Synergism between Apple Procyanidins and Lysosomotropic Drugs: Potential in Chemoprevention

NIKOLAUS SEILER[1], MEHDI CHAABI[2], STAMATIKI ROUSSI[1], FRANCINE GOSSÉ[1], ANNELISE LOBSTEIN[2] and FRANCIS RAUL[1]

[1]*INSERM U682, Laboratory of Nutritional Cancer Prevention, IRCAD, 67091 Strasbourg, cedex;*
[2]*CNRS-ULP UMR 7081, Faculty of Pharmacy, Laboratory of Molecular Pharmacochemistry, 67400 Illkirch cedex, France*

Abstract. *Background: Procyanidins are apple constituents with potential in colon cancer chemoprevention. Materials and Methods: Human colon cancer derived metastatic cells (SW620), growing under standardized conditions, were exposed to procyanidins and lysosomotropic compounds. Growth, apoptosis and lysosomal integrity was determined using published methods. Results: Lysosomotropic drugs (MDL 72527, phenylalanine methylester and chloroquine) amplified procyanidin-induced growth inhibition and apoptosis in SW620 cells at non-cytotoxic concentrations. The improved toxicity of the drug combinations relies primarily on the enhancement of lysosomal membrane permeability. Conclusion: Combinations with non-toxic concentrations of lysosomotropic compounds improve the anti-carcinogenic properties of apple procyanidins.*

Numerous polyphenols affect processes involved in carcinogenesis and cancer development. Procyanidins are the most abundant polyphenols in apples (1). They have antioxidant and radical scavenging properties, and prevent carcinogenesis by impairing molecular events in the initiation, promotion and progression stages (2). Among other effects, apple procyanidins inhibit melanogenesis in mouse melanoma cells (3) and induce apoptosis in stomach cancer (4) and colonic adenocarcinoma cells (5). Their ability to reduce the formation of pre-neoplastic colonic lesions (aberrant crypt foci) in a rat model of colon carcinogenesis (5, 6) suggests their suitability as chemopreventive agents. However, their low solubility in aqueous media restricts therapeutically useful concentrations. The enhancement of their anti-cancer effect by combination with non-toxic drugs, therefore, appears of interest and may improve their potential in colon cancer prophylaxis.

In the present work, an apple procyanidin fraction with enriched tetramer (designated PCy) was used. Figure 1 shows the structures of the compounds which were considered in this work. SW 620 human colon cancer-derived cells were employed because they are invasive and metastatic, and previous work on PCy cytotoxicity has been performed with this cell line (5, 7).

Correspondence to: Nikolaus Seiler, INSERM U682, Laboratory of Nutritional Cancer Prevention, IRCAD, 67091 Strasbourg, cedex, France. Tel: +33-3-88-11-90-23, Fax: +33-3-88-91-90-97, e-mail: nikolaus.seiler@ircad.u-strasbg.fr

Key Words: Procyanidins, chloroquine, MDL 72527, phenylalanine methylester, lysosomes, cytotoxicity, SW 620 cells.

Materials and Methods

Chemicals. Unless stated otherwise chemicals were from Sigma Chemical Co. (St. Louis, USA) or from Merck (Darmstadt, Germany). MDL 72527 (N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine dihydrochloride) was synthesized as described previously (8).

Isolation and characterisation of apple procyanidins. PCy were isolated from cider apple (Malus domestica, variety Antoinette) as described previously (5). They were characterized by thiolysis coupled with reverse-phase HPLC (9). On a weight basis, the PCy fraction contained 78.4% PCy, consisting of 95% (-)-epicatechin and 4% (+)-catechin. The mean degree of polymerisation was close to four.

Cell culture and treatments. SW620 cells were obtained from the European Collection of Animal Cell Culture (Salisbury, UK). They were seeded at $3x10^3$ cells per well in 96-well culture clusters, or at $1x10^6$ cells in culture dishes (10 cm diameter), and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (containing 25 mM glucose and glutamax) 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 polyamines by serum amine oxidase. Stock solutions of PCy (50 mg/ml) were prepared in dimethylsulfoxide (DMSO). Due to their limited solubility in aqueous media, the highest concentration of PCy in culture medium was 50 μg/ml, corresponding to 1 μl/ml DMSO. Cells were exposed for various times to PCy and/or lysomotropic drugs. All media contained 1 μl/ml DMSO and were replaced every 48 h. Cell growth was stopped by the addition of 50 μl/well trichloroacetic acid (50% v/v). Cell protein was assayed by staining with sulforhodamine B and

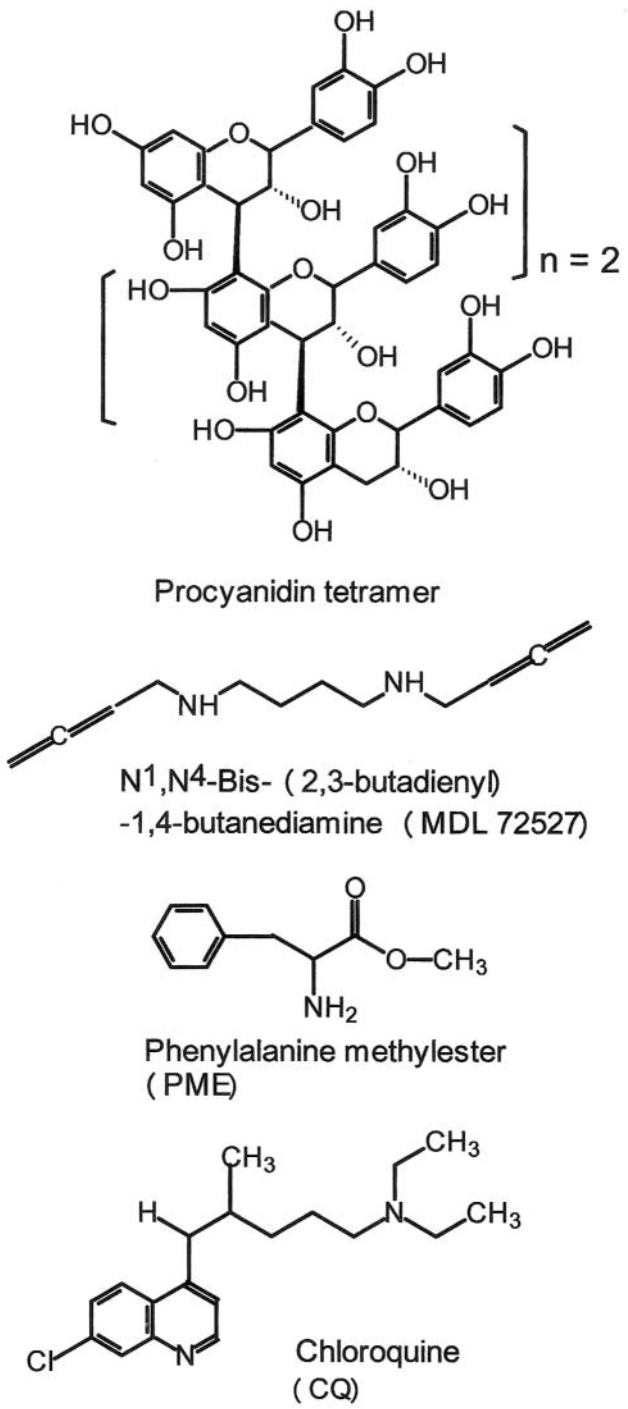

Figure 1. *Structural formulae of the procyanidin tetramer and other drugs employed in this work.*

determining absorbance at 590 nm (10). The relationship between cell number (protein content/well) and absorbance at 590 nm was linear between 0 and 200,000 cells per well. Since PCy have a limited stability, dilutions in culture medium were made at each treatment time.

Determination of apoptotic cells. After labelling cells with propidium iodide, the proportion of hypodiploid cells was determined according to Nicoletti *et al.* (11). The fluorescence of 10,000 cells was analyzed using the FACScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, USA).

Assay of lysosomal integrity. Lysosomal integrity was determined in acridine orange-stained cells according to Yuan *et al.* (12). Cells were grown in DMEM culture medium and 3% horse serum. Forty eight h after seeding, cells were exposed to 5 μg/ml acridine orange for 15 min at 37˚C and rinsed in complete medium. They were then exposed for 24 h to the drugs. After harvesting by trypsinisation, the cell pellet was re-suspended in 0.1 ml PBS. Increase in green fluorescence, as determined by flow cytometry (FL1 channel) from 10,000 cells per sample indicates release of acridine orange from lysosomes into the cytoplasm.

Statistical analysis. The dose-response curves and EC_{50} values were calculated from the experimental data using a four-parameter logistic, the Erithacus software, GraFit (version 3.0). Unless stated otherwise, data are means±S.D. Statistical differences between groups were evaluated by one-way ANOVA and specific differences were identified using the Student's *t*-test: differences with $p \leq 0.05$ were considered statistically significant.

Results

Effects of some lysosomotropic compounds on cell growth, alone and in combination with procyanidins. MDL 72527, a polyamine oxidase inactivator with lysosomotropic properties (13, 14), has recently been shown to sensitize human colon carcinoma (LoVo) and M14 human melanoma cells to the cytotoxic metabolites of spermine (15, 16), and enhances the cytotoxic effect of PCy on SW 620 cells (7). These effects of MDL 72527 are not due to its ability to inactivate polyamine oxidase, but rely, at least in part, on its lysosomotropy. In order to further investigate the presumed role of lysosomal membrane permeabilization in the enhancement of PCy toxicity, phenylalanine methylester (PME) (17) and chloroquine (CQ), the well known anti-malarial and anti-inflammatory drug, (18) were chosen.

PME is cytotoxic to SW 620 cells in the mM range. As is apparent from Figure 2, it inhibits SW 629 cell growth in a dose – dependent manner. After exposure for 96 h, the EC_{50} of PME was 16±1 mM. In the presence of 50 μg/ml PCy, the dose response curve shifted to the left (EC50=6±0.5 mM). However, since its low potency is a handicap in practical applications, no further experiments were carried out with PME.

CQ is a potent cytotoxic agent. After exposure of SW 620 cells to 100 μM CQ for 96 h, SW 620 cell numbers decreased almost to zero (Figure 3A). In the presence of 50 μg/ml PCy, the dose-response curve shifted from EC50=28±3 μM to 6±1 μM. Figure 3B shows the effect of 10 μM and 20 μM CQ on the dose-effect relationship of

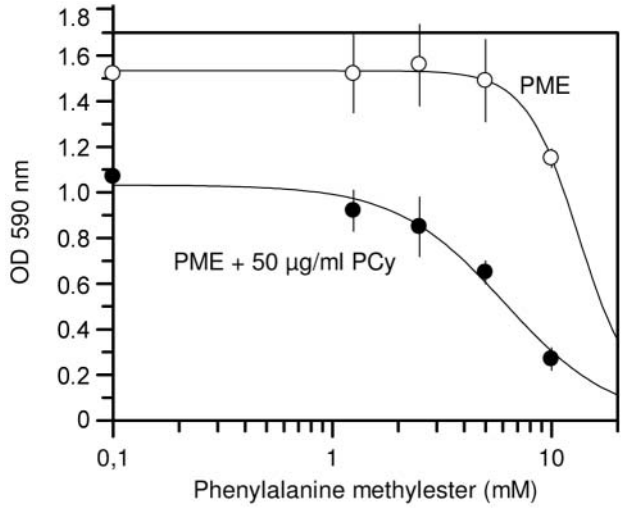

Figure 2. *Effect of phenylalanine methylester (PME) alone and in combination with 50 µg/ml procyanidins (PCy) on the growth of SW 620 cells. Dose – effect relationship of PEM after 96 h exposure. y-axis: absorbance at 590 nm after staining with sulforhodamine B. Error bars indicate±S.D. (n=8).*

PCy. Again the EC_{50} shifted to the left, from 32±2 µg/ml to 19±3 µg/ml and 13±2 µg/ml, respectively.

Effect of chloroquine and MDL 72527 on procyanidin-induced cell death. The ability of PCy to induce apoptosis has previously been demonstrated (4, 7). In the present work, combined treatments with MDL 72527 and CQ were studied at concentrations that caused only marginal cell death. The results of these experiments are shown in Figure 4. Fifty µg/ml PCy produced a significant number of hypodiploid cells, as determined by flow cytometry of propidium iodide stained SW 620 cells. At 50 µM MDL 72527 and 10 µM CQ, the proportion of hypodiploid (apoptotic) cells increased only marginally above control values. However, in combination with PCy, the proportion of apoptotic cells increased significantly, indicating a synergizing effect of these drugs on PCy cytotoxicity. These treatments only had a minor effect on the cell cycle phase distribution of the diploid cells (not shown).

Lysosomal membrane destabilization by MDL 7252, chloroquine and procyanidins. In order to test the idea that the enhancement of PCy toxicity by MDL 72527 and CQ is due to lysosomal destabilization, a method devised by Yuan *et al.* (12) was employed, which relies on the increase in green fluorescence of acridine orange-stained cells, following their exposure to compounds which increase the permeability of the lysosomal membrane. For methodical reasons, long-term incubations incorporating medium changes were not feasible using this method, hence the concentrations of the

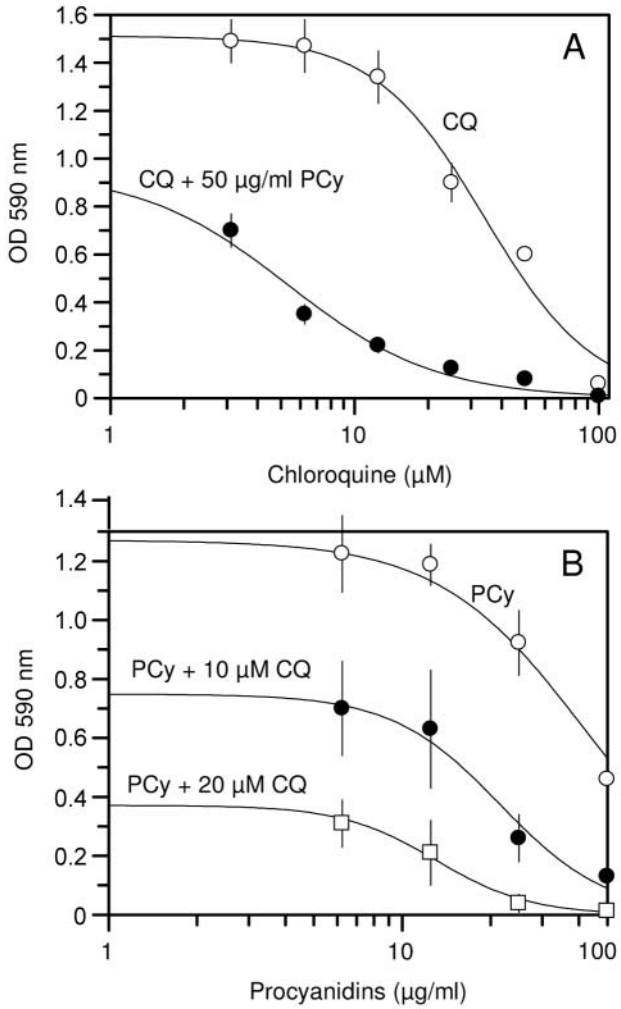

Figure 3. *Effect of chloroquine (CQ) alone and in combination with procyanidins (PCy) on the growth of SW 620 cells. A: Dose – effect relationship of CQ and of CQ + 50 µg/ml PCy after 4 days exposure. B. Dose – effect relationship of PCy and of PCy + 10 µM and 20 µM CQ after 96 h exposure. y-axis: absorbance at 590 nm after staining with sulforhodamine B. Error bars indicate±S.D. (n=8).*

lysosomotropic compounds had to be increased above those employed in the experiments discussed above. However, under such experimental conditions, the concentrations of the lysosomotropic compounds were sufficiently low to produce only a small effect on lysosomal permeability as shown in Figure 5 providing incubation time was limited to 24 h. PCy alone at 50 µg/ml produced a considerable increase in green fluorescence of acridine orange-stained cells. When combined with 300 µM MDL 72527 or 25 M CQ, green fluorescence increased 2- and 3-fold, respectively, that obtained with PCy, indicating the enhanced release of cathepsins due to increased permeability of the lysosomal membrane.

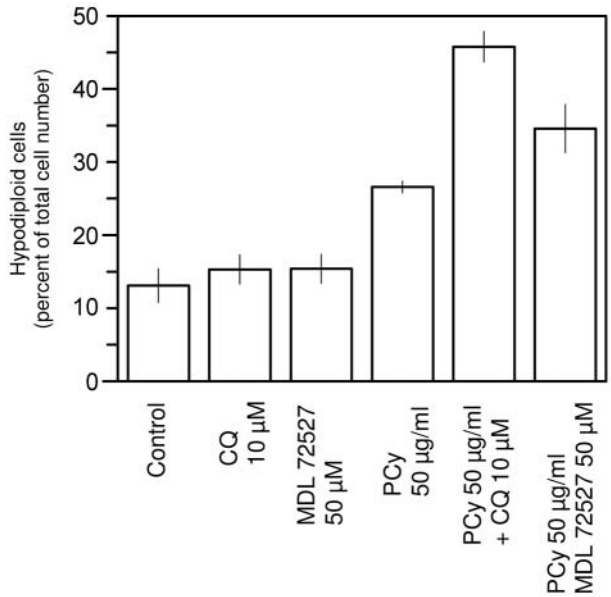

Figure 4. *Formation of hypodiploid cells due to exposure of SW 620 cells to procyanidins (PCy), MDL 72527 and chloroquine (CQ). Cells were exposed for 96 h to the drugs at the concentrations indicated in the graph. After harvesting by trypsinization and staining with propidium iodide, the proportion of hypodiploid cells was determined according to Nicoletti et al. (11). Error bars indicate±S.D. (n=2).*

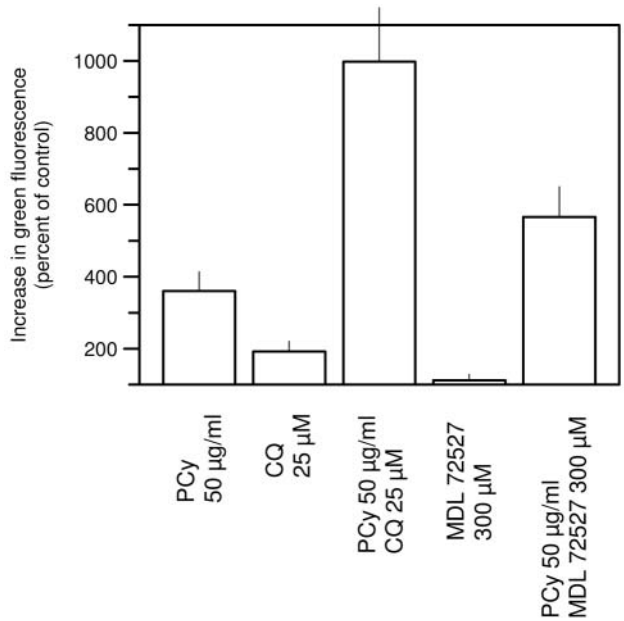

Figure 5. *Determination of lysosomal destabilization in SW 620 cells after exposure to procyanidins (PCy), MDL 72527 and chloroquine (CQ). After staining with acridine orange, cells were exposed for 24 h to the drugs at the concentrations indicated in the graph and lysosomal integrity was determined according to Yuan et al. (12) Error bars indicate±S.D. (n=2).*

Discussion

The abundance from natural sources and low toxicity suggest PCy as first rate chemopreventive agents, particularly for cancer prevention in the gastrointestinal tract. As a result of their size (FW of the tetramer=1150.99), most polymeric procyanidins do not traverse cell membranes (19) but bind to the surface of the gut mucosa cells and initiate death signaling pathways (5).

Our previous work on the cytotoxic properties of MDL 72527 (20, 21) led us to investigate its effect on cell viability in combination with potential anticancer treatments (15, 16), and with PCy. As a result of this work, the lysosomotropic properties of MDL 72527 emerged as a major cause of its sensitizing effect. A strong argument in this regard is the formation of cytoplasmic vacuoles by this compound (13, 20). Additional arguments in favor of this suggestion came from the present work. The fact that a well-known lysosomotropic compound (CQ) structurally different from MDL 72527, increases the apoptotic effect of PCy much like MDL 72527, though at a considerably lower concentration, argues in favour of the impairment of lysosome function by MDL 72527. The increase in green fluorescence of acridine orange-stained cells due to exposure to these drugs in combination with PCy is additional evidence for this assumption. Our observation on the enhancement of the cytotoxicity of PCy by lysosomotropic compounds has an analogy in the enhancement by CQ of the toxicity of vincristine and related drugs in multidrug resistant leukemia cells (22).

Based on our observations, we propose that lysosomal membrane permeabilization is a major cause for the observed increase in PCy toxicity by non-toxic concentrations of PME, CQ and MDL 72527. However, at present other toxic mechanisms cannot be excluded. Particularly CQ is known to have multiple effects on metabolism and signalling pathways. As an example CQ interferes with the mitogen-activated protein kinase (MAPK) signalling pathway (23), much like PCy (5).

It is known that exogenous (and endogenous) stress may lead to lysosomal membrane permeabilization, which may then mediate apoptosis-like cell death (24, 25). PCy is presumably a stressor due to its ability to down-regulate protein kinase C and affect MAPK. However, the direct impairment of lysosomal membrane stability by PCy, following intracellular accumulation by endocytosis is, also possible, but has not yet been studied.

CQ is a low cost drug with few side effects (18) that has proved to be useful in malaria prophylaxis, even in the treatment of children (26). In addition it has other

advantageous properties, among which the improvement of glioblastoma therapy should be mentioned (27). Our observations suggest CQ, in combination with procyanidins, as suitable for use in colon cancer chemoprevention. Other promising combinations of PCy with lysosomotropic compounds may be found in the future.

References

1 Guyot S, Le Bourvellec N, Marnet N and Drilleau JF: Procyanidins are the most abundant polyphenols in dessert apples at maturity. Lebensm Wiss Technol *35*: 289-291, 2002.

2 Yang CS, Landau JM, Huang MT and Newmark HL: Inhibition of carcinogenesis by dietary polyphenolic compounds. Annu Rev Nutr *21*: 381-406, 2001.

3 Shoji T, Masumoto S, Moriichi N, Kobori M, Kanda T, Shinmoto H and Tsushida T: Procyanidin trimers to pentamers fractionated from apple inhibit melanogenesis in B16 mouse melanoma cells. J Agric Food Chem *53*: 6105-6111, 2005.

4 Hibasami H, Shohji T, Shibuya I, Higo K and Kanda T: Induction of apoptosis by three types of procyanidins isolated from apple (Rosaceae, *Malus pumila*) in human stomach cancer KATO III cells. Int J Mol Med *13*: 785-799, 2004.

5 Gossé F, Guyot S, Roussi S, Lobstein A, Fischer B, Seiler N and Raul F: Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis. Carcinogenesis *26*: 1291-1295, 2005.

6 Barth SW, Fähndrich C, Bub A, Dietrich H, Watzl B, Will F, Briviba K and Rechkemmer G: Cloudy apple juice decreases DNA damage, hyperproliferation and aberrant crypt foci development in the distal colon of DMH-initiated rats. Carcinogenesis *26*: 1414-1421, 2005.

7 Gossé F, Roussi S, Guyot S, Schoenfelder A, Mann A, Bergerat JP, Seiler N and Raul F: Potentiation of apple procyanidin-triggered apoptosis by the polyamine oxidase inactivator MDL 72527 in human cancer-derived metastatic cells. Int J Oncol 2006, in press.

8 Bey P, Bolkenius FN, Seiler N and Casara F: N-2,3-Butadienyl-1,4-butanediamine derivatives. Potent irreversible inactivators of mammalian polyamine oxidase. J Med Chem *28*: 1-2, 1985.

9 Guyot S, Marnet N and Drilleau JF: Direct thiolysis of crude apple materials for high-performance liquid chromatography characterization and quantification of polyphenols in cider apple tissues and juices. *In*: Parker L (ed.). Methods in Enzymology - Flavonoids and other Polyphenols, Vol. 335, Academic Press, New York, pp. 57-70, 2001.

10 Skehan P, Storeng R, Scudero D, Monks A, McMahon J, Vistca D, Warran JT, Bokesch H, Kennedy S and Boyd MR: New colorimetric cytotoxicity assay for anticancer drug screening. J Natl Cancer Inst *83*: 1107-1112, 1990.

11 Nicoletti I, Migliorati G, Pagliacci MC, Grignani F and Riccardi C: A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunnol Methods *139*: 271-279, 1991.

12 Yuan XM, Dalen H, Lotem J, Kama R, Sachs L and Brunk LT: Lysosomal destabilization in p53-induced apoptosis. Proc Natl Acad Sci USA *99*: 6286-6291, 2002.

13 Dai H, Kramer DL, Yanag C, Murti KG, Porter CW and Cleveland H: The polyamine oxidase inhibitor MDL 72527 selectively induces apoptosis of transformed hematopoietic cells through lysosomotropic effects. Cancer Res *59*: 4944-4954, 1999.

14 Seiler N, Duranton B and Raul F: The polyamine oxidase inactivator MDL 72527. Prog Drug Res *59*: 1-40, 2002.

15 Agostinelli E, Dalla Vedova L, Belli F, Condello M, Arancia G and Seiler N: Sensitization of human colon adenocarcinoma cells (LoVo) to reactive oxygen species by a lysosomotropic compound. Int J Oncol 2006, in press.

16 Agostinelli E, Belli F, Molinari A, Condello M, Palmigiani P, Marra M, Seiler N and Arancia G: Toxicity of enzymatic oxidation products of spermine to human melanoma cells (M14): sensitization by heat and MDL 72527. Biochim Biophys Acta 2006, in press.

17 Rosenfeld CS: Antileukemic effect of phenylalanine methylester (PME): a lysosomotropic peptide methylester. Stem Cells *12*: 198-204, 1994.

18 Ginsburg H: Should chloroquine be laid to rest? Acta Trop *96*: 16-23, 2005.

19 Manach C, Scalbert A, Morand C, Rémésy C and Jimenez L: Polyphenols: food sources and bioavailability. Am J Clin Nutr *79*: 727-747, 2004.

20 Duranton B, Holl H, Schneider Y, Carnesecchi C, Gossé F, Raul F and Seiler N: Cytotoxic effects of the polyamine oxidase inactivator MDL 72527 to two human colon carcinoma cell lines SW 480 and SW 620. Cell Biol Toxicol *18*: 381-396, 2002.

21 Seiler N, Renault J, Gossé F, Roussi S and Raul F: Cytotoxicity of the polyamine oxidase inactivator MDL 72527 to cancer cells: Comparison with a saturated analogue. Int J Oncol *27*: 1669-1676, 2005.

22 Zamora JM and Beck WT: Chloroquine enhancement of anticancer drug cytotoxicity in multiple drug resistant human leukemic cells. Biochem Pharmacol *35*: 4303-4310, 1986.

23 Weber SM, Chen JM and Levitz SM: Inhibition of mitogen-activated protein kinase signalling by chloroquine. J Immunol *168*: 5303-5309, 2002.

24 Guicciardi ME, Leist M and Gores GJ: Lysosomes in cell death. Oncogene *23*: 2881-2890, 2004.

25 Kroemer G and Jäättelä M: Lysosomes and autophagy in cell death control. Nature Reviews Cancer *5*: 886-897, 2006.

26 Muller O, van Hensbroek MB, Jaffar S, Drakeley C, Okorie C, Joof D, Pinder M and Greenwood B: A randomized trial of chloroquine, amodiaquine and pyrimethamine-sulphadoxine in Gambian children with uncomplicated malaria, Trop Med Int Health *1*: 124-132, 1996.

27 Briceno K, Reyes S and Sotelo J: Therapy of glioblastoma multiforme improved by the antimutagenic chloroquine. Neurosurg Focus *14*: e3, 2003.

Received April 28, 2006
Accepted June 21, 2006