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Cellular and Molecular Targets of Silibinin, a Natural Flavonoid, in Colorectal Cancer Prevention and Therapy

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SUMMARY

Colorectal cancer (CRC) is the second most common cause for cancer-related deaths in Europe and in the USA. Because chemotherapeutic agents for the treatment of advanced CRC only possess limited efficacy and often show considerable toxicity, new approaches are urgently needed. The flavonolignan silibinin constitutes the major biologically active compound of the extract silymarin isolated from the milk thistle plant (*Silybum marianum*). Milk thistle extract has been used as a hepatoprotective substance for more than 2000 years, and during the last decade its main active agent silibinin has been shown to possess antineoplastic properties.

The objective of this thesis work was to study the molecular mechanisms of the anticancer properties of silibinin in CRC by using an *in vitro* model of cancer progression consisting of the primary adenocarcinoma cell line SW480 and its derived metastatic cell line SW620. Furthermore we aimed to assess its chemopreventive effects in an *in vivo* model of azoxymethane-induced colon carcinogenesis in the rat.

Our results showed that silibinin induced apoptotic cell death with DNA fragmentation and activation of caspase-3 in both cell lines. The expression of death receptors of the extrinsic apoptotic pathway was upregulated at the transcriptional and protein level, and the initiator caspase-8 was activated. Cleavage of Bid sustained the implication of the intrinsic mitochondrial pathway. The potential of the mitochondrial membrane was perturbed which permitted the release of the pro-apoptotic factor cytochrome c and the subsequent activation of caspase-9. Besides the activation of the extrinsic and the intrinsic apoptotic pathway in the two cell lines, silibinin also induced an autophagic response. With the aid of the inhibitor bafilomycin A1, we showed that silibinin-induced autophagy had a survival function.

Combination of silibinin and TRAIL, a promising anticancer agent which selectively induces apoptosis in cancer cells, induced synergistic cell death in SW480 and SW620 cells. The up-regulation of TRAIL death receptors 4 and 5 as well as the down-regulation of anti-apoptotic proteins such as Mcl-1 and XIAP were involved in the silibinin-mediated sensitization to TRAIL. A further synergy in cell death induction was observed by the combination of silibinin and the histone deacetylase (HDAC) inhibitors TSA and the clinically used SAHA.

In the preclinical model of colon carcinogenesis in the rat, silibinin administration was able to reduce preneoplastic lesions in the form of aberrant crypt foci by half.

In conclusion, silibinin is a promising natural agent for colon cancer chemoprevention and for combination therapy with TRAIL and HDAC inhibitors.

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LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
ACF	Aberrant Crypt Foci
AIF	Apoptosis-Inducing Factor
ALT	ALanine Transaminase
AOM	AzOxyMethane
AP-1	Activator Protein 1
Apaf-1	Apoptotic protease-activating factor-1
APC	Adenomatous Polyposis Coli
AST	ASpartate Transaminase
Atg	Autophagy-related proteins
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-Cell Lymphoma-2
BH	Bcl-2 homology domain
Bid	BH3 interacting-domain death agonist
BIR	Baculoviral IAP Repeat
CARD	CAspase Recruitment Domain
Caspases	Cysteine-ASPartate proteASES
CAT	CATalase
CDK	Cyclin-Dependent Kinase
CDKI	Cyclin-Dependent Kinase Inhibitor
cIAP	cellular IAP
CIN	Chromosomal Instability
CMC	CarboxyMethyl Cellulose
COX-2	CycloOXygenase-2
CRC	Colorectal Cancer
DCC	Deleted in Colon Cancer
DcR	Decoy Receptor
DD	Death Domain
$DiOC_2(3)$	3,3'-DiethylOxaCarboCyanine iodide
DISC	Death-Inducing Signaling Complex
DMH	1,2-DiMethylHydrazine
DMSO	DiMethylSulfOxide
DNMT	DNA MethylTransferase
DR	Death Receptor
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular signal-Regulated Kinase
FACS	Fluorescence-Activated Cell Sorting
FADD	Fas-Associated Death Domain
FAP	Familial Adenomatous Polyposis
FasL	Fas Ligand
FDA	Food and Drug Administration

FITC	Fluorescein isothiocyanate
FOBT	Fecal Occult Blood Test
FOLFIRI	Folinic acid/Fluorouracil/Irinotecan
FOLFOX	Folinic acid/Fluorouracil/Oxaliplatin
FSC	Forward Scatter
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione
GSK	Glycogen Synthase Kinase
GST	Glutathione S-Transferase
HCC	HepatoCellular Carcinoma
HDAC	Histone DeACetylase
HIF-1α	Hypoxia Inducible Factor 1α
HRP	HorseRadish Peroxidase
IAP	Inhibitor of APoptosis
IGF	Insulin-like Growth Factor
IGF1R	IGF Receptor
IGFBP	IGF Binding Protein
IKK	I-κB kinase
IL	InterLeukin
iNOS	inducible Nitric Oxide Synthase
I-κB	Inhibitor of κB
JNK	c-Jun N-terminal Kinase
LC3	Microtubule-associated protein Light Chain 3
LDH	Lactate DeHdyrogenase
LV	Leucovorin
MAPK	Mitogen-Activated Protein Kinase
Mcl-1	Myeloid Cell Leukemia Sequence 1
MMP	Matrix Metalloproteinase
MOMP	Mitochondrial Outer-Membrane Permeabilization
mTOR	mammalian Target Of Rapamycin
NAC	N-acetylcysteine
NAFLD	Non-Alcoholic Fatty Liver Disease
NF-κB	Nuclear Factor-κB
OD	Optical Density
PARP	Poly(ADP-ribose)polymerase
PC	PhosphatidylCholine
PCNA	Proliferating Cell Nuclear Antigen
PE	PhosphatidylEthanolamine
PE	Phycoerythrin
PGE2	ProstaGlandin E2
PI	Propidium Iodide
PI3K	PhosphatidylInositol-3-Kinase
PKB	Protein Kinase B
pNA	p-NitroAniline
PS	PhosphatidylSerine
qRT-PCR	quantitative Reverse Transcription Polymerase Chain Reaction

rhTRAIL	recombinant human TRAIL
RIP1	Receptor Interacting Protein 1
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
SAHA	SuberoylAnilide Hydroxamic Acid
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Smac/DIABLO	Second mitochondrial activator of caspases/Direct IAP Binding protein with LOw pI
SOD	SuperOxide Dismutase
SRB	SulfoRhodamine B
SSC	Side Scatter
STAT	Signal Transducers and Activators of Transcription
tBid	truncated Bid
TCF-4	T-Cell Factor-4
TGF-α	Transforming Growth Factor-α
TIMP	Tissue Inhibitor of MetalloProteinase
TMB	3,3',5,5'-TetraMethylBenzidine
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TRADD	TNFR-Associated Death Domain
TRAF2	TNFR-Associated Factor 2
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRAIL-R	TRAIL-Receptor
TSA	Trichostatin A
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling
UGT-1A1	Uridinediphosphoglucuronate-GlucuronosylTransferase-1A1
u-PA	Urokinase-Type Plasminogen Activator
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
XIAP	X-linked IAP
ΔΨm	Mitochondrial Membrane Potential

OBJECTIVES

Colorectal cancer (CRC) is the second most common reason for cancer mortality in Europe and the USA. Worldwide it is the third most common cancer with over one million new cancer cases per year. Its incidence keeps increasing on the course of western lifestyle spreading all over the world. As shown by migrant studies, diet is a very important factor in the etiology of CRC. Because fruits and vegetables seem to be involved in the chemoprevention of CRC, more and more studies focus on the chemopreventive properties of phytoconstituents. Indeed, many plant-derived polyphenols show anticancer activities.

The milk thistle plant has been used for hepatoprotective purposes for more than 2000 years, beginning in ancient Greece. The standardized extract of the fruit and the seeds of the milk thistle, silymarin, has been studied in a plethora of hepatic conditions: hepatitis, cirrhosis, mushroom poisoning, toxic exposure and prophylaxis in chemotherapy. The major biologically active component of silymarin is the flavonolignan silibinin which has started to be extensively studied for its pleiotropic effects on cancer during the last decade.

However, mechanisms of the action of silibinin in CRC have not been thoroughly explored. Therefore, the objective of this thesis work was to study the cellular and molecular mechanisms of silibinin-triggered cell death in CRC.

To this end, firstly two cell lines were used which constitute a validated *in vitro* model of colon cancer progression, the primary human colon adenocarcinoma SW480 cells and their derived metastatic SW620 cells which have been isolated from a mesenteric lymph node of the same patient.

Secondly, the anticancer effects of silibinin were studied in a pre-clinical model of colon carcinogenesis, the azoxymethane-induced colon carcinogenesis rat model.

The results should permit to evaluate the chemopreventive and chemotherapeutic potential of silibinin in CRC.

INTRODUCTION

I. COLORECTAL CANCER

1. Epidemiological and etiological aspects

1.1. Incidence

Colorectal cancer (CRC) is the second most common reason for cancer mortality in developed countries, for men and women combined (Figure 1) [1]. It is the third most common cancer worldwide with over one million new cancer cases and over half a million deaths per year [2]. The incidence of CRC correlates with the western lifestyle, industrialization and urbanization [3, 4]. Rates are higher in men than in women [2].

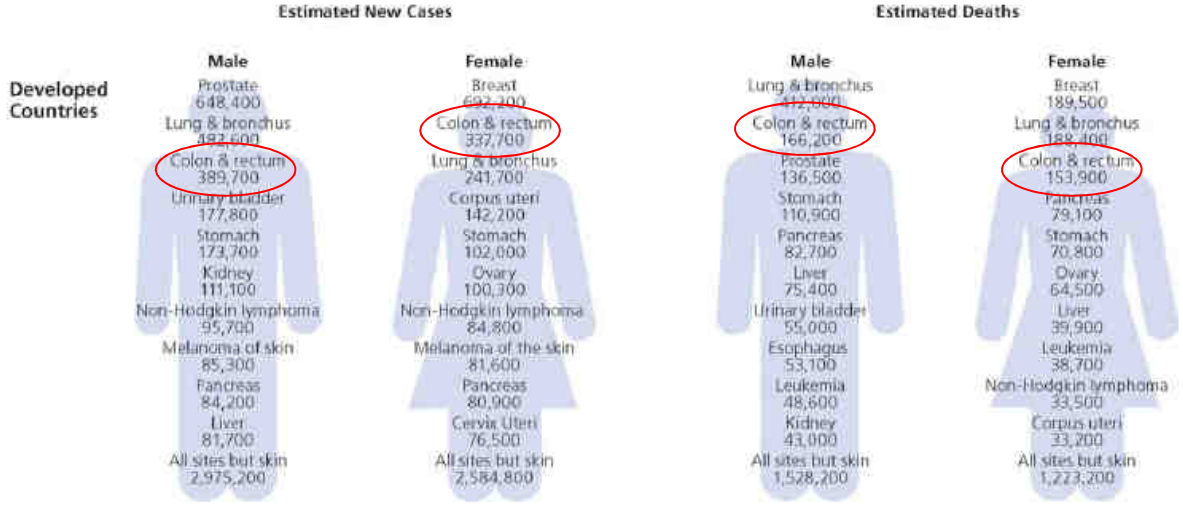


Figure 1: Estimated new cancer cases and deaths for leading cancer sites in economically developed countries [2]

1.2. Risk factors

It has been shown by migration studies that risk factors for CRC are mainly environmental exposures: When populations move from low- to high-risk areas (e.g. from Japan to the USA) the incidence of CRC already increases in the first generation of migrants [5]. CRC rates also rise in countries undergoing westernization, demonstrating the influence of exogenous factors such as dietary patterns, obesity and smoking [6]. Indeed, in the etiology of CRC, diet is a very important factor. In 2007, the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) have presented their report about the associations

between food, nutrition, physical activity and the risk of CRC. Their conclusions are summarized in Figure 2.

Matrices presented in the WCRF/AICR 2007 Expert Report

CANCER OF THE COLON AND RECTUM

	DECREASES RISK	INCREASES RISK
Convincing	Physical activity	Red meat Processed meat Alcoholic drinks (men) Body fatness Abdominal fatness Adult attained height
Probable	Food containing dietary fiber Garlic Milk Calcium	Alcoholic drinks (women)
Limited –suggestive	Non-starchy vegetables Fruits Food containing folate Foods containing selenium Fish Food containing vitamin D Selenium	Foods containing iron Cheese Foods containing animal fats Foods containing sugars
Limited –no conclusion	Cereals (grains) and their products; (grains) and their products; potatoes; poultry; shellfish and other seafood; other dairy products; total fat; fatty acid composition; cholesterol; sugar (sucrose); coffee; tea; caffeine; total carbohydrate; starch; vitamin A; retinol; vitamin C; vitamin E; multivitamins; non-dietary sources of calcium; methionine; beta-cartene; alpha-carotene; lycopene; meal frequency; energy intake	
Substantial effect on risk unlikely	None identified	

Figure 2: Dietary risk factors for CRC

(http://www.wcrf.org/PDFs/Colorectal-cancer-CUP-report-2010.pdf)

Non-dietary risk factors are tobacco smoking, colorectal diseases, the metabolic syndrome, genetic factors and advanced age [4].

The chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) seems to diminish CRC risk as it was found in a systematic review that aspirin reduces the recurrence of sporadic adenomatous polyps and even supported regression of polyps in familial adenomatous polyposis (FAP) (see next page) [7].

Inflammatory bowel diseases such as Crohn's disease and ulcerative colitis increase the risk of colon cancer [8]. The association of the metabolic syndrome with CRC can be attributed to abdominal obesity and abnormal glucose metabolism [9].

The main risk factor - as for almost all types of cancer - is advanced age: 90% of CRC occur after 50 years of age [10] and about 70% of patients with CRC are older than 65 years [4].

About 75% of people with CRC would have been classified as being at average risk for CRC before diagnosis, meaning they have passed 50 years of age but no other risk factors apply to them [10]. This means that CRC occurs sporadically most of the time. In fact, it can be traced back to identified genetic predispositions only in 5% of CRC cases [11]. About 1% of the patients suffer from inflammatory bowel disease, and the remaining 20% of persons at high risk have a family history of CRC without an identified genetic predisposition [10].

Congenital CRC can be divided into polyposis and non-polyposis syndromes. The main polyposis syndrome is FAP, associated with mutation or loss of FAP gene (synonymous to adenomatous polyposis coli (APC) gene) [4]. Hereditary non-polyposis CRC (HNPCC) syndrome results from a germline mismatch repair gene mutation [12]; this leads to a destabilization of the genome permitting other mutations to occur [4, 10]. In these patients, CRC develops much more rapidly, that is why screening must start at a young age and be performed in regular short intervals [10].

1.3. Prognosis

The survival rate entirely depends on the extent of tumor progression, i.e. the degree of penetration of the tumor through the bowel wall and the nodal involvement, at the time of diagnosis [3]. Diagnosis often takes place too late, when the cancer has already developed metastases. Early identification of CRC is especially important as the overall 5-year survival rate is about 60% [13] and for patients with advanced CRC less than 5% [4].

The median survival time of patients with advanced CRC without treatment is around 5-6 months, with 5-fluorouracil (5-FU)-based chemotherapy around 10-12 months, with additional irinotecan and oxaliplatin survival time increases to 18-24 months. Response rates to the treatment depend amongst others on the performance status of the patient, the presence of symptoms and extent of the disease [4].

Other prognostic factors are p53, ki-ras and Bcl-2 expression, TGF-α, epidermal growth factor (EGF), proliferative index and aneuploidy in tumor tissue [4].

1.4. Prevention and screening

Prevention by removal of polyps in colonoscopy is effective in reducing CRC mortality [3]. Population-wide screening programs are used in 19 of 27 EU countries [14]. Screening is focused on asymptomatic individuals past 50 years of age. There are two different screening strategies, fecal occult blood test (FOBT) and endoscopy. FOBT is the most frequently applied method. The advantage of screening by colonoscopy compared to FOBT is the visualization of the entire colon and the possibility to remove pathological lesions during a single examination [14]. Thus the goal of screening by colonoscopy is not only the detection of CRC but also the discovery and removal of premalignant polyps and thereby the interruption of the adenoma-adenocarcinoma sequence [10]. Polypectomy should be performed whenever possible [4]. The drawbacks of colonoscopy are the price, the discomfort for the patient and a very slight risk of complications (below 0.03%) [15]. Although there is no study reporting a reduction in CRC incidence with the use of screening colonoscopy, it is seen as "gold standard" for comparison with other methods. However, FOBT is the only test which meets the WHO criteria for screening. Thus FOBT is the test of choice in most of the European countries, and in case of positive results, colonoscopy is performed [14].

All screening methods require several criteria to work: information of the public by the media, patient compliance, sufficient funding, stratification of risks and the choice of the most suitable screening tests. The Council of Europe recommends FOBT in persons older than 50 years as age is the main risk factor for CRC.

In the United States decreasing incidence rates in the last years may be explained by the efficacy of screening and removal of precancerous lesions [2]. Likewise, in the UK, a recent randomized controlled trial has shown a reduction of CRC incidence by 33% and mortality by 43% by a one-time flexible sigmoidoscopy screening of 55 to 64 year-olds [15].

2. Development of CRC

2.1. Anatomy and function of the colon

The colon is the end of the digestive system and its function is to reabsorb water and sodium from the feces. The colon consists of four sections: the ascending, the transverse, the descending and the sigmoid colon, followed by the rectum (Figure 3).

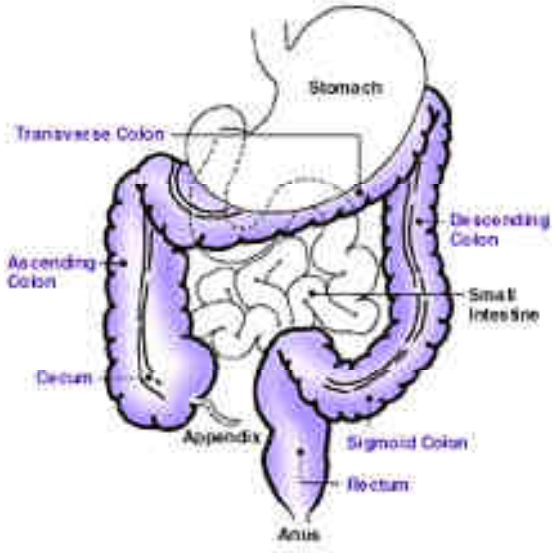


Figure 3: Diagram of the colon and rectum (www.cdc.gov)

The normal colon consists of millions of crypts each containing about 2000 differentiated cells and stem cells located at the bottom of the crypts (Figure 4). The stem cells produce differentiated cells which migrate to the top of the crypt where they undergo apoptosis [16]. This turnover is rapid: all cells except stem cells are replaced within a week. To achieve a homeostasis in the colon, the dividing rate has to match the apoptotic rate.

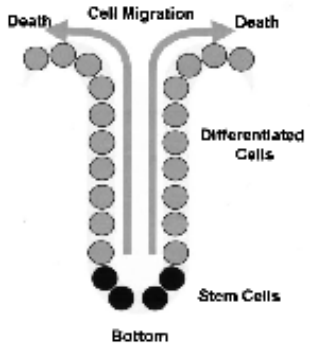


Figure 4: Colon crypt [16]

2.2. Colon carcinogenesis

The development of CRC is a multistep process, accompanied by multiple genetic and epigenetic alterations and associated with the progressive inhibition of apoptosis and an increase of proliferation.

Morphologically, it is characterized by the progression from normal colon tissue to cancer via the earliest neoplastic lesions called aberrant crypt foci (ACF) to precancerous adenomas (mostly polyps) to adenoma-containing carcinomas and adenocarcinomas [12, 17] (Figure 5). ACF are clusters of mucosal cells characterized by their elevated structure compared to the surrounding normal mucosa, increased size (at least twice of their normal adjacent counterparts) and thicker epithelial lining [12]. They can be classified into hyperplastic and into the larger dysplastic ACF which are also called microadenomas because of their histologic resemblance to adenomatous polyps and which are believed to be the actual precursor lesions of CRC. These dysplastic ACF and flat adenomas are easily missed during preventive colonoscopy and explain the cases where polypectomy does not prevent CRC [12].

Vogelstein *et al.* described the genetic alterations in the adenoma-carcinoma sequence model, studied in various stages of development of patient-derived tumors (Figure 5) [18]. These alterations include mutational activation of oncogenes, associated with mutational inactivation of tumor suppressor genes [19]. For the formation of a malignant tumor, at least four to five genes have to be mutated [19]. When the genome of the cell has reached a certain level of genomic instability due to the accumulated oncogenic mutations and silenced tumor suppressors, a malignant cell phenotype develops [12].

Frequent mutations or deletions concern the oncogene *KRAS* and the three tumor suppressor genes *APC*, deleted in colon cancer (*DCC*) and *TP53* [20]. *KRAS* and *APC* mutations occur in the early stages of colon carcinogenesis (ACF and adenoma) while *DCC* and *TP53* alterations are involved in later stages. Mutated *KRAS* results in the constitutive activation of its downstream signaling pathways, the Raf/MEK/MAPK and PI3K/Akt/PKB pathways. A mutation in *APC* renders APC protein unable to provide for the degradation of β-catenin [20]. This mutation is responsible for the FAP syndrome (see 1.2) and also occurs in 50-80% of sporadic CRC cases [21]. β-catenin normally plays a role in the cadherin-mediated cell-cell adhesion system [20]; however, its accumulation promotes the expression of c-myc, cyclin D1 and c-jun which are critical for cancer development [21].

Chromosomal instability (CIN) as a further trait of sporadic CRC is associated with *APC* mutations because mutated APC also runs the risk of losing its ability to connect chromosomes to microtubules [21].

The tumor suppressor p53 protein is a transcription factor in charge of cell cycle and apoptosis regulation [20]. Besides mutations, aberrant methylation of CpG islands in the promoter region of tumor suppressor genes is a common epigenetic event in tumor progression to silence their transcription [12].

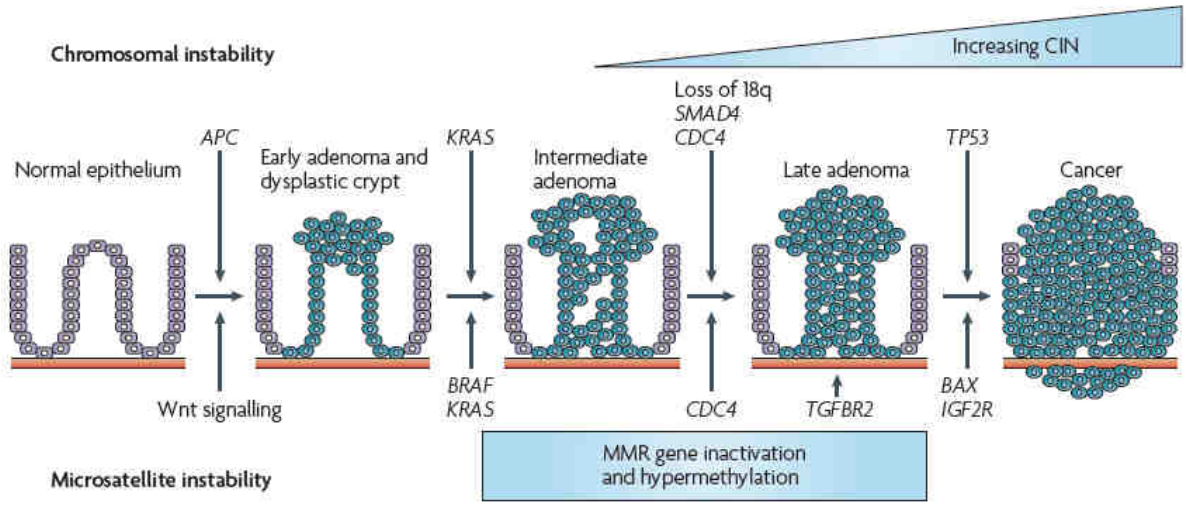


Figure 5: Adenoma-carcinoma sequence model [22]

CRC is characterized by a suppression or alteration of apoptosis which leads to the resistance to current chemotherapy because their mechanisms of action involve activation of apoptotic pathways [17]. Therefore approaches are needed which promote apoptosis, i.e. induce the pro-apoptotic and suppress the anti-apoptotic members of the Bcl-2 family [23].

Constitutive activation of mitogen-activated protein kinases (MAPK) as well as high expression and constitutive activation of the PI3K/Akt signaling pathway are often found in CRC and inhibit apoptosis [23].

Inhibitor of apoptosis (IAP) family proteins are generally over-expressed in CRC which heightens the resistance against the induction of apoptosis [23]. However, some chemotherapeutic agents work by down-regulating the IAP family members which sensitizes CRC cells to apoptosis.

The progression from a precursor lesion to CRC takes 10 to 15 years, thus rendering chemoprevention during this long time span possible [19].

3. Therapy

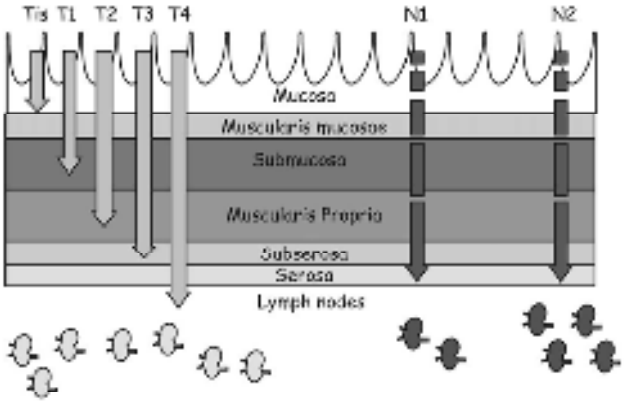
3.1. Diagnosis

CRC is either diagnosed in the frame of a screening program or when a patient starts to show symptoms [4]. As the symptoms of CRC occur only in an advanced state of the disease and are unspecific (tiredness, change in bowel habits, weight loss, abdominal pain), screening is of the utmost importance [4]. Bleeding, obstruction and perforation are further consequences of CRC and can lead to abdominal pain, nausea and vomiting.

The actual diagnosis generally takes place by sigmoidoscopy or colonoscopy.

3.2. Treatment

Treatment depends on the stage of the cancer. The recommended staging system uses TNM categories (Table 1) [22]. TNM classification includes a clinical (pretreatment, cTNM) and a pathological (postsurgical histopathological, pTNM) classification. The classification which accounts for the choice of treatment is the cTNM. With TNM classification, the stage can be determined (Figure 6).



Stage	TNM	5-year survival
I	T1, N0, M0, or T2, N0, M0:	93%
IIA	T3, N0, M0:	85%
IIB	T4, N0, M0:	72%
IIIA	T1-2, N1, M0:	83%
IIIB	T3-4, N1, M0:	64%
IIIC	Any T, N2, M0:	44%
IV	Any T, any N, M1:	8%

Figure 6: Staging system of CRC. T categories describe the extent of spread through the layers of the colon wall, N categories the involvement of lymph nodes and M categories the spread to distant organs [24]**.**

TNM classification.
Primary tumour (T)
TX: Primary tumour cannot be assessed
T0: No evidence of primary tumour
Tis: Carcinoma in situ: intraepithelial or invasion of the lamina propria*
T1: Tumour invades submucosa
T2: Tumour invades muscularis propria
T3: Tumour invades through the muscularis propria into the subserosa, or into the nonperitonealized pericolic or perirectal tissues
T4: Tumour directly invades other organs or structures and/or perforates the visceral peritoneum **,***
Regional lymph nodes (N)
NX: Regional nodes cannot be assessed
N0: No regional lymph node metastasis
N1: Metastasis in 1 to 3 regional lymph nodes
N2: Metastasis in 4 or more regional lymph nodes
Distant metastasis (M)
MX: Presence of distant metastasis cannot be assessed
M0: No distant metastasis
M1: Distant metastasis

* Note: This includes cancer cells confined within the glandular basement membrane (intra-epithelial) or lamina propria (intramucosal) with no extension through the muscularis mucosae into the submucosa.

** Note: Direct invasion in T4 includes invasion of other segments of the colorectum by way of the serosa; for example, invasion of the sigmoid colon by a carcinoma of the cecum.

*** Tumor that is adherent to other organs or structures, macroscopically, is classified T4. However, if no tumor is present in the adhesion, microscopically, the classification should be pT3. The V and L substaging should be used to identify the presence or absence of vascular or lymphatic invasion.

Table 1: TNM classification [4]

When localized to the bowel, CRC is often curable [4]. Surgery is the primary treatment for patients with localized CRC (stage I/II) and cures about 50% of patients. The aim of surgery is a wide resection of the concerned bowel segment and its lymphatic drainage.

A major problem and frequent cause of death is the recurrence of cancer following surgery [4]. Adjuvant (postoperative) therapy is a systemic treatment given after surgery to lower the risk of recurrence. Its application depends on the cancer stage of the patients; it constitutes a standard therapy in stage III patients and is recommended for high-risk stage II patients. An important criterion to consider stage II patients to be at high risk is the invasion of the serosa of the bowel wall by the neoplasm [4].

In stage III CRC, where lymph nodes are involved per definition, the standard treatment consists of wide surgical resection with anastomosis, followed by postoperative chemotherapy with a doublet schedule consisting of oxaliplatin, 5-FU and leucovorin (LV, folinic acid) (FOLFOX4) for 6 months. If for some reasons FOLFOX4 is not tolerated, different options are infusional 5-FU/LV by itself or capecitabine.

In metastatic disease (stage IV) the goal of therapy is to prolong survival and to maintain quality of life. Standard forms of treatment are surgical resection of the primary tumor, treatment of isolated metastases (liver, lung, ovaries), palliative chemotherapy, biological therapy and radiation therapy to the primary tumor and to metastases with palliative intent.

Once the primary tumor is resected, systemic chemotherapy is administered to treat the metastatic disease. However, as an operation frequently leads to complications and results in the postponement of chemotherapy, one might choose to administer systemic chemotherapy as the first treatment, especially when the tumor is asymptomatic. Standard systemic chemotherapy for advanced CRC is 5-FU/LV (infusional) in combination with irinotecan or oxaliplatin [4]. Oral capecitabine can replace 5-FU/LV.

The most common site of metastases from CRC is the liver, occurring in 40-70% of the patients with metastatic cancer. The percentage of liver metastases which are resectable ranges from 10 to 20%. Here, 5-year survival is about 30 to 40% [4]. Neo-adjuvant (preoperative) chemotherapies using oxaliplatin or irinotecan sometimes allow patients with unresectable disease to have a resection later on. 10-20% of patients with CRC show lung metastases which may also be resected under appropriate circumstances.

Even if CRC is not curable, chemotherapy can be given as palliative care only to decrease the tumor load. In this case, it is recommended to start chemotherapy before the start of the symptoms, as it has been shown to prolong life [4].

Targeted (biological) therapies which interrupt key pathways essential for tumor growth can convey additional benefit for patients with metastatic CRC [4]. The addition of bevacizumab, an inhibitor of VEGF, to chemotherapy seems to increase survival time. Monoclonal antibodies against the epidermal growth factor receptor (EGFR), such as cetuximab and panitumumab, also suggest promising activity by increasing the response rate of patients to FOLFOX or FOLFIRI (5-FU, LV and irinotecan) [4, 25]. These antibodies bind to EGFR with high specificity. This blocks phosphorylation of the receptor on ligand-binding and thus prevents the activation of signaling pathways, such as the G protein KRAS. KRAS plays an important role in the resistance to EGFR antibodies: when KRAS is continually activated by a mutation, the cancer is resistant to them. Approximately 40-45% of patients with metastatic CRC have mutated KRAS and thus do not benefit from cetuximab therapy. Wild-type BRAF, the effector of KRAS, is also required for cetuximab efficacy [4].

4. Nutritional chemoprevention of CRC

Chemoprevention is defined as the use of specific natural products or synthetic chemical agents to delay, prevent or even reverse premalignant lesions before the development of invasive cancer [26]. Ideal chemopreventive agents are non-toxic, highly effective in multiple sites, orally consumable, inexpensive, accepted by the population, and have well known mechanisms of action [26].

Per se, CRC is predestined for chemoprevention as the progression from a precursor lesion to CRC takes 10 to 20 years, allowing time for effective intervention and prevention [27]. Currently, only celecoxib is approved for chemoprevention of CRC and only for patients with FAP because of cardiovascular toxicity. Aspirin and sulindac have also demonstrated efficacy but are associated with gastrointestinal toxicity [27]. Indeed, a recent systematic review showed that daily aspirin reduces the incidence of CRC and the risk of distant metastasis [28].

Since the colon is exposed to ingested substances and because diet plays an important role in the etiology of CRC, chemoprevention with nontoxic phytochemicals seems the most “natural” option.

The World Cancer Research Fund/American Institute for Cancer Research in 2007 reported convincing evidence for decreased risk of CRC by increased physical activity, decreased abdominal adiposity and decreased red meat and processed meat consumption (see 1.2) but only “limited” evidence for increased consumption of fruits and vegetables, having

been downgraded from “convincing” (1997) after the completion of large prospective studies [29]. However, since then a meta-analysis of prospective dietary studies has shown a small but significant protective effect of a diet rich in fruits and vegetables on CRC risk [30]. Furthermore the EPIC (European Prospective Investigation into Cancer and Nutrition) study, a large prospective project carried out in 10 different European countries with over 500,000 participants, showed that a high consumption of fruit and vegetables was associated with a reduced risk of CRC [31]. Their authors propose that the discrepancy of previous results on fruit and vegetable consumption on CRC risk may be related to the bias of case-control studies and the choice of populations with fairly homogenous dietary habits. These biases were decreased in the EPIC study. Furthermore it is possible that only certain fruits or vegetables exert a protective effect against CRC and thus this effect is diluted when food groups are studied as a whole. A potential modifier of the protective effect is the smoking status as the positive effect of fruits and vegetables was stronger in never and former smokers [31]. Besides, studies often neglected the site of the cancer which seems to be important because the biological pathways leading to CRC depend on cancer subsites [32].

Indeed, there is a lot of evidence that dietary phytochemicals, the secondary plant metabolites contained in fruits and vegetables, have pleiotropic anticancer effects and are able to modulate various molecular pathways [26]. These phytochemical constituents include phenolics, flavonoids, carotenoids, alkaloids, nitrogen containing and organosulfur compounds. They exhibit biological activities such as the stimulation of the immune system, anti-bacterial, anti-viral, anti-hepatoxic, anti-inflammatory, antioxidant and anti-mutagenic effects [26]. Thus phytochemicals are able to interfere with various molecular pathways involved in CRC initiation and progression: cell cycle progression, apoptosis, cell proliferation, angiogenesis, tumor cell invasion, metastasis and tumor promoting signal transduction pathways such as cyclooxygenase 2 (COX-2)-induced PGE2 levels, Wnt/β-catenin pathway, ERK pathway and PI3K/Akt signaling pathway [26].

The efficacy of these constituents has been shown by *in vitro* and *in vivo* studies. For *in vivo* studies, 1,2-dimethylhydrazine (DMH)- or azoxymethane (AOM)-induced colon carcinogenesis models in rodents are mostly used. Furthermore, genetic models (APC Min) and xenograft models are employed.

However, confirmation of these results in humans faces the difficulties of all long-term intervention studies.

II. CELL DEATH

1. General Overview

The classification of mammalian cell death primarily includes apoptosis, necrosis and autophagic cell death (Figure 7) [33]. While apoptosis and autophagic cell death belong to the category of programmed cell death, necrosis has traditionally been considered an accidental and uncontrolled form of cell death but this perception is changing [34]. The kind of cell death that occurs depends on the nature and intensity of the noxious stimuli, ATP concentration, cell type, cell environment and other factors. Different forms of cell death can occur at the same time [34]. When one death pathway is blocked (by a genetic defect or pharmacological inhibitor) another may take over [33]. The type of cell death can be classified by morphological, biochemical and molecular criteria.

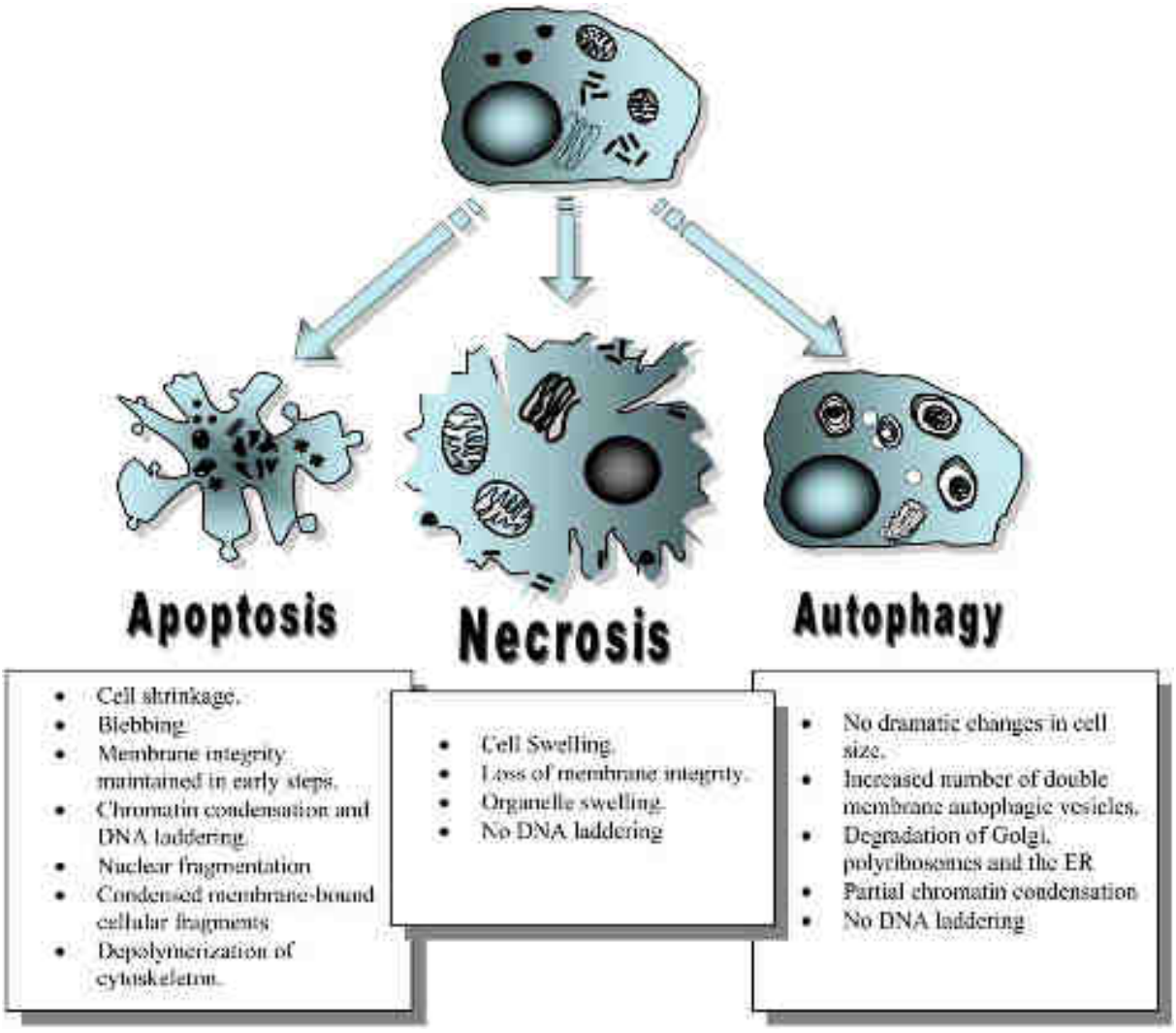


Figure 7: Morphological changes associated with the three ways of cell death [34]

2. Apoptosis

2.1. Characteristics

The word “apoptosis” derives from the Greek “απόπτωσις” (“apo” = “away” and “ptosis” = “fall”) and means “leaves falling from a tree”. Apoptosis is a programmed cell death necessary to maintain cellular homeostasis in the body; it plays an important role in organ development, tissue regeneration, immune response and tumor suppression by the removal of superfluous and irreparably damaged cells [23]. Apoptosis can be caused by many different stimuli, such as cellular stress or damage to cellular organelles by heat shock, radiation, cytotoxic drugs or infection [35]. Deficient signaling of apoptosis leads to diseases such as cancer, autoimmune diseases and metabolic disorders. In contrast, excessive apoptosis is involved in neurodegenerative disorders such as Alzheimer’s and Huntington’s disease [35].

Apoptosis is accompanied by certain morphological and biochemical characteristics: the shrinkage of the cell and its nucleus, chromatin condensation, DNA fragmentation and plasma membrane blebbing [33].

There are two distinct but frequently convergent pathways in apoptosis: the extrinsic (death receptor mediated) and the intrinsic (mitochondrial) pathways (see 2.2 and 2.3, Figure 8). Some signals are able to activate both pathways with a crosstalk between the two mechanisms (see 2.4) [23]. Both pathways ultimately lead to the activation of cysteine-aspartate proteases called caspases which results in apoptosis (see 2.5) [35].

A special feature of apoptosis is that the apoptotic bodies (the membrane-surrounded fragments of the dying cells) are engulfed and removed by phagocytes without the release of intracellular material, thus avoiding an inflammatory response which may occur in other forms of cell death [36].

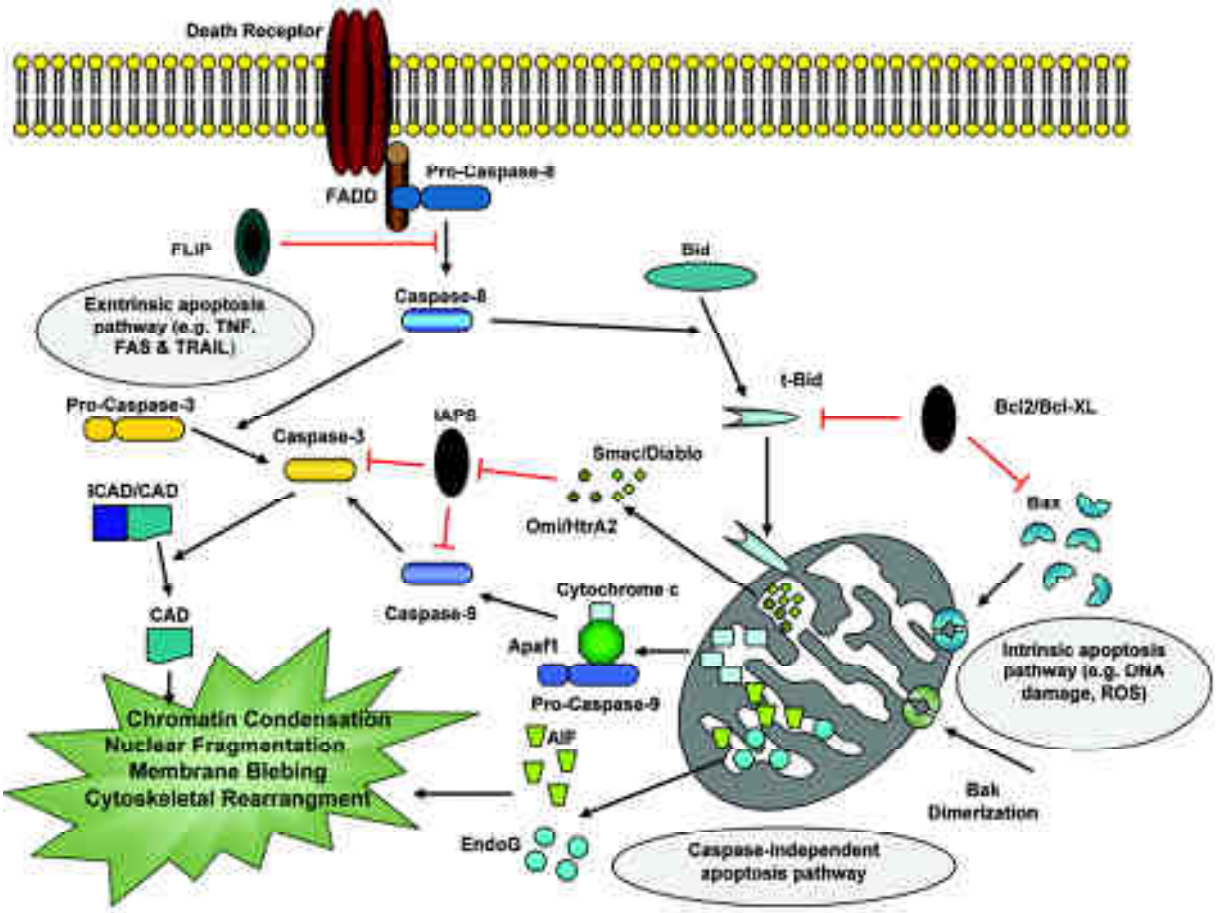


Figure 8: The extrinsic and intrinsic pathway of apoptosis [37]

2.2. The extrinsic pathway (death receptor pathway)

The extrinsic apoptotic pathway is activated by the binding of extracellular protein ligands to the death receptors on the surface of the cell (Figure 9). The extrinsic pathway has several functions in humans: the regulation of the immune response, selection and maintenance of the immune repertoire and the removal of infected, transformed or damaged cells. For the latter, pro-apoptotic ligands are expressed by immune cells such as natural killer cells and cytotoxic T lymphocytes [35].

Death ligands are tumor necrosis factor- α (TNF-α), Fas ligand (FasL) and apoptosis ligand 2/TNF-related apoptosis-inducing ligand (Apo2L/TRAIL), all of which belong to the TNF superfamily [35].

Death receptors (DR) are members of the TNF-receptor superfamily. They are transmembrane receptors which possess cystein-rich extracellular domains and a characteristic common ~80 amino acid sequence in their cytoplasmic domain which is called “death domain” (DD) [33, 38].

To this day, there are six identified DRs: TNF-R1 (receptor of TNF-α), Fas (receptor of FasL), DR3 (receptor of TL1A), DR4 and DR5 (receptors of TRAIL) and DR6 (receptor of N-APP (an amino-terminal fragment of the amyloid precursor protein) implicated in the pathology of Alzheimer) [35].

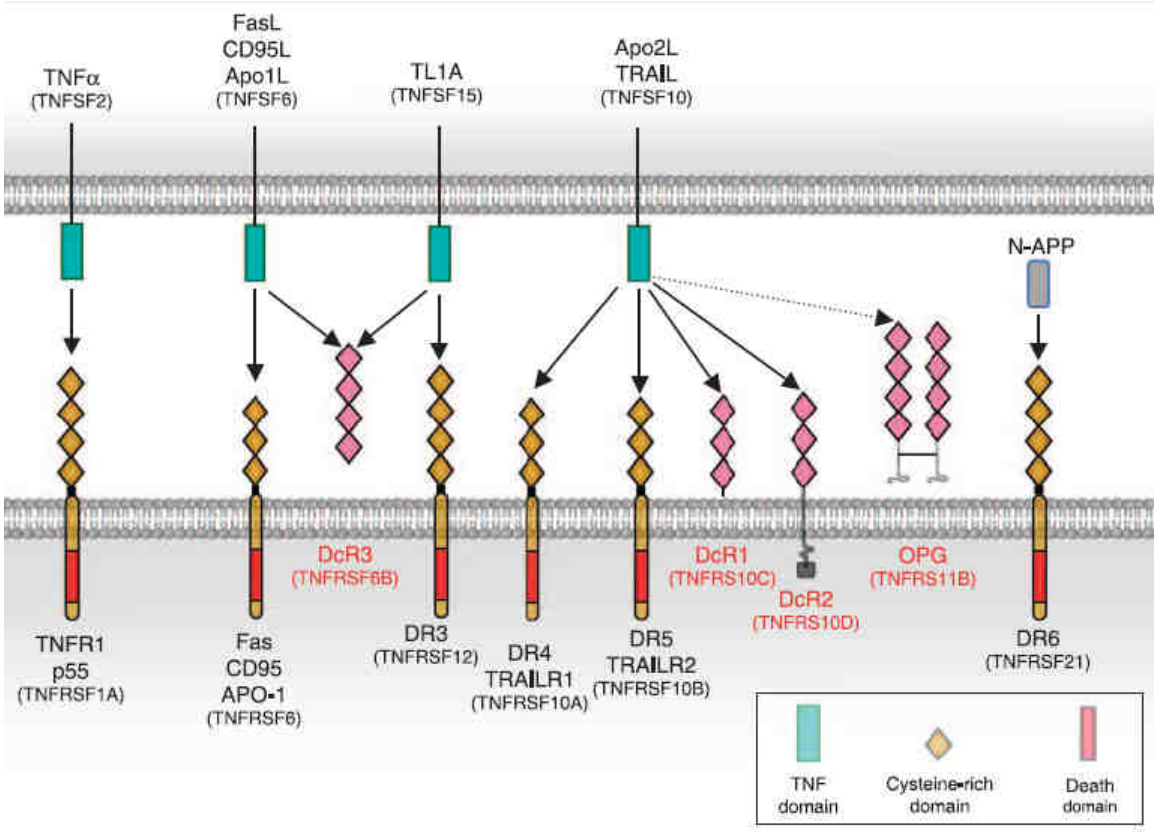


Figure 9: Death receptors and their ligands [35]

The binding of the receptors by their ligands induces receptor oligomerization and recruits death signal adaptor proteins - either the adaptor molecule FADD (Fas-associated death domain) for Fas and TRAIL receptors or TRADD (TNFR-associated death domain) for TNFR1 [23]. In the case of FasL and TRAIL, the recruited FADD forms the multi-protein death-inducing signaling complex (DISC) with the oligomerized receptors and their DDs [33]. In the DISC, FADD binds to caspase-8 and -10 and activates them by cleavage [33]. Caspase-8 and -10 activate the effector caspases-3, -6 and -7 which ultimately lead to apoptotic cell death [35].

A modulator of the extrinsic pathway is the cellular FLICE-inhibitory protein (c-FLIP) [34]. It is recruited to the DISC by interactions with FADD because its structure has a certain homology with caspase-8 and -10 and thus competes with their binding [35].

Signaling complexes, including TRADD, can either interact with FADD to induce apoptosis or with TRAF2 to activate anti-apoptotic NF-κB signaling (see 2.6.4) [39].

Under certain circumstances, DR4 and DR5 are able to promote the formation of a secondary signaling complex after the assembly of the DISC, the primary complex [40]. This secondary complex contains RIP1, TRAF2 and inhibitor of NF-κB kinase subunit gamma (IKK-γ) and is able to trigger NF-κB and MAPK signaling pathways [40].

2.2.1. TRAIL and its receptors

In contrast to the other pro-apoptotic family members such as Fas ligand and TNF-α, TRAIL has the unique feature of killing tumor cells, but sparing normal cells [35]. Thus TRAIL has emerged as a promising candidate for cancer therapy over the last decades since its discovery in 1995 [35, 41]. It is a type 2 transmembrane, zinc-coordinated glycoprotein that can be cleaved to form a soluble ligand [35]. TRAIL activates the extrinsic apoptotic pathway by binding in a homotrimeric form to pre-assembled death receptor trimers at the cell surface [35, 42]. Ligation of TRAIL induces further clustering of the receptors into high-molecular weight complexes [35, 42].

In addition to the agonistic receptors DR4 (also called TRAIL-R1) and DR5 (TRAIL-R2), there are two antagonistic TRAIL receptors at the membrane level, the decoy receptor 1 (DcR1) (TRAIL-R3) and DcR2 (TRAIL-R4) [43]. The decoy receptors are also members of the TNFR superfamily but do not have a functional DD. Therefore they are non-signaling receptors that compete with DR4 and DR5 for TRAIL binding [35]. A third decoy receptor of TRAIL is the soluble protein osteoprotegerin which is involved in bone metabolism.

The binding of TRAIL to its receptors provokes clathrin-mediated and dynamin-dependent DR4/DR5 internalization, which, however, is not required for TRAIL-mediated DISC formation and induction of apoptosis [35]. Besides the apoptotic pathway mediated by caspase-8, TRAIL is also able to activate kinase signaling cascades as IKK (inhibitor of κB kinase) leading to activation of NF-κB, of c-Jun N-terminal Kinase (JNK) and of p38 MAPK pathways (Figure 10) [40].

TRAIL-induced activation of the pro-survival and pro-proliferation transcription factor NF-κB can lead to the subsequent transcription of anti-apoptotic factors such as c-FLIP and XIAP [35]. Thus the TRAIL-induced activation of NF-κB is antagonistic to the apoptosis induction by TRAIL; hence its physiological relevance is not yet fully understood [35].

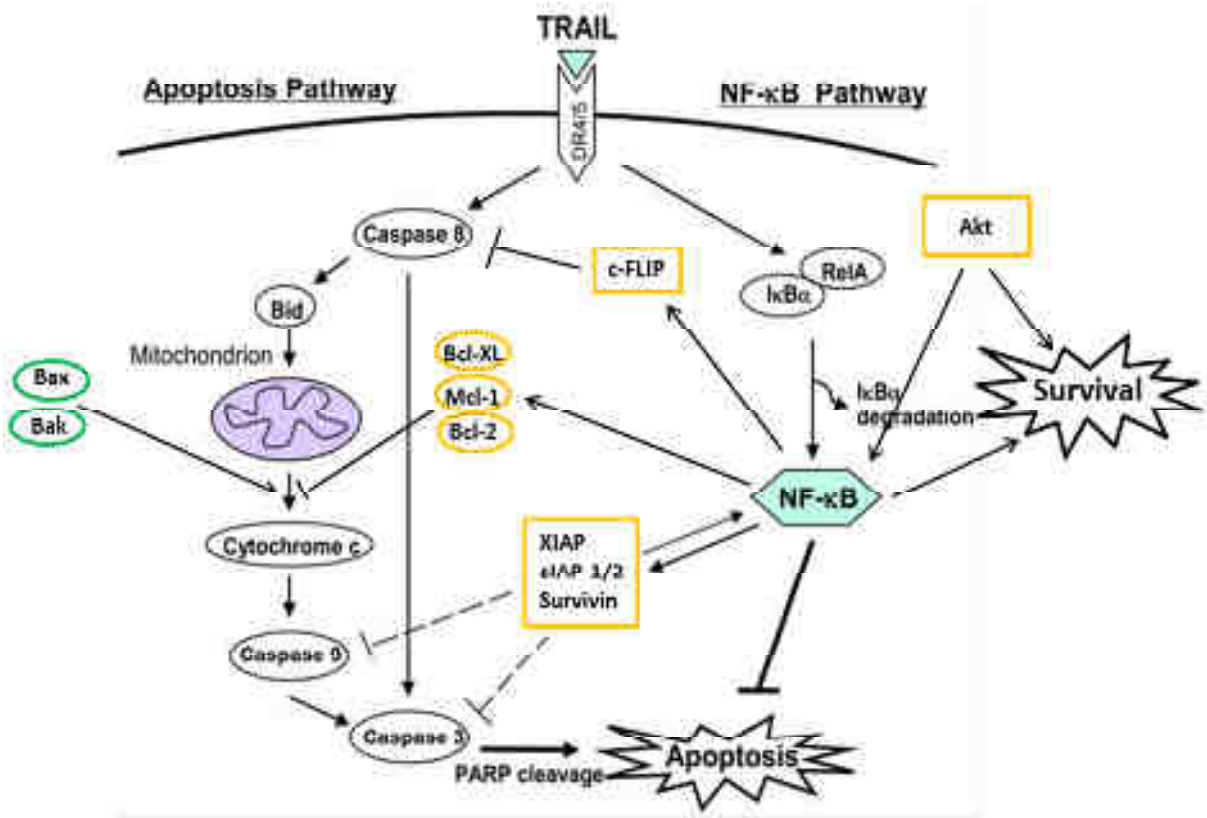


Figure 10: TRAIL-induced pathways, modified after [44]

TRAIL is expressed in many tissues and constitutively transcribed by some cell lines [41], it has been shown to be expressed in colonic adenocarcinoma cells [45] and in sporadic and hereditary colorectal tumors in patients [46]. Its cumulated expression on the surface of various cells of the immune system such as natural killer cells, cytotoxic T cells, macrophages and dendritic cells suggests a physiological function of TRAIL in the immune response [35]. Actually, studies have shown that TRAIL plays a role in the immune response to viral infections and may act as a tumor suppressor in the immune surveillance of tumors and immune-mediated tumor suppression [47, 48].

The activation of TRAIL death receptors is a promising approach to selectively targeting cancer cells because normal cells - in contrast to many cancer cells - are not sensitive to TRAIL [33]. Most normal cells express DR4 and DR5, though at a low degree [48]. In contrast, TRAIL receptors are often highly expressed in CRC cells [23].

Agonistic antibodies activating DR4 and DR5 as well as recombinant human TRAIL (rhTRAIL) are currently studied in phase II clinical trials to evaluate safety and efficacy in cancer patients [35].

However, resistance to TRAIL is a common problem in TRAIL-based therapies and therefore results from clinical trials show only small effects when TRAIL agonists are used as a monotherapy [48]. Mechanisms of resistance to TRAIL include (cf. Figure 10): lack of expression of TRAIL receptors, inefficient transportation of the receptors to the cell membrane, inhibition of caspase-8/10-activation by overexpression of FLIP, loss of pro-apoptotic and overexpression of anti-apoptotic Bcl-2 family members, overexpression of the inhibitor of apoptosis (IAP) proteins XIAP/survivin/cIAP-1/cIAP-2 (see 2.6.1) [48].

In TRAIL-resistant tumors, TRAIL can even promote proliferation because often TRAIL-induced NF-κB signaling is still functional. Thus it is crucial to identify biomarkers that can predict resistance to TRAIL, and to establish combination therapies to overcome resistance of cancer cells to TRAIL [48].

2.2.2. Other death ligands and their receptors

Fas (also called Apo1 or CD95) and FasL are constitutively expressed in colon cancer and therefore represent also a target for apoptosis induction [23]. However, unlike TRAIL-, Fas-induced cell death is not limited to tumor cells. The Fas/FasL system mainly plays a role in the following scenarios: activation-induced cell death of T cells, cytotoxic T lymphocyte-mediated killing of target cells, killing of inflammatory cells in immune privilege sites and killing of cytotoxic T lymphocytes by tumor cells [49].

TNF is mostly expressed in activated macrophages, T cells and some tumor cell lines. Interaction of TNF and TNFR-1 permits the recruitment of TRADD which may interact with FADD to induce apoptosis [49].

When TNF and FasL bind to their respective receptors, TNFR-1 and Fas are internalized by clathrin-mediated endocytosis which is required for apoptosis stimulation. In contrast, TNFR-1 and Fas ligation can also activate NF-κB and MAPK activation which does not need receptor internalization [35].

2.3. The intrinsic pathway (mitochondrial pathway)

The intrinsic apoptotic pathway is triggered by mitochondrial outer-membrane permeabilization (MOMP) induced by intracellular stress such as ROS formation, DNA damage, hypoxia, irradiation and cancer therapeutic agents [23, 33]. Changes in mitochondrial membrane potential and MOMP may lead to the release of pro-apoptotic proteins such as cytochrome c, second mitochondrial activator of caspases/direct IAP binding protein with low pI (Smac/DIABLO), apoptosis-inducing factor (AIF), endonuclease G or Omi/HtrA2 from the intermitochondrial membrane space into the cytosol (Figure 8) [34].

Released cytochrome c binds the adaptor protein Apaf-1 (apoptotic protease-activating factor-1), inducing the assembly of a multimeric complex called apoptosome which triggers proteolytic activation of procaspase-9 to caspase-9 [23]. Caspase-9 then activates effector caspases such as caspase-3.

Released Smac/DIABLO inactivates IAPs, thus indirectly increasing caspase activation (see 2.6.1) [23].

The mitochondria are also able to induce apoptosis in a caspase-independent way [50]. When AIF and endonuclease G are released from the mitochondrial intermembrane space after MOMP, these proteins translocate to the nucleus and induce chromatin condensation and DNA fragmentation.

Omi/HtrA2 is able to contribute to caspase-dependent (by neutralizing endogenous inhibitors of caspases) as well as caspase-independent apoptosis (as a protease) [50].

2.3.1. The Bcl-2 family

The activation of the mitochondrial pathway is regulated by the balance between pro- and anti-apoptotic members of the Bcl-2 (B-cell lymphoma-2) family which control the release of apoptogenic proteins from the mitochondria [33]. The Bcl-2 family consists of more than 20 proteins which can be divided into three groups based on their structure and their role in apoptosis (Figure 11) [23, 33].

Bcl-2 family members are defined by sharing at least one of the four evolutionarily conserved Bcl-2-homology domains (BH1-4) [33, 35]. These domains, mainly the BH3 domain, enable the proteins to regulate apoptosis by interaction with the Bcl-2 homology regions of other family Bcl-2 members. Thus members of the family can form homo- or heterodimers with each other (Figure 12) [23].

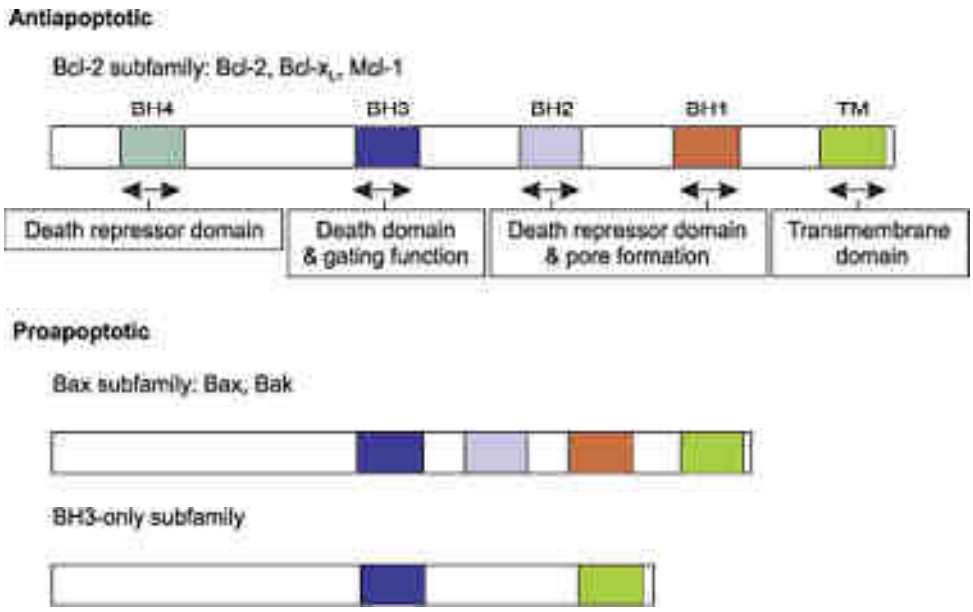


Figure 11: Structure of Bcl-2 family members

(NB: not all BH3-only subfamily members contain a TM domain), modified after [51]

Pro-apoptotic members

The pro-apoptotic Bcl-2 family proteins include multi-BH domain proteins and more than a dozen BH3-only pro-apoptotic proteins (Figure 11) [35, 52].

The oligomerization of the multi-BH domain proteins Bax (Bcl-2-associated X protein) and Bak (Bcl-2 homologous antagonist/killer) in the mitochondrial outer membrane induces MOMP and thus the release of pro-apoptotic factors (Figure 12) [33].

BH3-only proteins such as Bim, Bad, Puma, Noxa and Bid (BH3 interacting-domain death agonist) only possess the BH3 domain which allows them to bind to and inhibit the anti-apoptotic Bcl-2 family members, thereby indirectly promoting the oligomerization of the multi-domain pro-apoptotic proteins Bax and Bak (Figure 12) [33]. They are activated by transcriptional or post-translational mechanisms in response to different apoptotic stimuli such as endoplasmic reticulum stress, cytokine deprivation or DNA damage [35].

Anti-apoptotic members

Anti-apoptotic/pro-survival members include amongst others Bcl-2, Bcl-xL and Mcl-1 (Figure 11). They possess four Bcl-2 homology regions [33] and are able to inhibit MOMP induction by neutralizing pro-apoptotic family members such as Bax and Bak [35].

Increased levels of anti-apoptotic proteins such as Bcl-2 and Bcl-xL or reduced levels of pro-apoptotic proteins such as Bax and Bak accompany increased resistance of cancer to chemotherapy [23].

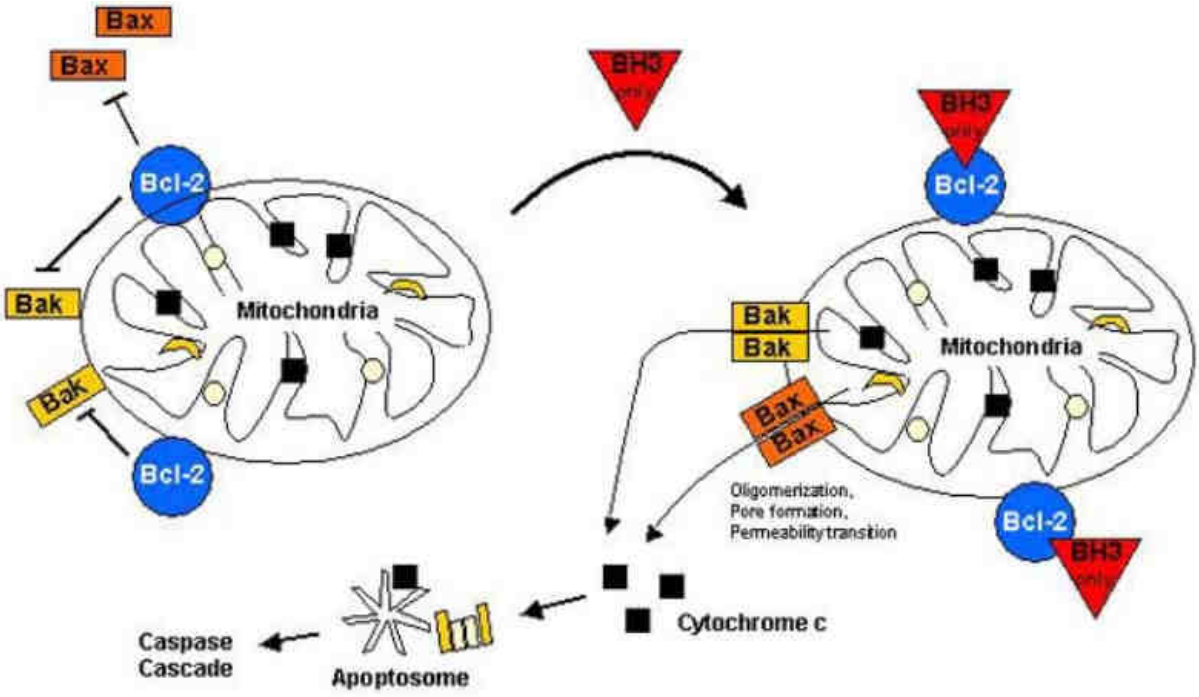


Figure 12: Regulation of apoptosis by the Bcl-2 family members (www.celldeath.de)

2.3.2. Oxidative stress

Cellular oxidative stress originates from an imbalance between ROS and the antioxidative systems of the cell. ROS - oxygen-containing chemical species with reactive chemical properties - consist of short-lived diffusible molecules such as hydroxyl ($^{\cdot}OH$), alkoxyl ($RO^{\cdot}$) or peroxyl ($ROO^{\cdot}$) radicals as well as superoxide (O_2^{-}), nitroxyl radical ($NO^{\cdot}$) and hydrogen peroxide (H_2O_2) [38]. Due to their high reactivity, ROS react with and thereby damage the cell structure including proteins, lipids, membranes and DNA [53]. Under normal conditions in a cell, ROS are detoxified by antioxidants and antioxidative enzymes like reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD). Besides their destructive role, ROS act as chemical messengers and are involved in receptor-mediated signaling pathways and transcriptional activation [38].

In cancer, ROS play an important dual role [54]. Cancer cells are under increased oxidative stress due to oncogenic transformation, increased metabolic activity and mitochondrial dysfunction. Mitochondria are the major source of ROS generation in cells through the respiratory chain but they are also a vulnerable target to oxidative stress [53]. Their dysfunction contributes to ROS-induced damage of the mitochondrial DNA which in turn amplifies their ROS production, creating a vicious cycle which promotes genetic instability, mutations and development of drug resistance [54].

On the other hand, ROS are able to kill cancer cells by various pathways [54]. Cytochrome c release from mitochondria seems to be a main mechanism of ROS-mediated cell death. The pathway of Fas receptor activation also uses ROS as secondary messengers which can also induce Fas receptor and Fas ligand expression.[38].

A further dualism in oxidative stress concerns the role of phytochemicals: Although they often exert remarkable antioxidant activity they are able to target the redox system of the cancer cells to induce ROS-mediated apoptosis, involving ROS-induced up-regulation of the TRAIL death receptors DR4 and DR5 as many recent studies have shown [55–58].

When ROS are at the origin of apoptosis, it can be inhibited *in vitro* by exogenous antioxidants and ROS scavengers such as GSH and N-acetylcysteine (NAC) [38].

2.4. Cross-talk of the extrinsic and intrinsic pathway

The cell type determines if successful apoptosis induction triggered by the extrinsic apoptotic pathway needs the activation of the intrinsic pathway [35]. In type I cells, the caspase cascade having been activated by the extrinsic pathway is sufficient for the activation of apoptosis, whereas type II cells depend on the additional activation of the mitochondrial pathway as an amplification loop [47].

The connection between the two pathways consists in the BH3-only protein Bid which is cleaved to truncated Bid (tBid) by caspase-8 or -10 [47, 52]. tBid then translocates to the mitochondria and activates either Bax by stimulating its translocation to mitochondrial membranes or Bak which in turn induces MOMP to trigger the release of pro-apoptotic factors [47, 52]. Most cancer cells respond in a type II manner [35].

2.5. Caspases

Caspases are specialized **c**ysteine-**asp**artate prote**ases** that cleave their substrates following an Asp residue [59]. They are synthesized as inactive precursors and have to be proteolytically processed to be activated.

In apoptosis, caspases are activated in a hierarchical order. The “initiator” caspases (-2, -8 and -10 in the extrinsic, -9 in the intrinsic pathway) proteolytically activate the downstream “effector” caspases -3, -6 and -7 which in turn cleave numerous cellular substrates and ultimately lead to the death of the cell by activating DNases [33].

To interact with other molecules, caspase-2 and -9 contain caspase recruitment domains (CARD), caspase-8 and -10 contain death effector domains (DED) which bind to FADD whereas caspase-3, -6 and -7 contain neither of them [59].

In cell death, caspase activation is responsible for the morphological manifestation of the hallmarks of apoptosis, including internucleosomal DNA fragmentation, chromatin condensation, cell shrinkage and plasma membrane blebbing [23].

There are also caspases that do not play a role in apoptosis execution, though in inflammation [59].

2.6. Regulation of apoptosis

2.6.1. The IAP family

The family of IAPs (Inhibitor of APoptosis) in humans is composed of eight anti-apoptotic proteins, including the X-linked inhibitor of apoptosis protein (XIAP), survivin and cellular inhibitor of apoptosis protein 1 and 2 (cIAP-1, cIAP-2) [23]. The hallmark of the IAP family is the BIR (baculoviral IAP repeat) domain [35]. IAPs promote survival signaling pathways and proliferation, are ubiquitously expressed and frequently overexpressed in cancer [60]. The IAP family plays an important role in the inhibition of apoptosis in response to various apoptotic stimuli [61]. Thus it mediates resistance to chemo- and radiation therapies and constitutes a therapeutic target for the treatment of cancer [61].

XIAP is the only direct inhibitor of caspases which acts downstream of the mitochondrial pathway by binding to caspases-3, -7 and -9 [35, 60]. The other IAPs block the assembly of apoptosis signaling complexes, e.g. the TNFR complexes in which the presence of cIAP-1/2 decides about cell death or survival [60, 61].

Survivin is the smallest member of the IAP family with a single BIR domain and plays a multifunctional role in cell division and inhibition of cell death [62]. It is abundantly upregulated in human tumors - while almost undetectable in normal adult tissue - and is involved in cancer progression and treatment resistance [63].

Smac/DIABLO is an endogenous IAP antagonist which is released from the mitochondria in apoptotic scenarios and binds to XIAP to prevent XIAP-mediated inhibition of caspases [60]. In turn, cIAP-1/2 bind to Smac/DIABLO to inhibit the binding of the latter to XIAP [64]. Besides controlling cell death, cIAP-1/2 also play a role in NF-κB signaling pathways (see 2.6.4).

2.6.2. The MAPK (mitogen-activated protein kinases) pathways

The MAPK pathways are serine-threonine kinase modules activated in response to extracellular signals (Figure 13). Their signal transduction cascades regulate elemental cellular processes such as cell proliferation, apoptosis, migration and differentiation [42, 65]. There are three major subgroups in humans: the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK) and the p38 MAPK [42]. Each cascade includes three kinases: a MAPK kinase kinase (MAP3K) which activates a MAPK kinase (MAP2K) by phosphorylating, which in turn activates a MAPK by phosphorylating. Finally, the activated MAPK phosphorylates substrate proteins including transcription factors such as p53, c-myc and c-jun [42, 64]. MAPKs play an important role in the development and progression of cancer as they are involved in the main events of tumorigenesis like proliferation, apoptosis, angiogenesis etc. [65].

The ERK pathway is mainly known for its proliferation signaling activated by growth factors and mitogens [65]. Signal transduction is mediated by members of the Ras family [64]. In colon cancer, K-Ras is frequently mutated leading to constitutive activation of the ERK signaling pathway. Likewise, mutations of B-Raf, a MAP3K of ERK signaling, result in constitutive activation. The ERK pathway is able to induce the expression of matrix metalloproteinases (MMPs) and thus promote the degradation of the extracellular matrix and tumor invasion [64].

JNK and p38 pathways are called stress-activated MAPK pathways [65]. Activators include the proinflammatory cytokines TNF-α and IL-1β, and hypoxic, metabolic, oxidative, genotoxic and pharmacological stress [42, 64]. In general, their effects are anti-proliferative and pro-apoptotic but their effect depends on the cellular environment [65].

Activated JNK can bind to and phosphorylate p53, leading to an increased p53 transcriptional activity and stability (see 2.6.3). The p38 MAPK is also involved in p53-activation and in its apoptotic signaling. There are many chemotherapeutic agents that induce apoptosis by activating p38 [65].

Ultimately, even if the role of JNK and p38 signaling is mostly pro-apoptotic and that of ERK anti-apoptotic, examples of the opposite have also been reported [66]. Many parameters such as the cell type, exposure time, status of other MAPKs and other cellular pathways are involved in the overall response to a given anticancer drug. The role of MAPKs in cancer is pleiotropic and has to be re-evaluated for each cellular setting [66].

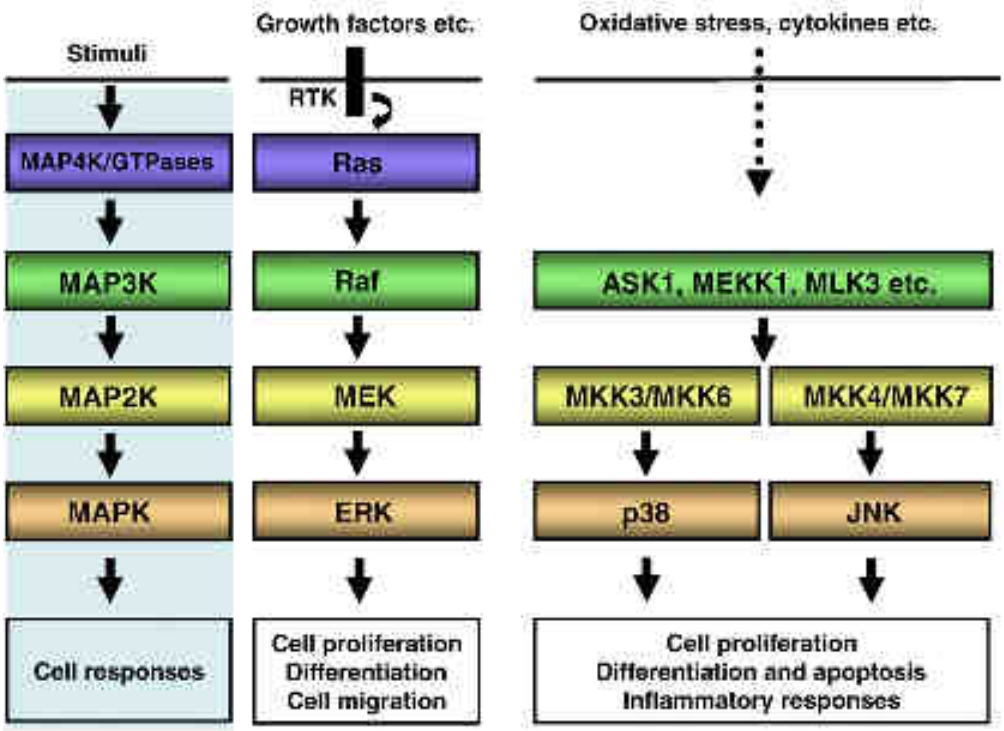


Figure 13: Mitogen-activated protein kinase (MAPK) signaling pathways [64]

2.6.3. The tumor suppressor p53

The tumor-suppressor gene *TP53* encoding the protein p53 is called the "guardian of the genome" [33]. The protein p53 triggers apoptosis or cell cycle arrest in response to DNA damage and further cellular stress induced for example by radiation, chemical agents or ROS. The objective is to preserve the integrity of the genome by the elimination of irreparably damaged cells and thus the protection against neoplasia [67].

The p53-initiated responses are mediated by its function as a transcriptional activator: For the induction of apoptosis p53 upregulates pro-apoptotic genes of both the extrinsic and the intrinsic pathway such as *PUMA*, *NOXA*, *BAX* and *FAS* at the transcriptional level and represses the expression of anti-apoptotic genes such as *BCL-2* [67, 68]. Furthermore p53 can upregulate the expression of antioxidant genes and induce autophagic cell death to maintain genomic integrity (see 3.3) [67].

In addition to its role as a transcription factor, p53 can directly promote MOMP by protein-protein interactions with the members of the Bcl-2 family [67].

More than half of human cancers have mutations in the *TP53* gene [67]. Loss of p53 provides an enormous growth advantage to tumor cells, as they lose p53-induced apoptosis and autophagy as possibilities of elimination [68].

2.6.4. The transcription factor NF-κB

The Nuclear Factor-κB (NF-κB) family of eukaryotic transcription factors regulates cellular responses to injury and infection. In cancer, its frequently constitutive activation accompanies cell proliferation, survival, angiogenesis and metastasis rendering NF-κB an attractive target for chemoprevention and chemotherapy [69].

The signal-activated NF-κB transcription factors are assembled through the dimerization of five different subunits (RelA (p65), c-Rel, RelB, p50 and p52). In the cytoplasm, they are bound by their inhibitors (inhibitor of κB, I-κB) and thus are inactive. Once released by the degradation of I-κB, they translocate to the nucleus to activate transcription [69].

For its degradation, I-κB is first phosphorylated by the complex of IKK which consists of IKK-α, IKK-β and IKK-γ, then degraded by the 26S proteasome [35, 69].

In cancer, activation of the NF-κB signaling is able to suppress apoptosis by inducing the transcription of anti-apoptotic genes such as Bcl-xL, c-FLIP, XIAP and cIAP-1 [23, 65].

NF-κB signaling frequently plays a role opposed to JNK signaling as NF-κB signaling can prevent oncogene-induced apoptosis, while JNK activation leads to apoptosis. Inhibition of NF-κB may represent a way to promote JNK-dependent apoptosis in some cells [65].

However, there is also evidence for a pro-apoptotic role of the NF-κB pathway: overexpression of the subunit c-Rel was shown to enhance expression of DR4, DR5, Fas and FasL and to inhibit cIAP-1, cIAP-2 and survivin [70], furthermore RelA (p65) can act as activator as well as repressor of anti-apoptotic gene expression [71]. This demonstrates the complexity of the role of NF-κB in apoptosis regulation.

2.7. Apoptosis and cancer

Apoptosis constitutes a possibility for the organism to eliminate genetically damaged cells which may otherwise develop into neoplastic cells [42]. Thus, disorders in apoptosis contribute to cancer development and treatment resistance [23].

More than 50% of neoplasms have defects in the apoptotic machinery. Frequent mutations are the overexpression of pro-survival Bcl-2 family proteins and mutations of the tumor-suppressor gene *TP53* [33].

Selective induction of apoptosis in cancer cells represents a promising approach for cancer therapy [23]. In fact, chemotherapeutic agents often work by inducing apoptosis in tumor cells [23, 42]. In addition, induction of apoptosis in a tumor does not lead to an immune response in the body [23].

Anticancer therapies directed toward several molecular targets are more promising, as apoptosis is a complex process controlled by many different signaling pathways [23].

3. Autophagy

3.1. Characteristics

The word “autophagy” derives from the Greek words “φαγεῖν” (“to eat”) and “αυτο” (“oneself”) and describes a process by which the cell generates energy and nutrients by digesting its own cellular components such as organelles and macromolecules [33].

There are three forms of autophagy: Microautophagy in which an invagination of the lysosomal membrane envelops the cargo; chaperone-mediated autophagy in which heat-shock cognate proteins deliver substrates to lysosomes; and macroautophagy. Macroautophagy describes the process of the formation of a double-membrane structure, the autophagosome, which engulfs bulk cytoplasm or organelles and then fuses with a lysosome [33].

In the following, “autophagy” stands for macroautophagy.

Autophagy can serve (1) as a basal housekeeping mechanism for the turnover of proteins and for the removal of redundant or defective organelles, (2) as a mechanism of cell survival induced by stress and starvation or (3) as a cell death mechanism [68].

More precisely, autophagy functions as a mechanism of cell survival as an adaptive response to sublethal stress such as privation of nutrients or growth factors, but can turn into a way of cell death in case of extensive self-digestion where the cell degrades all available substrates [68]. Cell death by autophagy is called “programmed cell death type II” [72].

3.2. Autophagic process

When autophagy is induced, autophagic vesicles are formed de novo in the cytosol through nucleation, assembly and elongation of the isolation membrane, to finally become double-membrane autophagosomes by the closure of the membrane structure (Figure 14). By the fusion of their outer membrane with the membrane of lysosomes autolysosomes are generated, the content of which is degraded together with the inner membrane by the lysosomal hydrolases [72].

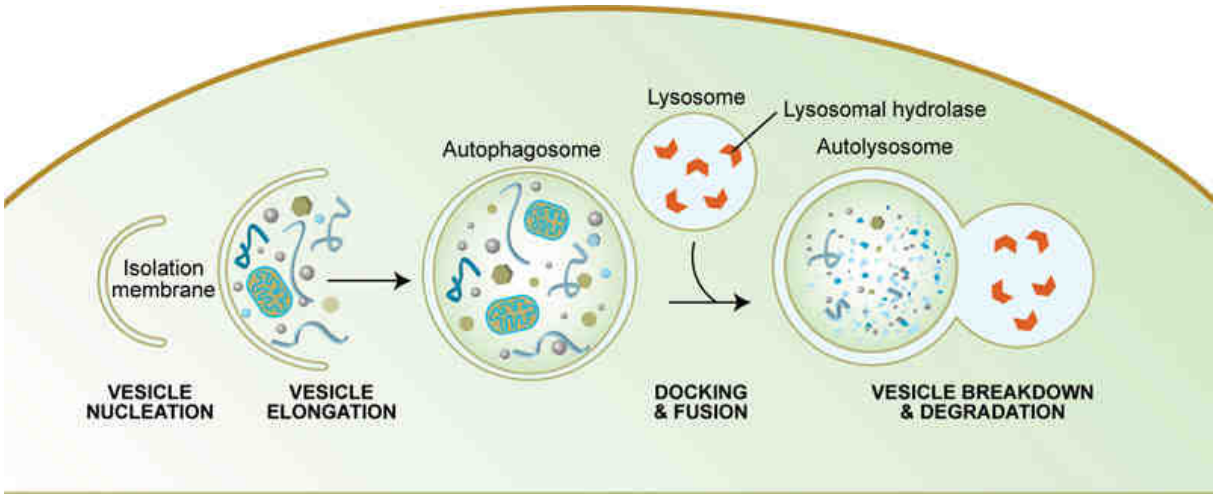


Figure 14: The cellular process of autophagy [73]

Autophagy is executed by the autophagy-related proteins (Atg). Two ubiquitin-like conjugation systems are essential for the elongation of the autophagosomes membrane during vesicle formation (Figure 15) [68]. First, Atg5 is conjugated to Atg12 in an ubiquitination-like manner, then the Atg12/Atg5 dimer binds to Atg16L. Through Atg16L, the multi-protein complex Atg12/Atg5/Atg16L is recruited to the forming outer membrane of the autophagosome [72].

The second conjugation system consists of Atg8 (mammalian microtubule-associated protein Light Chain 3, LC3) and phosphatidylethanolamine (PE) whose conjugation is mediated by Atg3 and Atg7. Consequently the carboxy-terminal glycine of LC3 is conjugated via an amide bond to an amino group of PE [72]. The site of LC3 lipidation - which occurs only during autophagy - is determined by the membrane localization of the Atg16L complex. The conversion of the soluble cytoplasmic form (LC3-I) to the autophagosome-membrane-bound form (LC3-II) is required for membrane expansion [68]. LC3-II can therefore be used as a marker of autophagy [74].

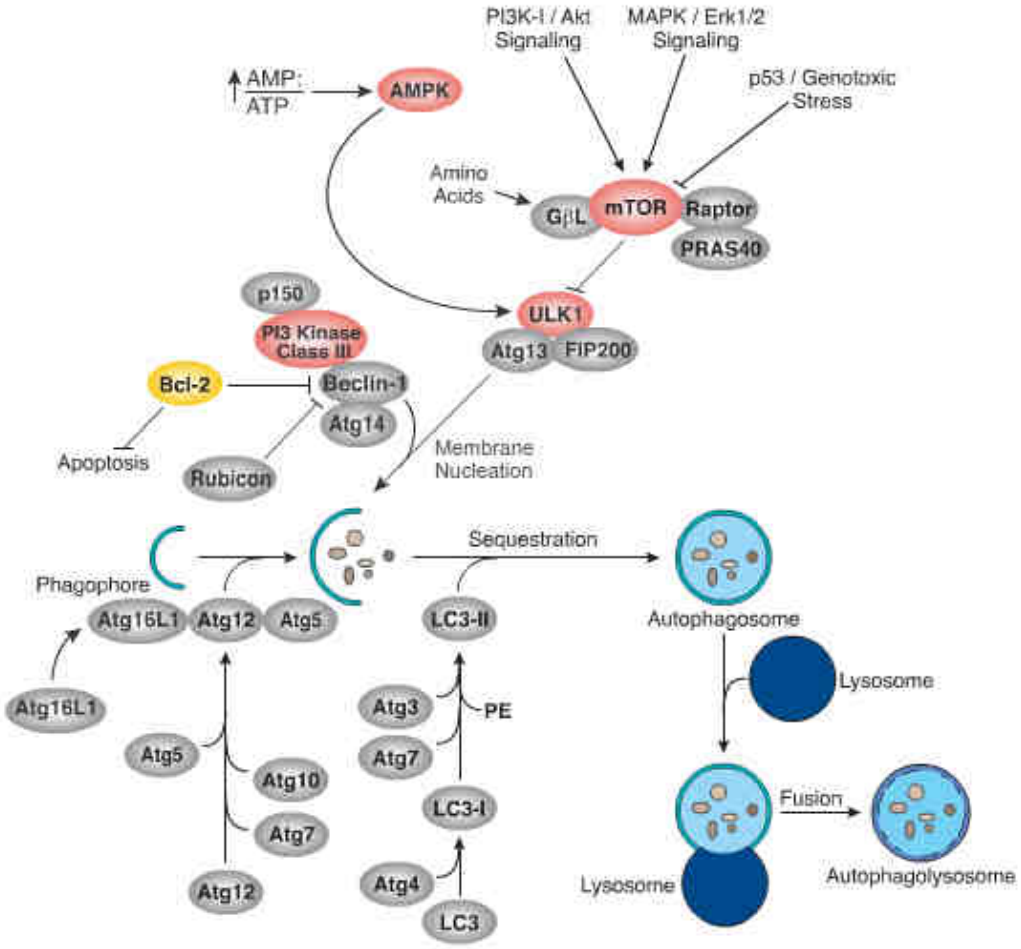


Figure 15: Autophagy signaling (www.cellsignal.com/reference/pathway/autophagy.html)

3.3. Regulation of autophagy

The Vps 34 kinase complex, a complex containing a class III phosphatidylinositol-3-kinase (PI3K) and regulatory proteins such as Beclin-1, is necessary for autophagosome formation in the canonical autophagic pathway [68]. Autophagy can be inhibited by inhibitors of PI3K such as 3-methyladenine or wortmannin [72].

The mTOR (mammalian target of rapamycin) kinase plays a major role as a molecular sensor of cellular energy and growth factor levels [68]. Thus the activation of the mTOR pathway inhibits autophagy while an inactivation of mTOR e.g. by rapamycin induces autophagy [72]. When energy grows scarce, mTOR is inactivated, thus autophagy is induced.

The tumor suppressor p53 plays a dual role in autophagy: nuclear p53 can upregulate autophagy leading to cell death, but cytoplasmic p53 suppresses autophagy with survival function. Thus the loss of the p53 function leads to the absence of autophagic cell death and to the activation of pro-survival autophagy [68].

3.4. Interplay of autophagy and apoptosis

Autophagy and apoptosis share a complex relationship (Figure 16). There are three possibilities of cross-talk between them: Apoptosis and autophagy can act as partners to induce cell death (Figure 16a), cooperatively or backing up each other in case of the defect of one pathway; autophagy can behave as an antagonist to suppress apoptosis by acting as a cell survival pathway (Figure 16b); or autophagy can enable apoptosis by maintaining cellular ATP levels necessary for apoptosis without inducing death itself (Figure 16c) [68].

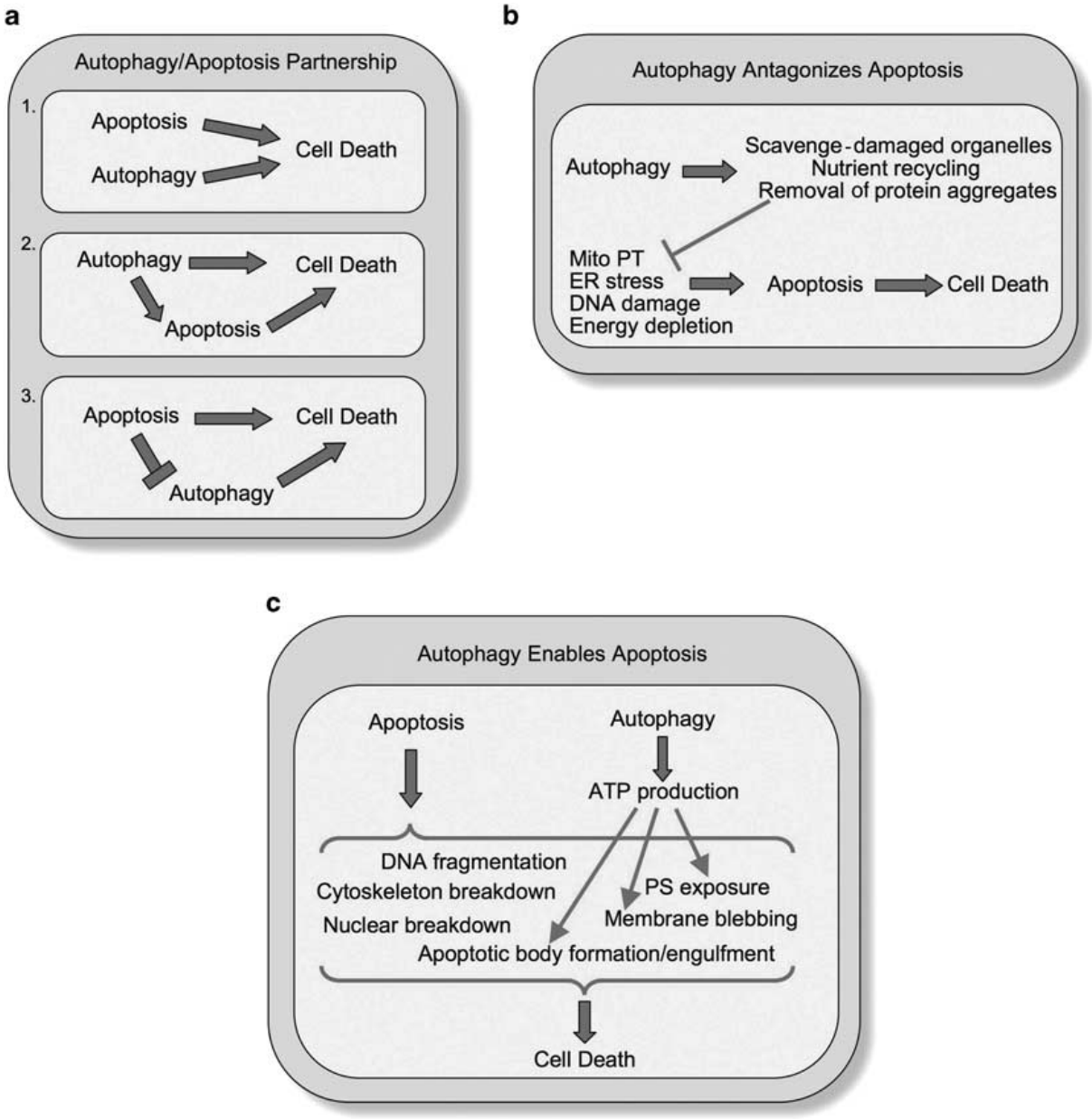


Figure 16: The relationship between apoptosis and autophagy [68]

Apoptosis and autophagy share numerous genes for the regulation of their pathways [68].

For example, Akt participates in positive regulation of mTOR activity, thereby inhibiting autophagy and at the same time, Akt can indirectly inhibit the pro-apoptotic functions of JNK and phosphorylate CREB (cAMP response element binding) to activate its transcriptional activity toward anti-apoptotic genes such as Bcl-2/xL [68].

The autophagic protein Atg5 also plays a role in apoptosis: Apoptotic stimuli lead to its cleavage by calpain, whereupon its cleaved product translocates to the mitochondria. There it interacts with Bcl-xL and promotes cytochrome c release [68].

The apoptosis-regulating Bcl-2 family members are also involved in autophagy, e.g. Bcl-2 which is not only anti-apoptotic but also anti-autophagic [74].

Beclin-1, which is also part of the BH3-only family of Bcl-2 proteins, can bind to Bcl-2/Bcl-xL through a BH3 domain which also interferes with the Beclin-1/Vsps34 complex formation. Thus autophagy is blocked while apoptosis is promoted [34].

It may be determined by their localization at the mitochondria or at the ER if Bcl-2 family members exert apoptotic or autophagic functions [68].

3.5. Autophagy and cancer

Autophagy has a dual role in cancer: It functions as a tumor suppressor by deleting protein aggregates and misfolded proteins and scavenging depolarized ROS-producing mitochondria and thereby maintaining genomic integrity [33]. Malignant transformation is often associated with the suppression of autophagy [72]. But autophagy can also contribute to tumor development by buffering metabolic stress of the tumor cell and thereby promoting its survival [75].

Several known inducers of apoptosis as well as anticancer drugs have been shown to activate autophagy in cancer cells, for example tamoxifen, arsenic trioxide, resveratrol and etoposide [74]. Thus inducing autophagic cell death may be a new way to treat cancer [72].

As autophagy can play different roles such as functioning as a survival mechanism or death mechanism it is important to know its role in a cellular setting before being able to act accordingly.

4. Necrosis

The word “necrosis” derives from the Greek “νεκρός” (“corpse”). In necrosis, the plasma membrane loses its integrity with an influx of extracellular ions and fluid, leading to a rapid swelling of the cell which results in the rupture of its membranes and the spillage of the intracellular contents [33]. This is frequently the result of overwhelming physical or chemical damage to the cell - hence necrosis was seen as an accidental non-programmed cell death [34]. Recently, reports have demonstrated that necrosis may also be an actively controlled process and a normal physiological event [76].

As necrotic cell death often occurs when other modes of cell death are inhibited, for example after caspase inhibition in an apoptotic scenario, it may function as an emergency form of cell death. Furthermore, necrosis frequently seems to be implicated in apoptotic and autophagic processes, but as there is no simple and reliable assay, it often passes unnoticed [34].

In contrast to apoptosis, necrosis may be accompanied by an inflammatory response provoked by cytosolic constituents released into the intercellular space [76]. However, this reaction may have adaptive significance under pathological conditions such as cancer [76].

Necrosis can be mediated by ROS that are able to activate monovalent cation and/or Ca^{2+} permeable channels [34]. Furthermore, TNF-α, TRAIL and Fas are able to induce caspase-independent death receptor-mediated necrosis which is also linked to ROS production [34, 76]. In lower concentrations ROS preferably induce apoptosis, in higher ones mostly necrosis [34].

When the DNA is profoundly damaged, excessive activation of the nuclear DNA repair enzyme Poly(ADP-ribose)polymerase (PARP) also leads to necrosis [76]. PARP then depletes ATP stores of the cell; and as about 25-30% of basal ATP levels have to be kept up for the execution of apoptosis [34], then the exhaustion of ATP causes a shift from apoptosis to necrosis [33]. When apoptosis occurs, PARP is normally cleaved to prevent PARP-caused depletion of ATP stores. However, necrosis is not always accompanied by a drop in ATP levels [34].

5. Epigenetic mechanisms and cell death

Epigenetic modifications such as DNA methylation and histone acetylation play an important role in the regulation of gene expression. Tumors often show altered epigenetic modification patterns affecting various cancer-related genes, e.g. tumor suppressor genes silenced by promoter hypermethylation [77]. Epigenetic cancer therapy exploits reactivation of these genes by DNA demethylation [77]. Another approach in epigenetic therapy is to reverse the deacetylation of histones that frequently occurs in cancer and also leads to transcriptional repression of tumor suppressor genes [78].

Hence, two drug classes are established in cancer treatment, histone deacetylase (HDAC) inhibitors and DNA methyltransferase (DNMT) inhibitors. Currently, there are four Food and Drug Administration (FDA)-approved drugs, and additional DNMT and HDAC inhibitors are studied in clinical trials [77]. Interestingly, the molecular basis of their anticancer activity is not completely elucidated, as there has been no study yet which has proven a direct relationship between their epigenetic effects and clinical responses [77, 78]. Histone deacetylation and DNA methylation have been shown to interact synergistically, providing a rationale of combination therapy.

Cell death is directly concerned with the epigenetic molecular events. Hypermethylation of DNA and deacetylation of histones is responsible for silencing of many genes in apoptosis, e.g. down-regulation of DR4/DR5 which is often caused through silencing by hypermethylation [48]. DNMT inhibitors reactivate these aberrantly silenced genes and thus promote apoptosis [77]. HDAC inhibitors are able to promote cell cycle arrest, to activate apoptosis selectively in tumor cells and to enhance synergistically the activity of many chemotherapeutic drugs [78]. However, the mechanisms of HDAC inhibitor-induced cell death are not yet completely understood [78]. HDACs do not only deacetylate histones but also non-histone targets, which play a role in cell death. Thus it is probable that several different mechanisms are implicated. HDAC inhibitors have been shown to upregulate TRAIL and its receptor DR5, FasL and its receptor Fas, and TNF-α [78]. Consequently, they have also been shown to sensitize cancer cells to TRAIL-induced apoptosis. Moreover, they are able to increase the expression of pro-apoptotic and to decrease the expression of anti-apoptotic genes, thus favoring apoptosis induced by chemotherapeutic agents [78].

III. SILIBININ, A FLAVONOLIGNAN OF THE MILK THISTLE

1. Milk thistle (*Silybum marianum*) and silymarin

The milk thistle plant (*Silybum marianum* (L.) Gaertner of the Asteraceae family)) is native to the Mediterranean region [79, 80]. The weed-like herb has large purple flowers and thorny leaves (Figure 17). It is cultivated as medicinal plant and as ornamental plant in Germany, Austria, Hungary and other countries of Eastern Europe [81]. Milk thistle leaves and flowers can be eaten in salads. The seeds have been used for more than 2000 years - starting in ancient Greece - for the protection of the liver against chemical and environmental toxins and the treatment of liver diseases like hepatitis and cirrhosis [82].

The standardized extract from the fruit and the seeds of the milk thistle is called silymarin which was first isolated for its putative hepatoprotective purposes in 1968 [83–85]. Since then, it has been studied in hepatitis, cirrhosis, *Amanita phalloides* mushroom poisoning, cytoprotection for toxic occupational exposure, and prophylaxis against chemotherapeutic adverse effects [85]. Silymarin contains approximately 65-80% flavonolignans and 20-35% fatty acids and other polyphenolic compounds [86]. The major component of the flavonolignans is silibinin (about 50 to 60%), along with other constituents such as isosilibinin (about 5%), silichristin (about 20%), silidianin (about 10%), taxifolin and quercetin [83].

Figure 17: The milk thistle plant (http://altmed.creighton.edu/MilkThistle)

2. Characteristics of silibinin

2.1. Chemical structure

The flavonolignan silibinin (synonymous with silybin) constitutes the major biologically active compound of the crude extract silymarin [87]. Silibinin is composed of two diastereomers (A and B) (Figure 18). The diastereomer B of silibinin reacts faster in conjugation reactions than A, indicating that metabolization of the diastereomers takes place at different rates.

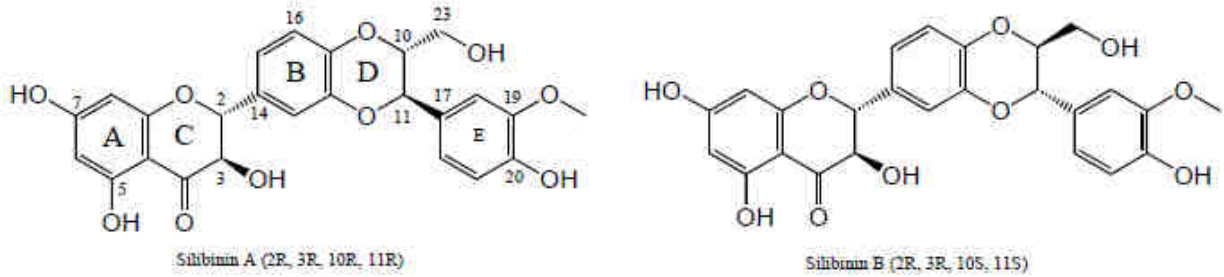


Figure 18 : Structure of silibinin [82]

The silibinin molecule possesses five hydroxyl moieties, three of which are of phenolic nature. The hydroxyl moieties are involved in conjugation reactions (see 2.2) [88]. Furthermore flavonoids with hydroxyl groups have been found to inhibit P450-dependent reactions which play a role in the activation of procarcinogens [89]. The 1,4-dioxane ring system has been shown to be important in anti-hepatotoxicity [86].

Silibinin has a very low solubility in water [90]. However, it is possible to create derivatives with improved hydrophilicity, for example by the enzymatic synthesis of its β-glycosides [90].

2.2. Bioavailability and metabolism

Preclinical and clinical studies have evaluated the bioavailability, the metabolism, the pharmacodynamics and the pharmacokinetics of silibinin. The bioavailability is important for the effectiveness of a drug as the limiting factor for the achievement of therapeutic concentrations in an organ.

Many flavonoids are poorly absorbed: because of their size (multiple-ring molecules) they cannot be absorbed by simple diffusion, furthermore they are not actively absorbed such as vitamins, and their lipophobicity prevents them from entering across the lipid-rich outer membranes of the enterocytes [91]. The absorption of silibinin lies between 20 and 50% [90]. However, its bioavailability can be improved: by the combination with phosphatidylcholine (PC) a lipid-compatible molecular complex is created, a so-called phytosome (defined as the complex of a natural active compound and a phospholipid, usually a flavonoid molecule linked with at least one PC molecule) [91]. Phytosome is a registered trademark of Indena S.p.A., Milan, Italy. Phytosomes facilitate the passage through the gastrointestinal mucosa because they are able to transit from a hydrophilic environment into the lipophilic cell membrane, onward into the cell and into the blood [91]. The commercial silybin-phytosome association uses PC from soy and is called IdB1016 (Silipide^{TM}) or Siliphos (see 3.8) [92]. PC is the principal component of the cell membrane, well absorbed when taken orally because it is miscible with water and oil, and it even shows clinical efficacy against liver diseases [93]. Comparative studies have proven the far superior bioavailability of silibinin from silybin-phytosome against silibinin from silymarin: when comparing the area under the curve in a study with 9 healthy volunteers, phytosomal silibinin was 4.6 times better absorbed [92]. In another study, the amount of silibinin in the bile was measured: 11% of the silibinin dose after silipide administration and 3% after silymarin administration were recovered after 48 h of bile collection, suggesting a 4-fold better bioavailability for phytosomal silibinin [94].

Silibinin quickly undergoes multiple conjugation reactions in humans so that most of the silibinin circulates in the bloodstream in conjugated form - as silibinin monoglucuronide, silibinin diglucuronide, silibinin monosulfate and silibinin glucuronide sulfate [88]. The half-life of plasma silibinin is short with less than 4 h [88, 95].

Pharmacokinetics of low-dose silybin-phytosome showed a peak concentration in the plasma of 298 ± 96 ng/mL (equivalent to 618 ± 199 nmol/L) 1.6 ± 0.3h after a single, oral dose of 360 mg silibinin [92]. The study of Hoh *et al*., using silybin-phytosome at dosages of 360, 720 and 1440 mg silibinin daily for 7 days, demonstrated levels of 0.3 to 4 µmol/L in the plasma of CRC patients with prominent interpatient variability [88]. In the colorectal tissue, Hoh *et al*. found that concentrations of 20 to 141 nmol/g tissue (equivalent to a concentration of 20 to 141 µmol/L) were obtained after 7 days of silybin-phytosome administration at dosages 360, 720 and 1440 mg, indicating a considerable accumulation of silibinin even after a short-term and low-dose therapy in the colorectal mucosa [88].

Flaig *et al*. investigated silibinin plasma concentrations in prostate cancer patients in a high-dose silybin-phytosome therapy [95]. After a daily dose of 13 g silybin-phytosome (equivalent to approximately 4 g silibinin) they reported peak plasma levels greater than 100 µmol/L.

2.3. Hepatoprotective properties and anti-oxidative activity

Silymarin and silibinin have traditionally been used for the treatment of hepatitis and cirrhosis and for the protection of the liver from toxic substances [86]. In the USA and Europe about 65% of patients with liver diseases take herbal preparations [90]. In modern medicine evidence partially confirms the benefits of silymarin and silibinin in the treatment of liver disorders such as hepatitis and cirrhosis, of diabetes mellitus, mushroom poisoning, neurodegenerative and neurotoxic diseases, nephrotoxicity and cancer (see 3) [82].

Liver disorders

In spite of the long history and its wide-spread use, the clinical efficacy of silibinin or milk thistle products in liver disorders is not fully ascertained [90]. This is mainly due to the limitations of the conducted clinical trials – typical for clinical studies on herbal products: small sample size, no sufficient randomization and no blinding, lack of characterization of the utilized product and of the inclusion/exclusion criteria [96]. Furthermore, most of the trials do not use the pure compound silibinin but rather the plant extract silymarin of which products differing in composition, in content of silibinin and in bioavailability are available and may lead to different clinical outcomes [90]. Therefore the Cochrane Collaboration group declared a need for randomized, high-quality clinical trials [97]. The need for drugs suitable for long-term use grows even more because today's patients with chronic liver diseases become younger as the frequency of non-alcoholic fatty liver disease (NAFLD) and alcoholic liver diseases increases [90]. In this respect silibinin is a very interesting drug because of the virtual absence of adverse effects, good compliance and its commercial availability. While the German Commission E recommends milk thistle for the treatment of liver damage due to toxins, cirrhosis of the liver and as a supportive therapy for chronic liver inflammation, the U.S. FDA does not approve the use of milk thistle for any medical use (www.cancer.gov).

However, some well-conducted studies with silibinin as a pure substance do exist and show its hepatoprotective potential (see Table 2): Consumption of a silybin-vitamin E-phospholipid complex significantly improved liver parameters, such as transaminase levels, in patients with chronic hepatitis C [98]. Silibinin showed strong antiviral action in patients with

chronic hepatits C virus infection by inhibiting its replication [99]. In patients with NAFLD the silybin-vitamin E-phospholipid complex succeeded in reducing liver fibrosis and steatosis and improved metabolic and liver parameters [100]. A meta-analysis about the clinical use of silymarin in liver diseases concluded that the use of silymarin is reasonable as a supportive element in the therapy of liver cirrhosis [101].

Mushroom poisoning

Administration of silibinin efficiently counteracts poisoning induced by the deathcap mushroom *Amanita phalloides*: a retrospective analysis of the amatoxin poisoning exposures from North America and Europe in the last 20 years showed an increased survival rate in persons treated with silibinin [102].

Antioxidant activity

The hepatoprotective activity of silymarin and silibinin is due to their strong antioxidant effects [84]. Silibinin supports redox homeostasis in the liver *in vitro* and *in vivo* models: It inhibits radical formation, scavenges reactive oxygen species (ROS), inhibits lipid peroxidation of membranes and malondialdehyde accumulation, activates superoxide dismutase (SOD) and cytochrome P450 enzymes, inhibits arachidonic acid metabolism, improves low density lipoprotein (LDL) removal and decreases the uptake of toxins [84, 103, 104]. In the presence of oxidative and nitrosative stress, silibinin inhibits the decrease of GSH, SOD, CAT, glutathione peroxidase (GPx) and glutathione reductase (GR) [90, 105]. Furthermore, silibinin acts as an iron chelator [106].

Anti-diabetic properties

Silibinin has anti-hyperglycemic properties [90]. It was shown to block glucose-6-phosphate hydrolysis, resulting in an inhibition of gluconeogenesis and glycogenolysis [107]. Silibinin β-cyclodextrin as an anti-diabetic drug significantly reduced glucose and triglyceride plasma levels in patients with alcoholic liver disease and non-insulin dependent diabetes mellitus without influencing liver function, probably due to a reduction in insulin resistance [108].

Further effects

In addition to its hepatoprotective effects, silibinin has recently been shown to possess bone-forming and osteoprotective effects in *in vitro* cell systems [109]. Moreover it seems to act as an inhibitor of Aβ aggregation, has been shown to reduce memory impairment *in vivo* and may thus be a potential therapeutic agent for the treatment of Alzheimer's disease [110, 111].

Table 2: Main studies on liver diseases performed with silibinin [90]

Authors	Type of study, number of patients	Drugs used, dose and duration of treatments	Outcomes	Results	Clinical relevance
Vailati *et al*	A phase II randomised, open trial on 60 patients with chronic alcoholic or viral hepatitis	Three doses (160, 240, 360 mg) of silybin and phosphatidylcholine (IdB 1016, Indena, Italy) for two weeks. No placebo or no intervention group was used	Liver tests	Improvement of liver enzymes with all used doses	Scarce
Buzzelli *et al*	Double blind with identical placebo. Twenty patients with HBV and/or HCV chronic active hepatitis	IdB1016 (complex with phosphatidylcholine and silybin) two capsules, twice a day (equivalent to 120 mg of silybin in each capsule) (480 mg/d). Duration of treatment and of follow-up: two months in total	Mortality. Liver biochemistry	Improvement of liver enzymes and bilirubin	Scarce
Buzzelli *et al*	Unclear, described as double blind, but the method to achieve this was not described. Trial characteristics: cross-over design. Patients were assigned to the Silipide group for two months treatment, and one month washout. Ten patients with chronic hepatitis C (non-responders) to a previous treatment with recombinant interferon α	Silipide (IdB1016) capsules 360 mg/d. Control group: placebo capsules. Duration of treatment and follow-up: two months of treatment and one month of washout	Mortality. Liver biochemistry	Results were not reported separately, only overall results. Improvement of liver tests	Data published only in abstract form
Lirussi *et al*	Blinding: adequate, double blind with placebo of identical appearance. Sixty out-patients with chronic alcoholic liver disease and non-insulin dependent type 2 diabetes	Silybin-β-cyclodextrin (135 mg silybin) sachets t.i.d. Duration of treatment: 6 mo	Mortality. Liver biochemistry	Decrease of fasting glucose and lipid peroxidation markers	Good
Bares *et al*	Randomised study to 1 of 3 oral doses. Thirty-seven patients with chronic hepatitis C non-responders to a previous IFN treatment	IdB1016 at 314, 628, 942 mg t.i.d. (120, 240 and 360 mg t.i.d. silybin equivalents, respectively) for 12 wk	Effects on serum markers of iron status	There was a significant decrease in serum ferritin, that was independently associated with the stage III-IV of liver fibrosi	Good
Falasca *et al*	Observational study on forty naïve HCV positive patients (30 treated and 10 observed without treatment)	Silybin-Vitamine E-Phospholipid Complex (Realsil®-Ibi-Lorenzini-Italy) in a dose of 4 pills per day (each pill: 47 mg of silybin) for 3 mo	Hepatoprotection and anti-inflammatory effect by determining cytokine pattern and markers of liver disease	Improvement of liver enzymes and of I12 plasma levels. Improvement of insulin resistance markers in patients with contemporaneous liver steatosis	Medium
Federico *et al*	Observational study on 85 out-patients: 59 with NAFLD and 26 with HCV related chronic hepatitis in combination with NAFLD, non-responders to previous antiviral treatment. 53 (39 NAFLD and 14 HCV) were treated, while the other 32 patients (20 NAFLD and 12 HCV) served as a control group (no treatment)	The complex silybin-vitamin E-phospholipids (Realsil®), 4 pieces/d for six months followed by another six months of follow-up	Effects on insulin resistance and liver damage	US steatosis, liver enzymes, hyperinsulinaemia, and indices of liver fibrosis were improved in both treated groups	Suggestive
Ferenci *et al*	Observational study on 36 patients with HCV chronic hepatitis non-responders to IFN + ribavirin. Duration of the study: 7 d	Silybin i.v. (Madaus, Germany) at 5, 10, 15 and 20 mg/kg per day for 14 d	Effect on viral load. Safety	Good compliance, no side effects and potent antiviral effect against HCV	High

2.4. Adverse effects

Silibinin is very well tolerated. At a typical daily dose of less than 1 g silibinin, there are no adverse effects, even in patients with compensated cirrhosis [112]. Most of the trials reported daily doses of silymarin (containing ~50% of silibinin) of 420-600 mg [101]. However, the choice of the daily dose of silibinin has not yet been based on a reasonable justification.

Flaig *et al*. conducted a phase I trial in prostate cancer patients (see 2.2) to determine the toxicity of high-dose silybin-phytosome therapy and to find a recommendation for an adequate phase II dose [95]. In their study the patients received daily doses ranging from 2.5 to 20 g silybin-phytosome in 3 divided doses for 4 weeks. They observed hyperbilirubinemia as the most prominent adverse event [95]. However, it was asymptomatic and improved after the end of treatment. Grade 1-2 bilirubin elevations were observed in 9 of the 13 patients, and 4 patients exhibited grade 1 lactate dehydrogenase (LDH) elevation. In one patient, grade 3 elevation of alanine transaminase (ALT) and grade 2 elevation of aspartate transaminase (AST) were found. However, this patient's transaminase levels normalized without having to reduce the dose. Another side effect of silybin-phytosome was a laxative effect leading to mild diarrhea which was generally not a problem. Grade 1 elevation in creatinine occurred in 6 patients but tended to disappear spontaneously without dose adjustment.

The explanation for the asymptomatic hyperbilirubinemia probably lies in the fact that silibinin inhibits the Uridinediphosphoglucuronate-glucuronosyltransferase-1A1 (UGT-1A1), the enzyme responsible for bilirubin glucuronidation [113]. In patients with Gilbert's syndrome, a common genetic disorder, functionality of UGT-1A1 is reduced, also resulting in hyperbilirubinemia. This syndrome is considered harmless [114]. However, irinotecan, a chemotherapy agent used in the treatment of CRC, is also metabolized by UGT-1A1. In consideration of the fact that patients with Gilbert's syndrome suffer from increased toxicity of irinotecan, caution should be taken when administering milk thistle products in association with irinotecan [95].

The conclusion of Flaig's study was that a daily amount of 13 g of oral silybin-phytosome (providing about 3.9 g of silibinin) is well tolerated in patients with advanced prostate cancer and is the recommended phase II dose. A recurring problem in these studies is the prominent interpatient variability [95].

In traditional toxicological tests, silybin-phytosome has also been proven safe: Oral acute toxicity is > 5,000 mg per kg in rats, dogs and monkeys [91].

3. Anticancer activity of silibinin

During the last decade silymarin and its major constituent silibinin have been discovered to possess anticancer effects in different *in vitro* and *in vivo* models of epithelial cancers such as skin [115], prostate [87], breast [116], cervix [117], lung [118], liver [119], bladder [120], kidney [121], and colon cancer [85]. Silibinin has pleiotropic effects targeting inhibition of multiple cancer cell signaling pathways such as anti-proliferative, anti-carcinogenic, anti-inflammatory, apoptotic, chemosensitizing, anti-metastatic, and anti-angiogenic effects [84].

3.1. Inhibition of cell cycle and cellular proliferation

Cell cycle progression is controlled by cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CDKIs). Furthermore, DNA damage or incomplete replication activates the DNA damage checkpoint which leads to the arrest of the cell cycle or even induces apoptosis. In cancer, mutations and deregulations of CDKs, cyclins and their regulators provoke uncontrolled cell cycle progression and thus cell proliferation.

Silibinin has been shown to inhibit cell growth by inducing G0-G1 and G2-M arrests, for example in human prostate carcinoma cells, primary stage colon carcinoma HT-29 cells, advanced-stage highly metastatic LoVo cells and bladder transitional cell carcinoma cells [122, 85]. The underlying effects of silibinin on the cell cycle are upregulation of the universal cell cycle inhibitors Cip1/p21 and Kip1/p27 and inhibition of CDKs and their corresponding cyclins [84, 82, 85, 123]. In summary, silibinin modulates the CDK-cyclin-CDKI complexes and their regulators to inhibit cell cycle progression.

Silibinin is able to inhibit cancer cell proliferation *in vitro* in a plethora of cancer types by targeting the cellular proliferative signaling pathways such as the downstream signaling mediators of receptor tyrosine kinases (RTKs) (the JAK-STAT pathway, the ERK-MAPK pathway and the PI3K-Akt-mTOR pathway) [82, 84] (Figure 19).

It was reported that silibinin inhibits ligand binding to EGFR, the internalization of EGFR into the cytoplasm, its dimerization and ERK1/2 activation, e.g. in advanced human prostate carcinoma cells [124]. Similar to EGFR signaling, insulin-like growth factor receptor (IGFR) signaling constitutes a possible therapeutic target in cancer therapy. Silibinin was observed to downregulate IGF1R signaling and increase mRNA and protein levels of IGF binding protein 3 (IGFBP-3) [125]. In a DU145 human prostate cancer cell xenograft model in athymic mice,

silibinin reduced tumor growth, associated with an increase of IGFBP-3 in the plasma [125]. Thus EGFR and IGFR signaling are involved in silibinin-induced anti-proliferative effects.

Signal transducers and activators of transcription (STAT) proteins are a family of latent cytoplasmic transcription factors which are implicated in cell proliferation, survival and apoptosis signaling by mediating the cellular response to cytokines, hormones and growth factors [126]. Deregulation of the STAT signaling pathway is often found in cancer. Silibinin showed inhibitory effects on STAT phosphorylation and a decrease in the total STAT level in human prostate carcinoma PC-3 orthotopic xenograft [127]. Silibinin also influences androgen/androgen receptor signaling in prostate cancer [84].

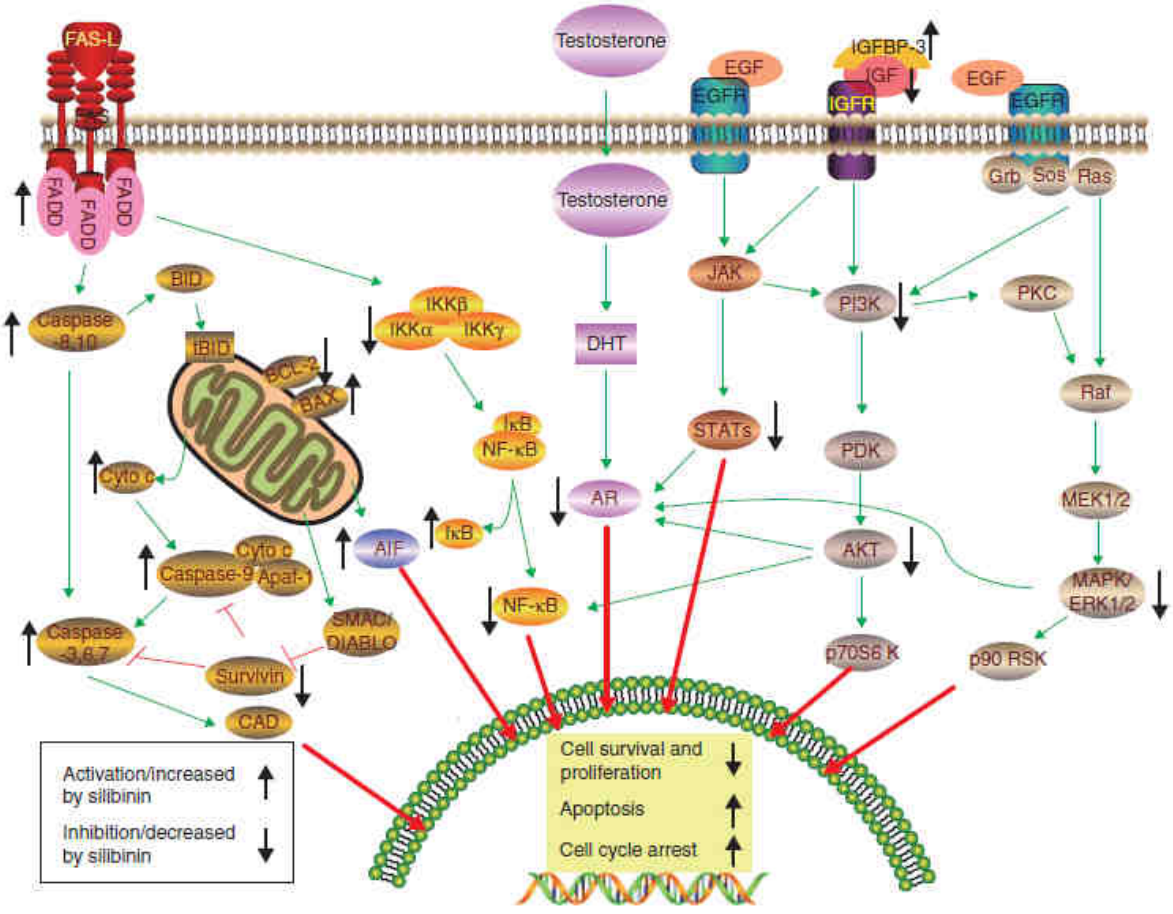


Figure 19: Cellular proliferative signaling pathways targeted by silibinin [84]

3.2. Induction of apoptosis

Deregulated apoptosis is one of the hallmarks of cancer. Conversely, activation of apoptosis represents an effective approach to eliminate cancer cells [17].

Silibinin has been shown to induce apoptosis in several models: For example, silibinin induced apoptosis in human bladder transitional cell papilloma RT4 cells via p53-caspase 2 activation [33] and in human breast cancer MCF-7 cells via both extrinsic and intrinsic apoptotic pathways [128]. For silibinin-induced activation of apoptosis in CRC, see 3.7.

Apoptosis is regulated by the balance and interaction of pro- and anti-apoptotic proteins (see Chapter 2). The anti-apoptotic proteins, of the Bcl-2 family as well as of the IAP family, represent a promising target for cancer therapy. It was reported that silibinin induced down-regulation of Bcl-2 and Bcl-2/Bax ratio and of the anti-apoptotic protein survivin [82], e.g. in hepatoma HuH7 cells [29].

3.3. Inhibition of inflammation

The Rel/NF-κB family of proteins consists of transcription factors which control many cellular processes in cancer such as inflammation, cell survival, transformation, proliferation, metastasis, and chemoresistance [21]. As the key transcription factor of the inflammatory pathway, NF-κB is often constitutively active in cancer cells, contributing to cancer progression and to chemotherapy resistance so that its deletion may inhibit the growth of tumor cells [22].

NF-κB transcription factors are composed of dimers formed of 5 subunits (see 2.6.4). In the cytoplasm they typically form inactive complexes with I-κBs which block their nuclear localization sequences. Upon stimulation, IKK complexes phosphorylate the I-κBs leading to their dissociation and rapid degradation. The liberated NF-κB dimers translocate to the nucleus and regulate transcriptional activation of their target genes [129].

Silibinin has been reported to inhibit NF-κB activation and translocation to the nucleus in prostate carcinoma DU145 cells, in endothelial ECV304 cells and in a chemically induced mouse urinary bladder tumor model [84, 130]. Furthermore, silibinin inhibited hepatic NF-κB activation in an acute model of liver damage in mice [131].

The suppression of the inflammatory molecules inducible nitric oxide synthase (iNOS) and COX-2, downstream targets of STAT and NF-κB via activator protein 1 (AP-1), contributing to inflammation, proliferation and reduced apoptosis, has become another target for cancer

chemoprevention. Silibinin has been shown to decrease iNOS expression in human lung carcinoma A549 cells and iNOS as well as COX-2 expression in an *in vivo* model of photocarcinogenesis [132, 133]. The inhibition of NF-κB signaling seems to be part of the anticancer effect of silibinin.

3.4. Inhibition of angiogenesis

Targeting angiogenesis, the growth of capillary vessels from existing blood vessels, is one of the fundamental methods of cancer treatment [134]. When there is a balance between pro-angiogenic and anti-angiogenic factors, the absence of tumor angiogenesis can lead to the dormancy of the tumor. Vascular endothelial growth factor (VEGF) is the most potent angiogenic factor, overexpressed by many solid tumors, and its inhibition has been reported to inhibit tumor growth. The hypoxia inducible factor 1α (HIF-1α) plays an important role in the transcriptional activation of VEGF [135].

Silibinin has been shown to decrease the secretion of VEGF and to inhibit *in vitro* capillary tube formation in colon cancer LoVo cells [136]. *In vivo*, silibinin decreased the nuclear level of HIF-α, the level of VEGF expression and microvessel density in HT29 xenograft tumors in nude mice [137]. Furthermore, Singh *et al.* showed a reduction of tumor microvessel density by up to 89% after silibinin treatment in A/J mice [118]. In hepatoma cell lines the anti-angiogenic effect of silibinin manifested itself by a downregulation of MMP-2 and CD34 [119].

3.5. Inhibition of metastasis

Cancer metastasis is responsible for more than 90% of cancer-associated deaths [138]. Metastasis depends on the acquisition of motility and invasiveness of cancer cells in which MMPs – because of their ability to degrade extracellular matrix - play a prominent role.

Silibinin inhibited the invasion and the motility of SCC-4 tongue cancer cells [16] as well as of highly metastatic lung cancer cells A549 [139] by reducing the expression of MMP-2 and urokinase-type plasminogen activator (u-PA) and enhancing the expression of tissue inhibitor of metalloproteinase-2 (TIMP-2) and PAI-1. In osteosarcoma MG-63 cells silibinin decreases cell adhesion and invasiveness, equally by inhibiting MMP-2 and u-PA expressions [140]. In MCF-7 human breast carcinoma cells silibinin suppressed MMP-9 expression by blocking AP-1 activation [116]. These results show the ability of silibinin to reduce invasion and metastasis of tumor cells.

3.6. Interaction with chemotherapeutic drugs and chemosensitization

Combination chemotherapy using cytotoxic agents and naturally occurring chemopreventive phytochemicals is a promising field of research. The criteria for a combined approach are 1) a synergistic response based on different mechanisms of action, 2) no shared common mechanisms of resistance and 3) no overlapping of major side effects [141]. Phytotherapeutic agents, especially silibinin because of its non-toxicity and hepatoprotective properties, have the double advantage of being effective against cancer without being toxic and are therefore appropriate candidates to be used with cytotoxic agents.

Silibinin has been studied in prostate, breast and lung cancer systems in combination with various chemotherapeutic agents [141]. In prostate carcinoma DU145 cells silibinin synergized the growth-inhibitory and apoptosis-inducing effects of the conventional cytotoxic agents doxorubicin, cisplatin and carboplatin [142, 143]. Silibinin and these three agents showed synergistic effects for cell growth inhibition in human breast carcinoma MCF-7 and MDA-MB468 cells [144]. In human ovarian and breast cancer cell lines silibinin potentiated the antitumor activity of cisplatin and doxorubicin [145, 146]. In a nude mouse model bearing the human A2780 ovarian cancer, silibinin increased the antitumor activity of cisplatin [146]. In A549 non-small cell lung carcinoma cells and tumor xenografts silibinin sensitized to doxorubicin [147]. These results show the capacity of silibinin to increase the efficacy of platinum compounds, providing a rationale for further investigations into combined therapies.

A common side effect of chemotherapeutic drugs is hepatotoxicity which may lead to dose reduction or even cessation of the treatment. Cancer patients often use milk thistle products in self-medication or through prescription because of their reputation as liver protectants [90]. However, there was only one randomized, double-blind study about the efficacy of silybin-phytosome to protect the liver. In children with acute lymphoblastic leukemia and grade ≥ 2 hepatic toxicity receiving cancer therapy, silibinin was effective in reducing AST levels significantly ($p < 0.05$) and showed a trend towards reducing ALT levels ($p < 0.07$) [90].

3.7. Anticancer activity against CRC

3.7.1. Studies *in vitro*

The first study about the effect of silibinin on CRC was conducted in 2003. In a co-culture of endothelial and colon cancer LoVo cells silibinin was shown to exert an **anti-angiogenic** effect [136]. A further study revealed that this effect of silibinin is associated with the upregulated mRNA expression of VEGF receptor-1 (Flt-1) [148].

The **anti-proliferative** and **pro-apoptotic** effects of silibinin on CRC were shown by Agarwal *et al*. for the first time [149]: In HT-29 cells, silibinin inhibited cell growth in a dose- and time-dependent fashion by a G0/G1 arrest. Higher dose (100 µg/mL, equivalent to ≈ 200 µM) and longer treatment time (72 h) induced an additional G2/M arrest. The reason for silibinin-induced **cell cycle arrest** was the upregulation of p27 and p21 protein as well as their mRNA levels and the decrease of CDK2, CDK4, cyclin E and cyclin D1 proteins. Silibinin induced up to 15% **caspase-independent** apoptotic cell death after 48 h without cytochrome c release [149].

Hogan *et al*. tested the effect of silibinin on the three human colon cancer cell lines Fet, Geo and HCT116 [85]: Like Agarwal's group, they showed a dose-dependent growth inhibitory effect by cell cycle arrest involving increased expression of p21 and p27 as well as inhibition of cyclin B1/D1 and CDK2 levels in all three cell lines. Yet they did not find relevant apoptotic cell death which may be explained by the relatively short treatment time (24 h) [85]. In LoVo cells, at concentrations ranging from 50 to 200 µM and treatment times ranging from 24 to 72 h, silibinin was able to induce apoptotic cell death [123]. Contrary to the results obtained in HT-29 cells, apoptosis was **caspase-dependent**. Furthermore, silibinin induced cell cycle arrest by the decrease of cyclins (D1, D3, A and B1) and CDKs (1, 2, 4 and 6) and the increase of p21 and p27 levels which confirmed the results obtained by the other groups. Silibinin treatment decreased phosphorylation of retinoblastoma protein [123].

Kaur *et al*. showed that silibinin inhibited cell growth and induced cell death in SW480 cells - harboring a mutant APC gene - at concentrations ranging from 100 to 200 µM and treatment time ranging from 24 h to 72 h and that silibinin was able to downregulate the β-catenin level [150]. Furthermore, silibinin decreased β-catenin-dependent T-cell factor-4 (TCF-4) transcriptional activity, the protein expression of the β-catenin target genes such as *c-Myc* and *cyclin D*, and expression of CDK8 and cyclin C. The effect of silibinin in the HCT116 cell line - harboring the wild-type APC gene - was weaker. This led to the conclusion that silibinin targeted the **Wnt/β-catenin pathway** [150].

3.7.2. Studies *in vivo*

Silibinin inhibits azoxymethane (AOM)-induced colon carcinogenesis

After the encouraging results in *in vitro* studies, the first studies about the potentially chemopreventive agent silibinin using *in vivo* models of CRC took place in 2008. However, Kohno *et al*. had already reported in 2002 that dietary feeding of the whole extract silymarin reduced the frequency of aberrant crypt foci (ACF) as well as the incidence and the multiplicity of colon adenocarcinoma in an experiment of AOM-induced colon carcinogenesis in male Fisher 344 rats [151]. In 2008, the group of Rajesh Agarwal studied the effect of silibinin on AOM-induced ACF in male F344 rats [152]. The rats were fed a silibinin-supplemented (0.033, 0.1, 0.33 or 1%, w/w) diet up to 11 weeks in two different treatment protocols (pre-/post- and post-AOM initiation). Silibinin dose-dependently **inhibited the development of ACF**, **diminished** the colonic cell **proliferation** (shown by the proliferation biomarker proliferating cell nuclear antigen (PCNA) and cyclin D1 immunohistochemical staining), and **induced apoptosis** (evidenced by TUNEL staining and cleaved PARP). Besides, silibinin decreased iNOS and COX-2 positive cells in colon tissue.

Silibinin inhibits 1,2-dimethylhydrazine (DMH)-induced carcinogenesis and modulates the biotransforming activity of microbial enzymes

In another study on chemically induced CRC (AOM is the metabolite of DMH), Sangeetha *et al*. investigated the effect of silibinin on DMH-induced colonic preneoplastic changes in a long-term preclinical model in male Wistar rats [153]. After 32 weeks of treatment (daily oral dose of 50 mg/kg body weight), silibinin had significantly reduced tumor incidence and inhibited the development of ACF and dysplastic ACF when given either for initiation, post initiation or the entire period, the latter being the most effective. Silibinin also **modulated the biotransforming activity of microbial enzymes**. As the colonic microflora mediates the formation of mutagens, carcinogens and tumor promoters, the activity of the concerned bacterial enzymes plays an important role in colon carcinogenesis. Supplementing silibinin during the postinitiation stage or during the entire period significantly decreased the activities of β-glucuronidase, β-glucosidase, β-galactosidase, nitroreductase and sulphatase. In 2011, Sangeetha *et al*. used the same model of DMH-induced tumorigenesis to show that silibinin decreases DMH-increased activities of phase I enzymes (cytochrome P450 isozymes) and restores DMH-decreased activities of phase II enzymes [89]. Furthermore silibinin alleviated

DMH-caused oxidative stress by enhancing levels of the endogenous antioxidant enzymes, demonstrating its antioxidant properties [89].

Silibinin inhibits CRC xenograft growth

A study in 2008 investigated the efficacy and the mechanisms of silibinin against HT29 xenograft – representing the primary localized stage of CRC [123] - growth in athymic nude mice [137]. The mice were fed with 200 mg/kg/d of silibinin or silybin-phytosome for 32 days. *In vivo* doses of 100 and 200 mg/kg body weight in mice extrapolate to 600 and 1200 mg silibinin/person daily, assuming a human body surface area of 1.8 m^2 for a body weight of 70 kg [123]. These doses are easily within the nontoxic range (see 2.4). Silibinin inhibited tumor growth without any adverse health effect [137]. Silibinin **decreased the proliferation index**, **induced apoptosis** and **reduced microvessel density**. Decreased ERK1/2 and Akt phosphorylation and reduction of cyclin D1 expression in tumors were identified as potential molecular targets of the anti-proliferative effects of silibinin. Anti-angiogenic effects of silibinin involved the decrease of iNOS, NOS3, COX-1, COX-2, HIF-1α, and VEGF. Silybin-phytosome preparation was even more effective in suppressing tumor growth, presumably by its improved bioavailability.

In a similar model of advanced-stage CRC, a LoVo xenograft in athymic nude mice, oral administration of silibinin for 30 days (at 100 and 200 mg/kg/d) significantly inhibited tumor growth without adverse side effects [123]. Silibinin inhibited proliferation (evidenced by immunohistochemistry for PCNA) and increased apoptosis (evidenced by TUNEL staining) along with a significant increase in p27 levels and a decrease in retinoblastoma phosphorylation.

Silibinin and the β-catenin pathway

The efficacy of silibinin in xenograft and chemically induced CRC models having been demonstrated, another study investigated the potential of silibinin in the $APC^{min/+}$ mouse model, a genetically predisposed animal model of human FAP [154]. $APC^{min/+}$ mice are heterozygous for a mutant allele of the tumor suppressor gene APC that has lost its function. Under normal circumstances, APC protein binds β-catenin in a destruction complex of Axin-APC-GSK-3β and promotes its proteolytic degradation by phosphorylation [155]. Thus, mutation of the APC gene leads to the excessive accumulation of β-catenin and the constitutive activation of the β-catenin pathway which is the major reason for polyp formation in this model. β-catenin plays an important role in cell proliferation, differentiation and

survival as a transcriptional coactivator. Therefore inhibition of the β-catenin pathway represents a chemotherapeutic approach.

This model displays genetic similarity to human CRC because APC is mutated in 70% of all cases of CRC [156] which renders the model appropriate to determine the efficacy of chemopreventive agents. However, mice develop tumors primarily in the small intestine while fewer tumors develop in the colon. In the study, the $APC^{min/+}$ mice were fed 250, 500 or 750 mg silibinin/kg body weight for 30 days. Silibinin significantly reduced the size and number of intestinal polyps at all three doses along with the inhibition of proliferation and activation of apoptosis [154]. At the same time, silibinin downregulated Wnt/β-catenin pathway proteins, showing its potential to **inhibit the β-catenin pathway**, and decreased phospho-Akt, COX-2, iNOS and nitrotyrosine levels. A further study in $APC^{min/+}$ mice examined the effect of 750 mg/kg silibinin daily for 5 days/week over the long-term period of 13 weeks [157]. Silibinin feeding significantly reduced polyp formation in the small intestine and in the colon. Most notably, the occurrence of large-size polyps decreased. Silibinin showed anti-proliferative (decreased PCNA), pro-apoptotic (increased cleaved caspase-3), anti-inflammatory and anti-angiogenic effects (reduced HIF-1α, VEGF, eNOS) on the molecular level. Furthermore, silibinin inhibited the β-catenin pathway by decreasing β-catenin levels and the expression of cyclin D1, it also downregulated the expression of COX-2 and PGE2 in polyps. Additionally, silibinin modulated the cytokine profile in intestinal polyps by decreasing the level of tumor-promoting cytokines such as TNF-α, IL-1α and IL-1β.

A study in SW480 tumor xenografts which overexpress β-catenin due to mutant APC showed that 100 and 200 mg/kg doses of silibinin feeding for 30 days inhibited tumor growth, decreased cell proliferation and expression of β-catenin, its downstream targets cyclin D1 and c-Myc and CDK8, a kinase which positively regulates β-catenin transcriptional activity. This confirmed the study in the $APC^{min/+}$ mice and the results the authors had found *in vitro* in SW480 cells (see 3.7.1) [150]. Another study by the same group on the effect of silibinin on AOM-induced colon tumorigenesis in A/J mice at a dose of 250 and 750 mg/kg for 16 weeks (starting after the last AOM injection, post-treatment regime) or 25 weeks (starting before AOM injection, pre-treatment regime) showed a dose-dependent decrease of AOM-induced tumor multiplicity and size in both protocols [158]. Silibinin significantly modulated proliferative (downregulation of PCNA and cyclin D1, upregulation of Cip1/p21) and decreased inflammatory (iNOS, COX-2) as well as angiogenic (VEGF) signaling molecules. In increasing caspase-3 and PARP levels, silibinin showed its pro-apoptotic potential.

Furthermore, silibinin lowered the high level of β-catenin, IGF1Rβ, p-GSKβ and phospho protein kinase B/pAkt proteins in AOM-treated mice. Lastly, it elevated the IGFBP-3 protein level. These results indicated that silibinin targeted β-catenin and IGFR1β pathways, resulting in chemopreventive efficacy.

Another study in SW480 xenograft in nude mice examined the effect of 200 mg/kg body weight silibinin on established tumors (silibinin treatment starting after 25 days of SW480 cell injection) and the effect of silibinin withdrawal after 28 days of silibinin feeding, starting at the same time as SW480 cell injection [159]. The aim of this study was to evaluate the therapeutic efficacy of silibinin on CRC rather than its preventive one. Silibinin inhibited tumor growth in both treatment protocols through anti-proliferative, pro-apoptotic and anti-angiogenic effects. Its suppressive effect on β-catenin was found again, this time in relation with a decrease in phosphorylation of its upstream regulator GSK3β as well as the decrease of expression of cyclin D1, c-Myc and survivin. Furthermore silibinin decreased tumor microvessel density and downregulated the angiogenesis signaling molecules VEGF and iNOS, also regulated by β-catenin.

Sangeetha *et al*. studied the effect of silibinin on the β-catenin pathway using DMH-induced colon carcinogenesis in male Wistar rats [160]. In this model deregulation of the β-catenin signaling pathway occurs frequently. Silibinin supplementation at 50 mg/kg body weight decreased tumor incidence and multiplicity along with decreasing levels of β-catenin, PCNA, agryophilic nucleolar organizer regions – correlated to the number of proliferative cells – and cyclin D1. Silibinin influenced the glutathione redox system, restoring the activities of the GSH-dependent enzymes glutathione-S-transferase (GST) and GR. A study by the same group investigated the effect of silibinin on ACF, tissue lipid peroxidation as a marker of oxidative stress and enzymatic antioxidant status (SOD, CAT, GPx) in the same model [161]. Administration of silibinin at 50 mg/kg body weight for 32 weeks reduced the occurrence and multiplicity of colonic ACF and restored the normal levels of the antioxidant enzymes. Thus the results seem to support the hypothesis of the authors that silibinin protects against DMH-induced carcinogenicity by reducing oxidative stress.

In summary, until now the studies about silibinin have consistently shown a tumor growth inhibition with downregulation of the β-catenin pathway and anti-angiogenic effects. However, no studies were conducted to investigate the detailed mechanisms of silibinin-induced apoptosis or autophagy *in vitro* and *in vivo*.

3.8. Silibinin in clinical trials

Many *in vivo* studies have already demonstrated the chemopreventive and chemotherapeutic efficacy of silibinin. Additionally, the non-toxic hepatoprotective character of silibinin, one of its most important properties, provides a rationale for its use in chemoprevention trials.

Because of the improved bioavailability of silibinin converted into a phytosome (silybin-PC), a lipid-compatible molecular complex [91], the most commonly used silymarin/silibinin products in clinical trials are Legalon, Thisilyn, Siliphos and Silipide (Table 3) [86]. These supplements are normally consumed in the form of capsules or tablets.

Herb	Products Used in Clinical Studies (Manufacturer)	Other Names (Manufacturer/Distributor) Herb
Milk Thistle	IdB 1016 Silipide (Indena S.p.A.)	Siliphos® (U.S.: Indena USA Inc.)
	Legalon® 35 Dragées (Madaus AG)	
	Legalon® 70 (Madaus AG)	
	Legalon® 140 (Madaus AG)	Thisilyn® (U.S.: Nature's Way Products, Inc.)

Table 3: Commercial products of milk thistle used in clinical studies [162]

MILK THISTLE SUMMARY TABLE

Product Name	Manufacturer/ U.S. Distributor	Product Characteristics	Dose in Trials	Indication	No. of Trials	Benefit (Evidence Level-Trial No.)
Legalon® (EU)	Madaus AG, Germany/None	Extract containing 80% silymarin	210-800 mg daily, usually in 3 doses	Alcoholic liver disease	10	Yes (III-1) Trend (I-1, II-1, III-2) Undetermined (III-3) No (I-2)
				Liver damage caused by toxins other than alcohol	2	Undetermined (III-2)
Silipide (EU)	Inverni della Beffa, Italy (Indena S.p.A., Italy)/None	Extract IdB 1060 (Siliphos®) complexed with phosphatidylcholine containing 29.7-36.3% silybin	240 or 480 mg daily	Chronic hepatitis	1	Yes (II-1)
				Viral hepatitis	1	Trend (II-1)
Silimarina® (Romania)	Biofarm, Romania/ None	Extract containing 5% silybin (said to be a copy of Legalon)	4.2 g (210 mg silybin) daily	Hepatitis or cirrhosis	1	Undetermined (II-1)
Generic	None/None	Silymarin	800 mg daily	Drug-induced liver damage	1	Trend (III-1)

*Products that contain the Indena S.p.A. Siliphos® extract as a single ingredient. The extract has been tested clinically but the following final formulations have not.

Product Name	Manufacturer	Product characteristics
Maximum Milk Thistle™	Natural Wellness	240 mg each capsule
Ultra thistle™	Natural Wellness	360 mg each capsule

Table 4: Preparations of milk thistle used in clinical studies [79]

Legalon® contains a standardized extract of milk thistle seeds consisting of 80% silymarin [79]. Legalon is produced in Germany by Madaus AG and was also sold in the United States by Nature's Way Products, Inc., under the name Thisilyn®. Siliphos® (the milk thistle extract IdB 1016), manufactured by Indena S.p.A., and Silipide, manufactured by Inverni della Beffa in Italy, are identical and distributed in the USA under the names Ultra Thistle™ and Maximum Milk Thistle™ by Natural Wellness [79, 163]. Siliphos is a complex of silybin and phosphatidylcholine with a molar ratio of 1:2.

These milk thistle preparations have been tested for their efficacy in clinical trials for liver diseases such as hepatitis, cirrhosis or hepatobiliary dysfunctions (Table 4, see also Table 2) [79].

In regard to cancer, there have been two trials using silibinin in prostate cancer patients and one trial in CRC patients. Flaig *et al*. conducted a phase I trial using Siliphos™ to determine the toxicity of high-dose silybin-phytosome in patients with advanced prostate cancer (see 2.2 and 2.4) [95]. Flaig *et al*. applied the resulting recommended phase II dose of 13 g daily in a study to determine tissue and blood effects of Siliphos™ in patients with localized prostate cancer and planning for a prostatectomy [164]. Peak blood concentrations of 35 µM were achieved but disappointingly, only low concentrations of silibinin were measured in prostate tissue after about 20 days of treatment. On the contrary, the only clinical study on CRC by Hoh *et al*. in patients with colorectal adenocarcinoma showed a considerable accumulation of silibinin in the colorectal mucosa after 7 days of therapy at a daily dose of 1.44 g (see 2.2) [88]. These results encourage further studies concerning CRC.

Currently, a phase I trial of silibinin (Siliphos™) in advanced hepatocellular carcinoma is ongoing (http://clinicaltrials.gov, ClinicalTrials.gov identifier: NCT01129570).

MATERIALS AND METHODS

I. STUDIES *IN VITRO*

1. Cell culture

SW480 and SW620 cells were purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). They were cultured in 75 cm^2 Falcon flasks in Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM glucose and supplemented with 10% heat-inactivated (56 °C) horse serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 1% non-essential amino acids (Invitrogen Corporation, Cergy Pontoise, France). Cells were kept at 37 °C in a humidified atmosphere with 5% CO_2. For maintaining cells in culture, they were seeded at 10^6 cells/ 15 mL medium and subcultured weekly. For passaging, they were washed (0.1 M PBS pH 7.2/2.6 mM EDTA) and detached by trypsinization (0.5% trypsin/2.6 mM EDTA). To stop the enzymatic reaction 4 volumes of DMEM supplemented with 20% horse serum, 100 U/mL penicillin and 100 µg/mL streptomycin were added. Cells were counted in a Malassez counting chamber after coloration with trypan blue.

For all experiments, cells were seeded at 10^6 cells/culture dish (10 cm internal diameter). The culture medium was DMEM supplemented with 3% heat-inactivated horse serum and 100 U/mL penicillin, 100 µg/mL streptomycin, and 1% non-essential amino acids. Transferrin (5 µg/mL), selenium (5 ng/mL) and insulin (10 µg/mL) were added to compensate for the lower serum concentration. The culture medium was replaced every 48 h.

The number of passages in our laboratory was limited at ten to avoid passage-number-related-effects. Every ten passages, new cells were thawed.

2. The cell lines SW480 and SW620

The human colon cancer SW480 cells and their derived metastatic SW620 cells have been validated as an *in vitro* model of colon cancer progression from a primary tumor to its metastatic spreading [165]. Their particularity is the fact that both cell lines were isolated from a single patient: the SW480 cell line was derived from a Dukes' stage B colon carcinoma of a 50-year-old male patient, and the SW620 cells were isolated from a mesenteric lymph node metastasis of the same patient one year later. SW480 cells have a spreading, epithelioid morphology while metastasis-derived SW620 cells resemble fibroblasts (Figure 20). Furthermore SW480 cells display higher epithelial and glandular differentiation than SW620 cells (e.g. more microvilli and more organelles), consistent with the fact that metastases are frequently undifferentiated.

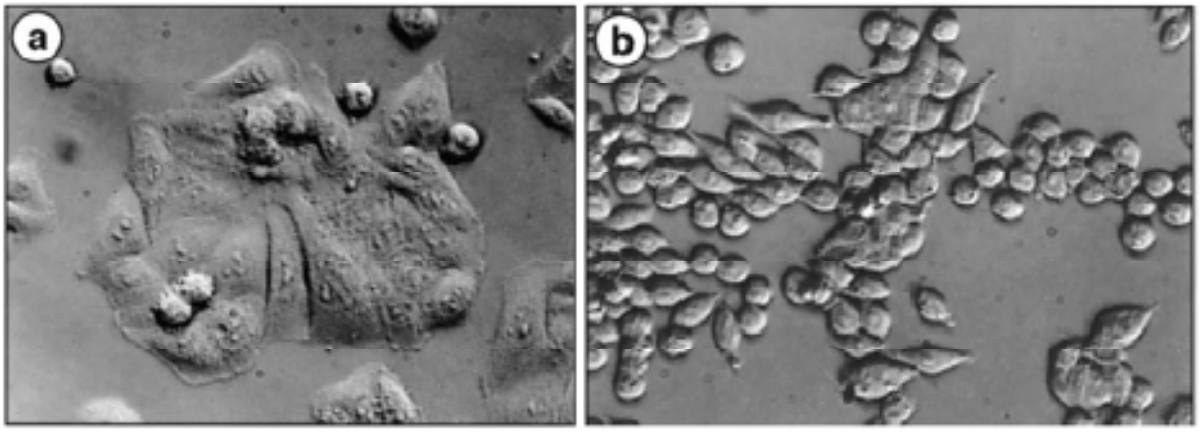


Figure 20: Cell morphology (a) SW480 cells (b) SW620 cells [165]

SW480 and SW620 cell lines also show phenotypic differences [165]. SW480 cells expresses more Fas death receptors, TNF-α receptors and TRAIL death receptors DR4 and DR5 [166], resulting in heightened sensitivity to FasL, to TNF-α and to TRAIL. This lacking sensitivity of SW620 cells to apoptosis-inducing agents plays an important role in resistance to chemotherapy and in tumorigenicity. SW480 cells have a higher capacity for adhesion and migration. *In vitro* and *in vivo* as a xenograft, SW620 cells have a higher growth rate and higher invasiveness than SW480 cells with higher secretion of the MMP matrilysin [165].

3. Tested molecules

Cells were exposed to silibinin (Sigma-Aldrich, Steinheim, Germany) 24 h after seeding. Silibinin was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich, Steinheim, Germany) and used at a final concentration of 300 µM. DMSO final concentration in culture medium did not exceed 0.1%. To study the potential activation of different pathways by silibinin further chemical molecules were used:

- TRAIL (50 ng/mL) (Enzo Life Sciences, Lausen, Switzerland) to study the influence of silibinin on TRAIL-induced apoptosis
- inhibitors of caspases to study the apoptotic pathway: caspase-8 inhibitor Z-IETD-FMK (20 µM) and pan-caspase-inhibitor Z-VAD-FMK (20 µM) (MBL International Corporation, Nagoya, Japan)
- inhibitors of MAP kinases to study the implication of these signaling pathways: SL327 (inhibitor of ERK, 10 µM), SP600125 (inhibitor of JNK, 10 µM) and SB203580 (inhibitor of p38 MAPK, 10 µM) (all from Sigma-Aldrich, Steinheim, Germany)
- an inhibitor of autophagy, bafilomycin A1 (2.5 nM) (Santa Cruz Biotechnology, Santa Cruz, CA), to clarify the role of autophagy in silibinin-induced cell death
- N-acetyl-cysteine (10 mM) (Enzo Life Sciences, Lausen, Switzerland) and reduced L-glutathione (5 mM) (Sigma-Aldrich, Steinheim, Germany) to inhibit oxidative stress and thus explain its role
- nystatin suspension (80 µg/mL) (Sigma-Aldrich, Steinheim, Germany) to study the role of lipid raft formation
- human recombinant DR4 Fc and DR5 Fc chimera protein (100 ng/mL) (R&D systems, Lille, France) to explain the role of the death receptors DR4 and DR5
- the specific inhibitor of p53, pifithrin-α (10 µM) (MBL International Corporation, Nagoya, Japan) to study the role of p53
- the InSolution^{TM} NF-κB Activation Inhibitor

  6-Amino-4-(4-phenoxyphenylethylamino)quinazoline in DMSO (3 µM) (Merck-Calbiochem, Darmstadt, Germany) to evaluate the involvement of NF- κB

- the inhibitor of O-linked-glycosylation benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (0.8 mM) (Merck-Calbiochem, Darmstadt, Germany) to study the involvement of glycosylation
- the small-molecule inhibitor of XIAP, embelin (25 µM) (Enzo Life Sciences, Lausen, Switzerland) to study the influence of XIAP in TRAIL resistance
- the HDAC inhibitors trichostatin A (TSA) (0.1 µM) and suberoylanilide hydroxamic acid (SAHA) (1 µM) (Sigma-Aldrich, Steinheim, Germany) to assess the involvement of histone acetylation

4. Flow cytometry

4.1. Principle

Flow cytometry is the measurement of the physical and chemical characteristics of single cells (cytometry) using a fluid stream (flow system) [167]. The stream aligns cells to pass one by one through a focused laser beam, the point of measurement. The advantage of this technique is the possibility to measure large quantities of cells in short periods of time [168].

Light is an electromagnetic radiation with wavelengths between 400 and 700 nm. The results of the encounter between the monochromatic laser light and the cell are scattering of the light (reflection and refraction at the same wavelength), absorption (energy taken up by the cell) and fluorescence (emission of light at a different wavelength).

The scattered light is measured at two different angles: Forward scatter (FSC) and side scatter (SSC) [168]. Scattered light collected in the forward direction (FSC) accrues mainly from the surface of the cell and is therefore proportional to its size. The light which is deflected from internal structures or granules supplies the SSC light, thus enabling the detection of granularity.

Fluorescent reagents introduced into the cell absorb the energy of the laser beam and emit fluorescence of a specific wavelength which is equally measured by the flow cytometer.

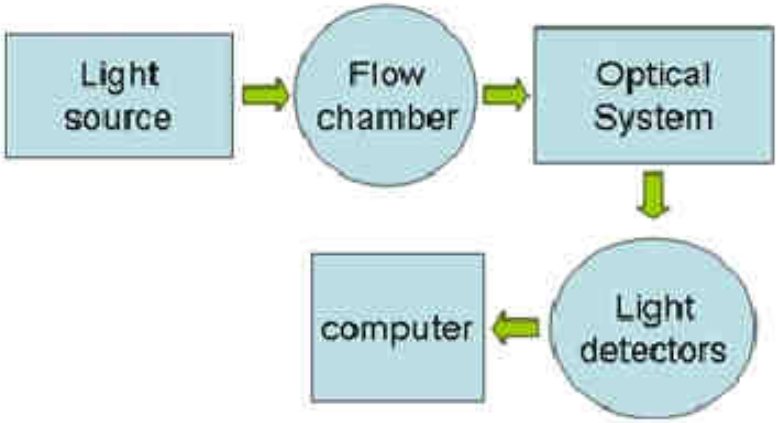


Figure 21: The building blocks of a flow cytometer [168]

The build-up of a flow cytometer can be seen in Figure 21. The sample containing the cells is injected into the sheath fluid which transports the cells to the point of measurement (flow chamber) without mixing with the sample (Figure 22).

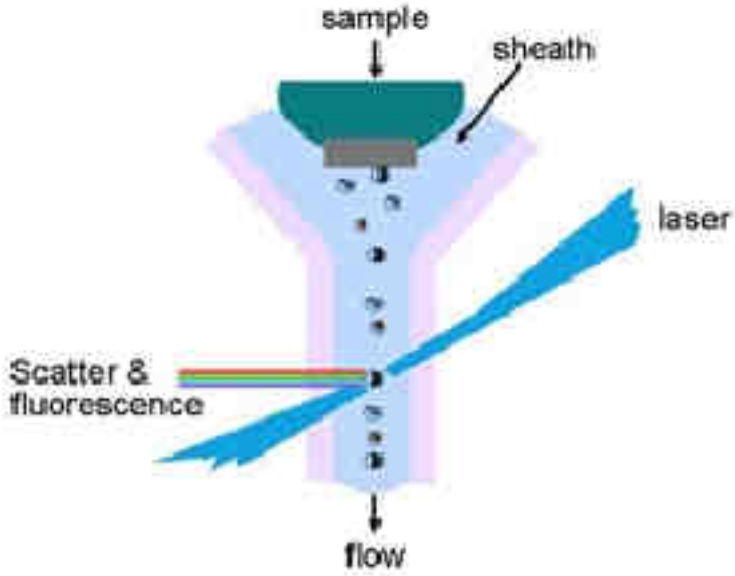


Figure 22: The flow chamber [168]

At this point, the laser beam encounters the cells one by one, generating scattered light and emission of fluorescence which are both measured by the optical system [2]. In the optical system, filters select and pass pre-defined wavelengths and direct them to sensors which usually are photomultiplier tubes (PMT) (www.coulterflow.com, accessed on 3rd of January, 2012). The PMT converts the light to a voltage pulse which is proportional to the intensity of the light. The pulse is further amplified by the system. Pulses which reach a minimum ("discriminator") level are converted into numbers called channels ("Analog to Digital Conversion"). The converted pulses increment counters for each channel. Finally, the computer displays histograms - the plots of these counts versus the channels. If two pulse heights are plotted against each other, a dot plot results.

Fluorescent reagents are applied to the cells to translate information about the structure or the function of the cell into a pulse of light [167]. Each fluorescent dye absorbs energy of specific wavelengths (excitation spectrum) and re-emits energy at different specific wavelengths (emission spectrum). For example, a blue laser will only excite dyes which absorb blue light. Examples for fluorescent reagents are antibodies labeled with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) and propidium iodide (PI). All experiments were conducted with the flow cytometer FACScan containing an argon-ion laser of 488 nm and three filters FL-1 (green: 515-545 nm), FL-2 (yellow: 564-606 nm) and FL-3 (red: 635 to about 720 nm) (Becton Dickinson, San Jose, CA, USA). The software used to analyze data was CellQuest (Becton Dickinson, USA).

4.2. Cell death analysis with propidium iodide: Hypodiploïd cells

The cell cycle consists of different phases: G0/G1, S and G2/M. These phases can all be characterized by their DNA content and thus analyzed by flow cytometry. When cells die by apoptosis or necrosis, DNA degradation occurs (in the case of apoptosis by DNA fragmentation) and leads to loss of nuclear DNA content. This phase of dying or dead cells is called Sub-G0/G1. These cells contain a quantity of DNA of less than 2n and therefore are called “hypodiploïd”.

To quantify the DNA content by flow cytometry, the DNA-intercalating fluorochrome propidium iodide (PI) is used [169]. When excited by the laser light at 488 nm, PI bound to nucleic acid gives off a strong emission in the red spectral region. Therefore the DNA content in a PI-marked cell correlates with the intensity of fluorescence (Figure 23). Every peak corresponds to a cellular population accumulated in a certain phase of the cell cycle.

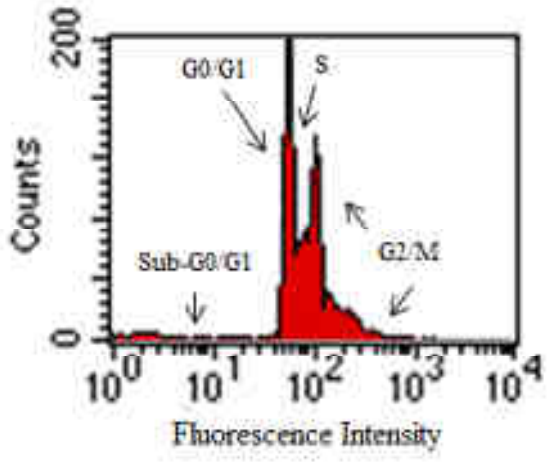


Figure 23: Cell cycle analysis with PI by flow cytometry

Cells were harvested by trypsinization after the desired treatment time and washed with PBS 0.1 M, pH 7.4. Then they were fixed in 70% ethanol at -20 °C for at least 30 min to permeabilize their membranes and thus facilitate the passage of PI in the cells. After the incubation they were washed twice in PBS and re-suspended in 200 µL PBS containing 0.25 mg/mL RNase A (Sigma-Aldrich, Steinheim, Germany) (to avoid the binding of PI to RNA) and 0.1 mg/mL PI (Sigma-Aldrich, Steinheim, Germany). After incubation in the dark at 37 °C for 30 min, the fluorescence of 10,000 cells per sample was analyzed by flow cytometry (channel FL-2) and histograms were analyzed by CellQuest software (BD Biosciences, Erembodegem, Belgium.

4.3. Cell death analysis with annexin V/PI: Phospatidylserine on the cell surface

Because PI-staining only serves at quantifying cell death but does not reveal the mode of cell death, other methods have to be used to qualify cell death. A clear difference between apoptotic and necrotic cell death is the externalization of the membrane phospholipid phosphatidylserine (PS) which is a hallmark of apoptosis [170]. This appearance of PS on the outer leaflet of the phospholipid bilayer can be monitored by using a fluorescent conjugate of annexin V, a Ca^{2+}-dependent PS binding protein. However, once the cells are dead, annexin V also marks cells which died by necrosis as a consequence of the loss of membrane integrity. This problem can be solved by double staining with annexin V and PI, as PI stains only cells whose membrane integrity is lost and thus allow PI to pass and bind to their cellular DNA. Three different cell populations can be observed:

a) cells not stained at all are live

b) cells only stained by annexin V are in the process of undergoing apoptosis

c) cells stained by annexin V and PI have already undergone apoptosis or necrosis

However, it is of note that cells undergoing autophagy also externalize PS [171].

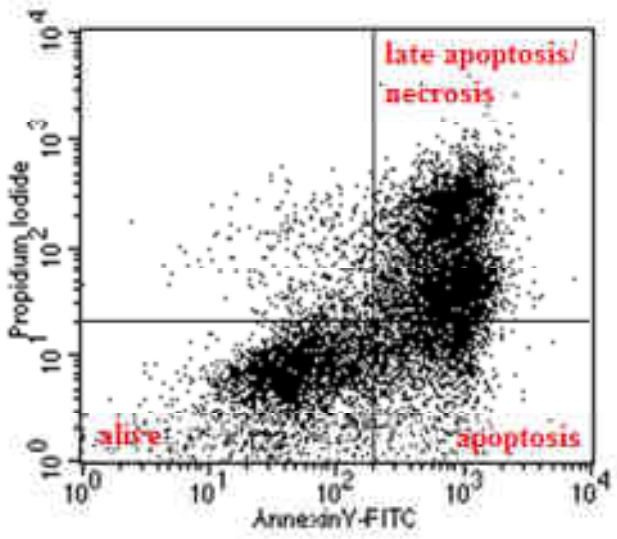


Figure 24: Doublestaining by annexin V and PI

We used the Annexin V-FITC Apoptosis Detection Kit (Enzo Life Sciences, Lausen, Switzerland) to perform the double-staining. After harvesting the cells by trypsinization, they were washed in cold PBS 0.1 M, pH 7.4 supplemented with 3% horse serum, incubated with 5 µL annexin V-FITC and 5 µL PI in 500 µL Binding Buffer for 5 min and then the fluorescence of 10,000 events per sample was analyzed using FACScan flow cytometer and

CellQuest Software (BD Biosciences, Erembodegem, Belgium) after having achieved correct fluorescence compensation by adjustment through passing unstained, single- and double-stained untreated cells. The FITC signal was detected using channel FL-1, PI staining byFL-2.

4.4. Mitochondrial membrane potential

The mitochondria play an important role in apoptosis as they are responsible for setting off the intrinsic pathway [172]. The collapse of the mitochondrial inner transmembrane potential ($\Delta\Psi_m$) often is associated to permeability of the outer mitochondrial membrane and therefore to a release of pro-apoptotic factors such as cytochrome c, AIF or Smac/DIABLO from the mitochondria [173].

We assessed changes in $\Delta\Psi_m$ by using the MitoProbe^{TM} $DiOC_2(3)$ (3,3'-diethyloxacarbocyanine iodide) Assay Kit (Invitrogen Corporation, Cergy Pontoise, France):

The kit provides the cyanine dye $DiOC_2(3)$ that accumulates in mitochondria with active membrane potentials. Emission increases due to dye stacking. The stain intensity decreases when agents disrupt the $\Delta\Psi_m$ [174]. Cells stained with $DiOC_2(3)$ are visualized by flow cytometry using 488 nm excitation and green (FL-1) or red (FL-3) emission filters. This method allows quantifying the cells with depolarized mitochondria.

Cells were harvested by trypsinization after the desired treatment time, washed once in PBS 0.1 M, pH 7.4 and incubated with $DiOC_2(3)$ at 37 °C for 30 min in the dark. Then cells were washed and re-suspended in PBS for flow cytometric analysis (10,000 events per sample). For positive controls, untreated cells were additionally incubated with a $\Delta\Psi_m$ -disrupter, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), at 50 µM during $DiOC_2(3)$ staining. Histograms were analyzed using the CellQuest Software (BD Biosciences, Erembodegem, Belgium).

4.5. Analysis of expression of death receptors (DR4, DR5, Fas, DcR1, and DcR2)

The expression of the death receptors on the cellular surface was determined by FITC-conjugated antibodies and detection by flow cytometry.

Cells were harvested by trypsinization, washed in PBS and incubated at 4 °C with the following antibodies:

- for **DR4** detection: 20 µL monoclonal anti-human DR4 (Mouse IgG1, clone HS101, 1:100) (Enzo Life Sciences, Vileurbanne, France) for 30 min
- for **DR5** detection: 20 µL monoclonal anti-human DR5 (Mouse IgG1, clone HS201, 1:100) (Enzo Life Sciences, Vileurbanne, France) for 30 min
- for **Fas** detection: 20 µL FITC-conjugated mouse anti-human Fas monoclonal antibody (clone DX2) (BD Biosciences, Erembodegem, Belgium) for 45 min
- for **DcR1** detection: 20 µL monoclonal anti-human TRAIL-R3 (Mouse IgG1, clone HS301, 1:50) (Enzo Life Sciences, Vileurbanne, France) for 30 min
- for **DcR2** detection: 20 µL monoclonal anti-human TRAIL-R4 (Mouse IgG1, clone HS402, 1:100) (Enzo Life Sciences, Vileurbanne, France) for 30 min

After a washing step, cells for DR4/DR5/DcR1/DcR2 detection were incubated in 50 µL FITC-conjugated goat anti-mouse IgG1 antibody (clone STAR81F, 1:50) (BD Biosciences, Erembodegem, Belgium) for 30 min at 4 °C. Then cells were washed twice and re-suspended in PBS. FITC-conjugated mouse IgG1 (BD Biosciences, Erembodegem, Belgium) monoclonal isotype control antibody was used for isotype control (incubation for 30 min at 4 °C) [175]. FL-1 filter (515 nm) was used for detection. The fluorescence of 10,000 events per sample was analyzed using FACScan flow cytometer and CellQuest Software (FACScan, BD Biosciences, Erembodegem, Belgium).

4.6. Analysis of expression of intracellular proteins (Bax, Mcl-1 and p53)

To determine the expression rates of the apoptotic regulatory proteins Bax, Mcl-1 and the tumor suppressor protein p53, specific FITC-coupled antibodies were used and detected by flow cytometry.

Cells were harvested by trypsinization after the desired treatment and washed twice with ice-cold PBS. Because the proteins of interest are intracellular, fixation and permeabilization of the cell membrane is necessary. Cells were fixed with 4% paraformaldehyde in PBS for 30 min at 4 °C and permeabilized in PBS/BSA 0.2%/Tween 20 0.5%. After a washing step with permeabilization buffer, cells were incubated with the specific antibodies:

- for **Mcl-1** detection: Cells were incubated with rabbit anti-human Mcl-1 polyclonal antibody (1:100; BD Biosciences, Erembodegem, Belgium) for 30 minutes at 4 °C, washed twice and incubated with FITC-conjugated swine anti-rabbit $F(ab')_2$ antibody (10 µL, 1:10, DakoCytomation, Glostrup, Denmark) for 30 min at 4 °C in the dark.

- for **Bax** detection: Cells were incubated with polyclonal rabbit anti-human (BD Biosciences Pharmingen, Erembodegem, Belgium) (10 µL, 1:100) for 30 min at 4 °C, washed twice and incubated with FITC-conjugated swine anti-rabbit $F(ab')_2$ antibody (10 µL, 1:10, DakoCytomation, Glostrup, Denmark) for 30 min at 4 °C in the dark.
- for **p53** detection: Cells were directly labeled with 20 µL of FITC-conjugated mouse anti-human p53 antibody (1:100) (Merck-Calbiochem, Darmstadt, Germany) for 1 h at 4 °C in the dark or with FITC-conjugated mouse IgG1 monoclonal isotype control antibody (clone MOPC-21, BD Biosciences, Erembodegem, Belgium).

After incubations with FITC-conjugated antibodies, cells were washed twice in permeabilization buffer and re-suspended in pre-heated 200 µL PBS. FL-1 channel was used for detection. The fluorescence of 10,000 cells was analyzed using FACScan flow cytometer and CellQuest software (BD Biosciences, Erembodegem, Belgium).

4.7. Cell acidification (Acridine orange)

Acridine orange is a fluorescent lysosomotropic agent (which means it accumulates preferentially in lysosomes) [176]. Staining with acridine orange allows quantifying accumulation of acidic vesicular organelles which happens during autophagy. In cells stained by acridine orange, the acidic compartments show a brightly red fluorescence while the cytoplasm and the nucleus fluoresce bright green because the pH influences the emission spectra of acridine orange [177]. The degree of red fluorescence (FL-3 channel) is proportional to the degree of acidity and the volume of the acidic compartment.

Cells were harvested by trypsinization, washed and stained with 10 µg/mL acridine orange (Acridine orange hydrochloride hydrate, Sigma-Aldrich, Steinheim, Germany) for 15 min at room temperature. Then they were washed, re-suspended in PBS and the red (FL-3) and green (FL-1) fluorescence of 10,000 events /sample was assessed by flow cytometric analysis. Histograms were analyzed by CellQuest Software (FACScan, BD Biosciences, Erembodegem, Belgium).

4.8. Autophagic activity

Autophagy is the process of self-digestion of a cell and may function as a survival or a cell death pathway [68].

Autophagic activity was detected by using the Cell-ID™ Autophagy Detection Kit (Enzo Life Sciences, Lausen, Switzerland). The 488 nm excitable Cell-ID™ Green autophagy dye serves as a selective marker of autolysosomes and earlier autophagic compartments because it becomes brightly fluorescent when incorporated in them.

Cells were harvested by trypsinization after the desired treatment, washed in Assay Buffer and incubated with Cell-ID™ Green Detection Reagent for 30 min at room temperature in the dark. After that, 10,000 events/sample were analyzed (FL-2 channel) by flow cytometry and CellQuest software (FACScan, BD Biosciences, Erembodegem, Belgium).

5. Colorimetric tests and ELISAs

5.1. Principle

The enzyme-linked immunosorbent assay (ELISA) is a biochemistry assay used to detect and quantify the presence of substances such as proteins, peptides, antibodies or hormones in a liquid sample in microplates. The sample is added to a so-called “ELISA plate” and its antigen of interest is immobilized to the bottom of the wells, either by direct adsorption to the surface or by a solid phase of a capture antibody, coated to the bottom (“sandwich ELISA”) (Figure 25).

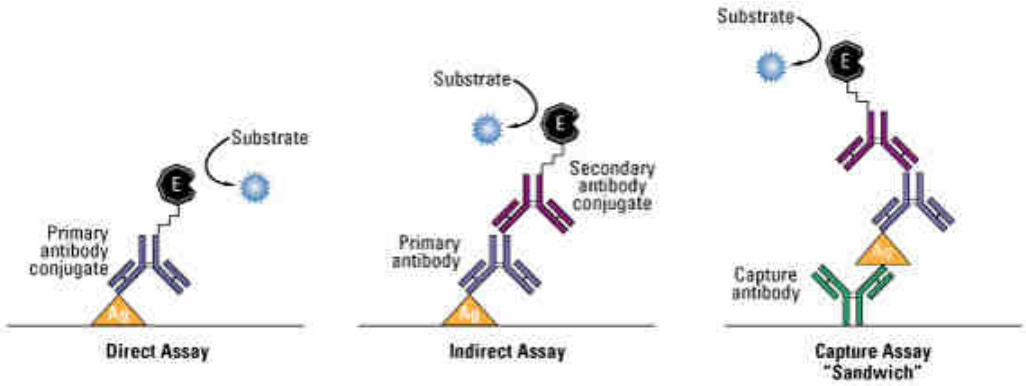


Figure 25: Common ELISA formats

(http://www.piercenet.com/browse.cfm?fldID=F88ADEC9-1B43-4585-922E-836FE09D8403)

Multiple reagents are added, incubated and the plate is washed between the binding steps to remove nonspecifically bound or unbound components. In most cases, at first an antibody, specific to the chosen antigen, is added. The characteristic element of an ELISA is this highly specific antibody-antigen interaction. This antibody ("direct assay") or a secondary antibody recognizing the primary antibody ("indirect assay") is coupled to an enzyme, usually horseradish peroxidase (HRP) or alkaline phosphatase (Figure 25). The final step, used for measurement, is a color development by addition of the enzyme's substrate. For quantitative detection, a spectrophotometric method is employed using quantification of transmission of light of a certain wavelength. Absorbance is also called optical density (OD).

Colorimetric assays determine the concentration of a substance in a solution with the help of a color reagent. In contrast to ELISAs, an enzymatic reaction is not obligatory.

5.2. Sulforhodamine B assay (cytotoxicity assay)

The sulforhodamine B (SRB) assay is a rapid and sensitive assay for determining cellular protein content in 96-well microtiter plates, i.e. suitable for measuring the cytotoxicity of anticancer drugs [178, 179]. In this method, cells are seeded in a 96-well plate, treated after 24h of incubation, fixed in trichloroacetic acid after the desired treatment time (2-8 days), stained with 0.4% SRB dissolved in 1% acetic acid, washed with 1% acetic acid to remove unbound dye and protein-bound dye is dissolved in 10 mM Tris base. OD is determined at 490 nm by a computer-interfaced microplate reader and correlates with protein content.

5.3. Activities of caspases-3, -8, -9 and -10

The activities of caspases-3, -8, -9 and -10 were measured by colorimetric assay kits (MBL International Corporation, Nagoya, Japan) in 96-well microplates, via colorimetric detection of the cleavage of caspase-specific substrates.

After harvesting by scraping, cells were washed in ice-cold PBS and lysed; proteins were extracted and stored at -80 °C. Protein content was determined by the method of Lowry. Twenty microliters of cell lysates were added to a buffer containing a p-nitroaniline (pNA)-conjugated substrate - either for caspase-3 (DEVD-pNA), caspase-8 (IETD-pNA), caspase-9 (LEHD-pNA) or caspase-10 (AEVD-pNA) - to a total of 100 µL reaction volume. Incubation was carried out at 37 °C. The caspases contained in the cell lysates cleave their specific tetrapeptide-pNA substrate and thus release pNA (Figure 26). The amount of the free pNA is

proportional to the caspase activity and can thus be calculated from the OD values at 405 nm and the calibration curve of defined pNA solutions. Results were adjusted to the protein content.

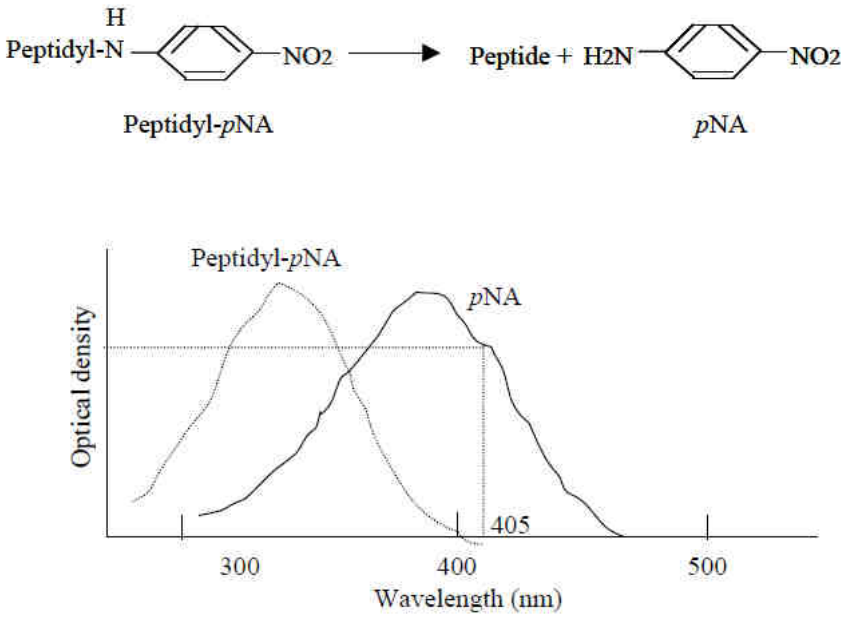


Figure 26: Comparison of UV-absorption spectrum of peptidyl-pNA with that of pNA
(APOCYTOTM Caspase-8 Colorimetric Assay Kit, MBL International Corporation)

5.4. Expression of survivin and XIAP

Survivin and XIAP concentrations in cell lysates were quantitatively determined with the aid of ELISA kits. Survivin was measured by the Survivin (human) ELISA Kit (Enzo Life Sciences, Lausen, Switzerland) and XIAP by the XIAP (human) EIA Kit (Enzo Life Sciences, Lausen, Switzerland) according to the manufacturer's instructions.

For both assays, cells were harvested by scraping, washed in ice-cold PBS and lysed. Protein content of the lysates was determined by Lowry, and lysates were stored at -80 °C.

Cell lysates were incubated on a microtiter plate coated with an immobilized monoclonal antibody to human survivin/XIAP. Recombinant human survivin/XIAP was used as standard. After a short incubation, excess sample or standard was washed out, then:

a) in the case of survivin, a rabbit polyclonal antibody was added to bind to the survivin captured on the plate. After washing to remove the excess antibody, a goat anti-rabbit IgG antibody conjugated to HRP was added to bind to the primary antibody.

b) in the case of XIAP, a biotinylated monoclonal antibody to human XIAP was added to bind to the XIAP captured on the plate. After a washing step, streptavidin conjugated to HRP was added which binds to biotin.

Excess conjugate was washed out and substrate was added. After a short incubation, the peroxidase reaction was stopped and OD was measured at 450 nm which is directly proportional to the concentration of survivin/XIAP in the standards and the samples. Thus survivin/XIAP concentration in the samples was calculated according to the standard curve, normalized to total protein content in the cell lysates and expressed as ng per mg of protein.

5.5. Enzymatic activity of MAP kinases

Mitogen-activated protein (MAP) kinases play an important role in signal transduction for cellular processes such as gene expression, differentiation, proliferation and apoptosis in response to extracellular and intracellular stimuli. Activation of MAPK leads to the activation of signaling cascades. The mammalian family of MAPK comprises the extracellular signal-regulated kinase (ERK1/2), p38 MAPK and c-Jun NH(2)-terminal kinase (JNK) [64].

The detection and quantification of phosphorylation of the MAPK was accomplished by use of the RayBio® Cell-Based ERK1/2 (activated) ELISA Sampler Kit.

Cells were seeded into a 96-well tissue culture plate. After the desired treatment, they were fixed and, after blocking, anti-phospho-protein specific antibody or anti-pan-protein specific antibody was added and incubated. After a washing step the secondary antibody in form of HRP-conjugated anti-mouse IgG was pipetted into the wells (indirect assay, see Figure 25). The wells were washed, then a TMB substrate solution was added and color developed. After stopping the reaction, OD correlating to the amount of protein of interest was measured at 450 nm. Results were presented as ratio of phosphorylation in the treated cells compared to the ratio in the control cells (phospho-MAPK : MAPK (treatment) / phospho-MAPK : MAPK (control)).

5.6. Liberation of cytochrome c

Cytochrome c is liberated from the mitochondria into the cytosol in the course of the activation of the intrinsic apoptotic pathway [36].

Concentrations in mitochondrial and cytoplasmic fractions were measured by using the Cytochrome c (human) ELISA Kit (Enzo Life Sciences, Lausen, Switzerland) to study the release of cytochrome c from the mitochondrial intermembrane space into the cytosol.

Cytoplasmic and mitochondrial fractions of the cells were isolated in the buffers provided in the kit, protein concentration was measured by the method of Lowry and cell lysates were stored at -80 °C.

The ELISA used was a "sandwich ELISA": the samples as well as a native human cytochrome c standard were incubated in a microtiter plate coated with an immobilized monoclonal antibody to cytochrome c. Excess sample/standard was washed out, then a biotinylated monoclonal antibody to cytochrome c was added to bind to the cytochrome c captured on the plate. After a washing step, streptavidin conjugated to alkaline phosphatase was added to bind the biotin. Excess conjugate was washed out, then substrate was added, the enzyme reaction was stopped and OD was measured at 405 nm. The OD is proportional to the concentration of cytochrome c in the samples.

5.7. Activity of NF-κB

To determine the activation of NF-κB, cells were harvested by scraping and nuclear extracts of the cells were prepared using the Nuclear Extract Kit (Active Motif Europe, Rixensart, Belgium). NF-κB p65 in the nucleus was quantified with the aid of the TransAM^{TM} NF-κB p65 Transcription Factor Assay Kit (Active Motif Europe, Rixensart, Belgium) in a 96-well plate according to the manufacturer's instructions. This ELISA-based kit detects and quantifies the activated NF-κB subunit p65 contained in the nuclear extract because it binds specifically to an immobilized oligonucleotide on the plate that contains the NF-κB consensus binding site.

First the nuclear extracts were incubated on the plate, followed by addition of a primary antibody directed against the activated NF-κB p65 subunit bound to the oligonucleotide. The addition of a secondary HRP-conjugated antibody and finally of the substrate of HRP enabled colorimetric detection of the reaction by reading OD at 450 nm.

5.8. Activity of HDAC (Histone Deacetylases)

To determine the activity/inhibition of the HDACs in nuclear samples, we used the colorimetric EpiQuik™ HDAC Activity/Inhibition Assay Kit (Epigentek-Euromedex, Strasbourg, France). First cells were harvested by scraping and nuclear extracts of the cells were prepared using the EpiQuik™ Nuclear Extraction Kit (Epigentek-Euromedex, Strasbourg, France). Protein content of the nuclear extracts was determined by Lowry, and nuclear extracts not immediately used were stored at -80 °C. Nuclear extracts were incubated with acetylated histone substrate in a 96-well plate, then the amount of remaining un-deacetylated histone which is inversely proportional to HDAC enzyme activity in the nuclear sample was colorimetrically quantified through an ELISA-like reaction with a capture and a detection antibody. OD was read at 450 nm. The activity of HDAC enzymes is inversely proportional to the OD.

5.9. Activity of DNMT (DNA Methyltransferases)

We used the colorimetric EpiQuik™ DNMT Activity/Inhibition Assay Ultra Kit (Epigentek-Euromedex, Strasbourg, France) to determine activity/inhibition of DNMTs in nuclear samples. First cells were harvested by scraping and nuclear extracts of the cells were prepared using the EpiQuik™ Nuclear Extraction Kit (Epigentek-Euromedex, Strasbourg, France). Protein content of the nuclear extracts was determined by Lowry, and nuclear extracts not immediately used were stored at -80 °C. Nuclear extracts were incubated on a microplate where a universal DNMT substrate was stably coated. DNMT enzymes from the nuclear sample methylate the DNA substrate during the incubation, and then the amount of methylated DNA which is proportional to enzyme activity was colorimetrically quantified through an ELISA-like reaction by reading the absorbance in a microplate spectrophotometer at a wavelength of 450 nm. The activity of DNMT enzymes is proportional to the OD.

6. Western Blot

Western Blot is an analytical technique of transferring ("blotting") proteins on a membrane to detect proteins of interest. Because the interaction antigen-antibody is used for the detection, this method is also called immunoblotting. Western blots produce qualitative and semi-quantitative data. Before a protein can be detected, a series of different steps is necessary:

Extraction of proteins

Cells were harvested by scraping, washed twice in PBS 0.1 M pH 7.4, lysed for 30 min at 4 °C in RIPA buffer (Sigma-Aldrich, Steinheim, Germany) with protease and phosphatase inhibitors to prevent degradation of the sample by therein contained enzymes and ultra-centrifugated at 16,000 g for 30 min at 4 °C.

Protein determination by Lowry (to load the same quantity onto the gel)

The protein content of the supernatant (the protein fraction) was determined by the Lowry assay [180].

Gel electrophoresis (to separate the proteins according to their physical properties)

Samples were diluted in the solution of Laemmli (containing β-mercaptoethanol to remove secondary and tertiary structure (e.g. to reduce disulfide bonds to sulfhydryl groups)) to denature the proteins. Between 10 and 20 µg were loaded onto a 12 or 15% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel for 1h30 to 2h30 at 150 V. SDS-PAGE separates proteins according to their molecular weight: SDS covers proteins which consequently become negatively charged and move to the positively charged electrode. The acrylamide mesh lets small proteins migrate faster (the lower the acrylamide concentration the better the resolution of higher molecular weight proteins).

Transfer (to bring the proteins onto a membrane)

Then proteins were transferred onto nitrocellulose membranes for 2 h at 200 mA (Bio-Rad, Marnes la Coquette, France).

Blocking (to prevent non-specific binding of the antibodies later on)

Membranes were blocked in Tris-Buffer Saline Tween 20 (TBST) buffer (10 mM Tris-HCl pH 7.5, 0.1 M NaCl, 0.1% Tween 20) containing 3% BSA for 1 h at room temperature with gentle shaking (50 rpm).

Immunodetection and Washing Steps (to detect the target protein and to remove unbound and nonspecifically bound reagents)

Membranes were incubated with the primary antibody at an appropriate dilution in TBST buffer overnight at 4 °C with gentle shaking. Subsequently the membranes were washed 3 times during 10 min in TBST buffer and incubated with the adequate dilution of HRP-conjugated secondary antibody, targeted at a species-specific epitope of the primary antibody, for 1 h at room temperature with gentle shaking.

After 3 washing steps in TBST buffer during 10 min and 2 washing steps in TBS buffer without Tween 20 during 5 min, the chemiluminescent reaction was performed by Super Signal West Pico Chemiluminescent Substrate System (Pierce, Brebières, France). Intensity of bands on a photographic film was analyzed by Gel Doc 2000 and Quantity One 1-D Analysis Software (Bio-Rad, Marnes la Coquette, France).

Secondary probing

To "strip" the membrane (remove the bound antibodies) in order to re-incubate it with different antibodies, it was heated at 50 °C during 30 min in a stripping solution (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl). Then 2 washing steps were performed in TBST buffer for 10 min and a washing step in TBS buffer without Tween 20 for 10 min. Then the procedure was started again at the blocking step.

Primary antibodies

- polyclonal rabbit anti-human Bid at 1:500 (BD Biosciences Pharmingen, Erembodegem, Belgium, Belgium)
- monoclonal mouse anti-human β-actin at 1:2,000 (0.1 µg/mL) (Chemicon Int., Hampshire, UK)
- monoclonal rabbit anti-rat Bax at 1:500 (BD Biosciences, Belgium)
- monoclonal mouse anti-rat Bcl-2 at 1:100 (Calbiochem, Merck Biosciences, Darmstadt, Germany)
- monoclonal rabbit anti-rat MMP7 at 1:500 (Santa Cruz Biotechnology, Santa Cruz, USA)
- monoclonal mouse anti-rat β-actin at 1:2,000 (Chemicon Int., Hampshire, UK)

Secondary antibodies

- monoclonal HRP-conjugated goat anti-rabbit IgG at 1:50,000 (0.02 µg/mL) (Merck-Calbiochem, Darmstadt, Germany)
- monoclonal HRP-conjugated goat anti-mouse IgG at 1:2,000 (0.02 µg/mL) (Pierce, Brebières, France)

7. DNA fragmentation (DNA laddering)

DNA fragmentation is a major biochemical hallmark of apoptosis, occurring later in the apoptotic process than PS externalization [181]. DNA ladders form because the ~ 200 base pairs of DNA around a histone core are protected by their conformation from endonuclease-mediated digestion.

We studied DNA fragmentation by using the Suicide-Track^{TM} DNA Ladder Isolation Kit (Merck-Calbiochem, Darmstadt, Germany). According to manufacturer's instructions, DNA was extracted from the cells after the desired treatment. In the process, apoptotic DNA was separated from high molecular weight, intact, genomic DNA, and the fragmented DNA was precipitated. Following resuspension, the samples were loaded onto a 1.5% (w/v) agarose gel containing ethidium bromide. DNA fragments were separated by electrophoresis (50 V, 1 h), and then DNA ladders were visualized under UV light by Transluminator Gel Doc 2000. HL60 cells, treated with 0.5 mg/mL Actinomycin D for 19 h, were used as a positive control.

8. Reverse transcription real-time - polymerase chain reaction (qRT-PCR)

The reverse transcription real-time polymerase chain reaction, also called quantitative reverse transcription PCR (qRT-PCR) is a technique of amplification and quantitation of a RNA sequence, based on PCR [182]. Firstly, the chosen messenger RNA (mRNA) sequence is converted into a DNA sequence, called cDNA, by reverse transcriptases (RT). RT are RNA-dependent DNA polymerases which are able to use a strand of RNA as a matrix to catalyze the synthesis of the complementary DNA strand. Secondly, the matching strand of the cDNA is synthesized and then PCR is used to amplify the cDNA. PCR is a method to amplify chosen DNA sequences within a longer DNA molecule, consisting in using primers that are complementary to the target sequence on either side of the DNA (forward and reverse) and a DNA polymerase which extends them to a copy of the whole sequence. After the first copy has been formed, primers can anneal either to the original DNA strand or to the newly synthesized copy to start a new cycle of duplication, consequently leading to exponential amplification of DNA. The DNA polymerase used must be thermostable because the temperature has to be raised to more than 90 degrees to separate the two DNA strands in each round of the amplification process to render the DNA single strands accessible for binding of

the primers. A thermostable DNA polymerase is provided by the bacterium *Thermus aquaticus* that lives in hot pools, the so called Taq polymerase. The number of amplification cycles (separation of the DNA strands, annealing of the primers, adding new complementary strands by Taq polymerase) usually is 20 to 40. In contrast to PCR, which finishes with the analysis of the PCR product by agarose gel electrophoresis and which is at best semi-quantitative, real-time PCR is a quantitative method [182].

The key feature of real-time PCR is that a labeled probe is added to the reaction mix, in our case the TaqMan® probe (Applied Biosystems), which also hybridizes to the target sequence (Figure 27). This probe contains a fluorescent reporter at the 5' end and a quencher of fluorescence at the 3' end, resulting in the prevention of fluorescence emission via fluorescence resonance energy transfer (FRET) while both are in proximity. When the Taq polymerase breaks down the probe annealed to the target DNA sequence due to its 5' nuclease activity during replication, the reporter is released and emits fluorescence when excited by a laser. Thus the fluorescence detected by the real-time PCR instrument at each PCR cycle is specific of the amplified target sequence. QRT-PCR focuses on the exponential phase during the first cycles of the reaction which yields the most accurate data.

The real-time PCR instrument calculates the threshold for detection of the emitted fluorescence intensity above the background. The number of cycles at which the fluorescence exceeds the threshold is measured and called cycle threshold (C_t).

An endogenous control - a stably expressed gene - is necessary for normalization to determine fold-change in the expression of the target gene, to correct variations in RNA quantity and quality and thus to ensure accuracy in the quantitation.

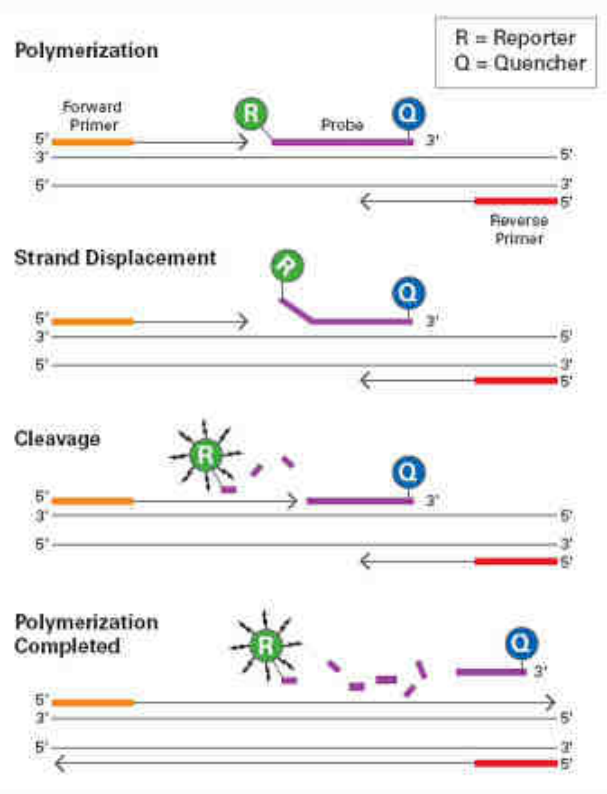


Figure 27: TaqMan® assay

(http://www.asuragen.com/Services/services/gene_expression/ab_taqman.aspx)

Total RNA was extracted from the cells using the RNAeasy Plus Mini Kit (Qiagen, VWR, Albertslund, Denmark) following the manufacturer's instructions. The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to reverse-transcribe RNA (1 µg) to DNA in 20 µl of reaction mix. The measurements of the transcription levels of the selected genes were performed with TaqMan Gene Expression assays (assay ID for DR4: Hs00269492, DR5: Hs00366272; Applied Biosystems). As an endogenous control β-actin was used (assay ID: Hs99999903; Applied Biosystems). Real-time RT-PCR was performed with TaqMan Universal PCR Master Mix and ABI Prism 7500 Sequence Detection System (Applied Biosystems Sequence detector) in triplicate wells. Data were analyzed with a comparative threshold cycle (ΔC_t) method. This method is used to determine the values of Δ cycle threshold (ΔC_t) by normalizing the average C_t value of each treatment with the value of each opposite endogenous control (β-actin). Then, calculation of $2\text{-}\Delta\Delta C_t$ of each treatment and statistical analysis were performed as described by Livak and Schmittgen [183].

II. *IN VIVO* STUDY: PRECLINICAL MODEL OF EXPERIMENTAL COLON CARCINOGENESIS

1. Description of the model

Azoxymethane (AOM)-induced colon carcinogenesis is the most frequently used animal model to examine the efficacy of putative chemopreventive agents against colorectal carcinogenesis [20]. The interest of this model lies in the fact that the AOM-induced carcinogenesis in rodents resembles the carcinogenesis in humans in regard to clinical, histopathologic and molecular features: As in humans, K-*ras* mutations occur frequently and Wnt/Apc/β-catenin signaling via accumulation of β-catenin is commonly activated, even if not *APC* is mutated in the animals but β-catenin itself [20, 21]. Inflammation-associated enzymes such as iNOS and COX-2 stimulating neovascularization are overexpressed [20, 21].

In the rat liver, AOM is converted to the ultimate carcinogen methylazoxymethanol by metabolic activation [184]. Then it starts a transformation process of the colonic epithelial tissue during which aberrant crypt foci (ACF) progress to adenoma (often polyps) and carcinoma [21].

ACF are intermediate biomarkers of increased CRC risk and in a first step allow the evaluation of cancer-promoting or -preventing properties of the tested agent [12, 185]. Three to 4 weeks after the AOM injection, these preneoplastic lesions can be observed while it takes 6 months for the first tumors to appear. Therefore the model can be used for short- (endpoint ACF) or for long-term evaluations (endpoint tumors) [20]. The fact that the development of tumors in rats takes such a short time compared to the development in humans where this process takes decades, contributes to the attractiveness of the model.

Furthermore, a meta-analysis has shown that carcinogen-induced rat models are better than the Min mouse model to predict the effect of chemopreventive agents in humans and are appropriate to screen potential chemopreventive agents [21].

2. Experimental design

In our experiment, we used male Wistar rats (n = 24) from the Charles River Laboratories (Les Oncins, France) weighing between 200 and 220 g which were kept under standardized

conditions: 22 °C, 60% relative air humidity, light/dark cycle of 12 h each, 20 air changes/h. They received a standard diet and had free access to drinking water.

Tumors were induced by intra-peritoneal injections of AOM (Sigma-Aldrich, Saint Quentin Fallavier, France) (15 mg/kg body weight) once a week for 2 weeks.

Rats were randomly divided into three groups: The first group (n = 8) consisted of AOM-injected rats which received daily 300 mg silibinin/kg body weight dissolved in 0.5% carboxymethyl cellulose (CMC) per intragastric feeding, the second group (n = 8) of AOM-injected rats received only the excipient CMC. The third group (n = 8) did not undergo AOM injections but saline injections (0.9% NaCl) and received the excipient.

One week after the last injection of AOM (post-initiation), treatment with silibinin/CMC started. All animals were sacrificed 7 weeks after the last AOM or saline injection.

All experiments were performed in accordance with the institutional guidelines of the French Ethical Committee (authorization no. A67-480, French Ministry of Agriculture).

3. Evaluation

After sacrifice of the animals, the colon was removed and hyperproliferative crypts as well as ACF were numbered on a 6-cm-long segment of the distal colon. This segment was washed with a physiological saline solution, cut open, pinned out flatly and fixed in 10% buffered formalin. Then the segment was stained in 0.2% methylene blue for 5 min and rinsed in Krebs-Ringer buffer. It was placed on a glass slide to be microscopically examined at 5-fold magnification. Criteria used for identification of hyperproliferative aberrant crypts and ACF were:

- increased size
- thicker epithelial lining
- increased pericryptal zone compared to normal crypts

Mucosal samples were scraped off with a glass slide and immediately frozen in liquid nitrogen. For flow cytometric analyses, mucosal cells were processed immediately by incubating the mucosa with 0.1 mg/mL collagenase for 40 min at 37 °C.

III. *IN VITRO* AND *IN VIVO* MODEL OF HEPATIC CANCER

1. Description of the model

Within 3 years of diagnosis, about a third of the CRC patients will develop liver metastases [186]. To investigate the effect of the hepatoprotective flavonolignan silibinin on hepatic tumor growth, we used Hep-55.1C hepatocellular carcinoma (HCC) cells *in vitro* and a murine orthotopic liver tumor model. The advantage of an orthotopic implantation of Hep-55.1C HCC cells in the liver is a better imitation of the usual tumor cell environment than by a subcutaneous implantation. Because of the employment of the syngeneic Hep-55.1C cells, immune-competent C57BL/6J mice can be used. The active host immune system contributes to a close resemblance to the real situation of the tumoral microenvironment and thus the transplanted cells histologically reproduce the tumors from which they are derived. To be able to study the tumor evolution non-invasively, we used micro-computed tomography (microCT) follow-up [187].

2. Experimental design

In our experiment, we used male C57BL/6J mice from Janvier breeding facilities (St Genest, France) which were housed under standard conditions (see II.2.).

For orthotopic tumor graft, 8 week-old C57BL/6J mice were anaesthetized by inhalation of isoflurane (Abbott, France). For orthotopic implantation, $2 \cdot 10^6$ Hep-55.1C cells resuspended in 30 µL of PBS (Sigma-Aldrich, Germany) were injected in the left liver lateral lobe after midline laparotomy. Following haemostasis, the abdomen was closed in two layers.

One week after tumor graft, the mice were randomly divided into 2 groups. The first group (n = 6) received a daily gavage (0.3 ml) of 700 mg silibinin/kg body weight in a vehicle solution (0.5% CMC and 0.025% Tween 20) 5 days/week, the control group (n = 6) received daily gavage of the vehicle solution. In both treated and control groups, tumor volumes were recorded by microCT imaging. All animals were sacrificed after 4 weeks of treatment.

All animal experiments were performed in accordance with the institutional guidelines of the French Ethical Committee (authorization no. A67-480, French Ministry of Agriculture).

3. Evaluation

Tumor evolution was followed-up by microCT imaging. This was performed with a micro CAT II scanner (Imtek Inc, Siemens) at 80 kVp X-ray voltage and 500 µA anode current under general gaseous anaesthesia with isoflurane. Respiratory-gated images were acquired with a resolution of 119 µm leading to a scanned volume of 6.1 x 6.1 x 6.1 cm. Four hours before first imaging, animals were injected intra-peritoneally with 6 µL/g Exitron Nano 6000 (Miltenyi, France) liver contrast agent. This nanoparticle based contrast agent is taken up by macrophages (Kuppfer cells), but not by tumor cells, and remains in the healthy liver for several weeks. Hep-55.1C tumors appear as a hypodense black area inside the white contrasted normal liver parenchyma. The presence of a tumor was assessed by microCT scan one week after tumor graft.

Images were analyzed with the VR Render software; 3D reconstruction and volume determination performed with the 3DVPM software developed at the IRCAD [188].

After autopsy, liver tumors were measured with a caliper, isolated and weighed. The evolution of tumor volume was calculated by normalizing the volume calculated at each time-point from microCT scan images after 3D reconstruction of the tumor, by the tumor volume calculated before the first treatment. Tumor volume at autopsy was determined using the semi-ellipsoid volume formula: $\pi/6$ (length · width · height).

RESULTS

CHAPTER I:

Silibinin triggers apoptotic signaling pathways and autophagic survival response in human colon adenocarcinoma cells and their derived metastatic cells

1. Abstract

Silibinin, a flavonolignan isolated from the milk thistle plant (Silybum marianum), possesses anti-neoplastic properties. Recent *in vitro* and *in vivo* studies have shown that silibinin inhibits the growth of colorectal cancer (CRC). This chapter investigates the mechanisms of silibinin-induced cell death using an *in vitro* model of human colon cancer progression, consisting of primary colon adenocarcinoma cells (SW480) and their derived metastatic cells (SW620) isolated one year later from a lymph node metastasis of the same patient.

Silibinin inhibited cell growth and induced apoptotic cell death evidenced by DNA fragmentation and activation of caspase-3 in both cell lines. Silibinin enhanced the expression (protein and mRNA) of TRAIL death receptors (DR4/DR5) on the cell surface in SW480 cells, and induced their expression in TRAIL-resistant SW620 cells normally not expressing DR4/DR5. Similarly, silibinin upregulated the Fas receptor on both mRNA and protein levels. Caspase-8 and -10 were activated, demonstrating the involvement of the extrinsic apoptotic pathway in silibinin-treated SW480 and SW620 cells. The protein Bid was cleaved in both cell lines indicating a cross-talk between the extrinsic (death receptor) and the intrinsic (mitochondrial) apoptotic pathway. In SW480 cells, silibinin was also able to downregulate survivin, an inhibitor of apoptosis (IAP) protein. We demonstrated that silibinin activated also the intrinsic apoptotic pathway in both cell lines, including the perturbation of the mitochondrial membrane potential, the release of cytochrome c into the cytosol and the activation of caspase-9.

By the use of various specific pharmacological inhibitors, we showed in both cell lines that silibinin induced apoptosis independently of MAPK and of p53. Antioxidants such as GSH and NAC were not able to prevent silibinin-induced cell death. Simultaneously to apoptosis, silibinin triggered an autophagic response. The inhibition of autophagy with a specific inhibitor enhanced cell death, suggesting a cytoprotective function for autophagy in silibinin-treated cells.

Taken together, our data show that in SW480 and SW620 cells silibinin initiated an autophagic-mediated survival response overwhelmed by the activation of both the extrinsic and intrinsic apoptotic pathways.

2. Introduction

Colorectal cancer (CRC) is the second most common cause for cancer-related deaths in the USA [189] and in Europe [190]. Due to the limited efficacy and the cytotoxicity of chemotherapeutic agents used for treatment of advanced CRC, prevention has gained in importance, based on the multistep sequence and the long latency of CRC. While CRC prevention by screening via faecal occult blood testing or colonoscopy is efficient, compliance of the population remains low. Therefore, chemoprevention may represent a valuable option for the population in general and for the intermediate-risk populations following polypectomy [191]. Non-toxic phytochemicals are especially attractive for chemoprevention because of their ability to target simultaneously several events related to carcinogenesis like apoptosis, inflammation and angiogenesis [192, 193]. As reduced rates of apoptosis in the colorectal mucosa are associated with higher risk of adenomas and tumorigenesis, the use of chemopreventive agents favoring apoptosis may constitute an efficient preventive strategy [194].

There are two main pathways of apoptotic cell death: the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway which converge into the activation of caspases, finally leading to cell death. The two pathways are linked by a cross-talk modulated by the protein Bid [195]. The extrinsic pathway is triggered by the binding of death-promoting ligands to their receptors such as DR4/DR5 and Fas leading to the activation of caspases-8 and -10. The intrinsic pathway is activated by intracellular signals mediated by the Bcl-2 protein family which increases the permeability of the mitochondrial membrane leading to a release of cytochrome c in the cytosol and to the activation of caspase-9 [17]. Ultimately the initiator caspases (-8, - 9 and -10) activate the effector caspase-3 that is responsible for DNA fragmentation and cell death [195].

It was shown that phytochemicals like resveratrol [196] as well as anticancer treatments like radiation [176] and 5-fluorouracil [197] do not only induce apoptosis but also autophagy in CRC cells. Autophagy is a process in which a part of the cytoplasm is engulfed in autophagosomes and digested by their fusion with lysosomes. Autophagy may constitute an alternative cell death pathway as well as a stress adaptive mechanism to avoid cell death [198].

The flavonolignan silibinin constitutes the major biologically active part of the crude extract silymarin isolated from the milk thistle plant (*Silybum marianum*) [199]. While milk

thistle extract was already used as a hepatoprotective substance for more than 2000 years and is of remarkable non-toxicity, it was reported recently that its main component, silibinin, exhibits anticancer and chemopreventive properties against many epithelial cancers like skin [115], prostate [87] and lung cancer [200] in different *in vitro* and *in vivo* models. Concerning CRC, it was shown that silibinin causes growth inhibition and cell cycle arrest in HT-29 [149], LoVo [123], Fet, Geo and HCT116 cells [85]. Silibinin induces apoptosis in HT-29 [149], LoVo [123] and SW480 cells [150]. However, these studies addressed only the intrinsic apoptotic pathway [123, 149] or the endpoint of apoptosis (by TUNEL staining) [150]. While Agarwal *et al*. found no caspase activation and no cytochrome c release [149], Kaur *et al*. found an activation of caspase-3 and -9 [123].

In this chapter, we aimed to investigate the mechanisms of the extrinsic and intrinsic apoptotic pathways and the induction of autophagy by silibinin in CRC cells. Until now, activation of autophagy by silibinin was only shown in human fibroblast HT1080 cells [201]. In order to address both primary and metastatic CRC cells we used the human colon cancer SW480 cells and their derived metastatic SW620 cells as a model of cancer progression. The SW480 cells correspond to a primary human colon adenocarcinoma, and the SW620 cells are derived from the primary tumor and isolated from a mesenteric lymph node metastasis of the same patient. These two cell lines have been validated as an *in vitro* model of colon cancer progression from a primary tumor to its metastatic spreading [165].

3. Results: Publication

Silibinin triggers apoptotic signaling pathways and autophagic survival response in human colon adenocarcinoma cells and their derived metastatic cells.

H Kauntz, S Bousserouel, F Gossé, F Raul.

Apoptosis 2011; 16: 1042-53

Link to the editor's website:

http://www.springerlink.com/content/cn6461q405110656

4. General Supplementary Results

4.1. Silibinin inhibits SW480 and SW620 cell growth

Silibinin has been shown to inhibit cell growth of several CRC cell lines [85] but its growth-inhibiting effect has not yet been shown in SW480 and SW620 cells. Here, we evaluated its cell growth inhibition and observed that it was both time- and dose-dependent (Figure 28).

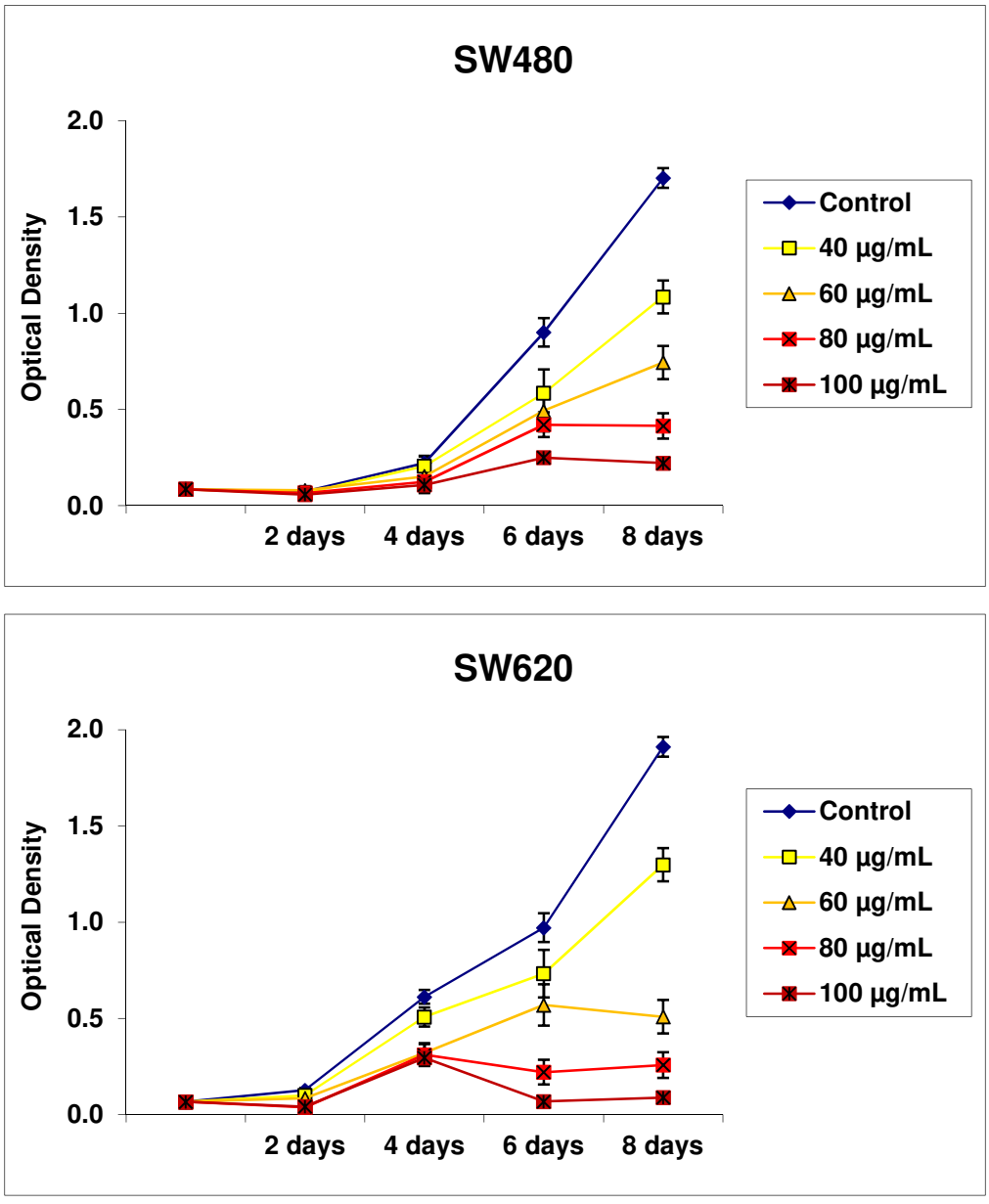


Figure 28: Time- and dose-dependent cell growth inhibition by silibinin. Cells were seeded in 96-well plates and treated with different concentrations of silibinin. After the indicated time, protein content was assessed by sulforhodamine B assay (see Materials and Methods). For each concentration, mean value ± SE of three separate experiments is represented.

4.2. Silibinin-induced cell death measured by annexin V/PI staining

Previously, we have shown that silibinin induced cell death by using propidium iodide (PI) staining. To confirm our results we also employed another method which is widely used to quantify apoptosis and cell death [202]: annexin V/PI double-staining.

When cells die by apoptosis, the membrane phospholipid phosphatidylserine (PS) translocates from the inner to the outer leaflet of the cell membrane. Because annexin V has a high affinity to PS, cells can be stained by a fluorescent conjugate of annexin V and hence be recognized as cells undergoing apoptosis. Once cells are dead, membrane integrity is lost and PI is able to enter the dead cells (in contrast to the PI-only staining method where cell membranes are permeabilized by 70%-ethanol to allow PI to enter all cells and quantify DNA content) and intercalate their DNA. Thus double staining with annexin V and PI allows distinguishing between live cells, dead cells and cells in the process of undergoing apoptosis.

Our results show that in the untreated cells, annexin V and PI fluorescence is low as expected (Figure 29); the cells are in the lower left quadrant. Cells treated by silibinin for 48 h have relocated to the lower right quadrant (annexin V-only marked cells - cells undergoing the process of apoptosis) and to the upper right quadrant (annexin V and PI-marked cells - cells already dead). At 72 h of treatment, there are even more cells in the right quadrants showing a time-dependent cell death. Even if exact percentages differ a bit, on the whole these results are in accordance with our results observed with the PI-only staining method.

However, exteriorization of PS also happens during autophagy [171] and thus labeling by annexin V is not specific for apoptosis, which is an explanation for the deviation of percentages. Because of this ambiguity we preferred to perform additional apoptosis- and autophagy-specific assays.

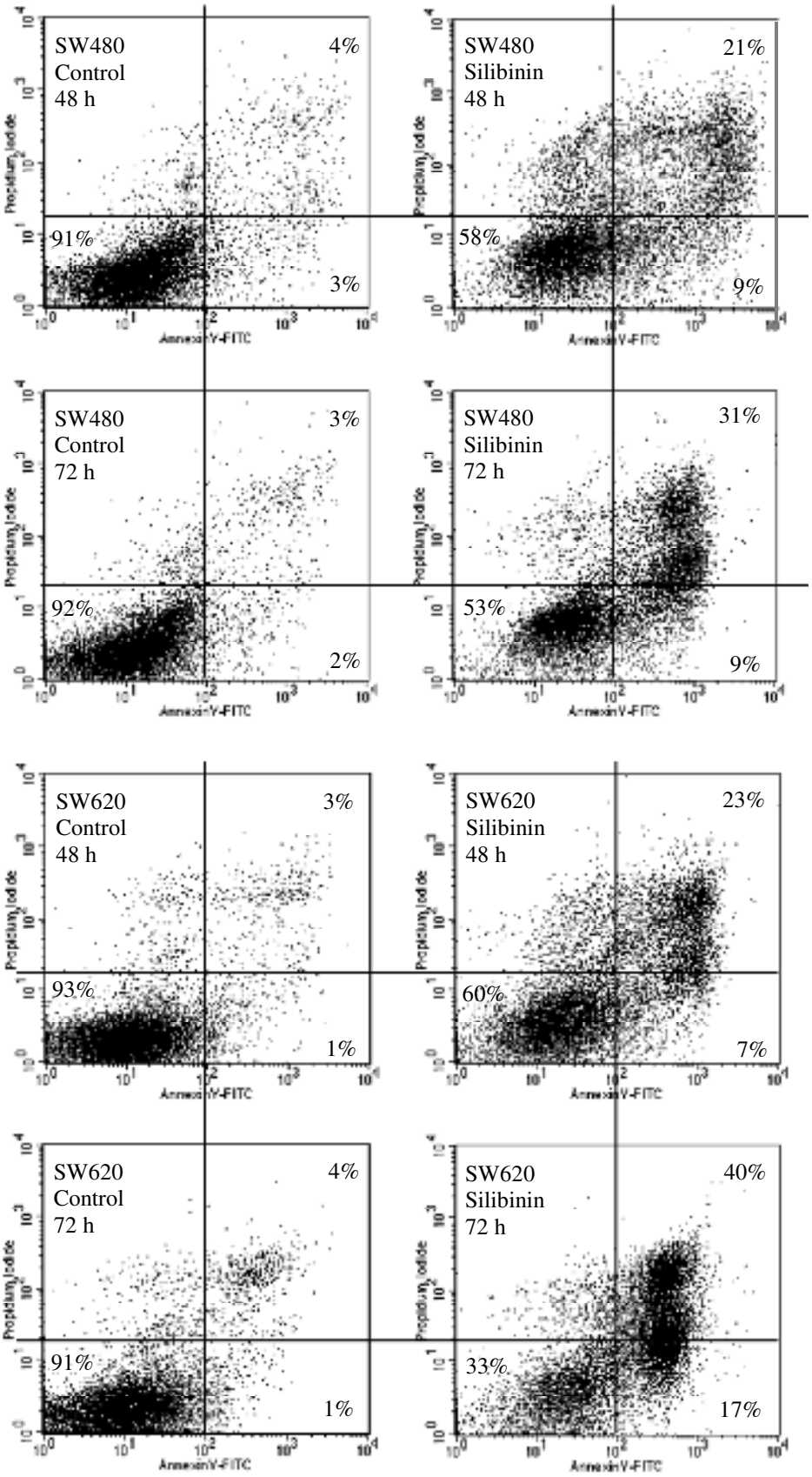


Figure 29: Silibinin-induced cell death measured by annexin V/PI double-staining. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 µM) for 48 or 72 h. Cells were stained with FITC-conjugated annexin V and PI and analyzed by flow cytometry as detailed in Materials and Methods. Data are the mean value of three separate experiments. Representative FACS dot plots are shown.

5. Supplementary Results concerning the Intrinsic Apoptotic Pathway

5.1. Silibinin-induced cell death is independent of ROS formation

ROS - oxygen-containing chemical species with reactive chemical properties - play an important dual role in cancer [54]. Oxidative stress is increased in cancer cells. The over-production of ROS damages the DNA and promotes genetic instability, mutations and drug resistance. On the other hand, it is possible to kill cancer cells by ROS-mediated mechanisms. Cytochrome c release from mitochondria appears to be one of the mechanisms of ROS-mediated cell death [38].

Phytochemicals have been shown to trigger apoptosis by inducing oxidative stress: The alkaloid berberine induces apoptosis in SW620 cells by the generation of ROS which can be reversed by the ROS scavenger NAC [203]. Isothiocyanates from cruciferous vegetables induce apoptosis in non-small cell lung cancer cells by generating oxidative stress [204]. Likewise, CRC chemopreventive agents such as nitric oxide-donating aspirin [205] induce oxidative stress as part of their chemopreventive action [206, 207]. Silibinin has also been shown to induce ROS-mediated autophagic death in human fibrosarcoma HT1080 cells [208] and ROS-dependent autophagic and apoptotic cell death in HeLa cells which was inhibited by NAC and by GSH [209]. Likewise, silibinin induced ROS-dependent cell death in glioma, preventable by NAC [210].

Here, we used the antioxidant molecules GSH and NAC to inhibit ROS formation before propidium iodide staining to measure cell death by flow cytometry. Our data show that inhibition of ROS formation did not modify silibinin-induced cell death (Figure 30). However, ROS-induction by silibinin was shown to be p53-dependent, as the inhibition of p53 reduced ROS production and reversed cell death in HeLa cells [211]. In SW480 and SW620 cells, p53 is mutated [212] which may be an explanation for the lacking involvement of ROS formation in silibinin-induced apoptosis.

We therefore conclude that ROS formation seems not to be the cause of silibinin-induced mitochondrial perturbation.

However, we showed that caspase activation may be important for mitochondrial perturbation because there was a significant reduction of perturbation of the mitochondrial membrane potential when cells were treated with the pan-caspase inhibitor Z-VAD-FMK (data not shown).

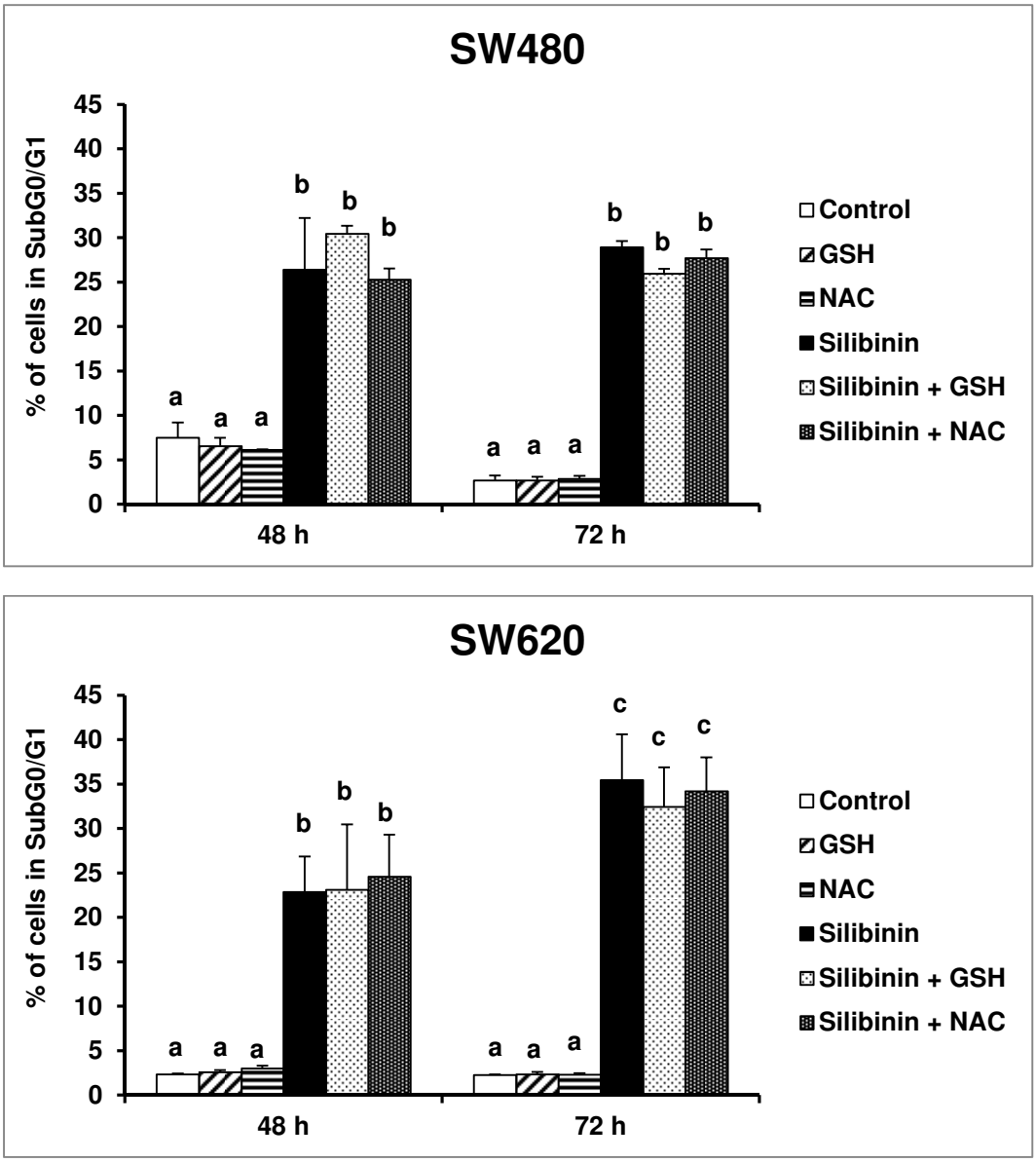


Figure 30: Silibinin-induced cell death after treatment with antioxidants. SW480 and SW620 cells were pre-treated for 1 h with GSH (5 mM) or NAC (10 mM) and then treated with DMSO 0.1% ± silibinin (300 µM) for 48 or 72 h. At each time point, SW480 and SW620 cells were harvested and stained with PI for the measurement of hypodiploïd bodies and analyzed by flow cytometry as detailed in Materials and Methods. Data are presented as mean ± SE of three separate experiments. For each cell line, columns not sharing the same superscript differ significantly: *P < 0.05.

5.2. Silibinin does not change the expression of Bax protein

The protein Bax is a pro-apoptotic member of the Bcl-2 family which can oligomerize to form pores in the outer mitochondrial membrane and thus induce the release of pro-apoptotic factors. Pro-apoptotic phytochemicals, among them silibinin, have been shown to increase the expression of Bax, especially the Bax/Bcl-2 ratio [84].

We used flow cytometric analysis to study the effect of silibinin on Bax expression in SW480 and SW620 cells and observed that silibinin did not significantly change the expression of Bax (Figure 31). These data indicated that silibinin-induced perturbation of the mitochondria was induced by other mechanisms, e.g. activation of Bak by tBid which was truncated by caspase-8 as shown before.

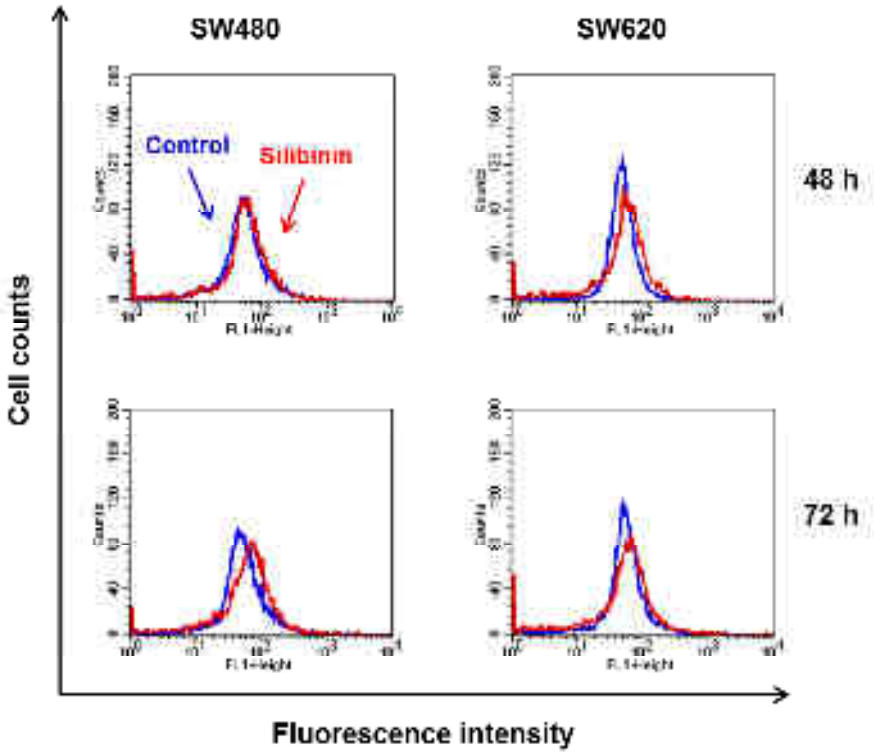


Figure 31: Expression of Bax after silibinin treatment. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 µM) for 48 or 72 h. Cells were fixed and permeabilized, then incubated with rabbit anti-human Bax polyclonal antibody, followed by FITC-conjugated secondary antibody and analyzed by flow cytometry as detailed in Materials and Methods. Representative FACS histograms of Bax analysis are displayed. The blue line represents DMSO-only treated cells, the red represents silibinin treatment.

5.3. Silibinin downregulates survivin expression in SW480 cells but does not change XIAP and Mcl-1 expression

Survivin and XIAP are anti-apoptotic proteins of the IAP family which inhibit cell death and hence contribute to treatment resistance in cancer. Survivin is overexpressed in human tumors, associated with poor prognosis and has thus become a target for anticancer therapies: survivin inhibitors are explored in clinical settings [63]. XIAP acts downstream of the mitochondrial pathway by binding and inhibiting caspases-3 and -9 [35].

Mcl-1 is an anti-apoptotic member of the Bcl-2 family which contributes to apoptosis resistance at the level of the mitochondria by binding to pro-apoptotic BH3-only proteins [213].

We wanted to explore the effect of silibinin on the expression of these anti-apoptotic proteins as silibinin has been found to downregulate survivin in prostate and in bladder cancer [82], and both survivin and Mcl-1 in endothelial cells [214] whereas nothing has been published about the effect of silibinin on XIAP.

Here, we measured survivin and XIAP expression after silibinin treatment using ELISA technique and Mcl-1 expression after silibinin treatment by flow cytometric analysis.

Our results show no significant modification in the expression of XIAP (Figure 32) and Mcl-1 (Figure 33) by silibinin treatment. However, silibinin was able to time-dependently reduce survivin expression starting after 24 h of treatment, but in SW480 cells only (Figure 34).

Our data suggest that XIAP or Mcl-1 downregulation did not seem to play a significant role in silibinin-induced apoptosis. In contrast, survivin appeared to play a role but only in the SW480 cell line.

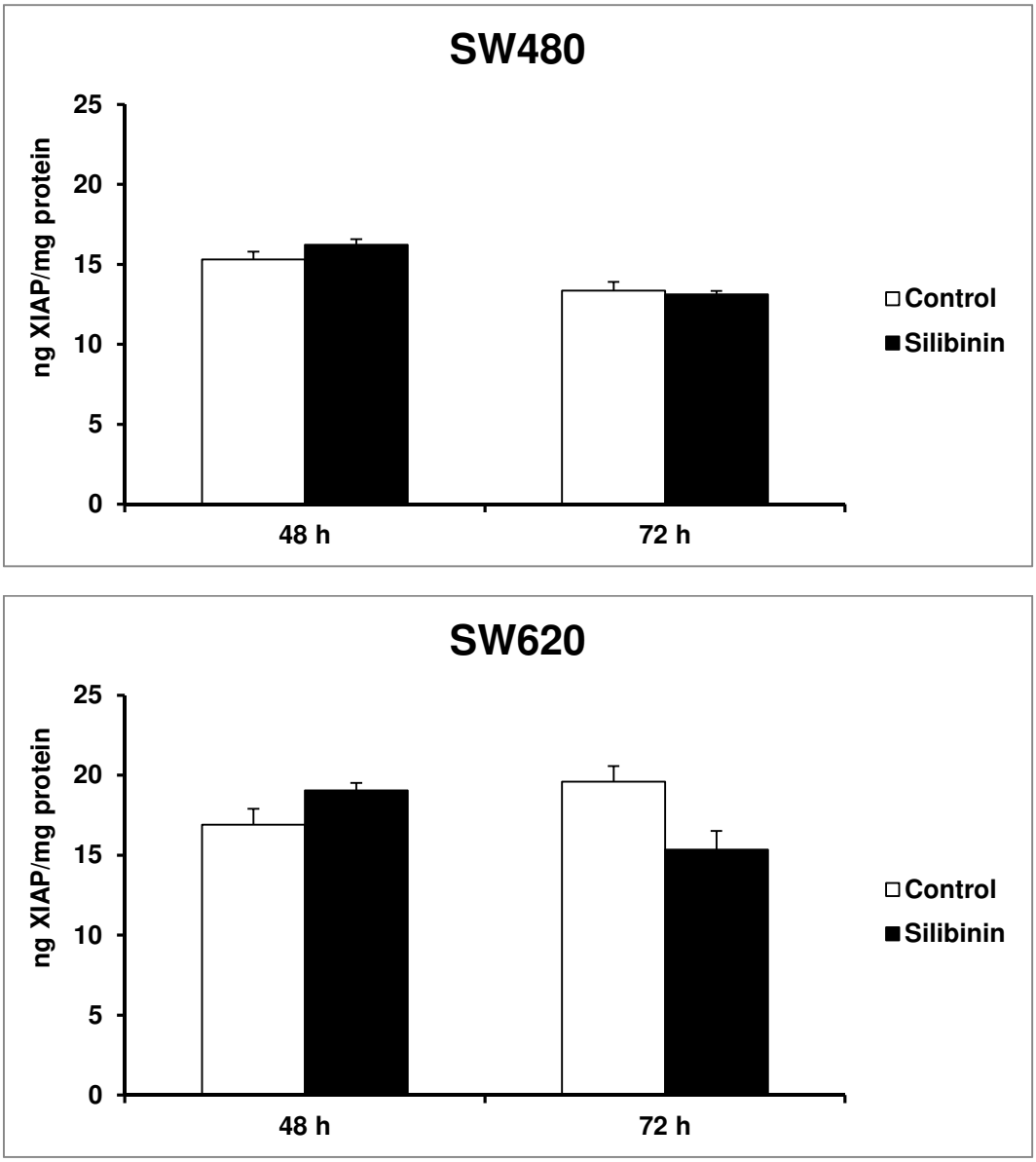


Figure 32: Silibinin does not modify XIAP expression. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 µM) for 48 or 72 h. Expression of XIAP was determined in cell lysates by ELISA as detailed in Materials and Methods. Data are the mean value ± SE of three separate experiments. For each cell line, silibinin treatment versus non-treated control: *P < 0.05.

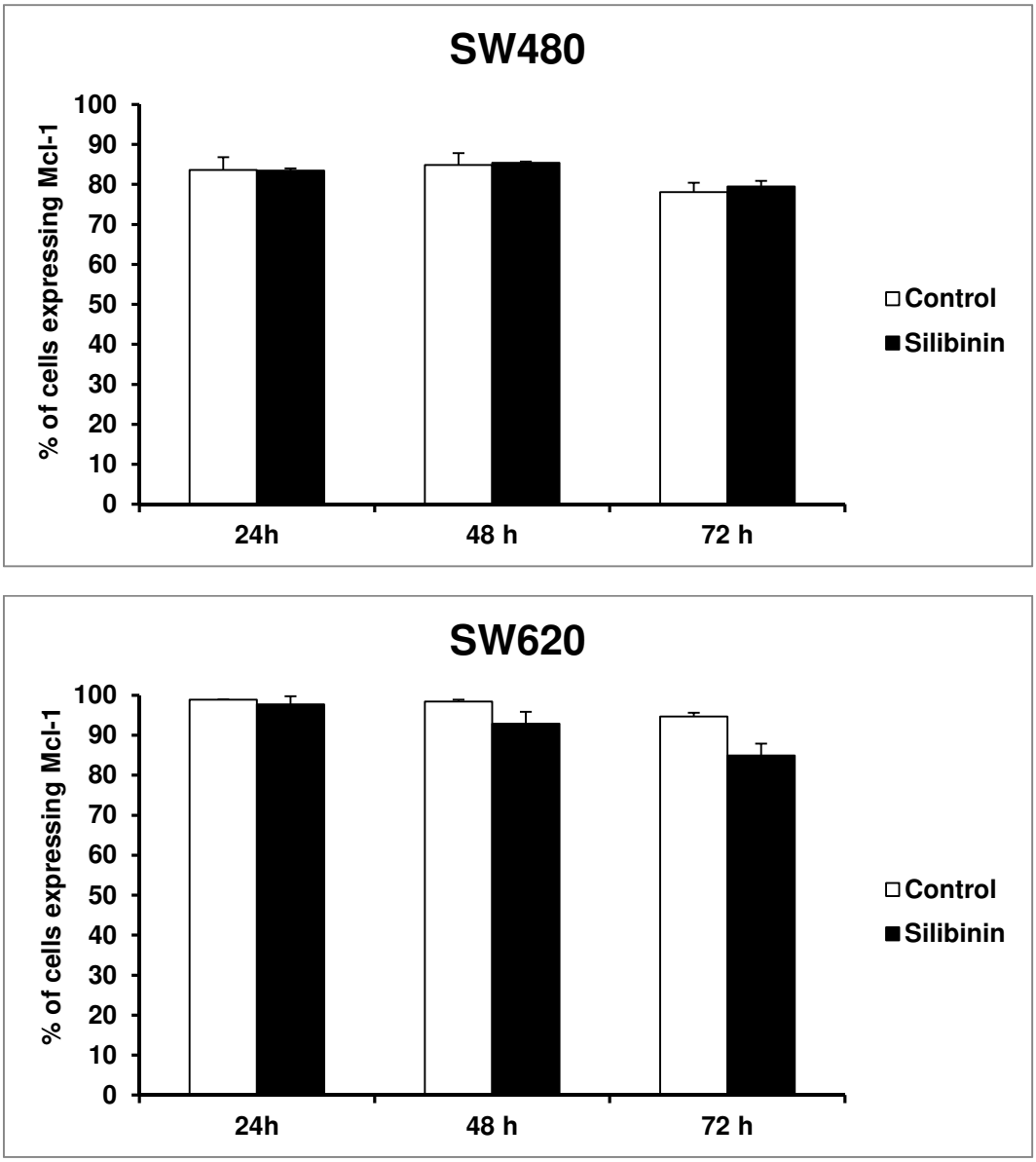


Figure 33: Silibinin does not modify Mcl-1 expression. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 µM) for 24, 48 or 72 h. Cells were fixed and permeabilized, then incubated with rabbit anti-human Mcl-1 polyclonal antibody, followed by incubation with FITC-conjugated secondary antibody and analyzed by flow cytometry as detailed in Materials and Methods. Data are the mean value ± SE of three separate experiments. For each cell line, silibinin treatment versus non-treated control: *$P < 0.05$.

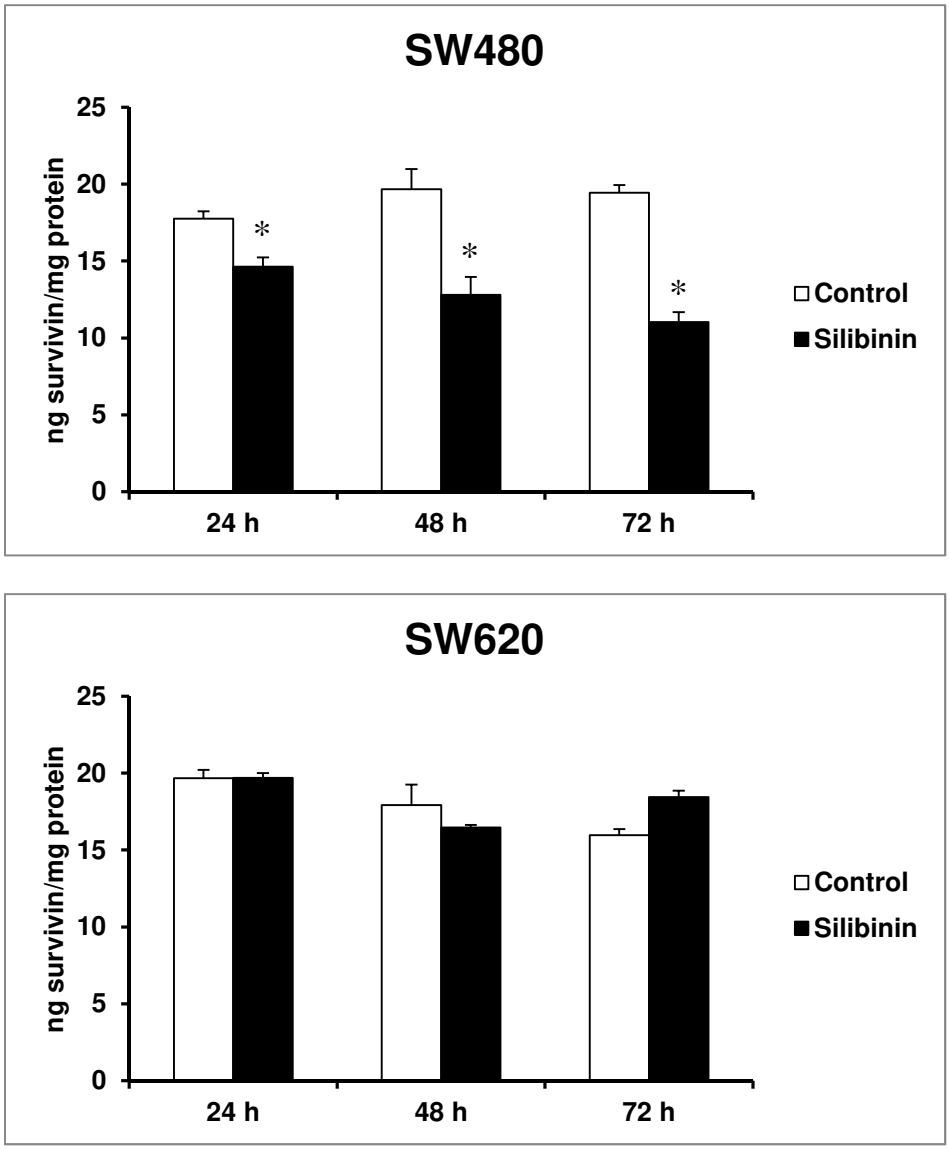


Figure 34: Silibinin downregulates the expression of the anti-apoptotic protein survivin in SW480 cells. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 µM) for 24, 48 or 72 h. Expression of survivin was determined in cell lysates by ELISA as detailed in Materials and Methods. Data are the mean value ± SE of three separate experiments. For each cell line, silibinin treatment versus non-treated control: *P < 0.05.

6. Supplementary Results concerning the Extrinsic Apoptotic Pathway

6.1. Silibinin increases Fas receptor expression at mRNA and protein level

Since silibinin was able to upregulate TRAIL death receptors, we were interested to see if silibinin was also capable of increasing the expression of Fas, another death receptor. Fas is the receptor of the death ligand FasL which is - like TRAIL - involved in the host cancer immunosurveillance system [215]. This makes targeting the Fas-mediated apoptosis pathway an attractive approach in cancer therapy.

The protein expression of Fas was measured by flow cytometry and the genetic expression of Fas by qRT-PCR. We observed a time-dependent increase in Fas receptor expression on the cell surface of SW480 cells (Figure 35). In SW620 cells, Fas expression was also increased, but after a maximum at 24 h Fas expression decreased. The Fas mRNA expression was time-dependently increased in the two cell lines, and this increase was higher in the SW620 cells (Figure 36).

Thus the death-receptor-upregulating effects of silibinin were not confined to TRAIL death receptors but also concerned Fas. The decreasing protein expression in SW620 cells in spite of time-dependent increase of Fas mRNA may result from the internalization of the receptors which takes place as a result of Fas binding [35].

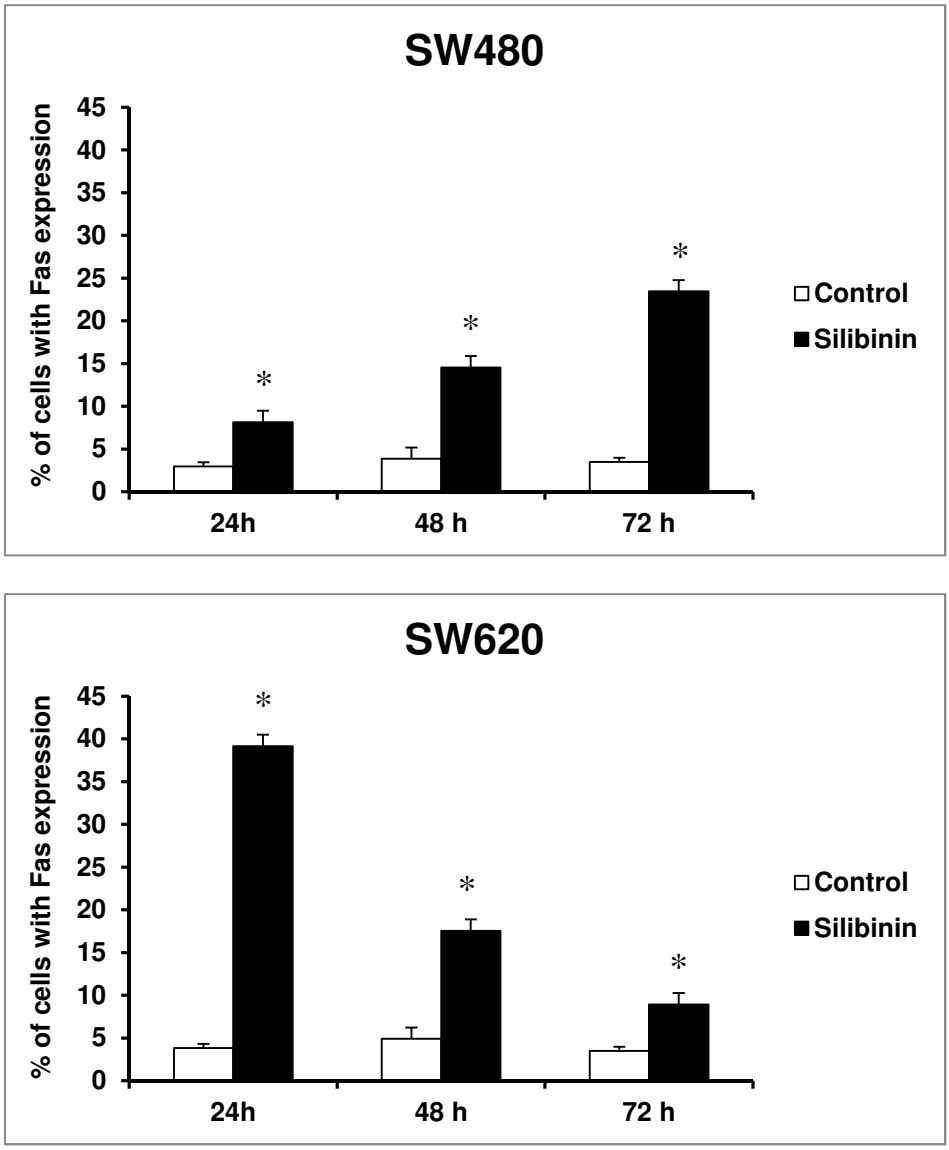


Figure 35: Fas receptor expression after silibinin treatment. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 µM) for 24, 48 or 72 h. Cells were harvested, incubated with FITC-conjugated anti-human Fas antibodies, and Fas protein expression was analyzed by flow cytometry. Data are the mean value ± SE of three separate experiments. For each cell line, silibinin treatment versus non-treated control: *P < 0.05.

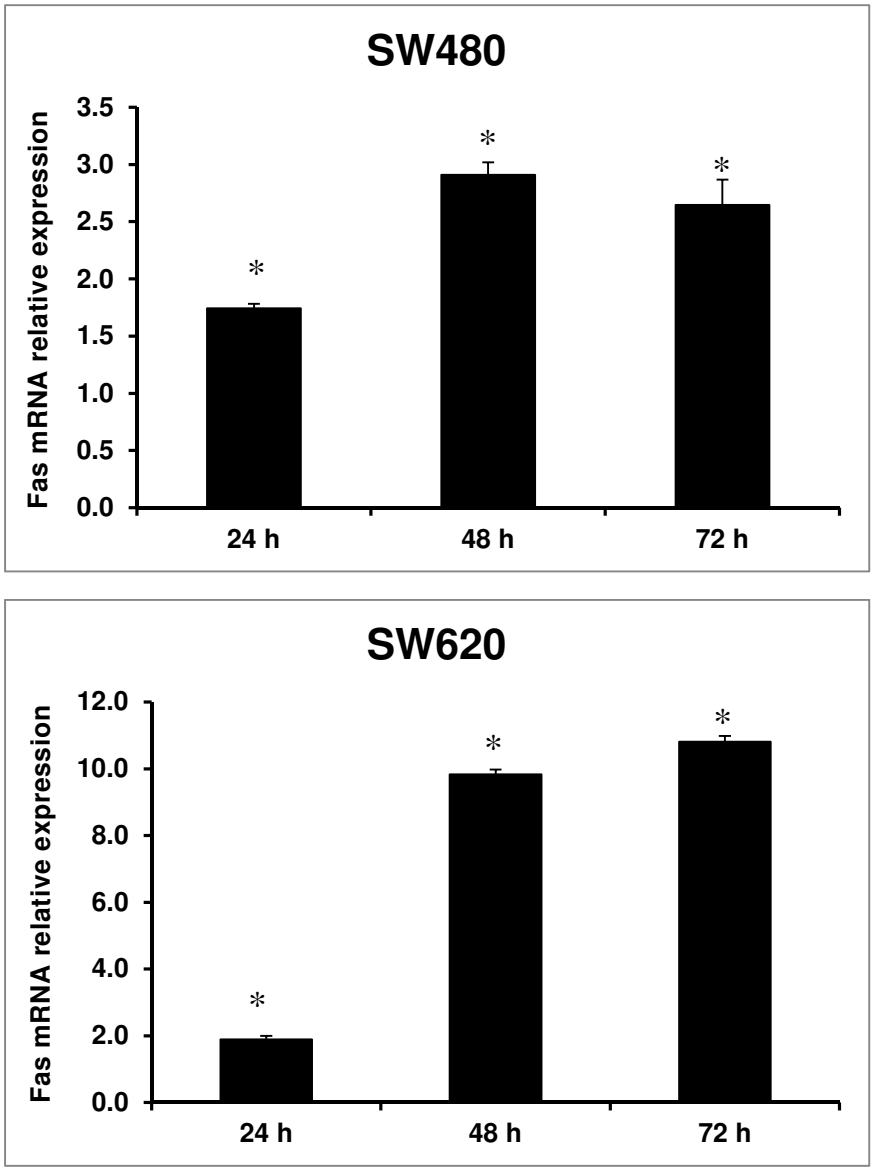


Figure 36: Fas mRNA expression after silibinin treatment. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 µM) for 24, 48 or 72 h. Total RNA was isolated and qRT-PCR was performed as detailed in Materials and Methods. Data are the mean value ± SE of three separate experiments. For each cell line, silibinin treatment versus non-treated control: *P < 0.05.

6.2. Silibinin does not directly bind to TRAIL death receptors

Previously, we have found that silibinin increased DR4 and DR5 expression on the cell surface and on the genetic level. To test the involvement of these receptors in silibinin-induced apoptosis, we used recombinant human DR4 and DR5 Fc chimera which enter in competition with the endogenous receptors and thus have a dominant-negative effect against the TRAIL receptors [216].

We added DR4/Fc and DR5/Fc chimeric protein to silibinin-treated cells and measured cell death by propidium iodide staining and flow cytometry. We observed no significant change in the cell death rate after addition of the chimeric proteins (Figure 37). The fact that the recombinant chimeric proteins of the death receptors are not able to inhibit silibinin-induced cell death suggests that silibinin does not activate these receptors by directly binding to them.

However, silibinin does activate the extrinsic pathway as we have shown by silibinin-induced caspase-8 activation.

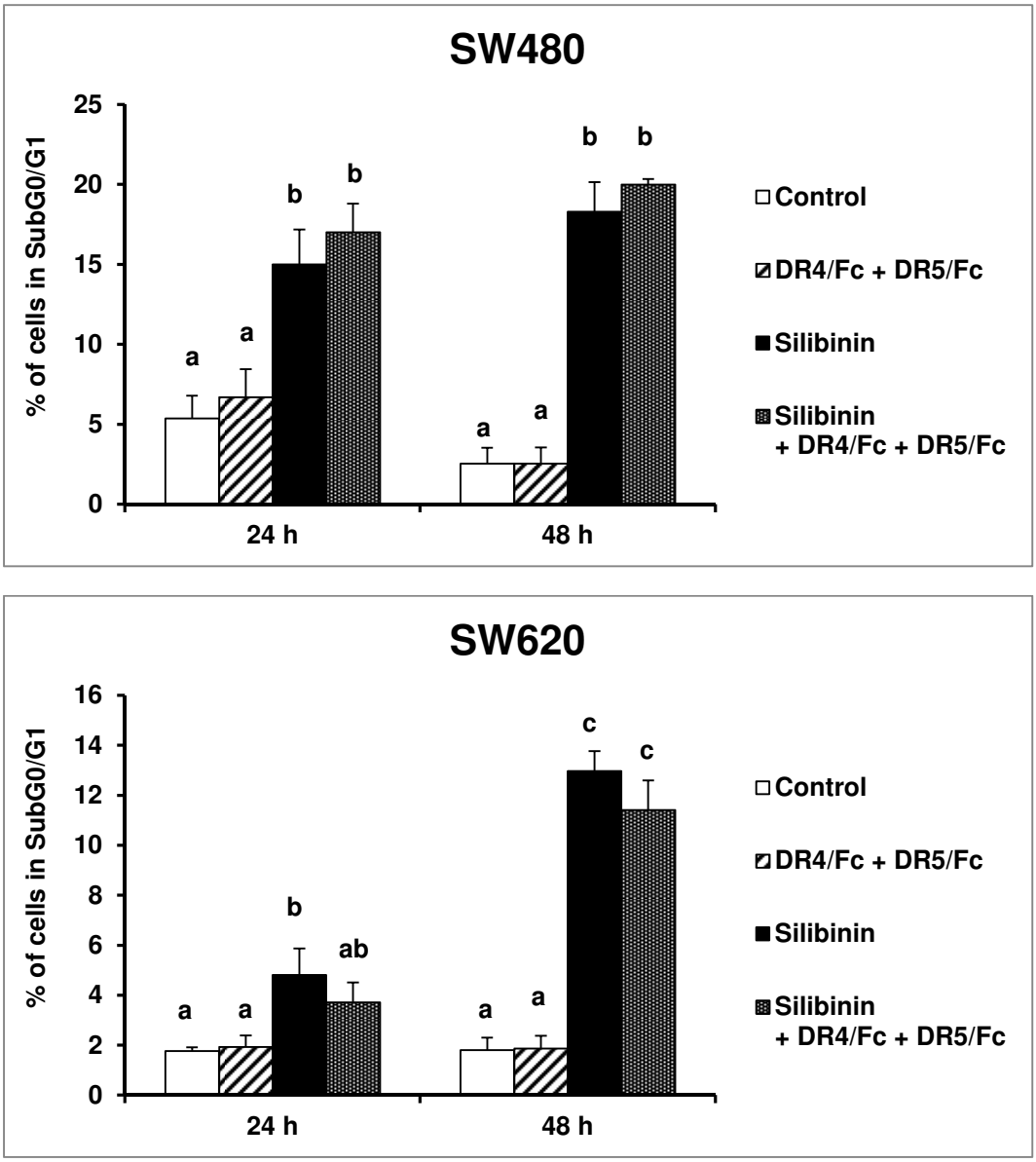


Figure 37: Role of death receptors in cell death induced by silibinin. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 μM) ± DR4/Fc chimera (100 ng/mL) ± DR5/Fc chimera (100 ng/mL) for 24 or 48 h. At each time point, SW480 and SW620 cells were harvested and stained with propidium iodide for the measurement of hypodiploïd bodies and analyzed by flow cytometry as detailed in Materials and Methods. Data are presented as mean ± SE of three separate experiments. For each cell line, columns not sharing the same superscript differ significantly: *P < 0.05.

7. Supplementary Results concerning the Regulation of Apoptosis

7.1. Role of MAPKs in silibinin-induced cell death

MAPKs play an important role in the regulation of cellular processes in response to extracellular signals: cell growth and proliferation, apoptosis, migration and differentiation. There are three major subgroups of MAPKs in humans: ERK, JNK and p38 MAPK. They have different functions - which are often altered in cancer - depending on the cellular context [65].

In general, the stress-activated JNK and p38 pathways usually exert pro-apoptotic functions whereas the ERK pathway, activated by growth factors and mitogens and often constitutively active in cancer cells, plays a role in cell proliferation and survival. However, the overall response to a given drug and the function of the MAPKs in this response depends on the cell type, the drug dose, the status of other MAPKs and other cellular pathways [66].

Silibinin has been reported to act on MAPK mitogenic signaling in prostate and in skin cancers [127, 217] and on MAPK signaling in migration and invasion of oral and hepatocellular cancer [218, 219]. However, no studies have yet been reported concerning the role of MAPKs in silibinin-treated CRC cells.

To get more insight into the implication of the MAPKs in silibinin-induced apoptosis in SW480 and SW620 cells, we determined first their activation by an ELISA kit which measures phosphorylated and total protein to calculate the phosphorylation ratio, and then we used the pharmacological inhibitors SL327 (inhibitor of ERK), SP600125 (inhibitor of JNK) and SB203580 (inhibitor of p38 MAPK) to inhibit each of the MAPKs individually.

We observed no significant activation of MAPKs in SW620 cells after 24 h (Figure 38). In SW480 cells, we observed a weak but significant activation of ERK and p38. When these MAPKs were inhibited, we observed a significant increase of SW480 cell death at 72 h (Figure 39). In SW620 cells, we found an increase of cell death at 48 h with inhibition of JNK or ERK; at 72 h cell death was likewise increased after inhibition of p38 MAPK.

MAPKs do not seem to play a role in the early phase of silibinin-induced apoptosis (24 h). However, the increased cell death after their inhibition for 72 h may suggest a pro-survival role for the three MAPKs.

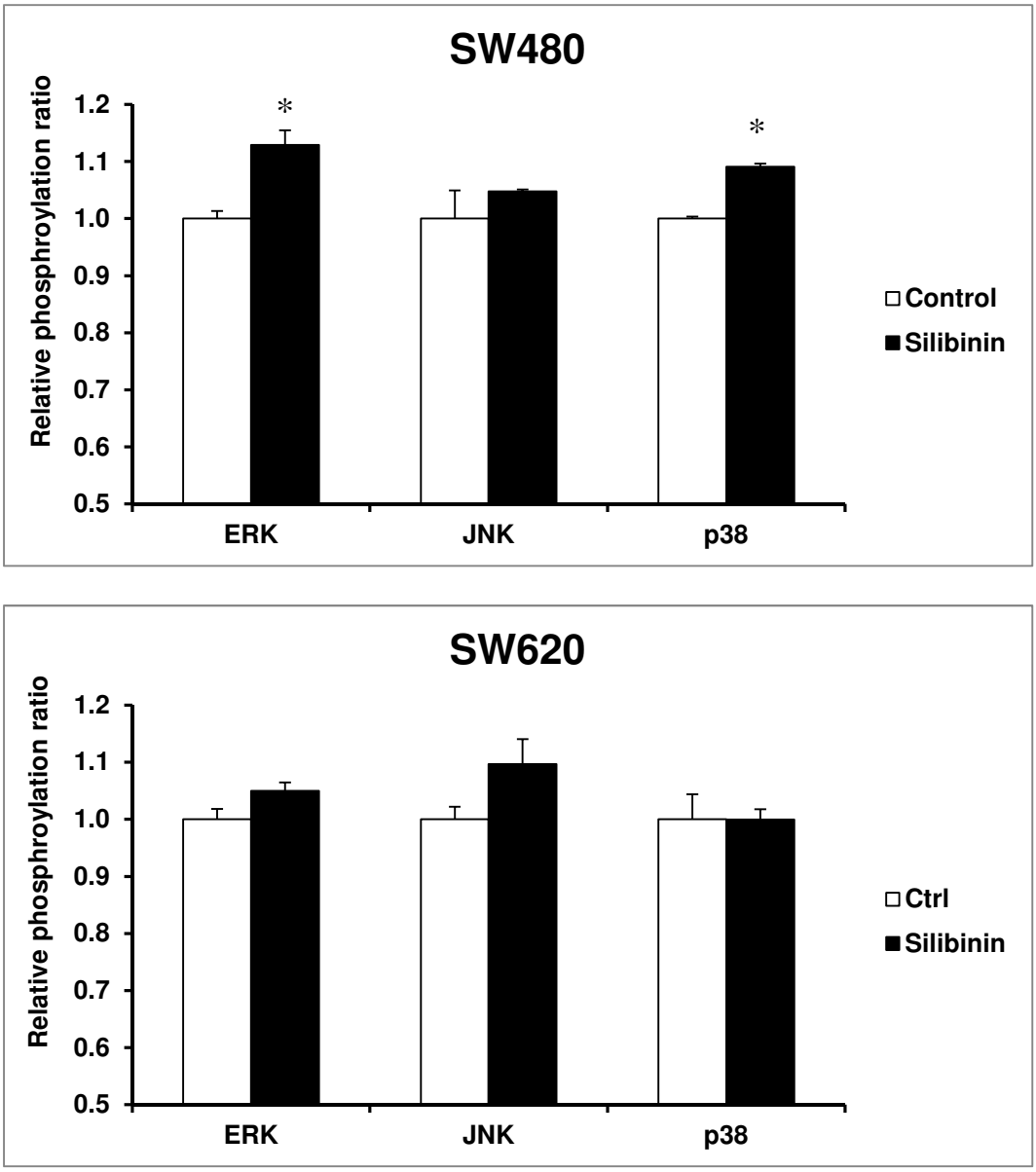


Figure 38: Activation of MAP kinases after silibinin treatment. SW480 and SW620 cells were seeded into 96-well tissue culture plates and treated with DMSO 0.1% ± silibinin (300 µM) for 24 h. Then they were fixed and blocked; anti-phospho-protein or anti-pan-protein specific antibody, then HRP-conjugated secondary antibody, then substrate solution were added and OD was measured as detailed in Materials and Methods. Data are presented as mean ± SE of three separate experiments. For each cell line, silibinin treatment versus non-treated control: *$P < 0.05$.

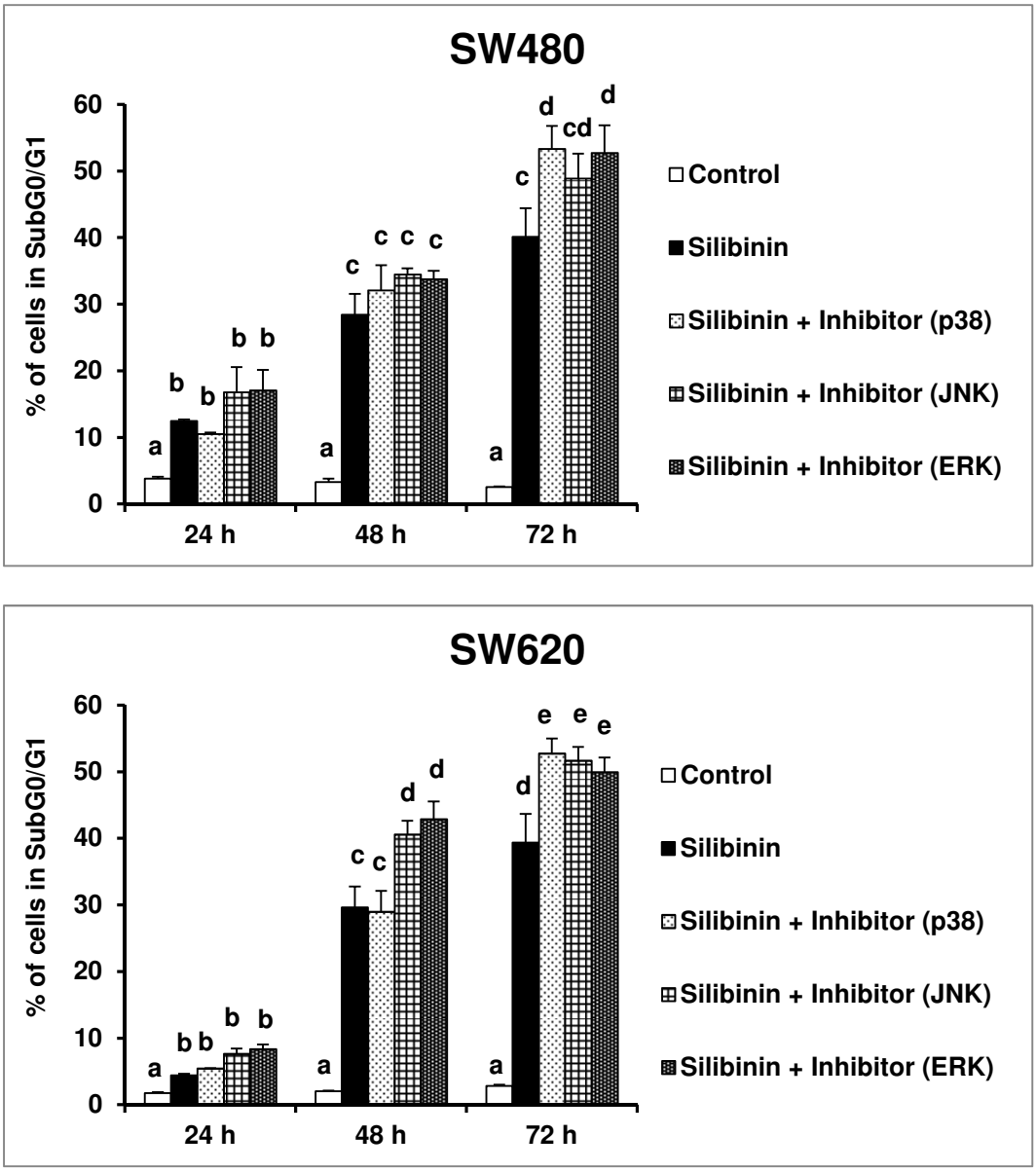


Figure 39: Silibinin-induced cell death after inhibition of MAP kinases. SW480 and SW620 cells were pre-treated for 1 h with inhibitors of p38, JNK or ERK (10 µM) and then treated with DMSO 0.1% ± silibinin (300 µM) for 24, 48 or 72 h. At each time point, SW480 and SW620 cells were harvested and stained with propidium iodide for the measurement of hypodiploïd bodies and analyzed by flow cytometry as detailed in Materials and Methods. Data are presented as mean ± SE of three separate experiments. For each cell line, columns not sharing the same superscript differ significantly: *P < 0.05.

7.2. Role of NF-κB activation in silibinin-induced cell death

NF-κB comprises a family of transcription factors which regulate cellular responses in injury and infection processes and often are constitutively activated in cancer [69]. In cancer, its activation is linked to cell proliferation, survival, angiogenesis and metastasis rendering NF-κB an attractive target for chemoprevention and chemotherapy. NF-κB transcription factors assemble through the dimerization of 5 different subunits: RelA (p65), c-Rel, RelB, p50 and p52. Normally bound in the cytoplasm by their inhibitors (I-κBs), they are released in case of stimulation and then translocate to the nucleus.

Silibinin has been shown to inhibit NF-κB activation in various epithelial cancer models [84]. To study the effect of silibinin on NF-κB activation in SW480 and SW620 cell lines, we prepared nuclear extracts of cells treated with silibinin for 24, 48 and 72 h and quantified their NF-κB p65 content by ELISA. Additionally, we inhibited NF-κB signaling in silibinin-treated cells by a cell-permeable quinazoline compound and measured cell death by the propidium iodide staining method. Surprisingly, we observed that silibinin did not significantly change the translocation of NF-κB p65 to the nuclear department (data not shown). Silibinin has been shown to decrease nuclear levels of p65 and p50 subunits in SW480 cells and other CRC cells, this, however, was observed after TNF-α-induced NF-κB activation [220]. On the other hand, silibinin has also been shown to activate NF-κB signaling in connection with autophagy [208].

Inhibition of NF-κB increased cell death in silibinin-treated SW620 cells but not in SW480 cells (Figure 40). Previous studies have already reported a higher constitutive activity of NF-κB in SW620 than in SW480 cells [221, 222] which may explain the difference of the effect of NF-κB inhibition on cell death in the two cell lines. In conclusion, silibinin did not seem to influence NF-κB activation but the inhibition of NF-κB promoted the pro-apoptotic effect of silibinin on the metastatic SW620 cells.

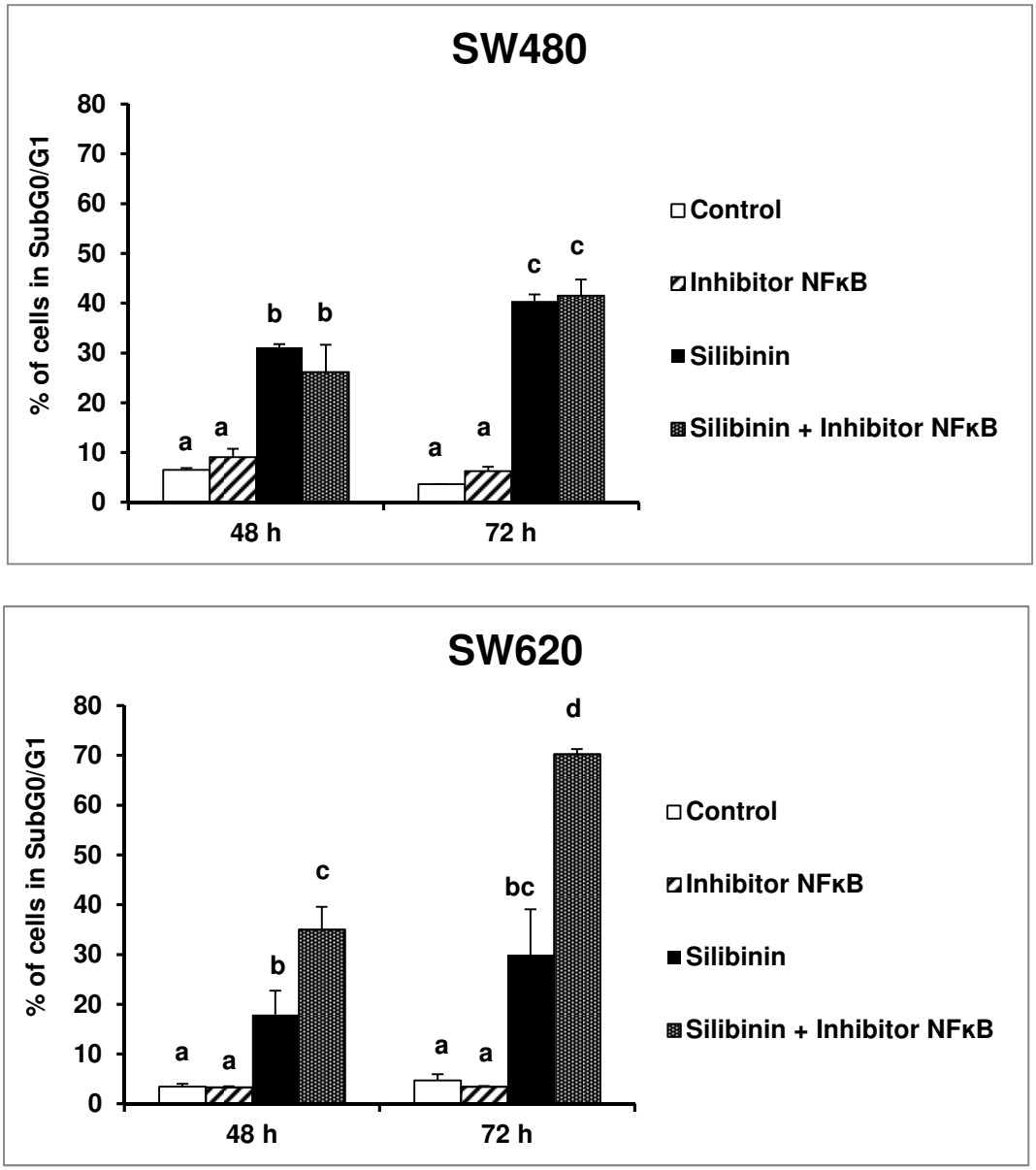


Figure 40: Silibinin-induced cell death after inhibition of NF-κB. SW480 and SW620 cells were pre-treated for 30 min with an inhibitor of NF-κB, 6-Amino-4-(4-phenoxyphenylethylamino)quinazoline (3 µM), and then treated with DMSO 0.1% ± silibinin (300 µM) for 48 or 72 h. At each time point, SW480 and SW620 cells were harvested and stained with propidium iodide for the measurement of hypodiploïd bodies and analyzed by flow cytometry as detailed in Materials and Methods. Data are presented as mean ± SE of three separate experiments. For each cell line, columns not sharing the same superscript differ significantly: *P < 0.05.

7.3. Role of p53 in silibinin-induced cell death

p53 is a tumor suppressor which induces cell cycle arrest and apoptosis in response to cellular stress signals and thus eliminates damaged or malignant cells in the ultimate goal of preserving genome integrity [67]. Its function as a transcription factor allows p53 to control the expression of many pro- and anti-apoptotic proteins. As in more than half of human cancers, p53 in SW480 and SW620 cells is mutated. The two cell lines share the same mutations in the p53 gene [212, 222]. Data from our laboratory [223] and other studies [224, 225] indicated that even though mutated, p53 may still exert some of its functions; therefore we studied its role in silibinin-induced cell death in SW480 and SW620 cells.

Silibinin has been shown to upregulate p53 and its phosphorylation in bladder cancer cells with intact p53 [120]. The p53 inhibitor pifithrin-α reversed caspase activation, demonstrating the p53-dependance of silibinin-induced apoptosis. In cervical carcinoma HeLa cells, silibinin promoted expression of phosphorylated p53 as well, and pifithrin-α was also able to inhibit silibinin-induced cell death [211]. In contrast, in breast cancer cells MCF7 and T47D, inhibition of p53 did not affect silibinin-induced cell death [226]. However, until now there have been no data about the role of p53 in silibinin-treated colon cancer cells.

Here, we measured expression of p53 by flow cytometry. Subsequently we inhibited p53 by pifithrin-α to evaluate the influence of p53 on silibinin-induced cell death by propidium iodide staining and flow cytometry. We observed no change at 24 h (Figure 41). After 48 h and 72 h there was a slight upregulation of p53 in the two cell lines. Still, when we used pifithrin-α, a p53 inhibitor, we observed no significant changes in the cell death rates (data not shown).

In SW480 and SW620 cells, silibinin-induced cell death appeared to be p53-independent.

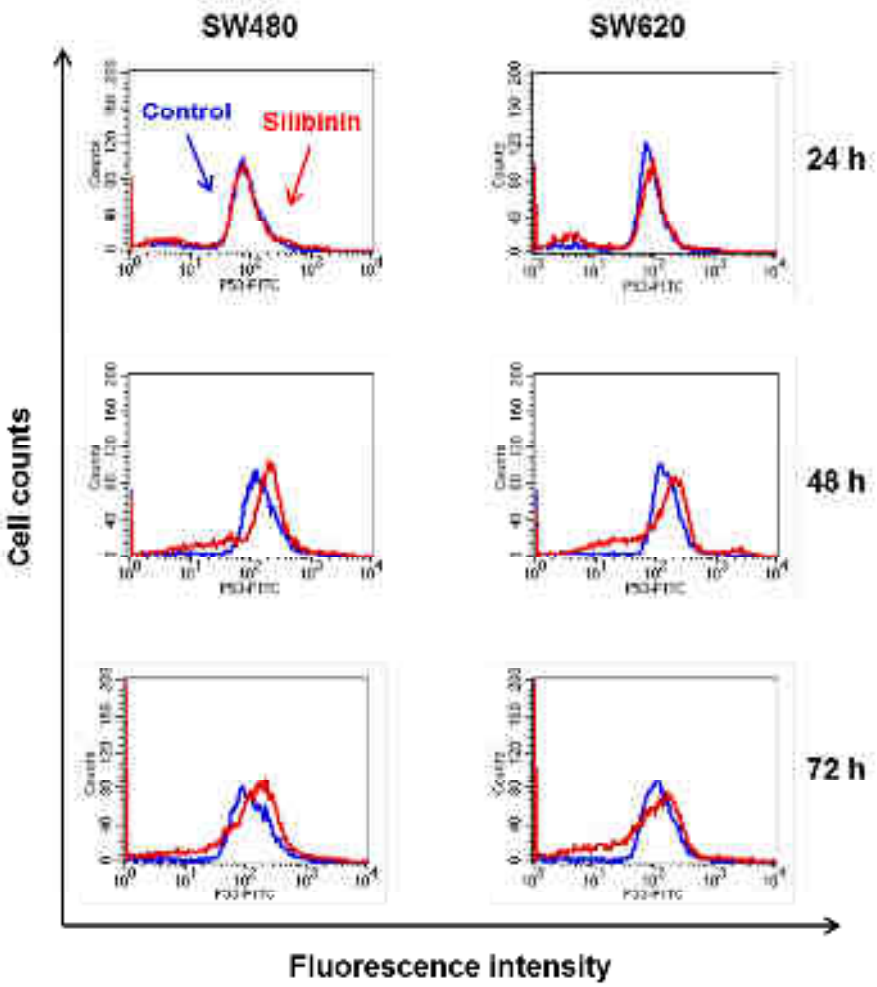


Figure 41: Effect of silibinin on p53 expression. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 µM) for 24, 48 or 72 h. Cells were fixed, permeabilized and incubated with FITC-conjugated mouse anti-human p53 antibody and analyzed by flow cytometry as detailed in Materials and Methods. Representative FACS histograms of p53 analysis are displayed. The blue line represents the DMSO-only treated cells while the red line represents the silibinin-treated cells.

8. Conclusion

In this chapter, we studied the mechanisms of silibinin-induced cell death in primary colon tumor SW480 cells and in their derived metastatic SW620 cells.

We observed that silibinin treatment for 24 to 72 h inhibited cell growth and induced apoptotic cell death with caspase-3 activation in both cell lines. Silibinin enhanced the expression of the death receptors DR4, DR5 and Fas on the cell surface on both transcript and protein level and activated their downstream caspases-8 and -10, confirming the activation of the extrinsic pathway. However, silibinin did not directly bind to these receptors as demonstrated by the use of recombinant human DR4 and DR5 Fc chimera.

Caspase-dependency of apoptosis was shown by the effective inhibition of cell death by pan-caspase-inhibitor Z-VAD-FMK and caspase-8-inhibitor Z-IETD-FMK in both cell lines.

Cross-talk between the extrinsic and the intrinsic pathway was evidenced by the cleavage of protein Bid which was more distinct in SW480 than in SW620 cells. A further difference between the two cell lines was the downregulation of the anti-apoptotic protein survivin only in SW480 cells. The silibinin-induced activation of the intrinsic pathway was shown by the perturbation of the mitochondrial membrane potential, the release of cytochrome c from the mitochondria and the activation of caspase-9.

Not involved in silibinin-induced apoptosis were MAPK activation, p53 activation, NF-κB activation, regulation of the anti-apoptotic proteins Mcl-1 and XIAP and formation of ROS.

Besides apoptotic pathways, autophagy was triggered in SW480 and SW620 cells. It carried out a pro-survival function, shown by increased cell death after inhibition of autophagy.

In conclusion, our study demonstrates that silibinin induces apoptosis through the activation of the extrinsic death receptor pathway and of the intrinsic mitochondrial pathway in the SW480 human colon cancer cells and in the derived metastatic SW620 cells. These observations show the potential of silibinin as an attractive chemoprotective as well as a potential chemotherapeutic agent against CRC and its metastatic spreadings.

CHAPTER II:

Epigenetic effects of silibinin in colon adenocarcinoma cells and their derived metastatic cells

1. Abstract

Epigenetic modifications play an important role in tumorigenesis. The most frequent epigenetic phenomena in cancer are histone deacetylation and DNA hypermethylation which lead to silencing of genes, especially of tumor suppressor genes.

In clinical trials of cancer treatment, histone deacetylase (HDAC) and DNA methyltranferase (DNMT) inhibitors are tested. HDAC inhibitors are promising for cancer therapy because they induce cell cycle arrest, promote differentiation and selectively induce apoptosis in tumor cells. DNMT inhibitors reverse DNA hypermethylation and thus reactivate silenced genes.

Natural substances also act on epigenetic signaling: soy isoflavones and apple polyphenols have been shown to inhibit DNMT activity. Green tea polyphenols inhibit both HDAC and DNMT activity. The flavonolignan silibinin has been shown to increase acetylation of histones in hepatic cancer *in vitro* and *in vivo*. Until now, the effect of silibinin on DNMT activity has not been studied.

The present study investigated the epigenetic effects of silibinin in a preclinical model of colon cancer progression, the primary adenocarcinoma cells SW480 and their derived metastatic cells SW620. Whereas silibinin did not change the activity of HDACs, it was able to inhibit DNMT activity significantly in SW480 cells after 48 and 72 h of treatment and in SW620 cells after 72 h. Potentially because of its DNMT inhibition properties, silibinin showed synergistic effects on cell death induction with the clinically-used HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) and the broad spectrum HDAC inhibitor trichostatin A (TSA).

2. Introduction

Epigenetic modification is a means of regulating gene expression. Tumors often show an aberrant epigenetic modification pattern including histone deacetylation and DNA hypermethylation which leads to the suppression of gene expression.

HDACs catalyze the removal of acetyl groups thereby stimulating chromatin condensation which promotes transcriptional repression, including the decrease of expression of tumor suppressor genes [78]. HDACs are also able to target numerous non-histone proteins, e.g. p53, even accounting for a majority of HDAC substrates [77]. Overexpression of HDAC and epigenetic silencing are often observed in CRC. As epigenetic changes are reversible, HDAC constitute promising targets for pharmacological inhibition in CRC [227].

By mechanisms not yet fully elucidated, inhibitors of HDAC are able to induce cell-cycle arrest, to promote differentiation and to selectively stimulate apoptosis in transformed cells via the extrinsic and/or intrinsic pathway [78]. Furthermore they synergistically enhance the anticancer activity of chemotherapeutic drugs, especially pro-apoptotic ones by shifting the balance between pro-and anti-apoptotic proteins. Examples for HDAC inhibitors are short-chain fatty acids, hydroxamic acids, benzamides and cyclic tetrapeptides [78] . The broad spectrum HDAC inhibitor suberoylanilide hydroxamic acid (SAHA, also called Zolinza® or Vorinostat) was the first HDAC inhibitor to have successfully completed clinical trials and is used for the treatment of cutaneous T-cell lymphoma. Numerous HDAC inhibitors are still undergoing clinical trials [77]. Trichostatin A (TSA) is another hydroxamic acid and broad spectrum HDAC inhibitor which targets class I, II and IV HDACs in the same way as SAHA [78].

DNA hypermethylation is also a frequent phenomenon in cancer which silences many genes for cell cycle regulation, receptors and apoptosis by DNA methylation of CpG islands in their promoter region.

DNA methyltransferase (DNMT) inhibitors are able to induce DNA demethylation and thus reactivation of epigenetically silenced genes. Currently there are two FDA-approved drugs with DNMT inhibitory activity: 5-azacytidine and decitabine, both of which are used in the treatment of the myelodysplastic syndrome and myeloid leukemias [77]. Global reduction of DNA methylation has been shown to have anticancer effects in intestinal tumorigenesis [228].

Histone deacetylation has been shown to act synergistically with DNA methylation in epigenetic silencing of tumor suppressor genes [229]. Therefore combinations of DNMT and HDAC inhibitors seem promising for further clinical trials [77].

Nutrition has been found to influence epigenetic signaling: Green tea polyphenols showed similar effects as the HDAC inhibitor TSA did in prostate cancer cells by inducing cell cycle arrest and apoptosis [230]: They were able to inhibit HDAC and induce their proteasomal degradation.

The tea polyphenol (-)-epigallocatechin-3-gallate was also able to inhibit DNMT activity and reactivate methylation-silenced genes in colon cancer cells [231]. Apple polyphenols reduced DNA methylation by inhibition of DNMT in CRC cells [232]. Soy isoflavones also reversed DNA hypermethylation and reactivated silenced genes in esophageal squamous cell carcinoma cells [233]. In combination with HDAC inhibitors their activity was enhanced [233]. The polyphenol curcumin has also been shown to inhibit DNMT activity [234] and to act synergistically with TSA in inducing cell death [235].

The effect of silibinin on epigenetic modification has only been studied in hepatic cancer: Silibinin increased acetylation of histone H3 and H4 *in vitro* in HuH7 cells [119] and *in vivo* in HuH7 xenografts in nude mice [236]. However, no studies have yet been completed about the effect of silibinin on DNMT activity.

Because of these data in the literature on epigenetic effects, we aimed to investigate if silibinin exerts its anti-proliferative and pro-apoptotic effects in SW480 and SW620 cells by modifying HDAC and DNMT activity.

3. Results

3.1. Effects of silibinin on DNMT and HDAC activity

To find out if silibinin induced epigenetic modifications in SW480 and SW620 cells, we determined HDAC and DNMT activities in nuclear extracts of cells treated with silibinin.

We measured HDAC and DNMT activity in the nuclear extracts of silibinin-treated and control cells with the aid of the colorimetric EpiQuik^{TM} HDAC Activity/Inhibition Assay Kit and the colorimetric EpiQuik^{TM} DNMT Activity/Inhibition Assay Ultra Kit. While the HDAC Activity Kit quantifies - by an ELISA-like reaction - the amount of un-deacetylated histone substrate which is inversely proportional to HDAC enzyme activity in the sample, the DNMT Activity Kit quantifies the amount of methylated DNA substrate which is proportional to DNMT enzyme activity.

After 48 h of treatment with silibinin, we observed a reduction of DNMT activity in silibinin-treated SW480 and SW620 cells (Figure 42). However, this reduction was significant only at 72 h for SW620 cells. In contrast, activity of HDACs in SW480 and SW620 cells was not changed by silibinin treatment (Figure 43).

Our data suggest that cell death induction by silibinin does not involve HDAC modulation but may be mediated in part by DNMT inhibition.

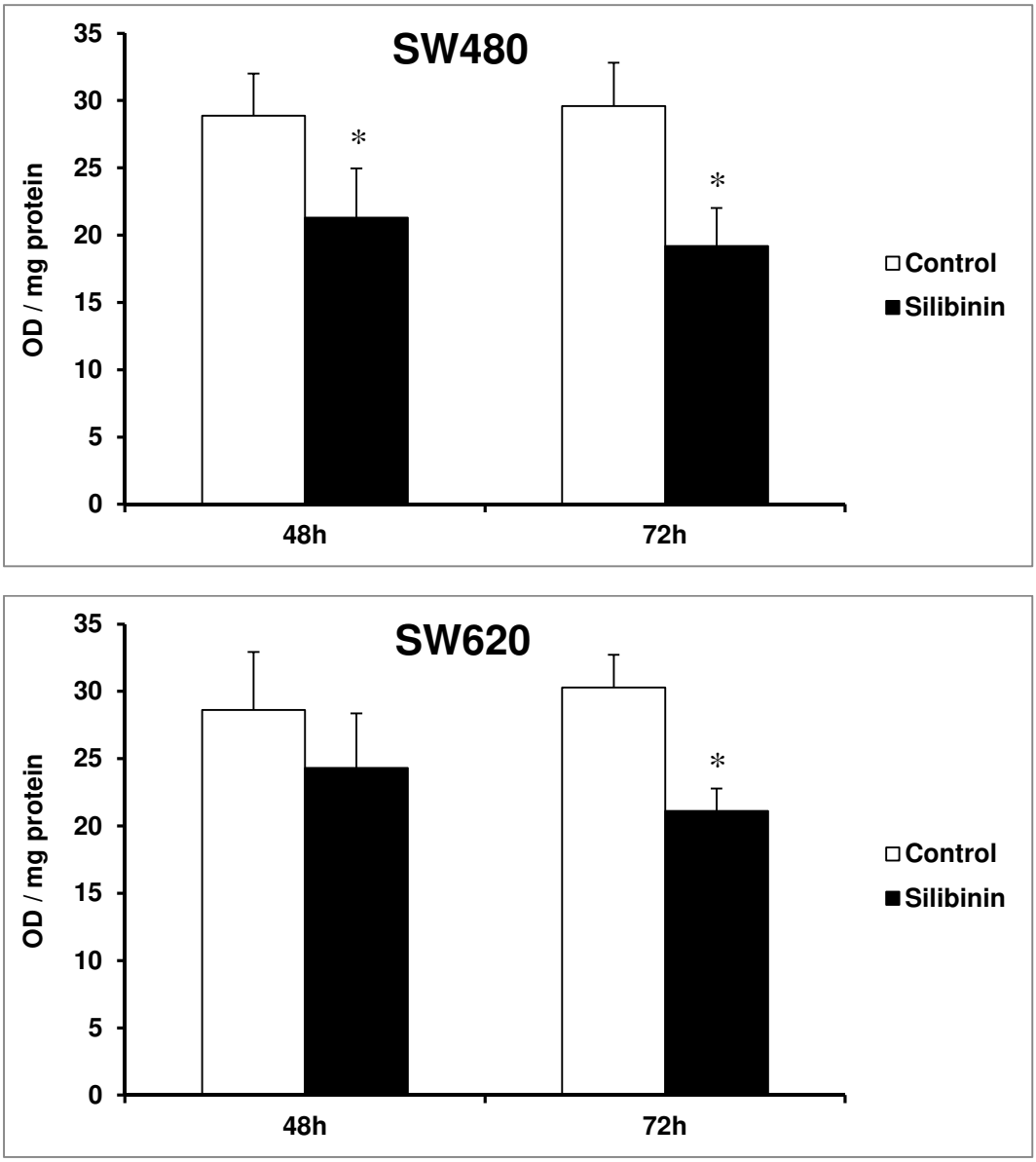


Figure 42: Effect of silibinin on DNMT activity. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 µM) for 24, 48 or 72 h. Nuclear extracts were prepared, and DNMT activity was measured by a colorimetric method as detailed in Materials and Methods. Data are the mean value ± SE of three separate experiments. For each cell line, silibinin treatment versus non-treated control: *$P < 0.05$.

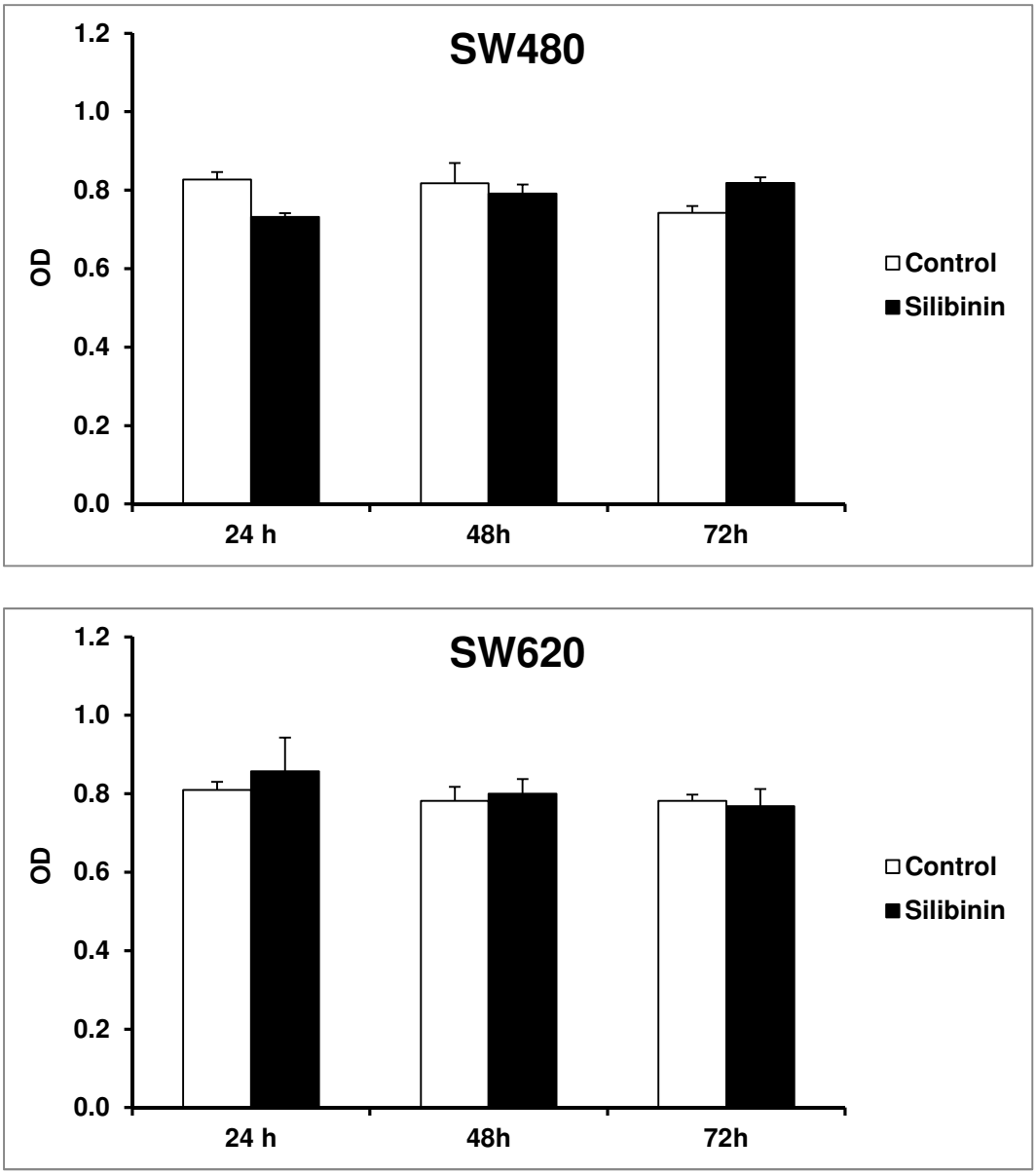


Figure 43: Effect of silibinin on HDAC activity. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 µM) for 24, 48 or 72 h. Nuclear extracts were prepared, and HDAC activity was measured by a colorimetric method as detailed in Materials and Methods. Data are the mean value ± SE of three separate experiments. For each cell line, silibinin treatment versus non-treated control: *P < 0.05.

3.2. Silibinin and HDAC inhibitors induce synergistic cell death

Because synergy between demethylation and HDAC inhibition has been reported in the re-expression of genes silenced in cancer [229], we were interested to see if there was a synergistic effect in cell death induction of silibinin - which inhibits DNMT as we have shown - and the HDAC inhibitors SAHA and TSA.

We measured cell death by the propidium iodide staining method after concomitant treatment with silibinin and HDAC inhibitors.

The broad spectrum HDAC inhibitor SAHA significantly enhanced silibinin-induced cell death (Figure 44) but showed no cell toxicity on its own. However, this effect was much stronger in SW620 cells. To verify the interaction between silibinin and HDAC inhibitors, we used the broad spectrum HDAC inhibitor TSA and observed a synergistic effect with silibinin on cell death induction in both cell lines (Figure 45). These effects were more important with TSA than with SAHA, and we found that SW620 cells were more sensitive than SW480 cells.

However, the synergistic effect of silibinin and HDAC inhibitors could not be entirely attributed to DNMT inhibition because at 48 h of treatment the silibinin-induced DNMT inhibition was not yet significant, while the percentage of dead cells induced by the combination in SW620 cells reached almost 90% (compared to about 20% when treated by silibinin alone).

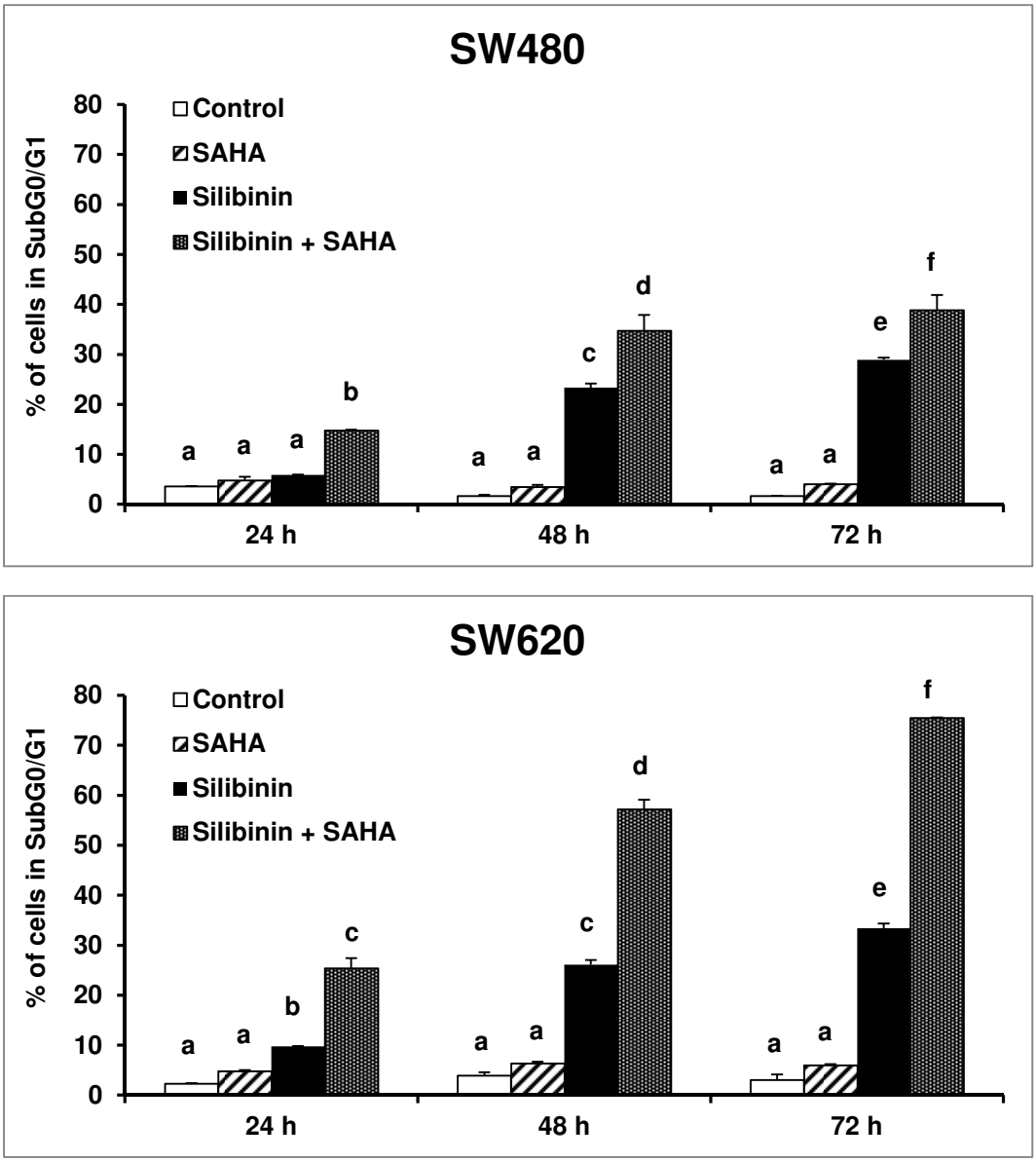
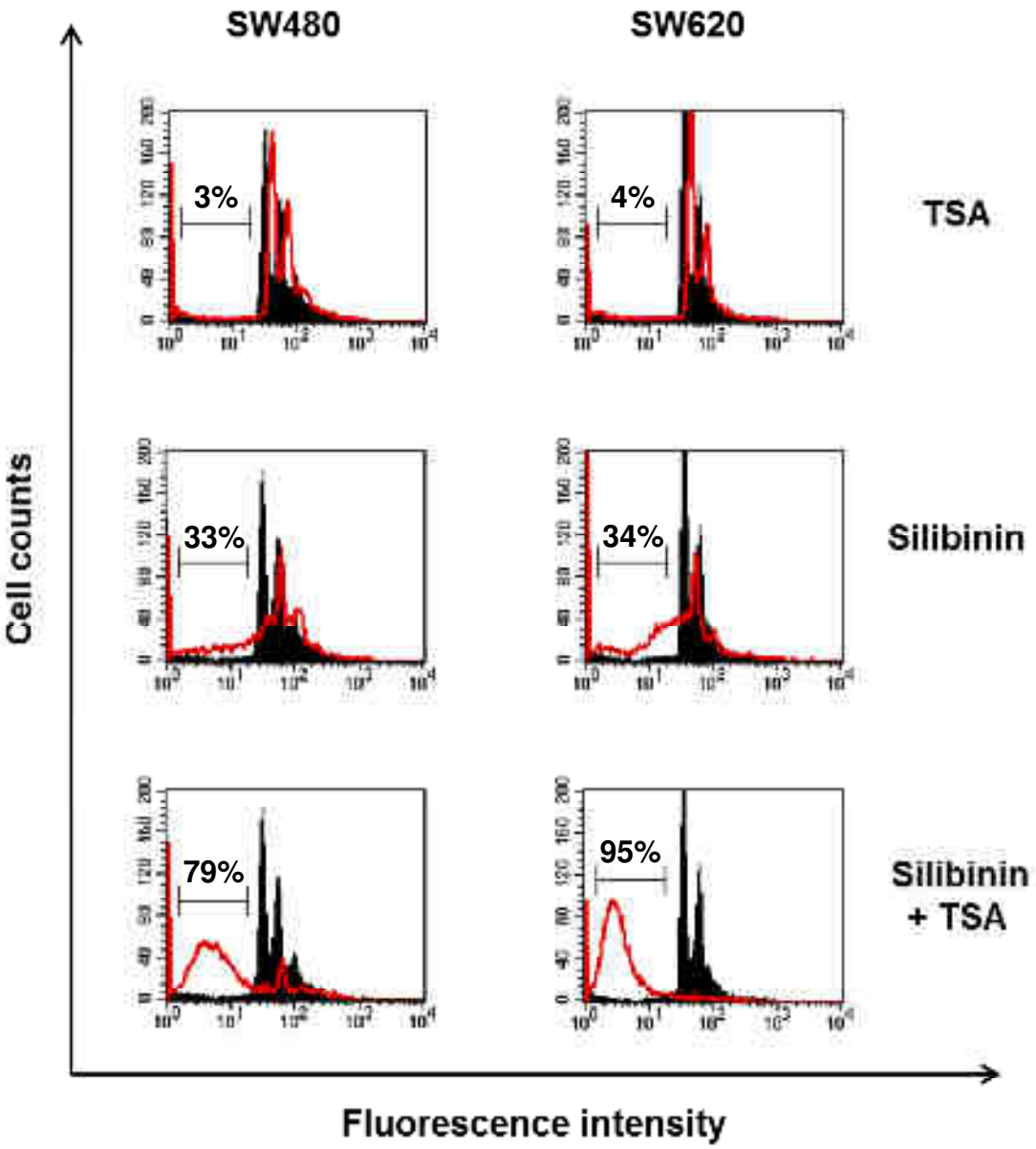


Figure 44: Cell death induced by silibinin and SAHA. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 µM) ± SAHA (1 µM) for 24, 48 or 72 h. At each time point, SW480 and SW620 cells were harvested and stained with propidium iodide for the measurement of hypodiploïd bodies and analyzed by flow cytometry as detailed in Materials and Methods. Data are the mean value ± SE of three separate experiments. For each cell line: columns not sharing the same superscript differ significantly: *P < 0.05.

A)


SW480
SW620
3%
4%
TSA
33%
34%
Silibinin
79%
95%
Silibinin
+ TSA
Cell counts
Fluorescence intensity

B)

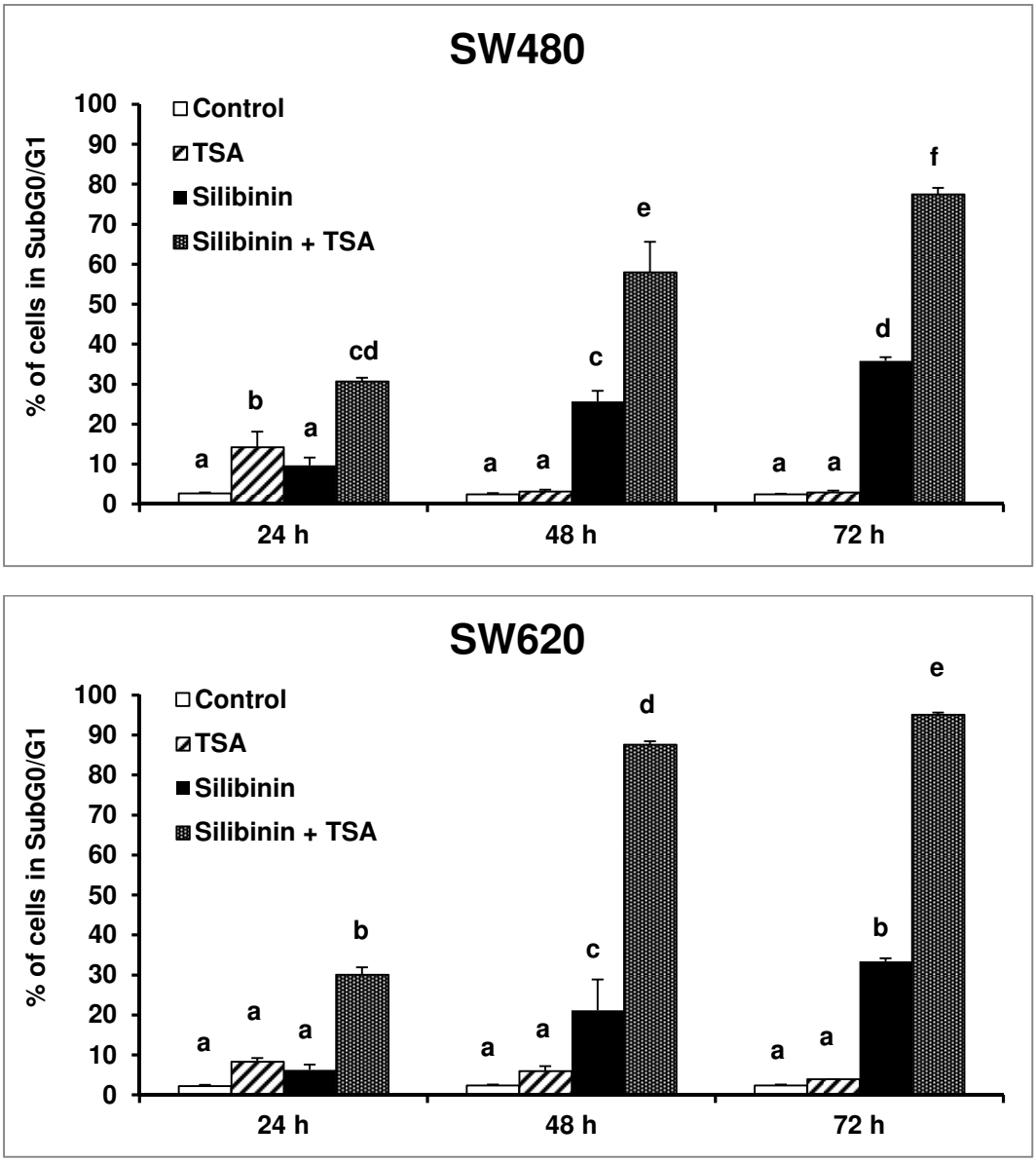


Figure 45: Cell death induced by silibinin and TSA. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 µM) ± TSA (0.1 µM) for 24, 48 or 72 h. At each time point, SW480 and SW620 cells were harvested and stained with propidium iodide for the measurement of hypodiploïd bodies and analyzed by flow cytometry as detailed in Materials and Methods. (A) shows representative FACS histograms at 72 h; the black line represents the DMSO-only treated cells while the red line represents the cells treated respectively. The percentage of cells in the subG0/G1 region is indicated. In (B), data are the mean value ± SE of three separate experiments. For each cell line: columns not sharing the same superscript differ significantly: *P < 0.05.

4. Conclusion

In chapter I, we have shown that silibinin inhibited cell growth and induced apoptosis in SW480 and SW620 cells. Polyphenols have been shown to modify histone deacetylation and DNA hypermethylation accompanying their chemopreventive effect. Here, we studied the effects of silibinin on epigenetic signaling.

We found that silibinin did not change HDAC activity, but suppressed DNMT activity in both cell lines after 72 h of treatment. Inhibition of DNMT activity was significant already at 48 h for SW480 cells.

As other polyphenols and DNMT inhibitors have been shown to act synergistically with HDAC inhibitors in cell death induction, we tested the effect of the combination of silibinin and two broad spectrum HDAC inhibitors, SAHA and TSA, on the two cell lines. Both combinations synergistically induced cell death. However, DNMT inhibition by silibinin does not seem to be the only reason for the synergy because the increase of cell death took place before DNMT inhibition by silibinin in SW620 cells.

Combination therapy by silibinin and HDAC inhibitors seems promising, given the non-toxic nature of silibinin and the fact that HDAC inhibitors selectively target cancer cells.

Next, we aimed at exploring a potential synergy of silibinin and TRAIL because of the silibinin-induced upregulation of the death receptors.

CHAPTER III:
Silibinin potentiates TRAIL-induced apoptosis in human colon adenocarcinoma and in their derived TRAIL-resistant metastatic cells

1. Abstract

Previously we showed that silibinin, the major active component of the milk thistle plant (*Silybum marianum*), possesses anti-neoplastic properties in SW480 and SW620 cells.

TNF-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent which selectively induces apoptosis in cancer cells. However, resistance to TRAIL-induced apoptosis is an important and frequent problem in cancer treatment.

In this chapter, we investigated the effect of silibinin and TRAIL on the primary colon tumor cells SW480 and on their derived TRAIL-resistant metastatic cells SW620. We showed that silibinin and TRAIL synergistically induced cell death in the two cell lines. Synergistic activation of caspase-3, -8, and -9 by silibinin and TRAIL was shown. When caspase inhibitors were used, cell death was blocked, showing the caspase-dependent apoptotic nature of cell death.

As a possible mechanism of silibinin-induced sensitization to TRAIL, upregulation of death receptor 4 (DR4) and DR5 by silibinin but not by TRAIL alone was observed at both mRNA and protein levels. Silibinin did not change expression levels of decoy receptors DcR1 and DcR2 on the cell surface. Human recombinant DR5/Fc and DR4/Fc chimera protein - which have a dominant-negative effect by competing with the endogenous receptors - abrogated cell death induced by silibinin and TRAIL with higher efficacy of the DR5/Fc chimera protein, demonstrating the importance of DR5 in the synergistic activation of cell death and the activation of the extrinsic pathway. Furthermore, silibinin and TRAIL potentiated and accelerated the activation of the mitochondrial apoptotic pathway. They downregulated the anti-apoptotic proteins Mcl-1 and XIAP. Downregulation of Mcl-1 and XIAP took place on the post-translational level. The involvement of XIAP in the sensitization of the two cell lines to TRAIL was demonstrated by using the XIAP inhibitor embelin. Sensitization was independent of glycosylation and lipid raft formation as inhibitors of glycosylation and lipid raft formation did not decrease cell death.

These findings demonstrate the synergistic action of silibinin and TRAIL, suggesting chemopreventive and therapeutic potential which should be further explored.

2. Introduction

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) is a member of the tumor necrosis factor (TNF) superfamily and known to induce apoptosis selectively in cancer cells without significant toxicity toward normal cells [35]. Therefore, TRAIL represents a promising and safe candidate for cancer prevention and treatment and is currently evaluated in clinical studies. TRAIL binds to its two agonistic cell surface death receptors 4 (DR4/TRAIL-R1) and 5 (DR5/TRAIL-R2), which induce cell death signaling by recruiting pro-caspase-8 to activate the extrinsic/death receptor apoptotic pathway. TRAIL can also bind to the two decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4) which have an antagonistic function because they lack the death domain to transmit the apoptotic signal [237]. TRAIL-induced cell death can also include the activation of the intrinsic/mitochondrial pathway through a caspase-8-mediated cleavage of Bid protein, leading to the permeabilization of the mitochondria and the activation of caspase-9. Such an activation of the intrinsic pathway may either represent an amplification loop for cell death or is even a necessary condition for it [43].

It is noteworthy that many types of cancer cells show resistance to TRAIL-induced apoptosis or can easily become resistant through survival mechanisms [238, 239]. Therefore, the identification of effective sensitizers able to overcome TRAIL resistance represents a valuable strategy to establish TRAIL-based cancer therapies. Recent work has shown that many flavonoids are able to restore TRAIL sensitivity of TRAIL-resistant cancer cells by various sensitizing mechanisms like upregulation of the agonistic TRAIL receptors DR4 and DR5, downregulation of anti-apoptotic proteins of the family of inhibitors of apoptosis (IAP) and/or Bcl-2 family or facilitation of TRAIL receptor aggregation [240, 43, 241]. Anti-apoptotic Bcl-2 family proteins such as Bcl-2 and Mcl-1 are able to inhibit apoptosis by inactivation of the mitochondrial pathway [242]. Members of the IAP family including XIAP, cIAP-1/2, and survivin can block apoptosis by inhibiting caspase activity [238].

The combination treatment of TRAIL and silibinin may be especially promising for cancer therapy as both compounds alone show efficiency and are proven to be non-toxic [243, 95, 244]. However, until now, only one study has reported that silibinin was able to sensitize human glioma cells to TRAIL-mediated apoptosis via DR5 upregulation and downregulation of the anti-apoptotic proteins c-FLIP and survivin [245].

In this chapter, we investigated whether silibinin could potentiate TRAIL-induced apoptosis in human colon adenocarcinoma SW480 cells and sensitize their derived metastatic TRAIL-resistant SW620 cells to TRAIL-induced apoptosis.

3. Results: Publication

The flavonolignan silibinin potentiates TRAIL-induced apoptosis in human colon adenocarcinoma and in derived TRAIL-resistant metastatic cells.

H Kauntz, S Bousserouel, F Gossé, F Raul.

Apoptosis 2012; 17:797-809

Link to the editor's website:

http://www.springerlink.com/content/p7265381572r8607/

4. Supplementary Results

4.1. Role of NFκB activation in silibinin/TRAIL-induced cell death

In chapter I, we showed that silibinin had no influence on nuclear translocation of NFκB p65. Here, we wanted to study effects of the combination of silibinin and TRAIL on NFκB activation and the potential effect of NFκB inhibition on silibinin/TRAIL-induced cell death.

NFκB activation in response to TRAIL has been frequently reported [246]. It is mediated by a secondary complex containing FADD and caspase-8 [247]. The biological significance of TRAIL-induced NFκB activation has not yet been fully elucidated but may play a role in resistance to TRAIL-induced apoptosis. As some substances which are able to sensitize cancer cells to TRAIL have been shown to exert their effect by the abrogation of NFκB activity [248, 249], and as silibinin is generally known for inhibiting elevated NFκB activity [84], it seemed possible that silibinin might act in a similar way in CRC cells.

We isolated nuclear extracts of cells after 24 h of treatment by silibinin ± TRAIL and measured their NFκB p65 content by ELISA. Furthermore, we inhibited NFκB signaling by a quinazoline compound and quantified cell death by the propidium iodide staining method.

As expected, TRAIL increased nuclear content of p65 in the two cell lines (Figure 46), even more so in SW480 cells which may be explained by their higher expression of TRAIL receptors. Contrary to our expectations, the addition of silibinin raised NFκB p65 content in the nucleus more than TRAIL alone and thus seemed to promote NFκB signaling. However, NFκB activation did not seem to matter for cell death induction as the inhibitor did not significantly change cell death in silibinin/TRAIL-treated cells (Figure 47).

NFκB activation is a double-edged sword in cancer: besides its well-studied anti-apoptotic effects, nuclear translocation of p65 can also mediate pro-apoptotic activity [71]. Cytotoxic stimuli have been shown to repress anti-apoptotic gene expression by NFκB p65 translocation to the nucleus, showing that p65 can be both activator and inhibitor of its target genes such as XIAP, Bcl-2 and Bcl-xL [71].

Thus silibinin/TRAIL-induced activation of NFκB may have led to the observed diminution of expression of XIAP. However, if this was the case, as NFκB inhibition did not change cell death, two conclusions are possible: either XIAP reduction was not necessary for cell death induction or SW480 and SW620 cells have multiple ways of downregulating XIAP.

We further investigated the downregulation of XIAP by measuring XIAP mRNA expression by qRT-PCR after treatment with silibinin/TRAIL as we hypothesized that this activation of NFκB signaling might have led to the repression of XIAP expression only observed with silibinin/TRAIL combination. We observed no downregulation of XIAP on the mRNA level (data not shown). Thus we conclude that silibinin/TRAIL-induced down-regulation of XIAP on the protein level did not take place on the transcription level and seems to be independent of NFκB signaling.

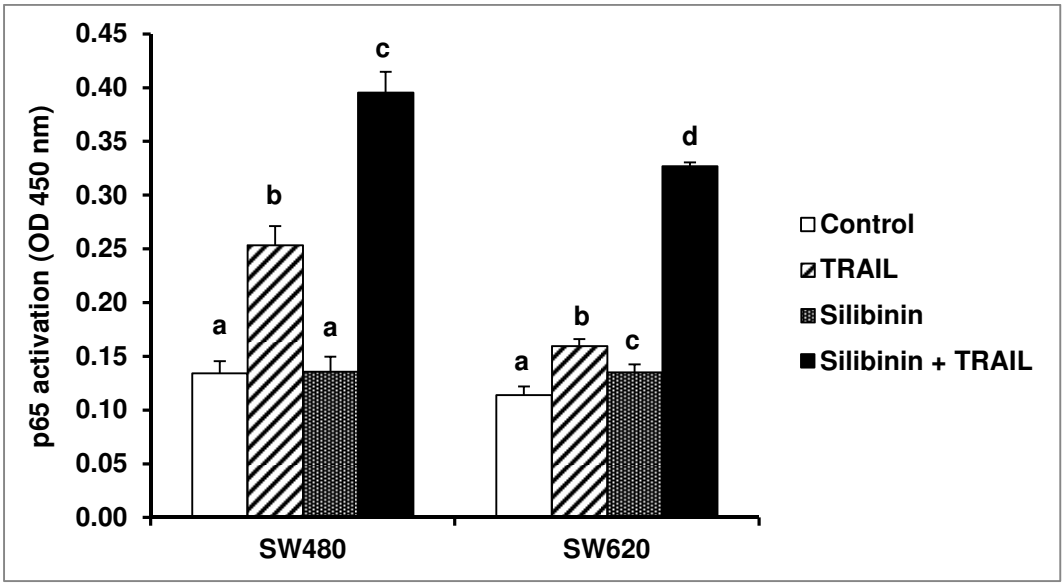


Figure 46: Activation of NFκB after treatment with silibinin and TRAIL. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 µM) ± TRAIL (50 ng/mL) for 24 h. SW480 and SW620 cells were harvested and nuclear extractions were performed. NFκB p65 in the nucleus was quantified in 12 µg nuclear protein by ELISA as detailed in Materials and Methods. Data are presented as mean ± SE of three separate experiments. For each cell line, columns not sharing the same superscript differ significantly: *P < 0.05.

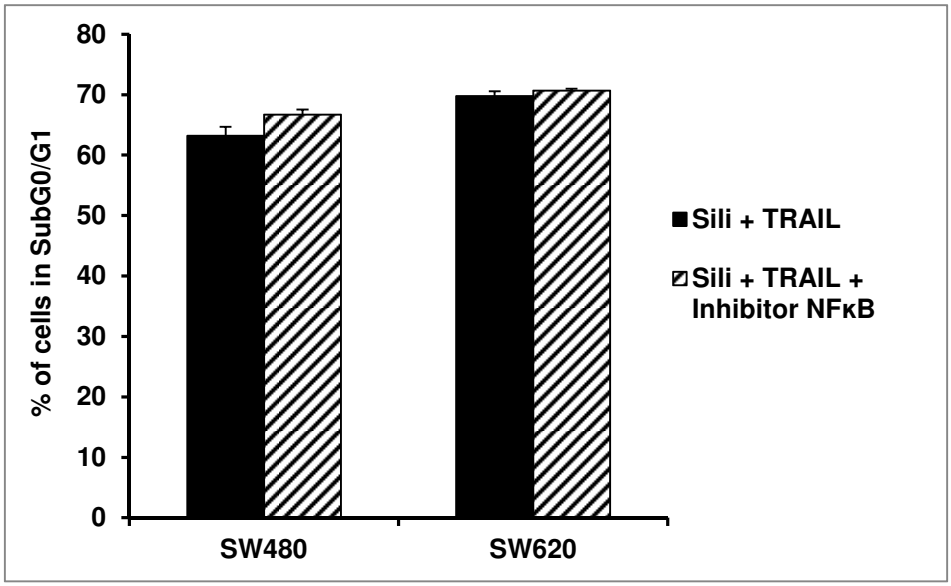


Figure 47: Effect of NFκB inhibition on silibinin/TRAIL-induced cell death. SW480 and SW620 cells were pre-treated for 30 min with an inhibitor of NFκB, 6-Amino-4-(4-phenoxyphenylethylamino)quinazoline (3 µM), and then treated with DMSO 0.1% ± silibinin (300 µM) ± TRAIL (50 ng/mL) for 24 h. SW480 and SW620 cells were harvested and stained with propidium iodide for the measurement of hypodiploïd bodies and analyzed by flow cytometry as detailed in Materials and Methods. Data are presented as mean ± SE of three separate experiments. For each cell line, silibinin +TRAIL + inhibitor NFκB treatment versus silibinin+TRAIL treated control: *P < 0.05.

4.2. Silibinin-induced sensitization to TRAIL is decoy receptor-independent

Not only the expression of the two agonist TRAIL death receptors DR4 and DR5 influences the TRAIL sensitivity of cancer cells, but also that of the two related decoy receptors, DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4) which abolish death signal transduction. They cannot transduce apoptotic signals because they lack the functional death domain [239]. As they compete with the functional death receptors for TRAIL, the ratio of DcRs to DRs considerably influences sensitivity to TRAIL [239].

The polyphenol resveratrol, e.g. has been shown to downregulate the cell-surface expression of DcR1 in human leukemia cells [250]. After our observation of the silibinin-induced increase of agonistic death receptor expression, we were interested in the effect of silibinin on TRAIL decoy receptors. To measure the cell-surface expression of DcR1 and DcR2, we used FITC-conjugated antibodies and performed analysis by flow cytometry.

We observed that both of the decoy receptors were expressed in the untreated two cell lines to a similar extent (Figure 48). Thus the decoy receptors do not seem to play a role in the TRAIL resistance of SW620 cells. In silibinin-treated cells, the results in the two cell lines were also the same: We observed a minimal increase of DcR1 expression and no change of DcR2 expression.

Our results suggest that the downregulation of the TRAIL decoy receptors is not part of the mechanisms of silibinin to sensitize the two cell lines to TRAIL. On the other hand, silibinin does change the ratio between the agonist and the antagonist TRAIL death receptors by increasing DR4 and DR5 expression which is a way of sensitizing cancer cells to TRAIL.

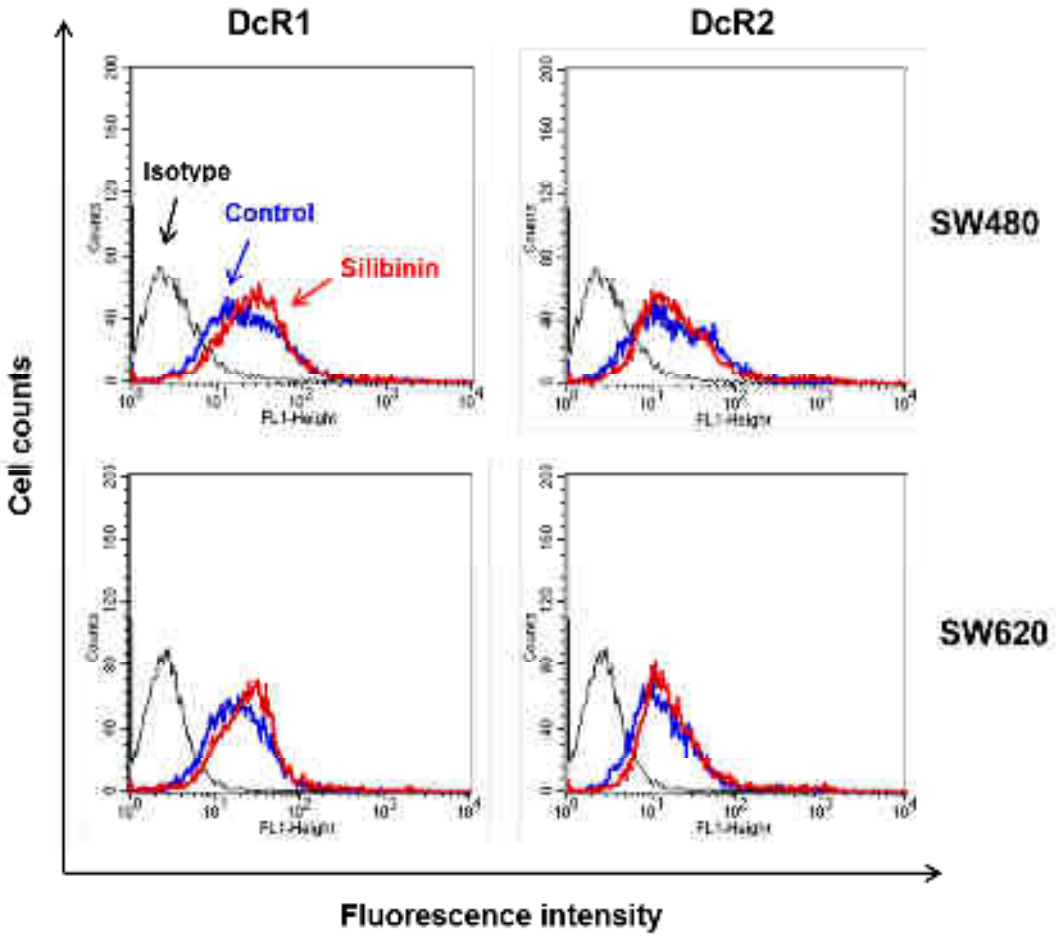


Figure 48: Effect of silibinin on DcR1/2 expression in SW480 and SW620 cells. SW480 and SW620 cells were treated with DMSO 0.1% ± silibinin (300 µM) for 24 h. The expression of TRAIL decoy receptors was analyzed by flow cytometry after staining with respective FITC-conjugated antibodies. A shift to the right of fluorescence intensity corresponds to an increase of TRAIL decoy receptor expression. Data are presented as cytometer histogram plots which are representative of at least three different experiments.

4.3. Silibinin-induced sensitization to TRAIL is glycosylation-independent

It has been shown that post-translational modifications of DR4 and DR5 such as glycosylation or palmitoylation are important modulators of sensitivity to TRAIL [35]. Elevated expression of the O-glycosyltransferase GALNT3 as well as the O-glycan processing enzymes FUT3 and FUT6 correlated with responsiveness to TRAIL in CRC cell lines [251]. The mechanism by which O-glycosylation of TRAIL receptors promotes sensitivity to TRAIL is the ameliorated ligand-induced clustering of DR4 and DR5 with more efficient DISC assembly and caspase-8 activation [251].

To evaluate a possible role of O-glycosylation in silibinin-induced sensitization to TRAIL, we used benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (benzyl-α-GalNAc) which is an inhibitor of O-linked glycosylation. We pre-incubated SW480 and SW620 cells with benzyl-α-GalNAc to inhibit O-linked glycosylation, then added silibinin and TRAIL. After 24 hours, we measured cell death by propidium iodide staining and flow cytometric analysis.

As shown in Figure 49, the inhibition of glycosylation did not significantly change cell death rates induced by the combination silibinin/TRAIL.

These data suggest that silibinin exerted its sensitization effect by different pathways such as increasing death receptor expression on the cell surface or downregulation of anti-apoptotic proteins as we have shown in the preceding publication.

However, it is possible that palmitoylation played a role in this context, as it has also been shown to be implicated in the regulation of the activation of the extrinsic apoptotic pathway and to be required for trimerization of DR4 [252].

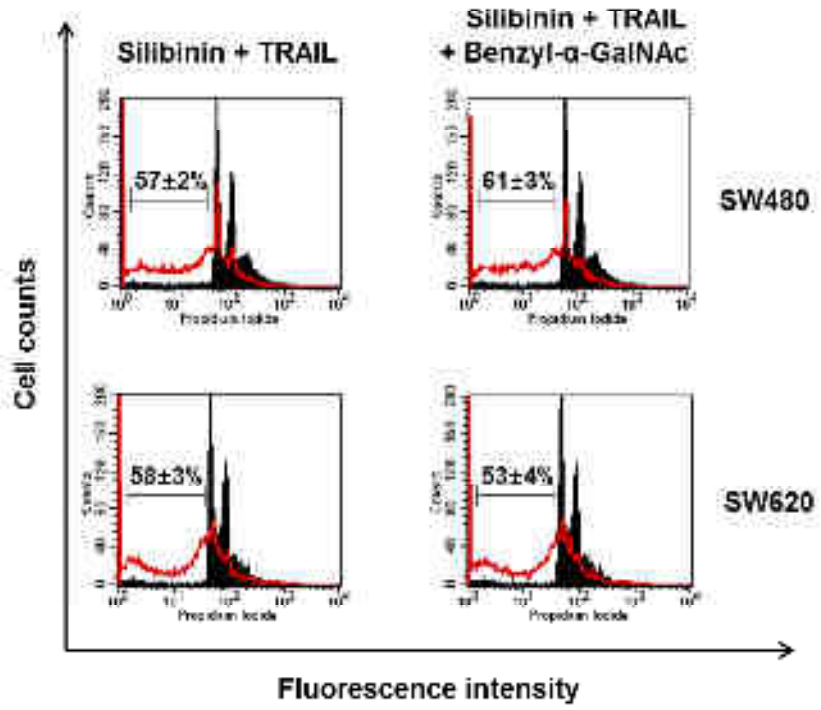


Figure 49: Effect of inhibition of glycosylation in silibinin/TRAIL-treated cells. SW480 and SW620 cells were pre-treated for 6 h with benzyl-α-GalNAc (0.8 mM) and then treated with DMSO 0.1% ± silibinin (300 µM) ± TRAIL (50 ng/mL) for 24 h. SW480 and SW620 cells were harvested and stained with propidium iodide for the measurement of hypodiploïd bodies and analyzed by flow cytometry as detailed in Materials and Methods. Data are presented as cytometer histogram plots which are representative of at least three different experiments with mean ± SE of three separate experiments.

4.4. Role of lipid rafts in silibinin/TRAIL-induced cell death

Lipid rafts are cholesterol- and sphingolipid-enriched microdomains in the plasma membrane able to exert various cellular functions. Their formation has been known to be implicated in death receptor-mediated cell death by clustering the death receptors on the cell surface [239]. This aggregation of death receptors in lipid rafts facilitates their TRAIL-induced activation, as TRAIL binds to pre-clustered receptor trimers. Consequently, the sensitivity of cancer cells to TRAIL is enhanced [239].

Some polyphenols have been shown to interact with lipid raft formation and to sensitize cancer cells to TRAIL: Epigallocatechin-3-gallate induced apoptosis by lipid raft clustering [253], quercetin enhanced TRAIL-induced apoptosis by redistribution of DR4 and DR5 into lipid rafts [254], and redistribution of Fas in lipid rafts of SW480 cells was involved in resveratrol-induced apoptosis [255]. Furthermore, resveratrol induced redistribution of death receptors into lipid rafts thus sensitizing the cells to death receptor-mediated apoptosis, even cells which were resistant to resveratrol-induced apoptosis [256].

The cholesterol-sequestering agent nystatin depletes cholesterol from lipid rafts thus leading to their disruption [257]. Hence, nystatin prevents redistribution of death receptors in lipid rafts and accordingly was shown to efficiently prevent quercetin-induced sensitization to TRAIL and resveratrol-induced sensitization to death receptor stimulation [254, 256].

We wondered if silibinin-induced sensitization to TRAIL was mediated by the redistribution of death receptors in lipid rafts. We inhibited lipid raft formation by pre-treatment with nystatin, and then used propidium iodide staining to analyze silibinin/TRAIL-induced cell death by flow cytometry.

We found that nystatin treatment did not significantly change cell death induced by silibinin/TRAIL (Figure 50). Thus the sensitizing effect of silibinin does not seem to have been caused by a redistribution of TRAIL death receptors in lipid microdomains.

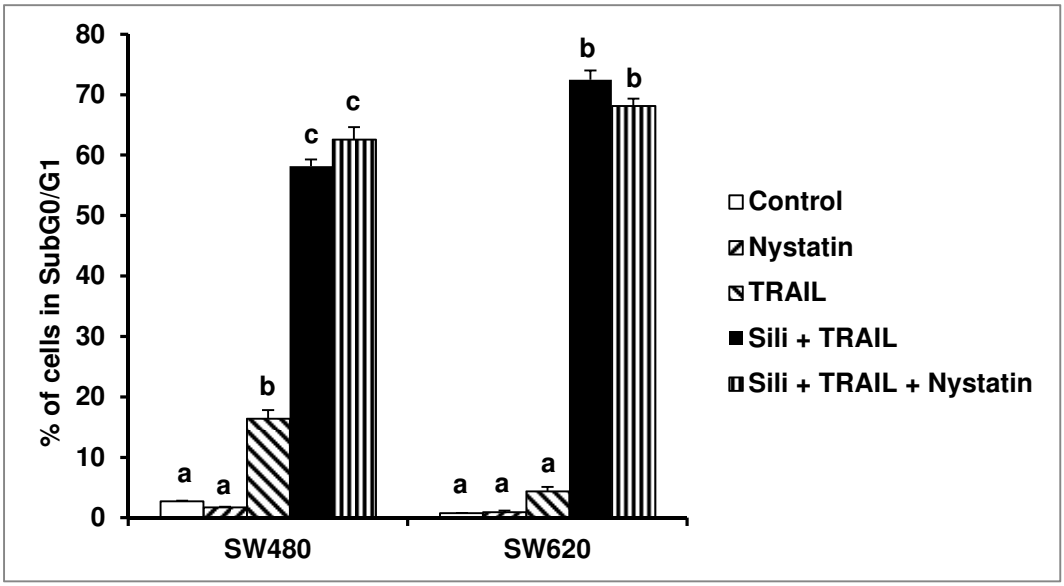


Figure 50: Effect of nystatin on silibinin/TRAIL-induced cell death. SW480 and SW620 cells were pre-treated for 30 min with nystatin and then treated with DMSO 0.1% ± silibinin (300 µM) ± TRAIL (50 ng/mL) for 24 h. SW480 and SW620 cells were harvested and stained with propidium iodide for the measurement of hypodiploïd bodies and analyzed by flow cytometry as detailed in Materials and Methods. Data are presented as mean ± SE of three separate experiments. For each cell line: Columns not sharing the same superscript differ significantly: *$P < 0.05$

Moreover, nystatin did not inhibit cell death induced by silibinin alone but rather showed a tendency - though not significant - to augment cell death (data not shown). These results are in agreement with data previously reported in our laboratory showing that nystatin did not inhibit but increase apoptosis induced by apple procyanidins in SW480 cells [258].

5. Conclusion

In the first chapter, we showed that silibinin was able to upregulate TRAIL death receptors DR4 and DR5. Therefore we studied the effects of the combination of silibinin and TRAIL on SW480 and the TRAIL-resistant SW620 cells.

Silibinin and TRAIL were able to induce synergistic and accelerated cell death with caspase-3 activation in both cell lines, involving synergistic activation of the extrinsic (activation of caspase-8) and the intrinsic pathway (perturbation of the mitochondrial membrane potential, activation of caspase-9). Cell death was utterly caspase-dependent, as shown by the complete inhibition of cell death by the pan-caspase-inhibitor Z-VAD-FMK.

The importance of upregulated DR4 and DR5 was shown by human recombinant DR4/Fc and DR5/Fc chimera which were able to block silibinin/TRAIL-induced cell death, in which DR5/Fc chimera proved to be more effective. Other factors involved in the sensitization of SW480 and SW620 cells to TRAIL were the downregulation of Mcl-1 and XIAP which took place on the post-translational level, there being no decrease on the transcriptional level. We proved the importance of downregulation of XIAP for sensitization to TRAIL by treatment with embelin, a small-molecule inhibitor of XIAP, which was also able to sensitize SW480 and SW620 cells to TRAIL.

Decoy receptor levels, glycosylation and lipid raft formation were not involved in silibinin-induced sensitization to TRAIL. Surprisingly, NFκB p65 nuclear translocation was observed in silibinin/TRAIL-treated cells but this activation did not have any influence on cell death as inhibition of NFκB did not change silibinin/TRAIL-induced cell death rates.

Taken together, our data support the use of silibinin in combination with TRAIL or TRAIL agonists in further studies of CRC and derived metastasis because of their non-toxic, synergistic, multi-targeted and p53-independent action in inducing cell death.

After these promising results *in vitro* of the three past chapters we aimed at a validation of our results in *in vivo* models which will be the subject of the next two chapters.

CHAPTER IV:
Silibinin modulates the early expression of chemopreventive biomarkers in a preclinical model of colon carcinogenesis

1. Abstract

Previously we showed that the flavonolignan silibinin possesses anticancer properties in primary adenocarcinoma SW480 cells and in their derived metastatic SW620 cells. Here, we investigated the potential of silibinin as a chemopreventive agent in colon carcinogenesis *in vivo*. The rat azoxymethane (AOM)-induced colon carcinogenesis model was used because of its molecular and clinical similarities to sporadic human colorectal cancer. One week after AOM injection (post-initiation), Wistar rats received daily intragastric feeding of 300 mg silibinin/kg body weight until their sacrifice after 7 weeks of treatment.

Silibinin-treated rats exhibited a 2-fold reduction in the number of AOM-induced hyperproliferative crypts and aberrant crypt foci (ACF) in the colon compared to AOM-injected control rats receiving the vehicle. As *in vitro*, silibinin induced apoptosis in the colon mucosal cells evidenced by flow cytometry after propodium iodide staining and by colorimetric measurement of caspase-3 activity. Mechanisms involved in silibinin-induced apoptosis included the downregulation of the anti-apoptotic protein Bcl-2 and upregulation of the pro-apoptotic protein Bax, inverting the Bcl-2/Bax ratio to < 1 when compared to non-treated AOM-injected rats. This modulation already takes place at the mRNA expression level as shown by real-time RT-PCR. Furthermore, silibinin treatment significantly decreased the genetic expression of biomarkers of the inflammatory response such as IL-1β, TNF-α and their downstream target MMP-7, all of them shown to be upregulated during colon carcinogenesis. The downregulation of MMP-7 protein was confirmed by western blot.

The present findings show the ability of silibinin to favorably shift the disturbed balance between cell renewal and cell death in colon carcinogenesis in rats previously injected with the carcinogen AOM. Silibinin administered via intragastric feeding exhibited potent pro-apoptotic, anti-inflammatory and multi-targeted effects at the molecular level. The effective reduction of preneoplastic lesions by silibinin supports its use as a natural agent for colon cancer chemoprevention.

2. Introduction

Colorectal carcinogenesis offers a large window spanning decades from cancer initiation to diagnosis making this disease suitable for chemopreventive approaches [259].

Reliable preclinical models allow evaluating the impact of potential chemopreventive agents on the carcinogenic process. The AOM-induced colon carcinogenesis rat model is frequently used for chemoprevention studies in colorectal cancer (CRC) [21]. AOM induces the transformation of normal colon epithelium into carcinoma via the multistage process of colon carcinogenesis. The resulting tumors show histopathological and genetical characteristics similar to sporadic colon tumors in humans [260]. Thus the earliest identifiable preneoplastic lesions during colon carcinogenesis, ACF, appear likewise in rodents and humans [261]. ACF have been shown to be valuable early biomarkers for the identification of chemopreventive agents against CRC and to allow a quantitative approach to assess the disease process [261, 262].

The efficacy of silibinin has been shown in the AOM-induced rat model: inhibition of AOM-induced ACF in male F344 rats was shown by Velmurugan *et al*. [152] and inhibition of DMH-induced ACF in male Wistar rats by Sangeetha *et al*. [153]. Silibinin-induced apoptosis has been observed in the AOM-induced rat model [152], in AOM-induced A/J mice [158], in the APC min/+ model [154, 157], and in HT29 [137], LoVo [123] and SW480 xenografts [159] in athymic nude mice. However, none of these studies investigated mechanisms of apoptosis; they mostly proved apoptosis by TUNEL assay, cleaved PARP or caspase-3.

In these studies, silibinin-induced decrease of inflammation-related enzymes such as COX-2 or iNOS was frequently observed [137, 152, 154, 157–159], but no further investigations concerning inflammation were undertaken. Thus studies about silibinin-induced mechanisms of apoptosis in CRC or silibinin-induced changes in other inflammatory response markers are lacking; and the efficacy of silibinin against CRC development in the AOM-induced rat model remains little understood.

We selected IL-1β, TNF-α and their downstream target MMP-7 as biomarkers for the inflammatory response all of which we had identified before as being upregulated in colon mucosa cells in the early post-initiation stage of tumor progression after AOM injection [263].

Matrix metalloproteinases (MMPs) are implicated in carcinogenesis not only by the degradation of extracellular matrix and basement membranes in tumor invasion and

metastasis but they are also involved in the early stages of CRC by interacting with various non-matrix proteins [264]. Especially MMP-7 (matrilysin) has been shown to play a role in colorectal tumorigenesis as it is one of the target genes of the β-catenin complex [264]. Its transcription is promoted by pro-inflammatory cytokines such as IL-1β and TNF-α [265]. Furthermore, high levels of circulating pro-inflammatory cytokines in plasma are associated with CRC in humans [266].

To study apoptotic mechanisms in detail we selected Bcl-2 and Bax which regulate apoptosis on the level of the mitochondria and the ratio of which is equally altered in colon carcinogenesis [263].

We aimed at determining the effects of dietary feeding of silibinin on the development of AOM-induced ACF formation, and on the expression of several gene and protein biomarkers involved in the inflammatory and apoptotic responses in the early post-initiation phases of colon carcinogenesis.

3. Results: Publication

Silibinin, a natural flavonoid, modulates the early expression of chemopreventive biomarkers in a preclinical model of colon carcinogenesis.

H Kauntz, S Bousserouel, F Gossé, J Marescaux, F Raul

International Journal of Oncology 2012; 41: 849-854

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http://www.spandidos-publications.com/10.3892/ijo.2012.1526

4. Conclusion

In the first chapter we showed the ability of silibinin to induce apoptosis in human colon adenocarcinoma cells SW480 and in their derived metastatic cells SW620. Here, we studied the potential chemopreventive effects of silibinin in an established *in vivo* model of colon carcinogenesis, the AOM-induced rat model.

We showed that silibinin-triggered apoptosis may at least in part be responsible for its overall efficacy in inhibiting AOM-induced ACF formation in the colon of the rats. At the molecular level, this effect was associated with silibinin-induced multi-targeted changes in the expression of apoptosis- and inflammatory-regulatory proteins and genes.

As to its pro-apoptotic effects, silibinin was able to change the Bcl-2/Bax ratio at the protein and the gene expression level. This ratio can be used as an indicator of chemopreventive efficacy which our laboratory previously reported for other pro-apoptotic drugs [267, 268].

Concerning inflammation, silibinin inhibited gene expression of pro-inflammatory cytokines such as TNF-α and IL-1β and decreased their downstream target MMP-7 which is involved in the early stages of colorectal tumor progression [264].

We report that intragastric feeding of silibinin to AOM-injected rats exerted various anti-carcinogenic and protective effects on the colonic mucosa at the early post-initiation stage. Silibinin treatment reduced AOM-induced crypt cell hyperproliferation and ACF formation, indicating the ability of silibinin to promote normal cellular homeostasis and thus its suitability to be used as a chemopreventive agent in CRC.

However, CRC is a disease which is especially lethal once it has metastasized [269]. The most common metastatic site is the liver. As silibinin and its parental extract silymarin have been used for hepatoprotective purposes for a long time, we were interested in potential anticancer effects of silibinin on liver cancer which we studied in a murine orthotopic hepatocarcinoma model, presented in the next chapter.

CHAPTER V:
Silibinin inhibits tumor growth in a murine orthotopic hepatocarcinoma model and activates the TRAIL apoptotic signaling pathway

1. Abstract

In the previous chapters, we have shown that silibinin exerts chemopreventive action on CRC *in vitro* and *in vivo* and may be a promising agent for use in a combination therapy with TRAIL in CRC.

As silibinin has long been known for its hepatoprotective properties, evaluating the efficacy of silibinin in chemoprevention of liver cancer seems logical.

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and often is refractory to chemotherapy. In this chapter, we used hepatocarcinoma Hep-55.1C cells *in vitro* and an orthotopic hepatocarcinoma model in mice to investigate the molecular mechanisms of silibinin-induced anti-tumoral effects.

Silibinin induced apoptosis in Hep-55.1C cells as attested by the detection of the accumulation of hypodiploïd cells and activation of caspase-3. TRAIL alone induced only transient cell death in Hep-55.1C cells at 24 h, while cells showed resistance to TRAIL-induced cell death at 48 and 72 h. The combination silibinin/TRAIL induced synergistic cell death of similar efficacy as in CRC cells. Silibinin activated the extrinsic apoptotic pathway in Hep-55.1C cells as attested by the upregulation of TRAIL and DR5 gene expression and by the activation of caspase-8. After treatment with silibinin/TRAIL combination, the activation of caspase-8 and -3 was potentiated at 24 h; however, at 48 h caspase activation had completely disappeared, probably due to switching from apoptosis to necrosis after the violent induction of cell death. Mitochondrial membrane perturbation was induced by neither silibinin nor TRAIL alone, only by the silibinin/TRAIL combination.

Orthotopic grafting of Hep-55.1C cells was performed in the liver of C57BL/6J mice, and tumor growth was followed by micro-computed imaging. Oral administration of silibinin for 4 weeks caused a significant time-dependent reduction of tumor growth, associated with the downregulation of inflammatory mediators (MMP-7, MMP-9, IL-1β), the upregulation of the immunoregulatory IFN-γ, the activation of caspase-3 and the upregulation of apoptotic markers (TRAIL, DR5).

Silibinin treatment exerted anti-carcinogenic effects, including the activation of the TRAIL receptor apoptotic signaling pathway in both Hep-55.1C cells *in vitro* and in liver tumors of mice. Furthermore silibinin sensitized Hep-55.1C cells to TRAIL treatment and may thus be of interest for a combination therapy with TRAIL in liver cancer.

2. Introduction

The most frequent site of metastases from CRC is the liver [4]. Hepatic metastases are involved in 40-70% of metastatic CRC and developed by up to 50% of CRC patients [4, 269]. Prognosis is poor with a median survival time with untreated hepatic colorectal metastases of approximately 6 months [269].

HCC is the most common primary malignancy of the liver and the third cause of cancer death [270]. Potential curative treatments for HCC represent surgical resection, transplantation or ablation [270]. However, this is mainly an option in early stages of HCC [270] whereas later stages as well as colorectal liver metastases are often unresectable [269]. In addition, recurrence after resection occurs in up to 80% of the HCC patients after 5 years [270]. Chemoembolization and chemotherapy to avoid recurrence have been shown to be inefficient [271]. Thus effective adjuvant treatments to prevent recurrence are urgently needed [270].

For unresectable HCC, new approaches are also needed - although trans-arterial chemoembolization (TACE) has shown some benefits it may lead to liver failure in patients with a deteriorated liver function [270]. TACE involves the administration of a chemotherapeutic agent such as doxorubicin or cisplatin in an emulsion applied directly intra-arterially to liver tumors which is recommended for the treatment of inoperable HCC and is being explored for CRC liver metastases [272]. However, HCC is frequently resistant to conventional chemotherapeutic agents and studies have not yet reported significant improvement of overall survival [272, 273].

Although silibinin has been traditionally used for its hepatoprotective properties, there are not many studies of its effects on liver cancer. A study in the human hepatoma cells HepG2 has shown that silibinin inhibits the activity of cytochrome P450 isoform CYP1A1, which apparently plays a role in carcinogenesis [274]. In the same cells, silibinin inhibited cell growth and proliferation through inhibition of ERK1/2 phosphorylation, furthermore it inhibited MMP-2 [218]. A third study has shown that silibinin led to growth inhibition in 4 different human HCC cell lines and induced apoptosis in HuH7 cells which was associated with activated caspase-3 and -9 and with a decrease of MMP-2 levels [119]. *In vivo*, there has been only one study on silibinin in hepatic cancer. The data showed that silibinin reduced HuH7 xenograft growth by the inhibition of cell cycle progression and promoted apoptosis in HuH7 xenografts [236].

Since we showed the silibinin-induced inhibition of pro-inflammatory mediators and the activation of pro-apoptotic processes in the AOM rat model, we aimed at determining whether silibinin activated similar mechanisms in an orthotopic mouse model of liver cancer.

The model we used was a syngeneic transplantable tumor model where a mouse HCC cell line is implanted in mice of the strain from which the implant derives. In this setting, we implanted Hep-55.1C cells in the liver of C57BL/6J mice.

An advantage of this model over the subcutaneous implantation of human HCC in immune-compromised nude mice is that it mimics the real tumor microenvironment of the active host immune system [275]. Orthotopic compared to subcutaneous implantation has further advantages in mimicking HCC development: tumor cells are able to interact with organ-specific factors such as endothelial and inflammatory cells. Thus processes in local invasion such as angiogenesis can also be better evaluated [275].

Normally, a disadvantage of orthotopic HCC implants is related to a more difficult assessment of tumor growth and response. However, we were able to study tumor evolution non-invasively by microCT follow-up as described previously [187].

We also studied the effect of the combination of silibinin and TRAIL in Hep-55.1C cells to know if the observed synergy in CRC can be reproduced in hepatic cancer cells.

3. Results: Publication

Silibinin inhibits tumor growth in a murine orthotopic hepatocarcinoma model and activates the TRAIL apoptotic signaling pathway

S Bousserouel, G Bour, H Kauntz, F Gossé, J Marescaux, F Raul

Anticancer Research 2012; 32 2455-2462

Link to the editor's website:
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4. Supplementary Results

4.1. Silibinin and TRAIL induce synergistic cell death in Hep-55.1C cells

HCC is frequently resistant to conventional chemotherapy and radiotherapy and patients with impaired liver function suffer even more from the toxicity of most anticancer agents. Therefore molecular targeted therapies with tolerable toxicity are urgently needed [273]. TRAIL and its agonists represent a promising therapy, targeting receptor-mediated apoptosis and being non-toxic for non-cancerous cells. However, HCC cell lines are mostly resistant to TRAIL-induced apoptosis [276, 277].

To investigate if silibinin, a hepatoprotective agent, was able to overcome TRAIL-resistance in HCC cells we treated Hep-55.1C cells with silibinin and TRAIL and determined cell death by the PI-staining method using flow cytometry analysis.

We observed that Hep-55.1C cells were indeed resistant to TRAIL-induced apoptosis (Figure 51): while there was significant cell death after 24 h of TRAIL treatment, it completely disappeared from 48 h on. Cells were sensitive to silibinin-induced cell death as shown before, and silibinin was able to sensitize them to TRAIL-induced apoptosis: while cell death at 24 h with the single agents did not exceed 20%, there was a cell death rate of over 80% with silibinin/TRAIL. From 48 h on, the rate exceeded 90%.

These results show that silibinin-induced sensitization to TRAIL-induced apoptosis was not limited to colon adenocarcinoma and their derived metastatic cells and that silibinin was also able to sensitize resistant HCC cells.

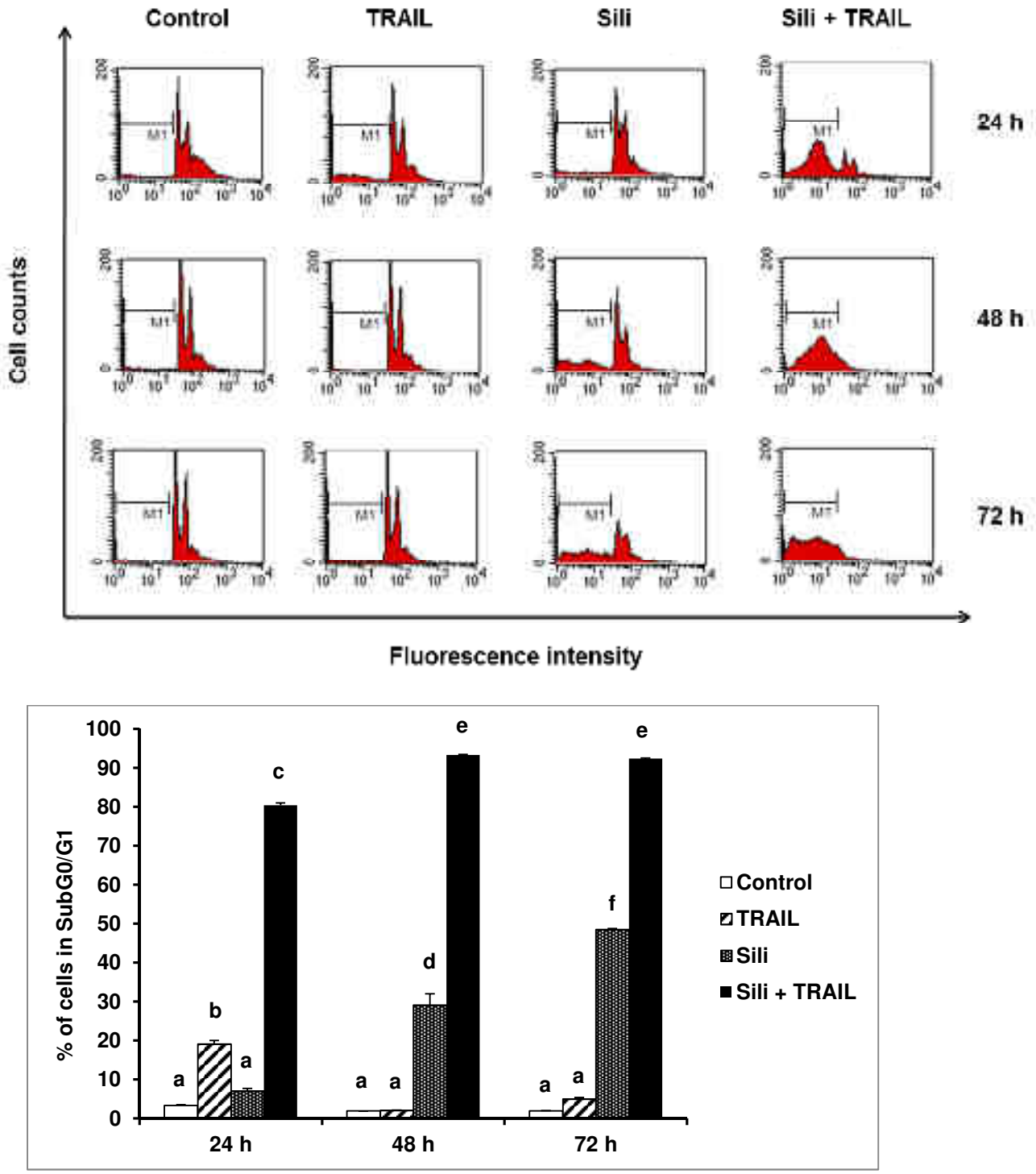

Figure 51: Cell death induced by silibinin and TRAIL. Hep-55.1C cells were treated with DMSO 0.1% ± silibinin (300 µM) ± TRAIL (50 ng/mL) for 24, 48 and 72 h. At each time point, cells were harvested and stained with propidium iodide for the measurement of hypodiploïd bodies and analyzed by flow cytometry as detailed in Materials and Methods. The upper panel shows representative FACS histograms, the lower panel displays the mean value ± SE of three separate experiments. Columns not sharing the same superscript differ significantly: *P < 0.05.

4.2. Silibinin and TRAIL induce synergistic activation of caspase-3 and -8

After the demonstration of synergistic cell death induction by the combination silibinin/TRAIL we wanted to know if the cell death was apoptotic. Therefore we studied the activation of caspase-3 which is considered a hallmark of caspase-dependent apoptosis. To verify if the activation of the extrinsic apoptotic pathway was potentiated in silibinin/TRAIL-induced cell death, we also determined the activation of caspase-8.

We determined the activities of caspase-3 and -8 by colorimetric assays measuring the amount of the colored reaction product (pNA) resulting from the cleavage of the specific substrates for caspase-3 (DEVD-pNA) and for caspase-8 (IETD-pNA). Our results show a synergistic activation of caspase-3 and -8 by silibinin/TRAIL after 24 h (Figure 52). However, after 48 h there was a complete loss of caspase activation by silibinin/TRAIL whereas caspase activation by silibinin alone was still on the rise.

These results are very similar to those that we obtained in SW480 and SW620 cells (see Chapter III): There we showed a synergistic activation of caspases-3, -8 and -9 by silibinin/TRAIL at 24 h. After 48 h of silibinin/TRAIL treatment, there was also a complete absence of caspase activation (data not shown).

A possible explanation for this absence of caspase activation is the ATP depletion of the cells. The extensive activation of caspases after 24 h in silibinin/TRAIL-treated cells is likely to consume much ATP. As neither apoptosis nor caspase activation can take place without sufficient ATP, the ATP depletion may stop the activation of caspases and cell death may switch to a caspase-independent form such as necrosis [278]. Nevertheless, it is also possible that silibinin/TRAIL-treated cells undergo secondary necrosis which follows the completed apoptotic program *in vitro* when apoptotic cells are not eliminated by phagocytosis by scavenger cells [279].

These results indicate that similar mechanisms of synergy as in CRC cells may be involved in hepatic cancer cells as the profiles of caspase activation are alike.

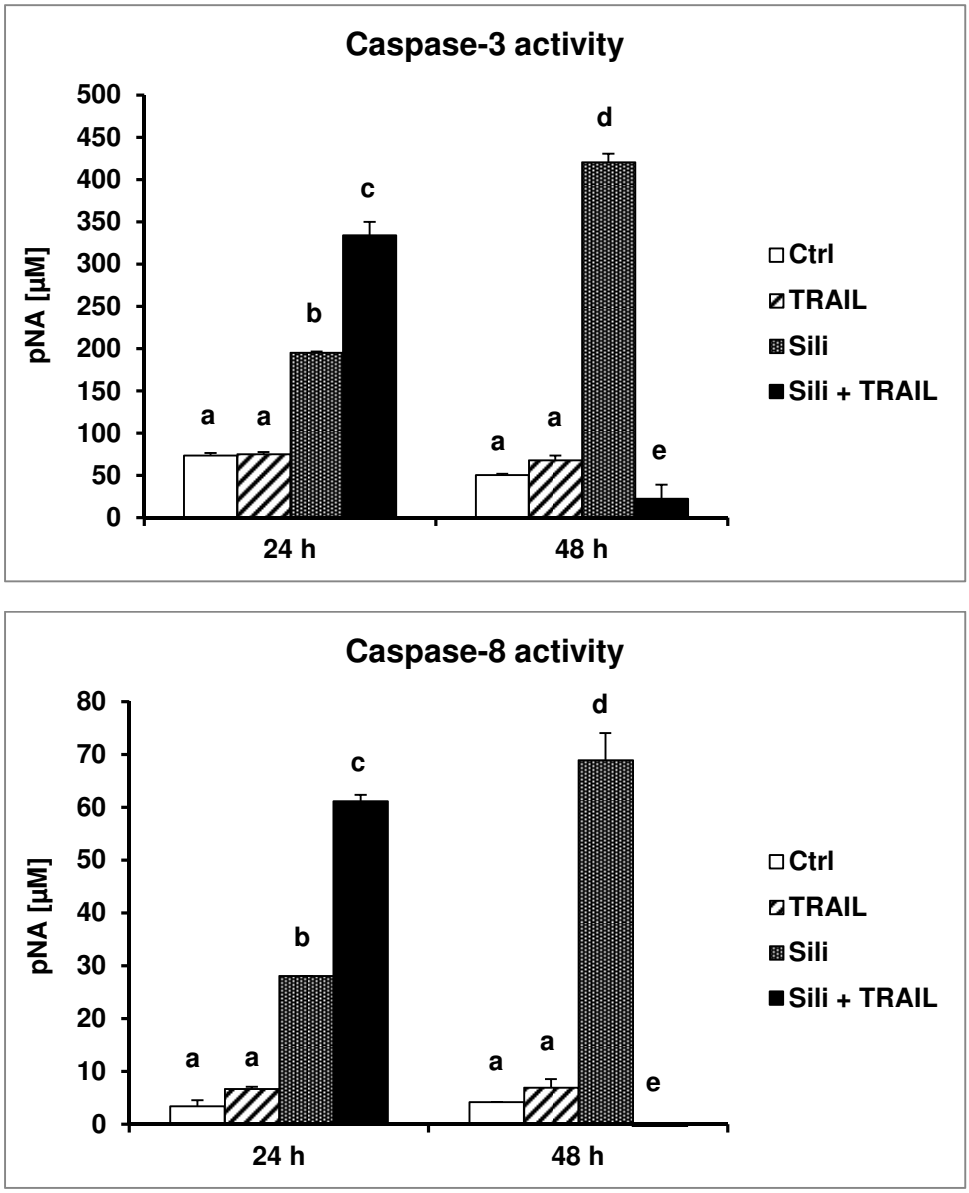

Figure 52: Caspase-3 and -8 activation by silibinin and TRAIL. Hep-55.1C cells were treated with DMSO 0.1% ± silibinin (300 µM) ± TRAIL (50 ng/mL) for 24 and 48 h. Caspase-3 and -8 activities were measured in cell lysates by a colorimetric test as detailed in Materials and Methods. Data are presented as mean ± SE of three separate experiments. Columns not sharing the same superscript differ significantly: *P < 0.05.

4.3. Silibinin and TRAIL induce synergistic perturbation of the mitochondrial membrane potential ($\Delta\Psi_m$)

To find out if the intrinsic mitochondrial pathway was also implicated in the synergistic cell death induction, we measured the perturbation of the mitochondrial membrane. To this end we assessed changes in $\Delta\Psi_m$ by flow cytometry after staining cells with the cyanine dye $DiOC_2(3)$.

While the profiles of TRAIL and silibinin-treated cells showed profiles such as the untreated control cells, cells treated by their combination emitted decreased fluorescence and showed profiles such as the positive control (Figure 53). This means that only the combination silibinin/TRAIL was able to perturbate mitochondria.

We conclude that the intrinsic pathway was implicated in silibinin/TRAIL-induced apoptosis and represented an amplification loop explaining higher death rates in comparison to cell death induction by silibinin alone which did not perturbate the mitochondria.

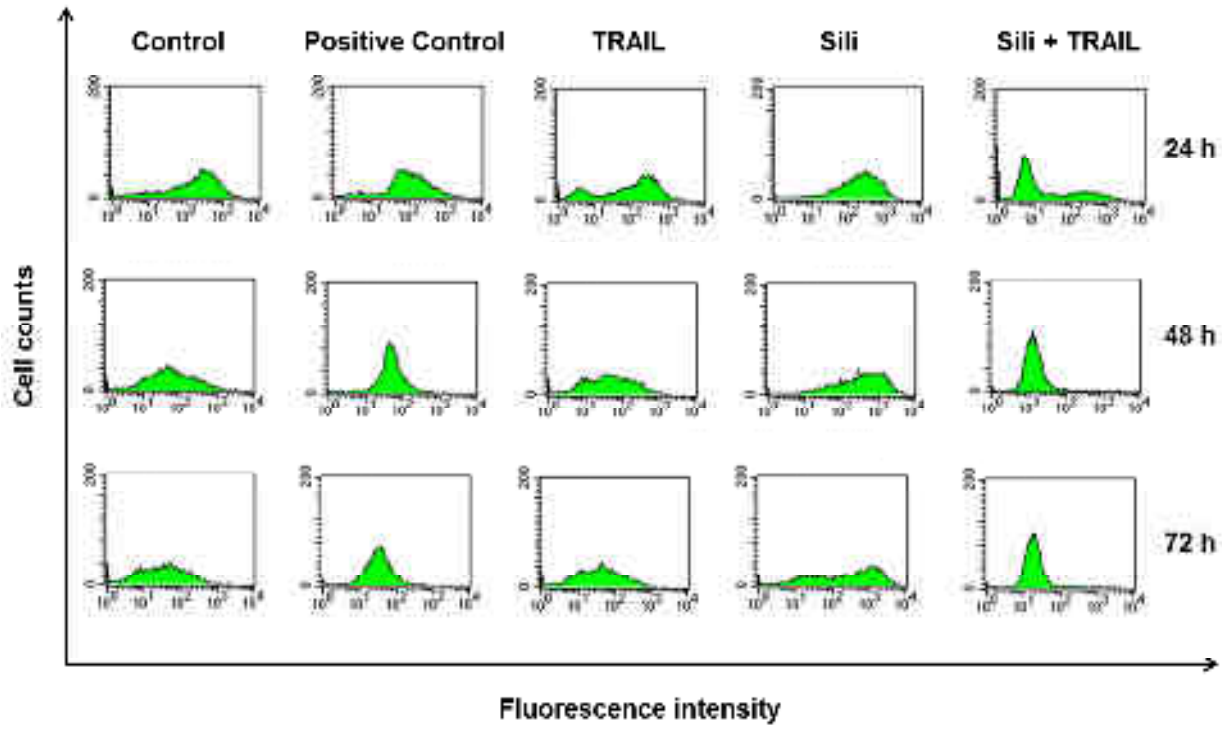

Figure 53: Silibinin and TRAIL induce mitochondrial membrane perturbation. Hep-55.1C cells were treated with DMSO 0.1% ± silibinin (300 µM) ± TRAIL (50 ng/mL) for 24, 48 and 72 h. Cells were stained with the cyanine dye $DiOC_2(3)$ and analyzed by flow cytometry as detailed in Materials and Methods. Data are presented as representative FACS histograms of green fluorescence.

5. Conclusion

We showed that silibinin administration for 4 weeks, starting one week after orthotopic implantation of Hep-55.1C cells in the liver of C57BL/6J mice, significantly reduced tumor growth by reducing tumor volume and weight. Silibinin induced apoptotic cell death in HCC cells with caspase-3 activation *in vivo* and *in vitro*. Furthermore, DR5 and TRAIL transcripts were upregulated *in vitro* and *in vivo* showing the implication of the extrinsic apoptotic pathway.

The genetic expression of pro-inflammatory molecules such as IL-1β, MMP-7 and -9 which was enhanced in liver tumors was downregulated by silibinin treatment whereas IFN-γ, a component of the innate immune system, was upregulated.

Silibinin sensitized TRAIL-resistant Hep-55.1C cells to TRAIL treatment *in vitro*. Potentiation of cell death also implicated the extrinsic pathway as shown by the potentiation of caspase-8 activation. But in contrast to the single agents, the combination silibinin/TRAIL was able to additionally perturbate the mitochondrial membrane potential.

Concerning the sensitizing mechanism of silibinin to TRAIL, just as in CRC, death receptors may be involved as supported by the silibinin-induced upregulation of DR5 transcription.

By using orthotopic syngeneic grafting of Hep-55.1C cells into the C57/BL/6J mouse liver and monitoring via micro-computed tomography, we were able to show that the flavonolignan silibinin induced anti-carcinogenic and protective effects on the liver of mice by activating the apoptotic TRAIL death receptor pathway. As the sensitizing effects of silibinin to TRAIL are not limited to CRC it may be interesting to evaluate the effects of silibinin in a combination therapy with TRAIL in the hepatic cancer model.

GENERAL DISCUSSION AND CONCLUSION

GENERAL DISCUSSION

Colorectal cancer (CRC) remains one of the leading causes of cancer deaths despite implementation of screening programs in many countries. Phytochemicals have been found to possess great chemopreventive potential in CRC.

The flavonolignan silibinin, the principal active constituent of the extract silymarin isolated from the milk thistle plant, has a good human acceptance through its long-established traditional use as a hepatoprotectant [149]. It is a very well tolerated substance and can thus be administered in high-dose therapy [95]. During the last decade, silibinin has been shown to possess anticancer activities against a plethora of *in vitro* and *in vivo* cancer models [82]. However, the silibinin-mediated mechanisms of cell death induction in CRC had not yet been elucidated.

In our study, we used a model of colon cancer progression consisting of the primary human colon adenocarcinoma cells SW480 and their derived metastatic cells SW620 to get insight into the apoptotic mechanisms activated by silibinin. With the aid of this model, we showed that silibinin was able to activate the extrinsic and the intrinsic apoptotic pathway as well as autophagy in both cell lines. To validate these results *in vivo*, we used a pre-clinical model of colon carcinogenesis, the rat azoxymethane (AOM)-induced colon cancer model, in which silibinin reduced the number of aberrant crypt foci (ACF) by half after 7 weeks of treatment and activated apoptosis. At last, because of the hepatoprotective properties of silibinin and as the liver is the first metastatic site of CRC, we studied the effects of silibinin on Hep-55.1C hepatocarcinoma cells both *in vitro* and as a syngeneic graft in an orthotopic mouse model in which silibinin also inhibited tumor growth and activated apoptosis. These results illustrate the high chemopreventive potential of silibinin in CRC.

Although silibinin has been studied in *in vitro* and *in vivo* models of CRC, no study had yet revealed the mechanisms of silibinin-mediated cell death. Previous studies mainly focused on silibinin-induced growth inhibition and cell cycle arrest [149, 123, 85, 150]. In our study, we investigated the implication of both the extrinsic and the intrinsic apoptotic pathway in the primary colon cancer SW480 cells and their derived metastatic SW620 cells.

In both cell lines, silibinin activated the extrinsic as well as the intrinsic apoptotic pathway (Figure 54). Silibinin induced time-dependent cell death accompanied by DNA fragmentation

and caspase-3 activation. Silibinin upregulated the expression of TRAIL and Fas death receptors on both protein and mRNA levels in SW480 and in SW620 cells. The effect was especially remarkable in SW620 cells as they are resistant to TRAIL and express significantly less death receptors than SW480 cells at their cell surface [166]. Activation of the death receptors was shown by activation of the initiator caspases of the extrinsic pathway, caspase-8 and caspase-10. Their activation can lead either directly to apoptosis via caspase-3 cleavage or to an amplification of the death signal via the activation of the intrinsic pathway by the cleavage of Bid [59]. However, we discovered that silibinin did not directly bind to the TRAIL receptors because the recombinant human DR4 and DR5 Fc chimeric proteins were not able to inhibit silibinin-induced cell death.

In SW480 cells, activation of caspase-8 was higher, leading to a more pronounced cleavage of Bid. But the activation of the intrinsic pathway took place to a similar extent in the two cell lines, demonstrated by an equal perturbation of the mitochondrial membrane potential, release of cytochrome c from the mitochondria into the cytosol and activation of caspase-9. The use of a specific caspase-8- and a pan-caspase-inhibitor led to a reduction of cell death in the two cell lines, demonstrating caspase-dependency of silibinin-induced apoptosis. At 24 h, the reduction of cell death was similar with both inhibitors, showing the initial importance of the extrinsic pathway involving crucial caspase-8 activation. At that time point, caspase-9 activation was not yet significant in the two cell lines. At 48 h, inhibition of caspase-8 did not reduce cell death as much as pan-caspase inhibition did, demonstrating the implication of the intrinsic pathway. Consistent with our data, Agarwal *et al*. found that silibinin induced apoptosis in LoVo cells with caspase activation [123]. In contrast, they found no caspase activation in HT-29 cells [149]. Caspase activation in CRC cells by silibinin seems to be dose- and time-dependent.

Autophagy induction is a common reaction to anticancer agents but plays a dual role in cancer therapy [280]: sometimes it protects cancer cells against apoptosis and provides them with the necessary energy for survival, then again autophagic cell death is able to eliminate cancer cells. Recently, it was shown that silibinin induced autophagic cell death in HT1080 human fibrosarcoma cells [201] but there had been no studies about silibinin-induced autophagy in CRC. In SW480 and SW620 cells, silibinin treatment elicited an autophagic response. When silibinin-treated cells were exposed to the autophagy inhibitor bafilomycin A1, we were able to clearly identify its pro-survival role because cell death increased in the two cell lines.

Various phytochemicals have been shown to trigger apoptosis in cancer cells by inducing oxidative stress [203, 204, 207]. ROS-dependent cell death can be inhibited by antioxidants such as NAC and GSH. However, these were not able to inhibit silibinin-induced apoptosis in SW480 and SW620 cells. This may be explained by the fact that ROS-induction by silibinin has been shown to be p53-dependent [211] and in SW480 and SW620 cells p53 is mutated [212].

The tumor suppressor p53 is frequently involved in apoptosis induction in response to cellular stress. Although there are studies which show that even mutated, p53 can still participate in apoptosis induction, inhibition of p53 did not alleviate silibinin-induced cell death as it has been shown to be the case in silibinin-treated cells with wild-type p53 [211].

The NF-κB transcription factor family is also involved in the regulation of apoptosis and a double-edged sword [71]. Besides its well-studied anti-apoptotic effects, it can also mediate pro-apoptotic activity. Silibinin has been reported to inhibit NF-κB signaling but here we observed no change in p65 nuclear translocation in silibinin-treated cells. When NF-κB signaling was inhibited, cell death increased only in SW620 cells. This may be attributed to a higher basal NF-κB activity in the metastatic SW620 cells [221].

Furthermore, MAPKs are mediators of apoptosis. In our study, inhibition of MAPKs had a tendency of increasing cell death which let us assume a pro-survival role.

(Re-)activation of apoptosis is an important approach for cancer therapy because tumor cells often evade apoptosis induction. To this end, they use various strategies including loss of tumor suppressor function, downregulation of pro-apoptotic and upregulation of anti-apoptotic factors [281]. Here, we showed that silibinin is able to induce apoptosis by modulating the extrinsic and intrinsic pathway in CRC cells and thus represents an interesting chemopreventive agent.

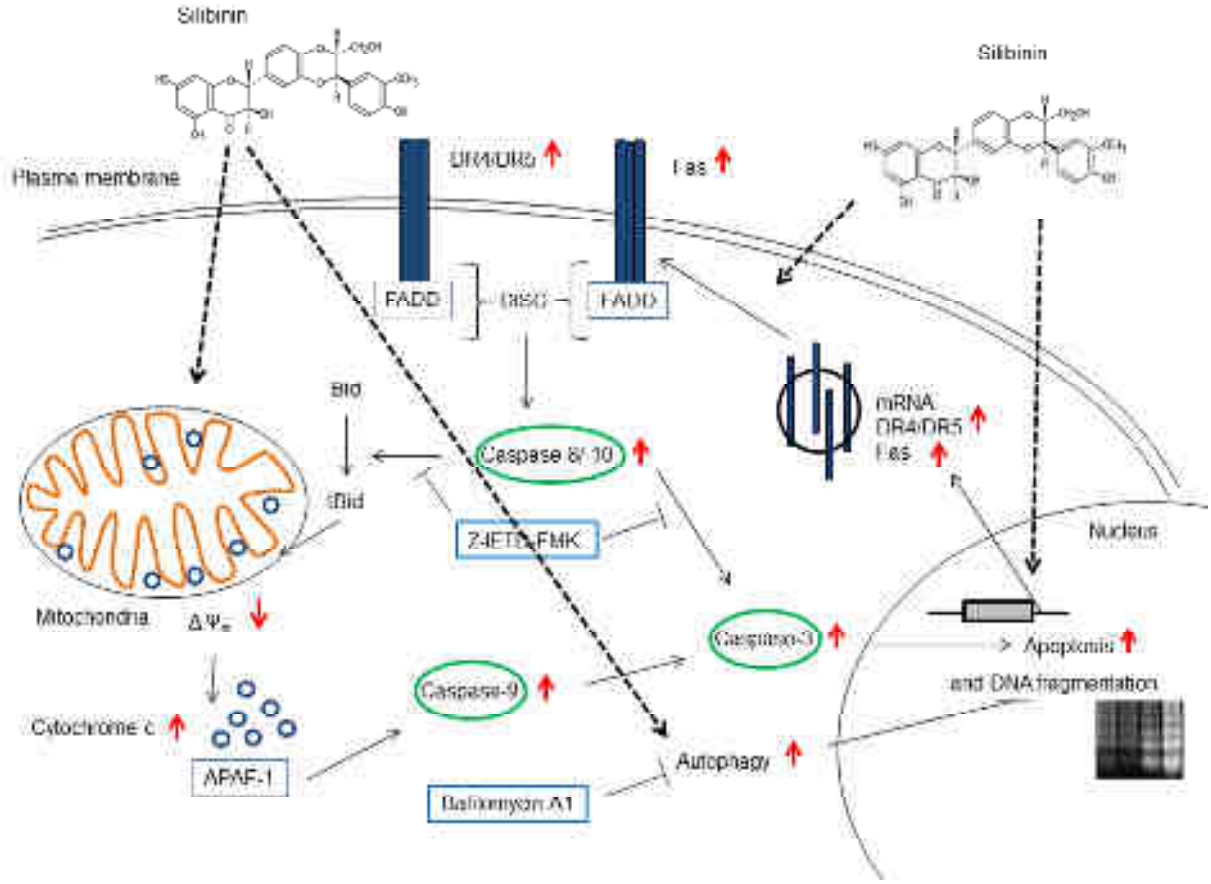

Figure 54: Mechanisms of silibinin-induced apoptosis in SW480 and SW620 cells. *Silibinin activated the extrinsic and the intrinsic pathway of apoptosis. Silibinin induced an upregulation of the TRAIL and Fas death receptors on both mRNA and protein level. The death receptors activate the initiator caspases of the extrinsic pathway, caspase-8 and -10, by recruiting Fas-associated death domain (FADD) protein and thus assembling the death-inducing signaling complex (DISC). Caspase-8 cleaved the protein Bid to the pro-apoptotic truncated Bid (tBid). Silibinin induced a perturbation of the mitochondrial membrane potential, leading to the release of the pro-apoptotic factor cytochrome c. Cytochrome c assembles with other factors such as Apaf-1 to form the apoptosome which activates the initiator caspase of the intrinsic pathway, caspase-9. Caspase-8 and caspase-9 then cleave the effector caspase-3, finally leading to apoptosis with its hallmark, DNA fragmentation. Silibinin also induced an autophagic response playing a pro-survival role but finally submerged by apoptosis. The autophagy inhibitor bafilomycin A1 increased cell death. The caspase-8 inhibitor Z-IETD-FMK was able to diminish silibinin-induced cell death.*

To confirm the anticancer properties of silibinin observed in the CRC cell lines, we tested its efficacy on the development of CRC in the widely-used AOM rat model of colon carcinogenesis [261]. AOM induces the transformation of normal colon epithelium into carcinoma via the multistage process of colon carcinogenesis [260]. The AOM rat model is attractive for evaluating the efficacy of chemopreventive substances because its clinical and molecular features resemble those occurring in the sporadic carcinogenesis in humans, accounting for 80% of CRC cases. Therefore the model allows elucidating the mechanisms implicated in chemopreventive effects [20].

ACF are the first preneoplastic lesions in colorectal carcinogenesis. Hence, they are used as endpoint in short-term chemopreventive studies to screen CRC preventive agents [282].

In our setting, male Wistar rats received daily intragastric feeding of 300 mg silibinin/kg body weight for 7 weeks, starting one week after AOM injection (post-initiation). Silibinin-treated rats showed a 2-fold reduction in the number of AOM-induced ACF and hyperproliferative crypts compared to AOM-injected rats receiving only the vehicle. In the past, studies have already shown the potential of silibinin in inhibiting preneoplastic lesions and tumors in models of carcinogen-induced colorectal carcinogenesis [152, 153]. In agreement with these results, our data confirmed the chemopreventive potential of silibinin.

Investigating the mechanisms, we found that silibinin induced apoptosis with activation of caspase-3 in the colonic mucosa. Induction of apoptosis and activation of caspase-3 was consistent with our results in the CRC cell lines *in vitro*.

In vivo, silibinin diminished the Bcl-2/Bax ratio by downregulating the anti-apoptotic protein Bcl-2 and upregulating the pro-apoptotic protein Bax both of which are responsible for the regulation of the activation of the intrinsic apoptotic pathway. We had shown before that the pro-inflammatory markers IL-1β, TNF-α and their downstream target MMP-7 which participates in early and late processes of carcinogenesis were upregulated in the early stages of colon carcinogenesis in the AOM model [263]. Expression of MMPs has been shown to be correlated to the progression of the disease and MMP-7 is expressed in 90% of colonic adenocarcinomas [283]. Here, silibinin downregulated the genetic expression of IL-1β, TNF-α and MMP-7.

The fact that silibinin exerted pro-apoptotic, anti-inflammatory and multi-targeted effects on the colonic mucosa indicates the ability of silibinin to inhibit colon carcinogenesis by promoting the normal balance between cell death and proliferation.

After obtaining evidence of the efficacy of silibinin in an *in vitro* model of CRC progression and in an *in vivo* rat model of colorectal carcinogenesis, we extended our research to evaluate the effects of silibinin in hepatic cancer. We chose hepatic cancer because of the well-known hepatoprotective properties of silibinin. Additionally, the liver is the first metastatic site of CRC.

In vitro, silibinin induced cell death in Hep-55.1C cells. As in SW480 and SW620 cells, the extrinsic apoptotic pathway was implicated, demonstrated by the activation of caspase-8 and -3 and the upregulation of DR5. Moreover, the death ligand TRAIL was upregulated.

Orthotopic grafting of the murine Hep-55.1C cells into the liver of C57BL/6J mice was performed, and tumor growth was followed by micro-computed imaging which permitted to non-invasively follow up the tumor growth. Orthotopic implantation better mimics the cell environment than subcutaneous implantation, and by using syngeneic grafts the host immune system remains active and thus the tumoral environment comes closer to reality. Administration of 700 mg silibinin/kg body weight for 4 weeks significantly reduced tumor growth, leading to a three-fold reduction of tumor weight and volume.

In vivo, the results were in agreement with those obtained *in vitro*: silibinin induced apoptotic cell death, shown by the activation of caspase-3, and activated the extrinsic apoptotic pathway, demonstrated by the upregulation of the gene expression of TRAIL and its receptor DR5.

Pro-inflammatory markers play an important role in carcinogenesis. In this model of hepatic cancer we found - just as in the AOM rat model - downregulation of the pro-inflammatory cytokines IL-1β which induce the overexpression of MMPs. In agreement with our findings in the AOM rat model, we also observed downregulation of MMP-7. Furthermore, silibinin induced downregulation of MMP-9 and upregulation of IFN-γ which plays an important role in the host defense against tumor development.

Mechanisms of the silibinin-mediated anticancer effects in hepatocellular carcinoma involved the activation of the extrinsic apoptotic pathway and anti-inflammatory effects and thus appear to be similar to those in the AOM rat model.

As a hepatoprotectant, silibinin is predestined for a combination therapy. The effectiveness of many chemotherapeutic agents is limited by their elevated systemic toxicity, often involving hepatotoxicity which is especially problematic in the case of colorectal liver metastases [284]. Milk thistle extract has been traditionally prescribed to provide support to the liver [90]. In the light of the recently discovered anticancer properties of silibinin, the interest of a combination therapy with silibinin is growing.

Because we observed that silibinin induced an increase of the expression of the TRAIL death receptors DR4 and DR5 in SW480 and SW620 cells and the expression of the murine DR5 in Hep-55.1C cells we were interested in potential synergistic effects of silibinin and TRAIL. Our results show remarkable synergistic effects in cell death induction by silibinin/TRAIL in the two CRC cell lines as well as in Hep-55.1C cells.

It has been shown that there is a rationale to use HDAC inhibitors in combination therapy because they synergistically enhance the anticancer activity of many chemotherapeutic agents [78]. We showed that silibinin worked synergistically with each of two broad spectrum HDAC inhibitors to induce cell death in SW480 and SW620 cells. Our data are promising for silibinin in combination therapies for cancer.

The physiological role of TRAIL signaling in tumor immunosurveillance and its selectivity for killing tumor cells render TRAIL a promising candidate for cancer therapy [48]. Indeed, recombinant human TRAIL (rhTRAIL) and its agonistic monoclonal antibodies against DR4 and DR5 are currently evaluated in clinical trials.

However, the results do not seem promising outside of combination therapies because unfortunately there are many cancers which are TRAIL-resistant and thus evade apoptosis: as it is the first-line defense of the body, many “successful” tumors have already acquired resistance against TRAIL-induced apoptosis [48]. Thus, strategies to re-sensitize tumor cells to TRAIL are urgently needed.

Here, we demonstrated that silibinin was able to sensitize the adenocarcinoma SW480 cells and their derived metastatic TRAIL-resistant SW620 cells to TRAIL-induced apoptosis (Figure 55).

The death receptors DR4 and DR5 played an important role in synergistic cell death induction: DR4/Fc and DR5/Fc chimeric proteins, which have a dominant-negative effect by respectively competing with endogenous DR4 and DR5, reversed synergistic cell death.

Flavonoids have been shown to sensitize cancer cells to TRAIL by upregulating DR4 and DR5 [285–287], this being the effect that we had observed for silibinin.

Treatment with the combination silibinin/TRAIL resulted in a major activation of caspase-3, -8, and -9. The pan-caspase-inhibitor Z-VAD-FMK was able to block cell death, proving its apoptotic nature and showing the caspase-dependency of the synergistic cell death. When only caspase-8 was inhibited by Z-IETD-FMK, cell death persisted, suggesting the activation of other initiator caspases. Indeed, TRAIL synergistically enhanced the perturbation of the mitochondrial membrane potential induced by silibinin, pointing to the implication of the intrinsic pathway. TRAIL-induced apoptosis often includes or even requires the activation of the mitochondrial pathway [239].

As the anti-apoptotic protein XIAP is an important regulator of caspase activation, we were interested in its expression. XIAP is the only member of the IAP family which inhibits caspases-3 and -9 by directly binding to them. Furthermore, its high expression represents a common feature of TRAIL-resistance in colon carcinoma [288]. We found a very significant downregulation of XIAP in SW480 and SW620 cells after silibinin/TRAIL treatment. This effect may be involved in silibinin-induced sensitization to TRAIL as inhibition of XIAP by embelin, a small-molecule inhibitor of XIAP, also succeeded in sensitizing the two cell lines to TRAIL. However, its effect was not as strong as that of silibinin, suggesting additional mechanisms in silibinin-mediated sensitization.

Mcl-1 is an anti-apoptotic member of the Bcl-2 family which plays a predominant role in the regulation of the mitochondrial apoptotic pathway [213]. Because of the synergistic perturbation of the mitochondrial potential by silibinin/TRAIL we investigated the expression of Mcl-1 and found a significant decrease after treatment with silibinin/TRAIL but not with silibinin or TRAIL alone. However, the decrease may also result from a degradation by the highly activated caspases [289]. Degradation of Mcl-1 may lead to a positive feedback loop to enhance apoptosis.

It seems that the ability of silibinin to sensitize cancer cells to TRAIL is not cancer-specific. The synergy of silibinin and TRAIL was reproducible in hepatic cancer. Hep-55.1C cells are resistant to TRAIL, but after administration of the combination silibinin/TRAIL, synergistic cell death was induced. As in SW480 and SW620 cells, cell death was accompanied by synergistic perturbation of the mitochondrial membrane potential and synergistic activation of caspase-3 and -8. Moreover, Son *et al.* showed that silibinin sensitized human glioma cells via DR5 upregulation and downregulation of survivin [245].

We also observed DR5 upregulation in SW480, SW620 and Hep-55.1C cells. In SW480 cells, but not in SW620 cells survivin was downregulated by silibinin.

An advantage of TRAIL-induced apoptosis compared to conventional chemotherapy is the independence of p53 [48]. Chemotherapy often necessitates activation of p53 to induce an apoptotic response which presents a problem because p53 is mutated in more than half of all cancer cells. Thus resistance to chemotherapy is often caused by loss or mutation of p53 [290]. Although the activation of the TRAIL pathway can be enhanced by upregulation of death receptors by p53, the function of p53 is not necessary for TRAIL to induce apoptosis [48]. Accordingly, we showed synergistic cell death induction of silibinin and TRAIL in SW480 and SW620 cells, which share the same mutated p53 alleles [291], and in Hep-55.1C cells that are also p53-negative tumor cells. It is noteworthy that silibinin was even able to upregulate the TRAIL death receptors DR4 and DR5 in a p53-independent manner. This suggests that silibinin/TRAIL treatment may be useful in the treatment of p53-deficient cancers.

Our data propose a multi-targeted mechanism for silibinin-induced sensitization to TRAIL, involving both the extrinsic pathway by upregulation of the death receptors DR4 and DR5, and the intrinsic pathway by downregulation of Mcl-1 and XIAP which are important anti-apoptotic mediators of resistance to TRAIL (Figure 55).

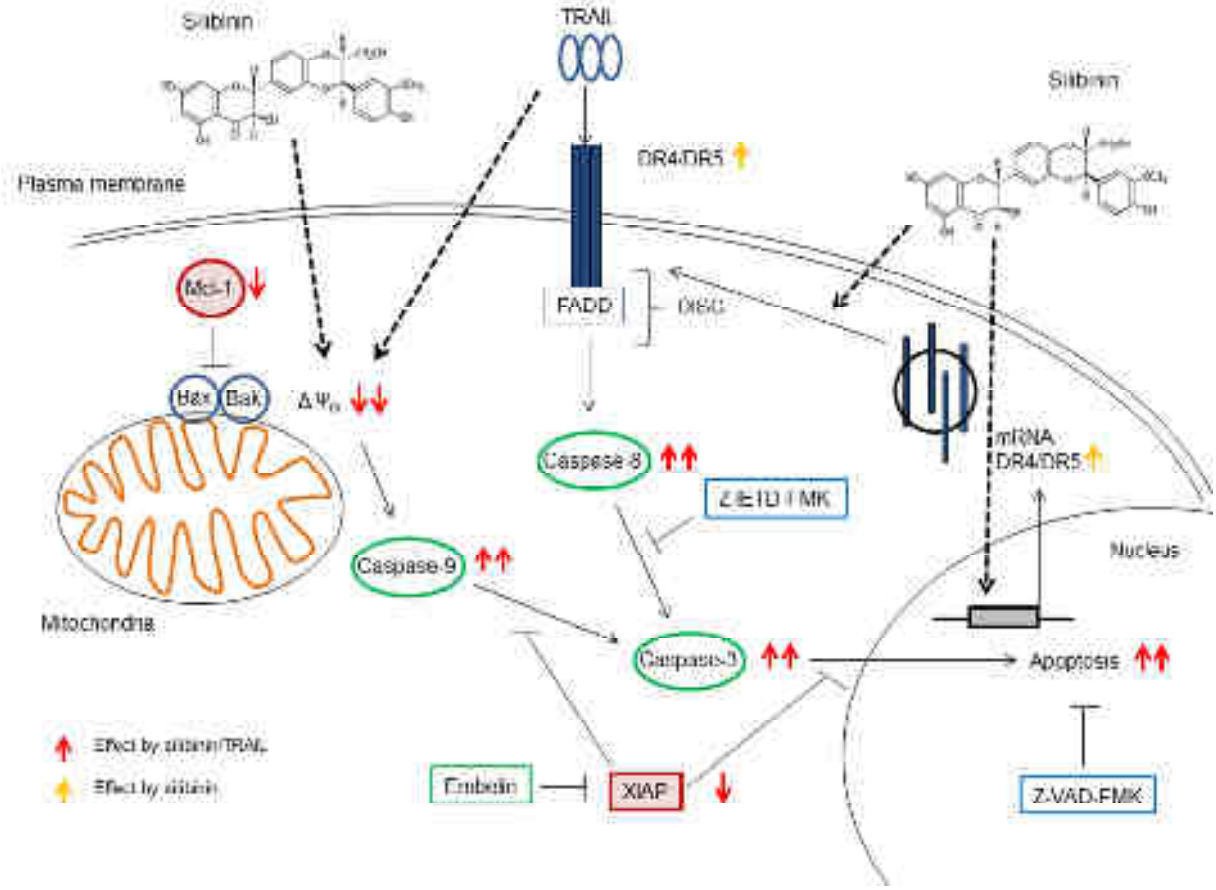

Figure 55: Mechanisms of silibinin/TRAIL-induced apoptosis in SW480 and SW620 cells. *Silibinin and TRAIL induced synergistic cell death by activating the extrinsic and the intrinsic pathway of apoptosis. Caspases-3, -8 and -9 were synergistically activated and the mitochondrial membrane potential was synergistically perturbated. The synergy was associated with silibinin-induced upregulation of TRAIL death receptors DR4 and DR5 on both mRNA and protein level, with downregulation of the anti-apoptotic Bcl-2 family member Mcl-1 and with downregulation of the X-linked inhibitor of apoptosis protein (XIAP). Inhibition of XIAP by the small-molecule inhibitor embelin was able to sensitize SW480 and SW620 cells to TRAIL-induced apoptosis. The caspase-8 inhibitor Z-IETD-FMK and the pan-caspase inhibitor Z-VAD-FMK were able to diminish silibinin/TRAIL-induced cell death.*

Polyphenols have been shown to modify histone deacetylation and DNA hypermethylation in the context of their chemopreventive effects [230–233] and this was the reason why we were interested in investigating the effect of silibinin on epigenetic signaling. The only study reported in the literature concerning the epigenetic effects of silibinin showed that silibinin increased acetylation of histones in hepatic cancer *in vitro* and *in vivo* [236, 292].

In our setting, silibinin did not show HDAC inhibitory activity, but it did diminish DNMT activity in SW480 and SW620 cells. As the TRAIL death receptors DR4 and DR5 have been shown to be frequently silenced by hypermethylation in (TRAIL-resistant) cancer cells, the DNMT inhibitory activity of silibinin may contribute to its upregulating effect on DR4/DR5 expression [48]. Other known methylation-silenced genes in cancer are TRAIL [293] and caspase-8 [294]; hence their activation may also be involved in silibinin-induced apoptosis. It would be interesting to explore these potential connections by further studies.

When we tested the effect of the combination of silibinin with each of two different broad spectrum HDAC inhibitors, Trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), we found that they worked synergistically to induce cell death.

A synergistic interaction between histone deacetylation and DNA methylation in the epigenetic silencing of cancer genes has been described [229], rendering combinations of HDAC and DNMT inhibitors attractive for combination therapy [77]. However, the cause of the synergy between HDAC inhibitors and silibinin did not seem to be entirely due to the DNMT inhibitory activity of silibinin because the onset of significant DNMT inhibition happened at a later time point than the observed synergistic effect. HDAC inhibitors have also been found to shift the balance of pro- and anti-apoptotic proteins which may enhance the pro-apoptotic activity of silibinin [78].

The preferential toxicity of HDAC inhibitors for tumor cells and the non-toxic character of silibinin render them an interesting combination therapy for CRC.

CONCLUSION

Our studies in the *in vitro* model of CRC progression consisting of the primary human adenocarcinoma SW480 cells and their derived metastatic SW620 cells have shown that silibinin induces apoptosis by activating the extrinsic and the intrinsic pathway. Silibinin also induces an autophagic survival response which is, however, overwhelmed by the activation of apoptosis.

Importantly, silibinin induces the expression of the TRAIL death receptors DR4 and DR5 in SW480 and in TRAIL-resistant SW620 cells and sensitizes both cell lines to TRAIL. The combination silibinin/TRAIL leads to a synergistic effect in cell death induction which was accompanied by synergistic perturbation of the mitochondrial membrane potential and synergistic activation of caspases.

In the AOM rat model, we showed that silibinin had a chemopreventive effect and reduced the number of preneoplastic lesions after 7 weeks by half. This effect was accompanied by induction of apoptosis and by inhibition of inflammatory processes.

Finally, we have demonstrated that silibinin also shows anticancer efficacy in an orthotopic model of hepatocarcinoma. Here, silibinin induced a three-fold reduction of tumor weight and volume which was also accompanied by activation of pro-apoptotic and anti-inflammatory pathways.

This work shows the efficacy of the polyphenol silibinin, the principal constituent of the milk thistle extract silymarin, in inducing apoptosis in CRC *in vitro* and *in vivo*.

Together, these results suggest that silibinin may be an effective chemopreventive agent against CRC. Combinations of silibinin with anticancer agents such as TRAIL and HDAC inhibitors are even more promising because of their non-toxicity and their synergistic action in cancer cell death induction.

REFERENCES

1. Xu R, Zhou B, Fung PCW, Li X (2006) Recent advances in the treatment of colon cancer. Histol Histopathol 21: 867–872
2. Jemal A et al. (2011) Global cancer statistics. CA Cancer J Clin 61: 69–90. doi:10.3322/caac.20107
3. Tenesa A, Dunlop MG (2009) New insights into the aetiology of colorectal cancer from genome-wide association studies. Nat Rev Genet 10: 353–358. doi:10.1038/nrg2574
4. Labianca R et al. (2010) Colon cancer. Crit Rev Oncol Hematol 74: 106–133. doi:10.1016/j.critrevonc.2010.01.010
5. Marchand LL (1999) Combined influence of genetic and dietary factors on colorectal cancer incidence in Japanese Americans. J Natl Cancer Inst Monographs 101–105
6. Le Marchand L, Wilkens LR, Hankin JH, Kolonel LN, Lyu LC (1997) A case-control study of diet and colorectal cancer in a multiethnic population in Hawaii (United States): lipids and foods of animal origin. Cancer Causes Control 8: 637–648
7. Asano TK, McLeod RS (2004) Nonsteroidal anti-inflammatory drugs and aspirin for the prevention of colorectal adenomas and cancer: a systematic review. Dis Colon Rectum 47: 665–673. doi:10.1007/s10350-003-0111-9
8. Ullman TA, Itzkowitz SH (2011) Intestinal inflammation and cancer. Gastroenterology 140: 1807–1816. doi:10.1053/j.gastro.2011.01.057
9. Aleksandrova K et al. (2011) Metabolic syndrome and risks of colon and rectal cancer: the European prospective investigation into cancer and nutrition study. Cancer Prev Res (Phila) 4: 1873–1883. doi:10.1158/1940-6207.CAPR-11-0218
10. Sidney J W (1999) Natural history of colorectal cancer. The American Journal of Medicine 106: 3–6. doi:10.1016/S0002-9343(98)00338-6
11. Kwak EL, Chung DC (2007) Hereditary colorectal cancer syndromes: an overview. Clin Colorectal Cancer 6: 340–344. doi:10.3816/CCC.2007.n.002
12. Orlando FA et al. (2008) Aberrant crypt foci as precursors in colorectal cancer progression. J Surg Oncol 98: 207–213. doi:10.1002/jso.21106
13. Coleman MP et al. (2003) EUROCARE-3 summary: cancer survival in Europe at the end of the 20th century. Ann Oncol 14 Suppl 5: v128–149
14. Zavoral M et al. (2009) Colorectal cancer screening in Europe. World J Gastroenterol 15: 5907–5915
15. Atkin WS et al. (2002) Single flexible sigmoidoscopy screening to prevent colorectal cancer: baseline findings of a UK multicentre randomised trial. Lancet 359: 1291–1300. doi:10.1016/S0140-6736(02)08268-5
16. (2002) Methylation reveals a niche: stem cell succession in human colon crypts. , Published online: 05 August 2002; | doi:101038/sj.onc1205604 21: . doi:10.1038/sj.onc.1205604
17. Yang SY, Sales KM, Fuller B, Seifalian AM, Winslet MC (2009) Apoptosis and colorectal cancer: implications for therapy. Trends Mol Med 15: 225–233. doi:10.1016/j.molmed.2009.03.003
18. Vogelstein B et al. (1988) Genetic alterations during colorectal-tumor development. N Engl J Med 319: 525–532. doi:10.1056/NEJM198809013190901
19. Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. Cell 61: 759–767
20. Takahashi M, Wakabayashi K (2004) Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. Cancer Sci 95: 475–480
21. Corpet DE, Pierre F (2005) How good are rodent models of carcinogenesis in predicting efficacy in humans? A systematic review and meta-analysis of colon chemoprevention in rats, mice and men. Eur J Cancer 41: 1911–1922. doi:10.1016/j.ejca.2005.06.006

22. Walther A et al. (2009) Genetic prognostic and predictive markers in colorectal cancer. Nat Rev Cancer 9: 489–499. doi:10.1038/nrc2645
23. Qiao L, Wong BCY (2009) Targeting apoptosis as an approach for gastrointestinal cancer therapy. Drug Resist Updat 12: 55–64. doi:10.1016/j.drup.2009.02.002
24. Bustin SA, Mueller R (2006) Real-time reverse transcription PCR and the detection of occult disease in colorectal cancer. Molecular Aspects of Medicine 27: 192–223. doi:10.1016/j.mam.2005.12.002
25. Peeters M, Cohn A, Köhne C-H, Douillard J-Y (2012) Panitumumab in combination with cytotoxic chemotherapy for the treatment of metastatic colorectal carcinoma. Clin Colorectal Cancer 11: 14–23. doi:10.1016/j.clcc.2011.06.010
26. Rajamanickam S, Agarwal R (2008) Natural products and colon cancer: current status and future prospects. Drug Dev Res 69: 460–471. doi:10.1002/ddr.20276
27. Half E, Arber N (2009) Colon cancer: preventive agents and the present status of chemoprevention. Expert Opin Pharmacother 10: 211–219. doi:10.1517/14656560802560153
28. Algra AM, Rothwell PM (2012) Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. The Lancet Oncology 13: 518–527. doi:10.1016/S1470-2045(12)70112-2
29. Johnson IT, Lund EK (2007) Review article: nutrition, obesity and colorectal cancer. Aliment Pharmacol Ther 26: 161–181. doi:10.1111/j.1365-2036.2007.03371.x
30. Aune D et al. (2011) Nonlinear reduction in risk for colorectal cancer by fruit and vegetable intake based on meta-analysis of prospective studies. Gastroenterology 141: 106–118. doi:10.1053/j.gastro.2011.04.013
31. van Duijnhoven FJB et al. (2009) Fruit, vegetables, and colorectal cancer risk: the European Prospective Investigation into Cancer and Nutrition. Am J Clin Nutr 89: 1441–1452. doi:10.3945/ajcn.2008.27120
32. Pusatcioglu CK, Braunschweig C (2011) Moving beyond diet and colorectal cancer. J Am Diet Assoc 111: 1476–1478. doi:10.1016/j.jada.2011.07.015
33. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE (2009) Cell death. N Engl J Med 361: 1570–1583. doi:10.1056/NEJMra0901217
34. Henriquez M, Armisén R, Stutzin A, Quest AFG (2008) Cell death by necrosis, a regulated way to go. Curr Mol Med 8: 187–206
35. Gonzalvez F, Ashkenazi A (2010) New insights into apoptosis signaling by Apo2L/TRAIL. Oncogene 29: 4752–4765. doi:10.1038/onc.2010.221
36. Reed JC (2000) Mechanisms of apoptosis. Am J Pathol 157: 1415–1430. doi:10.1016/S0002-9440(10)64779-7
37. Ghavami S et al. (2009) Apoptosis and cancer: mutations within caspase genes. J Med Genet 46: 497–510. doi:10.1136/jmg.2009.066944
38. Simon HU, Haj-Yehia A, Levi-Schaffer F (2000) Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis 5: 415–418
39. Hsu H, Shu HB, Pan MG, Goeddel DV (1996) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84: 299–308
40. Varfolomeev E et al. (2005) Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. J Biol Chem 280: 40599–40608. doi:10.1074/jbc.M509560200
41. Wiley SR et al. (1995) Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3: 673–682

42. Hu R, Kong A-NT (2004) Activation of MAP kinases, apoptosis and nutrigenomics of gene expression elicited by dietary cancer-prevention compounds. Nutrition 20: 83–88
43. Jacquemin G, Shirley S, Micheau O (2010) Combining naturally occurring polyphenols with TNF-related apoptosis-inducing ligand: a promising approach to kill resistant cancer cells? Cell Mol Life Sci 67: 3115–3130. doi:10.1007/s00018-010-0407-6
44. Dai Y et al. (2009) A Smac-mimetic sensitizes prostate cancer cells to TRAIL-induced apoptosis via modulating both IAPs and NF-kappaB. BMC Cancer 9: 392. doi:10.1186/1471-2407-9-392
45. Inoue H et al. (2002) Functional expression of tumor necrosis factor-related apoptosis-inducing ligand in human colonic adenocarcinoma cells. Lab Invest 82: 1111–1119
46. Koornstra JJ et al. (2005) Expression of tumour necrosis factor-related apoptosis-inducing ligand death receptors in sporadic and hereditary colorectal tumours: potential targets for apoptosis induction. Eur J Cancer 41: 1195–1202. doi:10.1016/j.ejca.2005.02.018
47. Falschlehner C, Schaefer U, Walczak H (2009) Following TRAIL's path in the immune system. Immunology 127: 145–154. doi:10.1111/j.1365-2567.2009.03058.x
48. Dimberg LY et al. (2012) On the TRAIL to successful cancer therapy? Predicting and counteracting resistance against TRAIL-based therapeutics. Oncogene . doi:10.1038/onc.2012.164
49. Ashe PC, Berry MD (2003) Apoptotic signaling cascades. Prog Neuropsychopharmacol Biol Psychiatry 27: 199–214. doi:10.1016/S0278-5846(03)00016-2
50. Fulda S, Debatin K-M (0000) Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 25: 4798–4811
51. Ogata Y, Takahashi M (2003) Bcl-xL as an antiapoptotic molecule for cardiomyocytes. Drug News Perspect 16: 446–452
52. Levine B, Sinha S, Kroemer G (2008) Bcl-2 family members: dual regulators of apoptosis and autophagy. Autophagy 4: 600–606
53. Acharya A, Das I, Chandhok D, Saha T (2010) Redox regulation in cancer: a double-edged sword with therapeutic potential. Oxid Med Cell Longev 3: 23–34. doi:10.4161/oxim.3.1.10095
54. Pelicano H, Carney D, Huang P (2004) ROS stress in cancer cells and therapeutic implications. Drug Resist Updat 7: 97–110. doi:10.1016/j.drup.2004.01.004
55. Su R-Y, Chi K-H, Huang D-Y, Tai M-H, Lin W-W (2008) 15-deoxy-Delta12,14-prostaglandin J2 up-regulates death receptor 5 gene expression in HCT116 cells: involvement of reactive oxygen species and C/EBP homologous transcription factor gene transcription. Mol Cancer Ther 7: 3429–3440. doi:10.1158/1535-7163.MCT-08-0498
56. Jung EM et al. (2006) Curcumin sensitizes tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis through CHOP-independent DR5 upregulation. Carcinogenesis 27: 2008–2017. doi:10.1093/carcin/bgl026
57. Lee T-J et al. (2009) Withaferin A sensitizes TRAIL-induced apoptosis through reactive oxygen species-mediated up-regulation of death receptor 5 and down-regulation of c-FLIP. Free Radic Biol Med 46: 1639–1649. doi:10.1016/j.freeradbiomed.2009.03.022
58. Yodkeeree S, Sung B, Limtrakul P, Aggarwal BB (2009) Zerumbone enhances TRAIL-induced apoptosis through the induction of death receptors in human colon cancer cells: Evidence for an essential role of reactive oxygen species. Cancer Res 69: 6581–6589. doi:10.1158/0008-5472.CAN-09-1161
59. Kumar S (2007) Caspase function in programmed cell death. Cell Death Differ 14: 32–43. doi:10.1038/sj.cdd.4402060

60. Fulda S, Vucic D (2012) Targeting IAP proteins for therapeutic intervention in cancer. Nat Rev Drug Discov 11: 109–124. doi:10.1038/nrd3627
61. Hunter AM, LaCasse EC, Korneluk RG (2007) The inhibitors of apoptosis (IAPs) as cancer targets. Apoptosis 12: 1543–1568. doi:10.1007/s10495-007-0087-3
62. Altieri DC (2008) New wirings in the survivin networks. Oncogene 27: 6276–6284. doi:10.1038/onc.2008.303
63. Pennati M, Folini M, Zaffaroni N (2008) Targeting survivin in cancer therapy. Expert Opin Ther Targets 12: 463–476. doi:10.1517/14728222.12.4.463
64. Kim EK, Choi E-J (2010) Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta 1802: 396–405. doi:10.1016/j.bbadis.2009.12.009
65. Dhillon AS, Hagan S, Rath O, Kolch W (2007) MAP kinase signalling pathways in cancer. Oncogene 26: 3279–3290. doi:10.1038/sj.onc.1210421
66. Fan M, Chambers TC (2001) Role of mitogen-activated protein kinases in the response of tumor cells to chemotherapy. Drug Resist Updat 4: 253–267. doi:10.1054/drup.2001.0214
67. Brady CA, Attardi LD (2010) p53 at a glance. J Cell Sci 123: 2527–2532. doi:10.1242/jcs.064501
68. Eisenberg-Lerner A, Bialik S, Simon H-U, Kimchi A (2009) Life and death partners: apoptosis, autophagy and the cross-talk between them. Cell Death Differ 16: 966–975. doi:10.1038/cdd.2009.33
69. Brown M, Cohen J, Arun P, Chen Z, Van Waes C (2008) NF-kappaB in carcinoma therapy and prevention. Expert Opin Ther Targets 12: 1109–1122. doi:10.1517/14728222.12.9.1109
70. Chen X, Kandasamy K, Srivastava RK (2003) Differential roles of RelA (p65) and c-Rel subunits of nuclear factor kappa B in tumor necrosis factor-related apoptosis-inducing ligand signaling. Cancer Res 63: 1059–1066
71. Campbell KJ, Rocha S, Perkins ND (2004) Active repression of antiapoptotic gene expression by RelA(p65) NF-kappa B. Mol Cell 13: 853–865
72. Gozuacik D, Kimchi A (2004) Autophagy as a cell death and tumor suppressor mechanism. Oncogene 23: 2891–2906. doi:10.1038/sj.onc.1207521
73. Meléndez A (2009) Autophagy in C. elegans. WormBook 1–26. doi:10.1895/wormbook.1.147.1
74. Kondo Y, Kondo S (2006) Autophagy and cancer therapy. Autophagy 2: 85–90
75. Degenhardt K et al. (2006) Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 10: 51–64. doi:10.1016/j.ccr.2006.06.001
76. Proskuryakov SY, Konoplyannikov AG, Gabai VL (2003) Necrosis: a specific form of programmed cell death? Exp Cell Res 283: 1–16
77. Rius M, Lyko F (2011) Epigenetic cancer therapy: rationales, targets and drugs. Oncogene . doi:10.1038/onc.2011.601
78. Carew JS, Giles FJ, Nawrocki ST (2008) Histone deacetylase inhibitors: mechanisms of cell death and promise in combination cancer therapy. Cancer Lett 269: 7–17. doi:10.1016/j.canlet.2008.03.037
79. Barrett M (2004) The handbook of clinically tested herbal remedies. Routledge, .
80. National Center for Complementary and Alternative Medicine ([date unknown]) Milk Thistle [NCCAM Herbs at a Glance]. http://www.nccam.nih.gov/health/milkthistle/ataglance.htm. Accessed 20 Sep 2011
81. Rottapharm | Madaus Heilpflanzen-Datenbank. http://www.rottapharm-madaus.de/heilpflanzen-datenbank/silybum_marianum/. Accessed 15 Jun 2011

82. Cheung CWY, Gibbons N, Johnson DW, Nicol DL (2010) Silibinin--a promising new treatment for cancer. Anticancer Agents Med Chem 10: 186–195
83. Abenavoli L, Capasso R, Milic N, Capasso F (2010) Milk thistle in liver diseases: past, present, future. Phytother Res 24: 1423–1432. doi:10.1002/ptr.3207
84. Li L, Zeng J, Gao Y, He D (2010) Targeting silibinin in the antiproliferative pathway. Expert Opin Investig Drugs 19: 243–255. doi:10.1517/13543780903533631
85. Hogan FS, Krishnegowda NK, Mikhailova M, Kahlenberg MS (2007) Flavonoid, silibinin, inhibits proliferation and promotes cell-cycle arrest of human colon cancer. J Surg Res 143: 58–65. doi:10.1016/j.jss.2007.03.080
86. Ramasamy K, Agarwal R (2008) Multitargeted therapy of cancer by silymarin. Cancer Lett 269: 352–362. doi:10.1016/j.canlet.2008.03.053
87. Singh RP, Agarwal R (2006) Prostate cancer chemoprevention by silibinin: bench to bedside. Mol Carcinog 45: 436–442. doi:10.1002/mc.20223
88. Hoh C et al. (2006) Pilot study of oral silibinin, a putative chemopreventive agent, in colorectal cancer patients: silibinin levels in plasma, colorectum, and liver and their pharmacodynamic consequences. Clin Cancer Res 12: 2944–2950. doi:10.1158/1078-0432.CCR-05-2724
89. Sangeetha N, Viswanathan P, Balasubramanian T, Nalini N (2011) Colon cancer chemopreventive efficacy of silibinin through perturbation of xenobiotic metabolizing enzymes in experimental rats. Eur J Pharmacol . doi:10.1016/j.ejphar.2011.11.008
90. Loguercio C, Festi D (2011) Silybin and the liver: From basic research to clinical practice. World J Gastroenterol 17: 2288–2301. doi:10.3748/wjg.v17.i18.2288
91. Kidd P, Head K (2005) A review of the bioavailability and clinical efficacy of milk thistle phytosome: a silybin-phosphatidylcholine complex (Siliphos). Altern Med Rev 10: 193–203
92. Barzaghi N, Crema F, Gatti G, Pifferi G, Perucca E (1990) Pharmacokinetic studies on IdB 1016, a silybin- phosphatidylcholine complex, in healthy human subjects. Eur J Drug Metab Pharmacokinet 15: 333–338
93. Hayashi H et al. (1999) Beneficial effect of salmon roe phosphatidylcholine in chronic liver disease. Curr Med Res Opin 15: 177–184. doi:10.1185/03007999909114089
94. Schandalik R, Gatti G, Perucca E (1992) Pharmacokinetics of silybin in bile following administration of silipide and silymarin in cholecystectomy patients. Arzneimittelforschung 42: 964–968
95. Flaig TW et al. (2007) A phase I and pharmacokinetic study of silybin-phytosome in prostate cancer patients. Invest New Drugs 25: 139–146. doi:10.1007/s10637-006-9019-2
96. Stickel F, Schuppan D (2007) Herbal medicine in the treatment of liver diseases. Dig Liver Dis 39: 293–304. doi:10.1016/j.dld.2006.11.004
97. Rambaldi A, Jacobs BP, Gluud C (2007) Milk thistle for alcoholic and/or hepatitis B or C virus liver diseases. Cochrane Database Syst Rev CD003620. doi:10.1002/14651858.CD003620.pub3
98. Falasca K et al. (2008) Treatment with silybin-vitamin E-phospholipid complex in patients with hepatitis C infection. J Med Virol 80: 1900–1906. doi:10.1002/jmv.21292
99. Polyak SJ et al. (2007) Inhibition of T-cell inflammatory cytokines, hepatocyte NF-kappaB signaling, and HCV infection by standardized Silymarin. Gastroenterology 132: 1925–1936. doi:10.1053/j.gastro.2007.02.038
100. Loguercio C et al. (2007) The effect of a silybin-vitamin e-phospholipid complex on nonalcoholic fatty liver disease: a pilot study. Dig Dis Sci 52: 2387–2395. doi:10.1007/s10620-006-9703-2

101. Saller R, Brignoli R, Melzer J, Meier R (2008) An updated systematic review with meta-analysis for the clinical evidence of silymarin. Forsch Komplementmed 15: 9–20. doi:10.1159/000113648
102. Enjalbert F et al. (2002) Treatment of amatoxin poisoning: 20-year retrospective analysis. J Toxicol Clin Toxicol 40: 715–757
103. Trouillas P et al. (2008) Mechanism of the antioxidant action of silybin and 2,3-dehydrosilybin flavonolignans: a joint experimental and theoretical study. J Phys Chem A 112: 1054–1063. doi:10.1021/jp075814h
104. Locher R, Suter PM, Weyhenmeyer R, Vetter W (1998) Inhibitory action of silibinin on low density lipoprotein oxidation. Arzneimittelforschung 48: 236–239
105. Ligeret H, Brault A, Vallerand D, Haddad Y, Haddad PS (2008) Antioxidant and mitochondrial protective effects of silibinin in cold preservation-warm reperfusion liver injury. J Ethnopharmacol 115: 507–514. doi:10.1016/j.jep.2007.10.024
106. Borsari M et al. (2001) Silybin, a new iron-chelating agent. J Inorg Biochem 85: 123–129
107. Guigas B et al. (2007) The flavonoid silibinin decreases glucose-6-phosphate hydrolysis in perfused rat hepatocytes by an inhibitory effect on glucose-6-phosphatase. Cell Physiol Biochem 20: 925–934. doi:10.1159/000110453
108. Lirussi F et al. (2002) Silybin-beta-cyclodextrin in the treatment of patients with diabetes mellitus and alcoholic liver disease. Efficacy study of a new preparation of an anti-oxidant agent. Diabetes Nutr Metab 15: 222–231
109. Kim J-L et al. (2011) Osteoblastogenesis and osteoprotection enhanced by flavonolignan silibinin in osteoblasts and osteoclasts. Journal of Cellular Biochemistry . doi:10.1002/jcb.23351
110. Yin F et al. (2011) Silibinin: A novel inhibitor of Aβ aggregation. Neurochem Int 58: 399–403. doi:10.1016/j.neuint.2010.12.017
111. Tota S, Kamat PK, Shukla R, Nath C (2011) Improvement of brain energy metabolism and cholinergic functions contributes to the beneficial effects of silibinin against streptozotocin induced memory impairment. Behav Brain Res 221: 207–215. doi:10.1016/j.bbr.2011.02.041
112. Bares JM et al. (2008) Silybin treatment is associated with reduction in serum ferritin in patients with chronic hepatitis C. J Clin Gastroenterol 42: 937–944. doi:10.1097/MCG.0b013e31815cff36
113. Sridar C, Goosen TC, Kent UM, Williams JA, Hollenberg PF (2004) Silybin inactivates cytochromes P450 3A4 and 2C9 and inhibits major hepatic glucuronosyltransferases. Drug Metab Dispos 32: 587–594. doi:10.1124/dmd.32.6.587
114. Strassburg CP (2008) Pharmacogenetics of Gilbert's syndrome. Pharmacogenomics 9: 703–715. doi:10.2217/14622416.9.6.703
115. Singh RP, Agarwal R (2005) Mechanisms and preclinical efficacy of silibinin in preventing skin cancer. Eur J Cancer 41: 1969–1979. doi:10.1016/j.ejca.2005.03.033
116. Lee S-O et al. (2007) Silibinin suppresses PMA-induced MMP-9 expression by blocking the AP-1 activation via MAPK signaling pathways in MCF-7 human breast carcinoma cells. Biochem Biophys Res Commun 354: 165–171. doi:10.1016/j.bbrc.2006.12.181
117. Bhatia N, Zhao J, Wolf DM, Agarwal R (1999) Inhibition of human carcinoma cell growth and DNA synthesis by silibinin, an active constituent of milk thistle: comparison with silymarin. Cancer Lett 147: 77–84
118. Singh RP et al. (2006) Effect of silibinin on the growth and progression of primary lung tumors in mice. J Natl Cancer Inst 98: 846–855. doi:10.1093/jnci/djj231

119. Lah J-J, Cui W, Hu K-Q (2007) Effects and mechanisms of silibinin on human hepatoma cell lines. World J Gastroenterol 13: 5299–5305
120. Tyagi A, Singh RP, Agarwal C, Agarwal R (2006) Silibinin activates p53-caspase 2 pathway and causes caspase-mediated cleavage of Cip1/p21 in apoptosis induction in bladder transitional-cell papilloma RT4 cells: evidence for a regulatory loop between p53 and caspase 2. Carcinogenesis 27: 2269–2280. doi:10.1093/carcin/bgl098
121. Li L et al. (2008) Silibinin inhibits cell growth and induces apoptosis by caspase activation, down-regulating survivin and blocking EGFR-ERK activation in renal cell carcinoma. Cancer Lett 272: 61–69. doi:10.1016/j.canlet.2008.06.033
122. Roy S et al. (2007) p21 and p27 induction by silibinin is essential for its cell cycle arrest effect in prostate carcinoma cells. Mol Cancer Ther 6: 2696–2707. doi:10.1158/1535-7163.MCT-07-0104
123. Kaur M et al. (2009) Silibinin suppresses growth and induces apoptotic death of human colorectal carcinoma LoVo cells in culture and tumor xenograft. Mol Cancer Ther 8: 2366–2374. doi:10.1158/1535-7163.MCT-09-0304
124. Sharma Y, Agarwal C, Singh AK, Agarwal R (2001) Inhibitory effect of silibinin on ligand binding to erbB1 and associated mitogenic signaling, growth, and DNA synthesis in advanced human prostate carcinoma cells. Mol Carcinog 30: 224–236
125. Singh RP et al. (2002) Dietary feeding of silibinin inhibits advance human prostate carcinoma growth in athymic nude mice and increases plasma insulin-like growth factor-binding protein-3 levels. Cancer Res 62: 3063–3069
126. Klampfer L (2006) Signal transducers and activators of transcription (STATs): Novel targets of chemopreventive and chemotherapeutic drugs. Curr Cancer Drug Targets 6: 107–121
127. Singh RP, Raina K, Deep G, Chan D, Agarwal R (2009) Silibinin suppresses growth of human prostate carcinoma PC-3 orthotopic xenograft via activation of extracellular signal-regulated kinase 1/2 and inhibition of signal transducers and activators of transcription signaling. Clin Cancer Res 15: 613–621. doi:10.1158/1078-0432.CCR-08-1846
128. Wang H-J, Tashiro S, Onodera S, Ikejima T (2008) Inhibition of insulin-like growth factor 1 receptor signaling enhanced silibinin-induced activation of death receptor and mitochondrial apoptotic pathways in human breast cancer MCF-7 cells. J Pharmacol Sci 107: 260–269
129. Luqman S, Pezzuto JM (2010) NFkappaB: a promising target for natural products in cancer chemoprevention. Phytother Res 24: 949–963. doi:10.1002/ptr.3171
130. Yoo HG et al. (2004) Involvement of NF-kappaB and caspases in silibinin-induced apoptosis of endothelial cells. Int J Mol Med 13: 81–86
131. Schümann J et al. (2003) Silibinin protects mice from T cell-dependent liver injury. J Hepatol 39: 333–340
132. Chittezhath M, Deep G, Singh RP, Agarwal C, Agarwal R (2008) Silibinin inhibits cytokine-induced signaling cascades and down-regulates inducible nitric oxide synthase in human lung carcinoma A549 cells. Mol Cancer Ther 7: 1817–1826. doi:10.1158/1535-7163.MCT-08-0256
133. Gu M, Singh RP, Dhanalakshmi S, Agarwal C, Agarwal R (2007) Silibinin inhibits inflammatory and angiogenic attributes in photocarcinogenesis in SKH-1 hairless mice. Cancer Res 67: 3483–3491. doi:10.1158/0008-5472.CAN-06-3955
134. Kerbel RS (2000) Tumor angiogenesis: past, present and the near future. Carcinogenesis 21: 505–515

135. López-Lázaro M (2006) Hypoxia-inducible factor 1 as a possible target for cancer chemoprevention. Cancer Epidemiol Biomarkers Prev 15: 2332–2335. doi:10.1158/1055-9965.EPI-06-0369
136. Yang S-H, Lin J-K, Chen W-S, Chiu J-H (2003) Anti-angiogenic effect of silymarin on colon cancer LoVo cell line. J Surg Res 113: 133–138
137. Singh RP, Gu M, Agarwal R (2008) Silibinin inhibits colorectal cancer growth by inhibiting tumor cell proliferation and angiogenesis. Cancer Res 68: 2043–2050. doi:10.1158/0008-5472.CAN-07-6247
138. Deep G, Agarwal R (2010) Antimetastatic efficacy of silibinin: molecular mechanisms and therapeutic potential against cancer. Cancer Metastasis Rev 29: 447–463. doi:10.1007/s10555-010-9237-0
139. Chu S-C, Chiou H-L, Chen P-N, Yang S-F, Hsieh Y-S (2004) Silibinin inhibits the invasion of human lung cancer cells via decreased productions of urokinase-plasminogen activator and matrix metalloproteinase-2. Mol Carcinog 40: 143–149. doi:10.1002/mc.20018
140. Hsieh Y-S et al. (2007) Silibinin suppresses human osteosarcoma MG-63 cell invasion by inhibiting the ERK-dependent c-Jun/AP-1 induction of MMP-2. Carcinogenesis 28: 977–987. doi:10.1093/carcin/bgl221
141. Raina K, Agarwal R (2007) Combinatorial strategies for cancer eradication by silibinin and cytotoxic agents: efficacy and mechanisms. Acta Pharmacol Sin 28: 1466–1475. doi:10.1111/j.1745-7254.2007.00691.x
142. Dhanalakshmi S, Agarwal P, Glode LM, Agarwal R (2003) Silibinin sensitizes human prostate carcinoma DU145 cells to cisplatin- and carboplatin-induced growth inhibition and apoptotic death. Int J Cancer 106: 699–705. doi:10.1002/ijc.11299
143. Tyagi AK, Singh RP, Agarwal C, Chan DCF, Agarwal R (2002) Silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth Inhibition, G2-M arrest, and apoptosis. Clin Cancer Res 8: 3512–3519
144. Tyagi AK, Agarwal C, Chan DCF, Agarwal R (2004) Synergistic anti-cancer effects of silibinin with conventional cytotoxic agents doxorubicin, cisplatin and carboplatin against human breast carcinoma MCF-7 and MDA-MB468 cells. Oncol Rep 11: 493–499
145. Scambia G et al. (1996) Antiproliferative effect of silybin on gynaecological malignancies: synergism with cisplatin and doxorubicin. Eur J Cancer 32A: 877–882
146. Giacomelli S et al. (2002) Silybin and its bioavailable phospholipid complex (IdB 1016) potentiate in vitro and in vivo the activity of cisplatin. Life Sci 70: 1447–1459
147. Singh RP et al. (2004) Oral silibinin inhibits lung tumor growth in athymic nude mice and forms a novel chemocombination with doxorubicin targeting nuclear factor kappaB-mediated inducible chemoresistance. Clin Cancer Res 10: 8641–8647. doi:10.1158/1078-0432.CCR-04-1435
148. Yang S-H et al. (2005) Silibinin inhibits angiogenesis via Flt-1, but not KDR, receptor up-regulation. J Surg Res 128: 140–146. doi:10.1016/j.jss.2005.04.042
149. Agarwal C et al. (2003) Silibinin upregulates the expression of cyclin-dependent kinase inhibitors and causes cell cycle arrest and apoptosis in human colon carcinoma HT-29 cells. Oncogene 22: 8271–8282. doi:10.1038/sj.onc.1207158
150. Kaur M et al. (2010) Silibinin suppresses growth of human colorectal carcinoma SW480 cells in culture and xenograft through down-regulation of beta-catenin-dependent signaling. Neoplasia 12: 415–424
151. Kohno H et al. (2002) Silymarin, a naturally occurring polyphenolic antioxidant flavonoid, inhibits azoxymethane-induced colon carcinogenesis in male F344 rats. Int J Cancer 101: 461–468. doi:10.1002/ijc.10625

152. Velmurugan B, Singh RP, Tyagi A, Agarwal R (2008) Inhibition of azoxymethane-induced colonic aberrant crypt foci formation by silibinin in male Fisher 344 rats. Cancer Prev Res (Phila) 1: 376–384. doi:10.1158/1940-6207.CAPR-08-0059
153. Sangeetha N, Felix AJW, Nalini N (2009) Silibinin modulates biotransforming microbial enzymes and prevents 1,2-dimethylhydrazine-induced preneoplastic changes in experimental colon cancer. Eur J Cancer Prev 18: 385–394. doi:10.1097/CEJ.0b013e32832d1b4f
154. Rajamanickam S, Kaur M, Velmurugan B, Singh RP, Agarwal R (2009) Silibinin suppresses spontaneous tumorigenesis in APC min/+ mouse model by modulating beta-catenin pathway. Pharm Res 26: 2558–2567. doi:10.1007/s11095-009-9968-1
155. van de Wetering M et al. (2002) The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 111: 241–250
156. Nagase H, Nakamura Y (1993) Mutations of the APC (adenomatous polyposis coli) gene. Hum Mutat 2: 425–434. doi:10.1002/humu.1380020602
157. Rajamanickam S, Velmurugan B, Kaur M, Singh RP, Agarwal R (2010) Chemoprevention of intestinal tumorigenesis in APCmin/+ mice by silibinin. Cancer Res 70: 2368–2378. doi:10.1158/0008-5472.CAN-09-3249
158. Ravichandran K, Velmurugan B, Gu M, Singh RP, Agarwal R (2010) Inhibitory effect of silibinin against azoxymethane-induced colon tumorigenesis in A/J mice. Clin Cancer Res 16: 4595–4606. doi:10.1158/1078-0432.CCR-10-1213
159. Velmurugan B et al. (2010) Silibinin exerts sustained growth suppressive effect against human colon carcinoma SW480 xenograft by targeting multiple signaling molecules. Pharm Res 27: 2085–2097. doi:10.1007/s11095-010-0207-6
160. Sangeetha N et al. (2010) Oral supplementation of silibinin prevents colon carcinogenesis in a long term preclinical model. Eur J Pharmacol 643: 93–100. doi:10.1016/j.ejphar.2010.05.060
161. Sangeetha N, Aranganathan S, Nalini N (2010) Silibinin ameliorates oxidative stress induced aberrant crypt foci and lipid peroxidation in 1, 2 dimethylhydrazine induced rat colon cancer. Invest New Drugs 28: 225–233. doi:10.1007/s10637-009-9237-5
162. Blumenthal M, Brinckmann JA, Wollschlaeger B (2003) The ABC clinical guide to herbs. Routledge, .
163. ([date unknown]) ephytosome.pdf (Objet application/pdf). http://www.indena.com/pdf/ephytosome.pdf. Accessed 13 Sep 2011
164. Flaig TW et al. (2010) A study of high-dose oral silybin-phytosome followed by prostatectomy in patients with localized prostate cancer. Prostate 70: 848–855. doi:10.1002/pros.21118
165. Hewitt RE et al. (2000) Validation of a model of colon cancer progression. J Pathol 192: 446–454. doi:10.1002/1096-9896(2000)9999:9999<::AID-PATH775>3.0.CO;2-K
166. Lamy V et al. (2008) Lupulone, a hop bitter acid, activates different death pathways involving apoptotic TRAIL-receptors, in human colon tumor cells and in their derived metastatic cells. Apoptosis 13: 1232–1242. doi:10.1007/s10495-008-0250-5
167. Shapiro H (2003) Practical flow cytometry. , 4th ed. edn. Wiley-Liss, New York.
168. Ormerod M (1999) Flow cytometry. , 2nd ed. edn. Bios Scientific Publishers;Springer-Verlag, Oxford UK ;New York.
169. Riccardi C, Nicoletti I (2006) Analysis of apoptosis by propidium iodide staining and flow cytometry. Nat Protoc 1: 1458–1461. doi:10.1038/nprot.2006.238
170. Hanshaw RG, Smith BD (2005) New reagents for phosphatidylserine recognition and detection of apoptosis. Bioorg Med Chem 13: 5035–5042. doi:10.1016/j.bmc.2005.04.071

171. Lockshin RA, Zakeri Z (2004) Apoptosis, autophagy, and more. The International Journal of Biochemistry & Cell Biology 36: 2405–2419. doi:10.1016/j.biocel.2004.04.011
172. Fulda S, Debatin K-M (2006) Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 25: 4798–4811. doi:10.1038/sj.onc.1209608
173. Green and John C. Reed DR (1998) Mitochondria and Apoptosis. Science 281: 1309 – 1312. doi:10.1126/science.281.5381.1309
174. Novo D, Perlmutter NG, Hunt RH, Shapiro HM (1999) Accurate flow cytometric membrane potential measurement in bacteria using diethyloxacarbocyanine and a ratiometric technique. Cytometry 35: 55–63
175. Fischer B et al. (2005) Fast neutrons-induced apoptosis is Fas-independent in lymphoblastoid cells. Biochem Biophys Res Commun 334: 533–542. doi:10.1016/j.bbrc.2005.06.125
176. Paglin S et al. (2001) A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. Cancer Res 61: 439–444
177. Millot C, Millot JM, Morjani H, Desplaces A, Manfait M (1997) Characterization of acidic vesicles in multidrug-resistant and sensitive cancer cells by acridine orange staining and confocal microspectrofluorometry. J Histochem Cytochem 45: 1255–1264
178. Skehan P et al. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82: 1107–1112
179. Vichai V, Kirtikara K (2006) Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protocols 1: 1112–1116. doi:10.1038/nprot.2006.179
180. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
181. Asai K, Buurman WA, Reutelingsperger CPM, Schutte B, Kaminishi M (2003) Low concentrations of ethanol induce apoptosis in human intestinal cells. Scandinavian Journal of Gastroenterology 38: 1154–1161. doi:10.1080/00365520310006252
182. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Research 6: 986 –994. doi:10.1101/gr.6.10.986
183. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408. doi:10.1006/meth.2001.1262
184. Bissonnette M et al. (2000) Mutational and nonmutational activation of p21ras in rat colonic azoxymethane-induced tumors: effects on mitogen-activated protein kinase, cyclooxygenase-2, and cyclin D1. Cancer Res 60: 4602–4609
185. Wali RK et al. (2002) Ursodeoxycholic Acid and F6-D3 Inhibit Aberrant Crypt Proliferation in the Rat Azoxymethane Model of Colon Cancer. Cancer Epidemiol Biomarkers Prev 11: 1653–1662
186. Spelt L, Andersson B, Nilsson J, Andersson R (2012) Prognostic models for outcome following liver resection for colorectal cancer metastases: A systematic review. European Journal of Surgical Oncology (EJSO) 38: 16–24. doi:10.1016/j.ejso.2011.10.013
187. Aprahamian M et al. (2011) Myo-InositolTrisPyroPhosphate treatment leads to HIF-1α suppression and eradication of early hepatoma tumors in rats. Chembiochem 12: 777–783. doi:10.1002/cbic.201000619
188. Soler L et al. (2001) Fully automatic anatomical, pathological, and functional segmentation from CT scans for hepatic surgery. Comput Aided Surg 6: 131–142. doi:10.1002/igs.1016
189. Jemal A et al. (2009) Cancer statistics, 2009. CA Cancer J Clin 59: 225–249. doi:10.3322/caac.20006

190. Ferlay J, Parkin DM, Steliarova-Foucher E (2010) Estimates of cancer incidence and mortality in Europe in 2008. European Journal of Cancer 46: 765–781. doi:16/j.ejca.2009.12.014
191. Cooper K et al. (2010) Chemoprevention of colorectal cancer: systematic review and economic evaluation. Health Technol Assess 14: 1–206. doi:10.3310/hta14320
192. Naithani R, Huma LC, Moriarty RM, McCormick DL, Mehta RG (2008) Comprehensive review of cancer chemopreventive agents evaluated in experimental carcinogenesis models and clinical trials. Curr Med Chem 15: 1044–1071
193. Gupta SC, Kim JH, Prasad S, Aggarwal BB (2010) Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. Cancer Metastasis Rev 29: 405–434. doi:10.1007/s10555-010-9235-2
194. West NJ, Courtney EDJ, Poullis AP, Leicester RJ (2009) Apoptosis in the colonic crypt, colorectal adenomata, and manipulation by chemoprevention. Cancer Epidemiol Biomarkers Prev 18: 1680–1687. doi:10.1158/1055-9965.EPI-09-0006
195. Ghobrial IM, Witzig TE, Adjei AA (2005) Targeting apoptosis pathways in cancer therapy. CA Cancer J Clin 55: 178–194
196. Opipari AW Jr et al. (2004) Resveratrol-induced autophagocytosis in ovarian cancer cells. Cancer Res 64: 696–703
197. Bijnsdorp IV, Peters GJ, Temmink OH, Fukushima M, Kruyt FA (2010) Differential activation of cell death and autophagy results in an increased cytotoxic potential for trifluorothymidine compared to 5-fluorouracil in colon cancer cells. Int J Cancer 126: 2457–2468. doi:10.1002/ijc.24943
198. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G (2007) Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol 8: 741–752. doi:10.1038/nrm2239
199. Gazák R, Walterová D, Kren V (2007) Silybin and silymarin--new and emerging applications in medicine. Curr Med Chem 14: 315–338
200. Tyagi A et al. (2009) Growth inhibition and regression of lung tumors by silibinin: modulation of angiogenesis by macrophage-associated cytokines and nuclear factor-kappaB and signal transducers and activators of transcription 3. Cancer Prev Res (Phila) 2: 74–83. doi:10.1158/1940-6207.CAPR-08-0095
201. Duan W et al. (2010) Silibinin induced autophagic and apoptotic cell death in HT1080 cells through a reactive oxygen species pathway. J Pharmacol Sci 113: 48–56
202. Moore A, Donahue CJ, Bauer KD, Mather JP (1998) Simultaneous measurement of cell cycle and apoptotic cell death. Methods Cell Biol 57: 265–278
203. Hsu W-H et al. (2007) Berberine induces apoptosis in SW620 human colonic carcinoma cells through generation of reactive oxygen species and activation of JNK/p38 MAPK and FasL. Arch Toxicol 81: 719–728. doi:10.1007/s00204-006-0169-y
204. Wu X et al. (2010) Isothiocyanates induce oxidative stress and suppress the metastasis potential of human non-small cell lung cancer cells. BMC Cancer 10: 269. doi:10.1186/1471-2407-10-269
205. Gao J, Liu X, Rigas B (2005) Nitric oxide-donating aspirin induces apoptosis in human colon cancer cells through induction of oxidative stress. Proc Natl Acad Sci USA 102: 17207–17212. doi:10.1073/pnas.0506893102
206. Sun Y, Rigas B (2008) The thioredoxin system mediates redox-induced cell death in human colon cancer cells: implications for the mechanism of action of anticancer agents. Cancer Res 68: 8269–8277. doi:10.1158/0008-5472.CAN-08-2010

207. Sun Y, Chen J, Rigas B (2009) Chemopreventive Agents Induce Oxidative Stress in Cancer Cells Leading to COX-2 Overexpression and COX-2-Independent Cell Death. Carcinogenesis 30: 93–100. doi:10.1093/carcin/bgn242
208. Duan W-J et al. (2011) Silibinin activated ROS-p38-NF-κB positive feedback and induced autophagic death in human fibrosarcoma HT1080 cells. J Asian Nat Prod Res 13: 27–35. doi:10.1080/10286020.2010.540757
209. Fan S et al. (2011) Silibinin induced-autophagic and apoptotic death is associated with an increase in reactive oxygen and nitrogen species in HeLa cells. Free Radic Res 45: 1307–1324. doi:10.3109/10715762.2011.618186
210. Kim KW et al. (2009) Silibinin inhibits glioma cell proliferation via Ca2+/ROS/MAPK-dependent mechanism in vitro and glioma tumor growth in vivo. Neurochem Res 34: 1479–1490. doi:10.1007/s11064-009-9935-6
211. Fan S et al. (2012) P53 activation plays a crucial role in silibinin induced ROS generation via PUMA and JNK. Free Radic Res 46: 310–319. doi:10.3109/10715762.2012.655244
212. Rodrigues NR et al. (1990) p53 mutations in colorectal cancer. Proc Natl Acad Sci USA 87: 7555–7559
213. Kim S-H, Ricci MS, El-Deiry WS (2008) Mcl-1: a gateway to TRAIL sensitization. Cancer Res 68: 2062–2064. doi:10.1158/0008-5472.CAN-07-6278
214. Singh RP, Dhanalakshmi S, Agarwal C, Agarwal R (2005) Silibinin strongly inhibits growth and survival of human endothelial cells via cell cycle arrest and downregulation of survivin, Akt and NF-kappaB: implications for angioprevention and antiangiogenic therapy. Oncogene 24: 1188–1202. doi:10.1038/sj.onc.1208276
215. Yang D et al. (2012) Decitabine and vorinostat cooperate to sensitize colon carcinoma cells to fas ligand-induced apoptosis in vitro and tumor suppression in vivo. J Immunol 188: 4441–4449. doi:10.4049/jimmunol.1103035
216. Taniguchi H et al. (2008) Baicalein overcomes tumor necrosis factor-related apoptosis-inducing ligand resistance via two different cell-specific pathways in cancer cells but not in normal cells. Cancer Res 68: 8918–8927. doi:10.1158/0008-5472.CAN-08-1120
217. Singh RP, Dhanalakshmi S, Mohan S, Agarwal C, Agarwal R (2006) Silibinin inhibits UVB- and epidermal growth factor-induced mitogenic and cell survival signaling involving activator protein-1 and nuclear factor-kappaB in mouse epidermal JB6 cells. Mol Cancer Ther 5: 1145–1153. doi:10.1158/1535-7163.MCT-05-0478
218. Momeny M et al. (2008) Effects of silibinin on cell growth and invasive properties of a human hepatocellular carcinoma cell line, HepG-2, through inhibition of extracellular signal-regulated kinase 1/2 phosphorylation. Eur J Pharmacol 591: 13–20. doi:10.1016/j.ejphar.2008.06.011
219. Chen P-N et al. (2006) Silibinin inhibits invasion of oral cancer cells by suppressing the MAPK pathway. J Dent Res 85: 220–225
220. Raina K, Agarwal C, Agarwal R (2011) Effect of silibinin in human colorectal cancer cells: Targeting the activation of NF-κB signaling. Mol Carcinog . doi:10.1002/mc.21843
221. Voboril R, Weberova-Voborilova J (2006) Constitutive NF-kappaB activity in colorectal cancer cells: impact on radiation-induced NF-kappaB activity, radiosensitivity, and apoptosis. Neoplasma 53: 518–523
222. Huerta S et al. (2007) Modification of gene products involved in resistance to apoptosis in metastatic colon cancer cells: roles of Fas, Apaf-1, NFkappaB, IAPs, Smac/DIABLO, and AIF. J Surg Res 142: 184–194. doi:10.1016/j.jss.2006.12.551

223. Lamy V et al. (2010) p53 Activates Either Survival or Apoptotic Signaling Responses in Lupulone-Treated Human Colon Adenocarcinoma Cells and Derived Metastatic Cells. Transl Oncol 3: 286–292
224. Rochette PJ, Bastien N, Lavoie J, Guérin SL, Drouin R (2005) SW480, a p53 double-mutant cell line retains proficiency for some p53 functions. J Mol Biol 352: 44–57. doi:10.1016/j.jmb.2005.06.033
225. Sugikawa E et al. (1999) Mutant p53 mediated induction of cell cycle arrest and apoptosis at G1 phase by 9-hydroxyellipticine. Anticancer Res 19: 3099–3108
226. Tiwari P, Kumar A, Balakrishnan S, Kushwaha HS, Mishra KP (2011) Silibinin-induced apoptosis in MCF7 and T47D human breast carcinoma cells involves caspase-8 activation and mitochondrial pathway. Cancer Invest 29: 12–20. doi:10.3109/07357907.2010.535053
227. Fulda S (2012) Histone deacetylase (HDAC) inhibitors and regulation of TRAIL-induced apoptosis. Experimental Cell Research . doi:10.1016/j.yexcr.2012.02.005
228. Laird PW et al. (1995) Suppression of intestinal neoplasia by DNA hypomethylation. Cell 81: 197–205
229. Cameron EE, Bachman KE, My|[ouml]|h|[auml]|nen S, Herman JG, Baylin SB (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nature Genetics 21: 103–107. doi:10.1038/5047
230. Thakur VS, Gupta K, Gupta S (2011) Green tea polyphenols causes cell cycle arrest and apoptosis in prostate cancer cells by suppressing class I histone deacetylases. Carcinogenesis . doi:10.1093/carcin/bgr277
231. Fang MZ et al. (2003) Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. Cancer Res 63: 7563–7570
232. Fini L et al. (2007) Annurca Apple Polyphenols Have Potent Demethylating Activity and Can Reactivate Silenced Tumor Suppressor Genes in Colorectal Cancer Cells. J Nutr 137: 2622–2628
233. Fang MZ et al. (2005) Reversal of hypermethylation and reactivation of p16INK4a, RARbeta, and MGMT genes by genistein and other isoflavones from soy. Clin Cancer Res 11: 7033–7041. doi:10.1158/1078-0432.CCR-05-0406
234. Shu L et al. (2011) Epigenetic CpG demethylation of the promoter and reactivation of the expression of Neurog1 by curcumin in prostate LNCaP cells. AAPS J 13: 606–614. doi:10.1208/s12248-011-9300-y
235. Yan G, Graham K, Lanza-Jacoby S (2012) Curcumin enhances the anticancer effects of trichostatin a in breast cancer cells. Molecular carcinogenesis . doi:10.1002/mc.21875
236. Cui W, Gu F, Hu K-Q (2009) Effects and mechanisms of silibinin on human hepatocellular carcinoma xenografts in nude mice. World J Gastroenterol 15: 1943–1950
237. Falschlehner C, Emmerich CH, Gerlach B, Walczak H (2007) TRAIL signalling: decisions between life and death. Int J Biochem Cell Biol 39: 1462–1475. doi:10.1016/j.biocel.2007.02.007
238. Zhang L, Fang B (2005) Mechanisms of resistance to TRAIL-induced apoptosis in cancer. Cancer Gene Ther 12: 228–237. doi:10.1038/sj.cgt.7700792
239. Mellier G, Huang S, Shenoy K, Pervaiz S (2010) TRAILing death in cancer. Mol Aspects Med 31: 93–112. doi:10.1016/j.mam.2009.12.002
240. Maldonado-Celis ME et al. (2009) Differential induction of apoptosis by apple procyanidins in TRAIL-sensitive human colon tumor cells and derived TRAIL-resistant metastatic cells. J Cancer Mol 5: 21–30

241. Rushworth SA, Micheau O (2009) Molecular crosstalk between TRAIL and natural antioxidants in the treatment of cancer. Br J Pharmacol 157: 1186–1188. doi:10.1111/j.1476-5381.2009.00266.x
242. Thorburn A, Behbakht K, Ford H (2008) TRAIL receptor-targeted therapeutics: resistance mechanisms and strategies to avoid them. Drug Resist Updat 11: 17–24. doi:10.1016/j.drup.2008.02.001
243. Bellail AC, Qi L, Mulligan P, Chhabra V, Hao C (2009) TRAIL agonists on clinical trials for cancer therapy: the promises and the challenges. Rev Recent Clin Trials 4: 34–41
244. (2011) Toxicology and carcinogenesis studies of milk thistle extract (CAS No. 84604-20-6) in F344/N rats and B6C3F1 mice (Feed Studies). Natl Toxicol Program Tech Rep Ser 1–177
245. Son Y-G et al. (2007) Silibinin sensitizes human glioma cells to TRAIL-mediated apoptosis via DR5 up-regulation and down-regulation of c-FLIP and survivin. Cancer Res 67: 8274–8284. doi:10.1158/0008-5472.CAN-07-0407
246. Hu WH, Johnson H, Shu HB (1999) Tumor necrosis factor-related apoptosis-inducing ligand receptors signal NF-kappaB and JNK activation and apoptosis through distinct pathways. J Biol Chem 274: 30603–30610
247. Martinez-Lostao L, Marzo I, Anel A, Naval J (2012) Targeting the Apo2L/TRAIL system for the therapy of autoimmune diseases and cancer. Biochem Pharmacol 83: 1475–1483. doi:10.1016/j.bcp.2011.12.036
248. Thanaketpaisarn O, Waiwut P, Sakurai H, Saiki I (2011) Artesunate enhances TRAIL-induced apoptosis in human cervical carcinoma cells through inhibition of the NF-κB and PI3K/Akt signaling pathways. Int J Oncol 39: 279–285. doi:10.3892/ijo.2011.1017
249. Kim M-O et al. (2010) beta-Ionone enhances TRAIL-induced apoptosis in hepatocellular carcinoma cells through Sp1-dependent upregulation of DR5 and downregulation of NF-kappaB activity. Mol Cancer Ther 9: 833–843. doi:10.1158/1535-7163.MCT-09-0610
250. Hu L, Cao D, Li Y, He Y, Guo K (2012) Resveratrol sensitized leukemia stem cell-like KG-1a cells to cytokine-induced killer cells-mediated cytolysis through NKG2D ligands and TRAIL receptors. Cancer biology & therapy 13: http://www.ncbi.nlm.nih.gov/pubmed/22406996. Accessed 10 May 2012
251. Wagner KW et al. (2007) Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. Nat Med 13: 1070–1077. doi:10.1038/nm1627
252. Rossin A, Derouet M, Abdel-Sater F, Hueber A-O (2009) Palmitoylation of the TRAIL receptor DR4 confers an efficient TRAIL-induced cell death signalling. Biochem J 419: 185–192, 2 p following 192. doi:10.1042/BJ20081212
253. Tsukamoto S et al. (2012) Green tea polyphenol EGCG induces lipid-raft clustering and apoptotic cell death by activating protein kinase Cδ and acid sphingomyelinase through a 67 kDa laminin receptor in multiple myeloma cells. Biochem J 443: 525–534. doi:10.1042/BJ20111837
254. Psahoulia FH, Drosopoulos KG, Doubravska L, Andera L, Pintzas A (2007) Quercetin enhances TRAIL-mediated apoptosis in colon cancer cells by inducing the accumulation of death receptors in lipid rafts. Mol Cancer Ther 6: 2591–2599. doi:10.1158/1535-7163.MCT-07-0001
255. Delmas D et al. (2003) Resveratrol-induced apoptosis is associated with Fas redistribution in the rafts and the formation of a death-inducing signaling complex in colon cancer cells. J Biol Chem 278: 41482–41490. doi:10.1074/jbc.M304896200

256. Delmas D et al. (2004) Redistribution of CD95, DR4 and DR5 in rafts accounts for the synergistic toxicity of resveratrol and death receptor ligands in colon carcinoma cells. Oncogene 23: 8979–8986. doi:10.1038/sj.onc.1208086
257. Grassme H et al. (2001) CD95 signaling via ceramide-rich membrane rafts. J Biol Chem 276: 20589–20596. doi:10.1074/jbc.M101207200
258. Maldonado-Celis ME, Bousserouel S, Gossé F, Lobstein A, Raul F (2009) Apple procyanidins activate apoptotic signaling pathway in human colon adenocarcinoma cells by a lipid-raft independent mechanism. Biochemical and biophysical research communications 388: 372–376
259. Boursi B, Arber N (2007) Current and future clinical strategies in colon cancer prevention and the emerging role of chemoprevention. Curr Pharm Des 13: 2274–2282
260. Reddy BS (2004) Studies with the azoxymethane-rat preclinical model for assessing colon tumor development and chemoprevention. Environ Mol Mutagen 44: 26–35. doi:10.1002/em.20026
261. Raju J (2008) Azoxymethane-induced rat aberrant crypt foci: relevance in studying chemoprevention of colon cancer. World J Gastroenterol 14: 6632–6635
262. Bird RP, Good CK (2000) The significance of aberrant crypt foci in understanding the pathogenesis of colon cancer. Toxicol Lett 112-113: 395–402
263. Bousserouel S et al. (2010) Identification of gene expression profiles correlated to tumor progression in a preclinical model of colon carcinogenesis. Int J Oncol 36: 1485–1490
264. Leeman MF, Curran S, Murray GI (2003) New insights into the roles of matrix metalloproteinases in colorectal cancer development and progression. J Pathol 201: 528–534. doi:10.1002/path.1466
265. Hayden DM, Forsyth C, Keshavarzian A (2011) The role of matrix metalloproteinases in intestinal epithelial wound healing during normal and inflammatory states. J Surg Res 168: 315–324. doi:10.1016/j.jss.2010.03.002
266. Kim S et al. (2008) Circulating levels of inflammatory cytokines and risk of colorectal adenomas. Cancer Res 68: 323–328. doi:10.1158/0008-5472.CAN-07-2924
267. Bousserouel S et al. (2010) Long-term administration of aspirin inhibits tumour formation and triggers anti-neoplastic molecular changes in a pre-clinical model of colon carcinogenesis. Oncol Rep 23: 511–517
268. Bousserouel S et al. (2011) Early modulation of gene expression used as a biomarker for chemoprevention in a preclinical model of colon carcinogenesis. Pathol Int 61: 80–87. doi:10.1111/j.1440-1827.2010.02621.x
269. Rocha FG, D'Angelica M (2010) Treatment of liver colorectal metastases: role of laparoscopy, radiofrequency ablation, and microwave coagulation. J Surg Oncol 102: 968–974. doi:10.1002/jso.21720
270. de Lope CR, Tremosini S, Forner A, Reig M, Bruix J (2012) Management of HCC. J Hepatol 56 Suppl 1: S75–87. doi:10.1016/S0168-8278(12)60009-9
271. Zhou W-P et al. (2009) A prospective, randomized, controlled trial of preoperative transarterial chemoembolization for resectable large hepatocellular carcinoma. Ann Surg 249: 195–202. doi:10.1097/SLA.0b013e3181961c16
272. Gadaleta CD, Ranieri G (2011) Trans-arterial chemoembolization as a therapy for liver tumours: New clinical developments and suggestions for combination with angiogenesis inhibitors. Crit Rev Oncol Hematol 80: 40–53. doi:10.1016/j.critrevonc.2010.10.005
273. Tanaka S, Arii S (2011) Molecular targeted therapy for hepatocellular carcinoma in the current and potential next strategies. J Gastroenterol 46: 289–296. doi:10.1007/s00535-011-0387-9

274. Dvorák Z, Vrzal R, Ulrichová J (2006) Silybin and dehydrosilybin inhibit cytochrome P450 1A1 catalytic activity: a study in human keratinocytes and human hepatoma cells. Cell Biol Toxicol 22: 81–90. doi:10.1007/s10565-006-0017-0
275. Leenders MWH, Nijkamp MW, Borel Rinkes IHM (2008) Mouse models in liver cancer research: a review of current literature. World J Gastroenterol 14: 6915–6923
276. Shin E-C et al. (2002) Human hepatocellular carcinoma cells resist to TRAIL-induced apoptosis, and the resistance is abolished by cisplatin. Exp Mol Med 34: 114–122
277. Yamanaka T et al. (2000) Chemotherapeutic agents augment TRAIL-induced apoptosis in human hepatocellular carcinoma cell lines. Hepatology 32: 482–490. doi:10.1053/jhep.2000.16266
278. Malhi H, Gores GJ, Lemasters JJ (2006) Apoptosis and necrosis in the liver: a tale of two deaths? Hepatology 43: S31–44. doi:10.1002/hep.21062
279. Silva MT (2010) Secondary necrosis: The natural outcome of the complete apoptotic program. FEBS Letters 584: 4491–4499. doi:10.1016/j.febslet.2010.10.046
280. Kondo Y, Kanzawa T, Sawaya R, Kondo S (2005) The role of autophagy in cancer development and response to therapy. Nat Rev Cancer 5: 726–734. doi:10.1038/nrc1692
281. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 646–674. doi:10.1016/j.cell.2011.02.013
282. Corpet DE, Taché S (2002) Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. Nutr Cancer 43: 1–21. doi:10.1207/S15327914NC431_1
283. Zucker S, Vacirca J (2004) Role of matrix metalloproteinases (MMPs) in colorectal cancer. Cancer Metastasis Rev 23: 101–117
284. Mikalauskas S et al. (2011) Dietary glycine protects from chemotherapy-induced hepatotoxicity. Amino Acids 40: 1139–1150. doi:10.1007/s00726-010-0737-6
285. Horinaka M et al. (2006) The dietary flavonoid apigenin sensitizes malignant tumor cells to tumor necrosis factor-related apoptosis-inducing ligand. Mol Cancer Ther 5: 945–951. doi:10.1158/1535-7163.MCT-05-0431
286. Horinaka M et al. (2005) The combination of TRAIL and luteolin enhances apoptosis in human cervical cancer HeLa cells. Biochem Biophys Res Commun 333: 833–838. doi:10.1016/j.bbrc.2005.05.179
287. Hasegawa H et al. (2006) Dihydroflavonol BB-1, an extract of natural plant Blumea balsamifera, abrogates TRAIL resistance in leukemia cells. Blood 107: 679–688. doi:10.1182/blood-2005-05-1982
288. Lippa MS et al. (2007) Expression of anti-apoptotic factors modulates Apo2L/TRAIL resistance in colon carcinoma cells. Apoptosis 12: 1465–1478. doi:10.1007/s10495-007-0076-6
289. Herrant M et al. (2004) Cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-induced apoptosis. Oncogene 23: 7863–7873. doi:10.1038/sj.onc.1208069
290. Velculescu VE, El-Deiry WS (1996) Biological and clinical importance of the p53 tumor suppressor gene. Clin Chem 42: 858–868
291. Jin Z, McDonald ER, Dicker DT, El-Deiry WS (2004) Deficient Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) Death Receptor Transport to the Cell Surface in Human Colon Cancer Cells Selected for Resistance to TRAIL-induced Apoptosis. Journal of Biological Chemistry 279: 35829 –35839. doi:10.1074/jbc.M405538200
292. Lah J-J, Cui W, Hu K-Q (2007) Effects and mechanisms of silibinin on human hepatoma cell lines. World J Gastroenterol 13: 5299–5305

293. Lund P et al. (2011) Transformation-dependent silencing of tumor-selective apoptosis-inducing TRAIL by DNA hypermethylation is antagonized by decitabine. Mol Cancer Ther 10: 1611–1623. doi:10.1158/1535-7163.MCT-11-0140
294. Kaminskyy VO, Surova OV, Vaculova A, Zhivotovsky B (2011) Combined inhibition of DNA methyltransferase and histone deacetylase restores caspase-8 expression and sensitizes SCLC cells to TRAIL. Carcinogenesis 32: 1450–1458. doi:10.1093/carcin/bgr135

LIST OF PUBLICATIONS

I. Kauntz H, Bousserouel S, Gossé F, Raul F (2011)
Silibinin triggers apoptotic signaling pathways and autophagic survival response in human colon adenocarcinoma cells and their derived metastatic cells.
Apoptosis 16: 1042–1053. DOI: 10.1007/s10495-011-0631-z

II. Kauntz H, Bousserouel S, Gossé F, Raul F (2012)
The flavonolignan silibinin potentiates TRAIL-induced apoptosis in human colon adenocarcinoma and in derived TRAIL-resistant metastatic cells.
Apoptosis 17: 797-809. DOI: 10.1007/s10495-012-0731-4

III. Kauntz H, Bousserouel S, Gossé F, Marescaux J, Raul F (2012)
Silibinin, a natural flavonoid, modulates the early expression of chemopreventive biomarkers in a preclinical model of colon carcinogenesis.
Int J Oncol 41: 849-854. DOI: 10.3892/ijo.2012.1526

IV. Bousserouel S, Bour G, Kauntz H, Gossé F, Marescaux J, Raul F (2012)
Silibinin inhibits tumor growth in a murine orthotopic hepatocarcinoma model and activates the TRAIL apoptotic signaling pathway.
Anticancer Research 32: 2455-2462.

V. Bousserouel S, Kauntz H, Gossé F, Bouhadjar M, Soler L, Marescaux J, Raul F (2010)
Identification of gene expression profiles correlated to tumor progression in a preclinical model of colon carcinogenesis.
Int J Oncol 36: 1485–1490. DOI: 10.3892/ijo_00000635

LIST OF COMMUNICATIONS

I. Kauntz H, Bousserouel S, Gossé F, Raul F.
La silybine active des voies apoptotiques et autophagiques dans les cellules cancéreuses coliques humaines et leurs dérivés métastatiques.
Séminaire Cancéropôle CLARA-NACRe Nutrition et Cancer: préventions primaire, secondaire et tertiaire.
19/05/2011, Lyon, France
(oral communication)

II. Kauntz H, Bousserouel S, Gossé F, Raul F.
The flavanolignan silibinin potentiates TRAIL-induced apoptosis in human colon adenoma and derived TRAIL-resistant metastatic cells.
4th International Congress on Nutrition & Cancer.
19-23/10/2011, Antalya, Turkey
(poster presentation)

III. Kauntz H, Bousserouel S, Gossé F, Raul F.
Cancer chemopreventive properties of the flavanolignan silibinin.
Tuesday Seminars in Biosciences at the Laboratory of Molecular and Cell Biology of Cancer (LBMCC).
20/03/2012, Luxembourg-Kirchberg, Luxembourg
(oral communication)