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Study report

Effects of *Haelan 951* on Tumor Cell Lines

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1. Introduction

Characteristics of Haelan 951

Haelan 951 is a concentrated fermented soybean protein beverage. The phytochemical substances contained in soybeans are broken down into their molecular units by a patented fermentation process. Haelan 951 contains among other things the bioactive isoflavones genistein and daidzein. The active agent MDT-13 (13-methyl-tetradecanoic acid), patented as an ingredient of Haelan 951, has shown to induce apoptosis (i.e. programmed cell death) in human tumor cell lines [1].

Objective of the study

In order to get more insight into the biological effects of Haelan 951 on tumor cells, the expression of selected genes in four different tumor cell lines treated with Haelan 951 was studied. For this, the influence of Haelan 951 on survival and the expression of genes involved in apoptosis and evolution of cancer were investigated. In each cell line, the differential expression of six genes was determined in relation to a housekeeping gene. The considered genes are shown in table 1.

Table 1: Considered genes of the study and their functions

Gene	Function	Description
GAPDH	Housekeeper	strong expression in equal amounts in all cell types. Used therefore as a reference gene on which the expression of the other genes is calibrated (= normalized). Since living cells are required for measuring GAPDH, dead cells do not disturb the normalization.
Bax	Apoptosis	mitochondrial membrane protein. Promotes apoptosis as an antagonist to Bcl2.
Bcl2	Apoptosis	mitochondrial membrane protein. Anti-apoptotic function. Is controlled by Bax. The relation of Bcl2 to Bax is important for assessment of the apoptotic status.
EGFR	Growth control	a cell surface receptor for the epidermal growth factor. Binding of the growth factor promotes cell proliferation. EGFR is deregulated in many tumors.
c-myc	cell proliferation	oncogene, transcription factor. Promotes cell division. Induces apoptosis in certain circumstances.
NF-kB	cell proliferation	transcription factor, Prevents cell death by induction of transcription of several anti-apoptotic and proliferative genes. The p65-subunit of NF-kB was measured in this study
Telomerase	cell division	prolongs the ends of the DNA of the chromosomes after each cell division. In contrast to normal cells which are limited in the number of cell division cycles, tumor cells are immortal. The expression of Telomerase in tumor cells is a crucial step for unlimited cell division.

2. Material and Methods

Cell lines

All used cell lines (table 2) were purchased from the German National Resource Centre for Biological Material, DSMZ (Deutsche Stammsammlung für Mikroorganismen und Zellen)

Table 2: Cell lines used for the study and their properties

Cell line	Origin and Description
BT474	human breast cancer cell line human breast ductal carcinoma DSMZ no.: ACC 64. Origin: established from a solid, invasive ductal carcinoma of the breast obtained from a 60-year-old woman; cells were reportedly tumorigenic in athymic mice and were found to be susceptible to mouse mammary tumor virus References: Lasfargues et al., J Natl Cancer Inst 61: 967 (1978) Depositor: Dr. H. Kirchner, Medical School of Hannover, Hannover, Germany DSMZ Cell Culture Data Morphology: adherent patches of epithelial cells; individual colonies with a thick center and a thin smooth margin Medium: 80% RPMI 1640 + 20% FBS + 10 µg/ml human insulin + 2 mM L-glutamine. Incubation: at 37 °C with 5% CO ₂ .
HepG2	human liver cancer cell line human hepatocellular carcinoma DSMZ no.: ACC 180. Origin: established from the tumor tissue of a 15-year-old Argentine boy with hepatocellular carcinoma in 1975; cell line is patented; cells were described not to harbor a hepatitis B virus genome; cells reportedly produce a variety of proteins: alpha-fetoprotein, albumin, alpha2-macroglobulin, alpha1-antitrypsin, transferrin, alpha1-antichymotrypsin, haptoglobin, ceruloplasmin, plasminogen, complement (C3, C4), C3 activator, fibrinogen, alpha1-acid glycoprotein, alpha2-HS glycoprotein, β-lipoprotein, retinol binding protein References: Aden et al., Nature 282: 615 (1979), Knowles et al., Science 209: 497 (1980). Depositor: obtained from ATCC (HB 8065), Rockville, Maryland, USA DSMZ Cell Culture Data Morphology: adherent, epithelial-like cells growing as monolayers and in small aggregates Medium: 90% RPMI 1640 + 10% FBS Incubation: at 37 °C with 5% CO ₂ .
LNCaP	human prostate carcinoma cell line human prostate carcinoma DSMZ no.: ACC 256. Origin: established from the left supraclavicular lymph node metastasis from a 50-year-old man with prostate carcinoma in 1977; cells were described to be androgen-sensitive References: Horoszewicz et al., Cancer Res 43: 1809 (1983). Gibas et al., Cancer Genet Cytogenet 11: 399 (1984). Horoszewicz et al., in Models for Prostate Cancer (ed. G.P. Murphy), Alan R. Liss, New York, 1981, p. 115-132 : Depositor: Prof. M. Motta, Universita di Milano, Milan, Italy DSMZ Cell Culture Data Morphology: adherent fibroblastoid cells growing in aggregates and as single cells Medium: 85-90% RPMI 1640 + 10-15% FBS. Incubation: at 37 °C with 5% CO ₂ .
SW480	human colorectal adenocarcinoma cell line human colon adenocarcinoma DSMZ no.: ACC 313. Origin: established from the tumor of a 50-year-old Caucasian man with colon adenocarcinoma (grade 4, Duke class B) References: Leibovitz et al., Cancer Res 36: 4562 (1976). Depositor: Dr. H. Kirchner, Medical School Hannover, Hannover, Germany DSMZ Cell Culture Data Morphology: endothelial-like adherent cells, mostly bipolar with microvilli growing in monolayers Medium: 90-95% RPMI 1640 + 5-10% FBS. Incubation: at 37°C with 5% CO ₂ .

Culturing conditions of cell lines

All cell lines were cultured according to the recommendations of the supplier DSMZ (see table above).

Cells were precultured in tissue culture flasks and harvested by trypsination when subconfluent. The number of cells was determined by microscopic visual counting in a Neubauer hemocytometer. The cell suspensions were then distributed in 24-well tissue culture plates

(Cellstar, Greiner, Germany) at a cell number of 2×10^5 cells per well. Cells were grown at 37° C, 5% CO₂ in 24-well plates until sub-confluency.

Incubation of cells with Haelan 951

Dose finding experiment: To determine the optimum active dosage of Haelan 951 on tumor cell lines, the cell lines were treated with different concentrations of the agent. The cells seeded at 2×10^5 cells per well in 24-well microtiter plates were grown until they reached sub-confluency. Then, culture medium containing different amounts of undiluted Haelan 951 was added. Cells were grown for 72 hours in 2 ml of medium containing final concentrations (% v/v) of Haelan 951 as depicted in table 3.

Table 3: Used concentrations of Haelan 951 in culture medium. First row, in % (v/v). Second row in μ l added to 2 ml culture medium

Used concentrations of Haelan 951 added to cell culture medium								
0.0 %	0.25 %	0.75 %	1.5 %	2.25 %	3.0 %	6.0 %	9.0 %	12 %
0 μ l	5 μ l	15 μ l	30 μ l	45 μ l	60 μ l	120 μ l	180 μ l	240 μ l

After 72h incubation in these media, attached cells were harvested from the bottom wells by trypsination and collected together with the cell in the supernatant by centrifugation (300 g, 10 min). The cell pellets were washed with PBS and afterwards processed for extraction of nucleic acids.

During the incubation intervals, cells on the plates were visually monitored under a light microscope for changes in morphology and detachment from the solid support.

Cell survival rate was determined by quantitative real-time RT-PCR for the gene GAPDH which served as “molecular cell counter”.

Extraction of Ribonucleic Acids (RNA) and Reverse Transcription

Total RNA was extracted from the cells with the commercial Qiaamp RNA Blood Minikit (Qiagen, Germany), employing Qia-shredder- and Qia-spin-columns according to the protocol of the manufacturer. Eluted total RNA was reverse transcribed into cDNA using random hexamers and MMLV-reverse transcriptase.

Analysis of gene expression

Quantitative mRNA expression was determined by real-time RT-PCR using 5'-nuclease („TaqMan“) chemistry (Applied Biosystems, USA). Each TaqMan run was accompanied by a serial log dilution of a control cell line for generation of the standard curve. Gene expression values of the Haelan – treated cell lines were set in relation to 2×10^6 cells according to the standard curve. Hence, all values in the following schedules are given in the unit of „cell equivalents according to the standard curve“ (CEQ).

To make the expression values comparable, the values were normalized to the housekeeper GAPDH, which is expressed in each cell line equally.

All gene expression measurements were done in duplicate.

3. Results

Determination of the effective dose optimum

The four cell lines were incubated under gradually increasing concentrations of Haelan 951 (0 % to 12 %) over 3 days. After harvesting the cells from the plates and extraction of RNA, GAPDH expression was determined by quantitative RT-PCR for each cell preparation. Since GAPDH is expressed only in viable cells, it served as a molecular cell counter for viability.

As shown in figure 1, all cells lines responded to a treatment with Haelan 951. LNCaP was most sensitive to Haelan 951, whereas HepG2 responded delayed and at somewhat higher concentrations.

At concentrations of 0.25% and 0.75%, no significant changes in cell morphology was observed. At these concentrations, there was only a minor reduction in cell viability measurable, especially in the case of HepG2 and SW480.

Increasing concentrations of Haelan 951 lead to a progressing decrease of measured GAPDH-values as well as a visual change in cell morphology and beginning detachment of the cells from the well bottom. At the concentration range of 2.25 – 3 % Haelan 951, an approximately 50 % reduction in cell viability was observed for all cell lines (fig. 1).

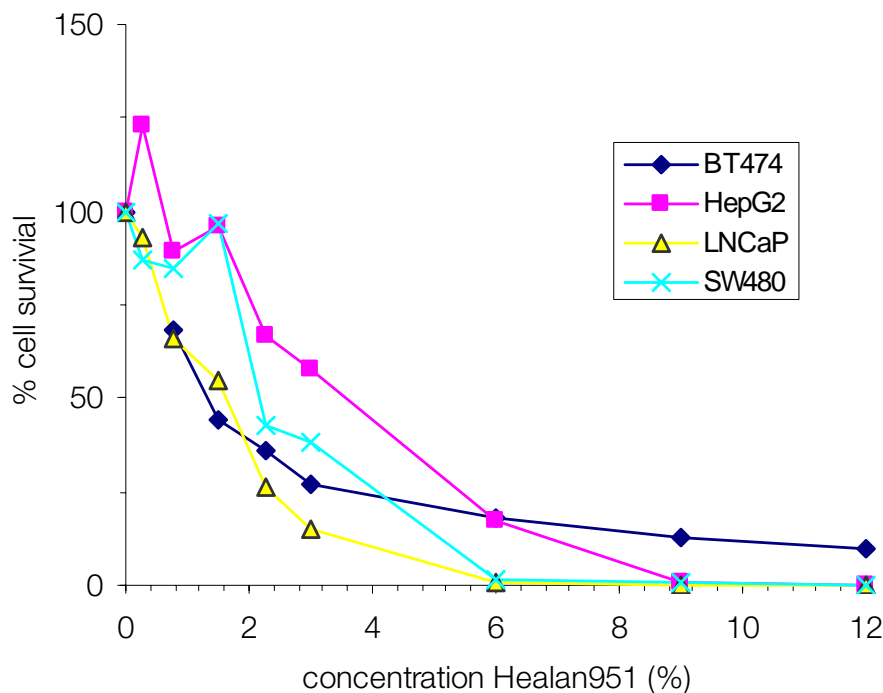
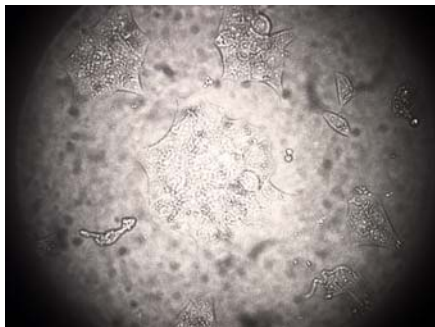


Figure 1: Determination of the cell survival rate in dependence of increasing concentrations of Haelan 951 in the culture media. Cell survival was measured by quantitative real-time PCR of the housekeeping-gene GAPDH. A reduction in the GAPDH-values correlates with cell death. The relative survival rate of untreated cells (0 % Haelan 951) was set to 100 % survival.

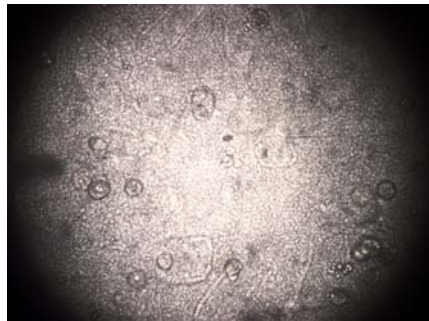
At 2.25 – 3 %, the cells rounded in their cell shape and started to detach from the solid support as was visible microscopically (fig. 2).

At concentrations $\geq 6.0\%$ of Haelan 951, the cell survival rate dropped below 20% or even lower for LNCaP and SW480 (fig. 1).

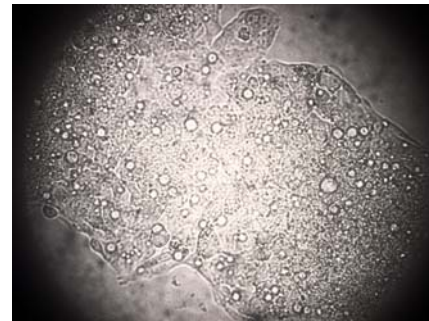
We therefore decided to perform the gene expression analysis of all further genes at Haelan-concentrations of 2.25 – 3 %, near the range of a 50 % rate of cell survival.



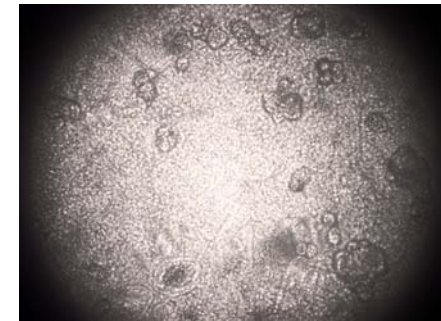
BT474 untreated



BT474, 2.25 % Haelan 951



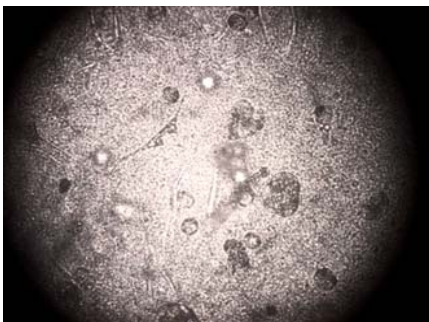
HEPG2 untreated



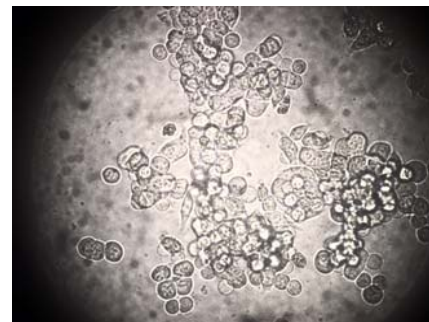
HepG2, 2.25 % Haelan 951



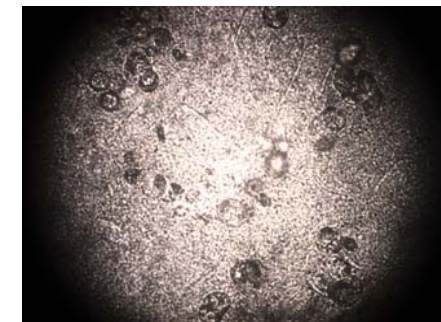
LNCaP untreated



LNCaP, 2.25 % Haelan 951



SW480 untreated



SW480, 2.25 % Haelan 951.

Figure 2.: Visual effects of Haelan 951 treatment over 72 h on tumor cell lines. Comparison between untreated and treated cells. The concentration of 2.25 % resulted from addition of 45 μ l undiluted Haelan 951 to 2 ml of culture medium.

Effects of Haelan 951 on the expression of tumor-associated genes

Prominent effects on the expression of the genes Bax, Bcl2, EGFR, c-myc, NF- κ B and Telomerase were observed in tumor cell lines grown for three days in presence of 2.25 and 3 % Haelan 951:

Effects in BT474 cells

Compared to the untreated cells, the most prominent effect observed in BT474 cell lines was an almost 5-fold drop in expression of Bcl2 (fig. 3). Despite of the slight increase (1.2 – 1.6-fold) in expression of Bax (an antagonist to Bcl2), the ratio of Bcl2/Bax decreased more than 5-fold compared to untreated cells (table 4). Obviously, the addition of Haelan 951 resulted in a suppression of an anti-apoptotic cell function like Bcl2 in BT474 cells.

There was only a minor increase (approx. 1.5-fold) in expression of the genes NF- κ B and telomerase. A minor decrease (ca. 1.6-fold) in expression of c-myc. EGFR levels were virtually unaffected by treatment with Haelan 951 (fig 3).

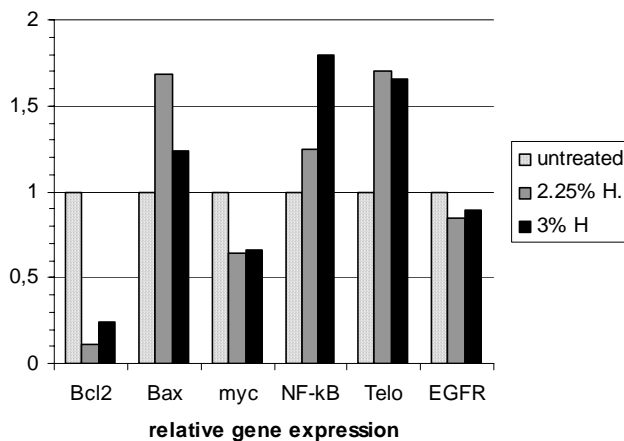


Figure 3: Effect of Haelan 951 on gene expression in BT474 cells. Relative expression of each analysed gene in untreated cells is set to 1. Overexpression, if values are >1. Underexpression, if values are <1. Haelan 951 is abbreviated as H.

Effects in HepG2 cells

The value of expression of Bax is nearly unchanged (1.2-fold decrease) compared to untreated HepG2 cells. However, Bcl2 decreased almost 3-fold upon treatment with Haelan 951 (fig 4). Consequently, the Bcl2/Bax ratio decreased (table 4), indicating the abrogation of the anti-apoptotic effect of Bcl2 induced by Haelan 951 as already observed in BT474 cells.

Expression of NF- κ B, EGFR and telomerase showed only minor changes generally lower than 1.5-fold. An obvious increase in the expression of c-myc was observed at 3 % Haelan 951, but not at 2.25 %. This is an interesting phenomenon in the context of the special relationship between c-myc and bcl2, which is discussed below.

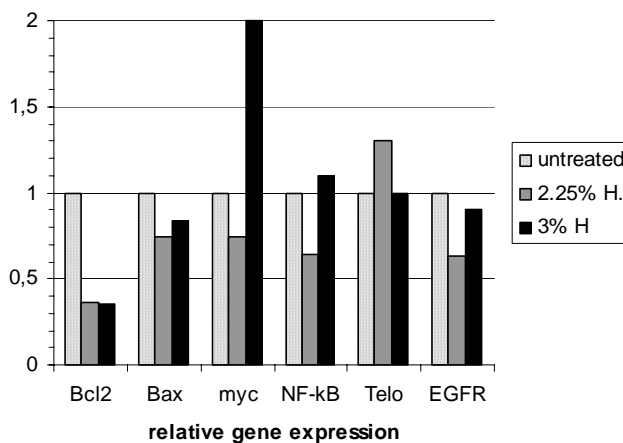


Figure 4: Effect of Haelan 951 on gene expression in HepG2 cells.

Effects in LNCaP cells

Generally, the changes of gene expression in LNCaP turned out to be more prominent at 3 % Haelan 951 than at 2.25% (fig. 5).

At 3% Haelan 951, the ratio Bcl2/Bax was not as drastically diminished as observed in BT474 and HepG2 cell lines (table 4). However, in contrast to these cells a four-fold reduction in the expression of the genes telomerase and EGFR was measured (fig. 5). In addition, a 1.5-fold reduction in the expression of NF-kB was observed (fig. 5). This implies an alternative mechanism of Haelan 951 in the induction of apoptosis in LNCaP cells compared to BT474 and HepG2. Perhaps a loss of ability of the cells to respond on growth signals by down-regulation of EGFR as well as reduced immortal properties by reduced telomerase function are responsible for killing of LNCaP by Haelan 951. The abrogation of anti-apoptotic signalling by downregulation of Bcl2/Bax may play a minor role in LNCaP cells.

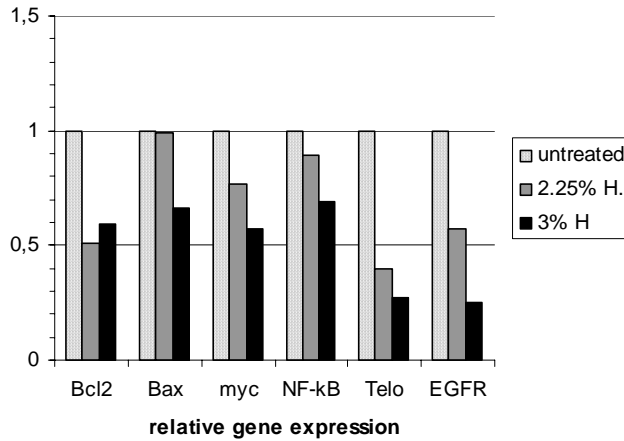


Figure 5: Effect of Haelan 951 on gene expression in LNCaP cells.

Effects in SW480 cells

A slightly lowered expression of Bax but a stronger, 2.5-fold down-regulation of Bcl2 implies a reduced anti-apoptotic activity as reflected by the Bcl2/Bax ratio.

This reduction in anti-apoptotic activity, accompanied by a clear, 2.5-fold increase in expression c-myc (fig. 6), probably drives the cells into apoptosis according to the oncogenic cooperation between bcl2 and c-myc as discussed below.

EGFR was highly overexpressed due to treatment with Haelan 951, rendering the cells more sensitive to growth signals. Obviously this did not aid to protect the cells from dying, probably due to the increased apoptotic activity induced by Haelan 951.

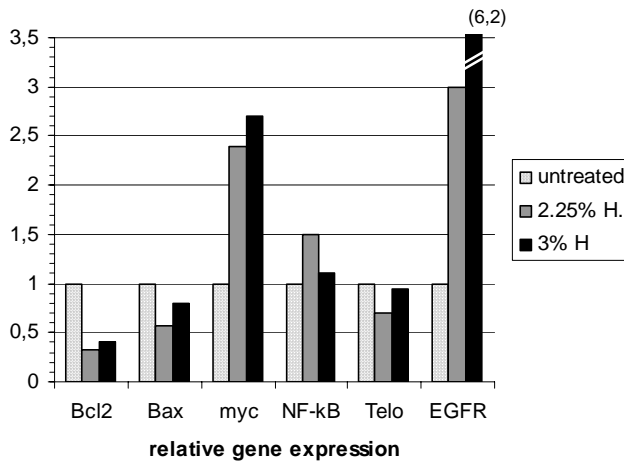


Figure 6: Effect of Haelan 951 on gene expression in SW480 cells. The bar for EGFR-expression at 3 % Haelan 951 is cut, the actual value was 6.2.

Effects observed in common between the cell lines

A common effect of Haelan treatment observed in all cell lines was a more or less prominent decline in the ratio of Bcl2/Bax. Lowering the anti-apoptotic effect of Bcl2 seems to be a common mechanism how Haelan 951 could induce apoptosis in tumor cells.

Table 4: Ratio of Bcl2/Bax after treatment with Haelan 951.

Conc. Haelan 951	Ratio Bcl2/Bax			
	BT474	HepG2	LNCaP	SW480
0 % (untreated)	1.0	1.0	1.0	1.0
2.25 %	0.06	0.49	0.5	0.56
3.0 %	0.19	0.42	0.89	0.5

4. Discussion

In all used cell lines, there was a clear relationship between the concentration of Haelan 951 in the culture medium and a proportional decline of the cell survival rate.

The most sensitive cell line was LNCaP, when cells began to die even at concentrations <1 %. The range where all cell lines showed a ~50 % survival rate turned out to be between 2.25 % and 3 % of Haelan 951 in the culture medium. The threshold concentration was at 6 % of Haelan 951, at which a sharp decline in cell viability occurred. At this concentration, more than 80 % of BT474 and HepG2 cells, and almost 100 % of LNCaP and SW480 tumor cells were killed.

Analysis of gene expression implied different mechanisms how Haelan 951 damages tumor cells. Three cells lines showed a clear reduction in Bcl2-expression and also an effective decline in the Bcl2/Bax ratio which brings the cells forward to apoptosis. Genistein, an isoflavone also included in Haelan 951 has already been described to reduce the Bcl2/Bax ratio in HT29 colon cancer cells [2] and primary gastric cancer cells [3]. Therefore, the observed effects on Bcl2/Bax could be possibly attributed to the flavonoid ingredients of Haelan 951.

Although SW480 and HepG2 cells did not show such a prominent reduction in the Bcl2/Bax ratio as BT474, apoptosis may be accelerated in these cells because of the Haelan-induced overexpression of c-myc. Oncogenic cooperation exists between Bcl2 and c-myc [4]. Normally, c-myc drives cell proliferation, but after a loss of Bcl2, c-myc facilitates apoptosis (fig. 7). Moderate reduction in Bcl2 may nonetheless lead to apoptosis if c-myc is concomitantly overexpressed, as seen in Haelan-treated SW480 and HepG2 cells.

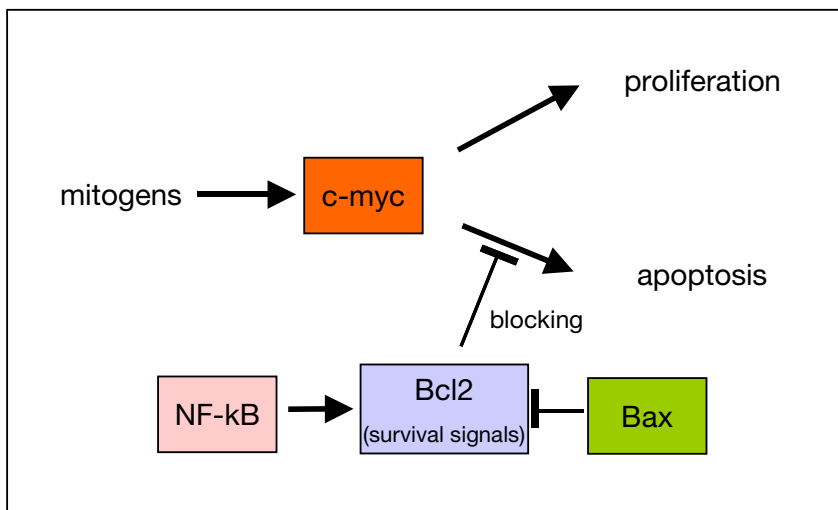


Figure 7: Dual signalling of c-myc (modified after Hueber and Evan, TIG 1998, 14, 364-7)

An alternative mechanism of Haelan-induced cell damage may work in LNCaP cells. At 3 % Haelan 951, the ratio of Bcl2/Bax in LNCAaP was not as diminished as in the other cell lines. However, these cells showed a strong Haelan-induced down-regulation of telomerase and EGFR. Haelan91 may inhibit tumor cell growth by i.) reduction of LNCaP's ability to respond on EGFR-transmitted growth signals and ii.) the reduced immortality by telomerase down-regulation. Equally, Ouchi H et al. described the isoflavone Genistein to be capable to down-regulate telomerase in LNCaP cells [5]. Although we did not observe down-regulation of telomerase in the other three cell lines, flavonoids may interfere with telomerase by other mechanisms than down-regulation of telomerase gene expression. For example, in MCF7-cells, Genistein has been reported to inhibit the translocation of telomerase to the nucleus [6].

Geistein has been connected to down-regulation of c-myc in LNCaP cells [5] and EGFR in DU145 prostate cancer cells [7], which is in concordance with our observed effects of Haelan 951 on LNCaP cells.

In contrast to LNCaP, the SW480 colon cancer cells showed a high overexpression of EGFR upon treatment with Haelan 951. This difference cannot fully explained by current knowledge from the literature, but differences in regulation of genetic pathways between the cell lines are possibly responsible for the varying responses. Indeed, tissue-dependent regulation of EGFR-expression upon treatment with Genistein in rats has been described by Brown & Lamartiniere [8].

Regarding the effect of Haelan 951 on the expression on NF- κ B, it is obvious that in three cells lines NF- κ B expression levels did not show significant change compared to the untreated cells. NF- κ B can activate gene expression of Bcl2 in many cells lines [9], however this effect was not observed in BT474 cells which slightly upregulated NF- κ B after treatment with Haelan 951. This may be explained by several inhibitory effects of Genistein on the NF- κ B pathway (fig. 8) described in the literature. As an effect of Genistein-treatment on LNCaP and PC3 cells, Davis et al. reported decreased NF- κ B DNA-binding capabilities and blocking of I- κ B phosphorylation upon cytotoxic stimuli, which is required for NF- κ B activation [10]. This post-translational inhibition of the NF- κ B by isoflavones suggests an additional pathway how Haelan 951 can promote apoptosis even if NF- κ B is upregulated as seen in BT474 cells.

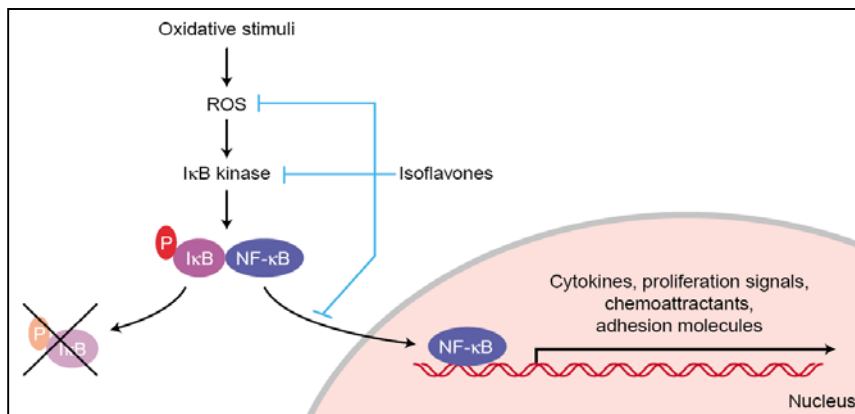


Figure 8: Inhibition of the NF- κ B pathway by isoflavones. I- κ B kinase is normally induced by reactive oxygen species (ROS) and phosphorylates I- κ B, the inhibitor of NF- κ B. This results in release of NF- κ B and transport to the nucleus, where NF- κ B initiates expression of various growth related genes. Isoflavones might counter activation of NF- κ B by reducing ROS, inhibiting I- κ B kinase and binding of NF- κ B to DNA.

5. Summary

- A clear relationship between the concentration of Haelan 951 in the culture medium and a proportional decline of the survival rate could be demonstrated for all four used tumor cell lines
- Visually, tumor cells showed detachment from the bottom of the culture wells and changes in cell morphology like rounding after treatment with Haelan 951.
- All four tumor cell lines responded with prominent changes in gene expression under the influence of Haelan 951 compared to untreated cells. The observed effects in the different cell lines varied for certain genes.
- A common effect observed more or less in all cell lines was down-regulation of Bcl2 and consequently a reduction of the Bcl2/Bax ratio, which generally leads to a pro-apoptotic condition of the tumor cells.

From this point of view, interesting perspectives arise. The inhibitor of apoptosis Bcl2 is overexpressed in more than half of all human cancers. The diminished apoptotic response caused by Bcl2 overexpression is associated with cellular resistance to many chemotherapeutic drugs which act by induction of apoptosis. Potential down-regulation of Bcl2 by Haelan 951 may sensitize drug-resistant tumor cells in which chemotherapy normally would be ineffective. A combinatorial use of Haelan 951 together with conventional chemotherapeutic drugs may therefore be very useful.

Further experiments could investigate a beneficial combinatorial effect of Haelan 951 and conventional chemotherapeutic drugs, especially in tumor cells overexpressing Bcl2 and being therefore less sensitive to the conventional drugs. In this context, measuring other parameters and drug targets associated with drug metabolism and apoptosis (e.g. the topoisomerase genes or p53) would be of interest.

6. Citations

- [1] Yang Z, et al. Induction of Apoptotic Cell Death and in Vivo Growth Inhibition of Human Cancer Cells by a Saturated Branched-Chain Fatty Acid, 13-Methyltetradecanoic Acid. *Cancer Res.* 2000, 60, 505-9
- [2] Lu QJ, Yu ZL. Effects of genistein on proliferation and apoptosis in HT-29 cells. *Wei Sheng Yan Jiu.* 2005 Sep;34:571-3.
- [3] Zhou HB, Chen JJ, Wang WX, Cai JT, Du Q. Apoptosis of human primary gastric carcinoma cells induced by genistein. *World J Gastroenterol.* 2004 Jun 15;10(12):1822-5.
- [4] Eischen CM, Woo D, Roussel MF, Cleveland JL. Apoptosis triggered by Myc-induced suppression of Bcl-X(L) or Bcl-2 is bypassed during lymphomagenesis. *Mol Cell Biol.* 2001 Aug;21(15):5063-70.
- [5] Ouchi H, Ishiguro H, Ikeda N, Hori M, Kubota Y, Uemura H. Genistein induces cell growth inhibition in prostate cancer through the suppression of telomerase activity. *Int J Urol.* 2005 Jan;12(1):73-80.
- [6] Chinni SR, Alhasan SA, Multani AS, Pathak S, Sarkar FH. Pleiotropic effects of genistein on MCF-7 breast cancer cells. *Int J Mol Med.* 2003 Jul;12(1):29-34.
- [7] Bhatia N, Agarwal R. Detrimental effect of cancer preventive phytochemicals silymarin, genistein and epigallocatechin 3-gallate on epigenetic events in human prostate carcinoma DU145 cells. *Prostate.* 2001 Feb 1;46(2):98-107.
- [8] Brown NM, Lamartiniere CA. Genistein regulation of transforming growth factor-alpha, epidermal growth factor (EGF), and EGF receptor expression in the rat uterus and vagina. *Cell Growth Differ.* 2000 May;11(5):255-60.
- [9] Fahy BN, Schlieman MG, Mortenson MM, Virudachalam S, Bold RJ. Targeting BCL-2 overexpression in various human malignancies through NF-kappaB inhibition by the proteasome inhibitor bortezomib. *Cancer Chemother Pharmacol.* 2005 Jul;56(1):46-54. Epub 2005 Mar 25.
- [10] Davis JN, Kucuk O, Sarkar FH. Genistein inhibits NF-kappa B activation in prostate cancer cells. *Nutr Cancer.* 1999;35(2):167-74.

Appendix

Biometrical data of the experiments

Appendix: Biometrical data

Experiment: Determination of the effective dose optimum

Cell line	Conc Haelan951 in %	GAPDH values (CEQ)	Mean GAPDH (CEQ)	% cell survival
BT474	0 (untreated control)	1464328 / 2097728	1781020	100
	0.75	1125177 / 1306945	1216061	68
	1.5	731383 / 829911	780647	44
	0 (untreated control)	1964568	1964568	100
	2.25	713830	713830	36
	0 (untreated control)	3113172	3113172	100
	3.0	856839	856839	27
	0 (untreated control)	9442784	9442784	100
	6.0	1731752	1731752	18
	9.0	1196590	1196590	13
12.0	980827	980827	10	
HepG2	0	5161707 / 5040976	5101342	100
	0.25	6633439 / 5942161	6287800	123
	0 (untreated control)	1378897 / 2302238	1840567	100
	0.75	1617976 / 1665941	1641958	89
	1.5	1490643 / 2029686	1760165	96
	0 (untreated control)	3761113	3761113	100
	2.25	2532114	2532114	67
	0 (untreated control)	2585966	2585966	100
	3.0	1491751	1491751	58
	0 (untreated control)	11009851	11009851	100
6.0	1868944	1868944	17	
9.0	120165	120165	1	
12.0	47925	47925	0	

Cell line	Conc Haelan951 in %	GAPDH values (CEQ)	Mean GAPDH (CEQ)	% cell survival
LNCaP	0	7221380 / 5732137	6476758	100
	0.25	6573992 / 5530932	6052462	93
	0 (untreated control)	3014721 / 3649088	3331904	100
	0.75	2285981 / 2113894	2199938	66
	1.5	1796114 / 1847891	1822003	55
	0 (untreated control)	4282286	4282286	100
	2.25	1133543	1133543	26
	0 (untreated control)	4520159	4520159	100
	3.0	704806	704806	15
	0 (untreated control)	10656020	10656020	100
	6.0	49309	49309	0,5
9.0	24016	24016	0,2	
12.0	15668	15668	0,1	
SW480	0	5518449 / 5028036	5273242	100
	0.25	4704821 / 4503817	4604319	87
	0 (untreated control)	2773886 / 3635535	3204710	100
	1.5	3014757 / 3208802	3111780	97
	0 (untreated control)	4540847	4540847	100
	2.25	1551238	1551238	43
	0 (untreated control)	4533390	4533390	100
	3.0	1718946	1718946	38
	0 (untreated control)	10462000	10462000	100
	6.0	155103	155103	1,5
	9.0	47768	47768	0,5
12.0	14020	14020	0,1	

Experiment: Effects of Haelan951 on the expression of tumor-associated genes

Cell line	Conc Haelan951 in %	Gene	CEQ gene	CEQ GAPDH	normalized	relative gene expression
BT474	0	Bcl2	5351206	1964568	2,720	1,00
	2.25		213007	713830	0,290	0,11
	0		6015108	3113172	1,930	1,00
	3.0		399150	856839	0,470	0,24
	0	Bax	580835	1964568	0,290	1,00
	2.25		355021	713830	0,490	1,68
	0		591542	2773453	0,210	1,00
	3.0		310863	1170471	0,260	1,24
	0	c-myc	289733	1964568	0,140	1,00
	2.25		69276	713830	0,090	0,64
	0		88812	2773453	0,030	1,00
	3.0		34646	1170471	0,020	0,66
0	NF-kB	4094899	1964568	2,080	1,00	
2.25		1848537	713830	2,590	1,25	
0		3332931	2943312	1,132	1,00	
3.0		2181669	1013655	2,152	1,90	
0	Telomerase	834029	1964568	0,430	1,00	
2.25		535022	713830	0,750	1,76	
0		742538	2943312	0,252	1,00	
3.0		400083	1013655	0,395	1,56	
0	EGFR	43067	1964568	0,021	1,00	
2.25		13252	713830	0,018	0,85	
0		53161	2773453	0,019	1,00	
3.0		20113	1170471	0,017	0,89	

Cell line	Conc Haelan951 in %	Gene	CEQ gene	CEQ GAPDH	normalized	relative gene expression
HepG2	0	Bcl2	48134	3761113	0,013	1,00
	2.25		11834	2532114	0,005	0,36
	0		79896	2585966	0,031	1,00
	3.0		16443	1491751	0,011	0,35
	0	Bax	3720984	3761113	0,989	1,00
	2.25		1861200	2532114	0,735	0,74
	0		4275874	4965771	0,860	1,00
	3.0		1149430	1591214	0,720	0,84
	0	c-myc	1395435	3761113	0,370	1,00
	2.25		729497	2532114	0,280	0,75
	0		458030	3775868	0,121	1,00
	3.0		377315	1541496	0,245	2,02
0	NF-kB	3815727	3761113	1,000	1,00	
2.25		1614813	2532114	0,640	0,64	
0		2862535	3775868	0,758	1,00	
3.0		1275294	1541496	0,827	1,09	
0	Telomerase	5354657	3761113	1,420	1,00	
2.25		4807936	2532114	1,890	1,33	
0		4037698	3775868	1,069	1,00	
3.0		1637522	1541496	1,062	0,99	
0	EGFR	44345	3761113	0,011	1,00	
2.25		19460	2532114	0,007	0,64	
0		56228	3775868	0,015	1,00	
3.0		22276	1541496	0,014	0,93	

Cell line	Conc Haelan951 in %	Gene	CEQ gene	CEQ GAPDH	normalized	relative gene expression
LNCaP	0	Bcl2	736014	4282286	0,170	1,00
	2.25		98256	1133543	0,087	0,51
	0		986611	2931160	0,340	1,00
	3.0		222644	1098614	0,200	0,59
	0	Bax	1295133	4282286	0,302	1,00
	2.25		338760	1133543	0,299	0,99
	0		830292	4520159	0,180	1,00
	3.0		86309	704806	0,012	0,66
	0	c-myc	1148881	4282286	0,270	1,00
	2.25		240991	1133543	0,210	0,77
	0		659328	4520159	0,140	1,00
	3.0		61033	704806	0,080	0,57
0	NF-kB	4479479	4282286	1,050	1,00	
2.25		1072609	1133543	0,940	0,89	
0		3272249	3725659	0,878	1,00	
3.0		561818	901710	0,623	0,70	
0	Telomerase	3369852	4282286	0,787	1,00	
2.25		354106	1133543	0,312	0,40	
0		1862220	2931160	0,635	1,00	
3.0		119814	1098614	0,109	0,27	
0	EGFR	346155	4282286	0,081	1,00	
2.25		52970	1133543	0,046	0,57	
0		579972	4520159	0,130	1,00	
3.0		22881	704806	0,032	0,25	

Cell line	Conc Haelan951 in %	Gene	CEQ gene	CEQ GAPDH	normalized	relative gene expression
SW480	0	Bcl2	922764	4540847	0,203	1,00
	2.25		98418	1551238	0,063	0,32
	0		2048789	3710616	0,550	1,00
	3.0		471281	2106466	0,220	0,40
	0	Bax	2677736	4540847	0,589	1,00
	2.25		516030	1551238	0,333	0,57
	0		1972603	4533390	0,430	1,00
	3.0		593222	1718946	0,340	0,79
	0	c-myc	2111062	4540847	0,460	1,00
	2.25		1736938	1551238	1,110	2,41
	0		1248911	4122003	0,303	1,00
	3.0		1573769	1912706	0,823	2,70
0	NF-kB	3951567	4540847	0,870	1,00	
2.25		2022752	1551238	1,300	1,50	
0		3432521	4122003	0,833	1,00	
3.0		1762733	1912706	0,922	1,11	
0	Telomerase	4661981	4540847	1,027	1,00	
2.25		1114977	1551238	0,719	0,70	
0		1267527	3710616	0,341	1,00	
3.0		674145	2106466	0,320	0,94	
0	EGFR	33205	4540847	0,007	1,00	
2.25		33735	1551238	0,021	3,00	
0		23478	4533390	0,005	1,00	
3.0		53499	1718946	0,031	6,20	