intracellular accumulation of N^1-acetyl-polyamines caused by MDL 72527 and procyanidins is somehow responsible for the observed effect. This idea is supported by the observation that 500 μM N^1-acetylspermidine in the presence of procyanidins increased the proportion of apoptotic SW620 cells in a similar way to the exposure of cells to MDL 72527 plus procyanidins (Fig. 7). N^1-acetylspermidine did not improve the apoptotic effect when added to MDL 72527/procyanidin combinations (not shown).

Discussion

Apple procyanidins diminish the formation of aberrant crypt foci in rat colon, trigger apoptosis, and alter signal transduction pathways in SW 620 cells (4). In order to study further the mechanistic basis of their chemopreventive effect, polyamine metabolism was studied as a potential target of procyanidins.

The diminution of cell growth rate is understandable on the basis that procyanidins reduce ODC and AdoMetDC activities, and decrease intracellular polyamine concentrations due to induction of SSAT. Inhibition of PKC activity by procyanidins contributes to these effects (4); since it is known that this enzyme is involved in the regulation of ODC (5). Its inhibition potentiates the antiproliferative effect of DL-2-(difluoromethyl)ornithine, a selective inactivator of ODC (30). The observed changes in enzyme activities and polyamine pools resemble effects of known inducers of SSAT inasmuch as putrescine, spermidine and spermine concentrations were diminished, together with an increase of N^1-acetylspermidine concentrations. However both, growth inhibition and polyamine pool changes, were considerably smaller than those usually reported for structural analogues of spermine (31), but resemble less potent SSAT inducers (example in ref. 32).

For inducers of SSAT it has repeatedly been shown that inactivation of PAO by MDL 72527 prevents apoptosis, because their apoptotic effect relies mainly on the PAO catalysed formation of hydrogen peroxide, and of 3-acetamidopropanal from the excessively formed N^1-acetyl derivatives of spermidine and spermine (33-35). The decrease in growth rate and the increased effects of treatments with procyanidins and MDL 72527 on SSAT, ODC, AdoMetDC and polyamine pools may not surprise, because they are in accordance with previous experience with SSAT inducers. However, it was surprising that MDL 72527 at an obviously non-toxic concentration improved growth inhibition and enhanced apoptosis in spite of the suppression of procyanidine-induced hydrogen peroxide formation. Since this effect of the PAO inactivator was mimicked by a high extracellular concentration of N^1-acetylspermidine in the presence of procyanidins, a key role of hitherto unobserved properties of MDL 72527 on apoptosis can be excluded. Depletion of acetyl-CoA due to the massive formation of N^1-acetylpolyamines may contribute to growth inhibition in the case of highly potent inducers of SSAT (36), and one may speculate about the possibility that accumulation of intracellular N-acetyl-polyamines affects histone acetylation by competing with acetylCoA:spermidine N^8-acetyltransferase, an enzyme that has also histone acetylating properties (37). However, these and related effects are more likely to be observed in situations of excessive SSAT induction than under the conditions of the present work, although they cannot be excluded at present.

MDL 72527 is a lysosomotropic compound (38). Its cytotoxicity at elevated concentrations relies most probably on its lysosomotropic property, as is suggested from the excessive formation of vacuoles in SW620 cells (17). As a weaker base with a higher lipophilicity than spermidine, N^1-acetylspermidine could also have hitherto unknown lysosomotropic properties. It seems not unlikely that the observed enhancement of procyanidin-triggered apoptosis by MDL 72527 and N^1-acetylspermidine is due to the sensitisation of cells to lysosomotropic compounds by the procyanidins. This effect may be initiated by the known binding of procyanidins to the cell surface (3), which affects multiple signalling pathways (4) and may affect transmembrane transport.

Apple procyanidins are non-toxic, and are therefore highly promising new drugs, particularly in colon cancer chemoprevention. The potentiation of their apoptotic effect is, therefore, a challenging target for anti-cancer drug development.

Acknowledgements

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