DOI 10.1002/mnfr.200600186 Mol. Nutr. Food Res. 2007, *51*, 192–200

Research Article

The epidermal growth factor receptor and human topoisomerases represent potential cellular targets of oligomeric procyanidins

Diana Fridrich[1], Melanie Kern[1], Jessica Fritz[1], Gudrun Pahlke[1], Nils Köhler[2], Peter Winterhalter[2] and Doris Marko[1]

[1] Institute of Applied Biosciences, Section of Food Toxicology, University of Karlsruhe (TH), Kaiserstrasse 12, Karlsruhe, Germany
[2] Institute of Food Chemistry, University of Braunschweig (TU), Schleinitzstraße 20, Braunschweig, Germany

Oligomeric procyanidins were found to inhibit the protein tyrosine kinase activity of the epidermal growth factor receptor (EGFR). The inhibitory potency was found to increase with the degree of oligomerisation (PA2 > PC1 >> PB1 = PB2 = PB3 = PB4 >> (–)-epicatechin). To address the question whether the interference with the activity of isolated EGFR preparations also plays a role within intact cells, effects on the phosphorylation status of the EGFR, as a measure of its activity, were determined in human colon carcinoma cells. Treatment of HT29 cells with the trimeric procyanidin PC1 resulted in a decrease of the EGFR autophosphorylation already at low micromolar concentrations. A respective procyanidin tetramer (PA2) failed to affect substantially the receptor phosphorylation status by up to 50 μM, indicating that the effectiveness of oligomeric procyanidins against EGFR activity within intact cells might be limited with increasing degree of polymerisation. Nevertheless, oligomeric procyanidins were identified as potential inhibitors of the EGFR, which might be of interest with respect to chemoprevention. However, PC1 and PA2 were also identified as potent inhibitors of the catalytic activity of human topoisomerase I and II, demanding further studies to elucidate whether the interference of procyanidins with topoisomerases might impair DNA integrity, thus limiting their usefulness in terms of chemoprevention.

Keywords: Autophosphorylation / Cinnamtannin / Colon carcinoma / ErbB1 / Receptor tyrosine kinase

Received: September 28, 2006; accepted: October 29, 2006

1 Introduction

Oligomeric procyanidins have been associated with a multitude of beneficial health effects including anti-inflammatory, antiatherosclerotic, antidiabetic and anticarcinogenic properties [1–6]. Even the promotion of hair growth has been repeatedly reported [7]. However, the underlying mechanisms of action have not been fully elucidated yet. We previously reported that a polyphenol-rich extract of a consumer-relevant apple juice blend potently inhibited the protein kinase activity of isolated epidermal growth factor receptor (EGFR) [8]. Activity-guided fractionation of the extract indicated that oligomeric procyanidins might contribute to the EGFR-inhibitory properties of the apple juice extract. From the apple constituents characterised so far, the procyanidin dimers B1 (PB1) and B2 (PB2) have already been identified as potential inhibitors of isolated EGFR preparations [8]. In the present study we addressed the question whether higher oligomeric procyanidins also affect EGFR activity and whether the interference with the receptor is of relevance within intact cells. Effective inhibition of the EGFR activity is expected to suppress the activation of the subsequent mitogen-activated protein kinase (MAPK) cascade, one of the key pathways in the regulation of cell growth. Thus, targeting of the EGFR is not only of interest in terms of chemotherapy, but might also be regarded as a promising mechanism of action with respect to chemoprevention. Several polyphenols of different

Correspondence: Professor Doris Marko, Institute of Applied Biosciences, Section of Food Toxicology, University of Karlsruhe (TH), Kaiserstrasse 12, 76131 Karlsruhe, Germany
E-mail: doris.marko@lmc.uni-karlsruhe.de
Fax: +49-721-608-7254

Abbreviations: EGFR, epidermal growth factor receptor; **HT29,** human colon carcinoma cell line; **PA2,** procyanidin tetramer cinnamtannin; **PB1-4,** procyanidin dimers; **PC1,** procyanidin trimer; **PTK,** protein tyrosine kinase

classes have already been described as EGFR inhibitors [9, 10, 11–13]. Evaluating the usefulness of such polyphenols in the prevention of carcinogenesis, the question has to be addressed whether enhanced intake of these polyphenols might also be associated with undesired health effects. Many polyphenols, potentially inhibiting EGFR activity, have concomitantly been reported to interfere with human topoisomerases [14–16]. Depending on the mode of interaction with the target enzyme, effective inhibition of topoisomerases might impair the maintenance of DNA integrity [14, 15]. Within the class of procyanidins PB2 has already been identified as a potent inhibitor of the catalytic activity of topoisomerase II [17]. Thus, in the present study we addressed the question whether, in addition to effects on the EGFR, higher oligomeric procyanidins also affect the activity of human topoisomerases.

2 Materials and methods

2.1 Chemicals

The EGFR specific inhibitor tyrphostin AG1478 was obtained from Sigma–Aldrich (Taufkirchen, Germany). For all assays, the compound solutions were freshly prepared in DMSO directly before starting the experiment. The grape seed extract (P100) that was used for the isolation of procyanidins was obtained from Breko (Bremen, Germany).

2.2 Cell culture

The human vulva carcinoma cell line A431 was cultured in minimum essential medium (MEM; Sigma) containing L-glutamine (4.5 g/L). The human colon carcinoma cell line (HT29) was cultivated in DMEM (DMEM with 4500 mg/L glucose, without sodium-pyruvate; Invitrogen™ Life Technologies, Karlsruhe, Germany) in a humidified incubator (37°C, 5% CO_2). Both cell culture media were supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin.

2.3 Tyrosine kinase assay

The EGFR was isolated from A431 cells and purified by affinity chromatography using wheat germ lectin agarose (Pharmacia Biotech, Uppsala, Sweden) according to [10]. The ELISA was carried out as described previously [8–10].

2.4 Western blot analysis

An aliquot of 1.0×10^6 HT29 cells were seeded *per* Petri dish and allowed to grow for 48 h. Thereafter, cells were serum-reduced (1% FCS) for 24 h and incubated with the respective compound for 45 min in serum free medium. The EGFR of HT29 cells was stimulated with EGF (100 ng/mL) within the last 15 min of incubation. Cells were abraded at 4°C in 0.2 mL RIPA buffer (50 mM Tris/HCl, pH 7.4, 250 mM NaCl 1 mM EDTA, 1 mM NaF, 1% v/v Igepal; 1 mM PMSF, 1 mM sodium orthovanadate and 2% v/v protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) were freshly added to RIPA buffer). Thereafter, the lysate was homogenised thoroughly and subsequently centrifuged for 10 min (20 000 *g*, 4°C). The supernatant was separated by SDS-PAGE (7% polyacrylamide gel) and the proteins were transferred onto a nitrocellulose membrane. Western blot was performed using mouse monoclonal antibodies against human EGFR and phospho-EGFR Tyr 1173 (175 kDa; Cell Signaling Technology, Beverly, MA, USA) and an antimouse IgG peroxidase conjugate (Santa Cruz, Heidelberg, Germany) as a secondary antibody. Alpha-tubulin was used as a loading control. The respective chemoluminescent signals (Lumi-GLO, Cell Signaling Technology) were analysed using the LAS 3000 system with the AIDA Image Analyzer 3.52 software for quantification (Raytest, Straubenhardt, Germany). Arbitrary Light Units were plotted as test over control (T/C) (%).

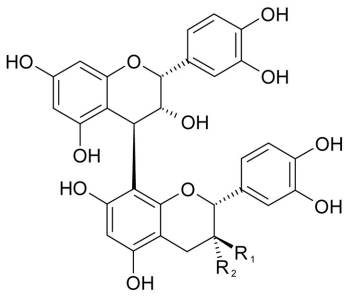
2.5 Preparation of nucleic extract

Human MCF-7 cells (3.0×10^7) were resuspended in 7.5 mL of lysis-buffer (0.3 M sucrose; 0.5 mM EGTA pH 8.0; 60 mM KCl, 15 mM NaCl; 15 mM HEPES pH 7.5; 150 μM spermine; 50 μM spermidine) and 0.5 mL of lysis buffer with Triton X-100 (13.5:1 v/v) was added. The cells were lysed on ice for 15 min. After centrifugation (150 *g*, 10 min), the supernatant was removed and the pellet was resuspended in extraction buffer (100 mM NaCl; 5 mM KH_2PO_4 pH 7.5; 1 mM PMSF, 1 μL/mL β-mercaptoethanol) to 3.0×10^7 nuclei *per* mL. 10% volume of 5 M NaCl was carefully added to precipitate the DNA on ice for 20 min. After centrifugation, the supernatant was collected, 1.5 × volume of glycerol was added and aliquots were stored at −20°C until use in relaxation assays.

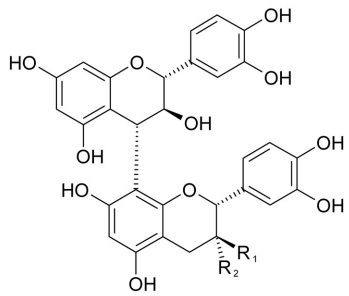
2.6 Topoisomerase activity

2.6.1 Topoisomerase I

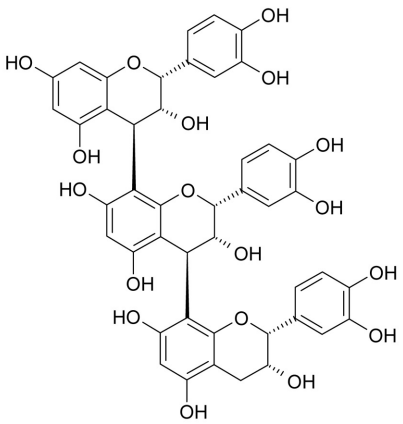
For the detection of catalytic topoisomerase I activity a plasmid relaxation assay was carried out. Plasmid DNA (250 ng pUC18) was incubated in a final volume of 30 μL (containing 0.3 μL nucleic extract; 10 mM Tris pH 7.9; 100 mM KCl; 10 mM $MgCl_2$; 0.5 mM DTT; 0.5 mM EDTA and 0.03 mg/mL BSA) for 30 min at 37°C. The reaction was stopped by the addition of 1/10 volume of 5% w/v SDS. The samples were incubated with 1 mg/mL proteinase K at 37°C for 30 min. Gel electrophoresis was performed at 4.5 V/cm in 1% w/v agarose gels with Tris acetate/EDTA buffer (40 mM Tris, 1 mM EDTA pH 8.5, 20 mM acetic acid). Subsequently, the gel was stained in 10 μg/mL ethidium bromide solution for 20 min. The fluorescence of



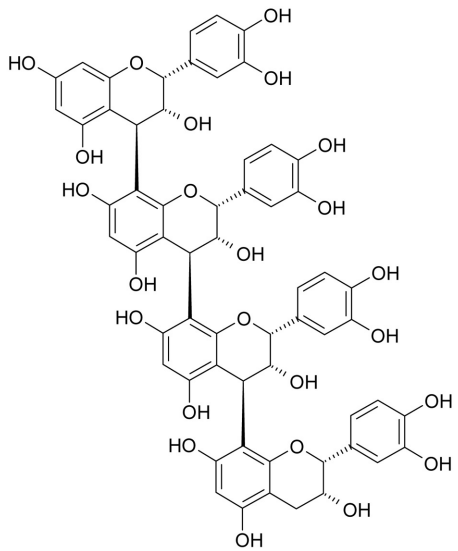
PB1: R_1 = OH, R_2 = H
PB2: R_1 = H, R_2 = OH



PB3: R_1 = OH, R_2 = H
PB4: R_1 = H, R_2 = OH



PC1: EC-4β→8-EC-4β→8-EC



PA2: Cinnamtannin A2
EC-4β→8-EC-4β→8-EC-4β→8-EC

Figure 1. Structures of di-, tri- and tetrameric procyanidins.

ethidium bromide was documented with the LAS 3000 system (Raytest, Straubenhardt, Germany).

2.6.2 Topoisomerase II

Effects on the catalytic activity of topoisomerase II were determined using a decatenation assay. Human recombinant topoisomerase IIβ was prepared as described previously [15]. Catenated kinetoplast DNA (200 ng, TopoGEN, Ohio) was used as a substrate. Kinetoplast DNA (kDNA) is an aggregate of interlocked DNA minicircles (mostly 2.5 kb), which can be released by topoisomerase II. The kinetoplast DNA was incubated in a final volume of 30 µL (containing 40 ng topoisomerase II; 50 mM Tris pH 7.9; 120 mM KCl, 10 mM $MgCl_2$; 1 mM ATP, 0.5 mM DTT, 0.5 mM EDTA and 0.03 mg/mL BSA) at 37°C for 60 min. The reaction was stopped by the addition of 1/10 volume of 1 mg/mL proteinase K in 10% w/v SDS and incubation at 37°C for further 30 min. Gel electrophoresis and detection were performed as described above.

3 Results

3.1 Isolation and structure elucidation

The procyanidins (Fig. 1) were isolated from grape seed extracts by the all-liquid chromatographic technique of countercurrent chromatography (CCC). A CCC-1000 apparatus (Pharma-Tech Research, Baltimore USA) was used to obtain strongly enriched fractions of dimeric procyanidins B1 (PB1), B2 (PB2), B3 (PB3), B4 (PB4), the trimeric pro-

cyanidin C1 (PC1) and procyanidin tetrameric cinnamtannin A2 (PA2), using the solvent system ethyl acetate/*n*-butanol/water (14:1:15 v/v/v). These fractions were further purified by preparative HPLC. All structure molecular weights were determined by LC-MS experiments using ESI in the negative ionisation mode [18]. Structure elucidation was carried out by NMR and acid catalysed degradation with phloroglucinol [19, 20]. The structures of the procyanidins B1–4 (PB1–PB4) were confirmed by 2-D NMR techniques (^{1}H-NMR, ^{13}C-NMR, HMBC, HMQC) and were compared with the literature [21–24]. Additionally, acid catalysed degradation of the compounds PB1–PB4 confirmed the absolute configurations of the flavan-3-ols within PB1-4 [19].

The structures of the trimer PC1 and the tetramer PA2 (Fig. 1) were confirmed by acid catalysed degradation. A full degradation produced flavan-3-ol-phloroglucinols and flavan-3-ols containing only 2,3-*cis* configurations. Therefore, PC1 and PA2 consist only of (–)-epicatechin units. According to [19, 25], a partial degradation confirmed the interflavanoid linkage. A fast cleavage and the determination of partially cleaved molecule fragments unambiguously proved only 4→8 connections within the molecules.

3.2 Inhibition of the protein tyrosine kinase (PTK) activity of the isolated EGFR

Effects on the PTK activity of isolated EGFR were determined using ELISA. We tested the isolated procyanidins representing different degrees of oligomerisation. The dimeric procyanidins (PB1–4) inhibited 50% of the enzyme activity at a concentration of about 10 μM, with no significant difference in effectiveness between the four different stereoisomers (Fig. 2A). Trimeric (PC1) and tetrameric (PA2) procyanidin inhibited the activity of isolated EGFR with IC_{50}-values of 1.3 ± 0.4 μM and 0.2 ± 0.06 μM, respectively (Fig. 2B). Based on the IC_{50}-values the potency of procyanidins for the PTK inhibition of isolated EGFR can be summarised as PA2 > PC1 >> PB1 = PB2 = PB3 = PB4.

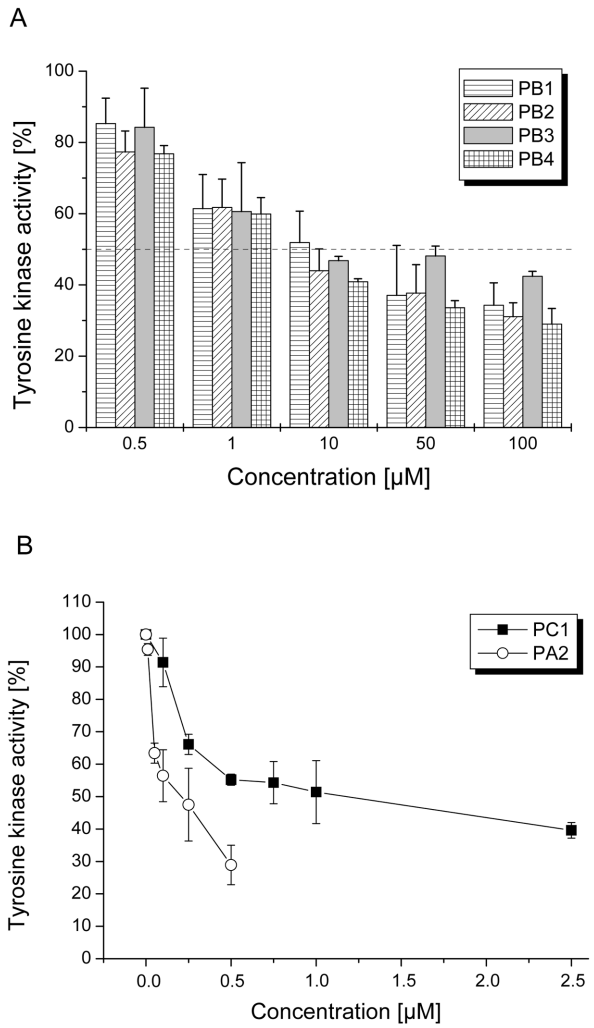


Figure 2. Inhibition of the tyrosine kinase activity of the EGF-receptor by (A) dimeric and (B) tri- and tetrameric procyanidins. Phosphorylation of tyrosine residues of a peptide poly(-Glu/Tyr) was determined by ELISA using an antiphosphotyrosine antibody linked to peroxidase. The data presented are the mean ± SD of at least two independent experiments, each performed in triplicate.

3.3 Modulation of EGFR autophosphorylation

We further addressed the question whether the inhibitory effects of procyanidins against the PTK activity of the EGFR are limited to the isolated receptor preparation in the ELISA or are also of relevance within intact colon tumour cells. Activation of the EGFR by respective ligands is mirrored by its autophosphorylation status. The effect of procyanidins on the phosphorylation status of the EGFR in HT29 cells was determined by Western blot analysis. With respect to their effectiveness against isolated EGFR preparations, the trimeric (PC1) and tetrameric (PA2) procyanidin were selected for testing. PC1 was found to effectively inhibit the EGFR-autophosphorylation (Figs. 3A and B) with an IC_{50}-value of 35 ± 15 μM. In contrast, PA2 failed to significantly affect the phosphorylation status of the EGFR up to 50 μM (Figs. 3C and D).

3.4 Interference with human topoisomerases

The catalytic activity of human topoisomerase I was determined as the relaxation of supercoiled pUC18 plasmid DNA by MCF-7 nucleic extract in the absence of ATP. Starting at a concentration of 0.5 μM, PC1 and PA2 were found to inhibit the relaxation of pUC18 (Fig. 4). In the presence of 1 μM of PC1 or PA2 the catalytic activity of topoisomerase I was substantially suppressed.

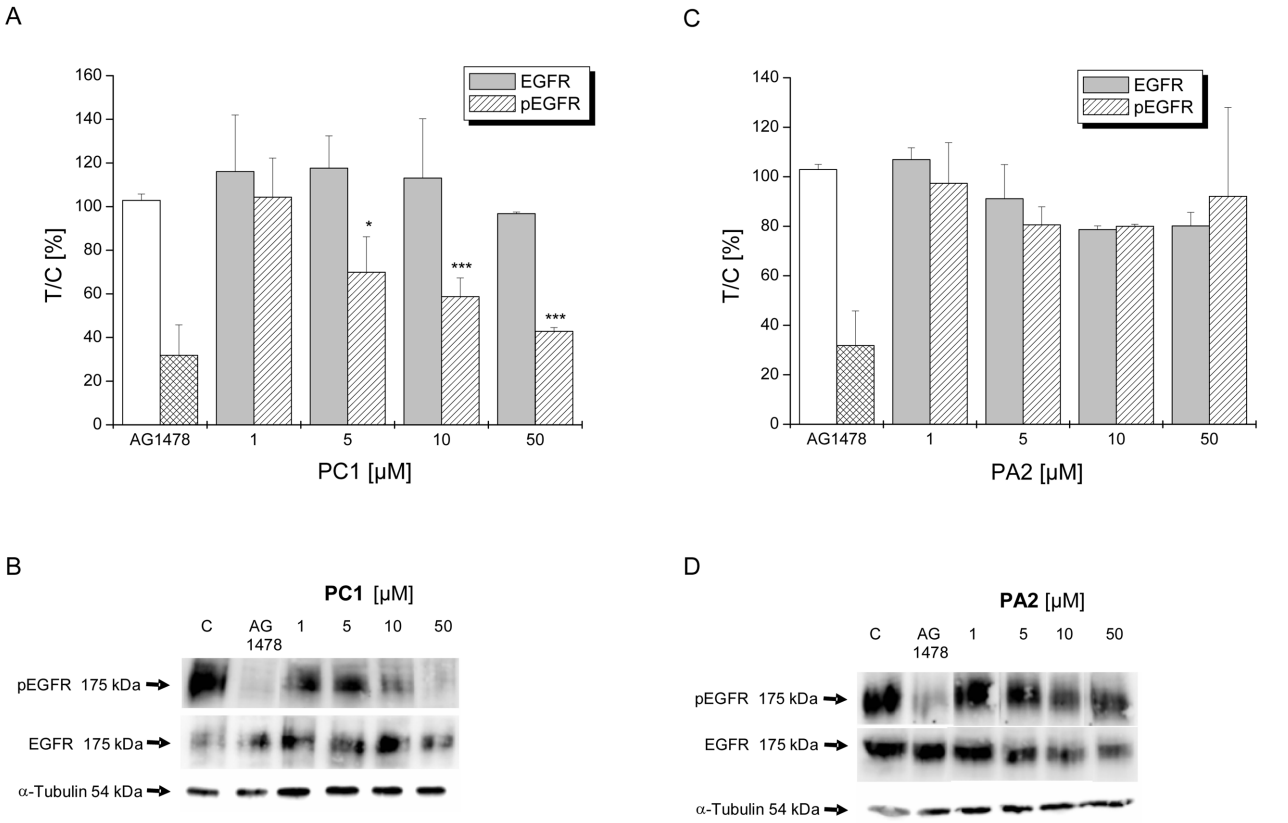


Figure 3. Western blot analysis of EGFR protein and phosphorylated EGFR (pEGFR) in HT29 cells stimulated by EGF (100 ng/mL) after 45 min treatment with (A) PC1 and (C) PA2. As a loading control α-tubulin was included in the test. The data presented are the mean ± SD of three independent experiments with similar outcome. The significances indicated refer to the comparison of the respective procyanidin concentration with the solvent control. *p <0.05, ***p <0.005; (C) solvent control (1% DMSO as final concentration); tyrphostin AG1478, EGFR specific inhibitor (5 μM). (B, D) Western blot of representative experiments.

To examine whether PC1 and PA2 have similar effects on human topoisomerase II, we determined the catalytic activity of recombinant human topoisomerase II in a decatenation assay. In the catenated form, kDNA cannot enter an agarose gel, whereas single circles released by catalytic active topoisomerase II from the catenated network will migrate into the gel (Fig. 5, compare lane 1 with 2).

PC1 was found to inhibit the catalytic activity of topoisomerase IIβ at concentrations exceeding 0.1 μM. At 10 μM PC1 the catalytic activity of topoisomerase IIβ was found to be completely blocked (Fig. 5, lane 6). PA2 even showed substantial inhibition of topoisomerase II activity already at 0.1 μM (Fig. 5, lane 7). At a concentration of 1 μM PA2 the catalytic activity of topoisomerase IIβ was completely blocked (Fig. 5, lane 9).

4 Discussion

Oligomeric procyanidins have been identified as a promising class of bioactive food constituents with respect to chemoprevention. Procyanidin-rich extracts have been reported to inhibit tumour cell growth *in vitro* and *in vivo* [5, 26, 27]. However, the underlying mechanism of action has not been fully elucidated yet. Furthermore, due to the limited availability of single procyanidin oligomers with defined structure, little is known so far about structure–activity relationships. In the present study, we demonstrated the isolation and structural characterisation of four stereoisomeric PB1–4 (PB1–PB4), the trimer PC1 and the tetramer PA2 from grape seed extract (Fig. 1). Using an ELISA-based approach, we showed that oligomeric procyanidins represent potential inhibitors of the EGFR. In a cell-free system the inhibitory potency of procyanidins was found to increase with the degree of oligomerisation (Figs. 2A and B). In previous studies, we showed that a respective monomeric flavan-3-ol, (–)-epicatechin, inhibits EGFR activity at concentrations >300 μM [8]. In contrast, the IC_{50} values of the dimeric procyanidins PB1–PB4 were localised at about 15 μM, with no significant differences in the effectiveness between the four different stereoisomers. The step from the procyanidin dimer to the trimer was found to further improve EGFR inhibition by an order of magnitude (compare Figs. 2A and B). The tetramer PA2 showed by far

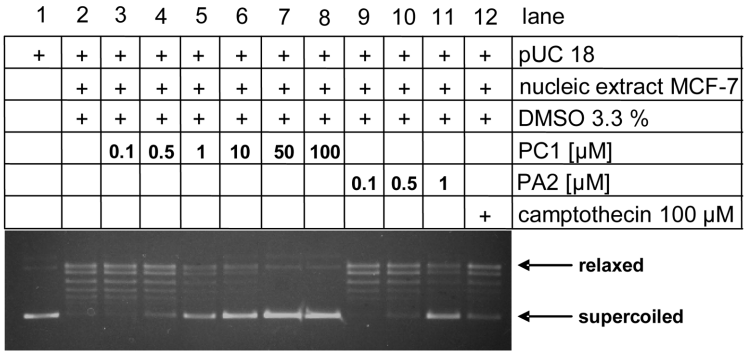


Figure 4. Catalytic activity of topoisomerase I measured by DNA relaxation. Supercoiled pUC18 plasmid DNA (lane 1) was incubated for 30 min at 37°C with nucleic extract from MCF-7 cells (lanes 2–12) in the absence (lane 2) or presence of increasing amounts of PC1 (lanes 3–8) or PA2 (lanes 9–11). Reaction was stopped with 1% SDS and after digestion with proteinase K, samples were subjected to submarine 1% agarose gel electrophoresis. UV-transilluminated gels were documented by digital photography. Active topoisomerase I converts the supercoiled pUC18 plasmid DNA into the relaxed form (see arrows). Shown is a representative gel out of two identical experiments with similar outcome.

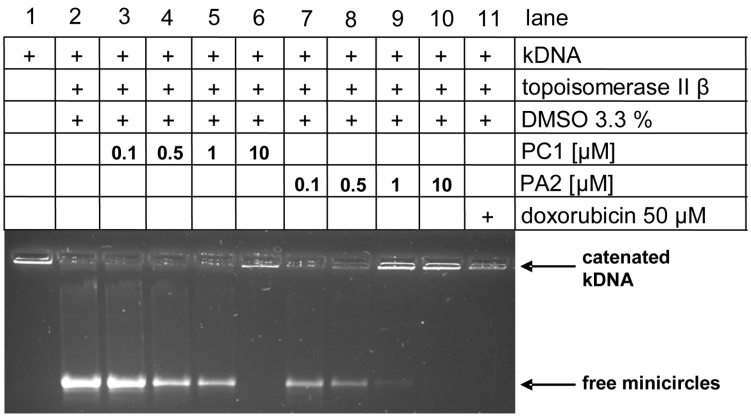


Figure 5. Topoisomerase IIß catalytic activity (recombinant enzyme) was determined as the decatenation of kDNA from *Crithidia fasciculata*. Recombinant topoisomerase IIß (40 ng) was added and reactions were carried out for 60 min at 37°C in the absence (lane 2) or presence of increasing amounts of PC1 (lanes 3–6) or PA2 (lanes 7–10). Reaction was stopped with 1% SDS and after digestion with proteinase K samples were subjected to submarine 1% agarose gel electrophoresis. UV-transilluminated gels were documented by digital photography. Lane 1 shows substrate not reacted with enzyme (catenated kDNA, see arrow). Only active topoisomerase II can release single DNA-circles from the catenated DNA-network (free DNA circles, see arrow). Shown is a representative gel of two independent experiments with similar outcome.

the strongest EGFR-inhibitory properties in the cell-free system (Fig. 2B). With respect to the inhibition of the PTK activity of isolated EGFR the inhibitory effectiveness of procyanidins can be summarised as PA2 > PC1 >> PB1 = PB2 = PB3 = PB4 >> (–)-epicatechin.

However, considering the increasing molecular weight, the question had to be addressed, whether these oligomeric procyanidins indeed affect the receptor activity within intact cells. The activity of the EGFR in intact cells is mirrored by its autophosphorylation status, which is crucial for the interaction with downstream elements of the MAPK cascade [28, 29]. The inhibition of the autophosphorylation of the EGFR has been already shown for several flavonoids such as the green tee catechin (–)epigallocatechin-3-gallate (EGCG), the flavonol quercetin or the flavone luteolin [12, 13, 30]. In the present study we showed that PC1 effectively suppressed the phosphorylation of the EGFR in HT29 cells (Figs. 3A and B). In contrast, studies with PA2 demonstrated up to 50 µM no significant effect on the phosphorylation status of the EGFR (Figs. 3C and D). These data indicate that the effectiveness of oligomeric procyanidins on the EGFR in intact cells might be limited upon increasing molecular weight. Thus, based on these results in cell culture only dimeric and trimeric analogues would be expected

to substantially contribute to the EGFR-inhibitory properties of complex procyanidin-containing mixtures such as *e. g.* apple extracts [8].

Considering the role of the EGFR in the regulation of cell growth, it is tempting to speculate that the inhibition of EGFR activity by procyanidins might be of relevance for the growth inhibitory effects of procyanidin-rich extracts. These results are in line with data from Gossè *et al.* [5] showing exclusively procyanidin-rich fractions of an apple juice extract to inhibit the growth of human colon carcinoma cells. Furthermore, procyanidins have been reported to mediate the onset of apoptosis [31, 32].

In the present study, we identified oligomeric procyanidins as potent EGFR inhibitors, thus presumably interfering with signalling cascades crucial for the regulation of cell proliferation. With respect to a risk/benefit assessment these effects on proliferation-associated signalling cascades might be taken into account as potentially beneficial, especially in terms of chemoprevention. However, as a potentially undesired effect with respect to the prevention of carcinogenesis, many flavonoids have been reported to interfere with human topoisomerases [14–16, 33–38]. Depending on the mode of interaction with the target enzyme, this effect might be of relevance for DNA integrity [15, 34–36, 38]. DNA topoisomerases are a group of enzymes that solve the topological problems caused by transcription, replication, chromosome condensation and segregation, and DNA repair [39, 40]. These enzymes change DNA topology by introducing transient breaks in the phosphodiester backbone of the DNA, enabling release of torsion stress. In mammals, two major classes of topoisomerases are expressed: topoisomerase I and II. Topoisomerase I introduces a transient single strand break in the DNA double helix by the formation of a covalent DNA-topoisomerase-intermediate, the so-called cleavable complex. Then torsion stress is released by strand rotation, followed by religation of the cleaved strand [41]. In contrast, topoisomerase II, an ATP-dependent enzyme, inserts a transient double strand break, through which a second DNA double helix is passed [42, 43]. The mode of interaction with the respective target enzyme is crucial with respect to potential consequences for DNA integrity. Catalytic inhibitors bind to the enzyme prior to DNA-binding, thus inhibiting the formation of the cleavable complex. However, the majority of topoisomerase targeting compounds does not interfere with the cleavable complex formation, but binds to and stabilises the DNA-topoisomerase-intermediate after it has formed, thus prohibiting the release and the resealing of the DNA strand. As a consequence, DNA double strand breaks are generated within cells due to collisions of the stabilised cleavable complexes with the replication forks [44–46]. With respect to the potential DNA damaging properties, those compounds are considered as topoisomerase poisons. Many flavonoids of different structural classes have been reported to target human topoisomerases, several of these acting as a topoisomerase poison [14–16, 33–38]. From the class of procyanidins PB2 has been already reported to inhibit the catalytic activity of topoisomerase II [17]. However, it has not been elucidated yet whether PB2 acts as a topoisomerase poison.

The majority of flavonoids targeting topoisomerases have been described to affect both topoisomerase I and II. Among the flavonoids EGCG and quercetin are some of the most potent compounds, exhibiting IC_{50}-values in the low micromolar range [15, 16, 36, 37]. Within the class of flavonoids vicinal hydroxy groups at the B-ring have been reported as an important structural element for efficient topoisomerase II targeting [15, 16]. These data are in line with the potent inhibition of topoisomerase activity mediated by PC1 and PA2 (Figs. 4 and 5). As the majority of flavonoids, PC1 and PA2 were found to affect both topoisomerase I and II. The inhibitory properties of PC1 and PA2 were comparable to EGCG and quercetin, some of the most potent flavonoids with respect to topoisomerase inhibition. Due to limitations in the availability of the isolated procyanidins the experiments had to be focussed on one topoisomerase II isoform. However, selectivity between the two different topoisomerase isoforms α and β is extremely rare and has not been reported so far for any flavonoids.

The potent inhibition of catalytic topoisomerase I and II activity by PC1 and PA2 in cell-free systems demands further studies on the underlying mechanism of action to elucidate whether oligomeric procyanidins might act as topoisomerase poisons. Furthermore, the question needs to be addressed whether the interference with topoisomerases is indeed of relevance in intact cells. These studies are crucial to clarify whether the targeting of topoisomerases by oligomeric procyanidins might impair the maintenance of DNA integrity, thus potentially limiting the usefulness of this class of compounds in terms of chemoprevention.

The potent bioactive properties of oligomeric procyanidins elucidated so far raises the question whether *in vivo* relevant substance concentrations might be achieved. However, studies on the bioavailability of oligomeric procyanidins are extremely limited. Experiments with human colonic carcinoma cells (Caco-2) showed that radiolabelled PB1–4 and trimers are absorbed to a similar extent as (+)-catechin. Furthermore, it has been reported that the absorption of oligomeric procyanidins depends on the M_r [47]. Because of their relatively high molecular weight, procyanidin oligomers are not easily absorbed in the small intestine. Evidence showing the absorption of procyanidins through the gut barrier is still scare.

In summary, oligomeric procyanidins were identified as potent inhibitors of the EGFR. In intact cells, however, the effectiveness of oligomeric procyanidins against EGFR activity appears to be limited upon increasing molecular weight. A procyanidin trimer (PC1) and tetramer (PA2) were found to potently inhibit the catalytic activity of human topoisomerase I and II in cell-free systems, demand-

ing further studies to determine whether the interference with topoisomerases might impair DNA integrity.

The study was performed as a part of the NutritionNet (www.nutrition-net.org), funded by the German Ministry of Research and Education (01EA0508). We thank F. Boege for generously providing us recombinant human topoisomerase IIβ. W. Lörsch (Breko, Bremen, Germany) is gratefully thanked for the contribution of the grape seed extract.

5 References

[1] Pinent, M., Blade, M. C., Salvado, M. J., Arola, L. *et al*., Metabolic fate of glucose on 3T3-L1 adipocytes treated with grape seed-derived procyanidin extract (GSPE). Comparison with the effects of insulin, *J. Agric. Food Chem.* 2005, *53*, 5932 – 5935.

[2] Kenny, T. P., Kenn, C. L., Jones, P., Kung, H. J. *et al*., Cocoa procyanidins inhibit proliferation and angiogenic signals in human dermal microvascular endothelial cells following stimulation by low-level H_2O_2, *Exp. Biol. Med.* 2004, *229*, 765 – 771.

[3] Pinent, M., Blay, M., Blade, M., Salvado, M. J. *et al*., Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines, *Endocrinology* 2004, *145*, 4985 – 4990.

[4] Saito, A., Mizushina, Y., Ikawa, H., Yoshida, H. *et al*., Systematic synthesis of galloyl-substituted procyanidin B1 and B2, and their ability of DPPH radical scavenging activity and inhibitory activity of DNA polymerases, *Bioorg. Med. Chem.* 2005, *15*, 2759 – 2771.

[5] Gossé, F., Guyot, S., Roussi, S., Lobstein, A. *et al*., Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis, *Carcinogenesis* 2005, *26*, 1291 – 1295.

[6] Zhang, W. Y., Liu, H. Q., Xie, K. Q., Yin, L., L, *et al*., Procyanidin dimer B2 [epicatechin-(4beta-8)-epicatechin]#suppresses the expression of cyclooxygenase-2 in endotoxin-treated monocytic cells, *Biochem. Biophys. Res. Commun.* 2006, *345*, 508 – 515.

[7] Kamimura, A., Takahashi, T., Morohashi, M., Takano, Y., Procyanidin oligomers counteract TGF-beta1- and TGF-beta2-induced apoptosis in hair epithelial cells: An insight into their mechanisms, *Skin Pharmacol. Physiol.* 2006, *19*, 259 – 265.

[8] Kern, M., Tjaden, Z., Ngiewih, Y., Puppel, N. *et al*., Inhibitors of the epidermal growth factor receptor in apple juice extract, *Mol. Nutr. Food Res*. 2005, *49*, 317 – 328.

[9] Marko, D., Puppel, N., Tjaden, Z., Jakobs, S., Pahlke, G., The substitution pattern of anthocyanidins affects different cellular signaling cascades regulating cell proliferation, *Mol. Nutr. Food Res.* 2004, *48*, 318 – 325.

[10] Meiers, S., Kemény, M., Weyand, U., Gastpar, R. *et al*., The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor, *J. Agric. Food Chem*. 2001, *49*, 958 – 962.

[11] Fritz, J., Kern, M., Pahlke, G., Vatter, S., Marko, D., Biological activities of malvidin, a red wine anthocyanidin, *Mol. Nutr. Food Res.* 2006, *50*, 390 – 395.

[12] Lee, L. T., Huang, Y. T., Hwang, J. J., Lee, P. P., *et al*., Blockade of the epidermal growth factor receptor tyrosine kinase activity by quercetin and luteolin leads to growth inhibition and apoptosis of pancreatic tumor cells, *Anticancer Res.* 2002, *22*, 1615 – 1627.

[13] Sah, J. F., Balasubramanian, S., Eckert, R. L., Rorke, E. A., Epigallocatechin-3-gallate inhibits epidermal growth factor receptor signalling pathway. Evidence for direct inhibition of ERK1/2 and AKT kinases, *J. Biol. Chem.* 2004, *279*, 12755 – 12762.

[14] Constantinou, A., Mehta, R., Runyan, C., Rao, K. *et al*., Flavonoids as DNA topoisomerase antagonists and poisons: Structure-activity relationships, *J. Nat. Prod.* 1995, *58*, 217 – 225.

[15] Habermeyer, M., Fritz, J., Barthelmes, H. U., Christensen, M. O. *et al*., Anthocyanidins modulate the activity of human DNA topoisomerases I and II and affect cellular DNA integrity, *Chem. Res. Toxicol*. 2005, *18*, 1395 – 1404.

[16] Austin, C. A., Patel, S., Ono, K., Nakane, H., Fisher, L. M., Site-specific DNA cleavage by mammalian DNA topoisomerase II induced by novel flavone and catechin derivatives, *Biochem. J.* 1992, *15*, 883 – 889.

[17] Jo, J. Y., Gonzalez de Mejia, E., Lila, M. A., Effects of grape cell culture extracts on human topoisomerase II catalytic activity and characterization of active fractions, *J. Agric. Food Chem.* 2005, *53*, 2489 – 2498.

[18] Hayasaka, Y., Waters, E. J., Cheynier, V., Herderich, M. J., Vidal, S., Characterization of proanthocyanidins in grape seeds using electrospray, *Rapid Commun. Mass Spectrom.* 2003, *17*, 9 – 16.

[19] Thompson, R. S., Jacques, D., Haslam, E., Plant Proanthocyanidins: Introduction – isolation, structure, and distribution in nature of plant procyanidins, *J. Chem. Soc. – Perkin Trans. 1*, 1972, *5*, 1387 – 1399.

[20] Kennedy, J. A., Jones, G. P., Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol, *J. Agric. Food Chem.* 2001, *49*, 1740 – 1746.

[21] Shoji, T., Mutsuga, M., Nakamura, T., Kanda, T. *et al*., Isolation and structural elucidation of some procyanidins from apple by low-temperature nuclear magnetic resonance, *J. Agric. Food Chem.* 2003, *51*, 3806 – 3813.

[22] Khan, M. L., Haslam, E., Williamson, M. P., Structure and conformation of the procyanidin B-2 dimer, *Magn. Reson. Chem.* 1997, *35*, 854 – 858.

[23] Debruyne, T., Pieters, L. A. C., Dommisse, R. A., Kolodziej, H. *et al*., Unambiguous assignments for free dimeric proanthocyanidin phenols from 2D NMR, *Phytochemistry* 1996, *43*, 265 – 272.

[24] Hatano, T., Hemingway, R. W., Conformational isomerism of phenolic procyanidins: Preferred conformations in organic solvents and water, *J. Chem. Soc. – Perkin Trans. 2*, 1997, *5*, 1035 – 1043.

[25] Morimoto, S., Nonaka, G., Nishioka, I., Tannins and related compounds.38. Isolation and characterization of flavan-3-ol glucosides and procyanidin oligomers from cassia bark, *Chem. Pharm. Bull.* 1986, *34*, 633 – 642.

[26] Faria, A., Calhau, C., de Freitas, V., Mateus, N., Procyanidins as antioxidants and tumor cell growth modulators, *J. Agric. Food Chem.* 2006, *54*, 2392 – 2397.

[27] Tourino, S., Selga, A., Jimenez, A., Julia, L. *et al*., Procyanidin fractions from pine (Pinus pinaster) bark: Radical scavenging power in solution, antioxidant activity in emulsion, and antiproliferative effect in melanoma cells, *J. Agric. Food Chem.* 2005, *53*, 4728 – 4735.

[28] Fan, Y. X., Wong, L., Johnson, G. R., EGFR kinase possesses a broad specificity for ErbB phosphorylation sites and ligand increases turnover number without affecting substrate binding affinity, *Biochem. J.* 2005, *392*, 417 – 423.

[29] Boonstra, J., Rijken, P., Humbel, B., Cremers, F. *et al*., The epidermal growth factor, *Cell Biol. Int*. 1995, *19*, 413 – 430.

[30] Liang, Y. C., Lin-Shiau, S. Y., Chen, C. F., Lin, J. K., Suppression of extracellular signals and cell proliferation through EGF receptor binding by (-)-epigallocatechin gallate in human A431 epidermoid carcinoma cells, *J. Cell Biochem.* 1997, *67*, 55 – 65.

[31] Nomoto, H., Iigo, M., Hamada, H., Kojima, S., Tsuda, H., Chemoprevention of colorectal cancer by grape seed proanthocyanidin is accompanied by a decrease in proliferation and increase in apoptosis, *Nutr. Cancer* 2004, *49*, 81 – 88.

[32] Hibasami, H., Shohji, T., Shibuya, I., Higo, K., Kanda, T., Induction of apoptosis by three types procyanidin isolated from apple (Rosaceae Malus pumila) in human stomach cancer KATO III cells, *Int. J. Mol. Med*. 2004, *13*, 795 – 799.

[33] Markovits, J., Linassier, C., Fosse, P., Couprie, J. *et al*., Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II, *Cancer Res.* 1989, *49*, 5111 – 5117.

[34] Martín-Cordero, C., López-Lázaro, M., Pinero, J., Ortiz, T. *et al*., Glucosylated isoflavones as DNA topoisomerase II poisons, *J. Enzyme Inhib. Med. Chem.* 2000, *15*, 455 – 460.

[35] Mittra, B., Saha, A., Chowdhury, A. R., Pal, C. *et al*., Luteolin, an abundant dietary component is a potent anti-leishmanial agent that acts by inducing topoisomerase II mediated kinetoplast DNA cleavage leading to apoptosis, *Mol. Med.* 2000, *6*, 527 – 541.

[36] López-Lázaro, M., Martín-Cordero, C., Toro, M. V., Ayuso, M. J., Flavonoids as DNA topoisomerase I poisons, *J. Enzyme Inhib. Med. Chem.* 2002, *17*, 25 – 29.

[37] Suzuki, K., Yahara, S., Hashimoto, F., Uyeda, M., Inhibitory activities of (−)-epigallocatechin-3-O-gallate against topoisomerase I and II, *Biol. Pharm. Bull.* 2001, *24*, 1088 – 1090.

[38] Strick, R., Strissel, P. L., Borgers, S., Smith, S. L., Rowley, J. D., Dietary bioflavonoids induce cleavage in the MLL gene and may contribute to infant leukaemia, *Proc. Natl. Acad. Sci.* 2000, *97*, 4790 – 4795.

[39] Wang, J. C., DNA topoisomerase, *Annu. Rev. Biochem.* 1996, *65*, 635 – 692.

[40] Champoux, J. J., DNA topoisomerases: Structure, function, and mechanism. *Annu. Rev. Biochem*. 2001, *70*, 369 – 413.

[41] Gupta, M., Fujimori, A., Pommier, Y., Eukaryotic DNA topoisomerases, I, *Biochem. Biophys. Acta.* 1995, *1262*, 1 – 14.

[42] Osheroff, N., Zechiedrich, E. L., Gale, K. C., Catalytic function of DNA topoisomerase II, *Bioassays* 1991, *13*, 269 – 273.

[43] Corbett, A. H., Zechiedrich, E. L., Lloyd, R. S., Osheroff, N., Inhibition of eukaryotic topoisomerase II by ultraviolet-induced cyclobutane pyrimidine dimers, *J. Biol. Chem.* 1991, *266*, 19666 – 19671.

[44] Hsiang, Y. H., Lihou, M. G., Liu, L. F., Arrest of replication forks by drug-stabilized topoisomerase poisons, *Cancer Res.* 1989, *49*, 5077 – 5082.

[45] Ryan, A. J., Squires, S., Strutt, H. L., Johnson, R. T., Camptothecin cytotoxicity in mammalian cells is associated with the induction of persistent double strand breaks in replicating DNA, *Nucleic Acids Res.* 1991, *19*, 3295 – 3300.

[46] Wu, J., Liu, L. F., Processing of topoisomerase I cleavable complexes into DNA damage by transcription, *Nucleic Acids Res.* 1997, *25*, 4181 – 4286.

[47] Deprez, S., Mila, I., Huneau, J. F., Tome, D., Scalbert, A., Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial Caco-2 cells, *Antioxid. Redox Signal*. 2001, *3*, 957 – 967.

