MODULATION OF POLYAMINE METABOLISM AS A CHEMOPREVENTIVE STRATEGY OF PHYTOCHEMICALS IN A CELL CULTURE MODEL OF COLORECTAL CANCERS

Dissertation zur Erlangung des Doktorgrades

Dr. oec. troph.

im Fachbereich Agrarwissenschaften, Ökotrophologie und Umweltmanagement

der Justus Liebig Universität Giessen

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"Let thy food be thy medicine and thy medicine be thy food."
Hippocrates (460-377 B.C.)

SUMMARY

Introduction: Resveratrol a natural occuring polyphenol present in red wine, peanuts and grapes, has been reported to exhibit a wide range of biological and pharmacological properties. In addition to cardioprotective and antiinflammatory effects, potent chemopreventive activities of resveratrol and its analogs in various carcinogenesis models are described and there has been a great deal of experimental effort directed toward defining these effects. We and others could previously demonstrate that resveratrol inhibits cell growth in several malignant cell lines via modulation of polyamine metabolism. In detail, resveratrol was shown to simultaneously inhibit biosynthetic ornithine decarboxylase (ODC) and activate catabolic spermine/spermidine acetyltransferase (SSAT). One aim of this work was to specify the underlying molecular mechanisms of resveratrol actions in colorectal cancer cells and especially to identify possible roles of transcription factor peroxisome-proliferator activated receptor γ (PPARγ) and the sphingolipid metabolite ceramide.

Previous studies could demonstrate that ursolic acid (UA), a pentacyclic triterpene found in berries and plants, has antiproliferative as well as proapoptotic activities on cancer cells. The objective of this second project was to elucidate the underlying molecular mechanisms of these chemopreventive effects.

Methods: The colorectal cancer cell lines Caco-2, HCT-116 and HT29 were cultured under standard conditions and were treated with miscellaneous agents for different time intervals. Cytotoxicity was excluded by commercial kit measuring lactate dehydrogenase activity in the supernatant of damaged cells. Cell growth was determined by BrdU incorporation and crystal-violet staining. Protein levels were examined by Western blot analysis. The activity of the enzyme SSAT was assayed with a radiometric technique measuring the amount of synthesized [acetyl-^{14}C]-spermidine. ODC activity was also assayed radiometrically measuring [$^{14}CO_2$]-liberation. Ceramide concentrations were detected by HPLC-coupled mass-spectrometry. A dominant-negative PPARγ mutant was transfected in Caco-2 cells to suppress PPARγ-mediated functions.

PPARγ ligand dependent transcriptional activity was measured via a luciferase assay. Apoptosis induction was detected by measuring DNA fragmentation. Caspase-3 induction was determined via an activity assay.

Results: Resveratrol [30-200 µmol/L] inhibits cell growth both in Caco-2- and HCT-116 cells in a dose- and time-dependent manner. In contrast to Caco-2-wildtype- resveratrol failed to increase SSAT activity in dominant-negative-PPARγ-cells. PPARγ involvement was further confirmed via ligand dependent activation as well as the induction of specific PPARγ-dependent target Cytokeratin 20 after resveratrol-treatment. Resveratrol further increases the expression of PPARγ coactivator PGC-1α as well as SIRT1 in a dose-dependent manner after 24h of incubation. Co-incubation with SB203580 abolishes SSAT activation significantly in both cell lines. The involvement of MAPK p38 was further confirmed by a resveratrol-mediated phosphorylation of p38 protein in both cell lines. Resveratrol further increases the expression of PPARγ coactivator PGC-1α as well as SIRT1 in a dose-dependent manner.

Moreover, the antiproliferative effects of resveratrol closely correlate with a dose-dependent increase of endogenous ceramides. Compared to controls the cell-permeable ceramide analogs C2- and C6-ceramide significantly inhibit ODC-activity in Caco-2 and HT29 cells. C6-ceramide further diminished protein levels of protooncogenes c-myc and ODC, which is strictly related to the ability of ceramides to inhibit cell growth in a time- and dose-dependent manner. These results were further confirmed using inhibitors of sphingolipid metabolism, where only co-incubation with a serine palmitoyltransferase inhibitor could significantly counteract resveratrol-mediated actions. These data suggest that the induction of ceramide *de novo* biosynthesis but not hydrolysis of sphingomyelin is involved in resveratrol-mediated inhibition of ODC. The relevance of intracellular polyamine depletion was further confirmed by exogenous polyamines which could counteract the growth inhibitory effects mediated by resveratrol. In contrast to the regulation of catabolic SSAT by resveratrol, inhibitory effects on ODC occur PPARγ-independently, indicating independent pathways of resveratrol-action.

We could also show, that treatment with UA leads to a significant time- and dose-dependent cell growth inhibition of Caco-2, HCT-116 as well as HT29 cells, coincident with the upregulation of the cell cycle regulators cyclin E,

$p21^{WAF1/Cip1}$ and $p27^{Kip1}$. In addition, UA significantly induces apoptosis, which is mediated by an increase of BAX/Bcl-2-protein-ratio as well as an upregulation of TRAIL protein which meets in an induction of caspase-3 activity. Furthermore, we could show that UA leads to a PPARγ-dependent induction of SSAT but in contrast to resveratrol does not inhibit biosynthetic ODC activity.

Conclusions: On the basis of these findings, p38 MAPK as well as transcription factor PPARγ can be considered as molecular targets of resveratrol in the regulation of cell proliferation and SSAT activity respectively in a cell culture model of colon cancer. Moreover, the results provide evidence for the involvement of ceramide *de novo* biosynthesis in resveratrol mediated inhibition of ODC activity.

The observed reduction of cell growth of colon cancer cell lines after treatment with ursolic acid presumably results from a large increase in the number of apoptotic cells. The induction of the catabolic enzyme SSAT via PPARγ-dependent mechanisms therby seems to present the major molecular target in the induction of programmed cell death.

Due to these results the phytochemicals resveratrol as well as ursolic acid could show great chemopreventive and therapeutic potential in the treatment of colorectal cancers.

ZUSAMMENFASSUNG

Einleitung: Resveratrol (3, 4‘, 5-Trihydroxy-trans-stilben) ist ein natürlich vorkommendes Polyphenol, welches vorwiegend in Trauben, Erdnüssen und Rotwein zu finden ist. Neben kardioprotektiven und antiinflammatorischen Wirkungen werden Resveratrol auch verschiedene chemopräventive Eigenschaften zugesprochen. In früheren Untersuchungen konnten wir bereits zeigen, dass Resveratrol die Hemmung des Zellwachstums in kolorektalen Karzinomzellen, zumindest teilweise, über die Modulation des Polyaminstoffwechsels vermittelt, zum einen über die Hemmung der bisynthetischen Ornithin Decarboxylase (ODC) und zum anderen über die Aktivierung der katabolen Spermidin/Spermin-Acetyltransferase (SSAT). Ein Ziel unserer weiterführenden Untersuchungen war es nun, die molekularen Mechanismen dieser Resveratrol-vermittelten Effekte genauer zu charakterisieren. Von besonderem Interesse waren dabei die mögliche Beteiligung des nukleären Transkriptionsfaktors Peroxisom-Proliferator aktivierter Rezeptor γ (PPARγ), sowie der Ceramide, eine zu den Lipiden zählende Untergruppe der Sphingolipide.

Die Ursolsäure (UA) ist ein pentacyclisches Triterpen, das überwiegend in Beeren, Früchten, Kräutern und der natürlichen Wachsschicht von Äpfeln und Birnen zu finden ist. Neben antiinflammatorischen und hepatoprotektiven Wirkungen weisen epidemiologische Untersuchungen auch auf chemopräventive Effekte der UA hin. Ein weiteres Ziel war es daher, auch die molekularen Mechanismen dieser Effekte näher zu charakterisieren

Methodik: Die kolorektalen Karzinomzelllinien Caco-2, HCT-116 und HT29 wurden unter Standardbedingungen kultiviert. Die Zellen wurden über definierte Zeiträume mit steigenden Konzentrationen verschiedener Substanzen inkubiert. Zytotoxische Wirkungen der eingesetzten Konzentrationen wurden in Vorversuchen mittels eines Zytotoxizitätstestes (Messung der Laktat Dehydrogenase-Freisetzung) ausgeschlossen. Die Zellzahl wurde anhand des BrdU Einbaus in die DNS, die Zellzahl mittels Kristallviolettfärbung ermittelt. Verschiedene Proteine wurden durch Western Blot Analyse dargestellt und densitometrisch ausgewertet. Die Aktivität der SSAT wurde mittels [Acetyl-^{14}C]-

Spermidin-Bindungsassay quantifiziert. Zur Aktivitätsbestimmung der ODC wird radioaktiv markiertes Substrat zum Zelllysat gegeben und die Freisetzung von [$^{14}CO_2$] gemessen. Zur Hemmung der PPARγ-vermittelten Funktionen wurde eine dominant-negative Mutante in Caco-2-Zellen transfiziert. Die Ceramidbiosynthese nach Resveratrol-Inkubation wurde mit HPLC-Massenspektrometrie erfasst. Die Ermittlung der Liganden-bindungsabhängigen PPARγ-Promotoraktivität erfolgte mittels Luziferase-Assay. Die Erfassung der Caspase-3-Aktivität sowie der DNA-Fragmentierung mittels ELISA dienten als Marker der Apoptose-Induktion.

Ergebnisse: Resveratrol [50-200µM] führte sowohl in Caco-2- als auch in HCT-116-Zellen zu einer zeit- und dosis-abhängigen Hemmung der Zellproliferation sowie einer Reduktion der Zellzahl. Resveratrol führte signifikant zu einem Anstieg der SSAT-Aktivität nach 24h sowohl in Caco-2-Wildtyp, als auch in Caco-2-Nullvektor-Zellen, während keine Effekte detektiert werden konnten, wenn die PPARγ-vermittelten Effekte unterdrückt sind. Eine Beteiligung von PPARγ konnte zudem durch die Liganden-abhängige Promotoraktivierung, sowie durch die Induktion des spezifischen PPARγ-Targets Cytokeratin 20 bestätigt werden. Zudem führte Resveratrol dosisabhängig zur Expressionssteigerung des PPARγ Coaktivators PGC1α sowie SIRT1 nach 24h Inkubation. Co-Inkubation mit dem spezifischen MAPK p38 Inhibitor SB203580 wirkte signifikant der Resveratrol-induzierten SSAT-Aktivierung entgegen. Eine Aktivierung von p38 konnte weiterhin durch einen Anstieg von phosphoryliertem p38 auf Proteinebene nachgewiesen werden.

Koinzident mit der zeit- und dosisabhängigen Zellwachstumshemmung führt Resveratrol signifikant zu einem Anstieg der intrazellulären Ceramidkonzentration in kolorektalen Karzinomzellen. Im Vergleich zur Kontrolle hemmt das membrangängige Ceramid-Analogon Hexanoylsphingosin (C6) signifikant die Expression der Protooncogene c-myc und ODC, sowie zusätzlich die ODC-Enzymaktivität in Caco-2 und HT29-Zellen, was signifikant mit einer dosisabhängigen Hemmung des Zellwachstums durch Ceramide korrelierte. Darüber hinaus führte die Co-Inkubation mit Ceramidsyntheseinhibitoren zu einer signifikant Verminderung der Resveratrol-induzierten Effekte, was auf eine Induktion der Ceramid-Neusynthese durch Resveratrol, nicht aber auf eine Hydrolyse von Sphingomyelin schließen lässt.

Im Gegensatz zur Aktivierung der SSAT erfolgte die Hemmung der ODC durch Resveratrol PPARγ-unahbängig.

Weiterhin konnten wir zeigen, dass UA [10-30µM] zu einer signifikanten zeit- und dosisabhängigen Hemmung der Zellproliferation, sowie einer Reduktion der Zellzahl von Caco-2-, HCT-116- und HT29-Zellen führt, was mit einer deutlichen Expressionssteigerung der Zellzyklusregulatoren Cyclin E, $p21^{WAF1/Cip1}$ und $p27^{Kip1}$ korreliert. UA führte zudem koinzident zu einer dosisabhängigen Expressionssteigerung von TRAIL, sowie einem gesteigerten BAX/Bcl-2-Protein-Quotienten, was wiederum in einer gesteigerten Caspase-3-Aktivität und darauf folgender DNA-Fragmentierung resultiert. Weiterhin konnten wir zeigen, dass die Inkubation mit UA zu einer PPARγ-abhängigen SSAT-Aktivierung führt, im Gegensatz zu Resveratrol aber nicht gleichzeitig auch die Aktivität der ODC hemmt.

Schlussfolgerung: Unsere Ergebnisse weisen darauf hin, dass sowohl die Aktivierung der p38 MAPK, als auch des Transkriptionsfaktors PPARγ essentielle Ereignisse für die Resveratrol-vermittelte Induktion der katabolen SSAT darstellen. Des weiteren scheint die Aktivierung der *de novo* Ceramidbiosynthese entscheidend an der Resveratrol-induzierten ODC-Hemmung beteiligt zu sein.

Weitere Daten lassen darauf schließen, dass die wachstumshemmenden Effekte der Ursolsäure überwiegend durch Zunahme apoptotischer Zellen vermittelt werden. Die Apoptose wird dabei sowohl über extrinsische, als auch intrinsische Signaltransduktionswege induziert, wobei die PPARγ-abhängige Aktivierung der SSAT eine zentrale Rolle einzunehmen scheint.

Diese *in vitro* Daten weisen auf potente chemopräventive und –therapeutische Eigenschaften der natürlichen Pflanzeninhaltsstoffe Resveratrol und Ursolsäure hin und könnten daher vielversprechende Kandidaten in der Entwicklung neuer Therapiekonzepte in der Behandlung des kolorektalen Karzinoms darstellen.

Diese Dissertation basiert auf den folgenden Veröffentlichungen und Manuskripten, auf die im Text mit römischen Ziffern verwiesen wird.

I. **ULRICH, S.,** LOITSCH, S., RAU O., VON KNETHEN, A., BRÜNE, B., SCHUBERT-ZSILAVECZ, M., STEIN, J. (2006) Peroxisome-Proliferator activated receptor γ as a molecular target of resveratrol-induced modulation of polyamine metabolism. Cancer Res 66(14): 7348-54.

II. **ULRICH, S.,** HUWILER, A., LOITSCH, S., SCHMIDT, H., STEIN J. (2007) *De novo* ceramide biosynthesis is assaociated with resveratrol-induced inhibition of ornithine decarboxylase activity. Biochem Pharmacol 74(2):281-9.

III. **ULRICH, S.,** JUNG, B., LOITSCH, S., KAMPAN, W., STEIN, J. (2007) Ursolic acid induces apoptosis through PPARγ mediated SSAT-activation in colon cancer cells. (submitted)

IV. WOLTER, F., **ULRICH, S.,** STEIN, J. (2004) Molecular mechanisms of the chemopreventive effetcs of resveratrol and its analogs in colorectal cancer: key role of polyamines. J Nutr 134(12): 3219-22. Review

V. **ULRICH, S.,** WOLTER, F., STEIN, J. (2005) Molecular mechanisms of the chemopreventive effects of resveratrol and its analogs in carcinogenesis. Mol Nutr Food Res 49(5): 452-61. Review

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LIST OF ABBREVIATIONS

ACF	aberrant crypt foci
AOM	azoxymethane
APAF-1	apoptotic protease activating factor-1
APC	adenomatous polyposis of the colon protein
ATP	adenosine triphosphate
BAX	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2 protein
BrdU	5-bromo-2-deoxyuridine
CDK	cyclin-dependent kinase
CKI	CDK inhibitor
CRC	colorectal cancer
DFMO	2-difluoromethylornithine
DISC	death inducing signaling complex
dnPPARγ	dominant negative PPARγ
ELISA	enzyme linked immunosorbent assay
ERK	extracellular signal-regulated kinase
5-FU	5-Fluorouracil
FADD	Fas-associated death domain
FAP	familial adenomatous polyposis
HNPCC	hereditary nonpolyposis colorectal cancer
HPLC	high performance liquid chromatography
IAP	inhibitor of apoptosis protein
JNK	c-Jun N-terminal kinase
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
MAPK	mitogen activated protein kinase
MTD	maximum tolerated dose
NCI	National Cancer Institute
NSAID	non-steroidal anti-inflammatory drug
ODC	ornithine decarboxylase
PAO	FAD-dependend polyamine oxidase
PGC-1α	PPARγ coactivator 1α
PPARγ	peroxisome-proliferator activated receptor γ

Rb	retinoblastoma
SAMDC	S-adenosylmethionine decarboxylase
SERM	selective estrogen receptor modulator
SIR2	silent information regulator 2
SIRT1	sirtuin 1
Skp2	S-phase kinase associated protein 2
Smac	second mitochondria-derived activator of caspases
SMase	sphingomyelinase
SPT	serine palmitoyltransferase
SSAT	spemine/spermidine acetyltransferase
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
UA	ursolic acid
UV	ultra violet

FIGURE INDEX

TABLE INDEX

1 INTRODUCTION

1.1 Colorectal Cancer

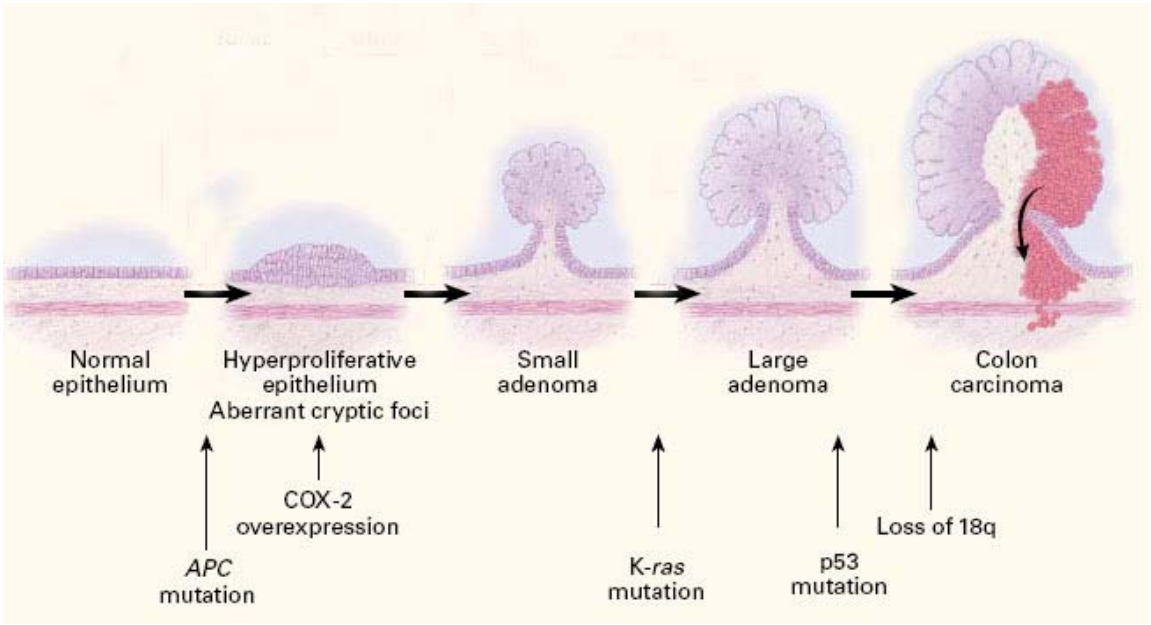


Figure 1: Adenoma-Carcinoma-Sequence

Colon cancer results from a series of pathologic changes that transform normal colonic epithelium into invasive carcinoma. Specific genetic events, shown by vertical arrows, accompany this multistep process [modified from Jänne [1]]

Colorectal cancer (CRC) accounts for approximately 10–15% of all cancers and is the second leading cause of cancer-related death in Western countries with an estimated 71400 new cases and 28868 deaths in Germany in 2002 [2] and 153760 new cases and 52180 deaths in the United States in 2007 [3]. As with many other cancers, the development of colorectal cancer typically results from a complex interaction between genetic and environmental influences. 25% of patients with colorectal cancer have a family history of the disease, which suggests the involvement of a genetic factor. Such inherited colon cancers can be divided into two main types: the well-studied but rare familial adenomatous polyposis (FAP) syndrome, which accounts for approximately 1% of cases of colon cancer annually, and the increasingly well-characterized, more common hereditary nonpolyposis colorectal cancer (HNPCC), which accounts for 5% to 15% of cases [4-7]. The remaining 75% of colorectal tumors develop sporadically or are caused by environmental or lifestyle factors such as physical

inactivity [8], obesity [9], smoking [10], alcohol consumption [11], a diet high in red meat [12], and inadequate intake of fruits and vegetables [13]. The World Cancer Research Fund estimates that 50% of colorectal tumors are avoidable through nutritional modifications [14;15].

The earliest model of pathogenesis of colorectal cancer - the adenoma-carcinoma hypothesis [16], is now widely accepted. It proposes that the initial colorectal lesion arises as a benign adenomatous polyp that later undergoes further disorganisation of cellular and tissue phenotype. Some earlier stages in the process have also been proposed: hyperproliferation of the upper crypt cells leading to the formation of aberrant crypt foci and microadenomas [17]. Vogelstein and colleagues [18-20] have provided a molecular basis for the adenoma-carcinoma sequence by describing the complex multistep process in which cells accumulate alterations of multiple genes that control cell growth and differentiation, resulting in the neoplastic phenotype (**Figure 1**).

Mutations in at least four or five genes are required for formation of a malignant tumour [14]. The first events inactivate the APC (adenomatous polyposis of the colon) gene (also the cause of FAP) in both chromosomes. APC is a tumor-suppressor gene, and when mutation eliminates its function cells are immediately launched on the pathway toward malignancy. This is followed by mutation in the oncogene *K-ras* and further mutation of the tumor suppressor genes *SMAD4* and *p53* [21]. Other genetic events also play a role, for example modulation of DNA methylation in CpG sequences of the promoter regions of tumor-suppressor and DNA-repair genes leading to inactivation or DNA amplification as a mechanism of oncogene activation [22]. These genetic alterations are associated with the development of preneoplastic lesions (aberrant crypt foci, polyps, adenomas) which can develop into carcinomas [23]. Up to 10% of all sporadic cancer types feature an additional pattern which is characterised by development of deficient DNA repair; this leads to genetic instability and, therefore, to an increased rate of mutations, prevalently in the oncogene K-*ras* [24].

1.2 Chemoprevention

Table 1 Causes of Death 1980-2003 (USA)

Cause of death	*1980*		*2003*	
	Death	*%*	*Death*	*%*
All causes	1 989 841	100.0	2 448 288	100.0
Diseases of Heart	761 085	38.3	685 089	28.0
Malignant neoplasms	416 509	20.9	556 902	22.7
Cerebrovascular diseases	170 225	8.5	157 689	6.4
Accidents	105 718	5,3	109 277	4.5
Chronic lower respiratory diseases	56 050	2.8	126 382	5.2
Influenza and pneumonia	54 619	2.7	65 163	2.7
Diabetes mellitus	34 851	1.8	74 219	3.0
Chronic liver disease and cirrhosis	30 583	1.5		
Atherosclerosis	29 449	1.5		
Suicide	26 869	1.4		
Alzheimer's disease			63 457	2.6
Nephritis, nephritic syndrome and nephrosis			42 453	1.7
Septicemia			34 069	1.4

Epidemiological data accumulated over the last 20 years show a significant decrease in the death rate within the US due to heart, cerebrovascular, and infectious diseases. However, cancer related mortality has slightly increased since 1980 [25;26]. Despite a better understanding of the disease and the advent of modern technology and rationally targeted drugs, the incidence and cure rate of cancer have not improved (**Table 1**). This failure to control cancer deaths from common epithelial malignancies provides the ultimate rationale for an approach based on prevention, before the complex series of genetic and epigenetic events that result in invasive and metastatic malignancy have occurred. It has been estimated that more than two-thirds of human cancers could be prevented through appropriate lifestyle modifications. Doll and Peto have reported that an average of 35% of human cancer mortality is attributable to diet [27], which has two rational explanations: (1) the presence of suspected carcinogens in the diet and (2) the absence in the diet of compounds possessing cancer preventing properties.

Cancer chemoprevention, as first defined in 1976 by Sporn, is the use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression [28]. Chemoprevention has been successfully

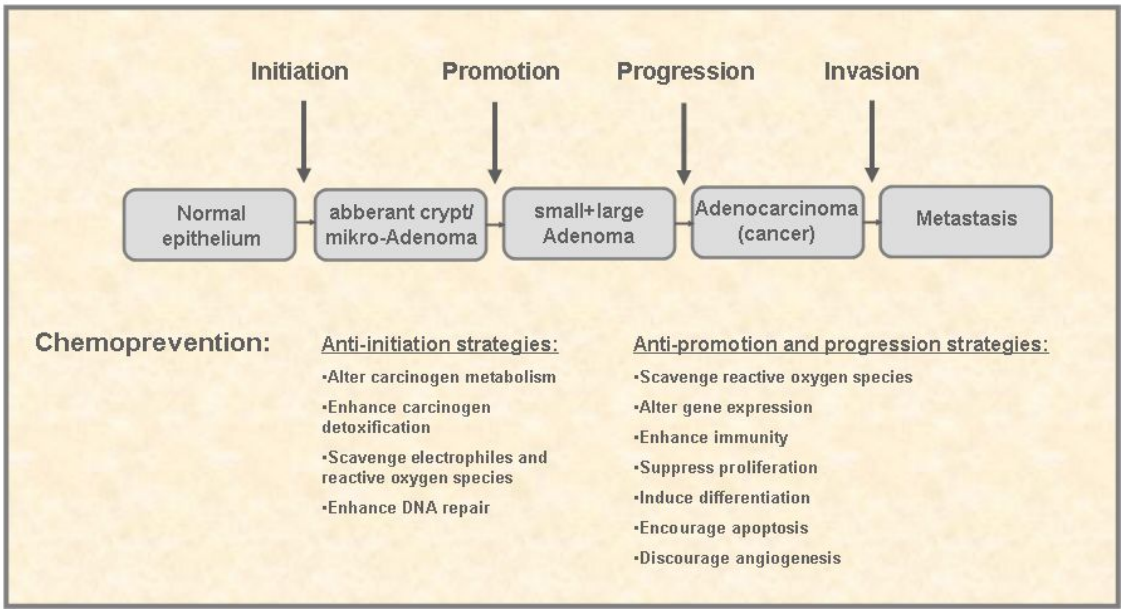


Figure 2: Chemopreventive strategies

achieved in numerous animal experiments over the past 25 years, and has been validated in several major clinical trials (for review see [29;30]) Based on these findings and following the indications of the National Cancer Institute (NCI), five classes of mainly chemically synthesized chemopreventive agents are considered of high priority: selective estrogen receptor modulators (SERMs), non-steroidal anti-inflammatory drugs (NSAIDs), calcium compounds, glucocorticoids and retinoids. In parallel, the NCI identified about 40 edible plants possessing potential chemopreventive compounds, globally known as phytochemicals [31]. These dietary agents are believed to block or reverse the premalignant stage (initiation, promotion and progression) of multistep carcinogenesis. In detail the cellular and molecular mechanisms affected by phytochemicals include carcinogen activation/detoxification by xenobiotic metabolizing enzymes; DNA repair, cell cycle progression, cell proliferation, differentiation and apoptosis, expression and functional activation of oncogenes or tumor-supressor genes, angiogenesis and metastasis and hormonal and growth-factor activity [32] (further chemopreventive strategies are reviewed in **Figure 2**).

To meet the requirements an effective and acceptable chemopreventive agent should have certain properties [33;34]:

- Little or no toxic effects in normal and healthy cells
- High efficacy against multiple sites
- Capability of oral consumption
- Known mechanism of action
- Low cost
- History of use by the human population
- Acceptance by human population

1.2.1 Cell cycle regulation

Cell division consists of two consecutive processes, mainly characterized by DNA replication and segregation of replicated chromosomes into two separate cells which is divided into two stages, namely mitosis (M), the process of nuclear division and interphase, the periode between two mitosis phases. The stages of mitosis consist of prophase, metaphase, anaphase and telophase while the interphase is subdivided in G1, S and G2 phases (reviewed in [35]) **(Figure 3)**. Each phase is characterized by distinct cellular processes that are required for proper cell division and is regulated by the activation of cyclins, which bind to cyclin-dependent kinases (CDKs) to induce cell-cycle progression towards S phase and later to initiate mitosis [36]. Different cyclins are required at different phases of the cell cycle. For the progression from G1 to S the D type cyclins bind to CDK4 and to CDK6 to form an active kinase that phosphorylates retinoblastoma (Rb) protein. In a hypophosphorylated state, Rb inhibits growth by sequestering the E2F transcription factor. Hyperphosphorylation of Rb by cyclin D-CDK4/6 results in the release of E2F, which activates transcription of cyclins for later phase transition as well as proteins required for DNA synthesis [37]. Another G1 cyclin is cyclin E which

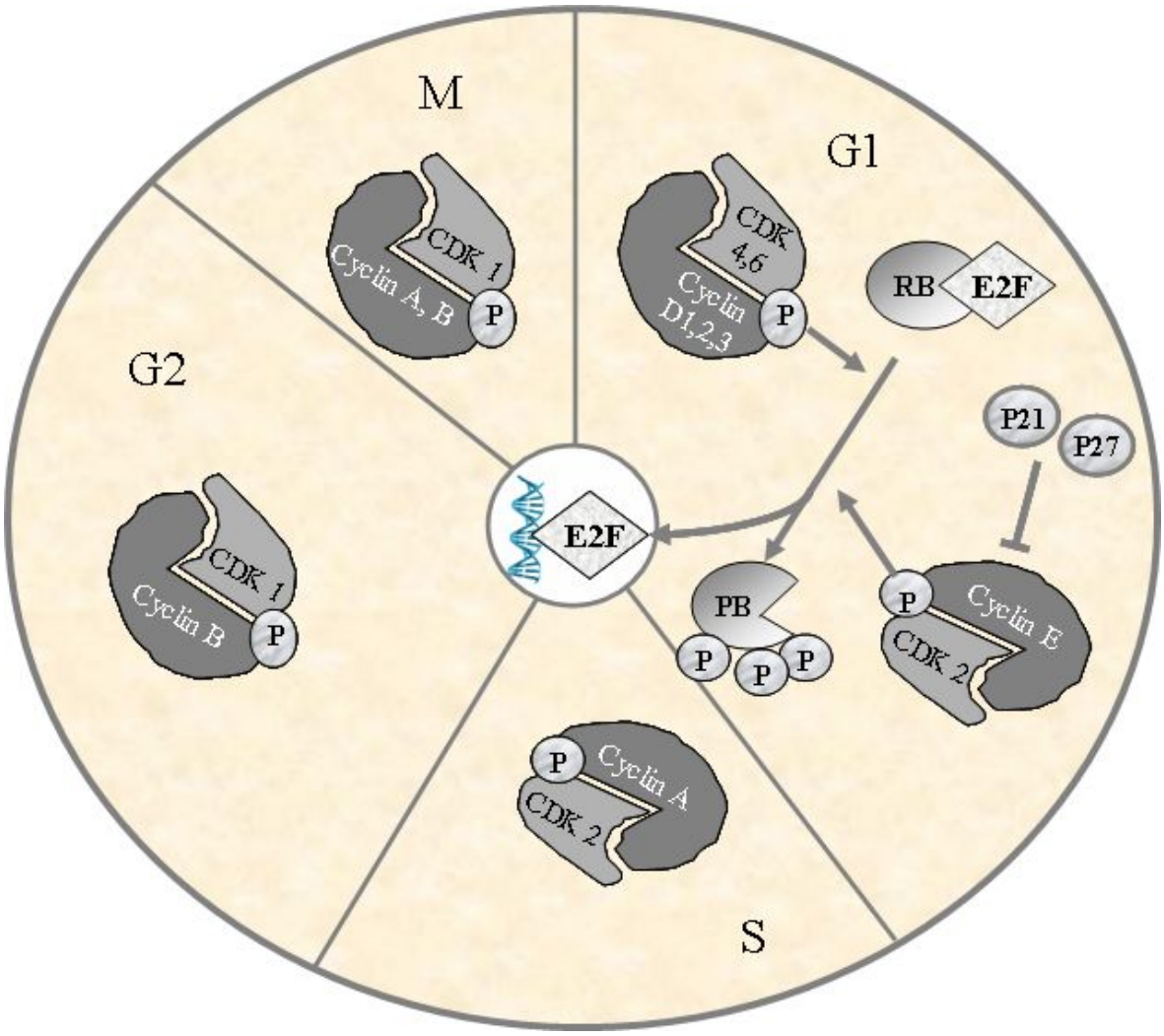


Figure 3: Phases of the cell cycle (adopted from MANUSCRIPT V)

associates with CDK2 to regulate progression from G1 into S phase [38]. Cyclin A binds with CDK2 and this complex is required during S phase [39]. In late G2 and early M, cyclin A complexes with CDK1 to promote entry into mitosis which is further regulated by cyclin B in complex with CDK1 [40]. CDK activity can be counteracted by cell cycle inhibitory proteins, called CDK inhibitors (CKI) which bind to CDK alone or to the CDK-cyclin complex. Two distinct families of CKI have been discovered, the INK4 family (p15 (INK4b), p16 (INK4a), p18 (INK4c) and p19 (INK4d)) and the Cip/Kip family (p21 (Waf1, Cip1), p27 (Cip2) and p57 (Kip2)) [41]. In cancer, there are fundamental alterations in the genetic control of cell division, resulting in an unrestrained cell proliferation. This cell cycle dysregulation occurs through mutation of proteins important at different levels of

the cell cycle, like CDKs, cyclins, CDK-activating enzymes, CKI, CDK substrates, and checkpoint proteins [42]. Hence, the control of cell proliferation represents an important preventive stragety in multistep carcinogenesis.

1.2.2 Induction of apoptosis

Apoptosis or programmed cell death is defined as an active physiologic process of cellular self-destruction, with specific morphologic and biochemical changes in the nucleus and cytoplasm [43]. The signaling events leading to apoptosis can be divided into two distinct pathways, namely the intrinsic and extrinsic pathway (**Figure 4**). Engagement of the intrinsic pathway results in altered mitochondrial membrane permeability and the release of pro-apoptotic factors including cytochrome c, caspase-9 and second mitochondria-derived activator of caspases (Smac)/DIABLO into the cytosol. Cytochrom c binds to the cytosolic protein apoptotic protease activating factor-1 (APAF-1) and procaspase-9 to form the “apoptosome”, which leads to activation of caspase-9 and subsequently caspase-3, resulting in apoptosis [44]. This pathway is primarily governed by proteins of the Bcl-2 family, which include anti- and pro-apoptotic molecules able to differentially affect mitochondrial homeostasis and cytochrome c release [45]. Moreover, other proteins belonging to the inhibitor of apoptosis protein (IAP) family are able to block a common step downstream of mitochondrial cytochrome c release by inhibiting terminal effector caspase-3 and caspase-7, and interfering with caspase-9 activity and processing [46]. A second caspase-independent pathway is characterized by the leakage of apoptosis-inducing factor (AIF) from mitochondria, resulting in direct chromatin condensation and DNA fragmentation [47]. The extrinsic pathway is characterized by ligand fixation to death receptors present on the cell surface. These death receptors are members of the TNF (tumor necrosis factor) receptor gene superfamily, which share similar, cysteine rich extracellular domains [48]. Ligation of death receptors results in recruitment of adapter

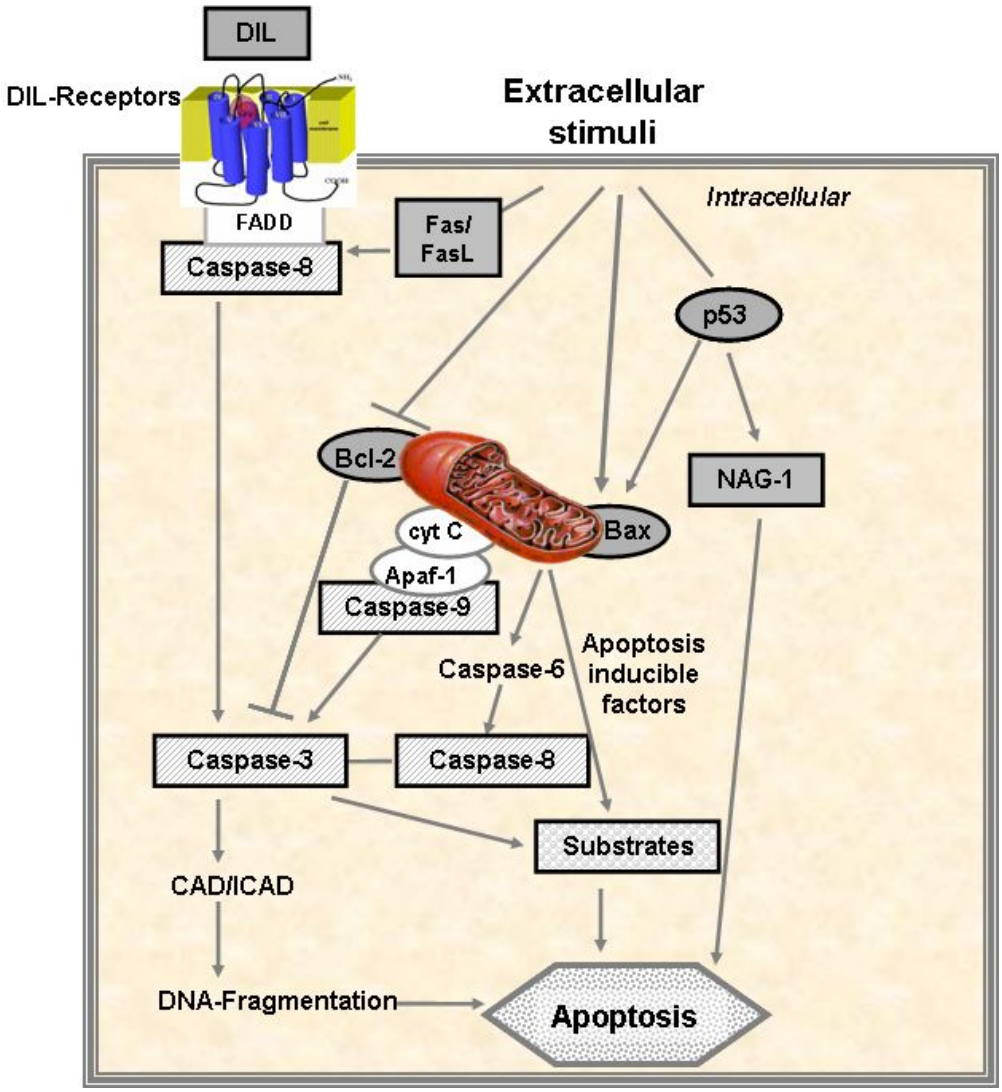


Figure 4: Apoptotic pathways (adopted from MANUSCRIPT V)

molecules such as FADD (Fas-associated death domain), which in turn recruits procaspase-8 to form the death inducing signaling complex (DISC) [49]. DISC releases caspase-8, which activates caspase-3 [50].

Apoptosis is the mechanism used by metazoans to regulate tissue homeostasis through the elimination of redundant or potentially deleterious cells. The disruption of this mechanism is observed in a variety of cancers. Therefore induction of apoptosis is arguably the most potent defense against cancer.

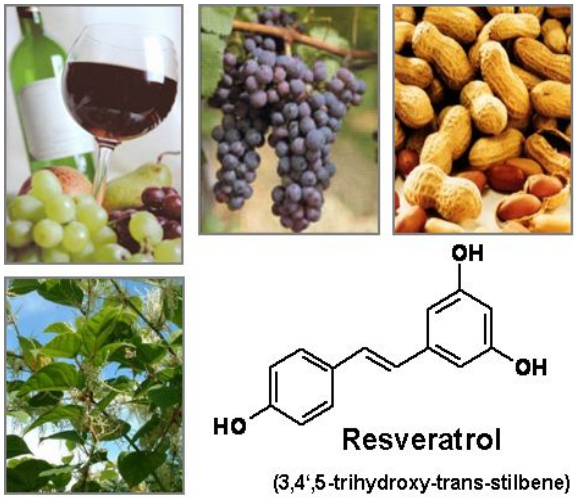
1.3 Phytochemicals

Figure 5: Dietary agents and their major biological active compounds

Information has been accumulated indicating that diets rich in vegetables and fruits can reduce the risk of a number of cancers [51]. Phytochemicals (non-nutrient components in the plant-based diet), such as carotenoids, antioxidative vitamins, phenolic compounds, terpenoids, steroids, indoles and fibers, have been considered responsible for risk reduction [52] (Dietary phytochemicals that most often appear to be protective against cancer are summarized in **Figure 5**). In this project we were focusing on the anti-carcinogenic properties of two different phytochemicals, namely the polyphenolic Resveratrol and the triterpenoid Ursolic acid which will be introduced in the following section.

1.3.1 Resveratrol

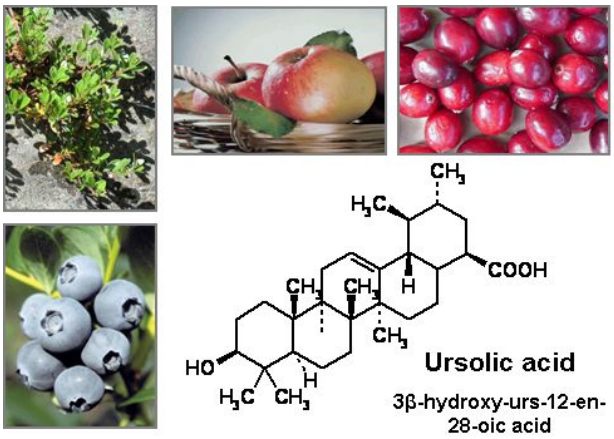
Resveratrol, chemically known as 3,5,4'-trihydroxytransstilbene (molecular weight=228.2), is a naturally occuring polyphenolic compound, also classified as a phytoalexin, which are herbal antibiotics produced in response to environmental stress factors including injuries, UV irradiation or fungal invasion [53]. Resveratrol-containing foods include grapes [54;55], wine [56], and peanuts [57;58]. An important factor for resveratrol in wine is the fermentation time in contact with grape skins because resveratrol is produced by the skin but not by the fruit flesh [59]. This explains the low concentrations in white wine because the grape skin is not fermented in the production process [56]. Resveratrol was first detected in the root extract of the weed *Polygonum cuspidatum* [60], which has been known in asian folk medicine under the name *Ko-jo-kon* and was traditionally used to treat liver, skin and circulatory diseases [61;62]. Resveratrol came to scientific attention as a possible explanation for the "French paradox" as it exhibits beneficial effects on the development of cardiovascular diseases [63]. It has been shown to inhibit platelet aggregation and eicosanoid synthesis [64], to interfere with arachidonate metabolism [65], to exert strong inhibitory effects on reactive oxygen species produced by human polymorphonuclear leukocytes [66], to be an antioxidant more powerful than vitamin E in preventing low-density lipoprotein (LDL) oxidation [67], and to exert vasorelaxing effects on endothelium-intact aorta rings of rats [68]. Further studies could show that resveratrol is an agonist for the estrogen receptor which may also be relevant to the reported cardiovascular benefits of drinking wine [69].



Anti-carcinogenic properties of reveratrol were first reported by Jang et. al demonstrating chemopreventive effectiveness against all the three major steps of carcinogenesis i.e. initiation, promotion and progression [70]. Several signal

transduction pathways have been examined to explain these effects [71;72]. We and others provide several lines of evidence, that resveratrol mediates these anti-carcinogenic effects partly through the modulation of polyamine metabolism [73-76].

1.3.2 Ursolic acid



Ursolic acid (3β-hydroxy-urs-12-en-28-oic-acid, molecular weight=456.7), the isomer of Oleanolic acid, is a pentacyclic triterpenoid compound which exists widely in natural plants in the form of free acid or as aglycones for triterpenoid saponins [77;78]. Ursolic acid naturally occurs in a large number of berries, like cranberries and blueberries [79], in the waxlike coatings of apples and pears [80] and other plants. It was also identified as the major biological active incredient in a large number of plants which are used in traditional east asian folk medicine as drugs exhibiting hepatoprotective, antiinflammatory and anti-tumor effects (for review see [81]), for example in the leaves of the Bearberry (uva ursi, urs=bear), which is the eponym of ursolic acid. Additionally, chemopreventive effects could be demonstrated in several cancer models comprising inhibition of proliferation as well as induction of apoptosis [82-84].

1.4 Polyamines

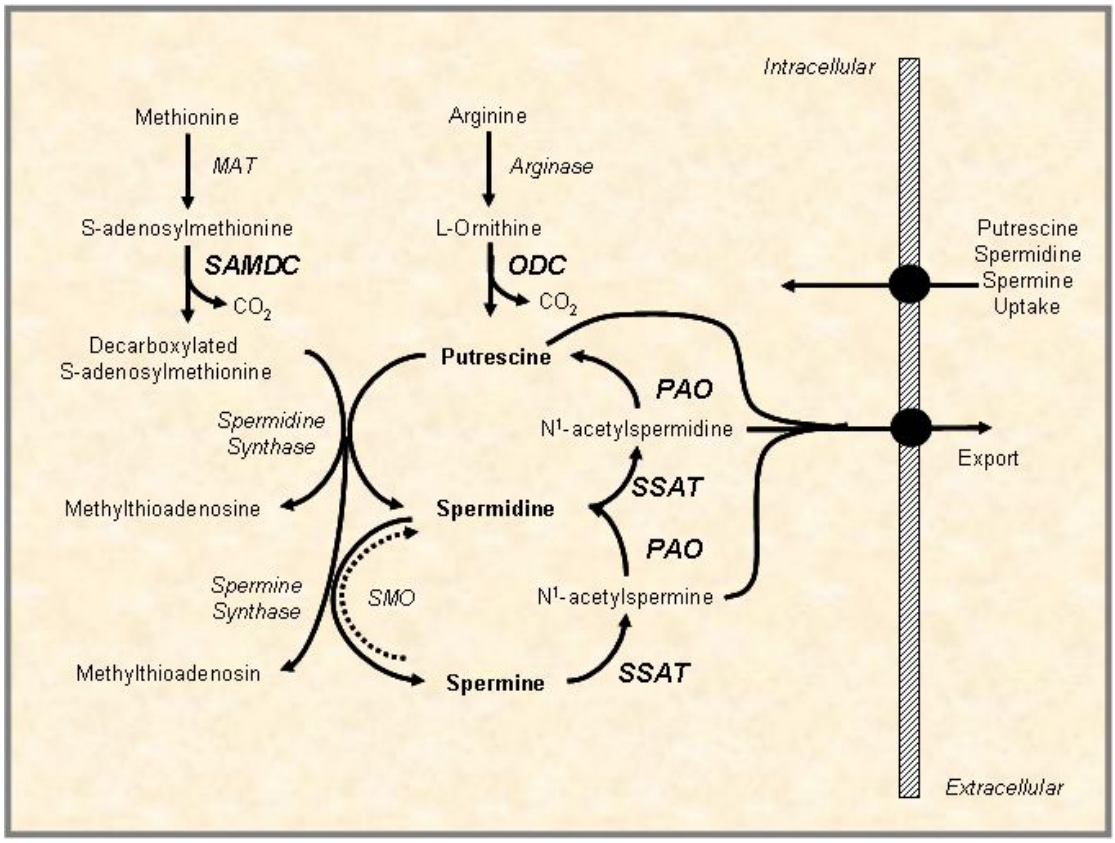


Figure 6: Polyamine metabolism

Regulation of intracellular polyamine content in mammalian cells is mediated by different key enzymes: the biosynthetic ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) and the catabolic spermidine/spermine acetyltransferase (SSAT) and FAD-dependent polyamine oxidase (PAO) (adapted from [85])

The polyamines spermidine, spermine as well as their precursor putrescine are ubiquitious polycationic metabolites in prokaryotic and eukaryotic cells which play an essential role in cell growth by stabilizing DNA structure [86], influencing membrane functions [87] and cell cycle regulating genes [88]. Increasing concentrations are generally associated with cell proliferation and cell transformations induced by growth factors, carcinogens, viruses and oncogenes [89]. Therefore intracellular polyamine pool size is controlled strictly by the combined action of de novo synthesis, catabolism, uptake and export of polyamines. This regulatory mechanism include reactions catalyzed by the biosynthetic enzymes ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) and the catabolic

spermidine/spermine acetyltransferase (SSAT) and FAD-dependent polyamine oxidase (PAO) [85] (**Figure 6**). An association of excess polyamine levels with cancer was first reported in the late 1960s, when Russel and Snyder reported high levels of ODC activity in several human cancers [90]. In colorectal cancer tissue polyamine contents are increased 3-4 fold over that found in the equivalent normal colonic tissue [91]. Based on these findings pharmacological or natural inhibitors of polyamine metabolism have been studied *in vitro* [75;92] and *in vivo* [93] as new potent therapeutic strategies in cancer treatment and prevention.

1.4.1 Inhibition of polyamine biosynthesis

The recognition that polyamines are required for cell growth and that their metabolic pathway is frequently dysregulated in cancers led to the development of inhibitors for each step of the polyamine biosynthetic pathway. 2-difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor, remains the prototypical inhibitor of ODC. DFMO initially competes with ornithine for binding to the active site of ODC and is then decarboxylated by ODC to create a highly reactive intermediate that in turn inhibits ODC activity. Even though triggering promising effects in vitro, DFMO has been less successful in cancer therapy, resulting in cytostatic rather than cytotoxic effects in vivo [94]. The limited success of DMFO has been attributed to *three factors*: (1) the tumour is able to obtain sufficient polyamines to sustain growth from the pool, since the diet, a major source of polyamines would provide a continuous exogenous supply; (2) intestinal bacteria may represent a potential limited source of polyamines; and (3) polyamines may be effectively reutilized following their release from dead cells, especially those of the gut. Consequently, it should not be surprising, that attaining the goal of reducing high polyamine levels in tissues during cancer development might require targeting more processes in the regulatory mechanism of polyamine metabolism. Studies in experimental models could already show that combinations of two drugs, specifically, combinations of DFMO and non-steroidal anti-inflammatory drugs (NSAIDs) were more effective than single-agent strategies [95;96]. One aspect of the rationale for combining ODC inhibitors and NSAIDs might be that

catabolic SSAT provides another transcriptional target for several NSAIDs [95;97].

Flamigni et al. could demonstrate that ODC expression could be regulated in leukaemia cells through exogenous ceramide-analogs. Ceramides are key compounds in the metabolism of sphingolipids and are emerging as important second messengers for various cellular processes including cell cycle arrest, differentiation and apoptosis.

1.4.2 Induction of polyamine catabolism

ODC is not the only polyamine metabolic gene that is regulated by oncogenes and tumour-suppressor genes as both SSAT and PAO activities were found to be decreased in human solid tumors [98]. In human colon and other gastrointestinal cancers the expression of spermidine/spermine acetyltranferase (SSAT) was described to be negatively regulated by the *K-ras* oncogene, which is commonly mutated – and, as a result, aberrantly activated [99]. K-ras activates the kinase Raf and suppresses expression of SSAT by inhibition of PPARγ which normally binds to PPARγ response elements in the promoter of the SSAT gene [95]. Peroxisome proliferator-activated receptors are ligand-inducible transcription factors belonging to the nuclear hormone receptor superfamily [100;101], which regulate transcription of target genes by heterodimerizing with the retinoid x receptor and binding to PPAR response elements [100;102]. PPARγ is expressed at high levels in colonic epithelial and colon cancer cells [103]. Girnun and Spiegelmann [104] hypothesize that PPARγ is exerting its effects early in the carcinogenic process by suppressing tumor formation. Activation of PPARγ therefore could function as an important molecular target of chemopreventive agents.

It was the discovery that specific antitumor polyamine analogs highly induce SSAT activity in a cell type specific manner that led to an increased interest in polyamine catabolism as a drug target. This ability of SSAT induction was suggested to correlate with the reduction of intracellular polyamines followed by cell growth inhibition and apoptosis [105-107].

1.5 Aims

Interest in the concept and practice of chemoprevention as an approach for the control of cancer has increased greatly in the past few years. Multiple natural agents have been shown to be effective for blocking carcinogenesis in certain human cancers and animal models. Using non-toxic chemical substances therefore is regarded as a promising alternative strategy to therapy for control of human cancer. The observed anti-carcinogenic effects may be due to blocking effects on the carcinogenesis stages of initiation, promotion, or progression. However, the precise underlying molecular mechanisms remain largely unknown. Thus, the aim of our study was to characterize chemopreventive effects of the two phytochemicals resveratrol and ursolic acid in a cell culture model of colorectal cancer.

2 RESULTS

2.1 PPARγ as a molecular target of resveratrol-induced modulation of polyamine metabolism (MANUSCRIPT I)

Based on our former findings that resveratrol induces cell growth inhibition of colon cancer cells via induction of catabolic SSAT, together with the identification of PPAR response elements in the promoter of the SSAT gene by Babbar et al., the aim of this work was to specify the underlying molecular mechanisms and to identify a possible role of transcription factor PPARγ. First, we determined the effects of resveratrol on cell proliferation and cell counts of the two colorectal cancer cell lines Caco-2 and HCT-116. Both cell lines were incubated with increasing concentrations of resveratrol [30-200 µmol/L] for 24, 48 and 72h. After each time interval, both cell proliferation ELISA (BrdU) and crystal violet staining were done. In HCT-116 cells, a significant time- and dose-dependent decrease in cell proliferation and cell counts could be measured. The same effects could be observed in Caco-2 cells, which is in accordance with our earlier studies [108]. Next, we tried to investigate the role of PPARγ in resveratrol-induced activation of SSAT. As no complete PPARγ antagonist have been described hitherto, we transfected a dominant-negative mutant receptor in Caco-2 cells to abolish PPARγ-mediated functions. In this mutant highly conserved hydrophobic and charged residues (Leu468 and Glu471) in helix 12 of the ligand-binding domain were mutated to alanine. So, this mutant retains ligand and DNA binding, but exhibits markedly reduced transactivation due to impaired coactivator recruitment [109]. Resveratrol leads to a significant increase of SSAT activity ($p<0.05$ vs. control) in Caco-2-wild type cells after 24h of incubation, which is in agreement with our previous data [75]. In Caco-2 empty vector cells, resveratrol also significantly increases SSAT-activity ($p<0.05$ vs. control) whereas no effects could be observed when PPARγ-mediated functions are suppressed in Caco-2-dnPPARγ mutant cells. To investigate the effects of resveratrol on PPARγ ligand-dependent activity, we did a chimeric Gal4-PPARγ transactivation assay. Because the chimeric receptor contained only hinge region and ligand binding domain of the PPARγ, any effect of

resveratrol affecting kinase-sensitive AF1 domain was ruled out. After incubation with resveratrol, we could generate similar effects of PPARγ agonist pioglitazone on PPARγ activity ($p<0.01$). To show evidence of resveratrol ability to increase PPARγ activity, we measured, after resveratrol treatment, the expression of cytokeratin 20, which is described to be a specific target gene of PPARγ activity in colorectal cancer cells [110]. Incubation with resveratrol led to an ~40% increase of cytokeratin 20 expression at after 72h ($p<0.001$ vs. control). We further did Western blot analysis to determine possible effects of resveratrol on translational level. However, no significant changes in PPARγ protein expression could be detected (unpublished data). In a next step Western blot analysis was done to determine possible effects of resveratrol on the expression of PGC1α (PPARγ coactivator 1α) and sirtuin homologue SIRT1, which exhibits PPARγ-suppressive effects in white adipocyte tissue. Resveratrol led to a significant dose-dependent increase in both PGC1α (~60%, $p<0.05$) and SIRT1 (~140%, $p<0.01$) expression after 24h of incubation.

There are several lines of evidence that resveratrol mediates its chemopreventive actions via modulation of mitogen-activated protein kinase (MAPK) pathways. To examine p38 MAPK-mediated actions, we used the specific inhibitor SB203580. This anti-inflammatory drug inhibits the catalytic activity of p38 MAPK by competitive binding in the ATP pocket [111]. Incubation with resveratrol augmented phosphorylated p38 in a time- and dose-dependent manner, both in Caco-2 and HCT-116 cells (~300% at 200 µmol/L after 16h; $p<0.01$), whereas p38 MAPK concentration remained unaffected. To characterize the role of p38 activation in resveratrol-mediated induction of SSAT, we pretreated Caco-2 and HCT-116 with p38 inhibitor SB203580 [10-20 µmol/L] for 1 hour and then added resveratrol for another 24 hours. Both in Caco-2 and HCT-116 cells, coincubation with SB203580 significantly diminished resveratol-induced SSAT activation ($p<0.05$ vs. resveratrol in Caco-2; $p<0.01$ vs. resveratrol in HCT-116). In summary, our data confirm our earlier studies showing that resveratrol-mediated gowth inhibition of colorectal cancer cells seem to involve SSAT-induced polyamine catabolism. Here, we further demonstrate that transcription factor PPARγ acts as a p38-dependent target in resveratrol-induced molecular mechanisms.

2.2 *De novo* ceramide biosynthesis is associated with resveratrol-induced inhibition of ornithine decarboxylase activity (MANUSCRIPT II)

Apart from inducing polyamine catabolism, resveratrol was also shown to inhibit key enzymes involved in the biosynthetic pathway of polyamine metabolism. And again we were interested in the underlying molecular mechanisms leading to these effects. In detail, the aim of this work was to study the potential involvement of ceramide biosynthesis in resveratrol mediated inhibition of biosynthetic ODC activity in colorectal cancer cells. First of all we examined the effect of resveratrol [50-200 µmol/L] on the intracellular ceramide concentrations of Caco-2 cells using mass-spectometry. After 24h of incubation we could observe a significant dose-dependent up-regulation of C16-ceramide levels ~6.5-fold at 200 µmol/L ($p<0.001$). Since natural ceramides are not permeant to cell membranes, our study has been carried out by using short chain cell-permeable analogs to determine the role of ceramides in these signal transduction pathways. Caco-2 and HT-29-cells were incubated with increasing concentrations of N-acetylsphingosine (C2-ceramide) and N-hexanoylsphingosine (C6-ceramide) [1-40 µmol/L] for 24-72h. After each time interval both cell proliferation ELISA (BrdU) and crystal violet staining were performed. Both in Caco-2- and HT-29-cells a significant time- and dose-dependent decrease in cell proliferation and cell counts could be measured. Resveratrol on the one hand induces intracellular ceramide synthesis and on the other hand reduces the protein levels of the protooncogenes ODC and c-myc, a transcription factor that directly regulates the expression of ODC [75]. To reveal a possible coherency, we first measured the effects of C2- and C6-ceramides in Caco-2- and HT-29 cells on ODC activity after 24h of treatment which both caused a significant inhibition in a dose-dependent manner ($p<0.001$). We further did Western blot analysis to measure effects on the protein levels of ODC and c-myc after treatment with the C6-ceramide. And actually a dose-dependent decrease both in c-myc ($p<0.001$) and ODC ($p<0.05$) protein levels comparable to the resveratrol-induced effects [75] could be observed after 6h of incubation. Two major pathways may contribute to intracellular ceramide accumulation: namely the sphingomyelinase (SMase)-

dependent catabolism of sphingomyelin, as well as the de novo synthesis catalyzed through serine palmitoyltransferase (SPT). Hence, we tested whether selective pharmacological inhibitors of these two key enzymes were able to prevent resveratrol-induced inhibition of ODC activity. While co-incubation with the SMase inhibitor manumycin [1 µmol/L] causes no changes in resveratrol action, blockade of de novo ceramide synthesis with the SPT-inhibitors L-cycloserine [1 mmol/L] and myriocin [5 µmol/L] counteracted inhibitory effects of resveratrol on ODC-activity. To further verify the involvement of ceramide synthesis in resveratrol-mediated effects we treated Caco-2 cells with resveratrol alone and in combination mith L-cycloserine and measured the protein levels of c-myc and ODC after 24h of incubation. As already shown in earlier studies resveratrol leads to a significant decrease of both c-myc ($p<0.001$) and ($p<0.001$) protein levels, which could be significantly reduced ($p<0.05$), when ceramide de novo synthesis was suppressed. To determine whether the decrease in c-myc and ODC are the cause of decreased growth rate or a result, we performed an add-back experiment with exogenous polyamines. For this we treated Caco-2 cells with spermine [50 µmol/L], resveratrol and the combination of both and measured cell counts after 48h of incubation. As spermine was able to counteract resveratrol-actions significantly, we conclude that the observed reduction of cell counts after resveratrol-treatment is due to a reduction of intracellular polyamine levels.

As shown in MANUSCRIPT I the activation of transcription factor PPARγ plays a crucial role in resveratrol-induced activation of catabolic SSAT. So we wanted to determine whether this receptor is also involved in ODC inhibition. In accordance to MANUSCRIPT I, we now investigated the effects of resveratrol on ODC activity in Caco-2-wildtype cells compared to Caco-2-cells transfected with either the empty vector or a dominant-negative PPARγ mutant after 24h. But in contrast to SSAT activation PPARγ seems not to be essential for resveratrol-induced ODC inhibition as no differences could be observed, when PPARγ mediated functions are suppressed.

2.3 Ursolic acid induces apoptosis through PPARγ mediated SSAT-activation in colon cancer cells (MANUSCRIPT III)

In addition to polyphenols, triterpenoid compounds, e.g. ursolic acid, were also described to show potent chemopreventive and anticarcinogenic properties in malignant cell lines. Little is known about the underlying molecular mechanisms of ursolic acid related effects on cell proliferation and apoptosis in colorectal cancers. Thus the major aim of this study was to analyze modulatory effects on cell cycle regulating proteins as well as pro- and anti-apoptotic factors and to further characterize signal transduction pathways leading to these chemopreventive actions.

Caco-2-, HCT-116- and HT29-cells were incubated with increasing concentrations of ursolic acid [5-30 µmol/L] for 24 h, 48 h and 72 h. After each time interval both cell proliferation ELISA (BrdU) and crystal violet staining were performed. While not effective in Caco-2 cells after 24h of incubation ursolic acid leads to significant time- and dose-dependent decrease in cell counts ($p<0.001$) as well as to a significant inhibition of cell proliferation ($p<0.001$) in all cell lines after 48h at the latest. To decipher the molecular mechanisms leading to cell growth inhibition we started to measure the expression status of several cell cycle regulating proteins, whereby the most prominent effects could be observed with the cell cycle inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$. While ursolic acid leads to a significant dose-dependent increase of $p21^{WAF1/Cip1}$ protein levels already after 24h of incubation (~ 30%, $p<0.05$), an increase of $p27^{Kip1}$ levels could not be observed until 48h of treatment (~ 40%, $p<0.05$). Another interesting change could be detected in the expression levels of cyclin E, which is essential for progression through the G1-phase of the cell cycle and for initiation of DNA replication by interacting with and activating its catalytic partner, the CDK 2 and therefore can be considered as a promotor of cell replication and proliferation. In contrast to our expectations, we could observe a significant dose-dependend increase in the protein levels of cyclin E in Caco-2 cells after 24h of incubation with ursolic acid (~ 40%, $p<0,05$).

To evaluate a possible influence of apoptosis induction on the cell growth inhibition of Caco-2, HCT-116 and HT-29 cells, we investigated DNA fragmentation as a marker of programmed cell death. Thereby, ursolic acid

causes a significant dose-dependent increase of DNA-fragments after 24h of incubation [p<0,001 vs. control in all cell lines]. Additionally, we measured caspase-3-activity in Caco-2 cells at the same point of time as a further marker of apoptotic actions. Again, ursolic acid seems to exhibit pro-apoptotic properties, as we could also observe a significant dose-dependent increase of caspase-3-activity in Caco-2-cells [p<0.001 vs. control]. To specify the underlying molecular mechanisms leading to apoptosis after incubation with ursolic acid, we examined several apoptosis regulating proteins in Caco-2 cells by Western blot analysis. To analyse the effects on the intrinsic pathway we have chosen members of the Bcl-2 family of proteins, which are known to regulate membrane permeability and cytochrome c release from mitochondria. While ursolic acid leads to an upregulation of proapoptotic BAX protein levels (up to ~20%), the expression of the antiapoptotic Bcl-2 was diminished after 24h of incubation (about ~40%). These single effects result in a significant increase of the BAX/Bcl-2 protein ratio up to 60% (p<0.001), which is generally known to trigger apoptosis. We further measured protein levels of TRAIL (TNF-related apoptosis-inducing ligand) an immunological inducer of extrinsic mechanisms leading to programmed cell death. This ligand, binding to specific death receptors on the cell surface, was also significantly upregulated after 24h of incubation with ursolic acid in a dose-dependent manner (p<0.05). As resveratrol was shown to mediate its chemopreventive effects at least partly through the modulation of polyamine metabolism (MANUSCRIPT I + II), we were interested if ursolic acid modulates similar intracellular mechanisms. Hence, we examined the effects of ursolic acid on ODC and SSAT activity in Caco-2-wildtype cells compared to Caco-2-cells transfected with either an empty vector or a dominant negative PPARγ mutant to investigate effects mediated by PPARγ. In contrast to resveratrol, ursolic acid only leads to a significant increase of SSAT activity (p<0,001 vs. control) in Caco-2-wildtype cells after 24 h of incubation but does not simultaneously inhibit ODC activity (unpublished data). In Caco-2-empty vector cells ursolic acid also significantly increases SSAT-activity (p<0,001 vs. control), whereas no effects could be observed when PPARγ mediated functions are suppressed in Caco-2-dnPPARγ mutant cells.

3 DISCUSSION

3.1 Resveratrol-induced modulation of polyamine metabolism

The phytoalexin resveratrol (3,4',5-trihydroxystilbene) exhibits multiple chemopreventive effects comprising cell growth inhibition [108;112], induction of apoptosis [113], and prevention of angiogenesis [114], whereby the underlying molecular mechanisms are only partly understood (MANUSCRIPT IV + MANUSCRIPT V). One of our theories is concerned with the role of polyamines or polyamine metabolism respectively. Intracellular polyamine levels are maintained within very narrow limits because decreases of polyamine concentrations interfere with cell growth, whereas an excess seems to be toxic [115]. The three key enzymes of polyamine metabolism are ODD and SAMDC, the rate-limiting enzymes of polyamine biosynthesis, and SSAT, which controls polyamine catabolism [116]. Wolter et al. showed that resveratrol-induced growth arrest of Caco-2 cells is accompanied by inhibition of polyamine biosynthesis as well as activation of polyamine catabolism [75]. One aim of our work was to further characterize molecular events leading to the observed modulation of polyamine metabolism.

3.1.1 Mitogen-activated protein kinases

Cells recognize and respond to extracellular stimuli by engaging specific intracellular programs, such as the signalling cascade, that leads to activation of the mitogen-activated protein kinases (MAPKs). All eukaryotic cells possess multiple MAPK pathways, which coordinately regulate diverse intracellular activities comprising gene expression, mitosis, survival and apoptosis, and differentiation. MAPKs are generally expressed in all cell types, yet their functions to regulate specific responses differ from cell type to cell type. To date, five distinct groups of MAPKs have been characterized in mammals: Extracellular signal-regulated kinase (ERK) 1/2, the p38 kinase (p38α, β, γ and δ), c-Jun N-terminal kinase (JNK) 1, 2, 3 and ERK3, ERK4, ERK5, which follow

the same principle of phosphorylation and activation cascades (Reviewed in [117]). Given the role of MAPKs in many critical responses reqired for cellular homeostasis, it is not surprising, that loss of fine control of MAPK regulation resulting from mutation or changes in expression of proteins regulating MAPK signalling contribute to cancer and thus the modulation of MAPK pathways presents an important anti-cancer strategy [118]. MAPK p38 for example has recently gained attention as a tumor suppressor, as it, upon activation, induced terminal differentiation in rhabdomyosarcoma cells [119]. Similarly, deletion of a p38-inhibitory phosphatase blocked Hras1- and erbB2-induced carcinogenesis in vivo, whereas inhibition of p38 promoted tumor formation [120]. Other studies have evaluated p38 activity in response to chemotherapy, as diverse chemotherapeutic agents stimulate apoptosis in a p38-dependent manner [121;122].

In several studies, resveratrol was also shown to mediate multiple functions by modulating MAPK pathways (Reviewed in MANUSCRIPT V and [52]). In MANUSCRIPT III we could show as well that incubation with resveratrol causes phosphorylation, and thus activation, of p38 MAPK in colon cancer cells. Furthermore, combination of resveratrol with an inhibitor of p38 MAPK leads to an inhibition of resveratrol-induced SSAT activation both in Caco-2 and HCT-116 cells. Consequently, an activation of MAPK cascade by resveratrol can be assumed in our system. But, while the activation of p38 plays a crucial role in resveratrol-induced SSAT-activation, an involvement in ceramide-mediated actions, and thus inhibition of ODC-activity (MANUSCRIPT II), is discussed controversially [123-125] and requires further investigations.

3.1.2 Peroxisome-proliferator activated receptor γ

The PPARγ is a nuclear receptor that controls the expression of a large array of genes involved in adipocyte differentiation [126], lipid metabolism [127], insulin sensitivity [128], inflammation [129] and artherogenesis [130]. There is much evidence that PPARγ plays another crucial role in carcinogenesis as it was shown to affect cell growth, differentiation and apoptosis in several malignant cell lines [131-133]. Additionally, an association between loss-of-function mutations of PPARγ with the development of colorectal cancer in humans was

documented [134]. According to the current findings, an essential role for PPARγ in enhancing SSAT enzyme activity is assumed [95]. In fact, we could show that, in contrast to Caco-2-wild type and Caco-2-empty vector cells, resveratrol failed to increase SSAT activity in Caco-2-dnPPARγ cells.

Here our results point out another important role of PPARγ in resveratrol-mediated actions whereas resveratrol-dependent PPARγ activation seem to be mediated at least partly by an activation of the ligand binding domain (LBD/ AF2) because a Gal4-PPARγ chimeric receptor was activated by resveratrol at a concentration of 100 µmol/L, and this concentration was sufficient to induce SSAT as well. In addition, our results suggest that activation of PPARγ by resveratrol is due to kinase activation, leading to phosphorylation-dependent activation of PPARγ coactivators like PGC-1α [135]. Coactivators all interact with a similar surface of the activated ligand binding domain of the receptors and have been suggested to mediate their transcriptional activity [136]. It is well established that, in addition to transcription factors, coactivators can also be targets of multiple signal transduction pathways in response to different stimuli [137]. Puigserver et al. [138] could show that PGC-1α is activated through p38 MAPK. The mechanism by which p38 activates PGC-1α is not yet clear, but it is suggested that p38 MAPK–mediated phosphorylation counteracts repressor effects, possibly by encouraging the release of a repressor from PGC-1α [139]. Upon activation, PGC-1α docks on PPARγ and thus can modulate its transcriptional activity [140]. In addition to PGC-1α, resveratrol further leads to an activation of SIRT1, a member of the silent information regulator 2 (Sir2) families of proteins (sirtuins; ref. [141]). SIRT1 is mainly linked to negative regulation of gene expression as a cofactor through protein deacetylation [142]. However, there is evidence that SIRT1 can act positively and negatively to control gene expression as a cofactor for PGC-1α. These opposite effects could possibly be due to the recruitment of a different set of coactivators and corepressors through PGC-1α/SIRT1 [143]. This could further be an explanation for the repressive effects of SIRT1 on PPARγ in white fat where PGC-1α is very low [144].

We were further interested, whether PPARγ plays another crucial role in resveratrol-induced ODC inhibition. But here, in contrast to SSAT induction,

PPARγ activation seems not to play a critical role as no differences could be observed when PPARγ mediated functions are suppressed.

3.1.3 Sphingolipid metabolism

Until the late 1970s lipids were primarily thought to serve as inert structural components of cellular membranes, but in recent years it has become more evident that lipids also act as signalling molecules to regulate fundamental cellular responses, such as cell death and differentiation, proliferation and certain types of inflammation [145-147]. One important class of membrane lipids acting as signaling molecules are the sphingolipids, which include ceramides and sphingosine. The generation of ceramide is either triggered by the action of sphingomyelinases, which hydrolyze the plasma membrane component sphingomyelin to yield ceramide and phosphorylcholine, or by de novo synthesis which is initiated by condensation of serine and palmitoyl-CoA catalyzed by SPT [148]. Various biological responses have been attributed to ceramide including cell growth inhibition and induction of apoptosis [149-151]. Furthermore, several chemotherapeutic agents have been shown to act, at least in part, by increasing tumor cell ceramide via de novo synthesis [152]. Ceramide can be metabolized by glycosylation, acetylation, or by catabolism to sphingosine, which can then be phosphorylated to the anti-apoptotic sphingosine-1-phosphate. Particularly the cellular balance between ceramide and sphingosine-1-phosphate seems to be crucial for a cell's decision to either undergo apoptosis or proliferate, two events which are implicated in tumor development and growth [153]. Thus, pharmacological manipulation of sphingolipid metabolism to enhance tumor cell ceramide offers a novel approach to cancer chemoprevention and therapy.

Interestingly, we could shown, that the antiproliferative effects of resveratrol closely correlate with a dramatic increase of endogenous ceramide levels. Similar effects could be observed in a metastatic breast cancer cell model, when ceramide levels increased ~5- and 10-fold after treatment with resveratrol

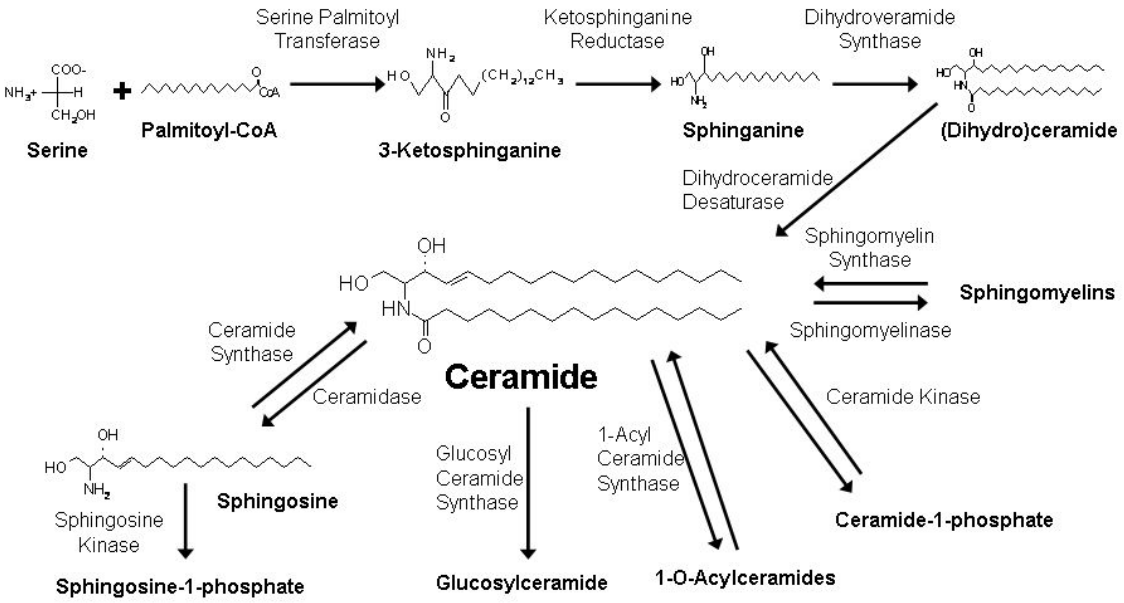


Figure 7: Ceramide metabolism

Major synthetic and metabolic pathways for ceramide. Ceramide can be produced via a de novo biosynthetic pathway which is initiated by condensation of serine and palmitoyl-CoA catalyzed by serine palmitoyltransferase as well as by sphingomyelinase-mediated hydrolysis of sphingomyelin (Adapted from [154]).

32 and 64 µmol/L, respectively, in comparison with untreated cells [155]. To further examine the cellular activities of ceramide, we worked with the exogenous cell-permeant ceramide-analogs N-acetylsphingosine (C2-Ceramide) and N-hexanoylsphingosine (C6-Ceramide). And actually, treatment with C2- or C6-ceramide caused distinct growth inhibition in our colorectal cancer cell model which seems to be the mechanistic explanation for observed growth inhibitory effects of resveratrol.

Nearly 70% of human cancers are associated with the activation of proto-oncogene c-myc [156], a transcription factor that directly regulates the expression of ODC by binding to specific CAGGTG sequence in the gene promoter [157] Based on our earlier findings that resveratrol regulates the expression of both c-myc and ODC genes [75], together with the results from Flamigni et al. [155] who demonstrated a reduction of c-myc and ODC expression in leukemia cells after ceramide-treatment, we tried to identify a possible involvement of ceramide synthesis in the regulatory pathway in colorectal cancer cells. We measured c-myc as well as ODC expression and

activity after treatment with C6-ceramide and with resveratrol in combination with the specific serine palmitoyltransferase inhibitors L-cycloserine and myriocin. While C6-ceramide led to an obvious decrease of both c-myc and ODC protein levels, L-cycloserine and myriocin but not sphingomyelinase-inhibitor manumycin conspicuously counteracted the inhibitory effects of resveratrol. These data suggest that the induction of ceramide de novo biosynthesis but not hydrolysis of sphingomyelin is involved in resveratrol-mediated inhibition of ODC.

3.1.4 Summary and conclusion

In summary, our data confirm our earlier studies showing that resveratrol-mediated growth inhibition of colorectal cancer cells seems to involve modulation of polyamine metabolism. Here we further show that transcription factor PPARγ acts as a p38-dependent target in resveratrol-induced activation of polyamine catabolism (MANUSCRIPT I). On the other hand we could demonstrate that the induction of de novo ceramide biosynthesis plays a crucial role in the inhibition of polyamine biosynthesis (MANUSCRIPT II) (For summary see **Figure 8**). Besides a decrease in cell proliferation, the observed reduction of cell growth is probably due to an induction of apoptosis, as Wolter et al. [108] showed an obvious increase of caspase-3-activity in resveratrol-treated cells. Recent studies further indicate that the activation of catabolic SSAT is related to an induction of programmed cell death [105]. These aspects of resveratrol-action require further investigations.

The identification of increased polyamine concentrations in a variety of cancer tissues has led to the design and development of inhibitors of polyamine metabolism as a new strategy for therapeutic or preventative interventions. The best-known inhibitor of polyamine biosynthesis is α-difluoromethylornithine (DFMO), a specific inhibitor of ornithine decarboxylase (See introduction). The failure of ODC monotherapy *in vivo* may at least partly also be due to inhibitory effects on catabolic SSAT activity, as DFMO effectively prevented SSAT-

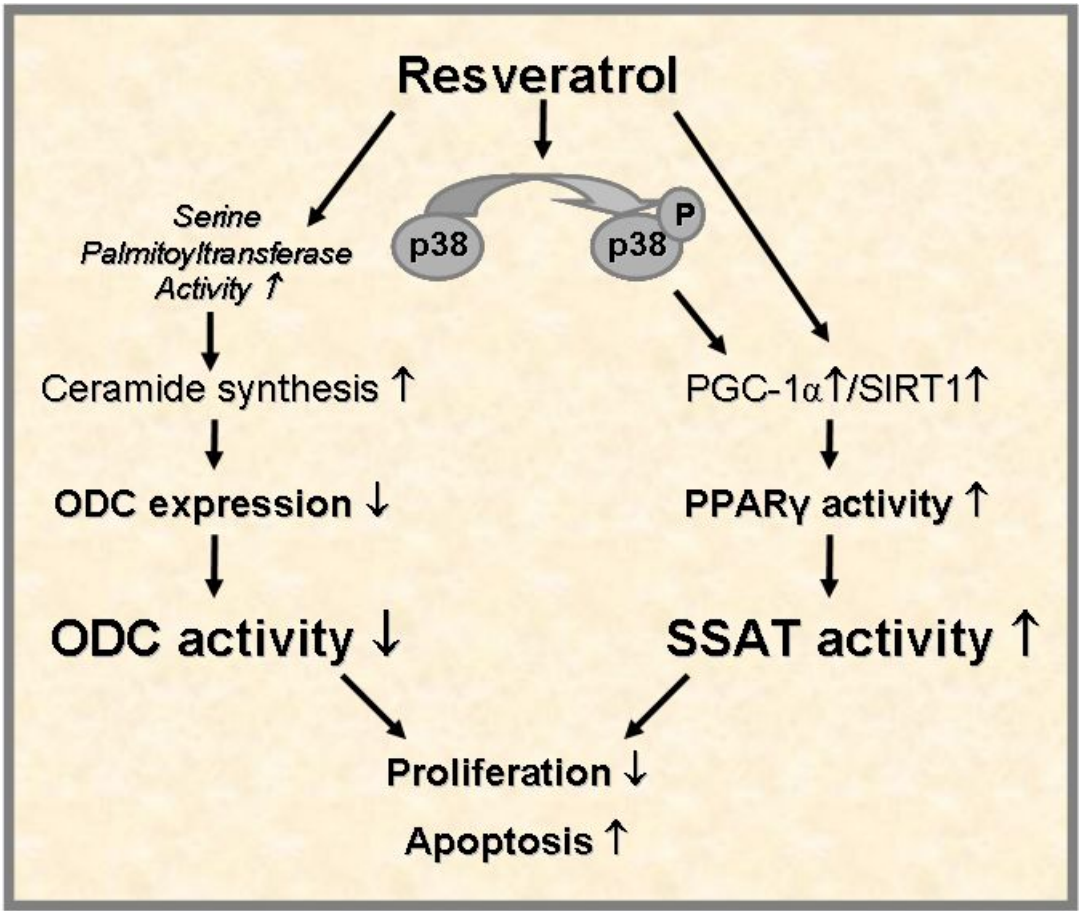


Figure 8: Possible Mechanisms of Resveratrol action
Further details are described in text

induced growth inhibition in a prostate cancer model while typically inhibiting cell growth rather than preventing cell growth inhibition [158]. Although much emphasis in the past has been on the biosynthetic pathway of polyamine metabolism, considerable interest has recently been generated with regard to the catabolic pathways, maintaining a properly balanced ratio of polyamines in cells. This suggestion may well explain the increased efficacy of combined chemopreventive therapy with non-steroidal anti-inflammatory drugs (NSAIDS) in animal models, as these agents recently have been shown, among others, to induce SSAT gene expression [92]. In this context resveratrol could show great potential in the chemoprevention and therapy of colorectal cancers, by simultaneously leading to SSAT activation as well as ODC inhibition.

3.2 Molecular characterization of the chemopreventive activities of pentacylic triterpene ursolic acid

Ursolic acid is a pentacyclic triterpenoid, with anti-cancer and anti-inflammatory properties. Attempts to show favourable effects in vitro have led to the identification of multiple direct targets for this compound. Inhibition of cell cycle progression in the G1 phase for example was known to be associated with a marked decrease in the protein expression of cyclins and their activating partners the cyclin-dependent kinases and with a concomintant induction of cell cycle inhibitors in miscellaneous cancer cell lines [83;84]. Unfortunately, little research is done in colon cancer cells thereby mainly focusing apoptotic mechanisms.

3.2.1 Cell cycle regulation

Each phase of the cell cycle is governed by a wide spectrum of protein families. Many of these proteins are synthesized and activated in a precise phase of the cell cycle. In the past decade, the critical role that cell cycle regulation plays in cancer development has been clearly established [159-161]. Indeed, the list of cell cycle perturbations involved in tumor progression has dramatically increased in the last years [159;162] and unscheduled cell proliferation has become one of the chief causes described for malignancy and carcinogenesis [163].

After detecting potent cell growth inhibitory properties of ursolic acid we started to measure the expression status of several cell cycle regulating proteins, whereby the most prominent effects could be observed in the upregulation of cell cycle inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$. The observed induction of $21^{WAF1/Cip1}$ is in accordance with results found in other malignant cell lines which could show the same effects ursolic acid [83;84;164], but we are the first to describe regulatory effects on $p27^{Kip1}$. Contrary to expectations incubation with ursolic acid also leads to a conspicuous increase of cell cycle progressor cyclin E, which is however consistent with earlier findings by Wolter et al. [108] and Schneider et al. [73], which could show the same effects after treatment with the polyphenol resveratrol and take this as a result of a cell cycle arrest in the S-

phase. However, cell cycle analysis of ursolic acid treated cells presents a predominant arrest in the G1 phase [83;165]. These controversial results will deserve further investigations. Moreover, there are several lines of evidence, that the induction of cyclin E by genotoxic stress, such as ionizing radiation [166] or chemotherapeutic agents [167] could play a functional role in the initiation phase of apoptosis in malignant cell lines, in addition to its reported key regulatory role on the control of the G1 to S-phase transition and the initiation of DNA replication [168]. It has recently become evident, that cyclin E can be processed proteolytically, with the resulting fragments playing important biological and pathological roles. In cyclin E-overexpressing tumor-derived cell lines, cyclin E is proteolytically cleaved to generate N-terminally truncated low molecular weight forms that are more resistant to the CKI $p21^{WAF1/Cip1}$ and $p27^{Kip1}$. Furthermore, cleavage of cyclin E results in loss of its CdK2 associated kinase activity, and consequently its cell cycle function. These low molecular weight forms in turn might exhibit more apoptosis-inducing properties [169]. Another interesting observation is the simultaneous accumulation in both $p27^{Kip1}$ and cyclin E levels, which normally correlate negatively as there exist interdependence regulatory mechanisms. These simultaneous events are known to be characteristic phenotypes in cells derived from mice lacking S-phase kinase associated protein 2 (Skp2) [170] suggesting a possible involvement of protooncogene Skp2 in the regulation of $p27^{Kip1}$ and cyclin E, which might provide another target of ursolic acid mediated actions.

3.2.2 Induction of apoptosis

Apoptosis, a form of programmed cell death, plays a fundamental role in the maintenance of tissues and organ systems by providing a controlled cell deletion to balanced cell proliferation. It is now apparent that many dietary chemopreventive agents with promise to human consumption can also preferentially inhibit tumor cell growth by targeting one or more signalling intermediates leading to induction of apoptosis. Two major apoptosis pathways have been identified, the death receptor or extrinsic pathway and the mitochondrial or intrinsic pathway. The mitochondrial pathway is regulated by members of the bcl-2 protein family, which can be divided in pro- and anti-

apoptotic groups [171]. Because the activation of mitochondria has been considered as the “point of no return” in the apoptotic process, the manipulation of mitochondrial activation with proapoptotic intentions has been envisaged as a potential therapeutic target. While ursolic acid leads to an upregulation of proapoptotic BAX protein levels, the expression of the antiapoptotic Bcl-2 was diminished after 24h of incubation. This results in a significant increase of the BAX/Bcl-2 protein ratio which is generally known to trigger apoptosis [172;173]. The extrinsic pathway of apoptosis is activated at the cell-surface when a specific ligand binds to its corresponding death receptor. Death receptors, like tumor necrosis factor (TNF) receptor, TRAIL receptor and Fas belong to the TNF receptor superfamily which consists of more than 20 proteins with a broad range of biological functions [174]. The TRAIL ligand and its receptors are of special interest for cancer therapy, since TRAIL has been shown to predominantly kill cancer cells, while sparing most other cells [175]. In certain tumor cell lines, TRAIL protein expression could be induced by chemopreventive agents resulting in TRAIL-mediated apoptosis in an autocrine or paracrine manner [176-178]. This suggests that endogenously expressed TRAIL, which we could also observe in our cells after ursolic acid treatment, may be at least partly responsible for the observed chemopreventive effects. Taken together ursolic acid seems to lead to activation both of extrinsic and intrinsic signaling pathways both resulting in caspase-3 activation, followed by DNA fragmentation and programmed cell death.

3.2.3 Modulation of polyamine metabolism

Based on the findings in Manuscript I and II we were interested whether the modulatory effects on the polyamine metabolism are specific for polyphenolic resveratrol or if reduction of intracellular polyamines is a common chemopreventive strategy used also by other phytochemicals like triterpene ursolic acid. A correlation between polyamines and cancer have been extensively studied for decades, pointing out the inhibition of polyamine biosynthetic enzymes ODC and SAMDC or activation of catabolic enzyme SSAT as a potential chemopreventive strategy [179;180]. An association between SSAT induction followed by catabolism of the ubiquitous intracellular

polyamines and subsequent apoptotic responses was first reported by Ha et al [105]. Furthermore, Chen et al. [181] could demonstrate that selective interference of polyamine-analogue induced SSAT prevents apoptotic signaling and apoptosis in human melanoma cells. Much on the focus on SSAT has been on the functional level, but the regulation of SSAT gene expression has also been a subject of recent investigations. As already mentioned, the transcription factor PPARγ was shown to be involved in this regulatory mechanisms [95] [MANUSCRIPT I]. We could show that also ursolic acid seems to activate SSAT in a PPARγ-dependent manner. But unlike resveratrol [MANUSCRIPT I], ursolic acid could not simultaneously inhibit the activity of biosynthetic ODC (unpublished data).

3.2.4 Summary and conclusion

Summarizing our results, the observed reduction of cell growth of colon cancer cell lines after treatment with ursolic acid presumably results from a large increase in the number of apoptotic cells. The modulation of the polyamine metabolism, especially the induction of the catabolic SSAT via PPARγ-dependent mechanisms thereby seems to present the major molecular target in the induction of programmed cell death.
Hence, providing potent chemopreventive activities in vitro, ursolic acid could in theory possibly serve as alternatives to chemically designed antineoplastic agents, as constituents of therapeutic drug combinations in advanced disease, or as adjuvant treatments.

3.3 Bioavailability of Phytochemicals

The bioavailability of a nutrient is defined by its degree to which it becomes available to the target tissue after administration. One important cause of failure in cancer therapies it due to a defect of drug accumulation in cancer cells. Indeed, the action of chemopreventive or chemotherapeutic agents can be nullified by a failure of their absorption, distribution, metabolism or an increase

in their excretion. Hence, the knowledge of absorption and metabolism of a compound in vivo is the precondition to determine its bioavailability.

The absorption and transport of resveratrol have been studied in several models: isolated rat intestine [182;183], rats and mice after oral administration [184-188], human colon carcinoma Caco-2 cell line [189], hepatocytes [190] and healthy human subjects [187;191;192].

These experiments showed, that jejunum and, to lesser extend, illeum are involved in the absorption of resveratrol. Using radiolabelled resveratrol administered orally, an appreciable fraction, 50-70% of the dose, was absorbed in rats [187], and radioactivity could be recovered from the stomach, liver, kidney, intestine, bile and urine in mice [188]. Intragastric administration of 2mg/kg, 20mg/kg and 50mg/kg resveratrol to rats generated peak values of 2μM, 1.2μM and 6.6μM in plasma. After oral administration to humans (25mg/70kg) the amount of free resveratrol in plasma and serum reached ~37nM (less than 2% of total resveratrol) [192]. The appearance of a new resveratrol peak 6h after consumption suggests enteric recirculation of conjugated metabolites by reabsorption after intestinal hydrolysis [193].

In contrast, most mechanistic studies in vitro suggest that carcinogenesis-modulating effects of resveratrol require the sustained presence of 5-100μM, which, according to the mentioned studies, seems hardly to be realizable *in vivo*.

Pharmacokinetic studies in mice and rats suggest consistently that resveratrol is well absorbed and rapidly glucuronidated and sulphated both in the liver and intestinal epithelial cells [183;187;188;194]. In humans, following its absorption, resveratrol is rapidly metabolized in the liver by phase-2 drug-metabolizing enzymes to water-soluble trans-resveratrol-3-o-glucuronide and trans-resveratrol-3-o-sulfate, accounting for its predominant urine excretion. Compared to resveratrol, which has a plasma half-life of 8-14min, these metabolites have a plasma half-life of about 9.2h [192]. Although modifications such as glucuronidation and sulphation typically reduce the cell permeability of drugs and aid in their excretion, the undeniable *in vivo* efficiacy of resveratrol (see next chapter), despite its low bioavailability, has led to the speculation that its metabolites could retain some activity. Research into the actions of metabolites has been hampered by the lack of commercial sources, but should

proceed more readily now that synthetic molecules have been established by several groups [192;193;195].

Most bioavailability studies on resveratrol concentrate on absorption rates and plasma levels, but in the case of colorectal cancers, it might also be interesting, which amount of resveratrol is not absorbed and reaches the colon. Unfortunately, there is a lack of data measuring fecal concentrations after resveratrol consumption. To our knowledge solely Walle et. al examined fecal recovery of [^{14}C]-Resveratrol after oral (25mg) and i.v. (0.2mg) doses in six volunteers which was highly variable (0.3-38%) [192]. Unfortunately, the number of participants in this study was very small, which is particularly critical due to the interindividual variability in xenobiotic metabolisms. The relevance of fecal concentrations becomes apparent in a recent study, when Karlsson et al. could show that fecal water from volunteers consuming a vegetarian diet potently reduced COX-2 protein levels and PGE_2 production in colorectal cancer cells [196]. This is in line with earlier publications demonstrating that dietary alterations can influence the biochemical composition of fecal water [197;198]. Hence, intraluminal accumulation of resveratrol might be another hypothesis to explain the discrepancy between low bioavailability and effectiveness *in vivo* which requires further investigations.

While the bioavailability of resveratrol is comparatively well characterized by now, little work was done on pharmacokinetic studies of ursolic acid. In one study plasma samples taken from rats that had received Lu-Ying extract, whose major effective constituent is ursolic acid, orally were analysed using a rapid and sensitive LC-MS method. These measurements demonstrated that ursolic acid exhibits a high binding activity in organs and a low blood distribution. This low bioavailability was due to a poor absorption rate and to rapid metabolization in entero- and hepatocytes [199]. But even though these results doubt the effectiveness of ursolic acid in vivo, a very recent study, examining the effect of orally administered ursolic acid on the formation of aberrant crypt foci (ACF) and intestinal SMase activity in azoxymethane (AOM)-treated rats, indicates that ursolic acid provides chemopreventive effects in the initiation phase of colon cancer associated with changes in SM metabolism, as the incidence of ACF could be significantly reduced after ursolic acid consumption when

compared to control animals [200]. These auspicious results raise hope for a clinical relevance of ursolic acid in therapeutic approaches of the future.

3.4 Red wine consumption as a chemopreventive strategy?

As red wine is the main dietary source of resveratrol in the western diet, one frequently asked question is: “So, how much of red wine do I have to drink then”? The concentrations of trans-resveratrol in red wine vary widely, but a reasonable estimate is about 5mg/L [201;202]. Assuming a consistent daily intake of 375ml, or about two glasses red wine, a person weighing 70kg would receive a dose of ~27µg/kg body weight each day. At higher doses, the detrimental effects of alcohol are likely to mask any health benefits. Consuming more than four drinks per day, for example, nullifies the beneficial effect of alcohol on the risk of myocardial infarction [203] and increases the development of alcoholic liver diseases [204]. Vitaglione et al. recently studied the bioavailability of trans-resveratrol after red wine consumption [195]. Resveratrol, its 3-glucuronide and its 4’-glucuronide were all detected sporadically in the plasma. This low recovery, due to a rapid metabolization and clearance of the compound, raises doubts about a possible association between the established beneficial health effects of moderate red wine consumption and the presence of resveratrol. But it is worth considering the potential interactions of resveratrol with other dietary constituents. Resveratrol synergistically induced apoptosis in leukaemia cells with both quercetin and ellagic acid [205], inhibited iNOS expression with ethanol [206], and prevented lipid peroxidation with tocopherol [207]. These results might help to explain how relatively low doses of resveratrol obtained from red wine or other dietary sources could produce a measurable health benefit. Thus, the protective effects associated with a moderate consumption of wine (e.g. the famous French paradox) could rather be due to the whole composition of compounds contained in wine and not to resveratrol alone, or to reasons different than wine consumption, such as a healthy lifestyle based on a correct dietary regime, practice of sports, and no smoking habit.

3.5 Therapeutic indications for Resveratrol in colorectal cancers – Future perspectives

Table 2 Resveratrol in Preclinical animal models

Model[a]	Daily Dose[b]	Route	Efficacy[c]	Plasma levels [µM]	Ref.
NMU-induced breast cancer in rat	100 mg/kg	Ig[d]	+	~14	[208]
	10 mg/kg	Ig	-	~1-2	
AOM-induced colon cancer in rat	200 µg/kg	Water	+	~0.02	[209]
DMBA-induced breast cancer in rat	1 mg/kg	Diet	+	~0.1	[210]
NMBA-induced oesophageal cancer in rat	1 or 2 mg/kg	i.g. or i.p.	+	~0.1-1	[211]
$APC^{Min+/+}$ mouse	15 mg/kg	Water	+	~1-2	[212]

Dose, chemopreventive efficacy, and putative peak plasma levels of resveratrol in preclinical animal models in vivo (adapted from [201])

[a] NMU, AOM, azoxymethane; DMBA, 7,12-dimethylbenzanthracene, NMBA, ;
[b] Doses of resveratrol admixed to the diet or drinking water are approximate,
[c] +, efficious; -, inefficious;
[d], Ig, immunoglobin

It was Dr. Michael Sporn who first coined the term chemoprevention in the 1970s as part of his pioneering effort to encourage research into preventing cancer before it begins rather than treating tumors once they appear [28]. According to a more modern and complete definition, chemoprevention includes the use of natural or pharmacological agents to suppress, arrest or reverse carcinogenesis, at its early stages. In this regard, a significant correlation between dietary intake and many types of cancer has been shown in epidemiological data generated throughout the world, pointing towards potent anticancer properties of many dietary substances. However, in many cases, the chemopreventive effects of natural occurring phytochemicals are primarily based on cell culture and animal model studies, and only few of them are entering clinical trials.

For many years, the naturally occurring polyphenol Resveratrol attracted little interest until it was postulated to explain some of the cardioprotective effects of red wine. Since then, more and more reports were published, showing, that resveratrol can prevent or slow the progression of a wide variety of diseases, including cancer. The observed effects of resveratrol on multiple signal

transduction pathways related to carcinogenesis has generated tremendous interest in evaluating its potential for use as a clinical chemopreventive and chemotherapeutic agent. By contrast results from pharmacokinetic studies indicate that circulating resveratrol is rapidly metabolized, and cast doubt on the physiological relevance of the high concentrations typically used for *in vitro* experiments. However, systemic administration of resveratrol has been shown to inhibit the initiation and growth of tumors in a wide variety of rodent cancer models (Summarized in **Table 2**).

Overall, these in vivo studies clearly show great promise for this molecule in the treatment and prevention of cancers. The observed efficacy of low doses, for example 200µg/kg body weight/day, which counteracted azoxymethane-induced carcinogenesis in a rat model of colon cancers, suggests that even concentrations of resveratrol, which might be achievable from dietary sources, such as red wine, could be therapeutic in some cases [209]. But protective effects of resveratrol are more dramatic at higher, but pharmacological achievable doses. Therefore, from a functional, pharmacological and clinical point of view it would be useful to distinguish between “pharmacological” and “dietary” chemoprevention.

The question remains whether the observed effects in vitro and in vivo using animal models are also conferrable and relevant for humans. Several phase I clinical trials are currently in progress for oral resveratrol administration in humans to respond this issue. At the National Cancer Institute chemopreventive effects of resveratrol are measured in a multicenter study, where cohorts of 10 participants receive escalating doses of resveratrol (up to 5g/day) until the maximum tolerated dose (MTD) is determined. (http://www.cancer.gov/clinicaltrials/CCUM-2004-0535).

In addition to chemopreventive properties a chemotherapeutic potential is presumed, as molecular targets of resveratrol are similar to those currently being used for the treatment of cancer [213]. At the University of California, phase I and II studies are in progress supplementing Resveratrol to Patients already diagnosed with colon cancer.
(http://www.clinicaltrials.gov/ct/show/NCT00256334).

Despite aggressive therapies, resistance of many tumors to established treatment procedures still constitutes a major problem in cancer therapy.

Recent evidence suggests that the use of resveratrol in combination with drugs, ionizing radiation or cytokines, can be effectively used for the sensitization to apoptosis. Resveratrol was shown to sensitize to various cytotoxic agents such as cyclosporine A [214], paclitaxel [215], 5-FU [216;217], cisplatin and doxorubicin [218]. Concerning cytokines, several works have shown that resveratrol is also able to sensitize to TRAIL-induced apoptosis in cancer cells [219;220]. Hence, and because of its pharmacological safety, resveratrol might be used in combination with chemotherapeutic agents to exert enhanced antitumor activity through synergic action or compensation of inverse properties. The combined treatment may also decrease the systemic toxicity caused by chemotherapies or radiotherapies because lower doses could be used.

As a result of the discovery of the interesting pharmacological properties of resveratrol, the trihydroxystilbene scaffold has become the subject of synthetic manipulations with the aim of generating novel congeners of pharmacological interest especially those with potential chemotherapeutic activities. In this context, efforts have been devoted to the detailed study of the structure-activity relationship. Some derivatives, for example 3,4,5,4'-tetrahydroxystilbene exhibits superior availability compared to resveratrol with potent biological effects [221]. A series of cis- and trans stilbene derivatives were prepared by Roberti et al. and were tested in vitro for cell growth inhibition and the ability to induce apoptosis in leukaemia cells. Interestingly, all the tested trans-stilbene analogues were less potent than their corresponding cis isomers exepting trans-resveratrol, whose cis counterpart was inactive [222]. This is in accordance with our findings showing 100fold higher effectiveness of a Trimethoxy-cis-stilbene compared to the parent compound in our colon cancer model (unpublished data). Deciphering the structural determinants which are responsible for the biological activity of resveratrol together with the observation that miscellaneous structural modifications generate higher chemopreventive activity and bioavailability provide useful information for new potential chemopreventive or chemotherapeutic drug design.

Due to all these properties, resveratrol seems to be an auspicious candidate in chemoprevention or in chemotherapeutic approaches and could be a potential compound in the development of new therapeutic strategies.

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ORIGINALPUBLIKATIONEN

1. **ULRICH, S.,** WACHTERSHAUSER, A., LOITSCH, S., VON KNETHEN, A., BRUNE, B., STEIN, J. (2005) Activation of PPARγ is not involved in butyrate-induced epithelial cell differentiation. Exp Cell Res 310(1): 196-204.

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5. **ULRICH, S.,** JUNG, B., LOITSCH, S., KAMPAN, W., STEIN, J. (2007) Ursolic acid induces apoptosis through PPARγ mediated SSAT-activation in colon cancer cells (submitted)

ÜBERSICHTSARBEITEN

1. WOLTER, F., **ULRICH, S.**, STEIN, J. (2004) Molecular mechanisms of the chemopreventive effects of resveratrol and its analogs in colorectal cancer: key role of polyamines. J Nutr 134(12): 3219-22. Review

2. **ULRICH, S.,** WOLTER, F., STEIN, J. (2005) Molecular mechanisms of the chemopreventive effects of resveratrol and its analogs in carcinogenesis. Mol Nutr Food Res 49(5): 452-61. Review

VORTRÄGE

I. **Ulrich, S.,** Loitsch, S., Huwiler, A., Rau, O., Stein, J. "Resveratrol modulates polyamine metabolism of colorectal cancer cells via two different signal transduction pathways", anlässlich der 2nd International Conference on Polyphenols and Health, Davis, CA, October 4-7, 2005

II. **Ulrich, S.,** Jung, B., Buettner, S., Stein, J (2006) "Molecular characterization of the chemopreventive activity of pentacyclic triterpene ursolic acid", anlässlich der Digestive Disease Week, Los Angeles, CA, May 20-25, 2006

III. **Ulrich, S.,** Huwiler, A., Loitsch, S., Schmidt, H., Stein, J. (2006) „De novo ceramide biosynthesis is involved in resveratrol-induced inhibition of ornithine decarboxylase activity", anlässlich der 3rd International Conference on Polyphenols Applications in Nutrition and Health, Malta, October 26-27, 2006

IV. **Ulrich, S.,** Büttner, S., Stein, J. (2007) "Molecular characterization of the anti-angiogenic properties of the pentacyclic triterpene ursolic acid, anlässlich der Digestive Disease Week, Washington, DC, May 19-24, 2007

V. **Ulrich, S.,** Kampan, W., Stein, J. (2007) "Resveratrol sensitizes colorectal cancer cells to oxaliplatin-induced cell growth inhibition", anlässlich der Digestive Disease Week, Washington, DC, May 19-24, 2007

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Frankfurt, den 26.09.2007

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre: Ich habe die vorgelegte Dissertation selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

Teile der vorliegenden Arbeit wurden in folgenden Publikationsorganen veröffentlicht:

I. **ULRICH, S.,** LOITSCH, S., RAU O., VON KNETHEN, A., BRÜNE, B., SCHUBERT-ZSILAVECZ, M., STEIN, J. (2006) Peroxisome-Proliferator activated receptor γ as a molecular target of resveratrol-induced modulation of polyamine metabolism. Cancer Res 66(14): 7348-54.

II. **ULRICH, S.,** HUWILER, A., LOITSCH, S., SCHMIDT, H., STEIN J. (2007) *De novo* ceramide biosynthesis is assaociated with resveratrol-induced inhibition of ornithine decarboxylase activity. Biochem Pharmacol 74(2):281-9

III. **ULRICH, S.,** JUNG, B., LOITSCH, S., KAMPAN, W., STEIN, J. (2007) Ursolic acid induces apoptosis through PPARγ mediated SSAT-activation in colon cancer cells. (submitted)

IV. WOLTER, F., **ULRICH, S.,** STEIN, J. (2004) Molecular mechanisms of the chemopreventive effetcs of resveratrol and its analogs in colorectal cancer: key role of polyamines. J Nutr 134(12): 3219-22. Review

V. **ULRICH, S.,** WOLTER, F., STEIN, J. (2005) Molecular mechanisms of the chemopreventive effects of resveratrol and its analogs in carcinogenesis. Mol Nutr Food Res 49(5): 452-61. Review

ABGRENZUNGSERKLÄRUNG

Frau Dipl. oec.troph. Sandra Ulrich hat die dem Promotionsamt des Fachbereichs Ernährungswissenschaften der Justus Liebig Universität Gießen vorgelegte Arbeit mit dem Titel:

„MODULATION OF POLYAMINE METABOLISM AS A CHEMOPREVENTIVE STRATEGY OF PHYTOCHEMICALS IN A CELL CULTURE MODEL OF COLORECTAL CANCERS"

als kumulative Dissertation verfasst.

Der Arbeit liegen folgende Veröffentlichungen zugrunde:

I. **ULRICH, S.,** LOITSCH, S., RAU O., VON KNETHEN, A., BRÜNE, B., SCHUBERT-ZSILAVECZ, M., STEIN, J. (2006) Peroxisome-Proliferator activated receptor γ as a molecular target of resveratrol-induced modulation of polyamine metabolism. Cancer Res 66(14): 7348-54.

II. **ULRICH, S.,** HUWILER, A., LOITSCH, S., SCHMIDT, H., STEIN J. (2007) *De novo* ceramide biosynthesis is assaociated with resveratrol-induced inhibition of ornithine decarboxylase activity. Biochem Pharmacol 74(2):281-9

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Frau Bettina Jung hat im Rahmen ihrer Doktorarbeit im Fachbereich Medizin der J.W. Goethe Universität (unter Betreung von Frau Sandra Ulrich)

verschiedene Versuche mit dem Triterpen Ursolsäure durchgeführt, die allesamt von Frau Sandra Ulrich wiederholt und verifiziert wurden.
Die dominant-negative PPARγ wurde freundlicherweise von Herrn Dr. V.K. Chatterjee (Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom) zur Verfügung gestellt und von Sandra Ulrich unter Anleitung von Herrn Dr. Stefan Loitsch in Caco-2-Zellen stabil transfiziert.
Die intrazelluläre Ceramidmessung wurde in Zusammenarbeit mit Frau Prof. Andrea Huwiler und Herrn Dr. Helmut Schmidt durchgeführt, wobei die Probenvorbereitung von Frau Sandra Ulrich durchgeführt wurde.
Die Messung der PPARγ Liganden-Aktivierung erfolgte in Zusammenarbeit mit der Arbeitsgruppe von Prof. Schubert-Zsilavecz, wo das Modell des Gal4-PPARγ Fusions-Rezeptors von Herrn Dr. Oliver Rau etabliert wurde
Weitere Versuche zur Messung der PPARγ-Aktivierung wurden von Frau Sandra Ulrich in der Arbeitsgruppe von Prof. Bernhard Brüne in Zusammenarbeit mit Dr. Andreas Knethen durchgeführt.
Prof. Dr. Dr. J. Stein hat die Manuskripte kritisch Korrektur gelesen und in Diskussionen hilfreiche Ideen für weitere Versuche geliefert.

Sandra Ulrich

ANHANG

Peroxisome Proliferator–Activated Receptor γ as a Molecular Target of Resveratrol-Induced Modulation of Polyamine Metabolism

Sandra Ulrich,[1] Stefan M. Loitsch,[1] Oliver Rau,[2] Andreas von Knethen,[3] Bernhard Brüne,[3] Manfred Schubert-Zsilavecz,[2] and Jürgen M. Stein[1]

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Abstract

Previous results indicate that the polyphenol resveratrol inhibits cell growth of colon carcinoma cells via modulation of polyamine metabolic key enzymes. The aim of this work was to specify the underlying molecular mechanisms and to identify a possible role of transcription factor peroxisome proliferator–activated receptor γ (PPARγ). Cell growth was determined by bromodeoxyuridine incorporation and crystal violet staining. Protein levels were examined by Western blot analysis. Spermine/spermidine acetyltransferase (SSAT) activity was determined by a radiochemical assay. PPARγ ligand–dependent transcriptional activity was measured by a luciferase assay. A dominant-negative PPARγ mutant was transfected in Caco-2 cells to suppress PPARγ-mediated functions. Resveratrol inhibits cell growth of both Caco-2 and HCT-116 cells in a dose- and time-dependent manner ($P < 0.001$). In contrast to Caco-2-wild type cells ($P < 0.05$), resveratrol failed to increase SSAT activity in dominant-negative PPARγ cells. PPARγ involvement was further confirmed via ligand-dependent activation ($P < 0.01$) as well as by induction of cytokeratin 20 ($P < 0.001$) after resveratrol treatment. Coincubation with SB203580 abolished SSAT activation significantly in Caco-2 ($P < 0.05$) and HCT-116 ($P < 0.01$) cells. The involvement of p38 mitogen-activated protein kinase (MAPK) was further confirmed by a resveratrol-mediated phosphorylation of p38 protein in both cell lines. Resveratrol further increased the expression of PPARγ coactivator PGC-1α ($P < 0.05$) as well as SIRT1 ($P < 0.01$) in a dose-dependent manner after 24 hours of incubation. Based on our findings, p38 MAPK and transcription factor PPARγ can be considered as molecular targets of resveratrol in the regulation of cell proliferation and SSAT activity, respectively, in a cell culture model of colon cancer. (Cancer Res 2006; 66(14): 7348-54)

Introduction

Resveratrol is a naturally occurring polyphenol present in red wine, peanuts, and grapes (1, 2). It has been speculated that dietary resveratrol could be an explanation for the so-called "French paradox," as it exhibits multiple cardioprotective properties (3, 4). Furthermore, we and others reported potent chemopreventive effects of resveratrol and its analogues in various carcinogenesis models (5–8). The polyamines spermidine and spermine as well as their precursor putrescine are essential for normal cell growth, development, and tissue repair (9, 10). Correlation of excess polyamine levels with cancer was first reported in the late 1960s, when Russel and Snyder (11) reported high levels of ornithine decarboxylase activity, the pivotal enzyme of polyamine biosynthesis, in several human cancers. In colorectal cancer tissue, polyamine contents are increased 3- to 4-fold over that found in the equivalent normal colonic tissue (12, 13). Based on these findings, pharmacologic or natural inhibitors of polyamine metabolism have been studied *in vitro* (13, 14) and *in vivo* (15) as new potent therapeutic strategies in cancer treatment and prevention. Peroxisome proliferator–activated receptors (PPARs) are ligand-inducible transcription factors belonging to the nuclear hormone receptor superfamily (16, 17), which regulate transcription of target genes by heterodimerizing with the retinoid X receptor and binding to PPAR response elements (16, 18). PPARγ is expressed at high levels in colonic epithelial cells and colon cancer cells (19). Girnun and Spiegelman (20) hypothesize that PPARγ is exerting its effects early in the carcinogenic process by suppressing tumor formation. Activation of PPARγ therefore could function as an important molecular target of chemopreventive agents such as resveratrol. Recently, Babbar et al. (21) identified two PPAR response elements in the promoter of the spermine/spermidine acetyltransferase (*SSAT*) gene. Based on our former findings that resveratrol induces cell growth inhibition of colon cancer cells via induction of catabolic enzyme SSAT (13), the aim of this work was to specify the underlying molecular mechanisms and to identify a possible role of transcription factor PPARγ.

Materials and Methods

Cell Culture and Materials

Caco-2 cells of passages 53 to 61 were kept in DMEM, supplemented with 10% FCS, 1% penicillin/streptomycin, 1% sodium pyruvate, and 1% nonessential amino acids. HCT-116 cells of passages 17 to 30 were cultured in McCoy's 5A supplemented with 10% FCS and 1% penicillin/streptomycin. Both cell lines were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. Cos7 cells were cultured in DMEM high-glucose supplemented with 10% FCS containing 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine, and 1 mmol/L sodium pyruvate at 37°C and 10% CO_2. The cells were passaged weekly using Dulbecco's PBS containing 0.25% trypsin and 1% EDTA. The medium was changed thrice per week. Cells were screened for possible contamination with mycoplasma at monthly intervals. For experiments, the cells were seeded onto plastic cell culture wells in serum-containing medium and allowed to attach for 24 hours. For the SSAT activity assay, the cells were synchronized in medium containing 1% FCS 24 hours before treatment. WY-14643, pioglitazone HCl, L-165,041, GW7647, and SB203580 were obtained from Calbiochem (San Diego, CA); resveratrol

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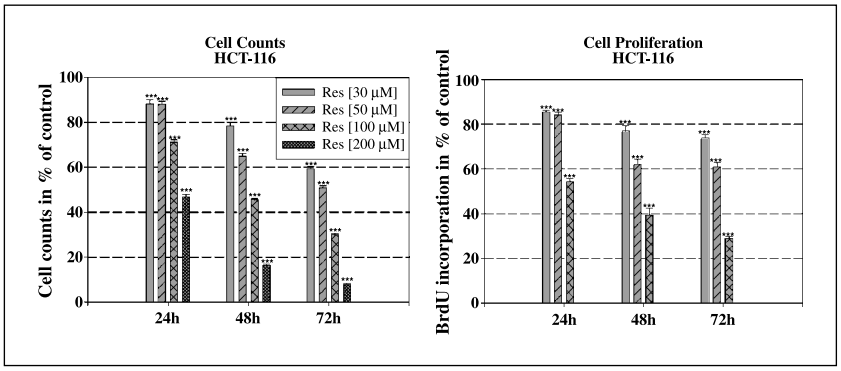

Figure 1. Cell counts and cell proliferation of HCT-116 cells 24, 48, and 72 hours after incubation without (control) or with resveratrol (30-200 μmol/L). Resveratrol leads to a conspicuous dose- and time-dependent reduction of cell counts as well as an inhibition of cell proliferation. *Columns,* mean (n = 8); *bars,* SE. ***, $P < 0.001$.

was obtained from Sigma-Aldrich; FCS and DMSO were obtained from Sigma; DMEM and Optimem I from Life Technologies, Inc.; sodium pyruvate solution, glutamine, penicillin, and streptomycin stock solutions from PAA Laboratories GmbH; Lipofectamine 2000 from Invitrogen; and Dual-glo Luciferase Assay system from Promega.

SDS-PAGE and Immunoblot Analysis

Caco-2 cells were seeded in 80-cm^2 flasks; 24 hours after plating, cells were incubated with substances for different time intervals. Cytosolic and nuclear extracts were obtained according to the instructions of the manufacturer (Active Motif, Rixensart, Belgium). Protein was quantified with the Bio-Rad protein colorimetric assay. After addition of sample buffer to the cellular extract and boiling samples at 95°C for 5 minutes, protein was separated on 10% SDS-polyacrylamide gel. Protein was transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and the membrane was blocked for 1 hour at room temperature with 3% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBST). Next, blots were washed and incubated overnight at 4°C in TBST containing either 5% bovine serum albumin or 3% milk skim powder with a 1:1,000 or 1:500 dilution of primary antibodies for p38 and phospho-p38 (all from Cell Signaling, Beverly, MA), SIRT1, PGC-1α, and cytokeratin 20 (all from Santa Cruz Biotechnology, Santa Cruz, CA), and PPARγ (Calbiochem). The horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology) was diluted at 1:2,000 and incubated with the membrane for another 30 minutes in skim milk. After chemiluminescence reaction (ECL; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), bands were detected after exposure to Hyperfilm-MP (Amersham International plc, Buckinghamshire, United Kingdom). Blots were reprobed with β-actin antibody (Santa Cruz Biotechnology). For quantitative analysis, bands were detected and evaluated densitometrically with ProViDoc system (Desaga, Wiesloch, Germany), normalized for β-actin density.

Cell Counts

Cells were suspended and cultured on 96-well dishes at a density of 10^4 per well (0.28 cm^2). Twenty-four hours after plating, cells were incubated for 24 to 72 hours with substances. At given time points following treatment, cell numbers were assessed by crystal violet staining. Medium was removed from the plates and cells were fixed with 5% formaldehyde for 5 minutes. After washing with PBS, cells were stained with 0.5% crystal violet for 10 minutes, washed again with PBS, and unstained with 33% acetic acid. Absorption, which correlates with the cell number, was measured at 620 nm.

Cell Proliferation

The effects of resveratrol on DNA synthesis of cells were assessed using the cell proliferation ELISA kit (Roche Diagnostics, Tokyo, Japan). This assay is a colorimetric immunoassay for quantification of cell proliferation based on the measurement of bromodeoxyuridine (BrdUrd) incorporation during DNA synthesis and is a nonradioactive alternative to the [^{3}H]thymidine incorporation assay. Cells were grown in 96-well culture dishes (10^4 per well), incubated with resveratrol for different time intervals, and then labeled with BrdUrd for a further 4 hours. Incorporated BrdUrd was measured colorimetrically.

SSAT Enzyme Activity Determination

Cells were washed twice with cold homogenizing buffer [10 mmol/L Tris-HCl (pH 7.5), 2.5 mmol/L DTT, 1 mmol/L EDTA], harvested by

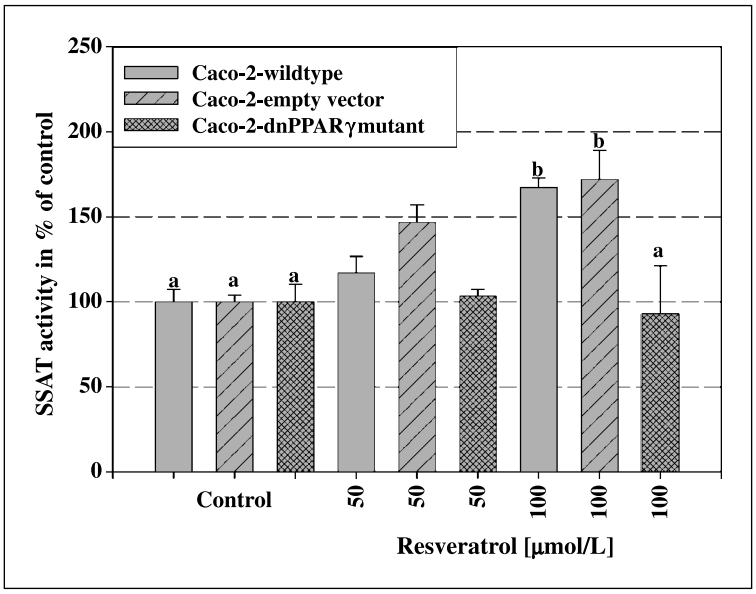

Figure 2. Activity of SSAT in Caco-2-wild type cells in comparison with transfected Caco-2-empty vector and Caco-2-dnPPARγ cells after incubation with resveratrol (50-100 μmol/L) for 24 hours. Resveratrol leads to a significant increase both in Caco-2-wild type and Caco-2-empty vector cells. However, no effects could be observed when PPARγ-mediated functions are suppressed. *Columns,* mean (n = 4); *bars,* SE. Values not sharing a letter differ significantly ($P < 0.05$).

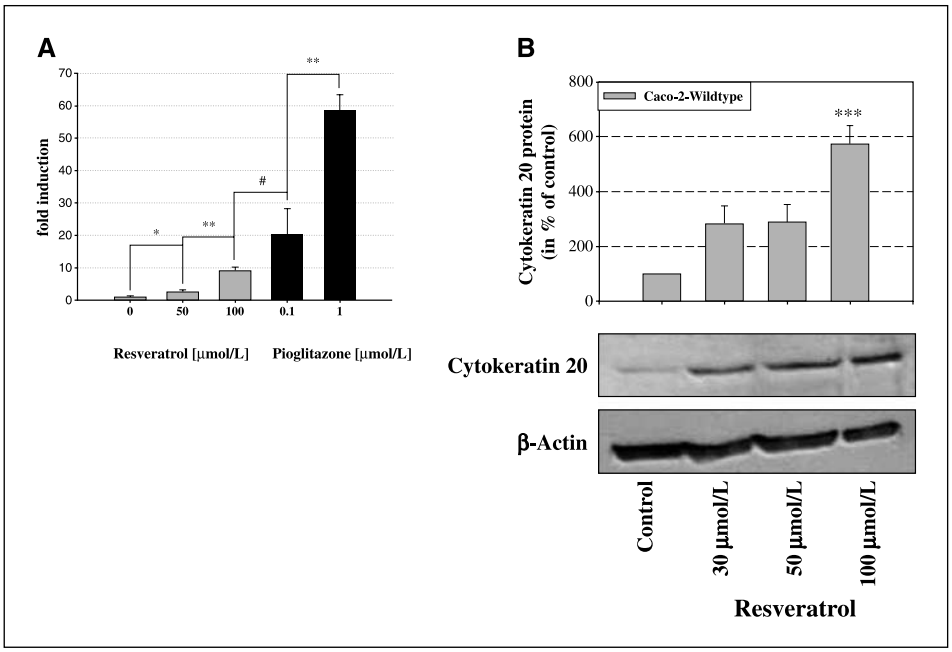

Figure 3. *A,* induction of a GAL4-driven luciferase reporter gene following ligand-dependent activation of a Gal4-PPARγ fusion receptor in transiently transfected Cos7 cells. Graph plots fold induction by resveratrol or pioglitazone at indicated concentration. Each experiment was done in triplicate and repeated in two independent experiments. *Columns,* mean; *bars,* SE. #, not significant; *, $P < 0.05$; **, $P < 0.01$. *B,* Western blot of cytokeratin 20 in Caco-2 cells after incubation with resveratrol (30-100 μmol/L) for 72 hours. Representative immunoblot of three independent experiments. Graph presents the densitometric analysis of cytokeratin 20 after 72 hours of incubation. *Columns,* mean ($n = 3$); *bars,* SE. ***, $P < 0.001$.

scraping, disrupted by sonification, and centrifuged at 15,000 × *g* at 4°C for 15 minutes. The radiochemical assay of the SSAT activity was done by the estimation of labeled N^1-acetylspermidine synthesized from [^{14}C]acetyl-CoA (Hartman Analytic, Braunschweig, Germany) and unlabeled spermidine (0.3 μmol/L) as described earlier (13).

Transfection Assay

The following plasmids were used for transfection: pcDNA3 (Invitrogen), as an empty vector for control transfection, and plasmid pcDNA3-PPARγL468A/E471A, a dominant-negative double mutant, which was kindly provided by V.K. Chatterjee (Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom; ref. 22). These constructs were transfected into subconfluent Caco-2 cells with Lipofectamine 2000 (Invitrogen). After 6 hours, the cells were fed with fresh medium containing 10% FCS. Twenty-four hours later, the cells were fed with medium containing G418 (400 μg/mL) and culture medium was replaced twice a week. G418-resistant colonies were collected and used for further analysis.

Gal4-PPARγ Transactivation Assay

Plasmids. The Gal4-fusion receptor plasmid pFA-CMV-PPARγ-LBD, containing hinge region and the LBD of PPARγ, was constructed by integrating cDNA fragment obtained from PCR amplification of human monocytes into the *Sma*I/*Xba*I (Promega) sites of the pFA-CMV vector (Stratagene). The cDNA fragment contained bps 610-1,518 (NM_015869) of the PPARγ coding sequence. Frame and sequence of the fusion receptors

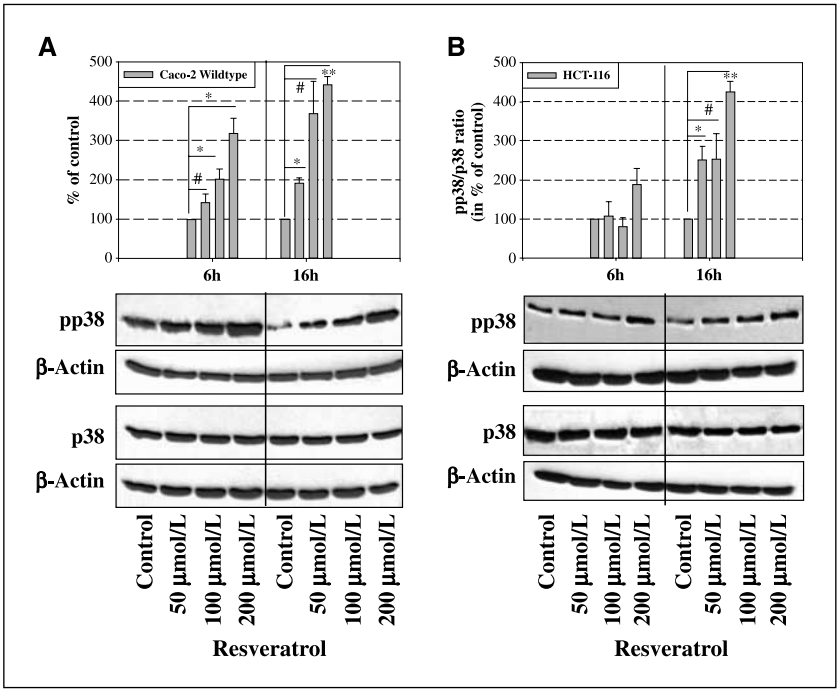

Figure 4. *A,* Western blot of p38 and phosphorylated p38 MAPK protein in Caco-2 cells after incubation with resveratrol (50-200 μmol/L) for 6 and 16 hours. Representative immunoblot of three independent experiments for both proteins. Graph presents the densitometric analysis of phospho-p38/p38 protein ratio after 6 and 16 hours. *Columns,* mean ($n = 3$); *bars,* SE. #, not significant; *, $P < 0.05$; **, $P < 0.01$. *B,* Western blot of p38 and phosphorylated p38 MAPK protein in HCT-116 cells after incubation with resveratrol (50-200 μmol/L) for 6 and 16 hours. Representative immunoblot of three independent experiments for both proteins. Graph presents the densitometric analysis of phospho-p38/p38 protein ratio after 6 and 16 hours. *Columns,* mean ($n = 3$); *bars,* SE. #, not significant; *, $P < 0.05$; **, $P < 0.01$.

were verified by sequencing. As reporter plasmids, we used pFR-Luc (Stratagene). For normalizing of transfection efficacy, we used pRL-SV40 (Promega).

Transfection. Cos7 cells were seeded at 30,000 per well in a 96-well plate. After 24 hours, transfection was carried out using Lipofectamine 2000 according to the protocol of the manufacturer. Transfection mixes contained 0.8 μL LF2000, 280 ng pFR-Luc, 2 ng pRL-SV40, and 14 ng of the PPARγ fusion receptor plasmid for each well. Four hours after transfection, medium was changed to DMEM without phenol red–containing 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, appropriate concentration of test substance, and 0.1% DMSO, testing each concentration in triplicate wells. Cells were incubated overnight and assayed for reporter gene activity with the Dual-Glo Luciferase Assay system. Luminescence of both luciferases was measured in GENiosPro Luminometer (Tecan). Each assay was repeated at least thrice.

Calculations. Luciferase activity for all assays was corrected by subtracting background activity obtained from nontransfected controls. Relative light units were calculated by dividing firefly light units by renilla light units. Activation factors are gained by dividing mean values of relative light units for each concentration of agonist by mean relative light unit values of the DMSO control. Relative activation is calculated by dividing activation factors by the maximum activation factor. Calculation of EC_{50} values was done using the four-parameter logistic regression function of SigmaPlot2001 (SPSS, Inc.) using the mean of relative activation for each tested concentration of at least three determinations.

Statistics

The data are expressed as means ± SE of at least three independent experiments. ANOVA was done when more than two groups were compared, and when significant ($P < 0.05$), multiple comparisons were done with the Turkey test. $P < 0.05$ was considered to be significant.

Results

Effects of resveratrol on cell proliferation and cell counts. Caco-2 and HCT-116-cells were incubated with increasing concentrations of resveratrol (30-200 μmol/L) for 24, 48, and 72 hours. After each time interval, both cell proliferation ELISA (BrdUrd) and crystal violet staining were done. In HCT-116 cells, a significant time- and dose-dependent decrease in cell proliferation and cell counts could be measured (Fig. 1). The same effects could be observed in Caco-2 cells, which is in accordance to our earlier studies (ref. 6; data not shown).

The role of PPARγ in resveratrol-induced activation of SSAT. Next, we examined the effects of resveratrol (50-100 μmol/L) on SSAT activity in Caco-2-wild type cells compared with Caco-2-empty vector and Caco-2-dnPPARγ mutant cells to investigate effects mediated by PPARγ. Resveratrol (100 μmol/L) leads to a significant increase of SSAT activity ($P < 0.05$ versus control) in Caco-2-wild type cells after 24 hours of incubation, which is in agreement with our previous data (13). In Caco-2-empty vector cells, resveratrol (100 μmol/L) also significantly increases SSAT-activity ($P < 0.05$ versus control) whereas no effects could be observed when PPARγ-mediated functions are suppressed in Caco-2-dnPPARγ mutant cells (Fig. 2).

Effects of resveratrol on PPARγ transcriptional activity. To investigate the effects of resveratrol (50-100 μmol/L) on PPARγ ligand–dependent activity, we did a chimeric Gal4-PPARγ transactivation assay. Because the chimeric receptor contained only hinge region and ligand binding domain of the PPARγ, any effect of resveratrol affecting kinase-sensitive AF1 domain was ruled out. After incubation with resveratrol (100 μmol/L), we could generate similar effects of PPARγ agonist pioglitazone on PPARγ activity

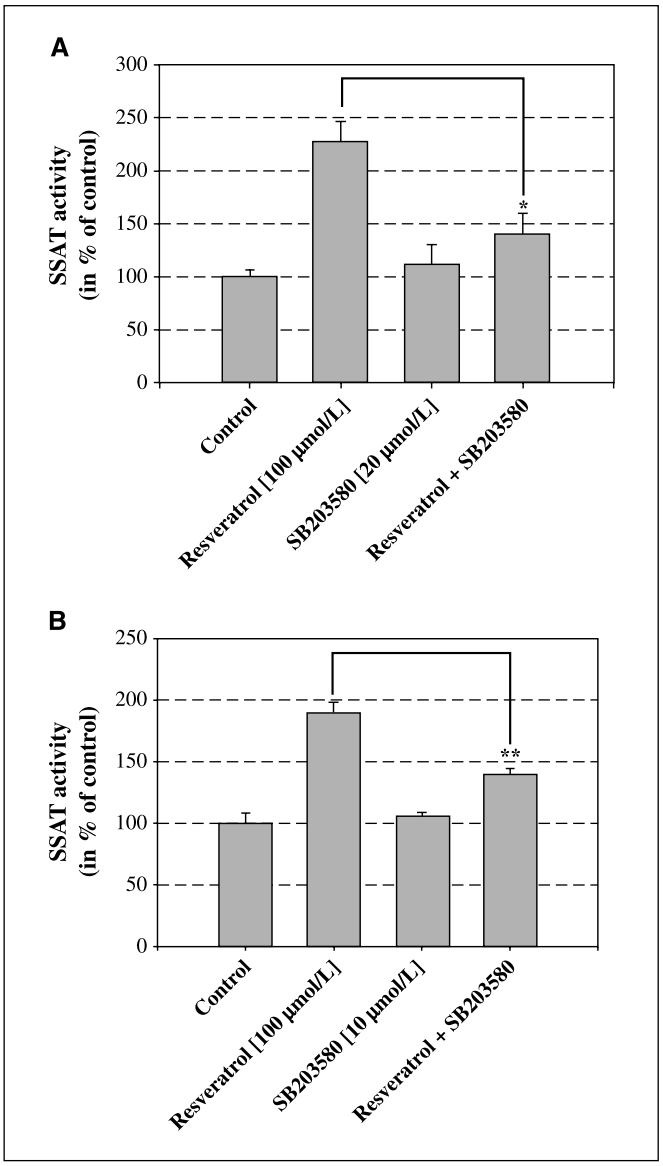

Figure 5. *A,* coincubation with SB203580 (20 μmol/L) abolishes resveratrol (100 μmol/L)–induced SSAT activation significantly in Caco-2 cells after 24 hours of incubation. *Columns,* mean ($n = 4$); *bars,* SE. *, $P < 0.05$. *B,* the same effects could be observed in HCT-116 cells when inhibition of p38 MAPK (10 μmol/L) significantly reduces resveratrol-induced SSAT activity after 24 hours of incubation, indicating the effects not to be cell specific. *Columns,* mean ($n = 4$); *bars,* SE. **, $P < 0.01$.

($P < 0.01$; Fig. 3*A*). To show evidence of resveratrol ability to increase PPARγ activity, we measured, after resveratrol treatment, the expression of cytokeratin 20, which is described to be a specific target gene of PPARγ activity in colorectal cancer cells (23). Incubation with resveratrol (30-100 μmol/L) led to an ~40% increase of cytokeratin 20 expression at 100 μmol/L after 72 hours ($P < 0.001$; Fig. 3*B*).

Effects of resveratrol on PPARγ protein levels. We further did Western blot analysis to determine possible effects of resveratrol on translational level. However, no significant changes in PPARγ protein expression could be detected (data not shown).

Involvement of mitogen-activated protein kinase p38 in resveratrol-induced inhibition of cell proliferation and SSAT activation. There are several lines of evidence that resveratrol

mediates its chemopreventive actions via modulation of mitogen-activated protein kinase (MAPK) pathways. To examine p38 MAPK–mediated actions, we used the specific inhibitor SB203580. This anti-inflammatory drug inhibits the catalytic activity of p38 MAPK by competitive binding in the ATP pocket (24). As shown in Fig. 4, incubation with resveratrol (50-200 μmol/L) augmented phosphorylated p38 in a time- and dose-dependent manner, both in Caco-2 and HCT-116 cells (~300% at 200 μmol/L after 16 hours; $P < 0.01$), whereas p38 MAPK concentration remained unaffected. To characterize the role of p38 activation in resveratrol-mediated induction of SSAT, we pretreated Caco-2 and HCT-116 with p38 inhibitor SB203580 (10-20 μmol/L) for 1 hour and then added resveratrol (100 μmol/L) for another 24 hours. Both in Caco-2-(Fig. 5*A*) and HCT-116-cells (Fig. 5*B*), coincubation with SB203580 significantly diminished resveratrol-induced SSAT activation [$P < 0.05$ versus resveratrol (100 μmol/L) in Caco-2; $P < 0.01$ versus resveratrol (100 μmol/L) in HCT-116].

Effects of resveratrol on PGC-1α and SIRT1 expression. Western blot analysis was done to determine possible effects of resveratrol on the expression of PPARγ coactivator 1α and sirtuin homologue SIRT1, which exhibits PPARγ-suppressive effects in white adipocyte tissue. Resveratrol (50-200 μmol/L) led to a significant dose-dependent increase in both PGC-1α (~60% at 200 μmol/L; $P < 0.05$; Fig. 6*A*) and SIRT1 (~140% at 200 μmol/L; $P < 0.01$; Fig. 6*B*) expression after 24 hours of incubation.

Discussion

The present study clearly shows that resveratrol mediates growth inhibitory effects in colorectal cancer cell lines, at least partly, via polyamine degradation, whereas activation of transcription factor PPARγ seems to play a pivotal role. The plant polyphenol resveratrol (3,4′,5-trihydroxystilbene) exhibits multiple chemopreventive effects comprising cell growth inhibition (6, 25), induction of apoptosis (26), and prevention of angiogenesis (27), whereby the underlying molecular mechanisms are only partly understood (28, 29). Intracellular polyamine levels are maintained within very narrow limits because decreases of polyamine concentrations interfere with cell growth whereas an excess seems to be toxic (30). The three key enzymes of polyamine metabolism are ornithine decarboxylase and S-adenosylmethionine decarboxylase, the rate-limiting enzymes of polyamine biosynthesis, and SSAT, which controls polyamine catabolism (31). Wolter et al. showed that resveratrol-induced growth arrest of Caco-2 cells is accompanied by inhibition of polyamine biosynthesis as well as activation of polyamine catabolism (13). The peroxisome proliferator–activated receptor γ (PPARγ) is a nuclear receptor that acts as a transcription factor controlling the expression of multiple genes involved in cell growth, differentiation, and apoptosis of several malignant cell lines, and therefore seems to play a crucial role in carcinogenesis (32, 33). To abolish PPARγ-mediated functions, we transfected a dominant-negative mutant in Caco-2 cells. In this PPARγ mutant, two charged amino acid residues (Leu^{468} and Glu^{471}) in helix 12 of the ligand binding domain are mutated to alanine, whereupon the mutant retains ligand and DNA binding but exhibits markedly reduced transactivation due to impaired coactivator recruitment (22). According to the current findings, an essential role for PPARγ in enhancing SSAT enzyme activity is assumed (21). In fact, we could show that, in contrast to Caco-2-wild type and Caco-2-empty vector cells, resveratrol failed to increase SSAT activity in Caco-2-dnPPARγ cells. The mitogen-activated protein kinase (MAPK) pathways have been recognized as a major signaling pathway by which cells transduce extracellular signals into an intracellular response. In several studies, resveratrol was shown to mediate multiple functions by modulating MAPK pathways (26, 34, 35). We could show as well that incubation with resveratrol causes phosphorylation, and thus activation, of p38 MAPK in colon cancer cells. Furthermore, combination of resveratrol with an inhibitor of p38 MAPK leads to an inhibition of resveratrol-induced SSAT activation both in Caco-2 and HCT-116 cells. Consequently, an activation of MAPK cascade by resveratrol can be assumed. As previously described, our results point out an

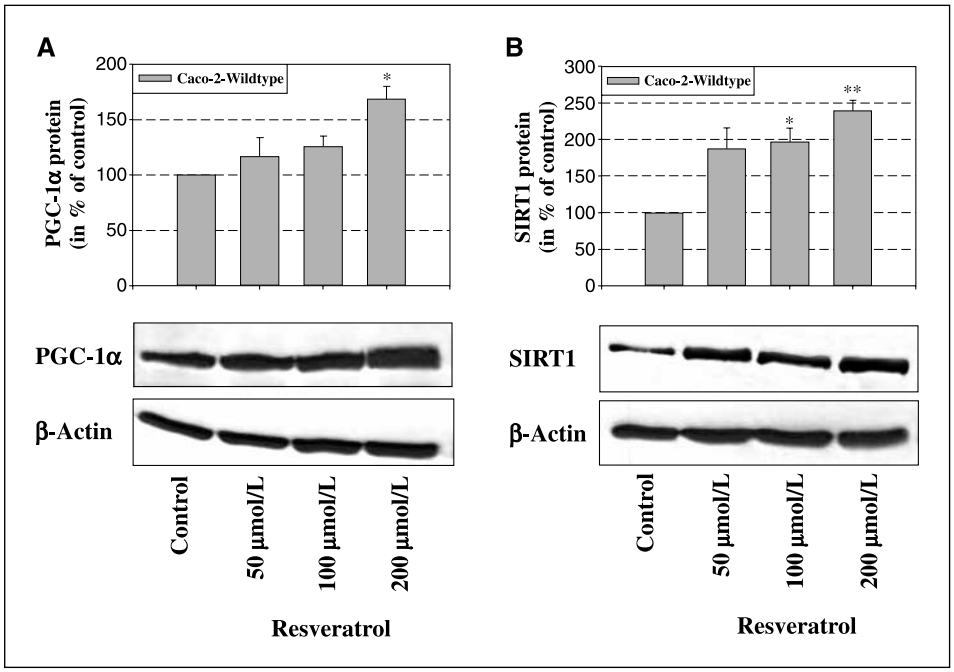

Figure 6. Western blot of PGC-1α protein in Caco-2 cells after incubation with resveratrol (50-200 μmol/L) for 24 hours. Graph presents the densitometric analysis of PGC-1α protein after 24 hours. Representative immunoblot of three independent experiments. *Columns,* mean ($n = 3$); *bars,* SE. *, $P < 0.05$. *B,* Western blot of SIRT1 protein in Caco-2 cells after incubation with resveratrol (50-200 μmol/L) for 24 hours. Graph presents the densitometric analysis of SIRT1 protein ratio after 24 hours. Representative immunoblot of three independent experiments. *Columns,* mean ($n = 3$); *bars,* SE. *, $P < 0.05$; **, $P < 0.01$.

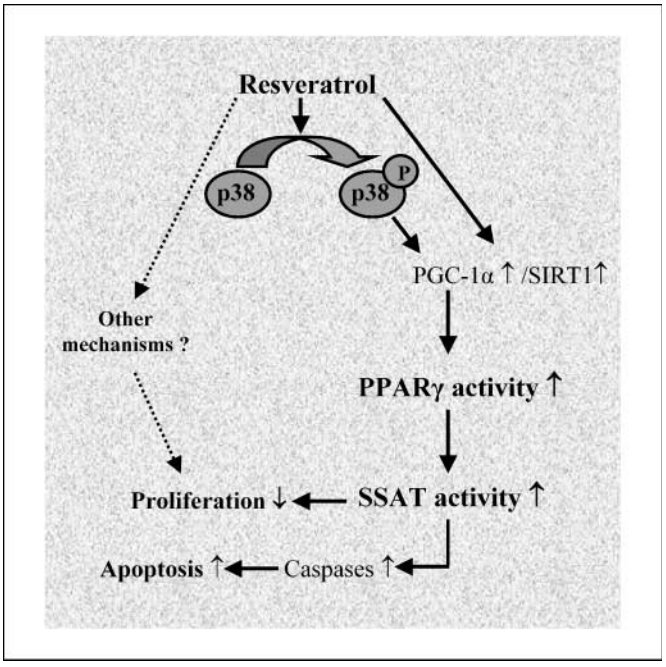

Figure 7. Possible molecular mechanism of resveratrol-induced inhibition of cell growth in colorectal carcinoma cell lines.

important role of PPARγ in resveratrol-mediated actions whereas resveratrol-dependent PPARγ activation seems to be mediated at least partly by an activation of the ligand binding domain (LBD/AF2) because a Gal4-PPARγ chimeric receptor was activated by resveratrol at a concentration of 100 μmol/L, and this concentration was sufficient to induce SSAT as well. In addition, our results suggest that activation of PPARγ by resveratrol is due to kinase activation, leading to phosphorylation-dependent activation of PPARγ coactivators like PGC-1α (36). Coactivators all interact with a similar surface of the activated ligand binding domain of the receptors and have been suggested to mediate their transcriptional activity (37). It is well established that, in addition to transcription factors, coactivators can also be targets of multiple signal transduction pathways in response to different stimuli (38). Puigserver et al. (39) could show that PGC-1α is activated through p38 MAPK. The mechanism by which p38 activates PGC-1α is not yet clear, but it is suggested that p38 MAPK–mediated phosphorylation counteracts repressor effects, possibly by encouraging the release of a repressor from PGC-1α (40). On activation, PGC-1α docks on PPARγ and thus can modulate its transcriptional activity (41). In addition to PGC-1α, resveratrol further leads to an activation of SIRT1, a member of the silent information regulator 2 (Sir2) family of proteins (sirtuins; ref. 42). SIRT1 is mainly linked to negative regulation of gene expression as a cofactor through protein deacetylation (43). However, there is evidence that SIRT1 can act positively and negatively to control gene expression as a cofactor for PGC-1α. These opposite effects could possibly be due to the recruitment of a different set of coactivators and corepressors through PGC-1α/SIRT1 (44). This could further be an explanation for the repressive effects of SIRT1 on PPARγ in white fat where PGC-1α is very low (45). In summary, our data confirm our earlier studies showing that resveratrol-mediated growth inhibition of colorectal cancer cells seems to involve SSAT-induced polyamine catabolism. Here we further show that transcription factor PPARγ acts as a p38-dependent target in resveratrol-induced molecular mechanisms (see Fig. 7). Besides a decrease in cell proliferation, the observed reduction of BrdUrd incorporation is probably due to an induction of apoptosis, as Wolter et al. (6) showed an obvious increase of caspase-3-activity in resveratrol-treated cells. Recent studies further indicate that the activation of catabolic SSAT is related to an induction of programmed cell death (46). Further projects directing towards these aspects of resveratrol action are going to proceed.

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De novo ceramide biosynthesis is associated with resveratrol-induced inhibition of ornithine decarboxylase activity

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ABSTRACT

Previous studies could demonstrate, that the naturally occuring polyphenol resveratrol inhibits cell growth of colon carcinoma cells at least in part by inhibition of protooncogene ornithine decarboxylase (ODC). The objective of this study was to provide several lines of evidence suggesting that the induction of ceramide synthesis is involved in this regulatory mechanisms.

Cell growth was determined by BrdU incorporation and crystal violet staining. Ceramide concentrations were detected by HPLC-coupled mass-spectrometry. Protein levels were examined by Western blot analysis. ODC activity was assayed radiometrically measuring [$^{14}CO_2$]-liberation. A dominant-negative PPARγ mutant was transfected in Caco-2 cells to suppress PPARγ-mediated functions.

Antiproliferative effects of resveratrol closely correlate with a dose-dependent increase of endogenous ceramides ($p < 0.001$). Compared to controls the cell-permeable ceramide analogues C2- and C6-ceramide significantly inhibit ODC-activity ($p < 0.001$) in colorectal cancer cells. C6-ceramide further diminished protein levels of protooncogenes c-myc ($p < 0.05$) and ODC ($p < 0.01$), which is strictly related to the ability of ceramides to inhibit cell growth in a time- and dose-dependent manner. These results were further confirmed using inhibitors of sphingolipid metabolism, where only co-incubation with a serine palmitoyltransferase (SPT) inhibitor could significantly counteract resveratrol-mediated actions. These data suggest that the induction of ceramide *de novo* biosynthesis but not hydrolysis of sphingomyelin is involved in resveratrol-mediated inhibition of ODC. In contrast to the regulation of catabolic spermidine/spermine acetyltransferase by resveratrol, inhibitory effects on ODC occur PPARγ-independently, indicating independent pathways of resveratrol-action. Due to our findings resveratrol could show great chemopreventive and therapeutic potential in the treatment of colorectal cancers.

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Abbreviations: BrdU, bromodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium; DFMO, alpha-difluoromethylornithine; dnPPARγ, dominant negative PPARγ mutant; ELISA, enzyme-linked immunosorbent assay; MAPK, mitogen-activated protein kinase; NSAID, non-steroidal anti-inflammatory drugs; ODC, ornithine decarboxylase; PAO, polyamine oxidase; PPARγ, peroxisome-proliferator activated receptor γ; RXR, retinoid X receptor; SAMDC, S-adenosylmethioninedecarboxylase; SMase, Sphingomyelinase; SPT, serine palmitoyltransferase; SSAT, spermine/spermidine acetyltransferase; TNF-α, tumor necrosis factor-α

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1. Introduction

Resveratrol, chemically known as 3,5,4′-trihydroxytransstilbene, is a naturally occuring polyphenolic antioxidant compound, also classified as a phytoalexin, which are herbal antibiotics produced in response to environmental stress factors including injuries, UV irradiation or fungal invasion [1]. Resveratrol was first detected in the root extract of the weed *Polygonum cuspidatum* [2], which has been known in Asian folk medicine under the name *Ko-jo-kon* and was traditionally used to treat liver, skin and circulatory diseases [3,4]. Anticarcinogenic properties of resveratrol were first reported by Jang et al. [5] demonstrating chemopreventive effectiveness against all the three major steps of carcinogenesis, i.e. initiation, promotion and progression. We and others provide several lines of evidence that resveratrol mediates these anticarcinogenic effects partly through the modulation of polyamine metabolism [6,7]. The major polyamines spermidine and spermine, and their diamine precursor, putrescine are organic cations with multiple functions in cell growth and cell death [8,9]. The intracellular polyamine pool size is controlled strictly by the combined action of *de novo* synthesis, catabolism, uptake and export of polyamines. This regulatory mechanism include reactions catalyzed by the biosynthetic enzymes ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC) and spermidine/spermine synthases and by the catabolic spermidine/spermine acetyltransferase (SSAT) and FAD-dependent polyamine oxidase (APAO) [10]. The finding that agents that inhibit polyamine biosythesis can prevent, or at least limit cell growth [6,11–13], together with the fact, that polyamine concentrations are elevated in multiple cancer tissues [14,15], has made the polyamine metabolism a promising target for cancer chemoprevention and therapy.

Ceramides are key compounds in the metabolism of sphingolipids and are emerging as important second messengers for various cellular processes including cell cycle arrest, differentiation and apoptosis (for review see Ref. [16]). Ceramides can be produced via a *de novo* biosynthetic pathway which is initiated by condensation of serine and palmitoyl-CoA catalyzed by serine palmitoyltransferase (SPT) as well as by sphingomyelinase-mediated hydrolysis of sphingomyelin. Our aim was to study the potential involvement of ceramide biosynthesis in resveratrol mediated inhibition of ODC activity in colorectal cancer cells.

2. Materials and methods

2.1. Cell culture and materials

Caco-2 cells of passages 53–61 were kept in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% sodium pyruvate and 1% nonessential amino acids. HT29 cells of passages 17–30 were cultured in McCoy's 5A supplemented with 10% FCS and 1% penicillin/streptomycin. Both cell lines were maintained at 37 °C in an atmosphere of 95% air and 5% CO_2. The cells were passaged weekly using Dulbecco's PBS containing 0.25% trypsin and 1% EDTA. The medium was changed three times per week. Cells were screened for possible contamination with mycoplasma at monthly intervals. For experiments, the cells were seeded onto plastic cell culture wells in serum containing medium and allowed to attach for 24 h. For the ODC activity assay the cells were synchronized in medium containing 1% FCS 24 h before treatment. Resveratrol, *N*-hexanoylsphingosine, *N*-acetylsphingosine, L-cycloserine, myriocin and manumycin were obtained from Sigma–Aldrich (St. Louis, MO); Dulbecco's modified Eagle's medium and Optimem™ I from Gibco (Invitrogen, Carlsbad, CA); fetal calf serum, sodium pyruvate solution, glutamine, penicillin and streptomycin stock solutions from PAA Laboratories GmbH (Ontario, Canada); Lipofectamine™ 2000 from Invitrogen (Carlsbad, CA).

2.2. SDS-polyacrylamide gel electrophoresis and immunoblot analysis

Caco-2 cells were seeded in 80 cm^3 flasks; 24 h after plating, cells were incubated with substances for different time intervals. Whole cell extract was obtained according to the manufacturer's instructions (Active Motif, Rixensart, Belgium). Protein was quantified with the Bio-Rad protein colorimetric assay. After addition of sample buffer to the total cellular extract and boiling samples at 95 °C for 5 min, protein was separated on a 10% SDS-polyacrylamide gel. Protein was transferred onto nitrocellulose membrane (Schleicher&Schuell, Dassel, Germany) and the membrane was blocked for 1 h at room temperature with 3% skim milk in tris-buffered saline containing 0.05% Tween 20 (TBST). Next, blots were washed and incubated overnight at 4 °C in TBST containing 3% skimmed milk powder with a 1:500 dilution of primary antibodies for ODC and c-myc (all from Santa Cruz Biotechnology, Santa Cruz, USA). The secondary, horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology) was diluted at 1:2000 and incubated with the membrane for another 45 min in skim milk. After chemoluminescence reaction (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK), band were detected after exposure to Hyperfilm-MP (Amersham International plc, Buckinghamshire, UK). Blots were reprobed with β-actin antibody (Santa Cruz Biotechnologies, Santa Cruz, USA). For quantitative analysis, bands were detected and evaluated densitometrically by ProViDoc system (Desaga, Wiesloch, Germany), normalized for the density of β-actin.

2.3. Cell counts

Cells were suspended and cultured on 96 well dishes at a density of 10^4/well (0.28 cm^2). Twenty-four hours after plating cells were incubated for 24–72 h with ceramides. At given time points following treatment cell numbers was assessed by crystal violet staining. Medium was removed from the plates and cells were fixed with 5% formaldehyde for 5 min. After washing with PBS cells were stained with 0.5% crystal violet for 10 min, washed again with PBS and unstained with 33% acetic acid. Absorption, which correlates with the cell number, was measured at 620 nm.

2.4. Cell proliferation

The effects of ceramides on DNA synthesis of cells were assessed using the cell proliferation ELISA kit (Roche Diag-

nostics, Tokyo, Japan). This assay is a colorimetric immunoassay for quantification of cell proliferation based on the measurement of bromodeoxyuridine (BrdU) incorporation during DNA synthesis, and is a non-radioactive alternative to the [^{3}H]-thymidine incorporation assay. Cells were grown in 96 well culture dishes (10^4 cells/well), incubated with C2- or C6-ceramide for different time intervalls, and then labelled with BrdU for a further 4 h. Incorporated BrdU was measured colorimetrically.

2.5. Lipid extraction and ceramide quantitation

Sub-confluent Caco-2-cells in 30 mm-diameter dishes were stimulated with increasing concentrations of resveratrol [50–200 μmol/L] for 24 h. Lipids were extracted according to the method established by Bligh and Dyer [17], and ceramide was quantitated by mass-spectrometry as previously described [18].

2.6. ODC-activity

The activity of the enzyme ODC was assayed with a radiometric technique in which the amount of $^{14}CO_2$ liberated from DL-[1-14C]ornithine (207.2_104 MBq/mol, Hartman Analytics Amersham Pharmacia Biotech, Freiburg, Germany) was estimated, as described earlier [13].

2.7. Transfection assay

The following plasmids were used for transfection: pcDNA3 (Invitrogen), as an empty vector for control transfection and the plasmid pcDNA3-PPARγL468A/E471A, a dominant-negative double mutant, that was kindly provided by VK Chatterjee (Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom) [19]. These constructs were transfected into subconfluent Caco-2 cells with lipofectamine 2000 (Invitrogen). After 6 h the cells were fed with fresh medium containing 10% FCS. Twenty-four hours later the cells were fed with medium containing Geneticine (G418) [400 μg/mL] and culture medium was replaced twice a week. G418-resistant colonies were collected and used for further analysis.

2.8. Statistics

The data are expressed as means ± S.E. of at least three independent experiments. Analysis of variance (ANOVA) was performed when more than two groups were compared, and when significant ($p < 0.05$), multiple comparisons were performed with the Turkey test. A $p < 0.05$ was considered to be significant.

3. Results

3.1. Effects of resveratrol on ceramide synthesis

First we examined the effect of resveratrol [50–200 μmol/L] on the intracellular ceramide concentrations of Caco-2 cells using mass-spectometry. After 24 h of incubation we could observe a significant dose-dependent up-regulation of C16-ceramide levels ~6.5-fold at 200 μmol/L [$^{***}p < 0.001$] (Fig. 1).

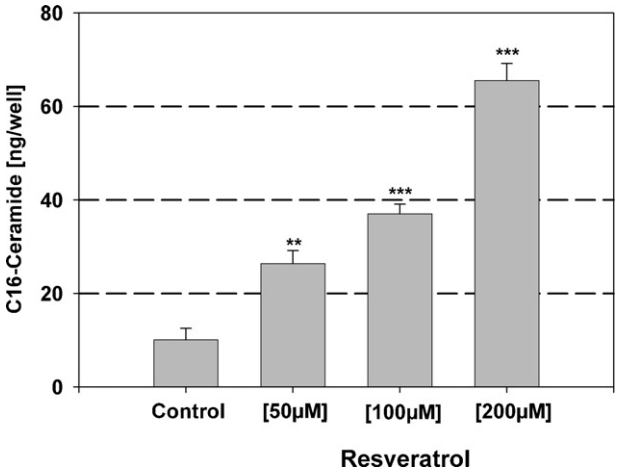

Fig. 1 – Synthesis of C16-ceramide in Caco-2-wildtype cells after incubation with resveratrol [50–200 μmol/L] for 24 h. Resveratrol significantly enhances the intracellular C16-ceramide concentration in a dose-dependent manner. Means ± S.E.; $n = 4$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. control.

3.2. Effects of C2- and C6-ceramides on cell proliferation and cell counts

Since natural ceramides are not permeant to cell membranes, our study has been carried out by using short chain cell-permeable analogs to determine the role of ceramides in this signal transduction pathways. Caco-2 and HT-29-cells were incubated with increasing concentrations of C2- and C6-ceramides [1–40 μmol/L] for 24, 48 and 72 h. After each time interval both cell proliferation ELISA (BrdU) and crystal violet staining were performed. Both in Caco-2- and HT-29-cells a significant time- and dose-dependent decrease in cell proliferation and cell counts could be measured, whereby we limit our illustrations on the effects after 48 h (Fig. 2).

3.3. Effects of ceramides on ODC activity as well as on c-myc and ODC protein expression

Resveratrol on the one hand induces intracellular ceramide synthesis and on the other hand reduces the protein levels of the protooncogenes c-myc and ODC [6]. To reveal a possible coherency, we first measured the effects of C2- and C6-ceramides [10–40 μmol/L] in Caco-2-cells (Fig. 3A and B) and C2- [10–30 μmol/L] and C6- [1–10 μmol/L] ceramides in HT-29-cells (Fig. 3C and D) on ODC activity after 24 h of treatment which both caused a significant inhibition in dose-dependent manner [$^{***}p < 0.001$]. We further have done Western blot analysis to measure effects on the protein levels of ODC and c-myc after treatment with the C6-ceramide *N*-hexanoylsphingosine [10–40 μmol/L]. And actually a dose-dependent decreases both in c-myc (Fig. 4B) [$^{**}p < 0.001$] and ODC (Fig. 4D) [$^{*}p < 0.05$] protein levels comparable to the resveratrol-induced effects [6] could be observed after 6 h of incubation.

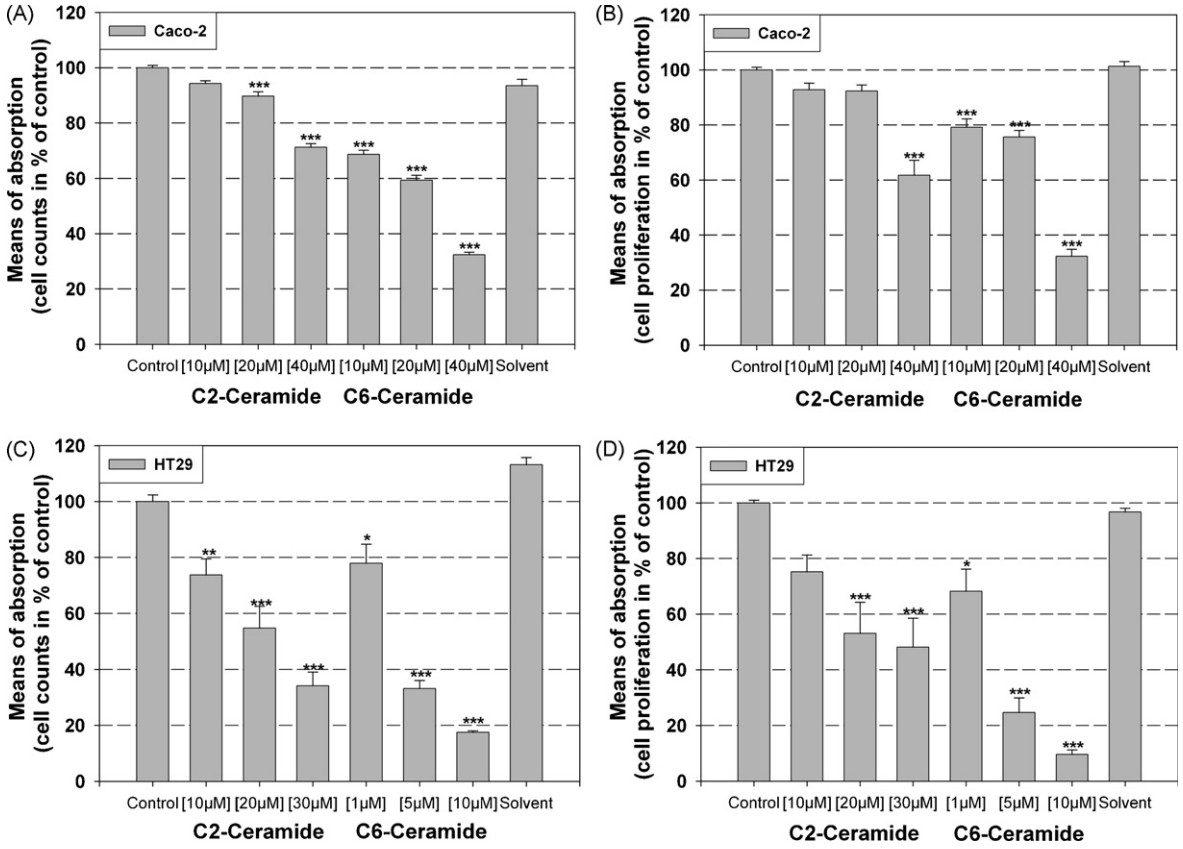

Fig. 2 – (A) Cell counts and (B) cell proliferation of Caco-2 cells 48 h after incubation without (control) or with C2-ceramide [10–40 μmol/L] or C6-ceramide [10–40 μmol/L]. The ceramides lead to a conspicuous dose- and time-dependent reduction of cell counts as well as an inhibition of cell proliferation. Means ± S.E., $n = 3$. (C) Cell counts and (D) cell proliferation of HT-29 cells 48 h after incubation without (control) or with C2-ceramide [10–30 μmol/L] or C6-ceramide [1–10 μmol/L]. The ceramides again lead to a conspicuous dose- and time-dependent reduction of cell counts as well as an inhibition of cell proliferation. Means ± S.E., $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. control.

3.4. *The role of ceramide biosynthesis in resveratrol-induced inhibition of ODC activity*

Two major pathways may contribute to intracellular ceramide accumulation: namely the sphingomyelinase (SMase)-dependent catabolism of sphingomyelin, as well as *de novo* synthesis catalyzed through serine palmitoyltransferase. Hence, we tested whether selective pharmacological inhibitors of these two key enzymes were able to prevent resveratrol-induced inhibition of ODC-activity in Caco-2- and HT-29-cells. While co-incubation with the SMase inhibitor manumycin [1 μmol/L] causes no changes in resveratrol action, blockade of *de novo* ceramide synthesis with the SPT-inhibitors L-cycloserine [1 mmol/L] and myriocin [5 μmol/L] counteracted inhibitory effects of resveratrol [100 μmol/L] on ODC-activity (Fig. 5). To further verify the involvement of ceramide synthesis in resveratrol-mediated effects we treated Caco-2 cells with resveratrol [100 μmol/L] alone and in combination with L-cycloserine and measured the protein levels of c-myc and ODC after 24 h of incubation. As already shown in earlier studies [6] resveratrol leads to a significant decrease of both c-myc [$^{***}p < 0.001$] (Fig. 4A) and ODC [$^{***}p < 0.001$] (Fig. 4C) protein levels, which could be significantly reduced [$^{*}p < 0.05$], when ceramide *de novo* synthesis was suppressed.

3.5. *The effect of exogenous spermine on resveratrol-induced reduction of cell counts*

To determine whether the decrease in c-myc and ODC are the cause of decreased growth rate or a result, we performed an add-back experiment with exogenous spermine [50 μmol/L]. For this we treated Caco-2-cells with spermine [50 μmol/L], resveratrol [50–100 μmol/L] and the combination of both and measured the cell counts after 48 h of incubation (Fig. 6). As spermine was able to counteract resveratrol-actions significantly, we conclude that the observed reduction of cell counts after resveratrol-treatment is due to a reduction of intracellular polymine levels.

3.6. *The role of PPARγ in resveratrol-induced inhibition of ODC activity*

As previously shown [20] the activation of transcription factor PPARγ plays a crucial role in resveratrol-induced activation of catabolic SSAT. So, we wanted to determine whether this receptor is also involved in ODC inhibition. In accordance to our recently published data, we now investigated the effects of resveratrol [50–100 μmol/L] on ODC activity in Caco-2-wild-type cells compared to Caco-2-cells transfected with either the

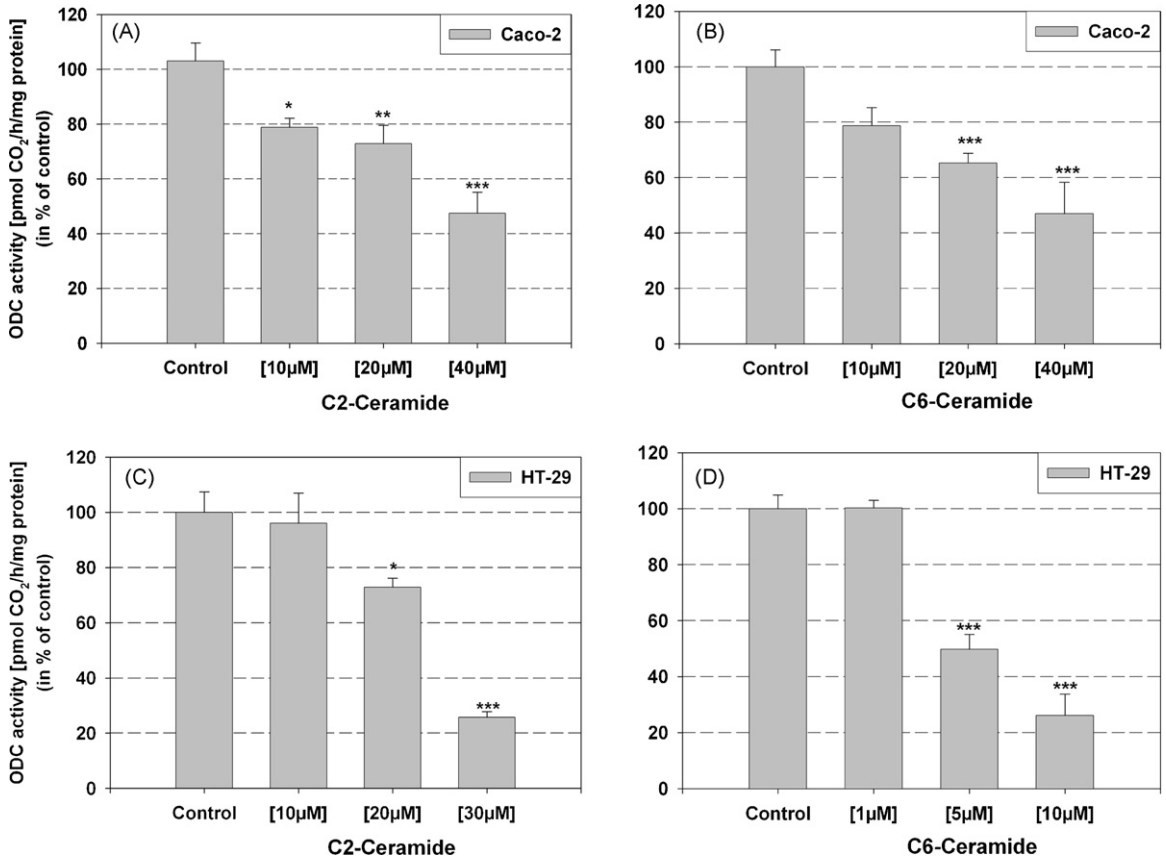

Fig. 3 – Influence of C2- and C6-ceramide on ODC activity in Caco-2- and HT-29-cells. Caco-2-cells were treated for 24 h with increasing concentrations of (A) C2-ceramide [10–40 μmol/L] and (B) C6-ceramide [10–40 μmol/L], HT-29-cells with (C) C2-ceramide [10–30 μmol/L] and (D) C6-ceramide [1–10 μmol/L]. ODC activity was determined by $^{14}CO_2$-release from labelled ornithine. Results (means ± S.E.; $n = 4$) are expressed in enzyme units (picomole of released CO_2) per milligram cellular protein per hour. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. control.

empty vector or a dominant-negative PPARγ mutant after 24 h. But in contrast to SSAT activation PPARγ seems not to be essential for resveratrol-induced ODC inhibition as no differences could be observed, when PPARγ mediated functions are suppressed (Fig. 7).

4. Discussion

Colorectal cancer is a major public health concern in all developed countries. Despite decades of advances in the treatment and prevention of colorectal cancer, it remains the second most common cause of cancer death [21]. Hence, interest in the concept and practice of chemoprevention as an approach to arrest or reverse carcinogenesis at its earliest stages has increased greatly in the past few years [22]. Therefore, dietary polyphenols are of great interest due to their antioxidative and anticarcinogenic activities. Resveratrol, present in red wines, peanuts and grapes, exhibits multiple chemopreventive effects comprising inhibition of cell growth [23,24] and angiogenesis [25] as well as induction of apoptosis [26], whereby the underlying molecular mechanisms are only partly deciphered [6,20].

It is now well established that ceramides are important second messengers for cell regulation which participate in signal transduction by activating specific serine/threonine kinases, or by stimulating protein phosphatases. An increase in intracellular ceramide concentrations could be induced by multiple exogenous agents comprising TNF-α, Fas ligand, 1α25-dihydroxyvitamin, chemotherapeutic agents, heat stress and interleukin-1 [27,28]. Over the past few years there has been an escalating interest in exploring the role of ceramide and its metabolites in tissue physiology and pathophysiology. Typically, strategies that elevate cellular ceramide are being used for therapies aimed to arrest growth or promote apoptosis [29,30]. Interestingly, we could show that also the antiproliferative effects of resveratrol closely correlate with a dramatic increase of endogenous ceramides. Similar effects could be observed in a metastatic breast cancer cell model, when ceramide levels increased ~5- and 10-fold after treatment with resveratrol 32 and 64 μmol/L, respectively, in comparison with untreated cells [31]. Treatment with C2- or C6-ceramide in turn, caused distinct growth inhibition in our colorectal cancer cell model. Sala et al. hypothesize that the phenolic moiety is critical for the ceramide-associated growth-inhibitory effects of resveratrol [32]. While the activation of mitogen-activated protein kinase

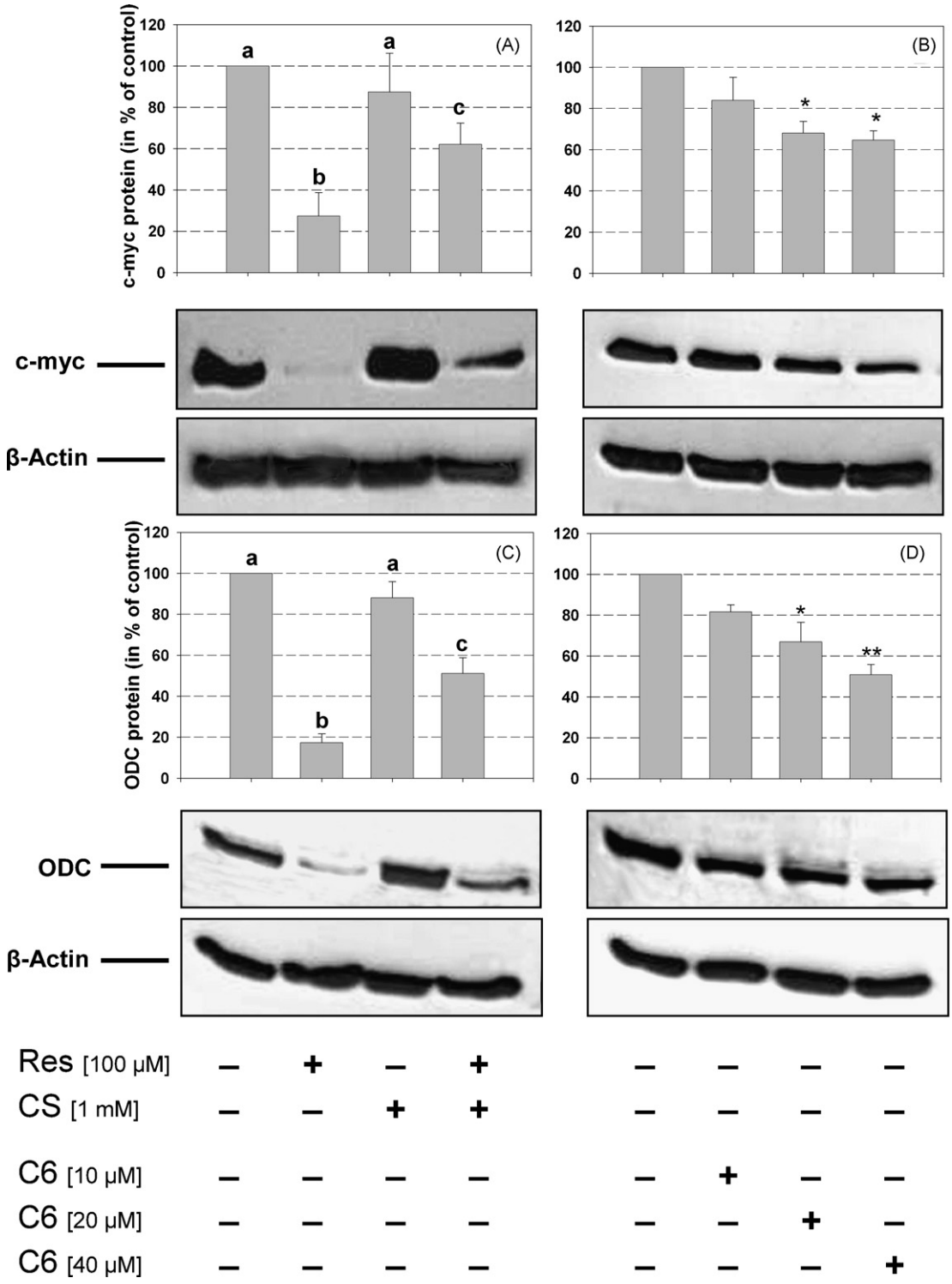

Fig. 4 – Western blot of c-myc (A) and ornithine decarboxylase protein (C) in Caco-2-cells after incubation with resveratrol [100 μmol/L] and cycloserine [1 mmol/L] alone and in combination for 24 h. Western blot of c-myc (B) and ornithine decarboxylase protein (D) in Caco-2 cells after incubation with increasing concentrations of C6-ceramide [10–40 μmol/L] for 6 h. A representative immunoblot of three independent experiments is shown. The graph presents the densitometric analysis. Means ± S.E.; $n = 3$; Values not sharing a letter differ significantly; $^{*}p < 0.05$, $^{**}p < 0.01$.

p38 plays a crucial role in resveratrol-induced SSAT-activation [20], an involvement in ceramide-mediated actions is discussed controversially [33–35] and requires further investigations.

Nearly 70% of human colon cancers are associated with the activation of proto-oncogene c-myc [36], a transcription factor that directly regulates the expression of ornithine decarboxylase (ODC) by binding to a specific CACGTG sequence in the

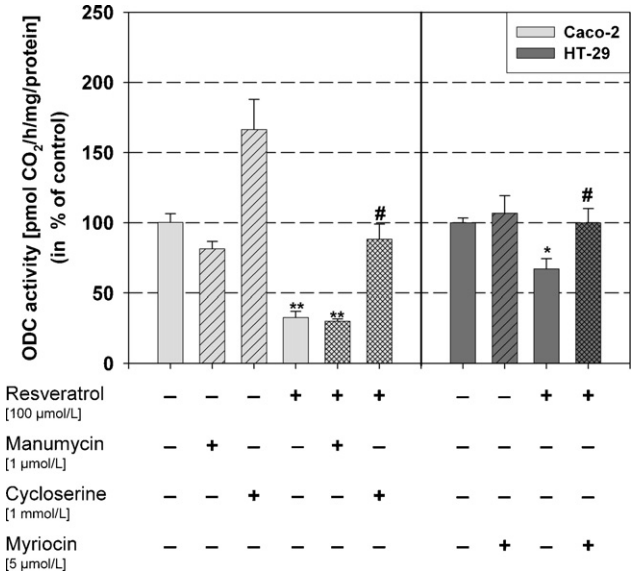

Fig. 5 – Effects of serine palmitoyltranferase inhibitors cycloserine [1 mmol/L] and myriocin [5 μmol/L] and sphingomyelinase-inhibitor manumycin [1 μmol/L] on resveratrol-induced ODC inhibition in Caco-2- and HT-29-cells after 24 h of incubation. Results (means ± S.E.; $n = 4$) are expressed in enzyme units (picomole of released CO_2) per milligram cellular protein per hour. # not significant, $^{*}p < 0.05$ $^{**}p < 0.01$ vs. control.

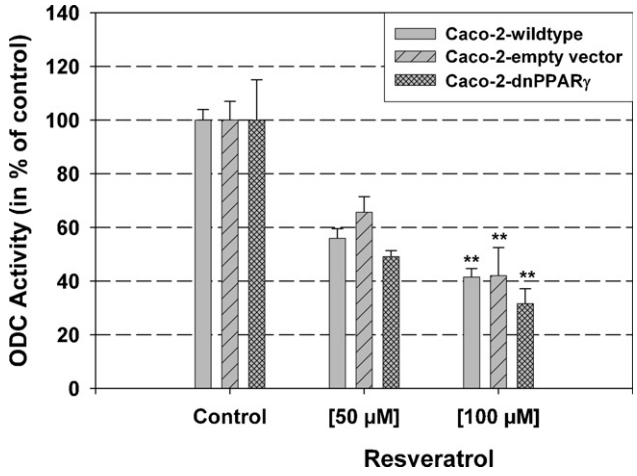

Fig. 7 – Activity of ornithine decarboxylase (ODC) in Caco-2-wildtype cells in comparison to transfected Caco-2 empty vector- and Caco-2-dnPPARγ cells after incubation with resveratrol [50–100 μmol/L] for 24 h. Means ± S.E.; $n = 4$; $^{**}p < 0.01$ vs. control.

gene promoter [37]. ODC in turn has long been known as a marker of carcinogenesis and tumor progression [38]. Based on our earlier findings that resveratrol regulates the expression of both c-myc and ODC genes [6], together with the results from Flamigni et al. [39] who demonstrated a reduction of c-myc and ODC expression in leukemia cells after ceramide-treatment, we tried to identify a possible involvement of ceramide *de novo* biosynthesis in the regulatory pathway in colorectal cancer cells. We measured c-myc as well as ODC expression after treatment with *N*-hexanoylsphingosine (C6-ceramide) and with resveratrol in combination with the specific serine palmitoyltransferase inhibitor L-cycloserine. While *N*-hexanoylsphingosine led to an obvious decrease of both c-myc and ODC protein levels, L-cyloserine but not sphingomyelinase-inhibitor manumycin conspicuously counteracted the inhibitory effects of resveratrol. Similar effects could be obtained in ODC activity.

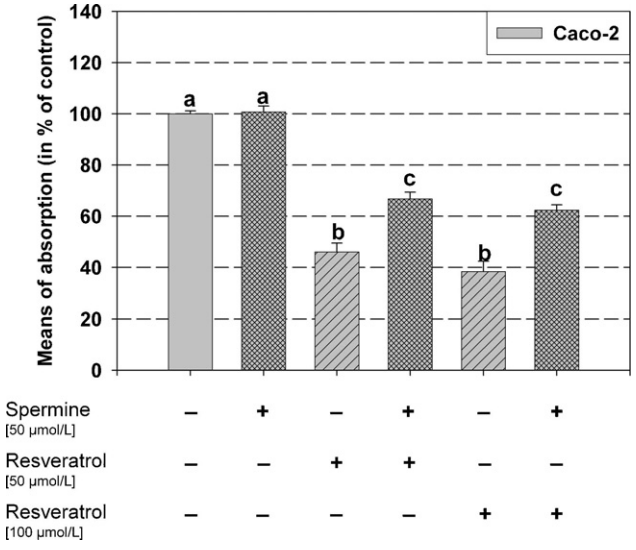

Fig. 6 – Cell counts of Caco-2 cells 48 h after incubation with spermine [50 mmol/L], resveratrol [50–100 μmol/L] and the combination of both. Spermine significantly counteracts Resveratrol-induced reduction of cell counts. Means ± S.E.; $n = 2$. Values not sharing a letter differ significantly.

Peroxisome-proliferator-activated receptors (PPARs) are ligand-activated transcription factors that heterodimerize with the RXRs and bind to peroxisomal proliferator response elements in the promoter region of multiple target genes [40–43]. Three PPAR isoforms have been described (α, β and γ). Several epidemiologic and *in vitro* studies suggest, that activation of PPARγ is associated with the prevention of colon cancer [43,44]. In a recently published study we could show, that activation of PPARγ is essentiell for resveratrol-induced activation of SSAT, the catabolic enzyme of polyamine metabolism [20]. Hence, we were interested, whether PPARγ activation plays another crucial role in resveratrol-induced ODC inhibition. To determine PPARγ-mediated functions we transfected a dominant-negative mutant in Caco-2 cells, which retains ligand and DNA binding, but exhibits markedly reduced transactivation due to impaired coactivator recruitment [19]. But in contrast to SSAT induction, PPARγ activation seems not to be critical for resveratrol-mediated ODC inhibition, as no differences in resveratrol-actions could be observed, when PPARγ mediated functions are suppressed (Fig. 7).

The identification of increased polyamine concentrations in a variety of cancer tissues has led to the design and development of inhibitors of polyamine metabolism as a new

strategy for therapeutic or preventative interventions. The best-known inhibitor of polyamine biosynthesis is alpha-difluoromethylornithine (DFMO), a specific inhibitor of ornithine decarboxylase [45]. Even though triggering promising effects *in vitro*, DFMO has been less successful in cancer therapy, resulting in cytostatic rather than cytotoxic effects *in vivo* [46]. Although much emphasis in the past has been on the biosynthetic pathway of polyamine metabolism, considerable interest has recently been generated with regard to the catabolic pathways, maintaining a properly balanced ratio of polyamines in cells [47]. This suggestion may well explain the increased efficacy of combined chemopreventive therapy with non-steroidal anti-inflammatory drugs (NSAIDS) in animal models, as this agents recently have been shown to induce SSAT gene expression [48]. In this context resveratrol could show great therapeutic potential in the chemoprevention and treatment of colorectal cancers, by simultanously leading to SSAT activation as well as ODC inhibition.

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Ursolic acid induces apoptosis through PPARγ mediated SSAT-activation in colon cancer cells

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Key words:
Ursolic acid, apoptosis, colorectal cancer, Spermidine/spermine acetyltransferase, PPARγ

*The **abbreviations** used are: BrdU, bromodeoxyuridine; CDK, cyclin dependent kinase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxid; dnPPARγ, dominant negative PPARγ mutant; DTT, Dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ELISA, Enzyme-linked immunosorbent assay; FADD, Fas Associated protein with Death Domain; PPARγ, peroxisome-proliferator activated receptor γ; RXR, retinoid X receptor; SSAT, spermine/spermidine acetyltransferase; TNF-α, tumor necrosis factor- α; TRAIL, Tumor necrosis factor-related apoptosis-inducing ligand; UA, ursolic acid

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ABSTRACT

Previous studies could demonstrate that ursolic acid (UA), a pentacyclic triterpene found in berries and plants has antiproliferative as well as proapoptotic activities on cancer cells. The objective of this study was to elucidate the underlying molecular mechanisms of these chemopreventive effects.
We could show, that treatment with UA leads to a significant time- and dose-dependent cell growth inhibition of colorectal cancer cells, coincident with the upregulation of the cell cycle

regulators cyclin E, $p21^{WAF1/Cip1}$ and $p27^{Kip1}$. In addition, UA significantly induces apoptosis, which is mediated by an increase of BAX/Bcl-2-protein-ratio as well as an upregulation of TRAIL protein which meets in an induction of caspase-3 activity. Furthermore, we could show that UA leads to a PPARγ-dependent induction of spermidine/spermine acetyltransferase (SSAT), a key enzyme of polyamine metabolism, which is associated with catabolism of intracellular polyamines and subsequent apoptotic responses.

In conclusion, the observed reduction of cell growth of colon cancer cell lines after treatment with ursolic acid presumably results from a large increase in the number of apoptotic cells. The induction of the catabolic enzyme SSAT via PPARγ-dependent mechanisms therby seems to present the major molecular target in the induction of programmed cell death.

INTRODUCTION

Ursolic acid is a pentacyclic triterpenoid compound which naturally occurs in a large number of berries, medicinal herbs and other plants. It was identified as the major biological active incredient in a large number of plants which are used in traditional east asian folk medicine as drugs exhibiting hepatoprotective, antiinflammatory and anti-tumor effects (for review see [1]). Additionally, chemopreventive effects could be demonstrated in several cancer models comprising inhibition of proliferation as well as induction of apoptosis [2-4].

Cell division relies on the activation of cyclins, which bind to cyclin-dependent kinases (CDKs) to induce cell-cycle progression towards S phase and later to initiate mitosis. Since uncontrolled cyclin-dependent kinase activity is often the cause of human cancer, their function is tightly regulated by cell-cycle inhibitors such as the $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ proteins [5]. They have been described to regulate the G1-S transition by interacting with specific cyclin/cyclin dependent kinase complexes, and have also been shown to mediate growth arrest when overexpressed in the cell.

Apoptosis or programmed cell death is defined as an active physiologic process of cellular self-destruction, with specific morphologic and biochemical changes in the nucleus and cytoplasm [6]. The signaling event leading to apoptosis can be divided into two distinct pathways, namely the intrinsic and extrinsic pathway. Engagement of the intrinsic pathway results in altered mitochondrial membrane permeability and the release of pro-apoptotic factors including cytochrome *c*, caspase-9 and second mitochondria-derived activator of caspases (Smac)/DIABLO into the cytosol. Cytochrome c binds to apoptosis-inducing factor-1 (Apaf1) and procaspase-9 to form the “apoptosome”, which leads to activation of caspase-9 and subsequently caspase-3, resulting in apoptosis [7]. A second caspase-independent pathway is characterized by the leakage of apoptosis-inducing factor AIF from mitochondria, resulting in direct chromatin condensation and DNA fragmentation [8]. The extrinsic pathway is characterized by ligand fixation to death receptors present on the cell surface. These death receptors are members of the TNF receptor gene superfamily, which share similar, cysteine rich extracellular domains [9]. Ligation of death receptors results in recruitment of adapter molecules such as FADD, which in turn recruits procaspase-8 to form the death inducing-signaling complex (DISC) [10]. DISC releases caspase-8, which activates caspase-3 [11].

Little is known about the underlying molecular mechanisms of ursolic acid related effects on cell proliferation and apoptosis in colorectal cancers. Thus the major aim of this study was to analyze modulatory effects on cell cycle regulating proteins as well as pro- and anti-apoptotic

factors and to further characterize signal transduction pathways leading to this chemopreventive actions.

MATERIALS AND METHODS

Cell culture. Caco-2 cells of passages 20-30 were kept in Dulbecco´s modified eagle medium (DMEM), supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% sodium pyruvate and 1% nonessential amino acids. HCT-116 and HT-29 cells of passages 17-30 were cultured in McCoy's 5A supplemented with 10% FCS and 1% penicillin/streptomycin. Both cell lines were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. The cells were passaged weekly using Dulbecco´s PBS containing 0.25 % trypsin and 1 % EDTA. The medium was changed three times per week. Cells were screened for possible contamination with mycoplasma at monthly intervals. For experiments, the cells were seeded onto plastic cell culture wells in serum-containing medium and allowed to attach for 24h. For the SSAT activity assay the cells were synchronized in medium containing 1% FCS 24h before treatment. Ursolic acid, Fetal calf serum (FCS) and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO), Dulbecco's modified Eagle's medium (DMEM) and Geneticin (G418) from Gibco/Invitrogen (Carlsbad, CA); sodium pyruvate solution, glutamine, penicillin and streptomycin stock solutions from PAA Laboratories GmbH (Ontario, Canada); Lipofectamine™ 2000 from Invitrogen (Carlsbad, CA).

Cell Counts

Cells were suspended and cultured on 96-well dishes at a density of 10^4/well (0.28 cm^2). Treatment started 24h after plating. At given time points following treatment cell numbers was assessed by crystal violet staining. Medium was removed from the plates and cells were fixed with 5% formaldehyde for 5 min. After washing with PBS cells were stained with 0.5% crystal violet for 10 min, washed again with PBS and unstained with 33% acetoic acid. Absorption, which correlates with the cell number, was measured at 620 nm.

Cell Proliferation

The effects of ursolic acid on DNA synthesis of cells were assessed using an cell proliferation ELISA kit (Roche Diagnostics, Mannheim, Germany). This assay is a colorimetric immunoassay for quantification of cell proliferation based on the measurement of bromodeoxyuridine (BrdU) incorporation during DNA synthesis, and is a non-radioactive

alternative to the [^{3}H]-thymidine incorporation assay. Cells were grown in 96-well culture dishes (10^4 cells/well), incubated with ursolic acid for different time intervalls, and then labelled with BrdU for a further 4 hours. Incorporated BrdU was measured colorimetrically.

SDS-polyacrylamide gel electrophoresis and immunoblot analysis

Caco-2 cells were seeded in 80 cm^2 flasks; 24h after plating, cells were incubated with substances for different time intervals. Western blot analysis using total protein extracts from cultured cells was performed as previously described [12]. Protein was quantified with the Bio-Rad (Bio-RAD Laboratories, Munich, Germany) protein colorimetric assay. Reprobing of blots for expression of β-actin was done routinely. Antibodies for $p21^{WAF1/CIP1}$, $p27^{KIP1}$, Cyclin E and BAX were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Bcl-2 was purchased from Cell Signaling (Danvers, MO) and anti-TRAIL from Cayman Chemicals (Ann Arbor, MI). For quantitative analysis, bands were detected and evaluated densitometrically by ProViDoc system (Desaga, Wiesloch, Germany), normalized for the density of β-actin.

Cell death detection assay

To determine and quantify the induction of apoptosis by ursolic acid in colon cancer cells, cytoplasmic histone-associated DNA fragments were measured using a commercially available Cell Death Detection ELISA kit (Roche Diagnostics, Mannheim, Germany). 24h after treatment with the mentioned substances, one µg of cell lysate was used for the ELISA procedure, following the manufacturer's instructions. DNA fragmentation was quantified photometrically at 405 nm.

Caspase-3-Activity assay

Activity of caspase 3 was determined using a fluorometric immunosorbent enzyme assay (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, cells grown in 6-well plates were incubated with increasing concentrations of ursolic acid for 24 h. Cells were washed with ice-cold PBS harvested in lysis buffer and centrifuged at 4°C for 2 minutes at 13000g. Caspase 3 from cellular lysates is captured by a monoclonal antibody on a coated microtiter plate. Following a washing step, subtrate is added that is cleaved proportionally to the amount of activated caspase 3. Due to proteolytic cleavage of the substrate, free fluorescent 7-amido-4-trifluoromethyl-coumarin (AFC) is generated.

Fluorescence was measured (excitation/emission, 430/535 nm) with the fluorescence microplate reader Tecan SpectraFluor PLUS.

SSAT-Activity

Cells were washed twice with cold homogenizing buffer (10mM Tris/HCl, pH 7.5, 2.5 mM DTT, 1mM EDTA), harvested by scraping, disrupted by sonification and centrifuged at 15.000 g at 4°C for 15 min. Sixty microlitre aliquots of the supernatant were incubated with 0.3 µmol/L spermidine, 10 µmol/L Tris/HCl (pH 7.8), and 3,7 kBeq [acetyl-^{14}C]CoA (Hartman Analytic GmbH, Braunschweig, Germany) at 37°C for 10 min. The reaction was terminated by chilling and the addition of 20 µl of 1M NH_2OH. Subsequently, samples were centrifuged at 15.000 g for 5 min. Thirty microlitres of the supernatant were spotted onto a Whatman P81 paper disc (2.4 cm in diameter). The paper disc was washed with aqua dest. and ethanol on a filter, dried and transferred to a vial containing 3 ml of scintillation cocktail (Packard Biosciences, Groningen, The Netherlands). Radioactivity was measured in a liquid scintillation counter (Packard Instruments, Meriden, CT). Controls included samples for measurement of non-enzymatic incorporation of [acetyl-^{14}C]CoA into monoacetylspermidine. For all treatments tested the assay was repeated without addition of spermidine, to estimate unspecific acetylation by other enzymes than SSAT. These values were subtracted from the results obtained from the SSAT-activity measurements.

Transfection assay

The following plasmids were used for transfection: pcDNA3 (Invitrogen, Carlsbad, CA), as an empty vector for control transfection and the plasmid pcDNA3-PPARγL468A/E471A, a dominant-negative double mutant, that was kindly provided by VK Chatterjee (Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom) [13]. These constructs were transfected into subconfluent Caco-2 cells with lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 6 h the cells were fed with fresh medium containing 10% FCS. G418 (400 µg/ml) and culture medium containing 10 % FCS. 24 hours later the cells were fed with medium containing G418 (400 µg/ml) and culture medium was replaced twice a week. G418-resistant colonies were collected and used for further analysis.

Statistics

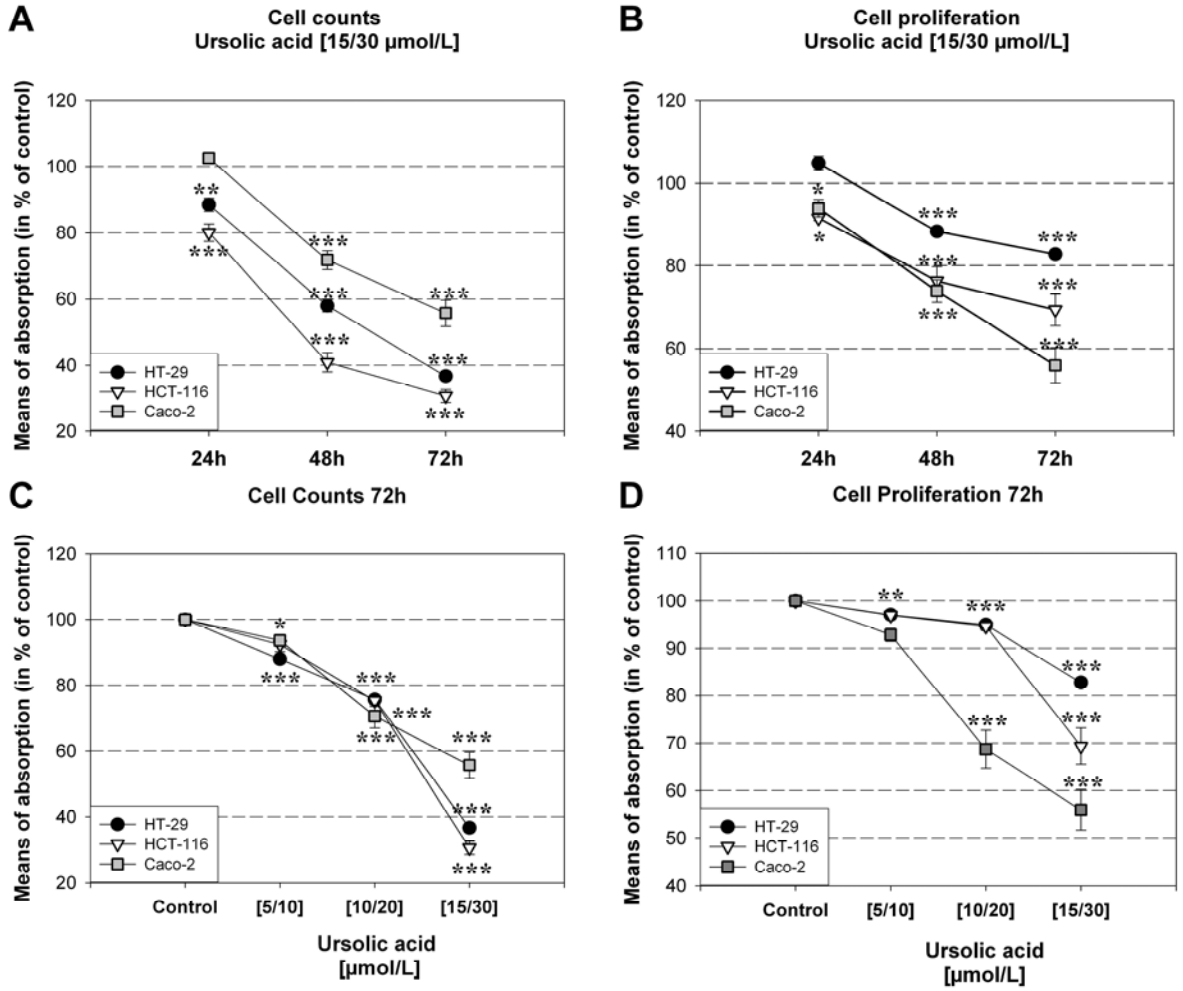
The data are expressed as means ± SE of at least three independent experiments. Analysis of variance (ANOVA) was performed when more than two groups were compared, and when

significant, multiple comparisons were performed with the Turkey test. A p value < 0.05 was considered to be significant.

RESULTS

Effects of ursolic acid on cell growth of colorectal cancer cell lines

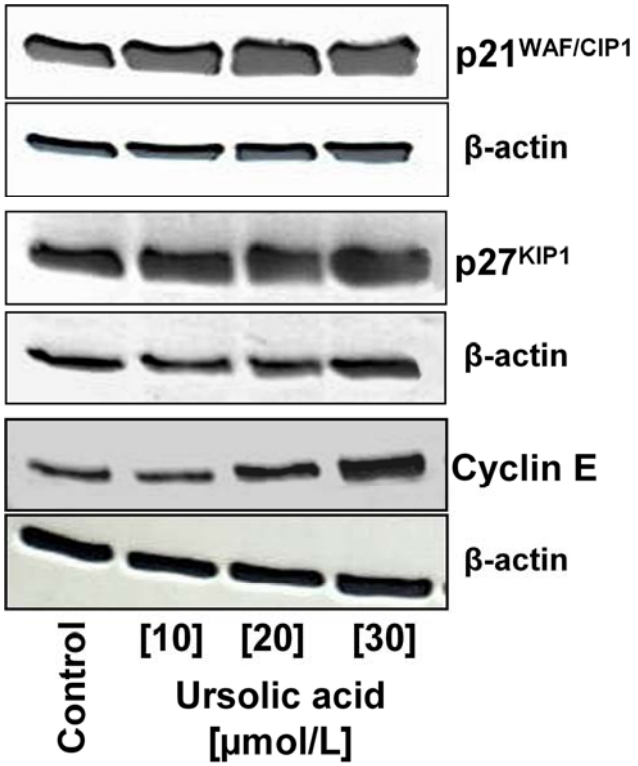
Caco-2 [10-30 µmol/L], HCT-116 [5-15 µmol/L] and HT29 [5-15 µmol/L] cells were incubated with increasing concentrations of ursolic acid for 24 h, 48 h and 72 h. After each time interval both cell proliferation ELISA (BrdU) and crystal violet staining were performed. While not effective in Caco-2 cells after 24h of incubation ursolic acid leads to significant time- and dose-dependent decrease in cell counts (***$p<0.001$) as well as to a significant inhibition of cell proliferation (***$p<0.001$) in all cell lines after 48h at the latest. (Figures 1A-D)



Effect of ursolic acid on cell cycle regulating proteins

To decipher the molecular mechanisms leading to cell growth inhibition we started to measure the expression status of several cell cycle regulating proteins (Figure 2), whereby the most prominent effects could be observed with the cell cycle inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$. While ursolic acid [10-30 µmol/L] leads to a significant dose-dependent increase of $p21^{WAF1/Cip1}$ protein levels already after 24h of incubation (~ 30% at 20 µmol/L, *$p<0.05$), an increase of $p27^{Kip1}$ levels could not be observed until 48h of treatment (~ 40% at 20 µmol/L, *$p<0.05$). Another interesting change could be detected in the expression levels of cyclin E, which is essential for progression through the G1-phase of the cell cycle and for initiation of DNA replication by interacting with and activating its catalytic partner, the cyclin dependend kinase 2 and therefore can be considered as a promotor of cell replication and proliferation. In contrast to our expectations, we could observe a significant dose-dependend increase in the protein levels of cyclin E in Caco-2 cells after 24h of incubation with ursolic acid [10-30 µmol/L] (~ 40% at 20 µmol/L, *$p<0,05$).

Figure 2



Induction of apoptosis by ursolic acid

To evaluate a possible influence of apoptosis induction on the cell growth inhibition of Caco-2, HCT-116 and HT-29 cells, we investigated DNA fragmentation as a marker of programmed cell death. Thereby, ursolic acid [5-30 µmol/L] causes a significant dose-dependent increase of DNA-fragments after 24h of incubation [***p<0,001 vs. control at 15/30 µmol/L in all cell lines] (Figure 3). Additionally, we measured caspase-3-activity in Caco-2 cells at the same point of time as a further marker of apoptotic actions. Again, ursolic acid seems to exhibit pro-apoptotic properties, as we could also observe a significant dose-dependent increase of caspase-3-activity in Caco-2-cells [***p<0.001 vs. control] (Figure 4).

Figure 3

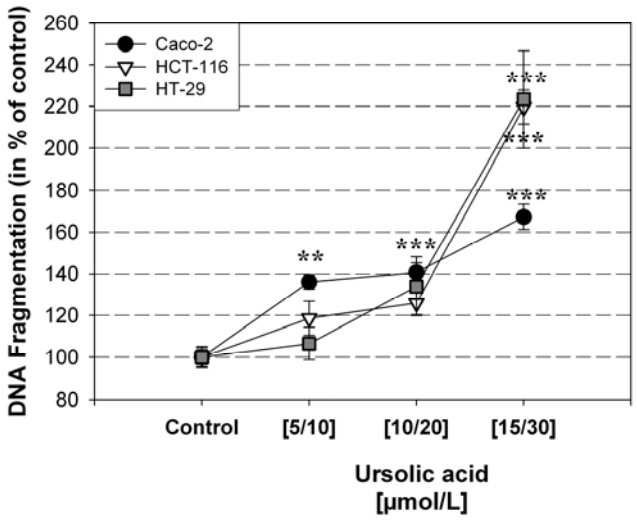
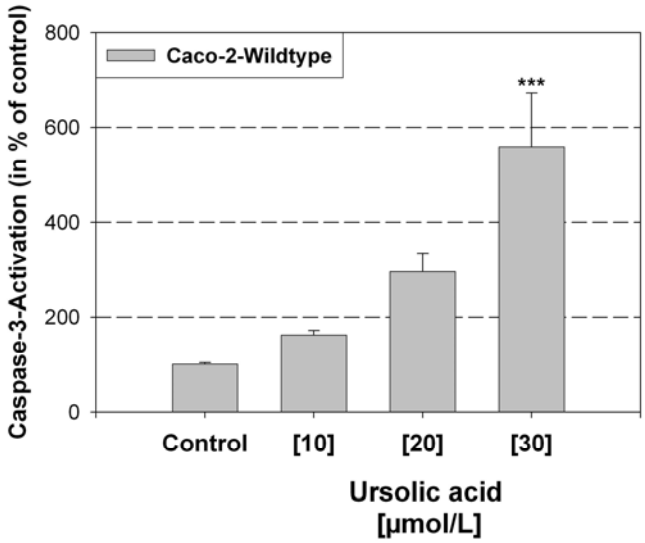


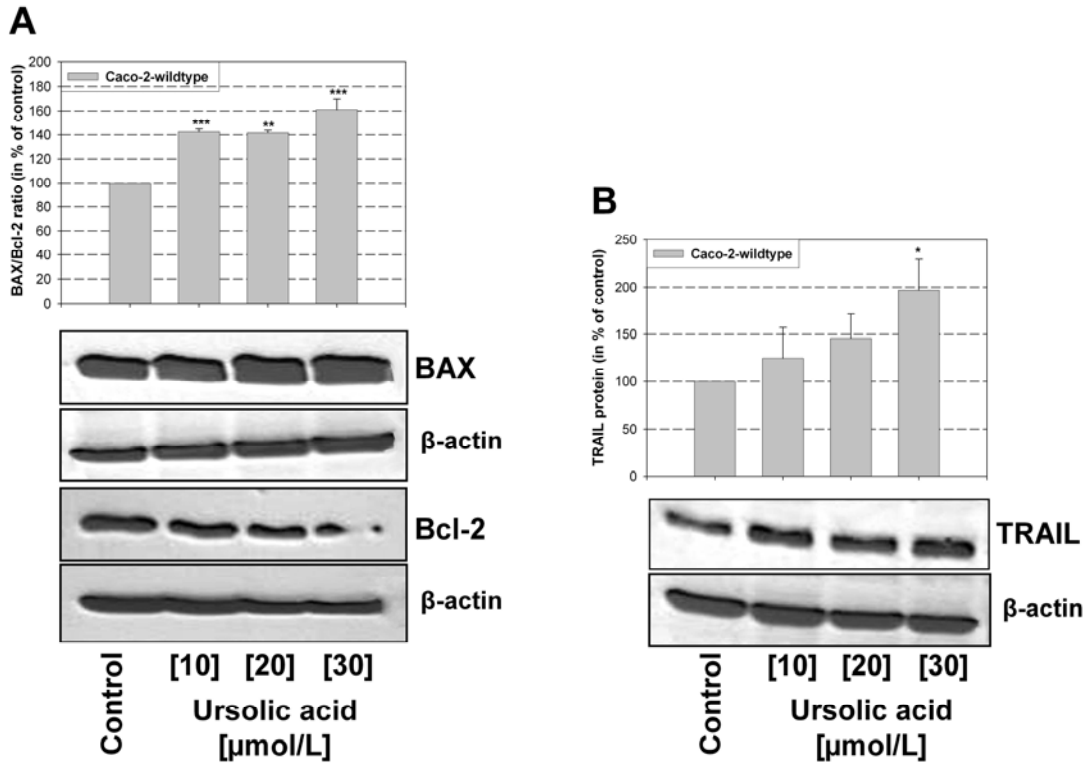
Figure 4



Expression of apoptosis regulating proteins

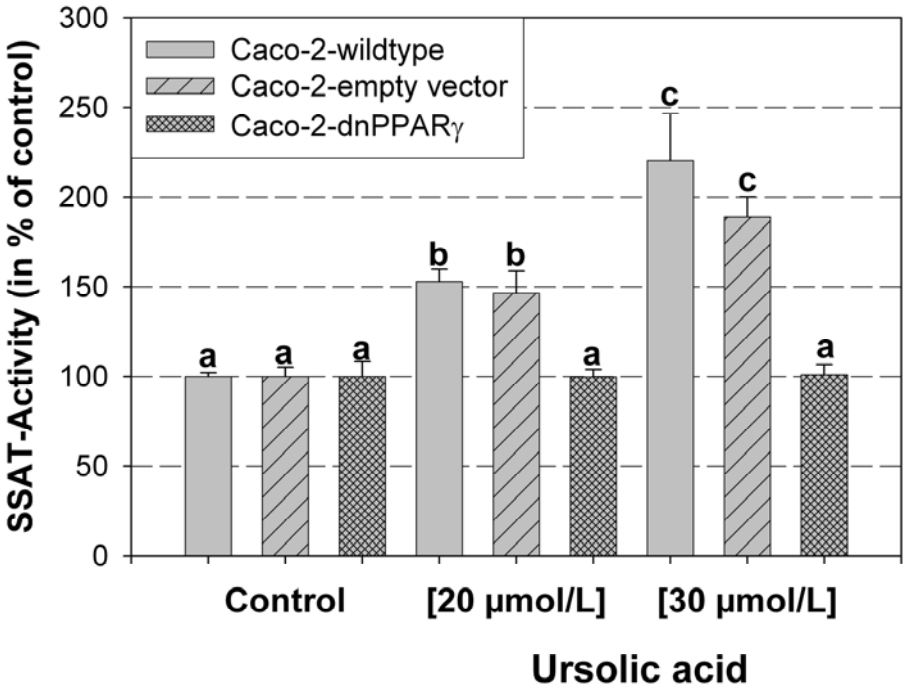
To specify the underlying molecular mechanisms leading to apoptosis after incubation with ursolic acid, we examined several apoptosis regulating proteins in Caco-2 cells by Western blot analysis. To analyse the effects on the intrinsic pathway we have chosen members of the Bcl-2 family of proteins, which are known to regulate membrane permeability and cytochrome c release from mitochondria. While ursolic acid [10-30 µmol/L] leads to an upregulation of proapoptotic BAX protein levels (up to ~20%), the expression of the antiapoptotic Bcl-2 was diminished after 24h of incubation (about ~40%). This results in a significant increase of the BAX/Bcl-2 protein ratio up to 60% (***$p<0.001$ at 30 µmol/L) (Figure 5A) which is generally known to trigger apoptosis [14,15]. We further measured protein levels of TRAIL an immunological inducer of extrinsic mechanisms leading to programmed cell death. This ligand, binding to specific death receptors on the cell surface, was also significantly upregulated after 24h of incubation with ursolic acid [10-30 µmol/l] in a dose-dependent manner (*$p<0.05$ at 30 µmol/L) (Figure 5B).

Figure 5



SSAT activation by ursolic acid

Next we examined the effects of ursolic acid [20-30 µmol/L] on SSAT activity in Caco-2-wildtype cells compared to Caco-2-cells transfected with either an empty vector or a dominant negative PPARγ mutant to investigate effects mediated by PPARγ. Ursolic acid leads to a significant increase of SSAT activity [***p<0,001 vs. control at 30 µmol/L] in Caco-2-wildtype cells after 24 h of incubation. In Caco-2-empty vector cells ursolic acid also significantly increases SSAT-activity [***p<0,001 vs. control at 30 µmol/L], whereas no effects could be observed when PPARγ mediated functions are suppressed in Caco-2-dnPPARγ mutant cells (Fig 6).



DISCUSSION

Interest in the concept and practice of chemoprevention as an approach for the control of cancer has increased greatly in the past few years [16]. Multiple natural agents have been shown to be effective for blocking carcinogenesis in certain human cancers and animal models. Using non-toxic chemical substances therefore is regarded as a promising alternative strategy to therapy for control of human cancers. The observed anti-carcinogenic effects may be due to blocking effects on the carcinogenesis stages of initiation, promotion, or progression [17,18]. However, the precise underlying molecular mechanisms remain largely unknown. The aim of our study was to characterize chemopreventive effects of ursolic acid in a cell culture model of colorectal cancer. Attempts to show favourable effects in vitro have let to the identification of multiple direct targets for this compound. UA blocked cell cycle progression in the G1 phase for example was shown to be associated with a marked decrease in the protein expression of cyclins and their activating partners the cyclin-dependent kinases [19-22] and with a concomitant induction of $p21^{WAF1/Cip1}$ in miscellaneous cancer cell lines [23-25]. Unfortunately, little reseach was done in colon cancer cells thereby mainly focusing apoptotic mechanisms [26,27]. After detecting potent cell growth inhibitory properties of ursolic acid we started to measure the expression status of several cell cycle regulating proteins, wherby the most prominent effects could be observed in the upregulation of cell cycle inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$. Contrary to expectations incubation with Ursolic acid also leads to a conspicious increase of cell cycle progressor cyclin E, which is however consistent with earlier findings by Wolter et al. [28] and Schneider et al. [29], which could show the same effects after treatment with the polyphenol resveratrol and take this as a result of a cell cycle arrest in the S-phase. However, cell cycle analysis of ursolic acid treated cells presents a predominant arrest in the G1 phase [30,31]. These controversial results will deserve further investigations. Moreover, there are several lines of evidence, that the induction of cyclin E by genotoxic stress, such as ionizing radiation [32] or chemotherapeutic agents [33] could play a functional role in the initiation phase of apoptosis in malignant cells lines, in addition to its reported key regulatory role in the control of the G1 to S-phase transition and the initiation of DNA replication [34]. Normally, the intracellular level and activities of $p27^{Kip1}$ and cyclin E correlate negatively as there exist interdependence regulatory mechanisms. Simultaneous accumulations in both $p27^{Kip1}$ and cyclin E are known to be characteristic phenotypes in cells derived from mice lacking S-phase kinase associated protein 2 (Skp2) [35] suggesting a possible involvement of protooncogene

Skp2 in the regulation of $p27^{Kip1}$ and cyclin E, which might provide a target of ursolic acid mediated actions.

Two major apoptosis pathways have been identified, the death receptor or extrinsic pathway and the mitochondrial or intrinsic pathway. The mitochondrial pathway is regulated by members of the bcl-2 protein family, which can be divided in pro- and anti-apoptotic groups. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family of cytokines, which can induce apoptotic cell death by engaging the death receptors DR4 and DR5, while sparing most normal cells [36]. In certain tumor cell lines, TRAIL protein expression could be induced by chemopreventive agents resulting in TRAIL-mediated apoptosis in an autocrine or paracrine manner [37-39]. This suggests that endogenously expressed TRAIL, which we could also observe in our colon cancer model after ursolic acid treatment, may be at least partly responsible for the observed chemopreventive effects. The targeting of pro- and anti-apoptotic members of the Bcl-2 family of proteins, is the focus of efforts to modulate the intrinsic pathway of apoptosis and effect tumor death [40,41]. While ursolic acid leads to an upregulation of proapototic BAX protein levels, the expression of the antiapoptotic Bcl-2 was diminished after 24h of incubation. This results in a significant increase of the BAX/Bcl-2 protein ratio which is generally known to trigger apoptosis [42,43]. Taken together ursolic acid seems to lead to an activation both of extrinsic and intrinsic signaling pathways both resulting in Caspase-3 activation, followed by DNA fragmentation and programmed cell death.

Ornithine-derived polyamines (putrescine, spermidine, and spermine) are biogenic organic polycations that are present in all living cells. They have pleiotropic effects, with a well-described role as major metabolic regulators of cell proliferation and cell death balance [44]. Intracellular levels of polyamines must be maintained within narrow limits, as a decrease is related to cell growth inhibition, whereas an excess appears to be toxic [45]. A correlation between polyamines and cancer have been extensively studied for decades, pointing out the inhibition of polyamine biosynthetic enzymes ornithinedecarboxylase and s-adenosylmethioninedecarboxylase or activation of catabolic enzyme spermidine/spermine acetyltransferase (SSAT) as a potential chemopreventive strategy [46-48]. An association between SSAT induction followed by catabolism of the ubiquitous intracellular polyamines and subsequent apoptotic responses was first reported by Ha et al. [49]. Furthermore, Chen et al. could demonstrate that selective interference of polyamine-analogue induced SSAT prevents apoptotic signaling and apoptosis in human melanoma cells [50]. Much on the focus on SSAT has been on the functional level, but the regulation of SSAT gene expression has also been a

subject of recent investigations. Several transcription factors have been shown to activate SSAT expression. One regulatory pathway is the peroxisome proliferator (PPARs)-dependent pathway. These ligand-inducible transcription factors belong to the nuclear hormone receptor superfamily and occur in three different isotypes termed α, β and γ [51,52]. We could show that PPARγ is essential for SSAT-activation mediated by ursolic acid, which is in accordance with our recently published data, presenting the same effects after treatment with the polyphenol resveratrol [53], possibly by binding to PPAR response elements, which could be identified on the promotor of the SSAT gene [54].

CONCLUSION

In conclusion, the observed reduction of cell growth of colon cancer cell lines after treatment with ursolic acid presumably results from a large increase in the number of apoptotic cells. The modulation of the polyamine metabolism, especially the induction of the catabolic enzyme SSAT via PPARγ-dependent mechanisms thereby seems to present the major molecular target in the induction of programmed cell death.

Therefore, providing potent chemopreventive activities, phytochemicals like ursolic acid could in theory possibly serve as alternatives to chemically designed antineoplastic agents, as constituents of therapeutic drug combinations in advanced disease, or as adjuvant treatments.

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FIGURE LEGENDS

Fig 1. Cell counts and cell proliferation of colorectal cancer cell lines after incubation without (control) or with ursolic acid (UA). UA leads to a conspicuous time (24-72h) dependent decrease in cell counts (A) and to an inhibition of cell proliferation (B) of Caco-2-[30 µmol/L], HCT-116-[15 µmol/L] and HT29-cells [15 µmol/L]. After 72h of incubation, also a dose-dependent reduction of cell counts (C) as well as cell proliferation (D) could be observed in the tested cell lines (Caco-2 [10-30 µmol/L, HCT-116 and HT29 [5-15 µmol/L]. Means ± SE, n = 3; *p<0.05, **p<0.01, ***p<0.001.

Fig 2. Expression of cell-cycle regulating proteins after ursolic acid treatment of Caco-2 cells. Ursolic acid [10-30 µmol/L] leads to a dose-dependent protein increase of cell cycle inhibitor $p21^{WAF1/Cip1}$ and Cyclin E after 24h of incubation. An up-regulation of $p27^{Kip1}$ protein could be observed after 48h of incubation. For all proteins, a representative immunoblot of three independent experiments is shown. Means ± SE; n=3

Fig 3. (A) DNA-Fragmentation in colorectal cancer cells.
Ursolic acid leads to a significant dose-dependent induction of apoptosis in Caco-2-[10-30 µmol/L], HCT-116-[5-15 µmol] and HT29-cells [5-15 µmol/L] after 24h of incubation. Means ± SE; **p<0.01; ***<0.001.

Fig. 4. Caspase-3-Activity in Caco-2 cells
Treatment with ursolic acid in increasing concentrations [10-30 µmol/L] significantly activates pro-apoptotic caspase-3 after 24h. Means ± SE, n=3, ***p<0,001.

Fig 5. Expression of apoptose-related proteins in Caco-2-cells.
(A) Western blot of BAX and Bcl-2 after incubation with ursolic acid [10-30 µmol/L] for 24h. For both proteins, a representative immunoblot of three independent experiments is shown. The graph presents the densitometric analysis of the BAX/Bcl-2 ratio after 24h. (B) TRAIL protein after incubation with ursolic acid [10-30 µmol/L] for 24h. A representative immunoblot of three independent experiments is shown. The graph presents the densitometric analysis of the TRAIL/β-actin ratio. Means ± SE; n=3; *p<0.05. Means ± SE; n=3, # = not significant, **p<0.01, ***p<0.001

Fig. 6 Activity of spermine/spermidine acetyltransferase (SSAT) in Caco-2-wildtype cells in comparison to transfected Caco-2 empty vector- and Caco-2-dnPPARγ cells.
Ursolic acid [10-30 µmol/L] leads to a significant increase in SSAT-activity both in Caco-2-wildtype- and Caco-2-empty vector-cells after 24h of incubation. But no effects could be observed, when PPARγ-mediated functions are suppressed. Means ± SE; n=4; Values not sharing a letter differ significantly. **$p<0.01$.

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IV

Molecular Mechanisms of the Chemopreventive Effects of Resveratrol and Its Analogs in Colorectal Cancer: Key Role of Polyamines?[1,2]

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ABSTRACT Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), a phytoalexin found in grape skins, peanuts, and red wine, has been reported to exhibit a wide range of biological and pharmacological properties. It has been speculated that dietary resveratrol may act as an antioxidant, promote nitric oxide production, inhibit platelet aggregation, and increase high-density lipoprotein cholesterol and thereby serve as a cardioprotective agent (the so-called "French paradox"). Recently, it was demonstrated that resveratrol can function as a cancer chemopreventive agent, and there has been a great deal of experimental effort directed toward defining this effect. It has been shown that resveratrol and some of its analogues interfere with signal transduction pathways. Thus the activities of various protein kinases are inhibited, the expression of nuclear proto-oncogenes declines, and the activity of ornithine decarboxylase (ODC) is reduced. ODC, which catalyzes the rate-limiting step in the biosynthesis of polyamines, is closely linked with cellular proliferation and carcinogenesis. This review summarizes the recent advances that have provided new insights into the molecular mechanisms underlying the promising properties of resveratrol focusing on the key role of the polyamine metabolism in colorectal cancer cells. J. Nutr. 134: 3219–3222, 2004.

KEY WORDS: • resveratrol • piceatannol • human cancer cells • polyamines

Colorectal cancer is not only the third most frequent cancer in the world but also one of the most common human malignancies in Western countries. It affects men and women almost equally, with about 400,000 new cases in men and 380,000 in women annually. Almost 400,000 deaths from colorectal cancer still occur worldwide every year. Conventional chemotherapy has no consistent benefit in overall survival. However, in recent years, multidisciplinary research in epidemiology, molecular biology, and laboratory animal model studies contributed much to the understanding of the etiology of colorectal cancer. More important, these studies enabled the design of highly promising preventive strategies, which are about to influence both the incidence and the prognosis in patients with a high risk of developing this disease (1).

Equally promising, however, are the preliminary data suggesting that various nutrients may act as chemopreventive agents as well (2). Indeed, a wide array of phenolic substances, particularly those present in dietary and medicinal plants, have been reported to possess substantial anti-carcinogenic activities.

Recently, we and others (3–7) reported that the plant polyphenol resveratrol and its analogs have a potent chemopreventive effect in multiple carcinogenesis models both in vivo and in vitro. Resveratrol (3,4′,5-trihydroxystilbene, molecular weight = 228.2) is a polyphenol that has been classified as a phytoalexin, because it is synthesized in spermatophytes in response to certain types of stress. It is the active ingredient of the dried roots of *Polygonum cuspidatum*, which has been known in traditional Asian medicine under the name *Ko-jo-kon* (8,9). Resveratrol-containing foods include grapes (10,11), wine (12), and peanuts (13,14). In grapes, especially when infected with *Botrytis cinerea*, resveratrol is exclusively synthesized in the grape skins, which contain 50–100 mg resveratrol/g when they are fresh. Because the grape skins are not fermented in the production process of white wines, only red wines contain considerable amounts of resveratrol. It has been proposed that resveratrol is at least in part responsible for the beneficial effects of a moderate red wine consumption on the development of cardiovasular diseases. Resveratrol inhibited platelet aggregation (15), protected porcine low-density lipoproteins against polyunsaturated fatty acid peroxidation (16), and exerted vasorelaxing effects on endothelium-intact aorta rings of rats (17).

Additionally, the inhibitory potency of resveratrol in various stages of tumor development has attracted much attention (3). This review will summarize our work on the mechanisms and activity of resveratrol and its derivative, focusing on polyamine metabolism as a possible target.

Polyamines and Colorectal Tumorgenesis. The naturally occurring polyamines putrescine, spermidine, and spermine are widespread in nature and they have been detected in all eukaryotic cells studied. Tissue polyamine levels are increased either by biosynthesis (generally from ornithine and methionine) or by uptake from extracellular fluids. Conversely, excess cellular polyamines are removed by catabolic reactions converting spermine to spermidine via a N^1-acetylspermine intermediate and eventually to putrescine via a N^1-acetylspermidine intermediate. The acetyl polyamines, along with excess putrescine and spermidine, can be excreted to the extracellular fluid. Cellular polyamine homeostasis is maintained through the concerted effort of feedback systems controlling polyamine transport as well as the 3 key enzymes in polyamine metabolism, namely the production of putrescine by ornithine decar-

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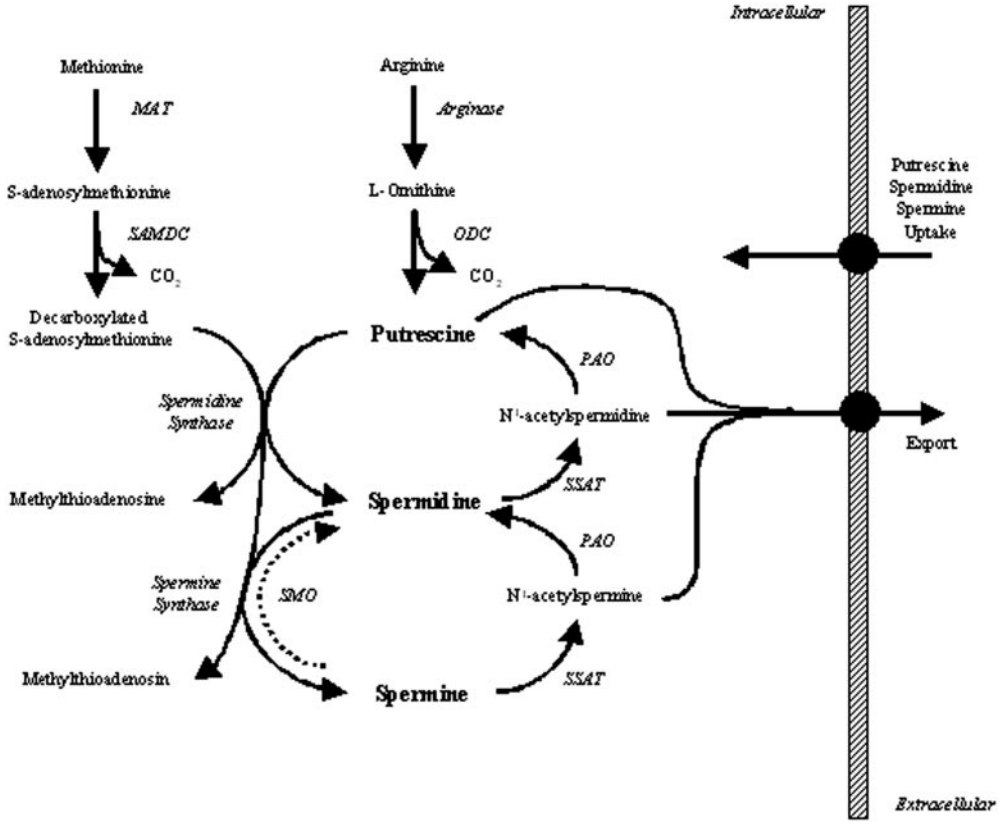

FIGURE 1 Regulation of intracellular polyamine content in mammalian cells [adapted from Ref. (51)].

boxylase (ODC),[4] the synthesis of decarboxylated *S*-adenosyl-L-methionine decarboxylase (SAMDC, AdoMet-DC), and the acetylation of spermidine and spermine by spermindine/spermine N^1-acetyltransferase (SSAT) (**Fig. 1**).

In colon cancer tissue the activities of polyamine-synthesizing enzymes and polyamine content are increased 10- to 15-fold in comparison to normal colonic epithelium (18,19), and polyamines have therefore been considered even as specific markers for neoplastic proliferation in the colon (20). ODC activity and expression have been among the first biomarkers of neoplastic proliferation: as early as 1984 it was shown that ODC activity correlates with risk for neoplastic transformation in patients with colonic adenomatous polyposis (21). ODC activity correlates also with degree of dysplasia in Barrett's esophagus (22) and different stages of colonic carcinogenesis (23). ODC activity is essential for cell proliferation and is required for progression into the S phase of the cell cycle (24). Studies suggest that ODC can be defined as a proto-oncogene (25) and overproduction of this enzyme results in malignant transformation (25–27). More important, however, is that polyamine uptake is upregulated by various mitogens and hormones, as well as by some known tumor promoters (28–31). On the other hand, it has been demonstrated that polyamines stimulated the transcription of c-myc and c-fos (32).

Involvement of Ornithine Decarboxylase in Cancer Prevention and Therapy by Resveratrol and Its Analogs. As stated above, polyamines and their biosynthetic enzyme ODC are intimately involved in carcinogenesis and malignant growth. It is possible that the anticancer and the chemopreventive activities of resveratrol and its analogs could also be explained by inhibiting ODC/SAMDC and/or increasing SSAT activity. To test this assumption, we and others investigated the effect of resveratrol on polyamine metabolism. TPA-induced ODC activity in mouse 308 cells was inhibited by 48% when treated with 10 μmol/L piceatannol, whereas resveratrol had no inhibitory effect at this concentration (33). Schneider et al. (34) incubated Caco-2 colorectal adenocarcinoma cells with 25 μmol/L resveratrol and detected decreased ODC activity (58% of the control value) after 24 h. The inhibitory effect intensified after 48 h. A direct enzyme inhibition of resveratrol on ODC activity was excluded. We could demonstrate that the decreased ODC activity in Caco-2 cells was accompanied by decreased levels of ODC protein, implicating either increased degradation by the 26S proteasome pathway or impaired ODC synthesis. When mRNA levels of the *odc* gene were determined, a reduction of the *odc* mRNA became evident. c-Myc protein was quantified because it controls the *odc* promoter. c-Myc was diminished by resveratrol treatment, demonstrating that decreased expression of the *odc* gene is responsible for the inhibition of ODC activity. Piceatannol also exerts an inhibitory effect on ODC activity, with decreased ODC and c-myc protein levels and with decreased *odc* mRNA levels, although the inhibitory effect of resveratrol was more potent (7). Whereas Schneider et al. (34) did not observe significant inhibition of SAMDC activity after treatment of Caco-2 cells with 30 μmol/L resveratrol (44), we demonstrated SAMDC inhibition when higher concentrations (≥50 μmol/L) were used. SAMDC was also inhibited by piceatannol, but the inhibitory effect was less pronounced (7).

[4] Abbreviations used: DFMO, difluoromethylornithine; MAPK, mitogen activated protein kinase; ODC, ornithine decarboxylase; PAO, polyamine oxidase; SAMDC, *S*-adenosyl-L-methionine-decarboxylase; SSAT, spermidine/spermine-*N*-1-acetyltransferase.

Polyamine-Catabolizing Enzymes [SSAT and Polyamine Oxidase (PAO)] as Molecular Targets for Resveratrol and Its Analogs. An early response of human tumor cells to etoposide-induced apoptosis was an increase in PAO activity (35), and increases in SSAT activity have been linked to cytotoxicity in neoplastic cells (36). Cancer cells exposed to polyamine analogs/inducers of SSAT arrest their growth in G_1 and induce the p53-p21$^{WAF1/CIP1}$-Rb pathway, ultimately undergoing apoptosis (37). The induction of both SSAT and PAO can produce an efficient system to generate locally high concentrations of hydrogen peroxide that could effectively induce a signaling pathway ultimately leading to cell death. Both SSAT and PAO activities were found to be decreased in human solid tumors (38). In this study both enzyme activities correlated with prognosis—PAO in a negative manner and SSAT in a positive manner—supporting the idea that polyamine catabolism, particularly oxidation, is linked to tumor growth potential. All these facts, although intriguing, have been studied only partially so far.

Resveratrol and to a lesser extent piceatannol potently upregulated SSAT activity, indicating that these hydroxystilbenes induce polyamine degradation. After 24 h of treatment with resveratrol we observed increased intracellular putrescine and N^1-acetylspermidine concentrations, whereas the levels of spermine and spermidine did not change significantly (7). Schneider et al. (34) detected reduced levels of putrescine and spermidine after 48 h of treatment with 25 μmol/L resveratrol. The resveratrol analogue *cis*-3,5,4′trimethoxystilbene decreased ODC and SAMDC activities at a concentration of 0.3 μmol/L with a concomitant reduction of putrescine concentrations after 24 h (39). c-Fos and c-jun are part of the transcription factor complex AP-1, which can consist of different Fos or Jun family proteins. The individual combination of these proteins seems to be responsible for the effect of the transcription factor. Proliferation, differentiation, or apoptosis can be the result of enhanced AP-1 binding activity. c-Fos is implicated in the process of differentiation and programmed cell death. Resveratrol treatment led to increased levels of *c-fos* and c-Jun in Caco-2 cells. Only the DNA-binding activity of c-fos increased, whereas c-jun binding activity remained unchanged (7). These results may be mediated by the increased putrescine levels measured after 24 h, which were caused by enhanced SSAT activity. Accumulation of intracellular putrescine concentration induces c-fos (40).

It has been well established that growth factors (mitogens) bind to specific receptors located on the cellular membrane. The mitogen-receptor complexes then trigger a cascade of events including the activation of Ras, which activates the kinase Raf and suppresses expression of SSAT by inhibition of peroxisome proliferator-activated receptor γ (41). The activation of protein kinases is regarded to be the next step in signal transduction. Mitogen activated protein kinases (MAPKs) are phosphorylated by MAPK/extracellular signal regulated kinases, which are, in turn, activated by Raf. MAPKs next trigger the expression of the nuclear oncogenes myc, jun, and fos (**Fig. 2**), which function as transcription factors, stimulating proliferation and the expression of the ODC gene (42,43). Polyamines, which are formed by ODC, enhance the expression of protein kinases (44) and nuclear oncogenes (32). Difluoromethylornithine (DFMO), which inhibits polyamine synthesis, prevents the expression of protein kinases (44) and nuclear oncogenes (32); resveratrol also inhibited MAPK activity (45). The inhibitory effect of resveratrol on polyamine metabolism in carcinoma cells is presumably mediated by a different pathway. Diminished ODC activity is very likely mediated by decreased c-myc protein levels as demonstrated in Caco-2 cells, because c-myc regulates *odc* expression (7). c-Myc expression is regulated by the transcription factor E2F1. We and others have demonstrated that resveratrol treatment leads to decreased retinoblastoma protein phosphorylation and thus inactivation of E2F family members (4,46). These data suggest that ODC inhibition could be a result of the cell cycle inhibitory effect of resveratrol. Most studies concerning polyamine metabolism as a target for chemoprevention or cancer treament have focused on inhibition of ODC. This approach has not proved efficient under in vivo conditions, because ODC inhibition led to an increased polyamine uptake from food, which neutralized cytostatic effects of DFMO (27,47). Results obtained from studies with novel polyamine analogs have suggested that induction of SSAT is a more promising approach to chemoprevention (48–50).

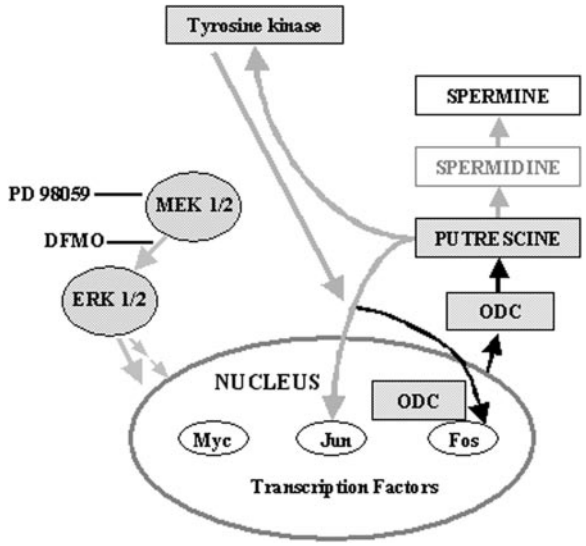

FIGURE 2 Effects of putrescine on signal transduction pathways involved in cell proliferation. The diamine putrescine, which is formed from ornithine by ODC, stimulates tyrosine kinases and the expression of the nuclear protooncogenes c-fos and c-jun [for extended review see (52)].

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Review

Molecular mechanisms of the chemopreventive effects of resveratrol and its analogs in carcinogenesis

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Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), a phytoalexin found in grape skins, peanuts, and red wine, has been reported to exhibit a wide range of biological and pharmacological properties. It has been speculated that dietary resveratrol could be an explanation for the so-called 'French paradox' as it may act as an antioxidant, promote nitric oxide production, inhibit platelet aggregation, and increase high-density lipoprotein cholesterol, and thereby serve as a cardioprotective agent. Recently, it has been demonstrated that resveratrol can function as a cancer chemopreventive agent, and there has been a great deal of experimental effort directed toward defining this effect. It has been shown that resveratrol and some of its analogs interfere with signal transduction pathways, modulate cell cycle-regulating proteins, and is a potent inducer of apoptosis in multiple carcinoma cell lines. This review summarizes the recent advances that have provided new insights into the molecular mechanisms underlying the promising properties of resveratrol.

Keywords: Apoptosis / Angiogenesis / Cancer / Cell cycle / Resveratrol / Signal transduction

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Contents

1 Introduction

In addition to cardiovascular diseases, cancer is one of the leading causes of death worldwide. Each year, the American Cancer Society estimates the number of new cancer cases and deaths expected in the United States in the current year, and compiles the most recent data on cancer incidence, mortality, and survival by using incidence data from the National Cancer Institute (NCI) and mortality data from the National Center for Health Statistics (NCHS). A total of 1 368 030 new cancer cases and 563 700 deaths were expected in the United States in 2004. The three leading cancer types for the estimated new cancer cases are at first cancers of the genital system, with 33% prostate cancers in male and 32% breast cancers in female, followed by about 13% cancers of lung and bronchus. Colorectal cancer is the third most common cancer. It affects both genders almost equally, with about 401 000 new cases in men annually and 381 000 in women [1]. It is estimated that 394 000 deaths from colorectal cancer still occur worldwide annually, and colorectal cancer is the second commonest cause of death from any cancer in men in the European Union [2]. Cancer is caused by both external (tobacco, chemicals, radiation, and infectious organisms) and internal factors (hormones, mutations, and immune conditions). One of the major risk factors is age as about 76% of cancers are diagnosed at age 55 and older. Other risk factors include smoking, alcohol consumption, obesity, physical inactivity, a high-fat diet, as well as inadequate intake of fruits and vegetables [1].

As conventional chemotherapy has no consistent benefit in overall survival, attention is focusing on preventative strate-

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Abbreviations: FGF-2, fibroblast growth factor 2; **MAPK**, mitogen-activated protein kinase; **MEK**, MAPK kinase; **NSAID**, nonsteroidal anti-inflammatory drug; **PMA**, phorbol ester myristate; **ROS**, reactive oxigen species; **TNF**, tumor necrosis factor

gies for multiple cancers [3 – 9]. Chemoprevention is defined as the employment of drugs or natural compounds to prevent malignant tumors [10]. Several epidemiological, clinical, and experimental studies established nonsteroidal anti-inflammatory drugs (NSAIDs) as promising cancer chemopreventive agents [11]. Long-term use of aspirin and other NSAIDs has been shown to reduce the risk of cancer of the colon and other gastrointestinal organs as well as of cancer of the breast, prostate, lung, and skin [12]. But also a large number of natural compounds have been linked to a possible decreased incidence of developing cancers [13 – 15]. Among others there is a special focus on polyphenols present in dietary and medicinal plants exhibiting anti-carcinogenic activities [16].

The plant polyphenol resveratrol (3,4′,5-trihydroxy-*trans*-stilbene; Fig. 1) has been classified as a phytoalexin, because it is synthesized in spermatophytes in response to certain types of stress, including injury, UV irradiation, or fungal attack [17, 18]. It was first described as a component in the root extracts of the weed *Polygonum cuspidatum*, which has been known in traditional Asian medicine under the name Ko-jo-kon [19]. Resveratrol naturally occurs in grapes [20 – 22], wine [23], and peanuts [24 – 26]. An important factor for resveratrol concentrations in wine is the fermentation time in contact with grape skins because resveratrol is produced by the skin but not by the fruit flesh [27]. This explains the low concentrations in white wine because the grape skins are not fermented in the production process [23]. Resveratrol came to scientific attention as a possible explanation for the "French paradox" as it has beneficial effects on the development of cardiovascular diseases [28]. It has been shown to inhibit platelet aggregation and eicosanoid synthesis [29], to interfere with arachidonate metabolism [30], to exert strong inhibitory effects on reactive oxygen species produced by human polymorphonuclear leukocytes [31], to be an antioxidant more powerful than vitamin E in preventing low-density lipoprotein (LDL) oxidation [32], and to exert vasorelaxing effects on endothelium-intact aorta rings of rats [33]. Further studies could show that resveratrol is an agonist for the estrogen receptor which may also be relevant to the reported cardiovascular benefits of drinking wine [34].

Additionally, we and others have examined that resveratrol and its analogs (Fig. 1) exhibit multiple properties including chemopreventive effects in several carcinogenesis models both *in vivo* and *in vitro* [35 – 41]. Several signal transduction pathways have been examined to explain these effects. One hypothesis is focusing on polyamine metabolism as a possible target of resveratrol activity [42]. Because many reviews regarding the preventive effect of resveratrol on cardiovascular diseases have been published [43, 44], this review will summarize our work on the mechanisms and activity of resveratrol and its derivatives in carcinogenesis.

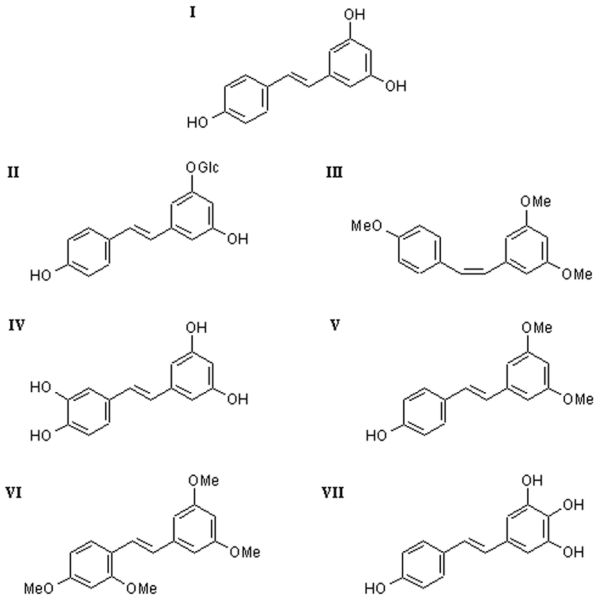

Figure 1. Chemical structures of stilbene compounds cited in this review. (I) 3,4′,5-trihydroxystilbene (resveratrol); (II) *trans*-resveratrol-3-*O*-D-glucoside (piceid); (III) *cis*-3,4′,5-trimethoxystilbene; (IV) 3,3′,4′,5-tetramethoxystilbene (piceatannol); (V) 3,5-dimethoxy-4′-hydroxystilbene (pterostilbene); (VI) 2′,3,4′,5-tetramethoxystilbene; (VII) 2,3,4′,5-tetrahydroxystilbene. Substituents are hydroxyl (OH) and methoxy (OCH_3) groups and *O*-β-D-glucose (OGlc).

2 Resveratrol and its analogs in carcinogenic *in vivo* models

Oral administration of resveratrol inhibited tumor growth of T241 fibrosarcoma in mice [45]. Rats inoculated with Yoshida AH-130 hepatoma cells and treated with resveratrol (intraperitoneal injection) had a decreased tumor cell number [46]. Lung cancer development in A/J mice induced by benzo[*a*]pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone was not inhibited by oral administration of resveratrol [47, 48], whereas in Balb/c mice resveratrol protects the lung from DNA damage and apoptosis caused by benzo[*a*]pyrene [49]. Additionally administration of resveratrol *per os* reduced the number of aberrant crypt foci in azoxymethane-induced tumorigenesis in the rat colon and led to enhanced expression of the proapoptotic protein Bax in these crypt foci [50]. In a 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinogenesis model in Sprague Dawley rats dietary administration of resveratrol had indeed no effects on body weight gain and tumor volume but produced reductions in the incidence, multiplicity, and extended latency period of tumor development [51]. The mean survival time of mice inoculated with 32Dp210 leukemia cells and treated with up to 80 mg resveratrol/kg body weight was not significantly different from untreated controls, even though resveratrol

exerted antileukemic properties on 32Dp210 cells *in vitro* [52]. In mice bearing highly metastatic Lewis lung carcinoma tumors resveratrol inhibited the DNA synthesis of tumor cells with an IC_{50} value of 6.8 μM. No effect could be monitored on CD4+, CD8+, and natural killer cells, from which the authors concluded that these cells are not responsible for the effects of resveratrol on DNA synthesis [53]. The *trans*-resveratrol-3-*O*-D-glucoside (piceid) also inhibited the proliferation of Lewis lung cancer cells, inoculated into mice, but only at a concentration of 1000 μM. 2,3,5,4′-Tetrahydroxystilbene-2-*O*-D-glucoside was more effective with an IC_{50} of 81 μM [54]. In addition, resveratrol treatment of mice (40 mg/kg daily for 28 days) suppressed the growth rate of subcutaneous neuroblastomas, resulting in 70% long-term survival [55]. The natural resveratrol analog pterostilbene (3,5-dimethoxy-4′-hydroxystilbene) inhibited the development of mammary lesions in a mouse mammary gland organ culture treated with 7,12-dimethylbenz[*a*]anthracene [56]. In $Apc^{Min/+}$ mice, an animal model for familiar adenomatous polyposis, the number of adenomas was reduced by 70% (colons contained no polyps following treatment) by a diet containing resveratrol. The intestinal mucosa of treated mice was subjected to DNA array analysis. Downregulation of the mRNAs encoding for cyclin D1, cyclin D2, DP-1, YB1, and RNA polymerase termination factor TTF-1 could be monitored along with an increase of transforming growth factor (TGF)-β, thrombopoietin, glutamate receptor, mitogen-activated protein kinase (MAPK), TSG101 tumor susceptibility protein, and other targets [36]. In contrast to these results, resveratrol did not inhibit tumorigenesis in $Apc^{Min/+}$ mice in a study conducted by Ziegler *et al.* [57], even though reduced PGE_2 levels could be observed in tumor tissue. Also in 4T1 breast cancer cells resveratrol had no effects on growth inhibition *in vivo*, although it exhibits potent inhibitory effects *in vitro* [58]. These controversial results may be due to metabolic processes, as resveratrol is absorbed in the small intestine as resveratrol glucuronide. Glucuronides of phenolic compounds have been assumed to be rapidly excreted *in vivo* and to be pharmacologically inactive [59].

3 Effects of resveratrol and its analogs *in vitro*

3.1 Resveratrol and MAPKs

The MAPKs convert extracellular signals (*e.g.*, growth factor signals) into intracellular events. Three kinase pathways (extracellular signal-regulated kinase (ERK), p38, and c-Jun kinase (JNK) have been identified, that follow the same principle of phosphorylation and activation cascades. Targets of the MAPK pathways are transcription factors like activator protein (AP)-1, c-Myc, and Elk-1. Tumor necrosis factor (TNF-α)-induced AP-1, JNK, and MEK (MAPK kinase) activation were inhibited in U937 lymphoma cells by pretreatment with resveratrol [60]. Resveratrol inhibited phosphorylation of ERK1 and ERK2 induced by fibroblast growth factor 2 (FGF-2) in bovine capillary endothelial cells [45] and by human serum in liver myofibroblasts [61]. In the cervical squamous cancer cell line HeLa, pretreatment with resveratrol inhibited phosphorylation of p38, ERK2, c-Src, and JNK and subsequently activation of AP-1 induced by UV irradiation. PMA-induced ERK2 and c-Src phosphorylation were strongly inhibited by resveratrol, wheras resveratrol had only a weak effect on epidermal growth factor (EGF)-induced ERK2-activation [62]. In undifferentiated SH-SY5Y neuroblastoma cells, treatment with resveratrol led to increased ERK1 and ERK2 phosphorylation. At a concentration of 50 μM and higher ERK phosphorylation was inhibited. Resveratrol treatment of SH-SY5Y cells caused to differentiate with retinoic acid decreased ERK phosphorylation at first, but then increased ERK phosphorylation markedly [63]. In porcine coronary arteries resveratrol inhibited ERK activation and tyrosine phosphorylation in a concentration-dependent manner. Pretreatment with resveratrol counteracted endothelin-1-stimulated ERK activity and tyrosine phosphorylation [64]. In another report it is shown that growth-inhibitory concentrations of the phytochemical resveratrol suppress endothelial growth factor receptor (EGFR)-dependent ERK1/2 activation pathways stimulated by EGF and phorbol ester (12-*O*-tetradecanoyl phorbol 13-acetate, TPA) in human AI PrCa PC-3 cells *in vitro*. These effects are mediated by protein kinase C (PKC) inhibition by resveratrol, the major cellular receptor for phorbol esters. The results provide evidence that resveratrol may have value as an adjuvant cancer therapeutic in advanced prostate cancer [65].

Resveratrol inhibited the activity of recombinant PKC prepared from sonicated vesicles induced by 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine with an IC_{50} value of 30 μM [66]. Resveratrol inhibited the PMA-induced redistribution of PKC from cytosol to membrane in mammary epithelial cells [67] and the autophosphorylation of isolated protein kinase D in a dose-dependent manner, whereas it had only negligible effects on PKC isozyme autophosphorylation [68].

The natural ocurring stilbene analog piceatannol (*trans*-3,4′,3′,5-tetrahydroxystilbene), which shares most of the structural moieties with resveratrol, was first identified as an inhibitor of the tyrosine kinase activity of $p72^{Syk}$ and $p56^{Lck}$ in lymphoid cells [69]. In addition, piceatannol inhibits the tyrosine kinase activity of human placenta [70] and the focal adhesion kinase and Src in thrombocytes [71]. In MCF-7 human breast cancer cells cAMP levels increased

after addition of resveratrol. This effect was demonstrated to be dependent on protein kinase A and phospholipase A_2 activities and independent of the estrogen receptor [72].

3.2 Cell cycle

Inhibition of cell cycle progression is a possible target for chemopreventive agents like resveratrol. The cell cycle is regulated by cyclins and cyclin-dependent kinases (Cdk), which are primarily regulated by their expression levels and by cell cycle-inhibiting proteins ($p21^{Waf1/Cip1}$, $p27^{Kip1}$, and members of the INK family of proteins) (Fig. 2). The effect of resveratrol on the cell cycle distribution of tumor cells seems to focus on the S-phase. A cell cycle arrest in the S-phase has been reported for different cell types [37, 73 – 81], except from HepG2 cells in which a G1 phase arrest could be observed [82]. An increased cyclin E and cyclin A expression was observed in HL-60 leukemia cells [83], U937 lymphoma cells [78], HCT-116, and Caco-2 colon cancer cells [37]. Ragione *et al.* [83] identified inactivation of Cdc2 by phosphorylation at tyrosine residue 15 as a possible pathway by which this S-phase arrest is mediated. A concentration-dependent decrease of the $p27^{Kip1}$ expression level was observed in LNCaP, U937, and Caco-2 cells [37, 77, 78]. In bovine pulmonary artery endothelial cells [75], HL-60 cells [83], A431 cells [80], and U937 cells [78] resveratrol treatment led to an increased $p21^{Waf1/Cip1}$ expression, whereas the protein level of the cell cycle inhibitor was unmodified in Caco-2 cells [37] and decreased in LNCaP cells [77] and neuroblastoma cells [55]. In the human prostate carcinoma cell line the antiproliferative effect of resveratrol was associated with the inhibition of D-type cyclins and Cdk 4 expression, and the induction of tumor suppressor p53 and Cdk inhibitor p21. Moreover, the kinase activities of cyclin E and Cdk2 were inhibited by resveratrol without alteration of their protein levels [84]. The retinoblastoma protein (pRb) sequesters the transcription factor E2F in the cytosol. Phosphorylation of pRb prevents binding of pRb to E2F which leads to the translocation of E2F into the nucleus. Dephosphorylation and thus activation of the tumorsuppressor pRb was observed in Caco-2 cells [37] and in A431 epidermoid carcinoma cells after treatment with resveratrol. In A431 cells this effect was accompanied by decreased protein levels of all E2F family members (1 – 5) and their binding partners DP-1 and DP-2 [85]. Resveratrol arrested the cell cycle of non-androgen responsive prostate cancer cell lines in the S-phase, but did not modify the cell cycle distribution of the androgen-responsive cell line LNCaP [86]. Stivala *et al.* [87] demonstrated that the cell cycle effects of resveratrol are dependent on certain structural determinants. The *trans*-configuration in combination with the hydroxy group in the 4′-position is essential for the effects of resveratrol on the cell cycle.

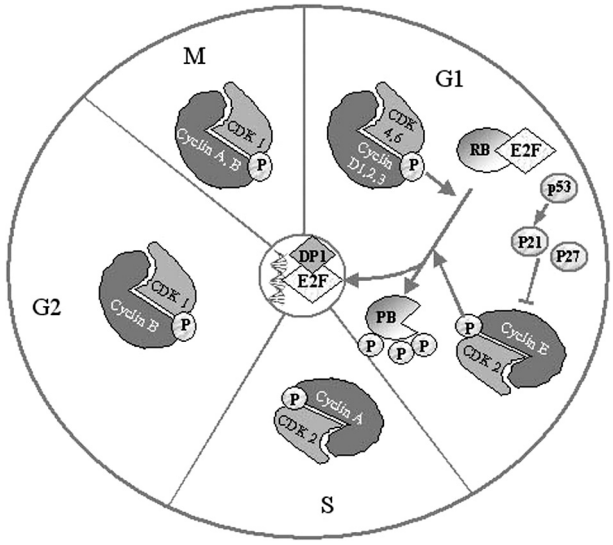

Figure 2. Cell cycle and possible modulation by resveratrol. The polyphenol resveratrol has been shown to inhibit the activity of cdc2 and cdk4. Additionally there is an accumulation of cyclins E and A, accompagnied by a decrease of D-type cyclins as well as an increase of p21 expression. Furthermore, resveratrol promotes dephosphorylation and thus activation of the tumor suppressor pRb. These activities contribute to the ability of resveratrol to inhibit cell progression at S-phase.

Cell cycle regulation was also observed in a few *in vivo* studies. In H22-bearing mice, resveratrol inhibited the growth of transplantable liver cancer by decreasing the expression of cyclin B and cdc2 protein [88]. In another study, resveratrol downregulated UVB-induced expression of Cdk2, 4, 6 and cyclin D1 and D2 in SKH-1 hairless mouse skin, which was accompanied by an upregulated UV-mediated increase in the expression of the Cdk inhibitor WAF1/p21 and the tumor supressor protein p53 [89]. In addition to the regulation of cell cycle proteins, the negative effect of resveratrol on proliferation has in part been attributed to inhibition of ribonucleotide reductase and DNA synthesis [90].

Piceatannol is also a cell cycle inhibitor that acts preferably in the S-phase. It has been demonstrated to inhibit the growth of Caco-2 and HCT-116 colon cancer cell lines. Following piceatannol treatment, the number of Caco-2 cells in the S-phase increased and reduced levels of Cdk4, Cyclin D1, Cyclin B1, and $p27^{Kip1}$ were detected. At the same time an increase in Cyclin E and Cyclin A expression could be shown. Taken together, these effects were comparable to those observed after treatment with resveratrol [38]. The methylated resveratrol analog *cis*-3,5,4′-trimethoxystilbene (0.3 μM) induces accumulation of Caco-2 cells in the G_2/M-phase with a diminished G_0/G_1-phase population. These effects were caused by depolymerization of the microtubule network [91].

3.3 Apoptosis

Apoptosis, also termed programmed cell death, is necessary for the maintenance of normal tissue homeostasis. Impaired apoptosis has been associated with hyperproliferation and tumorigenesis. Induction of apoptosis is accompanied by certain morphological and molecular changes in the cell, like DNA fragmentation, cleavage of caspases and caspase substrates, and breakdown of mitochondrial transmembrane potential (Fig. 3). Resveratrol has been demonstrated to induce apoptosis in a number of cell types [37, 75–77, 80, 82, 84–86, 92, 93]. The polyphenol not only induced apoptosis in leukemic hematopoietic cells, but also in normal activated peripheral blood lymphocytes. It had no apoptotic effect on nonactivated peripheral blood lymphocytes [94]. In HL-60 promyelocytic leukemia cells resveratrol-induced apoptosis was prevented by caspase inhibitors [95]. Resveratrol-induced apoptosis of CEM-C7H2 acute lymphoblastic leukemia cells was accompanied by cleavage of caspase-6, -3, and -2, but seemed to be independent of caspase-8 activation, because a caspase-8-deficient mutant Jurkat cell line was sensitive to resveratrol-induced cell death [96]. Activation of these caspases was inhibited by overexpression of the oncogene Bcl-2 [97]. Overexpression of Bcl-2 in U937 cells also attenuated apoptosis and prevented cleavage of caspase-3 and PARP (poly ADP-ribose polymerase) [98]. These findings could be confirmed by Zhou *et al.* [99] demonstrating an upregulation of Bax as well as a downregulation of Bcl-2 during resveratrol-induced apoptosis. Another study could show that resveratrol-induced apoptosis is accompanied by induction of p53, upregulation of Bax, activation of caspase 9, and decrease in Bcl-2 levels [100].

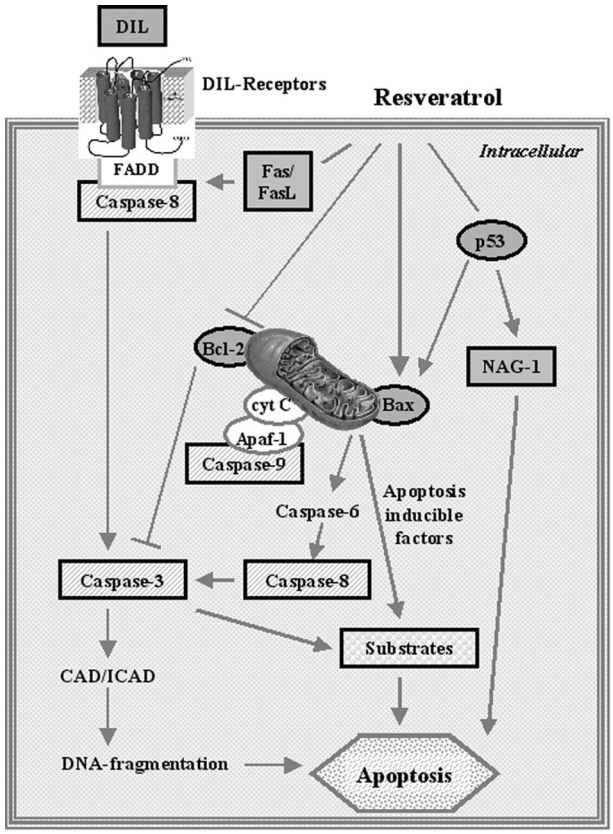

Figure 3. Apoptosis and possible stimulatory pathways by resveratrol. Resveratrol was shown to activate pro-apoptotic caspases-6, -3, and -9 but seems to be partly independent of caspase-8 activation. It also leads to an induction of tumor suppressor p53 and Bax as well as an inhibition of Bcl-2. Additionally, resveratrol leads to an upregulation of pro-apoptotic NAG-1. DIL, death-inducible ligands; NAG-1, NSAID-activated gene; CAD, caspase-activated Dnase; ICAD, inhibitor of CAD.

In human prostate carcinoma cells (DU145) resveratrol upregulated the Bax protein and mRNA expression in a dose-dependent manner, whereas Bcl-2 and Bcl-xL levels were not significantly affected. These results correlated with an activation of caspase-3 and caspase-9 [84]. Otherwise resveratrol-treated human liver cancer cells HepG2 and Hep3B had enhanced Bax expression but they were not involved in the Fas/Apo-1 apoptotic signal pathway [82]. In acute lymphoblastic leukemia cell lines, activation of caspase-9 and depolarization of mitochondrial membranes could be monitored after treatment with resveratrol [101]. Apoptosis can be induced by binding of proapoptotic proteins (TNF-α, Fas ligand) to their receptors. TNF-α-induced apoptosis, reactive oxygen species (ROS) generation and lipid peroxidation were also inhibited by pretreatment with 5 μM resveratrol in U937 cells [60]. Clément *et al.* [102] detected Fas-dependent apoptosis-signaling in HL-60 and T47D cells, whereas Fas-independent apoptosis could be demonstrated in CEM-C7H2 [96] and THP-1 monocytic leukemia cells [103]. In a human colon cancer cell line (SW480) resveratrol-induced apoptosis was also not mediated directly through modulation of Fas/FasL interaction, but was attributable to caspase activation and increased accumulation of Bax and Bak [104]. High levels of p53 expression in cells with fractional DNA content, *i. e.*, in apoptotic cells, strongly suggests that their apoptosis may be associated with upregulation of p53 [75]. Furthermore, Huang *et al.* [105] demonstrated that induction of apoptosis in JB6 mouse epidermal cells is dependent on the presence of the tumor suppressor p53. In HepG2 cells also a p53-dependent pathway involving an increased expression of Bax and upregulation of p21 was suggested [82]. In thyroid cancer cells (BHP 2–7, BHP 18–21, FTC 236, and FTC 238) the apoptosis induced by resveratrol was inhibited by p53 antisense oligonucleotide transfection or by addition of the p53 inhibitor pifithrin-α [106]. In DU 145 prostate cancer cells resveratrol-induced apoptosis was also inhibited by pifithrin-α. In addition, it was demonstrated that overexpression of p53 led to a higher apoptotic response [107]. In contradiction to these results induction of apoptosis by resveratrol has been shown in cell types deficient of func-

tional p53 [37, 108]. In the colorectal cancer cell line HCT-116, which possesses wild-type p53, apoptosis occurs after incubation with resveratrol *via* a p53-independent mechanism [109]. The MAPK activation by resveratrol was found to upregulate p53 in mouse epidermal JB6 cells [110, 111]. In thyroid cancer cells, apoptosis, c-Fos, and p53 induction induced by resveratrol were blocked by the MEK inhibitor PD98059 [106]. In DU 145 cells, Ser15 phosphorylation of p53 by resveratrol was also blocked by PD98059 [107]. Resveratrol induced NAG-1 (NSAID-activated gene), which has been demonstrated to induce apoptosis in the colorectal cancer cell line HCT-116 and the osteosarcoma cell line U2OS. NAG-1 induction was dependent on the presence of wild-type p53 which has been shown to activate the promoter of NAG-1 [112]. Further studies suggest that an involvement of the pRb-E2F/DP pathway is suggested as an important contributor of resveratrol-mediated cell cycle arrest and apoptosis [85].

The synthetic resveratrol analog 3,4,5,4′-tetrahydroxystilbene induced DNA fragmentation in SV40 transformed WI38 lung fibroblasts, but not in normal WI38 cells. This apoptosis induction was accompanied by increased p53 and Bax expression, enhanced p53-binding to the bax promoter, and decreased Bcl-xL, Bcl-xS, Bcl-2 expression. In addition, mRNA levels of BRCA1, BRCA2, and COX-2 were diminished [113]. Another analog, 3,5,2′,4′-tetramethoxy-*trans*-stilbene, was shown to induce the accumulation of cellular DNA contents in the sub-G0 phase of the cell cycle in a time-dependent manner, wheras the morphological changes were consistent with an apoptotic process [114]. The natural occuring resveratrol analog piceatannol (3,5,3′,4′-tetrahydroxy-*trans*-stilbene; PICE) was also shown to be a potent inducer of apoptosis in human SK-Mel-28 melanoma cells [115].

3.4 Angiogenesis and invasion

Neovascularization and thus supply of tumors with nutrients is essential for their growth. Endothelial cell migration and proliferation are as necessary for this process as the breakdown of existing basal membranes by matrix metalloproteinases (MMPs). These enzymes are also implicated in tumor cell invasion, which is the first step of metastasis development. Resveratrol was found to inhibit growth of bovine aorta endothelial cells in a dose-dependent manner. In addition, it suppressed migration of these cells in a wound assay and endothelial tube formation in a collagen matrix, which is considered to represent a marker for neoangiogenesis [116]. Resveratrol inhibited invasion, but not proliferation of the rat ascites hepatoma cell line AH109A pretreated with hypoxanthine and xanthine oxidase in a coculture model with mesothelial cells. Addition of sera from rats fed with resveratrol instead of calf serum also inhibited invasion, but not proliferation of AH109A cells, demonstrating a role for resveratrol in ROS-induced cell invasion [117]. Resveratrol also inhibited the growth of FGF-2-stimulated bovine capillary endothelial cells and induced avascular zones in developing chick chorioallantoic membranes in a dose-dependent manner. Corneal neovascularization induced by vascular endothelial growth factor (VEGF) and FGF-2 in mice was suppressed by oral administration of resveratrol. The inhibiting effects of resveratrol on angiogenesis were confirmed in a mouse skin model, where delayed wound healing could be demonstrated [45]. Resveratrol inhibited capillary-like tube formation of human umbilical vein cells (HUVEC) and inhibited the binding of VEGF to HUVEC [53]. VEGF expression did not significantly change when rat RT-2 glioma cells were treated with low-dose resveratrol, but it was suppressed when they were treated with 10, 25, or 100 μM resveratrol [118]. Although resveratrol did not affect HIF-1α mRNA levels in human ovarian cancer cells, it did dramatically inhibit both basal-level and growth factor-induced HIF-1α protein expression in the cells as well as VEGF expression [119]. Furthermore, resveratrol abolished VEGF-induced VE-cadherin tyrosine phosphorylation and redistribution and Src activity in HUVEC [120]. In contrast to these findings, resveratrol did not inhibit invasion of the murine melanoma cell line B16-BL6, as determined in a Boyden chamber invasion assay [121].

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Dedicated to the 65th Birthday of Prof. Dr. W. F. Caspary.

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