CHEMOPREVENTIVE AND SENSITIZING EFFECTS OF PHYTOCHEMICALS IN A CELL CULTURE MODEL OF COLORECTAL CANCER

Dissertation zur Erlangung des Doktorgrades

Dr. oec. troph.

im Fachbereich Agrarwissenschaften, Ökotrophologie und Umweltmanagement

der Justus Liebig Universität Gießen

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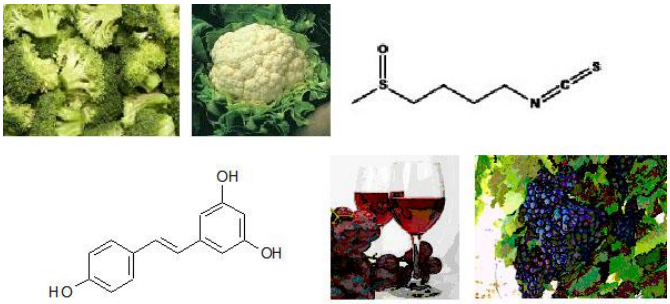

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SUMMARY

Introduction: Sulforaphane (SFN), a naturally occuring isothiocyante present in cruciferous vegetables, is an attractive target due to its wide range of biological and pharmacological properties. In addition to anti-inflammatory activities, potent chemopreventive effects of SFN in various carcinogenesis models are described whereas the underlying molecular mechanisms remain partially unknown. Previous studies could demonstrate that modulation of polyamine metabolism provides a chemopreventive strategy of different phytochemicals. Inhibition of the biosynthetic key enzyme ornithine decarboxylase (ODC) is associated with reduced cell growth in several human cancers. Thus, one aim of this work was to elucidate a possible regulation of ODC by sulforaphane and to further specify a possible role of the transforming growth factor-β (TGF-β)-signaling pathway.

Chemotherapy plays an important role in the overall treatment of colorectal cancer but the effectiveness is often limited by tumor drug resistance and severe side effects. Thus, the use of natural agents in combination with traditional chemotherapeutic drugs is a promising strategy for enhancing the therapeutic outcome. Due to their anticarcinogenic properties and their low toxicity, multiple phytochemicals may serve as potent agents for enhancing the therapeutic effect of classical chemotherapeutics. Resveratrol (Res), a natural occurring polyphenol present in red wine, peanuts and grapes, has been reported to exhibit a wide range of biological and pharmacological properties. In addition to cardioprotective and anti-inflammatory effects, also chemopreventive as well as chemosensitizing activities of Res in various carcionogenesis models are described. Therefore, another aim of this work was to investigate whether the isiothiocyante sulforaphane and the polyphenol resveratrol may potentiate the antitumor activities of the common chemotherapeutic oxaliplatin (Ox) in a cell culture model of colorectal cancer.

Methods: Caco-2 cells and human foreskin fibroblats were cultured under standard conditions and were treated with miscellaneous agents for different time intervals. For co-incubation, cells were pre-treated with SFN [10-50µM] or Res [50-100 µM] for 24 hours. Cell growth was determined by BrdU incorporation and crystal-violet staining. Cytotoxicity was analyzed by LDH release. Pro-

tein levels were examined by Western blot analysis. Reverse transcriptase-PCR was used for measuring mRNA expression. ODC activity was assayed radiometrically measuring [$^{14}CO_2$] liberation. Acetyl-Histone H3 and H4 immunoprecipitation (ChIP) assay was performed followed by PCR with TGF-β-receptor II promoter specific primers. Apoptotic events were characterized by different ELISA techniques. Annexin-V- and propidium-iodide (PI)-stained cells were detected by FACS analysis. Primary human macrophages were co-cultured with conditioned medium of treated tumor cells and cytokines were quantified by FACS. Drug interactions were assessed using the combination-index (CI) method as defined by median-effect analysis. CI<1 indicates synergism.

Results: SFN [1-50µmol/L] inhibites cell growth of Caco-2 cells in a dose- and time-dependent manner, which closely correlates with a dose-dependent reduction of ODC protein expression and activity after 24 h of incubation. This effect seems to be due to reduced protein levels and transactivation activity of transcription factor c-myc, a direct regulator of ODC expression, as a consequence of SFN-induced TGF-β/Smad signaling. The coherency of these results was further confirmed by using TGF-β receptor kinase inhibitor SB431542, which largely abolished inhibitory effects of SFN on both, ODC enzyme activity and tumor cell growth. Moreover, SFN, Res and Ox alone inhibited cell growth of Caco-2-cells in a dose-dependent manner, an effect, which could be synergistically enhanced, when cells were incubated with the combination of SFN/Ox or Res/Ox. Co-treated cells further displayed distinctive morphological changes that occurred during the apoptotic process, such as cell surface exposure of phosphatidylserine, membrane blebbing as well as the occurence of cytoplasmic histone-associated DNA fragments. Further observations thereby pointed towards simultaneous activation of both extrinsic and intrinsic apoptotic pathways. With increasing concentrations and treatment duration, a shift from apoptotic to necrotic cell death could be observed. Furthermore, cytotoxic effects of Res and Ox led to an altered cytokine profile of co-cultured macrophages. In addition, combinatorial treatment did not affect normal cells as cytotoxicity was not detected in human foreskin fibroblasts and in human platelets.

Conclusion: On the basis of these findings, diet-derived sulforaphane downregulated ODC expression and activity in colorectal cancer cells, whereby activation of the TGFβ signaling pathway seemed to play a pivotal role. Since ele-

vated ODC enzyme activity is associated with enhanced tumor development, SFN thus may be a dietary phytochemical with potential to prevent carcinogenesis.

Moreover, sulforaphane as well as resveratrol enhanced oxaliplatin-induced cell growth inhibition via induction of different modes of cell death without damaging non-transformed cells. In addition, resveratrol also showed the peculiarity to abrogate immunosuppressive properties of oxaliplatin-treated cells. Due to these results both phytochemicals show great chemopreventive and therapeutic potential in the treatment of colorectal cancer.

ZUSAMMENFASSUNG

Einleitung: Sulforaphan (SFN), ein natürlich vorkommendes Isothiocyanat, das vorwiegend in Kreuzblütlern zu finden ist, gilt aufgrund seiner potenten antikanzerogenen Wirkung als effektive, chemopräventiv wirkende Substanz. In früheren Studien konnte über eine Modulation des Polyaminstoffwechsels, insbesondere über eine Hemmung der biosynthetischen Ornithin Deacarboxylase (ODC), eine Hemmung des Zellwachstums in kolorektalen Karzinomzellen beobachtet werden. Ein Ziel unserer Arbeit war es daher, eine mögliche Sulforaphan-vermittelte Regulation der ODC sowie die Rolle des Transforming growth factor-β (TGF-β) in der SFN-induzierten Zellproliferationshemmung in Kolonkarzinomzellen näher zu charakterisieren.

In der Behandlung solider Tumore limitieren sowohl die Ausbildung von Resistenzen als auch die Toxizität der chemotherapeutischen Substanzen den Erfolg der Therapie. Ein kombinierter Einsatz sekundärer Pflanzenstoffe mit konventionellen Chemotherapeutika könnte durch synergistische Effekte auf verschiedene Signaltransduktionswege sowohl eine Steigerung der antikarzinogenen Wirkungen, sowie eine Verminderung von Chemoresistenzen bewirken. Resveratrol ist ein natürlich vorkommendes Polyphenol, das vorwiegend in Trauben, Erdnüssen und Rotwein zu finden ist. Neben kardioprotektiven und antiinflammatorischen Wirkungen werden Resveratrol auch verschiedene chemopräventive und chemotherapeutische Eigenschaften zugesprochen. Ein weiteres Ziel war es daher, mögliche synergistische Wirkungen von Sulforaphan und Resveratrol in Kombination mit Oxaliplatin in Kolonkarzinomzelllinien genauer zu charakterisieren.

Methoden: Die kolorektale Karzinomzelllinie Caco-2 und humane Vorhaut-Fibroblasten wurden unter Standardbedingungen kultiviert. Die Zellen wurden über definierte Zeiträume mit steigenden Konzentrationen verschiedener Substanzen inkubiert. Die Zellzahl wurde mittels Kristallviolett-Färbung, Zellproliferation anhand des BrdU-Einbaus in die DNA bestimmt. Zytotoxische Wirkungen der eingesetzten Substanzen wurden mittels eines Zytotoxizitätstestes (Messung der Laktat Dehydrogenase-Freisetzung) analysiert. Verschiedene Proteine wurden durch Western Blot-Analyse detektiert. RT-PCR wurde zur Messung der mRNA verwendet. Zur Aktivitätsbestimmung der ODC wird radioaktiv markier-

tes Substrat zum Zelllysat gegeben und die Freisetzung von [$^{14}CO_2$] gemessen. Ein Acetyl-Histon H3 und H4 Immunopräzipitations (ChIP) Assay gefolgt von einer PCR mit spezifischen TGF-β-Rezeptor II Promotor Primern wurde zur Ermittlung der HDAC-Wirkung von SFN verwendet. Zur Detektion apoptotischer Prozesse wurden verschiedene ELISA-Methoden angewandt. Mittels Annexin-V und Propidiumiodid-Anfärbung gefolgt von einer FACS-Analyse konnte zwischen Apoptose und Nekrose differenziert werden. Primäre humane Makrophagen wurden mit konditioniertem Medium von behandelten Tumorzellen kokultiviert. Anschließend wurden die Cytokine mittels FACS-Analyse quantifziert. Zur Identifikation synergistischer Effekte wurde der Kombinationsindex (CI) berechnet. CI<1 indiziert Synergismus.

Ergebnisse: Sulforaphan [1-50µM] hemmte konzentrationsabhängig Zellwachstum und –proliferation in Caco-2-Zellen. Weiterhin führte die Inkubation mit SFN zu einer dosisabhängigen Verminderung von ODC-Protein und –Aktivität nach 24 h, was eng mit den antiproliferativen Wirkungen korreliert. Zudem verminderte SFN sowohl die Proteinlevel als auch die Transaktivierung des Transkriptionsfaktors c-myc, der durch Bindung an eine spezifische DNA-Sequenz die ODC direkt reguliert. Eine Beteiligung des TGF-β Signalweges konnte durch die Zugabe des TGF-β Rezeptor Kinase Inhibitors SB431542, der die SFN vermittelten Wirkungen auf ODC und Zellwachstum weitestgehend aufgehoben hat, weiter bestätigt werden.

Des Weiteren hemmten SFN und Res in Kombination mit Ox synergistisch das Zellwachstum in Caco-2 Zellen. Nach kombinierter Behandlung mit SFN/Ox oder Res/Ox konnten zudem ausgeprägte morphologische Veränderungen, u.a. reduzierte ATP-Level, Caspase-3-Aktivierung und DNA Fragmentierung, die typischerweise im Zusammenhang mit Apoptose auftreten, beobachtet werden. Weiterhin konnte neben einer apoptotischen auch eine gesteigerte nekrotische Zellpopulation nachgewiesen werden, die im Falle von Resveratrol mit einer signifikant reduzierten Ausschüttung des anti-inflammatorischen Interleukin-10 einhergeht aus humanen Makrophagen. Ergänzend zeigten weder Sulforaphan noch Resveratrol in Kombination mit Oxaliplatin zytotoxische Effekte in nicht-transformiertem Zellen.

Schlussfolgerung: Die vorliegenden Daten zeigen erstmals, dass SFN seine antikanzerogenen Effekte, zumindest teilweise, über die Hemmung der ODC

vermittelt. Dabei scheint die TGF-β-Signalkaskade eine regulatorische Schlüsselrolle einzunehmen. Weitere Daten lassen darauf schließen, dass SFN und Res in Kombination mit Oxaliplatin synergistisch das Zellwachstum von Kolonkarzinomzellen durch Apoptose-Induktion hemmen. Diese *in vitro* Daten weisen auf potente chemopräventive und –therapeutische Eigenschaften der sekundären Pflanzeninhaltsstoffe Sulforaphan und Resveratrol hin, wodurch diese vielversprechende Substanzen in der Entwicklung neuer Therapiekonzepte in der Behandlung des kolorektalen Karzinoms darstellen.

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

I. **Kaminski, BM.**, Loitsch, SM., Ochs, MJ., Reuter, KC., Steinhilber, D., Stein, J., Ulrich S. (2010) Isothiocyanate sulforaphane inhibits protooncogenic ornithine decarboxylase activity in colorectal cancer cells via induction of the TGF-beta/Smad signaling pathway. Mol Nutr Food Res 54 (10): 1486-96

II. **Kaminski, BM.**, Weigert, A., Brüne, B., Schumacher M., Wenzel, U., Steinhilber, D., Stein, J., Ulrich, S. (2010) Sulforaphane potentiates oxaliplatin-induced cell growth inhibition in colorectal cancer cells via induction of different modes of cell death. Cancer Chemother Pharmacol. August 6. [Epub ahead of print]

III. **Kaminski, BM.**, Weigert, A., Ley, S., Brecht, K., Brüne, B., Steinhilber, D., Stein, J., Ulrich, S. (2010) Resveratrol-induced potentiation of the antitumor effects of oxaliplatin is accompanied by an altered cytokine profile of human-derived macrophages. Mol Nutr Food Res (under review)

IV. **Kaminski, BM.**, Steinhilber, D., Stein J., Ulrich S. (2010) Phytochemicals Resveratrol and Sulforaphane as potential agents for enhancing the anti-tumor activities of conventional cancer therapies. Curr Pharmaceut Biotechnol. Invited Review (in press)

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LIST OF ABBREVIATIONS

APAF-1	apoptotic protease activating factor-1
APC	adenomatous polyposis of the colon protein
ATP	adenosine triphosphate
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2 protein
BMP	bone morphogenic protein
BrdU	5-bromo-2-deoxyuridine
BSE	broccoli sprouts extracts
CDK	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
CI	combination index
CKI	CDK inhibitor
COX-2	cyclooxygenase II
CRC	colorectal cancer
DFMO	2-difluoromethylornithine
DISC	death inducing signaling complex
ELISA	enzyme linked immunosorbent assay
FAP	familial adenomatous polyposis
FITC	fluorescein isothiocyanate
5-FU	5-Fluorouracil
GSH	glutathione
GST	glutathione-S-transferase
HAT	histone acetyltransferase
HDAC	histone deacetylase

HFF	human foreskin fibroblasts
HNPCC	hereditary non-polyposis colorectal cancer
IAP	inhibitor of apoptosis
IC_{50}	half maximal inhibitory concentration
IL	interleukine
ITC	isothiocyanate
Keap1	Kelch-like ECH-associated protein 1
LDH	lactate dehydrogenase
LV	leucovorine
NAC	N-acetyl-cysteine
NCI	National Cancer Institute
Nrf2	nuclear factor E2-related factor 2
NSAID	non-steroidal anti-inflammatory drug
ODC	ornithine decarboxylase
Ox	oxaliplatin
PARP	poly (-ADP-ribose) polymerase
PAO	polyamine oxidase
PGE_2	prostaglandin E_2
PI	propidium iodide
Res	Resveratrol
RT	reverse transcriptase
SAMDC	S-adenosylmethionine decarboxylase
SBE	Smad binding element
SERM	selective estrogen receptor modulator
SFN	sulforaphane
Smac	second mitochondria-derived activator of caspases

SPD	spermidine
SSAT	spermidine/spermine acetyltransferase
TAMs	tumor associated macrophages
TGF-β	transforming growth factor-β
TGF-βR	TGF-β receptor
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
UV	ultra violet
XIAP	x-linked IAP

FIGURE INDEX

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1 INTRODUCTION

1.1 Colorectal cancer

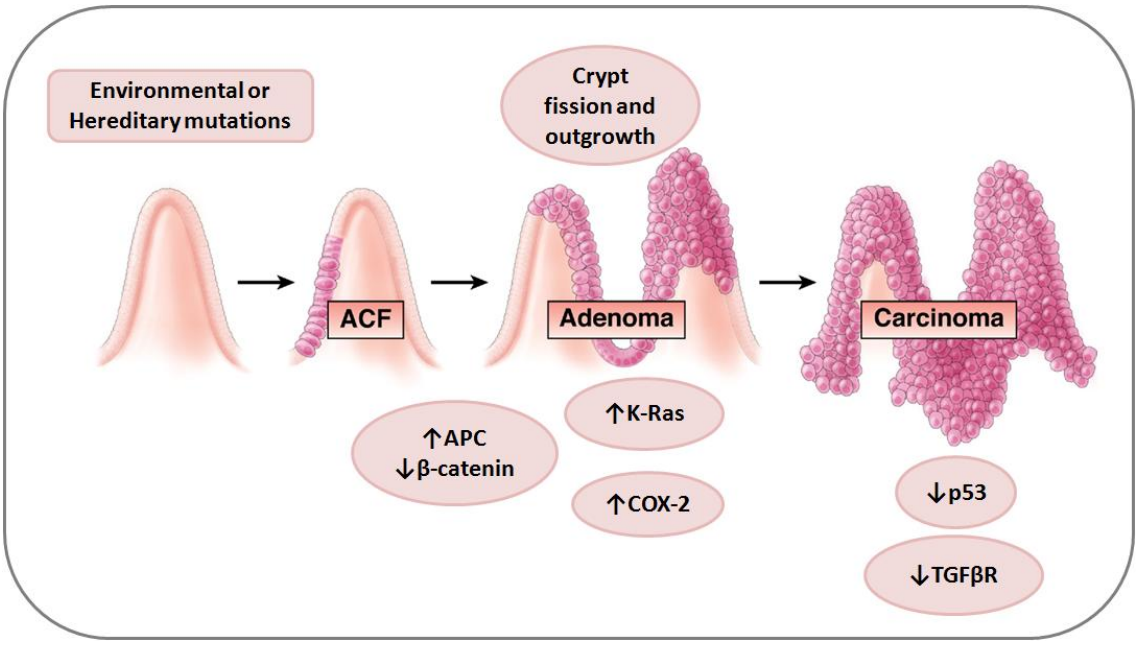


Figure 1: Adenoma-Carcinoma-Sequence

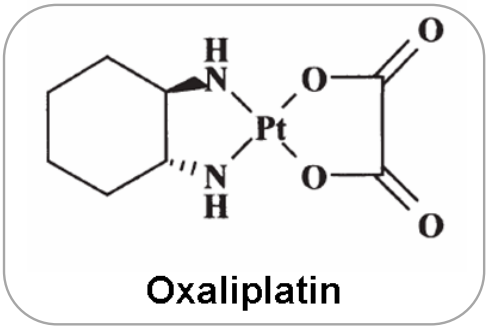
Colorectal cancer results from a series of pathologic changes that transform normal colonic epithelium into invasive carcinoma. Specific genetic events accompany this multistep process [modified from Terzic et al. [1]]

More than one million new cases of colorectal cancer (CRC) are diagnosed worldwide each year [2]. CRC is the second most common cause of cancer deaths in industrialized countries with 142.570 estimated new cases and 51.370 estimated deaths in 2010 in the United States [3] despite important advances in detection, surgery and chemotherapy [4, 5]. As with many other cancers, relative colorectal cancer risk is defined by a complex interaction between genetic and environmental influences. Only about 20% of CRC cases have a familial basis [6], which indicates the involvement of a genetic factor. There are two main inherited predisposition syndromes: the familial adenomatous polyposis (FAP) syndrome, which accounts for approximately 1% of cases of colon cancer annually, and the hereditary non-polyposis colorectal cancer (HNPCC); which accounts for 5% to 15% of cases [7-10]. The remaining 80% of colorectal tumors are attributed to so-called sporadic colorectal cancer or are caused by environmental or lifestyle factors such as physical inactivity [11], obesity [12],

smoking [13], alcohol consumption [14], a diet high in red meat [15] and inadequate intake of fruits and vegetables [16]. According to the World Cancer Research Fund 50% of colorectal cancer is avoidable through nutritional modifications [17, 18].

Although the timescale of appearance and the propensity for recurrence of these various forms of colorectal tumors differ, they share the common random pathway of the adenoma to carcinoma sequence. Vogelstein and colleagues [19] proposed a multistep model for the genetic events in the progression of sporadic colorectal cancer in which cells must accumulate a combination of four or five defects, including mutational activation of oncogenes and inactivation of tumor-suppressor genes, to undergo full malignant transformation [20] (**Figure 1**). The first event inactivates the tumor suppressor gene APC (adenomatous polyposis of the colon), which is also mutated in the case of FAP, followed by mutation of the oncogene *K-ras* and further mutations of the tumor suppressor genes *TGF-β*R and *p53* [21]. Other genetic events may also play a role in carcinogenesis of colorectal cancer, for example modulation of DNA methylation in CpG sequences of the promoter regions of tumor-suppressor and DNA-repair genes leading to inactivation or DNA amplification as a mechanism of oncogene activation [22]. All of these genetic alterations are associated with the development of preneoplastic lesions (aberrant crypt foci, polyps, adenomas) at least resulting in the development of carcinomas [23].

1.1.1 Chemotherapeutic agents in the treatment of colorectal cancer



Oxaliplatin

Despite a better understanding of the disease and the advent of modern technology and rationally targeted drugs over the past years, the prognosis for colorectal cancer patients in the advanced stage with distant metastasis still is very poor [24]. In the treatment of colorectal cancer, chemotherapy remains the cornerstone [25]. At this, diaminocyclohexane platinum compounds such as cisplatin, carboplatin, and oxaliplatin, have been used clinically for nearly 30 years as part of the treatment regimens of colorectal cancer. In general, the platinum compounds

are thought to predominantly target DNA [26]. Since Rosenberg and colleagues discovered cisplatin in the late sixties [27], around 3000 cisplatin analogs have been synthesized aiming to retain the wide antitumor spectrum of cisplatin, while at the same time reducing unwanted side toxicity. The main dose-limiting factors for the clinical outcome of cisplatin are toxicity on the nervous system, nephrotoxicity, myelotoxocity, nausea and vomiting [28-31] and auditory impairment. Other limitations are the mutagenic character of cisplatin as well as the development of resistance during clinical use [32, 33]. In the attempt to overcome these side-effects, carboplatin was tested and showed the best compromise between antitumor activity and tolerable side toxicity with respect to cisplatin [34, 35]. However, cardoplatin is a less efficient DNA-damaging agent than cisplatin, producing DNA adducts more slowly, and it is also 45 times less active than cisplatin [36]. Since then many cisplatin anlogs were made, and of these, the simple substitution of the two amino groups of cisplatin with the diaminocyclohexane group afforded agents with good antitumor activity and a lack of cross-resistance with cisplatin [37].

The best results were achieved with the third-generation platinum compound oxaliplatin, based on its water solubility [38] and promising antitumor activity with cisplatin. Oxaliplatin was first introduced into clinical trials by Mathé and colleagues in 1986 [39]. Molecular biology studies showed that oxaliplatin differs in the mechanism of action and resistance from cisplatin and carboplatin and is thereby effective in tumors with intrinsic and acquired resistance to both of these drugs [40]. The action of oxaliplatin is due to the formation of intrastrand cross-links with either two adjacent guanine or guanine-adenine residues of DNA resulting in the blockage of replication and transcription, cell cycle arrest and apoptosis [41, 42].

In addition to the approved platinum compounds, there are several substances with promising anticancer activity heading towards clinical use. Among these complexes, 5-fluorouracil (5-FU) is a fluorinated pyrimidine that acts primarily through inhibition of thymidylate synthetase, the rare-limiting enzyme in pyrimidine nucleotide synthesis. 5-FU is considered as the reference drug for the systemic treatment of metastatic CRC [43]. Its antitumor acitivity was subsequently shown to be enhanced when the drug was combined with leucovorin (LV), a

reduced folate that is thought to stabilize fluorouracil's interaction with thymidylate synthetase [44-47].

Remarkable and clinically relevant advances have been made in the last five years in the treatment of colorectal cancer, essentially because of the adoption of combination chemotherapy regimens containing oxaliplatin and irinotecan [48]. Irinotecan is a semisynthetic derivate of the natural alkaloid camptothecin and inhibits topoisomerase I, an enzyme that catalyzes breakage and rejoining of DNA strands during DNA replication [49, 50]. Both oxaliplatin combined with bolus and continuous infusion of 5-FU plus LV (FOLFOX) and irinotecan combined with bolus and continuous infusion of 5-FU plus LV (FOLFIRI) are recognized as standard first-line therapies for metastatic CRC in which FOLFOX is preferred in the upfront treatment option in the U.S. [51]. In particular, combination regimens that incorporate infusional schedules of FOLFOX show significantly improved clinical efficacy as related to overall response rates, time to tumor progression, and median overall survival in colorectal cancer patients [52, 53].

1.2 Chemoprevention

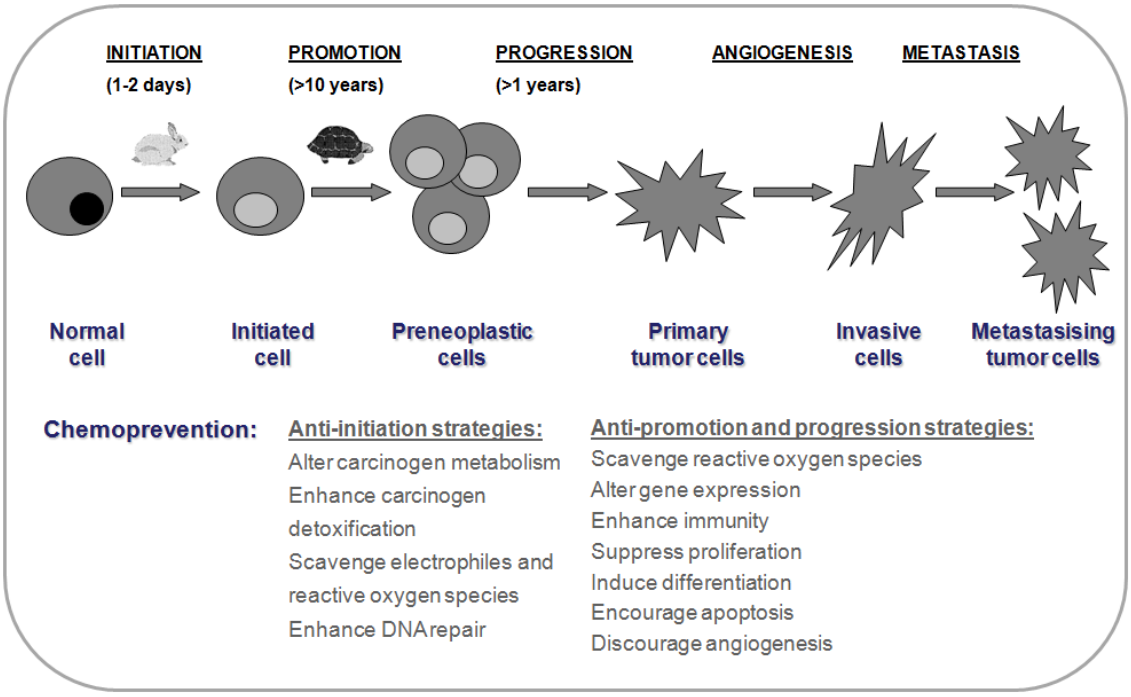


Figure 2: Chemopreventive strategies

Chemoprevention is defined as the employment of natural or synthetic agents that reverse, inhibit, or prevent the development of benign or malignant tumors [54]. In keeping the fact that more than two-third cancers could be prevented through appropriate lifestyle modifications, attention has recently focused on screening and chemoprevention as potential methods for reducing the number of cases of colorectal cancer [55]. Beside the five classes of mainly chemically synthesized chemopreventive agents like selective estrogen receptor modulators (SERMs), non-steroidal anti-inflammatory drugs (NSAIDs), calcium compounds, glucocorticoids and retinoids, indicated by the National Cancer Institute (NCI), diet-derived compounds may have significant impact on qualifying or changing recommendations for high-risk cancer patients and thereby increase their survival through simple dietary choices with easily accessible foods. This preventive measures are believed to target the multi-step process of carcinogenesis involving initiation, promotion and progression [56] and therefore decrease the incidence and mortality of cancer. In detail, the cellular and molecular mechanisms affected by chemopreventive agents include carcinogen activa-

tion/detoxification by xenobiotic metabolizing enzymes, DNA repair, cell cycle progression, cell proliferation, differentiation and apoptosis, expression and functional activation of oncogenes or tumor-supressor genes, angiogenesis and metastasis and hormonal and growth-factor activity [57] (further chemopreventive strategies are reviewed in **Figure 2**).

Following requirements for an effective and acceptable chemopreventive agent should be fulfilled [58]:

- Little or non toxic effects in normal and healthy cells
- High efficacy against multiple sites
- Capability of oral consumption
- Known mechanism of action
- Low cost
- History of use by the human population
- Acceptance by the human population

1.2.1 Induction of apoptosis

Apoptosis, the programmed cell death, is a tightly controlled cell suicide that occurs under a range of physiological and pathological conditions, resulting in cell shrinkage, chromatin condensation and DNA fragmentation [59]. These distinctive alterations are triggered by the proteolytic activity of a family of cysteinyl aspartate-specific proteases, known as caspases, which dismantle the cell by cleaving and thus inactivating key cellular proteins including the DNA repair enzyme poly(-ADP-ribose) polymerase (PARP) [60]. This cellular destruction results in the formation of apoptotic bodies that are subsequently eliminated by phagocytosis [59, 61]. Caspases are synthesized as inactive proenzymes, which are activated by cleavage at specific aspartate residues to activate enzymes comprising large (p20) and small (p10) units [62]. A subset of caspases, termed initiator caspases interact with specific adapter molecules that facilitate their autoprocessing.

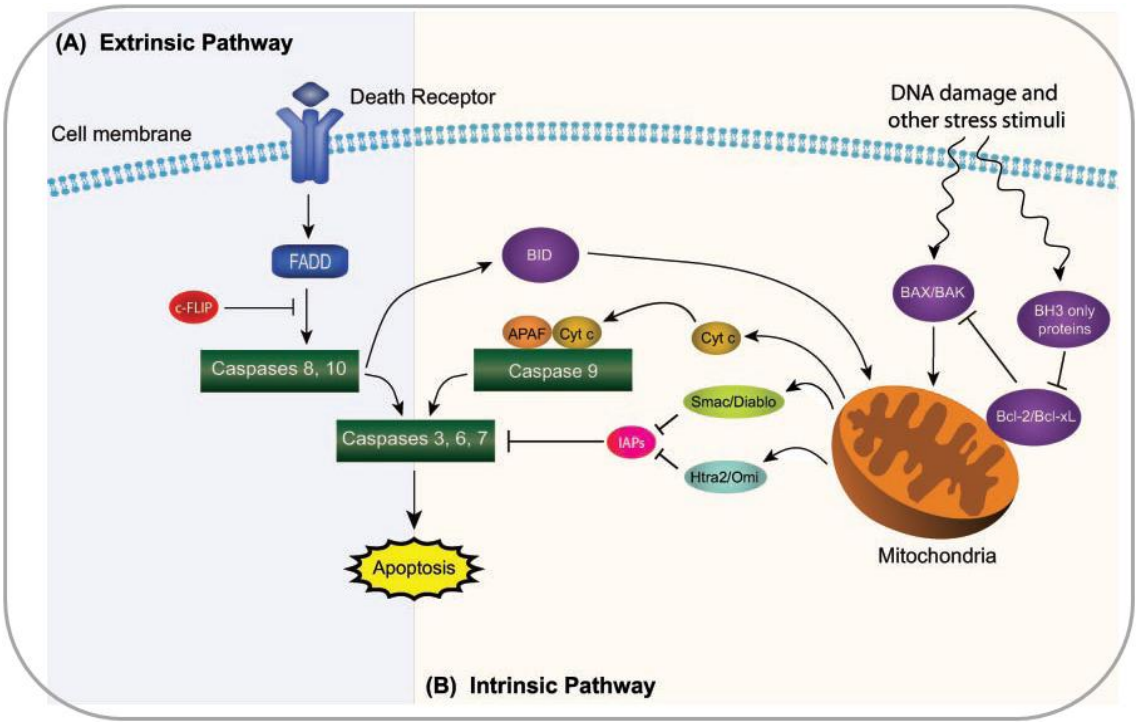


Figure 3: Extrinsic (a) and intrinsic (b) apoptotic pathway

Both pathways cause the activation of members of the caspase family which trigger the morphological process of cell death defined as apoptosis [adapted from Plati et al. [63]]

Upon activation, initiator caspases process a second class of caspases, termed effector caspases, which act on key cellular proteins, resulting in the dissolution of the cell [64, 65]. At present, two major apoptosis pathways have been identified: the death receptor or extrinsic pathway and the mitochondrial or intrinsic pathway (summarized in **Figure 3**). The extrinsic pathway is activated through cell surface death-receptors binding their respective cytokine ligands [59], followed by induction of the initiator caspase-8 and subsequent activation of the effector caspase-3. Death receptors, like tumor necrosis factor (TNF) receptor, TRAIL receptor and Fas belong to the TNF receptor superfamily which consists of more than 20 proteins with a broad range of biological functions [66]. The intrinsic pathway depends on mitochondrial membrane permeabilization, which causes the release of apoptogenic factors, such as cytochrome c and Smac/DIABLO from the intermembrane space to the cytoplasm [67]. Once released, cytochrome c directly activates Apaf-1 and, in the presence of dATP or ATP, induces the formation of a multimeric apoptosome complex, resulting in

the activation of the caspase-9 followed by the cleavage and activation of caspase-3 and -7. The mitochondrial pathway is controlled by members of the Bcl-2 family regulating mitochondrial membrane permeability and subsequent release of proapoptotic factors. They can be divided into a pro- and antiapoptotic group [68]. The antiapoptotic subclass consists of Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and A1, which act on the outer mitochondrial membrane by neutralizing the killer proteins Bax and Bak. In addition to their role in executing apoptosis, caspases also play important signaling roles in nonapoptotic processes, including regulation of actin dynamics, innate immunity, cell proliferation, differentiation, and survival [69]. Under such conditions, caspase activation does not lead to cell death. The required regulation of caspase activity is partly mediated by members of the inhibitor of apoptosis (IAP) protein family. The IAPs are a family of caspase inhibitors that directly bind caspase-3, -7 and/or -9 and thereby impair the activity of these critical effectors of apoptosis [70]. Elevated levels of IAPs, such as survivin, have been found in numerous types of malignant cells, and their over-expression is associated with chemoresistance and poor prognosis [70]. Apart from apoptosis, alternative forms of cell death can be activated, e.g. necrosis or autophagy, which might also lead to biological consequences differing from apoptosis [71].

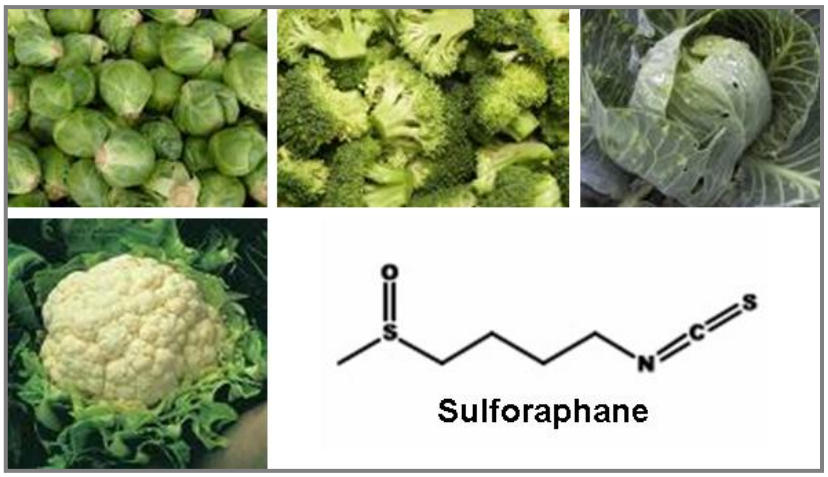
1.3 Phytochemicals

Figure 4: Dietary agents and their major biological active compounds [adopted from Ulrich S.[72]]

Epidemiological studies have consistently indicated that a high dietary intake of fruits and vegetables is strongly associated with reduced risk of developing chronic diseases, such as cancer and cardiovascular disease [73-75]. Phytochemicals, defined as bioactive nonnutrient plant compounds in fruits, vegetables, grains, and other plant foods, have been linked to risk reduction of major chronic diseases [76]. Examples of phytochemicals that show promise as cancer chemopreventive agents include sulforaphane, resveratrol, epigallocatechin gallate (EGCG), capsaicin, curcumin, 6-gingerol, and lycopene [77-79] (summarized in **Figure 4**). Using Phytochemicals is among the most promising chemopreventive and treatment options for the management of cancer. In this regard, the isothiocyanate sulforaphane as well as the polyphenolic resveratrol repre-

sent such ideal molecules, due to their relatively low toxicity and capacity to target multiple signaling molecules that collectively promote cancer cell survival and tumor growth. Both phytochemicals will be introduced in the following section.

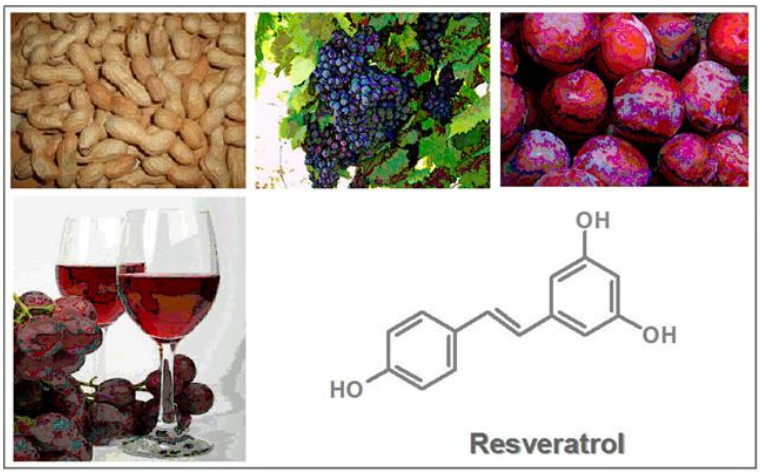
1.3.1 Sulforaphane



Increasing evidence suggests the importance of food and nutrition in the modification of the cancer development [80]. In particular, in vitro and in vivo data revealed that consumption of cruciferous vegetables is supposed to lower overall cancer risk, including colon cancer, especially during the early stages [81]. These anticarcinogenic activities have been suggested to be partly due to the relatively high levels of isothiocyanates accumulating within these vegetables. The content can vary between and within members of the cruciferous family depending on cultivation environment and genotype [82]. Isothiocyanates are generated from glucosinolates either by the action of plant thioglucosidases or, if the plant enzymes have been denatured by cooking, by the action of microbial enzymes in the colon. Sulforaphane (SFN) (1-isothiocyanato-4-methylsulfinyl butane), the major isothiocyanate, was firstly isolated from broccoli in the early 1990's and is also abundantly present in cauliflower, cabbage, and kale with the highest concentration found in broccoli sprouts [83]. It was initially identified as a potent phase 2 enzyme inducer, but in recent years numerous studies have implicated further chemopreventive effects, comprising cell growth inhibition, induction of apoptosis and reduction of angiogenesis [60, 84-86]. We and others provide several lines of evidence that sulforaphane mediates these anti-carcinogenic effects partly through inhibition of the protooncogenic ornithine decarboxylase via induction of the TGF-β pathway (MANUSCRIPT I and [87, 88]). In addition to the chemopreventive properties, we could demonstrate potent chemosensi-

tizing effects of sulforaphane in colorectal cancer cells making it to a prominent agent for enhancing the therapeutic outcome (MANUSCRIPT II).

1.3.2 Resveratrol



Another promising dietary phytochemical with chemopreventive and chemotherapeutic potential is the polyphenol resveratrol (*trans*-3,5,4 trihydroxy-stilbene) [89], which has first been isolated from the roots of white hellebore (Veratrum glandiflorum) [90]. Resveratrol is also classified as a phytoalexin, or plant antibiotic, produced in large quantitities in varius plants in reponse to environmental stresses such as injury, ultraviolet (UV) irradiation [91] and pathogenic attacks such as infection with Botrytis cinerea [92] and acts as a natural inhibitor of cell proliferation. Most widely known as a constituent of red wine [93], resveratrol has been detected in more than 70 plants species, including grapes, berries, plums, peanuts, and pines [94]. Fresh grape skin contains about 50 to 100 µg of resveratrol per gram wet weight [95], and the concentration in wine ranges from 0.2 mg/l to 7.7 mg/l. The epidemiological finding of an inverse relationship between consumption of red wine and incidence of cardiovascular disease has been called the "French paradox" [96, 97]. For a variety of reasons, the cardioprotective effects of red wine have been attributed to resveratrol [98]. These effects include suppression of lipid peroxidation [99] and eicosanoid synthesis [100], inhibition of platelet aggregation, and antioxidant, anti-inflammatory and vasorelaxant activities [101-103]. Extensive research during the last two decades has suggested that, besides cardioprotective effects, resveratrol exhibits potent chemopreventive and chemotherapeutic activities [92, 94, 104]. In a pioneering study, John M. Pezzuto and his colleagues [104] reported that resveratrol was effective in blocking all three stages (i.e., initiation, promotion and progression) of carcinogenesis. These properties of resveratrol have been explained mainly by its activities in several cell signal-

ing pathways including cell cycle arrest, suppression of tumor cell proliferation, induction of apoptosis and differentiation, reduction of inflammation and angiogenesis, and inhibition of adhesion, invasion, and metastasis [105-107]. We previously reported that chemopreventive properties of resveratrol are partly due to the inhibitory effects on polyamine metabolism [108-111]. In addtition to these findings, we could show that resveratrol is able to enhance the chemosensitvity of cancer cells with the distinctive feature to abrogate immunosuppressive properties of oxaliplatin-treated cells (MANUSCRIPT III) .

1.4 Aim

In addition to conventional therapy, preventive measures that target the multi-step progress of carcinogenesis involving initiation, promotion and progression are aimed at decrease the incidence and mortality of cancer. Indeed, multiple natural compounds have been shown to be effective for blocking carcinogenesis in certain human cancers and animal models. Using non-toxic chemical substances is regarded as a promising alternative for the prevention and control of human cancer. Moreover, recent studies have shown that the survival outcomes can possibly be further improved by adding biologic agents to combination chemotherapy regimens in first and subsequent lines of therapy. However, the precise underlying molecular mechanisms remain largely unknown. Thus, the aim of our study was to characterize chemopreventive and chemosensitizing effects of the two phytochemicals sulforaphane and resveratrol in a cell culture model of colorectal cancer.

2 RESULTS

2.1 Isothiocyanate sulforaphane inhibits protooncogenic ornithine decarboxylase activity in colorectal cancer cells via induction of the TGF-β/Smad signaling pathway (MANUSCRIPT I)

Regarding the role of polyamines in colonic carcinogenesis, inhibiting the polyamine metabolism, particularly the biosynthetic key enzyme ornithine decarboxylase (ODC), is considered to represent an attractive target for both cancer chemotherapy and cancer chemoprevention [111, 112]. Hence, the aim of this work was to elucidate a possible regulation of ODC by sulforaphane also with regard to a possible involvement of the TGF-β signaling pathway. In the present study, we could demonstrate that SFN [1-50µM] inhibited cell counts and proliferation significantly in Caco-2 cells in a dose and time dependent manner (***$p<0.001$), which closely correlated with a dose-dependent reduction of ODC protein levels after 3 and 6 h and activity after 24 h of incubation (***$p<0.001$). The crucial role of polyamine depletion was further suggested since addition of exogenous spermidine significantly (***$p<0.001$) counteracted growth inhibitory effects of SFN after 24 h. We also demonstrated that decreased ODC activity and protein levels in Caco-2 cells are accompanied by decreased protein levels of c-myc after 6 h of incubation, implicating a direct modulation of ODC gene expression by SFN. We previously reported that induction of the TGF-β signaling pathway in colon cancer cells is a crucial event in the anti-carcinogenic activities of butyrate, another natural occurring HDAC inhibitor [113]. This let us consider whether SFN possibly shows similar modes of action in our context. Thus, we measured protein as well as mRNA level of TGF-β in Caco-2 cells and could show an obvious increase of TGF-β precursor levels after 2 h and an increase of mRNA level after 1 and 3 h of incubation with SFN, indicating regulatory effects on the level of transcription. Not only TGF-β alone, but also the expression status of TGF-β receptors RI and II is essential for TGF-β-mediated actions. Therefore, protein levels of both receptors I and II were detected after incubation with SFN, and were found to be highly increased after 1 h

(***p<0.001). To further specify these regulatory mechanisms, we performed chromatin immunoprecipitation analysis and observed an accumulation of acetylated histone H3. These findings indicated histone acetylation to be involved in the transcriptional induction of RII and further suggested the importance of HDAC inhibitory properties of SFN in the regulation of TGF-β signaling. As both TGF-β receptor I and II protein levels were regulated by SFN, we were interested, whether co-incubation with exogenous TGF-β [20 nM] might amplify SFN-mediated reduction of cell proliferation in Caco-2 cells. For this, we analyzed BrdU incorporation after 24 h of treatment, but could only detect significant additive effects at a concentration of 5 µM SFN (**p<0.01). This, in accordance with our above-mentioned findings on intracellular TGF-β levels, let us hypothesize, that higher doses of SFN might lead to intracellular TGF-β-saturation and thus resistance to exogenous TGF-β. Upon ligand binding, receptors of the TGF-β family generally phosphorylate Smad proteins, which then move into the nucleus where they activate transcription of different target genes. For responding the question whether SFN-mediated TGF-β-signaling also involves an activation of Smads, we first analyzed the phosphorylation status of Smad2 and Smad3 in Caco-2 cells after 1 h of incubation with increasing concentrations of SFN, which was found to be significantly induced in a dose-dependent manner (***p<0.001). Then we performed reporter gene assays with SBE_4luc, where 4 Smad binding elements are cloned in the pGL3-vector. Reporter gene activity of SBE_4luc was significantly induced by SFN (**p<0.01), whereas the induction could be further enhanced when expression vectors pCGN-Smad3/pCGN-Smad4 were cotransfected (*** p<0.001). To give the direct evidence that sulforaphane mediates growth inhibitory effects, at least partly, via induction of the TGF-β-signaling pathway, we simultaneously treated the cells with SFN and the specific transforming growth factor superfamily type 1 activin receptor like kinase inhibitor SB431542 alone and in combination and measured BrdU incorporation after 24 and 48 h. And actually, cell growth inhibitory effects of SFN were largely abolished by SB431542 (***p<0.001). Since Smad proteins were able to suppress c-myc activity in human skin epithelial cells by directly binding to Smad-responsive elements in the *c-myc* promoter [114], we were interested whether the observed down-regulation of c-myc protein by SFN might also be due to an activation of Smad signaling. Therefore, we

have done reporter gene assays after 6 h of SFN-treatment on Caco-2 cells either transfected with wildtype c-myc-luc or with c-myc-luc bearing mutated Smad-binding-elements (SBEs). TGF-β was used as a positive control. While in Caco-2 cells transfected with the wild type construct both SFN and TGF-β significantly decreased reporter gene activity compared to untreated cells (***$p<0.001$), no effects could be observed in cells transfected with a mutated c-myc-promoter. So, we conclude that due to the mutation in the SBEs, Smads could not efficiently bind to the promoter resulting in an abolishment of the SFN- and TGF-β-mediated inhibition of c-myc gene activity. Since our results revealed an involvement of the TGF-β signaling pathway in SFN-mediated down regulation of the *c-myc* promoter, we suggested that modulation of TGF-β signaling might also affect downstream ODC expression and activity. Indeed we could observe that co-incubation of SFN with a specific TGF-β kinase inhibitor partly abolished SFN-induced reduction of ODC protein expression and activity, which might be due to direct TGF-β/Smad signaling-mediated transcriptional repression of transcription factor c-myc, upstream of the ODC gene. These data provide evidence for modulatory effects of SFN on intracellular polyamine levels by regulating gene expression of the protooncogene ODC in colorectal cancer cells, whereby the activation of the TGFβ/Smad signaling pathway seems to play a pivotal role.

2.2 Sulforaphane potentiates oxaliplatin-induced cell growth inhibition in colorectal cancer cells via induction of different modes of cell death (Manuscript II)

$$CI = \frac{\text{Dose of SFN}}{IC_{50}\ (SFN)} + \frac{\text{Dose of Ox}}{IC_{50}\ (Ox)}$$

Descriptive meanings of CI values	
Combination index range	Description
<0.1	Very strong synergism
0.1-0.3	Strong synergism
0.3-0.7	Synergism
0.7-1.0	Additive
1.0-3.3	Antagonism
3.3-10	Strong antagonism
>10	Very strong antagonsim

Figure 5: Descriptive meanings of CI values
[modified from Tumber et al. [115]]

Since several in vitro and in vivo studies show first promising results regarding the chemosensitizing capability of phytochemicals in different cancer models [116], we were interested, whether plant-derived SFN might be able to enhance Ox-induced anti-tumor activities in colorectal cancer cells. Thus, for MANUSCRIPT II, we first investigated possible synergistical antiproliferative effects of SFN and Ox in the colorectal cancer cell line Caco-2. Both substances significantly inhibited proliferation in Caco-2 cells in a dose-dependent manner (* $p<0.5$, ** $p<0.01$, *** $p<0.001$). The IC_{50}s for SFN and Ox in Caco-2 cells were 26.35 µM and 5.58 µM respectively. For studying combination effects, the cells were exposed to both SFN and Ox simultaneously for 24 h and co-treatment of the cells significantly reduced the IC_{50} values of the single drugs. The obtained data were analyzed by the Combination Index (CI)-method of Chou and Talalay

(**Figure 5**) [117]. In Caco-2 cells, we calculated a CI of 0.3, which indicates strong synergism. In the next experiments we investigated distinctive morphological changes that occurred during the apoptotic process. For a rapid screening of cell death we therefore treated Caco-2 cells for 1-24 h with SFN [10-20 µM] and Ox [500 nM] alone and in combination and measured effects on the ATP/ADP ratio. Changes in the intracellular ATP/ADP ratio are a useful indicator to distinguish between different modes of cell death and viability. Although, decreasing ATP and increasing ADP levels are generally found in apoptotic cells, cells will rather undergo necrosis when intracellular ATP levels fall below a critical threshold [118]. Incubation of the Caco-2 cells with SFN and Ox resulted in a significant time- and, at least after 6 h, also dose-dependent reduction of intracellular ATP which reaches a maximum after 24 h (*** $p<0.001$). As a positive control, we used staurosporine [0.5 µg/ml], a well-known inducer of apoptosis [119]. As DNA cleavage is another hallmark for apoptosis, we further quantified histone-complexed DNA fragments in Caco-2 after 24 h of treatment. SFN [20µM], in contrast to Ox [500 nM], thereby leads to a significant increase of cystoplasmic histone associated DNA fragments, an effect which could be further enhanced, when the drugs were used in combination (*** $p<0.001$). The activity of the effector caspase-3 was significantly activated 24 h after stimulation with SFN [20 µM] and Ox [500 nM], respectively, but these effects were not very prominent when compared to the positive control staurosporine. However, SFN could significantly enhance the Ox-induced effects (*** $p<0.001$), which is in agreement with the observed cleavage of PARP (*** $p<0.001$), a classical substrate for activated caspase-3. Proteolysis of PARP usually is an indicator for early apoptotic events. Further experiments could demonstrate that thereby apparently both, extrinsic and intrinsic apoptotic pathways were involved, as indicated by caspase-8-cleavage (*** $p<0.001$) and increased mitochondrial membrane permeabilization (*** $p<0.001$). For detecting mitochondrial membrane permealization, cells were incubated with SFN [20 µM] and Ox [500 nM] alone and in combination for 6 and 24 h before being stained with JC-1. JC-1 is a mitochondrial-selective dye and forms aggregates in normal polarized mitochondria resulting in a red-green emission of 590 nm after excitation at 490 nm. Upon depolarization of the mitochondrial membrane, JC-1 forms monomers that emit only green fluorescence at 527 nm. SFN induced a depolarization of the

mitochondrial membrane potential, which was significant after 24 h of treatment. In contrast to SFN, Ox-treatment did not show any detectable effects, neither after 6 nor 24 h of incubation. However, combinatorial treatment resulted in a distinct decrease of the red-green fluorescence intensity ratio after 6 h (** $p<0.01$) and 24 h (*** $p<0.001$). Interestingly, we could also observe an induction of TRAIL protein levels (*** $p<0.001$), a member of the TNF family of cytokines, which can induce apoptotic cell death in a variety of tumor cells [120-122]. This suggests that induction of endogenously expressed TRAIL after SFN/Ox-treatment for 6 h may further enhance their therapeutic outcome. To discriminate between different modes of cell death, Caco-2 cells treated with SFN [20 µM], Ox [500 nM-1 µM] alone and in combination for 5 and 24 h, were analyzed by Annexin V-FITC/PI labelling and flow cytometry. After 5 h of incubation, Annexin V-FITC positive but also PI-positive cells could be measured indicating direct necrotizing effects of SFN and Ox. Compared to the 5 h-treatment, the population of apoptotic cells in untreated and SFN [20 µM]- as well as Ox [500 nM-1 µM]-treated Caco-2 cells seemed to decline after 24 h. However, the rate of apoptosis was still significantly induced in the co-treated cells, but again this effect could not be further increased with a higher concentration of Ox [1 µM]. Rather, at this concentration, a distinct population of PI-stained cells could be observed, whereas a mixture of cells undergoing rapid primary as well as secondary necrosis/late apoptosis can be assumed. Obviously, apoptotic effects seemed to reach a maximum after 5 h of treatment, which is replaced by a shift towards an increased population of necrotic cells after 24 h. These observations could also be confirmed by SYTOX Green staining which was used to analyze late apoptosis. Binding of SYTOX Green stain to nucleic acids of Caco-2- cells incubated with SFN [20µM] and Ox [500nM], clearly presented signs of apoptotic events indicated by cell shrinkage, chromatin condensation and the formation of apoptotic bodies. However, again with increasing concentrations of Ox [1µM], necrotic alterations became more prominent. To analyze a possible toxicity of SFN and Ox on normal tissue cells, human foreskin fibroblasts were treated either with SFN [10-20µM] and Ox [500nM-1µM] alone or in combination and cell proliferation as well as LDH-release as a marker of direct cytotoxicity were measured after 24 h. Actually, SFN alone was found to significantly inhibit cell growth of HFF in a dose-

dependent manner (* $p<0.05$; ** $p<0.01$, *** $p<0.001$), but in contrast to Caco-2 cells, this effect was not further enhanced and no signs of cytotoxicity could be observed when SFN was combined with oxaliplatin. Taken together, these observations clearly indicate that depending on the applied concentration and the treatment duration, antiproliferative effects of SFN/Ox against Caco-2 cells can be associated with both apoptotic as well as necrotic events.

2.3 Resveratrol-induced potentiation of the anti-tumor effects of oxaliplatin is accompanied by an altered cytokine profile of human-derived macrophages (MANUSCRIPT III)

In MANUSCRIPT III, we explored the possible chemosensitizing capability of resveratrol in a cell culture model of colorectal cancer, also by analyzing possible effects on immune cells. Our first results quickly revealed Res-induced potentiation of cell growth inhibition mediated by Ox (*** $p<0.001$), which was more-than-additive as indicated by combination index analysis (CI=0.51). These cell-growth inhibitory effects of Res/Ox were accompanied by different hallmarks of apoptosis. First, changes in the intracellular ATP/ADP ratio, as a useful indicator to distinguish between different modes of cell death and viability, were analyzed. We could demonstrate that incubation with Res [50-100 µM] and Ox [1 µM] resulted in a significant dose-, and partially time-dependent reduction of intracellular ATP which reaches a maximum after 24 h (*** $p<0.001$). We further quantified histone-complexed DNA fragments in Caco-2 after 24 h of treatment. Res [50-100 µM] in contrast to Ox [1 µM] led to an increase of cytoplasmic histone-associated DNA fragments, an effect which could be significantly enhanced, when the drugs were used in combination (*** $p<0.001$ vs. Ox). Additionally, the effector caspase-3, which is one of the key proteases in the apoptotic pathway, was also induced by Res [100 µM]. This effect could be significantly enhanced by co-stimulation with Res and Ox (*** $p<0.001$), which is in agreement with the observed cleavage of PARP (*** $p<0.001$), a classical substrate for activated caspase-3. Further experiments could demonstrate that the intrinsic apoptotic pathway plays a major role in apoptosis-inducing efficacies of Res, as Res alone already significantly decreased the mitochondrial membrane potential (*** $p<0.001$). However, Res-induced depolarization of the mitochondrial membrane could not be further enhanced after combinatorial treatment. We also observed reduced protein levels of survivin after 24 h of drug exposure, whereas this effect was more prominent when the cells were co-treated with Res and Ox (*** $p<0.001$). To discriminate between different modes of cell death, Caco-2 cells treated with Res [50-100 µM], Ox [1 µM] alone and in combination for 5 and 24 h, were analyzed by Annexin V-FITC/PI labelling and

flow cytometry (also described in MANUSCRIPT II). Interestingly after 5 h of treatment, Annexin V-FITC positive but also PI-positive cells could be measured indicating direct necrotizing effects of Res and Ox. Compared to the 5 h-treatment, the population of apoptotic cells in untreated and Res [50-100 µM] as well as Ox [1 µM]-treated Caco-2 cells seemed to decline after 24 h. However, the rate of apoptosis was significantly induced in the co-treated cells (** $p<0.01$). Rather, at this time point, a distinct population of PI-stained cells could be observed, so that a mixture of cells undergoing rapid primary as well as secondary necrosis/late apoptosis can be suggested. Obviously, apoptotic effects seemed to reach a maximum after 5 h of treatment which is replaced by a shift towards an increased population of necrotic cells after 24 h. In the next experiments, possible effects of Res and Ox on the cytokine profile as a result of the observed necrotic events were investigated by co-culture experiments (**Figure 6**). Human primary macrophages were incubated with conditioned medium of treated or control tumor cells for 24 h followed by a quantification of production of the cytokines TNF-α, IL-10, IL-8 and IL-1β. We could show that macrophages that were incubated with the supernatant of Res- or Ox-treated Caco-2- cells released slightly elevated levels of the pro-inflammatory cytokines TNF-α, IL-8, and IL-1β. This effect was markedly counteracted when macrophages were incubated with the supernatant of Caco-2 cells treated with Res and Ox in combination (* $p<0.05$ vs. Ox). Interestingly, even though co-culture of macrophages with Res/Ox-treated Caco-2 cells significantly counteracted the release of IL-8 mediated by co-culture with Ox [1 µM] (* $p<0.5$), this combination still provoked a marked increase in IL-8 production as compared to control macrophages (*** $p<0.001$), similar to single drug exposure (*** $p<0.001$). Most importantly, release of the cytokine IL-10 by macrophages from Ox-treated co-cultures was increased, compared to control macrophages, an effect which could be significantly abolished when the Caco-2 cells were stimulated with Res and Ox in combination (* $p<0.05$). Thus, although tumor cells treated with a combination of Res and Ox did not elicit a strong production of pro-inflammatory mediators, production of immunosuppressive IL-10 was completely abolished.

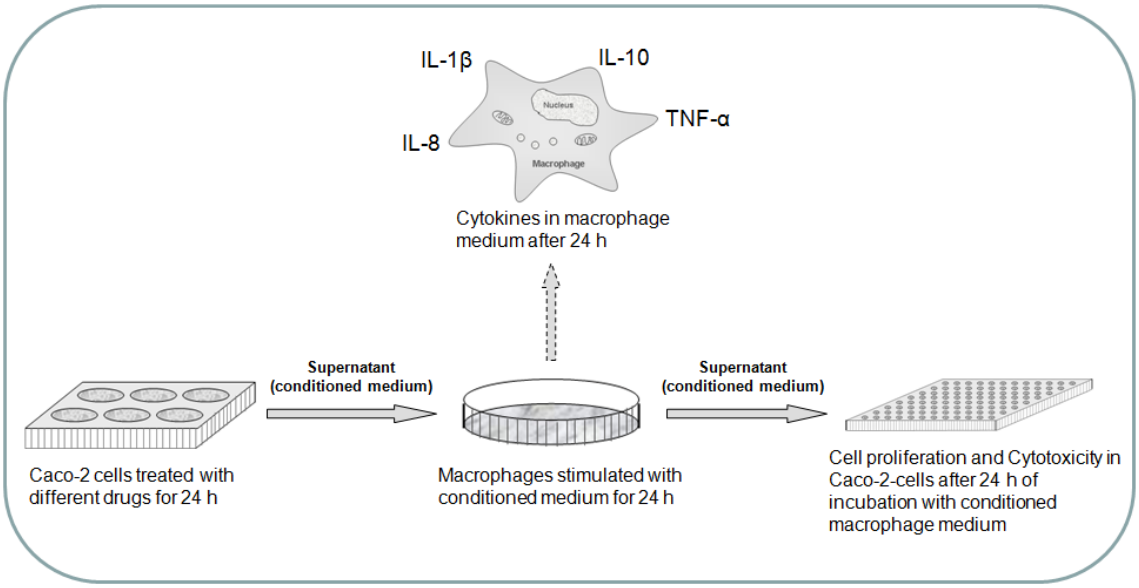


Figure 6: Co-culture experiments: Caco-2 cells were treated with Res [100 µM], Ox [1 µM] or their combination. After 24 h of drug exposure, supernatant (conditioned medium) was collected and human monocyte-derived macrophages were incubated with conditioned medium for further 24 h. Quantification of the cytokines TNF-α, IL-1β, IL-8 and IL-10 was done by FACS analysis with BD Cytometric Bead Array Flex Sets. Effects of conditioned medium on cell death of Caco-2 cells after 24 h of incubation were measured by BrdU incorporation and LDH release.

To investigate effects of conditioned medium on tumor growth in turn, we treated Caco-2 cells with the supernatant of co-cultured macrophages and measured cell proliferation as well as LDH release after 24 h of stimulation. We could demonstrate that cell growth inhibition (*** $p<0.001$) as well as lysis (*** $p<0.001$) of Caco-2 cells were significantly enhanced, especially when the cells were treated with the supernatant of co-cultured macrophages with Res/Ox-treated Caco-2 cells. Responding to the question whether Res and Ox, alone and in combination, cause severe toxicity to normal tissue cells, we treated human foreskin fibroblasts and human platelets with Res [50-100 µM], Ox [1 µM] alone and in combination and quantified LDH-release as a marker of direct cytotoxicity after 24 h. In healthy human fibroblasts, Res was found to inhibit cell growth in a dose-dependent manner. However, in contrast to Caco-2 cells, growth inhibitory effects on fibroblasts were not further enhanced and no signs of cytotoxicity could be observed when Res was combined with Ox, indicating a selective toxicity towards the tumor cell line while inducing only growth arrest in

normal fibroblasts. Additionally, neither Res or Ox alone nor their combination caused any signs of cytotoxicity in human platelets. In summary, the polyphenol resveratrol enhances oxaliplatin-induced cell growth inhibition with the feature to abrogate immunosuppressive properties of oxaliplatin-treated cells while showing no toxicity on non-transformed cells.

3 DISCUSSION

3.1 Chemopreventive strategies of the isothiocyanate sulforaphane

The plant-derived isothiocyanate sulforaphane exhibits multiple chemopreventive effects comprising cell growth inhibition, induction of apoptosis, inhibition of histone deacetylases, modulation of inflammation and inhibition of angiogenesis [reviewed in [60, 123]], whereby the underlying molecular mechanisms are only partly understood. One of our theories is concerning the role of polyamines or polyamine biosynthesis respectively (MANUSCRIPT I). Intracellular polyamine level are strictly controlled because decreases of polyamine concentrations interfere with cell growth, whereas an excess seems to be toxic [124]. Regulation of polyamine levels is governed primarily by activity of ornithine decarboxylase (ODC), the key enzyme of polyamine biosynthesis. Hence, regarding the role of polyamines in colonic carcinogenesis, modulation of polyamine metabolism and, in particular, of ODC activity has been studied as a new perspective in cancer prevention [111, 112]. One aim of our work was to investigate a possible regulation of ODC by the natural histone deacetylase inhibitor sulforaphane and to further identify a possible role of transforming growth factor-β (TGF-β) in this process.

3.1.1 Inhibition of histone deacetylases

In addition to genetic changes, epigenetic alterations of genes may contribute to cancer development [125, 126]. Among these modifications, histone acetylation of nuclear histones plays a central role in gene regulation resulting in transcriptional regulation [127]. In general, addition of acetyl groups to histones by histone acetyltransferases (HATs) results in an “open” chromatin conformation, thereby facilitating gene expression by allowing transcription factors access to DNA [128]. Removal of acetyl groups by histone deacetylases (HDACs) results in a “closed” conformation, which represses transcription (**Figure 7**).

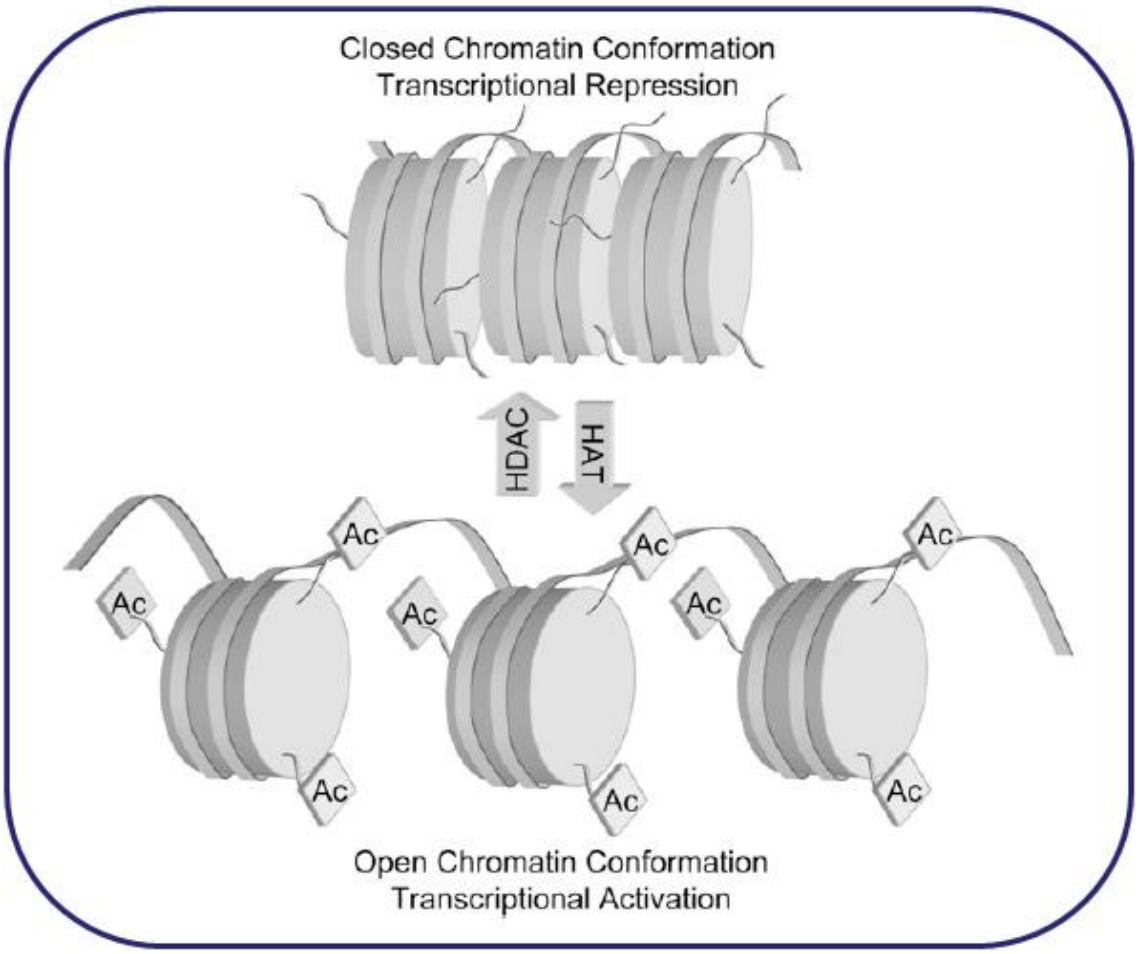


Figure 7: Modulation of chromatin conformation and transcriptional status by acetylation of lysine tails in histone core proteins [adopted from Ho et al. [129]]

In vivo, a tightly regulated balance exists between HAT and HDAC activities, and when this balance is interrupted, cancer development may ensue.

Currently, 18 HDACs have been identified in humans, which can be subdivided into four classes based on their homology to yeast HDACs, their subcellular expression and their enzymatic activities. All class I and II are zinc-dependent enzymes, whereas members of the III class, sirtuins, require NAD^+ for their enzymatic activity. Class IV HDAC is represented by HDAC11 [130]. HDAC inhibitors (HDACi), as a new class of targeted chemotherapeutic agents, show significant promise against a variety of cancers in clinical trials [131]. Most available HDACi inhibit all class I and II HDACs, thereby increasing acetylation of histone and nonhistone protein targets [132], resulting in cell cycle arrest, differentiation and apoptosis as well as reduced tumor volume and/or tumor number. Moreover, it has been shown that HDACi induce the expression of a small number of

tumor suppressive genes, such as $p21^{WAF1}$ [133], growth-differentiation factor 11, a member of the transforming growth factor-β (TGF-β) superfamily [134] as well as TGF-β -receptors I and II [135, 136]. Thus, the pro-tumorigenic role for HDACs in colon cancer, and the pre-clinical efficacy of these agents in colon cancer cells justify the use of HDACi in cancer prevention and therapy [137]. Indeed, it has been demonstrated that combinations of HDACi with well-established chemotherapeutics can synergise with their anti-tumor effects [138-140]. Recently, Myzak and colleagues identified SFN as a novel HDACi in colon and prostate cancer cells [141]. They have found that 3-15 µmol SFN already induces potent HDAC inhibition and G2/M arrest in PC3 cancer cells but have no effect on normal prostate epithelial cells (J. D. Clarke and E. Ho, un-published data). In addition to the *in vitro* studies, several dietary experiments in mice models supported these data [142, 143]. From these studies it can be concluded that HDAC inhibition represents a novel chemopreventive mechanism by which SFN might promote cell cycle arrest and apoptosis *in vivo*. In MANUSCRIPT I, it was also of interest whether sulforaphane-mediated effects on TGF-β signaling, observed in our study model, may be due to its ability to inhibit HDACs.

3.1.2 Polyamine metabolism

The intestinal mucosa is continuously renewed from the proliferative zone of undifferentiated stem cells within the crypts and has the most rapid turnover rate of any tissue in the body [144]. The cellular polyamines spermine and spermidine as well as their precursor putrescine are essential for cell growth by stabilizing DNA structure [145], influencing membrane functions [146] and cell cycle regulating genes [147], whereas increasing concentrations are generally associated with cell proliferation and cell transformation induced by growth factors [148], carcinogens [149] or oncogenes [150]. Therefore, cellular levels of polyamines are strictly controlled by the combined action of *the novo* synthesis, catabolism, uptake and export of polyamines.

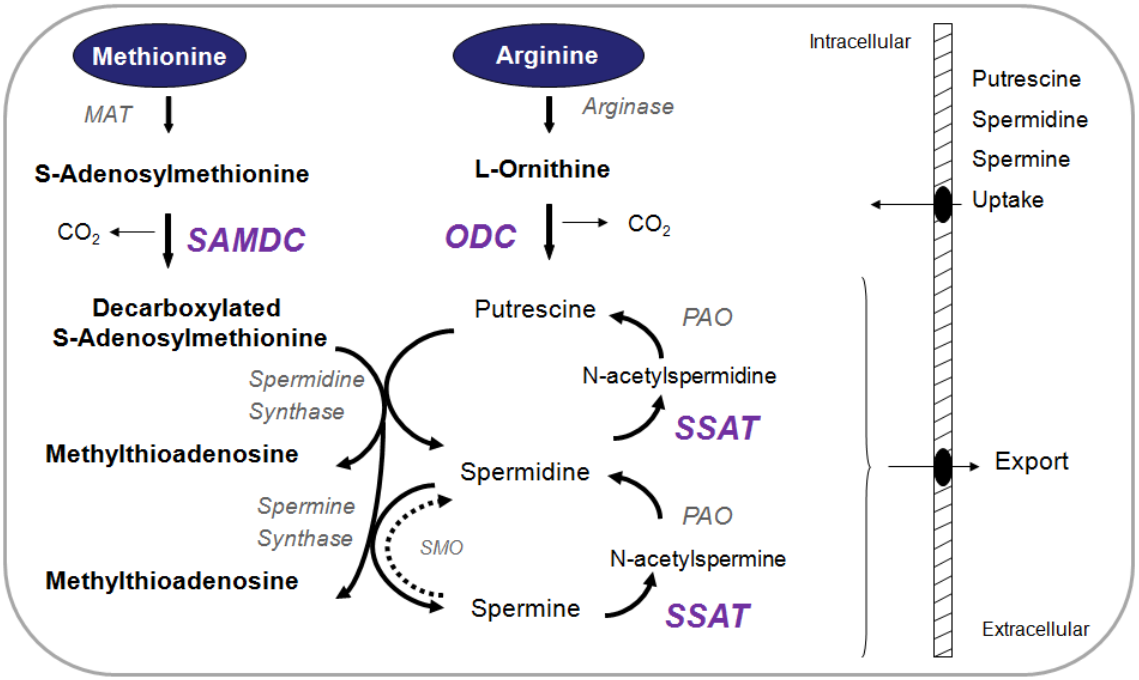


Figure 8: Polyamine metabolism

Regulation of intracellular polyamine content in mammalian cells is mediated by different key enzymes: the biosynthetic ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) and the catabolic spermidine/spermine acetyltransferase (SSAT) as well as the polyamine oxidase (PAO) [adapted from [151]].

Regulation of polyamine concentrations are governed by ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC), the key enzymes of the polyamine biosynthesis. Catabolism excretion or reconversion of higher polyamines is regulated by acetylation through spermine/spermine N^1-acetyltransferase (SSAT), the rare limiting enzyme in polyamine catabolism [152-154]. In combined action with polyamine oxidase (PAO), it converts spermine to spermidine and subsequently to putrescine (summarized in **Figure 8**). In colon cancer tissue, the activities of polyamine-synthesizing enzymes and polyamine content are increased 10- to 15-fold in comparison to normal colonic epithelium [155, 156], and polyamines seem to be involved in almost all steps of colonic tumorigenesis. Hence, regarding the role of polyamines in colonic carcinogenesis, modulation of polyamine metabolism and, in particular, of ODC activity has been studied as a new potent therapeutic strategy in cancer treatment and prevention [111, 112]. Pharmacological and natural inhibitors have been shown to decrease mucosal growth in vitro [111, 157] and in vivo [112].

However, the best known synthetic inhibitor α-difluoromethylornithine (DFMO), a specific inhibitor of ornithine decarboxylase [158], has been less succesfull in cancer therapy, resulting in cytostatic rather than cytotoxic effects in vivo [159]. Therefore, the use of natural substances to modulate polyamine metabolism is possibly a more promising strategy in cancer treatment and prevention.
Interestingly, in their most recent publication, Furniss et al. reported modulatory effects of broccoli extracts as well as SFN alone on polyamine metabolism in colorectal cancer cells. However, direct modes of action were not further specified [88]. In MANUSCRIPT I we could show that the antiproliferative effects of SFN closely correlate with a reduction of ODC protein expression and activity. Similar effects could be observed by the group of Lee et al. where SFN inhibited TPA-induced ODC activity in mouse epidermal ME308 cells [160]. ODC levels are tightly controlled either by transcriptional regulation or by rapid post-translational degradation. The proto-oncogene c-myc is a transcription factor that directly regulates the expression of ODC by binding to a specific CACGTG sequence in the gene promoter [161]. c-myc was actually found to be activated in various animal and human tumors and an overexpression of the c-myc gene has been described in ~15% of all human tumors [162]. Since c-myc is a ubiquitous promoter of cell growth and proliferation, it functions as a transcriptional activator or inhibitor depending on the target gene [163, 164]. We could demonstrate (MANUSCRIPT I) that decreased ODC activity and protein levels in Caco-2 cells are accompanied by decreased protein levels of c-myc after 6 h of incubation, implicating a direct modulation of ODC gene expression by SFN.

3.1.3 Transforming growth factor-β

The transforming growth factor-β (TGF-β) superfamily, comprising TGF-βs, bone morphogenetic proteins (BMPs), activins and related proteins, has been implicated in a wide variety of cellular processes, including regulation of cellular proliferation [165] and differentiation [166], immune modulation [167], and extracellular matrix remodelling [168]. It exhibits their antiproliferative functions by activating a signaling pathway that mediates cell cycle arrest and induction of apoptosis. TGF-β exerts its effects through heteromeric receptor complexes consisting of type I and type II serine/threonine kinase receptors.

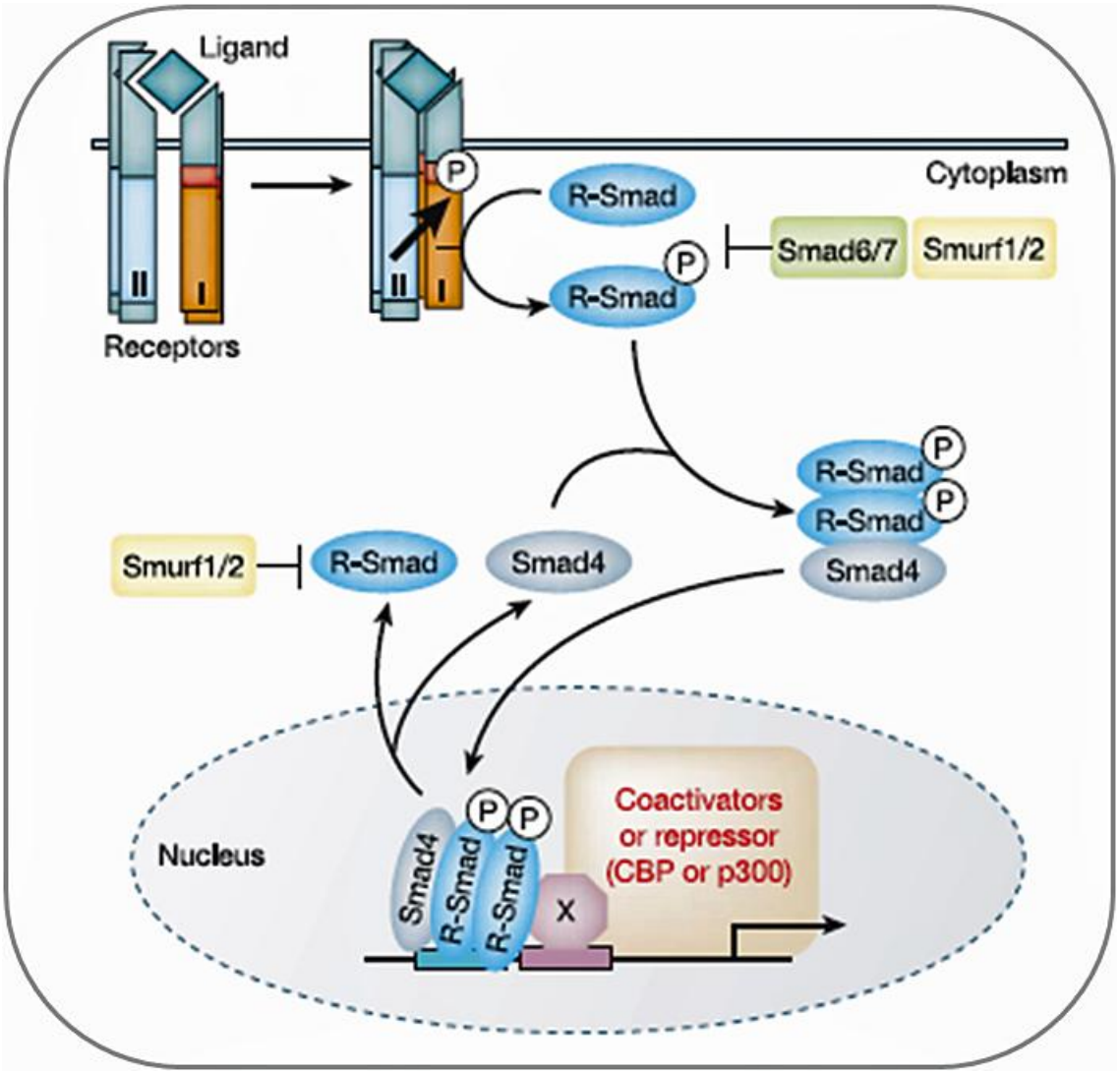


Figure 9 : Gerneral mechanism of TGF-β receptor and Smad activation (adopted from Derynck et al. [169])

The signaling is initiated by ligand-binding to the TGF-βRII cell surface receptor. This, in turn, recruits the TGF-βRI kinase, which then phosphorylates the R-Smad proteins, Smad2 and Smad3 [170]. Activated R-Smads form a complex with the Co-Smad, Smad4, which shuttles directly to the nucleus.

Here, the complex can either act as co-activator or DNA-binding transcription factor, participating in the regulation of target gene expression (e.g. P15, p21, c-myc) [170, 171]. TGF-β signaling is further controlled by a third class of Smads, the inhibitory Smad6 and Smad7 proteins, which negatively regulate R-Smad activation (**Figure 9**).

We previously reported that induction of the TGF-β signaling pathway in colon cancer cells is a crucial event in the anti-carcinogenic activities of butyrate, another natural occurring HDAC inhibitor [113]. This, together with the findings of

Traka et al., who could demonstrate, that TGF-β signaling also seems to play an important role in various SFN-mediated effects [172], let us consider, whether SFN possibly shows similar modes of action in our context. In colorectal cancers, the TGF-β signaling pathway plays a central but paradoxical role in the predisposition and progression of the disease. While on the one hand acting as a tumor suppressor in normal colonic epithelial cells, TGF-β on the other hand promotes the survival, invasion and metastasis of colorectal cancer cells, thereby rather acting as an oncogene [173, 174]. However, in our model we could demonstrate that incubation with SFN caused increased protein and mRNA levels of TGF-β, an enhancement of both TGF-βRI and -RII protein expression and an activation of Smads signaling. Furthermore, SB431542 largely abolished SFN-mediated effects on Caco-2-cell growth, which provides evidence that TGF-β seems to play a crucial role in the antiproliferative effects of SFN. In earlier works, Osada et al. already demonstrated that HDAC inhibitors can increase the transcriptional activity of TGF-βRII *in vivo* and *in vitro* in human cancer cell lines [136]. In addition to these findings, Lee et al. reported [175] that MS-275, another HDAC inhibitor, induces the accumulation of acetylated histones in the chromatin of the TGF-βRII gene, which is associated with an increase of TGF-βRII mRNA in human breast cancer cells, contributing to the restoration of TGF-β signaling. In terms of proving that the observed effects of SFN on the TGF-β pathway are due to its HDAC inhibitory properties, we further performed Acetyl-Histone H3 and H4 immunoprecipitation (ChIP) assay, followed by PCR with RII promoter specific primers and in fact, an accumulation of acetylated H3, but not acetylated H4, in chromatin associated with RII gene after incubation with SFN could be found.

Controversial data exist about the effects of TGF-β on the polyamine metabolism. On the one hand, TGF-β has been shown to induce ODC mRNA in H-ras-transformed fibrosarcoma cell lines on which TGF-β acts as a growth stimulator [176]. On the other hand, Motyl et al. [177] could demonstrate that TGF-β suppresses both cell growth and the activities of ODC and S-adenosyl-L-methionine decarboxylase in a human chronic myelogenous lymphoma cell line. Similar results were found in the group of Nishikawa et al. [178], showing inhibitory effects of TGF-β on polyamine metabolism. In accordance with these publications, we could observe, that co-incubation of SFN with a specific TGF-β ki-

nase inhibitor partly abolished SFN-induced reduction of ODC protein expression and activity, which might be due to direct TGF-β/Smad signaling-mediated transcriptional repression of transcription factor c-myc, upstream of the ODC gene. This effect was investigated in detail by Chen et al. who could demonstrate, that the repression of c-myc expression by TGF-β occurs by direct interaction of a repressor complex consisting of Smad3, the transcription factors E2F4/5 and DP1 and the retinoblastoma family member p107 with a regulatory Smad responsive element in the c-myc-promoter [179].

3.1.4 Summary and conclusion

In summary, the results in MANUSCRIPT I clearly demonstrate the first direct evidence that SFN mediates growth inhibitory effects in colorectal cancer cell lines, at least partly, via a TGF-β-dependent inhibition of c-myc and thus reduced protein expression and activity of protooncogenic ODC (summarized in **Figure 10**).

Noting the fact that colorectal cancer is stilll one of the most commonly occurring malignancies in the world, and the prognosis for patients with advanced colorectal cancer with distant metastasis is usually very poor, preventive measures that target the various steps involved in cancer initiation and progression could significantly decrease the incidence and mortality of cancer. Therefore, the use of non-toxic agents, e.g. phytochemicals like SFN, which inhibit specific molecular steps in the carcinogenic pathway, might be a promising strategy for cancer chemoprevention.

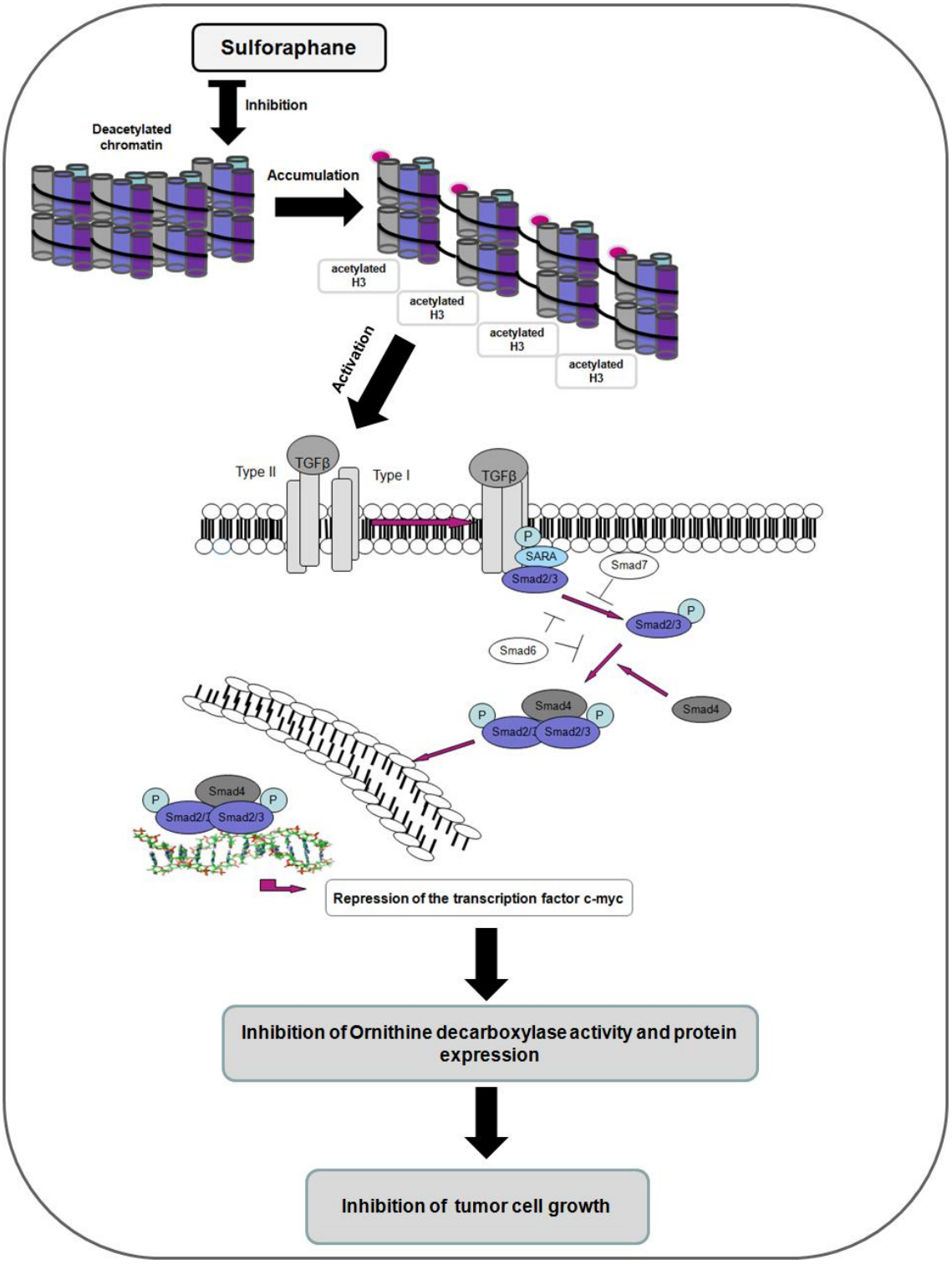


Figure 10: Possible mechanism of sulforaphane action

3.2 Chemosensitizing properties of phytochemicals in colorectal cancer cells

Even though conventional chemotherapy certainly plays an important role in the overall treatment of most solid tumors, successful cancer treatment is often limited due to severe side effects and the development of multi-drug resistance [116]. Therefore, there is a growing need for innovative anticancer therapies. In the last years, a new therapeutic concept of combining antitumor drugs with chemopreventive agents was introduced, which may lead to enhanced antitumor activity through synergistic action or compensation of inverse properties. Beside a multitude of synthetic substances, like non-steroidal anti-inflammatory drugs (NDAIDs) [180] or selective estrogen receptor modulators (SERMs) [181] also numerous phytochemicals have been identified to exhibit potent chemopreventive effects in different carcinogenesis models while, at the same time showing low toxicity [116]. Recent studies could further demonstrate that several of these plant-derived compounds, like capsaicin [182] from chilli peppers, epigallocatechine gallate [183, 184] extracted from green tea, genistein [185, 186] found in soybeans and other legume species or curcumin [187-189] the principal curcumoid of the popular Indian soice turmeric, are also capable of enhancing the efficacy of chemotherapy and radiotherapy in various in vitro and in vivo cancer models, predominantly by modulating intracellular cell signaling pathways, abrogating drug resistance and diminishing systemic toxicities [reviewed in MANUSCRIPT IV and [116, 190]]. Due to their wide range of biological and pharmacological effects, especially with regard to chemoprevention and the lack of toxicity in animal and human models, sulforaphane as well as resveratrol might also be possible agents for enhancing the effects of chemotherapy.

3.2.1 Chemosensitizing effects of sulforaphane

Most chemotherapeutic agents, including oxaliplatin, and irradiation act primarily by inducing apoptosis, accordingly, defects in the apoptotic pathway often account for chemotherapy resistance in different tumor cells, which could also

be demonstrated for drug resistance arising against oxaliplatin in colorectal cancer cells [24]. Novel targeted therapies that more potently induce cell death in cancer cells or sensitize them to established cytotoxic agents and radiation by modulating the apoptotic machinery might therefore not only enhance therapeutic outcome but can further help to reverse chemotherapeutic drug resistance [191]. In salivary gland adenoid cystic carcinoma high (ACC-M) and low (ACC-2) metastatic cell lines, treatment with sulforaphane and 5-flourouracil (5-FU) led to synergistic inhibition of cell growth, which was accompanied by decreased expression of nuclear NF-κB p65 protein [192]. In epithelial ovarian cancer cells (EOC), combination treatment with paclitaxel resulted in, at least, additive growth suppression [193]. Our first results in MANUSCRIPT II quickly revealed SFN-induced potentiation of cell growth inhibition mediated by oxaliplatin, which was more-than-additive as indicated by combination index analysis. In addition, these effects were accompanied by different hallmarks of apoptosis, such as reduced ATP levels, Caspase-3-activation, PARP cleavage and DNA Fragmentation. Further experiments could demonstrate that thereby apparently both, extrinsic and intrinsic apoptotic pathways were involved, as indicated by caspase-8-cleavage and increased mitochondrial membrane permeabilization. Interestingly, we could also observe an induction of TRAIL protein levels, a member of the TNF family of cytokines, which can induce apoptotic cell death in a variety of tumor cells by engaging the death receptors DR4 and DR5, while sparing most normal cells [194]. This might be due to the HDAC inhibitory properties of sulforaphane [141], since several other HDAC inhibitors were also shown to induce expression of TRAIL, DR4 or related proteins, which contributed to subsequent apoptotic events induced by these agents [195]. Since some drugs and radiation sensitize tumor cells to TRAIL-induced cell death, several studies have expectedly shown that combinations of recombinant TRAIL and some chemotherapeutic drugs exhibit synergistic effects in inducing tumor cell apoptosis *in vitro* and *in vivo* [196, 197]. Whether endogenously induced TRAIL possibly acts in a similar way remains to be elucidated. Another interesting aspect is that TRAIL, in contrast to DNA-damaging chemotherapeutic drugs or radiation, induces apoptosis independently of p53 [194], which might be helpful to circumvent resistance to conventional chemotherapy and radiotherapy. However, in contrast to known apoptosis inducers, such as staurosporin, apoptotic

events induced by SFN and Ox were not very prominent and may only partly account for the observed inhibition of cell proliferation. In fact, with increasing concentrations of oxaliplatin and increasing treatment duration, annexin-V and PI-staining revealed a shift from an apoptotic towards a distinct population of necrotic cells. Similar results were observed in Sytox Green-stained cells, which showed considerable signs of necrosis like swelling of the organelles or cell lysis with loss of membrane integrity. This is consistent with the almost complete ATP depletion after 24 h of incubation, which may further account for the low apoptotic response [198]. Currently, the majority of clinical chemotherapeutic agents ultimately induce tumor cell apoptosis following treatment, however, noting the fact that many cancers have defective apoptosis machinery or aquire apoptosis resistance during therapy [199], or the finding, that apoptosis may be reversed in cancer cells [200], it is reasonable to consider whether activating alternative cell death pathways, such as necrosis, may be another effective strategy for cancer therapy [201]. However, whether immunologic responses, typically associated with necrotic cell death might really be desirable in the context of cancer treatment or rather lead to further tumor growth or even overshooting inflammation resulting in autoimmunity remains to be elucidated. Interestingly, in healthy human fibroblasts, SFN was found to inhibit cell growth in a dose-dependent manner, an effect which was already reported for other HDAC inhibitors [202, 203]. But in contrast to Caco-2 cells, growth inhibitory effects on fibroblasts were not further enhanced and no signs of cytotoxicity could be observed when SFN was combined with oxaliplatin, indicating a selective toxicity towards the tumor cell line while inducing only growth arrest in normal fibroblasts.

3.2.2 Chemosensitizing effects of resveratrol

Resveratrol was examined in various studies to determine possible chemosensitizing properties when combined with established cancer treatments. And actually, sensitizing effects could be observed for different anticancer drugs in a variety of human cancer cell lines *in vitro* [204-210] as well as several *in vivo* tumor models [211-213], whereby some modes of resveratrol-action could already be identified. Investigations of Fulda et al. for example revealed, that pre-

treatment of different human cancer cell lines and primary tumor cells with resveratrol resulted in cell cycle arrest in the S phase and apoptosis induction preferentially out of S phase upon subsequent treatment with various anticancer drugs [204]. S phase arrest induced by resveratrol was also found in hepatoma22 transplanted to male BALB/c mice [213] and colon cancer cells [207], thus enhancing the anti-tumor effects of 5-fluorouracil (5-FU), a S phase specific pyrimidin analog typically used in the treatment of colorectal-, pancreatic-and hepato-carcinoma patients [214]. Gill et al. reported, that pre-treatment with resveratrol sensitized prostate cancer cells predominantly to agents, that specifically target death receptors but not agents that initiate apoptosis through other mechanisms [206]. In a mouse model of pancreatic cancer, resveratrol could potentiate the effects of gemcitabine through suppression of markers of proliferation, invasion, angiogenesis and metastasis, which seems to be mediated by inhibition of NF-kappa B activity [212], a transcription factor which plays a critical role in cancer development, progression and drug resistance [215]. Furthermore, the combination of chemotherapeutic agents with resveratrol might further be useful to limit the burden of related side effects, not only due to the fact that, with consistent efficiency, lower doses of the cytotoxic agents could be used, but also due to direct protective properties of resveratrol [210, 216-225]. In addtion, modulatory effects of resveratrol on most pathways which are involved in the development of multidrug resistances could be reported in different cellular and animal models (reviewed in MANUSCRIPT IV). In MANUSCRPIT III, we explored the possible chemosensitizing capability of resveratrol in a cell culture model of colorectal cancer, also by analyzing possible effects on immune cells. Similar to sulforaphane, cell-growth inhibitory effects of Res/Ox were accompanied by different hallmarks of apoptosis, such as reduced ATP levels, Caspase-3-activation, PARP cleavage and DNA Fragmentation. Further experiments could demonstrate that the intrinsic apoptotic pathway seems to play a major role in apoptosis-inducing efficacies as mitochondrial membrane potential was significantly decreased after combinatorial treatment. Recently, the anti-apoptotic protein survivin has been described as being selectively expressed at high levels in most human cancers and is related to clinical progression [226-229]. Survivin is a structurally unique member of the IAP (inhibitors of apoptosis proteins) family that is potentially involved in both control of cell divi-

sion and inhibition of apoptosis [229]. Specifically, its anti-apoptotic function seems to be related to an ability to directly/indirectly inhibit caspases [230], although the precise role of survivin in the modulation of the caspase cascade has not been fully elucidated [231]. In addition, survivin overexpression is accompanied with poor prognosis of carcinomas of the lung, breast, colon and esophagus [232-235]. Since inhibition of effector caspases by IAPs occurs at the core of the apoptotic machinery, therapeutic modulation of IAPs could target a key control point in cancer resistance [228]. Interestingly, in our studies we observed reduced protein levels of survivin after 24 h of drug exposure, whereas this effect was more prominent when the cells were co-treated with Res and Ox. Based on the above evidence, survivin is at present validated as a cancer therapeutic target [227] and in contrast to previous studies [236], we showed that Ox causes a slight induction of the protein. It is notified, that several anti-cancer agents, such as taxol, cisplatin and doxorubicin, show an upregulation of survivin in accordance with the cytoprotective function of survivin [237, 238].

Depending on the lethal stimulus, tumor cells can also die by necrosis which is characterized by swelling of the cell and the cytoplasma organells before the plasma membrane ruptures and the cellular content is shed into the intercellular space [239]. Similar to the results of sulforaphane and oxaliplatin, Res/Ox-mediated apoptotic effects seemed to reach a maximum after 5 h of treatment, which is replaced by a shift towards an increased population of necrotic cells after 24 h. Unlike apoptosis, which is considered immunosuppressive, therapy-induced necrotic cell death initiates an immune response [240]. This inflammatory response may help to recruit cytotoxic immune cells to the tumor site, thereby increasing the efficacy of the chemotherapeutic drugs. However, conversely the pro-inflammatory conditions might also damage normal tissue or induce the production of mitogenic or prosurvival cytokines, which can activate signaling pathways that promote cell excrescence in the damaged area and might induce tumor cell migration and metastasis [241, 242]. Interestingly, Ingenol-3-angelate, another plant-derived compound, was recently shown to mediate its in vitro anticancer activities via the induction of primary necrosis [243, 244], as displayed by plasma membrane and mitochondrial disruption leading to the activation of an antitumor immune response [245]. Thus, the success of Ingenol-3-angelate in phase IIa clinical trials against human skin cancer might

again support the importance and potential of cytotoxic agents that act through irreversible necrotic cell death [246].

3.2.3 Immunogenic cell death

Cells undergoing apoptosis are rapidly and specifically recognized and engulfed by phagocytes such as macrophages or immature dendritic cells [247]. Removal of apoptotic cells by macrophages seems to result in little or no production of inflammatory immune mediators by unstimulated macrophages [248]. On the contrary, necrosis is induced by external insults and is morphologically characterized by an increase in cell volume leading to the early rupture of the plasma membrane. Consequently, cytosolic, organelle, and nuclear components are spilled into the surrounding tissue [240]. Unlike apoptosis, necrosis is considered to be immunologically harmful at all times, because of the sudden release of so-called danger signals such as ATP or high mobility group box 1 (HMGB1), which elicit inflammation by activating toll like receptors (TLR) or the inflammasome in myeloid cells [249]. Besides, necrotic cells are able to act on fibroblasts to activate NF-κB and induce the expression of genes that are involved in inflammatory responses [250]. Classical activation of human macrophages is characterized by the production of superoxide (O_2 -), TNF-α, IL-1β and IL-6, also termed the M1 macrophage phenotype [251]. In contrast, the cytokines IL-4, IL-13 or IL-10 are usually released by the M2 phenotype [251, 252]. Since Virchow et al. observed the presence of leukocytes in human tumors in 1863, it seems accepted that macrophages are a major cell component infiltrating certain tumors [252]. High numbers of tumor-associated macrophages (TAMs) often predict a poor survival prognosis for patients with solid human tumors [253, 254]. Moreover, TAMs promote cancer metastasis through several mechanisms, including promotion of angiogenesis, induction of tumor growth, and enhancement of tumor cell migration and invasion (summarized in **Figure 11**) [255-257] while showing a similar molecular and functional profile, that is displayed by a polarized M2 phenotype [252, 258]. Therefore, reprogramming a M2 macrophage toward a M1 type seems to be beneficial with regard to tumor therapy [259].

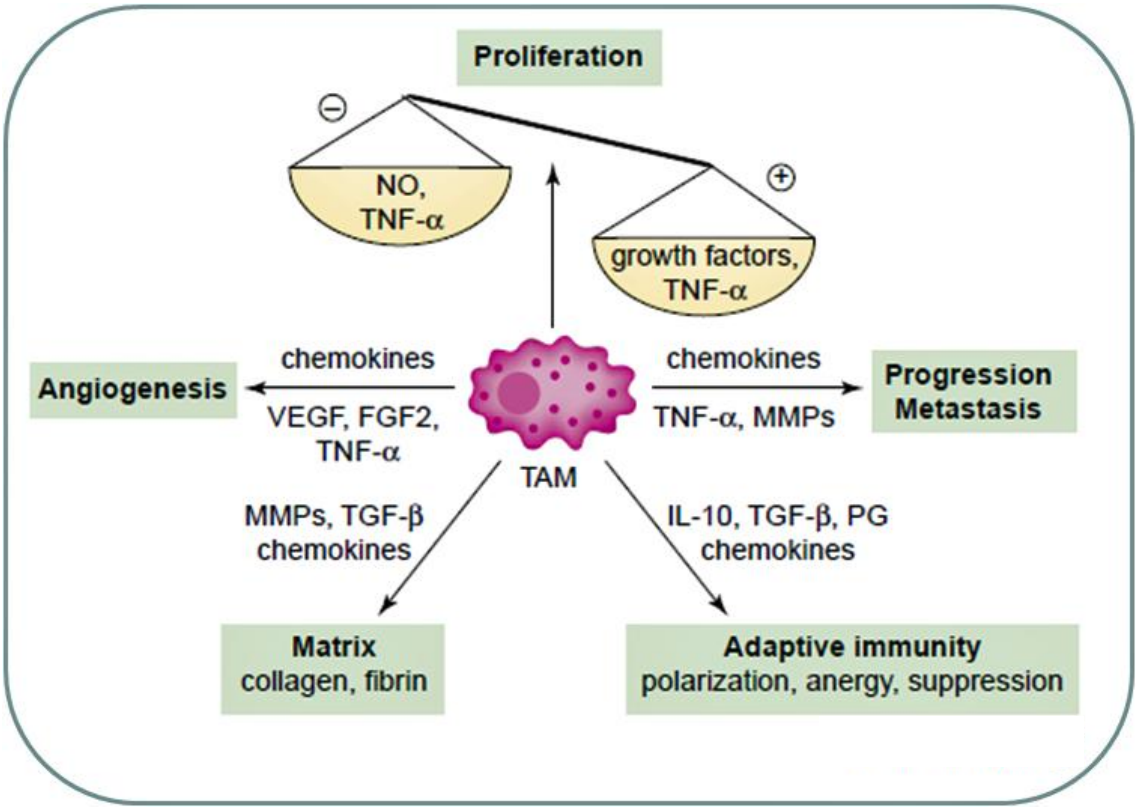


Figure 11: A simplified view of the role of TAMs in the immunology of tumors [adapted from Mantovani et al. [252]]

Among the chemotherapeutic agents, only a few have the capacity to stimulate immunogenic cell death. Recent studies have shown that EL4 thymoma, Glasgow osteosarcoma, and CT26 colon cancer cells treated with oxaliplatin, as well as CT26 colon cancers and MCA205 fibrosarcomas treated with anthracyclins, respond far better to chemotherapy when they are implanted into immunocompetent mice rather than into immunodeficient, athymic *(nu/nu)* hosts [260-262]. Therefore, the outcome of treatment with anthracyclins, oxaliplatin and radiotherapy depends on the active contribution of the host immune system [263, 264]. In our studies, tumor cells treated with a combination of Res and Ox did not elicit a strong production of pro-inflammatory mediators, however, production of immunosuppressive IL-10 was completely abolished. Considering the role of IL-10 in suppression of immunity against established tumors e.g. by inducing regulatory T cells [265, 266], these findings strengthen the rationale of using Ox in combination with Res for tumor therapy. Additionally, in MANUSCRIPT III we could show that cell growth inhibition as well as lysis of Caco-2 cells was significantly enhanced, especially when the cells were treated with the supernatant of co-cultured macrophages with Res/Ox-treated Caco-2 cells.

These findings correlate well with the observed effects of tumor cell supernatants on IL-10 production in macrophages, since IL-10 potently suppresses the cytotoxic potential of macrophages [267].

3.2.4 Summary and conclusion

In addition to the chemopreventive and chemosensitizing properties of SFN (MANUSCRIPT I+II), pharmacokinetic studies in both rats and humans also indicate, that dietary absorbed SFN can be distributed in the body, reach µM levels in the blood and is capable of reaching target tissues in an active form, which further supports the clinical relevance of the substance [268, 269]. However, further experiments focusing intramolecular mechanisms together with *in vivo* animal studies and clinical trials are needed for eventually translating the concept of phytochemicals in combination therapies of human colorectal cancer into clinical applications. As discussed in MANUSCRIPT III, the immune response against dying tumor cells can play a major role in determining therapeutic success. If tumor cell death occurs in a potentially immunogenic fashion and if the immune system is capable of perceiving this immunogenicity, a potent innate and cognate immune response raised against dying cancer cells can contribute to the control and elimination of residual cancer cells. Our findings demonstrate for the first time that the polyphenol resveratrol is capable of amplifying Ox-induced cell growth inhibition in colon cancer cells supposedly via induction of different modes of cell death. As a result of the apoptotic and necrotic effects of resveratrol and oxaliplatin, immunosuppressive potential in macrophages is prevented, which renders them potently tumoricidal (summarized in **Figure 12**).

Even though our results suggests that the phytochemicals sulforaphane and resveratrol show promise for serving as potent agents for enhancing the therapeutic effect of oxaliplatin, some reports point out that this strategy might also negatively affect classical treatment regimens under certain circumstances (reviewed in MANUSCRIPT IV). This should as well provide a cautionary note for the uncontrolled and non-critical supplementation of these compounds in patients with established cancer who are already undergoing chemo- or radiotherapeutic treatment. Further mechanistic studies, *in vivo* animal models and hu-

man clinical trials are therefore needed to certify the safety and efficacy of dietary compounds in combination with cancer therapeutics.

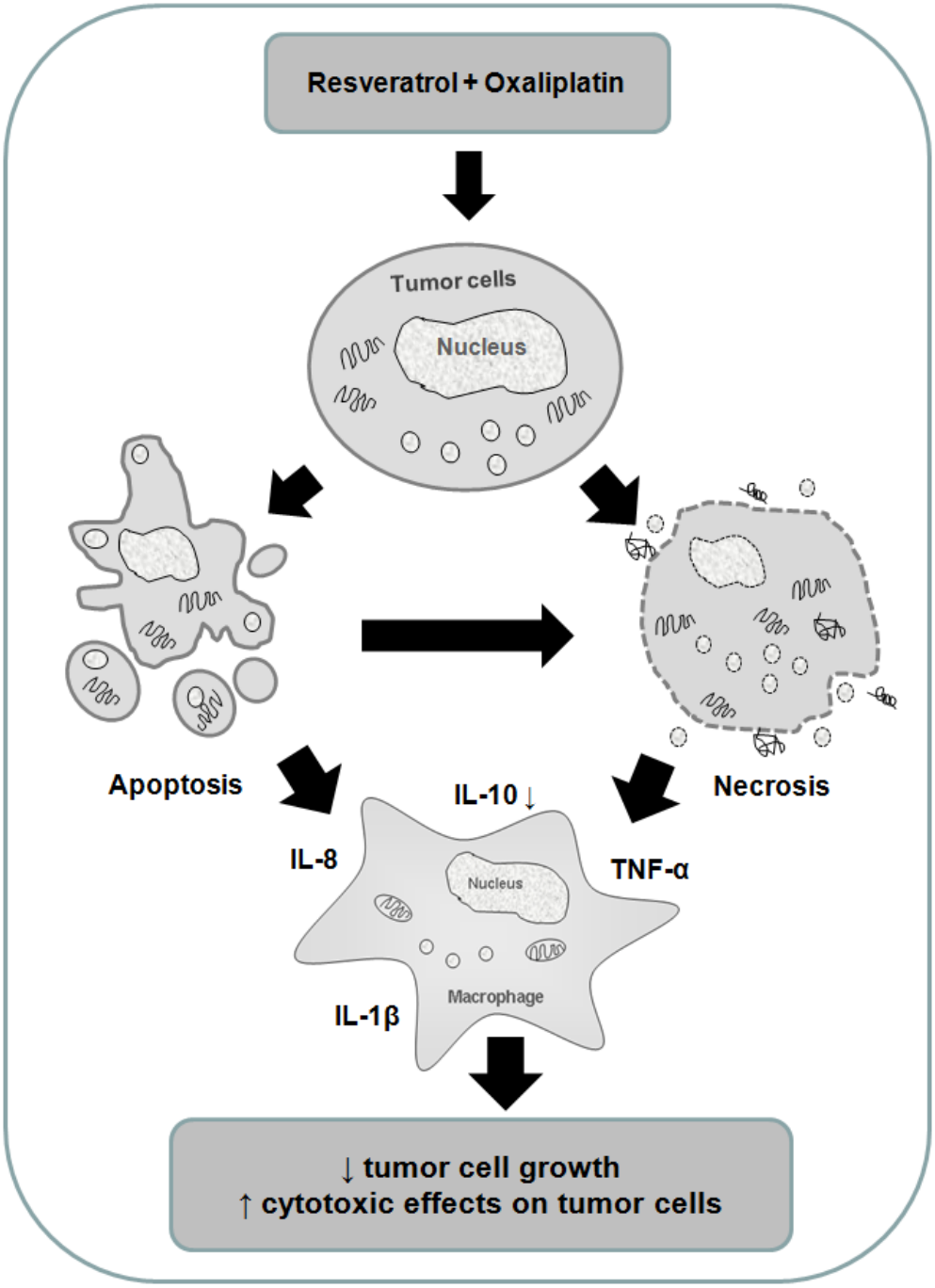


Figure 12: Possible effects of resveratrol and oxaliplatin in colorectal cancer cells

3.3 Bioavailability of phytochemicals

The bioavailability of a nutrient depends on the degree to which it becomes available to the target tissue after administration. Regarding cancer therapies, a defect of drug accumulation in cancer cells is an important cause of failure. Indeed, the action of chemopreventive and chemotherapeutic agents can be diminished by a failure of their absorption, distribution, metabolism or an increase in their excretion. Hence, the knowledge of absorption and metabolism of a compound *in vivo* is the precondition to analyze its bioavailability.

The initial step of SFN absorption involves enzymatic hydrolysis of glucoraphanin, the glucosinolate precursor of SFN, found in plants. The enzymatic reaction is catalyzed by myrosinase, a β-thioglucosidase, which cleaves the glycone from the glucosinolate forming glucose, hydrogen sulphate and one of many different aglycones (e.g. thiocyanate, ITC, or a nitrile). After absorption, SFN is predominantly metabolized via the mercapturic acid pathway, starting with glutathione (GSH) conjugation by glutathione-*S*-transferase (GST) [270] followed by generation of SFN-cysteine (SFN-Cys) and SFN-*N*-acetylcysteine (SFN-NAC) [123]. The absorption and bioavailability of SFN can be affected by several factors. For example, the initial step can be influenced by alterations in the myrosinase activity which is due to the fact that plant myrosinase is heat labile and thus cooking procedure can inactivate the enzyme and significantly reduce the bioavailability of SFN up to 3-fold [271]. Another source of myrosinase activity is the intestinal microbial flora. Data suggests that glucoraphanin can be converted by colonic microbial flora and that enterohepatic circulation is requisite for efficient metabolism [272]. However, it seems that the bioavailability is more depending on plant myrosinase activity as seen by the fact that bioavailability of SFN is six times less when metabolism of the glucosinolate to the ITC had not occurred prior to ingestion [273]. Further example for affecting SFN bioavailability is related to polymorphisms in Phase 2 SFN-metabolizing genes, such as GSTs, playing significant roles in determining the detoxifying ability of an organism. In general, GSTs are dimeric enzymes that catalyze the conjugation of GSH to electrophiles such as SFN, thereby facilitating their metabolism and excretion. Although the metabolism of SFN depends upon this GSTM1

genotype, approximately 50% of the population have a depletion of the GSTM1 gene (“GSTM1 nulls”). The group of Gasper et al. showed that after 24 h, while GSTM1 null individuals excreted almost 100% of SFN, GSTM1-positive individuals excreted only about 60% of ingested SFN [274]. Therefore, the observed protective effect of sulforaphane is influenced by GSTM1 genotype due to the fact that GSTM1-positive individuals gain greater cancer protection than GSTM1 Nulls [275-277].

Several pharmacokinetic studies in both rats and humans indicate, that dietary absorbed sulforaphane can be widely distributed in the body, reach µM levels in the blood and is capable of reaching target tissues in an active form [123, 268, 269]. Notably, the accumulation of SFN in colonic tissue corresponded with decreased adenoma formation in mice supplemented with 300 or 600 ppm SFN [278]. In clinical studies, it has been shown that 75% of SFN from broccoli is absorbed in the jejunum and a portion of that returns to the lumen of the jejunum as SFN-GSH [279]. When the metabolism of glucoraphanin efficiently occurs, SFN-NAC is the primary SFN metabolite excreted in the urine [270-272, 280]. In rats, nearly 72% of a single oral dose of SFN was recovered in the urine as NAC conjugates in 24 h [270], but only about 1% of the dose was detected in the second 24-h urine sample [281], indicating an extremely high bioavailability and a small inter-individual variation of SFN absorption and metabolism. Once SFN is distributed it can accumulate in tissues, and be maintained to achieve the anti-tumor effects [123]. In a recent pilot study in human mammary tissue, a oral dose of broccoli sprout preparation containing 200 µmol sulforaphane 1 h prior to tissue removal showed mean accumulation of 1.45 ± 1.12 pmol/mg tissue in the right breast and 2.00 ± 1.95 pmol/mg in the left breast [282].

The consumption of isothiocyanates, such as sulforaphane is actually expected to rise due to the use of dietary supplements and public health initiatives promoting the consumption of more fruits and vegetables. This together with its excellent bioavailability makes sulforaphane a potent candidate for food-drug interactions, whereby these interactions may result in both positive and negative consequences regarding cancer therapy.

In rats and humans, the bioavailability of resveratrol depends on its absorption and metabolism, which have been studied in several models, including isolated rat intestine [283, 284], rats and mice after oral administration [285-289], human colon carcinoma Caco-2 cell line [290], human hepatocytes [291] and healthy human subjects [292, 293]. Using radiolabelled resveratol administered by oral route, an considerable fraction, 50-75% of the dose, was absorbed in rats and radioactivity could be recovered in the stomach, liver, kidney, intestine, bile and urine in mice [289]. Intragastric administration of different amounts of resveratrol to rats revealed an absorption rate of 77-80% resveratrol in the intestine and reached µM levels in plasma [294]. In humans, after oral administration less than 2% of free resveratrol in plasma and serum was detected [294]. Obviously, with time, the plasma concentration diminished until a secondary peak appeared and this secondary peak was due to the recirculation of resveratrol after release from bile. The liver and gallbladder filtered resveratrol and its metabolites from the circulation and transported them back again into the intestine through the bile for a delayed absorption [286, 287]. According to pharmacokinetic studies in mice, rats, and dogs, resveratrol is rapidly absorbed and glucuronated in the human liver and sulphated in both the liver and the duodenum [105], whereas only very minute amounts of resveratrol are distributed to all organs [283]. In 2004, Walle [294] and colleagues showed that most of an intravenous dose of resveratrol is converted to sulphate conjugates within ~30 min in humans. A detailed analysis of plasma metabolites after oral dosing was not possible; however, both sulphate and glucuronide conjugates were detected. In addition, they also demonstrated that, compared to resveratrol, which has a plasma half-life of 8–14 min, the metabolites have a plasma half-life of about 9.2 hours, indicating that exposure to modified forms is much higher than that for unchanged resveratrol [294]. However, the bioavailability and efficacy of these resveratrol metabolites is unknown [94]. According to above-mentioned pharmacokinetic studies circulating resveratrol is rapidly metabolized [295], which cast doubts on the therapeutic relevance of resveratrol due to the discrepancy between the relatively low bioavailability *in vivo* and the biologically relevant concentrations used in *in vitro* studies [296]. However, systemic administration of resveratrol has been shown to inhibit the initiation and growth of tumors in a wide variety of rodent cancer models [297-301]. According to the literature, in-

traluminal accumulation of resveratrol might be a hypothesis to explain this discrepancy. In animals, resveratrol accumulated in the liver up to a concentration comparable to that which exerts biological effects in *in vitro* assays (micromolar range) [289] and several amounts are found in the colon, whereas its tissue distribution required a few hours. Thus, in the case of colorectal cancer, it might also be interesting, which amount of resveratrol is not absorbed and reaches the colon.

3.4 Future perspectives for the use of phytochemicals in cancer treatment

It is obvious that phytochemicals exhibit excellent anticancer and anti-inflammatory properties, but a majority of these studies were conducted in *in vitro* and in animal models. Due to differences in metabolism and genetics, there is a need to investigate these physiological effects in humans, as the activity observed in animal models cannot be easily extrapolated.

Several epidemiological trials recently suggested that cruciferous vegetables, including broccoli, may provide more protection than fruits and vegetables in general, against many different cancers [302]. Whereas epidemiological studies are excellent for hypothesis generation, a recent report even suggested that epidemiological studies are considerably less than 50% reproducible [303]. This low reproducibility might be due to our lack of knowledge of different aspects of the food under study, such as changes in the content of bioactive components with plant variety or cooking method. Hence, animal studies followed by clinical trials are necessary to clarify the data often based on epidemiological studies.

3.4.1 Sulforaphane: preclinical and clinical studies

While promising protective effects of broccoli are proposed, one frequently asked question is: “How many broccoli do I have to eat”? Most cultivars of broccoli accumulate between 2 and 10 µmol g^{-1} of 4-methylsulfinyl glucosinolate in their florets. Higher levels on a dry weight basis may sometimes be found within broccoli sprouts which are mostly used in animal or human trials, a few days after germination. A high-glucosinolate variety of broocoli has, however, been specially bred to accumulate about threefold higher levels of glucosinolates in its florets [304]. As Keum and colleagues [305] recently indicated, broccoli sprouts may serve as a good dietary source of SFN *in vivo* while showing significant inhibitory effects on prostate tumorigenesis. But it is difficult to determine whether such protective effects are related to isothiocyanates or other factors associated with cruciferous vegetables intake. As Hanlon et al. [306] recently demonstrated, fresh broccoli from retail sources generates very low levels of

SFN even when consumed raw, and that in humans, SFN is rapidly absorbed following consumption of liquidised broccoli, but repeated intake of vegetables does not lead to higher plasma levels. Nevertheless, the results of some prospective cohort studies suggest that adults should aim for at least 5 weekly servings of cruciferous vegetables for gaining preventive properties [307-309].

In preclinical rodent models, there is significant data supporting the chemopreventive effects of SFN at several stages of carcinogenesis [reviewed in Clarke, 2008]. As shown in suppression studies, supplementation with SFN decreased polyp formation in Apc^{min} mice [143, 278], decreased colonic aberrant crypt foci in azoxymethane (AOM)-induced rats [310] and also decreased tumor growth in prostate xengraft studies [142, 311]. To date, some clinical trials investigated the safety and tolerance of SFN when it was extracted from broccoli sprouts. A phase I clinical study, for example, examined the safety, tolerance, and metabolism of orally dosed broccoli sprout extracts as glucosinolates and isothiocyanates in healthy human volunteers over 7-day period [312]. No significant or consistent subjective or objective abnormal events like potential toxicities were observed with 25 µmol isothiocyanate (equivalent to ~4.4 mg as sulforaphane) per person per day, however, there have been little systematic evaluations of high doses and long-term administration of isothiocyanates in human studies. In order to move sulforaphane into large-scale clinical trials, more research into metabolism, bioavailability and the impact of this compound is needed. Dashwood et al. recently performed a small preliminary human study in the interest of determining whether the HDAC inhibition effects observed in cell culture and mice could be translated into humans. In normal healthy volunteers, 3-6 h after ingestion of 68 g of broccoli sprouts, a >50% significant decrease in HDAC activity was evident in peripheral blood mononuclear cells with a concomitant increase in acetylated H3 and H4 [142], which confirmed the findings in mice, and in cultured cells treated with SFN [313]. The extent to which acute inhibition of histone deacetylase activity plays a causative role in cancer prevention remains to be determined.

Rank	Phase	Study	Condition	Interventions	ClinicalTrials.gov Identifier
1	I	Sulforaphane as an Antagonist to human PXR-mediated drug-drug interactions	Adverse Drug Interactions	Drug: Rifampicin; Dietary Supplement: SFN plus rifampicin Dietary supplement: SFN alone	NCT00621309
2	I + II	Effects of Sulforaphane on normal Prostate Tissue	Prostate Cancer	Drug: High SFN Extract; Drug: Microcrystalline Cellulose NF (Placebo)	NCT00946309
3	II	Study to Evaluate the Effect of Sulforaphane in BSE on Breast Tissue	Breast Cancer	Drug: BSE; Dietary supplement: Mango juice without extract	NCT00982319
4	II	Total Application of Sulforaphane-containing BSE on Radiation Dermatitis	Breast Cancer; Dermatitis	Drug: SFN-containing BSE	NCT00894712
5	I	Cross-over Broccoli Sprouts Trial	Healthy	Dietary supplement: BSE	NCT01008826
6	I	Effects of 2 Different Broccoli Sprout Containing Supplements on Nasal Cells in Healthy Volunteers	Healthy adult volunteers	Dietary supplement: different preparations of SFN-containing nutritional supplements	NCT01129466
7	II	BSE in Treating Women With Newly Diagnosed Ductal Carcinoma In Situ and/or Atypical Ductal Hyperplasia	Breast Cancer; Precancer-ous conditions	Dietary supplement: BSE; Other: placebo	NCT00843167
8	II	Diet and Vascular Health Study	Cardiovas-cular Disease	Dietary supplement: broccoli	NCT01114399

Table 1: Clinical Trials with Sulforaphane

[[http://clinicaltrials.gov/ct2/results?term=sulforaphane] date: 08.2010]

BSE= Boccoli Sprout Extracts

To date, very few clinical studies have evaluated the effects of SFN on cancer outcome. However, some phase I-III trials are currently underway or finished (summarized in **Table 1**). Most of these studies are under the condition of breast cancer, none of them investigate the influence of broccoli sprout extracts or even pure sulforphane on colon cancer outcome. However, a cross-over broccoli sprouts trial in healthy subjects (NCT01008826) investigates whether broccoli sprouts are effective at altering the urinary levels of metabolites of the hepatocarcinogen aflatoxin B1 and of the air-borne pollutant phenanthrene in residents of Qidong. Residents of Qidong are at high risk for development of

heptacellular carcinoma, in part due to consumption of aflatoxin-contaminated foods, and are exposed to high levels of phenanthrene. In this study, an inverse correlation between SFN treatment and excretion of carcinogens was detected, suggesting induction of one or more Phase 2 enzymes. In summary, due to all these properties, also in regard to above described chemosensitizing properties, sulforaphane seems to be an auspicious candidate in chemoprevention or in chemotherapeutic approaches and could be a potential compound in the development of new therapeutic strategies. In future, also studies with purified sulforaphane are desirable and essential to verify the efficacy in humans suffering from cancer.

3.4.2 Resveratrol: preclinical and clinical studies

The initial reports of therapeutic potential to resveratrol led to the increased interest in this compound from 1997 [104]. Now, it is obvious that resveratrol exhibits excellent anticancer and anti-inflammatory properties including cell growth inhibition of a wide range of human cancer cells in culture as well as implanted tumors usually in mice [105, 314]. The compound inhibited experimental tumorigenesis in a wide range of animal models by targeting many components of intracellular signaling pathways including pro-inflammatory mediators, regulators of cell survival and apoptosis, and tumor angiogenic and metastatic switches by modulating a distinct set of upstream kinases, transcription factors, and their regulators [106, 315].

The potential association between red wine consumption and risk of cardiovascular disease mortality has been highlighted by the ‘‘French Paradox’’ where protective effects are observed for consumption levels up to 300 ml wine per day [316, 317], in which the reductions in risk from low to moderate consumption of alcoholic beverages are greater for red wine compared to white wine, beer, and most spirits [318]. Since concentration of *trans*-resveratrol in red wines estimate about 5 mg resveratrol per litre [319, 320], assuming a consistent daily intake of 375 ml, or about two glasses of wine, a person weighing 70 kg would receive a dose of ~27 µg per kg (body weight) each day. The problem is that at higher doses, the detrimental effects of alcohol are likely to mask any health benefits including the beneficial effect of alcohol consumption on

Alzheimer's disease which is maximal at 1–6 drinks per week [321] and consuming more than four drinks per day nullifies the beneficial effect of alcohol on the risk of myocardial infarction [322]. But apart from the negative effects of alcohol in higher concentrations, what about the safety aspect of resveratrol intake? As Planas et al. [323] recently reported no hematologic or histopathologic toxicity associated with daily oral administration of resveratrol at a high dose of 20 mg/kg in rats exists. This dosage represents a 1000-fold higher resveratrol dosage than typically consumed by humans at the rate of one glass of red wine a day. The results of Crowell et al. [324] further support the above finding where they have shown no adverse effects in rats when administered resveratrol at 300 mg/day for 4 weeks. Absence of toxicity has also been demonstrated in humans that received a single dosage of up to 5 g resveratrol [325]. These experiments clarify that this phytochemical could be applied as a chemopreventive agent without causing any adverse effects. However, it must be recognized that all of these observations have been made with doses of resveratrol that are well above those achievable in humans though normal diet. A recently concluded 10-year epidemiology study showed an inverse relationship between resveratrol and breast cancer risk in women with resveratrol consumption from grapes, but not from wine [326] which could not be explained by several potential confounding factors, including alcohol intake, nor was it attributable to a nonspecific favorable effect of fruit on breast cancer risk [326]. At high doses, orders of magnitude higher than would be achievable through red wine consumption by humans, orally ingested resveratrol is also able to extend lifespan greater than 50% in a number of organisms ranging from mono-cellular yeasts, nematode, insect, fish and mice [327-329]. But to achieve the equivalent dose of resveratrol that was fed to the fish in order to achieve a >50% lifespan extension [328] and to mice in order to prevent the detrimental effects of a high fat diet [330], one would need to consume around 60 l⁄d of high resveratrol red wine, which is clearly not feasible.

Preclinical trials clearly show great promise for resveratrol in the treatment and prevention of cancers [297-301]. The observed efficacy of low doses, for example 200µg/kg body weight/day, which counteracted azoxymethane-induced carcinogenesis in a rat model of colon cancers, suggests that even concentrations of resveratrol, which might be achievable from dietary sources,

Rank	Phase	Study	Condition	Interventions	ClinicalTrials.gov Identifier
1	I + II	Resveratrol for Patients With Colon Cancer	Colon Cancer	**Drug:** Resveratrol	**NCT00256334**
2	I	A Clinical Study to Assess the Safety, Pharmacokinetics, and Pharmacodynamics of SRT501	Colorectal Cancer; Hepatic Metastasis	**Drug**: SRT501; **Drug**: Placebo	**NCT00920803**
3	II	A Clinical Study to Assess the Safety and Activity of SRT501 Alone or in Combination with Bortezomib	Multible Myeloma	**Drug**: SRT501; Drug: Bortezomib	**NCT00920556**
4	I	Resveratrol in Treating Patients With Colorectal Cancer That Can Be Removed By Surgery	Colorectal Cancer	**Drug**: resveratrol	**NCT00433576**
5	I	Resveratrol in Preventing Cancer in Healthy Participants	Unspecified Adult Solid Tumor	**Drug:** resveratrol	**NCT00098969**
6	I	Phase I Biomarker Study of Dietary Grape-Derived Low Dose Resveratrol for Colon Cancer Prevention	Colon Cancer	**Dietary suplement**: grapes	**NCT00578396**
7	II	Dietary Intervention in Follicular Lymphoma	Follicular Lymphoma	**Drugs:** EPA; DHA; Selenium; Gralic extract; Promegranate juice; Grape juice; Green Tea	**NCT0000455416**

Table 2: Clinical trials with resveratrol in the condition of cancer [[http://clinicaltrials.gov/ct2/results?term=resveratrol]; date: 08.2010]

EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid

such as red wine, could be therapeutic in some cases [209]. But protective effects of resveratrol are more dramatic at higher, but pharmacological achievable doses. Therefore, from a functional, pharmacological and clinical point of view it would be useful to distinguish between “pharmacological” and “dietary” chemoprevention. Responding to the question whether the observed effects in *in vitro* and *in vivo* models are also conferrable and relevant for humans, several phase I and II clinical trials are currently in progress. One such study is a recently completed phase I clinical trial, supported by the US National Cancer Institute and the UK Medical Research Council, demonstrates that consumption of resveratrol (5 g) does not cause any serious adverse effects in healthy volunteers, but the peak plasma level (2.4 mol/L) remains much below the minimum required concentration (5 mol/L) of the compound to exert the chemopreventive effect in cultured cells [325]. The study also indicates the presence of several fold higher plasma levels of resveratrol monoglucuronides and resveratrol-3-sulfate. Several other clinical trials are in progress to demonstrate the cancer therapeutic effects of resveratrol in cancer patients (summarized in **Table 2**). One of these studies is a phase I trial (NCT00433576) conducted at the University of Michigan, investigating the bioavailability and toxicity profile of resvera-

trol, and its effects in the expression of COX-2 and in M/G cell cycle arrest in individuals with colorectal cancer. Phases I and II clinical trials in patients with colon cancer (NCT00256334) conducted from researchers at the University of California, will investigate the effects of resveratrol in modulating the Wnt-signaling pathway, a pathway that is implicated in the etiology of colon cancer. They also propose starting another clinical intervention study using dietary resveratrol in colon cancer prevention (NCT00578396). This study will investigate if a grape juice-supplemented diet will reduce the risk of colon cancer in healthy volunteers who are 18 years of age and older. In addition to these studies, resveratrol is part of a multicomponent dietary intervention phase II clinical trial in progress at the University of Oslo (NCT00455416). This study proposes to use dietary components, including resveratrol in the form of grape juice, in the induction of apoptosis, inhibition of cell proliferation, and modulation of tumor cell infiltrate in patients with follicular lymphoma. Results of these trials will address the issue of extrapolation from the results of resveratrol in animal studies to therapeutic potential for humans and also provide a basis for the prospective application of resveratrol in cancer chemoprevention. Recently, a clincal trial of a formulation of resveratrol has been suspended due to safety concerns. In this phase II trial safety and activity of a resveratrol-based drug alone or in combination with the proteasome inhibitor bortezomib in patients multiple myeloma should be assessed (NCT00920556). The trial was halted when 5 out of 24 patients developed a kidney condition called cast nephropathy. Interestingly, all patients who experienced kidney failure during the trial were being treated with only the resveratrol-based drug when their kidney problems developed. However, it is still uncertain whether the kidney failures were actually related to the resveratrol treatment, or were simply a manifestation of the underlying myeloma since cast nephropathy is so commonly associated with multiple myeloma that it is even called “myeloma kidney” [331]

Developing novel resveratrol derivatives is another possible approach for enhancing bioavailability. A series of cis-stilbenes and trans-stilbenes related to resveratrol with varying functional groups have been synthesized, and some of these compounds are more potent than resveratrol in suppressing the growth of human cancer cells in vitro (reviewed in [332]). Researchers have started to explore the anticancer effects of resveratrol derivatives in vivo [333-337] and at

least one study indicated that a tetramer of resveratrol (heyneanol) presented comparable or better anticancer efficacy than did resveratrol in a mouse lung cancer model [334]. The preclinical and clinical data examined in this report strongly suggest that resveratrol is a promising candidate in chemopreventive and chemotherapeutic strategies and a potential weapon in the effort to alleviate the burden of human cancer, even more when also regarding the chemosensitizing effects, described above. Considering its multivarious molecular targets, John Pezzuto has asserted very timely that resveratrol induces a ‘biologically specific tsunami’ [338].

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Eidesstattliche Erklärung

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

Teile der vorliegenden Arbeit wurden in folgenden Publikationsorganen veröffentlicht:

I. **Kaminski, BM.**, Loitsch, SM., Ochs, MJ., Reuter, KC., Steinhilber, D., Stein, J., Ulrich S. (2010) Isothiocyanate sulforaphane inhibits protooncogenic ornithine decarboxylase activity in colorectal cancer cells via induction of the TGF-beta/Smad signaling pathway. Mol Nutr Food Res 54 (10): 1486-96

II. **Kaminski, BM.**, Weigert, A., Brüne, B., Schumacher M., Wenzel, U., Steinhilber, D., Stein, J., Ulrich, S. (2010) Sulforaphane potentiates oxaliplatin-induced cell growth inhibition in colorectal cancer cells via induction of different modes of cell death. Cancer Chemother Pharmacol. August 6. [Epub ahead of print]

III. **Kaminski, BM.**, Weigert, A., Ley, S., Brecht, K., Brüne, B., Steinhilber, D., Stein, J., Ulrich, S. (2010) Resveratrol-induced potentiation of the antitumor effects of oxaliplatin is accompanied by an altered cytokine profile of human-derived macrophages. Mol Nutr Food Res (under review)

IV. **Kaminski, BM.**, Steinhilber, D., Stein J., Ulrich S. (2010) Phytochemicals Resveratrol and Sulforaphane as potential agents for enhancing the anti-tumor activities of conventional cancer therapies. Curr Pharmaceut Biotechnol. Invited Review (in press)

Abgrenzungserklärung

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

„ CHEMOPREVENTIVE AND SENSITIZING EFFECTS OF PHYTOCHEMICALS IN A CELL CULTURE MODEL OF COLORECTAL CANCER"

als kumulative Dissertation verfasst.

Der Arbeit liegen folgende Veröffentlichungen zugrunde:

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II. **Kaminski, BM.**, Weigert, A., Brüne, B., Schumacher M., Wenzel, U., Steinhilber, D., Stein, J., Ulrich, S. (2010) Sulforaphane potentiates oxaliplatin-induced cell growth inhibition in colorectal cancer cells via induction of different modes of cell death. Cancer Chemother Pharmacol. August 6. [Epub ahead of print]

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Die c-myc Promotoren (‚full lenght' und SBE-mutiert) wurden freundlicherweise von Herrn Prof. Ellenrieder (Universität Marburg) zur Verfügung gestellt. Anschließend wurden diese Konstrukte mittels Lipofektion von Frau Kaminski in Caco-2 Zellen transfiziert.

Die Annexin-V/PI-Anfäbung mit anschließender FACS-Analyse wurde in Zusammenarbeit mit der Arbeitsgruppe Prof. Brüne (Institut der Biochemie I/ZAFES, Goethe Universität, Frankfurt) unter der Anleitung von Herrn Dr. Andreas Weigert durchgeführt, wobei die Porbenvorbereitung durch Frau Kaminski erfolgte. Die Isolierung und Bereitstellung von Makrophagen erfolgte ebenfalls in der Arbeitsgruppe Prof. Brüne, mit der Hilfe von Frau Stefanie Ley und Frau Kerstin Brecht. Die anschließende Cytokin-Messung wurde von Frau Kaminski vorbereitet und mit der Hilfe von Herrn Dr. Andreas Weigert mittels FACS-Analyse quantifiziert.

Die Anfärbung der Caco-2 Zellen mit Sytox-Green erfolgte in Zusammenarbeit mit der Arbeitsgruppe Prof.Wenzel (Justus-Liebig-Universität Gießen) unter der Anleitung von Herrn Marco Schumacher.

Prof. Dr. Dr. J. Stein sowie Dr. Sandra Ulrich haben die Manuskripte kritisch Korrektur gelesen und in Diskussionen hilfreiche Ideen für weitere Versuche geliefert.

Bettina Kaminski

ANNEX

I

RESEARCH ARTICLE

Isothiocyanate sulforaphane inhibits protooncogenic ornithine decarboxylase activity in colorectal cancer cells *via* induction of the TGF-β/Smad signaling pathway

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Scope: The objective of this study was to elucidate molecular mechanisms behind the anti-tumor activities of the isothiocyanate sulforaphane (SFN) in colorectal cancer cells.
Methods and results: Cell growth was determined by BrdU incorporation and crystal violet staining. Protein levels were examined by Western blot analysis. Ornithine decarboxylase (ODC) activity was assayed radiometrically. Reverse transcriptase-PCR was used for measuring mRNA expression. For reporter gene assays plasmids were transfected into cells via lipofection and luciferase activity was measured luminometrically. Acetyl-histone H3 and H4 chromatin immunoprecipitation (ChIP) assays were performed followed by PCR with TGF-β-receptor II promoter specific primers. We could show that SFN-mediated cell growth inhibition closely correlates with a dose-dependent reduction of protein expression and enzymatic activity of ODC. This effect seems to be due to reduced protein levels and trans-activation activity of transcription factor c-myc, a direct regulator of ODC expression, as a consequence of SFN-induced TGF-β/Smad signaling. The coherency of these results was further confirmed by using TGF-β receptor kinase inhibitor SB431542, which largely abolishes inhibitory effects of SFN on both, ODC activity and cell growth.
Conclusion: Since elevated ODC enzyme activity is associated with enhanced tumor development, SFN may be a dietary phytochemical with potential to prevent carcinogenesis.

Keywords:
c-myc / Colorectal cancer / Ornithine decarboxylase / Sulforaphane / Transforming growth factor-β signaling

1 Introduction

Colorectal cancer accounts for approximately 10% of all cancers and is the second leading cause of cancer-related death in Western countries with an estimated 146 970 new cases and 49 900 deaths in 2009 in the United States [1]. In addition to conventional cancer therapy, preventive measures that target the multi-step progress of carcinogenesis involving initiation, promotion and progression [2] are aimed at decreasing the incidence and mortality of cancer. In this context, increasing evidence suggests the importance of food and nutrition in the modification of the cancer development [3]. In particular, *in vitro* and *in vivo* data revealed that consumption of cruciferous vegetables is supposed to lower overall cancer risk, including colon cancer, especially during the early stages [4]. These anti-carcinogenic activities have been suggested to be partly due to the relatively high levels of sulforaphane (SFN), the major biologically active compound [5]. SFN, first isolated from broccoli in the early 1990s, was initially identified as a potent

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Abbreviations: BrdU, bromodeoxyuridine; **FCS,** fetal calf serum; **HDAC,** histone deacetylase; **HDACi,** histone deacetylase inhibitor; **ODC,** ornithine decarboxylase; **SBE,** Smad binding element; **SFN,** sulforaphane; **TGF-β,** transforming growth facor-β; **TGF-β RI,** transforming growth factor-β receptor I; **TGF-β RII,** transforming growth factor-β receptor II

phase 2 enzyme inducer, but in recent years numerous studies have implicated further chemopreventive effects, comprising cell growth inhibition, induction of apoptosis and reduction of angiogenesis [6–9], whereby the underlying molecular mechanisms remain largely unclear.

Recently, Myzak and Colleagues identified SFN as a novel histone deacetylase inhibitor (HDACi) in colon and prostate cancer cells [10]. HDACi, as a new class of chemotherapeutic agents, show significant promise against a variety of cancers in clinical trials [11]. Most available HDACi inhibit all class I and II histone deacetylases (HDACs), thereby increasing acetylation of histone and nonhistone protein targets [12]. *In vivo*, histone acetylation depends on the balance between histone acetyltransferases and HDACs, which has been proposed to play an important role in transcriptional regulation by altering chromatin structure [13]. Histone acetylation by histone acetyltransferases is associated with an open chromatin conformation, promoting gene transcription, whereas HDACs maintain the chromatin in the closed, transcriptional inactive state. HDAC inhibitors have been shown to induce the expression of several tumor suppressive genes, such as $p21^{WAF1}$ [14], growth-differentiation factor 11, a member of the transforming growth factor-β (TGF-β) superfamily [15] as well as TGF-β receptors I and II (TGF-βRI and TGF-βRII) [16, 17]. Thus, the pro-tumorigenic role of HDACs in colon cancer and the pre-clinical efficacy of these agents in colon cancer cells justify the use of HDACi in cancer prevention and therapy [18].

TGF-β has been implicated in various cellular processes, including regulation of cellular proliferation [19] and differentiation [20], immune modulation [21] and extracellular matrix remodeling [22]. It exhibits its antiproliferative functions by activating a signaling pathway that mediates cell cycle arrest and induction of apoptosis. TGF-β exerts its effects through heteromeric receptor complexes consisting of type I and type II serine/threonine kinase receptors. The signaling is initiated by ligand binding to the TGF-βRII cell surface receptor. This, in turn, recruits the TGF-βRI kinase, which then phosphorylates the R-Smad proteins, Smad2 and Smad3 [23]. Activated R-Smads form a complex with the Co-Smad, Smad4, which shuttles directly to the nucleus. Here, the complex can either act as a co-activator or DNA-binding transcription factor, participating in the regulation of target gene expression (*e.g.* p15, p21, c-myc) [23, 24]. TGF-β signaling is further controlled by a third class of Smads, the inhibitory Smad6 and Smad7 proteins, which negatively regulate R-Smad activation. Recently, Daniel *et al.* [25] could demonstrate that butyrate, another natural occurring HDACi, mediates anticarcinogenic effects in colorectal cancer cells, at least partly, through the activation of the TGF-β signaling pathway. Furthermore, Traka *et al.* [26] provide evidence that SFN enhances TGF-β signaling in cell cultures.

Previous studies could demonstrate that modulation of polyamine metabolism provides a chemopreventive strategy of different phytochemicals [27–31]. Although cellular polyamines spermidine and spermine, as well as their precursor putrescine, are essential for growth and DNA synthesis, increasing concentrations are associated with hyperproliferation and cell transformations [32]. Overall, polyamines are involved in almost all steps of colonic tumorigenesis. Regulation of polyamine levels is governed primarily by activity of ornithine decarboxylase (ODC), the key enzyme of polyamine biosynthesis. Hence, regarding the role of polyamines in colonic carcinogenesis, modulation of polyamine metabolism and, in particular, of ODC activity has been studied as a potent therapeutic strategy in cancer treatment and prevention [30, 33].

Thus, the aim of this project was to provide insight into the molecular mechanisms of SFN-mediated anti-tumor activities, in particular by investigating regulatory functions of SFN on ODC activity, also with regard to a possible involvement of the TGF-β signaling pathway.

2 Materials and methods

2.1 Cell culture and materials

Caco-2 cells were kept in DMEM, supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% sodium pyruvate and 1% nonessential amino acids and were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. The cells were passaged weekly using Dulbecco's PBS containing 0.1% trypsin and 1% EDTA. The cells were screened for possible contamination with mycoplasma at monthly intervals. For experiments, the cells were seeded onto plastic cell culture wells in serum-containing medium and allowed to attach for 24 h. For the ODC activity assay, the cells were synchronized in a medium containing 1% FCS 24 h before treatment. SFN (Calbiochem) was dissolved in DMSO at a concentration of 100 mM, SB431542 (Sigma-Aldrich) was dissolved in DMSO at a concentration of 10 mM and TGF-β-1 (PeproTech) was dissolved in BSA at a concentration of 10 μg/mL. DMEM, DMEM/Ham's F-12 medium, McCoy's 5A, FCS, DMSO, Sodium pyruvate solution, penicillin and streptomycin stock solutions were all obtained from PAA Laboratories GmbH.

2.2 Cell counts

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

2.3 Cell proliferation

The effects of SFN on DNA synthesis of cells was assessed using a cell proliferation ELISA kit (Roche Diagnostics). This assay is a colorimetric immunoassay for the quantification of cell proliferation based on the measurement of bromodeoxyuridine (BrdU) incorporation during DNA synthesis. Cells were grown in 96 well culture dishes (10^4 cells/well), incubated with SFN for different time intervals and then labeled with BrdU for a further 4 h. Incorporated BrdU was measured colorimetrically.

2.4 Plasmids

The human pGL3-c-myc promoter (−62–93) with the sequence TTCTCAGAGGCTTGGCGGGAAAAAGAACGG and the pGL3-c-myc-promotor (−62–93) TIEm with the sequence TTCTCAGA**TTAAA**GGCGGGAAAAAGAACGG (Smad binding site mutated) were kindly provided by Prof. Ellenrieder (Marburg, Germany).

The pCGN-Smad3 and pCGN-Smad4 expression plasmids were obtained from Dr. X. F. Wang (Durham, USA) and the 4 × SBE (Smad binding element)-luc promoter was generously provided by B. Vogelstein (Johns Hopkins, Baltimore, MD, USA) [34].

2.5 Reporter assays

Caco-2 cells were transfected by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Medium was changed after 4 h of transfection and the cells were incubated in a medium containing 10% serum for another 16–20 h. Later, the cells were treated with SFN (20 μM) or TGF-β (20 nM) and then subjected to luciferase assay. pCGN and pGL3, as empty vector plasmids, were used as a negative control for transfection and luciferase assay. Luciferase assays were carried out by using luciferase assay kit (Promega) and a luminometer (TECAN Spectra-Fluor^{Plus}). A cytomegalovirus-Renilla luciferase plasmid or a Simian Virus-40-Renilla luciferase plasmid (both from Promega) was used as a control, to normalize the transfection efficiency, and was assayed as described [35].

2.6 SDS-polyacrylamide gel electrophoresis and immunoblot analysis

Caco-2 cells were seeded in 80-cm^2 flasks; 24 h after plating, cells were incubated with substances for different time intervals ranging from 1 to 48 h. After washing the cells with ice-cold PBS, followed by an incubation step with cell lysis buffer (Cell signalling) containing multiple protease inhibitors (Complete Mini®, Roche) for 20 min at 4°C, cells were harvested by scraping. Protein extracts were obtained after sonication of cell lysates (2 × 5 s) and centrifuged at 10 000 rpm for 10 min at 4°C. Samples were analyzed for their protein content using the BioRad® colorimetric assay according to the method of Bradford (BioRad Laboratories). After addition of sample buffer (Roti Load®, Roth) to the total cellular extract and boiling for 5 min at 95°C, 30–40 μg of total protein lysate was separated on a 10 or 12% SDS-polyacrylamide gel. Protein was transferred onto nitrocellulose membrane (Schleicher&Schuell) and the membrane was blocked for 1 h at room temperature with 5% skimmed milk in Tris-buffered saline containing 0.05% Tween-20. Next, blots were washed and incubated overnight at 4°C in Tris-buffered saline containing 0.05% Tween-20 containing either 5% BSA or 5% skimmed milk with a 1:1000 or 1:2000 dilution of primary antibodies for TGF-β1, TGF-βRI and TGF-βRII (all from Cell Signaling), c-myc and ODC (all from Santa Cruz Biotechnology). The secondary, horseradish peroxidase-conjugated, antibody (Santa Cruz Biotechnology) was diluted at 1:2000 or 1:4000 and incubated with the membrane for another 60 min in skimmed milk. Following chemoluminescence reaction (ECL, Amersham pharmacia biotech), bands were detected after exposure to Hyperfilm-MP (Amersham International plc). Blots were reprobed with β-actin antibody (Santa Cruz Biotechnology). For quantitative analysis, bands were evaluated densitometrically by ProViDoc system (Desaga), normalized for the density of β-actin.

2.7 Reverse transcriptase-PCR

Total cellular RNA was isolated by RNAzol BTM (Wak-Chemie), following manufacturer's instructions. Reverse transcription of total cellular RNA was carried out using Superscript II RNase H reverse transcriptase (Life Technologies) and random hexanucleotide primers (Promega). PCR was performed (on the cDNA) using the following sense and antisense primers, custom-synthesized by Biospring: *TGF-β1*: 5′-CAC GAT CAT GTT GGA CAA CTG CTC C-3′ and 5′-CTT CAG CTC CAC AGA GAA GAA CTG-3′; *GAPDH*: 5′-ATC TTC CAG GAG CGA GAT CC-3′ and 5′-ACC ACT GAC ACG TTG GCA GT-3′. Thermal cycling was performed as follows: denaturation for 30 s at 95°C, annealing for 30 s at 55°C and extension for 90 s at 72°C. Twenty-five to thirty-five cycles were performed. Primers were used at a final concentration of 10 μM each, dNTPs at 500 μM (Eurogentec) and $MgCl_2$ at 3 mM. Five units of Taq DNA Polymerase were used *per* 50 μL reaction. Ten microliter of PCR product were separated on a 1.5% agarose gel containing ethidium bromide and visualized by UV illumination.

2.8 ODC activity

The activity of ODC was assayed using a radiometric technique in which the amount of $^{14}CO_2$ liberated from

DL-[l-^{14}C]ornithine (207.2×10^4 MBq/mol, Amersham Pharmacia Biotech) was estimated, as described previously [36]. Briefly, after treatment, cell culture dishes were placed on ice and monolayers were washed three times with cold PBS. Cells were harvested by scraping in homogenizing buffer (50 mM Tris buffer, pH 7.2, 5 mM DTT, 100 μM EGTA), sonicated and centrifuged for 10 min at $15\,000 \times g$ at 4°C. Hundred microliter of the obtained supernatant was incubated in a stoppered tube with 74 μM DL-[l-^{14}C]ornithine in the presence of pyridoxal-5-phophate for 1 h at 37°C. $^{14}CO_2$, liberated by the decarboxylation of ornithine and trapped on filters impregnated with benzethonium hydroxide, was measured by liquid scintillation spectroscopy. Samples were analyzed for their protein content using the BioRad® colorimetric assay according to the method of Bradford (BioRad Laboratories). ODC is expressed in picomolar of released CO_2 *per* hour *per* milligram of protein. Controls always included samples for measurement of nonenzymatic release of $^{14}CO_2$.

2.9 Chromatin immunoprecipitation Assay

Chromatin immunoprecipitation assays were performed using Acetyl-Histone Immunoprecipitation Assay Kits (Upstate Biotechnology) following the manufacturer's instructions. Briefly, approximately 1×10^6 cells cultured in a 75 cm^2 dish were treated with different concentrations of SFN (10–20 μM). Histones were cross linked to DNA by adding 1% formaldehyde directly to culture medium for 10 min at 37°C. Each sample was lysed in SDS lysis buffer (kit component) containing protease inhibitors (1 mM PMSF, 1 μg/mL aprotinin and 1 μg/mL pepstatin A) followed by sonication on ice to shear DNA to lengths between 200 and 1000 bp and incubation with primary antibodies to acetylated histone H3 or acetylated histone H4 overnight at 4°C. Samples were incubated with Salmon Sperm DNA/Protein A Agarose-Slurry (kit component) for 1 h at 4°C with rotation. After washing several times with washing buffers (kit components), the pellet protein A agarose/antibody/histone complex was incubated with elution buffer (1% SDS and 0.1 M $NaHCO_3$) for 15 min at room temperature. Samples were incubated with 0.2 M NaCl for 4 h at 65°C. After DNA was recovered by phenol/chloroform extraction and ethanol precipitation, PCR was performed using the following primers for TGF-βRII promoter, sense: 5′-GAG AGA GCT AGG GGC TGG-3′; antisense: 5′-CTC AAC TTC AAC TCA GCG CTG C-3′; primer for β-actin gene promoter, sense: 5′-CCA ACG CCA AAA CTC TCC C-3′; antisense: 5′-AGC CAT AAA AGG CAA CTT TCG-3′ [17]. Results were finally normalized to β-actin.

2.10 Statistics

The data are expressed as mean ± SE of at least three independent experiments. Results were analyzed using GraphPad Prism software by a two-way ANOVA. A p-value < 0.05 was considered to be significant.

3 Results and discussion

3.1 Cell growth-inhibitory effects of SFN closely correlate with the reduction of ODC protein and activity

In colorectal cancer tissue, the activities of the polyamine-synthesizing enzymes ODC and *S*-adenosylmethionine decarboxylase as well as the content of polyamines are increased 3- to 4-fold over that found in the equivalent normal colonic tissue [37]. Increasing concentrations of polyamines are generally associated with cell proliferation and cell transformation induced by growth factors [38], carcinogens [39] or oncogenes [32]. Several studies suggest

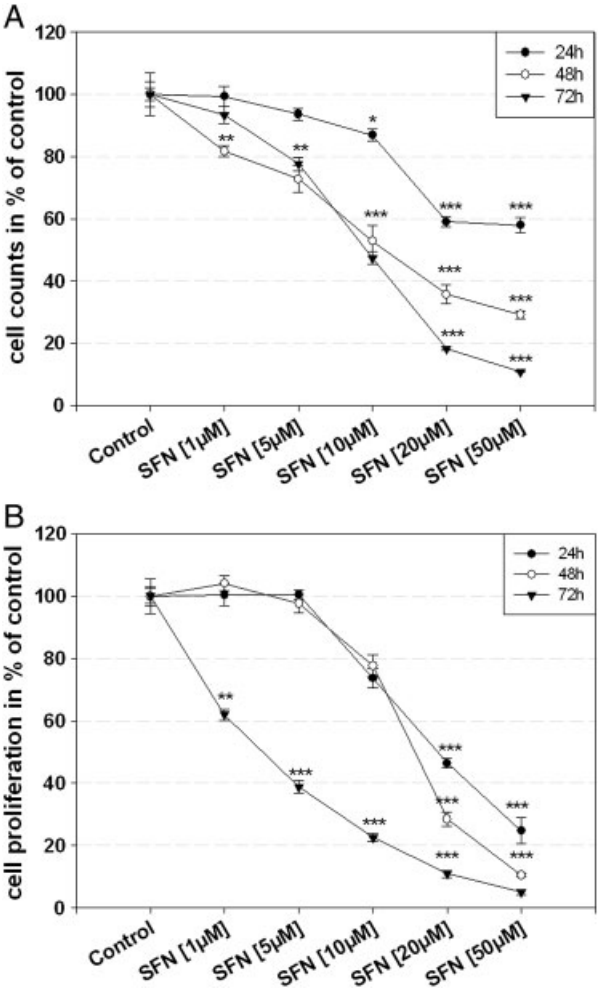

Figure 1. Cell counts and cell proliferation of Caco-2 cells (A, B) 24, 48, and 72 h after incubation without (control) or with SFN (1–50 μM). Values represent mean ± SE ($n = 3$); $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* control.

that a number of potential chemopreventive agents down-regulate ODC expression/activity and subsequently deplete polyamine content in colon cancer cells [40]. Hence, inhibiting the polyamine metabolism, particularly the biosynthetic key enzyme ODC, is considered to represent an attractive target for both cancer chemotherapy and cancer chemoprevention. Interestingly, in their most recent publication, Furniss *et al.* reported modulator effects of broccoli extracts as well as SFN alone on polyamine metabolism in colorectal cancer cells. However, direct modes of action were not further specified [41]. In the present study, we could demonstrate that SFN (1–50 μM) inhibits cell counts and proliferation significantly in Caco-2 cells in a dose- and time-dependent manner ($^{***}p<0.001$) (Fig. 1A and B), which closely correlates with a dose-dependent reduction of ODC protein levels after 3 and 6 h (Fig. 2A) and activity after 24 h of incubation (Fig. 2B) ($^{***}p<0.001$). The crucial role of polyamine depletion was further suggested as addition of exogenous spermidine significantly ($^{***}p<0.001$) counteracted growth inhibitory effects of SFN after 24 h (Fig. 2C). Similar effects could be observed by the group of Lee *et al.* where SFN inhibited TPA-induced ODC activity in mouse epidermal ME308 cells [42].

ODC levels are tightly controlled either by transcriptional regulation or by rapid post-translational degradation. The proto-oncogene c-myc is a transcription factor that directly regulates the expression of ODC by binding to a specific CACGTG sequence in the gene promoter [43]. c-myc was subsequently found to be activated in various animal and human tumors and an amplification of the c-myc gene has been described in ~15% of all human tumors [44]. Since c-myc is a ubiquitous promoter of cell growth and proliferation, it functions as a transcriptional activator or inhibitor depending on the target gene [45, 46]. We could demonstrate that decreased ODC activity and protein levels in Caco-2 cells are accompanied by decreased protein levels of c-myc after 6 h of incubation (Fig. 2D), implicating a direct modulation of ODC gene expression by SFN.

3.2 Effects of SFN on TGF-β signaling

We previously reported that induction of the TGF-β signaling pathway in colon cancer cells is a crucial event in the anti-carcinogenic activities of butyrate, another natural occurring HDAC inhibitor [25]. This, together with the

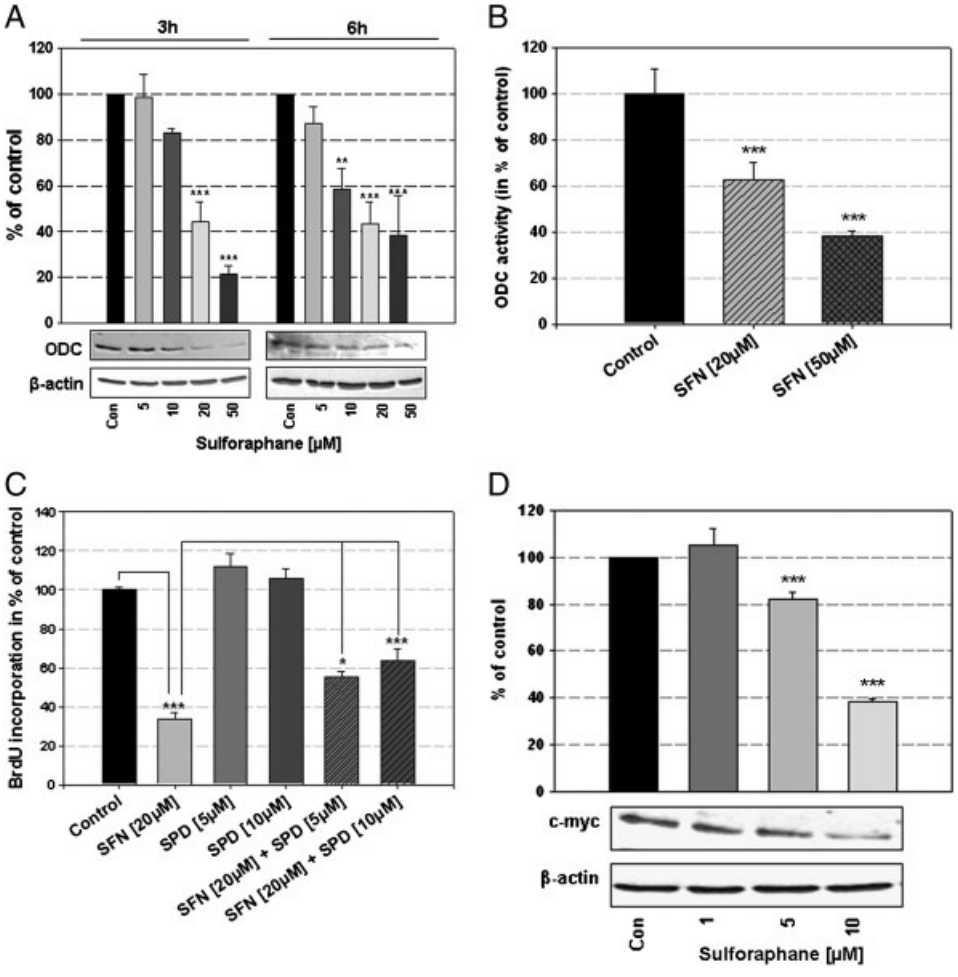

Figure 2. (A) Western blot of ODC (~53 kDa) in Caco-2 cells after incubation with SFN (5–50 μM) for 3 and 6 h. The graph presents densitometric analysis of the Western blot images normalized to β-actin (mean ± SE ($n=3$); $^{**}p<0.01$, $^{***}p<0.001$ *versus* control). (B) Activity of ODC in Caco-2 cells after incubation with SFN (20–50 μM) for 24 h. Bar graphs represent mean ± SE ($n=4$); $^{***}p<0.001$ *versus* control. (C) Add-back experiment with spermidine. Cell proliferation was measured after simultaneous treatment of Caco-2 cells with SFN (20 μM) and spermidine (SPD) (5–10 μM) for 24 h. Bar graphs represent mean ± SE ($n=3$); $^{*}p<0.05$, $^{***}p<0.001$ *versus* control. (D) Western blot of c-myc protein expression (~67 kDa) in Caco-2 cells after incubation with SFN (1–10 μM) for 6 h. The bar graph presents densitometric analysis of the Western blot images normalized to β-actin (mean ± SE ($n=3$); $^{*}p<0.05$, $^{***}p<0.001$ *versus* control). Representative immunoblots of three independent experiments are shown.

findings of Traka *et al.*, who could demonstrate that TGF-β signaling also seems to play an important role in various SFN-mediated effects [26] lets us consider whether SFN possibly shows similar modes of action in our context. Thus, we measured protein as well as mRNA level of TGF-β in Caco-2 cells and could show an obvious increase of TGF-β precursor levels after 2 h (Fig. 3A) and an increase of mRNA after 1 and 3 h of incubation with SFN, indicating regulatory effects on the level of transcription (Fig. 3B). As already mentioned, not only TGF-β alone but also the expression status of TGF-βRI and TGF-βRII is essential for TGF-β-mediated actions. Therefore, protein levels of both receptors I and II were detected after incubation with SFN, and were found to be highly increased after 1 h (Fig. 3C) ($^{***}p<0.001$).

To further specify these regulatory mechanisms, we used chromatin immunoprecipitation analysis to investigate possible effects of SFN-mediated HDAC inhibition on the

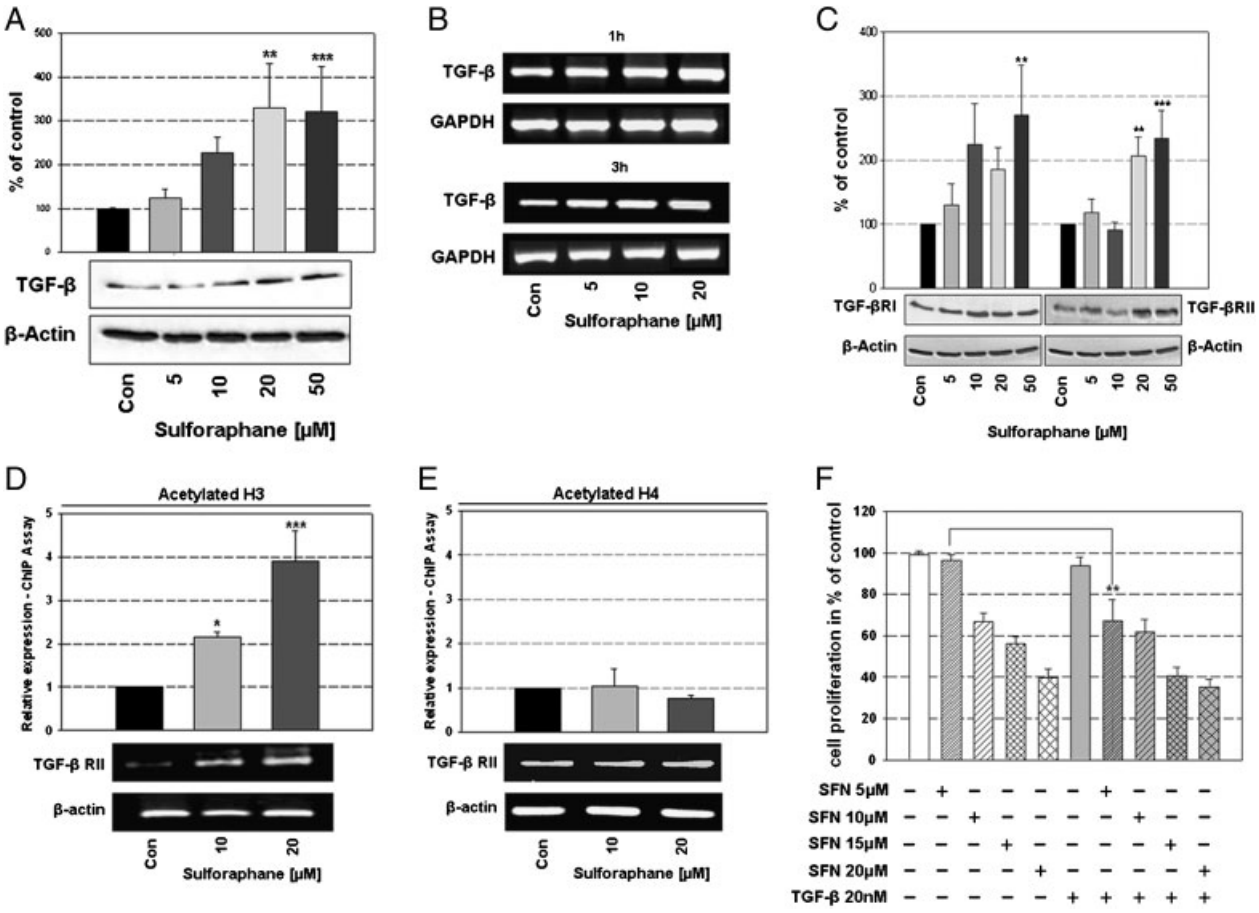

Figure 3. (A) Western blot of TGF-β1 precursor (~50 kDa) in Caco-2 cells after incubation with SFN (5–50 μM) for 2 h. A representative immunoblot of three independent experiments is shown. The bar graph presents densitometric analysis of the Western blot images normalized to β-actin (mean ± SE ($n=3$); $^{**}p<0.01$, $^{***}p<0.001$ *versus* control). (B) RT-PCR of TGF-β mRNA (298 bp) in Caco-2 cells after incubation with SFN (5–20 μM) for 1 and 3 h. Representative agarose gels showing PCR products of three independent experiments for both time-points are shown. (C) SFN-induced expression of TGF-β receptors I and II protein level after 1 h of incubation. Caco-2 cells were treated without (control) and with SFN (5–50 μM). Whole-cell lysates were prepared and TGF-β receptors I and II protein expression were determined by Western blotting using either anti-TGF-β receptors I (~52 kDa) and II (~110 kDa) antibodies. Representative immunoblots of three independent experiments are shown. $^{**}p<0.01$, $^{***}p<0.001$ *versus* control. (D) Caco-2 cells were treated with SFN (10–20 μM) or vehicle for 1 h of incubation. DNA was cross-linked to proteins before harvesting. Chromatin immunoprecipitation (ChIP Assay) was performed against acetylated histone H3, and after DNA isolation and reversal of cross-linking, primers specific for TGF-β receptor II (101 bp) were used during PCR amplification. Results were normalized to β-actin (156 bp), and expressed relative to control, which was assigned an arbitrary value of 1.0. Graph presents the densitometric analysis after 1 h (mean ± SE ($n=3$); $^{*}p<0.05$, $^{***}p<0.001$ *versus* control). (E) Caco-2 cells were treated with SFN (10–20 μM) or vehicle for 1 h of incubation. DNA was cross-linked to proteins before harvesting. ChIP Assay was performed against acetylated histone H4, and primers specific for TGF-β receptor II (101 bp) were used during PCR amplification. Results were normalized to β-actin (156 bp), and expressed relative to control, which was assigned an arbitrary value of 1.0. Graph presents the densitometric analysis after 1 h. (F) Cell proliferation of Caco-2 cells after simultaneous treatment with SFN (5–20 μM) and TGF-β (20 nM) for 24 h. Values represent mean ± SE ($n=3$); $^{**}p<0.01$, $^{***}p<0.001$ *versus* control.

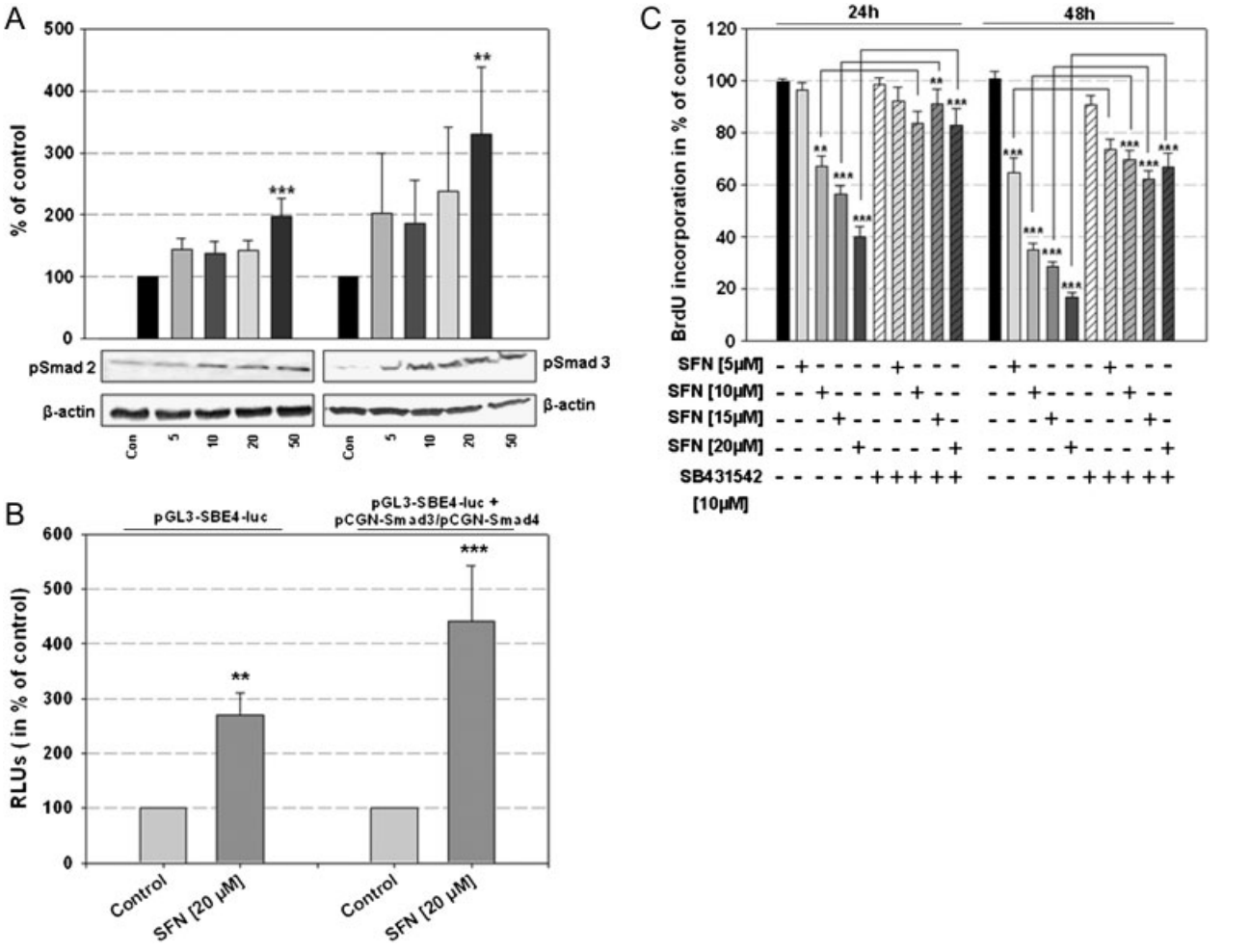

Figure 4. (A) Western blot of pSmad2 and pSmad3 in Caco-2 cells after incubation with SFN (5–50 μM) for 1 h. One representative immunoblot of three independent experiments for both proteins is shown. The bar graphs present densitometric analysis of the Western blot images normalized to β-actin (mean ± SE; $^{**}p<0.01$, $^{***}p<0.001$ *versus* control). (B) The pGL3-SBE4-luc reporter gene construct (800 ng) alone and in combination with the expression vectors pCGN-Smad3/pCGN-Smad4 (100 ng each) and 20 ng SV-40-renilla were transiently transfected into Caco-2 cells *via* lipofection. Cells were treated without (control) or with SFN (20 μM) for 6 h before luciferase activity was determined. Results are presented as relative light *per* units (RLUs) in percentage of control, normalized to transfection efficiency (cotransfection of SV-40-renilla) and normalized to effects of the empty vectors pCGN and pGL3basic (mean ± SE ($n = 3$); $^{**}p<0.01$, $^{***}p<0.001$ *versus* untreated cells). (C) Cell proliferation of Caco-2-cells treated with SB431542 (10 μM) and SFN (5–20 μM). BrdU incorporation was measured after 24 and 48 h of incubation (mean ± SE ($n = 3$); $^{**}p<0.01$, $^{***}p<0.001$ *versus* control).

acetylation status of histone H3 as well as histone H4 associated with the TGF-βRII promoter (Fig. 3D and E). After 1 h, accumulation of RII with highly acetylated histone H3 was observed in SFN-treated Caco-2 cells in comparison to untreated control cells ($^{***}p<0.001$) (Fig. 3D). This SFN effect on RII is selective because the β-actin gene was not affected and obviously specific for acetylated H3 since acetylated histone H4 was not associated with the TGF-βRII gene after incubation with SFN for 1 h (Fig. 3E). The observed accumulation of acetylated histone H3 indicated histone acetylation to be involved in the transcriptional induction of RII and further suggests the importance of HDAC inhibitory properties of SFN in the regulation of TGF-β signaling. In earlier works, Osada *et al.* already demonstrated that HDAC inhibitors can increase the transcriptional activity of TGF-βRII *in vivo* and *in vitro* in human cancer cell lines [17]. In addition to these findings, Lee *et al.* reported [47] that another HDAC inhibitor MS-275 induces the accumulation of acetylated histones in the chromatin of the TGF-βRII gene, which is associated with an increase of TGF-βRII mRNA in human breast cancer cells, contributing to the restoration of TGF-β signaling. As both TGF-βRI and TGF-βII protein levels were regulated by SFN, we were interested to know whether co-incubation with exogenous

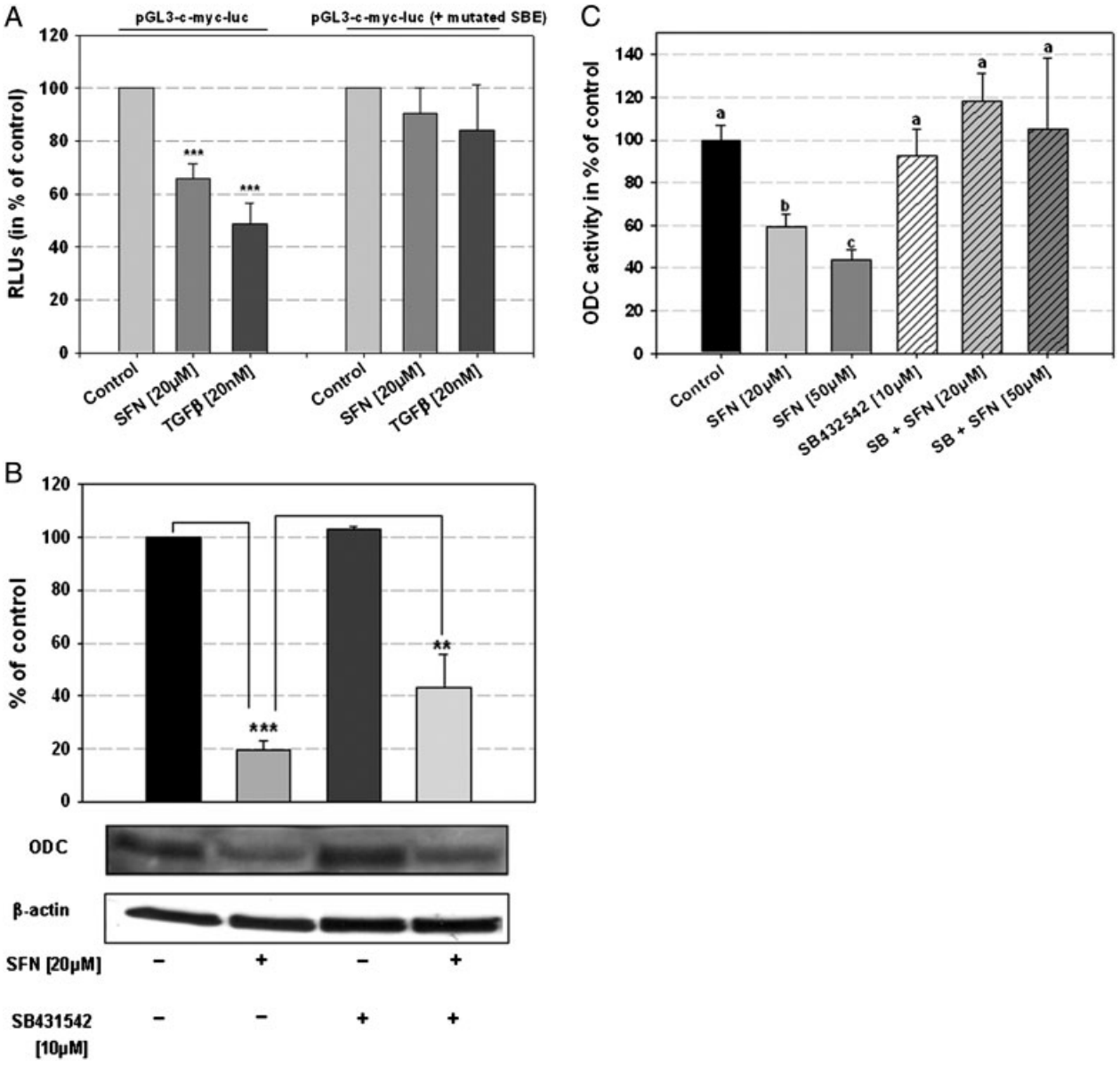

Figure 5. (A) Either pGL3-c-myc-luc or pGL3-c-myc-luc (ΔSBE) (400 ng) and 15 ng CMV-renilla were transiently transfected into Caco-2 cells *via* lipofection. Cells were treated with SFN (20 μM) or TGF-β (20 ng) for 6 h before luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of CMV-renilla. Results are expressed with respect to pGL3-cotransfected cells ($^{**}p<0.01$, $^{***}p<0.001$ *versus* control). (B) Western blot of ODC protein in Caco-2 cells after 6 h of incubation with SFN (20 μM) or/and SB431542 (10 μM). One representative immunoblot of three independent experiments for both proteins is shown. The bar graphs present densitometric analysis of the Western blot images normalized to β-actin (mean ± SE; ($n=3$); $^{***}p<0.001$ *versus* control; $^{**}p<0.01$ *versus* SFN). (C) Activity of ODC after simultaneous treatment with SFN (20–50 μM) and/or SB431542 (10 μM) in Caco-2 after 24 h of incubation (mean ± SE; ($n=3$); values not sharing a letter differ significantly, $^{*}p<0.05$).

TGF-β (20 nM) might amplify SFN-mediated reduction of cell proliferation in Caco-2 cells. For this, we analyzed BrdU incorporation after 24 h of treatment, but could only detect significant additive effects at a concentration of 5 μM SFN ($^{**}p<0.01$) (Fig. 3F). That, in accordance with our above-mentioned findings on intracellular TGF-β levels, lets us hypothesize that higher doses of SFN might lead to intracellular TGF-β-saturation and thus resistance to exogenous TGF-β.

Upon ligand binding, receptors of the TGF-β family generally phosphorylate Smad proteins, which then move into the nucleus where they activate transcription of different target genes. For responding to the question whether SFN-mediated TGF-β-signaling also involves an activation of

Smads, we first analyzed the phosphorylation status of Smad2 and Smad3 in Caco-2 cells after 1 h of incubation with increasing concentrations of SFN, which was found to be significantly induced in a dose-dependent manner (Fig. 4A) ($^{***}p<0.001$). Then, we performed reporter gene assays with SBE_4luc, where four SBEs are cloned in the pGL3-vector. Reporter gene activity of SBE_4luc was significantly induced by SFN ($^{**}p<0.01$), whereas the induction could be further enhanced when expression vectors pCGN-Smad3/pCGN-Smad4 were cotransfected ($^{***}p<0.001$) (Fig. 4B). To give the direct evidence that SFN mediates growth inhibitory effects, at least partly *via* induction of the TGF-β-signaling pathway, we simultaneously treated the cells with SFN and the specific TGF superfamily type 1 activin receptor-like kinase inhibitor SB431542 alone and in combination and measured the BrdU incorporation after 24 and 48 h (Fig. 4C). In fact, cell growth inhibitory effects of SFN were largely abolished by SB431542 ($^{***}p<0.001$).

3.3 Involvement of the TGF-β signaling pathway in SFN-induced inhibitory effects on ODC expression and activity in Caco-2 cells

Since Smad proteins were able to suppress c-myc activity in human skin epithelial cells by directly binding to Smad-responsive elements in the *c-myc* promoter [48], we were interested whether the observed downregulation of c-myc protein by SFN might also be due to an activation of Smad signaling. Therefore, we have done reporter gene assays after 6 h of SFN-treatment on Caco-2 cells either transfected with wild-type c-myc-luc or with c-myc-luc bearing mutated SBEs. TGF-β was used as a positive control. While in Caco-2 transfected with the wild-type construct, both SFN and TGF-β significantly decreased reporter gene activity compared to untreated cells ($^{***}p<0.001$), no effects could be observed in cells transfected with a mutated c-myc-promoter (Fig. 5A). We conclude that due to the mutation in the SBEs, Smads could not efficiently bind to the promoter resulting in an abolishment of the SFN- and TGF-β-mediated inhibition of c-myc gene activity.

Controversial data exist about the effects of TGF-β on polyamine metabolism. On the one hand, TGF-β has been shown to induce ODC mRNA in H-ras-transformed fibrosarcoma cell lines on which TGF-β acts as a growth stimulator [49]. On the other hand, Motyl *et al.* [50] could demonstrate that TGF-β suppresses both cell growth and the activities of ODC and *S*-adenosyl-L-methionine decarboxylase in a human chronic myelogenous lymphoma cell line. Similar results were found in the group of Nishikawa *et al.* [51], showing inhibitory effects of TGF-β on polyamine metabolism. In accordance with these publications and since our results revealed an involvement of the TGF-β signaling pathway in SFN-mediated downregulation of the *c-myc* promoter, we suggested that modulation of TGF-β signaling might also affect downstream ODC expression and activity. Indeed we could observe that co-incubation of SFN with a specific TGF-β kinase inhibitor partly abolished SFN-induced reduction of ODC protein expression (Fig. 5B) and activity (Fig. 5C), which might be due to direct TGF-β/Smad signaling-mediated transcriptional repression of transcription factor c-myc, upstream of the ODC gene. This effect was investigated in detail by Chen *et al.*, who could demonstrate that repression of c-myc expression by TGF-β occurs by direct interaction of a repressor complex consisting of Smad3 the transcription factors E2F4/5 and DP1 and the retinoblastoma family member p107 with a regulatory Smad responsive element in the *c-myc* promoter [52].

4 Concluding remarks

In summary, the present study clearly points out that SFN mediates growth inhibitory effects in colorectal cancer cell lines, at least partly, *via* TGF-β-dependent inhibition of c-myc and thus reduced protein expression and activity of proto-oncogene ODC. Noting the fact that colorectal cancer is still one of the most commonly occurring malignancies worldwide, the use of nontoxic agents like SFN, which inhibit specific molecular steps in the carcinogenic pathway, might be a promising strategy for cancer chemoprevention.

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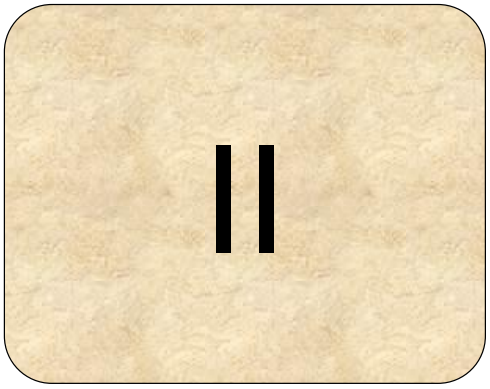
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ORIGINAL ARTICLE

Sulforaphane potentiates oxaliplatin-induced cell growth inhibition in colorectal cancer cells via induction of different modes of cell death

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Abstract The objective of this study was to investigate, whether the plant-derived isothiocyanate Sulforaphane (SFN) enhances the antitumor activities of the chemotherapeutic agent oxaliplatin (Ox) in a cell culture model of colorectal cancer. Caco-2 cells were cultured under standard conditions and treated with increasing concentrations of SFN [1–20 μM] and/or Ox [100 nM–10 μM]. For co-incubation, cells were pre-treated with SFN for 24 h. Cell growth was determined by BrdU incorporation. Drug interactions were assessed using the combination-index method (CI) (CI < 1 indicates synergism). Apoptotic events were characterized by different ELISA techniques. Protein levels were examined by Western blot analysis. Annexin V- and propidium iodide (PI) staining followed by FACS analysis was used to differentiate between apoptotic and necrotic events. SFN and Ox alone inhibited cell growth of Caco-2 cells in a dose-dependent manner, an effect, which could be synergistically enhanced, when cells were incubated with the combination of both agents. Co-treated cells further displayed distinctive morphological changes that occurred during the apoptotic process, such as cell surface exposure of phosphatidylserine, membrane blebbing as well as the occurence of cytoplasmic histone-associated DNA fragments. Further observations thereby pointed toward simultaneous activation of both extrinsic and intrinsic apoptotic pathways. With increasing concentrations and treatment duration, a shift from apoptotic to necrotic cell death could be observed. In conclusion, the data suggest that the isothiocyanate SFN sensitizes colon cancer cells to Ox-induced cell growth inhibition via induction of different modes of cell death.

Keywords Sulforaphane · Oxaliplatin · Colorectal cancer · Cell growth · Apoptosis

Abbreviations

CRC	Colorectal cancer
SFN	Sulforaphane
Ox	Oxaliplatin
5-FU	5-Fluorouracil
CI	Combination index
IC_{50}	Half maximal inhibitory concentration
FCS	Fetal calf serum
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylendiaminetetraacetic acid
DMSO	Dimethylsulfoxid
BrdU	Bromodeoxyuridine
TRAIL	TNF-related apoptosis-inducing ligand
PARP	Poly [ADP-ribose] polymerase
PI	Propidium Iodide
FITC	Fluorescein Isothiocyanate

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Introduction

Despite a markedly improved understanding of the disease, the advent of modern technology and rationally targeted drugs over the past years, colorectal cancer (CRC) remains a leading cause of cancer-related deaths worldwide [1]. Current treatment options involve the combination of a variety of chemotherapeutic drugs, more recently including the anticancer drug oxaliplatin (Ox). Ox is a third generation platinum-based drug, which has shown a broad spectrum of antitumor activities in a wide range of cancer cell lines by disrupting DNA replication and transcription by forming intrastrand DNA adducts [2]. It further demonstrates a better safety profile than platinum analogs of the first-(cisplatin) and second-(carboplatin)-generation and is typically administered in combination with different drugs as part of specific cancer-treatment regimens, e.g. Ox plus 5-Fluorouracil and leucovorin (referred to as FOLFOX), Ox plus capecitabine (XELOX) or Ox plus cetuximab (CAPOX). Such oxaliplatin-based combination regimens show improved clinical efficacy as related to overall response rates, time to tumor progression, median overall survival in patients with metastatic colorectal cancer and especially offer an alternative treatment option against cisplatin resistant tumors [3–5]. However, even though conventional cancer therapies play a major role in cancer treatment, successful therapeutic outcome is often limited due to high toxicity as well as the development of multidrug resistance. It is therefore of particular importance to investigate further drug combinations for the development of new therapeutic regimens obtaining higher efficacy, while, at the same time minimizing unwanted side effects, which could significantly improve patients outcome. Emerging evidence suggests that combining chemopreventive agents with chemotherapy or radiotherapy may lead to enhanced antitumor activity through synergistic action or compensation of inverse properties. Besides a multitude of synthetic substances, also numerous phytochemicals have been identified to exhibit potent chemopreventive effects in different carcinogenesis models while, at the same time showing low toxicity [6]. SFN is a naturally occuring isothiocyanate derived from cruciferous vegetables such as broccoli, cauliflower, cabbage and kale, which targets cancer initiation and progression both in vitro and in vivo, and further induces antiproliferative and cytotoxic effects in cells that are already transformed [7]. Recently, SFN was identified as a novel histone deacetylase inhibitor (HDACi) in colon and prostate cancer cells [8]. HDACi, as a new class of chemotherapeutic agents, show significant promise against a variety of cancers in clinical trials [9]. Most available HDACi inhibit all class I and II HDACs, thereby increasing acetylation of histone and nonhistone protein targets [10]. In vivo, histone acetylation depends on the balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC), which has been proposed to play an important role in transcriptional regulation by altering chromatin structure [11]. Histone acetylation by HATs is associated with an open chromatin conformation, promoting gene transcription, whereas HDACs maintain the chromatin in the closed, transcriptionally inactive state. HDAC inhibitors have been shown to induce the expression of several tumor suppressive genes, which justifies its use in cancer prevention and therapy [12].

Several studies indicate that SFN causes growth inhibition of human cancer cells predominantly by inducing apoptosis and/or blocking cell cycle progression [13, 14]. Apoptosis is defined as an active physiologic process of cellular self-destruction, with specific morphologic and biochemical changes characterized by DNA fragmentation, cell shrinkage, nuclear condensation and membrane blebbing [15, 16]. At present, two major apoptosis pathways have been identified: the intrinsic or mitochondrial pathway and the extrinsic or death receptor-related pathway. While the extrinsic pathway is activated through cell surface death receptors binding their respective cytokine ligands, such as TRAIL [17], the intrinsic pathway depends on mitochondrial membrane permeabilization, which causes the release of apoptogenic factors from the intermembrane space to the cytoplasm. Prevalently, both pathways result in the activation of members of the caspase family converging at the level of caspase-3 [18]. However, apoptotic events can also be observed in the absence of caspase-3 activation, depending on the cell type and the apoptosis initiating process [19, 20]. Apart from apoptosis, alternative forms of cell death can be activated, e.g. necrosis or autophagy, which might also lead to biological consequences differing from apoptosis [21]. In the present study, we addressed the question whether plant-derived sulforaphane is able to enhance the anticarcinogenic activities of the common chemotherapy drug oxaliplatin in colorectal cancer cells with special regard to regulatory effects on the cell death machinery.

Materials and methods

Cell culture

Caco-2 cells were kept in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% sodium pyruvate and 1% nonessential amino acids. Human foreskin fibroblasts (HFF) were cultured in DMEM/Ham's F-12 medium supplemented with 10% FCS and 1% penicillin/streptomycin. Both cell lines were maintained at

37°C in an atmosphere of 95% air and 5% CO_2. The cells were passaged weekly using Dulbecco's PBS containing 0.1% trypsin and 1% EDTA. The medium was changed thrice weekly. Cells were screened for possible contamination with mycoplasma at monthly intervals. For experiments, the cells were seeded onto plastic cell culture wells in serum-containing medium and allowed to attach for 24 h. Sulforaphane (Merck Chemicals, Darmstadt, Germany) was dissolved in DMSO at a concentration of 100 mM, oxaliplatin (Sigma–Aldrich, München, Germany) was dissolved in water at a concentration of 10 mM. DMEM, DMEM/Ham's F-12 medium, DMSO, sodium pyruvate solution, penicillin and streptomycin stock solutions were all obtained from PAA Laboratories GmbH. When synergistic effects were analyzed, the cells were pretreated with SFN for 24 h.

Cell proliferation assay

The effect of SFN, Ox or their combination on cellular DNA synthesis was assessed using a cell proliferation ELISA kit (Roche Diagnostics, Mannheim, Germany). This assay is a colorimetric immunoassay for quantification of cell proliferation based on the measurement of bromodeoxyuridine (BrdU) incorporation during DNA synthesis and is a nonradioactive alternative to the [^{3}H]-thymidine incorporation assay. Cells were grown in 96-well culture dishes (10^3 cells/well), incubated with SFN and/or Ox for different time intervals, and then labeled with BrdU for further four hours. Incorporated BrdU was measured colorimetrically.

Combination index (CI)

To assess the drug interactions of SFN and Ox, we used the combination-index (CI) method defined by median-effect analysis of Chou and Talalay [22]. The fractional inhibitory concentration was calculated by dividing the IC_{50} concentration of the drug in the combination by the amount of the drug that is required to reach the same degree of inhibition (IC_{50}) by itself.

$$\mathrm{CI} = \frac{\text{dose of SFN}}{\mathrm{IC}_{50}(\mathrm{SFN})} + \frac{\text{dose of Ox}}{\mathrm{IC}_{50}(\mathrm{Ox})}$$

In this equation, the sum of the dose of SFN and the dose of Ox give 50% inhibition of cell growth. $\mathrm{Cl} < 1$ indicates a synergistic effect; $\mathrm{Cl} = 1$, additive effect; and $\mathrm{Cl} > 1$, antagonistic effect [23].

Determination of ATP level

Caco-2 cells were grown in 96-well culture dishes (10^3 cells/well) and allowed to grow overnight. Cell Viability Assay Kit (ApoSENSOR^{TM}, BioVision, CA, USA) was used according to the manufacturer's instructions following a 6-h exposure to SFN and Ox alone and in combination. The assay utilizes luciferase to catalyze the formation of light from ATP and luciferin, and the light can be measured using a luminometer. Changes in ATP levels were determined by comparing the results with the levels of untreated cells (control).

Annexin V-FITC/PI double-labeled flow cytometry

To discriminate between apoptotic and necrotic cell subpopulations, simultaneous staining with Annexin V-FITC and propidium iodide was performed. Cells incubated with SFN and/or Ox drugs for 5 and 24 h were harvested with accutase for 30 min. After centrifugation, cells were resuspended in 100 µL binding buffer mixed with 5 µL Annexin V-FITC (ImmunoTools, Friesoythe, Germany) and 5 µl PI and then incubated in dark for 10 min at 4°C. Fluorescence was measured with a flow cytometer.

Determination of DNA fragmentation

Nuclear fragmentation as a late marker of apoptosis was determined by (1) DNA staining with SYTOX Green and (2) quantification of cytoplasmic histone-associated DNA fragments. For SYTOX® Green staining, cells (1×10^6) were seeded onto glass slides and incubated in Quadriperm wells with the test compounds for 48 h. Thereafter, glass slides were washed with PBS, and cells were fixed with 2% paraformaldehyde. DNA was stained with 0.25 µM SYTOX Green solution (Invitrogen, Karlsruhe, Germany) and then visualized under an epifluorescence microscope (Zeiss Axioskope 2).

Cytoplasmic histone-associated DNA fragments in control and treated cells were quantified using a commercially available ELISA kit (Roche Diagnostics). Briefly, Caco-2 cells were grown in 96-well culture dishes (10^3 cells/well) and allowed to grow overnight. The cells were then incubated with or without (control) the test substances. After 24 h of treatment, the cells were centrifuged with $200\times g$ for 10 min and the supernatant containing DNA from necrotic cells was removed. The cell pellet was resuspended in 200 µl lysis buffer and incubated for 30 min followed by centrifugation at $200\times g$ for 10 min. Then 20 µl of the supernatant were transferred to streptavidin-coated wells in a mircotiter plate. The supernatant aliquots were incubated for 2 h at room temperature with 80 µl of an immunoreagent containing monoclonal antibodies against histone (biotinlabeled) and DNA (peroxidase-conjugated), with which the nucleosomes in the supernatant bind. The immobilized antibody–histone complexes were washed three times with incubation buffer

to remove cell components that were not immunoreactive. Then 100 µl ABTS solution was added to each well, and the plates were incubated at room temperature on a plate shaker at 250 rpm for 20 min. Finally, the amount of colored product and thus of the immobilized antibody–histone complexes (DNA fragments) in the plate was measured spectrophotometrically at 405 nm on a microplate spectrophotometer (Fluostar Optima, BMG Labtech, Durham, NC) using ABTS solution as a blank control.

Mitochondrial membrane potential ($\Delta\Psi_m$) analysis

Cell cultures were seeded into cultivation flasks at a densitiy of 1×10^5 cells/well and allowed to grow overnight. Cells were stained with JC-1, as a component of the JC-1 Mitochondrial membrane potential Assay Kit (Cayman Chemical Company, Ann Harbor, MI) following a 6- and 24-h exposure to SFN and Ox alone and in combination. JC-1 is a lipophilic, cationic dye that can selectively enter into mitochondria and reversibly change color from green to red as the membrane potential increases. Changes in $\Delta\Psi_m$ were finally assessed by microfluorimetry analysis using TECAN SpectraFluor Plus (TECAN Austria GmbH, Grödig, Austria) and indicated as changes of red/green ratio.

Caspase-3 activity assay

Caco-2 cells were seeded in 80-cm^2 flasks, allowed to grow overnight. Caspase-3 activity was analyzed using a fluorometric immunosorbent enzyme assay (Roche Diagnostics) according to the manufacturer's instructions. Briefly, after 24 h of incubation with the test substances, the cells were incubated with lysis buffer for 10 min. After cell lysis and following centrifugation, samples were removed and transferred to the anti-caspase-3-coated wells of a microplate, capturing caspase-3. After 1 h, the immobilized antibody-caspase-3 complexes were washed three times to remove cell components that are not immunoreactive. Afterward, samples were incubated with caspase substrate (Ac-DEVD-AFC) for 120 min that is proteolytically cleaved into free fluorescent AFC. Then the AFC can be measured fluorometrically at excitation 430 nm and emission 535 nm. Finally, protein concentrations were measured and adapted to the activity.

SDS–polyacrylamide gel electrophoresis and immunoblot analysis

Caco-2 cells were seeded in 80 cm^2 flasks; 24 h after plating, cells were incubated with substances for 6 h. After washing the cells for three times with ice-cold PBS, followed by an incubation step with cell lysis buffer (Cell signalling, Berverly, MA) containing multiple protease inhibitors (Complete Mini®, Roche) for 20 min at 4°C, cells were harvested by scraping. Protein extracts were obtained after sonication of cell lysates (2×5 s) and centrifuged at 10,000 rpm for 10 min at 4°C. Samples were analyzed for their protein content using the BioRad® colorimetric assay according to the method of Bradford (BioRad Laboratories). After addition of sample buffer (Roti Load®, Roth, Karlsruhe, Germany) to the total cellular extract and boiling for 5 min at 95°C, 30–40 µg of total protein lysate was separated on a 10 or 12% SDS–polyacrylamide gel. Protein was transferred onto nitrocellulose membrane (Schleicher &Schuell, Dassel, Germany), and the membrane was blocked for one hour at room temperature with 5% skimmed milk in Tris–buffered saline containing 0.05% Tween 20 (TBS-T). Next, blots were washed and incubated overnight at 4°C in TBS-T containing either 5% bovine serum albuminate (BSA) or 5% skimmed milk with a 1:1,000 or 1:2,000 dilution of primary antibodies for PARP, full length as well as cleaved Caspase-8 (all from Cell Signaling, Berverly, MA) and TRAIL (from Santa Cruz Biotechnology, Heidelberg, Germany). The secondary, horseradish peroxidase-conjugated, antibody (Santa Cruz Biotechnology) was diluted at 1:2,000 or 1:4,000 and incubated with the membrane for another 60 min in skimmed milk. After chemoluminescence reaction (ECL, Amersham pharmacia biotech, Wien, Austria), bands were detected after exposure to Hyperfilm-MP (Amersham International plc, Buckinghamshire, UK). Blots were reprobed with β-actin antibody (Santa Cruz Biotechnologies).

Cytotoxicity

Cytotoxicity was analyzed by measuring lactate dehydrogenase (LDH) release using a commercially available kit (Cytotoxicity detection kit (LDH), Roche). For this, HFF were incubated with SFN and/or Ox for 24 h. Triton X-100 (2%) was used as a positive control. After centrifugation at $250 \times g$ for 10 min, the supernatant was carefully removed and transferred into corresponding wells of an optically clear 96-well flat bottom microplate. To determine the LDH activity in these supernatants, 100 µl reaction mixture was added and the samples were incubated for up to 30 min. Finally, the absorbance of the samples was measured at 490 nm.

Statistics

The data are expressed as means ± SE of at least three independent experiments. Results were analyzed using GraphPad Prism 4.01 (San Diego, CA, USA) by a two-way ANOVA. A P value <0.05 was considered to be significant.

Results

Effects of single-drug exposure on cell proliferation of Caco-2 cells

Effects of SFN [1–50 μM] and Ox [100 nM–10 μM] on Caco-2 cell proliferation were assessed after 24 h of drug exposure (Fig. 1a, b). Both substances significantly inhibit proliferation in Caco-2 cells in a dose-dependent manner ($*P < 0.5$, $**P < 0.01$, $***P < 0.001$ vs. control). The IC_{50}s for SFN and Ox in Caco-2 cells were 26.35 and 5.58 μM, respectively.

Synergistic antiproliferative effects of SFN and Ox

For studying combination effects, the cells were exposed to both SFN and Ox simultaneously for 24 h. As shown in Fig. 1c, co-treatment of the cells significantly reduced the IC_{50} values of the single drugs. The obtained data were analyzed by the CI method of Chou and Talalay [22]. In Caco-2 cells, we calculated a CI of 0.3, which indicates synergism (see "Materials and methods").

Effects of SFN/Ox on different apoptotic events

ATP/ADP ratio: Changes in the intracellular ATP/ADP ratio are a useful indicator to distinguish between different modes of cell death and viability. Although decreasing ATP and increasing ADP levels are generally found in apoptotic cells, cells will rather undergo necrosis when intracellular ATP levels fall below a critical threshold [24]. For a rapid screening of cell death, we therefore treated Caco-2 cells for 1–24 h with SFN [10–20 μM] and Ox [500 nM] alone and in combination and measured effects on the ATP/ADP ratio. As can be seen from Fig. 2a, incubation with SFN and Ox resulted in a significant time- and, at least after 6 h, also dose-dependent reduction in intracellular ATP, which reaches a maximum after 24 h ($***P < 0.001$). As a positive control, we used staurosporine [0.5 μg/ml], a well-known inducer of apoptosis [25].

DNA Fragmentation: As DNA cleavage is another hallmark for apoptosis, we further quantified histone-complexed DNA fragments in Caco-2 after 24 h of treatment. SFN [20 μM], in contrast to Ox [500 nM], thereby leads to a significant increase of cytoplasmic histone-associated DNA fragments, an effect which could be further enhanced, when the drugs were used in combination (Fig. 2b) ($***P < 0.001$).

Caspase-3 Activity: The activity of the effector caspase-3 was significantly activated 24 h after stimulation with SFN [20 μM] and Ox [500 nM], respectively, but this effects were not very prominent when compared to the positive control staurosporine. However, SFN could significantly enhance the Ox-induced effects (Fig. 2c) ($***P < 0.001$), which is in agreement with the observed cleavage of PARP (Fig. 2d) ($***P < 0.001$), a classical substrate for activated caspase-3. Proteolysis of PARP usually is an indicator for early apoptotic events.

Extrinsic and intrinsic apoptotic events: In the next step, we measured protein levels of the TNF-related apoptosis-inducing ligand (TRAIL) as well as of full length and cleaved caspase-8, both markers of the extrinsic or death receptor-mediated apoptotic pathway. In some tumor cell lines, TRAIL protein expression could be induced by chemopreventive agents resulting in TRAIL-mediated apoptosis in an autocrine or paracrine manner [26–28]. This suggests that induction of endogenously expressed TRAIL after SFN/Ox-treatment for 6 h (Fig. 3a) may further enhance their therapeutic outcome. Additionally, co-stimulation resulted in a decrease of procaspase-8 and an increase of cleaved caspase-8 after 6 h of treatment, a common upstream event of caspase-3 activation (Fig. 3b).

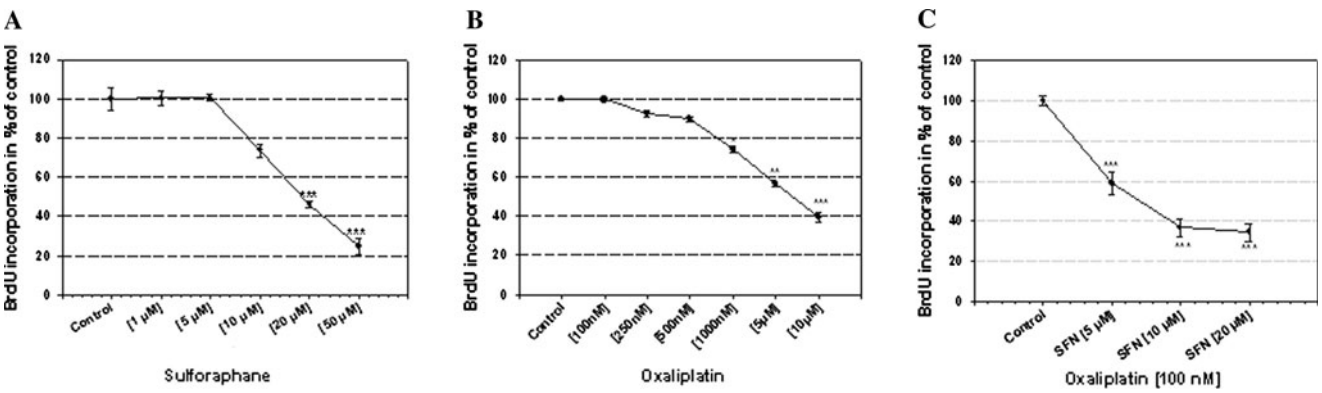

Fig. 1 **a**, **b** Inhibition of cell proliferation of Caco-2 by 24 h exposure to SFN and Ox alone. Values represent mean ± SE ($n = 8$); $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus control. **c** The combined effects of concurrent treatment with SFN and Ox on Caco-2 cells. Cell proliferation was measured by BrdU incorporation after 24 h of incubation. CI values were determined by the method of Chou and Talalay [22] described in "Materials and methods". Values represent mean ± SE ($n = 3$); $**P < 0.01$, $***P < 0.001$ versus control

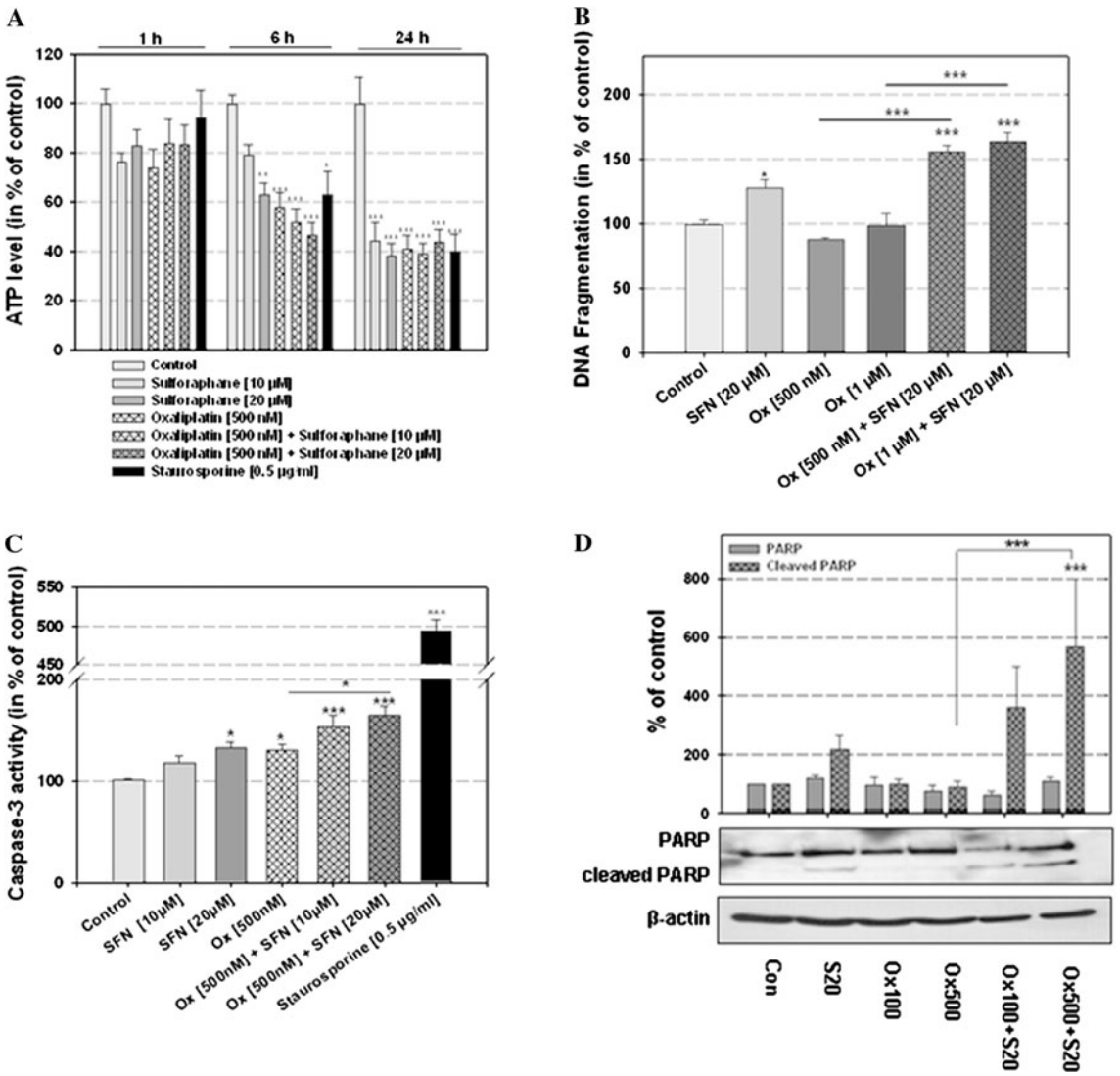

Fig. 2 **a** Intracellular content of ATP in control (untreated), SFN- and/or Ox- as well as staurosporine (0.5 µg/ml)-treated cells. Caco-2 cells were incubated with the test substances for 1–24 h. Results are expressed as the percentage of control. Values represent mean ± SE ($n = 4$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. **b** Quantification of cytoplasmic histone-associated DNA after 24 h of incubation with the test compounds. Values represent mean ± SE ($n = 4$); $^{***}P < 0.001$. **c** Effects of SFN, Ox, their combination and staurosporine on activation of caspase-3 in Caco-2 cells after 24 h of exposure. Results are expressed as the percentage of control. Values represent mean ± SE ($n = 4$); $^{*}P < 0.05$, $^{***}P < 0.001$. **d** Western blot analysis for PARP cleavage using whole cell extracts from Caco-2 cells exposed to SFN and Ox, separately or in combination, for 6 h. The bar graph presents densitometric analysis of the Western blot images normalized to β-actin (mean ± SE; ($n = 3$); $^{***}P < 0.001$. Representative immunoblots of three independent experiments are shown

Next, we determined whether both agents might also increase mitochondrial membrane depolarization as a consequence of the activation of the intrinsic apoptotic pathway. For this, cells were incubated with SFN [20 µM] and Ox [500 nM] alone and in combination for 6 and 24 h before being stained with JC-1 (Fig. 3c). JC-1 is a mitochondrial-selective dye and forms aggregates in normal polarized mitochondria that result in a green orange emission of 590 nm after excitation at 490 nm. Upon depolarization of the mitochondrial membrane, JC-1 forms monomers that emit only green fluorescence at 527 nm. As shown in Fig. 3c, SFN induced a depolarization of the mitochondrial membrane potential, which was significant after 24 h of treatment. In contrast to SFN, Ox-treatment did not show any detectable effects, neither after 6 nor 24 h of incubation. However, combinatorial treatment resulted in a distinct decrease of the red-green fluorescence intensity ratio after 6 h ($^{**}P < 0.01$) and 24 h ($^{***}P < 0.001$). These observations pointed toward involvement of both extrinsic and intrinsic apoptotic pathways in SFN/Ox-mediated induction of apoptosis.

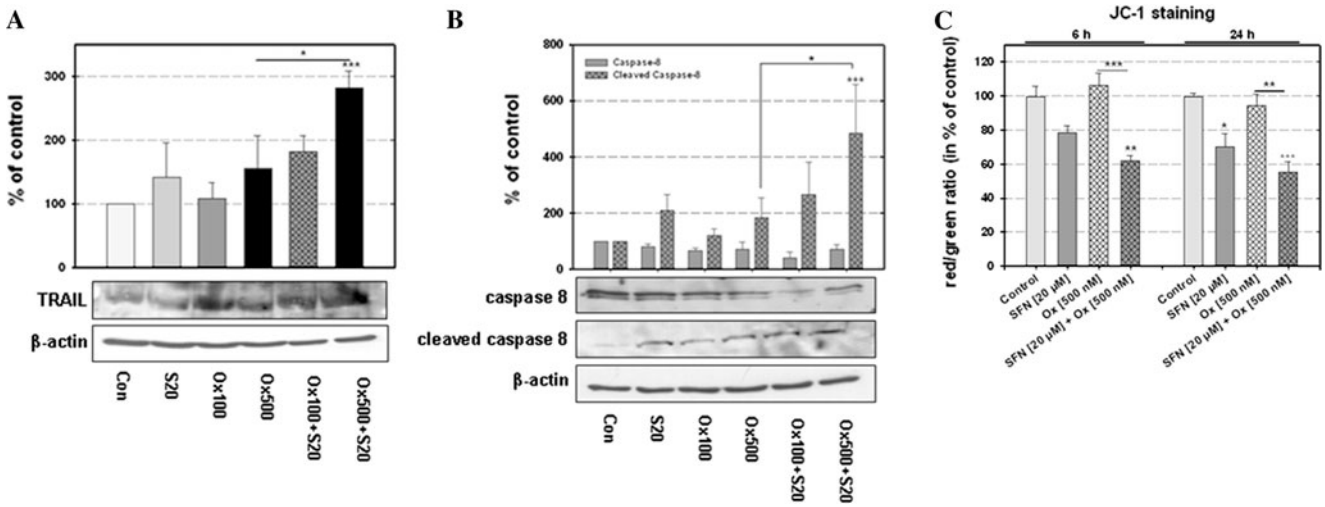

Fig. 3 **a** Western blot analysis for TRAIL in Caco-2 cells after incubation with SFN [20 μM] and/or Ox [100–500 nM] for 6 h. A representative immunoblot of three independent experiments is shown. The *bar graph* presents densitometric analysis of the Western blot images normalized to *β*-actin (mean ± SE ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). **b** Western blot analysis for caspase-8, full length and cleaved, using whole cell extracts from Caco-2 cells exposed to SFN and/or Ox for 6 h. A representative immunoblot of three independent experiments is shown. The *bar graph* presents densitometric analysis of the Western blot images normalized to *β*-actin (mean ± SE ($n = 3$); $^{*}P < 0.05$, $^{***}P < 0.001$). **c** Loss of mitochondrial membrane potential ($\Delta\Psi_m$) in Caco-2 cells exposed to SFN and Ox, alone or in combination, after 6 and 24 h of treatment. Results represent mean of at least three experiments, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Switching from apoptosis to necrosis with increasing concentrations of SFN/Ox

Even though increasing concentrations of Ox/SFN [e.g. 1/20 μM] can further potentiate the observed effects on cell growth inhibition shown in Fig. 1 (data not presented here), these effects seem not to be explainable by enhanced induction of apoptosis, as DNA fragmentation for example could not be further amplified with increasing concentrations of oxaliplatin (Fig. 2b), indicating other modes of action. Thus, to discriminate between different modes of cell death, Caco-2 cells treated with SFN [20 μM], Ox [500 nM–1 μM] alone and in combination for 5 and 24 h were analyzed by Annexin V-FITC/PI labeling and flow cytometry. The degree of apoptosis thereby was quantitatively expressed as a percentage of the Annexin V-FITC-positive but PI-negative cells, while necrosis or late apoptosis was quantitatively expressed as a percentage of PI-positive or Annexin V-FITC/PI double-stained cells. Analysis after 5 h was chosen in order to differentiate between primary and secondary necrosis as a result of late apoptosis. Interestingly at this time point, Annexin V-FITC-positive but also PI-positive cells could be measured indicating direct necrotizing effects of SFN and Ox (Fig. 4a, b). Compared to the 5-h treatment, the population of apoptotic cells in untreated and SFN [20 μM]- as well as Ox [500 nM–1 μM]-treated Caco-2 cells (Fig. 4c, d) seems to decline after 24 h. However, the rate of apoptosis was still significantly induced in the co-treated cells, but again this effect could not be further increased with a higher concentration of Ox [1 μM]. Rather, at this concentration, a distinct population of PI-stained cells could be observed, whereas a mixture of cells undergoing rapid primary as well as secondary necrosis/late apoptosis can be assumed (Fig. 4b). Obviously, apoptotic effects seem to reach a maximum after 5 h of treatment, which is replaced by a shift toward an increased population of necrotic cells after 24 h.

These observations could also be confirmed by SYTOX Green staining (Fig. 5), which was used to analyze late apoptosis. SYTOX Green nucleic acid stain is an unsymmetrical cyanine dye with three positive charges that is completely excluded from live eukaryotic and prokaryotic cells. Binding of SYTOX Green stain to nucleic acids of Caco-2 cells incubated with SFN [20 μM] and Ox [500nM] clearly presents signs of apoptotic events indicated by cell shrinkage, chromatin condensation and the formation of apoptotic bodies (see white arrows in Fig. 5a, c). However, again with increasing concentrations of Ox [1 μM], necrotic alterations like osmotic swelling and cell lysis with loss of membrane integrity became more prominent (Fig. 5c).

Taken together, these observations clearly indicate that depending on the applied concentration and the treatment duration, antiproliferative effects of SFN/Ox against Caco-2 cells can be associated with both apoptotic as well as necrotic events.

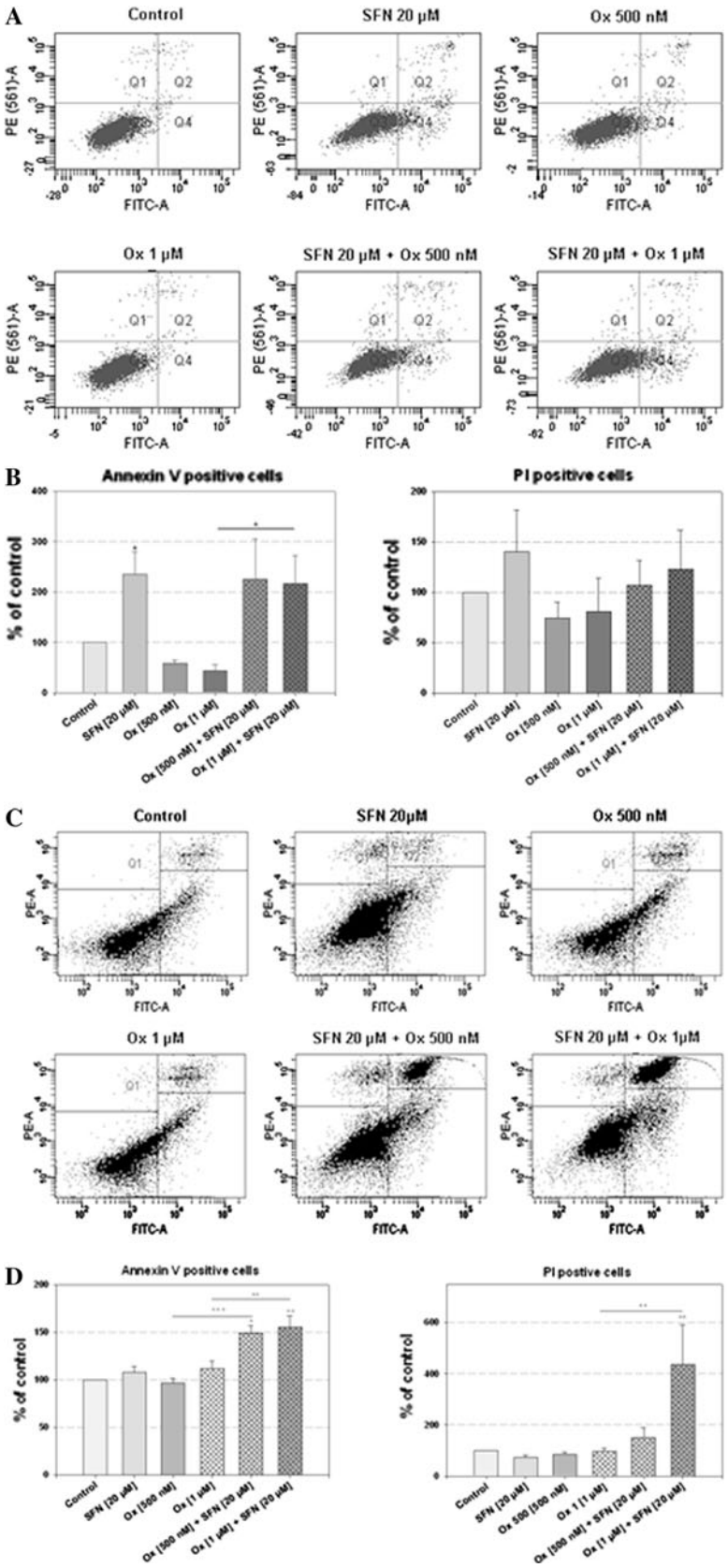

Fig. 4 Effects of SFN and Ox, separately and in combination, on Annexin V-FITC and/or PI staining of Caco-2 cells after 5 (**a**) and 24 h (**c**) of incubation. Cells were analyzed by flow cytometry as described in "Materials and methods". The percentage of apoptotic and necrotic (**b**, **d**) cells versus control as a result of the FACS analysis are presented by *bar graphs*. Values represent mean ± SE ($n = 4$), quantifying a minimum of 10,000 cells per treatment ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$)

Effects of SFN and Ox on cell proliferation and LDH release of human foreskin fibroblasts (HFF)

To analyze a possible toxicity of SFN and oxaliplatin on normal tissue cells, human foreskin fibroblasts were treated with either SFN [10–20 μM] and Ox [500 nM–1 μM] alone or in combination and cell proliferation as well as LDH release as a marker of direct cytotoxicity were measured after 24 h (Fig. 6). Actually, SFN alone was found to significantly inhibit cell growth of HFF in a dose dependent manner ($^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$) (Fig. 6a), but in contrast to Caco-2 cells, this effect was not further enhanced and no signs of cytotoxicity could be observed when SFN was combined with oxaliplatin (Fig. 6b).

Discussion

Oxaliplatin is now widely used in the treatment of advanced colorectal cancers mostly in combination with continuous intravenous infusions of 5-fluorouracil [3]. However, adverse effects, e.g. acute and persistent neuropathy as well as the development of chemotherapy resistance limit the overall success of oxaliplatin-based treatment regimens. Since several in vitro and in vivo studies show first promising results regarding the chemosensitizing capability of phytochemicals in different cancer models [6], we were interested, whether plant-derived SFN might be able to enhance Ox-induced antitumor activities in colorectal cancer cells, also concerning modes of action that might help to overcome drug resistance in cancer tissues.

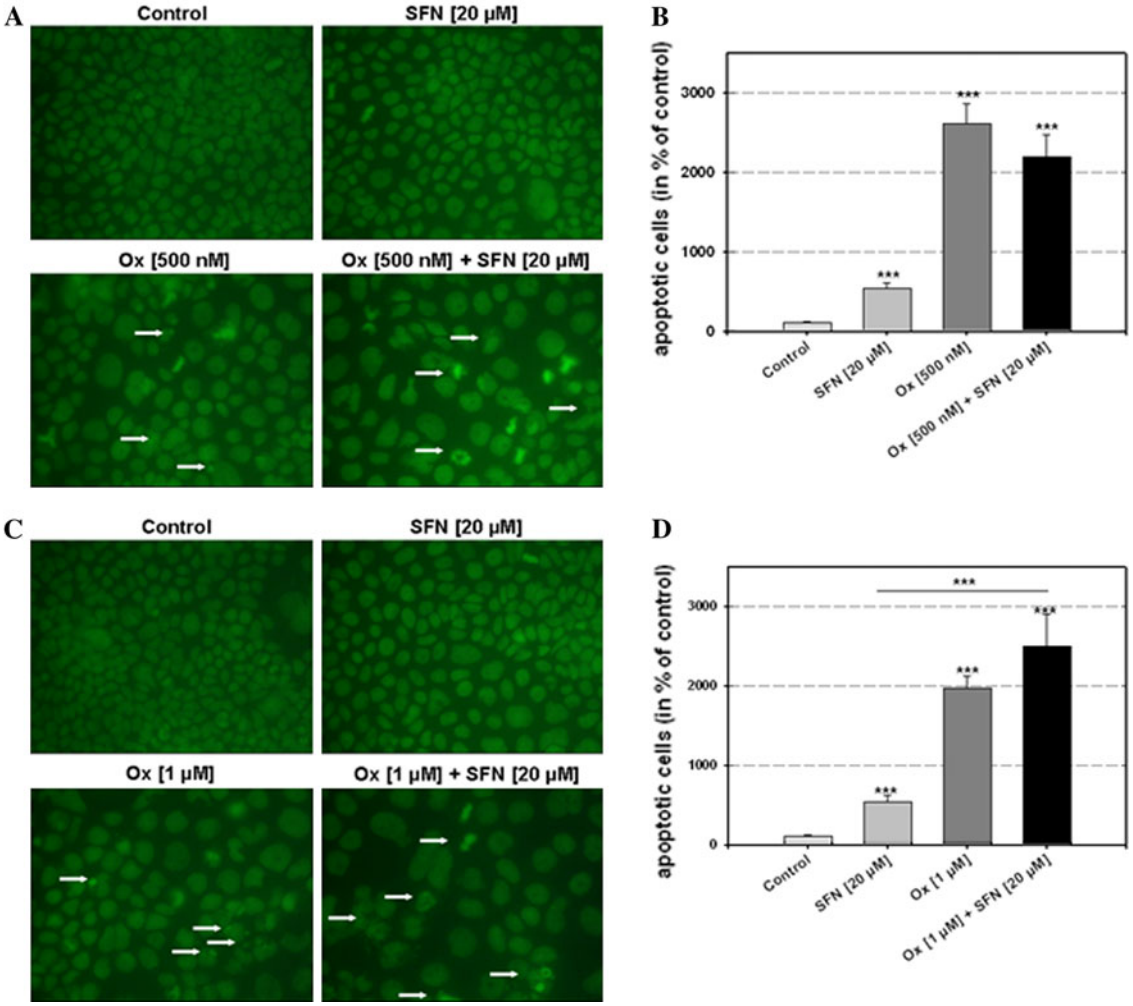

Fig. 5 Sytox Green® staining following a 48-h exposure to control or different concentrations of the test compounds (**a**, **c**). *Arrows* indicate morphological changes as a consequence of SFN/Ox-induced apoptosis. Experiments were repeated three times, and the results were comparable. Data from a representative experiment are shown. The percentage of apoptotic cells (**b**, **d**) cells versus control are presented by *bar graphs*. Values represent mean ± SE ($n = 3$), $^{***}P < 0.001$

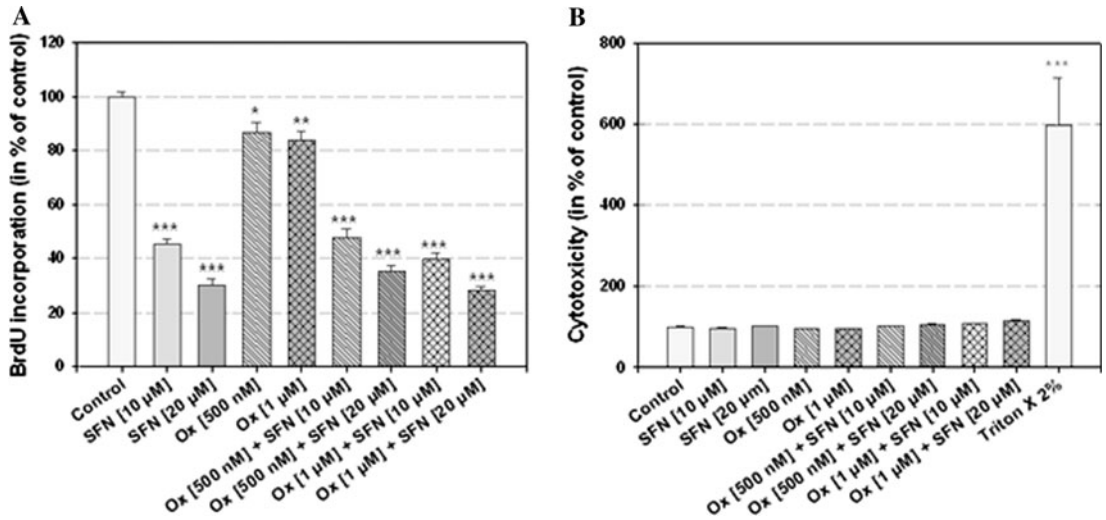

Fig. 6 **a** The combined effects of concurrent treatment with SFN and Ox on HFF cells. Cell proliferation was measured by BrdU incorporation after 24 h of incubation. Values represent mean ± SE ($n = 4$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. **b** Effects of SFN and Ox, separately and in combination, on cytotoxicity in HFF cells after 24 h of incubation. Results are expressed as the percentage of control. Values represent mean ± SE ($n = 4$); $^{***}P < 0.001$

Most chemotherapeutic agents, including oxaliplatin, and irradiation act primarily by inducing apoptosis, accordingly, defects in the apoptotic pathway often account for chemotherapy resistance in different tumor cells, which could also be demonstrated for drug resistance arising against oxaliplatin in colorectal cancer cells [29]. Novel targeted therapies that more potently induce cell death in cancer cells or sensitize them to established cytotoxic agents and radiation by modulating the apoptotic machinery might therefore not only enhance therapeutic outcome but can further help to reverse chemotherapeutic drug resistance [30].

Our first results quickly revealed SFN-induced potentiation of cell growth inhibition mediated by oxaliplatin, which was more-than-additive as indicated by combination-index analysis. In addition, these effects were accompanied by different hallmarks of apoptosis, such as reduced ATP levels, Caspase-3 activation, PARP cleavage and DNA Fragmentation. Further experiments could demonstrate that thereby apparently both, extrinsic and intrinsic apoptotic pathways were involved, as indicated by caspase-8 cleavage and increased mitochondrial membrane permeabilization. Interestingly, we could also observe an induction of TRAIL protein levels, a member of the TNF family of cytokines, which can induce apoptotic cell death in a variety of tumor cells by engaging the death receptors DR4 and DR5, while sparing most normal cells [31]. This might be due to the HDAC inhibitory properties of Sulforaphane [8], since several other HDAC inhibitors were also shown to induce expression of TRAIL, DR4 or related proteins, which contributed to subsequent apoptotic events induced by these agents [32]. Since some drugs and radiation sensitize tumor cells to TRAIL-induced cell death, several studies have expectedly shown that combinations of recombinant TRAIL and some chemotherapeutic drugs exhibit synergistic effects in inducing tumor cell apoptosis in vitro and in vivo [33, 34]. Whether endogenously induced TRAIL possibly acts in a similar way remains to be elucidated. Another interesting aspect is that TRAIL, in contrast to DNA-damaging chemotherapeutic drugs or radiation, induces apoptosis independently of p53 [31], which might be helpful to circumvent resistance to conventional chemotherapy and radiotherapy. However, in contrast to known apoptosis inducers, such as staurosporin, apoptotic events induced by SFN and Ox were not very prominent and may only partly account for the observed inhibition of cell proliferation.

In fact, with increasing concentrations of oxaliplatin and increasing treatment duration, Annexin V and PI staining revealed a shift from an apoptotic toward a distinct population of necrotic cells (Fig. 4). Similar results were observed in Sytox Green-stained cells, which showed considerable signs for necrosis-like swelling of the organelles or cell lysis with loss of membrane integrity (Fig. 5). This is consistent with the almost complete ATP depletion after 24 h of incubation, which may further account for the low apoptotic response [35].

Currently, the majority of clinical chemotherapeutic agents ultimately induce tumor cell apoptosis following treatment, however, noting the facts that many cancers have defective apoptosis machinery or aquire apoptosis resistance during therapy [36], or the finding, that apoptosis may be reversed in cancer cells [37], it is reasonable to consider whether activating alternative cell death pathways, such as necrosis, may be another effective strategy

for cancer therapy [38]. Unlike apoptosis that is largely immune silent or immunosuppressive, therapy-induced necrotic cell death initiates an immune response to tumor cells [39]. This inflammatory response may help to recruit cytotoxic immune cells to the tumor site, thereby increasing the efficacy of the chemotherapeutic drugs. However, conversely the pro-inflammatory conditions might also damage normal tissues or induce the production of mitogenic or prosurvival cytokines, which can activate signaling pathways that promote cell excrescence in the damaged area and might induce tumor cell migration and metastasis [40, 41]. Further studies are therefore required to address the question whether the inflammation associated with necrosis might be desirable in the context of cancer treatment or rather leads to further tumor growth or even overshooting inflammation, leading to autoimmunity. Interestingly, Ingenol-3-angelate, another plant-derived compound, was recently shown to mediate its in vitro anticancer activities via the induction of primary necrosis [42, 43], as displayed by plasma membrane and mitochondrial disruption leading to the activation of an antitumor immune response [44]. Thus, the success of Ingenol-3-angelate in phase IIa clinical trials against human skin cancer might again support the importance and potential of cytotoxic agents that act through irreversible necrotic cell death. [45]. In healthy human fibroblasts, SFN was found to inhibit cell growth in a dose-dependent manner, an effect that was already reported for other HDAC inhibitors [46, 47]. But in contrast to Caco-2 cells, growth inhibitory effects on fibroblasts were not further enhanced and no signs of cytotoxicity could be observed when SFN was combined with oxaliplatin, indicating a selective toxicity toward the tumor cell line while inducing only growth arrest in normal fibroblasts.

Summarizing our findings, we could demonstrate for the first time that the secondary plant-metabolite sulforaphane is capable of amplifying Ox-induced cell growth inhibition in colon cancer cells supposedly via induction of different modes of cell death. Sulforaphane might thereby not only be a promising candidate due to it's potent chemopreventive properties but also pharmacokinetic studies in both rats and humans indicate that dietary absorbed SFN can be distributed in the body, reach μM levels in the blood and is capable of reaching target tissues in an active form, which further supports the clinical relevance of the substance [48, 49]. However, further experiments focusing intramolecular mechanisms together with in vivo animal studies and clinical trials are needed for eventually translating the concept of phytochemicals in combination therapies of human colorectal cancer into clinical applications.

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III

RESEARCH ARTICLE

Resveratrol-induced potentiation of the antitumor effects of oxaliplatin is accompanied by an altered cytokine profile of human monocyte-derived macrophages

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Scope: The objective of this study was to investigate, whether the naturally occurring polyphenol resveratrol (Res) enhances the anti-tumor activities of the chemotherapeutic agent oxaliplatin (Ox) in a cell culture model of colorectal cancer, also with regard to a possible inflammatory response and cytotoxic side-effects.

Materials: Cell proliferation was measured by BrdU incorporation and cytotoxicity was analyzed by LDH release. Apoptotic events were characterized by different ELISA techniques. Protein levels were examined by Western blot analysis. Annexin-V- and propidium-iodide (PI)-stained cells were detected by FACS analysis. Primary human macrophages were co-cultured with conditioned medium of treated tumor cells and cytokines were also quantified by FACS. Res and Ox in combination synergistically inhibit cell growth of Caco-2 cells, which is accompanied by distinctive morphological changes that occurred during the apoptotic process. After 24 h of incubation a shift from apoptotic to necrotic cell death could be observed leading to an altered cytokine profile of co-cultured macrophages. Furthermore, combinatorial treatment did not affect normal cells as cytotoxicity was not detected in human foreskin fibroblasts and in human platelets.

Conclusion: The polyphenol resveratrol enhances oxaliplatin-induced cell growth inhibition with the distinctive feature to abrogate immunosuppressive properties of oxaliplatin-treated cells and without damaging non-transformed cells.

Keywords: Resveratrol / Oxaliplatin / Colorectal cancer / Apoptosis / Necrosis / Immunogenic cell death

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A**bbreviations**: **Res**, resveratrol; **Ox** , oxaliplatin; **LDH**, lactate dehydrogenase; **IL**, interleukine; **TNF-α**, tumor necrosis factor-α; **TAMs**, tumor associated macrophages; **CI**, combination index; **IC_{50}**, half maximal inhibitory concentration; **FCS**, fetal calf serum; **DMEM**, Dulbecco's modified–Eagle's medium; **EDTA**, Ethylendiaminetetraacetic acid; **DMSO**, Dimethylsulfoxid; **HFF**, human foreskin fibroblasts; **BrdU**, bromodeoxyuridine; **PARP**, Poly [ADP-ribose] polymerase; **PI**, Propidium Iodide; **FITC**, Fluorescein Isothiocyanate, **5-FU**, 5-fluorouracil; **IAPs**, Inhibitor of apoptosis proteins

1 Introduction

Colorectal carcinoma is one of the most prevalent human cancers in the world [1], and is frequently diagnosed at late stages that require chemotherapeutic treatment. Current treatment options involve the combination of a variety of chemotherapeutic drugs, more recently including the anticancer drug oxaliplatin (Ox). Ox is a third generation platinum-based drug demonstrating a better safety profile than platinum-analogs of the first-(cisplatin) and second-(carboplatin)-generation [2]. It is typically administered in combination with other drugs as part of specific cancer-treatment regimens, e.g. Ox plus 5-Fluoruracil and leucovorin (referred to as FOLFOX), Ox plus capecitabine (XELOX) or Ox plus cetuximab (CAPOX). Although oxaliplatin-based combination regimens show improved clinical efficacy as related to overall response rates, time to tumor progression and median overall survival in patients with metastatic colorectal cancer [3, 4], successful therapeutic outcome is often limited due to high toxicity as well as the development of multi-drug resistance. Therefore, there is a growing need for the development of innovative anticancer therapies. In the last years, a new therapeutic concept of combining antitumor drugs with chemopreventive agents was introduced, which may lead to enhanced antitumor activity through synergistic action or compensation of inverse properties. Beside a multitude of synthetic substances, also numerous phytochemicals have been identified to exhibit potent chemopreventive effects in different carcinogenesis models while, at the same time, showing low toxicity [5]. Due to its wide range of biological and pharmacological effects, especially with regard to chemoprevention and the lack of toxicity in animal and human models, resveratrol might be such a promising agent for enhancing the effects of chemotherapy. The chemopreventive properties of resveratrol have thereby been explained mainly by its activities in cell cycle control and apoptosis induction [6].

Apoptosis is morphologically defined by chromatin condensation, nuclear fragmentation, shrinkage of the cytoplasm, blebbing of the plasma membrane and formation of apoptotic bodies [7]. This physiological cell death is energy-dependent and usually, but not exclusively, associated with caspase activation [8] and mitochondrial membrane permeabilization [9]. Caspases are the major proteases responsible for the proteolytical cleavage of numerous substrates during this process [10]. Cells undergoing apoptosis are rapidly and specifically recognized and engulfed by phagocytes such as macrophages or immature dendritic cells [11]. Removal of apoptotic cells by macrophages seems to result in little or no production of inflammatory immune mediators by unstimulated macrophages [12]. On the contrary, necrosis is induced by external insults and is morphologically characterized by an increase in cell volume leading to the early rupture of the plasma membrane. Consequently, cytosolic, organelle, and nuclear components are spilled into the surrounding tissue [13]. Unlike apoptosis, necrosis is considered to be immunologically harmful at all times, because of the sudden release of so-called danger signals such as ATP or high mobility group box 1 (HMGB1), which elicit inflammation by activating toll like receptors (TLR) or the inflammasome in myeloid cells [14]. Besides, necrotic cells are able to act on fibroblasts to activate

NF-κB and induce the expression of genes that are involved in inflammatory responses [15].

Classical activation of human macrophages is characterized by the production of superoxide (O_2 -), TNF-α, IL-1β and IL-6, also termed the M1 macrophage phenotype [16]. In contrast, the cytokines IL-4, IL-13 or IL-10 are usually produced by the M2 phenotype [16, 17]. Since Virchow et al. observed the presence of leukocytes in human tumors in 1863, it seems accepted that macrophages are a major cell component infiltrating certain tumors [17]. High numbers of tumor-associated macrophages (TAMs) often predict a poor survival prognosis for patients with solid human tumors [18]. Moreover, TAMs promote cancer metastasis through several mechanisms, including promotion of angiogenesis, induction of tumor growth, and enhancement of tumor cell migration and invasion [19-21] while showing a similar molecular and functional profile, that is displayed by a polarized M2 phenotype [17, 22]. Therefore, reprogramming a M2 macrophage toward a M1 type seems to be beneficial with regard to tumor therapy [23].

Here, we investigate whether the polyphenol resveratrol enhances anti-tumor effects of the common chemotherapeutic oxaliplatin, with special regard to the induction of different modes of cell death. We also determine whether tumor cell death occurs in a potentially immunogenic fashion via stimulating macrophages towards the pro-inflammatory M1 phenotype, In addition, we determine possible cytotoxic effects on non-transformed cells.

2 Materials and Methods

2.1 Cell culture and materials

Caco-2 cells were kept in DMEM, supplemented with 10 % FCS, 1 % penicillin/streptomycin, 1 % sodium pyruvate and 1 % nonessential amino acids. Human foreskin fibroblasts (HFF) were cultured in DMEM/Ham's F-12 medium supplemented with 10 % FCS and 1 % penicillin/streptomycin. Both cell lines were maintained at 37 °C in an atmosphere of 95 % air and 5 % CO_2. The cells were passaged weekly using Dulbecco´s PBS containing 0.1 % trypsin and 1 % EDTA. Cells were screened for possible contamination with mycoplasma at monthly intervals. Resveratrol (Sigma-Aldrich, München, Germany) was dissolved in DMSO at a concentration of 100 mM, oxaliplatin (Sigma-Aldrich) was dissolved in water at a concentration of 10 mM. DMEM, DMEM/Ham's F-12 medium, DMSO, sodium pyruvate solution, penicillin and streptomycin stock solutions were all obtained from PAA Laboratories GmbH. When synergistic effects were analyzed, the cells were pre-treated with Res for 24 h.

2.2 Cell Proliferation Assay

The effect of the test subtances on DNA synthesis of cells was assessed using the cell proliferation ELISA kit (Roche Diagnostics, Mannheim, Germany). This assay is a colorimetric immunoassay for quantification of cell proliferation based on the measurement of bromodeoxyuridine (BrdU) incorporation during DNA synthesis, and is a non-radioactive alternative to the [^{3}H]-thymidine incorporation assay. Cells were grown in 96-well culture dishes (10^3 cells/well), incubated with Res and/or Ox for 24 h, and then labeled with BrdU for further four

hours. Incorporated BrdU was measured colorimetrically.

2.3 Combination index (CI)

To assess the drug interactions of Res and Ox, we used the combination-index (CI) method defined by median-effect analysis of Chou and Talalay [24]. The fractional inhibitory concentration was calculated by dividing the concentration of the drug in the combination at IC_{50} by the IC_{50} of the individual drug.

$$CI = \frac{\text{dose of Res}}{IC_{50}\,(\text{Res})} + \frac{\text{dose of Ox}}{IC_{50}\,(\text{Ox})}$$

In this equation, the sum of the dose of Res and the dose of Ox give 50% inhibition of cell growth. Cl < 1 indicates a synergistic effect; Cl = 1, additive effect; and Cl > 1, antagonistic effect [25].

2.4 Determination of ATP level

Caco-2 cells were grown in 96 well culture dishes (10^3 cells/well) and allowed to grow overnight. Cell Viability Assay Kit (ApoSENSOR^{TM}, BioVision, CA, USA) was used according to the manufacturer's instructions following a 1-24 h exposure to the substances. The assay utilizes luciferase to catalyze the formation of light from ATP and luciferin, and the light can be measured using a luminometer. Decrease in ATP levels was determined by comparing the results with the levels of untreated cells (control).

2.5 Determination of DNA Fragmentation

Caco.2 cells were seeded in 96-well culture dishes; 24 h after plating, cells were stimulated with substances for 24 h. Cytoplasmic histone associated DNA fragments, as a marker of apoptosis, in control and treated cells were quantified using a commercially available ELISA kit (Roche Diagnostics).

2.6 Caspase-3 activity Assay

Caco-2 cells, grown in 6-well plates, were stimulated with substances at 80% confluency. Fluorometric immunosorbent enzyme assay (Roche Diagnostics) was used according to the manufacturer's instructions. Subsequent to the test, protein concentrations were measured for adaption of caspase activity.

2.7 SDS-polyacrylamide gel electrophoresis and immunoblot analysis

Caco-2 cells were seeded in 80 cm^2 flasks; 24 h after plating, cells were incubated with substances for 6 or 24 hours. Western blot analysis were performed as described previously [26]. The blots were incubated overnight at 4°C in 5% skimmed milk with a 1:1000 dilution of primary antibodies for PARP (from Cell signaling, Berverly, MA) or survivin (from Santa Cruz Biotechnology, Heidelberg, Germany). The secondary, horseradish peroxidase-conjugated, antibody (Santa Cruz Biotechnology) was diluted at 1:5000 and incubated with the membrane for another 60 min in skimmed milk. After chemoluminescence reaction (ECL, Amersham pharmacia biotech, Wien, Austria), bands were detected after exposure to Hyperfilm-MP (Amersham International plc, Buckinghamshire, United Kingdom). Blots were reprobed with β-actin antibody (Santa Cruz Biotechnologies).

2.8 Mitochondrial membrane potential ($\Delta\Psi_m$) analysis

Caco-2 cells were seeded into cultivation flasks at a densitiy of 1 x 10^5 cells/well and allowed to grow overnight. Cells were stained with JC-1, as a component of the JC-1 Mitochondrial membrane potential Assay Kit (Cayman Chemical Company, Ann Harbor, MI) following a 6 and 24 h exposure to the subtances. JC-1 is a lipophilic, cationic dye that can selectively enter into mitochondria and reversibly change color from green to red as the membrane potential increases. Changes in $\Delta\Psi_m$ were finally assessed by microfluorimetry analysis using TECAN SpectraFluor Plus (TECAN Austria GmbH, Grödig, Austria) and indicated as changes of red/green ratio.

2.8 Annexin-V FITC/PI double labeled flow cytometry

To discriminate between apoptotic and necrotic cell subpopulations simultaneous staining with Annexin V-FITC and Propidium iodide was performed. Cells incubated with the drugs for 5 and 24 h were harvested with accutase for 30 min. After centrifugation, cells were resuspended in 100 µL binding buffer mixed with 5 µL Annexin V-FITC (ImmunoTools, Friesoythe, Germany) and 5 µl PI and then incubated in dark for 10 min at 4 ºC. Fluorescence was measured with a flow cytometer.

2.9 Cytotoxicity

Cytotoxicity was analyzed by measuring lactate dehydrogenase release using a commercially available kit (Cytotoxicity detection kit (LDH), Roche). For this, HFF were incubated with substances for 24 h. Triton X-100 (2%) was used as a positive control. After centrifugation at 250 x g for 10 min, the supernatant was carefully removed and transferred into corresponding wells of an optically clear 96-well flat bottom microplate. To determine the LDH activity in these supernatants, 100 µl Reaction mixture was added and the samples were incubated for up to 30 min. Finally, the absorbance of the samples was measured at 490 nm.

2.10 Cell Isolation and Culture

Human monocytes were isolated as described [27]. In brief, monocytes were isolated from buffy coats (DRK-Blutspendedienst Baden-Württemberg-Hessen, Institut für Transfusionsmedizin und Immunhämatologie, Frankfurt am Main, Frankfurt, Germany) using Ficoll-Hypaque gradients (PAA Laboratories, Karlsruhe, Germany). Peripheral blood mononuclear cells were washed twice with phosphate-buffered saline (PBS) and were allowed to adhere to culture dishes (Primaria 3072, Becton Dickinson, Lincoln Park, NJ) for 1 h at 37°C. Nonadherent cells were removed. Monocytes were then differentiated into macrophages with RPMI 1640 containing 10% AB-positive human serum (PAA Laboratories) for 7 d or more.

Purified platelets were resuspended in serum-free DMEM containing 1 % penicillin/streptomycin, 1 % sodium pyruvate and 1 % nonessential amino acids, seeded in 80 cm^2 flasks and immediately treated with subtances. After

24 h, LDH release in the supernatant was analyzed as described above.

2.11 Co-culture Experiments

Primary human monocyte-derived macrophages were seeded in 80 cm^2 flasks. After differentiation, supernatants of Res/Ox-treated tumor cells (conditioned medium) were added, and co-cultures were maintained for 24 h. After analysis of the cytokine pattern (see below), the conditioned macrophage medium/macrophage supernatant was further applied to Caco-2 cells, grown in 96-well culture dishes (10^3 cells/well) overnight, to investigate possible cell growth inhibitory and cytotoxic properties after 24h of treatment (see overview in Figure 5). Cell proliferation and cytotoxicity were measured as described elsewhere.

2.12 Quantification of Cytokines

Cytokine measurements were performed essentially as described previously [28]. To quantities cytokines in cell culture supernatants of macrophages, we performed FACS analysis using the BD Cytometric Bead Array System (CBA) Human Inflammation Kit to determine IL-10, TNF-α, IL-8 and IL-1β. Samples were analyzed with the FACSCanto (BD Biosciences) flow cytometer and processed with BD Biosciences FCAP software.

2.13 Statistics

All statistical analyses were performed using GraphPad Instat 4.01 (San Diego, CA, USA). The data are expressed as means ± SE of at least three independent experiments. Results were analyzed by a two-way ANOVA. A p value < 0.05 was considered to be significant.

3 Results and Discussion

3.1 Synergistic antiproliferative effects of Res and Ox on Caco-2 cells

Although chemotherapy still plays an important role in the treatment of most solid tumors, it can only contribute to overall patient's survival with compromised quality of life. Moreover, chemotherapy is falling behind on the fight against cancer because of an increasing trend of chemoresistance and the recurrence of secondary tumors. In this context, the use of phytochemicals as important enhancers of chemotherapy or radiotherapy, predominantly by modulating intracellular cell signalling pathways, abrogating drug resistance and diminishing systemic toxicities is considered to be an alternative for the management of this dread disease [5]. Resveratrol represents one of the promising dietary phytochemicals with chemopreventive and chemotherapeutic potential (3,5,40 trihydroxystilbene) [29], which has first been isolated from the roots of white hellebore (Veratrum glandiflorum O. Loes), and was subsequently identified in various food sources including red wine, grapes, peanuts, mulberries, etc. and in more than 70 other plant species [30]. Resveratrol was already examined in various studies to determine possible chemosensitizing properties when combined with established cancer treatments [31]. Here, we explored the possible chemosensitizing capability of

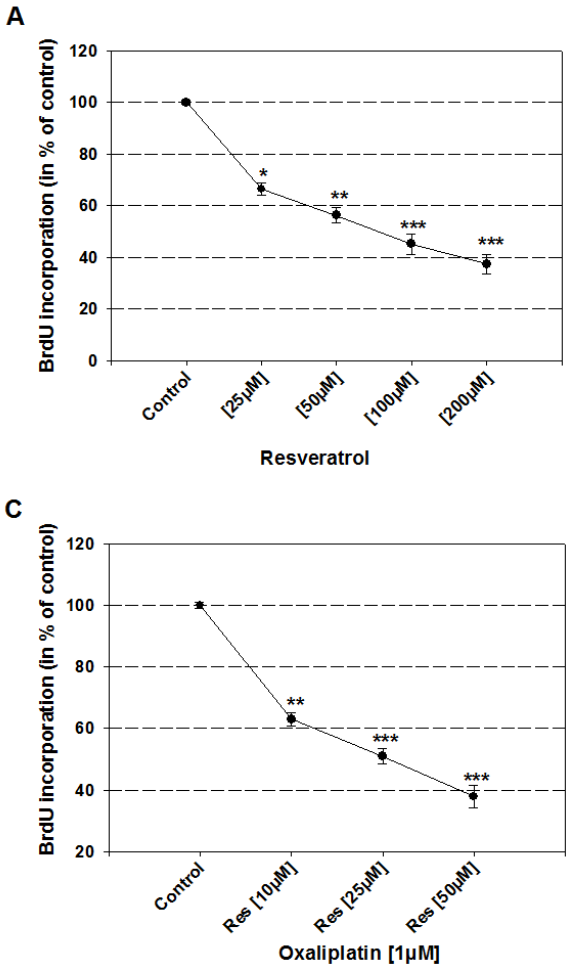

Fig. 1 A, B) Inhibition of cell proliferation of Caco-2 by 24 h exposure to Res and Ox alone. Values represent mean ± SE (n=8); * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control. The IC_{50}s for Res and Ox in Caco-2 cells were 75 µM (A) and 5.58 µM (B) respectively. C) The combined effects of concurrent treatment with Res and Ox on Caco-2 cells. Cell proliferation was mesaured by BrdU incorporation after 24 h of incubation. According to the CI method of Chou and Talalay [28], a CI of 0.51 could be calculated which indicates synergism (see "Material and Methods"). Values represent mean ± SE (n=3); * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control.

resveratrol in a cell culture model of colorectal cancer, also by anaylzing possible effects on immune cells. Our first results quickly revealed Res-induced potentiation of cell growth inhibition mediated by oxaliplatin (Figure 1) (*** $p<0.001$), which was more-than-additive as indicated by combination index analysis (CI=0.51) (C).

3.2 Apoptotic effects of Res/Ox-treatment in Caco-2 cells

These cell-growth inhibitory effects of Res/Ox were accompanied by different hallmarks of apoptosis. In Figure 2A, changes in the intracellular ATP/ADP ratio, as a useful indicator to distinguish between different modes of cell death and viability, were analyzed. Although, decreasing ATP and increasing ADP levels are generally found in apoptotic cells, cells will rather undergo necrosis when intracellular ATP levels fall below a critical threshold [32]. We could demonstrate that incubation with Res [50-100 µM] and Ox [1 µM] resulted in a significant dose-, and partially time-dependent reduction of intracellular ATP which reaches a maximum after 24 h (*** $p<0.001$). Staurosporine [0.5 µg/ml], a well-known inducer of apoptosis [33], was used as a positive control. As DNA cleavage is another hallmark for apoptosis, we further quantified histone-complexed DNA fragments in Caco-2 after 24 h of treatment. Res [50-100 µM], in contrast to Ox [1 µM], thereby led to an increase of cystoplasmic histone-associated DNA fragments, an effect which could be significantly enhanced, when the drugs were used in combination (Figure 2B) (***

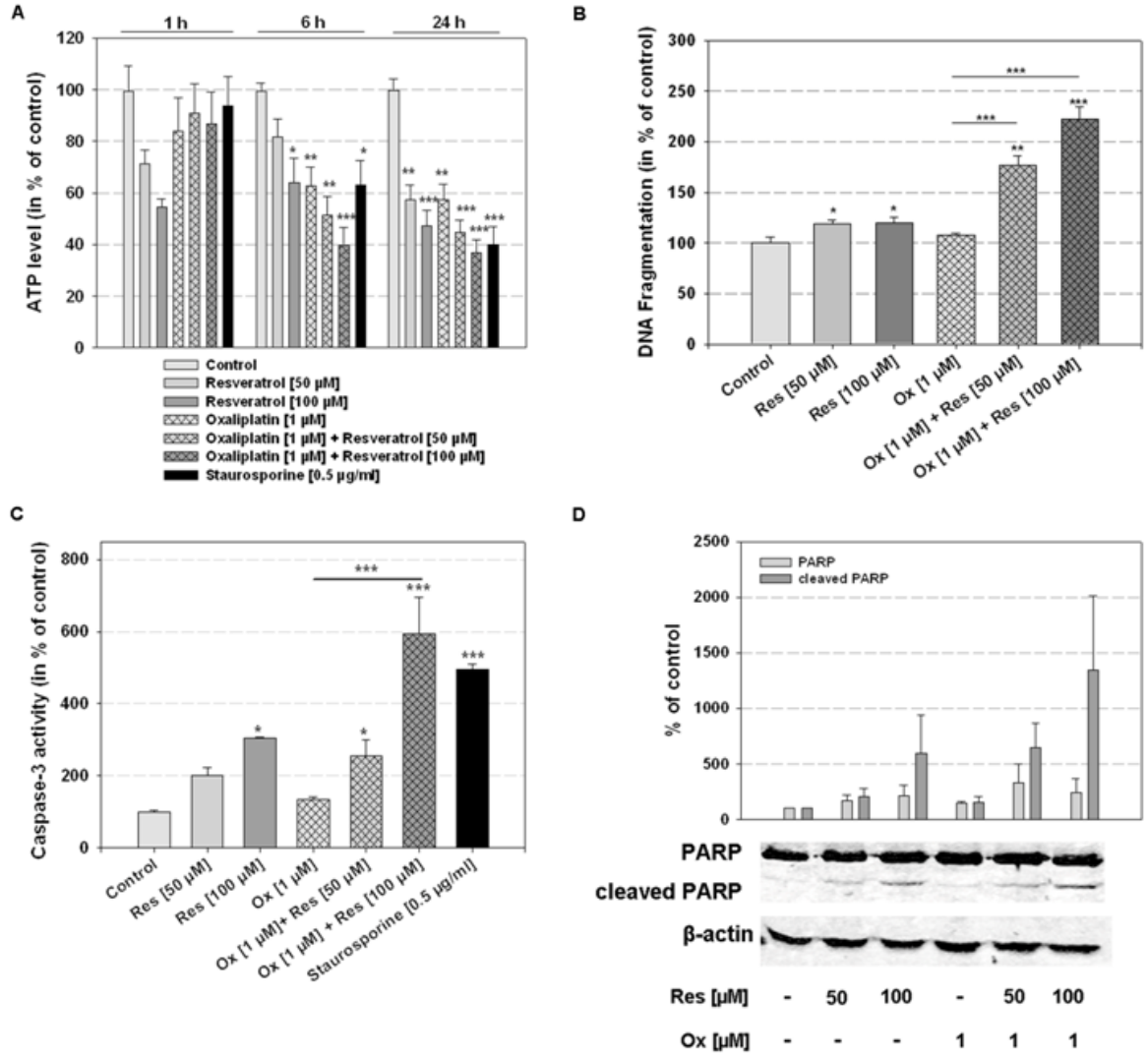

Fig. 2 A) Intracellular content of ATP in control (untreated), Res- and/or Ox- as well as staurosporine (0.5 µg/ml)-treated cells. Caco-2 cells were incubated with the test substances for 1 to 24 h. Results are expressed as the percentage of control. Values represent mean ± SE (n=4); * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control. B) Quantification of cytoplasmic histone associated DNA after 24 h of incubation with the test compounds. Values represent mean ± SE (n=4); * $p<0.05$, ** $p<0.01$, *** $p<0.001$. C) Effects of Res, Ox, their combination and staurosporine on activation of caspase-3 in Caco-2 cells after 24 h of exposure. Results are expressed as the percentage of control. Values represent mean ± SE (n=4); * $p<0.05$, *** $p<0.001$. D) Western blot analysis for PARP cleavage using whole cell extracts from Caco-2 cells exposed to Res and Ox, separately or in combination, for 6 h. Representative immunoblots of three independent experiments are shown. The bar graph presents densitometric analysis of the Western blot images normalized to β-actin (mean ± SE (n=3)).

$p<0.001$ vs. Ox). Additionally, as shown in Figure 2C, the effector caspase-3, which is one of the key proteases in the apoptotic pathway, was also induced by Res [100 µM]. This effect could be significantly enhanced by co-stimulation with Res and Ox (*** $p<0.001$), which is in agreement with the observed cleavage of PARP (Figure 2D) (*** $p<0.001$), a classical substrate for activated caspase-3. Proteolysis of PARP usually is an indicator for early apoptotic events. Further experiments could demonstrate that the intrinsic apoptotic pathway plays a major role in apoptosis-inducing properties of Res as shown in Figure 3A, where Res alone already significantly decreased the mitochondrial membrane (*** $p<0.001$). Here, Res-induced depolarization of the mitochondrial membrane potential could not be further enhanced after combinatorial treatment. Recently, the anti-apoptotic

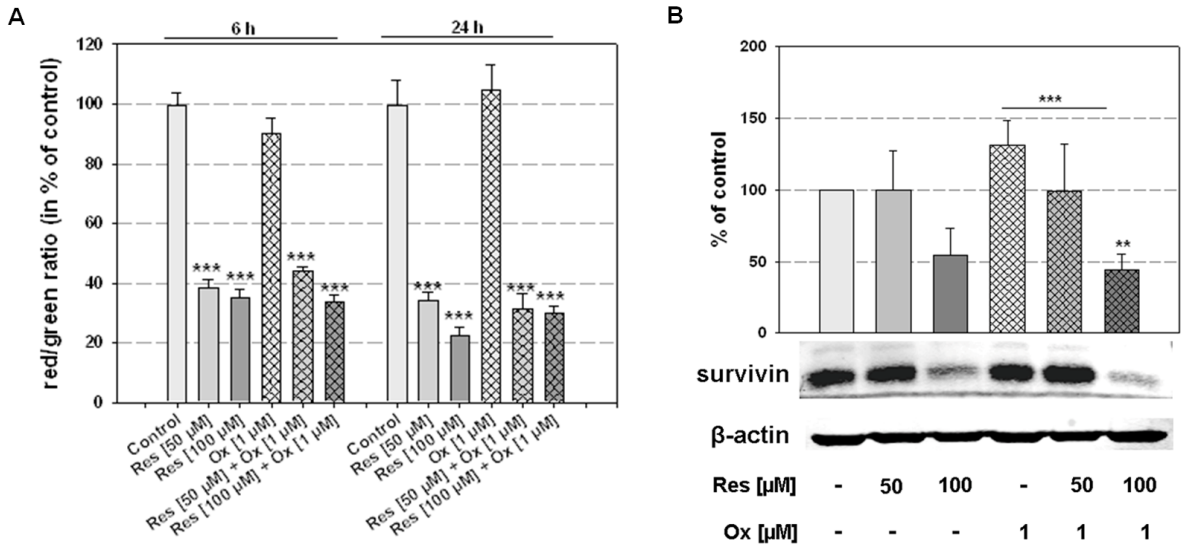

Fig. 3 A) Loss of mitochondrial membrane potential (ΔΨm) in Caco-2 cells exposed to Res and Ox, alone or in combination, after 6 and 24 h of treatment. Results represent mean of at least three experiments, *** p<0.001. B) Western blot analysis for survivin using whole cell extracts from Caco-2 cells exposed to Res and/or Ox for 24 h. A representative immunoblot of three independent experiments is shown. The bar graph presents densitometric analysis of the Western blot images normalized to β-actin (mean ± SE (n=3); ** p<0.01, *** p<0.001).

protein survivin has been described as being selectively expressed at high levels in most human cancers and is related to clinical progression.[34, 35]. Survivin is a structurally unique member of the IAP (inhibitors of apoptosis proteins) family that is potentially involved in both control of cell division and inhibition of apoptosis [36]. Specifically, its anti-apoptotic function seems to be related to an ability to directly/indirectly inhibit caspases [37], although the precise role of survivin in the modulation of the caspase cascade has not been fully elucidated [38]. In addition, survivin overexpression is correlated with poor prognosis of carcinomas of the lung, breast, colon and esophagus [39-42]. Since inhibition of effector caspases by IAPs occurs at the core of the apoptotic machinery, therapeutic modulation of IAPs could target a key control point in cancer resistance [43]. Thus, survivin is at present validated as a cancer therapeutic target [34] and actually in our studies we observed reduced protein levels of survivin after 24 h of drug exposure, whereas this effect was more prominent when the cells were co-treated with Res and Ox (*** p<0.001). Interestingly, in contrast to previous studies [44], we have shown that Ox causes a slight induction of the protein. It is by now well established that several anti-cancer agents, such as quercetin, arsenite and cisplatin show an upregulation of survivin [45].

3.3 Shifting from apoptosis to necrosis with increasing concentrations of SFN/Ox

Depending on the lethal stimulus, tumor cells may also die by necrosis which is characterized by swelling of the cell and the cytoplasma organells before the plasma membrane ruptures and the cellular content is shed into the intercellular space [46]. To discriminate between different modes of cell death, Caco-2 cells treated with Res [50-100 µM], Ox [1 µM] alone and in combination for 5 and 24 h, were analyzed by Annexin V-FITC/PI labelling and flow cytometry. The degree of apoptosis thereby was quantitatively expressed as a percentage of the Annexin V-FITC-

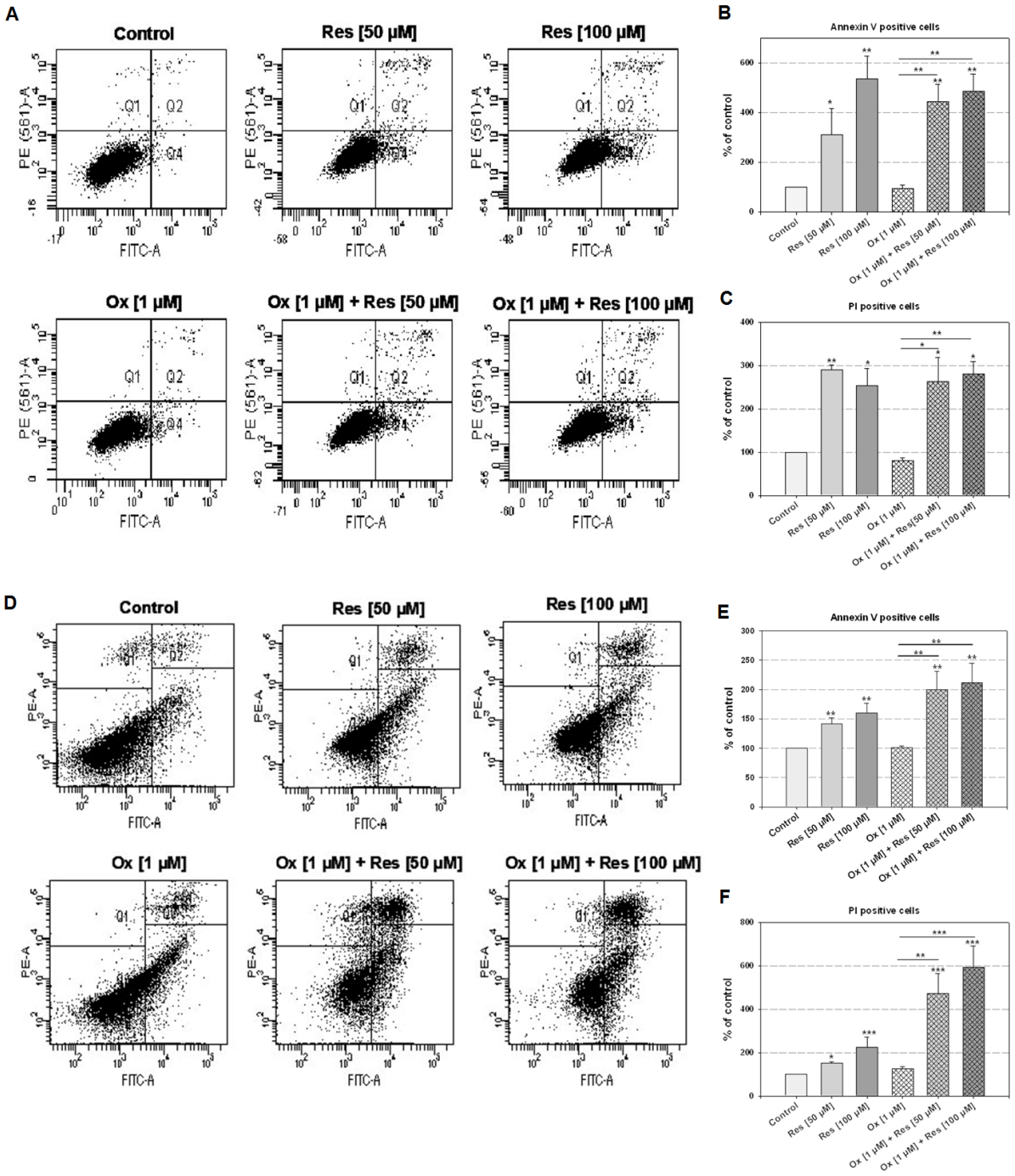

Fig. 4 Effects of Res and Ox, separately and in combination, on Annexin-V FITC/PI double labeled cells after 5 and 24 h of incubation. Cells were analysed by flow cytometry as described in „Material and Methods". The percentage of apoptotic (B, E) and necrotic (C, F) cells vs. control as a result of the FACS analysis is presented by bar graphs. Values represent mean ± SE (n=4), quantifying a minimum of 10.000 cells per treatment (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

positive but PI-negative cells, while necrosis or late apoptosis was quantitatively expressed as a percentage of PI-positive or Annexin V-FITC/PI double-stained cells. Analysis after 5 h was chosen in order to differentiate between primary and secondary necrosis. Interestingly at this time point, Annexin V-FITC positive but also PI-positive cells could be measured indicating direct necrotizing effects of Res and Ox (Figure 4A-C). Even though the population of apoptotic cells in the co-treated cells were still significantly increased after 24 h (** $p<0.01$), overall apoptotic events seemed to decline, particularly in Res [50-100 µM]- as well as Ox [1 µM]-treated cells (Fig. 4D-F), compared to the 5 h-treatment. Rather, at

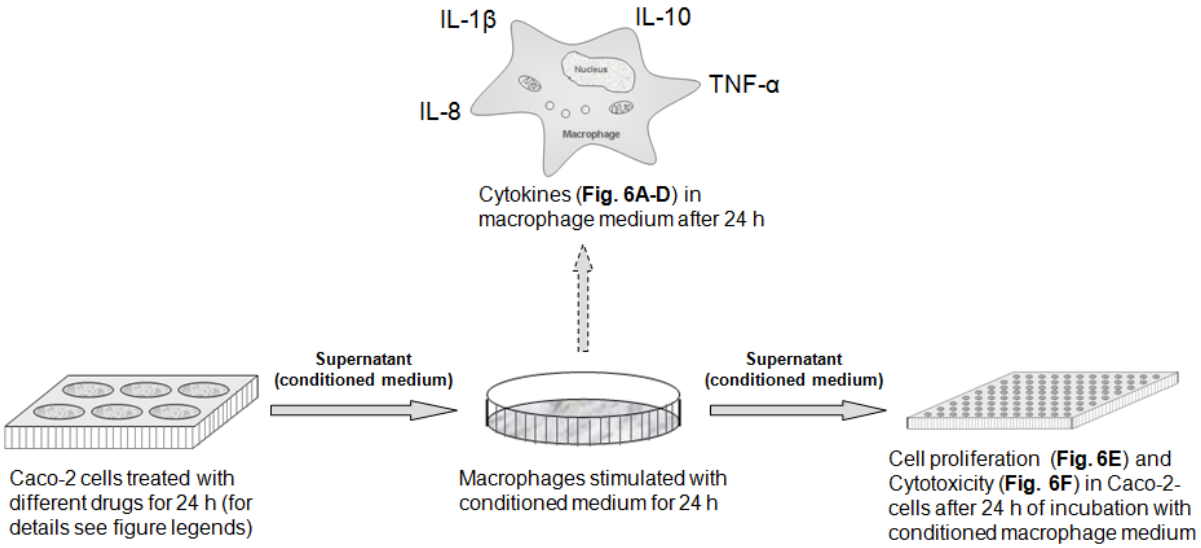

Fig. 5 The overview presents the experimental approach of the coculture experiments.

this time point, a distinct population of PI-stained cells could be observed, so that a mixture of cells undergoing rapid primary as well as secondary necrosis/late apoptosis can be assumed (Figure 4D). Obviously, apoptotic effects seemed to reach a maximum after 5 h of treatment, which is replaced by a shift towards an increased population of necrotic cells after 24 h. Noting the facts that many cancers have defective apoptosis machinery or aquire apoptosis resistance during therapy [47], or the finding that apoptosis may be reversed in cancer cells [48], it is reasonable to consider whether activating alternative cell death pathways, such as necrosis, may be another effective strategy for cancer therapy [49]. Unlike apoptosis, which is considered immunosuppressive, therapy-induced necrotic cell death initiates an immune response [13]. This inflammatory response may help to recruit cytotoxic immune cells to the tumor site, thereby increasing the efficacy of the chemotherapeutic drugs. Effects of Res and Ox on the cytokine profile as a result of the observed necrotic events were therefore investigated by co-culture experiments.

3.4 Conditioned medium of treated Caco-2 cells causes an alteration of cytokine profile in human macrophages

Human primary macrophages were incubated with conditioned medium of treated or control tumor cells for 24 h followed by a quantification of prodcution of the cytokines TNF-α, IL-10, IL-8 and IL-1β. As indicated in Figure 6, macrophages that were incubated with the supernatant of Res- or Ox-treated Caco-2-cells (see overview in Figure 5) released slightly elevated levels of the pro-inflammatory cytokines TNF-α (A), IL-1β (B) and IL-8 (C). This effect was markedly counteracted when macrophages were incubated with the supernatant of Caco-2 cells treated with Res and Ox in combination (* $p<0.05$ vs. Ox). Interestingly, even though co-culture of macrophages with Res/Ox-treated Caco-2 cells significantly counteracted the release of IL-8 mediated by co-culture with Ox [1 µM] (C) (* $p<0.5$), this combination still provoked a marked increase in IL-8 production as compared to control

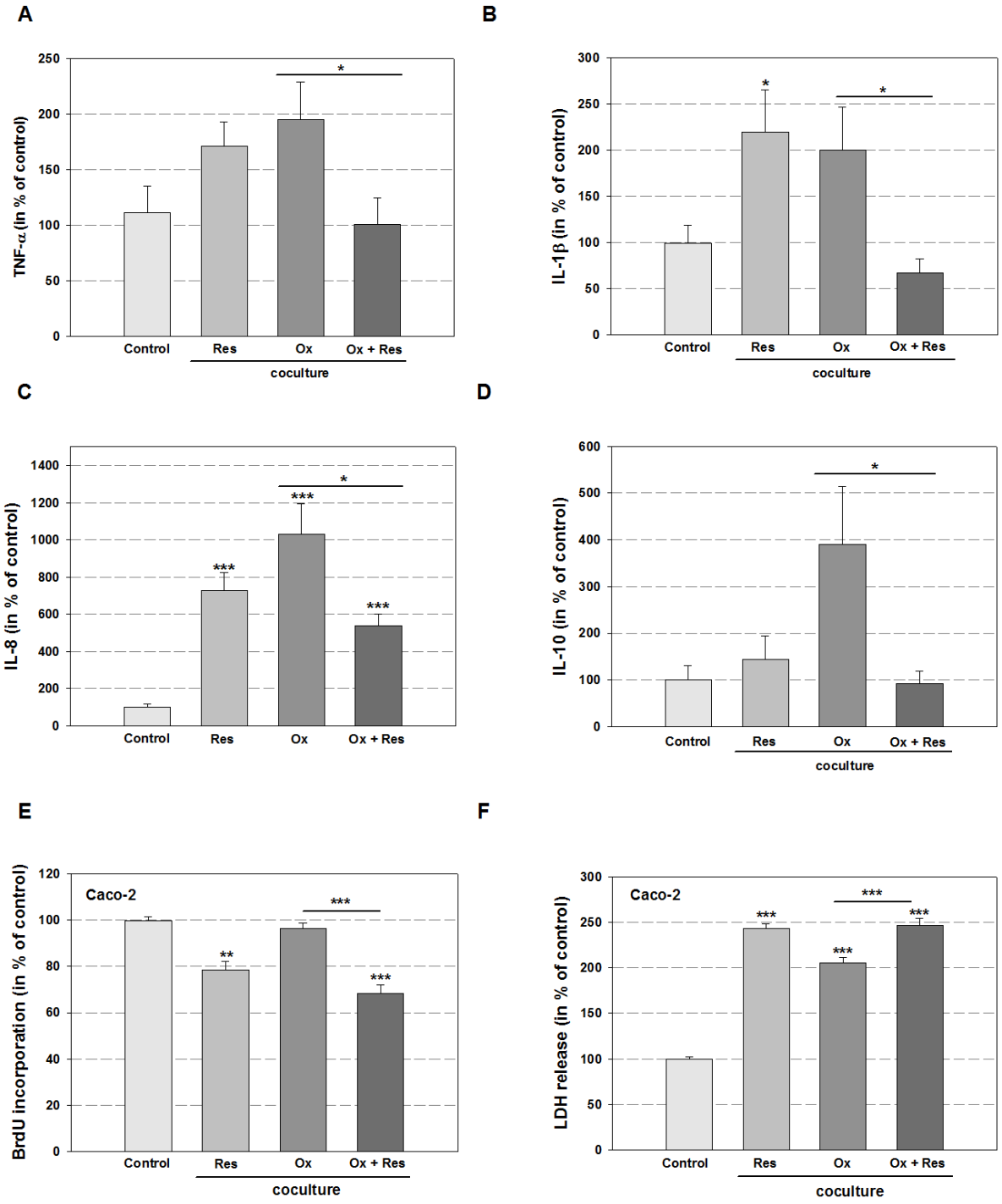

Fig. 6 A-D) Caco-2 cells were treated with Res [100 µM], Ox [1 µM] or their combination. After 24 h of drug exposure, supernatant (conditioned medium) was collected and human monocyte-derived macrophages were incubated with conditioned medium for further 24 h. Quantification of the cytokines TNF-α, IL-1β, IL-8 and IL-10 was by FACS with BD Cytometric Bead Array Flex Sets as described in "Material and Methods". Data are presented as the mean ± SE from six independent experiments. Differences between supernatants from control macrophages and cocultures marked with an asterisk are statistically significant (* $p<0.05$, *** $p<0.001$). Effects of conditioned medium on cell death of Caco-2 cells after 24 h of incubation were measured by BrdU incorporation (E) and LDH release (F). Results are expressed as the percentage of control. Values represent mean ± SE (n=4); ** $p<0.01$, *** $p<0.001$.

macrophages (*** $p<0.001$), similar to single drug exposure (*** $p<0.001$). Most importantly, release of the cytokine IL-10 by macrophages from Ox-treated co-cultures was increased, compared to control macrophages, an effect which could be significantly abolished when the Caco-2 cells were stimulated with Res and

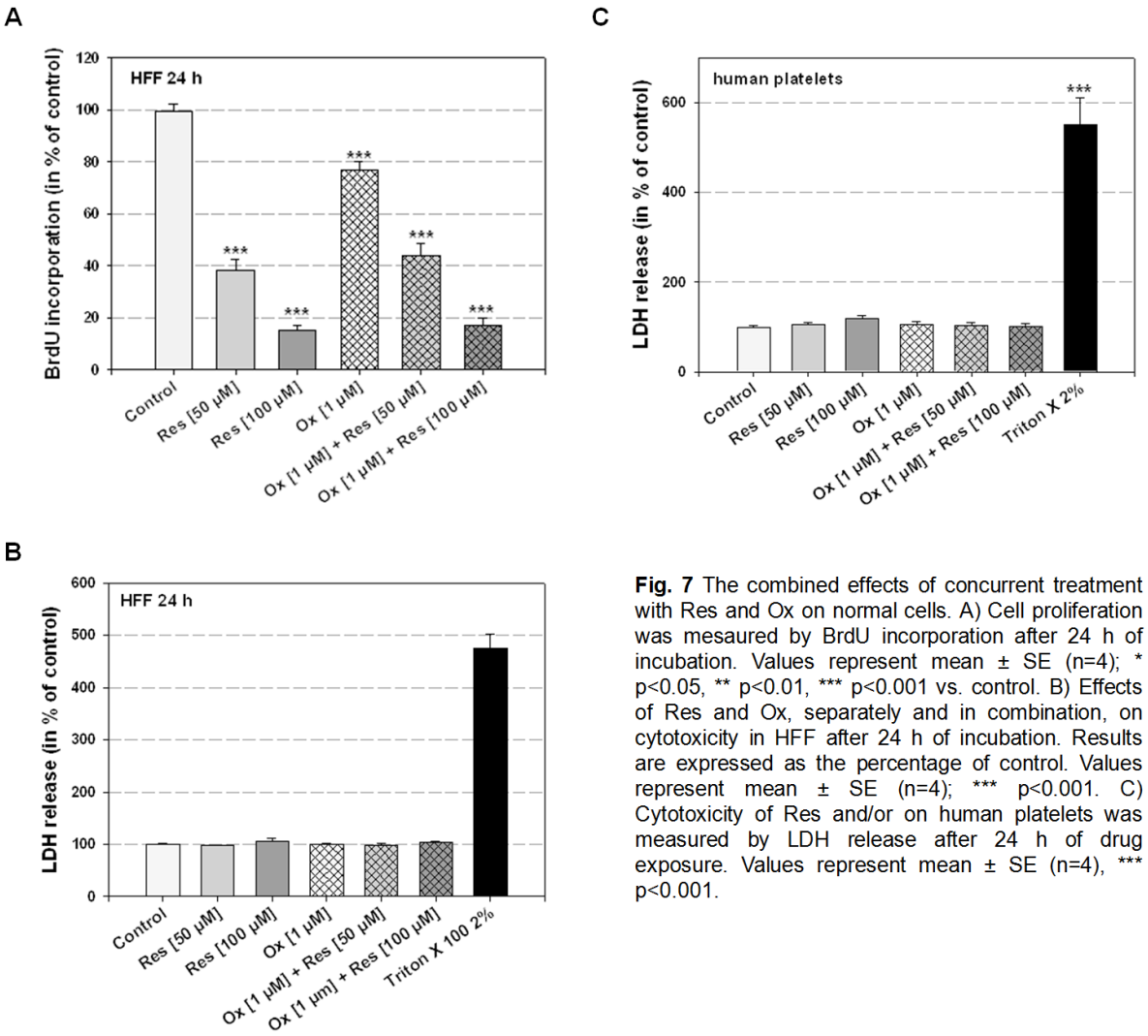

Fig. 7 The combined effects of concurrent treatment with Res and Ox on normal cells. A) Cell proliferation was mesaured by BrdU incorporation after 24 h of incubation. Values represent mean ± SE (n=4); * p<0.05, ** p<0.01, *** p<0.001 vs. control. B) Effects of Res and Ox, separately and in combination, on cytotoxicity in HFF after 24 h of incubation. Results are expressed as the percentage of control. Values represent mean ± SE (n=4); *** p<0.001. C) Cytotoxicity of Res and/or on human platelets was measured by LDH release after 24 h of drug exposure. Values represent mean ± SE (n=4), *** p<0.001.

Ox in combination (D) (* p<0.05). Thus, although tumor cells treated with a combination of Res and Ox did not elicit a strong production of pro-inflammatory mediators, production of immunosuppressive IL-10 was completely abolished. Considering the role of IL-10 in suppression of immunity against established tumors e.g. by inducing regulatory T cells [50, 51], these findings strengthen the rationale of using Ox in combination with Res for tumor therapy.

3.5 Apoptotic/necrotic tumor cells enhanced macrophage cytotoxicity against vital tumor cells

It has previously been shown that apoptotic tumor cells reduced macrophage cytotoxicity against vital tumor cells [52] and disrupting recognition of apoptotic cells by macrophages or dendritic cells in vivo induced tumor regression [53]. To investigate whether conditioned medium may further lead to tumor growth, we treated Caco-2 cells with the supernatant of co-cultured macrophages (for details see overview in Figure 5) and measured cell proliferation as well as LDH release after 24 h of stimulation. As indicated in Figure 6, cell growth inhibition (*** p<0.001) (E) as well as lysis (*** p<0.001) (F) of Caco-2 cells were significantly enhanced, especially when the cells were treated with the supernatant of co-cultured macrophages with Res/Ox-treated Caco-2

cells. These findings correlate well with the observed effects of tumor cell supernatants on IL-10 production in macrophages, since IL-10 potently suppresses the cytotoxic potential of macrophages [54].

3.6 Combinatorial treatment of Res and Ox failed to induce cytotoxicity in human foreskin fibroblasts (HFF) and human platelets

Responding to the question whether Res and Ox, alone and in combination, cause severe toxicity to normal tissue cells, we treated human foreskin fibroblasts and human platelets with Res [50-100 µM], Ox [1 µM] alone and in combination and quantified LDH-release as a marker of direct cytotoxicity after 24 h (Figure 7). [55]. In healthy human fibroblasts, Res was found to inhibit cell growth in a dose-dependent manner (B), an effect which was already reported for some HDAC inhibitors [56, 57]. However, in contrast to Caco-2 cells, growth inhibitory effects on fibroblasts were not further enhanced and no signs of cytotoxicity could be observed when Res was combined with oxaliplatin (A), indicating a selective toxicity towards the tumor cell line while inducing only growth arrest in normal fibroblasts. Additionally, neither Res or Ox alone nor their combination caused any signs of cytotoxicity in human platelets (C).

4 Concluding remarks

As discussed here, the immune response against dying tumor cells can play a major role in determining therapeutic success. If tumor cell death occurs in a potentially immunogenic fashion and if the immune system is capable of perceiving this immunogenicity, a potent innate and cognate immune response raised against dying cancer cells can contribute to the control and elimination of residual cancer cells. Our findings demonstrate for the first time that the polyphenol resveratrol is capable of amplifying Ox-induced cell growth inhibition in colon cancer cells supposedly via induction of different modes of cell death. As a result of the apoptotic and necrotic effects of resveratrol and oxaliplatin, immunosuppressive potential in macrophages is prevented, which renders them potently tumoricidal. Resveratrol might therefore not only be a promising candidate for chemoprevention, but also for chemotherapy options.

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IV

INVITED REVIEW

Phytochemicals resveratrol and sulforaphane as potential agents for enhancing the anti-tumor activities of conventional cancer therapies

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Key words: Cancer treatment, Chemosensitizers, Phytochemicals, Resveratrol, Sulforaphane,
Running title: Phytochemicals as Chemosensitizers in Cancer Therapy

Abstract:

Even though, conventional cancer therapies, comprising surgery and chemo- and radiotherapy, play an important role in the treatment of most solid tumours, successful therapeutic outcome is often limited due to high toxicity and related side-effects, as well as the development of multi-drug resistances. Therefore, there is need for new therapeutic strategies not only to obtain higher treatment efficacy, but also for the reduction of toxicity and adverse effects. Emerging evidence suggests that natural compounds with distinct anticarcinogenic activity, may be considered as potential agents for enhancing the therapeutic effects of common cancer treatments. By using the examples of resveratrol and sulforaphane this review will summarize the findings of recent investigations focusing this topic so far and the current knowledge of the molecular mechanisms by which these selected phytochemicals may potentiate the anti-tumor effects of different cancer therapies.

Introduction

Despite a markedly improved understanding of the disease, the advent of modern technology and rationally targeted drugs over the past years, cancer remains the second leading cause of death in industrialized countries, with a total of 1,529,560 new diagnoses and 569,490 deaths being projected in 2010 in the US [1]. Current treatment options involve the combination of a variety of chemotherapeutic drugs, radiation and surgery. However, successful therapeutic outcome is often limited due to high toxicity as well as the development of multi-drug resistance. It is therefore of particular importance to investigate other drug combinations for the development of new therapeutic regimens obtaining higher efficacy, and lower side effects, which could significantly improve patient survival rate. Emerging evidence suggests that combining chemopreventive agents with chemotherapy or radiotherapy may lead to enhanced antitumor activity through synergistic action or compensation of inverse properties. Combination treatment may also decrease the systemic toxicity of chemotherapy, because lower doses of radiation or anticancer drugs could be used. Beside a multitude of synthetic substances, like non-steroidal anti-inflammatory drugs (NSAIDs) [2] or selective estrogen receptor modulators (SERMs) [3], also numerous phytochemicals have been identified to exhibit potent chemopreventive effects in different carcinogenesis models, while, at the same time showing low toxicity [4]. Recent studies could further demonstrate that several of these plant-derived compounds are also capable of enhancing the efficacy of chemotherapy and radiotherapy in various *in vitro* and *in vivo* cancer models, predominantly by modulating intracellular cell signaling pathways, abrogating drug resistance and diminishing systemic toxicities. These findings have also been excellently reviewed by Sarkar and Li [5, 6]. Here, in particular we would like to give an overview on recent studies focusing the chemosensitizing activities of resveratrol and sulforaphane, two other interesting chemopreventive candidates, also with regard to possible risks and cons of this treatment strategy.

Resveratrol

Resveratrol (RSV) (Figure 1) is a naturally occuring polyphenol present in red wine, peanuts and grapes [7, 8] which exhibits multiple chemopreventive effects in various carcinogenesis models [9, 10], comprising cell growth inhibition, induction of apoptosis and prevention of angiogenesis. This effect of resveratrol on multiple signal transduction pathways related to carcinogenesis has generated tremendous interest in evaluating its potential for use as a clinical chemopreventive and chemotherapeutic agent.

Bioavailability:

Pharmacokinetic studies in mice and rats suggest consistently that resveratrol is well absorbed and rapidly glucuronidated and sulphated both in the liver and intestinal epithelial cells [11]. After oral administration of C^{14}-resveratrol to humans (25 mg/kg) measurement of total radioactivity also demonstrated high absorption (at least 70%) followed by rapid metabolisation of resveratrol in the liver by

phase-2 drug metabolizing enzymes. Compared to resveratrol, which has a half-life of 8-14 min, these metabolites have a plasma half-life of about 9.2 h. The amount of free resveratrol in plasma and serum reached ~37 nmol/L (less than 2% of total resveratrol). The appearance of a new resveratrol peak 6 h after consumption suggests enteric recirculation of conjugated metabolites by reabsorption after intestinal hydrolysis. Overall recovery in urine and feces was 71-98% after oral administration [12]. In 2007, Boocock et al. [13] reported the first phase I dose-escalation pharmacokinetics in 40 healthy volunteers. The doses administered ranged from 0.5 up to 5 g of resveratrol, which did not cause any serious adverse effects. However, even after high-dose *trans*-resveratrol, the plasma level of free resveratrol did not exceed 2,4 µmol/L. In contrast, most mechanistic studies suggest that carcinogenesis-modulating effects of resveratrol require a sustained presence of 5-100 µmol/L, which might raise doubts on the therapeutic relevance of resveratrol due to the discrepancy between the apparently low bioavailability *in vivo* and the biologically relevant concentrations used in *in vitro* studies [14]. However, contrary to all expectations, systemic and oral administration of resveratrol has been shown to inhibit the initiation and growth of tumors in a wide variety of rodent cancer models [15, 16] . To respond to the question whether the observed effects in *in vitro* and *in vivo* models are also conferrable and relevant for humans, several phase I and II clinical trials are currently in progress for oral resveratrol administration in humans for both prevention and treatment of different types of cancer (www.clinicaltrials.gov) .

Sulforaphane

Sulforaphane (SFN) (Figure 2) is a naturally occurring isothiocyanate derived from cruciferous vegetables such as broccoli, cauliflower, cabbage and kale [17], which targets cancer initiation and progression both *in vitro* and *in vivo,* and further induces antiproliferative and cytotoxic effects in cells that are already transformed [18].

Bioavailability:

Several pharmacokinetic studies in both rats and humans indicate, that dietary absorbed sulforaphane can be widely distributed in the body, reach µM levels in the blood and is capable of reaching target tissues in an active form [19, 20]. After absorption, sulforaphane is predominantly metabolized via the mercapturic acid pathway, starting with glutathione (GSH) conjugation by glutathione-*S*-transferase [21] followed by generation of sulforaphane-cysteine (SFN-Cys) and sulforaphane-*N*-acetylcysteine [22]. Notably, the accumulation of sulforaphane in colonic tissue corresponded with decreased adenoma formation in mice supplemented with 300 or 600 ppm sulforaphane [23]. In human experiments, it has been shown that 75% of sulforaphane from broccoli is absorbed in the jejunum and a portion of that returns to the lumen of the jejunum as SFN-GSH [24]. When the metabolism of glucoraphanin efficiently occurs, SFN-NAC is the primary sulforaphane metabolite excreted in the urine [25, 26]. In rats, nearly 72% of a single oral dose of sulforaphane was recovered in the urine as NAC conjugates in 24 h [21], but only about 1% of the dose was detected in the second 24-h urine sample [27], indicating an extremely high bioavailability and a small inter-individual variation of

sulforaphane absorption and metabolism. Once sulforaphane is distributed, it can accumulate in tissues and be maintained to achieve the anti-tumor effects [22]. In a recent pilot study in human mammary tissue, a oral dose of broccoli sprout preparation containing 200 µmol sulforaphane 1 h prior to tissue removal showed mean accumulation of 1.45 ± 1.12 pmol/mg tissue in the right breast and 2.00 ± 1.95 pmol/mg in the left breast [28].

The consumption of isothiocyanates, such as sulforaphane is actually expected to rise due to the use of dietary supplements and public health initiatives promoting the consumption of more fruits and vegetables. This together with its excellent bioavailability makes sulforaphane a potent candidate for food-drug interactions, whereby these interactions may result in both positive and negative consequences regarding cancer therapy.

Interactions of Resveratrol and Sulforaphane with chemotherapeutic drugs in different *in vitro* and *in vivo* tumor models

Due to their wide range of biological and pharmacological effects, especially with regard to chemoprevention, and the lack of toxicity in animal and human models, resveratrol and sulforaphane were also examined for chemosensitizing properties when combined with established cancer treatments. Actually both, synergistic as well as antagonistic effects could be observed for different anticancer drugs in *in vitro* as well as several *in vivo* tumor models (Table 1), whereby some modes of action could already be identified.

Modulation of cell signaling:

It is well known that chemopreventive agents exert inhibitory effects on the carcinogenesis process through modulation of multiple signaling pathways, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Akt, mitogen-activated protein kinase (MAPK), p53, cyclooxygenase-2 (COX-2), NF-E2-related factor-2 (Nrf-2) and many other molecules that are known to regulate cell cycle progression, apoptotis and cell survival and are frequently involved in the development of drug resistance and thus cancer treatment failure.

NF-κB is an inducible and ubiquitously expressed transcription factor which regulates cell survival, inflammation and differentiation and thus plays a critical role in cancer development and progression [29]. Moreover it is well known, that many chemotherapeutic agents and radiation may induce NF-κB activity in different cancer cells, which is mainly associated with the development of drug resistance [30]. Both resveratrol and sulforaphane have been reported to directly target NF-κB signaling in various tumor models [22, 31]. Moreover, resveratrol-induced inhibition of NF-κB activity seems to be critical for enhancing the antitumor activities of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and gemcitabine in melanoma and pancreatic cancer cells [32, 33]. Similar effects were described for sulforaphane, since NF-κB blockage was essential for enhancing the therapeutic potential of TRAIL in *in vitro* and *in vivo* models of pancreatic and prostate cancer [34]. In salivary gland adenoid cystic carcinoma high (ACC-M) and low (ACC-2) metastatic cell lines, treatment with sulforaphane and 5-fluorouracil (5-FU) led to synergistic inhibition of cell growth, which was accompanied by decreased expression

of nuclear p65 protein, a subunit of the NF-κB transcription complex [35].

Nrf-2 is a transcription factor which was originally identified to be a critical regulator of intracellular antioxidants and phase II detoxification enzymes by the transcriptional up regulation of many antioxidant response element (ARE)-containing genes, and thus defending cells against toxic and carcinogenic effects of many environmental insults. The efficacy of dietary or synthetic Nrf-2-activators in chemoprevention could also be verified both in animal models and in human clinical trials [36]. Conversely, Wang and colleagues demonstrated, that stable over expression of Nrf-2 in different cancer tissues resulted in cell survival and enhanced resistance of cancer cells to chemotherapeutic agents including cisplatin, doxorubicin and etoposide and they further discussed the feasibility of using Nrf-2 inhibitors as adjuvants to chemotherapeutic agents to maximize cancer cell death [37]. Actually, sulforaphane was reported to increase Nrf-2 and detoxification enzyme levels in breast cancer cell lines with very low basal Nrf-2 levels, which was suggested to be associated with significant chemoresistance to cytotoxic drugs like doxorubicin and paclitaxel [38].

The Akt pathway is another signaling pathway which is often involved in drug resistance. Both resveratrol and sulforaphane inhibit Akt phosphorylation and thus activation, thereby sensitizing different tumor cells to drug induced apoptosis [34, 39, 40].

Cell cycle effects:

Since the efficacy of most chemotherapeutic drugs is dependent on specific cell cycle phases, drug combinations with cell cycle modulating compounds may lead to both synergistic as well as antagonistic effects. Investigations by Fulda et al. for example revealed, that pretreatment of different human cancer cell lines and primary tumor cells with resveratrol resulted in cell cycle arrest in the S phase and apoptosis induction preferentially out of S phase upon subsequent treatment with various anticancer drugs [41]. Similar effects were described by Duraj et al. in drug-sensitive and drug-resistant leukemia cells [42]. S phase arrest induced by resveratrol was also found in hepatoma22 transplanted to male BALB/c mice [43] and colon cancer cells [44], thus enhancing the anti-tumor effects of 5-FU, a S phase specific pyrimidin analog typically used in the treatment of colorectal-, pancreatic- and hepato-carcinoma patients [45]. Zoberi et al. reported an early S phase cell cycle checkpoint arrest in cervical tumor cells induced by resveratrol, which also resulted in enhanced tumor cell killing by ionizing radiation [46]. G1 phase arrest, induced by resveratrol-mediated down-regulation of the Cyclin D1/cdk4-complex, in turn potentiated doxorubicin-cytotoxicity in B16 melanoma cells [47]. Mao et al. [48] recently reported an increased percentage of cisplatin-treated bladder cancer cells arrested in G1, when cells were cotreated with resveratrol. In constrast, antagonistic effects were reported in different cell systems when resveratrol was combined with paclitaxel, a mitotic inhibitor, which amongst others is approved for the treatment of ovarian, breast and lung cancer as well as HIV-associated Kaposi's sarcoma [49]. Fukui et al. found, that resveratrol strongly diminished the susceptibility of different breast cancer cell lines (MDA-MB-435s, MDA-MB-231 and SKBR-3) to paclitaxel-induced cell death in culture by inhibition of paclitaxel-induced G2/M-phase arrest. Solely in MCF-7 cells this effects was not observed [50]. Similar effects were described by

Nicolini and Rigolio et al. who not only demonstrated, that resveratrol was able to reduce paclitaxel-induced apoptosis in the human neuroblastoma cell line SH-SY5Y by direct modulation of different pro- and antiapoptotic proteins, but further suggested, that the resveratrol-induced S-phase block seems to prevent SH-SY5Y from entering into mitosis, the phase of the cell cycle in which paclitaxel exerts its core activity [51-53].

Apoptosis:

Both resveratrol and sulforaphane demonstrate potent apoptosis-inducing properties in various tumor models [10, 22, 31]. Morevover, most of the sensitizing effects, described here, are predominantly mediated by modulating the cell death machinery (summarized in Table 1). Gill et al. for example reported, that pre-treatment with resveratrol sensitized prostate cancer cells predominantly to agents, that specifically target death receptors but not agents that initiate apoptosis through other mechanisms [39]. Resveratrol also altered the expression of IAPs and Bax, leading to increased caspase activation and apoptosis. Resveratrol was also found to inhibit cell growth and induced apoptosis in TRAIL-resistant LNPCaP cells, while not effecting normal prostate epithelial cells. The expression of proapoptotic Bax, Bak, PUMA, Noxa, Bim, TRAIL-R1/DR4 and TRAIL-2/DR5 was upregulated, the expression of antiapoptotic Bcl-2, Bcl-xL, survivin and XIAP was downregulated on treatment with resveratrol [54]. By the way, similar observations could be found using sulforaphane, which was further confirmed in an orthotopic mouse model of prostate cancer [34]. In non-Hodgkin lymphoma, multiple myeloma and lung cancer lines, resveratrol significantly enhanced antiproliferative as well as apoptotic effects mediated by paclitaxel [55, 56]. Here, resveratrol was able to selectively down-regulate the expression of anti-apoptotic proteins such as Bcl-xL, Bcl-2 and myeloid cell differentiation factor-1 (Mcl-1) and to further upregulate both, pro-apoptotic proteins (Bax, Bid, apoptosis-protease activating factor-1 (Apaf-1)) as well as specific cell cycle inhibitors ($p21^{waf1}$, $p27^{kip1}$).

Most studies concerned with a possible sensitizing efficacy of sulforaphane are admittedly reported for interactions with TRAIL, a member of the TNF family of cytokines, which can induce apoptotic cell death in several *in vitro* and *in vivo* cancer models, while sparing most normal cells [57, 58]. Even though TRAIL is considered to be a novel promising anticancer agent, a multitude of primary tumors present a phenotype which is rather resistant to TRAIL-induced apoptosis [59, 60] so there is need for new strategies to overcome TRAIL-resistance. Sulforaphane was found to sensitize TRAIL-resistant human hepatoma [61], osteosarcoma [62] and lung carcinoma cell lines [40] but not peripheral blood mononuclear cells [62] to TRAIL-induced apoptosis, primarily by inducing death receptors DR4 and DR5. Similar effects could be detected in a mouse model of prostate cancer [34]. In another mouse xenograft model, using highly treatment-resistant tumor-initiating cells (TIC), which play a central role in the pathogenesis of different tumor entities, including pancreatic cancer, sulforaphane strongly blocked tumor growth and angiogenesis, while again combination with TRAIL leads to enhanced efficacy without obvious cytotoxicity in normal cells [63]. Moreover, we could recently demonstrate that sulforaphane, in addition to the reported synergistic activities, in combination with exogenous TRAIL also seems to be capable of

inducing endogenous TRAIL expression in colorectal cancer cells [64].

We could further show that sulforaphane synergistically amplifies oxaliplatin-induced cell growth inhibition in the colorectal cancer cell line Caco-2, which was accompanied by different hallmarks of apoptosis, whereby apparently both extrinsic as well as intrinsic pathways were involved. However, in contrast to known apoptosis inducers these apoptotic events were not very prominent and might only partly account for the observed cell growth inhibition. In fact, with rising concentrations of oxaliplatin not only an increase of apoptotic but also a distinct population of necrotic cells could be revealed [64]. Noting the facts, that many cancers exhibit defective apoptosis machineries or aquire apoptosis resistance during therapy [65], or the finding, that apoptosis may be reversed in cancer cells [66], it is reasonable to consider, whether activating alternative cell death pathways, such as necrosis, may be another effective strategy for cancer therapy [67]. However whether immunologic responses, typically associated with necrotic cell death might really be desirable in the context of cancer treatment or rather lead to further tumor growth or even overshooting inflammation resulting in autoimmunity remains to be elucidated.

Abrogation of multidrug resistance

A major obstacle to effective chemotherapy is the development of multidrug resistances (MDR), which can be generated by different mechanisms.

By now, modulatory effects of resveratrol on most of these pathways could be reported in different cellular and animal models. In drug resistant human oral epidermoid carcinoma KB cells (KBv200) and acute myeloid leukemia cells, resveratrol was capable of reversing multidrug resistance by decreasing the expression levels of membrane efflux pumps, namely multidrug-resistance gene 1 (MDR-1) and Multiple Drug Resistance protein 1 (MRP-1) [68, 69]. In human multidrug-resistant carcinoma KB-C2 cells inhibitory effects of resveratrol on P-glycoprotein (MDR-1 gene product) resulted in increased intracellular accumulation of daunorubicin, a natural relative of doxorubicin [70]. In addition, more recent studies could demonstrate that resveratrol regulates activity and expression levels of different members of the cytochrom P450 superfamily of enzymes *in vitro* and *in vivo*, and thus is able to directly modulate intracellular drug metabolism and bioavailability of various drugs [71, 72]. Moreover, in multidrug-resistant human non-small cell lung, breast and oral epidermoid cancer cells, resveratrol was reported to induce apopotosis by down-regulating the expression of anti-apoptotic proteins (Bcl-2, Bcl-xL, survivin) [68, 73], whereby evidence suggests, that again resveratrol-activated SIRT1 deacetylase might play a pivotal role in regulating the balance of multidrug-resistance and sensitivity [74].

Sulforaphane was also found to increase expression levels of multidrug resistance proteins 1 (MRP1) and 2 (MRP2) in different human carcinoma cell lines [75, 76]. Owing to the major role of these membrane transporters in elimination of drugs, comprising cytostatics, modulation of its expression may also lead to adverse effects or to changes in drug pharmacokinetics, possibly resulting in therapeutic resistance. On the other hand, Fimognari et al. re-

ported, that sulforaphane was able to reverse resistance to doxorubicin in mouse fibroblasts transfected with a p53 (Ser220) mutation, which is typically found in doxorubicin-resistant phenotypes of different human cancers [77].

Conclusion

Resveratrol and sulforaphane definitely show promise for serving as potent agents for enhancing the therapeutic effects of chemotherapy or radiotherapy in cancer treatment. However, on the other hand this strategy might also negatively affect classical treatment regimens under certain circumstances. These converse findings might at least partly be due to discrepancies in drug concentrations, experimental settings, tumor models and treatment procedures used in the different studies, whereby overall regularities could not yet be defined. Ahmad et al. for example could demonstrate, that contrary to the apoptosis-inducing activity at relatively high concentrations of resveratrol (30-100µM), prior exposure to low concentrations of resveratrol (4-8µM) could create an intracellular milieu resistant to apoptosis induced by either H_2O_2 or anti-cancer drugs [78, 79], which could provide cancer cells with a survival advantage by impeding death execution signals. The relevance of the applied concentration was further confirmed by Chan et al. who could show that resveratrol synergistically promotes 5-FU-mediated apoptosis at higher concentrations, but counteracts 5-FU-activity when used at lower concentrations in a cell culture model of colorectal cancers [80]. Another reason for different findings might be related to varying modes of treatment. In their most recent publication, Mao et al could demonstrate, that resveratrol effectively prevented tumor death induced by taxol (paclitaxel) in 5637 bladder cancer when the drugs were added simultaneously, but in turn, induced synergistic cytotoxicity, when cells were pre-treated prior to addition of taxol [48]. Also cell cycle modulatory effects frequently observed in the presence of phytochemicals seem to be critical for discriminating between synergistic or antagonistic effects when combined with cell cycle specific anti-tumor drugs. This should as well provide a cautionary note for the uncontrolled and non-critical use of biologically active food supplements in patients with established cancer who are already undergoing chemo- or radiotherapeutic treatment.

However, the most important fact that should be noted about all of these studies is that the efficacy of most phytochemicals on anti-tumor therapies has been tested only in preclinical conditions, either *in vitro* or *in vivo*. Whether benefical or detrimental effects can be also seen in humans is largely unknown.

Recently, a clincal trial of a formulation of resveratrol has been suspended due to safety concerns. In this phase 2 trial safety and activity of a resveratrol-based drug alone or in combination with the proteasome inhibitor bortezomib in patients multiple myeloma should be assessed (http://clinicaltrials.gov/ct2/show/NCT00920556). The trial was halted when 5 out of 24 patients developed a kidney condition called cast nephropathy. Interestingly, all patients who experienced kidney failure during the trial were being treated with only the resveratrol-based drug when their kidney problems developed.

However, it is still uncertain whether the kidney failures were actually related to the resveratrol treatment, or were simply a

manifestation of the underlying myeloma since cast nephropathy is so commonly associated with multiple myeloma that it is even called “myeloma kidney” [81]

Further mechanistic studies, *in vivo* animal models and most important human clinical trials are therefore needed to certify the safety and efficacy of dietary compounds in combination cancer therapies paving the way for eventually transferring this concept to clinical applications.

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Table 1 Possible interactions of Resveratrol and Sulforaphane with Chemo- and Radiotherapy in different *in vitro* and *in vivo* tumor models

Resveratrol		
Tumor models/Major findings	**Targets/Proposed Mechanisms**	**References**
Lung cancer		
RSV enhanced paclitaxel-induced apoptosis of lung cancer cells (A549, EBC-1, Lu65)	DNA Fragmentation ↑, caspases↑, $p21^{WAF1}$↑	[56]
RSV induces apoptosis in human multidrug-resistant non-small cell lung cancer cell lines SPC-A-1/CDDP and in a nude mice xenograft model	Cell Proliferation ↓, Apoptosis ↑, Tumor growth ↓, Cell Cycle Arrest, Survivin ↓	[73]
Breast cancer		
RSV attenuated efficacy of taxol in human breast cancer cells MDA-MB-435s, MDA-MB-231 and SKBR-3 cells and in a athymic nude mice xenograft model, but not in MCF-7 cells	Paclitaxel-induced G2/M cell cycle arrest ↓, Paclitaxel-induced ROS accumulation ↓	[50]
RSV augmented cytotoxicity of cisplatin in Ehrlich-acites-bearing mice	Tumor size ↓, Serum CRP↓, TNF-α ↓, leucocytes ↓, malondialdehyde ↓	[82]
Prostate cancer		
RSV sensitized androgen independent prostate cancer PC-3 and DU145 cells to death-receptor mediated apoptosis	Caspases ↑, PARP cleavage ↑, Nuclear condensation ↑, BAX ↑, Akt phosphorylation ↓	[39]
RSV enhanced the therapeutic potential of TRAIL in LNCaP cells	Bax ↑, Bak ↑, PUMA ↑, Noxa ↑, Bim ↑, TRAIL-R1/DR4 ↑, TRAIL-R2/DR5 ↑, Bcl-2 ↓, Bcl-xL ↓, survivin ↓, XIAP ↓, ROS ↑ , mitochondrial membrane potential, release of mitochondrial proteins, caspase-3 ↑, caspase-9 ↑, apoptosis ↑	[54]

Neuroblastoma		
RSV cooperated with anticancer agents VP16, doxorubicin, cytarabine (AraC), actinomycin D, taxol or methotrexate to induce apoptosis in SEHP neuroblastoma cells in a time-and dose-dependent manner	S-Phase arrest, p53-independent, Survivin ↓, Activation of caspase-3, -8, -9 and PARP ↑	[41]
RSV reduced paclitaxel-induced apoptosis in human neuroblastoma SH-SY5Y cells	Caspase-7-activation, Poly-(ADP-ribose)-polymerase degradation, Raf-1 phosphorylation, Bcl-2 phosphorylation, S-phase block prevents cells to enter mitosis	[51-53]
Leukemia		
RSV inhibited H_2O_2-induced apoptosis in promyelocytic leukemia HL60 cells	Caspase-Activity ↓, DNA Fragmentation ↓, Cell proliferation ↑	[78]
RSV inhibited H_2O_2-, vincristine-, daunorubicin- and C2-induced apoptosis in human leukemia HL60 cells	Caspase Activation ↓, DNA Fragmentation ↓, cytochrome C translocation ↓, intracellular superoxide ↓, drug induced acidification ↓, activation of NADPH oxidase complex ↓	[79]
RSV sensitized sensitive (HL60) and resistant (HL60/VCR (Pg-positive)) leukemia cells to doxorubicin (DOX), cycloheximide (CHX), busulfan (BUS), gemcitabine (GEM) and paclitaxel (PTX)-induced apoptosis	S-phase arrest, apoptosis ↑	[42]
RSV reverses doxirubicin-resistance in acute myeloid leukemia cells	Cell growth arrest ↑, apoptosis ↑, MRP1 ↓	[69]
RSV reduced H_2O_2-induced apoptosis in erythroleukemia	Leukotriene B4 ↓, Prostaglandine E2 ↓, 5-lipoxygenase ↓, 15-	[83]

K562 cells by inhibiting leukotriene B4 and prostaglandin E2	lipoxygenase ↓, Prostaglandin H synthase ↓	
Skin cancer		
RSV sensitized melanoma cells (WM9, LU1205, LOX) to TRAIL induced apoptosis	STAT3 ↓, NF-κB ↓, JNK-c-Jun ↑, c-FLIP ↓, Bcl-XL ↓	[32]
RSV reverses multidrug resistance against vincristine, adriamycin and paclitaxel in KBv200 cell lines	Apoptotic cells ↑, Bcl-2 ↑, MDR-1 ↑	[68]
RSV induced the accumulation of daunorubicin in KB-C2 epidermoid cancer cells	P-glycoprotein ↓	[70]
RSV inhibits the growth of a doxorubicin-resistant B16 melanoma in vitro and in vivo and further potentiates doxorubicin-cytotoxicity in B16 melanoma cells	Cyclin D1/cdk4 ↓, p53↑, G1-phase-arrest, apoptosis ↑	[47]
Gastrointestinal cancers		
RSV sensitized cholangiocarcinoma cells (Mz-ChA-1, HuCC-T1, CCLP1, and SG231) to chemotherapeutic agents (5-FU, gemcitabine, or mitomycin C) and further potentiates 5-FU-cytotoxicity in an *in vivo* xenograft model using Mz-ChA-1 cells	Cell proliferation ↓, Apoptosis ↑, Tumor size ↓, Cyp1b1 ↓	[72]
RSV enhanced antitumor activity of gemcitabine pancreatic cancer cells (AsPC-1, MIA-PaCa-2, Panc-1, Panc-28) and in an orthotopic mouse model of human pancreatic cancer	NF-κB activation ↓, bcl-2 ↓, bcl-xL ↓, COX-2 ↓, cyclin D1 ↓, MMP-9 ↓, VEGF ↓, cyclin D1 ↓, ICAM-1 ↓, survivin ↓	[33]
RSV enhanced the anti-tumor effects of 5-FU in a transplantable murine hepatoma22 model and antagonized 5-FU toxicity	Tumor size ↓, survival time ↑, S-phase-arrest	[43]

RSV and Res-Analogs potentiate 5-FU cytotoxicity in SW480, SW620 and HCT116 colon cancer cells	S-Phase-arrest, Cyclin A/cdk2-complex ↑, cell proliferation ↓	[44]
RSV synergistically promotes 5-FU-mediated apoptosis at higher concentrations irrespective of p53 but inhibits 5-FU-triggered apoptosis at lower concentrations in p53+/+ colorectal cancer cells (HCT-116)	Caspase-6-activity ↑, p53, apoptosis ↑↓	[80]
Gynecologic cancers		
RSV enhanced γ-radiation-induced apoptosis and cell cycle arrest of Hela and SiHa cells	S-phase-arrest, COX1 activity ↓, cell growth ↓	[46]
RSV improved the effectiveness of cisplatin and doxorubicin in human gynecologic cancer cells (OVCAR-3, Ishikawa)	Cell growth ↓	[84]
Multiple Myeloma		
RSV sensitized multiple myeloma (8226/S and 8226/Dox40) cells and non-Hodgkin's lymphoma (Raji and Ramos) cells to paclitaxel-induced apoptosis	Bcl-xL ↓, myeloid cell differentiation factor (Mcl-1) ↓, formation of tBid, mitochondrial membrane depolarization ↑, cytosolic release of cytochrome c and Smac/DIABLO ↑, activation of caspase cascade ↑, PARP cleavage ↑	[55]
Bladder cancer		
RSV prevented taxol- and vinblastine-induced cell death in 5637 bladder cancer cells, but enhanced the cytotoxicity of cisplatin	Taxol, viniblastine: Cell viability ↑, colony formation ↑, PARP cleavage ↓, Caspase-3-cleavage ↓, p-Bcl-2 ↓, p-Akt ↑, pERK ↑, pJNK ↓, p-p38 ↓, p-IκB ↑, NF-κB ↑, cyclin D1 ↑, Cyclin E ↑, S-phase-arrest Cisplatin: Cell viability ↓, G1-phase-arrest	[85]

Sulforaphane

Tumor models/Topic	Targets/Major findings	References

Lung cancers		
SFN sensitized lung adenocarcinoma A549 cells to TRAIL-induced apoptosis	Caspase-3-activity ↑, PARP cleavage ↑, pp38 ↑, pJNK ↑, pERK ↓, pAkt ↓	[40]
In contrast to other isothiocyanates, SFN did not sensitize non-small cell lung cancer cells to cisplatin-induced cytotoxicity	Cell viability →, β-tubulin →	[86]
SFN enhanced the expression of phase II detoxification enzymes in and lung (A549) cancer cells	MRP1 and MRP2 mRNA and protein levels ↑	[76]
Gastrointestinal Cancers		
SFN potentiates oxaliplatin-induced anti-tumor-activities in the colorectal cancer cell line Caco-2	Cell proliferation ↓, ATP levels ↓, DNA Fragmentation ↑, caspase-3-activity ↑, PARP cleavage ↑, TRAIL ↑, caspase-8 cleavage ↑, mitochondrial membrane potential ↓, necrotic cells ↑	[64]
SFN sensitized TRAIL-resistant hepatoma cells to TRAIL-induced apoptosis	DR5 ↑, Caspase cleavage ↑, PARP cleavage ↑, ROS ↑, IAPs →	[61]
The combination of SFN and TRAIL blocked tumor growth and angiogenesis in vitro (AsPC-1, BxPc-3, Ca-pan-1 and MIA-PaCa2) and in vivo using a mouse xenograft model (MIA-PaCa2) of pancreatic cancer without obvious cytotoxicity in skin fibroblasts	NF-κB activity ↓, XIAP ↓, cIAP ↓, FLIP ↓, Cell viability ↓, clonogenicity ↓, Tumor size ↓, Caspase-activity ↑, apoptosis ↑	[63]
SFN enhanced the expression of phase II detoxification enzymes in liver (HepG2) and colon (Caco-2) cancer cells	MRP1 and MRP2 protein levels ↑	[76]
Prostate cancer		

SFN enhanced the therapeutic potential of TRAIL in vitro (PC-3 and LNCaP) and in vivo in a prostate cancer orthotopic mouse model	In vitro: reactive oxygen species (ROS) ↑, mitochondrial membrane potential ↓, caspase-3 ↑, caspase-9 ↑, DR4 ↑, DR5 ↑, Bax ↑, Bak ↑, Bim ↑, Noxa ↑, Bcl-2 ↓, Bcl-xL ↓, Mcl-1 ↓ In vivo: TRAIL-R1/DR4 ↑,TRAIL-R2/DR5 ↑, Bax ↑, Bak ↑, NF-κB pathway ↓, P13K/AKT pathway ↓, MEK/ERK pathway ↓	[34]
Gynecological cancer		
Combination treatment with SFN and paclitaxel resulted in additive growth suppression in epithelial ovarian cancer cells	Cell viability ↓	[87]
Other systems		
SFN and 5-FU synergistically inhibit cell growth of high (ACC-M) and low (ACC-2) metastatic cell lines of salivary gland adenoid cystic carcinoma	Cell growth ↓, NF-κB p65 expression ↓	[35]
SFN enhanced TRAIL-induced apoptosis in human osteosarcoma cells (Saos2, MG63)	Bid cleavage ↑, Caspase-8 ↑, Caspase-9 ↑, Caspase-10 ↑, Caspase-3 ↑, DR5 mRNA and protein levels ↑, p53-independent	[62]
Combination of SFN and doxorubicin reversed doxorubicin-resistant phenotype in mouse fibroblasts with p53Ser220 mutation	Apoptosis ↑	[77]