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MOLEKULARE MECHANISMEN DER ANTIPROLIFERATIVEN WIRKUNG SEKUNDÄRER PFLANZENINHALTSSTOFFE AM BEISPIEL VON RESVERATROL

Inauguraldissertation zur Erlangung des Doktorgrades *Dr. oec. troph.*
im Fachbereich Agrarwissenschaften, Ökotrophologie und Umweltmanagement
der Justus Liebig Universität Gießen

Vorgelegt von

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Frankfurt am Main September 2002

Disputationstermin 14.02.2003

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ZUSAMMENFASSUNG

Einleitung: Das pflanzliche Polyphenol Resveratrol kommt unter anderem in Rotwein, Weintrauben und Erdnüssen vor und wirkt chemopräventiv. Ziel der vorliegenden Arbeit war es, die molekularen Mechanismen der durch Resveratrol induzierten Hemmung des kolorektalen Karzinomzellwachstums näher zu charakterisieren.

Methodik: Die Versuche wurden an den kolorektalen Karzinomzelllinien Caco-2 und HCT-116 durchgeführt, die unter Standardbedingungen kultiviert wurden. Zytotoxische Effekte wurden durch Laktatdehydrogenasemessung im Überstand ausgeschlossen. Die Zellzahl wurde mittels Kristallviolettfärbung und die Proliferation mit dem Einbau von [^{3}H]-Thymidin und [^{14}C]-Leucin bestimmt. Die Aktivitäten der alkalischen Phosphatase und der Caspase 3 wurden mit einem Enzymassay bestimmt, die Proteine wurden mittels Western blot und die Zellzyklusdistribution wurde mit Hilfe eines Durchflusszytometers analysiert. Die Sekretion des transformierenden Wachstumsfaktors (TGF)-β1 wurde mit einem ELISA-Test quantifiziert. Die Aktivitäten der Ornithindecarboxylase (ODC) und der S-Adenosylmethionindecarboxylase (SAMDC) wurden durch Freisetzung von [$^{14}CO_2$] aus [^{14}C]-Ornithin bzw. [^{14}C]-S-Adenosylmethionin bestimmt. Die Aktivität der Spermidin/Spermin-N^1-Acetyltransferase (SSAT) wurde durch [^{3}H]-Acetyl-CoA-Transfer auf Spermidin bestimmt und die intrazelluläre Polyaminkonzentration wurde mittels HPLC quantifiziert. Die DNA-Bindungsaktivitäten von c-Jun und c-Fos wurden mit einem auf dem ELISA-Test basierenden Verfahren gemessen.

Ergebnisse Resveratrol unterdrückte das Wachstum der Zelllinien Caco-2 und HCT-116 (12,5 - 200 µM). Es war eine Hemmung der Proliferation zu verzeichnen, die Differenzierung von Caco-2-Zellen wurde jedoch nicht gefördert. Unter Resveratrolbehandlung verschob sich die Zellzyklusdistribution zugunsten der S-Phase-Population. Bei Caco-2-Zellen wurde dies nach Inkubation mit 50 µM Resveratrol am deutlichsten, bei höheren Konzentrationen war der Effekt schwächer. Diese Wirkungen waren spezifisch für Resveratrol, da die Strukturanaloga Rhapontin und Stilbenmethanol keine Alteration der Zellzyklusverteilung bewirkten. Die Inkubation mit Resveratrol

hatte bei Caco-2- und HCT-116-Zellen eine dosisabhängige Reduktion der zellzyklusregulierenden Proteine Cyclin D1, cyclinabhängige Kinase (Cdk)4 und $p27^{Kip1}$ zur Folge. Während $p21^{Waf1/Cip1}$, Cdc2, Cdk2, Cdk6 und das proliferierende Zellkernantigen durch diese Behandlung nicht beeinflusst wurden, nahm die Expression von Cyclin E und Cyclin A zu. Der intrazelluläre Retinoblastomaprotein-Spiegel wurde konzentrationsabhängig reduziert und bei einer Resveratrolkonzentration von 200 µM konnte durch Bandenverschiebung eine Hypophosphorylierung des Proteins detektiert werden. Apoptose mit Caspase 3-Aktivierung erfolgte ebenfalls bei Behandlung von Caco-2-Zellen mit 200 µM Resveratrol.

Auch das Resveratrolanalogon Piceatannol wirkte wachstumshemmend auf Caco-2- und HCT-116-Zellen. Die Proliferation von Caco-2-Zellen wurde gehemmt, es konnte jedoch keine differenzierungsfördernde Wirkung verzeichnet werden. Eine Zellzyklushemmung in der S-Phase fand koinzident zur konzentrationsabhängigen Abnahme der Proteine Cyclin D1, Cyclin B1, Cdk4 und $p27^{Kip1}$ statt. Die Proteinmenge von Cdk2, Cdk6, Cdc2 und $p21^{Waf1/Cip1}$ wurde nicht beeinflusst, wohingegen die Cycline E und A vermehrt exprimiert wurden. Die gleichen Veränderungen der zellzyklusregulierenden Proteine nach Piceatannolbehandlung konnten bei HCT-116-Zellen beobachtet werden.

Im Folgenden sollte untersucht werden, ob Resveratrol die chemopräventiven Wirkungen der kurzkettigen Fettsäure Butyrat beeinflusst. Im Vergleich mit Butyrat (2 mM) war die wachstumshemmende und antiproliferative Wirkung des Resveratrols (50 µM) ausgeprägter. Diese ließ sich auch bei gleichzeitiger Gabe beider Substanzen nicht verstärken. Während Resveratrol alleine weder die alkalische Phosphatase-Aktivität noch die E-Cadherinexpression beeinflusste, beides sind Marker der Differenzierung von Kolonozyten, verstärkte es den induzierenden Effekt von Butyrat auf diese Proteine. Die durch Butyrat hervorgerufene Exkretion von TGF-β1 wurde dagegen durch Resveratrol gehemmt, wohingegen das Stilben die butyratinduzierte $p21^{Waf1/Cip1}$-Expression potenzierte. Der Zellzyklusinhibitor $p27^{Kip1}$ wurde nach Resveratrolinkubation vermindert exprimiert, was auch durch Butyrat nicht verhindert werden konnte.

Auf die Polyaminsynthese in Caco-2-Zellen wirkte Resveratrol inhibitorisch, indem es, ebenso wie Piceatannol und (-)-Epigallocatechin-Gallat (EGCG), die ODC-Aktivität hemmte. Beide Stilbene wirkten über Reduktion des ODC-Proteins, jedoch war nur durch Resveratrol eine deutliche *odc*-mRNA-Abnahme zu verzeichnen. Eine konzentrationsabhängige Abnahme des c-Myc-Onkoproteins durch Resveratrol fand statt. Piceatannol, nicht aber EGCG verminderte ebenfalls das Vorkommen von c-Myc. Resveratrol und in geringerem Maße Piceatannol, nicht jedoch EGCG hemmten die SAMDC-Aktivität. Das an der Polyamindegradierung beteiligte Enzym SSAT wurde durch Resveratrol, in geringem Maße durch EGCG, jedoch nicht durch Piceatannol stimuliert. Intrazellulär kam es zu einem konzentrationsabhängigen Anstieg von Putreszin und N^8-Acetylspermidin, zweier Marker des vermehrten Polyaminkatabolismus. Resveratrol führte zu einer Zunahme des c-Fos- und des c-Jun-Proteins. Piceatannol erhöhte ebenfalls die c-Jun-Expression. Beide Stilbene induzierten die DNA-Bindungsaktivität von c-Fos, wohingegen die Bindung von EGCG gehemmt wurde. Keines der Polyphenole übte einen Einfluss auf die Aktivität von c-Jun aus.

Schlussfolgerung: Die Ergebnisse demonstrieren, dass Resveratrol, das natürlicherweise durch die Nahrung im Darmlumen präsent ist, eine Reihe zellulärer Prozesse in Kolonkarzinomzellen beeinflusst, die schließlich in einer Proliferationshemmung sowie Apoptose der Zellen resultieren. In Kombination mit der kurzkettigen Fettsäure Butyrat wirkt das Polyphenol jedoch differenzierungsfördernd. Auch das Resveratrolanalogon Piceatannol hemmt die Proliferation. Die vorliegenden Daten weisen darauf hin, dass Resveratrol von pharmakologischem Interesse hinsichtlich der Chemoprävention des Kolonkarzinoms sein könnte.

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

I. **WOLTER, F.**, AKOGLU, B., CLAUSNITZER, A. & STEIN, J. (2001) Down-regulation of the cyclin D1/Cdk4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines. Journal of Nutrition 131: 2197-2203.

II. **WOLTER, F.**, CLAUSNITZER, A., AKOGLU, B. & STEIN, J. (2002) Piceatannol, a natural analog of resveratrol, inhibits progression through the S phase of the cell cycle in colorectal cancer cell lines. Journal of Nutrition 132: 298-302.

III. **WOLTER, F.** & STEIN, J. (2002) Resveratrol enhances the differentiation induced by butyrate in Caco-2 colon cancer cells. Journal of Nutrition 132: 2082-2086.

IV. **WOLTER, F.**, TURCHANOWA, L. & STEIN, J. (2003) Resveratrol-induced spermidine/spermine N^1-acetyltransferase activity is accompanied by induction of c-Fos. Carcinogenesis (reviewed version in press).

V. **WOLTER, F. &** STEIN, J. (2002) Biological activities of resveratrol and its analogs. Drugs of the Future 27(10): 949-959 (Invited review).

DANKSAGUNG

Zutiefst dankbar bin ich allen Personen, die mich während der Erstellung dieser Arbeit fachlich, psychisch, emotional und finanziell gefördert und unterstützt haben.

Mein besonderer Dank gilt:

meinem Doktorvater Herrn Prof. Dr. Dr. J. Stein,

Frau Prof. Dr. K. Becker-Brandenburg,

Herrn Prof. Dr. W. F. Caspary,

Frau Dr. Lyudmila Turchanowa,

meinen Diplomandinnen Antje Clausnitzer und Caroline Gabriel,

allen Mitarbeitern und Mitarbeiterinnen des Gastrolabors und der Ernährungsambulanz,

meinen Eltern Ute und Hans Jürgen Wolter und

meinem Lebenspartner Holger Rüth.

Diese Arbeit wurde aus Mitteln der Else Kröner-Fresenius-Stiftung unterstützt.

"Of all the plants we've tested for cancer chemopreventive activity and all the compounds we've seen, this one has the greatest promise."
(Dr. John M. Pezzuto über Resveratrol)

INHALTSVERZEICHNIS

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VERZEICHNIS DER ABKÜRZUNGEN

$\Delta\Psi_m$	mitochondriales Transmembranpotential
AP	Alkalische Phosphatase
AP-1	Aktivatorprotein-1
Apc	adenomatöses Polyposis Coli Protein
ATP	Adenosin-Triphosphat
BAX	Bcl-2 assoziiertes X Protein
Bcl-2	B-Zell Lymphom 2- / B-Zell Leukämie 2-Protein
bp	Basenpaare
Caspase	Cystein Aspartat-spezifische Protease
Cdk	cyclinabhängige Kinase
Cip-1	cyclininhibierendes Protein 1
CKI	Inhibitor cyclinabhängiger Kinasen
COX	Cyclooxygenase
dcc	fehlend in kolorektalen Karzinomen
dcSAM	Decarboxyliertes S-Adenosylmethionin
DGE	deutsche Gesellschaft für Ernährung
DMBA	7,12-Dimethylbenz(*a*)anthracen
DMSO	Dimethylsulfoxid
DNA	Desoxyribonukleinsäure
EGCG	(-)-Epigallocatechin-Gallat
EGF	epidermaler Wachstumsfaktor
ELISA	enzymgekoppelter Immunassay
ER	Östrogenrezeptor
ERK	durch extrazelluläre Signale regulierte Kinase
FAP	familiäre adenomatöse Polyposis
Fas	Fas-Rezeptor
FasL	Fas-Ligand
FCS	fötales Kälberserum
FGF	Fibroblastenwachstumsfaktor
HPLC	Hochdruck-Flüssigkeitschromatographie
HUVEC	humane Endothelzellen der Umbilikalvene
HWZ	Halbwertszeit
IKK	IκB-Kinase
INK	Kinase-Inhibitoren
iNOS	induzierbare NO-Synthase
Jak	Januskinase
JNK	c-Jun-N-terminale Kinase
Kip1	Kinase inhibierendes Protein 1
KRK	kolorektales Karzinom
LDH	Laktatdehydrogenase

LPS	Lipopolysaccharide
MAPK	mitogenaktivierte Kinase
mcc	mutiert in kolorektalen Karzinomen
MEK	MAPK Kinase
MMP	Matrixmetalloproteinase
mRNA	Boten-Ribonukleinsäure
NAG-1	NSAR-aktiviertes Gen
NF-κB	Zellkernfaktor κB
NSAR	nichtsteroidales Antirheumatikum
ODC	Ornithindecarboxylase
PAO	Polyaminoxidase
PARP	Poly-ADP-Ribose-Polymerase
PBS	Phosphat-gepufferte isotonische NaCl-Lösung
PCR	Polymerasekettenreaktion
PCNA	Zellkernantigen proliferierender Zellen
PDGF	plättchenassoziierter Wachstumsfaktor
PG	Prostaglandin
PKC	Proteinkinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristat 13-acetat
pRb	Retinoblastomaprotein
RNA	Ribonukleinsäure
ROS	reaktive Sauerstoffspezies
SAMDC	S-Adenosylmethionindecarboxylase
SSAT	Spermidin/Spermin-N^1-Acetyltransferase
STAT	Aktivator der Signaltransduktion und Transkription
SV40	Affenvirus 40
TGF-β	transformierender Wachstumsfaktor-β
TNF	Tumornekrosefaktor
TPA	12-*O*-Tetradecanoyl-phorbol-13-acetat
VEGF	vaskulärer endothelialer Wachstumsfaktor
Waf1	Wildtyp-p53-aktivierter Faktor 1

VERZEICHNIS DER ABBILDUNGEN

1 Einleitung

1.1 Kolorektales Karzinom

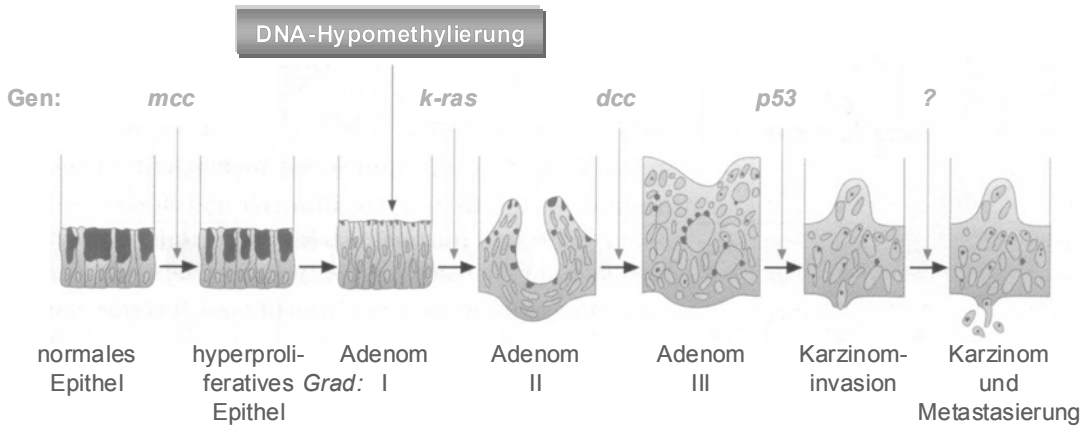


Abb. 1: Adenom-Karzinom-Sequenz [modifiziert nach FEARON & VOGELSTEIN 1990]

Derzeit stellt das *kolorektale Karzinom* (KRK) noch die zweithäufigste Todesursache durch Tumorerkrankungen weltweit dar. Allein in Deutschland versterben jährlich 31.000 Menschen am KRK, während 52.000 neu erkranken [AG KREBSREGISTER 2002]. Zu 95% tritt das KRK nach dem 50. Lebensjahr auf. Nur 5 - 10% der KRK sind genetisch bedingt, an der Ätiologie der restlichen 90% sind Umweltfaktoren beteiligt [CASPARY *et al.* 1999]. Der World Cancer Research Fund schätzt, dass bis zu 50% der kolorektalen Tumoren durch eine Ernährungsmodifikation zu vermeiden wären [SCHEPPACH *et al.* 2000].

Im Verlauf der Karzinogenese kommt es zu einer Reihe von Mutationen, die Tumorzellen gegenüber gesunden Zellen einen Überlebensvorteil verschaffen. Zunächst entsteht ein *hyperproliferierender Phänotyp*, der sich nachfolgend durch Entdifferenzierung, Störungen der Apoptoseregulation, Neoangiogenese und Invasion in andere Gewebe auszeichnet (Abb. 1). Besonders häufig treten Mutationen der Gene *apc* (adenomatöses Polyposis Coli Protein) und *dcc* (engl. Abk. für fehlend in kolorektalen Karzinomen), die an der Zelladhäsion beteiligt sind, *mcc* (engl. Abk. für mutiert in kolorektalen Karzinomen), *k-ras,* das die Proliferation positiv beeinflusst und *p53,* das zur Apoptoseregulation dient, auf. Die Adenome sind durch Hypomethylierung der DNA und Genominstabilität gekennzeichnet [FEARON & VOGELSTEIN 1990].

1.2 Chemoprävention

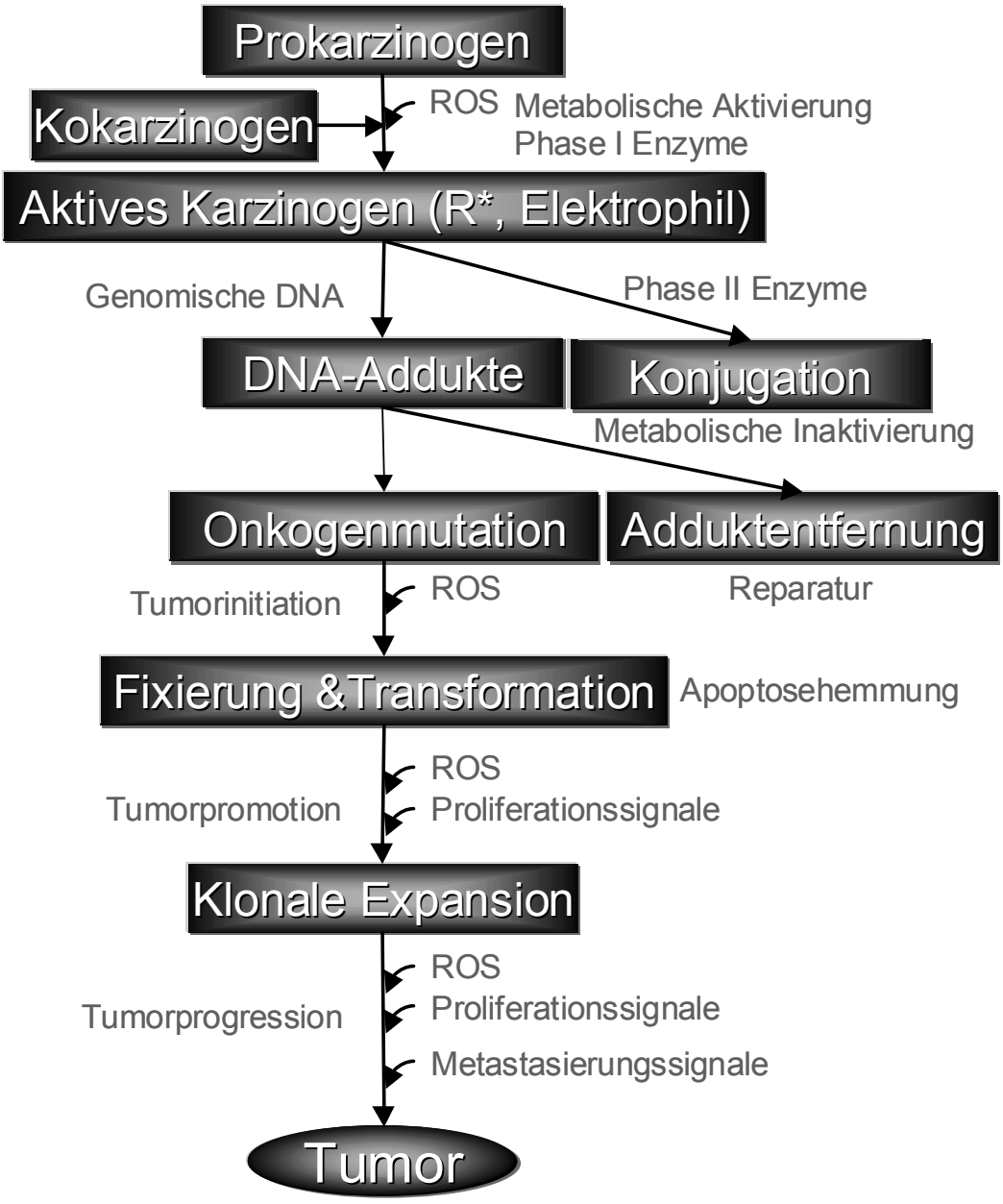


Abb. 2: Prozess der Karzinogenese (ROS – reaktive Sauerstoffspezies)

Der Terminus *Chemoprävention* wurde 1976 von SPORN geprägt. Der Begriff ist als Hemmung oder Umkehrung der Karzinogenese durch die Verwendung nichttoxischer Nahrungsmittelbestandteile oder pharmakologischer Substanzen, die vor der Entstehung oder Progression mutierter Klone maligner Zellen schützen, definiert [SPORN 1976]. Dieser Ansatz der Tumorprävention berücksichtigt, dass ein Karzinom nicht Folge eines einzelnen Geschehnisses, sondern eines mehrstufigen molekularen und zellulären Entwicklungsprozesses

ist, der von der Initiation bis zur Bildung eines invasiven und metastasierenden Phänotyps mehrere Jahre umfassen kann (Abb. 2).

Die Mehrzahl sporadisch auftretender Kolonkarzinome folgt dieser *Adenom-Karzinom-Sequenz* [SCHEPPACH *et al.* 2000]. Chemopräventiv wirkende Substanzen können in unterschiedliche Stadien der Karzinogenese eingreifen und differenzierbare Wirkmechanismen aufweisen:

1. Verhinderung der Karzinogenbildung oder ihrer Bindung an die DNA,
2. als Antioxidans, das die Interaktion zwischen Zellbestandteilen mit elektrophilen Karzinogenen verhindert,
3. als Modulatoren des Xenobiotika-Metabolismus,
4. durch Umkehrung der Entdifferenzierung,
5. als molekulare Modulatoren der Tumorbiologie,
6. durch Hemmung der Hyperproliferation und
7. durch Apoptoseinduktion [HONG & SPORN 1997].

An eine chemopräventiv wirkende Substanz werden eine Reihe von Anforderungen gestellt. Das ideale Agens zur Chemoprävention ist

- kostengünstig,
- oral konsumierbar,
- nicht oder nur gering toxisch,
- äußerst wirksam,
- wird vom Konsumenten akzeptiert und
- wirkt über einen bekannten Mechanismus [AHMAD & MUKHTAR 1999].

Zahlreiche epidemiologische Studien belegen, dass der regelmäßige Verzehr pflanzlicher Produkte entscheidend zur Prävention von Tumorerkrankungen beitragen kann [STEINMETZ & POTTER 1991A, STEINMETZ & POTTER 1991B]. Bereits 1996 zeigten CLIFFORD *et al.*, dass die Fütterung HTLV-1-transgener Mäuse mit Rotweinextrakt die Tumorentwicklung hinauszögert.

1.3 Signaltransduktion

1.3.1 Zellzyklusregulation

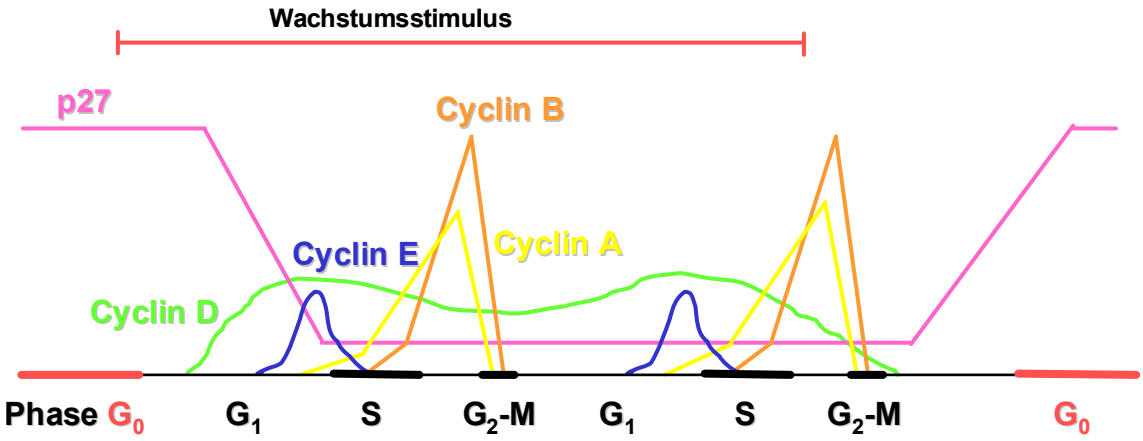


Abb. 3 : Intrazelluläre Cyclinspiegel in Abhängigkeit von der Zellzyklusphase

Im Gegensatz zu ausdifferenzierten Zellen, die dauerhaft in der G_0-Phase des Zellzyklus verharren, durchlaufen proliferierende Zellen den gesamten Zellzyklus, der hauptsächlich von Cyclinen und cyclinabhängigen Kinasen (Cdk) reguliert wird. Während die Konzentration der Cdks weitgehend konstant bleibt, oszilliert die Proteinkonzentration der Cycline in Abhängigkeit von der jeweiligen Zellzyklusphase (Abb. 3).

Cyclin D wird als Antwort auf mitogene Reize synthetisiert und assoziiert in der G_1-Phase mit Cdk4 und Cdk6. In der späten G_1-Phase wird Cyclin E synthetisiert, bildet mit Cdk2 Komplexe und ist für den Eintritt in die S-Phase verantwortlich. Die Cyclin A-Bildung erfolgt parallel zur DNA-Synthese und führt zur Komplexbildung mit Cdk2 und später mit Cdc2 (synonym: Cdk1). Cdc2 kontrolliert mit Cyclin B in der G_2-Phase den Beginn sowie das Ende der M-Phase [LUNDBERG & WEINBERG 1999].

Die Aktivität der Cdk/Cyclin-Komplexe ist zunächst von der Menge intrazellulär verfügbarer Cycline abhängig und von aktivierenden und deaktivierenden Phosphorylierungen der Cdk-Untereinheit, die durch verschiedene Kinasen und Phosphatasen reguliert werden. Außerdem beeinflussen Cdk-inhibitorische Proteine (CKI) die Cdk/Cyclin-Aktivität negativ. Zu den CKIs werden zum einen die Familie der Kinase-Inhibitoren (INK-Familie) gezählt, die inhibitorisch auf die Cdk4- und Cdk6-Aktivität wirken, sowie die Cip/Kip-Familie, denen $p21^{Waf1/Cip1}$

(Cip1: cyclininhibierendes Protein 1; Waf1: Wildtyp p53 aktivierter Faktor 1) und $p27^{Kip1}$ (Kip1 - Kinase inhibierendes Protein 1) zugeordnet werden. Diese können sowohl Cyclin D-abhängige Kinasen, als auch die Interaktion zwischen Cdk2 und den Cyclinen E und A negativ beeinflussen [MORGAN 1995]. Die intrazelluläre $p27^{Kip1}$-Konzentration ist ebenfalls zellzyklusabhängig und in der S-Phase am niedrigsten [MONTAGNOLI *et al.* 1999].

Cdk4/Cyclin D phosphorylieren das Retinoblastomaprotein (pRb), wodurch es inaktiv im Zytosol festgehalten wird. Im hypophosphorylierten Zustand transloziert pRb in den Zellkern und hemmt Transkriptionsfaktoren der E2F-Familie. Die transkriptionelle Aktivität von E2F führt zur Transaktivierung von Genen, deren Produkte für die Initiation der S-Phase des Zellzyklus essentiell sind (u.a. Cyclin A und E), so dass ein Übergang von der G_1- in die S-Phase prinzipiell nur bei hyperphosphoryliertem pRb möglich ist. Die Überexpression von Cyclin D ist jedoch ein Mechanismus, mit dem sich Karzinomzellen der Zellzyklusregulation durch pRb entziehen können [BARTKOVA *et al.* 1994].

1.3.2 Apoptose

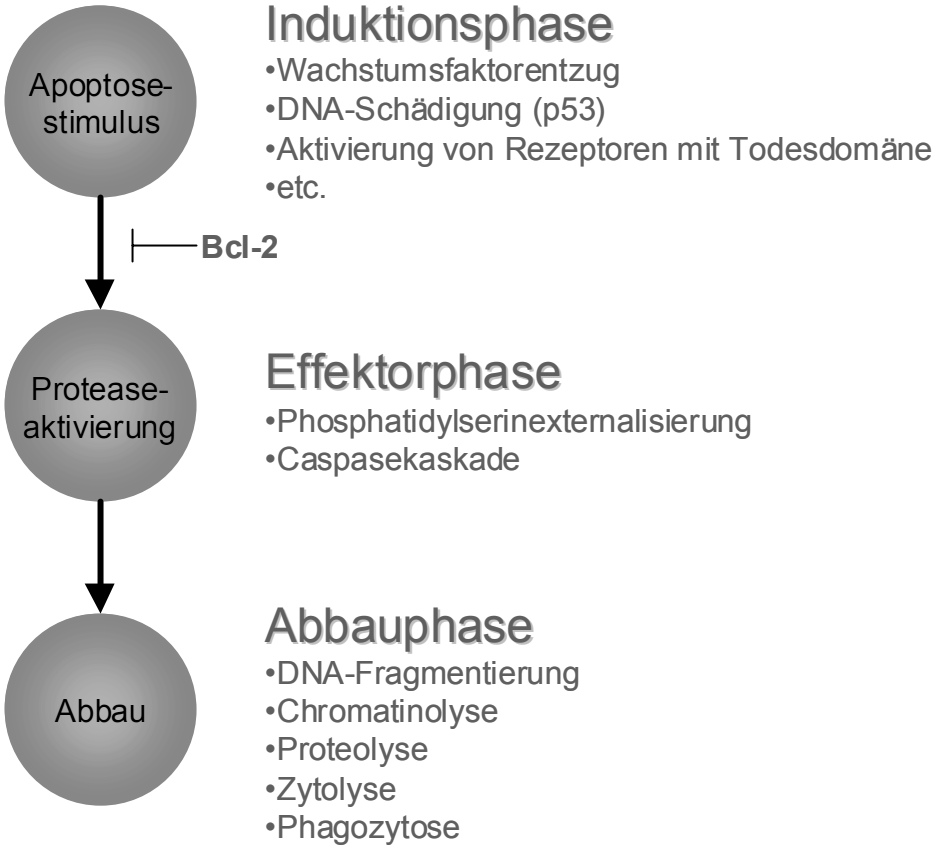


Abb. 4: Schematische Darstellung der Apoptosephasen [modifiziert nach KROEMER *et al.* 1995]

Der Terminus *Apoptose* ist eine Kombination der griechischen Wörter *apo* „herab“ und *ptosis* „fallen“ und steht für das Fallen der Blätter im Herbst [KERR *et al.* 1972]. Apoptose ist eine aktive Form des Zelltodes mit spezifisch ablaufenden morphologischen und biochemischen Prozessen (Abb. 4), bei der die Zelle selbst ein genetisch kontrolliertes Programm startet, das zu ihrem Untergang führt [QUE & GORES 1996]. Dieser programmierte Zelltod reguliert die Zellzahl und die gezielte Entfernung einzelner geschädigter oder infizierter Zellen aus dem Zellverband. Apoptose ist im frühen Stadium von Chromatinkondensation und einer Externalisierung des normalerweise auf der Zytosolseite der Plasmamemebran lokalisierten Phosphatidylserins, das durch Färbung mit Annexin sichtbar gemacht werden kann, gekennzeichnet. Später geht sie mit einer spezifischen DNA-Fragmentierung in 180 Basenpaar-(bp)-bruchstücke, einer Aktivierung von *Caspasen* (Cystein Aspartat-spezifische Proteasen), die im nachfolgenden für das Zellüberleben wichtige Proteine, wie

z.B. die Poly-ADP-Ribose-Polymerase (PARP), spalten und der Bildung sogenannter „apoptotic bodies“, abgeschnürter Zellbestandteile, einher [LINCZ 1998].

1.3.3 Differenzierung

Im Kolon sind die Stammzellen, aus denen die proliferierenden Kolonepithelzellen hervorgehen, am Grunde der Krypten angesiedelt. Die proliferative Zone reicht von der Kryptenbasis bis etwa zum zweiten Drittel der Kryptentiefe. Oberhalb der proliferativen Zone nimmt der Differenzierungsgrad der Kolonozyten zum Darmlumen hin zu. Am äußeren Ende der Krypten werden Zellen abgeschilfert und gehen durch „Anoikis“, eine besondere Form der Apoptose, die durch Kontaktverlust zur extrazellulären Matrix gekennzeichnet ist, zugrunde. Der Migrationsprozess von der Kryptenbasis bis zum Darmlumen dauert zwischen drei und acht Tagen [POTTEN *et al.* 1997].

Differenzierung bezeichnet die Entwicklung einer unreifen, teilungsfähigen Zelle zu einer funktionellen, spezifische Aufgaben übernehmenden Zelle, die dauerhaft in der G_0-Phase des Zellzyklus verharrt und somit teilungsunfähig ist. Kolonozytendifferenzierung geht mit Ausbildung eines polarisierten Phänotyps einher, der Mikrovilli, Zellgerüste, Zell-Zell-Kontakte und spezifische Bürstensaumenzyme bildet [TIAN & QUARONI 1999].

Bestimmte Substanzen können den Grad der Differenzierung von Karzinomzellen erhöhen und dadurch die Hyperproliferation dieser Zellen hemmen. Daher kann die Redifferenzierung ein Ziel für chemopräventive Substanzen darstellen. Eine Substanz, die *in vitro* und *in vivo* den Differenzierungsgrad von Kolonozyten erhöht, ist die kurzkettige Fettsäure Butyrat.

Butyrat

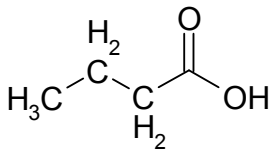


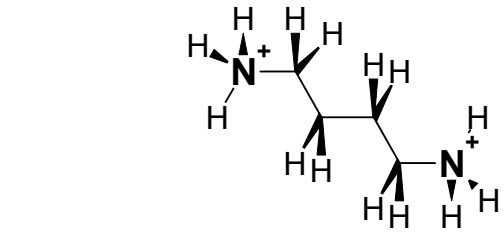
Abb. 5: Chemische Struktur von Butyrat

Butyrat ensteht im Darm bei der bakteriellen Fermentation unverdaulicher Kohlenhydratbestandteile (Abb. 5). Es dient Kolonozyten als primäre Energiequelle aus dem Lumen und fördert die Proliferation im basalen Kompartiment der Krypten ohne die Proliferation des lumenwärts gerichteten Kryptenkompartiments zu stimulieren [VELÁZQUEZ *et al.* 1996]. Ein Absinken der luminalen Butyratkonzentration führt zur mukosalen Atrophie, die durch Applikation kurzkettiger Fettsäuren, insbesondere Butyrat, im Kolon reversibel ist. Im Gegensatz dazu hemmt Butyrat jedoch die DNA-Synthese neoplastischer Kolonozyten [EBD.]. Dieses „Butyratparadoxon“ kann auch in Kolonkarzinomzellkulturen (u.a. HT-29- und Caco-2-Zellen) beobachtet werden. Hier führt Butyrat zu einer deutlichen Wachstumshemmung, wobei die wirksame Butyratkonzentration bei ca. 1 - 5 mM und damit deutlich unterhalb der im Kolon anzutreffenden physiologischen Konzentration liegt [AUGERON & LABOISSE 1984, GUM *et al.* 1987]. Butyrat verhindert die Zellzyklusprogression in der G_0/G_1-Phase [DING *et al.* 1998, CORADINI *et al.* 2000]. Dieser Effekt wird wahrscheinlich durch die Induktion der Zellzyklusinhibitoren $p21^{Waf1/Cip1}$ und $p27^{Kip1}$ [LITVAK *et al.* 1998] sowie der Modulation weiterer zellzyklusrelevanter Proteine, beispielsweise einer Abnahme von Cyclin D1 [CORADINI *et al.* 2000], vermittelt.

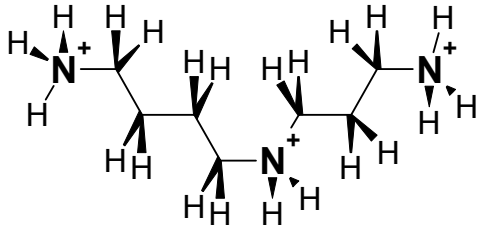
Eine Butyratgabe induziert in Kolonkarzinomzellen Differenzierung mit den damit einhergehenden morphologischen Alterationen. So erhöht Butyrat u.a. die Sekretion des intestinalen Bürstensaumenzyms alkalische Phosphatase (AP) [SUSSMAN *et al.* 1989] sowie der Disaccharidasen Saccharase-Isomaltase, Laktase und Dipeptidylpeptidase IV. Zudem induziert es die E-Cadherin-Expression [HAGUE *et al.* 1996] und erhöht den transepithelialen Widerstand [MARIADASON *et al.* 1997] in der kolorektalen Karzinomzelllinie Caco-2.

Ein Wirkmechanismus von Butyrat ist die Hemmung der Histondeacetylase, was zu einer Hyperacetylierung von Histonen, einer Auflockerung des Chromatingerüstes und damit zur erhöhten transkriptionellen Aktivierung, Phosphorylierung und Methylierung der Histon-DNA führt. Dadurch wird eine veränderte Onkogenexpression (*c-myc*, *c-myb*, *c-ras*) und eine verminderte Urokinaseaktivität bewirkt [SCHRÖDER & STEIN 1997].

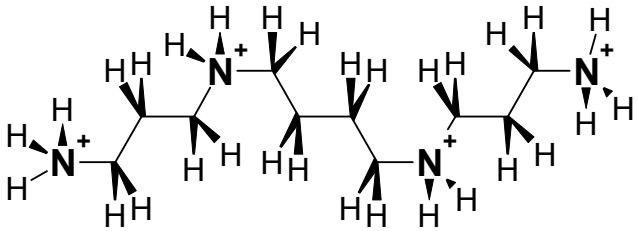
1.3.4 Polyaminhomöostase



Putreszin



Spermidin



Spermin

Abb. 6: Chemische Struktur der Polyamine

Polyamine sind unter physiologischen Bedingungen positiv geladen und haben eine hohe Affinität zu anionischen zellulären Bestandteilen (Abb. 6). Aufgrund ihrer stabilisierenden Wirkung auf die DNA-Struktur [MARQUET & HOUSSIER 1988, MCLEAN UND WELL 1988], ihrer Wirkungen auf die Membran [Schuber 1989] und auf wachstumsregulierende Gene [BALASUNDARAM & TYAGI 1991] sind Polyamine essentiell für das Zellwachstum. Die intrazellulären Polyaminspiegel werden durch Polyaminbiosynthese, -aufnahme und –abbau reguliert (Abb. 7). Konzentrationsänderungen verursachen gegenregulierende Mechanismen.
Mukosazellen des Gastrointestinaltraktes sind Polyaminen mit Herkunft aus dem Lumen in hohen Konzentrationen ausgesetzt. Diese stammen aus der Nahrung, der bakteriellen Synthese, der gastrointestinalen Sekretion und sind zudem in abgeschilferten intestinalen Epithelzellen vorhanden [MCEVOY & HARTLEY 1975].

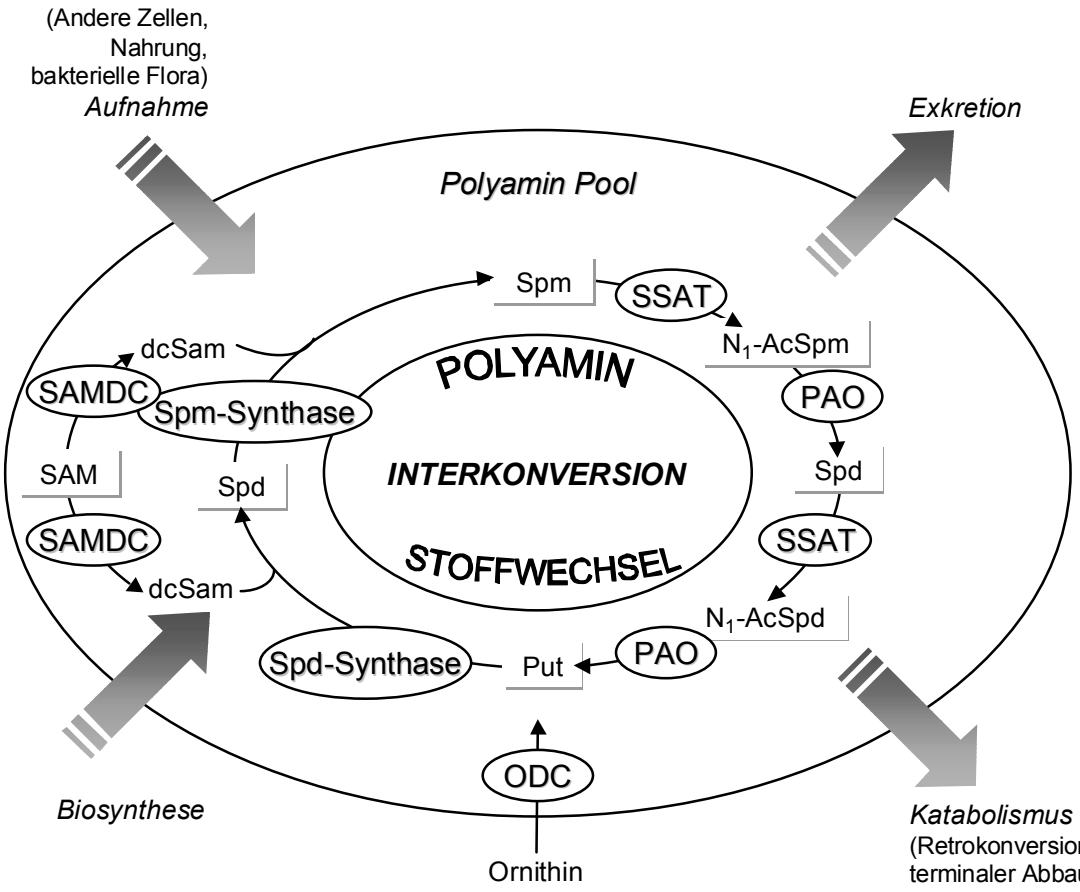


Abb. 7: Polyaminstoffwechsel (dcSAM – decarboxyliertes S-Adenosylmethionin; N_1-AcSpd – *N_1*-Acetylspermidin; N_1-AcSpm – *N_1*-Acetylspermin; ODC - Ornithindecarboxylase; PAO - Polyaminoxidase; Put - Putreszin; SAM – S-Adenosylmethionin; SAMDC – S-Adenosylmethionindecarboxylase; Spd - Spermidin; Spm - Spermin; SSAT - Spermidin/Spermin-*N^1*-Acetyltransferase)

Ornithindecarboxylase (ODC)

Das Schlüsselenzym der Polyaminsynthese stellt die ODC (E.C. 4.1.1.17) dar, deren Funktion in der Bildung von Putreszin aus Ornithin besteht. Die ODC-Aktivität kann innerhalb kurzer Zeit durch den Einfluss verschiedener Wachstumsfaktoren extrem stark induziert werden [JOHNSON *et al.* 1989]. Beim Menschen erfolgt die Regulierung des Enzyms auf Ebene der Gentranskription [GREENBERG *et al.* 1985], der mRNA-Degradierung [HÖLTTÄ *et al.* 1988], der mRNA-Translation [KAMEJI & PEGG 1987] und des Enzymabbaus [MURAKAMI & HAYASHI 1985].

Ornithin wird entweder aus dem Plasma bereitgestellt [LIAPPIS 1972] oder im Harnstoffzyklus gebildet. Die Darmepithelzellen können mit Hilfe von Arginase II Ornithin aus Arginin synthetisieren [CYNOBER 1994]. In Kolon-karzinomzellen ist die ODC-Aktivität gegenüber normalem Gewebe drei- bis vierfach erhöht

[LÖSER *et al.* 1990, ELITSUR *et al.* 1992]. Die Polyaminaufnahme und -synthese werden durch spezifische Aktivierung des Proteins Antizym im Sinne eines negativen Feedbacks kontrolliert [MURAKAMI *et al.* 1988]. Das Antizym bindet mit hoher Affinität reversibel an die ODC, hemmt nichtkompetitiv deren Aktivität [FONG *et al.* 1976] und bewirkt einen ubiquitinunabhängigen Abbau durch das zytosolische 26S-Proteasom [MURAKAMI *et al.* 1992]. Die Halbwertszeit (HWZ) einer an Antizym gebundenen ODC beläuft sich auf < 5 Minuten [MURAKAMI *et al.* 1994].

Das Protoonkoprotein c-Myc gilt neben den Transkriptionsfaktoren c-Fos und c-Jun als Schlüsselregulator des Zellwachstums. Die Expression von *c-myc* verhindert nicht nur eine Zellzyklushemmung, sondern fördert vielmehr den Eintritt in die S-Phase. AMATI *et al.* [1993] konnten zeigen, dass das Myc-Onkoprotein in Interaktion mit Max an die CACGTG-Hexanukleotid-Sequenz der DNA bindet, die in ODC-Promotoren vorhanden ist.

S-Adenosylmethionindecarboxylase (SAMDC)

S-Adenosylmethionin dient als Propylamindonor zur Synthese von Spermidin und Spermin. Während der Putreszinsynthese durch die ODC wird die SAMDC (E.C.4.1.1.40) aktiviert. Decarboxyliertes S-Adenosylmethionin (dcSAM) und Putreszin dienen der Spermidinsynthese und Spermidin wird durch Addition von dcSAM in Spermin umgewandelt. Die SAMDC hat eine HWZ von ~1 Stunde [PEGG & MCCANN 1982], wird *in vitro* spezifisch durch Putreszin und verwandte Diamine stimuliert und durch Produkthemmung reguliert [MAMONT & DAUZIN 1981].

Im Gegensatz zu ODC und SAMDC werden die Propylamintransferasen Spermidin- und Sperminsynthase, konstitutiv exprimiert und sind zudem stabile Enzyme mit langen HWZ [JÄNNE *et al.* 1978]. Ihre Aktivität ist hauptsächlich von der Verfügbarkeit des Substrates dcSAM abhängig und auch sie werden durch Produkthemmung reguliert [PÖSÖ *et al.* 1976].

Die Interkonversion wird von zwei Enzymen kontrolliert: der Spermidin/Spermin-N^1-Acetyltransferase (SSAT; E.C.2.3.1.57) und der Polyaminoxidase (PAO; E.C.1.5.3.11). Durch Interkonversion können einzelne Polyamine ineinander umgewandelt werden. Die PAO ist im Überschuss vorhanden, weshalb die Reaktion der SSAT geschwindigkeitsbestimmend ist. Die SSAT transferiert Acetyl-CoA an die *N*-Position sowohl des Spermidins als auch des Spermins

[PEGG & MCCANN 1982]. Die Acetylierung dient dazu, die positive Ladung der Polyamine zu verringern und sie so aus Bindungen zu lösen [SEILER 1987]. Die Produkte der SSAT-Reaktion, N^1-Acetylspermin und N^1-Acetylspermidin, werden von der PAO in Spermidin und 3-Acetamidpropanal bzw. Putreszin und 3-Acetamidpropanal konvertiert [HÖLTTÄ 1977]. Die Diaminoxidase katalysiert die Decarboxylierung von Putreszin zu γ-Aminobutyraldehyd, welches nachfolgend zu γ-Aminobutyrat und Δ^1-Pyrrolidin abgebaut wird.

Folglich wird nicht nur die Polyaminsynthese, sondern auch der Polyaminabbau in der Zelle komplex reguliert. Der Abbau findet durch die direkte Oxidierung durch Polyaminoxidasen zu Aldehyden [ALLAN *et al.* 1979], den Umbau von Spermin zu Spermidin und Putreszin durch eine Kombinierung von Acetylierung und Oxidation [MATSUI *et al.* 1982] und die direkte Freisetzung als Polyamin oder acetyliertes Derivat statt [SEILER *et al.* 1981, WALLACE & KEIR 1981].

1.4 Stilbene

Resveratrol (5-[(1E)-2-(4-Hydroxyphenyl)-ethenyl]-1,3-benzendiol bzw. 3,4‘,5-Stilbentriol oder 3,5,4‘-Trihydroxy-*trans*-stilben) hat ein Molekulargewicht von 228,2 g/mol und gehört zur Klasse der Phytoalexine (*phyton*, griech. für: Pflanze, *alexein*, griech. für: abwehren). Diese zeichnen sich dadurch aus, dass sie zum Schutz der Pflanze erst als Antwort auf einen Reiz biologischer (Pilzbefall), chemischer (Ozon) oder physikalischer Natur (UV-Strahlung, mechanische Schädigung) gebildet werden. Chemisch ist Resveratrol der Gruppe der Stilbene zuzuordnen, deren Grundgerüst ein Diphenylethylen darstellt und die sich durch ein niedriges Molekulargewicht (meist 200 - 300 g/mol) sowie ihre fungizide Wirkung auszeichnen [BAVARESCO *et al.* 1999].
Piceatannol ((E)-4-[2-(3,5-Dihydroxyphenyl)-ethenyl]1,2-benzendiol bzw. *trans*-3,4,3‘,5‘-Tetrahydroxystilben), auch 3-Hydroxyresveratrol, 3,3‘,4,5‘-Stilbentetrol oder Astringinin genannt, ist im Gegensatz zu Resveratrol mit einer vierten Hydroxylgruppe konjugiert, hat ein Molekulargewicht von 244,2 g/mol und gehört zu den konstitutiv gebildeten Stilbenen, die vor allem in holzigen Pflanzenanteilen gebildet werden. Diese haben vermutlich die Aufgabe, Wurzeln, Halme, Rinde und Kernholz vor mikrobiellen Zersetzungsprozessen und Insektenfraß zu schützen [BAVARESCO *et al.* 1997].

1.4.1 Vorkommen der Stilbene Resveratrol und Piceatannol

Resveratrol wurde bisher in mindestens 72 Spermatophytenarten nachgewiesen (Abb. 8). Hohe Konzentrationen wurden vor allem in Wein und dort speziell in Rotwein gemessen. Dies liegt daran, dass beim Prozess der Rotweinherstellung, im Gegensatz zur Herstellung von Weißwein, die Schale in der Maische mitvergoren wird. In der Schale der *Vitis vinifera*-Beeren (Weintrauben) sind wiederum die höchsten Konzentrationen an Resveratrol nachzuweisen. Die Resveratrolsynthese in der Traubenschale findet vor allem nach Befall der Pflanze mit *Botrytis cinerea*, einem Schimmelpilz, statt [FRÉMONT 2000]. LANGCAKE und PRYCE detektierten Resveratrol erstmals 1976 in den Blättern von *Vitis vinifera.* Seine Existenz im Wein selbst wurde erst 1992 nachgewiesen [SIEMANN & CREASY].

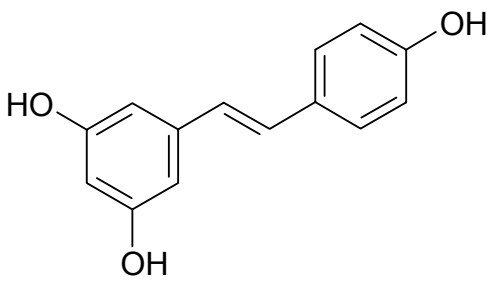


Abb. 8: Chemische Struktur Resveratrols

Der Resveratrolgehalt im Wein wird von unterschiedlichen Faktoren, wie

- der Rebsorte,
- der Stärke des Reizes zur Stilbensynthese,
- dem Klima,
- dem Reifegrad der Trauben,
- den Kelterungsmethoden und
- der Lagerung beeinflusst.

Neben *trans*-Resveratrol kommen im Wein noch das biologisch inaktive *cis*-Resveratrol, Piceid und Astringin (Stilbenglykosid), Pterostilben (3,5-methyliertes Resveratrol), Tetrahydroxystilben (Piceatannol) und ε-Viniferin (Resveratrolpolymer) vor. Quantitativ macht hierbei ε-Viniferin den Hauptanteil aus [SOLEAS *et al.* 1997A]. Insgesamt ist Resveratrol zu ca. 1% des Gesamtpolyphenolgehalts im Wein enthalten [FRÉMONT 2000], der wiederum 0,2% des Rotweins ausmacht [SOLEAS *et al.* 1997B]. Die durchschnittliche Resveratrolkonzentration im Rotwein liegt bei 2 - 40 µM [ELATTAR & VIRJI 1999]. In frischer Traubenhaut sind 50 bis 100 µg Resveratrol pro Gramm enthalten [JANG *et al.* 1997].
Eine weitere Nahrungsquelle für Resveratrol stellen Erdnüsse mit 0,02 - 1,79 µg Resveratrol pro Gramm [SANDERS 2000] und in geringerem Maße Eukalyptus dar [HEGNAUER 1990].

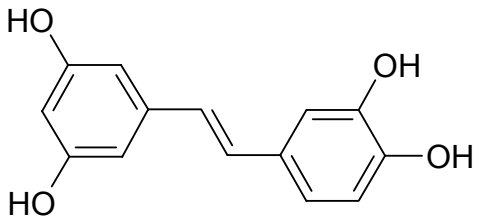


Abb. 9: Chemische Struktur Piceatannols

Piceatannol wurde aus der Rinde von Fichten (lat. *Picea abies (L.)*) [HEGNAUER 1990] und dem Kernholz verschiedener Leguminosen, wie *Vouacapoua* und *Cassia garretiana* isoliert (Abb. 9) [SCHRÖDER & SCHRÖDER 1990].
LAMIKANRA *et al.* [1996] wiesen erstmals Tetrahydroxystilbene im Rotwein, vor allem in *Cabernet Sauvignon* und *Merlot*, nach. Da die Tetrahydroxystilbenkonzentration in Wein, der in Holzfässern gelagert wurde, besonders hoch war, geht man davon aus, dass die Tetrahydroxystilbene entweder aus dem Holz bereitgestellt werden oder durch Begleitstoffe aus dem Holz stabilisiert werden. TEGUO *et al.* [1996] detektierten Piceatannol und Astringin (Piceatannol-3-*O*-β-D-Glukosid) zudem direkt in Suspensionskulturen von *Vitis vinifera*.

1.4.2 Die Metabolisierung Resveratrols und Piceatannols

Hydroxystilbene permeieren durch Zellmembranen, sind *stabil* und *nicht zytotoxisch* [BISHOP *et al.* 1997]. Die orale Gabe von 20 mg Resveratrol pro kg Körpergewicht täglich, über einen Zeitraum von 28 Tagen, führte bei Ratten zu keinerlei toxischen Wirkungen [JUAN *et al.* 2002]. Im isolierten Rattendarm konnte gezeigt werden, dass Resveratrol zu 96,5 $\pm$ 4,6% in Enterozyten glucuronidiert wird [KUHNLE *et al.* 2000]. Die *Glucuronidierung* fand UDP-Glucuronosyltransferase 1A1-vermittelt, präferentiell an Position 3, statt [AUMONT *et al.* 2001]. Eine Konjugation mit Sulfat wurde ebenso beschrieben [DE SANTI *et al.* 2000A & 2000B]. Gleichfalls im isolierten Rattendarm betrug die Absorptionsrate von Resveratrol aus einer Pufferlösung 20,5%. Hauptsächlich wurde Resveratrol konjugiert als Glucuronid (16,8%), weniger als Sulfat (0,3%) und zu 3,4% als freies Resveratrol absorbiert. Der Transportweg der Resveratrolkonjugate in die Kolonozyten ist bislang unbekannt, es wird jedoch ein carrierproteinvermittelter Mechanismus vermutet [ANDLAUER *et al.* 2000]. Nach *ad libitum*-Gabe von Rotwein bekannter Resveratrolkonzentration an

Ratten wurden die höchsten Plasmakonzentrationen nach 1 Stunde erreicht. Diese Konzentrationen waren ausreichend um physiologische Wirkungen, wie eine Hemmung der Thrombozytenaggregation, auszuüben [BERTELLI *et al.* 1998A]. In Leber und Nieren wurden ähnliche Konzentrationen wie im Blutplasma detektiert. Auch im Herzgewebe war eine signifikante Absorption feststellbar [BERTELLI *et al.* 1998B]. Bei kontrollierter Resveratrolgabe an Ratten wurde eine Bioverfügbarkeit von 38,1 ± 13,5% ermittelt. Messbare Mengen glucuronidierten und unkonjugierten Resveratrols wurden mit der Galle ausgeschieden und gelangten so in den enterohepatischen Kreislauf. Dieser scheint die Plasma-HWZ entscheidend zu beeinflussen [MARIER *et al.* 2002]. Durch täglichen moderaten Rotweinkonsum sollte es grundsätzlich möglich sein, biologisch aktive Resveratrolkonzentrationen im Plasma und in verschiedenen Geweben zu erreichen [BERTELLI *et al.* 1998A, PALMIERI *et al.* 1999]. Nach oraler Gabe von 25 mg Resveratrol war die höchste Plasmakonzentration beim Menschen nach 30 Minuten feststellbar (7,1 µg/L freies Resveratrol und 338 µg/L konjugiertes Resveratrol) und kehrte nach 2 Stunden zum Ausgangswert zurück. Der Anteil konjugierten Resveratrols war 30 - 50fach höher als die Konzentration freien Resveratrols. Innerhalb von 24 Stunden wurden 24,6% des Stilbens im Urin ausgeschieden [SOLEAS *et al.* 2001A]. Zur Bioverfügbarkeit glykosylierter Stilbene liegen bislang keine Daten vor. Da Flavonoidglykoside resorbierbar sind, kann angenommen werden, dass dies auch bei Stilbenen der Fall ist [FRÉMONT 2000]. Über die Metabolisierung von Piceatannol ist bisher nichts Näheres bekannt.

1.4.3 Die biologischen Wirkungen Resveratrols

Die antikanzerogenen Eigenschaften Resveratrols wurden erstmals von der Arbeitsgruppe um John M. Pezzuto 1997 in der Zeitschrift *Science* beschrieben [JANG *et al.* 1997]. In dieser Arbeit wurde eine direkte Hemmung der Cyclooxygenase (COX)-1 (nicht aber der COX-2) mit einer ca. 60fach höheren Potenz als Acetylsalicylsäure demonstriert. Im Gegensatz zu anderen nichtsteroidalen Antirheumatika (NSAR) hemmte Resveratrol zudem die Hydroperoxidaseaktivität der COX-1 und der COX-2. Der Einfluss des Stilbens auf die Tumorinitiation wurde an promyelozytischen HL-60 Leukämiezellen untersucht. Resveratrol wirkte der Produktion freier Radikale nach Behandlung der Zellen mit dem Tumorpromoter 12-*O*-tetradecanoylphorbol-13-acetat (TPA)

entgegen. Eine antimutagene Potenz Resveratrols wurde am Modell des *Salmonella typhimurium*-Bakterienstammes TM677, nach Behandlung mit dem Tumorinitiator 7,12-Dimethylbenz(*a*)anthracen (DMBA) belegt. Zudem induzierte Resveratrol das Phase II-Enzym Quinonreduktase in Mäuse-hepatomzelllinien und führte zur Proliferationshemmung und Induktion der Differenzierung von HL-60-Zellen. In einem Mammadrüsenzellkulturmodell der Maus hemmte das Stilben die Entstehung präneoplastischer Läsionen. Bei einem zweistufigen Karzinogenesemodell an Mäusehaut, bei dem DMBA als Initiator und TPA als Promotor eingesetzt wurden, reduzierte Resveratrol (25 µM) die Anzahl der Tumoren um 98% [EBDA.].

Zellzyklus

In HL-60-Zellen bewirkte Resveratrol bereits bei einer Konzentration von 30 µM eine vollständige, jedoch reversible Wachstumshemmung. Diese ging mit einer Akkumulation der Zellen, die sich in der S-Phase des Zellzyklus befanden, einher. In diesem Zellkulturmodell blieb jedoch die gesamte G_1-Phase-Maschinerie (Cycline D1, D2, D3, Cdk4, Cdk6, Cdk2, $p21^{Waf1/Cip1}$ und $p27^{Kip1}$) sowie der Phosphorylierungsgrad des pRb unbeeinflusst. Dagegen konnte eine Induktion der Cycline A und E nach 48 Stunden beobachtet werden. Zusätzlich kam es zu einer Akkumulation der inaktiven Tyrosin 15-phosphorylierten Cdc2-Form, was für eine Zellzyklushemmung am Übergang von der S-Phase in die G_2/M-Phase spricht [RAGIONE *et al.* 1998].

Auch das Wachstum Kupffer'scher Sternzellen, isoliert aus der Rattenleber, wurde durch Inkubation mit Resveratrol gehemmt. Diese Wachstumshemmung erfolgte koinzident zu einer Reduktion des Cyclins D1, wurde jedoch nicht von einer Abnahme der Cycline A und E oder einer Veränderung der Cdk2- oder Cdk4-Spiegel begleitet [KAWADA *et al.* 1998].

Der Effekt Resveratrols auf das Wachstum von Prostatakarzinomzellen ist von der Androgensensitivität abhängig. Während Resveratrol das Wachstum androgenunsensitiver Zelllinien hemmte und deren Zellzyklusdistribution zugunsten der S-Phase verschob, fand sich bei androgensensitiven Zellen zwar keine Änderung der Zellzyklusverteilung, aber eine verminderte pRb-Expression [HSIEH & WU 1999]. HSIEH und Mitarbeiter [1999A] zeigten ausserdem, dass Resveratrol auf hochinvasive Mammakarzinomzelllinien (MDA-MB-435) stärker wachstumshemmend wirkt als auf weniger invasive Brustkarzinomzellen (MCF-

7). Das Wachstum endothelialer Zellen aus der Pulmonalarterie von Kälbern wurde dagegen in der S- und der G_2/M-Phase des Zellzyklus gehemmt. Zudem war eine Induktion des Tumorsuppressors p53 und des Zellzyklusinhibitors $p21^{Waf1/Cip1}$ zu verzeichnen [HSIEH *et al.* 1999B]. ZOU und Mitarbeiter [1999] untersuchten die Auswirkungen Resveratrols auf das Wachstum glatter Muskelzellen, die aus Kälberaorta gewonnen wurden und entweder mit PDGF (engl. für plättchenassoziierter Wachstumsfaktor) oder mit Endothelin stimuliert wurden. Hierbei konnte eine Hemmung der wachstumsfaktorstimulierten Proliferation und eine Reduktion der Zellen in der S-Phase zugunsten der Zellen in der G_0/G_1-Phase detektiert werden. Die antiproliferative Wirkung Resveratrols auf humane Epidermoidkarzinomzellen (A431) ist $p21^{Waf1/Cip1}$-abhängig, was zur Zellzyklushemmung in der G_0/G_1-Phase führte. Eine Abnahme der Proteine Cyclin D1, D2, E, Cdk2, 4 und 6 wurde beobachtet [AHMAD *et al.* 2001] und ging mit einer Abnahme und Dephosphorylierung von pRb einher. Die intrazellulären Proteinkonzentrationen der Interaktionspartner des Tumorsuppressorproteins pRb im Zellkern, E2F 1 bis 5, sowie DP1 und 2 waren ebenfalls reduziert [EBD.].

Dass die Wirkung Resveratrols auf die Zellzyklusdistribution nicht konzentrationsabhängig ist, zeigten erstmals PARK *et al.* an U937 Lymphomzellen. Sie demonstrierten, dass Resveratrol bis zu einer Konzentration von 60 µM die Anzahl der U937-Zellen in der S-Phase erhöht, dieser Effekt jedoch bei höheren Konzentrationen nicht mehr zu beobachten ist. Während die Menge an Cdk4, Cdc2 und Cyclin B1 nicht beeinflusst wurde, war $p27^{Kip1}$ leicht vermindert. Cyclin A, D3 und E wurden durch Resveratrol-behandlung induziert [PARK *et al.* 2001A].

Eine resveratrolvermittelte Zellzyklusblockade in der S-Phase von HCE7- (Ösophaguskarzinom), MCF-7- (Mammakarzinom), SW480- (Kolonkarzinom) und HL-60-Zellen wurde von JOE *et al.* [2002] demonstriert. Bei Adeno-karzinomzelllinien aus Barrett's-Ösophagusgewebe wurde entweder keine Veränderung der Zellzyklusdistribution (Seg-1-Zellen) oder eine Zunahme der G_0/G_1-Phase-Population (Bic-1-Zellen) gemessen. In SW480-Zellen kam es zur Abnahme der Cycline A, D1 und B1. Reduzierte Cyclin B1-Spiegel ließen sich auch in den Zelllinien Bic-1, HCE7, SW480, MCF-7, Seg-1 und HL-60 detektieren [EBD.].

Apoptose

In HL-60-Zellen bewirkte Resveratrol eine Externalisierung des normalerweise intrazellulär angesiedelten Phosphatidylserins, eines Markers der frühen Apoptose, und einen Anstieg subdiploider DNA. Die proteolytische Spaltung der PARP und eine Hemmbarkeit der resveratrolinduzierten Apoptose durch Caspaseinhibitoren sprechen für eine caspaseabhängige Apoptose. Die Induktion des CD95-Liganden, auch Fas-Ligand (FasL) genannt, trat sowohl in HL-60- als auch in T47D-Brustkarzinomzellen, jedoch nicht in untransformierten Lymphozyten auf. Die Apoptose war durch Antikörper gegen FasL hemmbar [CLÉMENT *et al.* 1998]. HL-60-Zellen, die mit 100 µM Resveratrol 8 Stunden inkubiert wurden, wiesen die charakteristischen Zeichen apoptotischer Zellveränderungen wie Zellschrumpfung, Bildung von „apoptotic bodies“ (engl. für Apoptosekörper), Chromatinkondensation und Schwellung der Zellorganellen auf. Zudem war eine Fragmentierung der DNA in die für Apoptose typischen 180 bp-Nukleosomen nachweisbar, und die Bcl-2 (B-Zell-Lymphom 2)-Expression war reduziert [SURH *et al.* 1999]. In akuten T-lymphoblastischen Leukämiezellen wurde die resveratrolinduzierte Apoptose durch Überexprimierung von Bcl-2 und durch Gabe von *N*-Acetylcystein, das reaktive Sauerstoffspezies quencht, antagonisiert [TINHOFER *et al.* 2001]. In humanen monozytischen THP-1-Leukämiezellen induzierte Resveratrol unabhängig von Fas Apoptose [TSAN 2000]. Auch in akuten lymphoblastischen Leukämiezellen, die resistent gegenüber Fas-vermittelter Apoptose sind, induzierte Resveratrol Apoptose, die mit einer Caspase 9-Aktivierung und dem Verlust des mitochondrialen Transmembranpotentials ($\Delta\Psi_m$) einherging [DÖRRIE 2001]. In MS1418-Lymphoblasten induzierte Resveratrol eine sphingomyelinaseunabhängige Apoptose. In *p53*$^{-/-}$-Mäusefibroblasten führte eine Exposition der Zellen gegenüber Resveratrol im Gegensatz zu *p53*$^{+/+}$-Mäusefibroblasten nicht zur Apoptose [HUANG *et al.* 1999], was auf eine p53-abhängige Apoptose hindeutet. Während in U937-Leukämiezellen nach der Inkubation mit Resveratrol eine Phospholipase C (PLC)-γ-Spaltung, eine Caspase 3-Aktivierung und Apoptoseinduktion stattfanden, wurde die resveratrolinduzierte Apoptose in *bcl-2*-transfizierten U937-Zellen gehemmt [PARK *et al.* 2001A]. Apoptose wurde auch in humanen epidermoiden Karzinomzellen (A431) [AHMAD *et al.* 2001] und androgensensitiven

Prostatakarzinomzellen (LNCaP) [HSIEH & WU 1999], die mit Resveratrol inkubiert wurden, nachgewiesen.

Differenzierung

RAGIONE *et al.* [1998] verzeichneten in HL-60-Zellen durch Resveratrol-behandlung einen signifikanten Anstieg der Differenzierung zu einem monozytären Phänotyp, was die Ergebnisse von Jang *et al.* [1997] unterstützt. In der Osteoblastenzelllinie MC3T3-E1 induzierte Resveratrol die AP- und die Prolylhydroxylaseaktivität [MIZUTANI *et al.* 1998]. Auf die erythrozytische Leukämiezelllinie K562 wirkte es hinsichtlich der Hämoglobinproduktion prodifferenzierend [RODRIGUE *et al.* 2001]. Kein differenzierungsfördernder Effekt konnte bei THP-1-Zellen (monozytischen Leukämiezellen) festgestellt werden [TSAN *et al.* 2000].

Die Wirkungen Resveratrols auf den Darm

TESSITORE *et al.* [2000] untersuchten erstmals die Wirkung Resveratrols auf die azoxymethaninduzierte Kolonkarzinogenese von Ratten. Das Stilben reduzierte die Anzahl hyperproliferativer Krypten im Kolon der Versuchstiere. Im Bereich dieser präneoplastischen Zonen war, im Gegensatz zu unbehandelten Tieren, in der Resveratrolgruppe eine Zunahme des proapoptotisch wirkenden Proteins Bax (Bcl-2 assoziiertes Protein X) zu verzeichnen. In hyperproliferativen Krypten resveratrolbehandelter Ratten kam es zu einer Abnahme von $p21^{Waf1/Cip1}$, während der Zellzyklusinhibitor in der normalen Mukosa der Resveratrolgruppe nicht mehr exprimiert wurde [EBD.]. Bei *ApcMin*-Mäusen reduzierte die orale Gabe Resveratrols über einen Zeitraum von 6 Wochen die Tumorrate um 70%. Im Kolon konnten nach Behandlung keine Tumoren nachgewiesen werden. In der intestinalen Mukosa dieser Tiere wurde mittels cDNA-Expressions-Array eine Abnahme von *cyclin D1*, *cyclin D3*, *dp-1*, *RNA-polymerase 1* und *y-box DNA-bindendes protein1* demonstriert. Demgegenüber kam es zu einer Zunahme der mRNA von Glutamatrezeptor, zytotoxischem T-Lymphozytenantigen-4, Desmocollin 2, Monozyten-Chemoattraktionsprotein 3, Homeobox-Protein 4.2, mitogenaktivierter Protein-Kinase, Follistatin, der βA-Untereinheit von Inhibin, Thrombopoietin, leukämieinhibitorischem Faktor, transformierendem Wachstumsfaktor-β (TGF-β), Mas und TSG101 [SCHNEIDER *et al.* 2001].

In Caco-2-Kolonkarzinomzellen hemmte Resveratrol dosisabhängig das Wachstum. Caco-2-Zellen verharrten in der S-Phase des Zellzyklus und zeigten keine apoptoseassoziierte DNA-Fragmentierung. Eine zeitabhängige Hemmung der ODC, nicht aber der SAMDC, mit einer intrazellulären Abnahme von Putreszin und Spermidin wurde bei einer Behandlung mit 25 µM Resveratrol ebenfalls demonstriert [SCHNEIDER *et al.* 2000]. MAHYAR-ROEMER und ROEMER [2001] zeigten mittels Durchflusszytometeranalyse, dass Resveratrol in HCT-116-Kolonkarzinomzellen Apoptose induziert, die unabhängig von p53 und $p21^{Waf1/Cip1}$ stattfindet. Es kam zu einer gesteigerten Bax-Expression und vor Einsetzen der Apoptose waren Anzeichen für epitheliale Differenzierung und mitochondriale Proliferation zu ersehen [MAHYAR-ROEMER *et al.* 2001].

In SW480 Kolonkarzinomzellen führte Resveratrol zur Wachstumshemmung, Zellzyklushemmung in der S-Phase und verminderter Expression von Cyclin A, B1, D1 und β-Catenin. Trotz Abnahme der *cyclin D1*-mRNA war die Cyclin D1-Reduktion durch einen Proteasomeninhibitor hemmbar und Resveratrol hatte keinen Einfluss auf die Promotoraktivität des *cyclins D1* [JOE *et al.* 2002].

1.4.4 Die biologischen Wirkungen Piceatannols

Erstmals wurde Piceatannol als spezifischer Inhibitor der Tyrosinkinase $p72^{Syk}$ bekannt. Zudem hemmte es die Aktivität von $p56^{Lck}$ in lymphoiden Zellen [GEAHLEN & MCLAUGHLIN 1989]. Die fokale Adhäsionskinase und die Src-Kinase aus Thrombozyten waren ebenfalls durch Piceatannol hemmbar [LAW *et al.* 1999]. Auch einige Serin-/Threoninkinasen, die Proteinkinase A aus Rattenleber und Proteinkinase C aus Rattengehirn, wurden durch Piceatannol gehemmt [WANG *et al.* 1998]. Die Interferon-α-induzierte Phosphorylierung der STATs 3 und 5 (Aktivatoren der Signaltransduktion und Transkription), sowie der Tyrosinkinase Jak (Januskinase)1 wurde in B- und T-Lymphozyten, HeLa-Zellen und primären Fibroblasten gehemmt [SU & DAVID 2000].

Piceatannol hemmte die F0/F1-ATPase (ATP: Adenosintriphosphat) aus Rattengehirn und Rattenleber [ZHENG & RAMIREZ 1999]. Ebenso wie Resveratrol übt auch Piceatannol antioxidative Wirkungen aus. Beim Eliminieren freier 2,2-Diphenyl-1-Picrylhydrazyl-Radikale sowie bei der Hemmung der Lipid-peroxidation war Piceatannol jedoch effizienter als Resveratrol [WANG *et al.* 1999]. Auf kultivierte Lebermyofibroblasten übte Piceatannol eine

antiproliferative Wirkung aus, bewirkte aber keine morphologischen Veränderungen dieser Zellen und hemmte im Gegensatz zu Resveratrol nicht die Expression des α-Aktins glatter Muskelzellen. In Konzentrationen über 25 µM wirkte es zytotoxisch [GODICHAUD *et al.* 2000]. In der Lymphomzelllinie BJAB induzierte Piceatannol unabhängig von der Fas-assoziierten Todesdomäne Apoptose, die mit Caspase 3-Aktivierung und mitochondrialer Permeabilisierung einherging. Auch in primären leukämischen Lymphoblasten induzierte Piceatannol Apoptose. Der Effekt war sogar ausgeprägter als der von Resveratrol [WIEDER *et al.* 2001].

Ein synthetisches Tetrahydroxystilben (3,4,5,4'-Tetrahydroxystilben) hemmte das Wachstum SV40 (engl. Abk. für Affenvirus 40)-transformierter WI38-Zellen (humane Lungenfibroblastenzelllinie), jedoch nicht „normaler" WI38-Zellen. Ebenso induzierte das Piceatannolanalogon, im Gegensatz zu untransformierten Zellen, in transformierten WI38-Zellen Apoptose. Diese ging mit einer Induktion von p53, GADD45 und Bax einher. Auch die DNA-Bindungsaktivität von p53 und das Bax:Bcl-2-Verhältnis wurden erhöht [LU *et al.* 2001].

1.5 Zielsetzung

Im Rahmen der vorliegenden Arbeit sollte erstmals untersucht werden:

- welchen Einfluss Resveratrol auf Proliferation, Zellzyklusregulation, Differenzierung und Apoptose kolorektaler Karzinomzellen ausübt,
- ob das natürlich vorkommende Strukturanalogon Piceatannol ähnlich wie Resveratrol wirkt,
- inwieweit Resveratrol die Wirkungen Butyrats auf die Differenzierung der Zelllinie Caco-2 moduliert und
- welche Auswirkungen eine Resveratrolinkubation auf die Polyamin-homöostase der Zelllinie Caco-2 hat.

2 Ergebnisse

2.1 Der Einfluss Resveratrols auf die Proliferation, Differenzierung, Apoptose und Zellzyklusregulation kolorektaler Karzinomzelllinien (MANUSKRIPT I)

Als Zellkulturmodell dienten Caco-2- und HCT-116-Zellen, die sich aufgrund verschiedener Mutationen genetisch unterscheiden. Während HCT-116-Zellen einem Patienten mit hereditärem nichtpolypösem Kolonkarzinom entnommen wurden und Wildtyp-p53 exprimieren, liegt *p53* in Caco-2-Zellen, das einem sporadischen Kolonkarzinom entstammt, auf beiden Allelen mutiert vor [DJELLOUL *et al.* 1997]. Caco-2-Zellen exprimieren keine COX-1, sondern nur COX-2, während HCT-116-Zellen weder messbare COX-1- noch COX-2-Aktivität aufweisen [KAMITANI *et al.* 1998, SHENG *et al.* 1997].

Bis zu Konzentrationen von 200 µM erwies sich Resveratrol nicht als zytotoxisch, was mittels Laktatdehydrogenase (LDH)-Freisetzungstest überprüft wurde. Resveratrol hemmte die Zunahme der *Zellzahl* von Caco-2 über 72 Stunden konzentrations- und zeitabhängig, was durch Färbung mit Kristallviolett demonstriert wurde. Dies beruht zumindest teilweise auf einer Hemmung der *Proliferation*, die nach 24 Stunden durch Einbau von [^{3}H]-Thymidin bzw. [^{14}C]-Leucin determiniert wurde.

Ein Einfluss auf die *Differenzierung*, anhand einer Aktivitätsbestimmung der AP von Caco-2-Zellen quantifiziert, konnte auch über einen Zeitraum von 12 Tagen nicht nachgewiesen werden.

Mit Hilfe eines Durchflusszytometers wurden die propidiumjodidgefärbten Zellkerne von Caco-2- und HCT-116-Zellen nach 24 Stunden auf ihren DNA-Gehalt hin untersucht, was Aufschluss über die *Zellzyklusphase*, in der sich die Zellen gerade befanden, geben sollte. Bei beiden Zelllinien kam es zu einer Zunahme der S-Phase-Population und einer Abnahme der Zellen, die sich in der G_2/M-Phase befanden, die am deutlichsten bei einer Resveratrolkonzentration von 50 µM in Erscheinung trat. Bei höheren Konzentrationen war dieser Effekt weniger ausgeprägt. Weder Stilbenmethanol noch das glykosylierte Stilbenanalogon Rhapontin, das im Rhabarber enthalten ist, bewirkten in einer Konzentration von 100 µM eine Verschiebung der

Zellzyklusphasendistribution, was darauf hinweist, dass die beobachteten Effekte auf den Zellzyklus spezifisch für Resveratrol sind.

Die Quantifizierung zellzyklusassoziierter Proteine mit dem Western blot-Verfahren zeigte, dass nach 24 Stunden weder PCNA (Zellkernantigen proliferierender Zellen) noch Cdc2, Cdk2 oder Cdk6 quantitativ durch Resveratrol beeinflusst wurden. Die intrazellulären Cyclin D1- und Cdk4-Konzentrationen wurden dosisabhängig reduziert. Die Cyclin A-Spiegel waren bis zu einer Resveratrolkonzentration von 100 µM erhöht, wobei der maximale Effekt bei 25 µM zu verzeichnen war. Dagegen wurde Cyclin E bis zu einer Resveratrolkonzentration von 200 µM vermehrt exprimiert. Der CKI $p21^{Waf1/Cip}$ war in behandelten Zellen konstitutiv vorhanden, während $p27^{Kip1}$ konzentrationsabhängig abnahm. Unter Behandlung mit 200 µM Resveratrol wurde eine Hypophosphorylierung des Tumorsuppressors pRb verzeichnet, was einer Aktivierung der inhibitorischen Wirkungen des Proteins gleichkommt. Untersuchungen an der Zelllinie HCT-116 zeigten, dass sich die Menge an Cdk2 und Cdk6 ebenfalls nicht durch Resveratrolbehandlung verändern ließ, während Cdk4, Cyclin D1 und $p27^{Kip1}$ abnahmen und Cyclin E zunahm.

Um den Einfluss Resveratrols auf die Induktion von *Apoptose* in Caco-2-Zellen zu untersuchen, wurde die Aktivität des Apoptosemarkers Caspase 3 determiniert. Durch Behandlung mit 200 µM Resveratrol nahm die Caspaseaktivität bereits nach 24 Stunden zu und war nach 48 Stunden noch ausgeprägter.

Diese Daten belegen, dass Resveratrol die Proliferation kolorektaler Karzinomzellen unabhängig von ihrem p53- oder COX-Status hemmt, ohne die Differenzierung zu fördern. Zudem hemmt es den Zellzyklus und modifiziert die Expression zellzyklusassoziierter Proteine. In höherer Konzentration induziert das Stilben Apoptose. Dies deutet darauf hin, dass Resveratrol seine chemopräventiven Wirkungen auch in Kolonkarzinomzellen entfaltet.

2.2 Der Einfluss Piceatannols auf die Proliferation, Differenzierung und Zellzyklusregulation kolorektaler Karzinomzelllinien (MANUSKRIPT II)

Basierend auf den Ergebnissen des MANUSKRIPTES I sollte untersucht werden, ob Piceatannol, als ebenfalls natürlich vorkommendes Stilben, ähnliche Effekte

auf Kolonkarzinomzellen ausübt wie Resveratrol. Hemmende Wirkungen des Piceatannols auf Tyrosinkinasen und STATs (somit chemopräventive Eigenschaften) wurden bereits demonstriert [GEAHLEN & MCLAUGHLIN 1989, SU & DAVID 2000]. Obwohl für die Resveratrolanaloga Rhapontin und Stilbenmethanol (s.o.) derartige Eigenschaften nicht belegt werden konnten, stellte Piceatannol aufgrund seiner dem Resveratrol vergleichbaren Struktur, es hat eine Hydroxylgruppe mehr als Resveratrol, eine weitere Substanz mit potentieller chemopräventiver Wirkung dar.

Eine konzentrations- und zeitabhängige Hemmung der *Zellzahlzunahme* von Caco-2- und HCT-116-Zellen über einen Zeitraum von 72 Stunden bewirkte auch Piceatannol, wenngleich mit geringerer Potenz als dies mit Resveratrol gezeigt werden konnte. Piceatannol hemmte nach einer Inkubationsdauer von 24 Stunden signifikant die *Proliferation* von Caco-2-Zellen.

Eine *differenzierung*sfördernde Wirkung wies das Tetrahydroxystilben während zehntägiger Inkubation, ebenso wie Resveratrol, nicht auf. Piceatannol (100 µM) übte jedoch nach 24 Stunden eine ausgeprägte Hemmung auf den *Zellzyklus* in der S-Phase aus.

Während die Cdc2-, Cdk2-, Cdk6- und $p21^{Waf1/Cip1}$-Expression piceatannol-behandelter Caco-2-Zellen konstant blieb, sanken die intrazellulären Cyclin B1- und D1-, Cdk4- und $p27^{Kip1}$-Spiegel. Cyclin E wurde ebenso wie Cyclin A vermehrt exprimiert. Die maximale Konzentration des Cyclins A wurde nach Behandlung mit 50 µM verzeichnet. In HCT-116-Zellen waren Cdk4, Cyclin A, B1 und D1 sowie $p27^{Kip1}$ nach Behandlung mit 200 µM Piceatannol in geringerer Menge nachzuweisen, während die Cyclin E-Expression deutlich zunahm.

Dies bedeutet, dass Piceatannol, ebenso wie Resveratrol, die Proliferation und den Zellzyklus kolorektaler Karzinomzelllinien hemmt, ohne differenzierungs-fördernd zu wirken. Aus diesen Daten kann geschlussfolgert werden, dass auch Piceatannol bezüglich der Kolonkarzinogenese chemopräventive Eigenschaften aufweist.

2.3 Synergistische Wirkungen der Kombination von Resveratrol und Butyrat auf die Differenzierung von Caco-2-Zellen (MANUSKRIPT III)

Besonders bei der Kolonkarzinogenese, auf die unzählige promovierende und auch hemmende Substanzen Einfluss nehmen und die eine große Variabilität der genetischen Modifikationen aufweist, sind kombinierte Chemopräventionskonzepte von Interesse. Neue onkologische Konzepte, die diesen Ansatz verfolgen, haben sich bereits als äußerst effektiv erwiesen [TORRANCE *et al.* 2000].

Die chemopräventiven Wirkungen Butyrats sind unter dem Begriff des „Butyrat-Paradoxons“ bekannt und basieren in erster Linie auf einer Hemmung des Wachstums entarteter Zellen und auf einer Umkehrung der Entdifferenzierung, die sich im Rahmen der Kolonkarzinogenese manifestiert. Die gut charakterisierte Zelllinie Caco-2 eignet sich ideal für Untersuchungen, die die intestinale Differenzierung betreffen, da die Zellen nach Erreichen eines konfluenten Stadiums spontan ausdifferenzieren [ZWEIBAUM *et al.* 1983].

Im Rahmen dieser Untersuchung wurden Caco-2-Zellen gleichzeitig mit 50 µM Resveratrol und 2 mM Butyrat behandelt. Beide Konzentrationen wurden so gewählt, dass keine Apoptoseinduktion stattfand.

Die Substanzen wirkten über 72 Stunden hemmend auf die *Zellzahlzunahme*, wobei der inhibitorische Effekt Resveratrols sehr viel ausgeprägter war. Durch die Behandlung mit beiden Substanzen zugleich wurde die Hemmung auf das Wachstum nicht verstärkt. Auch der inhibitorische Effekt Resveratrols auf die *Proliferation* wurde durch Butyrat nicht potenziert. Alleine appliziert hemmte die kurzkettige Fettsäure die Inkorporation von [^{14}C]-Leucin nach 24 Stunden in geringerem Maße als das Trihydroxystilben und beeinflusste den [^{3}H]-Thymidineinbau nicht.

Wie bereits demonstriert [MANUSKRIPT I], übte Resveratrol keinen Einfluss auf die AP-Aktivität aus. Wurde es jedoch zusammen mit Butyrat über einen Zeitraum von 8 Tagen appliziert, verstärkte es die Induktion, die durch Butyrat alleine induziert wurde. Ein synergistischer Effekt Resveratrols und Butyrats auf die Proteinexpression des *Zell-Zellkontaktproteins* und *Differenzierungsmarkers* E-Cadherin wurde zudem nach 48 Stunden mittels Western blot nachgewiesen,

während Resveratrol alleine keinen quantitativen Einfluss auf E-Cadherin ausübte. Da bekannt ist, dass TGF-β1 bei der butyratinduzierten Differenzierung sezerniert wird [SCHRÖDER *et al.* 1999], wurde untersucht, ob Resveratrol oder die Kombination ebenfalls die TGF-β1-Sekretion stimulieren. Hierbei wurde deutlich, dass Resveratrol die butyratinduzierte Exkretion des Zytokins hemmt.

Da davon ausgegangen wird, dass die *CKIs* $p21^{Waf1/Cip1}$ und $p27^{Kip1}$ an der Differenzierung beteiligt sind [EVERS *et al.* 1996, DESCHÊNES *et al.* 2001, QUARONI *et al.* 2000], wurden diese ebenfalls mittels Western blot detektiert. Bekannt ist, dass Resveratrol $p21^{Waf1/Cip1}$ in Caco-2-Zellen nicht beeinflusst und zur Reduktion von $p27^{Kip1}$ führt [MANUSKRIPT I]. Butyrat dagegen erhöht beide CKIs [LITVAK *et al.* 1998, WANG & FRIEDMAN 1998]. Während Resveratrol die butyratinduzierte $p27^{Kip1}$-Induktion hemmte, war die Wirkung auf die $p21^{Waf1/Cip1}$-Expression synergistisch [MANUSKRIPT III].

Diese Ergebnisse zeigen, dass sich die antiproliferativen Wirkungen Resveratrols und Butyrats zwar nicht addieren, jedoch gemeinsam die Differenzierung, über die von Butyrat alleine vermittelte Wirkung hinaus, stimulieren. Dies spricht dafür, dass hier eine kombinierte Chemoprävention stattfindet, wobei sich die beiden Substanzen hinsichtlich der differenzierungsfördernden Wirkung potenzieren.

2.4 Der Einfluss Resveratrols auf die Polyaminhomöostase in Caco-2-Zellen (MANUSKRIPT IV)

Da Polyamine essentiell für das Zellwachstum sind und sowohl die Synthese als auch die Aufnahme von Polyaminen in Karzinomzellen stark erhöht sind, stellt der Polyaminmetabolismus einen interessanten Angriffspunkt für die Chemoprävention dar.

Resveratrol hemmte konzentrationsabhängig die *ODC-Aktivität*, was bereits von SCHNEIDER *et al.* [2000] demonstriert werden konnte. Diese Hemmung wurde von einer konzentrationsabhängigen Abnahme sowohl des ODC-Proteins, als auch der *odc*-mRNA begleitet. Auch Piceatannol und in geringerem Maße Epigallocatechin-Gallat (EGCG), ein Polyphenol, das vor allem in grünem Tee enthalten ist, hemmten die ODC, es war jedoch nur eine geringe Abnahme der *odc*-mRNA zu verzeichnen.

c-Myc dient als Transkriptionsfaktor für den *odc*-Promoter. Daher wurde die Proteinexpression des Onkoproteins ebenfalls determiniert. Mittels Western blot konnte eine konzentrationsabhängige Abnahme des c-Myc-Proteins festgestellt werden.

Zudem wurde eine dosisabhängige Hemmung der *SAMDC*-Aktivität durch Resveratrol demonstriert. Auch Piceatannol, nicht jedoch EGCG wirkte inhibitorisch auf die SAMDC.

Resveratrol erhöhte die *Aktivität der SSAT*, eines Enzyms des Polyaminkatabolismus. Auch EGCG wirkte schwach induzierend, während Piceatannol keine Wirkung auf die SSAT ausübte. Mittels HPLC (Hochdruck-Flüssigkeitschromatographie) wurde anschließend die intrazelluläre *Polyamin-konzentration* resveratrolbehandelter Zellen untersucht. Während die Spermidin- und Sperminspiegel konstant blieben, stiegen die Putreszin- und N^8-Acetylspermidinkonzentrationen unter Resveratrolbehandlung dosisab-hängig.

Der immunologische Nachweis der Aktivatorprotein (AP)-1-Bestandteile *c-Fos* und *c-Jun* ergab eine konzentrationsabhängige Zunahme beider Proteine durch Resveratrol und eine Zunahme von c-Jun durch Piceatannol. EGCG veränderte weder die c-Jun- noch die c-Fos-Expression. Um zu überprüfen, ob die Proteinzunahme mit einer gesteigerten *DNA-Bindungsaktivität* der Trans-kriptionsfaktoren einherging, wurde mit einem auf dem ELISA-Test basierenden Verfahren die Bindungsaktivität an eine AP-1-spezifische Konsensusnukleotid-sequenz bestimmt. Während c-Jun durch Behandlung mit den pflanzlichen Polyphenolen nicht vermehrt an die Oligonukleotide band, kam es durch Resveratrol- und Piceatannolbehandlung zu einer gesteigerten c-Fos-Aktivität. Im Gegensatz dazu hemmte EGCG die Bindungsaktivität von c-Fos.

Diese Daten belegen, dass Resveratrol seine chemopräventiven Wirkungen auch über Modulation der Polyaminhomöostase ausübt. Zum einen hemmt es die Polyaminsynthese, zum anderen stimuliert es den Polyaminkatabolismus. Interessant ist hierbei, dass Piceatannol nicht exakt die gleichen Wirk-mechanismen wie Resveratrol aufweist. Die Wirkungen des als potent geltenden Polyphenols EGCG waren hinsichtlich des Polyaminstoffwechsels weitaus schwächer ausgeprägt als die der Hydroxystilbene.

3 Diskussion

3.1 Zellzyklushemmung durch Resveratrol in der S-Phase

In Analogie zu den Daten anderer Untersucher [RAGIONE *et al.* 1998, HSIEH & WU 1999, HSIEH *et al.* 1999A, HSIEH *et al.* 1999B, BERNHARD *et al.* 2000, PARK *et al.* 2001A, KUWAJERWALA *et al.* 2002, JOE *et al.* 2002] konnte in der vorliegenden Arbeit demonstriert werden, dass Resveratrol das Wachstum transformierter Zellen in der S-Phase des Replikationszyklus hemmt [MANUSKRIPT I]. Da Resveratrol in HL-60-Zellen als selektiver COX-1-Inhibitor agiert [JANG *et al.* 1997], sollte untersucht werden, ob die wachstumshemmenden Effekte des Polyphenols COX-abhängig sind. In MANUSKRIPT I wurde der Einfluss Resveratrols auf Wachstum, Proliferation, Zellzyklus und Apoptose untersucht. Die Experimente wurden an den Kolonkarzinomzelllinien Caco-2 und HCT-116 durchgeführt. Caco-2-Zellen exprimieren eine aktive COX-2, verfügen jedoch nicht über COX-1 [KAMITANI *et al.* 1998]. HCT-116-Zellen zeigen dagegen so gut wie keinerlei COX-Aktivität [SHENG *et al.* 1997]. Hierdurch konnte demonstriert werden, dass die Hemmung des Wachstums, der Proliferation und des Zellzyklus, sowie die Induktion der Apoptose durch Resveratrol unabhängig von seiner COX-1-hemmenden Aktivität sind [MANUSKRIPT I].

Während in promyelozytischen HL-60-Zellen keine Beeinflussung der G_1-Phase-Maschinerie beobachtet werden konnte [RAGIONE *et al.* 1998], kam es in Caco-2- und HCT-116-Zellen zu einer Verminderung der Cyclin D1- und Cdk4-Spiegel [MANUSKRIPT I]. Die Abnahme des Cyclins D1, nicht aber von Cdk4 wurde auch in Kupffer'schen Sternzellen beobachtet [KAWADA *et al.* 1998]. In der epidermoiden Karzinomzelllinie A431 bewirkte Resveratrol eine Abnahme der Cycline D1 und D3 sowie der Proteine Cdk2, Cdk4 und Cdk6. Koinzident kam es zu einer Reduktion von Cyclin E [AHMAD *et al.* 2001], während in anderen Arbeiten eine Zunahme dieses Cyclins verzeichnet wurde [MANUSKRIPT I, RAGIONE *et al.* 1998, KAWADA *et al.* 1998]. Auch Cyclin A nahm sowohl in Kolonkarzinomzellen, als auch in HL-60-Zellen zu [MANUSKRIPT I, RAGIONE *et al.* 1998]. Die Anwesenheit von Cyclin D1 ist von mitogenen Stimuli abhängig und bei Ausbleiben von Wachstumssignalen tritt eine rasche Proteolyse des Cyclins ein [SHERR 1996]. Eine Cyclin D1-Hemmung unterdrückt

das Wachstum transformierter Zellen und wirkt antitumorigen [ARBER *et al.* 1997].

Während bei HL-60-Zellen keine Veränderung der CKIs $p21^{Waf1/Cip1}$ und $p27^{Kip1}$ durch Resveratrolbehandlung zu erkennen war [RAGIONE *et al.* 1998], kam es in A431-Zellen und bovinen arteriellen Endothelzellen zu einer Zunahme von $p21^{Waf1/Cip1}$ [HSIEH *et al.* 1999B, AHMAD *et al.* 2001]. Die $p21^{Waf1/Cip1}$-Expression resveratrolbehandelter Caco-2-Zellen änderte sich nicht, es kam jedoch zur konzentrationsabhängigen Abnahme des $p27^{Kip1}$-Proteins [MANUSKRIPT I]. Auch in U937-Zellen bewirkte die Resveratrolinkubation eine $p27^{Kip1}$-Reduktion [PARK *et al.* 2001A]. DOKI *et al.* [1997] zeigten, dass die Expression von Cyclin D1 und $p27^{Kip1}$ in einem engen Zusammenhang stehen. Besonders in *cyclin D1*-überexprimierenden Zellen sind die $p27^{Kip1}$-Spiegel von der Anwesenheit des Cyclin D1-Proteins abhängig [HAN *et al.* 1996]. Diese Beobachtungen könnten eine Erklärung für die reduzierten $p27^{Kip1}$-Spiegel resveratrolbehandelter Caco-2-Zellen sein.

In nm23-H1-überexprimierenden MDA-MB-435-Mammakarzinomzellen waren durch Resveratrolbehandlung (72 Stunden mit 25 µM) reduzierte PCNA-Spiegel nachzuweisen [HSIEH *et al.* 1999A], während in Caco-2-Zellen keine Änderung der PCNA-Expression verzeichnet werden konnte [MANUSKRIPT I]. PCNA ist eine Untereinheit der DNA Polymerase δ und assoziiert mit $p21^{Waf1/Cip1}$ [CHEN *et al.* 1995]. Vermutlich wird PCNA in diesem Zellkulturmodell nicht durch das Stilben reguliert, da auch $p21^{Waf1/Cip1}$ nicht durch Resveratrol beeinflusst wurde. Dies ist wahrscheinlich auf das Fehlen von Wildtyp-p53 in Caco-2-Zellen zurückzuführen [DJELLOUL *et al.* 1997].

In HL-60-Zellen beeinflusste Resveratrol die pRb-Expression nicht [RAGIONE *et al.* 1998], wohingegen in Prostatakarzinomzellen und in Caco-2-Zellen eine pRb-Abnahme verzeichnet wurde [MANUSKRIPT I, HSIEH & WU 1999]. In A431-Zellen kam es zur Reduktion hyperphosphorylierten pRbs parallel zur Zunahme hypophosphorylierten pRbs. Zudem war die Expression aller fünf Proteine der E2F-Familie sowie DP1 und DP2 vermindert [ADHAMI *et al.* 2001]. Auch in Caco-2-Zellen war eine Hypophosphorylierung pRbs zu beobachten. Diese erfolgte jedoch erst bei einer Konzentration von 200 µM [MANUSKRIPT I]. Das Cyclin D1/Cdk4-Dimer sorgt in proliferierenden Zellen für eine Aufrechterhaltung der pRb-Hyperphosphorylierung, wodurch das Protein inaktiviert wird

[SHERR 1996]. Die nach Resveratrolinkubation verzeichnete Reduktion des Cyclin D1/Cdk4-Komplexes fördert somit die Hypophosphorylierung des Tumorsuppressors. Die Arbeit von PARK *et al.* demonstrierte an U937-Lymphomzellen, ähnlich wie dies an Caco-2-Zellen beobachtet wurde [MANUSKRIPT I], dass die Zellzyklushemmung in der S-Phase ebenso wie die vermehrte Cyclin A-Expression am deutlichsten bei 60 µM Resveratrol auftritt und bei höheren Konzentrationen weniger ausgeprägt ist [PARK *et al.* 2001A]. Cyclin E vermittelt den Eintritt der Zellen in die S-Phase, während Cyclin A am Ende der S-Phase akkumuliert [SHERR 1993]. Diese Daten, in Zusammenhang mit der beobachteten Zunahme der Zellpopulation in der S-Phase, deuten auf eine Hemmung des Zellzyklus vor dem Eintritt in die G_2-Phase hin. Diese ist jedoch nur bei Konzentrationen von 25 - 50 µM zu beobachten [SCHNEIDER *et al.* 2000, MANUSKRIPT I]. Bei höheren Konzentrationen nimmt das Cyclin A wieder ab und pRb liegt hypophosphoryliert vor. Dies führt zu einem Austritt der Zellen aus der S-Phase.

Wird die Gesamtheit der Daten, die bislang zur Wirkung Resveratrols auf den Zellzyklus erhoben wurden, in Betracht gezogen, lässt sich daraus schließen, dass sich eine Resveratrolbehandlung auf die Zellzyklusmaschinerie verschiedener Zellkultursysteme unterschiedlich auswirkt. Ursächlich hierfür könnte sein, dass einige der Untersuchungen mit nur einer Resveratrol-konzentration durchgeführt wurden und somit nicht das gesamte Spektrum seiner Wirkung demonstrieren konnten. Zudem reagieren nicht alle Zelllinien gleich sensitiv auf das Polyphenol. So war der wachstumshemmende Effekt Resveratrols auf die Zelllinie HCT-116 ausgeprägter als der auf Caco-2-Zellen [MANUSKRIPT I]. Dies könnte unter anderem daran liegen, dass HCT-116-Zellen Wildtyp-p53 exprimieren, während der Tumorsuppressor in Caco-2-Zellen mutiert vorliegt [DJELLOUL *et al.* 1997, KAMITANI *et al.* 1998, SHENG *et al.* 1997]. Tatsächlich führt die Behandlung von HCT-116-Zellen mit 50 µM Resveratrol zur Induktion von p53 [BAEK *et al.* 2002]. Zu berücksichtigen sind außerdem weitere mögliche Mutationen, die das Zellzyklusverhalten zusätzlich beeinflussen können. Insgesamt scheint die Wirkung Resveratrols auf den Zellzyklus durch den Zelltyp beeinflusst zu werden. Da der Effekt Resveratrols auf Caco-2- und HCT-116-Zellen qualitativ vergleichbar war, kann spekuliert werden, dass die Wirkung auf den Zellzyklus von Kolonkarzinomzellen

möglicherweise weitgehend gleich ist. Auch in der kolorektalen Karzinomzelllinie SW 480 führte eine Behandlung mit 300 µM Resveratrol zu einer Abnahme der Cycline D1 und A, wobei bei letzterem Protein die Expression initial zunahm und erst später niedriger als die unbehandelter Zellen war [JOE *et al.* 2002]. Diese Daten decken sich weitgehend mit den Ergebnissen aus MANUSKRIPT I.

3.2 Apoptoseinduktion durch Resveratrol

Bei einer Konzentration von 200 µM Resveratrol konnte in Caco-2-Zellen mittels Aktivitätsbestimmung der Caspase 3 eine Apoptoseinduktion nachgewiesen werden [MANUSKRIPT I]. SCHNEIDER und Mitarbeiter [2000] konnten im Gegensatz dazu im gleichen Zellkulturmodell keine DNA-Fragmentation als Marker für die Apoptose feststellen. Dies kann mit der geringen Konzentration (25 µM), die bei diesem Experiment eingesetzt wurde erklärt werden. Zu kritisieren ist jedoch bei dieser Untersuchung, dass als Positivkontrolle keine apoptotischen Caco-2-Zellen (z.B. durch den Topoisomeraseinhibitor Camptothecin induziert) verwendet wurden. Der Nachweis eines „DNA-Laddering“ in Caco-2-Zellen ist nur in nichtadhärenten Zellen möglich und daher in diesem Zellkulturmodell nicht geeignet, um Apoptose zu detektieren [KAMITANI *et al.* 1998]. Im Gegensatz zu den Daten aus MANUSKRIPT IV ist vermutlich auch das Fehlen einer SAMDC-Hemmung und einer Veränderung der intrazellulären Polyaminkonzentration nach 24 Stunden in dieser Arbeit durch eine für diese Effekte zu niedrige Resveratrol-konzentration zu erklären [SCHNEIDER *et al.* 2000]. Eine DNA-Fragmentation konnte an monozytischen Leukämiezellen (THP-1) nach dreitägiger Behandlung mit 30 µM Resveratrol demonstriert werden [TSAN *et al.* 2000]. In A431-Zellen trat bereits bei geringeren Konzentrationen als 25 µM eine apoptoseassoziierte PARP-Spaltung auf [AHMAD *et al.* 2001]. Huang *et al.* [1999] zeigten, dass die resveratrolinduzierte Apoptose in Epidermiszellen der Maus von der Anwesenheit von Wildtyp-p53 abhängig ist. In Caco-2-Zellen liegt p53 mutiert und inaktiv vor (s.o.). Dass trotzdem eine Caspase 3-vermittelte Apoptose detektiert werden konnte [MANUSKRIPT I], spricht dafür, dass Resveratrol in Caco-2-Zellen unabhängig von p53 Apoptose induziert. Diese Vermutung wird dadurch unterstützt, dass Resveratrol in erythroleukämischen Zellen ohne p53 ebenso Apoptose induziert [RODRIGUE *et al.* 2001] wie in einer epidermoiden

Karzinomzelllinie mit nur einem funktionalen p53-Allel [AHMAD *et al.* 2001]. In HCT-116-Zellen induziert Resveratrol zwar p53, der Verlauf der Apoptose ist jedoch nicht von der Anwesenheit des Tumorsuppressors abhängig [MAHYAR-ROEMER *et al.* 2001]. In unterschiedlichen akuten lympho-blastischen Leukämiezellen konnte Apoptose durch einen Breitspektrum-Caspaseinhibitor unterdrückt werden, nicht jedoch der apoptoseassoziierte Verlust des $\Delta\Psi_m$ [DÖRRIE *et al.* 2001]. Dies spricht dafür, dass die resveratrolvermittelte Apoptose zwar in Abhängigkeit von der Caspaseaktivierung stattfindet, aber parallel eine mitochondrienassoziierte Apoptose abläuft. Diese Vermutung wird dadurch unterstützt, dass der Verlust des $\Delta\Psi_m$ in akuten lymphoblastischen Leukämiezellen der Caspaseaktivierung vorgeschaltet ist und dass die Unterdrückung des $\Delta\Psi_m$-Verlustes durch *N*-Acetylcystein die Apoptose verhindert [TINHOFER *et al.* 2001].

3.3 Der Einfluss Resveratrols auf die butyratinduzierte, nicht aber auf die spontane Differenzierung von Caco-2-Zellen

In MANUSKRIPT I wurde demonstriert, dass Resveratrol alleine keine Erhöhung der AP-Aktivität, eines Markers für Differenzierung, verursacht. Dafür verstärkte Resveratrol den differenzierungsfördernden Effekt der kurzkettigen Fettsäure Butyrat [MANUSKRIPT III]. In verschiedenen Zelllinien konnte ein positiver Effekt Resveratrols alleine auf die Differenzierung gezeigt werden [RODRIGUE *et al.* 2001, MIZUTANI *et al.* 1998, RAGIONE *et al.* 1998]. Dass in Caco-2-Zellen eine Koinkubation mit Butyrat für eine differenzierungsfördernde Wirkung Resveratrols notwendig ist, spricht dafür, dass bestimmte Gene „an- oder abgeschaltet“ sein müssen, damit das Stilben derart wirken kann. Butyrat bewirkt eine Hemmung der Histondeacetylase, wodurch es zur Hyper-acetylierung der Histone kommt [ARCHER *et al.* 1998]. Eine modifizierte Onkogenexpression ist die Folge. Diese könnte prinzipiell dazu führen, dass Resveratrol nun prodifferenzierend wirkt. Die Redifferenzierung kann ein Ziel der Sekundärprävention sein. Hierbei sollen hyperproliferierende Zellen dazu gebracht werden, dauerhaft in die G_0-Phase des Zellzyklus einzutreten und letztendlich für Kolonozyten typische Funktionen wiederaufzunehmen. Dieser Ansatz muss kritisch hinterfragt werden, da es Hinweise auf eine verminderte Chemotherapeutikasensitivität differenzierter Zellen gibt. Beispielsweise wurde

in vitro bei konfluenten kolorektalen Karzinomzellen eine verminderte Cisplatinsensitivität demonstriert [DIMANCHE-BOITREL *et al.* 1992]. Bei dieser erhöhten Resistenz scheint $p27^{Kip1}$ eine wichtige Rolle zu spielen [DIMANCHE-BOITREL *et al.* 1998]. Wie in MANUSKRIPT I und II demonstriert wurde, hemmen sowohl Resveratrol als auch Piceatannol die $p27^{Kip1}$-Expression und stoppen das Wachstum kolorektaler Karzinomzellen in der S-Phase des Zellzyklus. Die Abnahme des CKI wird möglicherweise durch Cyclin E vermittelt, das in Kooperation mit Cdk2 $p27^{Kip1}$ phosphoryliert, wodurch $p27^{Kip1}$ für den proteasomalen Abbau markiert wird [SHEAFF *et al.* 1997]. Die Verminderung von $p27^{Kip1}$ fand selbst dann statt, wenn die Zellen gleichzeitig mit Resveratrol und Butyrat inkubiert wurden [MANUSKRIPT III]. Dies schließt eine vermehrte Resistenzentwicklung durch $p27^{Kip1}$ weitgehend aus. Zudem unterstützt diese Beobachtung die Hypothese, dass $p27^{Kip1}$ nicht an der butyratinduzierten Differenzierung beteiligt ist [WÄCHTERSHÄUSER & STEIN 2001]. Die Hemmung des Zellzyklus in der S-Phase könnte außerdem dazu führen, dass die Zellen gegenüber Chemotherapeutika sensitiver sind, als Zellen, die in der G_0/G_1-Phase gehemmt werden.

3.4 Modulation der Polyaminhomöostase durch Resveratrol

Während der Entartung von Zellen kommt es zur Erhöhung der intrazellulären Polyaminkonzentration [BAUSKE *et al.* 2000, MILOVIC *et al.* 2000, PEGG 1988]. Die Hemmung der Polyaminsynthese und –aufnahme führt zur Wachstumshemmung. Mit Difluoromethylornithin wird dieser Wirkmechanismus in der onkologischen Therapie in die Praxis umgesetzt. Wie in MANUSKRIPT IV demonstriert wurde, hemmt Resveratrol sowohl die ODC als auch die SAMDC. Diese Enzyme stellen die geschwindigkeitsbestimmenden Schritte der Polyaminsynthese dar. Die reduzierte ODC-Aktivität erfolgt durch Abnahme sowohl des Proteins als auch der mRNA. Der Transkriptionsfaktor c-Myc bindet an den *odc*-Promotor, wodurch die ODC-Synthese eingeleitet wird. Die beobachtete Reduktion von c-Myc spricht dafür, dass die hemmende Wirkung Resveratrols auf die ODC über eine Abnahme von c-Myc vermittelt wird.

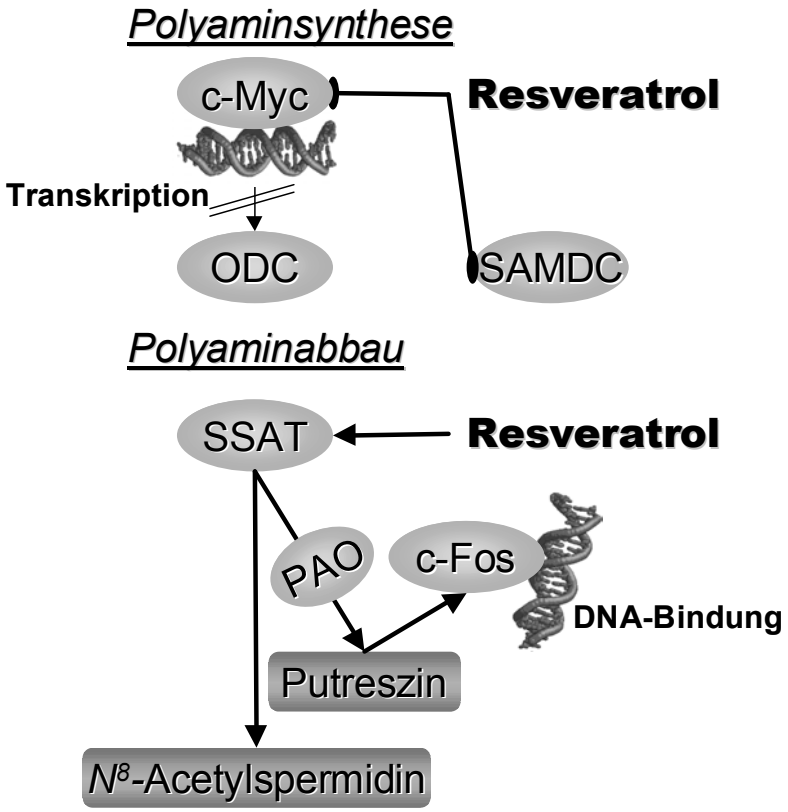


Abb. 10: Möglicher Mechanismus der Wirkung Resveratrols auf die Polyaminhomöostase und c-Fos. (ODC – Ornithindecarboxylase; PAO – Polyaminoxidase; SAMDC – S-Adenosylmethionin-decarboxylase; SSAT - Spermidin/Spermin-N^1-Acetyltransferase)

Parallel dazu induziert das Stilben die SSAT, durch die der Polyaminabbau eingeleitet wird. Gemeinsam mit der PAO bildet die SSAT acetylierte Polyamine und Putreszin. Durch die Bestimmung der intrazellulären Polyaminkonzentration mittels HPLC konnte gezeigt werden, dass es durch Resveratrolbehandlung zu einer Zunahme der N^8-Acetylspermidin- und Putreszinkonzentration kam [MANUSKRIPT IV]. TABIB und BACHRACH [1999] zeigten, dass Putreszin c-Fos induziert. So konnte auch nach Behandlung mit Resveratrol eine Zunahme der AP-1 Proteine c-Jun und c-Fos detektiert werden. Im Gegensatz zu c-Jun nahm die DNA-Bindungsaktivität von c-Fos ebenfalls zu [MANUSKRIPT IV]. Dies könnte indirekt durch die Zunahme der SSAT-Aktivität vermittelt werden (Abb. 10).

Um die Spezifität der Wirkungen Resveratrols zu untersuchen, wurden diese Versuche auch mit dem Grüntee-Polyphenol EGCG durchgeführt. Dieses wirkte ebenfalls antiproliferativ und entfaltete seine hemmende Wirkung auch auf die ODC-Aktivität. Im Gegensatz zu Resveratrol konnte jedoch weder eine Hemmung der SAMDC-Aktivität noch eine Induktion des Transkriptionsfaktors

c-Fos verzeichnet werden. Der Wirkmechanismus von EGCG scheint demnach ein anderer zu sein als der Resveratrols. Insgesamt waren die hemmenden Effekte des Stilbens wesentlich ausgeprägter als die des Catechins.

3.5 Die Wirkungen Piceatannols im Vergleich zu den Wirkungen Resveratrols

Nach der Behandlung von Caco-2-Zellen mit Piceatannol kam es ebenso wie nach der Inkubation mit Resveratrol zu einer Akkumulation der Caco-2-Zellen in der S-Phase. Gleichzeitig wurden Cdk4, Cyclin D1 und $p27^{Kip1}$ reduziert. Dagegen stiegen die Spiegel der Cycline E und A. Auch die Cyclin B1-Expression nahm ab [MANUSKRIPT II]. In SW480-Zellen führte die Resveratrol-behandlung ebenfalls zur Reduktion von Cyclin B1 [JOE *et al.* 2002]. Ein weiteres Ergebnis der vorliegenden Arbeit ist demnach, dass Piceatannol und Resveratrol ähnliche Wirkungen auf die Zellzyklusregulation kolorektaler Karzinomzellen ausüben, nicht aber die Stilbenanaloga Rhapontin und Stilbenmethanol, die keine Veränderung der Zellzyklusdistribution bewirkten [MANUSKRIPT I]. STIVALA und Mitarbeiter [2001] untersuchten die Abhängigkeit der antioxidativen und zellzyklushemmenden Effekte Resveratrols von seiner Struktur. Ein Ergebnis dieser Arbeit war, dass die Hydroxylgruppe in 4‘-Position im Zusammenhang mit der *trans*-Konformation essentiell für die Wirksamkeit Resveratrols ist. Auch Piceatannol verfügt über eine Hydroxylgruppe an der Position 4, was eine Erklärung für die ähnlichen Effekte Piceatannols und Resveratrols liefern könnte. Der proliferationshemmende Effekt Piceatannols ist nicht so stark ausgeprägt wie der Resveratrols [MANUSKRIPTE I & II]. Möglicherweise hat das Carrierprotein, das den Transport der Stilbene in die Zelle katalysiert, eine geringere Affinität zu Piceatannol. Durch die vierte Hydroxylgruppe ist Piceatannol weniger lipophil als Resveratrol. Dies könnte zudem bei der Diffusion durch Phospholipidmembranen die Durchlässigkeit für Piceatannol herabsetzen.

Ebenso wie bei Resveratrol (s.o.) konnte demonstriert werden, dass die Hemmung von Wachstum, Proliferation und Zellzyklus der kolorektalen Karzinomzelllinien Caco-2 und HCT-116 durch Piceatannol nicht abhängig vom COX- oder p53-Status der Zellen sind [MANUSKRIPT II].

Auch Piceatannol hemmte die ODC-Aktivität und führte zu einer deutlichen Abnahme von ODC und c-Myc. Die *odc*-mRNA wurde weniger stark reduziert. Auf die SAMDC hatte Piceatannol einen geringeren Einfluss als Resveratrol, während es die SSAT-Aktivität nicht signifikant induzierte. Auch Piceatannol verstärkte die DNA-Bindungsaktivität von c-Fos, nicht aber von c-Jun.
Das Cytochrom P450 1B1, das in KRK häufig überexprimiert wird, kann Resveratrol zu Piceatannol hydroxylieren, was die Frage aufwirft, ob Resveratrol nicht zumindest zum Teil über Piceatannol wirkt [POTTER *et al.* 2002]. Auch dies könnte als Erklärung für ähnliche Wirkungen dienen. Andererseits wurde gezeigt, dass Resveratrol das Cytochrom P450 1B1 hemmt und in MCF-7 Mammakarzinomzellen zu einer Abnahme der *p450 1B1*-mRNA führt [CHANG *et al.* 2000]. Dadurch wird die Übertragbarkeit der Ergebnisse von Potter *et al.* auf Zellkultur- oder *in vivo*-Verhältnisse in Frage gestellt. *In vivo* wird Resveratrol vor allem glucuronidiert [MARIER *et al.* 2002, KUHNLE *et al.* 2000]. Hinweise auf eine intrazelluläre Hydroxylierung zu Piceatannol gibt es bisher nicht. Über die Pharmakokinetik von Piceatannol ist bisher ebenfalls nichts bekannt. Zudem sollte in zukünftigen Untersuchungen determiniert werden, wieviel Resveratrol intrazellulär in Piceatannol oder *vice versa* umgewandelt wird.

3.6 Die Bioverfügbarkeit Resveratrols

Eine wichtige Voraussetzung für die Wirkung chemopräventiver Substanzen ist ihre Verfügbarkeit. Es ist daher von Interesse, ob Kolonozyten ausreichenden Resveratrolkonzentrationen ausgesetzt werden. Da Resveratrol nur zu ca. 20% im Dünndarm absorbiert wird [BERTELLI *et al.* 1998B], gelangen die restlichen 80% ins Kolon. Zudem ist nicht bekannt, inwieweit glykosylierte Stilbene (z.B. Piceid) oder Stilbenpolymere (z.B. ε-Viniferin) aus dem Rotwein durch bakterielle Hydrolyse der Darmflora im Dickdarm bioverfügbar werden. Die Verweildauer des Nahrungsbreies im Kolon ist außerdem länger als im Dünndarm, wodurch unter Umständen mehr Resveratrol aufgenommen werden kann. Letztlich fällt bei Substanzen, die Kolonozyten aus dem Darmlumen aufnehmen, der first-pass-Effekt der Leber weg. Es ist daher möglich, dass Kolonozyten aus dem Lumen höheren Resveratrolkonzentrationen als andere Gewebe im Körper ausgesetzt werden.

3.7 Mögliche Risiken durch Resveratrol

Da einige pflanzliche Polyphenole trotz Erhöhung der antioxidativen Kapazität des Serums DNA binden und mutagen wirken können (z.B. Quercetin) [FERGUSON 2001], ist die Bewertung möglicher Risiken einer resveratrolreichen oder –angereicherten Ernährung von Bedeutung. Direkte mutagene Wirkungen von Resveratrol wurden ausgeschlossen, da es im Ames-Test mutationshemmend wirkt und Phase II-Enzyme, die zur Entgiftung von Karzinogenen dienen, induziert [JANG *et al.* 1997]. Auf der anderen Seite hemmt Resveratrol die Induktion von Enzymen, die Prokarzinogene zu Karzinogenen umwandeln. Dies umfasst eine Hemmung der Cytochrom P450 1A1-Aktivität und –mRNA-Expression [CIOLINO & YEH 1999, CHUN *et al.* 1999], die Inaktivierung des Cytochroms P450 3A4 [CHAN & DELUCCHI 2000] und die Hemmung der Cytochrom P450 1B1-Aktivität und –Expression [CHANG *et al.* 2000]. Von einer mutagenen Wirkung Resveratrols kann demnach nicht ausgegangen werden. Da jedoch in Caco-2-Zellen bei einer Dosierung von 1 mM zytotoxische Effekte beobachtet wurden (unpublizierte Daten), sollte sorgfältig abgeschätzt werden, in welcher maximalen Dosierung das Stilben verabreicht werden darf.

Ein weiteres Problem bei der Evaluierung chemopräventiver Substanzen ist, dass *in vivo*, wenn bereits Polypen vorhanden sind, möglicherweise ein tumorfördernder Effekt überwiegt. Dies wurde in einem Darmkarzinogenese-Mausmodell für die Gabe von Folat demonstriert [SONG *et al.* 2000]. Ein weiteres Risiko besteht darin, dass ein Agens, das auf ein Organ chemopräventiv wirkt, prinzipiell in einem anderen Organ karzinogen wirken kann. So schützt z.B. Genistein im Tiermodell zwar vor Mammakarzinomen, erhöht jedoch das Kolonkarzinomrisiko [RAO *et al.* 1997].

3.8 Moderater Rotweinkonsum als chemopräventive Maßnahme?

Moderater Weinkonsum beeinflusst Duodenalulzera, Gallensteine, infektiöse Enteritiden, rheumatoide Arthritis, Osteoporose und Diabetes mellitus Typ 2 positiv, jedoch ist die Festlegung der optimalen Dosis für diese Wirkungen im Hinblick auf eine mögliche Leberschädigung umstritten. Leberzirrhose, Pankreatitis, Schädigung des zentralen und peripheren Nervensystems, sowie des Gehirns, der Muskulatur und des Myokards, aber auch Tumorerkrankungen

können Folgen eines zu hohen Alkoholkonsums sein [GOLDBERG *et al.* 1999]. Die Empfehlungen zum täglichen Alkoholgenuss variieren in verschiedenen Ländern zwischen 13,5 - 70 g Ethanoläquivalenten für Männer und 10 - 50 g Ethanoläquivalenten für Frauen [EBD.]. Das entspricht ca. 150 - 700 mL bzw. 150 - 500 mL Rotwein pro Tag. Im Allgemeinen werden in Deutschland Empfehlungen ausgesprochen, die für Frauen ein Glas und für Männer zwei Gläser Rotwein am Tag gestatten. Die deutsche Gesellschaft für Ernährung e.V. (DGE) publizierte im Jahr 2000 erstmals einen Richtwert für Alkohol. Danach sollten Männer nicht mehr als 20 g Alkohol pro Tag und Frauen nicht mehr als 10 g zu sich nehmen. Diese Menge gelte als verträglich für die Gesundheit und berücksichtige die präventive Wirkung von Alkohol im Hinblick auf das Herzinfarktrisiko [DGE 2000]. In den USA gelten bis zu ein alkoholisches Getränk für Frauen und zwei alkoholische Getränke für Männer täglich als moderater Alkoholkonsum. Hierbei ist ein alkoholisches Getränk mit einem Alkoholgehalt von 12 g definiert. Dies entspricht ca. 150 mL Wein [DUFOUR 1999]. Ausgenommen von Empfehlungen zum moderaten Weinkonsum sind Kinder und Jugendliche, Menschen, die nicht in der Lage sind, ihren Alkoholkonsum auf ein moderates Maß zu beschränken, Schwangere und Frauen, die eine Schwangerschaft planen, Menschen, die Auto fahren oder Tätigkeiten ausführen, die Können oder Aufmerksamkeit erfordern und Personen, die Medikamente einnehmen, die mit Alkohol interagieren [EBD.]. Besonderes Augenmerk sollte hier den Schwangeren gewidmet werden, da bereits ein moderater Alkoholkonsum der Mutter zu intellektuellen und sozialen Defiziten beim Fötus führt [JACOBSON & JACOBSON 1999].

Nicht zuletzt gibt es deutliche Unterschiede bei der Resveratrolkonzentration verschiedener Rotweinsorten. Dies wird häufig bei Empfehlungen zum gesundheitsfördernden moderaten Rotweinkonsum außer Acht gelassen.

Aufgrund der zahlreichen Daten zur Wirkung Resveratrols müsste angenommen werden, dass Rotweinkonsumenten gesünder und länger leben. In Dänemark wurden in einer epidemiologischen Studie die Einflussfaktoren auf die Mortalität durch ischämische Herzerkrankungen an ca. 50.000 Probanden untersucht. Interessanterweise ergab diese Studie, dass Weinkonsumenten, im Gegensatz zu Bier- oder Spirituosentrinkern signifikant häufiger Obst, Fisch,

Gemüse, Salat und Olivenöl verzehren und seltener Fette als Brotaufstrich verwenden [TJØNNELAND *et al.* 1999]. Da diese diätetischen Präferenzen protektiv hinsichtlich Herz-Kreislauferkrankungen sowie Tumorerkrankungen wirken, lässt sich epidemiologisch nur schwer nachweisen, ob der Konsum von Rotwein an sich vor malignen Erkrankungen schützt. Dies weist wiederum darauf hin, das zwar durchaus eine negative Korrelation zwischen dem Verzehr eines bestimmten Lebensmittels und der Inzidenz von Tumorerkrankungen bestehen kann. Dies bedeutet aber noch nicht, dass ein kausaler Zusammenhang besteht. Somit kann die Epidemiologie zwar Hinweise auf die chemopräventive Wirkung bestimmter Substanzen liefern, sie kann jedoch nicht den Beweis für eine derartige Wirkung erbringen.

3.9 Vor- und Nachteile eines gezielten Einsatzes isolierter Polyphenole zur Chemoprävention

In der traditionellen orientalischen Medizin werden resveratrolhaltige Präparate schon seit langem zur Heilung unterschiedlicher Erkrankungen angewendet. Weinblätter wurden diesen Zubereitungen zur Beeinflussung des Lipidstoffwechsels und bei Durchblutungsstörungen zugesetzt. In hohen Konzentrationen ist Resveratrol auch in den Wurzeln von *Polygonum cuspidatum* nachzuweisen, die in der traditionellen japanischen und chinesischen Heilkunde verwendet werden [SOLEAS *et al.* 1997B]. *Darakchasava*, eine Kräutermischung aus der indischen Heilkunde, enthält als Hauptingredienz getrocknete Weinbeeren und wird bei Tumoren, Herzerkrankungen, Wurminfektionen und Tuberkulose verabreicht [PAUL *et al.* 1999]. Es existieren jedoch zurzeit keine Studien bezüglich der Wirksamkeit oder eventueller Nebenwirkungen dieser Präparationen.

Die isolierte Gabe pflanzlicher Polyphenole kann unter Umständen Nachteile mit sich bringen. Zunächst ist die Synthese meist kostspieliger als die Verwendung natürlicher Produkte, in denen hochwirksame Substanzen enthalten sind. Außerdem erweist sich die isolierte Gabe im Vergleich zur Verabreichung des Naturproduktes häufig als toxischer oder unter Umständen als weniger effektiv. Häufig kommt es auf die Interaktionen mit anderen Inhaltsstoffen an, die für die Wirksamkeit des Ursprungsproduktes notwendig sind (z.B. mehrfach ungesättigte Fettsäuren und Vitamin E). So kann vermutet

werden, dass Resveratrol, trotz der beschriebenen Wirkungen lediglich einen kleinen Teil der positiven Wirkungen des Rotweins vermittelt, da er nur einen geringen Prozentsatz der Gesamtpolyphenole darstellt [FRÉMONT *et al.* 2000]. Trotzdem könnte die isolierte Gabe pflanzlicher Polyphenole für spezielle Risikogruppen (z.B. familiäre adenomatöse Polyposis (FAP) oder hereditäres nichtpolypöses Kolonkarzinom) sinnvoll sein.

Im Rahmen einer kombinierten Chemoprävention ist vor allem der Aspekt eines breiteren Wirkungsspektrums interessant. Werden zwei unterschiedliche Signaltransduktionswege gehemmt, kann es zu einer Potenzierung der antikarzinogenen Wirkung kommen, es besteht jedoch auch die Gefahr der addierten Toxizität. TORRANCE *et al.* [2000] demonstrierten in *ApcMin*-Mäusen, einem Modell für FAP, einen Polypenrückgang bis zu 100% durch Kombination eines EGF-Rezeptorinhibitors (EGF: epidermaler Wachstumsfaktor) mit einem NSAR. Die Daten zur synergistischen Wirkung Resveratrols und Butyrats auf die Differenzierung kolorektaler Karzinomzellen [MANUSKRIPT III] unterstützen prinzipiell einen ganzheitlichen diätetischen Ansatz, nach dem eine gesunde ausgewogene Ernährung der isolierten Gabe einzelner Vitamine, Mineralien oder Phytochemikalien vorzuziehen wäre. Hinreichend bekannt sind die positiven Wirkungen der „kretischen Diät“, einer Kost, die reich an einfach ungesättigten Fettsäuren, Ω-3-Fettsäuren, Vitamin E, Vitamin C, Folsäure, Ballaststoffen und Polyphenolen wie Resveratrol und dagegen arm an gesättigten Fettsäuren und Fleisch ist [SIMOPOULOS 2001]. Diese Diät spiegelt die traditionelle Ernährungsweise der Bewohner Kretas wider und geht mit einer verminderten Mortalität für Tumor- und Herz-Kreislauferkrankungen einher. Ein großer Vorteil der Verwendung chemopräventiver Substanzen in ihrer natürlichen Matrix ist, dass Nebenwirkungen praktisch ausgeschlossen werden können [GESCHER *et al.* 2001].

Grundsätzlich erfüllt Resveratrol die Anforderungen, die an eine ideale chemopräventiv wirkende Substanz gestellt werden. Es ist kostengünstig, oral konsumierbar, nicht oder nur gering toxisch, äußerst wirksam, wird in seiner natürlichen Matrix vom Konsumenten akzeptiert und wirkt über einen weitgehend bekannten Mechanismus [s.o.].

3.10 Ausblick

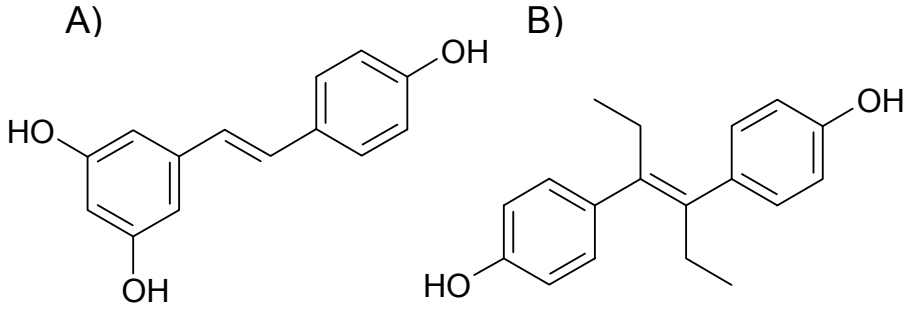


Abb. 11: Vergleich der Strukturformeln von A) Resveratrol und B) Diethylstilbestrol

Neben den bislang untersuchten Wirkungen Resveratrols auf Kolonkarzinomzellen könnten noch andere Wirkmechanismen des Stilbens einen chemopräventiven Effekt vermitteln. Epidemiologische Studien zeigten, dass bei Frauen, die postmenopausal Östrogene substituieren, eine 20%ige Reduktion des Kolonkarzinomrisikos und eine 19%ige Reduktion des Rektumkarzinomrisikos erfolgt. Diese Risikoverminderung wurde nicht durch die Dauer der Therapie, die Dosis oder die Art der Östrogengabe beeinflusst [PAGANINI-HILL 1999]. Aufgrund seiner Strukturähnlichkeit zum synthetischen Östrogen Diethylstilbestrol (4,4'-Dihydroxy-*trans*-α,β-diethylstilben) wird Resveratrol auch als Phytoöstrogen angesehen (Abb. 11).

In verschiedenen Mammakarzinomzelllinien konnte demonstriert werden, dass Resveratrol als Östrogenrezeptoragonist wirkt und Östradiol aus seiner Bindung verdrängt, wobei die aktivierende Wirkung auf östrogenabhängige Transkription in einigen Zelllinien stärker war, als die von Östradiol [GEHM *et al.* 1997]. Im Gegensatz dazu zeigten LU und SERRERO [1999], dass Resveratrol das Wachstum der östrogenrezeptorpositiven Zelllinie MCF-7 und den wachstumsfördernden Effekt von 17-β-Östradiol hemmt. Dies weist darauf hin, dass Resveratrol selbst zwar ein Östrogenrezeptoragonist ist, in Gegenwart von Östrogenen jedoch als Rezeptorantagonist fungieren kann [EBD.]. Resveratrol konkurriert mit Östrogen um die Bindung an den Östrogenrezeptor aus Rattenuteri [ASHBY *et al.* 1999]. BOWERS *et al.* [2000] bestätigten, dass Resveratrol eine östrogenabhängige Transkription induziert und demonstrierten, dass das Stilben die Östrogenrezeptoren α und β zwar mit gleicher Affinität bindet, aber mit 7.000fach geringerer Affinität als Östradiol. Zudem konnten sie

zeigen, dass Östrogenrezeptor β-gebundenes Resveratrol die Transkription stärker aktiviert als Östradiol. Als Östrogenrezeptoragonist könnte Resveratrol ebenfalls chemopräventiv hinsichtlich des kolorektalen Karzinoms wirken. Inwieweit die Wirkungen Resveratrols über Östrogen-rezeptoren vermittelt werden, ist bisher unbekannt. Vergleichende Versuche mit östrogenrezeptor-negativen Kolonkarzinomzellen oder Knockout-Mausmodellen könnten hier zur Aufklärung beitragen.

Abgesehen von seinen chemopräventiven Eigenschaften könnte Resveratrol auch eine Bedeutung in der Chemotherapie erlangen. Aufgrund der demonstrierten Zellzyklushemmung durch Resveratrol in der S-Phase wäre es möglich, dass es die Wirkung zytostatisch wirkender Substanzen verstärkt. Dies würde möglicherweise dazu führen, dass geringere Mengen von Chemo-therapeutika eingesetzt werden könnten, womit eventuell Nebenwirkungen verringert würden. Insofern wären Untersuchungen, bei denen Resveratrol in Kombination mit chemotherapeutisch wirksamen Medikamenten getestet wird, vor allen solchen, die in der S-Phase des Zellzyklus wirken, von großem Interesse. In den Zervixkarzinomzelllinien HeLa und SiHa wurde durch Präinkubation mit Resveratrol eine erhöhte Sensitivität gegenüber ionisierender Strahlung festgestellt, was diese Hypothese prinzipiell unterstützt [ZOBERI *et al.* 2002].

Bislang wurden zwar vielfältige Wirkungen Resveratrols demonstriert, der Stand der Untersuchungen lässt jedoch zum derzeitigen Zeitpunkt keine Empfehlungen bezüglich einer resveratrolreichen oder –angereicherten Ernährung zu. Die Anzahl der Arbeiten, die über positive Wirkungen Resveratrols berichtet, überwiegt. Jedoch sind weitere Tierversuche und Langzeituntersuchungen notwendig, um schwere Nebenwirkungen des Stilbens auszuschließen.

HAIN *et al.* [1993] transfizierten Tabakpflanzen mit dem Gen für die Stilbensynthase, die Resveratrol synthetisiert. Dieses Verfahren war hinsichtlich einer erhöhten Widerstandsfähigkeit gegenüber Pilzinfektionen erfolgreich. Prinzipiell müsste auf diese Weise auch die Resveratrolanreicherung anderer pflanzlicher Lebensmittel möglich sein, wenn dies beim Verbraucher Akzeptanz findet. Andererseits sind vermutlich noch nicht alle Nahrungsmittel, die Resveratrol enthalten, bekannt. Der Trend bei der Rotweinherstellung wird

möglicherweise dahin gehen, Kelterungs- und Lagerungsmethoden anzuwenden, die höhere Resveratrolkonzentrationen erzielen (Zugabe pektinolytischer Enzyme zur Maische, schonende Schönungs- und Klärverfahren).

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Frankfurt, den 24. September 2002

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Agrarwissenschaften, Ökotrophologie und Umweltmanagement der Justus-Liebig-Universität in Gießen eingereichte Inauguraldissertation mit dem Titel:

MOLEKULARE MECHANISMEN DER ANTIPROLIFERATIVEN WIRKUNG SEKUNDÄRER PFLANZENINHALTSSTOFFE AM BEISPIEL VON RESVERATROL

in der Medizinischen Klinik II, Schwerpunkte Gastroenterologie und Pneumologie / Allergologie (Direktor: Prof. Dr. W. F. Caspary) unter Leitung von Herrn Prof. Dr. Dr. J. Stein ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel verwendet habe.

Ich habe bisher weder an einer in- oder ausländischen Fakultät ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende Arbeit als Dissertation vorgelegt.

Teile der vorliegenden Arbeit wurden in folgenden Publikationsorganen veröffentlicht:
Journal of Nutrition 2001; 131: 2197-2203.
Journal of Nutrition 2002; 132: 298-302.
Journal of Nutrition 2002; 132: 2082-2086.
Carcinogenesis 2003; (reviewed version in press)
Drugs of the Future 2002; 27(10): 949-959.

Frankfurt, den 24. September 2002

Freya Wolter

ANHANG

ABGRENZUNGSERKLÄRUNG

Frau Dipl. oec. troph. Freya Wolter hat die dem Promotionsamt der Ernährungswissenschaften der Justus Liebig Universität Gießen vorgelegte Arbeit mit dem Titel „Molekulare Mechanismen der antiproliferativen Wirkung sekundärer Pflanzeninhaltsstoffe am Beispiel von Resveratrol“ als kumulative Dissertation verfasst. Der Arbeit liegen folgende Veröffentlichungen zugrunde:

Wolter F, Stein J. Resveratrol enhances the differentiation induced by butyrate in caco-2 colon cancer cells. J Nutr. 2002 Jul;132(7):2082-6.

Wolter F, Clausnitzer A, Akoglu B, Stein J. Piceatannol, a natural analog of resveratrol, inhibits progression through the S phase of the cell cycle in colorectal cancer cell lines. J Nutr. 2002 Feb;132(2):298-302.

Wolter F, Akoglu B, Clausnitzer A, Stein J. Downregulation of the cyclin D1/Cdk4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines. J Nutr. 2001 Aug;131(8):2197-203.

Wolter F, Turchanova L, Stein J. Resveratrol-induced spermidine/spermine N^1-acetyltransferase activity is accompanied by induction of c-fos. Carcinogenesis 2003 (Überarbeitete Version befindet sich im Druck)

Wolter F, Stein J. Biological activities of resveratrol and related analogs. Drugs of the future 2002 Oct;27(10): 949-959 (Eingeladener Übersichtsartikel).

Frau A. Clausnitzer hat im Rahmen ihrer Diplomarbeit (unter Betreuung von Frau F. Wolter) Versuche mit den Strukturanaloga Rhapontin und Stilbenmethanol durchgeführt (Manuskript I). Zusätzlich hat Frau Clausnitzer mit Piceatannol Versuche zur Zytotoxizität (LDH-Freisetzung) und zur Differenzierung (Aktivität der alkalischen Phosphatase) gemacht. Sie führte zudem Western blots mit Caco-2 Zellen durch (cdc2, cdk2, cdk6, cyclinA, cyclinD1), die allesamt von Frau F. Wolter wiederholt und verifiziert wurden (Manuskript II).

Herr Dr. B. Akoglu hat Frau F. Wolter bei der Zellzyklusanalyse die Bedienung des Durchflusszytometers erklärt und nahm die Auswertung der Zellzyklusanalysen mit der Software ModFit vor (Manuskript I und II).

Frau Dr. L. Turchanova hat die Polyaminkonzentrationsmessungen an der HPLC-Anlage durchgeführt, wobei die Probenaufbereitung von Frau F. Wolter vorgenommen wurde.

Prof. Dr. Dr. J. Stein hat die Manuskripte kritisch Korrektur gelesen und in Diskussionen hilfreiche Ideen für weitere Versuche geliefert.

Freya Wolter

I

Nutrition and Cancer

Downregulation of the Cyclin D1/Cdk4 Complex Occurs during Resveratrol-Induced Cell Cycle Arrest in Colon Cancer Cell Lines[1]

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ABSTRACT Resveratrol is a naturally occurring polyphenol with cancer chemopreventive properties. The objective of the current study was to investigate the effect of resveratrol on the human colonic adenocarcinoma cell line Caco-2. The compound inhibited cell growth and proliferation of Caco-2 cells in a dose-dependent manner (12.5–200 μmol/L) as assessed by crystal violet assay, [^{3}H]thymidine and [^{14}C]leucine incorporation. Furthermore, apoptosis was determined by measuring caspase-3 activity, which increased significantly after 24 and 48 h of treatment with 200 μmol/L resveratrol. Perturbed cell cycle progression from the S to G2 phase was observed for concentrations up to 50 μmol/L, whereas higher concentrations led to reversal of the S phase arrest. These effects were specific for resveratrol; they were not observed after incubation with the stilbene analogs stilbenemethanol and rhapontin. Levels of cyclin D1 and cyclin-dependent kinase (cdk) 4 proteins were decreased, as revealed by immunoblotting. In addition, resveratrol enhanced the expression of cyclin E and cyclin A. The protein levels of cdk2, cdk6 and proliferating cell nuclear antigen were unaffected. Similar results were obtained for the colon carcinoma cell line HCT-116, indicating that cell cycle inhibition by resveratrol is independent of cyclooxygenase inhibition. The phosphorylation state of the retinoblastoma protein in Caco-2 cells was shifted from hyperphosphorylated to hypophosphorylated at 200 μmol/L, which may account for reversal of the S phase block at concentrations exceeding 50 μmol/L. These findings suggest that resveratrol exerts chemopreventive effects on colonic cancer cells by inhibition of the cell cycle. J. Nutr. 131: 2197–2203, 2001.

KEY WORDS: • *resveratrol* • *Caco-2 cells* • *cell cycle* • *colon cancer*

The polyphenol resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is naturally produced in several plants in response to environmental stress and challenges by plant pathogens (1). Red wine [contains up to 13.4 mg resveratrol/L (2)] and peanuts [contain 0.02–1.79 μg resveratrol/g (3)] represent the main sources in the human diet. The compound has been shown to exhibit cancer chemopreventive properties in different cell culture and animal models. Resveratrol induces HL-60 promyelocytic leukemia cell differentiation, suppresses prostanoid synthesis by inhibition of cyclooxygenases and induces phase II carcinogen detoxifying enzymes. In addition, it has been reported to inhibit the growth of breast cancer cells in vitro and to reduce the number of preneoplastic lesions in a mouse mammary gland culture model (4). Moreover, resveratrol induces apoptosis by enhancing the expression of Fas-ligand and activating caspases in HL-60 cells (5), whereas it decreases the level of the antiapoptotic protein Bcl-2 (6). Recent studies have demonstrated the inhibitory effect of resveratrol on cell cycle progression (7–9). Most studies report perturbation of the S/G2 phase transition with a decrease of cells in G0/G1 phase of the cell cycle and an increase of cells in S phase (10–14). Despite a large amount of data on the antiproliferative and proapoptotic properties, the exact mechanism by which resveratrol exerts its effects on tumor cells is unknown. Even fewer data exist concerning inhibition of tumorigenesis and cell growth in the colon. Schneider et al. (15) demonstrated reduced ornithine decarboxylase activity and growth inhibition at the S/G2 phase transition of Caco-2 cells after treatment with 25 μmol/L resveratrol. In addition, it has been shown that resveratrol inhibits growth of colorectal aberrant crypt foci and upregulates bax in azoxymethane-induced carcinogenesis of the rat colon, whereas it reduces $p21^{WAF1/CIP1}$ protein level overall in the normal surrounding mucosa (16).

Colonic epithelial cells undergo a sequential process of proliferation, differentiation, apoptosis and exfoliation as they migrate along the crypt-villus axis, which is deregulated in carcinogenesis. This process is largely regulated by periodical activation and inactivation of a highly conserved family of cyclin-dependent kinases (cdk)[3] (17). Cdk activity is modulated by the cyclins, which bind to and activate the cdk (18). They are regulated primarily by their expression levels. A critical event in the development of malignant colorectal

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[3] Abbreviations used: AP, alkaline phosphatase; cdk, cyclin-dependent kinase; cki, cyclin-dependent kinase inhibitor; COX, cyclooxygenase; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; LDH, lactate dehydrogenase; PCNA, proliferating cell nuclear antigen; pRb, retinoblastoma protein.

Manuscript received 8 March 2001. Initial review completed 12 April 2001. Revision accepted 17 May 2001.

carcinomas from benign adenomas is mutation of the tumor suppressor p53 (19). Loss of p53 function leads to impaired control of cell cycle and apoptosis. Another common characteristic among colon carcinomas is overexpression of cyclooxygenase (COX)-2. The chemopreventive effects of nonsteroidal anti-inflammatory drugs have been associated with their ability to inhibit COX (20). Because resveratrol is a COX inhibitor, it might exert its effects on colon cancer cells via this pathway.

The primary objective of the present study was to elucidate the underlying molecular mechanisms of inhibition of cell cycle progression by resveratrol. Because of the importance of cell cycle regulators in carcinogenesis, we determined whether they can be affected by resveratrol. Therefore, we assessed the influence of resveratrol on positive and negative regulators of the cell cycle in Caco-2 cells and HCT-116, which present well-established cell culture models and differ in p53 and COX expression. Caco-2 cells express mutated p53 and possess active COX-2, whereas HCT-116 cells express wild-type p53, but have no detectable COX activity (21–23). Our interest was in determining whether effects of resveratrol on the cell cycle would differ in these two cell lines.

MATERIALS AND METHODS

Cell culture. The human colon cancer cell lines Caco-2 and HCT-116 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Caco-2 cells of passages 45–55 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), penicillin (1000 U/L) and streptomycin (1 mg/L). HCT-116 cells of passages 31–37 were cultured in McCoy's 5A supplemented with 10% FCS, penicillin (1000 U/L) and streptomycin (1 mg/L). Both cell lines were maintained at 37°C under an atmosphere of 5% CO_2 in air. The medium was changed three times per week. All cell culture reagents were obtained from Gibco (Eggenstein, Germany). A 1 mol/L stock solution of resveratrol (Sigma Chemical, Deisenhofen, Germany) was prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. Control cells not receiving resveratrol routinely had equal amounts of DMSO added to the culture media (≤ 0.1%). For treatment with resveratrol, cells were cultured until nearly confluent. The medium was then removed and replaced by a medium containing either solvent or 12.5–200 μmol/L resveratrol. Cytotoxicity was excluded by lactate dehydrogenase (LDH) release assay (Boehringer Mannheim, Mannheim, Germany). Stilbenemethanol and rhapontin were obtained from Sigma Chemical.

Cell number. Determination of cell numbers was carried out using a modification of the method of Matsubara et al. (24). Briefly, cells were plated at a densitiy of 7×10^3 cells per well in 96-well microtiter plates. Treatment with increasing concentrations of resveratrol was carried out for 24, 48 and 72 h (resveratrol-containing medium was changed after 48 h). At the end of the incubation period, the medium was removed and any adherent cells were fixed to the plate with 5% formaldehyde in PBS. The cells were then stained with an aqueous solution of crystal violet (5 g/L) followed by elution of the dye with 33% aqueous acetic acid. Absorbance at 570 nm was determined with a Tecan Spectrafluor Plus microplate reader (Tecan, Crailshaim, Germany) and the number of cells was determined from a standard curve of absorbance against cell numbers calculated from a mean of six experiments.

Western blot analysis. Cells were plated on 80 cm^2 flasks at a density of 2×10^6 cells per flask, allowed to attach overnight and then exposed to resveratrol vs. DMSO for 24 h. After the cells were washed three times with ice-cold PBS, they were incubated with cell lysis buffer (New England Biolabs, Beverly, MA) containing multiple protease inhibitors (Boehringer Mannheim, Germany) for 20 min at 4°C. Cells were sonicated on ice and centrifuged at 10,000 × *g* for 5 min to sediment the particulate material. Aliquots of the supernatant were assayed for total protein (BioRad Laboratories, Muenchen, Germany) according to the method described by Bradford (25). Protein (20 μg/lane) was separated by SDS-PAGE along with prestained molecular weight markers (BioRad Laboratories). The separated proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) with a semidry blotting device. Membranes were reversibly stained with Ponceau-S red to verify homogeneity of protein blotting. The blots were blocked overnight with Tris-buffered saline containing 0.05% Tween-20 and 30 g/L nonfat milk at 4°C. The level of proteins was assayed using the primary antibodies for 1 h with agitation at room temperature. Immunoreactivity was demonstrated by enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK) using appropriate horseradish peroxidase conjugated secondary antibodies (1: 2000). Reprobing of blots for expression of actin was done routinely. Antibodies against $p27^{KIP1}$, cdc2, cdk2, cdk4, cdk6, cyclin A and cyclin D1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-$p21^{WAF1/CIP1}$ (Ab-1) and anti-proliferating cell nuclear antigen (anti-PCNA) were purchased from Oncogene (Cambridge, MA) and anti-pRb and anti-cyclin E from Pharmingen (Becton-Dickinson, Heidelberg, Germany). For quantification, the density of the bands was detected with scanning densitometry, using a Desaga CabUVIS scanner and Desaga ProViDoc software (Wiesloch, Germany). Results were expressed as a percentage of the control.

Incorporation of [^{3}H]thymidine and [^{14}C]leucine. Caco-2 cells cultured in 24-well plates (5×10^4/well) were treated with resveratrol for 24 h. During treatment, the cells were pulsed with 1.8×10^7 Bq/well [^{3}H]thymidine and 9.2×10^8 Bq/well [^{14}C]leucine (Amersham Pharmacia Biotech, Freiburg, Germany). The medium was discarded, the monolayers were washed three times with PBS and the cellular macromolecules were precipitated using 5% trichloroacetic acid. The acid was aspirated, cells were washed with absolute methanol and formic acid (2.5 mol/L) was used to solubilize the precipitated macromolecules. Probes were transferred to scintillation vials; 3.0 mL scintillation fluid (Packard Biosciences, Groningen, Netherlands) was added and measurements were carried out with a liquid scintillation counter (Packard Instruments, Meridien, CT). Cellular protein concentrations were determined as described in the Western blot analysis section.

Determination of alkaline phosphatase (AP) activity. Alkaline phosphatase activity, a marker of differentiation was measured using *p*-nitrophenylphosphate as substrate according to the manufacturer's instructions (Merck, Darmstadt, Germany). Cell lysates of Caco-2 cells treated for 1, 4, 8 or 12 d with 12.5 μmol/L resveratrol were analyzed in the assay. Cellular protein concentrations were determined as described in the Western blot analysis section. AP activity was calculated in units per milligram protein (U/mg protein).

Cell cycle analysis. Cells were seeded 24 h before treatment in 6-well plates at a density of 15×10^4/well. Cells were washed 24 h after treatment with PBS and harvested by trypsinization (2.5% trypsin/EDTA solution, Gibco). DNA contents of cells were measured using a DNA staining kit (CycleTEST PLUS DNA Reagent Kit, Becton Dickinson). Propidium iodide–stained nuclear fractions were obtained by following the kit protocol. Data were acquired using CellQuest Software (Becton Dickinson) with a FACScalibur (Becton Dickinson) flow cytometry system using 10,000 cells per analysis. Cell cycle distributions were calculated using ModFit LT 2.0 software (Verity Software House, Topsham, ME).

Apoptosis assay. The EnzCheck caspase-3 assay kit #2 (Molecular Probes, Leiden, Netherlands) was used according to the manufacturers suggestions. Briefly, cells grown in 80 cm^2 cell culture flasks were incubated with 200 μmol/L resveratrol, vehicle or 50 μmol/L camptothecin for 8, 24 and 48 h. Floating cells were collected with the medium, and attached cells were collected by trypsinization. Both fractions were counted together with a hemocytometer. Cells (1×10^6/sample) were collected in PBS and centrifuged at 200 × *g* for 10 min. The supernatant was discarded and the cell pellets were frozen at −80°C until all samples were collected. The pellets were thawed on ice and resuspended in lysis buffer. After complete lysis of the cells, the particulate material was sedimented by centrifugation at 2000 × *g* for 5 min. The supernatant was incubated with the Z-

DEVD-R110 substrate for 30 min. Fluorescence was measured (excitation/emission, 496/520 nm) with the fluorescence microplate reader Tecan SpectraFluor PLUS.

Statistical analysis. Data are expressed as means ± SD Differences were tested for statistical significance using two-way ANOVA. Individual differences between groups were assessed using the least significant differences (LSD) test (Microsoft Excel, Microsoft, Roselle, IL). A P-value < 0.05 was considered to indicate a significant difference.

RESULTS

To investigate the ability of resveratrol to inhibit growth of colon cancer cells, various amounts of resveratrol were added to the cell culture medium. Resveratrol significantly reduced the growth rate of Caco-2 cells in a dose- and time-dependent manner (**Fig. 1**). When Caco-2 cells were treated with 100 μmol/L resveratrol, cell number did not increase after 72 h of incubation, whereas 200 μmol/L treatment reduced cell counts at this time ($P < 0.05$). To discriminate between an effect of resveratrol on the rate of cell growth as opposed to cell death, thymidine and leucine uptake were used to estimate any net change in biomass. **Figure 2** shows the effect of increasing concentrations of resveratrol on the proliferation of Caco-2 cells over 24 h when both thymidine and leucine uptake were measured and related to protein content of the cells. Treatment resulted in a dose-dependent inhibition of [^{3}H]thymidine and [^{14}C]leucine incorporation. A significant inhibitory effect was observed with resveratrol concentrations ≥12.5 μmol/L, and a concentration of 100 μmol/L completely abolished proliferation of Caco-2 cells. The effects were not associated with unspecific toxicity of the compound because LDH activity in the cell culture medium was unaffected by resveratrol treatment (data not shown). AP activity, a marker of differentiation, was assessed in Caco-2 cells, which differentiate spontaneously after 1 wk in culture (increase of AP activity from 10.35 ± 2.23 at d 1 to 117.4 ± 14.06 U/mg protein at d 12). Treatment with 12.5 μmol/L resveratrol increased AP activity from 9.73 ± 4.25 at d 1 to 128.66 ± 4.34 U/mg protein at d 12 (values are means ± SD, $n = 6$, $P < 0.05$ vs. control at d 12).

DNA flow cytometry was performed to ascertain the cell cycle distribution of exponentially growing Caco-2 and HCT-116 cells after treatment with resveratrol for 24 h (**Fig. 3**). Untreated subconfluent Caco-2 cells revealed a constant cell cycle phase distribution of cells in G0/G1 (42.27 ± 0.27%), in S (44.57 ± 0.38%) and G2/M (13.17 ± 0.60%) phases (Fig. 3A). At 50 μmol/L, resveratrol led to a 54% decline ($P < 0.05$) of cells in G2/M phase. This effect was associated with a 14% increase ($P < 0.05$) in proportion of cells in S phase. At higher concentrations (100 and 200 μmol/L), the S phase arrest of the cell cycle was reversed, with values of 44.13 ± 0.13 and 43.93 ± 0.47% in the S phase population, respectively. Similar results were obtained for HCT-116 cells, which possess wild-type p53, and the effects were more dramatic (Fig. 3*B*). Under control conditions, the distribution of cells in G0/G1 (37.00 ± 0.84%), S (44.66 ± 0.46%) and G2/M (18.35 ± 0.38%) phases of the cell cycle remained constant during a 24-h incubation. The distribution profile for cells incubated with 50 μmol/L for 24 h recorded 13.23 ± 0.35% of cells in G0/G1, 83.60 ± 2.02% in S and 3.19 ± 2.37% in G2/M. This effect was reversible at higher concentrations. The cell cycle distribution of HCT-116 cells treated with 200 μmol/L resveratrol (32.45 ± 0.39% G0/G1, 48.36 ± 2.81% S, 19.20 ± 3.20% G2/M) differed little from values obtained for controls. To test the specificity of the cell cycle inhibitory effect of resveratrol, we treated Caco-2 cells with the glycosylated stilbene analogs rhapontin (100 μmol/L; 41.37 ± 0.23% G0/G1, 43.60 ± 0.70% S, 15.04 ± 0.30% G2/M) and stilbenemethanol (25 μmol/L; 41.73 ± 0.69% G0/G1, 44.58 ± 0.24% S, 13.70 ± 0.93% G2/M). No significant change in cell cycle distribution was observed ($n = 2$).

Resveratrol did not affect the protein levels of PCNA,

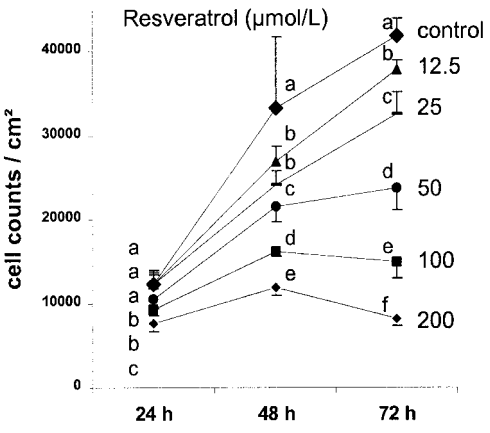

FIGURE 1 Effect of increasing concentrations of resveratrol on the growth of Caco-2 colonic adenocarcinoma cells over a 3-d period. Cell numbers were measured using the crystal violet technique. Values are means ± SD, $n = 6$. Values at a time point not sharing a letter differ significantly, $P < 0.05$.

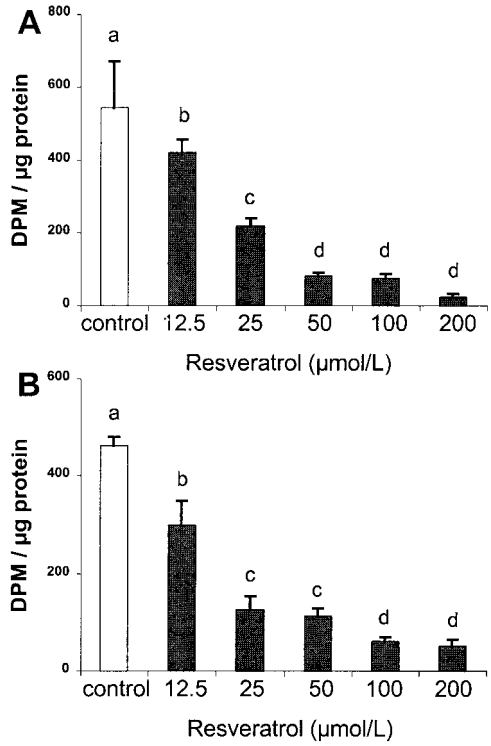

FIGURE 2 Proliferation of Caco-2 cells after resveratrol treatment using [^{3}H]thymidine (*panel A*) and [^{14}C]leucine incorporation (*panel B*). Cells were labeled with either [^{3}H]thymidine or [^{14}C]leucine for 24 h. Radioactivity was analyzed as described in Materials and Methods and is related to protein content. Values are means ± SD, $n = 5$. Values not sharing a letter differ significantly, $P < 0.01$.

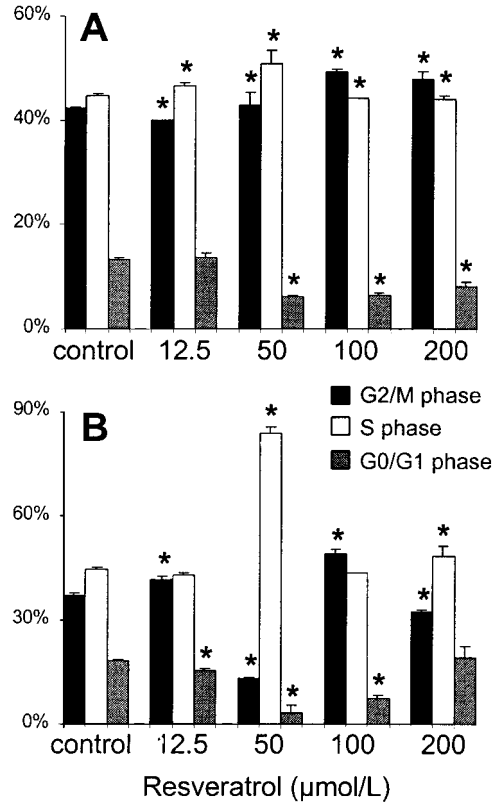

FIGURE 3 Cell cycle analysis of Caco-2 (*panel A*) and HCT-116 colon adenocarcinoma cells (*panel B*) after treatment with resveratrol at the indicated concentrations for 24 h. On the basis of DNA content, cells in G0/G1 can be distinguished from those in S and G2/M. The percentages of cells in each phase are shown. Values are means ± SD, $n = 2$. *Significantly different from control values of the corresponding cell cycle phase, $P < 0.05$.

cdc2, cdk2 or cdk6 (**Figure 4**A). A dose-dependent reduction in cdk4 and cyclin D1 protein levels was apparent. At concentrations ≥100 μmol/L, no cyclin D1 protein was detected. Cyclin A protein level was enhanced at concentrations up to 100 μmol/L. The maximal effect was observed after the addition of 25 μmol/L resveratrol. The amount of cyclin E protein was elevated to a concentration of 200 μmol/L. We further examined the expression of the cell cycle inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ by immunoblot (Fig. 4*B*). A dose-dependent decrease of $p27^{KIP1}$ was observed. The $p21^{WAF1/CIP1}$ protein was constitutively present in resveratrol-treated Caco-2 cells. As an indication that resveratrol inhibits cdk activity, we determined the phosphorylation state of pRb by immunoblot. The retinoblastoma protein (pRb), as observed by a shift of the band in Western blot analysis, was changed from the hyperphosphorylated to the hypophosphorylated form at the highest concentration of resveratrol and total pRb protein was decreased. To exclude the possibility that the observed effects of resveratrol on the cell cycle–regulating proteins were cell line specific we incubated HCT-116, a human colorectal cancer cell line with 200 μmol/L resveratrol vs. vehicle for 24 h and performed Western blots with the cellular lysates. As observed in Caco-2 cells, resveratrol treatment decreased cyclin D1, cdk4 and $p27^{KIP1}$ levels, whereas cdk2 and cdk6 levels were unmodified. The increase in cyclin E protein expression was also apparent in HCT-116 cells (**Fig. 5**).

To evaluate a possible influence of apoptosis induction on cell growth of Caco-2 cells, we assessed the activity of caspase-3 (**Fig. 6**). The topoisomerase inhibitor camptothecin, which induces capase-3–dependent apoptosis, was used as a positive control at 50 μmol/L. After 24 h, a significant increase in caspase-3 cleaving activity was found in resveratrol-treated cells, which was even more apparent after 48 h. Camptothecin induced capase-3 activity significantly after 8 h and led to higher values of capase-3 activity after 24 and 48 h compared with resveratrol.

DISCUSSION

The present study was designed to test whether resveratrol, a polyphenol present in the human diet in grapes, peanuts and red wine, may exert chemopreventive effects on colon cancer cells. Interest in the antitumorigenic properties of resveratrol has been elicited from the work of Jang et al. (4) in which an inhibitory effect has been shown in different stages of tumorigenesis.

This study demonstrates that resveratrol significantly inhibits the growth and proliferation of the human colonic adeno-

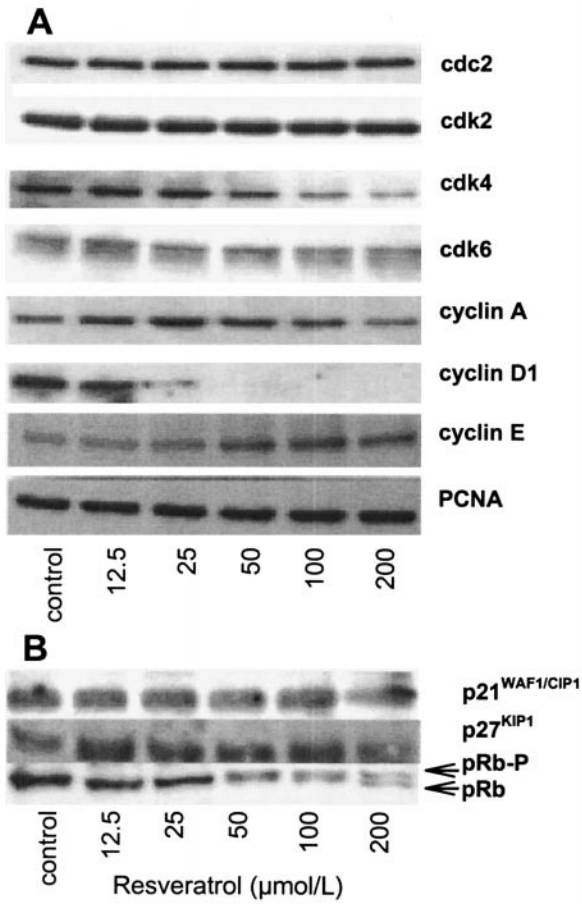

FIGURE 4 Western blot analysis of cell cycle regulatory protein expression in Caco-2 cells treated for 24 h with increasing amounts of resveratrol. Equal volumes of whole-cell extracts containing 20 μg (for $p21^{WAF1/CIP1}$ 40 μg) of protein were separated and electrophoretically blotted. For each protein a representative immunoblot is shown ($n = 3$). *Panel A*: Influence of resveratrol treatment on cdc2, cyclin-dependent kinase (cdk)2, cdk4, cdk6, cyclin A, cyclin D1, cyclin E, and proliferating cell nuclear antigen (PCNA) levels. *Panel B*: Influence of resveratrol on the protein expression of the cell cycle inhibitors $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and tumor suppressor retinoblastoma protein (pRb).

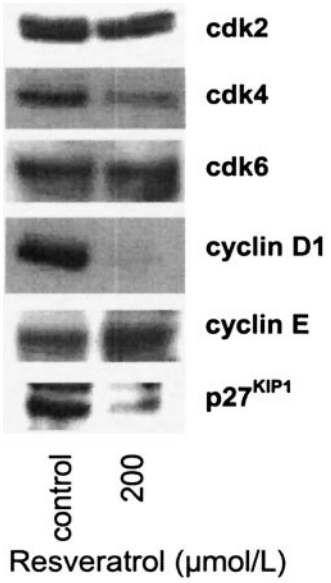

FIGURE 5 Western blot analysis of cell cycle regulatory proteins cyclin-dependent kinase (cdk)2, cdk4, cdk6, cyclin A, cyclin D1, cyclin E, and $p27^{KIP1}$ expression in HCT-116 cells treated for 24 h with increasing amounts of resveratrol. Equal volumes of whole-cell extracts containing 20 μg of protein were separated and electrophoretically blotted. For each protein, a representative immunoblot is shown ($n = 3$).

carcinoma cell line Caco-2 in vitro without influencing differentiation. Even though the rise in AP activity, a hallmark of differentiation, was significant after 12 d of treatment with resveratrol, these values cannot be considered physiologically relevant. Comparison with the differentiation-inducing agent butyrate showed that the short-chain fatty acid enhanced Caco-2 cell AP activity at 2 mmol/L six- to sevenfold (26).

Flow cytometry results indicated a significant reduction of cells in the G2/M phase of the cell division cycle, whereas the S phase population increased, which is consistent with the results obtained by Schneider et al. (15). These effects are specific for resveratrol because addition of the stilbene analogs stilbenemethanol and rhapontin did not change cell cycle distribution. This prompted us to investigate in further detail the mechanism of action of resveratrol on the cell cycle machinery. Western blot analysis of positive cell cycle regulators (cdc2, cdk2, cdk4, cdk6, cyclin A, cyclin D1 and cyclin E) showed a dose-dependent increase in cyclin E levels and an increase in cyclin A levels only at concentrations up to 100 μmol/L, suggesting the presence of an S to G2 block. Resveratrol treatment significantly reduced cyclin D1 levels and its related serine/threonine kinase cdk4. As a positive regulator of cdk4 and cdk6, cyclin D1 has been implicated in controlling the G1 phase of the cell cycle and is frequently overexpressed in human colon adenocarcinomas (27,28). Overexpression of an antisense cyclin *D1* cDNA construct in a human colon carcinoma cell line leads to impaired cell growth and tumorigenicity, implying that cyclin *D1* presents an oncogene (29). The cyclin D1/cdk4 complex is responsible for cell cycle progression in early G1 phase and for phosphorylation and thus inactivation of the tumorsuppressor pRb (30,31). Hypophosphorylated pRb is able to sequester the transcription factor E2F in the cytosol, which suppresses protein expression of the cell cycle machinery, thus causing a blockade in G1 phase. As revealed by immunoblot, dephosphorylation of pRb was observed in response to resveratrol treatment at a concentration of 200 μmol/L. Hypophosphorylation of pRb has been associated with inhibition of growth and arrest of cells in G1 phase (32). This might be a possible explanation for the increase in G1/S ratio, a marker of G1 arrest, observed at concentrations exceeding 50 μmol/L. It is tempting to speculate that the reduction of cdk4, cyclin D1 and cyclin A levels is responsible for this effect. Previous studies indicate that primary human tumors and tumor-derived cell lines often display a correlation between the levels of expression of cyclin D1 and the pRb tumor suppressor protein (33). This could contribute to the observed reduction of total pRb protein expression. The kinase activity of the cdk is negatively regulated by binding of the cyclin-dependent kinase inhibitors (cki) $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (34), which are positive regulators of differentiation (35). Subsequently, we determined protein expression of cki $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. The $p21^{WAF1/CIP1}$ level was unmodified by resveratrol addition. In contrast, $p27^{KIP1}$ expression was inhibited in treated cells, consistent with the results obtained by Doki et al. (33) that expressions of cyclin D1 and $p27^{KIP1}$ are closely correlated. The level of $p27^{KIP1}$ in cyclin *D1* overexpressing cells is largely dependent on levels of cyclin D1 protein (36), possibly contributing to the observed effect.

Expression of PCNA, a protein involved in cell cycle regulation, DNA synthesis and DNA repair, was not affected by resveratrol addition. As revealed by flow cytometry, the cells accumulate in S phase at a concentration of 50 μmol/L. This cell cycle arrest was reversed when higher concentrations of resveratrol were used. The cell cycle distribution corresponds well with expression of cyclin A, which is synthesized during S phase (37). Disruption of cyclin A function can inhibit chromosomal DNA replication (38). The growth inhibitory effect of resveratrol seems to be more pronounced at concentrations ≥50 μmol/L, which correlates with a reduction of cyclin A levels. This effect, together with accumulation of cyclin E, was also observed by Park et al. (10) in U937 human leukemia cells. This might lead to the conclusion that the effects of resveratrol on the cell division cycle are mainly a result of hampered DNA synthesis. The mechanism of action of resveratrol on tumor cells proposed to date includes inhibition of ribonucleotide reductase (39), DNA polymerase (40) and COX-1 (4) and inhibition of COX-2 transcription (41,42). Because Caco-2 cells express little or no COX-1 (22) and HCT-116 cells lack COX-1 or COX-2 activity (23), we

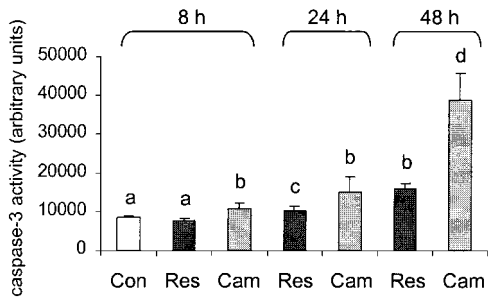

FIGURE 6 Effect of 200 μmol/L resveratrol (Res) vs. control (Con) on caspase-3 activity in Caco-2 cells at the times indicated. The topoisomerase inhibitor camptothecin (Cam) was used at a concentration of 50 μmol/L as a positive control. Activity of caspase-3 was determined on the basis of the cleavage of the fluorogenic substrate Z-DEVD-Rhodamine. Values are means ± SD, $n = 3$. Values not sharing a letter differ significantly, $P < 0.05$.

can conclude that the observed effects are not mediated by COX inhibition.

It has been assumed that the cell cycle inhibition in S and G2 phase is mediated by upregulation of p53 and induction of $p21^{WAF1/CIP1}$ (43). The resveratrol-mediated cell cycle inhibition we observed was clearly independent of p53 because Caco-2 cells possess mutated p53 (21). Our finding that resveratrol exerts the same effects on HCT-116 cells, which express wild-type p53, confirms earlier work showing that inhibition of cell cycle progression by resveratrol can function independently of p53 (10,44). In addition, no changes in the $p21^{WAF1/CIP1}$ protein levels were observed in Caco-2 cells.

Another possible mechanism by which the antiproliferative activity of resveratrol on Caco-2 cells may be exerted is induction of apoptosis. Indeed it has been demonstrated that resveratrol induces apoptosis in promyelocytic leukemia cells (HL-60) (6) via upregulation of CD95 ligand (5) through a strictly p53-dependent pathway in a mouse epidermal cell line (45), and independent of the CD95 pathway in CEM-C7H2 acute leukemia cells (44). We therefore determined whether resveratrol could induce apoptosis in Caco-2 cells. This was done by measuring the activity of caspase-3, a key protease in apoptosis. The increase of caspase-3 activity in Caco-2 cells was significant after 24 h. However, compared with camptothecin, this increase is very small. Because caspase-3 cleaving activity represents an early event in apoptosis, this might explain the reduction of cell counts, which was observed in the crystal violet assay after 72 h with 200 μmol/L resveratrol. Elimination of transformed cells via apoptosis is considered to be crucial for restoration of normal epithelial growth in the colon (46).

Epidemiologic studies reveal a strong inverse association between frequency of intake of plant-derived foods and cancer incidence. Resveratrol, widely distributed in red wine, peanuts and other sources of the human diet, exerts broad and potent anticarcinogenic and antitumor activities when applied at a pharmacologic level. The fact that the intestinal epithelium might be confronted with much higher concentrations than cells in other tissues, because the absorption rate of resveratrol in the perfused small intestine of the rat was estimated to be only 20.5% (47), implies that resveratrol could have an important role in the prevention of colon cancer by blocking hyperproliferation of the epithelium and by promoting apoptosis.

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II

Nutrition and Cancer

Piceatannol, a Natural Analog of Resveratrol, Inhibits Progression through the S Phase of the Cell Cycle in Colorectal Cancer Cell Lines[1]

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ABSTRACT Piceatannol, a naturally occurring analog of resveratrol, was previously identified as the active ingredient in herbal preparations in folk medicine and as an inhibitor of $p72^{Syk}$. We studied the effects of piceatannol on growth, proliferation, differentiation and cell cycle distribution profile of the human colon carcinoma cell line Caco-2. Growth of Caco-2 and HCT-116 cells was analyzed by crystal violet assay, which demonstrated dose- and time-dependent decreases in cell numbers. Treatment of Caco-2 cells with piceatannol reduced proliferation rate. No effect on differentiation was observed. Determination of cell cycle distribution by flow cytometry revealed an accumulation of cells in the S phase. Immunoblotting demonstrated that cyclin-dependent kinases (cdk) 2 and 6, as well as cdc2 were expressed at steady-state levels, whereas cyclin D1, cyclin B1 and cdk 4 were downregulated. The abundance of $p27^{Kip1}$ was also reduced, whereas the protein level of cyclin E was enhanced. Cyclin A levels were enhanced only at concentrations up to 100 μmol/L. These changes also were observed in studies with HCT-116 cells. On the basis of our findings, piceatannol can be considered to be a promising chemopreventive or anticancer agent. J. Nutr. 132: 298–302, 2002.

KEY WORDS: • *piceatannol* • *Caco-2 cells* • *cell cycle* • *colon cancer*

Piceatannol (*trans*-3,4,3′,5′-tetrahydroxystilbene, also known as 3-hydroxyresveratrol or astringinine) is a naturally occurring polyphenol (**Fig. 1**) and an analog of the cancer chemopreventive agent resveratrol (*trans*-3,5,4′-trihydroxystilbene). Both substances are synthesized in plants in response to fungal or other environmental stress, classifying them as phytoalexins. Piceatannol has been identified as the active ingredient of *Melaleuca leucadendron* (white tea tree), *Cassia garretiana* (Asian legume) and *Rheum undulatum* (Korean rhubarb), which are used in traditional herbal medicine (1–4), and as the antileukemic compound in the seeds of *Euphorbia lagascae*, which is used in folk medicine to treat cancer, tumors and warts (5). Teguo et al. (6) detected piceatannol in cell suspension cultures of *Vitis vinifera* (wine grapes).

To date, piceatannol has been known as a specific inhibitor of the protein tyrosine kinase $p72^{Syk}$, but it also inhibits the activity of $p56^{lck}$ (7). The molecule acts as an inhibitor of the focal adhesion kinase and Src in murine platelets (8) and exerts an inhibitory effect on the catalytical subunit of rat liver protein kinase A and rat brain protein kinase C (9). In addition, piceatannol potently reduces rat brain mitochondrial F0F1-ATPase activity (10). It was demonstrated recently that the compound prevents interferon-α–induced Stat3, Stat5 and Jak1 phosphorylation in B and T lymphocytes as well as in primary fibroblasts and HeLa cells (11). These results indicate that piceatannol interferes with neoplastic growth by modifying multiple cellular targets.

The primary objective of the present study was to elucidate the underlying molecular mechanisms of the antiproliferative action of piceatannol. Because of the importance of positive and negative cell cycle regulators in carcinogenesis, we determined whether they can be modulated by piceatannol. The data demonstrated that piceatannol inhibits the growth of colorectal cancer cell lines and arrests Caco-2 cells in the S phase of the cell cycle.

MATERIALS AND METHODS

Cell culture. The human colon cancer cell lines Caco-2 and HCT-116 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Caco-2 cells of passages 57–64 were cultured in Dulbecco's modified Eagle medium and HCT-116 cells of passage 17 were cultured in McCoy's 5A. Both cell lines were supplemented with 10% fetal calf serum, penicillin (1000 U/L) and streptomycin (1 mg/L) and incubated at 37°C under an atmosphere of 5% CO_2 in air. A stock solution of piceatannol (Alexis Biochemicals, Grünberg, Germany) was prepared in dimethyl sulfoxide (DMSO)[3]. The compound was added directly to cell cultures at the indicated concentrations, whereas untreated cells received the solvent alone ($\leq$ 0.1% DMSO). Cytotoxicity was excluded by lactate dehydrogenase (LDH) release assay (Roche Molecular Biochemicals, Mannheim, Germany).

Cell number. Determination of cell numbers was carried out using a modification of the method of Matsubara et al. (12). Briefly, cells were plated at a density of 7×10^3 cells/well in 96-well microtiterplates. Treatment with increasing concentrations of piceat-

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[3] Abbreviations used: AP, alkaline phosphatase; cdk, cyclin-dependent kinase; COX, cyclooxygenase; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; pRb, retinoblastoma protein

Manuscript received 29 June 2001. Initial review completed 7 September 2001. Revision accepted 12 November 2001.

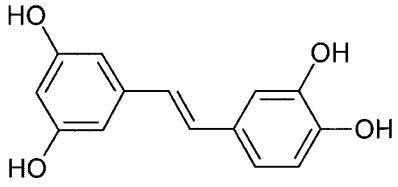

FIGURE 1 Chemical structure of piceatannol.

annol was carried out for 24, 48 and 72 h (piceatannol-containing medium was changed after 48 h). At the end of the incubation period, the medium was removed and any adherent cells were fixed to the plate with 5% formaldehyde in PBS. The cells were then stained with a 0.5% aqueous solution of crystal violet followed by elution of the dye with 33% aqueous acetic acid. Absorbance at 570 nm was determined with a Tecan Spectrafluor Plus microplate reader (Tecan, Crailshaim, Germany) and the number of cells was determined from a standard curve of absorbance against cell numbers calculated from a mean of six experiments.

Western blot analysis. Cells were seeded in 80 cm^2 flasks and incubated with increasing concentrations of piceatannol (25–200 μmol/L) for 24 h. Western blot analysis using total protein extracts from cultured cells was performed as previously described (13). Protein content was quantified with the Bio-Rad (Bio-Rad Laboratories, Munich, Germany) colorimetric assay. Reprobing of blots for expression of actin was done routinely. Antibodies against $p27^{KIP1}$, cdc2, cyclin-dependent kinase (cdk)2, cdk4, cdk6, cyclin A, cyclin B1 and cyclin D1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-$p21^{WAF1/CIP1}$ was purchased from Oncogene (Cambridge, MA) and anti-cyclin E from Pharmingen (Becton Dickinson, Heidelberg, Germany).

Incorporation of [^{3}H]thymidine and [^{14}C]leucine. Caco-2 cells were seeded in 24-well plates (5×10^4/well). During treatment with piceatannol, cells were pulsed with 18.5 MBq/well [^{3}H]thymidine and 0.925 MBq/well [^{14}C]leucine (Amersham Pharmacia Biotech, Freiburg, Germany). Medium was discarded, monolayers were washed three times with PBS and the cellular macromolecules were precipitated using 5% trichloroacetic acid. The acid was aspirated, cells were washed with absolute methanol and formic acid (2.5 mol/L) was used to solubilize the precipitated macromolecules. Probes were transferred to scintillation vials, 3.0 mL scintillation fluid (Packard Biosciences, Groningen, Netherlands) was added and measurements were carried out with a liquid scintillation counter (Packard Instruments, Meridien, CT). Cellular protein concentrations were determined as described in the Western blot analysis section.

Determination of alkaline phosphatase (AP) activity. Alkaline phosphatase activity was measured using *p*-nitrophenylphosphate as substrate according to the manufacturer's instructions (Merck, Darmstadt, Germany). Before treatment, cells were seeded in 6-well plates at a density of 25×10^4/well and allowed to attach overnight. Cell lysates of Caco-2 cells treated for 1, 4, 8 or 12 d with 12.5 μmol/L piceatannol were analyzed in the assay. Cellular protein concentrations were determined as described in the Western blot analysis section. AP activity was calculated in units per milligram protein.

Cell cycle analysis. Cells were seeded in 6-well plates at a density of 15 x 10^4/well 24 h before treatment; 24 h after treatment, they were washed with PBS and harvested by trypsinization (0.5 g/L trypsin 0.2 g/L EDTA solution, Gibco, Eggenstein, Germany). DNA contents of cells were measured using a DNA staining kit (CycleTEST PLUS DNA Reagent Kit, Becton Dickinson). Propidium iodide–stained nuclear fractions were obtained by following the kit protocol. Data were acquired using CellQuest Software (Becton Dickinson) with a FACScalibur (Becton Dickinson, Heidelberg, Germany) flow cytometry system using 10,000 cells per analysis. Cell cycle distributions were calculated using ModFit LT 2.0 software (Verity Software House, Topsham, ME).

Statistical analysis. Data were expressed as means ± SD. Differences between two values were tested for statistical significance using the Student's unpaired *t* test (SigmaPlot, SPSS Chicago, IL). A *P*-value < 0.05 was considered to indicate a significant difference.

RESULTS

To investigate the effect of piceatannol on the growth of colon cancer cells, Caco-2 cells incubated with piceatannol for 24, 48 and 72 h were analyzed by crystal violet assay. In the presence of piceatannol, cell growth was reduced in a dose- and time-dependent manner (**Fig. 2***A*). After 24 h of treatment, piceatannol (200 μmol/L) significantly reduced cell counts to 83.7 ± 2.9% of control values. After 72 h, the growth rate of cells decreased to 94.3 ± 1.5 and 60.0 ± 3.2% of the control level with 12.5 and 200 μmol/L piceatannol, respectively. A growth inhibitory effect was also demonstrated for the colorectal cancer cell line HCT-116. After 72 h, the growth rate of HCT-116 cells was 91.7 ± 2.2 and 58.3 ± 3.1% of the control level with 12.5 and 200 μmol/L piceatannol, respectively (Fig. 2*B*).

In addition to cell counts, DNA and protein synthesis were examined. Caco-2 cells were treated with piceatannol for 24 h. During treatment, cells were pulsed with [^{3}H]thymidine and [^{14}C]leucine. **Figure 3** shows the effect of increasing concentrations of piceatannol on the proliferation of Caco-2 cells over a period of 24 h, when both thymidine and leucine uptake were measured and related to protein content of the cells. Treatment caused a dose-dependent inhibition of [^{3}H]thymidine (75.5 ± 18.8, 61.3 ± 22.7, 54.8 ± 18.3, 39.9 ± 9.4 and 23.7 ± 4.8% of control, with 12.5, 25, 50, 100 and 200 μmol/L, respectively) and [^{14}C]leucine incorporation (72.9 ± 15.1, 67.3 ± 14.8, 54.3 ± 15.2, 52.4 ± 9.5 and 48.8 ± 6.3% of control, with 12.5, 25, 50, 100 and 200 μmol/L, respectively). The effects were not associated with unspecific toxicity of the compound because LDH activity in the cell culture medium was unchanged after piceatannol treatment

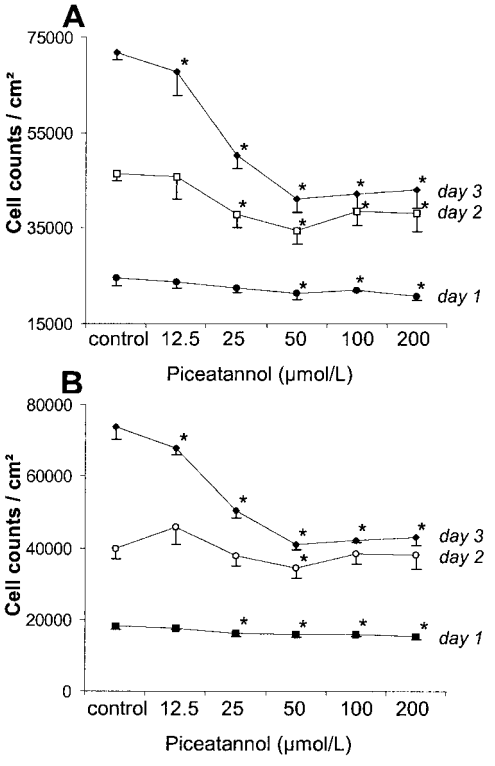

FIGURE 2 Effect of increasing concentrations of piceatannol on cell growth of Caco-2 (*panel A*) and HCT-116 (*panel B*) cells over a 3-d period. Cell numbers were measured using the crystal violet technique. Values are means ± SD, $n = 6$, $^*P < 0.01$ vs. time-matched control.

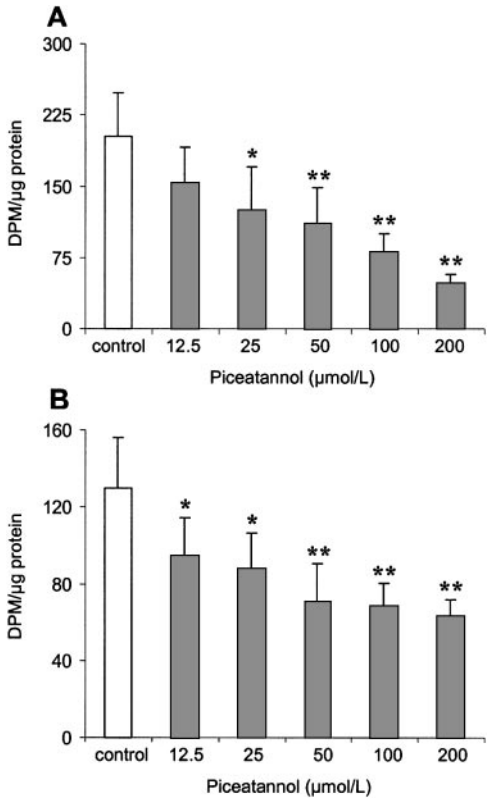

FIGURE 3 Proliferation of Caco-2 cells after piceatannol treatment using [^{3}H]thymidine (*panel A*) and [^{14}C]leucine incorporation (*panel B*). Cells were labeled with [^{3}H]thymidine or [^{14}C]leucine for 24 h. Radioactivity was analyzed as described in Materials and Methods and related to protein content. Values are means ± SD, $n = 5$, $^*P < 0.05$ and $^{**}P < 0.01$ vs. control.

(data not shown). AP activity, a well-established marker of differentiation was assessed in Caco-2 cells, which differentiate spontaneously after 1 wk in culture. Assessment of AP activity was carried out after 4, 7 or 10 d of incubation with 5 µmol/L piceatannol. No significant effect on AP activity was observed under these conditions (data not shown).

Subsequently, we investigated the effect of piceatannol on the cell cycle distribution profile of Caco-2 cells. **Figure 4** shows the sequence of these changes. Control cells revealed a distribution of cells in G0/G1 (36.39 ± 0.74%), in S (46.53 ± 0.65%), and G2/M (17.09 ± 1.38%) phases of the cell cycle. At 100 µmol/L, piceatannol led to an accumulation of cells in the S phase (76.23 ± 0.82% in S phase, 13.33 ± 0.22% in G0/G1, and 10.44 ± 1.03% in G2/M phase).

To elucidate the underlying mechanism of the S phase arrest, Western blot analysis was performed to detect changes in the expression of cell cycle regulating proteins. As shown in **Figure 5**, the proteins cdc2, cdk2 and cdk6 were constitutively present in Caco-2 cells incubated with piceatannol. Immunoblot analysis revealed a dose-dependent decrease in cyclin B1, cyclin D1, and cdk4 protein levels. Cyclin A protein expression was enhanced. The maximal effect was observed after the addition of 100 µmol/L piceatannol. We further examined the effect of piceatannol on the cyclin E abundance in Caco-2 cells. The protein was increased in a dose-dependent manner. Subsequently, we examined the expression of the cell cycle inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. A dose-dependent decrease of $p27^{KIP1}$ was observed, whereas $p21^{WAF1/CIP1}$ was unmodified after exposure to piceatannol. To ascertain whether piceatannol-induced effects on cell cycle regulating proteins was a general phenomenon or whether it was exclusive for Caco-2 cells, we investigated the effect of piceatannol on HCT-116 colon cancer cells. Our data show that after 24 h of exposure to 200 µmol/L piceatannol, protein levels of cyclin A, cyclin B1, cyclin D1, cdk4 and $p27^{KIP1}$ were diminished, whereas the cyclin E level was enhanced (**Fig. 6**).

DISCUSSION

The present study was designed to test how piceatannol, a naturally occurring polyphenol and an analog of the well-characterized polyphenol resveratrol, exerts its antiproliferative effect on intestinal tumor cells. Various investigators have demonstrated inhibition of the cell cycle progression by resveratrol (14–16). Extensive in vitro cell culture studies have demonstrated this effect at the S to G2 phase transition with a decreased G0/G1 phase population in favor of an increase of cells in the S phase of the cell cycle (15,17–22). Schneider et al. (22) described these effects for the colorectal adenocarci-

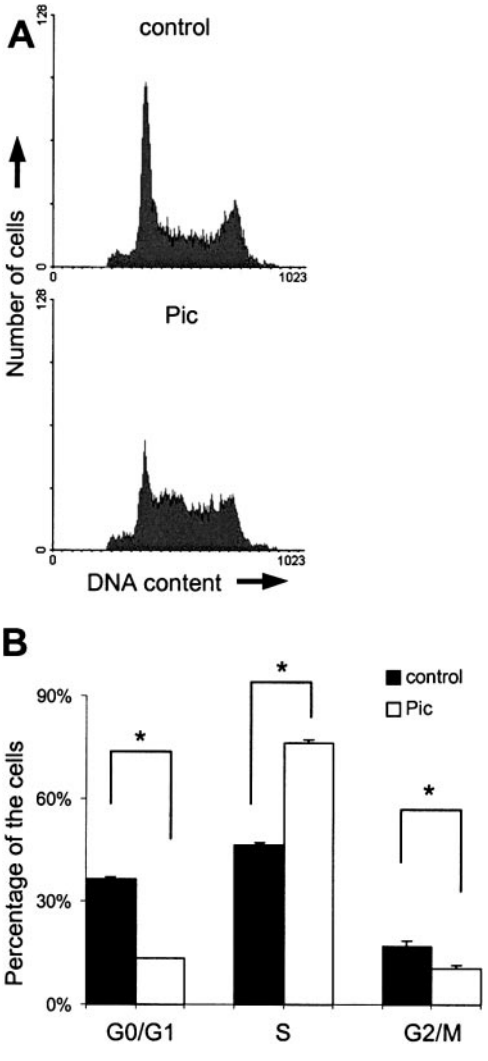

FIGURE 4 Cell cycle analysis of Caco-2 cells after treatment with 100 µmol/L piceatannol (Pic) for 24 h. The figure shows results of one of these experiments. (*Panel A*): cellular DNA content frequency histogram of a representative measurement that reveals the cell cycle distribution in Caco-2 cells. On the basis of DNA content, cells in G0/G1 can be distinguished from those in S and G2/M, as shown in *panel B*. Values are means ± SD, $n = 3$, $^*P < 0.01$, vs. control.

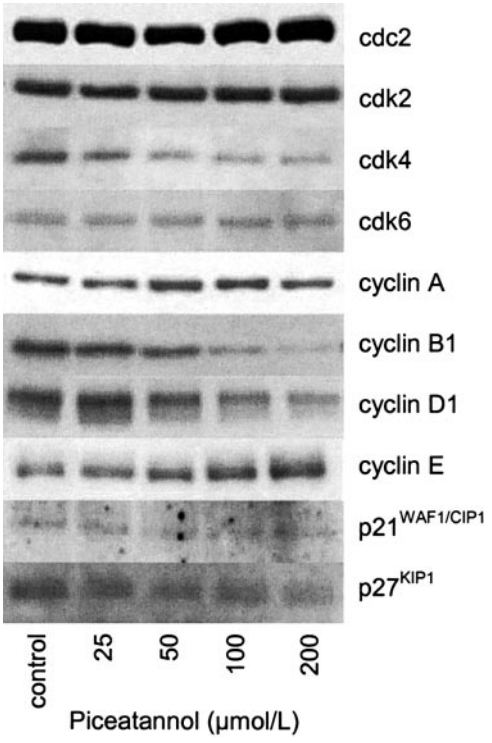

FIGURE 5 Western blot analysis of cell cycle regulatory proteins cdc2, cyclin-dependent kinase (cdk)2, cdk4, cdk6, cyclin A, cyclin B1, cyclin D1, cyclin E, $p21^{WAF1/CIP1}$, and $p27^{KIP1}$ expression in Caco-2 cells treated for 24 h with 0–200 μmol/L piceatannol. Equal volumes of whole-cell extracts containing 20 μg of protein (40 μg for $p21^{WAF1/CIP1}$) were separated and blotted electrophoretically. For each protein, a representative immunoblot is shown (n = 3).

noma cell line Caco-2 after treatment with 25 μmol/L resveratrol. Most authors attributed the S phase arrest to an inhibition of ribonucleotide synthase and DNA synthesis, which have been demonstrated by Fontecave et al. (23).

As shown by crystal violet assay, [^{3}H]thymidine- and [^{14}C]leucine-incorporation, piceatannol inhibits growth and proliferation of Caco-2 cells in a dose- and time-dependent manner. However, compared with resveratrol, the cytostatic effect is lower. Schneider et al. (22) demonstrated >70% growth inhibition for 25–30 μmol/L resveratrol in Caco-2 cells. One of the mechanisms by which resveratrol exerts its antitumorigenic effects on cancer cells is inhibition of cyclooxygenases (COX) (24). With respect to these data, we tested whether piceatannol affects growth of HCT-116 cells, which do not express COX-2 and lack COX-1 activity (25,26). Piceatannol also hampered growth of these cells, suggesting that the mechanism of growth inhibition is independent of COX activity.

Flow cytometry results revealed a reduction of Caco-2 cells in the G0/G1 and G2/M phases of the cell division cycle, whereas the S phase population increased. Data regarding resveratrol also demonstrated an increase of cells in the S phase (15,17–19,21–22,27). We further examined expression of certain cell cycle–associated proteins. Western blot analysis of positive cell cycle regulators (cdc2, cdk2, cdk4, cdk6, cyclin A, cyclin B1, cyclin D1, and cyclin E) demonstrated a reduction of cyclin D1 levels and its related serine/threonine kinase cdk4 in Caco-2 cells as well as in HCT-116 cells. The cyclin D1/cdk4 complex mediates progression of the cell cycle in early G1 phase and inactivates the retinoblastoma protein (pRb), a tumor suppressor by phosphorylation (28–29). Negative control of cdk activity is exerted by inhibitors of cyclin-dependent kinases $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (30), which are positive regulators of differentiation (31). Whereas $p21^{WAF1/CIP1}$ was unmodified by piceatannol addition, $p27^{KIP1}$ levels were diminished in both cell lines tested. The cdk inhibitor $p27^{KIP1}$ has its peak activity in G1 phase, whereas it is phosphorylated through the cyclin E-cdk2 complex in late G1 (32) and afterwards subjected to ubiquitin-proteasome–dependent degradation (33,34). Treated Caco-2 cells progress over this restriction point with elevated cyclin E protein levels, which could account for the observed downregulation of $p27^{KIP1}$. Another possible explanation is that expression of $p27^{KIP1}$ is largely dependent on cyclin D1, especially in cyclin *D1* overexpressing cells (35,36). Cyclin E mediates entry into S phase, whereas cyclin A accumulates later during S phase (29). Piceatannol treatment led to a dose-dependent increase in cyclin E levels and an elevation of cyclin A levels in Caco-2 cells only at concentrations up to 100 μmol/L, suggesting the presence of an S phase arrest. Cyclin E protein expression of HCT-116 cells was also enhanced, whereas cyclin A was diminished. Similar changes in expression of cell cycle regulatory proteins were observed after incubation of Caco-2 cells with resveratrol (27). Cyclin B is synthesized as a regulatory subunit of cdc2 as cells progress from S into G2/M phase (37), and cdk2 is largely responsible for the induction of cyclin B observed at the G2/M transition (38). Thus, the downregulation of cyclin B in piceatannol-treated cells likely reflects the inhibition of cdk2 activity. After exposure of Caco-2 cells to resveratrol, the same effect on cyclin B1 levels can be observed (data not shown).

These effects are specific for piceatannol and resveratrol because incubation of Caco-2 cells with the stilbene derivatives rhapontin and stilbene-methanol did not mimic the effects of resveratrol and piceatannol on S phase arrest (27).

Our data showed for the first time that piceatannol suppresses growth by perturbing progression through the S phase. These effects could be mediated by the upregulation of positive cell cycle regulators, cyclin E and A, which reach their maximal activity and protein levels in the S phase of the cell cycle

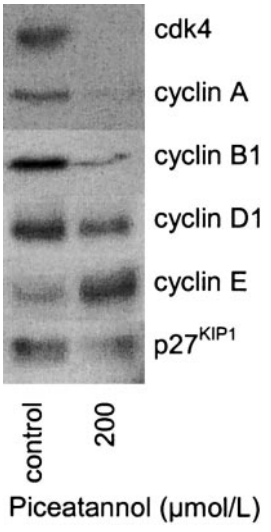

FIGURE 6 Western blot analysis of cell cycle regulatory proteins cyclin-dependent kinase (cdk4), cyclin A, cyclin B1, cyclin D1, cyclin E and $p27^{KIP1}$ expression in HCT-116 cells treated for 24 h with 200 μmol/L piceatannol vs. control. Equal volumes of whole-cell extracts containing 20 μg of protein were separated and electrophoretically blotted. For each protein, a representative immunoblot is shown (n = 3).

(39,40). At the same time, G0/G1 phase–regulating proteins, cyclin D1, cdk4 and $p27^{KIP1}$, and G2/M regulating cyclin B1 are expressed at lower levels. Although the growth inhibition exerted by piceatannol is not as pronounced as the cytostatic effect of resveratrol, piceatannol seems to induce the same changes in cell cycle distribution and cell cycle regulatory proteins. The amount of cells that accumulate in the S phase of the cell cycle is even higher than that observed after addition of resveratrol.

Plant polyphenols such as genistein, quercetin, curcumin and green tea polyphenols cause growth inhibition either by arresting cells in G0/G1 phase or in G2/M phase (41–44). There is evidence that colon carcinoma cells arrested in G0/G1 phase are less susceptible to chemotherapeutics, which has been attributed to elevated $p27^{Kip1}$ expression (45). It is tempting to speculate that piceatannol, as a molecule that downregulates $p27^{Kip1}$ and arrests cells in the S phase, might be utilized to enhance the effect of chemotherapeutic drugs that exert their effects specifically in the S phase of the cell cycle, like 5-fluorouracil, which is used to treat colon carcinoma. However, further studies are warranted to specify the effects of piceatannol and evaluate whether it can be used as an anticancer drug.

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III

Nutrition and Cancer

Resveratrol Enhances the Differentiation Induced by Butyrate in Caco-2 Colon Cancer Cells[1]

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ABSTRACT Butyrate, a short-chain fatty acid produced in the colon by microbial fermentation of fiber, inhibits growth of colonic carcinoma cells while inducing differentiation. Resveratrol, a plant polyphenol found in red wine and peanuts, has been shown to exert chemopreventive properties on colon cancer cells. The aim of this study was to determine whether resveratrol modulates the effects of butyrate on Caco-2, a colonic adenocarcinoma cell line. The growth inhibitory effect of resveratrol (50 μmol/L) was more powerful than that of butyrate (2 mmol/L). Butyrate did not intensify the inhibition of proliferation exerted by resveratrol. Although the polyphenol enhanced the differentiation-inducing effect of butyrate, it did not elevate alkaline phosphatase activity or E-cadherin protein expression, markers of epithelial differentiation, when applied alone. Butyrate-induced transforming growth factor-β1 secretion was inhibited by resveratrol. Treatment with the combination of resveratrol and butyrate attenuated levels of $p27^{Kip1}$, whereas resveratrol enhanced butyrate's effect on the induction of $p21^{Waf1/Cip1}$ expression. These data demonstrate a possible combined chemopreventive effect of two substances naturally occurring in the colonic lumen after ingestion of fibers and resveratrol-containing food. J. Nutr. 132: 2082–2086, 2002.

KEY WORDS: • *resveratrol* • *Caco-2 cells* • *differentiation* • *butyrate* • *colon cancer*

Colorectal tumors are remarkably common in Western populations. Chemopreventive agents occurring in the diet offer great potential to reduce the incidence of cancer. An association between reduced risk of colorectal cancer and diets high in fruit, fiber or vegetables has been well-established in epidemiologic studies (1).

Butyrate is synthesized by colonic bacteria from the unabsorbed complex carbohydrates present in dietary fiber (2). The short-chain fatty acid (SCFA)[3] stimulates growth of normal epithelial cells, whereas it inhibits proliferation and induces rapid cell differentiation in colon cancer cell lines (3–5). At higher concentrations, induction of apoptosis occurs in carcinoma cells treated with butyrate (6). Histone deacetylase inhibition resulting in modified oncogene expression has been considered to be a molecular mechanism by which butyrate modulates growth and differentiation (7). Induction of peroxisome proliferator-activated receptor γ (8) and upregulation of the vitamin D receptor have also been implicated in butyrate-induced differentiation (9).

The polyphenol resveratrol (*trans*-3,5,4′-trihydroxystilbene) is produced in plants in response to environmental or fungal stress. Dietary sources of this phytoalexin are red wine, grapes and peanuts. The molecule has been shown to inhibit proliferation of Caco-2 cells (10). In addition, treatment with resveratrol leads to accumulation of colon cancer cells in the S phase of the cell cycle and, at high concentrations, to induction of apoptosis (11). Proposed mechanisms of action include inhibition of cyclooxygenases (12–14), ribonucleotide reductase (15) and DNA polymerase (16).

The objective of the present study was to examine whether the combination of these two substances with different modes of action has a beneficial chemopreventive effect over the monotherapeutic application. The approach of combined chemoprevention has already proved to be efficient in the prevention of polyps in an $APC^{Min/+}$ mouse model, in which an inhibitor of cyclooxygenases was combined with an epidermal growth factor receptor kinase inhibitor (17). We chose Caco-2 cells as an in vitro model because this colorectal cancer cell line displays an undifferentiated phenotype under normal cell culture conditions, but can express an "enterocyte-like" phenotype either in response to various inducers of differentiation, including sodium butyrate or spontaneously after reaching confluence (4).

MATERIALS AND METHODS

Cell culture. The human colon cancer cell line, Caco-2, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Caco-2 cells of passages 52–58 were cultured in Dulbecco's modified Eagle medium, supplemented with 10% fetal calf serum, penicillin (1000 U/L) and streptomycin (1 mg/L) and incubated at 37°C under an atmosphere of 5% CO_2 in air. A stock solution of resveratrol (Sigma, Deisenhofen, Germany) was prepared in dimethyl sulfoxide (DMSO) and of sodium butyrate (Merck, Darmstadt, Germany) in PBS. Resveratrol was used at a concentration of 50 μmol/L, which has been shown not to be cytotoxic (11)

[1] Supported by the Else Kröner-Fresenius Foundation, Bad Homburg, Germany.

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[3] Abbreviations used: AOM, azoxymethane; AP, alkaline phosphatase; cki, cyclin-dependent kinase inhibitor; DMSO, dimethyl sulfoxide; SCFA, short-chain fatty acid; TGF, transforming growth factor.

Manuscript received 21 February 2002. Initial review completed 30 March 2002. Revision accepted 26 April 2002.

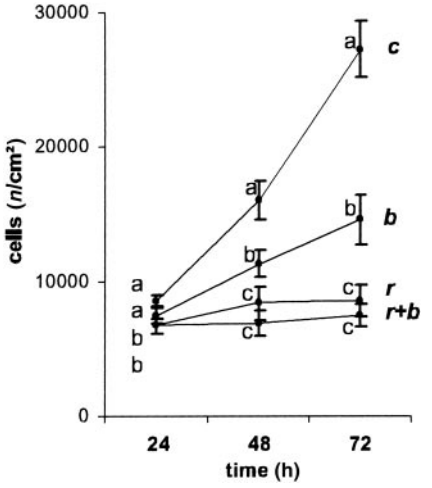

FIGURE 1 Effect of 50 μmol/L resveratrol (r) and 2 mmol/L butyrate (b), alone and in combination (r + b) compared with controls (c) on cell growth of Caco-2 cells over 72 h. Cell numbers were measured using the crystal violet technique. Values are means ± SD, $n = 6$. Means at a time not sharing a letter differ, $P < 0.01$.

and butyrate at a concentration of 2 mmol/L; untreated cells received the solvent alone (≤0.1% DMSO).

Cell number. Determination of cell numbers was carried out using a modification of the method of Matsubara et al. (18). Briefly, cells were plated at a density of 7×10^3 cells/well in 96-well microtiter plates and were allowed to attach overnight. After treatment for 24, 48 and 72 h (resveratrol- and butyrate-containing media were changed after 48 h), the medium was removed and any adherent cells were fixed to the plate with 5% formaldehyde in PBS. The cells were then stained with a 0.5% aqueous solution of crystal violet followed by elution of the dye with 33% aqueous acetic acid. Absorbance at 570 nm was determined with a Tecan Spectrafluor Plus microplate reader (Tecan, Crailshaim, Germany) and the number of cells was determined from a standard curve of absorbance against cell numbers calculated from a mean of six experiments.

Incorporation of [^{3}H]thymidine and [^{14}C]leucine. Caco-2 cells were seeded in 24-well plates (5×10^4/well) and were allowed to attach for 24 h. Together with treatment 18.5 MBq/well [^{3}H]thymidine and 0.925 MBq/well [^{14}C]leucine (Amersham Pharmacia Biotech, Freiburg, Germany) were applied to the cells for 24 h. Medium was discarded, monolayers were washed three times with PBS and the cellular macromolecules were precipitated using 5% trichloroacetic acid. The acid was aspirated, cells were washed with absolute methanol, and formic acid (2.5 mol/L) was used to solubilize the precipitated macromolecules. Probes were transferred to scintillation vials, 3.0 mL scintillation fluid (Packard Biosciences, Groningen, Netherlands) was added and measurements were carried out with a liquid scintillation counter (Packard Instruments, Meridien, CT). Cellular protein concentrations were determined as described in the Western blot analysis section.

Determination of alkaline phosphatase (AP). Alkaline phosphatase activity was measured using *p*-nitrophenylphosphate as substrate according to the manufacturer's instructions (Merck, Darmstadt, Germany). Cells were seeded in 6-well plates at a density of 25×10^4/well 24 h before treatment. Cell lysates of Caco-2 cells treated for 24, 96 or 192 h were analyzed in the assay. Cellular protein concentrations were determined as described in the Western blot analysis section. AP activity was calculated in units/mg protein (U/mg protein).

Transforming growth factor (TGF)-β1 secretion. TGF-β1 secretion was determined by use of an immunoassay kit (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions. Caco-2 cells were seeded in 6-well plates at a density of 25×10^4/well and allowed to attach overnight. Substances were added with serum-free medium and media were collected 24 or 48 h later.

Western blot analysis. Caco-2 cells were seeded in 80 cm^2 flasks; 24 h after plating, cells were incubated for 24 or 48 h with substances. Western blot analysis using total protein extracts from cultured cells was performed as previously described (11). Protein content was quantified with the Bio-Rad (Bio-Rad Laboratories, Munich, Germany) colorimetric assay. Reprobing of blots for expression of actin was done routinely. The antibody against p27^{KIP1} was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), anti-p21$^{WAF1/CIP1}$ was purchased from Oncogene (Cambridge, MA), and anti-E-cadherin was obtained from Becton Dickinson (Heidelberg, Germany).

Statistical analysis. Data are expressed as means ± SD. One-way ANOVA was used to compare means; *P*-values were corrected by the Bonferroni method for multiple comparisons (Microsoft Excel, Microsoft, Roselle, IL). A *P*-value < 0.01 was considered to indicate a significant difference.

RESULTS

Resveratrol was more potent than butyrate in reducing Caco-2 cell number as assessed by crystal violet assay. After 72 h of treatment, cell counts were reduced to 53% of control values with butyrate and to 31% with resveratrol (**Fig. 1**). When applied in combination, butyrate did not add to the growth inhibitory effect of resveratrol (27% of control). DNA synthesis rate, measured by [^{3}H]thymidine incorporation, was not affected by butyrate treatment alone for 24 h, whereas resveratrol alone and the combination of both substances exerted an inhibitory effect with comparable potency (**Fig. 2***A*). Protein synthesis rate (as measured by [^{14}C]leucine incorporation) was reduced to 79% of control values after 24 h of incubation with butyrate, whereas resveratrol diminished the rate to 32% of control (Fig. 2*B*). There was no difference between growth inhibition exerted by resveratrol or the combination (35% of control).

Figure 3*A* shows AP activity, a marker of differentiation. We demonstrated earlier that treatment with resveratrol did not increase AP activity in Caco-2 cells (11), whereas butyrate enhanced it after 192 h approximately twofold ($P < 0.01$). Compared with the spontaneous differentiation occurring in

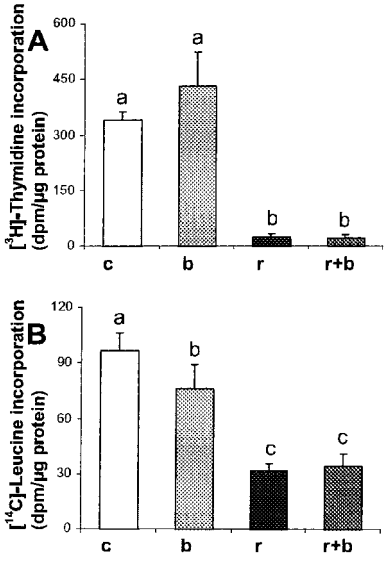

FIGURE 2 Proliferation of Caco-2 cells after treatment with 50 μmol/L resveratrol (r) and 2 mmol/L butyrate (b), alone and in combination (r + b) compared with controls (c) using [^{3}H]thymidine (*panel A*) and [^{14}C]leucine incorporation (*panel B*). Radioactivity was related to cellular protein content. Values are means ± SD, $n = 4$. Means in a panel not sharing a letter differ, $P < 0.01$.

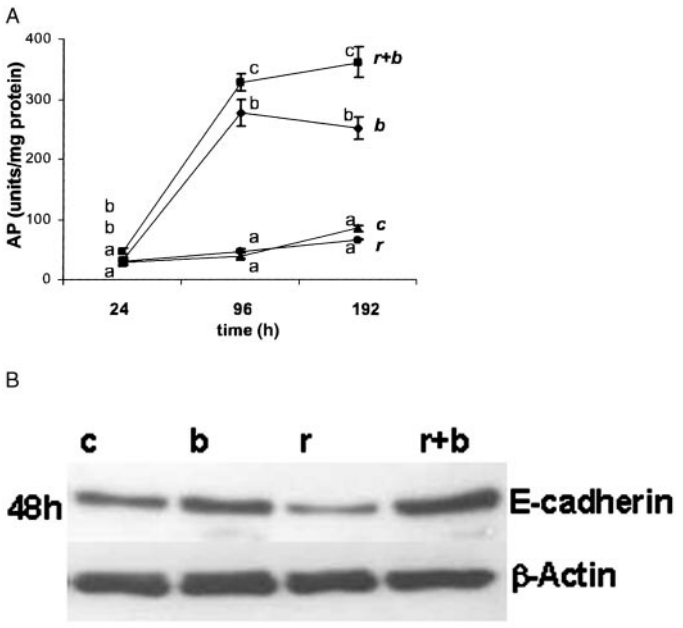

FIGURE 3 Effect of 50 μmol/L resveratrol (r) and 2 mmol/L butyrate (b), alone and in combination (r + b) compared with controls (c) on Caco-2 cell differentiation. Alkaline phosphatase (AP) activity assay (*panel A*) was used to assess differentiation over time. Values for AP are expressed as units/mg cellular protein (means ± SD, $n = 6$. Means at a time not sharing a letter differ, $P < 0.01$. E-cadherin protein expression was detected by Western blot 48 h after treatment (*panel B*).

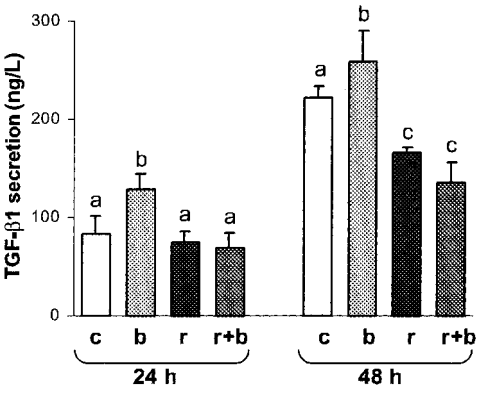

FIGURE 4 Transforming growth factor (TGF)-β1 secretion of Caco-2 cells treated for 24 or 48 h with 50 μmol/L resveratrol (r) and 2 mmol/L butyrate (b), alone and in combination (r + b) compared with controls (c). Values are means ± SD, $n = 6$. Means at a time not sharing a letter differ, $P < 0.01$.

untreated cells, the combination of the SCFA with resveratrol induced AP activity 3.3-fold ($P < 0.01$). E-cadherin protein expression is also a specific marker for differentiation of intestinal epithelial cells (19–21). Western blot analysis was used to detect E-cadherin. Treatment with resveratrol alone for 48 h attenuated E-cadherin protein expression, whereas butyrate alone had the opposite effect. In comparison with butyrate-treated cells, the combined treatment enhanced E-cadherin protein levels (Fig. 3*B*). Transforming growth factor (TGF)-β1 has also been implicated in epithelial differentiation (22). Therefore, TGF-β1 secretion into the medium was measured. Although butyrate induced secretion of the cytokine, TGF-β1 secretion was inhibited in Caco-2 cells treated with resveratrol as well as in cells treated with the combination of resveratrol and butyrate for 48 h (**Fig. 4**).

To examine the effect on cyclin-dependent kinase inhibitors (cki), Caco-2 cells treated with resveratrol and butyrate alone and in combination were harvested after 24 and 48 h and subjected to Western blot analysis. As shown in **Figure 5**, butyrate increased $p27^{Kip1}$ levels, whereas resveratrol, alone and in combination with butyrate, attenuated expression of the cki after 24 h as well as after 48 h. Protein expression of $p21^{Waf1/Cip1}$ was enhanced by the addition of butyrate, whereas resveratrol alone did not change expression of the protein (**Fig. 6**). Although $p21^{Waf1/Cip1}$ levels were not modified 24 h after combined treatment, an increase greater than that due to butyrate alone was observed after 48 h.

DISCUSSION

The results of the current study clearly indicate that treatment of colonic cancer cells with the SCFA butyrate and the plant polyphenol resveratrol inhibits proliferation. The growth-inhibiting effect of resveratrol is not potentiated by butyrate, whereas resveratrol enhances the increase of differentiation markers AP activity and E-cadherin protein expression by butyrate in colorectal cancer cells.

Other studies have demonstrated that resveratrol treatment alone leads to differentiation of erythroleukemic (23), osteoblastic (24) and promyelocytic cells (25). In contrast to these findings we did not observe effects on differentiation by resveratrol or its analog, piceatannol, in Caco-2 cells, as previously demonstrated (11,26). TGF-β1 has been implicated in butyrate-induced differentiation (22,27). Butyrate-induced TGF-β1 secretion was inhibited by resveratrol. Therefore we assumed that TGF-β1 was not involved in the combined effect of resveratrol and butyrate on differentiation. Induction of the cell cycle inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ has been suggested to be involved in butyrate-induced differentiation of Caco-2 cells (28,29). Wächtershäuser and Stein (30) demonstrated that differentiation also occurs independently of $p27^{Kip1}$ induction. Because we observed an attenuation of $p27^{Kip1}$ expression along with the increase of AP activity after combined treatment with butyrate and resveratrol, $p27^{Kip1}$ is

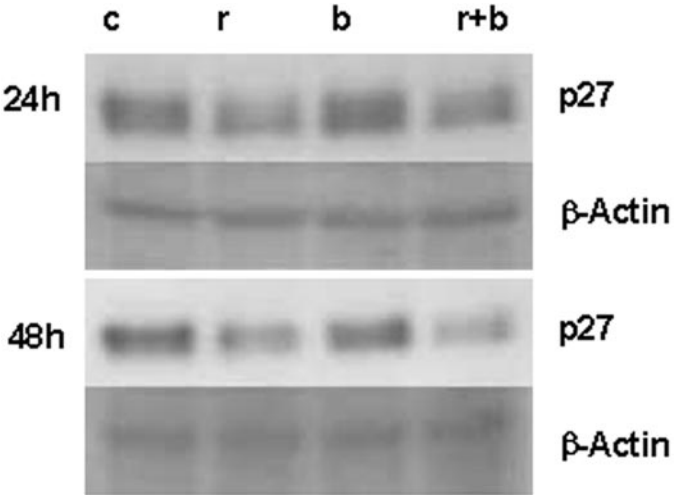

FIGURE 5 Western blot analysis of cell cycle regulatory protein $p27^{KIP1}$ expression in Caco-2 cells treated for 24 or 48 h with 50 μmol/L resveratrol (r) and 2 mmol/L butyrate (b), alone and in combination (r + b) compared with controls (c).

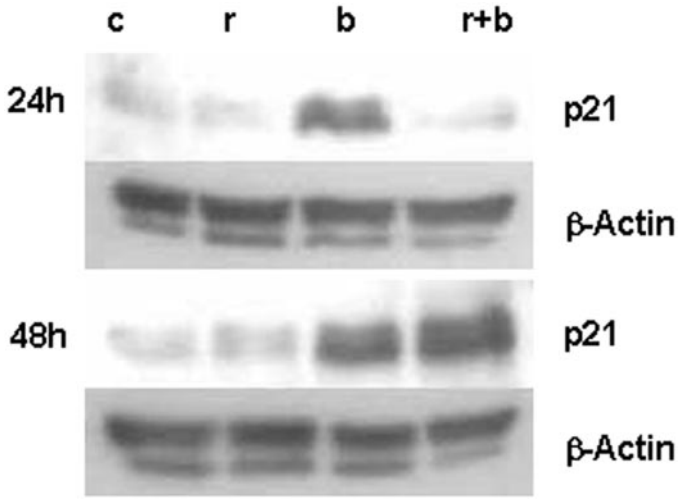

FIGURE 6 Effect of 50 μmol/L resveratrol (r) and 2 mmol/L butyrate (b), alone and in combination (r + b) compared with controls (c) on expression of the cyclin-dependent kinase inhibitor (cki) $p21^{Waf1/Cip1}$ in Caco-2 cells treated for 24 or 48 h, as determined by Western blot.

unlikely to play a role in this process. We also showed that the combination of butyrate and resveratrol increased butyrate's induction of $p21^{Waf1/Cip1}$ expression, implying that $p21^{Waf1/Cip1}$ rather than $p27^{Kip1}$ was involved in the induction of differentiation in this context.

The tumor suppressor p53 is one of the most important regulators of $p21^{Waf1/Cip1}$-induction, but is frequently mutated in colorectal carcinomas during the development from adenomas to carcinomas (31). Mutation of p53 has been implicated in the defective response to several growth inhibiting agents and this occurs also in Caco-2 cells (32). Activation of the *waf1/cip1* promoter by butyrate is independent of p53 induction (33). A likely cause for induction of $p21^{Waf1/Cip1}$ mediated by butyrate could be the histone deacetylase–inhibiting effect (7). It has been shown that resveratrol treatment alone enhances $p21^{Waf1/Cip1}$ expression in erythroleukemic cells lacking p53 (23), an epidermoid carcinoma cell line with only one functional p53 allele (34), and cultured bovine pulmonary artery endothelial cells possessing wild-type p53 (35), ruling out an obligatory role for p53 in the induction of $p21^{Waf1/Cip1}$ by resveratrol. In contrast to these findings, $p21^{Waf1/Cip1}$ levels in azoxymethane (AOM)-induced colorectal aberrant crypt foci of rats are attenuated by resveratrol treatment (36). This leads us to speculate that the effect of resveratrol on $p21^{Waf1/Cip1}$ might be dependent on a specific genetic pattern that is altered in the process of carcinogenesis. It has been suggested that chemopreventive substances can compensate for genetic and biochemical alterations in neoplastic lesions. In our work, butyrate modified gene and protein expression in ways that allowed resveratrol to enhance differentiation. This might mean that butyrate creates the intracellular conditions for the differentiation-inducing action of resveratrol, in spite of genetic lesions.

Epidemiologic studies investigating a possible correlation between fiber intake and reduced risk of colorectal carcinoma were rather disconcerting (37). The conflicting results may relate to the heterogeneity of the fibers and the basal diet because almost complete colonic fermentation is achieved solely with soluble fibers. Several studies have demonstrated that the production ratio of SCFA is decreased in patients with colorectal adenomas and cancers [for a review see (38)]. Perrin et al. (39) demonstrated that only fibers that lead to a constant butyrate production decreased the rate of aberrant crypt foci in rats injected with AOM. Direct application of butyrate with enemas reduced the incidence and size of colorectal tumors in the same animal model, even when colitis was induced with trinitrobenzensulfonic acid before AOM injection (40). Further in vivo studies with defined fibers are required to clarify the role of butyrate in chemoprevention of colorectal carcinoma.

Taken together, our findings provide evidence that resveratrol intensifies the differentiation-inducing effect of butyrate. Aberrancy of differentiation occurs during carcinogenesis and the altered states of cell and tissue differentiation are characteristic of premalignant lesions long before they become invasive and metastatic. The principal of using combined strategies may provide dramatic improvements over monotherapeutic regimens in chemoprevention of colorectal carcinoma.

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IV

Resveratrol-induced spermidine/spermine N^1-acetyltransferase activity is accompanied by induction of c-Fos.

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Running title: Resveratrol induces SSAT

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Abbreviations used in this paper:

AP-1, activator protein 1; cdk, cyclin dependent kinase inhibitor; DMSO, dimethyl sulfoxide; EGCG, (-)-epigallocatechin gallate; ODC, ornithine decarboxylase; PAO, polyamine oxidase; PBS, phosphate buffered saline; SAMDC, S-adenosylmethionine decarboxylase; SCFA, short chain fatty acid; SSAT, spermidine/spermine N^1-acetyltransferase

Abstract

The objective of the current study was to investigate the effect of resveratrol, a naturally occurring polyphenol with cancer chemopreventive properties, on polyamine metabolism in the human colonic adenocarcinoma cell line Caco-2. We demonstrated that inhibition of ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis was due to attenuated ODC protein and mRNA levels (50-200 μM). The naturally occuring resveratrol analog piceatannol (100 μM) also dimished ODC activity, protein and mRNA levels, whereas the green tea polyphenol (-)-epigallocatechin gallate (EGCG; 100 μM) exerted only weak effects on ODC. The transcription factor c-Myc, a positive regulator of the *odc* gene was attenuated by resveratrol treatment and to a lesser extent by piceatannol and EGCG. S-adenosylmethionine decarboxylase, an enzyme which synthesizes higher polyamines, was concomitantly inhibited by resveratrol and piceatannol treatment, whereas EGCG did not affect its activity. In addition resveratrol and EGCG, but not piceatannol enhanced spermidine/spermine N^1-acetyltransferase activity, an enzyme which degrades polyamines in cooperation with polyamine oxidase. Intracellular levels of spermine and spermidine were not affected, whereas putrescine and N^8-acetylspermidine concentrations increased after incubation with resveratrol. These events were paralleled by an increase of the activator protein-1 constituents c-Fos and c-Jun. Whereas DNA-binding activity of c-Jun remained unchanged, DNA-binding activity of c-Fos was significantly enhanced by resveratrol and piceatannol, but inhibited by EGCG. These data suggest, that growth arrest by resveratrol is accompanied by inhibition of polyamine synthesis and increased polyamine catabolism. C-Fos seems to play a role in this context. Effects of piceatannol on polyamine synthesis were similar, but not as potent as those exerted by resveratrol, whereas the impact of EGCG on polyamine homeostasis was comparably weak.

Key words: resveratrol, Caco-2 cells, polyamine, ornithine decarboxylase, c-Fos

Introduction

The cellular polyamines spermidine and spermine, as well as their precursor putrescine are essential for growth and DNA-synthesis (1-2). Increases in the levels of these polycations are generally associated with cell proliferation and cell transformation induced by growth factors (3), carcinogens (4) or oncogenes (5). Therefore polyamine metabolism is considered to represent an attractive target for both cancer chemotherapy and chemoprevention. Ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) are the key enzymes in polyamine biosynthesis. Inhibition of ODC by *DL*-α-difluoromethylornithine has been shown to decrease mucosal growth *in vivo* and *in vitro* (6-7). Catabolism, excretion or reconversion of higher polyamines is preceded by acetylation through spermidine/spermine N^1-acetyltransferase (SSAT), the rate-limiting enzyme in polyamine catabolism which can be induced by reactive oxygen species (8). In combined action with polyamine oxidase (PAO) it converts spermine to spermidine and the latter to putrescine.

Resveratrol (*trans*-3,4‘,5-trihydroxystilbene) is a plant polyphenol naturally occuring in grapes, red wine, and peanuts (9-10) with chemopreventive properties (11). The red wine polyphenol has been demonstrated to inhibit ODC activity and progression of the cell cycle in Caco-2 colorectal cancer cells (12). In previous studies we have shown that this cell cycle arrest is accompanied by reduced cyclin D1 and cyclin dependent kinase (cdk)4 levels (13). The number of aberrant crypt foci in azoxymethane-induced carcinogenesis of the rat colon is significantly reduced by treatment with resveratrol (14). In the *ApcMin*-mouse model for familiar adenomatous polyposis oral application of resveratrol reduced adenomas by 70% (15). The natural resveratrol analog piceatannol (*trans*-3,4,3‘,5‘-tetrahydroxystilbene) also inhibits cell cycle progression with decreased cyclin D1 and cdk4 levels of colorectal carcinoma cell lines (16). It has been demonstrated to inhibit formation of 7,12-dimethylbenz[a]anthracene-induced preneoplastic lesions in a mouse mammary gland model.

In contrast to resveratrol it does not significantly inhibit cyclooxygenase activities (17). The underlying molecular mechanisms for the antineoplastic effects of resveratrol and piceatannol have not been fully clarified.

The objective of the present study was to further elucidate the effect of resveratrol on polyamine metabolism and to test whether the analog piceatannol exerts the same effects as resveratrol. These results were compared with the effects of (-)-epigallocatechin gallate (EGCG), a green tea polyphenol implied in chemoprevention (18), to evaluate the specificity of the data obtained with the stilbenes.

Materials and Methods

Cell culture. The human colon cancer cell line Caco-2 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Caco-2 cells of passages 45-55 were cultured in Dulbecco's modified Eagle medium, supplemented with 10% fetal calf serum, penicillin (1000 U/l), and streptomycin (1 mg/l) and incubated at 37° C under an atmosphere of 5% CO_2 in air. All cell culture reagents were obtained from Life Technologies (Eggenstein, Germany). Stock solutions of resveratrol (Sigma, Deisenhofen, Germany) and piceatannol (Alexis Biochemicals, Grünberg, Germany) were prepared in dimethyl sulfoxide (DMSO). EGCG (Sigma, Deisenhofen, Germany) was diluted in phosphate buffered saline (PBS). The compounds were directly added to cell cultures at a concentration of 100 μM for piceatannol and EGCG and at concentrations ranging from 50-200 μM for resveratrol, whereas untreated cells received the solvent alone (≤ 0.1% DMSO). Cytotoxicity was excluded by lactate dehydrogenase release assay (Roche Molecular Biochemicals, Mannheim, Germany).

Western blot analysis. Cells were plated in 80 cm^2 flasks and incubated with plant polyphenols for 24 h. Western blot analysis using total protein extracts from cultured cells was performed as previously described (13). Protein content was quantified with the Bio-Rad (Bio-Rad Laboratories, München, Germany) colorimetric assay. Reprobing of blots for

expression of actin was done routinely. Antibodies against c-Myc and c-Fos were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), anti-ODC was purchased from Sigma (Deisenhofen, Germany), and anti-c-Jun from BD Transduction Laboratories (Heidelberg, Germany).

Reverse transcriptase-PCR. Cells were seeded in 6-well plates, allowed to attach overnight and treated with polyphenols for 24 h. RNA was isolated from cells by lysis in RNAzol B (Tel-Test, Friendswood, TX, USA) followed by phenol extraction and ethanol precipitation. Reverse transcription of total cellular RNA was carried out using Superscript II RNase H reverse transcriptase (Life Technologies, Inc., Karlsruhe, Germany) and random hexanucleotide primers (Promega, Madison, WI, USA). PCR was performed on the cDNA using the following sense and antisense primers, respectively (primers were custom-synthesized by Biospring, Frankfurt, Germany): ODC: AATCAACCCAGCGTTGGACAA and ACATCACATAGTAGATCGTCG; GAPDH: ATCTTCCAGGAGCGAGATCC and ACCACTGACACGTTGGCAGT. Thermal cycling was performed as follows: denaturation at 95° C for 30 s, annealing at 55° C for 30 s, and extension at 72° C for 90 s. 35 cycles were performed. Primers were used at a final concentration of 10 µM each, dNTPs at 500 µM (Eurogentec, Seraing, Belgium), and $MgCl_2$ at 3 mM. 5 U of Taq DNA Polymerase were used per 50 µl reaction. 10 µl of PCR product were electrophoresed on a 2% agarose-gel containing ethidium bromide and visualized by UV illumination.

ODC and SAMDC activity. The activities of the enzymes ODC and SAMDC were assayed with a radiometric technique in which the amount of $^{14}CO_2$ liberated from *DL*-[1-^{14}C]ornithine ($207{,}2 \cdot 10^4$ MBq/mol, Amersham Pharmacia Biotech, Freiburg, Germany) or *S*-adenosyl[carboxyl-^{14}C]-L-methionine ($222 \cdot 10^4$ MBq/mol, Amersham Pharmacia Biotech, Freiburg, Germany) was estimated, as described previously (19). Briefly, after treatment was carried out as described above, the cell culture dishes were placed on ice; the monolayers

were washed three times with cold PBS, and the cells were harvested by scraping in homogenizing buffer (50 mM Tris buffer, pH 7.2, 5 mM DTT, 100 µM EGTA), sonicated, and centrifuged at 15,000 *g* at 4° C for 10 min. 100 µl of the supernatant was incubated in a stoppered tube with 74 µM *DL*-[1-^{14}C]ornithine in the presence of pyridoxal-5-phosphate for 60 min at 37° C. For the SAMDC assay, instead of *DL*-[1-^{14}C]ornithine, S-adenosyl[carboxyl-^{14}C]-L-methionine was used and putrescine instead of pyridoxal-5-phosphate. $^{14}CO_2$ liberated by the decarboxylation of ornithine or S-adenosyl-methionine and trapped on filters impregnated with benzethonium hydroxide was measured by liquid scintillation spectroscopy. The supernatant was assayed for protein as described in the Western blot analysis section. ODC and SAMDC activity are expressed in pmol of released CO_2 per h per mg of protein. Controls always included samples for measurement of non-enzymatic release of $^{14}CO_2$.

SSAT activity. Cells were washed twice with cold homogenizing buffer (10 mM Tris-HCl, pH 7.5, 2.5 mM DTT, 1 mM EDTA), harvested by scraping, disrupted by sonification and centrifuged at 15,000 *g* at 4° C for 15 min. 60 µl aliquots of the supernatant were incubated with 0.3 µM spermidine, 10 µM Tris-HCl (pH 7.8), and 3,7 kBq [acetyl-^{3}H]-CoA ($495{,}8 \cdot 10^6$ MBq/mol, Moravek, Brea, CA) at 37°C for 10 min. The reaction was terminated by chilling and the addition of 20 µl of 1 M NH_2OH. Subsequently samples were centrifuged at 15,000 *g* for 5 min. 30 µl of the supernatant was 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, Netherlands). Radioactivity was measured in a liquid scintillation counter (Packard Instruments, Meridien, CT). Controls included samples for measurements of nonenzymatic incorporation of [acetyl-^{3}H]-CoA into monoacetylspermidine. SSAT activity is expressed in fmol of synthesized [acetyl-^{3}H]-spermidine per min per mg protein.

Determination of polyamines with HPLC. 24 h after plating in 6-well plates, resveratrol was added in FCS-free medium. After an incubation period of 24 h cells were washed with cold PBS and harvested by scraping. Cells were sonicated and centrifuged at 10,000 *g* for 10 min. Aliquots of the supernatant were used for protein determination. 0.2 M perchloric acid was added to the remaining supernatant. Polyamines were analyzed by the HPLC method according to Hyvonen *et al.* (20). Separation was carried out on a Hypersil (ODS 3 µm) 150 x 3 mm column (MZ analytical, Mainz, Germany) by isocratic elution with 0.1% potassium phosphate plus 0.01 M sodium octane sulfonate with acetonitrile (10:3, v/v) and a flow rate of 0.8 ml/min.

Activator protein-1 (AP-1) activation assay. Cells were plated in 80 cm^2 flasks and incubated with substances for 24 h. Nuclear extracts from total protein were prepared with a nuclear extract kit (Active Motif, Rixensart, Belgium). 20 µg of nuclear protein were either used for TransAM c-Fos or TransAM c-Jun transcription factor assay kit (Active Motif, Rixensart, Belgium). Activity of the transcription factors was determined according to the manufacturers instructions. AP-1 from nuclear cell extracts specifically binds to an oligonucleotide containing a 12-*O*-tetradecanoylphorbol-13-acetate-responsive element that is attached to a 96-well plate. By using an antibody directed against either c-Fos or phosphorylated c-Jun, the AP-1 dimer bound to the oligonucleotide is detected. A secondary antibody conjugated to horseradish peroxidase provides colorimetric visualisation. Absorbance was determined with a microplate reader (Tecan, Crailshaim, Germany).

Statistical analysis. Data were expressed as means ± SD. Differences between two values were tested for statistical significance using the Student's unpaired *t*-test (SigmaStat, SPSS Inc., Chicago, IL). A *P* value less than 0.05 was considered to indicate a significant difference.

Results

As demonstrated earlier (12), resveratrol inhibits ODC, the key enzyme in polyamine synthesis. At a concentration of 100 μM resveratrol reduced ODC activity to 42% of control values (Figure 1A). Compared to the effects of resveratrol, inhibition of ODC by EGCG was moderate with a reduction to 71% vs. control, whereas the inhibitory effect of piceatannol was more pronounced (47% of control). Diminished ODC activity after treatment with resveratrol or piceatannol was accompanied by reduced ODC protein levels, as monitored by Western blotting (Figure 1B). EGCG treatment did not affect ODC expression. To evaluate whether attenuation of ODC was caused on the level of transcription, *odc* mRNA was determined with PCR (Figure 1C). Treatment with resveratrol attenuated *odc* mRNA in a concentration-dependent manner. Piceatannol and EGCG exerted only weak effects on *odc* mRNA. As shown in figure 2 the polyamine-synthesizing enzyme SAMDC was also inhibited in a dose-dependent manner by treatment with resveratrol (54% of control with 100 μM). Piceatannol diminished SAMDC activity to 85% of control, whereas EGCG had no impact on SAMDC.

The transcription factor c-Myc is known to be an activator of the *odc* promoter.Therefore we determined whether the oncoprotein is influenced by treatment with resveratrol. As shown in Figure 3 performance of Western blot revealed diminished intracellular levels of the oncoprotein in a dose-dependent manner. Piceatannol was almost as effective as resveratrol in attenuating c-Myc expression, whereas the effect of EGCG was less pronounced.

SSAT activity was measured to evaluate whether polyamine degradation is also modulated by resveratrol (Figure 4A). Addition of resveratrol (100 μM) increased SSAT activity to 217% of control value. Piceatannol and EGCG augmented SSAT activity to 198% and 170% of control, respectively. In accordance with these results we could show that the intracellular polyamine concentrations changed after incubation with resveratrol (Figure 4B). There was

an increase in putrescine (651% of control with 200 μM resveratrol) and N^8-acetylspermidine (242% of control with 200 μM resveratrol), which are markers for enhanced SSAT activity.

As shown in Figure 5, resveratrol, but not piceatannol or EGCG was found to augment the abundance of c-Fos protein in Caco-2 cells. To determine whether this upregulation was accompanied by enhanced AP-1 activity, an AP-1 DNA binding activity assay was performed. Both stilbenes, resveratrol as well as piceatannol, markedly increased DNA binding of c-Fos. The effect of resveratrol was most prominent with a concentration of 100 μM (362% of control values). Piceatannol induced c-Fos binding to 216% of control values. Moreover, this binding was specific, because addition of wild type oligonucleotides containing the AP-1 binding motif, but not of a mutated oligonucleotide prevented DNA-binding of AP-1 from nuclear cell extracts. EGCG significantly inhibited c-Fos DNA-binding (80% compared to controls). Figure 6 reveals that protein levels of c-Jun increased after treatment with resveratrol and piceatannol, but not after incubation with EGCG. The DNA binding activity of c-Jun was not influenced by incubation with resveratrol, piceatannol or EGCG.

Discussion

The present study demonstrates that resveratrol modifies polyamine homeostasis on the level of synthesis as well as on the level of degradation of polyamines. As already shown by others (12) resveratrol significantly inhibited ODC activity. In addition we could demonstrate that this effect was accompanied by reduced protein and mRNA expression of *odc*. Transcription of the human *odc* gene is directly mediated by the Myc/Max transcriptional complex (21). In order to evaluate whether c-Myc is involved in resveratrol-induced growth inhibition, c-Myc protein content was determined. Resveratrol attenuated levels of the transcription factor, implying that downregulation of ODC might be mediated by reduction of c-Myc levels. Deregulation of *c-myc* with prolonged half life has been implicated in a number of

malignancies including tumors of the colon (22), classifying it as an oncogene. Constitutive expression of c-Myc has a profound effect on the cell cycle. C-Myc represents a therapeutic target of chemoprevention and its downregulation might contribute to the cell cycle inhibitory effect exerted by resveratrol.

In contrast to an earlier published study which investigated the effects of resveratrol on polyamine metabolism (12), a concomitant and dose-dependent (50-200 μM) inhibition of SAMDC activity, an enzyme involved in spermidine and spermine synthesis was observed. Whereas we observed an increase in putrescine and N^8-acetylspermidine concentrations, no significant changes in polyamine content of Caco-2 cells after treatment of cells with 25 μM resveratrol for 24 h was detected by Schneider *et al*. An explanation for these conflicting results could be the difference of concentrations used (12).

Furthermore, resveratrol potently upregulated SSAT activity in Caco-2 colon cancer cells. SSAT acetylates spermidine and spermine, which can either be secreted from the cell or are degraded by PAO forming putrescine or spermidine. Vujcic *et al.* postulated, that induction of SSAT can negatively affect cell growth (23). Accumulation of SSAT mRNA is accompanied by augmented intracellular putrescine levels (24). After treatment of Caco-2 cells with resveratrol an increased intracellular concentration of putrescine, the result of enhanced SSAT activity, was confirmed. Accumulation of N^8-acetylspermidine, a sign for active polyamine catabolism could also be monitored. Putrescine is also a product of polyamine catabolism, mediated by SSAT and PAO and is an effective inhibitor of ODC activity in IEC-6 intestinal crypt cells (25-26). It has been stated previously that whereas spermidine induces c-Myc, c-Fos is preferentially induced by putrescine (27). Elevated protein levels of c-Fos were observed together with an increased DNA binding activity of c-Fos after treatment with resveratrol. This leads us to hypothesize, that the increase in c-Fos might be due to enhanced putrescine levels. C-Fos is part of the dimeric transcription factor AP-1, which is composed of

members of c-Jun and c-Fos families that bind the AP-1 site as either homo- or heterodimers. There is increasing evidence that the AP-1 complex plays an important role not only in proliferation but also in differentiation of several cell types. In Caco-2 cells the chemopreventive agent 1,25-dihydroxyvitamin D_3 stimulates cell differentiation, which is dependent on AP-1 (28). Butyrate is also considered to represent a chemopreventive substance and triggers differentiation. Concomitantly the short chain fatty acid (SCFA) butyrate rapidly induces c-Fos at a posttranslational level (29). EGCG inhibits AP-1 activity of transformed keratinocytes induced by UV irradiation (30-31), whereas it increases AP-1-dependent gene expression in normal keratinocytes (32) and augments protein levels of AP-1 constituents in HepG2 cells (33). It has also been shown, that AP-1 activation occurs during differentiation of Caco-2 cells, before alkaline phosphatase and disaccharidase activities increase (34). These data imply that AP-1 is associated with differentiation and/or growth inhibition. Resveratrol alone does not induce differentiation of Caco-2 cells (12), whereas, when applied in combination with butyrate, significantly enhances the differentiation induced by the SCFA (35). Resveratrol has been demonstrated to inhibit AP-1 activation by tumor necrosis factor (36), UV irradiation, and by phorbol 12-myristate 13-acetate (37). This suggests that the induction of AP-1 by resveratrol could be limited to processes of growth inhibition and that resveratrol is able to inhibit AP-1 activation when it is associated with hyperproliferation.

Although the natural resveratrol analog piceatannol also inhibited polyamine synthesis, it had no significant influence on SSAT activity. We demonstrated recently that the growth inhibiting effect of piceatannol is not as pronounced as that of resveratrol (16), suggesting that higher concentrations of piceatannol could possibly induce SSAT. In order to test specificity of these results all experiments were also performed with EGCG, the most abundant

polyphenol in green tea, because ODC-inhibition by EGCG has been demonstrated earlier (38).

Taken together our findings demonstrate that resveratrol not only impairs polyamine synthesis and decreases the oncoprotein c-Myc, but also increases polyamine catabolism by SSAT. At the same time induction of c-Fos and its DNA binding activity take place. Modulation of polyamine homeostasis seems to be an additional target of the antiproliferative effects of resveratrol. In this context resveratrol is more potent than EGCG. The mechanism of the growth inhibitory action of resveratrol seems to differ from that of the green tea polyphenol, because effects on c-Fos activity were the opposite. The impact of piceatannol on polyamine synthesis was similar to that of resveratrol, although less pronounced.

Acknowledgments

This work was supported by the Else Kröner-Fresenius-Foundation, Bad Homburg, Germany.

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Figures

Fig. 1. Influence of resveratrol on ODC in Caco-2 cells. Cells were treated for 24 h with resveratrol, piceatannol (pic) or EGCG. ODC activity (panel A) of Caco-2 cells was determined by $^{14}CO_2$-release from labelled ornithine. Results (means ± SD, $n = 4$) are expressed in enzyme units (pmol of released CO_2) per milligram cellular protein per hour. *$P < 0.01$, **$P < 0.001$ vs. control (con). To determine ODC protein expression in Caco-2 cells volumes of whole cell extracts containing 20 µg of protein were separated and electrophoretically blotted (panel B). Total RNA from Caco-2 cells was analyzed by reverse transcriptase-polymerase chain reaction for *odc*-mRNA content and *gapdh* was used as a control for initial RNA loading (panel C).

Fig. 2. Influence of resveratrol on SAMDC activity in Caco-2 cells. Cells were treated for 24 h with resveratrol, piceatannol (pic) or EGCG. SAMDC activitiy of Caco-2 cells was determined by $^{14}CO_2$-release from labelled S-adenosylmethionine. Results (means ± SD, $n = 4$) are expressed in enzyme units (pmol of released CO_2) per milligram cellular protein per hour. #$P < 0.05$, *$P < 0.01$, **$P < 0.001$ vs. control (con).

Fig. 3. Western blot of c-Myc in Caco-2 cells. Caco-2 cells treated with resveratrol, EGCG or piceatannol (pic) for 24 h were harvested, subjected to Western blotting and compared with controls (con).

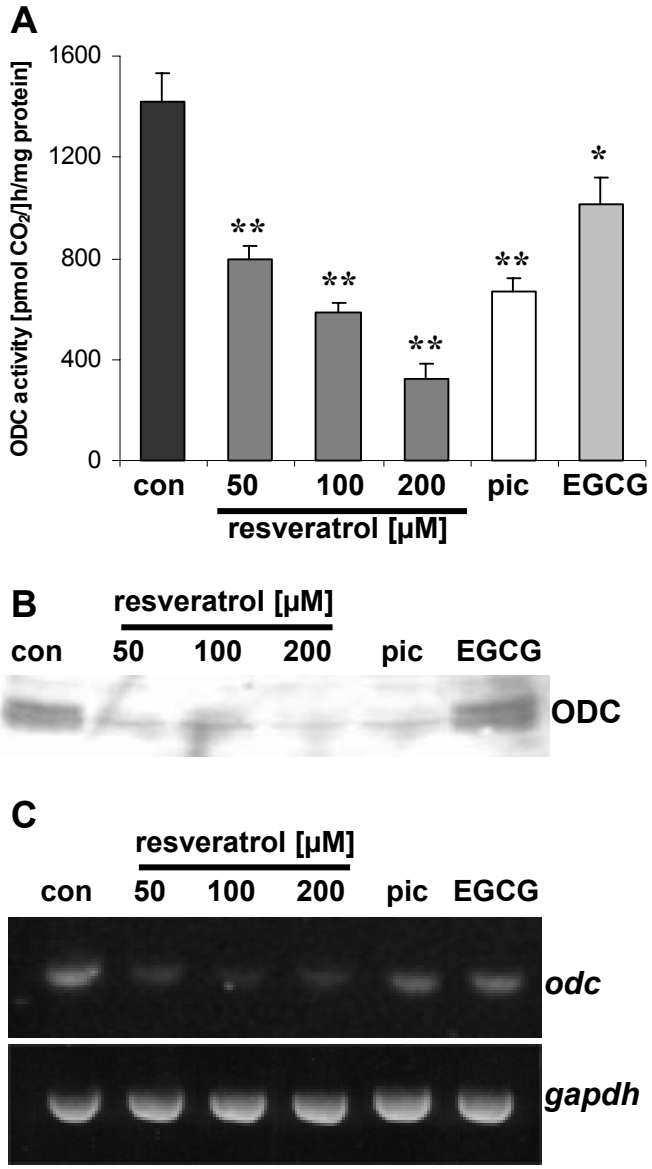
Fig. 4. SSAT activity of Caco-2 cells was determined to monitor polyamine catabolism (panel A). Cells were incubated in the absence or presence of resveratrol, piceatannol (pic) or EGCG for 24 h, and SSAT activity was determined by formation of [acetyl-^{3}H]-spermidine after the addition of labelled acetyl-CoA. Values are means ± SD, $n = 4$, #$P < 0.05$, *$P < 0.01$, **$P < 0.001$ vs. control (con). Panel B shows intracellular polyamine concentrations of Caco-2 cells treated with increasing concentrations of resveratrol for 24 h. Measurements were performed

with HPLC and values were related to protein content of the cells. Values are means ± SD, $n = 3$, $*P < 0.01$ vs. control (con).

Fig. 5. Protein expression and DNA-binding activity of AP-1 constituent c-Fos after treatment of Caco-2 cells with resveratrol, piceatannol (pic) or EGCG. Treated Caco-2 cells were harvested after 24 h. Equal volumes of whole cell extracts containing 20 µg of protein were separated and electrophoretically blotted to detect c-Fos protein expression (panel A). Treated cells were subjected to an AP-1 DNA-binding assay (panel B). Equal volumes of nuclear extracts from Caco-2 cells containing 20 µg of protein were used and binding activity was determined according to the kit protocol. Values are means ± SD, $n = 3$, $**P < 0.001$ vs. control (con).

Fig. 6. Protein expression and DNA-binding activity of AP-1 constituent c-Jun after treatment of Caco-2 cells with resveratrol, piceatannol (pic) or EGCG. Treated Caco-2 cells were harvested after 24 h. Equal volumes of whole cell extracts containing 20 µg of protein were separated and electrophoretically blotted to detect c-Jun protein expression (panel A). Treated cells were subjected to an AP-1 DNA-binding assay (panel B). Equal volumes of nuclear extracts from Caco-2 cells containing 20 µg of protein were used and binding activity was determined according to the kit protocol. Values are means ± SD, $n = 3$, compared to control (con), no significant changes in c-Jun DNA-binding could be detected.

Fig. 1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gapdh

Fig. 2

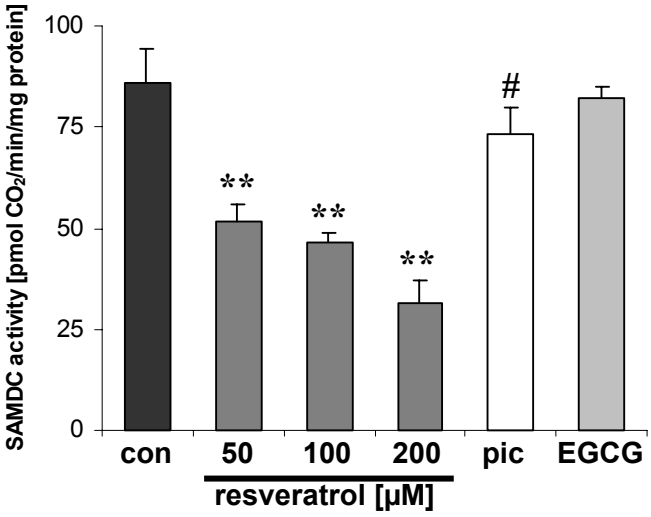
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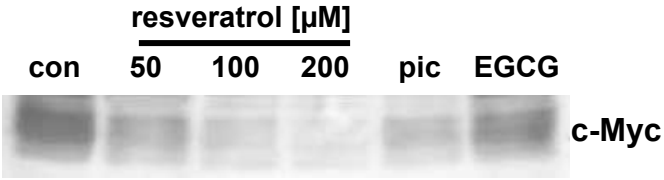
Fig. 3



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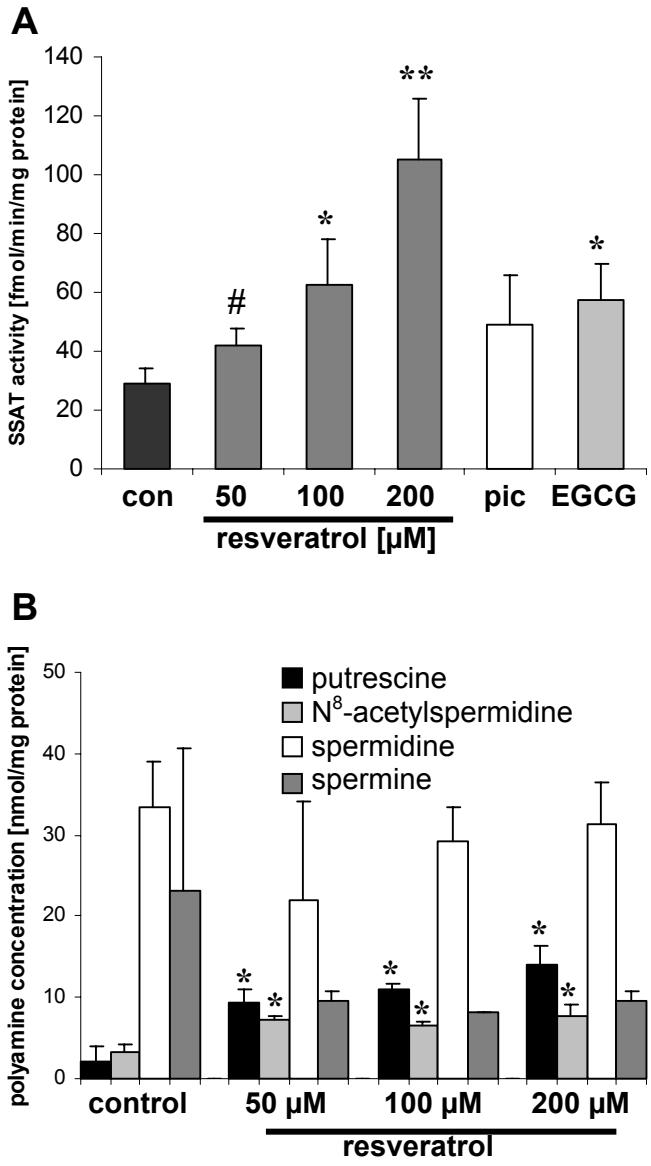
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EGCG
resveratrol [µM]
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polyamine concentration [nmol/mg protein]
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40
30
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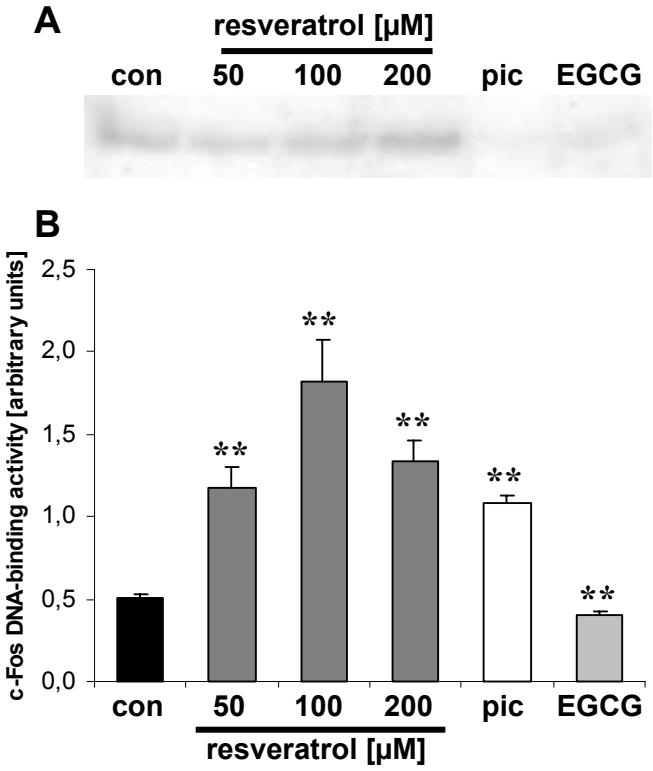
resveratrol

Fig. 5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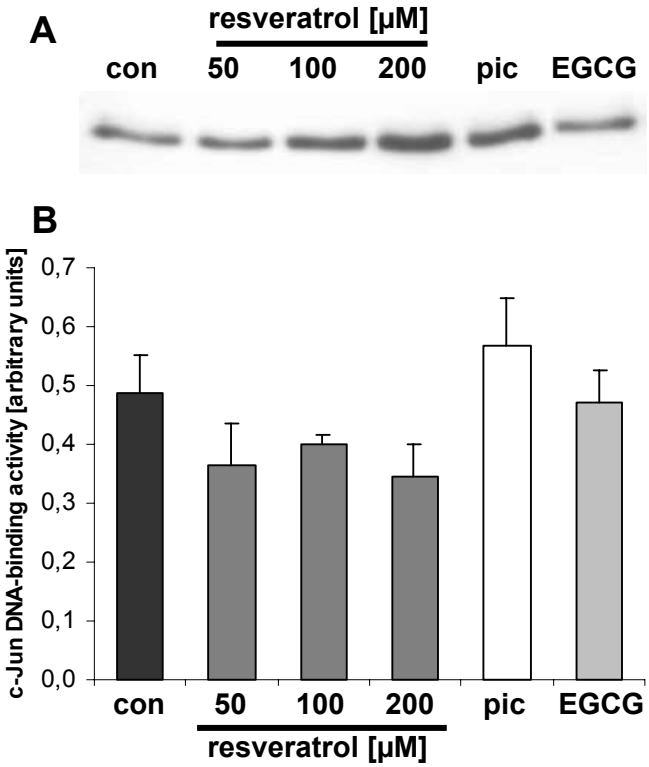
200
pic
EGCG
resveratrol [µM]

Fig. 6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Drugs of the Future 2002, 27(10)

CCC: 0377-8282/2002

Review Article

Biological activities of resveratrol and its analogs

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CONTENTS

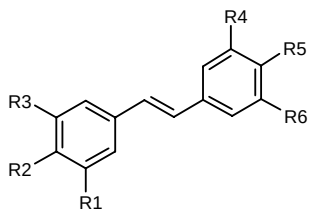
Abstract

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, thereby serving as a cardioprotective agent. Recently, resveratrol was shown to function as a cancer chemopreventive agent, and there has been a great deal of experimental effort directed toward defining this effect. In addition, resveratrol exhibits antiinflammatory, neuroprotective and antiviral properties. This review summarizes the recent advances that have provided new insights into the molecular mechanisms underlying the promising properties of resveratrol, including cyclooxygenase, nitric oxide synthase and cytochrome P450 inhibition, as well as cell cycle effects, apoptosis modulation and hormonal activity.

Introduction

Resveratrol (3,4',5-trihydroxystilbene; molecular weight = 228.2; Fig. 1) 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 is known in traditional Asian medicine as Ko-jo-kon (1, 2) (Table I). Resveratrol-containing foods include grapes (3, 4), wine (5) and peanuts (6, 7). In the case of grapes, especially when infected with *Botrytis cinerea*, resveratrol is exclusively synthesized in grape skins, which contain 50-100 mg resveratrol/g when they are fresh. Because 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 moderate red wine consumption on preventing the development of cardiovascular diseases. Resveratrol inhibited platelet aggregation (8), protected porcine low density lipoproteins against polyunsaturated fatty acid peroxidation (9) and exerted vasorelaxing effects on endothelium-intact aorta rings of rats (10).

The inhibitory potency of resveratrol in various stages of tumor development has attracted much attention (11).



	R1	R2	R3	R4	R5	R6
Stilbene	-H	-H	-H	-H	-H	-H
Resveratrol	-H	-OH	-H	-OH	-H	-OH
Piceatannol	-OH	-OH	-H	-OH	-H	-OH
Piceid	-H	-OH	-H	-OH	-H	-O-Gluc
Pterostilbene	-H	-OH	-H	$-OCH_3$	-H	$-OCH_3$
Rhapontigenin	-OH	$-OCH_3$	-OH	-OH	-H	-OH

Fig. 1. Chemical structures of resveratrol and its analogues.

Table I: Resveratrol and piceid concentrations of selected foods and beverages.

Product	*Trans*-resveratrol	*Trans*-piceid	Ref.
Red grape juice	0.5 mg/l	3.38 mg/l	4
White grape juice	0.05 mg/l	0.18 mg/l	4
Pinot noir red wine, 1994	10.57 mg/l	ND	2
Merlot red wine, 1994	0.48 mg/l	ND	2
Cabernet Sauvignon white wine, 1995	0.53 mg/l	ND	2
Natural peanut butter	0.652 μg/g	0.143 μg/g	7
Blended peanut butter	0.409 μg/g	0.128 μg/g	7
Ko-jo-kon root	523 μg/g	1653 μg/g	2

ND = not detectable

Because many reviews regarding the preventive effect of resveratrol on cardiovascular diseases have been published, this review will focus mainly on the cancer chemopreventive effects of the compound.

Metabolism

Hydroxystilbenes permeate through cell membranes and are stable and not cytotoxic (12). Oral daily administration of 20 mg/kg body weight of resveratrol over a period of 28 days did not lead to any toxic effects in rats (13). In the perfused small intestine of the rat, a model of intestinal absorption, the bioavailability of luminally administered resveratrol was 20.5%. On the vascular side, 16.8% of the resveratrol was found to be conjugated to yield resveratrol glucunoride, 0.3% was found conjugated as resveratrol sulfate and 3.4% was absorbed as free resveratrol (14). Kuhnle *et al.* used the same model to show that 96.5% of the absorbed resveratrol is conjugated as a glucuronide in enterocytes (15). The glucuronidation is mediated by UDP-glucuronyltransferase 1A1, preferentially at the 3-hydroxygroup (16). A conjugation with sulfate has also been reported (17, 18).

After feeding rats red wine with known resveratrol concentrations *ad libitum* the highest plasma concentration was reached after 1 h. The concentration was sufficient to induce physiological effects such as inhibition of thrombocyte aggregation (19). The concentrations detected in the liver and kidneys were comparable to the plasma concentration. A significant absorption could also be found in the heart tissue (20). When known amounts of resveratrol were administered to rats, a bioavailability of 38.1 ± 13.5% was determined. Measurable amounts of glucuronidated and unglucuronidated resveratrol were secreted with bile and reached the enterohepatic cycle. The enterohepatic circulation of resveratrol seemed to influence the plasma half-life (21). After oral administration of 25 mg resveratrol, the highest plasma concentration in humans was detected after 30 min (7.1 μg/l free resveratrol and 338 μg/l conjugated resveratrol) which returned to baseline after 2 h. The rate of conjugated resveratrol was 30-50 times higher than the concentration of free resveratrol. During 24 h, 24.6% of the stilbene was excreted with urine (22). To our knowledge, no data exist concerning the bioavailability of stilbene glucosides. Because glucosides of flavonoids are absorbable, it is likely that stilbene glucosides are bioavailable as well (23). So far no data are available regarding the absorption and metabolism of piceatannol. The extent of absorption of resveratrol from the normal diet is largely unknown and may also depend on the degradability of resveratrol glucosides (*e.g.,* piceid) and resveratrol polymers (*e.g.*, viniferins) in the gut.

In vivo effects

Oral administration of resveratrol inhibited tumor growth of T241 fibrosarcoma in mice (24). Rats inoculated with Yoshida AH-130 hepatoma cells and treated with resveratrol (i.p.) had a decreased number of tumor cells (25). 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 (500 ppm) (26), but the compound did reduce the number of aberrant crypt foci in azoxymethane-induced tumorigenesis in the rat colon, which led to enhanced expression of the proapoptotic protein Bax in these crypt foci (27). In Min mice, an animal model of familiar adenomatous polyposis, the number of adenomas was reduced by 70% (colons contained no polyps following treatment) in animals given 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, MAPK (mitogen activated protein kinase), TSG101 tumor susceptibility protein and other targets (28). The mean survival time of mice inoculated with 32Dp210 leukemia cells and treated with up to 80 mg/kg body weight resveratrol was not significantly different from untreated controls, even though resveratrol exerted antileukemic properties on 32Dp210 cells *in vitro* (29).

In mice bearing highly metastatic Lewis lung carcinoma tumors, resveratrol inhibited DNA synthesis of tumor cells with an IC_{50} value of 6.8 μM. No effects were detected on $CD4^+$, $CD8^+$ and natural killer cells, leading the authors to conclude that these cells are not responsible for the effects of resveratrol on DNA synthesis (30). The

Table II: Overview of the effects of resveratrol on human cancer cell lines.

Cell line	Proliferation	Apoptosis	Cell cycle arrest	Differentiation	$p21^{Waf1/Cip1}$ expression	Ref.
Acute lymphoblastic leukemia cells from patients	–	↑	–	–	–	71
HL-60 (leukemia)	↓	↑	–	–	–	70
HL-60 (leukemia)	↓	↑	S-phase	↑	Unmodified	33
CEM-C7H2 (leukemia)	–	↑	S-phase	–	–	56
THP-1 (leukemia)	↓	↑	S-phase	–	–	74
U937 (leukemia)	–	↑	S-phase	–	–	75
U937 (leukemia)	↓	–	S-phase	–	–	60
LNCaP (prostate cancer)	↓	↑	–	–	↓	68
LNCaP (prostate cancer)	↓	–	S-phase	–	↓	59
LNCaP (prostate cancer)	↓	↑	Unmodified	–	–	65
DU 145 (prostate cancer)	–	↑	–	–	↑	78
Caco-2 (colon carcinoma)	↓	↑	S-phase	Unmodified	Unmodified	62
Caco-2 (colon carcinoma)	↓	No	S-phase	–	–	60
HCT-116 (colon carcinoma)	–	↑	–	↑	↑	35
HCT-116 (colon carcinoma)	↓	↑	–	–	–	81
HCT-116 (colon carcinoma)	–	–	S-phase	–	–	62
MCF-7 (mammary carcinoma)	↓	–	S-phase	–	–	55
MDA-MB-435 (mammary carcinoma)	↓	–	S-phase	–	–	55
A431 (epidermoid carcinoma)	↓	↑	G_0/G_1-phase	–	↑	63
Diverse (thyroid carcinoma)	–	↑	–	–	↑	77

↑ induction; ↓ inhibition; – not investigated.

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 (31). 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 (32).

Differentiation

Resveratrol induced differentiation of promyelocytic HL-60 cells (33). Whereas resveratrol did not affect differentiation when applied alone, it potentiated the differentiation-inducing effect of the short-chain fatty acid butyrate on Caco-2 colonic carcinoma cells and enhanced butyrate-upregulated E-cadherin and cell cycle inhibitor $p21^{Waf1/Cip1}$ expression without increasing $p27^{Kip1}$ levels (34). In HCT-116 colon cancer cells treatment with 100 μM resveratrol led to an increase in microvilli density and villin expression, both signs of terminal differentiation (35).

An overview of *in vitro* anticancer effects of resveratrol is given in Table II.

Arachidonic acid metabolism

Arachidonic acid is released from phospholipid membranes by phospholipase A_2 and subsequently converted by two major pathways. The lipoxygenase pathway results in production of immunomodulatory leukotrienes, whereas the cyclooxygenase (COX) pathway leads to production of prostaglandins, prostacyclins and thromboxanes. COX has been implicated in inflammatory processes and tumorigenesis of the colon and the mammary gland. Resveratrol is an inhibitor of the constitutively expressed COX-1 isoenzyme. This inhibition is exerted on the COX activity as well as on the hydroperoxide activity of COX-1. The hydroperoxide activity of the inducible COX-2 isoform was also inhibited, but only with 20-fold higher concentrations of resveratrol than for COX-1; the COX activity of COX-2 was not affected (11). Whereas inhibition of COX-1 by resveratrol was confirmed by other authors, stimulation of COX-2 activity was also reported (36). In contrast to these results, Subbaramaiah *et al.* demonstrated that resveratrol inhibits prostaglandin (PG) production via recombinant COX-2. In addition, resveratrol suppressed basal and phorbol 12-myristate 13-acetate (PMA)-induced COX-2 activity of 184B5/HER mammary epithelial cells. This effect was accompanied by reduced COX-2 mRNA levels and inhibition of COX-2 promoter activity. Resveratrol also inhibited COX-2 promoter activity induced by overexpression of ERK1, c-Jun and PKCα (37). In DLD-1 colon cancer cells COX-2 promoter activity induced by TGF-α was also inhibited by resveratrol (38). Treatment with resveratrol led to diminished PGE_2 production in murine peritoneal macrophages stimulated with lipopolysaccharides (LPS). In addition, resveratrol downregulated COX-2 levels induced by PMA, LPS or O^-_2 (39). In the erythroleukemia cell line K562, resveratrol suppressed H_2O_2-induced leukotriene

B_4 and PGE_2 production. Dioxygenation of linoleic acid by purified 5-lipoxygenase or 15-lipoxygenase was inhibited as well (40). Induction of phospholipase A_2-association with membranes induced by fetal calf serum (FCS) or platelet derived growth factor (PDGF) was inhibited by resveratrol in 3T6 murine fibroblasts. Resveratrol also suppressed [^{3}H]-arachidonic acid release from membranes and PGE_2 synthesis induced by FCS and PDGF (41).

Inflammation

Resveratrol has been demonstrated to exhibit antiinflammatory properties by suppressing carrageenan-induced paw edema (11). The nuclear factor (NF)-κB is a mediator of inflammation and exerts antiapoptotic activities. Normally, transcription factor NF-κB is sequestered in the cytoplasm by its inhibitor IκB. NF-κB release and translocation to the nucleus occurs when IκB is degraded by proteasomes after phosphorylation by IKK (IκB kinase) following an inflammatory stimulus. Tumor necrosis factor (TNF)-α-induced NF-κB-activation was inhibited by pretreatment of U937 histiocytic lymphoma cells, Jurkat T-cells, HeLa uterus carcinoma cells and H4 glioma cells with resveratrol. In U937 cells, NF-κB-induction by PMA, LPS, okadaic acid, ceramide and H_2O_2 was suppressed as well. Resveratrol did not modify the ability of NF-κB to bind to DNA of U937 cells and did not directly interfere with TNF-α-induced NF-κB-binding to DNA (42). In contrast to these results, Holmes-McNary and Baldwin (43) demonstrated that resveratrol inhibits DNA-binding of NF-κB induced by TNF-α and LPS in U937 cells and THP-1 monocytes. NF-κB-dependent transcription, IκBα degradation, and IKK activation induced by TNF-α in THP-1 cells were inhibited by pretreatment with resveratrol. In LPS-activated RAW 264.7 macrophages, resveratrol inhibited generation of nitric oxide (NO). This effect was due to downregulation of the inducible NO synthase (iNOS) protein and mRNA. An upstream event of LPS-induced iNOS-activation is the activation of NF-κB. Resveratrol treatment inhibited LPS-induced nuclear localization and DNA-binding of NF-κB and suppressed phosphorylation and degradation of the NF-κB-inhibitory protein IκBα (44).

Signal transduction

The MAPK 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. TNF-α-induced AP-1, JNK and MEK (MAPK kinase) activation were inhibited in U937 lymphoma cells by pretreatment with resveratrol (42). Resveratrol inhibited phosphorylation of ERK1 and ERK2 induced by fibroblast growth factor 2 (FGF-2) in bovine capillary endothelial cells (24) and by human serum in liver myofibroblasts (45). Pretreatment of the cervical squamous cancer cell line HeLa 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, whereas resveratrol had only a weak effect on epidermal growth factor (EGF)-induced ERK2-activation (46). In undifferentiated SH-SY5Y neuroblastoma cells, treatment with resveratrol led to increased ERK1 and ERK2 phosphorylation. ERK phosphorylation was inhibited at concentrations of 50 μM and higher. Resveratrol treatment of retinoic acid-differentiated SH-SY5Y cells decreased ERK phosphorylation at first, but then subsequently markedly increased it (47). 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 (48).

Resveratrol inhibited recombinant protein kinase C (PKC) activity induced by sonicated vesicles prepared from 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 (49). Resveratrol inhibited the PMA-induced redistribution of PKC from cytosol to membrane (37) and the autophosphorylation of isolated PKD in a dose-dependent manner, whereas it had only negligible effects on PKC isozyme autophosphorylation (50).

The naturally occurring 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 (51). In addition, piceatannol inhibits the tyrosine kinase activity of human placenta (52) and the focal adhesion kinase and Src in thrombocytes (53).

Cell cycle

The negative effect of resveratrol on proliferation has in part been attributed to inhibition of ribonucleotide reductase and DNA synthesis (54). 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 modulated by their expression levels and by cell cycle inhibiting proteins ($p21^{Waf1/Cip1}$, $p27^{Kip1}$, and members of the INK family of proteins). The effect of resveratrol on the cell cycle distribution of tumor cells appears to affect the S-phase with a cell cycle arrest in the S-phase reported for different cell types (21, 55-62). Increased cyclin E and cyclin A expression was observed in HL-60 leukemia cells (21), U937 lymphoma cells (60), HCT-116 and Caco-2 colon cancer cells (62). Ragione *et al.* identified inactivation of cdc2 by phosphorylation at tyrosine

residue 15 as a possible pathway by which this S-phase arrest is mediated (21). A concentration-dependent decrease of the $p27^{Kip1}$ expression level was observed in LNCaP, U937 and Caco-2 cells (59, 60, 62). In bovine pulmonary artery endothelial cells (57), HL-60 cells (21), A431 cells (63) and U937 cells (60), 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 (62) and decreased in LNCaP cells (59). 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 tumor suppressor pRb was observed in Caco-2 cells (62) 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 (64). Resveratrol arrested the cell cycle of nonandrogen-responsive prostate cancer cell lines in the S-phase, but did not modify the cell cycle distribution of the androgen-responsive cell line LNCaP (65). Stivala *et al.* 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 are essential for the effects of resveratrol on the cell cycle (66).

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 were shown. Taken together, these effects were comparable to those observed after treatment with resveratrol (67).

Apoptosis

Apoptosis or 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 such as DNA fragmentation, cleavage of caspases and caspase substrates and breakdown of mitochondrial transmembrane potential. Resveratrol was demonstrated to induce apoptosis in a number of cell types (62, 68). 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 (69). In HL-60 promyelocytic leukemia cells resveratrol-induced apoptosis was prevented by caspase inhibitors (70). 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, since a caspase-8 deficient mutant Jurkat cell line was sensitive to resveratrol-induced cell death (44). Activation of these caspases was inhibited by overexpression of the oncogene Bcl-2 (71). In acute lymphoblastic leukemia cell lines, activation of caspase-9 and depolarization of mitochondrial membranes could be monitored after treatment with resveratrol (72). Resveratrol, but not stilbene or stilbene oxide, prevented H_2O_2-induced apoptosis of K562 erythroleukemia cells (40). 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 (42). Clément *et al.* detected Fas-dependent apoptosis-signaling in HL-60 and T47D cells (73), whereas Fas-independent apoptosis could be demonstrated in CEM-C7H2 (44) and THP-1 monocytic leukemia cells (74). Induction of apoptosis by resveratrol was also monitored in leukemia cell lines that are resistant to Fas-induced cell death (72). In CEM-C7H2 cells, resveratrol-induced breakdown of the mitochondrial transmembrane potential was independent of caspase-8 activation and Bid-cleavage, which are arguments against Fas involvement in this context (71).

In THP-1 cells, overexpression of $p16^{INK4}$ with subsequent cell cycle arrest in G0/G1-phase abrogated apoptosis (74). The same effect could be observed in the acute lymphoblastic leukemia cell line CEM-C7H2 demonstrating the dependence of resveratrol-induced apoptosis on the S-phase arrest (56). In CEM-C7H2 cells overexpression of the antiapoptotic protein Bcl-2 prevented resveratrol-induced apoptosis and mitochondrial transmembrane potential breakdown (71). Overexpression of Bcl-2 in U937 cells also attenuated apoptosis and prevented cleavage of caspase-3 and PARP (poly ADP-ribose polymerase) (75).

Huang *et al.* demonstrated that induction of apoptosis in JB6 mouse epidermal cells is dependent on the presence of the tumorsuppressor p53 (76). In thyroid cancer cells (BHP 2-7, BHP 18-21, FTC 236, and FTC 238), apoptosis induced by resveratrol was inhibited by *p53* antisense oligonucleotide transfection or by addition of the p53 inhibitor pifithrin-α (77). In DU145 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 (78). In contrast to these results, induction of apoptosis by resveratrol has been seen in cell types deficient in functional p53 (34, 52, 79). In the colorectal cancer cell line HCT-116, which possesses wild-type p53, apoptosis occurred after incubation with resveratrol via a p53-independent mechanism (35). Stabilization of p53 by phosphorylation at Ser15 in mouse JB6 epidermal cells induced by resveratrol was shown to be dependent on activated ERKs and p38 kinase, but not on activated JNK (80). In thyroid cancer cells apoptosis, c-Fos and p53 induction induced by resveratrol were blocked by the MEK inhibitor PD-98059 (77). In DU145 cells, Ser15 phosphorylation of p53 by resveratrol was also blocked

by PD-98059 (78). Resveratrol induced nonsteroidal anti-inflammatory drug-activated gene (NAG)-1 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 (81).

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 induction of apoptosis was accompanied by an increase in p53 and Bax expression, enhanced p53-binding to the *bax* promoter and decreased Bcl-x_L, Bcl-x_S, Bcl-2 expression. In addition, mRNA levels of BRCA1, BRCA2 and COX-2 were reduced (82).

Angiogenesis and invasion

Neovascularization is essential for tumor growth. Endothelial cell migration and proliferation are required for the process as well as the breakdown of existing basal membranes by matrix metalloproteinases (MMP). These enzymes are also implicated in tumor cell invasion which is the first step in 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 collagen matrix, which represents a marker for neoangiogenesis (83). 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, indicating a role for resveratrol in ROS-induced cell invasion (84). 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 inhibitory effects of resveratrol on angiogenesis were confirmed in a mouse skin model, where delayed wound healing could be demonstrated (24). Resveratrol inhibited capillary-like tube formation of human umbilical vein cells (HUVEC) and inhibited the binding of VEGF to HUVEC (30). 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 (85). The direct effects of resveratrol on MMP-2 and MMP-9 were weak (86), whereas resveratrol decreased the secretion of MMP-2 by 40% and inhibited migration of liver myofibroblasts (45).

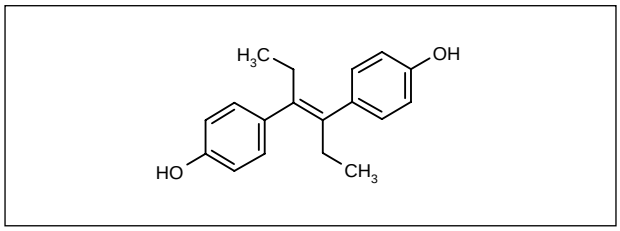


Fig. 2. Chemical structure of diethylstilbestrol.

Estrogenic/antiestrogenic properties

The stilbene structure of resveratrol is related to the synthetic estrogen diethylstilbestrol (Fig. 2). Therefore, the effect of resveratrol on estrogen receptors (ER) has been evaluated although the results obtained are inconsistent. Resveratrol bound to the human ER, inhibited receptor binding of estradiol and initiated transcriptional activity of a reporter gene construct in MCF-7 mammary carcinoma cells containing an ER-responsive element. The proliferation of T47D, an estrogen-dependent breast cancer cell line, was stimulated by resveratrol, but the stilbene was shown to possess proestrogenic activity that was weaker than that of estradiol (87). In MCF-7 cells, resveratrol inhibited growth and antagonized 17β-estradiol-induced growth, thus acting as an antiestrogen (88). In rat uterine cytosol, resveratrol acted as a weak ER ligand and transactivated the ER in ER-α and luteinizing hormone-beta cotransfected Cos-1 cells (89). Resveratrol has been shown to bind ER-α as well as ER-β, but with 7000-fold lower affinity than estradiol. Resveratrol exhibited growth inhibiting properties in CHO-K1 Chinese hamster ovary cells transfected with either ER-α or ER-β. In addition, resveratrol induced reporter gene activity of ER-responsive elements with ER-α and ER-β with equal potency. Resveratrol-liganded ER-β had a higher transcriptional activity than estradiol-liganded ER-β (90).

A study has shown that resveratrol also has effects on the androgen receptor. Treatment of the androgen-stimulated prostate cancer cell line LNCaP with resveratrol led to reduced levels of the androgen-inducible proteins $p21^{Waf1/Cip1}$, prostate specific antigen (PSA) and hK2. Experiments with a reporter gene construct revealed that resveratrol abolished activation of the PSA promoter and the androgen receptor binding site as well as activation of the androgen receptor promoter (68).

Allergies

Resveratrol and the stilbene analog rhapontigenin exhibited a potent inhibitory effect on β-hexoaminidase release from RBL-2H3 mast cells (91). Piceatannol inhibited the IgE-mediated histamine release in human basophils with an IC_{50} value of 3-5 μM (92).

Neuroprotection

Administration of resveratrol to rats protected the olfactory cortex and hippocampus from kainic acid-induced excitotoxic damage (93). Cultured hippocampal cells from rats treated with sodium nitroprusside, an NO-donor, were protected from NO-induced damage by cotreatment and posttreatment with resveratrol (94).

Antioxidant properties

Preincubation of murine peritoneal macrophages with resveratrol prevented LPS-induced production of ROS (39). Resveratrol inhibited iron and UV irradiation-catalyzed lipid peroxidation in microsomes prepared from rat liver. In addition, resveratrol scavenged 2,2'-diphenyl-*p*-picrylhydrazyl radicals whereas *trans*-stilbene did not induce any of these effects (95). Incubation of thrombocytes with 2 μM resveratrol led to reduced intracellular ROS levels. This effect was more pronounced than that observed after addition of 3 mM ascorbic acid (96). Pterostilbene was shown to possess antioxidant properties with a higher total reactive antioxidant potential than trolox, but with less potential than resveratrol (31).

HIV

Resveratrol has been shown to synergistically enhance the anti-HIV activity of the nucleoside analogs zidovudine, zalcitabine and didanosine. In infected cells with decreased susceptibility to didanosine, combination treatment with resveratrol and didanosine decreased viral replication by 80%. When administered alone, resveratrol reduced viral replication in monocyte-derived macrophages by 30% (97).

P450 and aryl hydrocarbon receptor

Whereas Frötschl *et al.* demonstrated that resveratrol is a potent inducer of cytochrome P450 1A1-mRNA in HeLa cells (98), Chun *et al.* demonstrated that resveratrol is a selective inhibitor of P450 1A1, but not of P450 1A2 and NADPH-P450 reductase (99). Aryl hydrocarbon-induced P450 1A1 and P450 1A2 activities were inhibited dose-dependently in microsomes and HepG2 hepatoma cells. Expression of the *cyp1A1* gene was also inhibited in HepG2 and MCF-7 mammary carcinoma cells. Resveratrol abolished binding of the activated aryl hydrocarbon receptor to the xenobiotic-responsive element of the *cyp1A1* promoter (100). Recombinant CYP1B1 activity was inhibited in a dose-dependent fashion when treated with resveratrol. Expression of *cyp1B1*-mRNA was also inhibited in MCF-7 cells (101). Interestingly, Potter *et al.* showed that microsomal CYP1B1 converts resveratrol to piceatannol (102). Resveratrol inhibited dioxin-mediated transactivation in an aryl hydrocarbon receptor-positive breast cancer cell line (T-47D) transfected with a dioxin response element (103).

Liver

Resveratrol downregulates smooth muscle alpha-actin in human liver myofibroblasts but not in skin fibroblasts or vascular smooth muscle cells, indicating possible activity for the management of chronic liver diseases. Neither piceatannol nor piceid induced these effects. Resveratrol also inhibited the expression of type I collagen mRNA which is a sign of antifibrogenic activity (45). In rat hepatic stellate cells, resveratrol inhibited proliferation and also suppressed the expression of smooth muscle α-actin as well as that of cyclin D1 (104). Piceatannol inhibited proliferation of liver myofibroblasts but was cytotoxic at concentrations exceeding 25 μM. On the other hand, piceid had no inhibitory effect on proliferation up to concentration of 100 μM. (45).

Conclusions

Resveratrol could be valuable in improving cancer therapies as well as chemopreventive strategies. The compound has been demonstrated to be a potent inhibitor of cancer cell growth and might therefore be useful in cancer treatment. Beneficial effects might be achieved in combination with other chemopreventive substances for new chemopreventive strategies or with chemotherapeutic drugs to improve cancer therapy. Because resveratrol reversibly inhibits the cell cycle in the S-phase, it is possible that it could enhance the effect of chemotherapeutic drugs that act specifically in the S-phase. Zoberi *et al.* have demonstrated that resveratrol sensitizes cervical cancer cell lines against ionizing radiation, which leads to a decreased cell survival following radiation (105). Resveratrol also enhances the chemopreventive effects of the short-chain fatty acid butyrate. Butyrate, a product of microbial fermentation of fibers in the colon, serves as an energy source for untransformed colonocytes but inhibits growth and induces differentiation and apoptosis of colon cancer cells. Resveratrol enhances the differentiation-inducing effect of butyrate (34). These data support the use of combinatorial strategies including resveratrol. Animal studies have shown that oral supplementation with resveratrol is nontoxic. Clinical trials are needed to evaluate the anticarcinogenic potential of resveratrol *in vivo*.

Acknowledgements

The authors would like to thank the Else Kröner-Fresenius-Foundation for financial support.

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