Proteome response in HT-29 human colorectal cancer cells to two apoptosis-inducing compounds with different mode of action

ARTICLE *in* INTERNATIONAL JOURNAL OF CANCER · MAY 2008

Impact Factor: 5.09 · DOI: 10.1002/ijc.23387 · Source: PubMed

4 AUTHORS, INCLUDING:

Hannelore Daniel
Technische Universität München
450 PUBLICATIONS **10,583** CITATIONS

SEE PROFILE

Int. J. Cancer: **122,** 2223–2232 (2008)

Proteome response in HT-29 human colorectal cancer cells to two apoptosis-inducing compounds with different mode of action

Isabel Winkelmann[1], Anna-Maria Näßl[1], Hannelore Daniel[1] and Uwe Wenzel[2]*

[1]*Molecular Nutrition Unit, Department of Food and Nutrition, Technical University of Munich, Am Forum 5, Freising, Germany*
[2]*Molecular Nutrition Research, Interdisciplinary Research Center, Justus-Liebig-University of Giessen, Heinrich-Buff-Ring 26-32, Giessen, Germany*

Flavone and camptothecin were both shown to potently induce apoptosis in HT-29 human colon cancer cells. Whereas camptothecin acts on the basis of topoisomerase-I inhibition, flavone appears to burst mitochondrial production of reactive oxygen species by increasing respiratory chain activity. In our study, we searched for similarities and differences in the proteome response of HT-29 cells when treated with the two different compounds. The accessible proteome of HT-29 cells was separated subsequent to the exposure to flavone or camptothecin by 2D-polyacrylamide-gel electrophoresis using pH-gradients between 4 and 7 and 6 and 11 in the first dimension and proteins with changed expression level were identified by peptide mass fingerprints of tryptic digests of the protein spots. Whereas there was a high congruence with regard to the identities of regulated proteins and their grade of regulation, a number of spots changed specifically only in response to either flavone or camptothecin. Nuclear envelope proteins were specifically increased by camptothecin indicating the intervention of this drug with cell division processes. Increased levels of coproporphyrinogen III oxidase, involved in cytochrome synthesis, and ubiquinol-cytochrome-c reductase suggest adaptations to flavone in order to enable a higher substrate flux through the respiratory chain. In conclusion, HT-29 cells respond to camptothecin and flavone with regulations of many proteins in a similar manner suggesting those alterations to be caused by apoptosis induction. Some protein regulations, however, were specific for each compound and point to the mechanism of their action.

Key words: HT-29 human colon cancer cells; proteome; apoptosis; nuclear membrane; mitochondria

The resistance of transformed cells towards apoptotic signals is regarded as a key parameter in promoting tumor cell development.[1–3] Accordingly, one of the major goals in cancer therapy is to restore the sensitivity of transformed cells towards apoptotic signals for allowing the execution of apoptotic cell death.[1,4,5] Camptothecin and its derivatives have emerged in this regard as chemotherapeutic agents and are used for second line treatment of ovarian cancer and metastatic colorectal cancer.[6] As camptothecins bind at the interface of the topoisomerase-I-DNA complex, they represent a paradigm for interfacial inhibitors that reversibly trap macromolecular complexes.[7] However, those interactions generally target processes that are crucial for cell division and thus have unwanted side effects in all tissues with a high rate of cell turnover.[8] It is imperative therefore to find chemotherapeutic agents with excellent tumor killing qualities and low normal tissue toxicity. One of such compounds could be the flavonoid flavone that has been shown previously to potently and selectively induce apoptosis in transformed cells of the colon *in vitro* and *in vivo*.[9–11] Mechanistically, flavone enhances the uptake of the monocarboxylates pyruvate or lactate into mitochondria thus providing substrates for the respiratory chain. Increased respiration is associated with an increased production of reactive oxygen species (ROS) and finally with efficient induction of apoptosis in tumor cells.[12] Accordingly, flavone overcomes a metabolic alteration that is typical for most cancer cells, the so-called "Warburg-effect," describing glycolysis as the prime energy delivery pathway even in the presence of sufficient oxygen. Since almost all nontransformed cells already rely on respiratory energy production with a considerable production of ROS flavone is unable to induce apoptosis in these cells.[10,11]

For better understanding apoptosis execution or resistance in cancer cells it is important to know the mechanisms of action and the targets of the drugs, as well as the cellular response towards the drugs. We have used a proteomic approach in HT-29 cells exposed to concentrations of flavone or camptothecin that are equally effective in inducing apoptosis to dissect the different modes of actions from common responses to both compounds by employing 2D-PAGE and peptide mass fingerprinting for identification of proteins with changed steady state level.

Materials and methods

Chemicals

All standard laboratory chemicals and the solvents for mass spectrometry, of highest grade available, were purchased from Merck (Darmstadt, Germany). Flavone, camptothecin and α-cyano-4-hydroxy-cinnamic acid were obtained from Sigma Aldrich (Steinheim, Germany), Coomassie Brilliant Blue G250 and silicone oil for the first dimension from Serva Electrophoresis GmbH (Heidelberg, Germany). Other chemicals and materials for 2D-PAGE, like IPG buffers, pharmalyte, Immobiline Dry Strips and DeStreak were from GE Healthcare (Munich, Germany). The CompleteMini protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Mannheim, Germany), sequencing grade trypsin for mass spectrometry from Promega (Mannheim, Germany), and RPMI cell culture medium from Biochrom AG (Berlin, Germany). All other cell culture chemicals were obtained from the PAA Laboratories GmbH (Pasching, Austria). The Bio-Rad Protein Assay was from Biorad Laboratories GmbH (Munich, Germany).

Cell culture

The human colon cancer cell line HT-29 was purchased from the American Type Culture Collection (ATCC, Rockville, MD). The cells were maintained in RPMI-1640 containing 10% FCS supplemented with 25 mM Hepes-buffer, 100 U/ml penicillin and 100 μg/ml streptomycin and cultured in 75 cm^2 tissue culture flasks (TPP Laboratories, Switzerland) in a water-saturated 5% CO_2 atmosphere at 37°C. Cells were passaged using a solution containing 0.05% trypsin and 0.5 mM EDTA. The stock solutions of flavone and camptothecin were made in DMSO with the solvent

Abbreviations: 2D-PAGE, two-dimensional gel electrophoresis; DTT, 1,4-dithiothreit; FAST, fragment analysis and structural time-of-flight; FCS, fetal calf serum; HCCA, α-cyano-4-hydroxy-trans-cinnamic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; IEF, isoelectric focusing; IPG, immobilized pH-gradient; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; pI, isoelectric point; PMF, peptide mass fingerprint; PSD, post source decay; ROS, reactive oxygen species; SDS, sodium dodecylsulfate; TFA, trifluoracetic acid.

Grant sponsor: Deutsche Forschungsgemeinschaft; Grant number: WE 2684/3-1 and 3-2.

***Correspondence to:** Interdisciplinary Research Center, Molecular Nutrition Research, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany. Fax: +49-641-99-39229.
E-mail: uwe.wenzel@ernaehrung.uni-giessen.de

Received 11 September 2007; Accepted after revision 3 December 2007
DOI 10.1002/ijc.23387
Published online 23 January 2008 in Wiley InterScience (www.interscience.wiley.com).

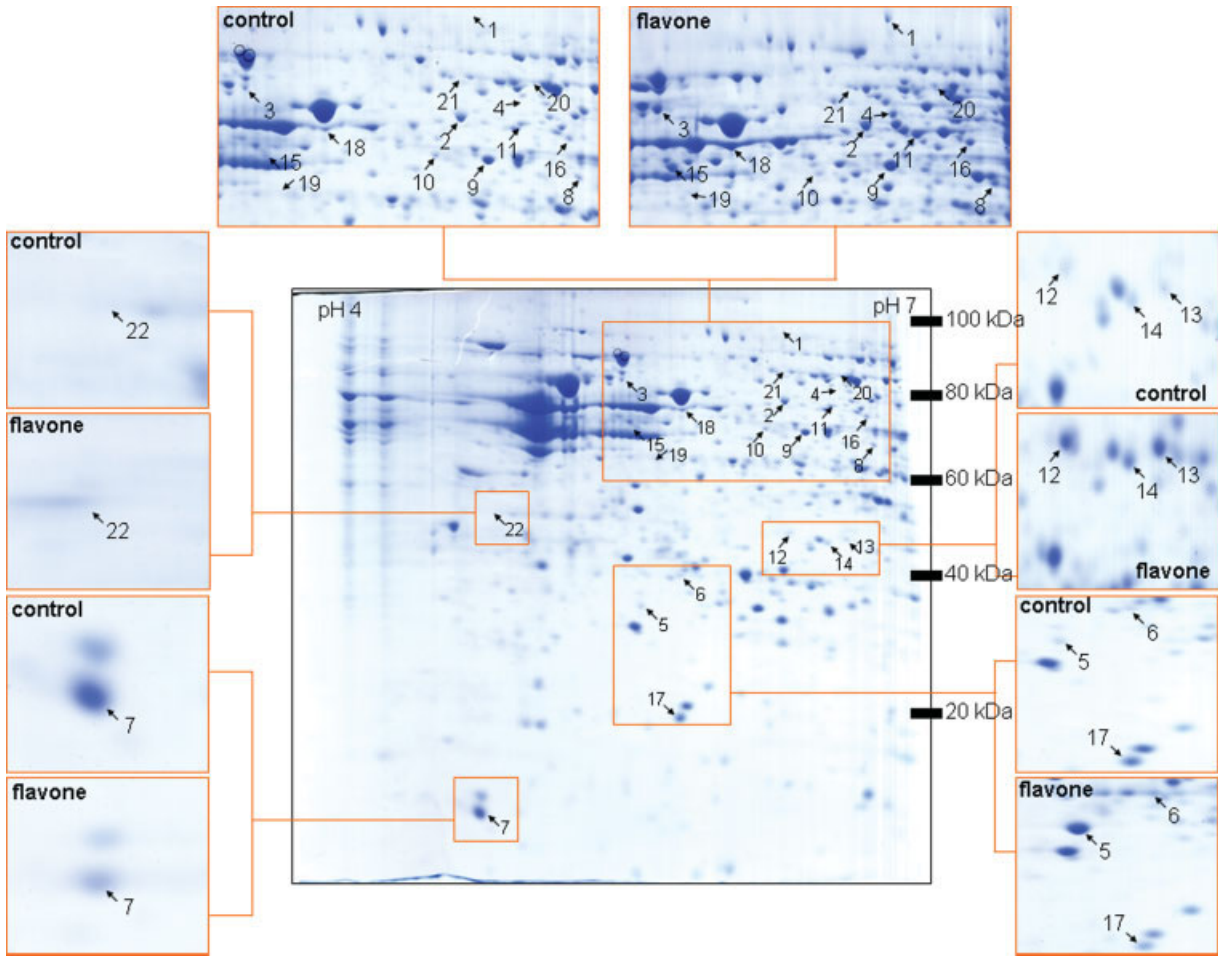

FIGURE 1 – Two-dimensional map of proteins derived from HT-29 cells treated for 24 hr with flavone. Proteins were separated on a linear pH 4–7 IPG-strip in the first dimension and on a 12.5% SDS-polyacrylamide gel in the second dimension. Enlargements from identical sections of gels derived from separations of HT-29 proteins from control cells or cells treated with 150 μM flavone are shown around a typical gel derived from control cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

not exceeding 1.0% in the final medium. Controls were treated with the same amount of DMSO. All cells were exposed for 24 hr to flavone, camptothecin or the solvent only.

Protein extraction

Cells were rinsed twice with ice-cold 0.35 M sucrose buffer and then scraped off from the flasks in the sucrose solution containing protease inhibitor. After pelleting the cells at 1,500*g* for 7 min they were lysed according to the method of Rabbilloud[13] in a buffer containing 7 M urea, 2 M thiourea, 65 mM DTT, 2% CHAPS (w/v), 2% pharmalyte (pH 3–10) and protease inhibitor cocktail tablets. Homogenization of the resultant cell lysate was achieved by ultrasonication for 6 × 10 s (amplitude 45) using an ultrasonic processor (Hielscher Ultrasonics GmbH, Germany). After 30 min on ice the homogenate was centrifuged at 14,000*g* for 1 hr to collect the supernatant. Proteins were precipitated from the supernatant with 5 volumes of ice-cold acetone overnight at −20°C. The precipitated protein was pelleted at 14,000*g* for 10 min and lysed again as described earlier. The concentration of the solubilized proteins was determined using the Bradford method with the Bio-Rad Protein Assay, measuring extinction at OD 595 nm.

Two dimensional gel electrophoresis

For the first dimension proteins were focused using the Ettan IPG Phor II from GE Healthcare and a standard running protocol described by Görg *et al.*,[14,15] with slight modifications. Focusing was achieved at the following conditions: 500 V (10 min, gradient), 4,000 V (2.5 hr, gradient), 8,000 V (30,000 Vh, step-n-hold). A total protein amount of 750 μg was applied by cup-loading at the anodic end of an 18 cm long IPG strip with an immobilized pH-gradient 4–7. IPG-strips were rehydrated prior to cup-loading for 12 hr in 340 μl solubilization buffer per strip, containing 8 M urea, 2% CHAPS, 2% pharmalyte 3–10, 1% IPG buffer 4–7 and 13 mM DTT. IPG strips with pH 6–11 gradients were passively rehydrated overnight with 750 μg protein per strip in solubilization buffer with additional 1% IPG buffer 6–11 and 1.2% DeStreak. The gel strips with the focused proteins were either frozen at −80°C or directly processed for the second dimension. Therefore, the strips had to be equilibrated with a buffer containing 6 M urea, 30% glycerol, 0.4% SDS, 50 mM Tris-buffer (pH 8.8) and in addition 1% DTT for the first 10 min incubation and 4% iodacetamide for the second equilibration step, respectively. The equilibrated IPG strips were transferred onto a 12.5% acrylamide gel for the second dimension by coating them with 0.5% agarose containing bromphenolblue and treated according to the method of Laemmli.[16] Second dimension was run in the ETTAN Dalt II system (GE Healthcare) with 4 mA/gel for 1 hr and 12 mA/gel afterwards. For subsequent protein mass identification a low molecular weight standard ranging from 14.4 to 97 kDa was used. Gels were fixed in 40% (v/v) ethanol and 10% (v/v) acetic acid at least for 6 hr before they were transferred to the Coomassie staining solution

TABLE I – PROTEINS OF HT-29 CELLS WITH PI BETWEEN 4 AND 7 REGULATED IN STEADY-STATE LEVEL BY FLAVONE TREATMENT

Spot no.	Protein description	Swiss prot acc. No.	Sequence-cover. (%)	Mw/pI theor.	Mw/pI exp.	Subcellular location	Reg.-factorF/ control
	Apoptosis						
1	*Programmed cell death 6-interacting protein*	Q8WUM4	37	97/6.1	90/6.2	Cytoplasm	Only in F
	Chaperones						
2	T-complex protein 1, beta subunit	P78371	53	58/6.0	62/6.4	Cytoplasm	2.19
3	T-complex protein 1, epsilon subunit	P48643	23	61/5.5	68/5.5	Cytoplasm	2.49
4	T-complex protein 1, zeta subunit	P40227	52	58/6.3	68/6.6	Cytoplasm	Only in F
	Detoxification						
5	Glutathione *S*-transferase P	P09211	52	23/5.4	29/5.6	Cytoplasm	5.57
6	Peroxiredoxin 4	Q13162	50	31/5.9	32/5.8	Cytoplasm	2.61
7	*Thioredoxin*	P10599	66	12/4.8	12/4.7	Cytoplasm	0.51
	Gene regulation						
8	Elongation factor Tu, mitochondrial [Precursor]	P49411	52	50/7.3	53/6.9	Mitochondrion	6.72
9	*Proliferation-associated protein 2G4*	Q9UQ80	26	44/6.1	55/6.5	Cytoplasm, nucleus; nucleolus	2.16
10	*Proliferation-associated protein 2G4*	Q9UQ80	50	44/6.1	56/6.3	Cytoplasm, nucleus; nucleolus	2.31
11	RuvB-like 1	Q9Y265	48	51/6.0	61/6.6	Nucleus (mainly), associated with nuclear matrix or in the nuclear cytosol; cytoplasm, associated with the cell membranes	3.18
	Metabolism						
12	Delta3,5-delta2,4-dienoyl-CoA isomerase, mitochondrial [Precursor]	Q13011	42	36/8.2	38/6.4	Mitochondrion, peroxisome	2.60
13	Delta3,5-delta2,4-dienoyl-CoA isomerase, mitochondrial [Precursor]	Q13011	36	36/8.2	37/6.7	Mitochondrion, peroxisome	2.75
14	Proteasome (Prosome, macropain) subunit, alpha type, 1	Q53YE8	44	30/6.2	37/6.6	Cytosol, proteasome core complex (sensu Eukaryota), protein complex	2.43
15	*Ubiquinol-cytochrome-c reductase complex core protein I, mitochondrial [Precursor]*	P31930	51	53/5.9	52/5.3	Mitochondrion; mitochondrial inner membrane	Only in F
	Cytoskeletal						
16	Septin-11	Q9NVA2	36	55/7.3	59/6.8	Localized along stress fibers	2.70
17	Stathmin	P16949	41	17/5.8	19/5.8	Cytoskelet associated	0.47
18	Keratin, type II cytoskeletal 8	P05787	34	56/5.6	60/5.9	Intermediate filament associated	8.20
19	*Keratin, type I cytoskeletal 19*	P08727	72	44/5.0	48/5.4	Intermediate filament associated	Only in F
20	Lamin C	P02545	49	65/6.4	66/6.4	Nucleus	Only in F
21	Lamin C	P02545	45	65/6.4	72/6.1	Nucleus	Only in F
	Others						
22	WD repeat protein 61	Q9GZS3	52	34/5.2	39/5.0	Membrane associated	Only in F

The spot numbers are identical to those given in Figure 1. Protein descriptions are according to the Swiss-Prot website (www.expasy.org/sprot/) with their associated primary accession numbers. Proteins altered significantly by 24 hr flavone treatment of HT-29 cells, as derived from analysis with the Proteomweaver software, were identified by MALDI-TOF-MS: Those that responded only to flavone but not to camptothecin are shown in italics; Sequence cover. [%], sequence coverage obtained by the identified peptides; Mw/pI theor., mass and pI values taken from the MSDB database; Mw/pI exp., mass values calculated by the Proteomweaver software referring to the low molecular weight standard and calculated pI values; Subcellular location is indicated according to www.expasy.org, www.harvester.embl.de and literature; Reg.-factor F/control, regulation of intensities of protein spots derived from treated cells compared to those derived from the control ($p < 0.05$; Student's t test); only in F, associated protein spot was only detectable in flavone treated cells; ($n = 6$).

(10% (w/v) $(NH_4)_2SO_4$, 2% (v/v) phosphoric acid, 25% (v/v) methanol and 0,625% (w/v) Coomassie brilliant blue G250) overnight. The destaining was performed in Milli Q water until the desired contrast was obtained.

Protein detection, analysis, and in-gel digestion

Two gels were prepared for each treatment and for each experiment out of three independent experiments, resulting in six gels per treatment group. Gels with stained protein spots were scanned with an Umax scanner Power Look III (software: Magic Scan version V4.5, UMAX) and computer-assisted image analysis was performed by means of the Proteomweaver software version 3.1 (BioRad). Background subtraction, volume normalization and matching of the detected protein spots were performed by the software automatically. Those spots differing significantly ($p < 0.05$, Student's t test) in their intensities were used for further analysis. Spots were excised from the Coomassie-stained gels with a 2 mm skin-picker with at least four spots of a distinct protein from gels derived from different groups. Spots were transferred into 0.2 ml Eppendorf tubes loaded with 50 µl of 50 mM ammonium bicarbonate solution. The destaining of the gel pieces was performed with alternating washing procedures in 50 µl of acetonitrile/50 mM ammonium bicarbonate solution (1/1, v/v) and pure 50 mM

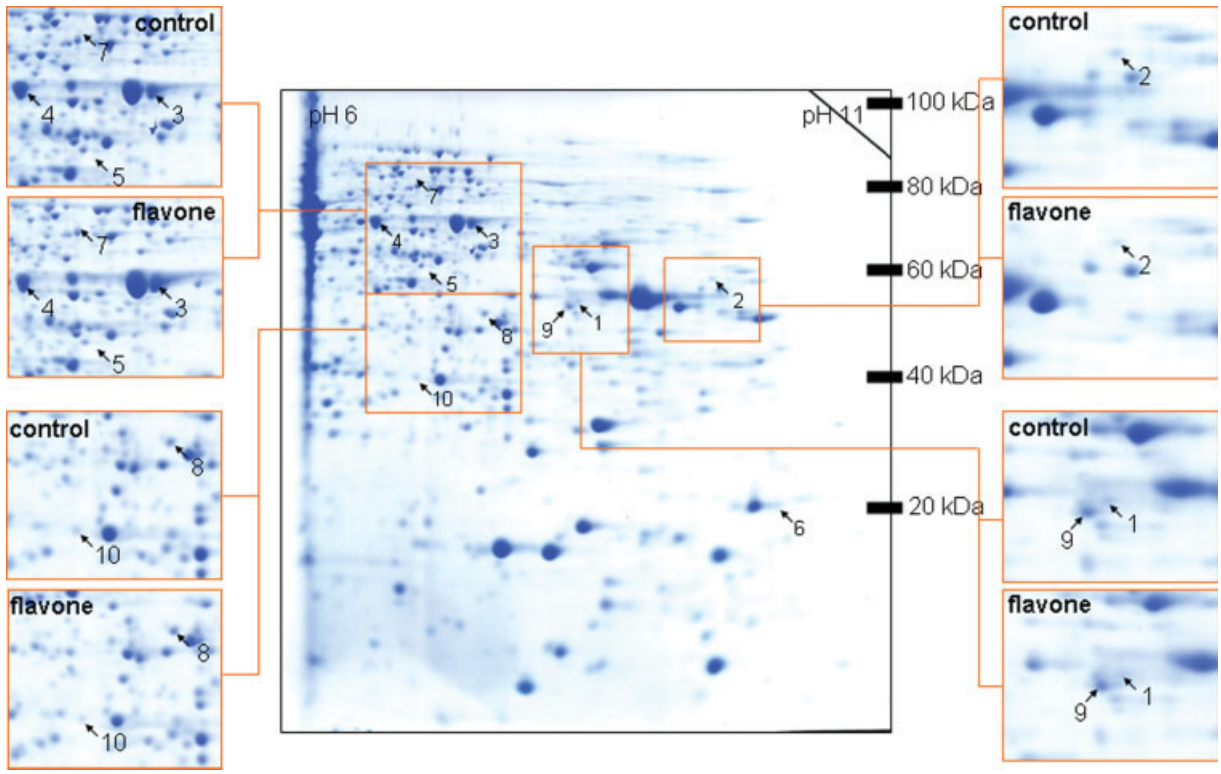

FIGURE 2 – 2D-PAGE of proteins from HT-29 cells treated for 24 hr with medium alone (control) or with 150 μM flavone. Proteins were separated in the first dimension on IPG-strips with linear pH-gradients between 6 and 11. Enlargements around the mid panel show corresponding sections of gels with proteins derived either from control or flavone-treated cells, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II – PROTEINS WITH PI BETWEEN 6–11 REGULATED IN STEADY-STATE LEVEL BY FLAVONE TREATMENT

Spot no.	Protein description	Swiss prot acc. no.	Sequence-cover. (%)	Mw/pI theor.	M_w/pI exp.	Subcellular location	Reg.-factor F/ control
	Gene regulation						
1	Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	42	37/9.0	40/8.6	Nuclear; component of ribonucleosomes	2.18
2	RNA-binding protein Raly	Q9UKM9	31	33/9.2	44/9.9	Nucleus	0.50
	Metabolism						
3	Alpha-enolase	P06733	51	47/7.0	54/7.7	Cytoplasm, cell membrane, nucleus	2.78
4	Alpha-enolase	P06733	53	47/7.0	53/6.7	Cytoplasm, cell membrane, nucleus	0.37
5	*Coproporphyrinogen III oxidase, mitochondrial [Precursor]*	P36551	56	41/6.7	44/7.5	Mitochondrion; mitochondrial intermembrane space	Only in F
6	Cyclophilin B, Chain A		61	20/9.2	20/10.5	Endoplasmic reticulum (ER); ER lumen	11.84
7	*Glucose-6-phosphate 1-dehydrogenase*	P11413	60	60/6.4	62/7.1	Cytoplasm	Only in F
8	Glyceraldehyde-3-phosphate dehydrogenase	P04406	46	36/8.3	38/7.7	Cytoplasm	2.00
9	Glyceraldehyde-3-phosphate dehydrogenase	P04406	49	36/8.6	40/8.4	Cytoplasm	2.33
	Cytoskeletal						
10	Keratin, type I cytoskeletal 18	P05783	41	48/5.3	30/7.0	Intermediate filament associated	3.45

The spot numbers are identical to those given in Figure 2. Abbreviations are explained in the legend to Table I. Identical proteins that were identified as regulated at both pH-gradients are indicated only once, i.e., in Table I and not in addition in Table II; Those proteins that responded only to flavone but not to camptothecin are shown in italics; ($n = 6$).

ammonium bicarbonate until the Coomassie was removed. Subsequently, spots were dehydrated in 50 μl 100% acetonitrile for 10 min and dried in a vacuum table centrifuge (Thermo Electron, Dreieich, Germany). The dried gel pieces were stored at −20°C until preparation. Swelling of the spots with 6 μl of 0.02 μg/μl sequencing-grade modified trypsin was done on ice. After 1 hr in the fridge the excess enzyme was removed and the in gel digest was performed at 37°C for 8–10 hr. The generated peptide mixtures were extracted with 8 μl of 1% TFA solution per spot and 10 min in the sonication bath.

Mass spectrometry and database searching

The generated peptide mixture samples were spotted by hand onto an Anchor Chip™ MALDI-target 400/384 by using the HCCA thin layer affinity method.[17] The samples were acidified by using aqueous 0.1% TFA as washing solution and air-dried at room temperature. Analysis was performed with an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics) operating in reflectron mode with a 20-kV accelerating voltage and a 130-ns delayed extraction. Peptide mass fingerprint spectra were acquired in the automatic mode using the AutoXecute module of flexControl software version 2.4 (Bruker Daltonics). The spectra gained from the protein samples were processed with flexAnalysis 2.4 (Bruker Daltonics) by using the smoothing option and calibrating both external, and internally with the autoproteolysis peptide of trypsin (m/z 2211.10). Background peaks like keratin, Coomassie etc., were removed and a signal to noise threshold (S/N) of 3 was applied for the samples and 6 for the Peptide Calibration Standard (1,000–4,000 Da, Bruker Daltonics). Peptides were selected in the mass range of 800–3,500 Da. The resulting mass list was evaluated using Bio Tools 3.0 with the search engine Mascot (version 1.9.00, www.matrixscience.com) and the MSDB database. Following search criteria were applied: trypsin as digestion enzyme, ±50–150 ppm peptide mass tolerance, 1 missed cleavage, carbamidomethyl modification of cysteine as global and methionine oxidation as variable modification, and charged state as MH^+. A protein was seen as validated when 3 samples satisfactorily showed the same results with a probability based mowse score being significant ($p < 0.05$) and showing a root mean square (RMS) error below 100 ppm.

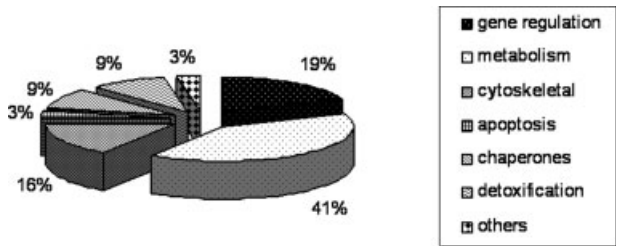

Figure 3 – Pie chart showing the allocation of proteins from HT-29 cells affected in their steady state level by exposure to 150 μM flavone for 24 hr to different protein classes. Protein classes were built on a functional basis.

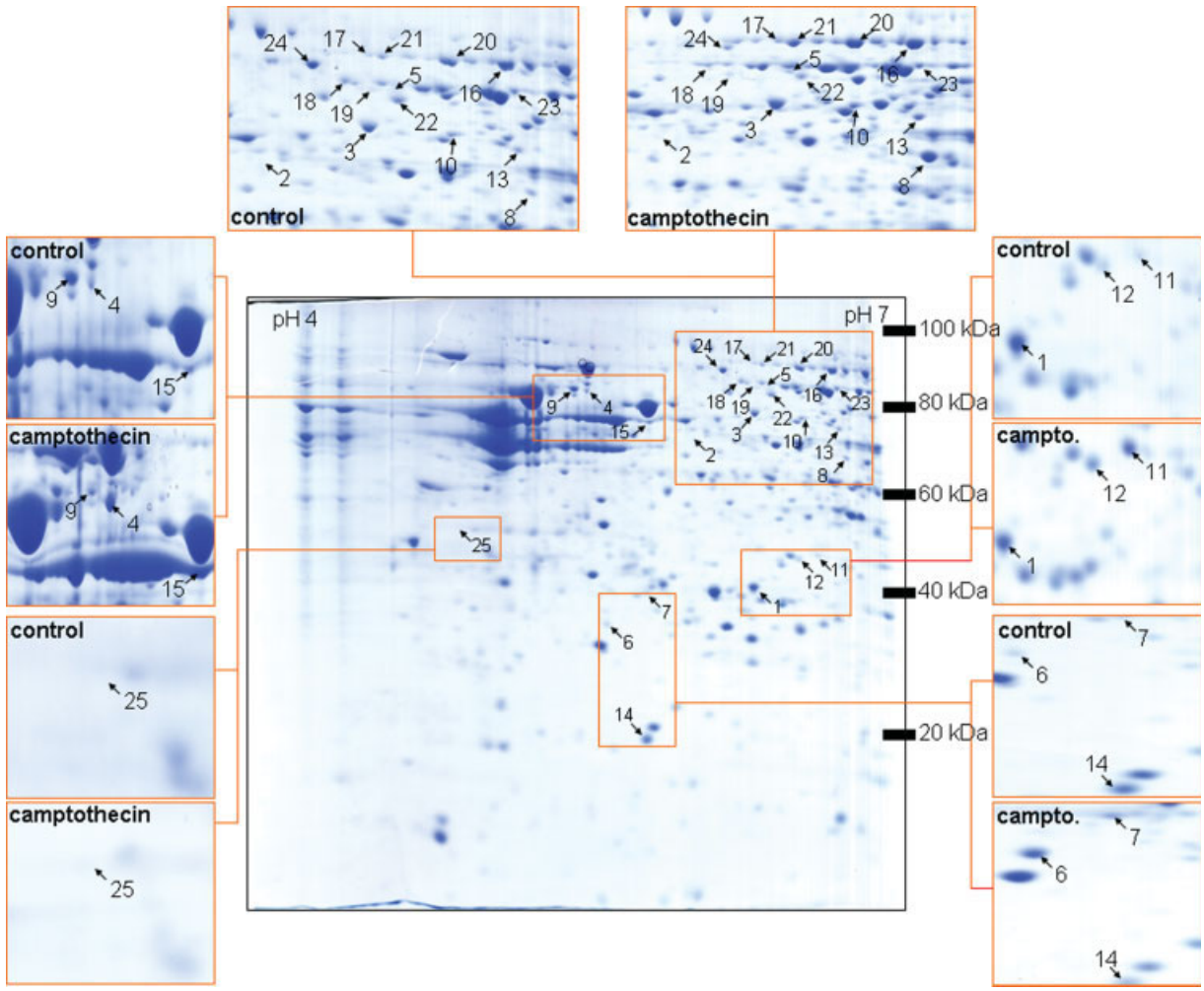

Figure 4 – Two-dimensional map of proteins from camptothecin-treated HT 29 cells. Proteins were isolated after exposure of the cells to 50 μM camptothecin for 24 hr and separated on IPG-strips with pH 4–7 in the first dimension. Enlargements around the control shown in the mid panel, display corresponding sections of gels with proteins derived from control cells or camptohecin-exposed cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE III – PROTEINS WITH PI BETWEEN 4 AND 7 REGULATED IN STEADY-STATE LEVEL BY CAMPTOTHECIN IN HT-29 CELLS

Spot no.	Protein description	Swiss prot acc. no.	Sequence-cover. (%)	Mw/pI theor.	Mw/pI exp.	Subcellular location	Reg.-factor C/control
	Chaperones						
1	*Endoplasmic reticulum protein ERp29 [Precursor]*	P30040	53	29/6.8	33/6.3	Endoplasmic reticulum (ER); ER lumen	0.55
2	*Protein disulfide-isomerase A3 [Precursor]*	P30101	37	57/6.0	54/5.9	Endoplasmic reticulum (ER); ER lumen	Only in C
3	T-complex protein 1, beta subunit	P78371	53	58/6.0	62/6.4	Cytoplasm	2.35
4	T-complex protein 1, epsilon subunit	P48643	23	61/5.5	68/5.5	Cytoplasm	2.96
5	T-complex protein 1, zeta subunit	P40227	52	58/6.3	68/6.6	Cytoplasm	Only in C
	Detoxification						
6	Glutathione *S*-transferase P	P09211	52	23/5.4	29/5.6	Cytoplasm	5.51
7	Peroxiredoxin 4	Q13162	50	31/5.9	32/5.8	Cytoplasm	2.38
	Gene regulation						
8	Elongation factor Tu, mitochondrial [Precursor]	P49411	52	50/7.3	53/6.9	Mitochondrion	4.61
9	*Heterogeneous nuclear ribonucleoprotein K*	P61978	49	51/5.2	68/5.5	Cytoplasm; nucleus, nucleoplasm	0.49
10	RuvB-like 1	Q9Y265	48	51/6.0	61/6.6	Nucleus (mainly), associated with nuclear matrix or in the nuclear cytosol; cytoplasm, associated with the cell membranes	3.31
	Metabolism						
11	Delta3,5-delta2,4-dienoyl-CoA isomerase, mitochondrial [Precursor]	Q13011	36	36/8.2	37/6.7	Mitochondrion peroxisome	2.40
12	Proteasome (Prosome, macropain) subunit, alpha type, 1	Q53YE8	44	30/6.2	37/6.6	Cytosol, proteasome core complex (sensu Eukaryota), protein complex	2.36
13	Septin-11	Q9NVA2	36	55/7.3	59/6.8	Localized along stress fibers	2.04
14	Stathmin	P16949	41	17/5.8	19/5.8	Cytoplasm	0.36
	Cytoskeletal						
15	Keratin, type II cytoskeletal 8	P05787	34	56/5.6	60/5.9	Intermediate filament associated	6.33
16	*Lamin A*	P02545	47	74/6.6	76/6.8	Nucleus	2.43
17	*Lamin A*	P02545	47	74/6.6	71/6.0	Nucleus	Only in C
18	Lamin C	P02545	49	65/6.4	72/6.0	Nucleus	Only in C
19	Lamin C	P02545	45	65/6.4	65/6.3	Nucleus	Only in C
20	*Lamin A/C transcript variant 1*	Q5I6Y4	48	74/6.4	77/6.6	Primordial components of the cytoskeleton and the nuclear envelope	2.88
21	*Lamin A/C transcript variant 1*	Q5I6Y4	30	74/6.4	71/6.1	Primordial components of the cytoskeleton and the nuclear envelope	Only in C
22	*Progerin*	Q6UYC3	45	69/6.2	70/6.6	Primordial components of the cytoskeleton and the nuclear envelope	2.99
23	*Progerin*	Q6UYC3	42	69/6.2	71/6.8	Primordial components of the cytoskeleton and the nuclear envelope	2.03
	Others						
24	*Premature ovarian failure, 1B*	Q5H9E9	45	70/5.9	77/6.2		0.35
25	WD repeat protein 61	Q9GZS3	52	34/5.2	39/5.0	Membrane associated	Only in C

The spot numbers are identical to those given in Figure 4. Proteins altered significantly by 24 hr camptothecin treatment of HT-29 cells were identified by MALDI-TOF MS; Proteins that responded only to camptothecin but not to flavone are shown in italics; Reg.-factor C/control, regulation of the spot intensities observed between camptothecin treated cells versus control cells according to Proteomweaver software analysis with $P < 0.05$ (Student's *t* test); only in C, associated protein spot was only detectable in the camptothecin exposed cells. Other abbreviations are given in the legend to Table I; ($n = 6$).

Results

Responses of the HT-29 cell proteome to flavone exposure

HT-29 cells were exposed for 24 hr to 150 μM flavone, a concentration that has been shown to cause apoptosis in about 50% of the cell population.[9] In 2D-PAGE with proteins from these cells about 800 spots could be detected by the Proteomweaver software. After normalization and statistical analysis by the software 29 proteins differed significantly ($p < 0.05$) in their intensities between flavone-treated cells and the control in a pI-range between 4 and 7. Of these, 22 could be identified by MALDI-TOF MS (Fig. 1, Table I).

Using IPG-strips with a pH-gradient of 6–11 another 14 proteins were shown to be significantly affected in their levels by flavone of which 10 were identified (Fig. 2, Table II). Classification

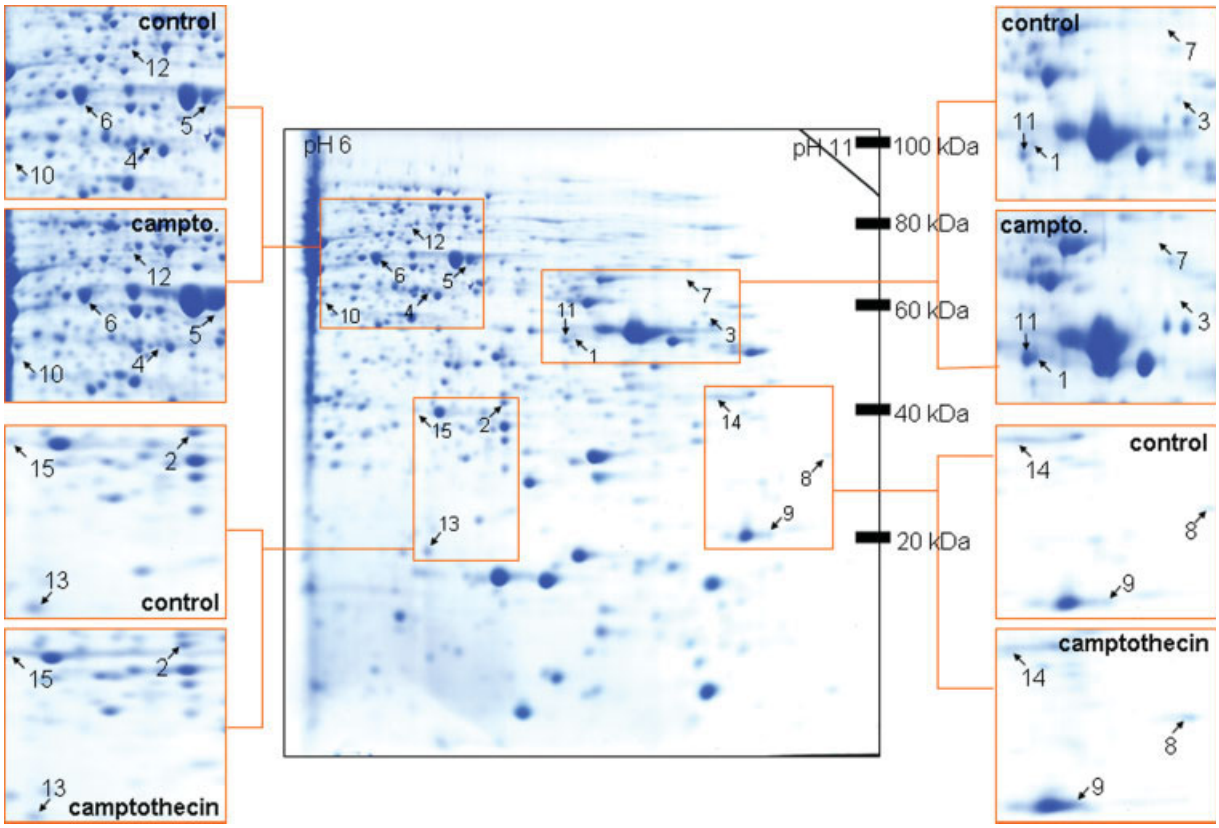

FIGURE 5 – 2D-PAGE of proteins from control HT-29 cells or those treated for 24 hr with 50 μM camptothecin separated on a linear pH-gradient between 6 and 11 in the first dimension. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of the proteins that responded to flavone exposure with altered steady state levels according to www.expasy.org and www.harvester.embl.de revealed that 41% of the responsive proteins play a role in intermediary metabolism (Fig. 3). Nineteen percentage of the regulated proteins are involved in gene regulation. Another 16% represent cytoskeletal proteins that are probably changed as an indicator of ongoing apoptosis (Fig. 3) which can be triggered by changes in the levels of proteins with a direct link to programmed cells death (3%). Finally 18% of the responses are observed in proteins that enhance the stress response of cells such as chaperones and detoxification proteins indicating adaptations of the cancer cell to the death-inducing agent.

Camptothecin- and apoptosis-specific effects in the response of the HT-29 proteome

Camptothecin was applied to the cells at a concentration of 50 μM since apoptosis induction under these conditions is equal to that observed with 150 μM flavone.[9] 2D-PAGE of proteins from camptothecin-treated cells revealed the regulation of 36 protein in the pI-range 4–7, with the identification of 25 by MALDI-TOF MS (Fig. 4, Table III) and of 20 proteins in the pI range 6–11 with the identification of 15 (Fig. 5, Table IV). Surprisingly, many of the proteins that were altered in steady state levels by flavone were responsive in a very similar manner to camptothecin (Tables I–IV). However, a shift in regulated proteins could be observed in camptothecin-treated cells as compared to flavone-treatment from those playing a role in metabolism (31 *vs.* 41%) to those belonging to the class cytoskeleton (32 *vs.* 16%) (Fig. 6). In cells exposed to camptothecin especially the increase of lamins, which are proteins of the nuclear envelope, appeared as a specific response to camptothecin (Table III).

Discussion

Colorectal cancer is still the second leading cause of cancer-related deaths for both men and women in Western countries and improved therapies with reduced side effects are therefore needed.[18] Flavone could be such a therapeutic or adjuvant drug targeting specifically colon cancer cells through its interference with aerobic glycolysis,[12] a metabolic alteration that is typical for transformed but not for nontransformed cells.[19–21] In our study we used a proteomic approach to identify cellular adaptations that mediate those apoptosis-inducing effects that are specific for this flavonoid in HT-29 human colon cancer cells. The proteome changes were compared to those exerted by a classical antitumor drug, the topoisomerase-I inhibitor camptothecin. We used a split pH-gradient in the first dimension of 2D-PAGE to enhance resolution of separated proteins and thereby sensitivity in the identification of responding proteins.

An interesting finding was that out of 27 protein entities affected significantly in amount by flavone and out of 34 that responded to camptothecin 20 proteins were regulated by both compounds in an almost identical manner. Many of these proteins could be linked directly to apoptosis and should be regarded consequently as markers of an apoptotic response which is independent on the apoptotic stimulus. In this regard, those proteins affected by both antitumor drugs could possibly serve as markers to indicate the efficiency of chemotherapy. Of the T-complex protein 1, e.g., three subunits were upregulated by flavone and also

TABLE IV – PROTEINS WITH PI BETWEEN 6 AND 11 AFFECTED IN STEADY-STATE LEVEL BY CAMPTOTHECIN

Spot no.	Protein description	Swiss prot acc. no.	Sequence-cover. (%)	Mw/pI theor.	Mw/pI exp.	Subcellular location	Reg.-factor C/control
	Gene regulation						
1	Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	42	37/9.0	40/8.6	Nuclear; component of ribonucleosomes	3.56
2	Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	44	36/8.7	31/7.9	Nuclear; component of ribonucleosomes	0.49
3	RNA-binding protein Raly	Q9UKM9	31	33/9.2	44/9.9	Nucleus	0.32
	Kinases						
4	*Phosphoglycerate kinase 1*	P00558	24	45/8.3	47/7.2	Cytoplasm	0.42
	Metabolism						
5	Alpha-enolase	P06733	51	47/7.0	54/7.7	Cytoplasm, cell membrane, nucleus	2.76
6	Alpha-enolase	P06733	53	47/7.0	53/6.7	Cytoplasm, cell membrane, nucleus	0.37
7	*Aspartate aminotransferase, mitochondrial [Precursor]*	P00505	45	48/9.1	48/9.9	Mitochondrion; mitochondrial matrix	Only in C
8	*ATP synthase O subunit, mitochondrial [Precursor]*	P48047	62	23/10.0	26/11.0	Mitochondrion; mitochondrial matrix	2.30
9	Cyclophilin B, Chain A	P23284	61	20/9.2	20/10.5	Endoplasmic reticulum	9.39
10	*GIPC PDZ domain-containing protein 1*	O14908	56	36/5.9	44/6.3	Cytoplasm, membrane; peripheral membrane protein	0.49
11	Glyceraldehyde-3-phosphate dehydrogenase	P04406	49	36/8.6	40/8.4	Cytoplasm	2.11
12	*Inosine-5′-monophosphate dehydrogenase 2*	P12268	31	56/6.4	63/6.9	Mitochondrion	0.49
13	*Low molecular weight phosphotyrosine protein phosphatase*	P24666	40	18/7.0	19/7.3	Cytoplasm	0.41
14	*NAD(P)H dehydrogenase [quinone] 1*	P15559	38	31/8.9	34/9.9	Cytoplasm	2.07
	Cytoskeletal						
15	Keratin, type I cytoskeletal 18	P05783	41	48/5.3	30/7.0	Intermediate filament associated	2.32

The spot numbers are identical to those given in Figure 5. Abbreviations are explained in the legend to Table III. Identical proteins that were identified as regulated at both pH-gradients are indicated only once, i.e., in Table III and not in addition in Table IV; Proteins that responded only to camptothecin but not to flavone are shown in italics; ($n = 6$).

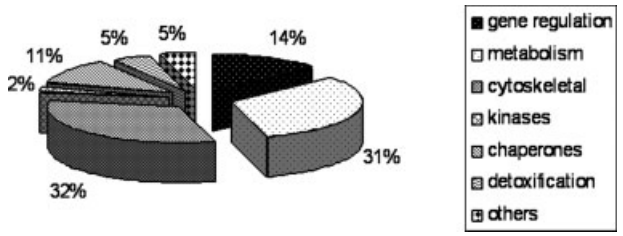

FIGURE 6 – Pie chart with the percentage of proteins affected in amount by 50 μM camptothecin belonging to individual protein classes.

camptothecin. T-complex proteins are essential in the folding of proteins to produce stable and functionally competent protein conformations.[22] In this context, their increased expression must be regarded as a cellular stress response of the cells to the exposure with flavone or camptothecin. Indeed we have previously found the same response in endothelial cells that were stressed with oxidized-LDL or homocysteine and both stressors caused endothelial cell apoptosis.[23,24] Nevertheless it must be emphasized that those T-complex subunits that displayed increased expression were suggested also to play a role in colorectal cancer progression.[22] Indicative for a cellular defense mechanism against the apoptosis-inducing agents appears also the increased levels of glutathione-*S*-transferase P and peroxiredoxin 4. Glutathione-*S*-transferase P has been implicated in the development of resistance toward chemotherapy agents[25] and increased levels of peroxiredoxin 4 were found in a variety of cancerous tissues when compared to nontumor tissues.[26]

Metabolic alterations by flavone and camptothecin became also evident by increased levels of delta 3,5-delta 2,4-dienoyl-CoA isomerase, an enzyme facilitating the flux of unsaturated fatty acids through beta-oxidation.[27] We have previously shown that flavone can increase the supply of long-chain fatty acids to mitochondria of HT-29 cells end promote their β-oxidation which is also followed by increased generation of mitochondrial ROS.[28] Such a higher throughput of oxidizable substrates through citric acid cycle and respiratory chain, however, is not only suggested for fatty acids but also for glycolysis products. An increased expression of glyceraldehyde-3-phosphate dehydrogenase and alpha-enolase may be taken as an indicator for this.

Finally a number of cytoskeletal proteins were increased in expression level by flavone as well as camptothecin. Cytoskeletal proteins are known to be cleaved when apoptosis is initiated.[29] It is therefore interesting that the levels of some increased rather than decreased. However, lamins for example are released from the nucleus into the cytosol during apoptosis and they appear to be more effectively extracted from the cytosol than from nuclear sources during protein isolation for proteome analysis. This assumption is substantiated by the increased levels of proliferation-associated protein 2G4 found in flavone-treated cells in spite of a reduced proliferation of the cells. Indeed proliferation-associated protein 2G4 is present in the nucleus only in the G1 and mid S phase, followed by a low nuclear abundance at the end of S phase, and its nuclear absence at the S/G2 transition.[30] A cytoskeleton-associated protein, stathmin, was reduced in levels by both apoptosis-inducing compounds. Stathmin is involved in cell cycle progression and has been shown to be linked to tumor progression.[31] An almost 12- and 10-fold increased expression of cyclophilin B in flavone- and camptothecin-treated cells may also be

taken as a robust marker for efficient apoptosis induction since this protein has been shown to participate in the induction of chromosomal DNA-degradation during cell death execution.[32]

In addition to these common changes in cellular proteins caused by flavone as well as camptothecin there were a few proteins that changed in amount specifically in response to either of the two compounds. Those alterations might serve as more specific indicators resembling the different mechanisms underlying their apoptosis-inducing activity. Flavone actions were characterized not only by increased expression of enzymes of the β-oxidation and glycolysis pathways but also of proteins which are relevant for electron transport in the respiratory chain. Those encompass ubiquinol cytochrome-c reductase and coproporphyrinogen III oxidase. Whereas the first is a constituent of complex III of the respiratory chain,[33] the latter is involved in porphyrin and thus cytochrome synthesis.[34] Both adaptations may enable a higher flux of electrons through the respiratory chain associated with an increased one-electron transfer onto molecular oxygen giving rise to ROS and finally ROS-induced apoptosis. The increased levels of glucose-6-phosphate dehydrogenase in flavone-treated cells may be taken as a mechanism to compensate for the enhanced production of ROS by increased delivery of NADPH for regeneration of oxidized thiols including glutathione-*S*-transferase-P and peroxiredoxin-4 related processes. However, the flavone-specific down-regulation of thioredoxin may allow ROS-driven apoptosis to prevail, as enhanced thioredoxin expression was shown to inhibit apoptosis.[35] The apoptosis induction by flavone may also be transmitted through the increased expression of programmed cell death 6-interacting protein, which appears to be involved in mediating cell sensitivity to cytotoxic drugs.[36]

Camptothecin-specific effects on the proteome of HT-29 cells are predominantly characterized by increased levels of lamin A, lamin A/C transcript variants and lamin A mutant forms (progerins).[37] As all of them are proteins of the nuclear envelope it must be suggested that they are released from their original location into the cytosol because of nuclear fragmentation as a consequence of topoisomerase-I inhibition. However, although nuclei from flavone-treated cells fragment as well,[9,12] increased levels of lamin-type proteins seem a specific marker for agents that initiate apoptosis within the nucleus. Further camptothecin-specific effects relate to an increased expression of NAD(P)H dehydrogenase, which generally protects cells against ROS-damage but is also able to trigger apoptosis by activating cytotoxic agents in tumor cells,[38] and to reduced levels of inosine-5′-monophosphate dehydrogenase 2, whose inhibition was found to induce differentiation of cancer cells.[39]

In conclusion, HT-29 human colon cancer cells display a very similar response in their proteome to the exposure *versus* flavone or camptothecin. Many of the alterations point to an induction of programmed cell death. There are, however, also agent-specific responses pointing at an increased substrate oxidation by flavone and disturbances of the nuclear envelope by camptothecin as the main mechanisms underlying the specific induction of apoptosis by these compounds.

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft (WE 2684/3-1 and 3-2) to Dr. U. Wenzel.

References

1. Pajak B, Orzechowski A. Overview how adenocarcinoma cancer cells avoid immune- and chemotherapy-induced apoptosis. Adv Med Sci 2006;51:39–45.
2. Hengartner MO. The biochemistry of apoptosis. Nature 2000;407: 770–6.
3. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100:57–70.
4. Nicholson DW. ICE/CED3-like proteases as therapeutic targets for the control of inappropriate apoptosis. Nat Biotechnol 1996:14:297–301.
5. Nicholson DW. From bench to clinic with apoptosis-based therapeutic agents. Nature 1996;407,810–16.
6. Legarza K, Yang LX. New molecular mechanisms of action of camptothecin-type drugs. Anticancer Res 2006;26:3301–5.
7. Pommier Y. Topoisomerase I inhibitors: camptothecins and beyond. Nat Rev Cancer 2006;6:789–802.
8. Giles GI, Sharma RP. Topoisomerase enzymes as therapeutic targets for cancer chemotherapy. Med Chem 2005;1:383–94.
9. Wenzel U, Kuntz S, De Sousa UJ, Daniel H. Nitric oxide suppresses apoptosis in human colon cancer cells by scavenging mitochondrial superoxide anions. Int J Cancer 2003;106:666–75.
10. Wenzel U, Kuntz S, Daniel H. Nitric oxide levels in human preneoplastic colonocytes determine their susceptibility toward antineoplastic agents. Mol Pharmacol 2003;64:1494–502.
11. Winkelmann I, Diehl D, Oesterle D, Daniel H, Wenzel U. The suppression of aberrant crypt multiplicity in colonic tissue of 1,2-dimethylhydrazine-treated C57BL/6J mice by dietary flavone is associated with an increased expression of Krebs-cycle enzymes. Carcinogenesis 2007;28:1446–54.
12. Wenzel U, Schoberl K, Lohner K, Daniel H. Activation of mitochondrial lactate uptake by flavone induces apoptosis in human colon cancer cells. J Cell Physiol 2005;202:379–90.
13. Rabilloud T. Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis. Electrophoresis 1998; 19:758–60.
14. Gorg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 2000;21:1037–53.
15. Gorg A, Obermaier C, Boguth G, Weiss W. Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: wide pH gradients up to pH 12, longer separation distances and simplified procedures. Electrophoresis 1999;20:712–17.
16. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227,680–5.
17. Gobom J, Schuerenberg M, Mueller M, Theiss D, Lehrach H, Nordhoff E. Alpha-cyano-4-hydroxycinnamic acid affinity sample preparation. A protocol for MALDI-MS peptide analysis in proteomics. Anal Chem 2001;73:434–8.
18. Schulmann K, Reiser M, Schmiegel W. Colonic cancer and polyps. Best Pract Res Clin Gastroenterol 2002;16:91–114.
19. Brooks GA. Lactate shuttles in nature. Biochem Soc Trans 2002;30: 258–64.
20. Gatenby RA. The potential role of transformation-induced metabolic changes in tumor-host interaction. Cancer Res 1995;55:4151–6.
21. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? Nat Rev Cancer 2004;4:891–9.
22. Coghlin C, Carpenter B, Dundas SR, Lawrie LC, Telfer C, Murray GI. Characterization and over-expression of chaperonin t-complex proteins in colorectal cancer. J Pathol 2006;210:351–7.
23. Fuchs D, Erhard P, Turner R, Rimbach G, Daniel H, Wenzel U. Genistein reverses changes of the proteome induced by oxidized-LDL in EA.hy 926 human endothelial cells. J Proteome Res 2005;4:369–76.
24. Fuchs D, Erhard P, Rimbach G, Daniel H, Wenzel U. Genistein blocks homocysteine-induced alterations in the proteome of human endothelial cells. Proteomics 2005;5:2808–18.
25. Salinas AE, Wong MG. Glutathione *S*-transferases—a review. Curr Med Chem 1999;6:279–309.
26. Khalil AA, James P. Biomarker discovery: a proteomic approach for brain cancer profiling. Cancer Sci 2007;98:201–13.
27. Ren Y, Schulz H. Metabolic functions of the two pathways of oleate β-oxidation double bond metabolism during the β-oxidation of oleic acid in rat heart mitochondria. J Biol Chem 2003;278:111–16.
28. Wenzel U, Nickel A, Daniel H. Increased mitochondrial palmitoylcarnitine/carnitine countertransport by flavone causes oxidative stress and apoptosis in colon cancer cells. Cell Mol Life Sci 2005;62:3100–5.
29. Broers JL, Bronnenberg NM, Kuijpers HJ, Schutte B, Hutchison CJ, Ramaekers FC. Partial cleavage of A-type lamins concurs with their total disintegration from the nuclear lamina during apoptosis. Eur J Cell Biol 2002;81:677–91.
30. Radomski N, Jost E. Molecular cloning of a murine cDNA encoding a novel protein, p38-2G4, which varies with the cell cycle. Exp Cell Res 1995;220:434–45.

31. Yuan RH, Jeng YM, Chen HL, Lai PL, Pan HW, Hsieh FJ, Lin CY, Lee PH, Hsu HC. Stathmin overexpression cooperates with p53 mutation and osteopontin overexpression, and is associated with tumour progression, early recurrence, and poor prognosis in hepatocellular carcinoma. J Pathol 2006;209:549–58.
32. Nagata T, Kishi H, Liu QL, Yoshino T, Matsuda T, Jin ZX, Murayama K, Tsukada K, Muraguchi A. Possible involvement of cyclophilin B and caspase-activated deoxyribonuclease in the induction of chromosomal DNA degradation in TCR-stimulated thymocytes. J Immunol 2000;165:4281–9.
33. Schagger H, Pfeiffer K. Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. EMBO J 2000;19:1777–83.
34. Woodard SI, Dailey HA. Multiple regulatory steps in erythroid heme biosynthesis. Arch. Biochem. Biophys. 2000;384:375–8.
35. Welsh SJ, Bellamy WT, Briehl MM, Powis G. The redox protein thioredoxin-1 (Trx-1) increases hypoxia-inducible factor 1α protein expression: Trx-1 overexpression results in increased vascular endothelial growth factor production and enhanced tumor angiogenesis. Cancer Res 2002;62:5089–95.
36. Stewart JJ, White JT, Yan X, Collins S, Drescher CW, Urban ND, Hood L, Lin B. Proteins associated with Cisplatin resistance in ovarian cancer cells identified by quantitative proteomic technology and integrated with mRNA expression levels. Mol Cell Proteomics 2006;5:433–43.
37. Dechat T, Shimi T, Adam SA, Rusinol AE, Andres DA, Spielmann HP, Sinensky MS, Goldman RD. Alterations in mitosis and cell cycle progression caused by a mutant lamin A known to accelerate human aging. Proc Natl Acad Sci USA 2007;104:4955–60.
38. Cavelier G, Amzel LM. Mechanism of NAD(P)H:quinone reductase: ab initio studies of reduced flavin. Proteins 2001;43:420–32.
39. Floryk D, Tollaksen SL, Giometti CS, Huberman E. Differentiation of human prostate cancer PC-3 cells induced by inhibitors of inosine 5′-monophosphate dehydrogenase. Cancer Res 2004;64:9049–56.