MECHANISMS OF CELL CYCLE ARREST AND APOPTOSIS INDUCTION BY SULFORAPHANE AND COMBINATORIAL EFFECTS OF SULFORAPHANE AND 3,3'-DIINDOLYLMETHANE ON CANCER CELL GROWTH INHIBITION

DISSERTATION

zur Erlangung des akademischen Grades
doctor rerum naturalium (Dr. rer. nat.)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät
der Friedrich-Schiller-Universität Jena

von

Diplom-Ernährungswissenschaftlerin

Gerlinde Pappa

geboren am 19.08.1978 in Hamburg

Meiner Familie und Alexander

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Der Langsamste,
der sein Ziel nicht aus den Augen verliert,
ist immer noch schneller,
als jener, der ohne Ziel umher irrt.

- Gotthold Ephraim Lessing -

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LIST OF ABBREVIATIONS

40-16	human colon carcinoma cell line, random clone derived from HCT116
AhR	aryl hydrocarbon receptor
ARE	antioxidant response element
AOM	azoxymethane
BCA	bicinchoninic acid
BSA	bovine serum albumin
BSO	L-buthionine-*S,R*-sulfoximine
Cdk	cyclin-dependent kinase
c-FLIP	cellular FLICE-inhibitory protein
Cox-2	cyclooxygenase-2
CYP450	cytochrome P450 (Phase I-enzymes)
DIM	3,3'-diindoylmethane
DISC	death-inducing signaling complex
DMBA	7,12-Dimethyl-benz[a]anthracene
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTNB	5,5-dithiobis(2-nitrobenzoic) acid
EGCG	epigallocatechin gallate
ELISA	enzyme-linked immunoabsorbant assay
FCS	fetal calf serum
Fig.	figure
GSH	glutathione
GST	glutathione-*S*-transferases (Phase II-enzymes)
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HCT116	human colon carcinoma cell line
I3C	indole-3-carbinol
IAP	inhibitor of apoptosis protein
IC_{50}	halfmaximal inhibitory concentration
IκB	inhibitor of NF-κB

IKK	IκB kinase
ITC	isothiocyanate
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide
JNK	c-Jun-NH_2-terminale Kinase
kDa	kilodalton
Keap1	kelch ECH associating protein 1
MMS	methyl methanesulfonate
MPM-2	mitotic protein monoclonal-2
NF-κB	nuclear factor κappa B
Nrf-2	nuclear factor-E2-related factor 2
PAGE	polyacrylamide gelelectrophoresis
PARP	poly (ADP-ribose) polymerase
PEITC	phenethyl isothiocyanate
PI	propidium iodide
RNA	ribonucleic acid
ROS	reactive oxygen species
SD	standard deviation
SFN	sulforaphane
SRB	sulforhodamin B
TCA	trichloroacetic acid
TPA	12-O-tetradecanoylphorbol-13-acetate
wt	wildtype
XIAP	X-linked mammalian inhibitor of apoptosis protein
XRE	xenobiotic response element

I. INTRODUCTION

1. The Formation and the Prevention of Cancer

1.1 Cancer in general

Cancer - the uncontrolled growth and spread of abnormal cells – causes more than 6 million deaths every year worldwide concerning almost any tissue of the human body. In industrialized countries, prostate, lung and colorectal cancer are the most common types in men, whereas breast and colorectal cancer appear to be predominant in females. Worldwide, colorectal cancer is the fourth common cause of cancer deaths after lung, stomach and liver cancer [Parkin *et al.*, 2005].

Neoplasias can be caused by genetic as well as exogenous factors. To the current knowledge only 5% of all tumors are linked to genetic predisposition. Thus, in more than 95% of all tumor formations environmental factors like tobacco smoke, alcohol, infections, nutrition and occupational exposure to carcinogens are involved [Becker & Wahrendorf, 1998]. With regard to this awareness, it appears desirable to inhibit cancer formation by preventive activities as far as possible. With more cancer prevention one would need less drastic medical interventions like surgery, radio- or chemotherapy and one would save costs in the health care system.

1.2 Cancer Prevention

Carcinogenesis is a multistage process involving individual steps including initiation, promotion and progression (Figure 1), and offers a considerable time frame for preventive agents to interfere [Pitot, 1993]. The initiation describes the transformation of one or more cells upon exposure towards exogenous or endogenous carcinogens. Usually pro-carcinogens have low reactivity and they have to become activated by phase I enzymes of the biotransformation system of xenobiotics, which largely belong to the family of Cytochrome p450–dependent enzymes (CYPs) [Sheweita, 2000]. Activated carcinogens then react with and damage the DNA. These transformed cells undergo a promotion phase in which they hyperproliferate and accumulate additional mutations, leading to the

formation of pre-neoplasias. Yet, the process of promotion can be reversed. In the progression phase preneoplastic cells differ increasingly from normal cells with regard to morphology, function and growth behavior while they develop into a tumor that can become metastatic in advanced stages.

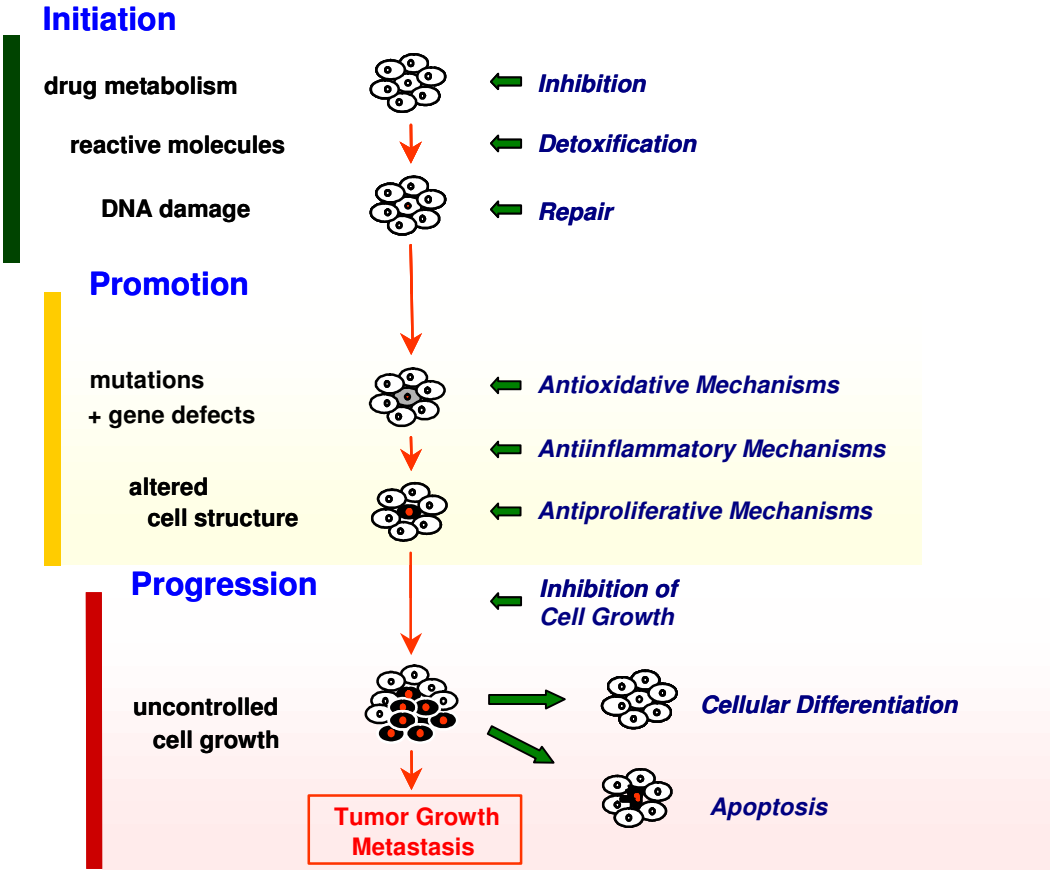


Fig. 1: Cellular carcinogenesis and chemopreventive mechanisms

As shown in Figure 1, each step in the cascade of carcinogenesis can theoretically be inhibited, representing a target for preventional interventions.

Primary cancer prevention involves the avoidance of cancer-causing behavior and exposure, whereas secondary prevention directs at the cessation of exposure to carcinogenic agents of individuals at risk as well as at screening to detect malignancies at an earlier stage. Tertiary chemoprevention focuses on the administration of agents intended to prevent the development of cancer and to inhibit or delay the occurrence of metastases [Zanker, 1999].

1.3 Chemoprevention

Chemoprevention is a form of secondary prevention. The aim of chemoprevention as cancer management is defined as preventing, delaying or reversing carcinogenesis by intervention with nontoxic compounds, synthetic chemicals, or natural compounds before malignancy [Sporn & Newton, 1979].

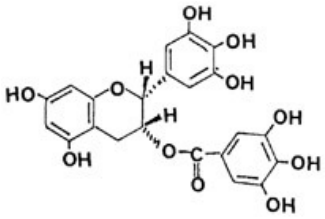
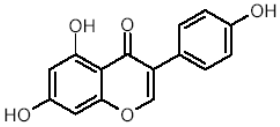
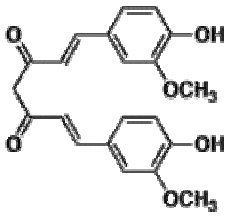
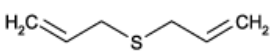
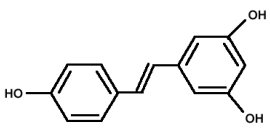
Chemopreventive compounds taken in by humans should in general fulfill certain criteria: 1. low costs and high availability, 2. activity following oral consumption, 3. high efficacy at achievable doses with little or no adverse effects and 4. a known mechanism of action [Katiyar & Mukhtar, 1996]. So far numerous natural compounds and synthetic derivates with potential chemopreventive efficacy have been identified. Some of them are already tested in clinical trials, e.g. retinoids against breast, selenium and vitamin E against prostate, fiber and calcium against colon, and green tea polyphenols against lung cancer development [Kelloff *et al.*, 2006].

The compounds can be catagorized according to the stage of carcinogenesis they inhibit, initiation, promotion or progression. Tumor initiation, which is usually linked to DNA damage, can be reduced by so-called blocking factors. These are most effective when administered before the carcinogen. Blocking factors change the profile of phase I and phase II metabolizing enzymes. Further blocking mechanisms comprise the modulation of DNA repair rate or the scavenging of free radicals. So-called suppressing agents interfere with tumor promotion and progression by inhibiting cell growth of transformed cells or by inducing apoptosis. Thereby the accumulation of damaged cells is prevented (Fig. 1) [Wattenberg, 1992; Kelloff *et al.*, 1994; Manson *et al.*, 2000].

1.4 The role of nutrition in cancer formation and development

Dietary behavior has been identified as one of the most important modifiable determinants of cancer risk. Thereby nutrition can influence cancer development in a positive, as well as in a negative way. For example, while a diet rich in saturated fats, red meat and alcohol is associated with an elevated cancer incidence, the consumption of fruits and vegetables is generally considered as protective against the development of different types of cancers [Steinmetz & Potter, 1991; WCRF & AICR, 1997]. However, evidence for a protective role of fruit and vegetable intake in cancer development varies

Table 1: Examples of chemopreventive compounds and their major mechanisms of action ([Surh, 2003] and literature cited therein)

Compound	Source	Mechanisms
EGCG 	*Thea chinesis* L.	• inhibition of carcinogen activation • control of cell cycle and proliferation • induction of apoptosis and/or differentiation • inhibition of oncogene expression/activity
genistein 	*Glycine max* L.	• inhibition of carcinogen activation • stimulation of carcinogen detoxification • control of cell cycle and proliferation • induction of apoptosis and/or differentiation • inhibition of the activity of oncogene products • modulation of hormonal and growth-factor activity
curcumin 	*Curcuma longa* L.	• inhibition of carcinogen activation • stimulation of carcinogen detoxification • control of cell cycle and proliferation • induction of apoptosis and/or differentiation • inhibition of oncogene expression/activity
diallyl sulfide 	*Allium sativum* L.	• inhibition of carcinogen activation • stimulation of carcinogen detoxification • induction of apoptosis and/or differentiation
resveratrol 	*Vitis vinifera* L.	• inhibition of carcinogen activation • control of cell cycle and proliferation • induction of apoptosis and/or differentiation

considerably depending on the type of fruit/vegetable and the type of cancer. For example, of six case-control studies investigating the association of tomato intake and the risk of developing colon cancer, four showed a negative and two a positive correlation. In the case of citrus fruits, evidence is strong for a decreased stomach cancer risk, while the colon cancer incidence is elevated [WCRF & AICR, 1997].

The impact of nutrition on cancer risk is further strongly affected by genetic polymorphisms, which can alter the response to dietary components by influencing the absorption, metabolism, or site of action. In order to understand the controversial outcomes from epidemiological and human studies, more research is needed in the fields of genetic polymorphisms and epigenetics, absorption, metabolism, distribution and excretion of dietary components, and interactions among dietary bioactive compounds and/or food combinations. Based on knowledge in these areas, future mechanistic *in vitro* and *in vivo* studies should be conducted in a more focused manner.

Since approximately 35% of all cancer cases can be attributed to nutritional failures [Doll & Peto, 1981], cancer prevention by nutrition is of major importance. Chemopreventive compounds in food stuff mainly comprise micronutrients like secondary plant ingredients or vitamins. Examples of chemopreventive compounds are listed in Table 1. Through the identification of these compounds with screening tests and through the investigation of the molecular mechanisms responsible for their effects, targeted prevention of malignant transformation will be alleviated in the future.

2. Brassica Vegetables and Cancer Prevention

2.1 *Brassicaceae* and Glucosinolates

Vegetables of the plant family *Brassicaceae/Cruciferae* are considered to be cancer preventive, mainly due to their content of glucosinolates [Jongen, 1996]. Many well-known sorts of vegetables are subspecies or variations of the genus *Brassica oleracea*. These vegetables comprise white cabbage, red cabbage, broccoli, cauliflower, brussels sprouts or kohlrabi. A characteristic feature of this plant family is the occurrence of glucosinolates (thioglucosides). Glucosinolates are secondary plant ingredients with a common basic structure consisting of glucose, a sulfur-containing group with an aglycon residue and a sulphate group (Fig. 2).

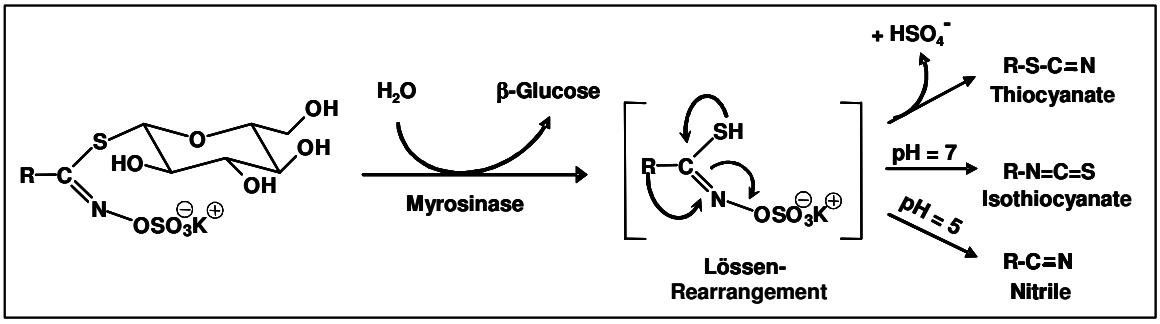


Fig. 2: General structure of glucosinolates and their cleavage by myrosinase (adopted from [Krul *et al.*, 2002])

So far more than 120 different glucosinolates have been identified, 10 to 12 of them occurring in *Brassica* [Fahey *et al.*, 2001]. They differ in the aglycon residue featuring alkylic, alkenylic, arylic or indolylic structures. Hydrolytic cleavage of glucosinolates mainly leads to the formation of reactive isothiocyanates (ITCs) which are responsible for the hot and bitter taste of radish or mustard. In evolution glucosinolates serve as a protection against being eaten as well as against pathogens. The enzyme myrosinase (thioglucoside glucohydrolase *EC 3.2.3.1.*) is responsible for thioglucosidases cleavage. It is located separate from its substrates in a different plant cell compartment. Once the plant cell is destroyed mechanically (e.g. by chewing or cutting) glucosinolates are broken down enzymatically. Thereby the glucose molecule is released, resulting in an instable intermediate, which is rearranged forming isothiocyanates, thiocyanates and nitriles. Formation of the latter two depends on pH and on the presence of heavy metals (Fig. 2) [Krul *et al.*, 2002]. ITCs from indolyl glucosinolates like glucobrassicin are chemically instable and are rearranged forming indole-3-carbinol and other indole derivates.

The outcome of glucosinolate cleavage by myrosinase is further dependent on the presence and activity of epithiospecifier protein (ESP) which has been found in several brassica vegetables [Foo *et al.*, 2000]. ESP directs myrosinase-catalyzed hydrolysis of alkenyl glucosinolates toward epithionitrile formation.

The glucosinolate content of *Brassicaceae* ranges from 150 to 5000 mg/kg fresh weight. It depends on the species and variation, as well as on the conditions for growth, maturation and storage. In addition, the glucosinolate content varies between the different plant organs. Highest levels are found in sprouts [Fahey *et al.*, 1997; Verhoeven *et al.*, 1997].

2.2 Uptake and Metabolism of Glucosinolates

ITC bioavailability from brassica vegetables ranges from 10 to 90% and depends on several factors [Holst & Williamson, 2004]. Glucosinolates cannot be absorbed from the intestine due to their water solubility. In contrast, glucosinolates cleavage products have a high bioavailability. The enzyme myrosinase is solely present in the plant itself as well as in the gut microflora. Glucosinolate cleavage in the human body is therefore restricted to the colon. Hence, systemic uptake and exposure of different organs to ITCs strongly depends on the preparation of brassica vegetables prior to consumption.

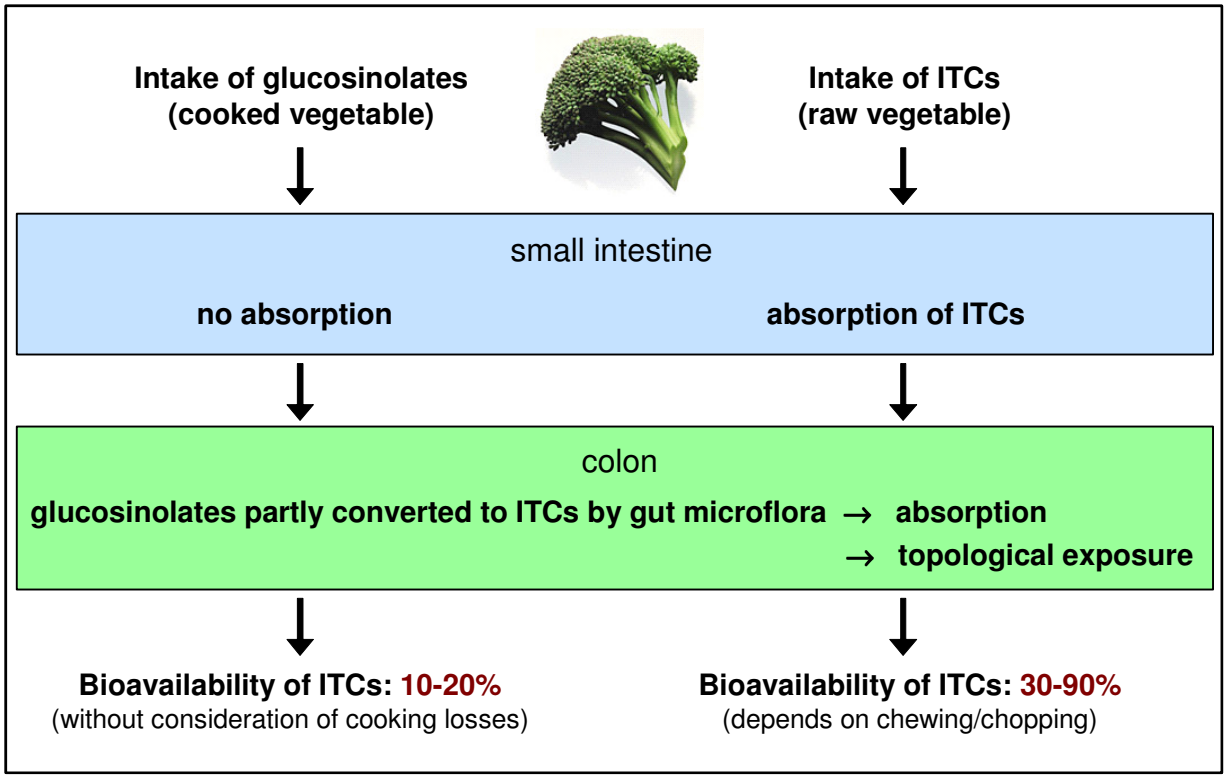


Fig. 3: Determinants of ITC bioavailability

Chopping and chewing of raw vegetables leads to a relatively high systemic uptake of ITCs, whereas cooking inactivates myrosinase and favors the uptake of intact glucosinolates, leading to a topological exposure toward glucosinolate cleavage products in the colon [Conaway *et al.*, 2000; Shapiro *et al.*, 2001; Johnson, 2002]. The length of this direct exposure is however unclear and is probably limited due to intestinal transit. Figure 3 shows a scheme of ITC bioavailability in the gastrointestinal tract.

One additional determinant of ITC bioavailability is the activity of ESP in some brassica vegetables. While nitrile formation can be favored when raw vegetables are consumed,

mild heat treatment abrogates ESP activity so that ITCs are formed exclusively [Matusheski *et al.*, 2006].

Following absorption ITCs are rapidly metabolized by the mercapturic pathway, as depicted in Figure 4. An initial conjugation with glutathione (GSH) promoted by glutathione-*S*-transferases (GSTs) gives rise to the corresponding GSH-ITC conjugates.

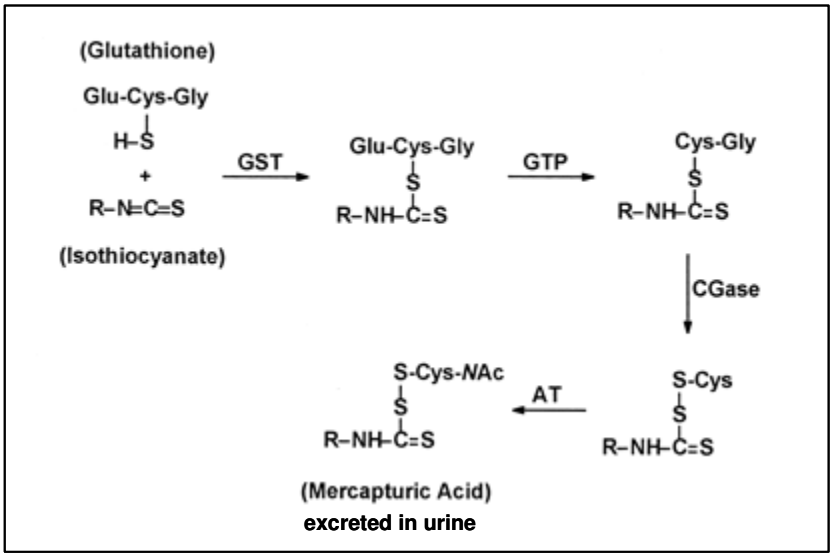


Fig. 4: Metabolism of ITCs via the mercapturic acid pathway [Shapiro *et al.*, 2001]

These undergo further enzymatic modification to give rise sequentially to the cysteinylglycine-, cysteine- and N-acetylcysteine-ITC conjugates, the latter being excreted in the urine. After consumption of brassica vegetables urinary excretion of the corresponding dithiocarbamates peaks between 2 and 4 hours. The amount of excreted ITC-metabolites directly correlates with the amount of ITC taken up [Chung *et al.*, 1998; Getahun & Chung, 1999].

2.3 Epidemiology

Five out of seven prospective cohort studies on the association between the consumption of brassica vegetables and cancer risk showed a protective effect [van Poppel *et al.*, 1999]. Another overview of cohort studies in relation to colorectal cancer and *Brassicaceae* demonstrated that out of twelve studies eight disclosed an inverse, three none, and one a positive correlation between the intake of cruciferous vegetables and

colon cancer risk [Steinmetz & Potter, 1996]. 67% of case-control studies reported of a correlation between brassica vegetable consumption and a decreased cancer risk, whereby this coherence was strongest for lung, colon, rectum and stomach [Verhoeven *et al.*, 1996].

More recent studies provide evidence that cancer risk can substantially be influenced by genetic polymorphisms of drug-metabolizing enzymes, together with the consumption of cruciferous vegetables. This is due to the fact that ITCs are not only metabolized by GSTs, but can also induce phase II-enzyme activity and gene expression, which leads to enhanced detoxification rates [Zhang & Talalay, 1994; Pool-Zobel *et al.*, 2005]. Especially the effect of *GSTM1*- and *GSTT1*-null polymorphisms on cancer risk in relation to brassica vegetable intake has been investigated, since these gene defects are relatively common in humans [Wiencke *et al.*, 1990; Pemble *et al.*, 1994]. The frequency of the homozygous null genotype of *GSTM1* varies between 39% and 63%. The homozygous null *GSTT1* genotype can be found in 10% to 21% of the Caucasian populations, while in some Asian populations the frequency can be as high as 64% [Cotton *et al.*, 2000]. Epidemiological studies conducted in the United States have concluded that *GSTM1*-positive persons gain greater cancer protection from either broccoli consumption or total cruciferous vegetable consumption than do *GSTM1*-null persons [Spitz *et al.*, 2000; Joseph *et al.*, 2004; Wang *et al.*, 2004]. In contrast, studies conducted in Asia concluded that *GSTM1*- and *GSTT1*-null persons may profit more from crucifer consumption than do *GSTM1*- and *GSTT1*-positive persons [London *et al.*, 2000; Zhao *et al.*, 2001; Fowke *et al.*, 2003; Seow *et al.*, 2005]. The major cruciferous component of the diets in these Asia-based studies would have been Chinese cabbage, as opposed to broccoli. Moreover, the predominant isothiocyanate derived from broccoli, sulforaphane, is mainly metabolized by GSTM1-1, which may be one explanation for the observed discrepancies [Kolm *et al.*, 1995; Zhang *et al.*, 1995; Gasper *et al.*, 2005]. In summary, whether subjects with *GSTM1*- or *GSTT1*- null-polymorphisms, or subjects positive for either gene are more protected against cancer formation depends on several factors like the ethnical origin as well as the amount and type of cruciferous vegetables consumed.

2.4 Sulforaphane and 3,3'-Diindolylmethane: two promising cancer chemopreventive compounds from brassica vegetables

Of all known glucosinolate cleavage products sulforaphane (SFN) and 3,3'-diindolylmethane (DIM) are among the most promising and intensively studied substances. The chemical structures of SFN and DIM are depicted in Figure 5. SFN is an aliphatic ITC derived from the glucosinolate glucoraphanin, which is found in substantial quantities in broccoli. DIM on the other hand is a dimeric condensation product of indole-3-carbinol, which is derived from the indolyl glucosinolate glucobrassicin, present in most cruciferous vegetables. Because of the different chemical nature of these two compounds and because both concomitantly occur in broccoli, SFN and DIM were chosen to be investigated in the present thesis regarding their anti-proliferative potential.

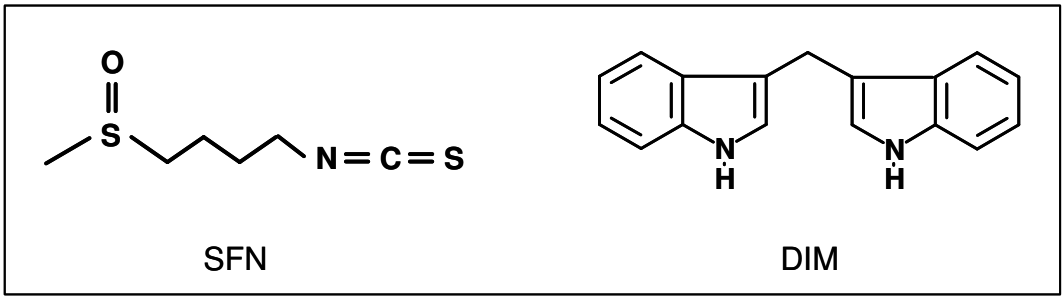


Fig. 5: Chemical structures of sulforaphane (SFN) and 3,3'-diindolylmethane (DIM)

2.4.1 Sulforaphane

In 1992, Zhang *et al.* initially identified sulforaphane (SFN) as a principal inducer of phase II enzymes including NAD(P)H:quinone oxidoreductase in broccoli [Zhang *et al.*, 1992]. In the following years numerous studies demonstrated SFN's potencies to modulate carcinogen metabolism *via* inhibition of phase I (CYPs) and induction of phase II enzymes [Talalay & Zhang, 1996]. Due to its feature to induce the expression of phase II- but not of phase I enzymes, SFN is a so-called monofunctional inducer [Prestera *et al.*, 1993]. The underlying mechanism involves an electrophilic interaction with cysteine residues of Kelch ECH associating protein 1 (Keap1), which leads to the liberation of the transcription factor nuclear factor-E2-related factor 2 (Nrf2), culminating in the induction of phase II enzyme genes and antioxidative stress protein genes via the antioxidant response element (ARE) [Talalay *et al.*, 1995; Dinkova-Kostova *et al.*, 2002].

Cancer preventive activity of SFN could be proven in several animal models. SFN inhibited 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumorigenesis in rats, azoxymethane-induced colonic aberrant crypt foci formation in rats, benzo[a]pyrene-induced forestomach cancer in mice, DMBA-induced skin tumorigenesis in mice and intestinal adenoma formation in $Apc^{Min/+}$ mice [Zhang *et al.*, 1994; Chung *et al.*, 2000; Fahey *et al.*, 2002; Hu *et al.*, 2006; Myzak *et al.*, 2006; Xu *et al.*, 2006].

Apart from the ability of SFN to block initiation of carcinogenesis by modulation of drug metabolism it can also interfere with tumor promotion. One way of suppressing carcinogenesis in the promotion stage is by anti-inflammatory mechanisms. In cultured cells, SFN was shown to inhibit the activation of nuclear factor-κB (NF-κB), a transcription factor regulating a series of proinflammatory and prosurvival genes [Heiss *et al.*, 2001; Heiss & Gerhauser, 2005; Xu *et al.*, 2005]. On the other hand, immunomodulatory activity of SFN in mice was reported, in that markers indicating a fortified immune system were increased after administration of SFN [Thejass & Kuttan, 2006; Thejass & Kuttan, 2006]. An effective way to interfere with tumor growth is to halt cell cycle progression in cancer cells by the induction of a cell cycle arrest, either in G_1 or in G_2/M phase. SFN was shown to mainly induce a G_2/M cell cycle arrest in various cell lines. Moreover, the induction of apoptosis - programmed cell death - could be demonstrated after treatment of cultured cells with SFN [Gamet-Payrastre, 2006]. Another anti-tumor property was described for SFN: the inhibition of new blood vessel formation, angiogenesis. In the immortalized human microvascular endothelial cell line HMEC-1 SFN interfered with all essential steps of neovascularization [Bertl *et al.*, 2006]. Also in human umbilical vein endothelial cells (HUVECs) SFN's antiangiogenic activity could be demonstrated [Asakage *et al.*, 2006].

So far, mostly animal and *in vitro* studies have been conducted to investigate the anti-cancer properties of SFN. One clinical phase I study was performed with broccoli sprouts that are known to have a very high content of glucosinolates, predominantly glucoraphanin [Shapiro *et al.*, 2006]. In volunteers receiving daily doses of either 75 µmol glucosinolates, 300 µmol glucosinolates or 75 µmol isothiocyanates from broccoli sprout extract for the duration of 7 days did not show any signs of toxicity. Thus, it is safe for humans to consume 50.4 g broccoli sprouts per day, which is equivalent to 300 µmol glucosinolates.

2.4.2 3,3'-Diindolylmethane – a condensation product of Indole-3-Carbinol

Myrosinase-mediated cleavage of the glucosinolate glucobrassicin yields several indole derivates, of which indole-3-carbinol (I3C) is the predominant one [Fahey *et al.*, 2001]. I3C is especially known for its cancer-protective effects in reproductive organs, both *in vitro* and *in vivo*, which are supposed to be due to the induction of specific CYP enzymes involved in estrogen metabolism [Bradlow *et al.*, 1991; Kojima *et al.*, 1994; Cover *et al.*, 1998]. In addition to the induction of phase I, I3C is also able to induce phase II detoxification enzymes, for which it is termed bifunctional inducer [Prestera *et al.*, 1993; Shertzer & Senft, 2000]. Modulation of gene expression by indoles is known to take place via the aryl hydrocarbon receptor (AhR) that directly interacts with the xenobiotic response element (XRE), which is present in the regulatory regions of the genes for many phase II-, as well as phase I enzymes [Kim & Milner, 2005].

Despite of the evidence for I3C's chemopreventive efficacy a tumor-promoting effect of this indole derivate in animal models has been described as well [Pence *et al.*, 1986; Bailey *et al.*, 1987; Kim *et al.*, 1997].

Under low pH conditions, as in the stomach, several condensation reactions of I3C occur which result in the formation of DIM as a major condensation product [Grose & Bjeldanes, 1992]. Therefore, it has been suggested that DIM and not I3C is responsible for the physiological effects of dietary indole-3-carbinol *in vivo* [Stresser *et al.*, 1995; Takahashi *et al.*, 1995].

With I3C already several clinical trials have been conducted confirming the effect on estrogen metabolism by an increase of 2-hydroxylation of estrogens [Rogan, 2006]. Concerns have been raised that I3C might increase the formation of estrogen metabolites that induce or promote cancer, but this has not been demonstrated.

In recent years, numerous *in vitro* studies have investigated the mechanisms responsible for the anticancer activity of DIM, apart from the well-described inducing effects on drug metabolizing enzymes. DIM was suggested to be an androgen-antagonist in human prostate cancer cells, it inhibited NF-κB activation and angiogenesis, stimulated interferon-gamma secretion, acted as a topoisomerase IIα catalytic inhibitor and potently induced cell cycle arrest in G_1 phase as well as apoptosis [Aggarwal & Ichikawa, 2005; Chang *et al.*, 2005; Rahman & Sarkar, 2005; Xue *et al.*, 2005; Bhuiyan *et al.*, 2006; Gong *et al.*, 2006].

Table 2: Comparison of characteristics of SFN and DIM as chemopreventive agents

	SFN	**DIM**
chemical structure	aliphatic isothiocyanate	indole derivate
effects on xenobiotic metabolizing enzymes	monofunctional inducer	bifunctional inducer
mode of action on gene expression	via Keap1–Nrf2 and ARE	via AhR and XRE
effect on the cell cycle	G_2/M arrest	G_1 arrest
cancer sites most affected	colon, rectum, lung, stomach	hormone-dependent cancers (breast, cervix, prostate)

Taken together, SFN and DIM, both derived from broccoli, exert their anticancer activity by different mechanisms. Table 2 gives an overview of the differences between these two chemopreventive compounds.

3. Anti-proliferative Mechanisms for Cancer Prevention and Treatment

Cancer cells are characterized by deregulated growth control, which can either be due to deactivating mutations in tumor suppressor genes or the activation of oncogenes. Genes belonging to these two classes of cancer-related factors are often associated with cell cycle regulation, apoptosis or signaling pathways involved in cell growth control like the NF-κB pathway. These pathways therefore represent targets for chemopreventive or –therapeutic agents in order to interfere with uncontrolled tumor cell growth.

3.1 Arrest of the Cell Cycle

3.1.1 Cell Cycle Regulation

The eukaryotic cell cycle can be divided into four distinct phases. Mitosis and cell division occur during M phase (mitosis). This is followed by the G_1 phase (for gap). G_1 gives way to the S phase (for synthesis) which is the period in the cell cycle when DNA is replicated. During G_2 phase the now tetraploid cell prepares for mitosis. It then enters M phase once again and thereby commences a new round of the cell cycle. Many terminally differentiated cells, such as neurons or muscle cells, never divide; they assume a quiescent state known as the G_0 phase.

The progression of a cell through the cell cycle is regulated by proteins known as cyclins and cyclin-dependent protein kinases (Cdks). Cyclins are synthesized during one phase of the cell cycle and completely degraded during a succeeding phase. A particular cyclin specifically binds to and thereby activates its corresponding Cdk(s) to phosphorylate its target proteins [Voet & Voet, 2004]. The different cell cycle phases and the involved cyclin-Cdk complexes are depicted in Figure 6.

3.1.2 Cell Cycle Checkpoints

In order to enter a new phase in the cell cycle, a cell must satisfy a corresponding checkpoint, which monitors whether the cell has satisfactorily completed the preceding phase (e.g. the attachment of all chromosomes to the mitotic spindle must precede mitosis). If the cell has not met the criteria of the checkpoint, the cell cycle is slowed or arrested until it does so. If cellular damage or mistakes that have led to the cell cycle arrest are too severe, cells die *via* apoptosis.

3.1.2.1 The DNA damage- or G_2 Checkpoint

The DNA damage checkpoint prevents cells from entry into mitosis when they experience DNA damage during G_2. The result is an accumulation of cells in G_2 to allow DNA repair. Sensors of the DNA damage checkpoint are the kinases ATM (ataxia telangiectasia mutated) and ATR (AMT- and Rad3-related), which activate the signal transduction pathway by phosphorylating the checkpoint kinases Chk1 and Chk2. Targets of Chk1 and Chk2 are members of the Cdc25 family of phosphatases that normally activate Cdk1, the cyclin-dependent kinase needed for the G_2-M transition.

Phosphorylation of Cdc25 phosphatases results in their subcellular sequestration, degradation and/or inhibition so that the cyclin B/Cdk1 complex cannot be activated and cells are not able to enter mitosis [Kastan & Bartek, 2004].

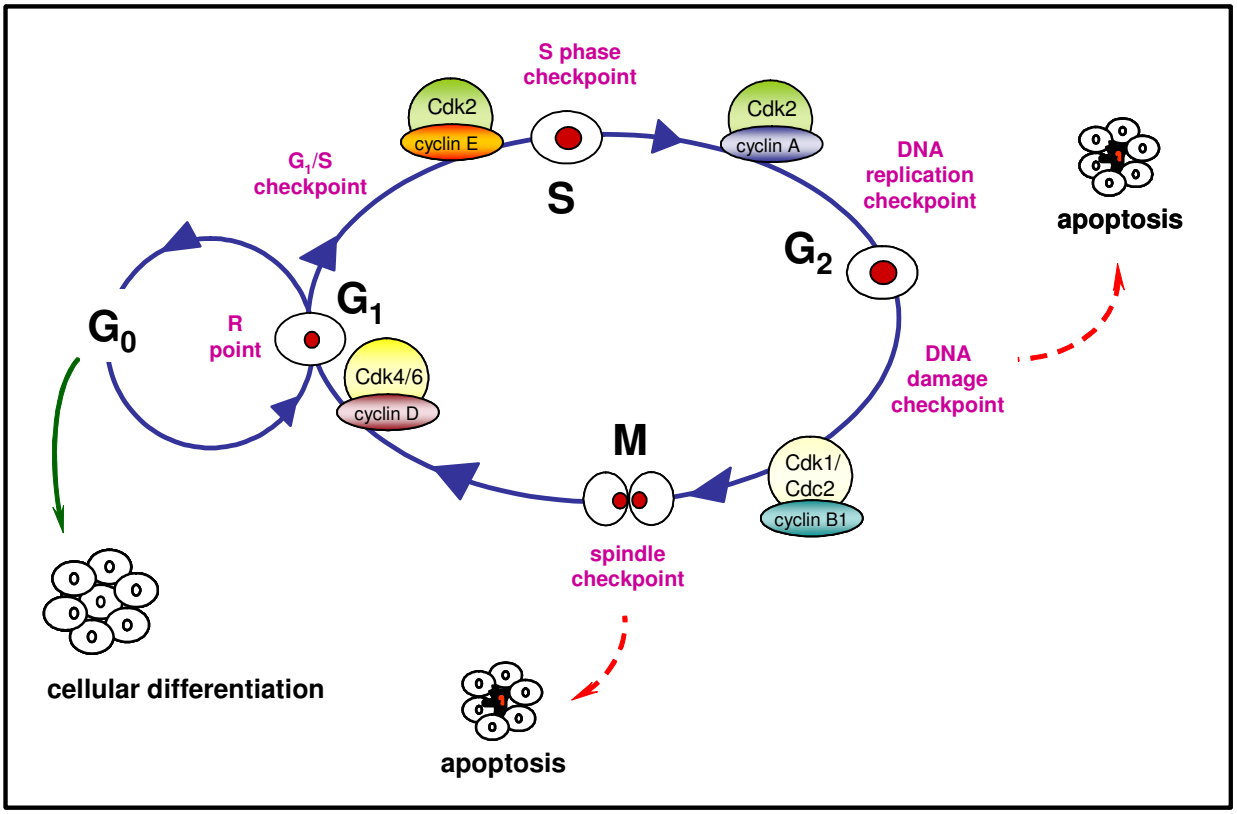


Fig. 6: Cell cycle regulation. Refer to text for descriptions. M, mitosis; G_0, Gap phase 0; G_1, Gap phase 1; S, synthesis phase; G_2, Gap phase 2; Cdk, cyclin-dependent protein kinases; Cdc, cell division cycle; R point, restriction point.

3.1.2.2 The Spindle- or Metaphase Checkpoint

The spindle checkpoint detects loss or impairment of functional connections between kinetochores and spindle microtubules during mitosis. Activation of the spindle checkpoint leads to a temporary or permanent cell cycle arrest in mitosis to allow time for correction of chromosome connections to the spindle. This is accomplished by the inhibition of the anaphase-promoting complex/cyclosome (APC/C), which controls mitotic progression and sister-chromatid segregation. The APC/C is an ubiquitin ligase complex that catalyzes polyubiquitinylation of a variety of targets including securin and mitotic cyclins like cyclin B1 during mitosis. The inhibition of the APC/C causes high cyclin levels, sustained Cdk1 activity, and prolonged mitotic arrest [Kops *et al.*, 2005; Yamada & Gorbsky, 2006].

Dynamic microtubules continue to be one of the most successful cancer chemotherapeutic targets. Many new drugs that target microtubules are in clinical trials and large numbers of microtubule-active compounds are being developed. Microtubule-targeted antimitotic drugs are usually classified into two main groups. One group, known as the microtubule-destabilizing agents, inhibits microtubule polymerization at high concentrations and includes several compounds such as the Vinca alkaloids (vinblastine, vincristine, vinorelbine, vindesine and vinflunine), cryptophycins, colchicine and combretastatins. The second main group is known as the microtubule-stabilizing agents. These agents stimulate microtubule polymerization and include paclitaxel (the first agent to be identified in this class), docetaxel (Taxotere), the epothilones, discodermolide and many more [Jordan & Wilson, 2004].

Mitotic arrest is usually followed by cell death which has been shown to involve apoptosis. Studies with cultured cells have even suggested that the spindle checkpoint-mediated mitotic arrest may be a requirement for the subsequent cell death induced by antimitotic drugs. However, the precise signaling pathways connecting checkpoint activation and apoptosis initiation remain unknown [Yamada & Gorbsky, 2006].

3.2 Apoptosis - Programmed Cell Death

3.2.1 Apoptosis in general

Apoptosis is the ability of a cell to eliminate itself upon external or internal signals. This programmed cell death is strictly controlled and involves different signaling cascades. Apoptosis is indispensable for the development of multicellular organisms. Mistakes in apoptosis regulation can have detrimental consequences. Too little apoptosis can result in cancer and autoimmune diseases, whereas neurodegenerative diseases are characterized by too much apoptosis [Thatte & Dahanukar, 1997; Fesik, 2005]. The term “apoptosis” (apo = from, ptosis = falling) has greek origin and describes the falling of leaves in autumn [Kerr *et al.*, 1972].

Apoptosis must be distinguished from necrosis, which is caused by external injury like mechanical damage. Characteristic morphological features of apoptosis include cell shrinkage, chromatin condensation, defined DNA degradation, membrane blebbing and the formation of several vesicles called apoptotic bodies, which are then phagocytosed

[Savill *et al.*, 1993]. Necrosis on the other side is less orderly compared to apoptosis. Events during necrosis involve cell swelling, followed by disruption of the plasma membrane and release of the cytosolic content which results in an inflammatory reaction [Vercammen *et al.*, 1998].

Possible triggers for programmed cell death are DNA damage, cytotoxic T cells or nutrient deficiency. However, the initiation of apoptosis strongly depends on the cellular context and on the cell type. In general, apoptosis can be initiated via two different pathways, the extrinsic, death receptor pathway, and the intrinsic, mitochondria-mediated pathway (Fig. 7). Both pathways lead to the activation of the caspase cascade, which irreversibly results in cell death [Kim *et al.*, 2002].

Caspases (cysteinyl-aspartate specific proteases) are responsible for the execution of apoptosis. They are synthesized as inactive precursors (zymogenes) and activated through proteolytic cleavage, in most cases by upstream caspases in the caspase cascade [Thornberry, 1999]. Different caspases fulfill different functions during the initiation and execution of apoptosis. Initiator caspases like caspase-8, -9 and -10 transfer early apoptotic signals, whereas so-called effector caspases including caspase-3, -6 and -7, cleave cellular substrates, leading to the typical morphologic features of apoptosis [Earnshaw *et al.*, 1999; Slee *et al.*, 1999]. A very prominent substrate of effector caspases is the DNA repair enzyme PARP [poly (ADP-ribose) polymerase], the cleavage of which is considered as a hallmark of apoptosis [Soldani & Scovassi, 2002].

3.2.2 The Mitochondrial Pathway

The mitochondrial pathway of apoptosis induction is initiated by an internal stress signal resulting in the permeabilization of the outer mitochondrial membrane. This leads to the release of Cytochrome *c* and other proapoptotic factors like Smac/Diablo from the intermembrane space into the cytosol. Once in the cytosol, Cytochrome *c* participates in the formation of the apoptosome complex together with its adaptor molecule, Apaf-1 (apoptotic protease activating factor-1), resulting in the recruitment, processing and activation of procaspase-9 in the presence of dATP or ATP. Subsequently, caspase-9 cleaves and activates pro-caspase-3 and -7. The release of Cytochrome *c* is considered a key initiative step in the apoptotic process, although the precise mechanisms regulating this event remain unclear [Gogvadze & Orrenius, 2006].

Proteins of the Bcl-2 family, which comprise pro-apoptotic (e.g. Bax, Bak) as well as anti-apoptotic (e.g. Bcl-2, Bcl-x_L) members, are major regulators of the intrinsic pathway. They form homo- and heterooligomers, which act directly at the outer mitochondrial membrane. The ratio of pro- to anti-apoptotic oligomers has been suggested to play an important role in determining commitment to cell death [Kuwana & Newmeyer, 2003].

Further regulatory proteins involved in controlling the mitochondrial pathway of apoptosis induction include the family of inhibitors of apoptosis (IAPs), which bind and inhibit specific caspases. The IAPs themselves are also regulated by the IAP-inhibitor proteins like Smac/DIABLO (second mitochondria-derived activator of caspases/ direct IAP-binding protein with low pI) and HtrA2/Omi (high temperature requirement protein A2) [Armstrong, 2006].

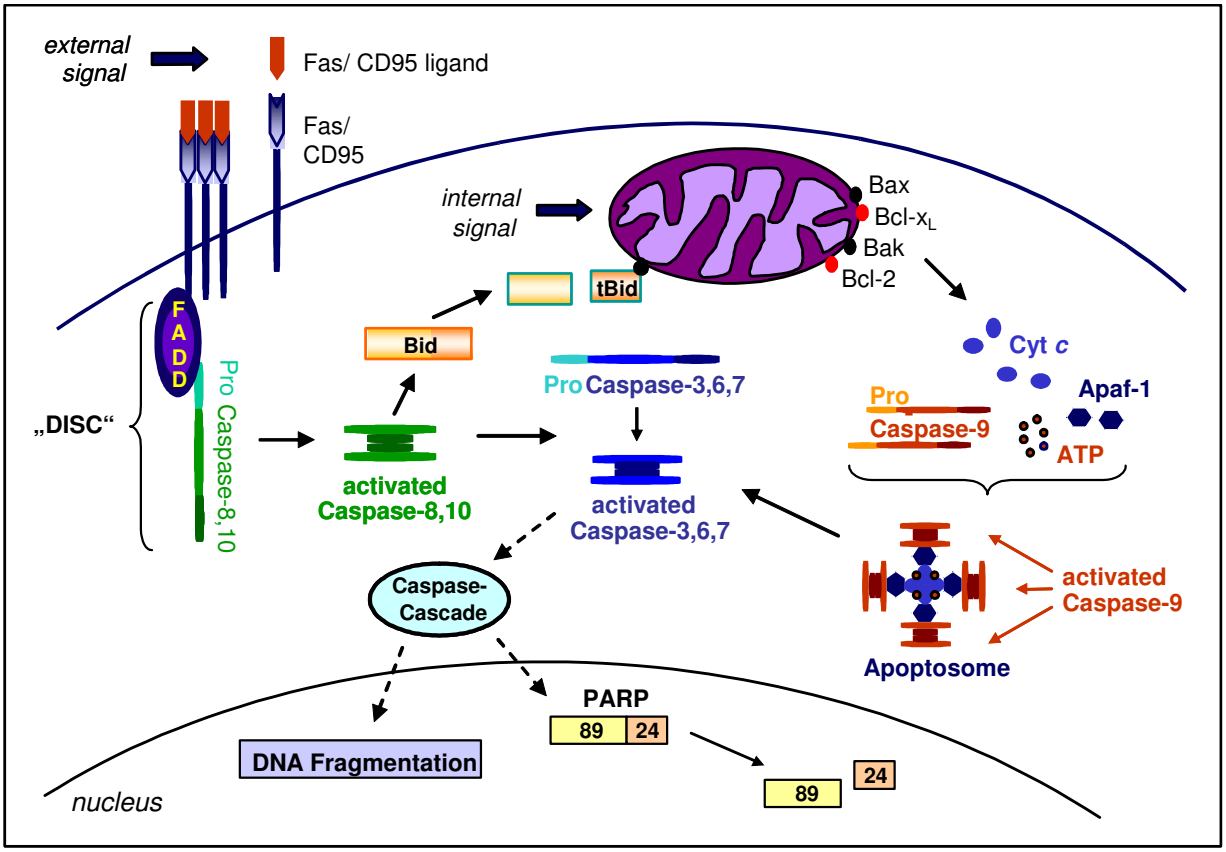


Fig. 7: Apoptosis - Mechanisms of Initiation and Regulation. Refer to text for descriptions. DISC, death-inducing signaling complex; FADD, Fas-associated death domain; tBid, truncated Bid; Apaf-1, apoptotic protease-activating factor 1; Cyt *c*, Cytochrome *c*; ATP, adenosine triphosphate; PARP, poly (ADP-ribose) polymerase.

3.2.3 The Death Receptor Pathway

The extrinsic pathway of apoptosis is induced by death receptors, a subgroup of the TNF1/nerve growth factor receptor superfamily. The best characterized member of the death receptor subfamily is CD95, also known as APO-1 or Fas. Stimulation of CD95 with its cognate ligand, CD95L, leads to the trimerization of the monomeric receptor. This enables binding of the adapter molecule FADD/MORT1 and of procaspase-8 to CD95 *via* homophilic death domain and death effector domain (DED) interactions, respectively, forming the death-inducing signaling complex (DISC). Recruitment of procaspase-8 to the DISC leads to its proteolytic activation through several cleavage steps followed by direct cleavage of caspase-3 which in turn leads to apoptosis [Lavrik *et al.*, 2005]. Important regulators of death receptor-induced apoptosis are the c-FLIP proteins. c-FLIPs are recruited to the DISC by DED interactions and thereby inhibit procaspase-8 activation [Golks *et al.*, 2005; Golks *et al.*, 2006].

Apart from this direct pathway *via* caspase-8-mediated cleavage of caspase-3 (also called type I, mitochondria-independent pathway) a second possibility exists as a link between the death receptor- and the mitochondria-mediated pathway, which involves the Bcl-2 family protein member Bid. Upon CD95-mediated stimulation activated caspase-8 cleaves Bid resulting in truncated Bid (tBid) and the release of Cytochrome *c* from mitochondria, which is mediated by dimerizing with the proapoptotic Bcl-2 family member Bak (type II, mitochondria-dependent pathway) [Kim *et al.*, 2002].

3.3 Inhibition of Nuclear factor-κB

The transcription factor nuclear factor-κB (NF-κB) plays a central role in cell proliferation, apoptosis and cancer development [Karin, 2006]. NF-κB consists of homo- and heterodimeric complexes formed from the Rel family of proteins, which consists of five proteins, named c-Rel, RelA (p65), RelB, NF-κB1/p50 and NF-κB2/p52. In quiescent cells the transcription factor is sequestered in the cytosol, but it rapidly translocates into the nucleus upon stimulation [Ghosh & Karin, 2002]. This process, known as NF-κB activation, can be initiated by two different signaling pathways. The classical NF-κB pathway involves p65 and p50 as well as the inhibitor protein IκB (inhibitor of NF-κB), which retains the p50:p65 dimer in the cytoplasm. Cell stimulation

activates the IκB kinase (IKK) complex, which is composed of two catalytic subunits (IKK-α and IKK-β) and a regulatory subunit (IKK-γ /NEMO) [Rothwarf & Karin, 1999]. Activated IKK phosphorylates NF-κB-bound IκB proteins, and targets them for polyubiquitination and rapid degradation by creating a binding site for $SCF^{\beta TrCP}$ ubiquitin ligase complex. Freed NF-κB dimers translocate to the nucleus where they coordinate the transcriptional activation of several hundred target genes [Karin, 2006].

The second pathway of NF-κB activation, the alternative pathway, results in specific activation of p52:RelB heterodimers and is not required for activation of the more ubiquitous p50:p65 dimers. Unlike the classical pathway, which is dependent on IKK-γ and to a large extent on the activity of IKK-β, the alternative pathway is based on IKK-α homodimers, the preferred substrate of which is the precursor of p52-p100/NF-κB2. This protein binds RelB through its amino-terminal Rel homology domain, and keeps itself and its partner in the cytoplasm through its carboxy-terminal IκB-like domain. Activation of IKK-α homodimers results in degradation of the latter and nuclear entry of p52:RelB dimers [Senftleben *et al.*, 2001; Karin, 2006].

Signals that lead to NF-κB activation include many proinflammatory and prosurvival factors like LPS, inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), UV light or oxidative stress. Among the genes that are regulated by NF-κB are cyclin D1, vascular endothelial growth factor (VEGF), Bcl-x_L, Bcl-2, c-FLIP, COX-2 and MMP-9, which are involved in a variety of cellular events including tumor cell proliferation, cell death, angiogenesis and metastasis [Amit & Ben-Neriah, 2003; Karin, 2006].

4. Aim of the Study

The broccoli-derived isothiocyanate SFN is a highly promising chemopreventive compound described to interfere with tumor cell growth by inducing cell cycle arrest and apoptosis. The underlying molecular mechanisms of cell cycle arrest and apoptosis induction are only partially analyzed and need to be fully clarified in order to benefit from the positive effects of SFN as a chemopreventive-/therapeutic agent. The aim of the present study was to further elucidate these mechanisms in cultured human colon cancer cells.

With respect to cell cycle arrest the literature describes an accumulation of cells in the G_2/M phase of the cell cycle, but it is still a matter of controversy whether cells are arrested in G_2 phase or mitosis. In addition, the causative events for this arrest have not been identified yet. In order to analyze the specific phase of cell cycle arrest, the mitosis-specific antibody MPM-2 represented a valuable tool. Also, the comparison of the effect of SFN with that of microtubule- and DNA-damaging agents was a useful approach.

Programmed cell death (apoptosis) can be initiated via the mitochondrial and the death-receptor pathway. This study focused on investigating which pathway plays a role in SFN-induced apoptosis and what the inducing factors are. The problem was approached by analyzing critical proteins involved in upstream- and downstream events of both pathways like Cytochrome *c*, Bcl-2 proteins and caspases, as well as by utilizing a leukemia cell line stably overexpressing the anti-apoptotic protein Bcl-2, a crucial factor in the regulation of mitochondria-dependent apoptosis.

The transcription factor NF-κB plays a key role in the regulation of cell survival and cell death since the expression of many important anti-apoptotic and pro-survival genes depends on its activity. SFN is a strong inhibitor of NF-κB activation, but reports about the responsible mechanisms are contradictory. This study aimed at shedding more light on how and in which step of the signal transduction cascade SFN interferes with the activation of NF-κB.

The cell line utilized in this study, the human colon carcinoma cell line 40-16, is a clone of the parental cell line HCT116. A colon cancer cell line was chosen for the mechanistical investigations due to the fact that the colon is one of the organs most affected by nutrition. In addition, the colon is the only organ in the human body where - in addition to the systemic exposure by absorbed SFN - a topological exposure to SFN takes place due to the thioglucosidase activity of the colonic microflora. Since isothiocyanates underlie a rapid metabolism, and topological exposure in the colon is limited due to intestinal transit, the aim of the study was to analyze time-dependency of SFN-induced anti-proliferative effects. Thus, the impact of short exposure times (3, 6 and 12 hours) and the reversibility of G_2/M cell cycle arrest and apoptosis induction were investigated.

In broccoli, apart from many other ingredients like fiber, carbohydrates, proteins and vitamins, the glucosinolate glucobrassicin - the precursor of DIM - is present in considerable amounts next to glucoraphanin, the precursor of SFN. Significant

differences exist between SFN and DIM regarding the mechanisms of modulation of drug metabolism and cell growth inhibition. Therefore, potential synergistic or antagonistic interactions between SFN and DIM in terms of anti-proliferative activity were investigated using the method of Chou and Talalay (1984).

II. MANUSCRIPTS - OVERVIEW

1. Sulforaphane-induced mitotic cell cycle arrest is followed by mitochondria-mediated apoptosis induction

Authors:	Contribution (%):
Gerlinde Pappa	72.5
Alexander Golks	5
Carina Pforr	2.5
Julia Strathmann	2.5
Helmut Bartsch	2.5
Clarissa Gerhäuser	15

Manuscript is in preparation for submission to "*Molecular Cancer Therapeutics*"

In this manuscript the chemopreventive isothiocyanate sulforaphane was shown to induce a mitotic cell cycle arrest in the human colon cancer cell line 40-16. This arrest was followed by caspase-dependent apoptosis, which was mediated by the mitochondria-dependent pathway of apoptosis induction. Sulforaphane potently inhibited NF-κB activation upstream of its nuclear translocation and decreased the expression of a series of anti-apoptotic, NF-κB-dependent genes, which supposedly facilitated apoptosis induction.

Own Contribution

- Planning, performing and evaluation of all practical work, except for performing the caspase-8 and c-FLIP Western Blots of the 40-16 cell line, the caspase-8 and PARP Western Blots of the Jurkat cell lines and the cell cycle analyses of the Jurkat cell lines
- Generation and revision of the manuscript

2. Biphasic modulation of cell proliferation by sulforaphane at physiologically relevant exposure times in a human colon cancer cell line

Authors:	Contribution (%):
Gerlinde Pappa	75
Helmut Bartsch	5
Clarissa Gerhäuser	20

accepted for publication in "*Molecular Nutrition & Food Research*" on April 27th 2007

Transient exposure of 40-16 human colon carcinoma cells to sulforaphane for up to 6 h resulted in reversible G_2/M cell cycle arrest and cytostatic growth inhibition, whereas a minimum continuous exposure time of 12 h was necessary for sulforaphane to irreversibly arrest cells in G_2/M and subsequently induce apoptosis. Low concentrations of sulforaphane caused a transient decrease in cytoplasmic glutathione levels followed by glutathione induction after 24 h. However, depletion of glutathione did not seem to play a role for sulforaphane-induced apoptosis.

Own Contribution

- Planning, performing and evaluation of all practical work
- Generation and revision of the manuscript

3. Quantitative combination effects between sulforaphane and 3,3'-diindolylmethane on proliferation of human colon cancer cells *in vitro*

Authors:	Contribution (%):
Gerlinde Pappa	75
Julia Strathmann	2.5
Maria Löwinger	2.5
Helmut Bartsch	2.5
Clarissa Gerhäuser	17.5

accepted for publication in "*Carcinogenesis*" on February 22nd 2007, available online since February 28th 2007 (doi:10.1093/carcin/bgm044)

Combination effects of sulforaphane (SFN) and 3,3'-diindolylmethane (DIM) were investigated in terms of cell growth inhibition in the 40-16 human colon carcinoma cell line. SFN and DIM inhibited cell proliferation antagonistically at low concentrations, but synergistically at cytotoxic concentrations. A threshold concentration of 10 µM SFN in the mixture resulted in strong G_2/M cell cycle arrest.

Own Contribution

- Planning, performing and evaluation of all practical work, except for the Western Blot
- Generation and revision of the manuscript

III. MANUSCRIPTS

1. Pappa G., Golks A., Pforr C., Strathmann J., Bartsch H., Gerhauser C., Sulforaphane-induced mitotic cell cycle arrest is followed by mitochondria-mediated apoptosis induction. Manuscript in preparation for *Molecular Cancer Therapeutics.*

Sulforaphane-induced mitotic cell cycle arrest is followed by mitochondria-mediated apoptosis induction

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Abbreviations:

SFN, Sulforaphane; ROS, reactive oxygen species; PI, propidium iodide; MMS, methyl methanesulfonate; TPA, 12-O-tetradecanoylphorbol-13-acetate

Abstract

Sulforaphane (SFN) is a naturally occurring cancer chemopreventive isothiocyanate found as its glucosinolate precursor in cruciferous vegetables like broccoli. Apart from its ability to modulate carcinogen metabolism SFN also acts by anti-proliferative and apoptosis-inducing activities. The objective of the present study was to elucidate the mechanisms of SFN-induced cell cycle arrest and apoptosis induction in molecular terms, using the human colon carcinoma cell line 40-16. SFN induced a cell cycle arrest in G_2/M phase after 24 h with subsequent apoptosis induction after 48 h of exposure. SFN treatment led to a significant increase of mitotic cells indicating that SFN activated the spindle checkpoint, possibly due to microtubule damage. Subsequent cell death is mediated via the mitochondria-dependent apoptosis pathway shown by mitochondrial membrane potential disruption, Cytochrome *c* release and resistance of Bcl-2 overexpressing Jurkat cells toward SFN-induced apoptosis. In addition, SFN inhibits NF-κB activation and thereby downregulates NF-κB-dependent anti-apoptotic proteins like Bcl-2, XIAP and c-FLIP facilitating apoptosis induction.

1. Introduction

The isothiocyanate sulforaphane (SFN) is a well-known, highly promising cancer chemopreventive agent. SFN was first identified in 1992 as a principal inducer of phase II enzymes including NAD(P)H:quinone oxidoreductase in broccoli (1). Subsequently, numerous studies were conducted demonstrating chemopreventive efficacy of SFN. For example, SFN was shown to inhibit 7,12-dimethylbenz[a]anthracene-induced mammary tumorigenesis (2), azoxymethane-induced colonic aberrant crypt foci formation in rats (3) and intestinal adenoma formation in $Apc^{Min/+}$ mice (4, 5). Apart from its modulatory effects on carcinogen metabolism, current research indicates that inhibition of cell proliferation contributes to the chemopreventive activities of SFN (6, 7). In particular, SFN was shown to arrest few cancer cell lines in G_1- and the majority of cultured cancer cells in G_2/M phase of the cell cycle (8-15). With respect to the SFN-mediated G_2/M arrest, Singh *et al.* proposed that SFN-induced reactive oxygen species (ROS) may cause DNA damage in PC-3 human prostate cancer cells and consequently activate the DNA damage checkpoint. This was shown to be mediated via phosphorylation of cell division cycle 25 C (Cdc25C) by checkpoint kinase 2 (16). On the contrary, Jackson and Singletary demonstrated that SFN halted human mammary carcinoma cells in early mitosis by disrupting microtubule polymerization (17, 18). In the HCT116-derived colon cancer cell line 40-16, we recently demonstrated that transient exposure to SFN at a concentration of 15 µM for short incubation times up to 6 h caused a reversible G_2/M arrest, whereas a minimum continuous exposure time of 12 h was necessary for SFN to irreversibly arrest cells in G_2/M phase and subsequently induced apoptosis (Pappa *et al.*, *Mol. Nutr. Food Res.*). Apoptosis induction in the 40-16 cell line as well as its p53 -/- derivative 379.2 was demonstrated by cleavage of PARP [poly(ADP-ribose)polymerase] (19). As a responsible mechanism the mitochondrial pathway of apoptosis induction appeared to be involved as demonstrated by the activation of caspases-7 and -9 as well as changes in the protein expression of anti-apoptotic Bcl-x_L and pro-apoptotic Bax and Bak proteins. Similarly, SFN induced cell death *via* the mitochondrial pathway in various other cancer cell lines (reviewed in (7, 20, 21)).

Despite of these numerous investigations, there is still limited understanding on whether the mechanisms of SFN-induced cell cycle arrest and apoptosis are causally linked.

A recent body of evidence indicates that the transcription factor nuclear factor-κB (NF-κB) plays a central role in the regulation of cell proliferation, apoptosis and cancer development (22). This was of interest for the present investigations since NF-κB was identified as a key target of SFN-mediated anti-inflammatory mechanisms (23). NF-κB consists of homo- and heterodimeric complexes formed from the Rel family of proteins, which consists of five proteins, namely c-Rel, Rel A (p65), Rel B, p50 and p52. In quiescent cells the transcription factor is sequestered in the cytosol by interaction with the inhibitor protein IκB (inhibitor of NF-κB). Upon exposure of cells to stress signals like inflammatory cytokines or oxidative stress, activation and nuclear translocation of NF-κB are controlled by rapid phosphorylation, ubiquitination and proteolytic degradation of IκB (22). Once in the nucleus, NF-κB dimers bind to consensus DNA elements and transactivate transcription of genes encoding numerous different proteins, which are involved in a variety of cellular events including tumor cell proliferation, cell death, angiogenesis and metastasis (22, 24). The inhibition of NF-κB activation could represent an important mechanism contributing to SFN-induced apoptosis induction. Indeed, SFN was shown to inhibit NF-κB activation in conjunction with apoptosis induction in the PC-3 human prostate cancer cell line (25, 26). We sought to investigate this mechanism in the 40-16 colon cancer cell line and to highlight important NF-κB-dependent anti-apoptotic factors like Bcl-2 and XIAP that are affected by SFN treatment.

Here, we present novel results on SFN-induced cell cycle arrest and apoptosis induction using the human colon cancer cell line 40-16 as a model. Our aim was to elucidate whether both mechanisms occurred in parallel, or whether we could detect a cause-consequence relation between both. We show that SFN-induced G_2/M cell cycle arrest is not a result of DNA damage. Instead, the mitotic checkpoint is activated, of which caspase-dependent apoptosis induction is a consequence. As apoptotic mechanisms we found the mitochondrial pathway to be involved, which is accompanied by caspase-8 activation and the inhibition of NF-κB.

2. Materials and Methods

Cell Culture

The human colon cancer cell line 40-16, derived from a random HCT116 clone, was kindly provided by B. Vogelstein from Johns Hopkins Oncology Center (Baltimore, USA). Cells were cultured as described earlier (19). The Jurkat cell line is a human T cell leukemia cell line. Jurkat Bcl-2 is a Jurkat cell line overexpressing anti-apoptotic Bcl-2 (27). Jurkat cells were maintained in RPMI 1640 medium with 10% fetal bovine serum and penicillin/streptomycin (100 units/ml).

Chemicals

Sulforaphane (SFN, CAS no. 4478-93-7) was synthesized as described earlier (23). Cell culture material was obtained from Invitrogen (Eggenstein, Germany). Fetal bovine serum (FBS) was purchased from PAA Laboratories (Pasching, Austria). McCoy's 5A cell culture medium, propidium iodide (PI), RNase A, JC-1, caffeine, methyl methanesulfonate (MMS) and paclitaxel were obtained from Sigma-Aldrich (Taufkirchen, Germany). ZVAD-fmk was from Bachem (Heidelberg, Germany). All material required for Flow cytometry was purchased from Becton Dickinson (Franklin Lakes, NJ USA). Anti-MPM-2 monoclonal antibody (#05-368) was obtained from Upstate Biotechnology (Lake Placid, NY USA), and rabbit anti-mouse Alexa Fluor 488 conjugated secondary antibody (A-11059) was from Molecular Probes (Eugene, Oregon USA). NF-κB anti-p65 (sc-109), anti-p50 (sc-1190), anti-Bid (sc-6538) and anti-Bcl-2 (sc-492) antibodies were from Santa Cruz (Heidelberg, Germany) and anti-XIAP (610762) and anti-PARP (556362) antibodies were from BD Pharmingen (Heidelberg, Germany). Anti-caspase-8 monoclonal antibody (#9746) was obtained from Cell Signaling Technology (Beverly, USA). Anti-caspase-8 monoclonal antibody C15 (mouse IgG2b) recognizes the p18 subunit of caspase-8 (28). Anti-c-FLIP monoclonal antibody NF6 (mouse IgG1) was described in (29) and recognizes both the c-$FLIP_L$ and the c-$FLIP_S$ isoforms. Anti-β-Actin antibody AC-15 (A5441) was purchased from Sigma (Taufkirchen, Germany). Anti-mouse- and anti-rabbit-secondary antibodies were obtained from Santa Cruz (Heidelberg, Germany). Jurkat cell lysates as positive- (camptothecin-

treated) and negative controls for apoptosis were provided by BD Pharmingen (Heidelberg, Germany). Materials and equipment for gel electrophoresis were from Bio-Rad (Munich, Germany). All other chemicals were from Sigma-Aldrich (Taufkirchen, Germany).

Cell Cycle Analysis

40-16 cells were plated in 150 mm tissue culture dishes (5 x 10^4 cells/ml; 35 ml) and treated as indicated in Figure legends after overnight growth. Cell cycle analyses were performed as described previously (30).

PI exclusion assay

Cells were plated in 100 mm tissue culture dishes (5 x 10^4 cells/ml; 10 ml) and treated as indicated in Figure legends after overnight growth. Attached and floating cells were collected and immediately incubated with cell culture medium containing propidium iodide (2 µg/ml) for 15 min at RT. DNA staining as an indication for necrotic cells was measured by Flow cytometry.

MPM-2/PI bivariate flow cytometry

40-16 cells were plated in 150 mm tissue culture dishes as described for cell cycle analysis and treated as indicated in the Figure legends after overnight growth. Cells were collected and fixed as for cell cycle analysis. After washing twice with PBS containing 0.05% Tween 20 and 2% FBS, cells were labeled with MPM-2 antibody (final concentration of 5 µg MPM-2 antibody/ml PBS containing 2% FBS) for 1 h at 4 °C. Cells were washed three times with PBS containing 0.05% Tween 20 and 1% FBS and incubated with rabbit anti-mouse Alexa Fluor 488 secondary antibody (final concentration of 10 µg /ml PBS containing 2% FBS) for 1 h at RT in the dark. After washing three times with PBS containing 0.05% Tween 20 and 1% FBS, cells were resuspended in 50 µg/ml PI containing 100 µg/ml RNase A and incubated for 30 min at RT in the dark. Samples were analyzed using Flow cytometry.

NF-κB DNA-binding assay

Cells were plated in 100 mm tissue culture dishes with 5 x 10^4 cells/ml; 10 ml and treated as indicated in the Figure legends after overnight growth. Cells were scraped into 1 ml of ice cold PBS, spun down and resuspended in hypotonic buffer (10 mM sodium phosphate buffer, pH 7.0, containing 10 mM NaF, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF and 1% NP-40), followed by a 30 min incubation on ice. After centrifugation supernatants were collected representing the cytosolic fraction. Nuclei were lysed with hypertonic buffer (hypotonic buffer + 0.5 mM NaCl). After centrifugation the supernatant was collected, which represented the nuclear fraction. To analyze NF-κB DNA-binding, the "TransAM" NF-κB family kit from ActiveMotif (Rixensart, Belgium) was used. The assay was performed according to the manufacturer's instructions. Absorbance readings were normalized to the protein content of each sample determined using the bicinchoninic acid (BCA) method (31).

Preparation of mitochondrial extracts

Cells were plated in 100 mm tissue culture dishes with 5 x 10^4 cells/ml; 10 ml and treated as indicated in the Figure legends after overnight growth. Floating and adherent cells were collected and washed once with PBS. The separation of cellular mitochondrial and cytosolic fractions was performed with the "ApoAlert Cell fractionation Kit" (Clontech Laboratories Inc., Palo Alto, CA, USA) according to the manufacturer's instructions.

Western Blot Analysis

For the preparation of whole cell lysates cells were plated in 100 mm tissue culture dishes (5 x 10^4 cells/ml; 10 ml) and treated as indicated in the Figure legends after overnight growth. Preparation of cell lysates, SDS PAGE and electroblotting was performed as described previously (30). For quantitation of protein expression, band densities were semi-quantitatively measured using TINA software version 2.09a (Raytest Isotopenmessgeräte GmbH, Staubenhardt, Germany). Background staining was subtracted, values were normalized to β-actin expression, and expressed

in relation to control values, which were set as 1.0 for each time point or subcellular fraction, respectively.

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential ($\Delta\Psi m$) was measured using the dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide). Briefly, cells were plated in 100 mm tissue culture dishes (5×10^4 cells/ml; 10 ml) and treated as indicated in the Figure legends after overnight growth. Cells were incubated with medium containing JC-1 (2.5 µg/ml, diluted 1:1000 from stock prepared in DMSO) for 30 min at 37 °C. Cells were washed with PBS and analyzed unsing a FACS Calibur Flow cytometer. Loss of $\Delta\Psi m$ was quantified by quadrant analysis with the lower right quadrant representing cells with disrupted membrane potential (loss of red fluorescence).

Statistical Analysis

Results are presented as mean ± standard deviation. For statistical evaluation one-way ANOVA analysis and Bonferroni multiple comparison test were applied. Values of $p < 0.01$ were considered as statistically significant.

3. Results

Dose-dependent effects of SFN on the cell cycle

SFN is known to have anti-proliferative and apoptosis-inducing activity (21). We have demonstrated previously that 40-16 cells are arrested in the G_2/M phase of the cell cycle upon treatment with SFN [Pappa *et al.*, *Mol. Nutr. Food Res.*]. Although 6 h of incubation with SFN already led to a significant G_2/M arrest and cytostatic cell growth inhibition, incubation for 12 h and longer was necessary to irreversibly halt the cell cycle in G_2/M and to induce apoptosis. To further clarify the mechanisms of action, we first investigated the dose-dependency of cell cycle effects induced by SFN after 24 h as well as 48 h of treatment. As indicated in **Fig. 1A**, treatment

of 40-16 cells for 24 h and 48 h led to interesting effects on cell cycle progression, which did not follow a strict dose-response pattern. After 24 h, the sub-G_1 fraction representing apoptotic cells increased dose-dependently reaching the highest value at a concentration of 15 µM of SFN. In parallel, the percentage of cells in G_2/M phase was increased dose-dependently and the maximum was observed for 15 µM of SFN, indicating a marked G_2/M arrest. G_0/G_1 and S phase cells were concomitantly reduced. A further increase in the SFN concentration to 25 and 50 µM surprisingly led to a reversal of the effects observed with lower concentrations: The fraction of cells in sub-G_1 declined, G_1- and S-phase increased, and the G_2/M peak was reduced almost to control levels.

After 48 h, results for sub-G_1 phase were even stronger compared to 24 h of treatment. SFN potently induced apoptosis dose-dependently up to 15 µM (**Fig. 1A**). Again, higher concentrations of SFN were less effective in inducing apoptotic cell death. G_0/G_1 and S phase were both reciprocally regulated in relation to sub-G_1, whereas the G_2/M fraction showed a slight dose-dependent increase. Thus, the occurrence of a G_2/M cell cycle arrest and subsequent apoptosis induction after cell treatment with SFN depends on a relatively narrow concentration window, which is around 15 µM in the 40-16 cell line. Increasing or decreasing the concentration leads to less pronounced responses. Interestingly, the G_2/M arrest appears to directly correlate with cell death, since both phenomena are strongest after treatment with 15 µM, the first after 24 h, and the latter after 48 h.

Since the effects seen with a SFN concentration of 50 µM were quite unexpected, we next investigated why high concentrations of SFN had relatively little effects on cell cycle distribution and apoptosis induction. Since the number of cells was nevertheless dramatically diminished after treatment, we hypothesized that necrosis could be induced. Therefore we analyzed necrosis induction by positive PI staining and subsequent Flow cytometry analysis. As shown in **Fig. 1B**, SFN at the concentration of 15 µM marginally induced necrosis after 48 h, whereas at 50 µM, the percentage of necrotic cells was significantly induced after 24 h and especially after 48 h of treatment. These data indicate that in the 40-16 cell line high concentrations of SFN induce necrosis, whereas concentrations around 15 µM induce a cell cycle arrest in G_2/M phase, followed

by apoptosis induction after 48 h. As these effects were strongest for the concentration of 15 µM we used this concentration for the following experiments.

SFN-induced G_2/M arrest is not abrogated by caffeine

Next we aimed to shed more light onto the mechanism of G_2/M arrest induction by SFN. Cell cycle arrest in G_2/M phase could be the result of two main causes: i) DNA damage during G_2 leading to the activation of the DNA damage checkpoint and halt of the cell cycle before mitosis entry, or ii) spindle perturbation in early mitosis, for instance due to tubulin hyperpolymerization. It is not possible to distinguish between those two mechanisms using cell cycle analysis. Therefore we used caffeine which is well-known for its ability to abrogate the DNA damage checkpoint (32, 33). As positive control for DNA damage induction we used the alkylating agent methyl methanesulfonate (MMS). Results of cell cycle analysis are depicted in **Fig. 2**. Single treatments using SFN and MMS both induced a significant G_2/M arrest, whereas caffeine induced a weak accumulation of cells in G_0/G_1. The combination of SFN and caffeine further enhanced the G_2/M fraction. In contrast, MMS plus caffeine led to a significant attenuation of the G_2 arrest. It is evident that caffeine is able to abrogate MMS-induced, but not SFN-induced G_2/M cell cycle arrest.

These results suggest that SFN does not induce DNA damage and does not activate the DNA damage checkpoint in the 40-16 cell line.

SFN induces accumulation of cells in mitosis

To clarify if SFN-induced G_2/M arrest was due to an accumulation of cells in mitosis, we utilized the mitosis-specific monoclonal antibody MPM-2. This antibody binds to phospho-epitopes that are phosphorylated only during mitosis on many mitotic proteins (34). As a positive control for mitotic spindle disruption we treated 40-16 cells with the microtubule-interfering agent paclitaxel. Cell cycle analyses shown in **Fig. 3** revealed that both SFN and paclitaxel strongly induced a G_2/M arrest. Both paclitaxel and SFN increased the number of mitotic cells about 10-fold. Thus,

SFN appears to act as a microtubule-interfering agent which activates the spindle checkpoint leading to an arrest of cells in mitosis.

SFN inhibits the expression of NF-κB dependent anti-apoptotic genes

We have shown previously that SFN inhibits the lipopolysaccharide-induced activation of pro-inflammatory genes through inhibition of NF-κB DNA binding in murine macrophages (23). Due to its important role in the induction of anti-apoptotic genes, we were interested in whether SFN might inhibit NF-κB-dependent effects also in the 40-16 cell line. We quantified binding of each of the five NF-κB subunits in the nuclear extracts of treated cells to the corresponding consensus sequences using an Elisa-based method. As a positive control for NF-κB activation we treated the cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) for 1 h.

As the signal intensity for the subunits Rel B and p52 was very low, we assume that they are not expressed in the 40-16 cell line. Results for the subunits p50, p65 and c-Rel are depicted in **Fig. 4A**. Treatment of cells with 15 µM SFN for 3 h inhibited the activation of NF-κB as measured via binding of the p50 chain of NF-κB to the consensus sequence by ~60%. TPA as a positive control significantly enhanced p50-DNA binding about 3-fold. When cells were pretreated with SFN for 2 h prior to the addition of TPA for an additional hour, DNA-binding of p50-NF-κB was reduced to control levels. The results were similar for p65-NF-κB, although the overall signal intensity was weaker compared to p50, either due to lower expression or inefficient nuclear translocation. In the case of c-Rel values were very low, regardless of the treatment regime. Thus, c-Rel does not appear to play a role in NF-κB dimerization in the 40-16 cell line. Notably, the endogenous background NF-κB activity observed in the control cells can be efficiently blocked via SFN treatment of the cells.

In order to determine whether SFN inhibits the translocation of NF-κB into the nucleus we performed Western blotting with nuclear and cytosolic extracts of cells treated with SFN, TPA or the combination thereof. As shown in **Fig. 4B**, TPA induced the translocation of both p50 and p65

into the nucleus, whereas SFN and SFN plus TPA did not. In summary, we show that SFN inhibits the nuclear translocation of NF-κB and thereby inhibits NF-κB activation.

The transcription factor NF-κB is involved in the regulation of numerous cellular proteins including many anti-apoptotic and pro-survival factors (22). Therefore we addressed the question whether apoptosis induction upon SFN treatment is facilitated by the downregulation of NF-κB-dependent proteins involved in apoptosis regulation.

We investigated protein levels of Bcl-2, the X-linked mammalian inhibitor of apoptosis protein (XIAP), as well as of c-FLIP using immunoblotting (**Fig. 4C**). Bcl-2 is an anti-apoptotic member of the Bcl-2 family of proteins, the major regulators of the mitochondrial pathway of apoptosis (35). XIAP belongs to the family of inhibitors of apoptosis proteins (IAPs), endogenous caspase inhibitors (36). XIAP selectively inhibits the mitochondrial pathway of apoptosis via binding to caspases-3, -7 and -9 (36). C-FLIP, also described to be NF-κB-dependent, is an anti-apoptotic protein acting at the death-inducing signaling complex (DISC), where it prevents caspase-8 activation (37). As can be seen in **Fig. 4C**, SFN markedly downregulated XIAP, Bcl-2 as well as c-FLIP after 24 h and especially after 48 h. In a previous study we showed that SFN leads to a down-regulation of Bcl-x_L, another anti-apoptotic member of the Bcl-2 protein family (19).

In summary, we conclude that SFN potently inhibits NF-κB activation, which results in decreased expression levels of the anti-apoptotic proteins Bcl-2, XIAP and c-FLIP.

SFN causes disruption of the Mitochondrial Membrane Potential and Cytochrome c release

The mitochondria play a central role in apoptosis induction (35). Disruption of the Mitochondrial Membrane Potential is an irreversible trigger of the mitochondrial cell death cascade leading to Cytochrome *c* release from the intermembrane space of the mitochondria into the cytosol, the activation of initiator caspase-9 and subsequent activation of the effector caspases-3, -6 and -7. As a result of caspase activation a number of cellular substrates including DNA is cleaved, resulting in apoptotic cell death (35). We have previously shown that SFN treatment led to an increased ratio of pro-apoptotic Bax and Bak proteins in relation to anti-apoptotic Bcl-x_L protein expression

and the activation of caspase-9 (19). After semi-quantitative analysis of Bcl-2 protein expression (compare **Fig. 4C**), and comparison with protein expression data of Bax and Bak from our previous work (19), expression ratios between these proteins were calculated. After 24 h Bax:Bcl-2 and Bak:Bcl-2 ratios were ~0.5 and 1.0, respectively, whereas after 48 h the expression ratio for both pro-apoptotic proteins in relation to Bcl-2 was ~2.0 (data not shown). These results indicate that at the later time point protein expression of Bcl-2 family proteins is in favor of the pro-apoptotic members Bax and Bak, which is consistent with the marked increase of the apoptotic fraction after 48 h as presented in **Fig. 1A**.

Next we sought to determine mitochondrial membrane depolarization and release of Cytochrome *c* from the intermembrane space into the cytosol. **Fig. 5A** depicts representative dot plots of cells stained with JC-1 and **Fig. 5B** gives a summary of results obtained after 6 h, 12 h, 24 h and 48 h of incubation with SFN. The percentage of cells with disrupted Mitochondrial Membrane Potential was significantly augmented already after 6 h of SFN treatment (5.3% *vs.* 2.3% in the control). Time-dependently the fraction of cells with depolarized mitochondrial membrane increased, reaching ~35% after 48 h. Usually the opening of the outer mitochondrial membrane is accompanied by the release of Cytochrome *c* into the cytosol. To test whether this took place in 40-16 cells as well, mitochondrial and cytosolic fractions were prepared and protein levels of Cytochrome *c* were visualized by immunoblotting. Proper separation of mitochondrial and cytosolic extracts was assured by probing for the mitochondria-specific Cytochrome Oxidase subunit 4 (COX4). As shown in **Fig. 5C**, treatment with SFN caused an increase of Cytochrome *c* in the cytosolic fraction after 24 h and more pronounced after 48 h, while the amount of Cytochrome *c* in the mitochondrial fraction was slightly decreased. Collectively, these results indicate that SFN potently induces the mitochondrial, intrinsic pathway of apoptosis in the 40-16 cell line.

Apoptosis feedback mechanisms are also induced by SFN treatment: caspase-8 activation and Bid cleavage

In addition to the mitochondrial pathway of apoptosis induction also an extrinsic, death receptor-mediated pathway has been described (38-40). Death-receptor-mediated apoptosis is normally induced via extracellular death ligand stimuli, but recent literature suggests that death-receptor-associated events can also be induced as a feedback mechanism of the activation of downstream effector caspases like caspases-3 and -7 (41). Since we have shown that SFN triggers the instrinsic apoptosis signaling we aimed to address the question whether these feedback mechanisms might also be affected by SFN treatment.

Thus, we incubated 40-16 cells with SFN for 24 or 48 h and analyzed the processing of procaspase-8. As visualized in **Fig. 6**, SFN induced the processing of procaspase-8 to the p43/p41 cleavage products and to the catalytically active subunit p18 (caspase-8), presumably facilitated by down-regulation of NF-κB-dependent c-FLIP. SFN-induced caspase-8 activation was completely blocked by the pan-caspase inhibitor zVAD-fmk.

Bid belongs to the pro-apoptotic members of the Bcl-2 protein family and represents a link between death receptor- and mitochondria-mediated apoptosis. As a substrate for caspase-8, Bid is cleaved into truncated Bid (tBid), which then translocates into the mitochondria to facilitate the permeabilization of the outer mitochondrial membrane (35). In a Western blot we visualized the levels of full-length Bid that were reduced after 24 h and 48 h of SFN treatment, probably due to caspase-8-mediated cleavage (**Fig. 6**). Cotreatment of cells with SFN and zVAD-fmk partly blocked this cleavage. In summary, SFN-induced caspase-8 activation is a feedback mechanism which in turn leads to cleavage of Bid into tBid. As a control and hallmark for apoptosis induction we analyzed poly(ADP-ribose)polymerase (PARP) cleavage. Also shown in **Fig. 6**, SFN induced the cleavage of PARP in 40-16 cells after 24 h and 48 h, which was inhibited by co-incubation with the caspase inhibitor zVAD-fmk. Thus, SFN-induced apoptosis is entirely dependent on caspases.

Chronology of events induced by SFN

SFN was shown to induce a cell cycle arrest in mitosis after 24 h as well as apoptosis via the mitochondria-dependent pathway after 48 h of treatment. Yet the detailed interplay between these two events is unclarified. Therefore we investigated if SFN-induced apoptosis is a secondary event after the cell cycle arrest in mitosis or if both signaling pathways are initiated in parallel.

We treated 40-16 cells with SFN alone or in combination with zVAD-fmk for 48 h and analyzed cell cycle distribution. We observed that zVAD-fmk potently abrogated the increase of sub-G_1 fraction seen with SFN alone (**Fig. 7**). In contrast, the G_2/M arrest observed with SFN incubation was unaltered by cotreatment with zVAD-fmk. Hence, it is likely that the mitosis arrest precedes apoptosis induction in 40-16 colon carcinoma cells.

To analyze if the chronology of events is not only limited to a single cell line, we also used Jurkat cells for additional analysis. Since the Jurkat cells were more sensitive toward SFN, we used 10 µM instead of 15 µM for treatment. In wildtype (wt) Jurkat control cells apoptosis was also induced after 48 h of treatment with SFN (**Fig. 8A,** left panels). Apoptosis could be blocked using the pan-caspase inhibitor zVAD-fmk, whereas the mitosis arrest was still evident. To unravel whether apoptosis in Jurkat cells after SFN treatment is also induced via mitochondria-dependent signaling, Jurkat cells over-expressing anti-apoptotic Bcl-2 protein were utilized (**Fig. 8A**, right panels). Upon treatment of Jurkat Bcl-2 cells with SFN for 48 h no apoptosis induction was detected, but the mitosis arrest was still present. These data suggest that apoptosis induction by SFN is a secondary event following mitosis arrest.

To assure that the chronology of events is indeed true, we investigated PARP cleavage as well as the activation of caspase-8 in Bcl-2- and wt control Jurkat cells. Again we also tested the effect of the caspase inhibitor zVAD-fmk. As a positive control for apoptosis we used a camptothecin-treated Jurkat cell lysate. In Jurkat wt cells SFN induced PARP cleavage as well as caspase-8 activation, which was both blocked by co-treatment with zVAD-fmk (**Fig. 8B**). In contrast, Jurkat Bcl-2 cells were completely resistant toward apoptosis induction and caspase-8 activation by SFN.

Collectively these data show that not only in 40-16 cells, but also in Jurkat cells the chronology of events induced by SFN is the same. SFN induces a mitosis arrest that precedes mitochondria-dependent apoptosis induction.

4. Discussion

SFN is known for its anticancer-activities including the induction of cell cycle arrest and apoptosis in tumor cells (21). Despite intense research on this field the mechanisms underlying SFN's anti-proliferative effects are still a matter of controversy. In the present study we provide evidence that SFN acts as an anti-mitotic agent in the human colon cancer cell line 40-16, shown by two approaches. 1) Caffeine, able to abrogate DNA-damage induced cell cycle arrest in G_2, blocked alkylating agent MMS-induced, but not SFN-induced G_2/M cell cycle arrest. 2) SFN treatment led to the accumulation of cells in mitosis, similar to microtubule-damaging paclitaxel, shown by the binding intensity of the mitosis-specific antibody MPM-2. Moreover, we demonstrate that apoptosis induction is a consequence of the mitotic arrest, since the pan-caspase inhibitor zVAD-fmk blocked SFN-induced apoptosis, but not the mitotic arrest. This notion is supported by the kinetics of events induced by SFN, being a marked cell cycle arrest after 24 h followed by apoptosis after 48 h. With regard to the mechanism of apoptotic cell death initiation we demonstrate here, that apoptosis is initiated via the mitochondrial pathway leading to the disruption of the mitochondrial membrane potential and Cytochrome *c* release into the cytosol. Furthermore, Bcl-2 overexpressing Jurkat cells were completely resistant toward SFN-induced apoptosis, but not toward the mitotic arrest. Thus, M phase arrest as the primary effect of SFN treatment is not unique for the 40-16 cell line. Others have also reported about spindle perturbations upon SFN treatment: Jackson and Singletary demonstrated a cell cycle arrest in early mitosis and aberrant mitotic spindles, accompanied by elevated p34cdc2 (cdc2) kinase activity upon treatment of the BALB/c mouse mammary carcinoma cell line F3II with 15 µM SFN (17). The authors also reported that SFN inhibited tubulin polymerization *in vitro*, but at non-physiologically high doses. Similar results were shown for MCF-7 human mammary

carcinoma cells, in which cyclin B1, the cyclin responsible for the G_2-M transition together with cdc-2 kinase, was elevated by SFN treatment, indicating that cells had entered mitosis (18). Mitotic spindle disruption was also described to occur in the human bladder cancer cell line UM-UC-3 upon incubation with a broccoli sprout extract (containing mainly SFN) (42). In that study, apoptosis induction was mitochondria-mediated and evident only at later time points (48 h) compared with the mitotic arrest, that was strongest after 24 h of treatment. Thus, the behaviour of UM-UC-3 cells resembles that of 40-16 cells as presented in this study. Pham *et al.* showed SFN-induced mitotic arrest to be accompanied with an increase in mitotic marker phospho-histone H3 and cyclin B1 in human pancreatic adenocarcinoma cells (39). Interestingly, consistent with our results, G_2/M phase arrest could not be blocked by the checkpoint kinase 1 (Chk 1) inhibitor UCN-01, which is able to override the DNA damage checkpoint similar to caffeine, whereas γ-radiation-induced G_2 arrest was blocked by UCN-01. Still the authors detected apoptosis only after treatment with higher concentrations of SFN and indepenent of the G_2/M phase arrest which is in contrast to the present report.

Mechanisms responsible for SFN-induced spindle perturbation still need to be explored. However, one can speculate that SFN's thiol-reactivity could play a decisive role, since tubulin contains many cysteine residues (43). It is known that the accessible thiol groups of tubulin are very sensitive toward oxidation and that oxidation or binding to these residues leads to the inhibition of tubulin polymerization (44).

Importantly, SFN was shown to induce DNA damage and subsequent cell cycle arrest in G_2 in other cultured cell lines. Singh *et al.* demonstrated reactive oxygen species (ROS)-induced double-strand breaks of the DNA to be the trigger for an accumulation of PC-3 human prostate cancer cells in G_2 phase upon treatment with SFN (16). In another study SFN-induced ROS formation was also associated with G_2/M cell cycle arrest (11). We investigated cellular GSH levels as well as the formation of ROS in the 40-16 cell line [Pappa *et al.*, *Mol. Nutr. Food Res.*]. We could not detect any significant enhancement of ROS after treatment with SFN for several time points. In addition, SFN-induced transient GSH depletion was not associated with sustained

G_2/M cell cycle arrest and apoptosis. Thus, SFN is able to activate distinct signaling cascades, one involving spindle disruption and mitotic arrest, the other involving ROS formation and the activation of the DNA damage checkpoint. Whether one or the other outcome occurs, most likely depends on the genetic background of the cells treated with SFN, mainly in terms of cell cycle checkpoint functionality. Interestingly, in a very recent report the induction of autophagy has been described as a defense mechanism against apoptosis and supposedly the reason for the delayed cell death upon treatment with SFN (45).

In the present study, SFN-induced mitotic arrest was followed by mitochondria-dependent cell death shown by disruption of $\Delta\Psi m$, Cytochrome *c* release into the cytosol and apoptosis-resistance of Bcl-2 overexpressing cells. However, caspase-8 – a caspase mainly involved in death receptor-mediated apoptosis – was also activated by SFN treatment which we assumed to be a feedback mechanism of the activation of downstream effector caspases like caspases-3 and -7. In other studies SFN-induced apoptosis was caspase-8-dependent, suggestive of a more important role for this protease (11, 38). Death receptor-induced apoptosis has also been described to be induced by SFN. In MDA-MB-231 breast cancer cells activation of apoptosis by SFN seemed to be initiated through induction of Fas ligand (46). An enhancement of Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by SFN was reported to involve upregulation of death receptor 5 (DR5), a receptor for TRAIL (47, 48). On the other hand, the majority of reports demonstrate mitochondria-mediated cell death by SFN (reviewed in (21)).

The molecular mechanisms involved in cell death after prolonged mitotic checkpoint activation have not been defined so far (49). Microtubule-damage is often associated with Bcl-2 phosphorylation which is considered to inactivate anti-apoptotic properties of Bcl-2 (50, 51). This could lower the threshold for apoptosis induction. Another possible mechanism could involve direct interaction of mitochondria-bound tubulin with Bcl-2 proteins and the induction of apoptosis upon treatment with anti-mitotic drugs (52).

We show here that SFN is a strong inhibitor of NF-κB activation in 40-16 cells acting upstream of the translocation of NF-κB into the nucleus, which is consistent with the current literature. Xu *et*

al. proposed SFN-mediated NF-κB inhibition to be due to a reduction of IKK phosphorylation, particularly IKKβ in PC-3 human prostate cancer cells (25). Consequently, IκBα phosphorylation and degradation was inhibited. Of note, in murine Raw 264.7 macrophages SFN did not inhibit the nuclear translocation of NF-κB but blocked NF-κB DNA binding in the nucleus (23). Thus, different mechanisms of action seem to exist which could involve redox- and thiol-mediated interactions of SFN with NF-κB subunits or with further upstream targets of NF-κB signaling like IKK or IκB.

NF-κB regulates the expression of a variety of survival and anti-apoptotic genes. We investigated protein levels of important NF-κB dependent apoptosis-related factors and found Bcl-2, XIAP and c-FLIP to be downregulated upon SFN treatment. It remains unclear whether NF-κB inhibition is essential for SFN-induced apoptosis induction, but it definitely contributes to facilitate cell death upon cell damage. Taking the NF-κB dependent, anti-apoptotic protein Bcl-2 as an example, downregulation of Bcl-2 is accompanied by apoptosis induction and Bcl-2 overexpression prevents cell death.

Taken together, we have comprehensively demonstrated that the isothiocyanate SFN exerts its anti-proliferative activity by mechanisms involving mitotic spindle perturbation and subsequent apoptosis induction in 40-16 human colon carcinoma cells. Cell death was clearly mediated via the mitochondria-dependent pathway of apoptosis. These results contribute to a better understanding of the molecular mechanisms responsible for SFN's anticancer potential with regard to cell cycle arrest and apoptosis induction. Knowledge of these mechanisms is crucial for a further development of this highly promising compound into a widely used cancerpreventive-/therapeutic agent.

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Figure legends:

Figure 1. (**A**) Dose-dependent effects of SFN on the cell cycle. 40-16 cells were treated with 0.5% DMSO as a solvent control (c) or with SFN at the indicated concentrations in µM for 24 h or 48 h. Cell cycle analyses of fixed cells were performed by Flow cytometry after staining DNA with PI. Bars represent the percentage of cells in the cell cycle phases sub-G_1, G_0/G_1, S and G_2/M. Data are means ± SD of at least three independent experiments. * Significantly ($p < 0.01$) different from respective control.

(**B**) PI exclusion for necrosis detection. 40-16 cells were treated with 0.5% DMSO as solvent control (c) or with SFN at the concentrations of 15 µM (S 15) or 50 µM (S 50) for 24 h (black bars) or 48 h (white bars). Percentages of PI positive cells represent those that have undergone necrotic cell death. Data are means ± SD of three independent experiments. * Significantly ($p < 0.01$) different from respective control.

Figure 2. Effects of caffeine on G_2/M cell cycle arrest. 40-16 cells were treated with 0.2% DMSO as solvent control, SFN (15 µM), MMS (250 µM), caffeine (1.5 mM), the combination of SFN + caffeine or the combination of MMS + caffeine for 24 h. Cell cycle analyses of fixed cells were performed by Flow cytometry after staining DNA with PI. Upper panel: representative histograms of cell cycle distribution. Lower panel: means and SD of percentages of cell cycle distribution. Data are from three independent experiments. [a] Significantly ($p < 0.01$) different from respective control. [b] Significantly ($p < 0.01$) different from the respective treatment without caffeine within the same cell cycle phase.

Figure 3. Determination of mitotic cells. 40-16 cells were treated with 0.2% DMSO as solvent control, SFN (15 µM) or paclitaxel (0.1 µM) for 24 h. Fixed cells were probed with mitosis-specific MPM-2 monoclonal antibody followed by an Alexa Fluor 488-conjugated secondary antibody, staining of DNA with PI and Flow cytometry analysis. Left panel: representative dot

plots for discrimination of mitotic cells (MPM-2 positive cells with 2n DNA content) with mean percentages of mitotic cells ± SD from five independent experiments. Right panel: respresentative histograms of cell cycle distribution with mean percentages of cells in G_2/M phase ± SD from five independent experiments. * Significantly ($p < 0.01$) different from respective control.

Figure 4. SFN inhibits NF-κB activation and the expression of NF-κB-dependent proteins. (**A**) Transactivation of NF-κB subunits p50, p65 and c-Rel. 40-16 cells were treated with 0.2% DMSO as solvent control for 3 h (black bars), SFN at a concentration of 15 µM for 3 h (white bars), TPA at a concentration of 0.15 µM for 1 h (dark grey bars) or with SFN (15 µM) for 2 h before the addtion of TPA (0.15 µM) and incubation for another hour (light grey bars). Binding of activated NF-κB subunits present in the nuclear extracts of treated 40-16 cells to the NF-κB consensus sequence was quantified with specific antibodies against p50, p65 and c-Rel and colorimetric visualisation. Absorbance readings were normalized to protein concentrations. Data are means ± SD of three independent experiments. * Significantly ($p < 0.01$) different from control. # Significantly ($p < 0.01$) different from TPA treatment. (**B**) Nuclear translocation of NF-κB. Immunoblotting for NF-κB p65 and p50 was performed using cytosolic and nuclear extracts of 40-16 cells treated with 0.2% DMSO for 3 h, SFN (S; 15 µM) for 3 h, TPA (T; 0.15 µM) for 1 h or SFN for 2 h + TPA for 1 h. Equal loading was confirmed by using an anti-β-actin antibody. (**C**) Immunoblotting for XIAP, Bcl-2 and c-FLIP using whole cellular lysates of 40-16 cells treated with 0.2% DMSO as control or SFN (15 µM) for 24 h or 48 h. Equal loading was confirmed by using an anti-β-actin antibody.

Figure 5. SFN induces mitochondrial membrane permeabilisation and release of Cytochrome *c* into the cytosol. (**A**) Representative Flow histograms depicting JC-1 fluorescence in 40-16 cells treated with 0.2% DMSO (control) or SFN (15 µM) for 48 h. Cells with disrupted $\Delta\Psi m$ are located in the lower right quadrant. (**B**) Percentages of cells with disrupted $\Delta\Psi m$ in 40-16 cells

treated with 0.2% DMSO as control (black bars) or SFN at a concentration of 15 µM (white bars) for 6 h, 12 h, 24 h or 48 h. Data are means ± SD of five independent experiments. * Significantly ($p < 0.01$) different from respective control. (**C**) Immunoblotting for Cytochrome *c* (Cyt c) using cytosolic (cyto) and mitochondrial (mito) fractions of 40-16 cells treated with 0.2% DMSO (c) or SFN (15 µM) for 24 h or 48 h. Efficient separation of cytosolic and mitochondrial cell fractions was confirmed by using an anti-COX4 antibody. Equal loading was confirmed by using an anti-β-actin antibody.

Figure 6. SFN induces activation of caspase-8, Bid cleavage and caspase-dependent apoptosis. Immunoblotting for procaspase-8 and activated caspase-8, Bid and PARP was performed with lysates from 40-16 cells treated with 0.5% DMSO as control, SFN (15 µM), zVAD-fmk (zV; 20 µM) or the combination of SFN + zVAD-fmk (S+zV) for 24 h or 48 h. Caspase-8 activation was investigated using an antibody directed against procaspase-8 (55/53 kDa and 43/41 kDa) and the catalytically active subunit of caspase-8 (18 kDa). PARP cleavage was investigated using an antibody directed against full length PARP (uncl. PARP; 116 kDa) and the cleavage product (cl. PARP; 89 kDa). Equal loading was confirmed by using an anti-β-actin antibody.

Figure 7. Cell cycle analyses of 40-16 cells treated with 0.5% DMSO as solvent control, SFN (15 µM) or SFN (15 µM) + zVAD-fmk (20 µM) for 48 h. Representative Flow histograms are depicted with percentages for each cell cycle phase in the upper right corner. Data are means ± SD of three independent experiments. [a] Significantly ($p < 0.01$) different from control. [b] Significantly ($p < 0.01$) different from SFN treatment. [c] Significantly ($p < 0.01$) different from control and SFN treatment.

Figure 8. Effects of SFN on Jurkat cells. (**A**) Cell cycle analysis of wildtype (wt) or Bcl-2- (Bcl-2) transfected Jurkat cells treated with 0.5% DMSO as solvent control, SFN (10 µM) or SFN (10 µM) + zVAD-fmk (20 µM) for 48 h. Representative Flow histograms are depicted with

percentages for each cell cycle phase in the upper right corner. Data are means of two independent experiments. (**B**) Immunoblotting for PARP and procaspase-8 was performed with lysates from Jurkat wt and Jurkat Bcl-2 cells treated with 0.5% DMSO as control, SFN (10 µM) or the combination of SFN (10 µM) + zVAD-fmk (20 µM; S+zV) for 48 h. Additionally, negative (nc) and positive (pc) control lysates were analyzed. Caspase-8 activation was investigated using an antibody directed against procaspase-8 (Pro-C8; 55/53 kDa and 43/41 kDa). PARP cleavage was investigated using an antibody directed against full length PARP (uncl. PARP; 116 kDa) and the cleavage product (cl. PARP; 89 kDa). Equal loading was confirmed by using an anti-β-actin antibody.

Fig. 1

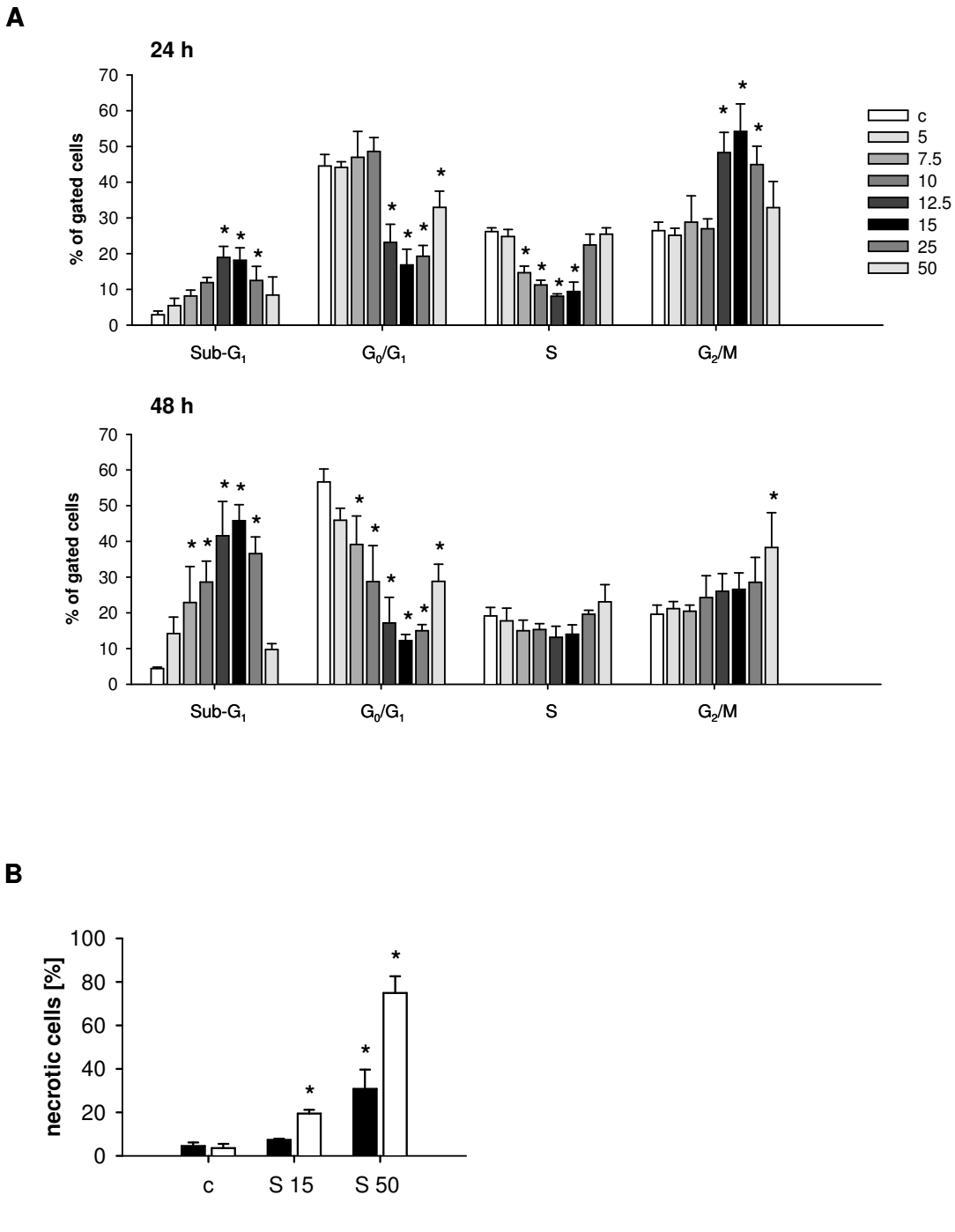
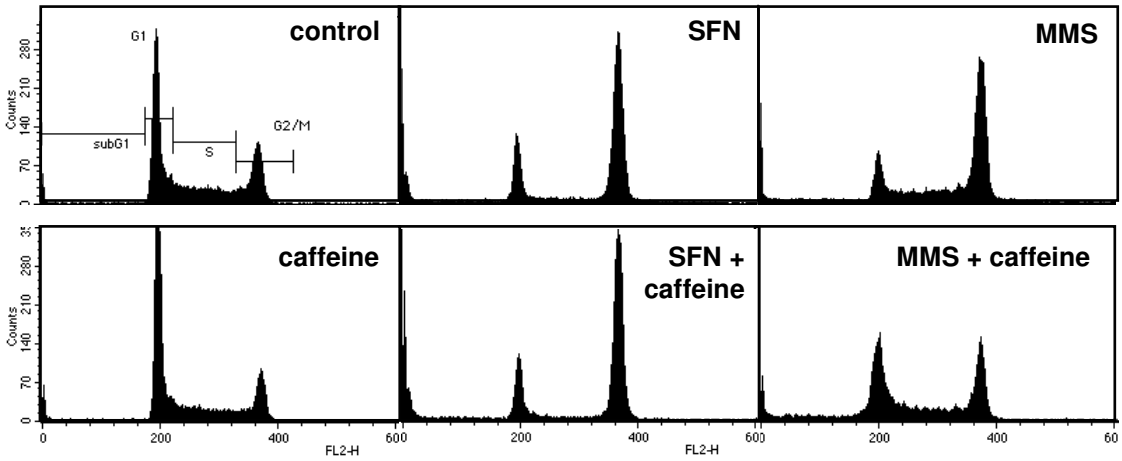

A
24 h
% of gated cells
70
60
50
40
30
20
10
0
Sub-G1
G0/G1
S
G2/M
c
5
7.5
10
12.5
15
25
50
48 h
% of gated cells
Sub-G1
G0/G1
S
G2/M
B
necrotic cells [%]
100
80
60
40
20
0
c
S 15
S 50

Fig. 2



Treatment	subG$_1$ [%]	G$_0$/G$_1$ [%]	S [%]	G$_2$/M [%]
control	2.2 ± 0.6	45.2 ± 0.9	25.5 ± 0.3	26.5 ± 1.1
caffeine	4.4 ± 1.0	55.0 ± 2.1[a]	21.3 ± 0.7[a]	19.1 ± 0.7[a]
SFN	14.1 ± 0.4[a]	16.4 ± 0.4[a]	7.1 ± 0.4[a]	59.6 ± 1.1[a]
SFN + caffeine	15.2 ± 1.1[a]	16.1 ± 1.2[a]	5.0 ± 0.4[b]	61.9 ± 0.7[a]
MMS	7.6 ± 2.5[a]	18.2 ± 3.2[a]	17.5 ± 1.0[a]	55.3 ± 2.1[a]
MMS + caffeine	10.7 ± 3.2[a]	37.2 ± 1.9[b]	20.5 ± 0.9[b]	31.0 ± 1.3[b]

Fig. 3

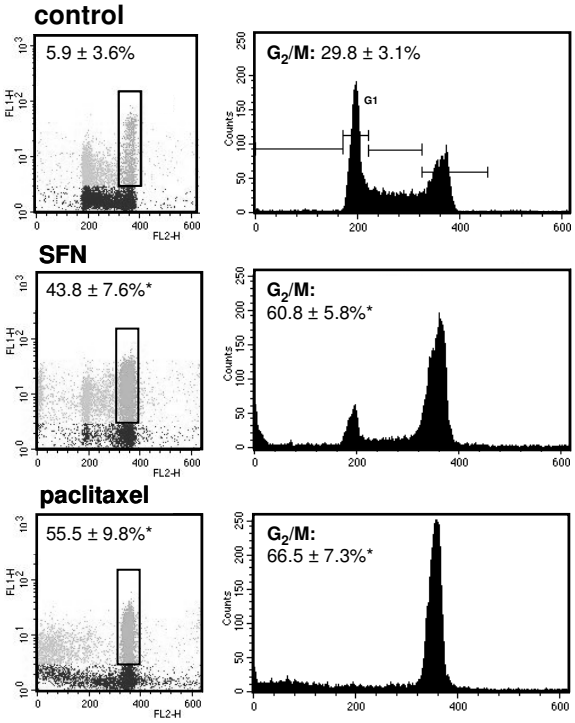

control
5.9 ± 3.6%
G2/M: 29.8 ± 3.1%
G1
SFN
43.8 ± 7.6%*
G2/M:
60.8 ± 5.8%*
paclitaxel
55.5 ± 9.8%*
G2/M:
66.5 ± 7.3%*
FL1-H
FL2-H
Counts

Fig. 4

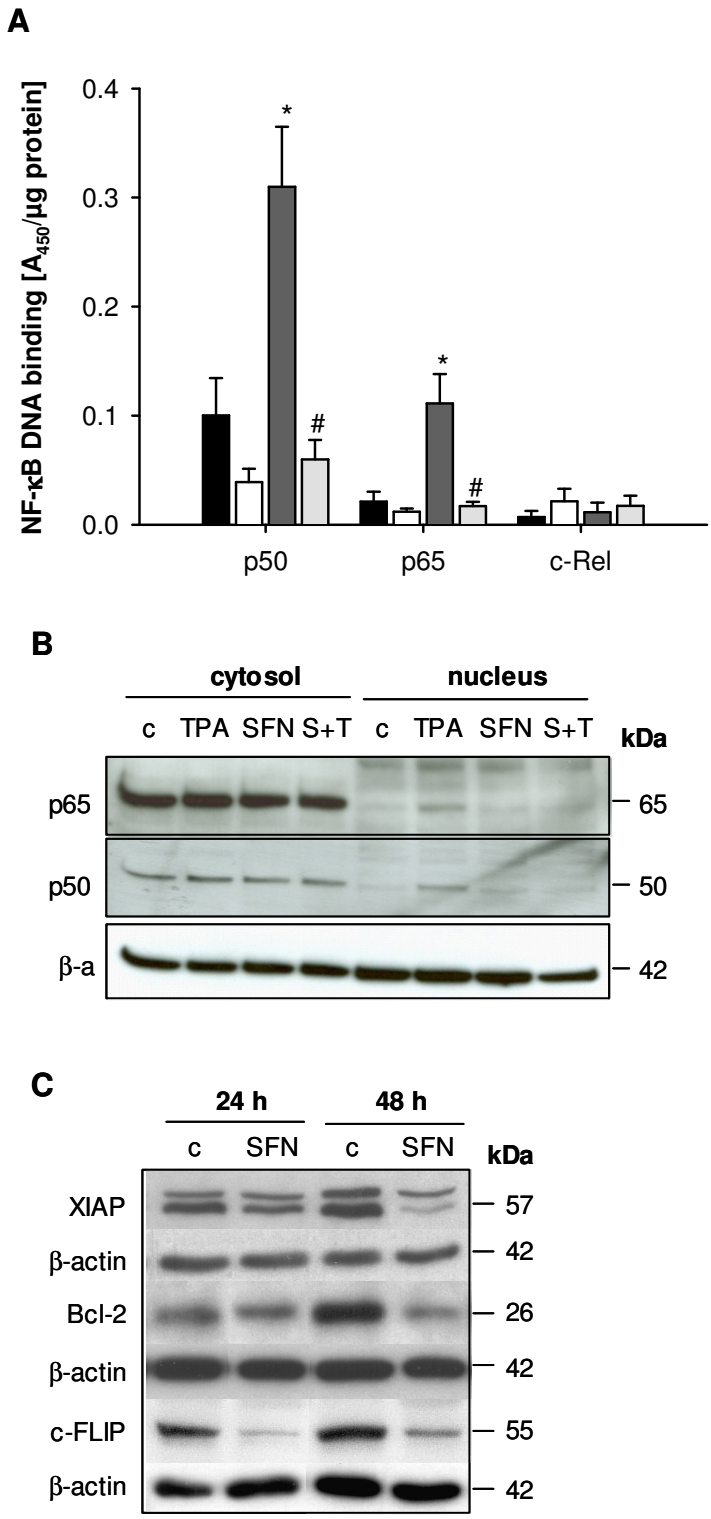

A
NF-κB DNA binding [A450/µg protein]
0.4
0.3
0.2
0.1
0.0
*
*
#
#
p50
p65
c-Rel
B
cytosol
nucleus
c TPA SFN S+T c TPA SFN S+T
kDa
p65
p50
β-a
65
50
42
C
24 h
48 h
c SFN c SFN
kDa
XIAP
β-actin
Bcl-2
β-actin
c-FLIP
β-actin
57
42
26
42
55
42

Fig. 5

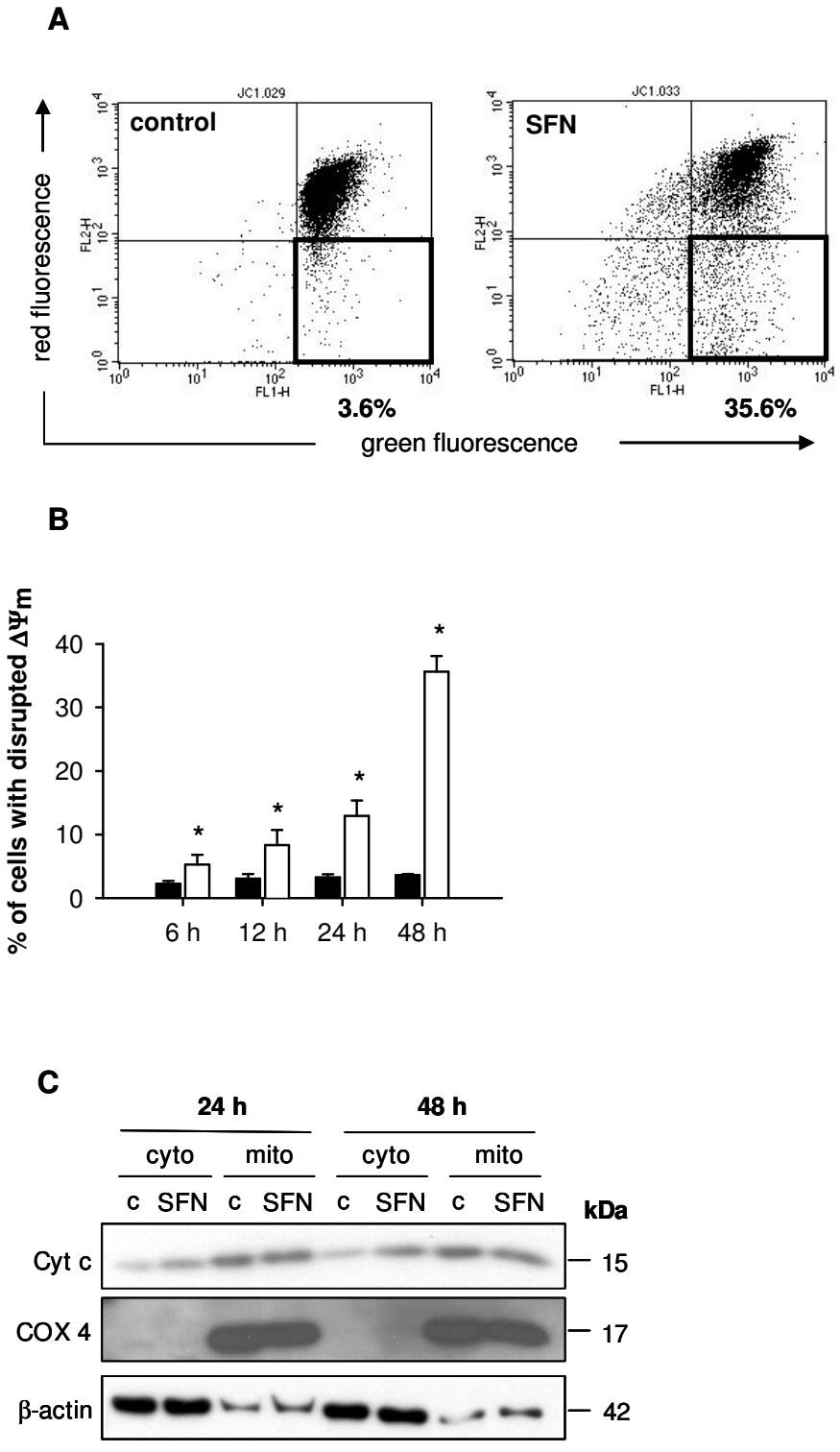

A
JC1.029
control
JC1.033
SFN
red fluorescence
FL2-H
FL1-H
3.6%
35.6%
green fluorescence
B
% of cells with disrupted ΔΨm
40
30
20
10
0
*
6 h
12 h
24 h
48 h
C
24 h
48 h
cyto
mito
cyto
mito
c
SFN
c
SFN
c
SFN
c
SFN
kDa
Cyt c
15
COX 4
17
β-actin
42

Fig. 6

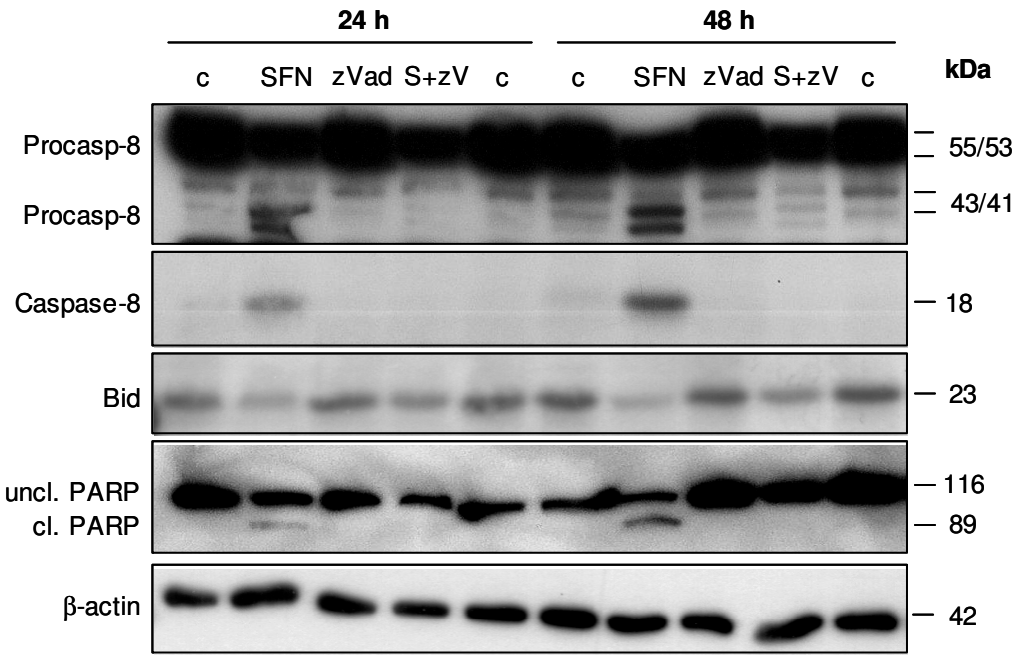

24 h
48 h
c
SFN
zVad
S+zV
c
c
SFN
zVad
S+zV
c
kDa
Procasp-8
Procasp-8
55/53
43/41
Caspase-8
18
Bid
23
uncl. PARP
cl. PARP
116
89
β-actin
42

Fig. 7

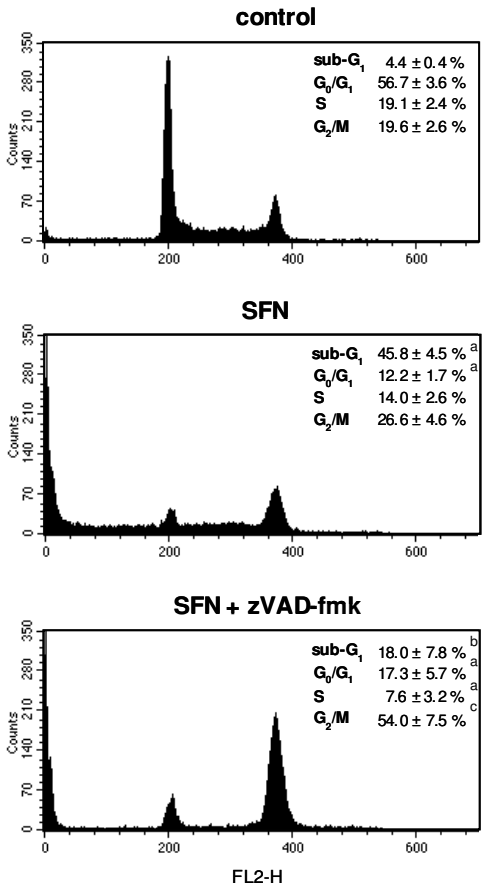
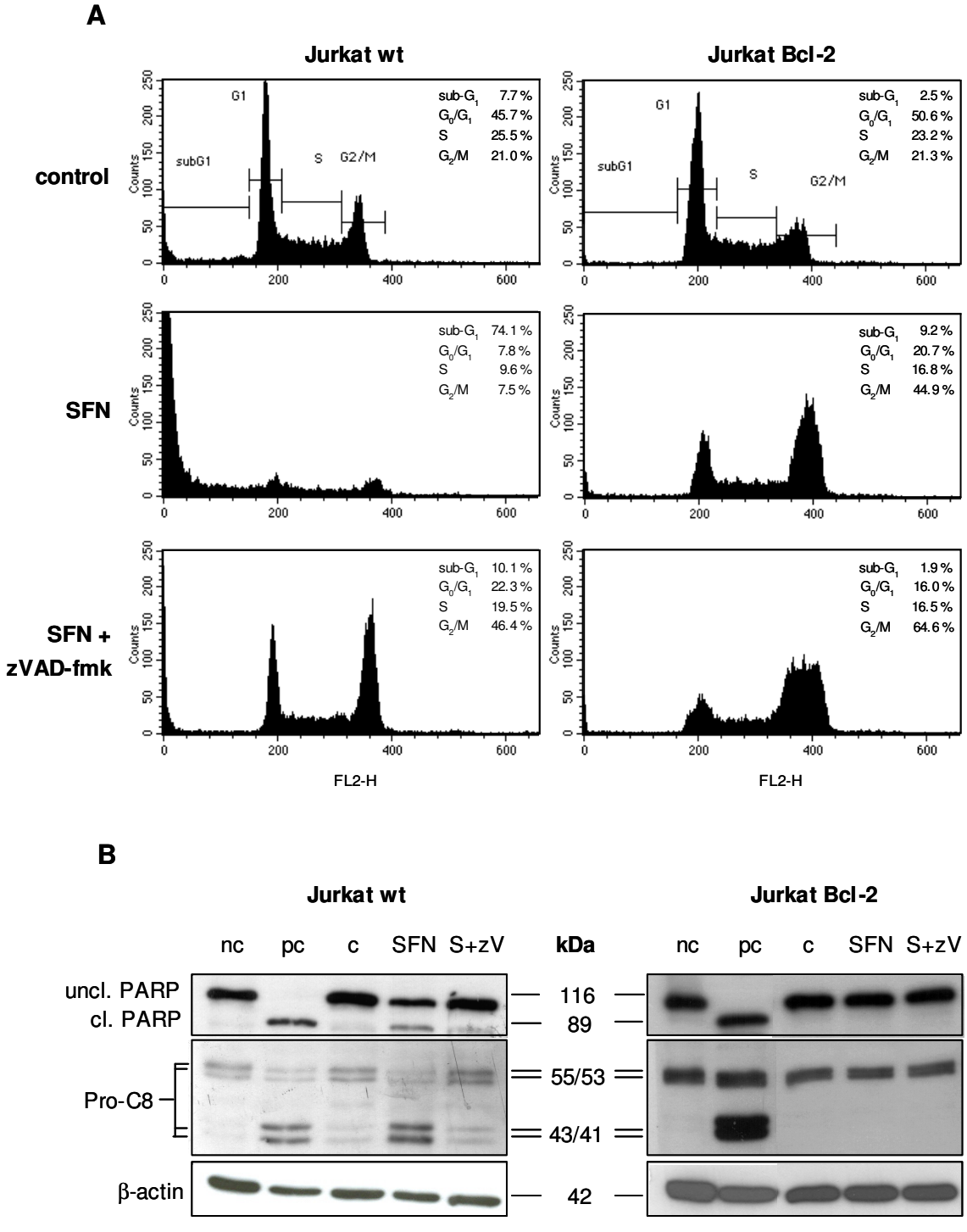

control
sub-G1 4.4 ± 0.4 %
G0/G1 56.7 ± 3.6 %
S 19.1 ± 2.4 %
G2/M 19.6 ± 2.6 %
SFN
sub-G1 45.8 ± 4.5 % a
G0/G1 12.2 ± 1.7 % a
S 14.0 ± 2.6 %
G2/M 26.6 ± 4.6 %
SFN + zVAD-fmk
sub-G1 18.0 ± 7.8 % b
G0/G1 17.3 ± 5.7 % a
S 7.6 ± 3.2 % a
G2/M 54.0 ± 7.5 % c
Counts
0
70
140
210
280
350
0
200
400
600
FL2-H

Fig. 8


A
Jurkat wt
Jurkat Bcl-2
control
SFN
SFN +
zVAD-fmk
G1
subG1
S
G2/M
sub-G1 7.7 %
G0/G1 45.7 %
S 25.5 %
G2/M 21.0 %
sub-G1 2.5 %
G0/G1 50.6 %
S 23.2 %
G2/M 21.3 %
sub-G1 74.1 %
G0/G1 7.8 %
S 9.6 %
G2/M 7.5 %
sub-G1 9.2 %
G0/G1 20.7 %
S 16.8 %
G2/M 44.9 %
sub-G1 10.1 %
G0/G1 22.3 %
S 19.5 %
G2/M 46.4 %
sub-G1 1.9 %
G0/G1 16.0 %
S 16.5 %
G2/M 64.6 %
Counts
FL2-H
B
Jurkat wt
Jurkat Bcl-2
nc
pc
c
SFN
S+zV
kDa
uncl. PARP
cl. PARP
Pro-C8
β-actin
116
89
55/53
43/41
42

2. **Pappa G.,** Bartsch H., Gerhauser C. (2007), Biphasic modulation of cell proliferation by sulforaphane at physiologically relevant exposure times in a human colon cancer cell line. Accepted for publication in *Molecular Nutrition & Food Research.*

Biphasic modulation of cell proliferation by sulforaphane at physiologically relevant exposure times in a human colon cancer cell line

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Abbreviations:

SFN, Sulforaphane; ITC, isothiocyanate; IC_{50}, half-maximal inhibitory concentration; GSH, glutathione; BSO, L-buthionine-*S,R*-sulfoximine; SD, standard deviation

Keywords: broccoli, G_2/M arrest, glutathione, reversibility, sulforaphane

Abstract

Sulforaphane (SFN), a cancer chemopreventive compound derived from broccoli, is able to induce cell cycle arrest and apoptosis in various tumor cell lines. Here we show that cell growth inhibition by SFN follows a biphasic pattern: Transient exposure of 40-16 human colon carcinoma cells for up to 6 h resulted in reversible G_2/M cell cycle arrest and cytostatic growth inhibition even at elevated concentrations, whereas a minimum continuous exposure time of 12 h was necessary for SFN to irreversibly arrest cells in G_2/M phase and subsequently induce apoptosis. IC_{50} values after 12 h of exposure followed by drug-free recovery up to 72 h (6.4 – 8.1 µM) were indistinguishable from those of chronic exposure for 24 h to 72 h (5.4 – 6.6 µM). Low concentrations of SFN caused a transient decrease in glutathione (GSH) levels followed by GSH induction, which may be related to reversible G_2/M arrest and cytostatic effects. Depletion of GSH does not seem to play a role in SFN-mediated apoptosis induction. Our data clearly contribute to a better understanding of the kinetics of anti-proliferative activity of SFN.

Introduction

Colorectal cancer is the second leading cause of cancer-related deaths in industrialized countries [1]. Strong evidence exists from epidemiological studies that high intake of cruciferous vegetables is associated with a lower risk of developing this disease [2, 3]. Cancer preventive potential of cruciferous vegetables such as broccoli has been attributed to their content of glucosinolates. Through catalytic mediation of myrosinase (β-thioglucosidase), which is released upon physical damage of plant cells (e.g. during cutting or chewing), glucosinolates are hydrolyzed, releasing the corresponding isothiocyanates (ITCs) [4]. ITCs are reactive Michael acceptor type-compounds which rapidly bind to thiol groups, leading to dithiocarbamate formation [5]. Intracellularly, ITCs are conjugated to the tripeptide glutathione (GSH), the most abundant thiol present in the cytoplasm. This generally leads to a transient depletion of GSH, which in turn activates the transcription of γ-glutamylcysteine synthetase (γ-GCS), the key regulatory enzyme in glutathione synthesis [6]. Concomitantly, thiol modification of the sensor protein Keap1 activates the Nrf2 pathway, leading to antioxidant responsive element-mediated induction of phase II detoxifying enzymes [7].

The ITC sulforaphane (SFN) was initially identified as the principal inducer of anticarcinogenic protective enzymes from broccoli [8]. Subsequently, chemopreventive efficacy of SFN was demonstrated by inhibition of carcinogen-induced mammary and colon tumorigenesis and of intestinal adenoma formation in $Apc^{Min/+}$ mice [9, 10]. Apart from its modulatory effects on carcinogen metabolism, induction of cell cycle arrest and apoptosis were identified as potential mechanisms contributing to the chemopreventive activities of SFN [4, 11]. In particular, SFN was shown to induce G_2/M cell cycle arrest in a series of cancer cell lines derived from human colon, breast, prostate, and pancreas as well as in human leukemia cell lines [11-13]. On the other hand, non-transformed human T-lymphocytes and cultured human prostate and colon cancer cells were arrested by SFN treatment in G_1 phase [11, 14-16]. In many of these cell lines, SFN-mediated cell cycle arrest was accompanied by induction of apoptosis (reviewed in [17-19]).

Under *in vivo* conditions, ITCs like SFN are readily absorbed, metabolized, and excreted *via* the mercapturic acid pathway with peak urinary levels at 1 and 1.5 h after ingestion [4, 11, 20]. The colon is one of the organs with highest exposure to ITCs, both systemically after absorption of free ITC, as well as topologically after ingestion of cooked cruciferous vegetables and subsequent release of ITCs from glucosinolates by the intestinal microflora [4, 11, 21]. In cell culture experiments, SFN and other ITCs accumulate intracellularly in the form of their GSH conjugates and can reach millimolar concentrations. Intracellular accumulation is dependent on sustained uptake; otherwise, ITC-glutathione conjugates are rapidly exported from the cells *via* a transporter-mediated process [22]. In this respect, we considered whether the results of *in vitro* studies with incubation times of 24 h or longer would be relevant for the human situation with short transient exposure to ITCs. In particular, using the human colon cancer cell line 40-16 we investigated how i) the duration of SFN treatment and ii) drug removal and recovery for up to 72 h would influence cell proliferation and cell cycle progression. In addition, dose-dependent kinetics of GSH depletion and induction were analysed for a potential correlation with cytotoxic mechanisms.

Materials and methods

Chemicals. Sulforaphane (SFN, CAS no. 4478-93-7) was synthesized as described earlier [23]. All cell culture material was obtained from Invitrogen (Eggenstein, Germany). All material required for flow cytometry was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Fetal bovine serum was provided by PAA Laboratories (Pasching, Austria). McCoy's 5A cell culture medium, sulforhodamin B (SRB), propidium iodide (PI) and RNase A and all other chemicals were obtained from Sigma (Taufkirchen, Germany).

Inhibition of cell proliferation. The human colon cancer cell line 40-16 derived from HCT116 was provided by B. Vogelstein. We have previously investigated apoptosis-inducing

mechanisms of SFN in this cell line [24] and decided to utilize the same cell line for further studies for better comparison of results. McCoy's 5A cell culture medium containing 5% fetal bovine serum was used throughout all experiments. Cells (2.5 x 10^4 cells/ml) were plated in 96-well plates (200 µl/well). After overnight growth, cells were treated in duplicate with eight serial dilutions of SFN dissolved in DMSO (final DMSO concentration 0.5%) in a concentration range of 0.4 to 50 µM or with 0.5% DMSO as solvent control for 24 h, 48 h and 72 h. Alternatively, after treatment for either 3 h, 6 h, 12 h, or 24 h, cell culture medium was changed to SFN-free medium, and cells were incubated for up to 24 h, 48 h or 72 h, respectively, to allow recovery. Then the medium was discarded, and cells were fixed using 50 µl of 10% aqueous trichloroacetic acid (TCA) for 30 min at 4 °C. Sulforhodamin B staining was performed as described earlier [24]. Calculation of anti-proliferative activity was based on the ratio of absorbance readings of treated cells to those of solvent controls (set as 100%) after correction for absorbance of cells present at the time of treatment (day 0 values). Negative values indicate cytotoxic effects. In these cases, day 0 values of the control were used to calculate the percentage of cytotoxicity. IC_{50} values (half maximal inhibitory concentrations) were computed from the results of eight serial two-fold dilutions of SFN tested in duplicate from at least three independent experiments, using TableCurve Windows version 1.0 software (Jandel Scientific, San Rafael, USA) with the equation for a logistic dose-response curve.

Cell Cycle Analysis. 40-16 cells were plated in 150 mm tissue culture dishes (1.75 x 10^6 cells/35 ml). After overnight growth cells were treated with SFN at a concentration of 15 µM or with 0.2% DMSO as solvent control and incubated for 6 h, 12 h, 24 h or 48 h. For recovery experiments, cells were treated with SFN for 3 h, 6 h, 12 h or 24 h before cell culture medium was replaced by SFN-free medium. Incubation with drug-free medium was continued up to 48 h. Attached and floating cells were collected, washed with phosphate-buffered saline, pH 7.4 (PBS), fixed in ice cold 70 % ethanol and stored at -20 °C. After washing twice with PBS, cells were incubated with PBS containing propidium iodide (50 µg/ml) and RNase A (100 µg/ml) for 30 min

at 37 °C. Then they were analyzed by flow cytometry using a FACS Calibur with CELL QUEST PRO software (Becton Dickinson, Franklin Lakes, NJ, USA).

Determination of GSH levels. 40-16 cells were seeded in 96-well plates (1 x 10^4 cells/well). SFN (final concentration 0.4-50 µM in 0.5% DMSO) was added 0.5-24 h prior to determination of total GSH levels. Plates were washed with PBS and stored at –80 °C until analyzed. GSH was measured as described previously using 5,5´-dithiobis-(2-nitrobenzoic acid) and normalized to protein concentrations determined using bicinchoninic acid [25].

Statistical Analysis. Results are presented as means ± standard deviation (SD) of data originating from at least three independent experiments. For statistical evaluation, one-way ANOVA followed by Bonferroni's multiple comparison test was applied. Values of $p < 0.01$ were considered as statistically significant.

Results

SFN-induced inhibition of cell proliferation: Influence of exposure and total incubation time. We have shown previously that SFN exhibits cytotoxic effects towards the 40-16 human colon cancer cell line with IC_{50} values of 7.7 µM, 6.6 µM and 5.7 µM after 24 h, 48 h and 72 h of continuous treatment, respectively [24]. In the present study, we analyzed SFN-induced effects on cell proliferation after shorter exposure times, and determined the reversibility of the observed effects after drug removal. In this respect, we treated 40-16 cells for 3 h to 24 h with SFN in a concentration range of 0.4 to 50 µM before cell culture medium was replaced by SFN-free medium. Cells were then allowed to recover for 24 h, 48 h and 72 h. Alternatively, SFN-containing medium was not removed. As shown in **Fig. 1A**, treatment of 40-16 cells with SFN for 3 h followed by recovery up to 72 h resulted in IC_{50} values of 21 µM, 22.3 µM and 22.6 µM. IC_{50} values were not significantly influenced by the duration of recovery. After 6 h of SFN treatment,

IC_{50} values (10.8 to 13.1 µM) were about 50% lower than those measured after 3 h of incubation, but still twice as high as without recovery (5.4 to 6.6 µM). In contrast to these short treatment periods, 12 h and 24 h incubation with SFN resulted in irreversible cytotoxicity. Specifically, IC_{50} values after 12 h of SFN treatment followed by recovery up to 72 h (6.4 to 8.1 µM) did not differ significantly from IC_{50} values obtained after 24 h to 72 h exposure without drug removal.

A comparison of profiles of cell growth inhibition after SFN exposure for 3 to 24 h revealed obvious differences between short (up to 6 h) and longer (12 and 24 h) exposure times. An example of 48 h total incubation is given in **Fig. 1B**. SFN treatment for 3 h and 6 h resulted in cytostatic effects. Even at a concentration of 50 µM, cell numbers (*i.e.* absorbance values after SRB staining) were still above the initial baseline, indicating inhibition of cell proliferation, but no cytotoxicity. In contrast, after 12 h and 24 h exposure, SFN displayed cytotoxic effects at concentrations higher than 12.5 µM, measured by a reduction in cell numbers to values below the initial baseline.

Effects of SFN on cell cycle cycle progression. SFN has been described to cause G_1 or, more frequently, G_2/M phase cell cycle arrest in various cancer cell lines [11-16]. Therefore, we were interested how effects of SFN on cell cycle progression in 40-16 cells would relate to the observed biphasic pattern in cell growth inhibition. Cells were treated with SFN at a concentration of 15 µM for 6 h to 48 h. Cell cycle distribution was assessed by flow cytometry after DNA staining with propidium iodide. Results are depicted as representative histograms in **Fig. 2**.

SFN induced a cell cycle arrest in G_2/M phase that was already visible after 6 h (41.4 ± 4.3% *vs.* 29.5 ± 5.2% in the control). The accumulation of cells in G_2/M phase increased with incubation time, with mean percentages of 51.2 ± 3.8% *vs.* 33.3 ± 2.9% after 12 h and 47.0 ± 7.3% *vs.* 27.8 ± 2.1% after 24 h, respectively. After 48 h, the G_2/M arrest was no longer detectable. In contrast, we observed a marked increase of cells in subG_1 phase. This was in good agreement with our earlier investigations on the induction of apoptosis in 40-16 cells by 15 µM SFN [24].

To further explain the results described in **Fig. 1**, cell cycle analyses were also performed to investigate the influence of the duration of SFN exposure and recovery time on cell cycle distribution. 40-16 cells were exposed to SFN at a concentration of 15 µM for increasing time periods from 3 h to 48 h and then harvested or allowed to recover for total incubation times up to 48 h. The results are summarized in **Table 1**. Most relevant changes in sub-G_1 and G_2/M fractions are depicted in **Fig. 3**.

SFN treatment for only 3 h already induced a significant increase in the G_2/M fraction of the cells (36.4 ± 4.0%) compared to untreated control cells (25.1 ± 2.4%), concomitant with a reduction of cells in G_0/G_1 phase. After drug removal, cells were able to recover quickly: further incubation for 3 h in drug-free medium (6 h total incubation time) restored the percentage of cells in G_2/M phase to control values.

Continuous exposure to SFN for 6 h provoked a significant G_2/M arrest (41.4 ± 4.3 %; compare **Fig. 2 and 3**), whereas G_0/G_1 and S phases were significantly reduced (for statistical evaluation see Table 1 and footnotes for explanation). The observed G_2/M arrest was still evident after 6 h of recovery in SFN-free medium (12 h total incubation time: 43.8 ± 7.1%). The percentage of cells in S phase as an indicator of cell proliferation was further reduced at this time point (6 h: 26.8 ± 2.0% *vs.* 12 h: 19.5 ± 3.1%). Importantly, another 12 h of recovery (at a total incubation time of 24 h) abolished the G_2/M arrest (25.8 ± 4.1%), although it was clearly evident after 24 h of continuous exposure to SFN (47.0 ± 7.3%; compare **Fig. 2 and 3**). These results indicated that the G_2/M arrest induced by SFN treatment for 6 h was almost completely reversible.

Continuous SFN exposure for 12 h resulted in the highest accumulation of cells in G_2/M phase (51.2 ± 3.8% *vs.* 33.3 ± 2.9% in the untreated control; compare **Fig. 2 and 3**). Further incubation in drug-free medium up to 48 h led to a gradual decrease of G_2/M-arrested cells, paralleled by an increase of cells in subG_1 phase, indicative of apoptosis induction (total incubation time 24 h: 14.9 ± 6.9%; 48 h: 27.7 ± 8.8%; compare **Fig. 3**). Nevertheless, 48 h continuous exposure to SFN was significantly more effective in inducing apoptosis than 12 h treatment plus 36 h recovery time (45.5 ± 5.1% *vs.* 27.7 ± 8.8%). Of notice, we have demonstrated

recently by Western Blotting *via* the detection of poly (ADP-ribose) polymerase cleavage that the percentage of cells in $subG_1$ phase caused by SFN treatment directly correlated with apoptosis induction in the 40-16 cell line [26].

After 24 h incubation with SFN, G_2/M phase arrest similar to that observed after 12 h was evident. At the same time point, a substantial portion of cells was apoptotic (23.2 ± 1.8% $subG_1$ *vs.* 4.3 ± 0.9% in the untreated control). When cells were incubated with SFN for 24 h and allowed to recover for further 24 h without SFN, there was no significant difference in the percentage of cells in $subG_1$ phase compared to 48 h SFN treatment (40.2 ± 8.5% *vs.* 45.5 ± 5.1%; compare **Fig. 3**).

Taken together, short treatment times with SFN for 3 h and 6 h led to a transient cell cycle arrest in G_2/M phase, which was reversible after drug removal. After exposure for 12 h and 24 h, the early G_2/M arrest turned into apoptosis induction with total incubation times of 24 h and 48 h, indicated by an increase in the percentage of cells in $subG_1$ phase. Exposure of 40-16 cells to SFN for 12 h was sufficient for an irreversible commitment to cell death, which confirmed the results obtained with the cytotoxicity experiments depicted in **Fig.1**.

Effects of SFN on cellular GSH levels. GSH depletion was suggested to play a role in SFN-induced cell cycle arrest and apoptosis [13, 27]. We therefore tested whether the kinetics of thiol depletion would be related to the growth inhibition profile observed with SFN. 40-16 cells were treated for 30 min to 24 h with SFN at concentrations of 6.25 µM, 12.5 µM and 25 µM. In addition, we used L-buthionine-*S,R*-sulfoximine (BSO), a specific inhibitor of γ-GCS [6], as positive control for GSH depletion.

Treatment with BSO at a concentration of 10 µM for 16 h markedly lowered GSH levels to 26.4 ± 1.5% of the untreated control (**Fig. 4**). Of note, BSO exerted no cytotoxicity, since protein levels were not changed compared to control cells (data not shown). SFN treatment led to a rapid depletion in GSH levels within 30 min, especially at the two higher concentrations. After treatment with SFN at a concentration of 6.25 µM, GSH levels transiently decreased up to 4 h,

followed by gradual elevation to 159.5 ± 11.7% of the untreated control after 24 h, indicative of an induction of GSH synthesis. A similar trend was observed with the intermediate concentration, although the drop in GSH was more pronounced and longer lasting (minimum 49.5 ± 4.1% after 6 h), and GSH resynthesis was slower with lower maximum induction, at least up to 24 h. In contrast, when cells were treated with 25 µM SFN, GSH levels dropped to a minimum of 29.5 ± 3.2% after 10 h and did not recover, suggestive of irreversible cytotoxicity.

Discussion

In the present study we demonstrated that the time-dependent profile of cell growth inhibition by SFN was biphasic. Short exposure times up to 6 h led to cytostatic inhibition of cell proliferation, which was observed with concentrations up to 50 µM and was sustained up to 72 h even after drug-removal. Further, short exposure times resulted in a transient G_2/M arrest, which was rapidly reversible when exposure was terminated after 3 h followed by drug-free incubation, and longer-lasting, but still reversible when cells were treated for 6 h with 15 µM of SFN. On the other hand, treatment for at least 12 h caused cytotoxic inhibition of cell proliferation and was necessary to efficiently induce cell death. Detailed analyses of molecular mechanisms and a potential causal relation between cell cycle arrest in G_2/M phase and apoptosis induction are currently underway (Pappa *et al*., in preparation).

Gamet-Payrastre *et al.* investigated reversibility of SFN-induced apoptosis in HT29 human colon cancer cells. A minimum 24 h treatment was required for irreversible induction of cell death [28]. Synchronized cells treated with SFN were unable to reenter the cell cycle, but cell proliferation was initiated without signs of cytotoxicity after SFN removal. Thus, resting cells in G_0 seemed to be resistant towards SFN. Zhang *et al.* [29] treated various cancer cell lines with SFN, phenethyl-ITC, allyl-ITC and benzyl-ITC for 72 h or for 3 h followed by 69 h drug-free incubation. Interestingly, while IC_{50} values of SFN were significantly lower after 72 h continuous treatment compared to 3 h plus 69 h recovery time, IC_{50} values of the other three ITCs were similar for both

time regimes. Thus, SFN's biphasic growth inhibition profile with delayed initiation of cytotoxicity seems to be unique for this ITC.

Consistent with our data, SFN-induced G_2/M arrest was reported to be irreversible after 16 h in PC-3 human prostate cancer cells [30]. In that study, the authors postulated that G_2/M cell cycle arrest was due to oxidative stress-induced DNA damage. Using the dye 2',7'-dichlorodihydrofluorescein diacetate sensitive to reactive oxygen species, we could not detect any significant enhancement of oxidative stress after treatment of 40-16 cells with 15 µM SFN for several time points (data not shown).

A recent report demonstrated that significant oxidative stress and resulting DNA damage was correlated with depleted pools of mitochondrial and nuclear GSH, but not with cytoplasmic GSH levels [31]. Consistently, in the present study, reduced GSH levels due to inhibition of GSH synthesis by BSO [32] did not result in toxic effects or cell growth inhibition. Also, time- and dose-dependent depletion and resynthesis of GSH by SFN was not clearly related with cytotoxic potential. SFN treatment for 6 h at a concentration of 12.5 µM, which we assume to produce comparable effects on cell cycle and apoptosis induction as 15 µM used for the data summarized in **Table 1**, reduced GSH levels by 50%. This was associated with a transient cell cycle arrest in G_2/M (**Table 1**) and cytostatic inhibition of cell proliferation (**Fig. 1**), but was not sufficient to induce cell death. In contrast to short incubation times, continuous exposure to 12.5 µM SFN for 12 h and 24 h resulted in GSH resynthesis, while causing sustained G_2/M arrest and commitment to undergo apoptosis. Higher concentrations of SFN resulted in stronger GSH depletion, but were merely cytostatic when exposure was discontinued after 3 and 6 h (**Fig. 1B**). Taken together, these data indicate that GSH depletion does not play a major role in SFN-induced apoptosis in the 40-16 cell line, but may be related to the observed G_2/M cell cycle arrest and cytostatic cell growth inhibition. Cytoplasmic GSH depletion may also be an indicator for intracellular SFN accumulation, which needs to be sustained for at least 12 h to cause irreversible cytotoxicity.

Further pharmacokinetic investigations need to clarify which organs may be affected by SFN or its metabolites for 12 h or longer after ingestion of cruciferous vegetables. Regarding the colon,

the situation is unique due to the fact that this organ is exposed to SFN both systemically and topologically. The length of exposure to SFN and its metabolites then depends on the way in which vegetables are consumed (raw *vs*. cooked) and on intestinal transit time.

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Figure legends:

Fig. 1. Time-dependent inhibition of cell proliferation by SFN and the effect of drug removal. (**A)** Inhibition of cell proliferation was determined by sulforhodamine B staining. Cells were treated with eight different concentrations of SFN (0.4 to 50 µM) or 0.5% DMSO as a solvent control for 3 h, 6 h, 12 h, 24 h before replacement of SFN-containing cell culture medium by SFN-free medium and further incubation for up to 24 h (black bars), 48 h (grey bars) or 72 h (stippled bars). Alternatively cells were treated continuously (nr, no removal) for 24 h (black bar), 48 h (grey bar) or 72 h (stippled bar), respectively. IC_{50} values were calculated as described in "Materials and methods". Data are means ± SD of three independent experiments. [a] significantly different ($p < 0.01$) compared with 3 h SFN treatment within the same total incubation time. [b] significantly different ($p < 0.01$) compared with 3 h and 6 h SFN treatment within the same total incubation time.

(B) Profiles of cell growth inhibition after treatment with SFN for 3 h to 24 h as indicated, before cell culture medium was changed to drug-free medium (nr, no removal). Data were taken from one representative cell proliferation experiment with a total incubation time of 48 h. Dose-dependent inhibition of proliferation is shown by values above the baseline, whereas values below the baseline represent cytotoxic effects.

Fig. 2. Cell cycle effects of SFN. 40-16 cells were treated with SFN (15 µM) or 0.2% DMSO (untreated control) for 6 h, 12 h, 24 h or 48 h, respectively. Cell cycle analyses of fixed cells were performed by flow cytometry after staining DNA with propidium iodide. Representative histograms of one out of four independent experiments are depicted.

Fig. 3. SFN-induced cell cycle effects and the influence of exposure time and drug removal. Data were taken from Table 1. 40-16 cells were treated with SFN (15 µM) or 0.2% DMSO for 3 h, 6 h, 12 h or 24 h before medium change and drug-free incubation for up to 48 h total incubation time.

Alternatively, cells were treated with SFN continuously for 3 h, 6 h, 12 h, 24 h or 48 h, respectively (nr, no removal, represented by stippled bars). Cell cycle analyses of fixed cells were performed by flow cytometry after staining DNA with propidium iodide. Data for G_2/M and subG_1 phase are shown as means ± SD of four independent experiments. Letters indicate significant differences as in Table 1.

Fig. 4. Effects of SFN on total intracellular GSH levels. 40-16 cells were treated with SFN for 0.5 - 24 h. Results obtained with SFN at 6.25 µM (black bars), 12.5 µM (light grey bars) and 25 µM (stippled bars), and BSO (10 µM, 16 h, dark grey bar) as a positive control are shown as percentage of the respective solvent control. Means ± SD significantly different ($p < 0.01$) from [a] respective control, [b] control and 6.25 µM, [c] control, 6.25 µM and 12.5 µM, [d] preceding time point with identical SFN treatment ($n = 3$). GSH levels in the controls were in the range of 55 to 80 pmol/µg protein.

Table 1 Summary of reversibility experiments of SFN-induced cell cycle effects (mean ±SD)

total incubation time [h]	subG$_1$ [%]	G$_0$/G$_1$ [%]	S [%]	G$_2$/M [%]
Control (no SFN treatment)				
3	2.1 ± 0.5	47.0 ± 3.5	25.9 ± 3.0	25.1 ± 2.4
6	2.3 ± 0.7	36.5 ± 3.7 [a]	31.9 ± 3.9 [a]	29.5 ± 5.2
12	3.1 ± 0.5	37.0 ± 1.9	26.4 ± 0.9 [a]	33.3 ± 2.9
24	4.3 ± 0.9	42.8 ± 2.3	24.4 ± 2.1	27.8 ± 2.1
48	4.1 ± 0.7	56.1 ± 4.5 [a]	19.1 ± 3.3	20.4 ± 1.8 [a]
Treatment with SFN (15 µM) for 3 h				
3	2.4 ± 0.2	36.9 ± 1.6 [b]	24.4 ± 3.2	36.4 ± 4.0 [b]
6	2.4 ± 0.5	42.4 ± 1.7 [a]	25.9 ± 1.6 [b]	29.3 ± 3.0 [a]
12	2.8 ± 0.4	42.8 ± 4.8	19.4 ± 2.1 [b]	33.9 ± 4.0
24	6.3 ± 4.1	37.8 ± 3.0	22.7 ± 3.7	31.1 ± 1.4
48	6.1 ± 1.8	50.7 ± 2.5 [a]	20.2 ± 2.6	22.1 ± 2.0 [a]
Treatment with SFN (15 µM) for 6 h				
6	3.4 ± 0.5 [c]	28.2 ± 6.0 [c]	26.8 ± 2.0 [b]	41.4 ± 4.3 [c]
12	4.2 ± 1.1	30.9 ± 10.1	19.5 ± 3.1 [a,b]	43.8 ± 7.1 [b]
24	7.1 ± 3.6	37.0 ± 10.1	23.4 ± 5.4	25.8 ± 4.1 [a]
48	9.6 ± 2.5	43.4 ± 7.3 [b]	18.6 ± 3.5	26.9 ± 4.0
Treatment with SFN (15 µM) for 12 h				
12	7.4 ± 2.4 [d]	21.6 ± 3.6 [c]	18.7 ± 2.0 [b]	51.2 ± 3.8 [c]
24	14.9 ± 6.9 [b]	35.7 ± 10.6	11.1 ± 1.5 [a,d]	33.6 ± 4.7 [a]
48	27.7 ± 8.8 [d]	23.2 ± 6.3 [d]	16.0 ± 3.1	25.4 ± 2.9 [a]
Treatment with SFN (15 µM) for 24 h				
24	23.2 ± 1.8 [d]	16.1 ± 2.6 [e]	12.2 ± 4.4 [d]	47.0 ± 7.3 [e]
48	40.2 ± 8.5 [a,e]	12.3 ± 1.4 [d]	16.0 ± 1.4	25.0 ± 5.9 [a]
Treatment with SFN (15 µM) for 48 h				
48	45.5 ± 5.1 [e]	14.0 ± 6.8 [d]	14.4 ± 3.5	24.4 ± 5.2

[a] mean significantly different ($p < 0.01$) from preceding total incubation time without SFN treatment (control) or within the same SFN treatment time

[b] significantly different ($p < 0.01$) from untreated control of the same total incubation time

[c] significantly different ($p < 0.01$) from untreated control and from 3 h treatment with SFN within the same total incubation time

[d] significantly different ($p < 0.01$) from untreated control and from 3 h and 6 h treatment with SFN within the same total incubation time

[e] significantly different ($p < 0.01$) from untreated control and from 3 h, 6 h and 12 h treatment with SFN within the same total incubation time

Fig. 1

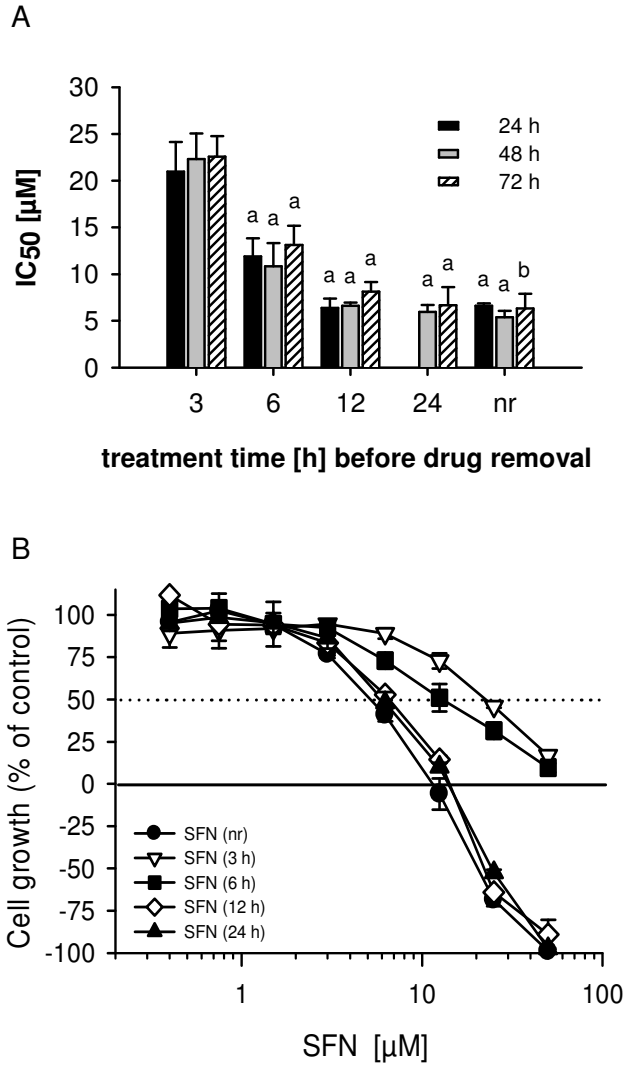

A
IC50 [µM]
30
25
20
15
10
5
0
24 h
48 h
72 h
a a a
a a a
a a
a a b
3
6
12
24
nr
treatment time [h] before drug removal
B
Cell growth (% of control)
100
75
50
25
0
-25
-50
-75
-100
SFN (nr)
SFN (3 h)
SFN (6 h)
SFN (12 h)
SFN (24 h)
1
10
100
SFN [µM]

Fig. 2

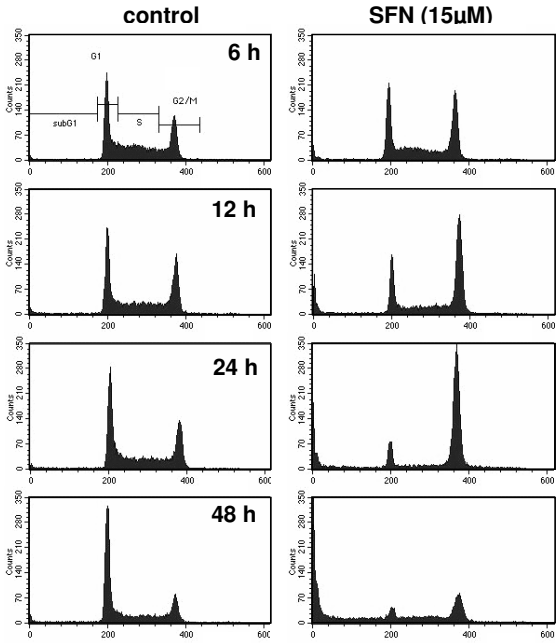

control
SFN (15µM)
6 h
12 h
24 h
48 h
G1
G2/M
subG1
S
Count
0
70
140
210
280
350
200
400
600

Fig. 3

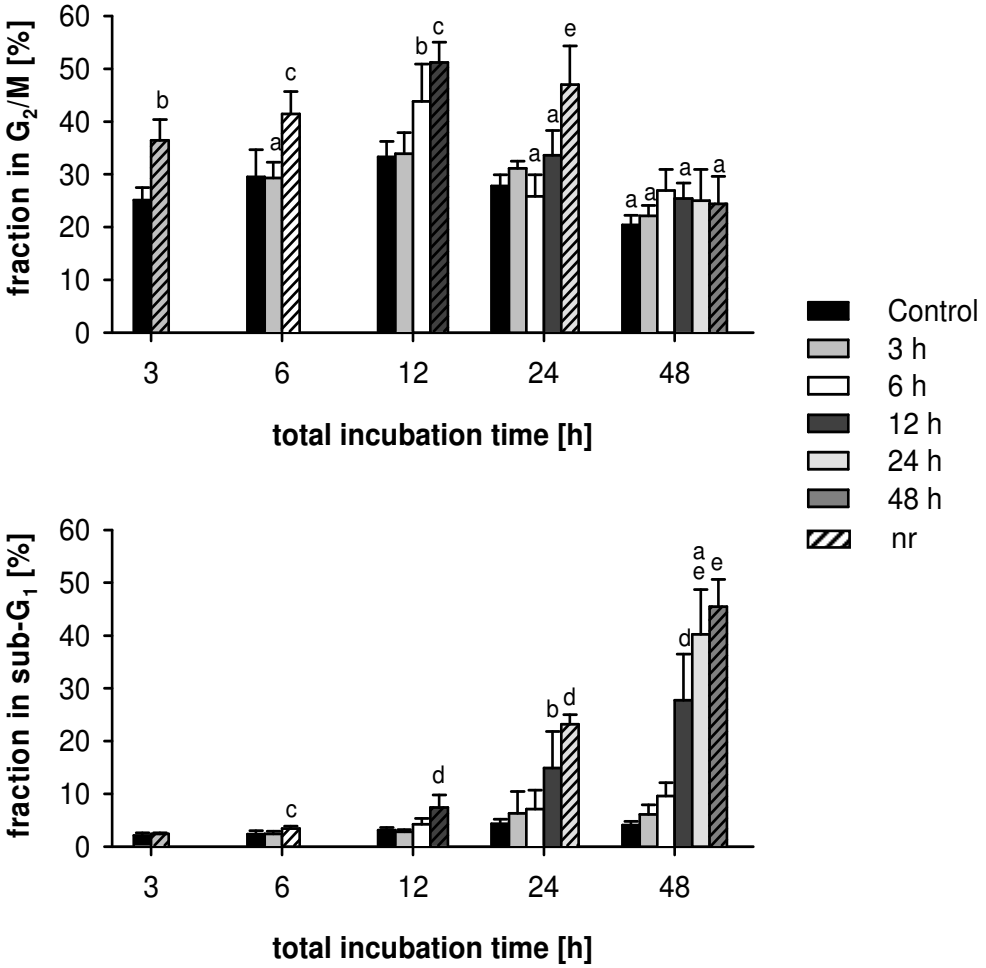
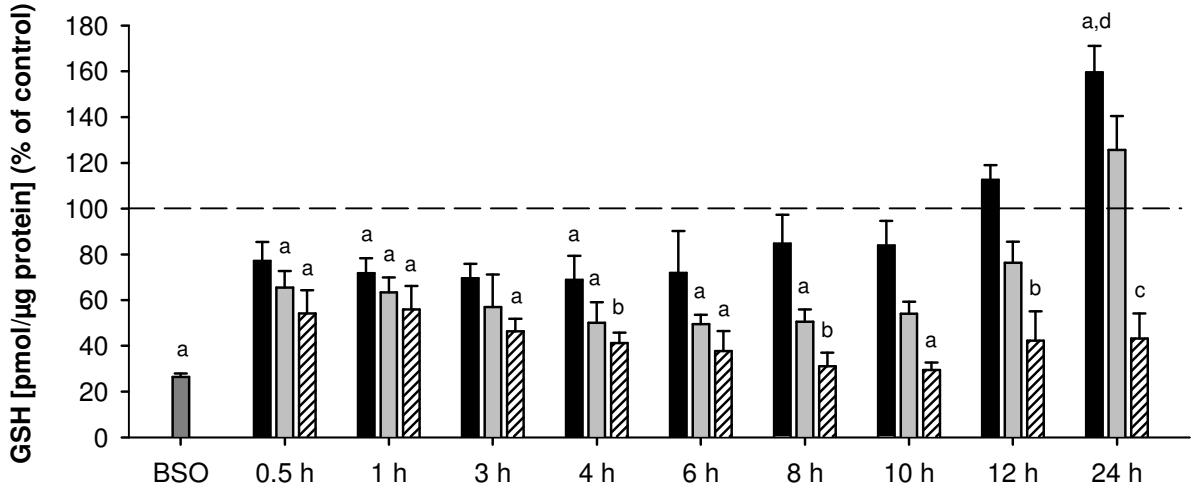

fraction in G2/M [%]
60
50
40
30
20
10
0
3
6
12
24
48
total incubation time [h]
Control
3 h
6 h
12 h
24 h
48 h
nr
fraction in sub-G1 [%]
total incubation time [h]

Fig. 4


GSH [pmol/µg protein] (% of control)
180
160
140
120
100
80
60
40
20
0
a
a
a
a
a
a
a
a
a
a
a
b
a
a
a
b
a
a
b
a,d
c
BSO
0.5 h
1 h
3 h
4 h
6 h
8 h
10 h
12 h
24 h

3. Pappa G., Strathmann J., Löwinger M., Bartsch H., Gerhauser C. (2007), Quantitative combination effects between sulforaphane and 3,3'-diindolylmethane on proliferation of human colon cancer cells in vitro. Accepted for publication in *Carcinogenesis* (doi:10.1093/carcin/bgm044).

Quantitative combination effects between sulforaphane and 3,3'-diindolylmethane on proliferation of human colon cancer cells *in vitro*

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Abbreviations:

SFN, Sulforaphane; DIM, 3,3'-diindolylmethane; ITC, isothiocyanate; IC_{50}, half-maximal inhibitory concentration; CI, combination index; *f*a, affected fraction; *f*u, unaffected fraction; *D*, concentration; PARP, poly (ADP-ribose) polymerase

Abstract

Isothiocyanates and indoles derived from cruciferous vegetables possess growth-inhibiting and apoptosis-inducing activities in cancer cell lines *in vitro*. Isothiocyanates like sulforaphane (SFN) are cytotoxic, whereas indoles including indole-3-carbinol or its condensation product 3,3'-diindolylmethane (DIM) are acting by cytostatic mechanisms in human colon cancer cell lines. In the present study, we have investigated the impact of defined combinations of SFN and DIM (ratio 1:4, 1:2, 1:1, 2:1 and 4:1) on cell proliferation, cell cycle progression and apoptosis induction in cultured 40-16 colon carcinoma cells. Calculations of combination effects were based on the method of Chou and Talalay (1984), and were expressed as a combination index (CI) with CI<1, CI=1 or CI>1 representing synergism, additivity or antagonism, respectively. Interestingly, at a total drug concentration of 2.5 µM, all combinations of SFN and DIM were antagonistic. With increasing concentrations, the antagonistic effect gradually turned into a synergistic interaction at the highest combined cytotoxic concentration of 40 µM. Cell cycle analyses with SFN:DIM ratios of 1:1, 1:2 and 1:4 and total concentrations between 10 and 25 µM confirmed antagonism at low, and additive effects at higher doses. SFN (10 µM) in combination with DIM (10 µM) resulted in strong G_2/M cell cycle arrest which was not observed with either compound alone. Our results indicate that cytotoxic concentrations of SFN:DIM combinations affect cell proliferation synergistically. At low total concentrations (below 20 µM), which are physiologically more relevant, the combined broccoli compounds showed antagonistic interactions in terms of cell growth inhibition. These data stress the need for elucidating mechanistic interactions for better predicting beneficial health effects of bioactive food components.

1. Introduction

Cruciferous vegetables, in particular those of the *Brassica* genus (broccoli, cabbage, cauliflower, radish, mustard etc.) possess cancer preventive potential *in vitro* and *in vivo* that has been attributed to their content in thioglucoside conjugates, namely glucosinolates [1,2]. Through catalytic mediation of myrosinase (β-thioglucosidase), which is released upon physical damage of plant cells (e.g. during cutting or chewing), glucosinolates are hydrolyzed, releasing the corresponding isothiocyanates (ITCs).

The ITC sulforaphane (SFN) is particularly abundant in broccoli (*Brassica oleracea* L. var. *italica*) as its corresponding glucosinolate glucoraphanin [3]. In 1992, Zhang *et al.* initially identified SFN as a principal inducer of phase II enzymes including NAD(P)H:quinone oxidoreductase in broccoli [4]. Subsequently, chemopreventive efficacy of SFN was demonstrated by inhibition of 7,12-dimethylbenz[a]anthracene-induced mammary tumorigenesis, of azoxymethane-induced colonic aberrant crypt foci formation in rats [5,6] and of intestinal adenoma formation in $Apc^{Min/+}$ mice [7,8]. Apart from its modulatory effects on carcinogen metabolism, induction of cell cycle arrest and apoptosis in various cancer cell lines were identified as mechanisms underlying the chemopreventive activities of SFN [9-13].

Mature broccoli is also rich in the indole-based glucosinolate glucobrassicin. Cleavage by myrosinase results in an unstable ITC that is further converted to indole-3-carbinol and other indole derivatives [3]. Under low pH conditions, as in the stomach, several condensation reactions of indole-3-carbinol occur which result in the formation of 3,3'-diindolylmethane (DIM) as a major condensation product [14]. Based on this observation, it has been suggested that DIM rather than indole-3-carbinol is responsible for the physiological effects of dietary indole-3-carbinol observed *in vivo* [15,16].

Both indole-3-carbinol and DIM possess cancer protective effects in reproductive organs, which are supposed to be due to the induction of specific cytochrome P450 enzymes involved in estrogen metabolism [17-19]. Indole-3-carbinol and DIM were also found to suppress cell

proliferation and to induce apoptosis in breast, prostate, cervical and colon cancer cell lines [10,20,21], partly mediated by cell cycle arrest in the G_1 phase [22-24].

Numerous bioactive plant constituents are concertedly consumed with a diet rich in fruits and vegetables that is supposed to reduce cancer risk. It has been suggested that synergistic effects of combined low doses of these phytochemicals may account for the observed health benefits [25]. Although intense research is being conducted to reveal molecular mechanisms of chemopreventive activities and to demonstrate chemopreventive efficacy of single pure plant constituents, only few studies have been undertaken to systematically quantitate combination effects of these compounds. With respect to glucosinolate cleavage products and other substances found in Brassica vegetables, El-Bayoumy *et al.* demonstrated that a mixture of 1,4-phenylenebis(methylene)selenocyanate, phenethyl isothiocyanate, indole-3-carbinole and *d*-limonene was significantly more potent in inhibiting lung tumor multiplicity in A/J mice than indole-3-carbinol alone, but not than either of the other single compounds [26]. A synergistic induction of phase II enzymes was shown in rats treated with indole-3-carbinol and crambene (derived from the glucosinolate progoitrin) [27,28]. In a later study the same group reported that only a relatively high, non-physiological dose of a combination of indole-3-carbinol and crambene was able to reduce aflatoxin B1-induced expression of the preneoplastic marker glutathione *S*-transferase π in rats [29].

In extension of these studies focusing on the impact of combined broccoli compounds on the initiation stage of carcinogenesis, we were interested in how later stages would be affected by a mixture of SFN and DIM, using cell proliferation as an example. Interestingly, ITCs and indole derivates exert their anti-proliferative effects - at least in part - by different mechanisms. Recently, we have demonstrated that ITCs like SFN are cytotoxic in human colon cancer cell lines, whereas indoles like indole-3-carbinol or DIM are acting by a cytostatic mechanism [30]. Moreover, indoles were shown to halt the cell cycle in G_1 [21], while ITCs induce cell cycle arrest in G_2/M phase [31].

Thus, the aim of the present report was to quantify combination effects between the broccoli compounds SFN and DIM as representatives for ITCs and indoles, respectively, with regard to cell growth inhibition, cytotoxicity and on cell cycle distribution in the human colon cancer cell line 40-16.

2. Materials and Methods

Chemicals

Sulforaphane (SFN, CAS no. 4478-93-7) was synthesized as described earlier [32]. 3,3'-Diindolylmethane (DIM, CAS no. 1968-05-4) was purchased from LKT Laboratories Inc. (Minnesota, USA). All cell culture material was obtained from Invitrogen (Eggenstein, Germany). Fetal bovine serum was provided by PAA Laboratories (Pasching, Austria). McCoy's 5A cell culture medium, sulforhodamin B (SRB), propidium iodide (PI) and RNase A were obtained from Sigma (Taufkirchen, Germany). All material required for flow cytometry was purchased from Becton Dickinson (Franklin Lakes, NJ USA). The antibody against PARP (#9542) was obtained from Cell Signaling Technology (Beverly, USA). Anti-mouse- and anti-rabbit-secondary antibodies were obtained from Santa Cruz (Heidelberg, Germany). β-Actin antibody AC-15 (A5441) was purchased from Sigma (Taufkirchen, Germany). Materials and equipment for gel electrophoresis were purchased from Bio-Rad (Munich, Germany). All other chemicals were from Sigma (Taufkirchen, Germany).

Inhibition of cell proliferation

The human colon cancer cell line 40-16, derived from a random HCT116 clone, was generously provided by B. Vogelstein from Johns Hopkins Oncology Center (Baltimore, USA). Cells were cultured as described earlier [30].

For combination experiments 40-16 cells (2.5 x 10^4 cells/ml in McCoy's 5A medium) were plated in 96-well plates (200 µl/well). After overnight growth, cell culture medium was changed and cells were treated with SFN or DIM dissolved in DMSO (final DMSO concentration 0.5%) in a

concentration range of 0.3 to 40 µM, respectively. Alternatively, cells were treated with the combination of SFN and DIM (molar ratios 1:4, 1:2, 1:1, 2:1 and 4:1). Eight serial two-fold dilutions of the following starting concentrations were applied: 10 µM SFN + 40 µM DIM (1:4), 20 µM SFN + 40 µM DIM (1:2), 20 µM SFN + 20 µM DIM (1:1), 40 µM SFN + 20 µM DIM (2:1) and 40 µM SFN + 10 µM DIM (4:1). Cells were treated with 0.5% DMSO as solvent control. After incubation for additional 24 h, 48 h or 72 h, respectively, the medium was discarded and cells were fixed using 50 µl of 10% aqueous trichloro acetic acid (TCA) for 30 min at 4 °C. Sulforhodamin B staining was performed as described by Skehan *et al.* [33]. Calculations of anti-proliferative activities were based on the ratio of absorbance readings of treated cells to those of solvent controls. Absorbance readings were corrected for absorbance of cells present at the time of treatment (day 0 values) in order to distinguish between cytostatic and cytotoxic effects. Negative values indicate cytotoxicity. In these cases, day 0 values of the control were used to calculate the percentage of cytotoxicity. IC_{50} values (half maximal inhibitory concentrations) were computed from the results of eight serial two-fold dilutions of test compound tested in duplicate from at least three independent experiments, using Table Curve Windows version 1.0 software (Jandel Scientific, San Rafael, USA) with the equation for a logistic dose-response curve. The percentage of cell growth inhibition was alternatively expressed as affected fraction (*f*a), with values between 0 and 0.5 for cytostatic effects, and 0.5 to 1 for cytotoxic effects.

Calculation of combination effects

Combination effects of SFN and DIM on cell growth inhibition were calculated according to Chou and Talalay [34]. Briefly, for median-effect plots, log (*f*a/*f*u) was plotted against log (*D*), where *D* represents the concentration of each single compound alone or the mixture of both, and *f*a and *f*u stand for the affected (values between 0 and 1) and unaffected (1-*f*a) fraction, respectively, at each concentration *D*.

Using CalcuSyn software (Biosoft, Ferguson, MO USA), which is based upon the method by Chou and Talalay [34], a combination index (CI) was computed for every fraction affected. CI<1, CI=1 or CI>1 represent synergism, additivity or antagonism of SFN and DIM, respectively. For the generation of CI-effect plots, original data points were taken and simulation curves were calculated for each experiment using Table Curve Windows version 1.0 software (Jandel Scientific, San Rafael, USA). These simulation curves were also used for adjusting CI values to fixed total concentrations.

Cell Cycle Analysis

40-16 cells were plated in 150 mm tissue culture dishes (1.75×10^6 cells/35 ml) and treated as indicated in figure legends after overnight growth. Attached and floating cells were collected, washed with PBS, fixed in ice cold 70% ethanol and stored at –20 °C. After washing twice with PBS, cells were incubated with PBS containing propidium iodide (50 µg/ml) and RNase A (100 µg/ml) for 30 min at 37 °C. Then they were analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA). After discrimination of doublets, the percentage of cells in each phase of the cell cycle was determined by setting markers with CELL QUEST PRO software (Becton Dickinson, Franklin Lakes, NJ, USA).

Western Blot Analysis

40-16 cells were plated in 100 mm tissue culture dishes (2.5×10^5 cells/10 ml) and treated as indicated in the figure legend after overnight growth. Attached and floating cells were collected, lysed and homogenized in SDS lysis buffer (62.5 mM Tris HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 50 mM DTT, 0.01% (w/v) bromphenol blue). The protein content was determined using the bicinchoninic acid method [35] after precipitation with cold 10% TCA. Total protein (ca. 15 µg/lane) was subjected to 10% acrylamide SDS-PAGE under standard conditions and electroblotted onto polyvinylidene difluoride membranes in 15% methanol, 25 mM Tris, and

192 mM glycine. The membranes were blocked with 5% nonfat milk in Tris-buffered saline (10 mM Tris, pH 7.4, 100 mM NaCl) containing 0.05% Tween 20 for 1.5 h at RT and incubated with anti-PARP antibody (1:1000) overnight at 4 °C. After washing, the membranes were incubated with secondary antibody (1:10 000) conjugated with horseradish peroxidase for 1 h at RT. The membranes were developed using a chemiluminescence system. Equal protein loading per lane was ensured by using an anti-β-actin antibody (1:10 000).

Statistical Analysis

Results are presented as means ± standard deviation of data originating from at least three independent experiments. For statistical evaluation one-way ANOVA and Tukey's studentized range test was applied. Values of $p < 0.05$ were considered as statistically significant.

3. Results

Anti-proliferative effects of SFN and DIM

Incubation of 40-16 human colon cancer cells with SFN or DIM (**Figure 1**) at a concentration range of 0.3 to 40 µM led to a dose-dependent inhibition of cell proliferation. IC_{50} values obtained after 24 h, 48 h and 72 h of treatment were 6.8 ± 0.4 µM, 6.6 ± 0.5 µM, and 8.2 ± 0.4 µM for SFN, and 11.6 ± 1.7 µM, 10.1 ± 1.9 µM, and 6.8 ± 0.9 µM for DIM, respectively. Interestingly, after an incubation time of 24 h, SFN was significantly more effective in suppressing cell growth than DIM ($p = 0.009$), whereas after 72 h, DIM had a lower IC_{50} value than SFN. Accordingly, SFN was less toxic after 72 h compared to 24 h and 48 h incubations, whereas DIM's effectiveness increased with elongated treatment times.

Comparison of growth inhibition profiles clearly indicated that SFN was cytotoxic at concentrations higher than 10 µM, demonstrated by *f*a values higher than 0.5 (as described in *Materials and Methods*). On the other hand, the dose-response curves obtained with DIM revealed a cytostatic profile, as shown in a representative dose-effect plot (**Figure 2**). These results are in congruence with our recently reported observations [30].

Anti-proliferative effects of combinations of SFN and DIM

To determine combination effects of SFN and DIM on cell proliferation, cells were treated with constant molar ratios of the two drugs (1:4, 1:2, 1:1, 2:1 and 4:1) using eight serial two-fold dilutions of each mixture for 24 h, 48 h and 72 h. As an example, a dose-effect curve of the 1:1 mixture after 48 h of incubation is included in **Figure 2**. Notably, at the two highest total concentrations (20 and 40 µM), the fraction *fa* affected by the mixture was lower than that of SFN, but higher than that of DIM. In contrast, total concentrations below 20 µM inhibited cell growth less than either compound alone.

Quantification of combination effects according to Chou and Talalay

The method by Chou and Talalay is a widely accepted approach to analyze synergistic, additive or antagonistic effects of two compounds [34]. The first step of the procedure is to assess mutual exclusivity of the test compounds by generating median-effect plots, with log (*fa*/*fu*) plotted against log (*D*) (see *Material and Methods*). Parallelism of the regression lines obtained for either compound alone and the mixture of both indicates that two drugs are mutually exclusive, *i.e.* they have the same target or mode of action. If the lines are not in parallel, the tested compounds are mutually non-exclusive, *i.e.* they act independently or have different modes of action. As shown in **Figure 3**, the regression lines of SFN, DIM and the 1:1 mixture were not in parallel. We therefore concluded that SFN and DIM are mutually non-exclusive and inhibit cell proliferation by different mechanisms. Additional median-effect plots generated for all combination experiments with molar ratios of SFN and DIM of 4:1, 2:1, 1:1, 1:2, and 1:4, and for 24 h, 48 h, and 72 h of incubation, respectively, further confirmed these results (data not shown).

In order to determine whether SFN and DIM influenced each other in a synergistic, additive or antagonistic manner, we used the CalcuSyn software to determine combination indices (CI) for all combinations and incubation times described above. CI values lower and higher than 1 indicate synergism and antagonism, respectively, whereas additive effects result in a combination index of

1. A representative CI-effect plot for the 1:1 combination of SFN and DIM after 48 h of treatment is depicted in **Figure 4**. At low effects provoked by high dilutions of the SFN:DIM combination, CI values were between 1 and 3, indicating that cell proliferation was affected in an antagonistic manner. However, CI values decrease with increasing effects. At the highest effects observed with highest SFN:DIM concentrations, CI values were even below 1, demonstrating that cytotoxic concentrations of the 1:1 combination inhibit cell proliferation synergistically.

SFN and DIM are antagonistic at low and synergistic at high concentrations

Since the effects of SFN:DIM combinations could not be directly compared due to different total concentrations, we adjusted the obtained CI values to the fixed total concentrations of 2.5, 5, 10, 20 and 40 µM by generating simulation curves from the original results. Data for five SFN:DIM combinations and increasing incubation times are summarized in **Figure 5**. In agreement with the results described above, CI values computed for low combined drug concentrations were higher than 1, indicating antagonism in terms of cell growth inhibition. The highest CI value of 4.2 ± 2.2 was calculated for the 1:4 combination after 24 h of treatment. Increasing concentrations resulted in decreasing CI values. Combinations of SFN and DIM showed synergistic effects (CI < 1) only at the highest total drug concentration (40 µM), except for the 1:4 combination after 72 h of treatment. Synergism was strongest when the cells were treated with the 1:2 mixture of SFN and DIM for 24 h. At a total concentration of 20 µM, the two compounds influenced each other either additively (e.g., CI = 1.0 for the 1:2 combination after 24 h) or antagonistically (e.g., CI = 1.6 for the 1:1 combination after 72 h).

Taken together, low to moderate total concentrations of SFN:DIM combinations (2.5 to 20 µM) appear to inhibit proliferation of 40-16 cells in an antagonistic manner, whereas at cytotoxic total concentrations of 40 µM or higher, the compounds act in a synergistic manner. Overall, depending on the total concentration, strongest antagonistic and synergistic effects were observed i) after short incubation times, and ii) when DIM was present in excess of SFN.

Cell cycle effects of SFN, DIM and their combinations

Previous studies have established that SFN and DIM both halt cell cycle progression in cultured tumor cells. Notably, while in many cancer cell lines SFN induces cell cycle arrest in G_2/M phase [31], DIM provokes a stop in G_1 [21]. We therefore addressed the question how SFN:DIM combinations would affect cell cycle progression of 40-16 cells. We performed cell cycle analyses with cells treated with either SFN (5 µM or 10 µM) or DIM (10 µM) alone, or mixtures thereof. We used the same fixed molar ratios as described for cytotoxicity experiments, with the exception of combinations with an excess of SFN (SFN:DIM 2:1 and 4:1). Preliminary experiments had indicated that SFN in excess of DIM completely abrogated DIM's effects on cell cycle progression. Cells were arrested in G_2/M phase similarly as with SFN alone (data not shown). Thus, we tested 5 µM SFN in combinations with increasing concentrations of DIM (5, 10 and 20 µM, molar ratios of 1:1, 1:2, and 1:4). Additionally, cells were treated with a 1:1 mixture of 10 µM of each compound.

After 24 h of treatment, DIM (10 µM) and the lower dose of SFN (5 µM) as well as the combination of 5 µM SFN + 5 µM DIM had no effect on the sub-G_1 fraction, whereas SFN at a 10 µM concentration significantly induced a sub-G_1 peak indicative of apoptosis induction (**Figure 6**). Addition of increasing concentrations of DIM to 5 µM of SFN resulted in increasing effects. Strongest apoptosis induction was observed with the combination containing 10 µM SFN + 10 µM DIM (23.9 ± 2.2% sub-G_1). Increases in sub-G_1 fractions were accompanied by significant reduction in S phase as a further sign of cell growth inhibition. Neither treatment with the compounds alone nor with combinations influenced the fraction of cells in G_1 or in G_2/M phase, with the exception of the 10 µM SFN + 10 µM DIM mixture, which provoked a strong G_2/M arrest (52.8 ± 5.2% *vs.* 26.8 ± 2.3% in untreated control cells). Consequently, the fraction of cells in G_1 was significantly reduced (15.5 ± 5.1%) in comparison with the control (44.0 ± 3.1%).

After 48 h of treatment, SFN (5 µM) and DIM (10 µM) had weak, but non-significant effects on cell cycle distribution. Further, combining 5 µM SFN with 5 µM or 10 µM DIM did not lead to a significant enhancement of effects when compared with the untreated control or with 5 µM SFN

or 10 µM DIM, respectively. This may point to an antagonistic interaction. SFN at a 10 µM concentration was more potent in inducing the percentage of cells in sub-G_1 (28.6 ± 5.8%) than 5 µM SFN + 20 µM DIM. Addition of 10 µM DIM to 10 µM SFN further increased the sub-G_1 peak induced by SFN (41.1 ± 6.5%). Concomitantly, the percentage of cells in G_1 and S phase was significantly reduced by these treatment regimens. As observed after 24 h, the combination of 10 µM SFN with 10 µM DIM caused a significant G_2/M cell cycle arrest.

Effects of SFN, DIM and their combinations on PARP cleavage

To confirm the apoptosis-inducing potential of SFN and DIM combinations, we analyzed cleavage of the DNA repair enzyme PARP [poly (ADP-ribose) polymerase] by Western blotting (**Figure 7**). After 24 h of treatment, SFN alone at a concentration of 10 µM, as well as the 10 µM SFN + 10 µM DIM mixture induced PARP cleavage, which is consistent with the increase of the sub-G_1 fraction observed with cell cycle analyses. Treatment with 5 µM SFN + 20 µM DIM for 24 h was not sufficient to trigger PARP cleavage.

After 48 h, PARP cleavage was markedly induced by SFN (10 µM) and the mixtures of 10 µM SFN + 10 µM DIM and 5 µM SFN + 20 µM DIM. The effect produced by the latter combination was approximately equivalent to the 10 µM SFN + 10 µM DIM mixture after 24 h, which is consistent with sub-G_1 peak induction.

4. Discussion

In the present study, we have demonstrated that combinations of SFN and DIM, two chemopreventive compounds derived from cruciferous vegetables like broccoli, inhibit the growth of cultured human colon cancer cells antagonistically at low concentrations, but synergistically at cytotoxic concentrations. First, we determined the anti-proliferative potential of SFN and DIM alone in the 40-16 human colon carcinoma cell line. IC_{50} values as low as 6.6 to 11.6 µM underline the strong ability of SFN and DIM to inhibit tumor cell growth. We confirmed that SFN is a cytotoxic agent, whereas DIM has a cytostatic profile of cell growth inhibition [30]. Also,

SFN and DIM were found to be mutually non-exclusive, which is not surprising, since fundamental differences in the growth inhibition profiles of the two compounds as well as different effects on cell cycle distribution have been reported previously [21,30,31].

A dose-response curve obtained with a 1:1 mixture of SFN and DIM in comparison with the single compounds clearly indicated that at high total concentrations (20 µM SFN and 20 µM DIM), the mixture was more active than either compound alone. In contrast, at low total concentrations, the dose-response curve of the mixture was below those of SFN and DIM alone, suggesting antagonistic interaction. This observation prompted us to quantitate combination effects of SFN and DIM using the method of Chou and Talalay [34]. Results of these analyses indicated that combinatory interactions between SFN and DIM are strongly dose-dependent, in that low combined concentrations are antagonistic, whereas cytotoxic concentrations are synergistic, regardless of the ratio applied. A possible explanation for this phenomenon might be that at low concentrations, mechanisms other than cell cycle arrest or apoptosis induction play a more prominent role for chemopreventive potential. In a majority of *in vitro* studies on apoptosis induction, including our investigation in the 40-16 cell line, SFN or DIM had to be applied at concentrations of at least 10 µM to induce apoptosis [30,36,37]. In contrast, SFN at concentrations as low as 1 - 5 µM was very effective in inducing phase II detoxification enzymes [38-40], whereas DIM affected biotransformation enzymes only at concentrations higher than 20 µM [21,38,41]. Therefore, treatment with low doses of SFN might cause increased 'detoxification' of the compounds themselves or of reactive intermediates involved in inhibition of cell proliferation, resulting in the observed antagonistic effects.

In the present study, only relatively high concentrations of combinations of SFN and DIM were able to inhibit cell proliferation or induce cell death in a synergistic manner, whereas intermediate concentrations (SFN + DIM = 20 µM) were weakly antagonistic or additive. Since SFN and DIM alone induce G_2 and G_1 cell cycle arrest, respectively [21,31], antagonism could also be explained by these opposing effects. To address this question, we performed cell cycle analyses with mixtures of SFN and DIM in ratios of 1:4, 1:2 and 1:1, with total drug concentrations ranging

from 10 to 25 µM. Due to the distribution of analyzed cells to four different fractions (sub-G_1, G_0/G_1, S, and G_2/M), flow cytometry data were not suitable to calculate combination indices. Therefore, influences on cell cycle phases had to be compared directly.

Again, effects of SFN, DIM and their combinations strongly depended on total drug concentrations tested. Most interesting effects were observed with SFN alone at a concentration of 10 µM and mixtures of either 5 µM SFN + 20 µM DIM or 10 µM SFN + 10 µM DIM, whereas DIM at a 10 µM concentration did not cause any change in cell cycle distribution. Notably, addition of 10 µM DIM to 10 µM SFN significantly arrested cells in G_2/M and enhanced the effect of SFN alone (10 µM) with respect to reduction of the percentage of cells in G_1 and S phase as well as induction of a sub-G_1 peak. Since the 10 µM SFN + 10 µM DIM combination affected all phases of the cell cycle, the net result may be inhibition of cell proliferation, but not necessarily a strong enhancement of cytotoxic effects. This was confirmed when cell death was analyzed by the detection of PARP cleavage with Western Blotting. Cell cycle effects of the 5 µM SFN + 20 µM DIM mixture resembled those of SFN at a 10 µM concentration, although PARP cleavage was only detectable after 48 h of treatment with the combination. These data indicate that a threshold concentration of around 10 µM of SFN is necessary to sensitize cells to cell death, but DIM addition might modulate cell growth inhibition by enhancing the G_2/M arresting potential of SFN. Numerous attempts have been made to elucidate the mechanism how SFN induces G_2/M arrest. Reactive oxygen species-mediated DNA damage as well as disruption of tubulin polymerization have been proposed [12,42]. Further investigations have to clarify how DIM interacts with these mechanisms. Since SFN has also been described to cause G_1 arrest, e.g. in the HT-29 colon cancer cell line [43], further investigations with other cells line will be required to confirm the general validity of these results.

In terms of synergistic or antagonistic interactions between SFN and DIM, data obtained from cell cycle and Western blot analyses are consistent with data of the cell growth experiments. Low concentrations of combinations seem to be antagonistic, whereas higher total concentrations seem to act rather additive or weakly synergistic, especially in the case of the 10 µM SFN + 10 µM

DIM combination. Although the impact on cell cycle phases differed depending on the ratio of SFN and DIM, CI values obtained from cytotoxicity data were similar regardless of the applied ratio of the two drugs.

A dose-dependent switch from antagonistic to synergistic interaction between two compounds has been reported before. Khafif *et al.* observed synergism of the green tea compound (-)-epigallocatechin-3-gallate (EGCG) and the spice curcumin in inhibiting cell growth of differentially transformed human oral epithelial cell lines [44]. The authors described antagonistic interactions at low doses of the drug combination, which were selective for normal or less progressed cells, but a synergistic growth-inhibitory effect on malignant cells, implicating a protective effect for normal tissues. In PC-3 human prostate cancer cells, curcumin in combination with phenethyl isothiocyanate induced apoptosis in an additive manner [45]. In this study, experiments were limited to concentrations relevant for apoptosis induction; therefore combination effects at lower concentrations were not determined. The same group also investigated the combined inhibitory effects of curcumin and PEITC on the growth of human PC-3 prostate xenografts in immunodeficient mice. While PEITC or curcumin alone had little effect, the mixture of both significantly reduced the growth of PC-3 xenografts [46].

Broccoli extracts represent a natural combination of ITCs and indoles. In mature broccoli about 70% of the glucosinolates are indole-based. The main compound is glucobrassicin as the precursor of DIM. The rest consists of different other glucosinolates, especially glucoraphanin, the cleavage of which leads to the formation of SFN [47]. In a very recent study broccoli extracts induced G_2/M cell cycle arrest and apoptosis in cultured rat glial cells [48]. Although the authors did not quantify concentrations of glucosinolate cleavage products, in comparison with the results presented here it can be speculated that either SFN was present in excess or that its concentration was high enough to induce a halt of the cell cycle in G_2/M. Another report described mitochondria-mediated apoptosis and S-/M phase arrest in human bladder carcinoma cells induced by broccoli sprout extract [49]. Broccoli sprouts primarily (> 90%) contain glucoraphanin and consequently SFN after conversion to ITCs [47]. Notably, comparison with synthetic SFN

revealed that the anti-proliferative potency of the extract was almost identical to that of the single compound, suggesting that non-ITC substances in the extract may not interfere with growth-inhibitory activity of ITCs [49]. In future studies, combinations of SFN or DIM together with other chemopreventive compounds should be conducted to investigate potential synergistic effects. With this respect, DIM and paclitaxel were shown to synergistically induce apoptosis in HER2/Neu human breast cancer cells [50].

Although progress is made in understanding combinatory effects between bioactive compounds present in natural foods, more research efforts are needed in order to elucidate mechanistic interactions and dose-dependent differences in the outcomes of combination treatments. The concept of combination chemoprevention should be paid more attention as it holds great potential for targeted prevention or preventory treatment of malignancies while causing little side effects. Transfer of promising findings achieved with single food components to the consumption of whole foods should however be done with caution.

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Figure legends:

Fig. 1. Chemical structures of SFN and DIM.

Fig. 2. Profiles of cell growth inhibition. Growth curves of SFN (○), DIM (▼) and the 1:1 mixture of SFN and DIM (□), respectively, with cultured 40-16 cells. Inhibition of cell proliferation was determined by sulforhodamine B staining. Cells were treated with eight different concentrations of SFN and DIM alone (0.3 to 40 µM), a 1:1 mixture of SFN and DIM (0.15 to 20 µM of each drug) or 0.5% DMSO as a solvent control for 48 h. Mean values from three independent experiments (± SD) are expressed as affected fraction (*f*a) compared with control cells. Dose-dependent inhibition of proliferation is indicated by *f*a values between 0 and 0.5, whereas values above 0.5 represent cytotoxic effects.

Fig. 3. Median-effect plot of 40-16 cells treated for 48 h with SFN (○), DIM (▼), a 1:1 mixture of SFN and DIM (□), respectively, or 0.5% DMSO as a solvent control. Data were taken from one representative cell proliferation experiment. Log (*f*a/*f*u) was plotted against log (*D*), whereby *f*a and *f*u stand for affected fraction and unaffected fraction, respectively, and *D* stands for the concentration.

Fig. 4. CI-effect plot. CI (combination index) values obtained from cell proliferation experiments with 40-16 cells treated for 48 h with a 1:1 mixture of SFN and DIM or 0.5% DMSO as a solvent control were plotted against the effects (equivalent to affected fraction *f*a) mediated by five different concentrations (2.5, 5, 10, 20 and 40 µM total concentration) of this drug combination, respectively. Data are derived from three independent experiments.

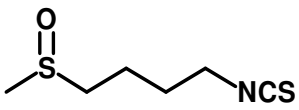
Fig. 5. CI values of combinations of SFN and DIM. 40-16 cells were treated with combinations of SFN and DIM in ratios of 1:4, 1:2, 1:1, 2:1 and 4:1 or 0.5% DMSO as a solvent control for 24 h

(**A**), 48 h (**B**) and 72 h (**C**). CI values were calculated as described in Materials and Methods. All original data were normalized to fixed total concentrations of 2.5, 5, 10, 20 and 40 µM as indicated. Data are means ± SD from three independent experiments (exception: n=2 for the 1:4 combination after 72 h).

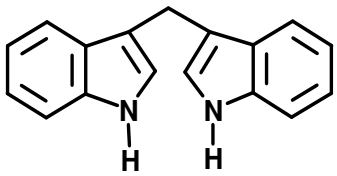
Fig. 6. Cell cycle analyses. 40-16 cells were treated with SFN alone, DIM alone, combinations of SFN and DIM at ratios of 1:4, 1:2 and 1:1, or 0.5% DMSO as a solvent control for 24 h (**A**) and 48 h (**B**). The concentrations used are indicated in the figure. Cell cycle analyses of fixed cells were performed by flow cytometry after staining DNA with propidium iodide. Bars represent the percentage of cells in sub-G_1, G_0/G_1, S and G_2/M cell cycle phases. Data are means ± SD from at least three independent experiments. *Means significantly ($p < 0.05$) different compared to respective control using one-way ANOVA und Tukey's studentized range test. [$]Means significantly ($p < 0.05$) different compared to SFN at a 5 µM concentration in the case of the 5 µM SFN + 20 µM DIM combination or to SFN at a 10 µM concentration in the case of the 10 µM SFN + 10 µM DIM combination, respectively. [d]Means significantly ($p < 0.05$) different compared to DIM at a 10 µM concentration.

Fig. 7. Detection of apoptosis induction by PARP cleavage. Immunoblotting for PARP using lysates from 40-16 cells treated with 0.2% DMSO (–), SFN, and DIM, alone and in combinations, respectively, for 24 h and 48 h as indicated. PARP cleavage was investigated by Western blotting using an antibody directed against full length PARP (116 kDa) and the cleavage product (89 kDa). Equal loading was confirmed by using an anti-β-actin antibody. One of two blots is shown.

Figure 1



Sulforaphane (SFN)



3,3'-Diindolylmethane (DIM)

Figure 2

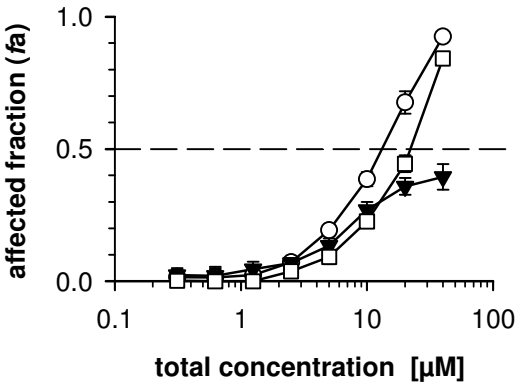

1.0
0.5
0.0
affected fraction (fa)
0.1
1
10
100
total concentration [µM]

Figure 3

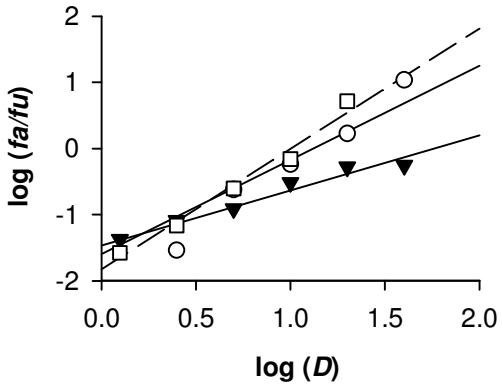


Figure 4

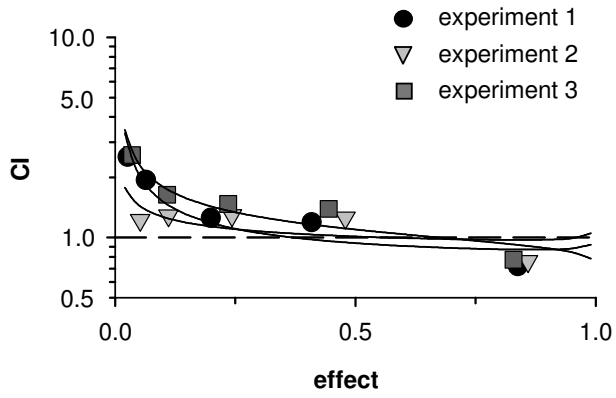

experiment 1
experiment 2
experiment 3
10.0
5.0
1.0
0.5
CI
0.0
0.5
1.0
effect

Figure 5

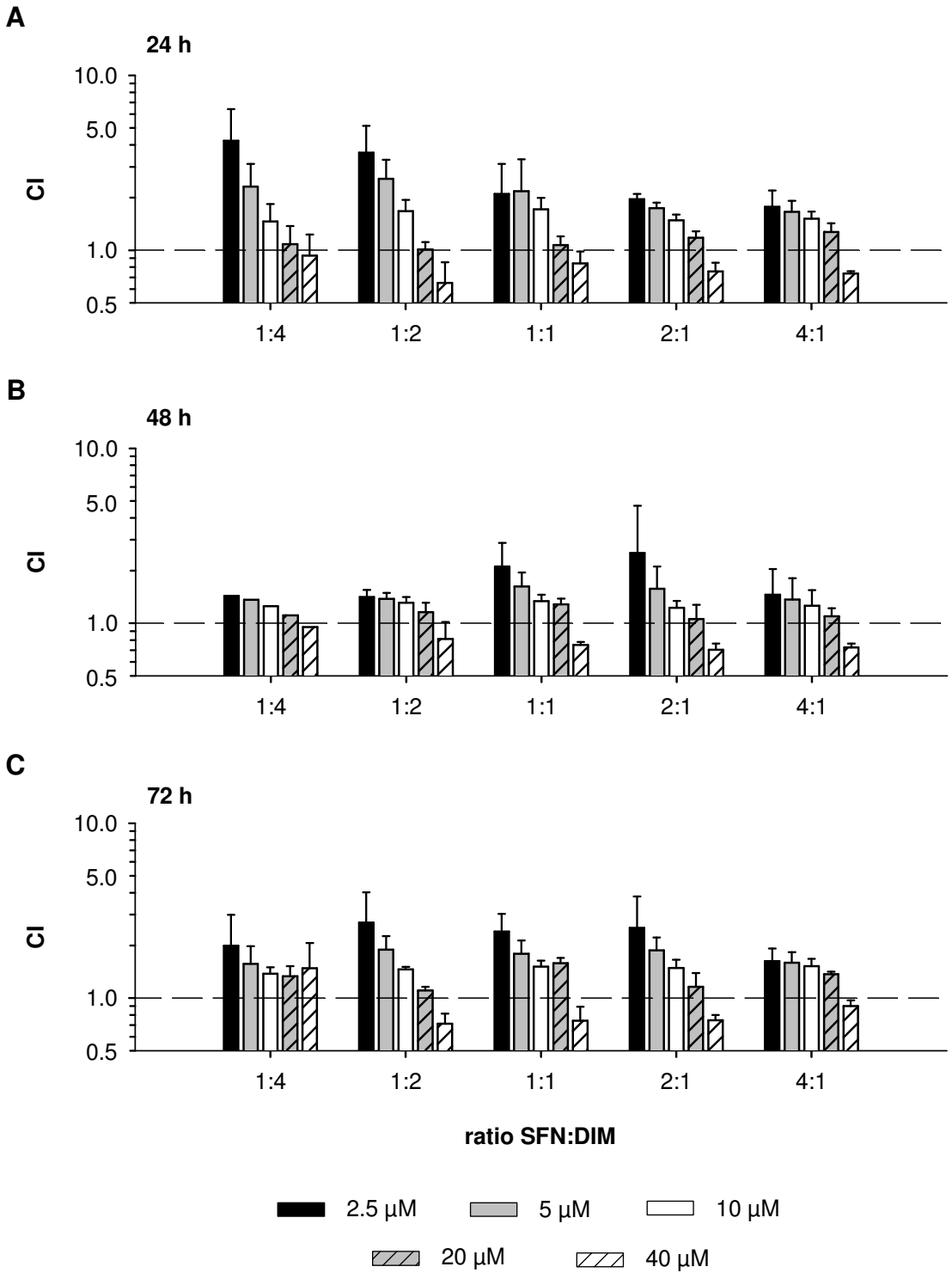
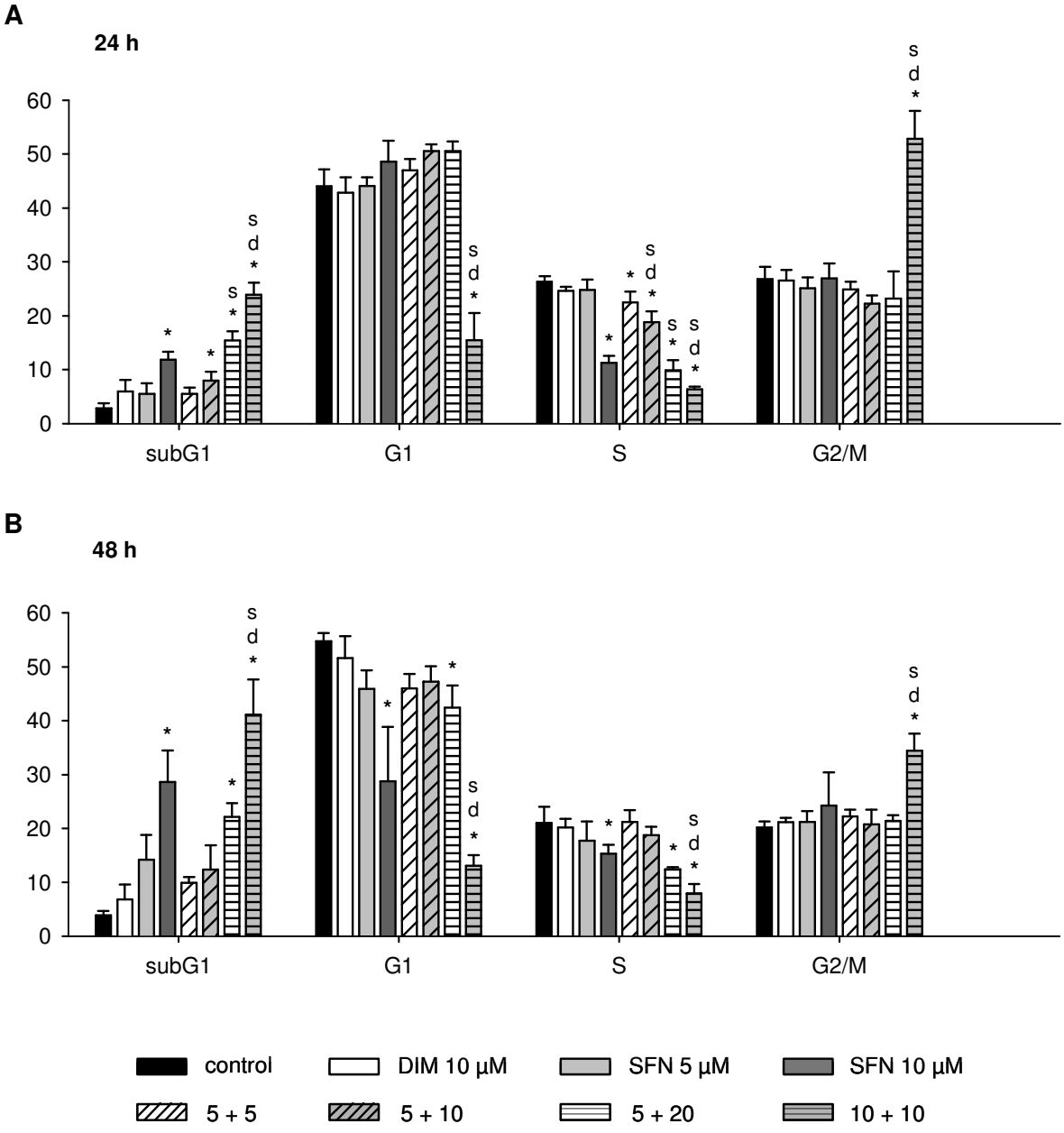

A
24 h
B
48 h
C
72 h
CI
10.0
5.0
1.0
0.5
1:4
1:2
1:1
2:1
4:1
ratio SFN:DIM
2.5 µM
5 µM
10 µM
20 µM
40 µM

Figure 6


A
24 h
60
50
40
30
20
10
0
subG1
G1
S
G2/M
B
48 h
control
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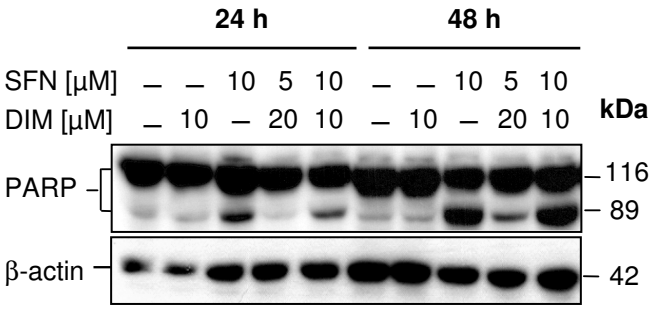
SFN 10 µM
5 + 5
5 + 10
5 + 20
10 + 10

Figure 7


24 h
48 h
SFN [µM] – – 10 5 10 – – 10 5 10
DIM [µM] – 10 – 20 10 – 10 – 20 10
kDa
PARP
116
89
β-actin
42

IV. DISCUSSION

According to epidemiological studies, a high consumption of brassica vegetables like broccoli, cabbage, Brussels sprouts or cauliflower is associated with a lower risk of developing cancer. This has mainly been attributed to secondary plant ingredients present in cruciferous vegetables, namely glucosinolates. Glucosinolates are thioglucosides, which are cleaved into isothiocyanates and indole derivates by the plant-endogenous enzyme myrosinase upon plant cell damage. One of the most prominent and intensively studied isothiocyanates is sulforaphane (SFN). SFN is especially known for its ability to modulate xenobiotic biotransformation by inhibiting phase I- and inducing phase II enzymes. Chemopreventive efficacy of SFN has been demonstrated in several animal models. In recent years, SFN was shown to affect other anticancer mechanisms as well, including the induction of cell cycle arrest and apoptosis in cultured cancer cells. To elucidate the molecular mechanisms of SFN's action is essential for the development of functional food/agents suitable for chemopreventive or -therapeutic applications.

The results of the present thesis contribute to a better understanding of the mechanisms involved in SFN-induced apoptosis induction. In the human colon cancer cell line 40-16 SFN is shown to induce a marked cell cycle arrest in M phase due to microtubule disruption and activation of the spindle checkpoint. This mitotic arrest was followed by apoptosis, which was demonstrated to depend on caspase activity. Further mechanistic investigations revealed that the mitochondrial pathway of apoptosis was activated, since SFN-induced apoptosis was blocked in Bcl-2 overexpressing Jurkat leukemia cells. The inhibition of NF-κB activation facilitated apoptosis induction with the result of a decreased expression of anti-apoptotic proteins like XIAP, Bcl-2 and c-FLIP **(Manuscript 1)**.

In the literature it is still a matter of controversy whether SFN-induced cell cycle arrest is due to an activation of the DNA damage checkpoint or the spindle checkpoint. The primary effect of SFN for cell cycle arrest or apoptosis induction is also unclarified: Do these two effects occur independently from each other or is one the prerequisite for the other? In the present study, it could clearly be shown that the mitotic arrest was SFN's

primary effect, and apoptosis a consequence of spindle disruption (**Manuscript 1**). The applied methodology was Flow cytometric analysis of cells stained with the mitosis-specific antibody MPM-2. The results were confirmed by using the well-known antimitotic agent paclitaxel as positive control. In extension of these experiments and to further confirm the results, immuncytochemistry with an anti-tubulin antibody might be performed to score the abundance of prophase/promethaphase-like mitotic figures. In addition, it would be interesting to analyse factors involved in the regulation in G_2/M transition like Cdk1 and cyclin B1 in terms of gene expression, protein levels and phosphorylation status. To exclude the possibility that SFN induces the DNA damage checkpoint in the 40-16 cell line, the DNA damaging agent Methyl methanesulfonate (MMS)-induced G_2/M arrest was demonstrated to be abrogated by caffeine, which is known to block DNA damage-induced cell cycle arrest in G_2. Since the G_2/M phase arrest induced by SFN was not inhibited by caffeine it was concluded that SFN does not induce the DNA damage checkpoint. However, these investigations were of rather indirect nature. To confirm the results, the genotoxic potential of SFN should be analyzed directly, e.g. by performing the Comet assay.

In a recent report Singh *et al.* suggested that the formation of reactive oxygen species (ROS) may be the initial signal for SFN-induced apoptosis [Singh *et al.*, 2005]. The authors used cultured PC-3 prostate cancer cells for their experiments and found mitochondrial- as well as nonmitochondrial mechanisms involved in ROS production. However, the same authors also reported a ROS-mediated G_2 cell cycle arrest and the induction of autophagy by SFN preceding apoptosis, all in the PC-3 cell line [Singh *et al.*, 2004; Herman-Antosiewicz *et al.*, 2006]. It was suggested that apoptosis and the G_2 arrest were induced by SFN independently, and that apoptosis could also be a result of the cell cycle arrest. Autophagy was suggested to inhibit apoptosis induction by SFN and might be the reason for the delayed cell death.

In the present study, ROS formation after SFN treatment for 1 h, 3 h and 6 h was investigated in the 40-16 cell line using the ROS-sensitive dye 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and Flow cytometric analysis. No significant elevation of ROS was detectable for any time point, in contrast to the above-mentioned studies. Thus, SFN's mechanisms of action seem to depend strongly on the cell type. A reduction of intracellular glutathione (GSH) levels is often associated with

oxidative stress and the formation of ROS [Townsend *et al.*, 2003]. Although no increase in ROS was observed in the present thesis, a significant GSH depletion was detected, peaking in the time range of four to six hours of treatment with SFN. At subtoxic concentrations, GSH levels were induced again after 24 h due to enhanced resynthesis **(Manuscript 2)**. These results led to the conclusion that GSH depletion was not a prerequisite for SFN-induced apoptosis. Supporting this, significant GSH depletion induced by L-buthionine-S,R-sulfoximine (BSO), a specific inhibitor of γ-glutamyl-cysteine synthetase, did not result in any cytotoxic effects in the 40-16 cell line.
For determining GSH levels, a colorimetric assay based on the oxidation of thiols by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; also called Ellman's reagent) was performed. This is a well-accepted method to measure the cellular GSH content, although strictly speaking not only GSH, but also other thiols present in the cell are detected. To get a more precise picture of changes in the levels of reduced and oxidized GSH, either HPLC analysis or the fluorescence dyes monochlorobimane or monobromobimane should be used, which form fluorescent adducts with reduced GSH upon catalytic activity of GSTs. For the analysis of ROS formation, cell staining with the dye H_2DCFDA could be performed in 96-well plates rather than for Flow cytometric analysis in order to reduce trypsinization stress and the consequent high background levels of ROS.

In the present study, as well as in other studies, SFN was shown to inhibit the activation of NF-κB **(Manuscript 1)**. Among others, oxidative stress within the cell is a common trigger for NF-κB activation. Therefore the question remains whether an increase in ROS and the inhibition of NF-κB activation can be induced by SFN simultaneously. In most studies, this has not been investigated in a single cell line with the same SFN concentration. At relatively high concentrations (25 and 50 µM) SFN even activated NF-κB along with increased ROS production in Caco-2 human colon cancer cells [Jakubikova *et al.*, 2006].
Due to these discrepancies, it can be hypothesized that not ROS formation but rather thiol reactivity may be the main mode of action of SFN. SFN is known to be a reactive Michael acceptor type-compound, which rapidly binds to thiol groups, leading to dithiocarbamate formation [Zhang & Talalay, 1994]. SFN-mediated induction of phase II enzymes was found to be due to its thiol reactivity. Binding of SFN to specific sulfhydryl groups of the sensor protein Keap1 activates the Nrf2 pathway, leading to ARE-mediated

induction of phase II detoxifying enzymes [McMahon *et al.*, 2001; Dinkova-Kostova *et al.*, 2002].

An interaction of SFN with thiol groups has also been proposed as a mechanism underlying SFN-mediated NF-κB inhibition. In Raw 264.7 macrophages SFN prevented active NF-κB from binding to its nuclear DNA sites via a thiol-mediated mechanism, presumably by dithiocarbamoylation of NF-κB subunits or of factors involved in the redox regulation of NF-κB [Heiss *et al.*, 2001]. NF-κB requires oxidative conditions for activation and nuclear translocation, but a reductive environment for DNA binding in the nucleus [Anderson *et al.*, 1994]. However, in the present study SFN inhibited NF-κB upstream of its translocation into the nucleus **(Manuscript 1)**. Thus, the above-mentioned mechanism can not apply for the 40-16 cell line. Since others have shown that SFN treatment resulted in reduced phosphorylation of IKK-β [Xu *et al.*, 2005], it can be speculated that SFN may interfere with the most upstream signaling events of the NF-κB pathway by specific thiol-modifications. Future investigations should verify that these mechanisms are not cell-type specific and e.g. also apply for the cell line used in this study.
In order to understand the importance of NF-κB inhibition in SFN-induced apoptosis induction more mechanistic investigations are needed. One possibility would be to generate cell lines stably overexpressing p65- or p50 NF-κB and to see whether a strong constitutive NF-κB activation renders the cells resistant toward SFN treatment.

In the present thesis kinetic experiments were performed with SFN in order to investigate the effects of short exposure times, as well as the effect of drug removal on cell cycle arrest and apoptosis in the 40-16 cell line. It could be demonstrated that cell growth inhibition by SFN follows a biphasic pattern: transient exposure for up to 6 h resulted in reversible G_2/M cell cycle arrest and cytostatic growth inhibition even at elevated concentrations, whereas a minimum continuous exposure time of 12 h was necessary for SFN to irreversibly arrest cells in G_2/M and subsequently induce apoptosis **(Manuscript 2)**. These findings are of major importance when results of *in vitro* studies are transferred to the human situation. Due to rapid metabolism of SFN in the human body it is unlikely that a continuous exposure of 12 h or longer is achievable. In the colon however, also

taking the local release of SFN from intact glucosinolates into account, a longer exposure time could be feasible. These matters should be investigated in future studies. Apart from the effect of a single treatment with SFN, the impact of repeated dosing in well-defined time intervals should also be analyzed *in vitro* to see whether that could be equally effective compared with continuous dosing. In most animal studies treatments are performed in intervals to observe long-term effects. As an alternative, the agent to be administered is given with the food, so that dosing intervals depend on the eating habits of the animals. With both treatment regimens, chemopreventive efficacy of SFN in the intestine could be demonstrated: by the suppression of AOM-induced ACF formation in the rat colon, and by the inhibition of tumorigenesis in $Apc^{Min/+}$ mice, respectively [Chung *et al.*, 2000; Hu *et al.*, 2006; Myzak *et al.*, 2006]. In one of the studies conducted with $Apc^{Min/+}$ mice, gene expression analysis of small intestinal polyps revealed that genes involved in apoptosis and cell growth regulation were modulated strongest by SFN treatment [Khor *et al.*, 2006]. Moreover, SFN selectively induced apoptosis in the intestinal adenomas but not in the normal mucosa [Hu *et al.*, 2006]. In conclusion of these findings, apoptosis induction definitely plays a role *in vivo*. Consistently, Conaway *et al.* detected a significant induction of apoptosis markers in lung tumors of mice treated with SFN after tumor initiation by tobacco carcinogens [Conaway *et al.*, 2005]. Taken together, these results clearly show that concentrations relevant for apoptosis induction can be reached in tumor cells of animals orally receiving SFN, and that the duration of exposure to the chemopreventive agent was obviously long enough to effectively induce apoptosis.

One concern often raised with respect to apoptosis induction as a chemopreventive mechanism is whether cell death induction selectively takes place in initiated cells while normal cells are left unaffected. Studies with cultured cells have indicated that those cell lines with a higher malignancy status were more sensitive toward SFN treatment than more differentiated cell lines. Gamet-Payrastre *et al.* reported that the colon cancer cell line HT29 was significantly more susceptible toward SFN than undifferentiated Caco-2 cells, also derived from a colon cancer, indicating selective cytotoxicity [Gamet-Payrastre *et al.*, 1998]. In another study the nontumorigenic colon cell line HCEC was resistant to SFN treatment with no signs of apoptosis induction as opposed to the transformed colon cancer cell lines LS-174 and Caco-2 [Bonnesen *et al.*, 2001]. In contrast to these studies,

others observed marked apoptosis induction in non-transformed human T lymphocytes after treatment with SFN, similar to that in Jurkat T leukemia cells [Fimognari *et al.*, 2003]. Nevertheless, in animal studies no adverse effects were observed in rats or mice treated with SFN [Chung *et al.*, 2000; Hu *et al.*, 2006; Myzak *et al.*, 2006].

SFN appears to exert its anti-proliferative effects selectively in tumor cells. A possible reason for this phenomenon could be the fact that tumor cells are actively dividing, whereas normal cells usually have a lower mitotic index, especially when they are differentiated. In the colon, epithelial cells undergo a rapid turnover and are constantly renewed by newly differentiated cells derived from stem cells located in the crypts of the colon. Thus, those cells exposed to the lumen are non-dividing differentiated cells and presumably relatively resistant toward ITC cytotoxicity. Since cells with a high mitotic index appear to be particularly susceptible to SFN, it is likely that SFN affects components of the mitotic machinery, as already proposed above. Supporting this assumption, synchronized HT29 colon cancer cells treated with SFN were not able to reenter cell cycle, but cell proliferation was initiated without signs of cytotoxicity after SFN removal [Gamet-Payrastre *et al.*, 1998]. Thus, resting cells in G_0 seemed to be resistant toward SFN treatment.
Further research is needed to elucidate the molecular mechanisms of cancer cell growth inhibition by SFN. From what is known so far, mechanisms appear to differ substantially between cell types and depend on the genetic background of the cell line used. These discrepancies limit the possible appliance of SFN for the prevention of cancers in humans and further underline the need for sophisticated *in vitro*- as well as *in vivo* studies.

Natural foods are composed of a variety of different ingredients. Apart from water, fiber, carbohydrates, proteins, fats, vitamins and minerals, various different secondary plant metabolites with a potential accessory function can be found in vegetables. Unique and characteristic for brassica vegetables are glucosinolates, which are thought to be responsible for the cancer preventive effects of a diet rich in cruciferous vegetables. For the investigation of the mechanisms underlying these effects, mostly single isolated glucosinolate cleavage products are being used. Promising findings achieved with single compounds are then often transferred to the whole food. Thereby possible combinatory interactions between the different compounds present in whole vegetables are often

disregarded, mainly because there is a lack of knowledge. In the present study, combination effects were investigated between SFN and the indole derivate 3,3'-diindolylmethane (DIM), both derived from broccoli (**Manuscript 3**). These two compounds were chosen, since their precursor glucosinolates are the predominant glucosinolates in broccoli, and since SFN and DIM were found to exert their chemopreventive activities by different mechanisms [Fahey *et al.*, 2001].

Both SFN and DIM have the ability to modulate gene expression of drug metabolizing enzymes. Indoles like indole-3-carbinol and DIM are known to be bifunctional inducers of biotransformation enzymes, whereas ITCs like SFN are monofunctional inducers and thus only associated with the induction of phase II enzymes [Prestera *et al.*, 1993].

Not only with respect to modulation of biotransformation, but also in terms of cancer cell growth inhibition the modes of action of SFN and DIM appear to be different. It was recently demonstrated that ITCs like SFN are cytotoxic in human colon cancer cell lines, whereas indoles like DIM are acting by a cytostatic mechanism [Pappa *et al.*, 2006]. Although IC_{50} values of SFN and the aromatic ITC phenethyl isothiocyanate were similar to those of indole-3-carbinol and DIM, the indole derivates inhibited cell growth without cytotoxicity, whereas the ITCs reduced the original cell number to almost zero. Another striking difference between these two groups of glucosinolate cleavage product is that indoles were shown to halt the cell cycle in G_1 [Aggarwal & Ichikawa, 2005], while ITCs induce cell cycle arrest in G_2/M phase [Zhang *et al.*, 2006].

As an endpoint for the investigation of combination effects between SFN and DIM, anti-proliferative activity was taken into account using a cytotoxicity assay. 40-16 cells were treated with defined ratios of the two compounds or SFN and DIM alone **(Manuscript 3)**. For quantitative evaluation of combinatorial interactions Chou and Talalay have developed a method which has become the "golden standard" for these type of investigations [Chou & Talalay, 1984].

The first step of this procedure is to assess whether the two compounds of the combination are mutually exclusive (same receptor or same mode of action) or mutually non-exclusive (different modes of action). This is accomplished by generating a median-effect plot, in which parallelism of the regression lines of the two single compounds and the combination indicates mutual exclusivity. If the three regression lines are not in parallel the drugs are mutually non-exclusive and thus act by distinct modes of action. In the case of SFN and DIM the regression lines were not in parallel for any of the mixtures

applied (**Manuscript 3**). Thus, SFN and DIM are mutually non-exclusive. This finding was in congruence with the earlier mentioned differences between SFN and DIM with regard to mechanisms of gene expression modulation and cell growth inhibition.
The next step of the Chou-Talalay approach is the calculation of the combination index (CI). Thereby the doses of each drug in the combination needed to produce a given effect, e.g. 50% growth inhibition, are calculated in relation to the doses of each drug alone needed to produce the same effect. A CI < 1, CI = 1 and CI > 1 indicates synergism, additivity and antagonism, respectively. For the combinations of SFN and DIM the CI values corresponding to fixed total drug concentrations were calculated to be able to compare different mixtures. It was found that combinatory interactions between SFN and DIM were strongly dose-dependent, in that low concentrations were antagonistic, whereas cytotoxic concentrations were synergistic, regardless of the ratio applied (**Manuscript 3**). An explanation for these results could be the strong ability of SFN to enhance detoxification at low concentrations. Also consistent with this assumption, synergism between ITCs and indoles was reported in terms of phase II enzyme induction [Staack *et al.*, 1998; Nho & Jeffery, 2001]. This is probably due to the different mechanisms of modulating gene expression of these two types of glucosinolate cleavage products.

In the present study, mixtures of SFN and DIM were not only tested in a cytotoxicity assay, but also in Flow cytometric analysis of cell cycle distribution. Quantification of combination effects by CI calculations was not possible with cell cycle data, because values between 0 and 1 are needed. Due to the distribution of cells in four cell cycle phases this kind of quantitative evaluation could not be performed. Nevertheless, results clearly confirmed the data obtained with the cytotoxicity experiments (**Manuscript 3**). Low concentrations of the mixtures were antagonistic and the highest concentrations were synergistic with respect to the induction of cell cycle arrest and apoptosis. Interestingly, when the SFN concentration in the mixture exceeded 5 µM, a marked G_2/M arrest was observed. Thus, it can be hypothesized that a certain threshold concentration of SFN is necessary to induce spindle damage. Below that concentration SFN's main effect is the modulation of gene expression. The threshold concentration of SFN to induce mitotic arrest was lowered in combination with DIM, although DIM alone was reported to rather induce a cell cycle arrest in G_1 phase. Considering these results, it is even more likely that

the consumption of brassica vegetables can give rise to ITC and indole concentrations sufficient for cancer cell growth inhibition in humans.
Synergistic interactions of DIM in combination with a microtubule-damaging agent have been described before. In HER2/Neu human breast cancer cells DIM and paclitaxel were shown to synergistically induce apoptosis [McGuire *et al.*, 2006]. This finding is of great clinical relevance since some tumors are resistant to treatment with antimitotic agents [Geney *et al.*, 2005]. The mechanisms underlying this synergistic interaction have to be elucidated in future studies.

Combination chemotherapy is increasingly investigated and already successfully applied in the clinic. Especially in tumors with acquired resistance to certain agents a sensitization can offer great treatment advantages. Moreover, in most cases of combination therapy adverse side effects are reduced.
In chemoprevention research, clinical trials conducted with isolated compounds have often led to disappointing outcomes. For instance, in the α-tocopherol, β-carotene cancer prevention study (ATBC) lung cancer incidences were increased in subjects taking β-carotene [1994]. As another example, two large randomized trials that examined the effects of fiber supplementation on colorectal adenoma recurrence failed to show a chemopreventive effect [Schatzkin *et al.*, 2000]. Possible synergistic interactions among the different ingredients in whole foods could explain the lack of efficacy obtained with single compounds as well as the fact that numerous case control studies showed inverse correlations between cancer incidences and the consumption of certain types of food. Therefore the field of combination chemoprevention should be paid more attention. Clinical trials conducted with defined combinations of chemopreventive agents could lead to more promising results. Moreover, the molecular mechanisms of chemopreventive action as well as of combinatorial interactions should be the focus of future research.

V. SUMMARY

Introduction: Isothiocyanates and indole derivates derived from brassica vegetables are promising chemopreventive compounds. Sulforaphane (SFN), the predominant isothiocyanate from broccoli, is known to interfere with all phases of carcinogenesis, including the ability to induce cell cycle arrest and apoptosis in cancer cells. However, the molecular mechanisms of these effects are largely unresolved so far and were a matter of investigation in the present thesis. Since isothiocyanates and indole derivates occur concomitantly in broccoli, combination effects between SFN and 3,3'-diindolylmethane (DIM) were investigated in terms of cell growth inhibition.

Methods: In the human colon cancer cell line 40-16, cell cycle effects were analyzed using Flow cytometry after staining the DNA with propidium iodide. To investigate necrosis induction and the accumulation of cells in mitosis, the propidium iodide exclusion assay and labeling of cells with the mitosis-specific antibody MPM-2 were utilized, respectively. NF-κB activation was determined by an ELISA-based assay. The potential-sensitive fluorescent dye JC-1 was used to investigate the mitochondrial membrane potential. The expression of proteins involved in apoptosis and NF-κB activation was analyzed by Western Blotting. A Jurkat cell line stably overexpressing Bcl-2 was a tool to explore the involvement of the mitochondrial pathway of apoptosis. Sulforhodamin B staining was used to assess the inhibition of cell proliferation. Cellular glutathione levels were measured with a colorimetric assay using Ellman's reagent. Combination effect between SFN and DIM were calculated base on the method of Chou and Talalay (1984).

Results: SFN induced a cell cycle arrest in G_2/M phase, which was demonstrated to be due to the activation of the spindle checkpoint. Apoptosis was a consequence of this mitotic arrest. Cell death induction was found to be due to the activation of the mitochondria-mediated pathway. In addition, SFN strongly inhibited NF-κB activation upstream of its translocation into the nucleus, leading to the downregulation of different anti-apoptotic proteins. Kinetic experiments revealed that a minimum continuous exposure time of 12 h was necessary for SFN to irreversibly arrest cells in mitosis and subsequently induce apoptosis. Depletion of glutathione does not seem to play a role in SFN-mediated apoptosis induction. Combinations of SFN and DIM at different molar

ratios inhibited cell growth in an antagonistic manner at low concentrations, whereas cytotoxic concentrations were synergistic.

Conclusions: The results of this thesis contribute significantly to the knowledge about the molecular mechanisms and kinetics of SFN-induced cell cycle arrest and apoptosis in human cancer cells. Moreover, this study provides insights into combinatorial interactions between the two broccoli compounds SFN and DIM, which represent a valuable contribution to the understanding of possible combined effects of food compounds, an important aspect of chemoprevention research.

VI. ZUSAMMENFASSUNG

Einleitung: Isothiocyanate und Indolderivate aus Kohlgemüse sind Erfolg versprechende chemopräventive Substanzen. Sulforaphan (SFN), das dominierende Isothiocyanat aus Brokkoli, interferiert mit allen Phasen der Karzinogenese, einschließlich der Fähigkeit in Krebszellen Zellzyklusarrest und Apoptose auszulösen. Die molekularen Mechanismen dieser Effekte sind jedoch bisher zum Großteil unbekannt und stellen daher den Untersuchungsgegenstand der vorliegenden Arbeit dar. Da Isothiocyanate und Indolderivate gemeinsam in Brokkoli vorkommen, wurden Kombinationseffekte zwischen SFN und dem Indol 3,3'-Diindolylmethan in Bezug auf die Hemmung des Zellwachstums untersucht.

Methoden: In der humanen Kolonkrebszelllinie 40-16 wurden Zellzykluseffekte mittels Färbung der DNA mit Propidiumiodid und anschließender Durchflusszytometrie analysiert. Für die Erfassung nekrotischer und mitotischer Zellen wurde der "propidium iodide exclusion assay", bzw. das Markieren der Zellen mit dem mitosespezifischen Antikörper MPM-2 durchgeführt. Die Aktivierung von NF-κB wurde mittels einer ELISA-basierten Methode erfasst. Der potentialsensitive Fluoreszenzfarbstoff JC-1 wurde für die Untersuchung des mitochondrialen Membranpotentials verwendet. Die Expression von Proteinen, die bei der Apoptose und der NF-κB Aktivierung eine Rolle spielen wurden mittels Western Blot analysiert. Als Hilfsmittel, um die Beteiligung des mitochondrialen Signalwegs der Apoptose zu erforschen, wurde eine Bcl-2 überexprimierende Jurkat Zelllinie eingesetzt. Sulforhodamin B Färbung wurde für die Erfassung von Zellwachstumshemmung verwendet. Die Messung von zellulärem Glutathion wurde mit Ellman's Reagenz und colorimetrischer Bestimmung erfasst. Kombinationseffekte zwischen SFN and DIM wurden nach der Methode von Chou and Talalay (1984) ausgewertet.

Ergebnisse: SFN induzierte einen G_2/M Zellzyklusarrest, der auf die Aktivierung des Spindel-Kontrollpunkts zurückgeführt werden konnte. Apoptose war eine Konsequenz dieses mitotischen Arrests. Es konnte gezeigt werden, dass Apoptose über den mitochondrialen Signalweg ausgelöst wurde. Weiterhin hemmte SFN die Aktivierung von NF-κB vor dessen Translokation in den Nukleus, was zur Herunterregulation verschiedener anti-apoptotischer Proteine führte. Kinetische Experimente ergaben, dass eine minimale Expositionszeit von 12 h nötig war, um unumkehrbar Mitosearrest und

Apoptose auszulösen. Die Depletion von Glutathion scheint keine Rolle für die durch SFN ausgelöste Apoptose zu spielen. Kombinationen von SFN und DIM in verschiedenen molaren Verhältnissen hemmten das Zellwachstum auf antagonistische Art und Weise bei niedrigen Konzentrationen, wogegen cytotoxische Dosen synergistisch wirkten.

Schlussfolgerungen: Die Ergebnisse dieser Arbeit leisten einen signifikanten Beitrag zum Wissen über die molekularen Mechanismen und die Kinetik von SFN-induzierter Zellzyklusarrettierung und Apoptoseinduktion in humanen Krebszellen. Des Weiteren bietet diese Studie Erkenntnisse über kombinatorische Interaktionen zwischen den zwei Brokkoli-Substanzen SFN und DIM, was zu einem besseren Verständnis von möglichen Kombinationseffekten zwischen Lebensmittelinhaltsstoffen beiträgt, einem wichtigen Aspekt der Chemopräventionsforschung.

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PRESENTATIONS AT SCIENTIFIC MEETINGS

Pappa G., Bartsch H., Gerhäuser C. (2006), Combination effects of the broccoli compounds sulforaphane and 3,3'-diindolylmethane on cell growth inhibition of cultured 40-16 colon carcinoma cells. (Poster) 10th Karlsruhe Nutrition Congress, Karlsruhe, Germany

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Pappa G., Iori R., Barillari J., Bartsch H., Gerhäuser C. (2004), Mechanisms of sulforaphane-mediated apoptosis induction in colon carcinoma cell lines. (Poster) 18th Meeting of the European Association for Cancer Research (EACR), Innsbruck, Austria

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Gerlinde Pappa