DOI 10.1002/mnfr.200500228 Mol. Nutr. Food Res. 2006, *50*, 390 – 395

Biological activities of malvidin, a red wine anthocyanidin

Jessica Fritz[1], Melanie Kern[1], Gudrun Pahlke[1], Sandra Vatter[2] and Doris Marko[1]

[1]Section of Food Toxicology, Institute of Applied Biosciences, University of Karlsruhe (TH), Karlsruhe, Germany
[2]Division of Food Chemistry and Environmental Toxicology, Department of Chemistry, University of Kaiserslautern, Kaiserslautern, Germany

Malvidin (mv) has been identified as a potential inhibitor of 3′,5′-cyclic adenosine monophosphate (cAMP) phosphodiesterases (PDE). This study was to investigate if, as a possible consequence of intracellular PDE inhibition, the activity of the mitogen-activated protein kinase (MAPK) cascade is affected by mv treatment. At a concentration of 5 μM of mv a significant decrease of phosphorylated ERK1 and ERK2 (ERK, extracellular regulated kinase) in HT29 cells was observed. However, an increase in substance concentration led to a substantial recurrence of the phosphorylated enzymes. Cell cycle analysis underlined that indeed G_1-relevant targets are only marginally affected by mv. The recurrence of phosphorylated ERK1/2 and the lack of effectiveness on the G_1-passage up to 100 μM indicated that the inhibition of cAMP-specific PDEs is of minor relevance for the growth-inhibitory properties of mv in HT29 cells. In contrast, the release of cells, synchronised in the G_2/M-phase of the cell cycle by nocodazole treatment, was effectively blocked in the presence of 1 μM mv. These results suggest that mv interferes with cellular targets relevant for G_2/M-progression which have not been identified so far.

Keywords: Anthocyanin / Cell cycle / 3′,5′-Cyclic nucleotide phosphodiesterase / MAPK cascade / Red wine

Received: October 30, 2005; revised: November 29, 2005; accepted: November 30, 2005

1 Introduction

Anthocyanins are naturally occurring coloured plant constituents, which are found in many fruits and vegetables in our daily diet. Depending on nutrition customs, the intake of anthocyanins in Germany was estimated in 2002 to be 2.7 mg/day, with a high interindividual variability of 0–76 mg depending on individual food patterns [1]. Anthocyanins have been associated with potentially beneficial effects on various diseases, such as diabetic retinopathy [2], and have been suggested to possess anti-inflammatory as well as chemopreventive properties [3]. Based on these proposed health benefits, anthocyanin preparations have gained increasing popularity on the fast-expanding market of food supplements within the last years. However, solid epidemiological data on these proposed health effects are not abundant. Some reports hint at a potential anticarcinogenic activity, *e.g.* the consumption of coloured fruits and vegetables has also been associated with a reduced risk to develop breast cancer [4] or colorectal polyps [5]. Furthermore, anthocyanins have been suggested to contribute to the apparent decreased risk of cardiovascular diseases despite a high-fat diet in certain French populations, the so-called 'French paradox' [6]. In several animal studies chemopreventive properties of anthocyanins have been postulated [7–10], raising the question towards the underlying mechanism of action. With respect to the inhibition of neoplastic cell survival, anthocyanidins have been shown to possess greater potency than their glycosylated counterparts [11]. Among anthocyanidins, delphinidin (del) and malvidin (mv) were found to exhibit the highest growth-inhibitory potential [12–14], but appear to differ substantially with respect to their cellular activity profile. Del, as a potent inhibitor of the epidermal growth factor receptor (EGFR), has been shown to affect subsequent cellular signalling cascades [11, 14] and also interferes with human topoisomerases [15]. In contrast, these cellular targets remain unaffected by mv, which was found to represent a potent inhibitor of 3′,5′-cyclic adenosine monophosphate

Correspondence: Professor Doris Marko, Division of Food Toxicology, Institute of Applied Biosciences, University of Karlsruhe (TH), Fritz-Haber-Weg 2, 76131 Karlsruhe, Germany
E-mail: doris.marko@lmc.uni-karlsruhe.de
Fax: +49-721-608-7254

Abbreviations: cAMP, 3′,5′-cyclic adenosine monophosphate; **del**, delphinidin; **EGFR**, epidermal growth factor receptor; **ERK**, extracellular regulated kinase; **FCS**, fetal calf serum; **mv**, malvidin; **MAPK**, mitogen-activated protein kinase; **PDE**, 3′,5′-cyclic nucleotide phosphodiesterase; **PKA**, protein kinase A

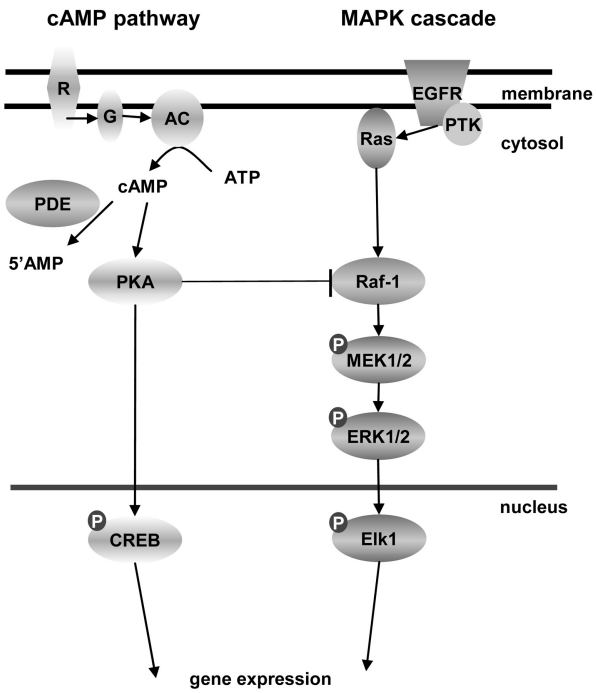

Figure 1. Simplified scheme of the crosstalk between the MAPK cascade and the cAMP pathway. EGFR, epidermal growth factor receptor; PTK, protein tyrosine kinase; Ras, GTP-binding protein; Raf-1, serine/threonine kinase; MEK, MAPK/ERK kinase; ERK, extracellular regulated kinase; Elk-1, Ets-like kinase (transcription factor); R, receptor; G, GTP-binding protein; AC adenylate cyclase; cAMP, 3′,5′-cyclic adenosine monophosphate; PKA, protein kinase A; PDE, 3′,5′-cyclic nucleotide phosphodiesterase; CREB. cAMP-responsive element binding protein.

(cAMP)-specific phosphodiesterases (PDE). PDEs are key enzymes in the maintenance of cAMP homeostasis. Many tumour cells have been shown to overexpress cAMP-specific PDEs [16]. Effective PDE inhibition increases the intracellular cAMP level, activating the subsequent protein kinase A (PKA). The serine/threonine kinase Raf-1 is one of the substrates of PKA, representing a key interlink between the cAMP- and the mitogen-activated protein kinase (MAPK) pathway. Raf-1 is phosphorylated by PKA in a deactivating fashion, suppressing the activation of the subsequent MAPK cascade (Fig. 1) [11, 14]. The objective of this study was to investigate the relevance of PDE inhibition for the growth-inhibitory properties of mv.

2 Materials and methods

2.1 Chemicals

Mv and del (Fig. 2) were purchased from Extrasynthèse (Genay, France). For all assays the compound solutions were freshly prepared in DMSO directly before the beginning of the experiment, without using stored stock solutions.

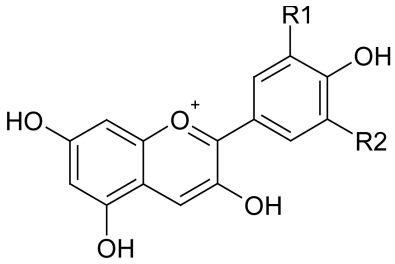

Figure 2. Chemical structure of mv (R_1, $R_2 = OCH_3$) and del (R_1, $R_2 = OH$).

2.2 Cell culture

The human colon carcinoma cell line HT29 was cultivated in DMEM (with 4500 mg/L glucose, without sodium pyruvate). Cell culture medium was supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. DMEM and the supplements were obtained from Invitrogen™ Life Technologies (Karlsruhe, Germany).

2.3 Western blot analysis

HT29 cells (10^6) were seeded *per* Petri dish in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. After 48 h, FCS was reduced from 10 to 1% for 24 h. Mv or tyrphostin AG1478 were dissolved in DMSO and adopted in serum-free medium to a final DMSO concentration of 1%. Cells were incubated with mv or tyrphostin AG1478 for 30 min, prior to the addition of EGF (Calbiochem, VWR, Germany), resulting in a final EGF concentration of 1 ng/mL. After 15 min of stimulation by EGF, the medium was removed and the cells were washed with ice-cold PBS. Cells were abraded at 4°C with 0.2 mL lysis buffer (25 mM Tris/HCl, pH 7.4, 3 mM EDTA, 3 mM EGTA, 50 mM NaF, 0.27 M sucrose, 10 mM Na-β-glycerophosphate, 5 mM Na-pyrophosphate, 0.5% v/v Triton-X 100, 5 mM Na_3VO_4). Protease inhibitor cocktail (2% (v/v); Roche Diagnostics, Mannheim, Germany) and β-mercaptoethanol (0.1% v/v) were freshly added to 2 mL lysis buffer. Thereafter the lysate was homogenized thoroughly and subsequently centrifuged for 10 min (20 000 × *g*, 4°C). The supernatant was subjected to SDS-PAGE (12% polyacrylamide gel). The proteins were transferred onto an nitrocellulose membrane. Western blot was performed using a rabbit polyclonal antibody against human p42/p44 (Cell Signaling Technology, USA) and an antirabbit IgG peroxidase conjugated secondary antibody (New England Biolabs, Frankfurt, Germany). Alpha tubulin was used as loading control. The respective

chemoluminescent signals (LumiGLO, New England Biolabs GmbH, Frankfurt, Germany) were analysed using the Lumi-Imager™ with the LumiAnalyst 3.0 software for quantification. Arbitrary light units were plotted as test over control T/C (%).

2.4 Flow cytometry

HT29 cells (5×10^5) were seeded into tissue culture plates (∅ 6 cm) in serum containing medium and allowed to grow for 48 h. For synchronisation, the cells were kept either for 48 h serum-deprivated or for 16 h in nocodazole-containing medium (0.5 µg/mL) prior to drug treatment. Thereafter, incubation with mv, delphinidin or the solvent control (1% DMSO) was performed for 36 h in serum-containing medium. Sample preparation and flow cytometry were carried out as previously described [16].

3 Results

3.1 Modulation of ERK1/2 (ERK, extracellular regulated kinase) phosphorylation

Mv was identified as a potent inhibitor of cAMP-hydrolysing PDE (IC_{50} of 23 ± 5 µM) [14]. Effective PDE inhibition is expected to result in the activation of PKA, enabling a crosstalk to the MAPK cascade by a deactivating phosphorylation of Raf-1. We investigated whether mv treatment affects the activity of the subsequent MAPK cascade in HT29 cells. The activity of this signalling pathway is reflected by the phosphorylation status of the MAP kinases ERK1/2 (p42/p44). Phosphorylated ERK1 and ERK2 in HT29 cells were determined by Western blot analysis. After 45 min incubation mv was found to significantly diminish phosphorylated ERK1 and ERK2 already at a concentration of 5 µM (Fig. 3). However, an increase in substance concentration failed to enhance this effect, but led to the recurrence of the phosphorylated enzymes (Fig. 3).

3.2 Effects on cell cycle distribution

The analysis of perturbations of the cell cycle passage provides a useful tool to achieve a further insight into the impact of different targets on the fate of the cell. We therefore investigated the influence of mv in comparison to del on cell cycle distribution in HT29 cells. To pronounce the effect of the compounds on the respective cell cycle phases, the cells were either synchronised in the G_1-phase by serum deprivation or in the G_2/M-phase by nocodazole treatment. After release from the respective block, the cells were treated with mv or del for 36 h. Treatment with del suppressed the release of HT29 cells from G_1-arrest at concentrations

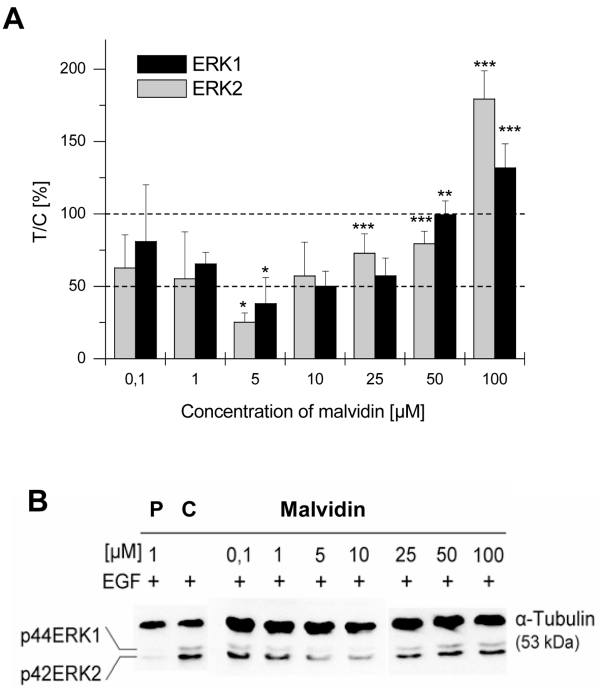

Figure 3. Western blot analysis of phosphorylated p42/p44 (ERK1/ERK2) in HT29 cells stimulated by EGF after 45 min treatment with mv. P, positive control with tyrphostin AG1478; C, solvent control stimulated by EGF. α-tubulin was included in the test as a loading control. (A) Data presented are the mean ± SD of three independent experiments with similar outcome. Significances indicated refer to the comparison of the respective concentration with the solvent control (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). (B) Western blot of a representative experiment.

of 10 and 50 µM (Fig. 4A). At 100 µM of del the G_1-arresting effect was lost, while concomitantly the proportion of cells in G_2/M was enhanced. In contrast, the release from the G_1-block remained mainly unaffected by mv treatment up to 100 µM (Fig. 4B). An inverted pattern of activity was observed for effects of the compounds on the release from G_2/M-arrest. Mv was found to effectively sustain cells synchronized by nocodazole treatment in the G_2/M-phase of the cell cycle at low micromolar concentrations (≥1 µM) (Fig. 4D), whereas del treatment only slightly enhanced the proportion of cells in G_2/M (Fig. 4C).

4 Discussion

Mv, a characteristic red wine anthocyanidin, is known to inhibit the growth of human tumour cells *in vitro* [11, 14]. Investigating the potential underlying mechanism of action, we focused on central signalling cascades crucial for the regulation of cell growth. The ERK/MAPK pathway (Fig. 1) and the cAMP-signalling pathway are among the central signalling cascades regulating cell proliferation.

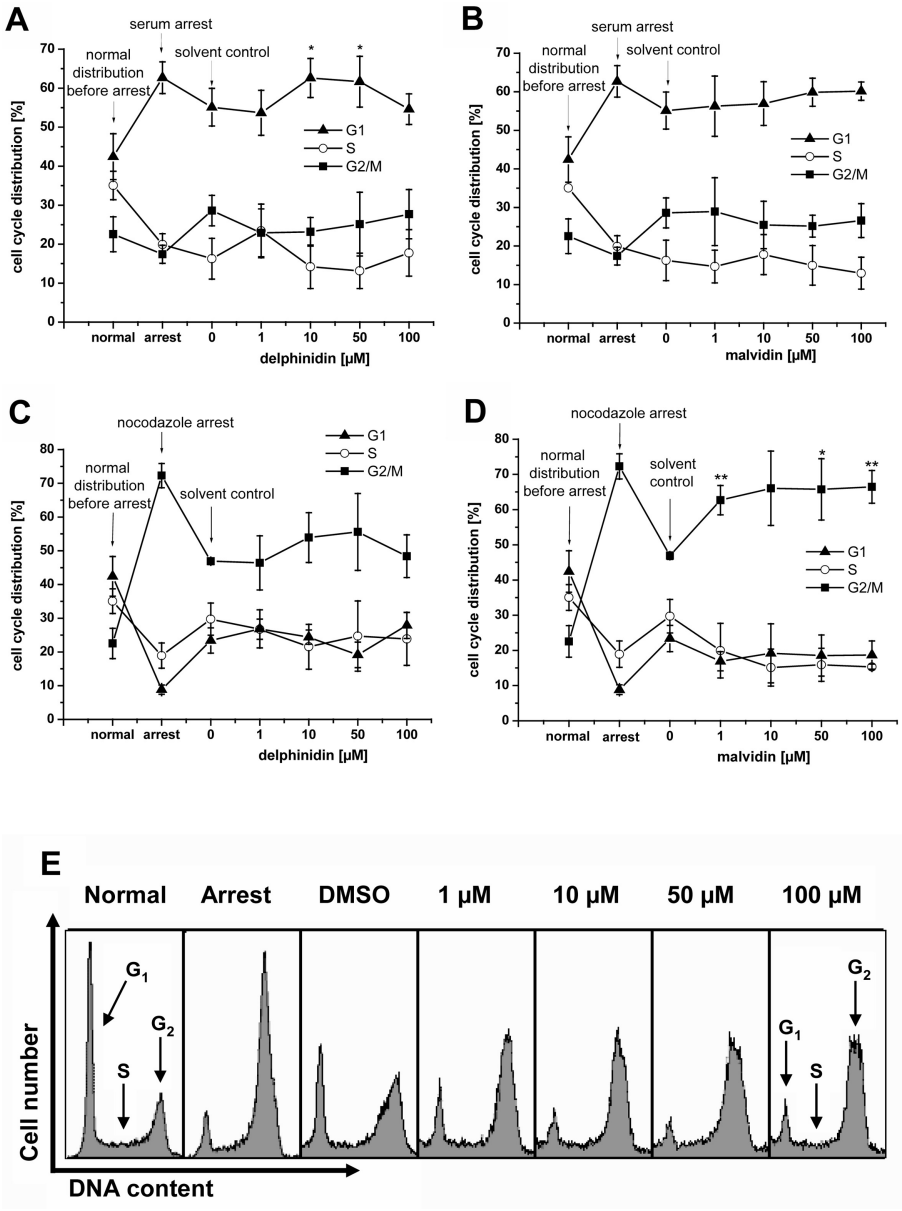

Figure 4. Flow cytometrical analysis of the effect of del (A and C) and mv (B and D) on the cell cycle distribution of HT29 cells synchronised by serum-deprivation (A and B) or nocodazole treatment (C and D). After synchronisation, the cells were incubated with the respective anthocyanidin or the solvent control (DMSO) for 36 h in the presence of serum. (A)–(D) Quantitative analysis of cell cycle distribution. Flow cytometry was performed with DAPI for DNA staining. Data presented are the mean ± SD of at least three independent experiments, each performed in duplicate. (E) Representative histograms from cell cycle analysis of HT29 cells synchronised by nocodazole treatment and subsequently treated with mv for 36 h in the presence of serum. Normal: normal distribution of HT29 cells prior to nocodazole treatment; arrest: distribution immediately after serum deprivation or nocodazole treatment; DMSO: distribution of synchronised cells after incubation for 36 h with the solvent control (1% DMSO).

Key elements of the MAPK cascade are ERK1 and ERK2. Effects on the phosphorylation state of ERK1/2 reflects the targeting of upstream elements belonging to the MAPK cascade itself or effects mediated by crosstalks with other pathways, *e.g.* the deactivating phosphorylation of Raf-1 as a result of PKA activation [17–20]. We previously showed that mv only marginally affects the tyrosine kinase activity of the EGFR [11, 14]. In contrast to the anthocyanidins bearing vicinal hydroxy groups at the B-ring, mv was found to effectively inhibit isolated cAMP-specific PDE, suggesting an activation of PKA and probably subsequent Raf-1 mediated inhibition of ERK1/2 phosphorylation as a potential mechanism of action leading to the inhibition of cell growth. The effect of mv on ERK1/2 phosphorylation was

found to represent an U-shaped curve with an apparent minimum at about 5 μM. At mv concentrations ≥50 μM a significant recurrence of phosphorylated ERK1/2 was observed (Fig. 3). The inhibition of the MAPK cascade at low mv concentrations might indeed represent an antiproliferative signal. The enhanced phosphorylation status of ERK1/2 at concentrations ≥50 μM *per se* has to be considered as a proliferative stimulus. However, from the results on the growth-inhibitory properties of mv in the sulforhodamine B assay [11, 14], it can be concluded that this potentially growth-stimulating signal apparently is not associated with enhanced cell growth in the respective concentration range. These results suggest that other antiproliferative factors are affected by mv, probably compensating the potentially proliferative stimulation of ERK1/2 activity. With respect to the targeting of cAMP-specific phosphodiesterases by mv, the results show that either the PDE-inhibitory effect is limited to the isolated protein preparation without relevance within intact cells, or the effect on cellular PDE is not mediated *via* crosstalk on the MAPK cascade, thus possibly affecting cell growth by alternative downstream effectors. The interference with signalling cascades crucial for cell proliferation such as the cAMP pathway or the MAPK cascade is known to block the cells mainly in the G_1-phase of the cell cycle. Accordingly, treatment of cells synchronised in G_1 with the EGFR-inhibitory del significantly sustained cells in the G_1-phase of the cell cycle (Fig. 4A). However, despite the effective inhibition of isolated cAMP-specific PDE, no significant effect of mv on cell cycle progression of HT29 cells synchronised in the G_1-phase (serum-deprivation) was observed (Fig. 4B). The recurrence of phosphorylated ERK1/2 together with the lack of effectiveness on the G_1-passage up to 100 μM, led to conclude that the inhibition of cAMP-specific PDEs is of minor relevance for the growth-inhibitory properties of mv in HT29 cells.

In contrast to the results on the G_1-passage, mv was found to effectively arrest synchronized HT29 in the G_2/M-phase of the cell cycle (Fig. 4D), whereas del mediated only marginal effects (Fig. 4C). We showed recently that the inhibition of topoisomerases I and II, which represents one mechanism to be expected to interfere with G_2/M-passage, is within the class of anthocyanidins limited to analogues bearing vicinal hydroxy groups at the B-ring, *e.g.* del or cyanidin [15]. These results suggest that mv interferes with cellular targets relevant for G_2/M-progression other than topoisomerases, which have not been identified so far.

We previously reported the affinity of anthocyanidins to dsDNA [15]. At concentrations ≥50 μM mv was found to induce DNA strand breaks [15]. In cells with an intact DNA integrity checkpoint, such as HT29 [21], this loss of DNA integrity might result in the accumulation of the cells in the G_2-phase of the cell cycle. However, the effects on DNA integrity were determined after 1 h of incubation in serum-free medium, whereas effects on cell cycle distribution were measured after 36 h of incubation in the presence of serum. Therefore, the effective concentrations are difficult to compare. However, del, exhibiting a higher DNA-strand breaking potential than mv and, additionally, representing a potent topoisomerase inhibitor, showed no effect on the G_2/M-passage at a concentration of 1 μM (Fig. 4C), whereas the effects of mv were already highly significant (Fig. 4D). Based on this discrepancy, we propose that other, yet unknown G_2/M-relevant targets are affected by mv treatment, at least contributing to the substantial G_2/M-arrest.

In summary, our data indicate that the inhibition of cAMP-specific PDEs is of minor relevance for the growth-inhibitory effects of mv. The effective arrest of cells in the G_2/M-phase of the cell cycle leads to the assumption that yet unknown cellular targets, relevant for the respective cell cycle passage, are affected. Further studies on the cellular mechanisms of mv are warranted to elucidate the cellular targets responsible for the growth-inhibitory properties of the compound.

The study was performed as a part of the FlavoNet, funded by the Deutsche Forschungsgemeinschaft (MA 1659/4–1/2).

5 References

[1] Watzl, B., Briviba, K., Rechkemmer, G., *Ernährungs-Umschau.* 2002, *49*, 148 – 150.

[2] Scharrer, A., Ober, M., *Klein. Monatsbl. Augenheilkd.* 1981, *178*, 386 – 389.

[3] Seeram, N. P., Zhang, Y., Nair, M. G., *Nutr. Cancer* 2003, *46*, 101 – 106.

[4] Adlercreutz, H., *Baillieres. Clin. Endocrinol. Metab.* 1998, *12*, 605 – 623.

[5] Almendingen, K., Hofstad, B., Vatn, M. H., *Nutr. Cancer* 2004, *49*, 131 – 138.

[6] Renaud, S., de Lorgeril, M., *Lancet* 1992, *339*, 1523 – 1526.

[7] Kang, S. Y., Seeram, N. P., Nair, M. G., Bourquin, L. D., *Cancer Lett.* 2003, *194*, 13 – 19.

[8] Singletary, K. W., Stansbury, M. J., Giusti, M., Van Breemen, R. B. *et al.*, *J. Agric. Food Chem.* 2003, *51*, 7280 – 7286.

[9] Hagiwara, A., Yoshino, H., Ichihara, T., Kawabe, M. *et al.*, *J. Toxicol. Sci.* 2002, *27*, 57 – 68.

[10] Harris, G. K., Gupta, A., Nines, R. G., Kresty, L. A. *et al.*, *Nutr. Cancer* 2001, *40*, 125 – 133.

[11] Meiers, S., Kemény, M., Weyand, U., Gastpar, R. *et al.*, *J. Agric. Food Chem.* 2001, *49*, 958 – 962.

[12] Hou, D. X., Ose, T., Lin, S., Harazoro, K. *et al.*, *Int. J. Oncol.* 2003, *23*, 705 – 712.

[13] Lazze, M. C., Savio, M., Pizzala, R., Cazzalini, O. *et al.*, *Carcinogenesis* 2004, *25*, 1427 – 1433.

[14] Marko, D., Puppel, N., Tjaden, Z., Jakobs, S., Pahlke, G., *Mol. Nutr. Food Res.* 2004, *48*, 318 – 325.

[15] Habermeyer, M., Fritz, J., Barthelmes, H. U., Christensen, M. O. *et al.*, *Chem. Res. Tox.* 2005, *18*, 1395 – 1404.

[16] Marko, D., Romanakis, K., Zankl, H., Fürstenberger, G. *et al.*, *Cell Biochem. Biophys.* 1998, *28*, 75 – 101.

[17] Lewis, T. S., Shapiro, P. S., Ahn, N. G., *Adv. Cancer Res*. 1998, *74*, 49 – 139.

[18] Marais, R., Marshall, C. J., *Cancer Surv.* 1996, *27*, 101 – 125.

[19] Dhillon, A. S., Pollock, C., Steen, H., Shaw, P. E. *et al.*, *Mol. Cell. Biol.* 2002, *22*, 3237 – 3246.

[20] Cook, S. J., McCormick, F., *Science* 1993, *262*, 1069 – 1072.

[21] Parsels, L. A., Parsels, J. D., Chung-Ho Tai, D., Coughlin, D. J., Maybaum, J., *Cancer Res.* 2004, *64*, 6588 – 6594.

