

Gemeinschaftspraxis für Laboratoriumsmedizin

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Study report

Effects of combined treatment with *Haelan 951* and Doxorubicin on the breast cancer cell line BT474

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1. Introduction

1.1. Objective of the study

The aim of this study was to investigate the combined action of Haelan 951 and the drug doxorubicin on the breast cancer cell line BT₄₇₄. The analyses mainly focused on how these substances altered the expression of selected genes as a consequence of treatment.

In succession to a former study done at Gemeinschaftspraxis Recklinghausen which covered the effects of Haelan 951 on four different tumor cell lines, the present work should elucidate the following questions:

- the effects of doxorubicin in monotherapy on the expression of selected genes in cultured BT₄₇₄ cells
- the effects of doxorubicin combined with Haelan 951 on the expression of the selected genes in BT₄₇₄ cells
- if it is possible to reduce the dosage of doxorubicin when combined with Haelan 951 to achieve comparable efficacy of higher doses of doxorubicin alone

1.2. Characteristics of doxorubicin

Doxorubicin (adriamycin) is pharmacologically an antibiotic cytostatic agent and belongs to the substance-class of anthracyclines. It is used as a chemotherapeutic drug for treatment of metastatic breast cancer and several other tumor types. Anthracyclines are DNA intercalating agents and interfere with the function of topoisomerase II. Topoisomerases are nuclear enzymes that regulate DNA topology during multiple DNA functions (including transcription, replication, and recombination), mediated by cleavage and relegation of DNA strands. Drug interference with these functions leads to lethal DNA damage like double-strand breaks (Wiese L, 2001).

Tumor cells with an enhanced proliferation rate and impaired repair functions are particularly vulnerable to cytostatic drugs. However, these drugs are also harmful to rapidly proliferating normal cells like bone marrow or hair follicle, leading to serious side-effects. Moreover, anthracyclines like doxorubicin are mutagenic and teratogenic.

1.3. Characteristics of Haelan 951

Haelan 951 is a fluidic, concentrated soy protein beverage, manufactured from specially cultured soybeans. The phytochemical substances contained in the soybeans are broken down into their molecular units by a patented fermentation process to achieve an improved bioavailability for the human organism. For one bottle of Haelan 951, 12 kg of soybean are processed.

Among other components, Haelan 951 contains the bioactive isoflavones genistein, daidzein, genistin as well as the saturated branched-chain fatty acid MDT-13 [13-methyl-tetradecanoic acid] (Kousidou O, et al., 2006). The active agent MDT-13 has shown to induce apoptosis in tumor cells and possesses anti-tumor activity (Yang Z, et al., 2000).

1.4. Description and function of the selected genes of this study

The study focused on induction of differential gene expression by treatment of the cell line BT474 with test-substances doxorubicin and/or Haelan 951. The genes which were included in the analysis are summarized in table 1.

Table 1: Function of the analyzed genes of the present study

Gene	Function	Description
GAPDH	Housekeeping gene	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, controlled by Bax. The ration of Bcl2 to Bax is important for assessment of the apoptotic status.
Topo IIa	Nucleic acid synthesis	Nuclear enzyme that regulates DNA topology during transcription, replication, and recombination. Catalyses the relaxation of supercoiled DNA by cleaving and rejoining of DNA double-strands. Altered expression of topoisomerase IIa has been reported in many tumor types.
MDR1	Efflux pump	MDR1 (P-glycoprotein) is a membrane proteine involved in detoxification acting as an efflux pump for various drugs (including doxorubicin)and xenobiotics. Overexpression of MDR1 in tumor cells leads to multi-drug resistance.
ERBB2	Growth signal receptor	ERBB2 (Her2/neu) is a cell-surface receptor tyrosine kinase involved in regulation of cell growth and differentiation. ERBB2 is frequently overexpressed in various tumor entities, including breast cancer.

2. Material and Methods

2.1. Test-substances / drugs

Haelan 951: Organic NON-GMO Soy. Fermented Soy Beverage. Made in China. Distributed by Haelan Products. INC. 18568 142nd Ave. N.E. Bldg.F. Woodinville, WA 98072 USA. www.Haelan951.com

Doxorubicin: Doxorubicin 10 HEXAL solution. Active agent: doxorubicin hydrochloride 10 mg in 5 ml bottle (2 µg/µl). Antibiotic, anti-tumor agent. HEXAL AG. 83607 Holzkirchen, Germany.

2.2. Cell lines

The cell line BT₄₇₄ was purchased from the German National Resource Centre for Biological Material, DSMZ (Deutsche Stammsammlung für Mikroorganismen und Zelllinien). The characteristics of the BT₄₇₄ cell line are summarized in table 2:

Table 2: Characteristics of the BT₄₇₄ cell line

Cell line	Origin and Description
BT ₄₇₄	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 .

2.3. Culturing conditions of cell lines

The BT₄₇₄ cells were cultured according to the recommendations of the supplier (tab. 2). 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 density of 1×10^5 cells per well. Medium containing the test-substances was added and the cells were grown at 37° C, 5% CO₂ for three days (72 h). Afterwards, cells were harvested by trypsination and ribonucleic acids extracted.

The culturing was regularly monitored microscopically for morphologic changes induced by the test-substances.

2.4. Extraction of ribonucleic acids (RNA) and reverse transcription

Total RNA was extracted from the cells with the commercial Qiamp 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.

2.5. Gene expression analysis

Quantitative mRNA expression was determined by real-time RT-PCR using 5'-nuclease ("TaqMan") chemistry (Applied Biosystems, USA). Standard curves were determined by a serial log dilution of cDNA generated from 2×10^6 cells of control cell lines known to express the genes of interest.

Gene expression values of the cells treated with test-substance were set in relation to the measured values of the standard curve. Hence, all gene expression measurements in the following schedules are given in the relative unit of „cell equivalents compared to the standard curve“ (CEQ).

For normalization, the CEQ-values of the gene of interest were set in relation to the measured CEQ-values of the housekeeping gene GAPDH (i.e. '**normalized gene expression**').

Finally, the **relative gene expression** was calculated by setting the normalized gene expression value of the untreated cells to unity ('1').

2.6. Dosage-determination of the applied test-substances

Preliminary experiments had to be performed to determine the dosage of the test-substances to be applied in monotherapy and in combination on the tumor cell cultures. For this, several concentration series of either Haelan 951 or doxorubicin were applied to BT474 cells cultured in 24-well microtiter plates. As mock-treated controls (cell cultures without any test-substance), PBS (phosphate buffered saline) was used instead of test-substances. The finally employed concentration series were chosen according to the concentrations of visible morphologic changes and noticed decline of GAPDH-expression during the preliminary experiments. Examples of micrographs of such morphologic changes are shown in figure 2.

The finally applied concentration series of the test-substances as determined by the preliminary experiments are shown in table 3, table 4 and table 5:

Table 3: Added amounts of the test-substance doxorubicin (DOX) [$2 \mu\text{g}/\mu\text{l}$] to 2 ml of culture medium.

Concentrations of DOX added to 2 ml of cell culture medium						
0 DOX	10 DOX	25 DOX	50 DOX	75 DOX	100 DOX	[μl] DOX
0	0.5	1.25	2.5	3.75	5	% (v/v)
0	20	50	100	150	200	[μg]

Table 4: Added amounts of the test-substance Haelan 951 (HAL) in 2 ml of culture medium.

Concentrations of HAL added to 2 ml of cell culture medium				
0 HAL	10 HAL	30 HAL	60 HAL	[μl] HAL
0	0.5	1.5	3.0	% (v/v)

Table 5: Used combinations of doxorubicin (DOX) and Haelan 951 (HAL). To each of the four different amounts of DOX three different amounts of Haelan 951 had been added which resulted in four combination series. The numbers indicate the amount (μ l) of test-substance added to 2 ml of cell culture medium.

1.)	10 DOX 10 HAL	10 DOX 30 HAL	10 DOX 60 HAL
2.)	25 DOX 10 HAL	25 DOX 30 HAL	25 DOX 60 HAL
3.)	50 DOX 10 HAL	50 DOX 30 HAL	50 DOX 60 HAL
4.)	100 DOX 10 HAL	100 DOX 30 HAL	100 DOX 60 HAL

3. Results

3.1. Influence of the test-substances on cell viability

The BT₄₇₄ cells were cultured for 3 days under gradually increasing concentrations of the test-substances doxorubicin, Haelan 951, and combinations of doxorubicin/Haelan 951 (tables 3 – 5).

After harvesting the cells from the plates and extraction of RNA, the expression of GAPDH was determined by quantitative RT-PCR for each cell culture. Since GAPDH is expressed only in living cells, it served as a molecular cell counter for viability and survival-rate.

As shown in figure 1, the viability of BT₄₇₄ cells declined in response to treatment with all test-substances in a dose-dependent manner:

a. Doxorubicin: The number of viable cells, as reflected in the measured expression of GAPDH, declined significantly under the treatment with doxorubicin (fig. 1a). Compared to the untreated cell cultures, the expression values of GAPDH dropped almost two-fold after the addition of 25 μ l doxorubicin and ca. 40-fold after the addition of 75 μ l doxorubicin.

b. Haelan 951: Under the influence of Haelan 951 the number of viable cells also decreased with increasing concentrations of applied test-substance. Compared to the untreated cell cultures, 5-fold and 10-fold reductions in viable cell numbers were observed after the addition of 30 μ l and 60 μ l Haelan 951, respectively.

c. Doxorubicin/Haelan 951 combinations: To constant amounts of doxorubicin, increasing amounts of Haelan 951 were added (tab. 5). Obviously, the dosage increment of Haelan 951 resulted in a steadily reduction of viable tumor cells (fig 1c). Evidently, there exists a synergy regarding cytotoxicity between the two substances. If higher doses of Haelan 951 were added, the concentration of doxorubicin could have been reduced to achieve comparable levels of cytotoxicity. E.g., the combination of 50 DOX 10 HAL showed the same effect as the combination 10 DOX 30 HAL (fig. 1c).

Synergistic action was observed at each of the three applied concentrations of doxorubicin (10 μ l, 25 μ l, and 50 μ l) if this drug was combined with Haelan 951 (fig 1c).

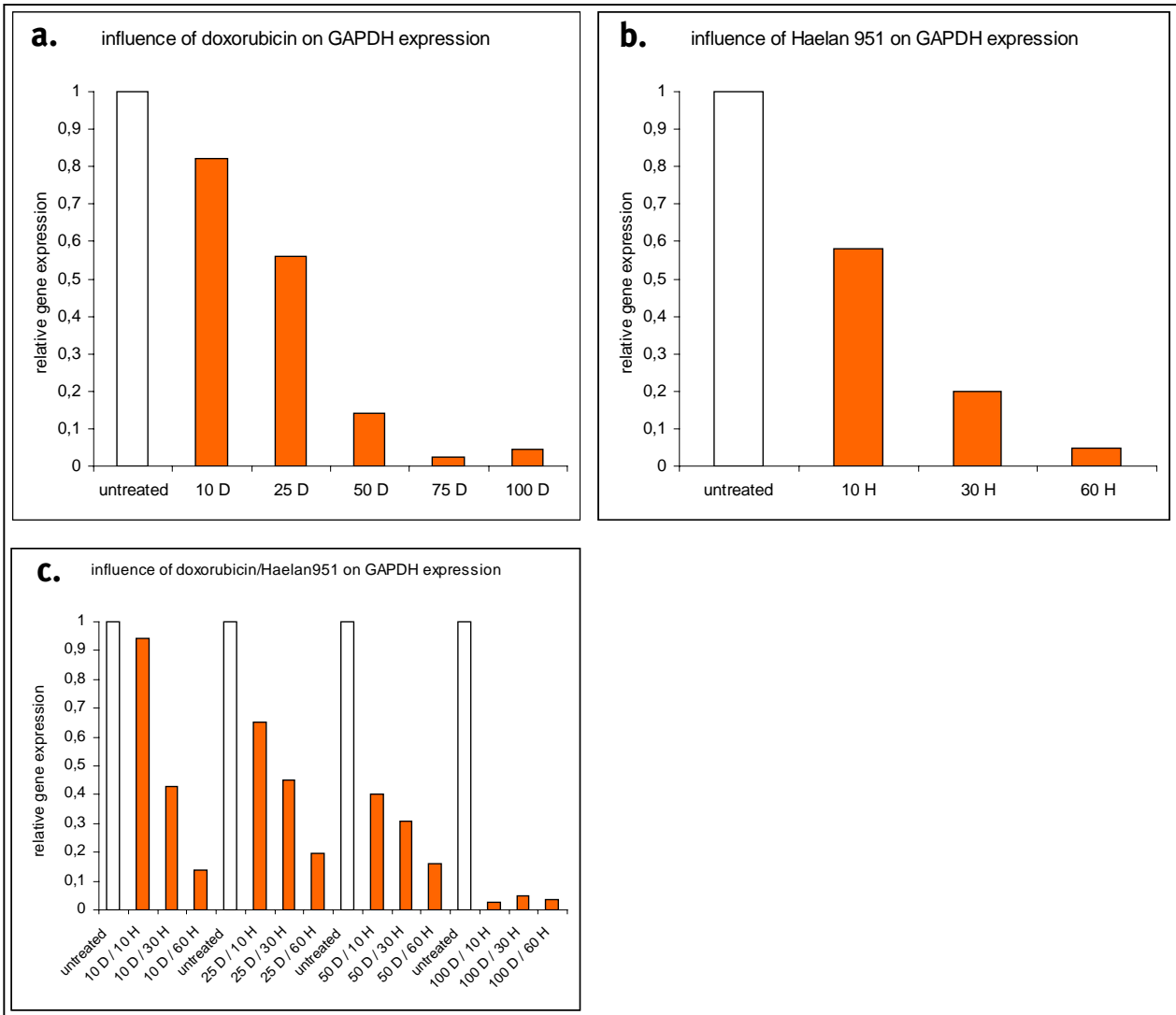


Figure 1: Effect of the test-substances on the viability of BT474 cells. Loss of viability is reflected in a decline of relative gene expression of the housekeeping gene GAPDH. Both doxorubicin and Haelan 951 induced a dose-dependent cytotoxic effect if these substances were added to the culture media (1a; 1b). Synergistic action of cytotoxicity was observed if doxorubicin and Haelan 951 were combined (1c). The underlying values of these graphs are listed in table A1 in the appendix. D = doxorubicin; H = Haelan 951.

3.2. Influence of the test-substances on cell morphology

If BT₄₇₄ cells are cultured under normal conditions, they grow in large, irregular shape (fig. 2a). Culturing of the cells under the test-substances influenced cell shape and cell size. During the treatment, cells became smaller and more rounded (fig. 2b).

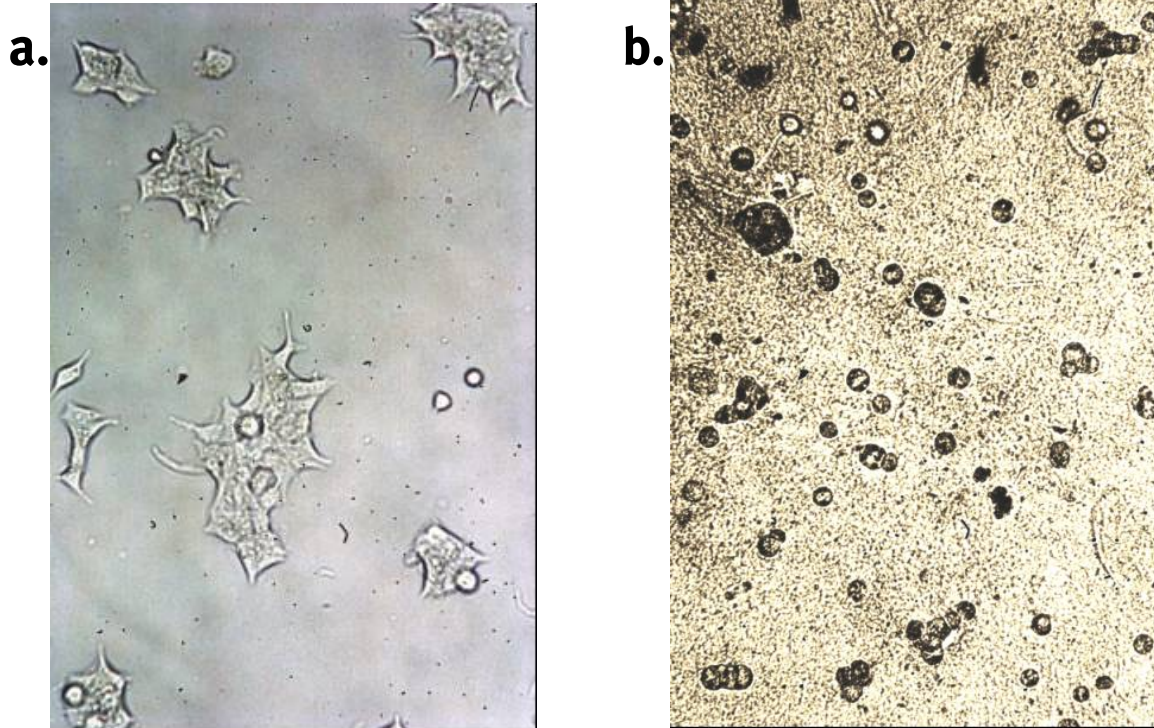


Figure 2: Morphology of the Bt₄₇₄ cells cultured under normal conditions (a.) and under the influence of the combination treatment (30 HAL25 DOX) with doxorubicin and Haelan 951 (b.).

The cells showed shrinking, which is typical for the process of apoptosis. Apoptotic cells shrink without losing the cell contents, whereas necrotic cells swell and eventually burst with leakage of the cell contents (Williams GT, et al., 1992).

3.3. Influence of the test-substances on Bcl2-expression

a. Doxorubicin: Doxorubicin showed a prominent effect on expression of Bcl2. Under increasing concentrations of doxorubicin, the Bcl2 expression values declined sharply (fig. 3a) and dropped more than 10-fold under the value of the untreated cells.

b. Haelan 951: Also, the exposure of the cells to Haelan 951 resulted in a strong downregulation of the antiapoptotic gene Bcl2 up to 18-fold under the values of the untreated cells (fig 3b).

c. Doxorubicin/Haelan 951 combinations: At the comparatively low concentration of 10 µl doxorubicin, the addition of 30 µl and 60 µl Haelan 951 enhanced the downregulation of Bcl2 (fig 3c). The combination of 10 DOX 60 HAL led to almost identical reduction of Bcl2-expression as the combinations of 25 DOX 30 HAL or 50 DOX 30 HAL. Hence, combinatorial use with Haelan 951 allowed the reduction of the doxorubicin dosage to achieve almost identical suppression of Bcl2-expression.

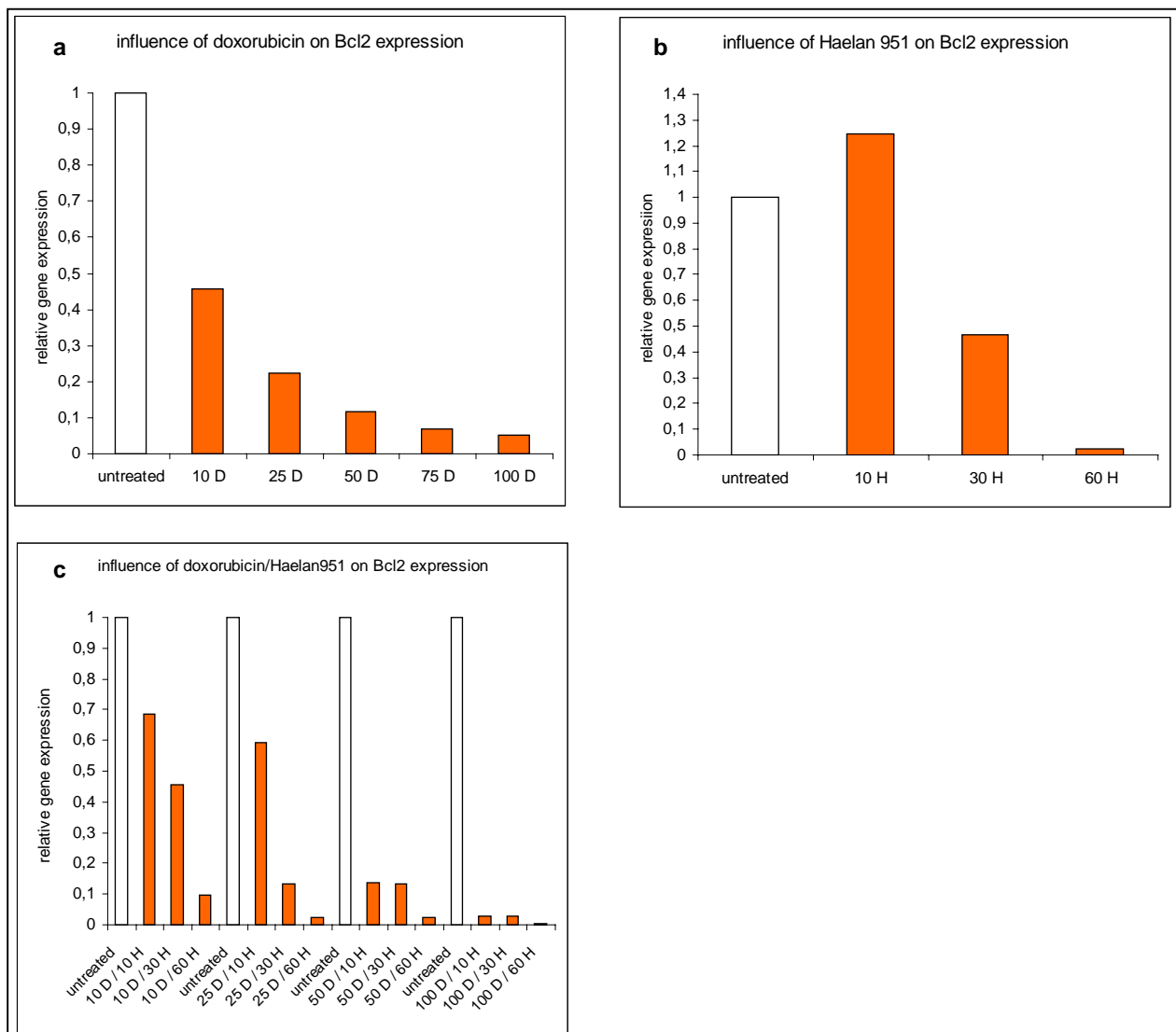


Figure 3: Effect of the test-substances on the expression of Bcl2. The underlying values of these graphs are listed in table A2 in the appendix. D = doxorubicin; H = Haelan 951.

3.4. Influence of the test-substances on Bax-expression

a. Doxorubicin: Doxorubicin had only a marginal effect on the expression of proapoptotic Bax (fig. 4a).

b. Haelan 951: In contrast to doxorubicin, Haelan 951 induced a dose-dependent upregulation of proapoptotic Bax-expression in BT₄₇₄ (fig. 4b). The application of 60 µl Haelan 951 to the cell culture induced almost four-fold overexpression of Bax compared to the untreated cell culture.

c. Doxorubicin/Haelan 951 combinations: The Bax-inducing effect of Haelan 951 also persisted if applied in combination with doxorubicin (fig. 4c). Although the same increase of Bax-expression observed with Haelan 951 monotherapy was not fully achieved, the combination treatment resulted in two- to three-fold upregulation of proapoptotic Bax (fig. 4c).

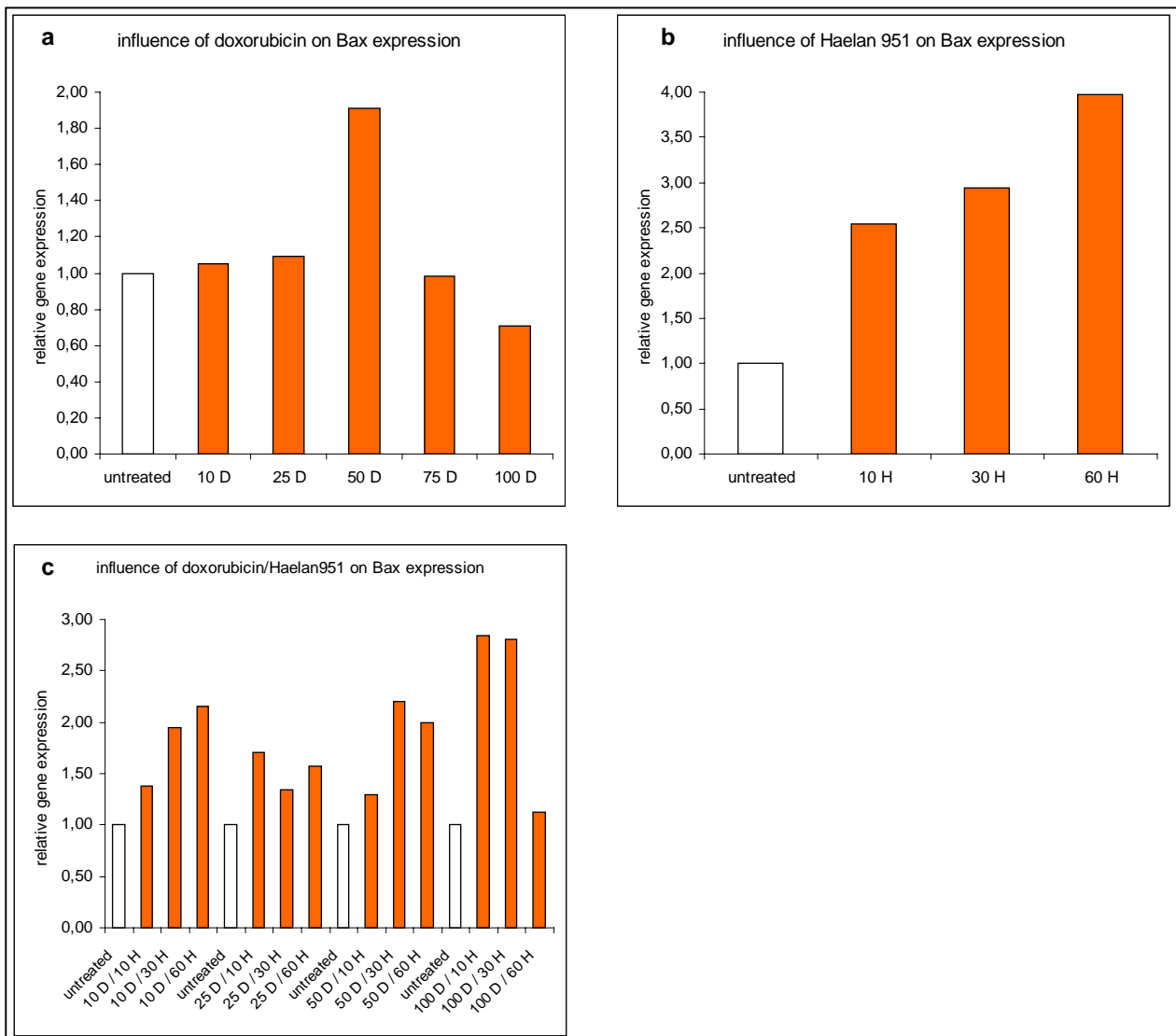


Figure 4: Effect of the test-substances on the expression of Bax. The underlying values of these graphs are listed in table A3 in the appendix. D = doxorubicin; H = Haelan 951.

3.5. Influence of the test-substances on topoisomerase II-expression

a. Doxorubicin: Only higher concentrations of doxorubicin caused a decrease in gene expression of topoisomerase IIa, whereas lower concentrations caused even a slight increase (fig. 5a). Decreases of gene expression in the range of 0.6-fold to 0.4-fold were observed after addition of 75 μ l and 100 μ l doxorubicin, respectively.

b. Haelan 951: Unlike doxorubicin, medium amounts of Haelan 951 readily induced a prominent downregulation of topoisomerase IIa. Furthermore, an upregulation of topoisomerase II was generally not observed. A drop to 0.2-fold level relative to untreated cell cultures was measured after the addition of 30 μ l Haelan 951, which means 5-fold downregulation of gene expression (fig. 5b).

c. Doxorubicin/Haelan 951 combinations: Similar trends already shown with the monotherapies emerged when doxorubicin and Haelan 951 was applied in combination. Upregulation of topoisomerase II expression occurred at low/medium doxorubicin amounts of combined with low Haelan 951 (fig 5c). However, following the addition of 30 HAL or 60 HAL, the gene expression levels began to fall. That means the addition of medium amounts of Haelan 951 outweighed the gene-inductive effect of low doxorubicin concentrations.

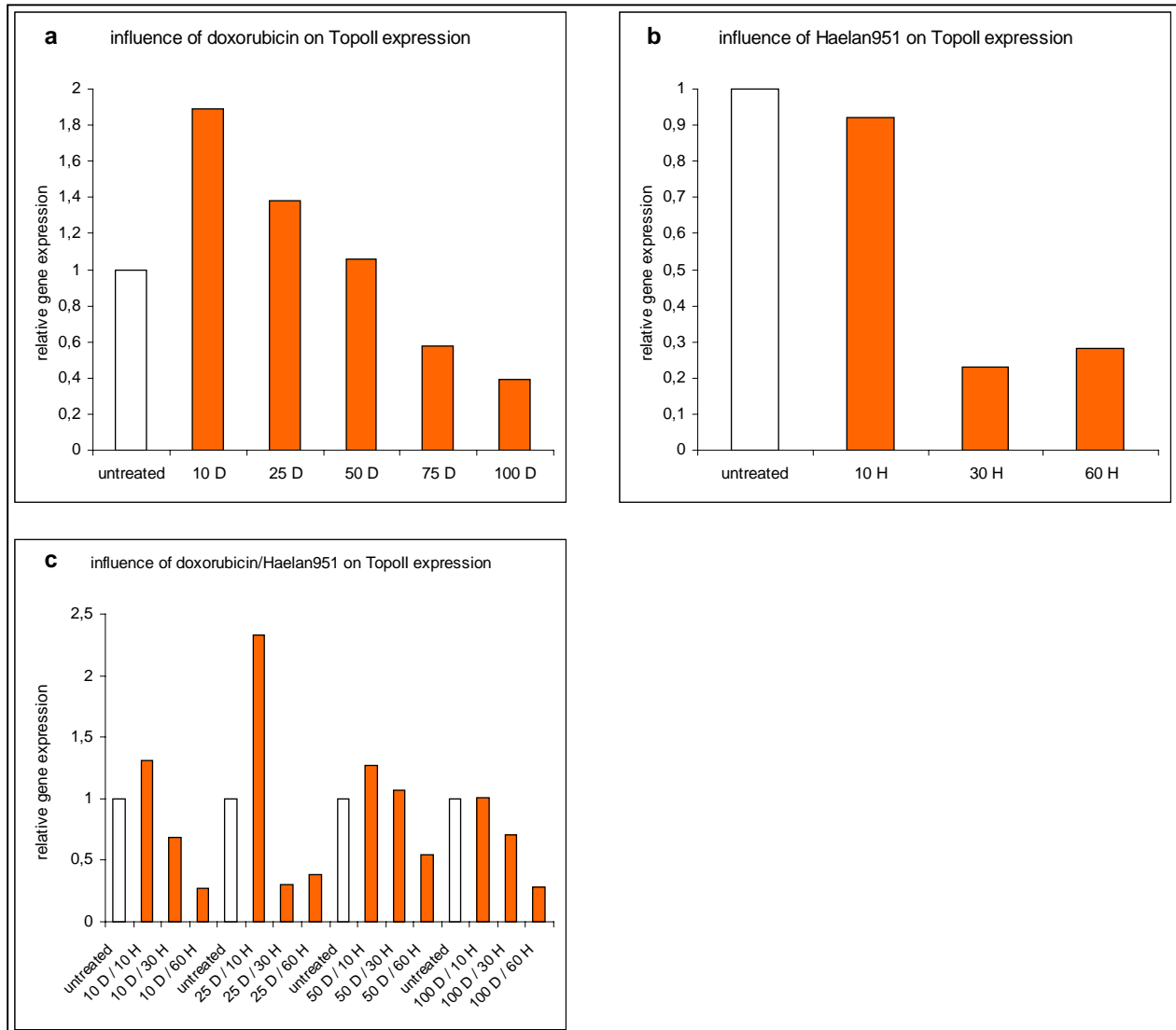


Figure 5: Effect of the test-substances on the expression of topoisomerase IIa. The underlying values of these graphs are listed in table A4 in the appendix. D = doxorubicin; H = Haelan 951.

3.6. Influence of the test-substances on MDR1-expression

a. Doxorubicin: Increasing concentrations of doxorubicin led to a strong upregulation of the multi-drug resistance gene MDR1. While at the lowest used concentration an effect was not yet visible, a steadily raise of MDR1-expression was measured with increasing doxorubicin amounts, finally reaching a 17-fold upregulation at 100 DOX (fig. 6a).

b. Haelan 951: In contrast, Haelan 951 did not induce MDR1 in such extents as doxorubicin. Only at the highest applied concentration of Haelan 951, a 4.5-fold increase in gene expression was seen. The lower amounts of Haelan 951 even caused downregulation of MDR1 (fig. 6b).

c. Doxorubicin/Haelan 951 combinations: Interestingly, the addition of Haelan 951 to doxorubicin predominantly abrogated the MDR1-inductive effect of doxorubicin in BT474 cells. Whilst 25 DOX as well as 50 DOX in monotherapy induced two- to ten-fold overexpression of MDR1, the addition of Haelan 951, even at the lowest used concentration, abrogated the doxorubicin-related induction of MDR1 (fig. 6c). Moreover, the strong upregulation of MDR1 at high doxorubicin concentrations (100 DOX) could be diminished if treatment was combined with Haelan 951 (fig. 6c).

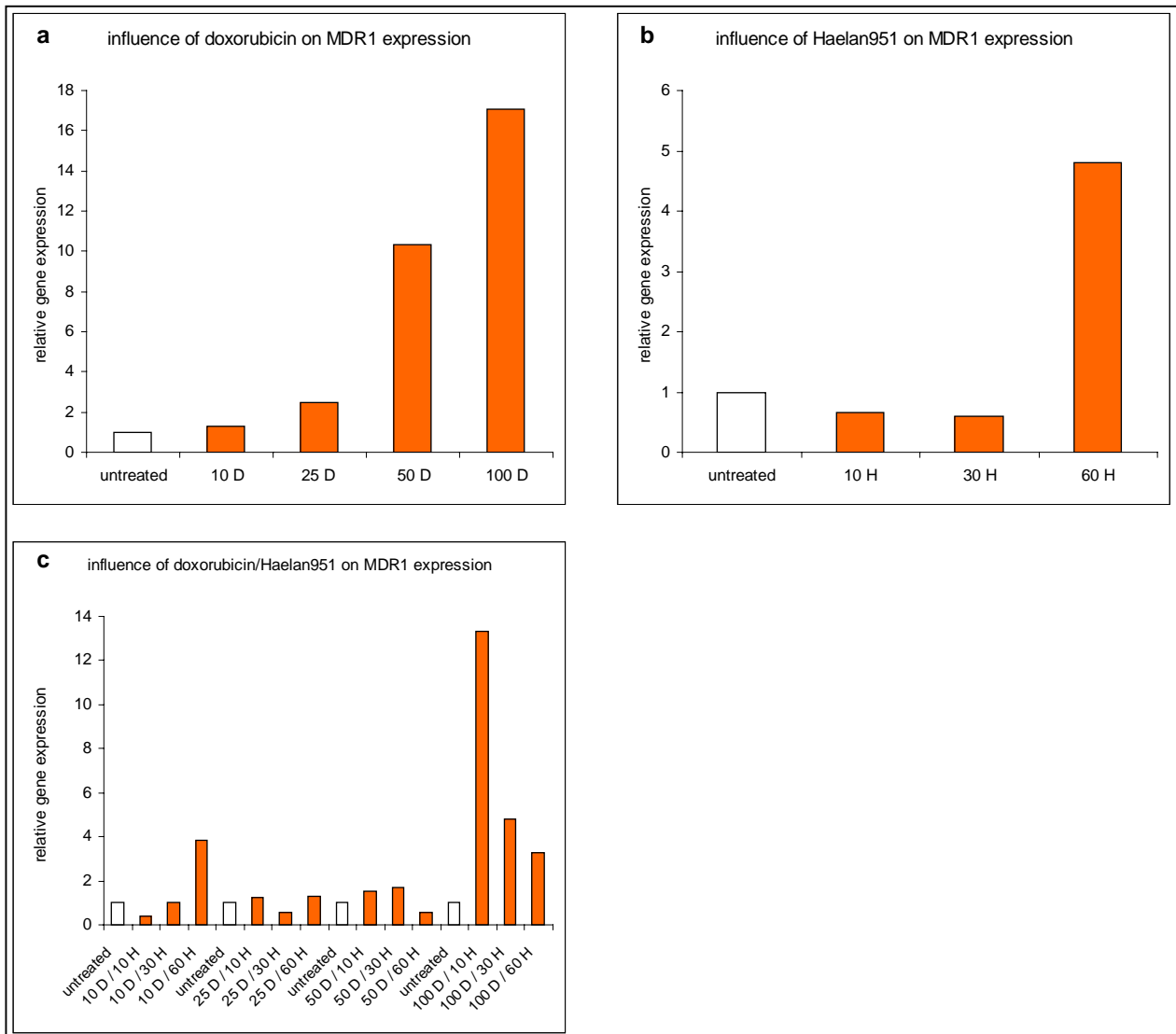


Figure 6: Effect of the test-substances on the expression of MDR1. The underlying values of these graphs are listed in table A5 in the appendix. D = doxorubicin; H = Haelan 951.

3.7. Influence of the test-substances on ERBB2-expression

a. Doxorubicin: A dose-dependent reduction in ERBB2-expression was noticed in the doxorubicin-treated BT474 cell cultures. A steadily decline of the gene expression values was measured if doxorubicin increases, finally reaching only one tenth of the expression levels of the untreated cell culture at ≥ 75 DOX (fig. 7a).

b. Haelan 951: The effects of Haelan 951 on ERBB2 were not as prominent as of doxorubicin. Low and medium amounts (10 HAL, 30 HAL) of Haelan 951 had no or little impact. Only at the highest concentration (60 HAL) an approximately two-fold reduction of the ERBB2-level occurred (fig. 7b).

c. Doxorubicin/Haelan 951 combinations: Generally, the changes of ERBB2-expression after combination treatment resembled those of single doxorubicin use. Haelan 951 did not further enhance the downregulation of ERBB2 beyond to doxorubicin alone. However, the impact of doxorubicin on ERBB2-downregulation was otherwise not inhibited by Haelan 951 (fig. 7c).

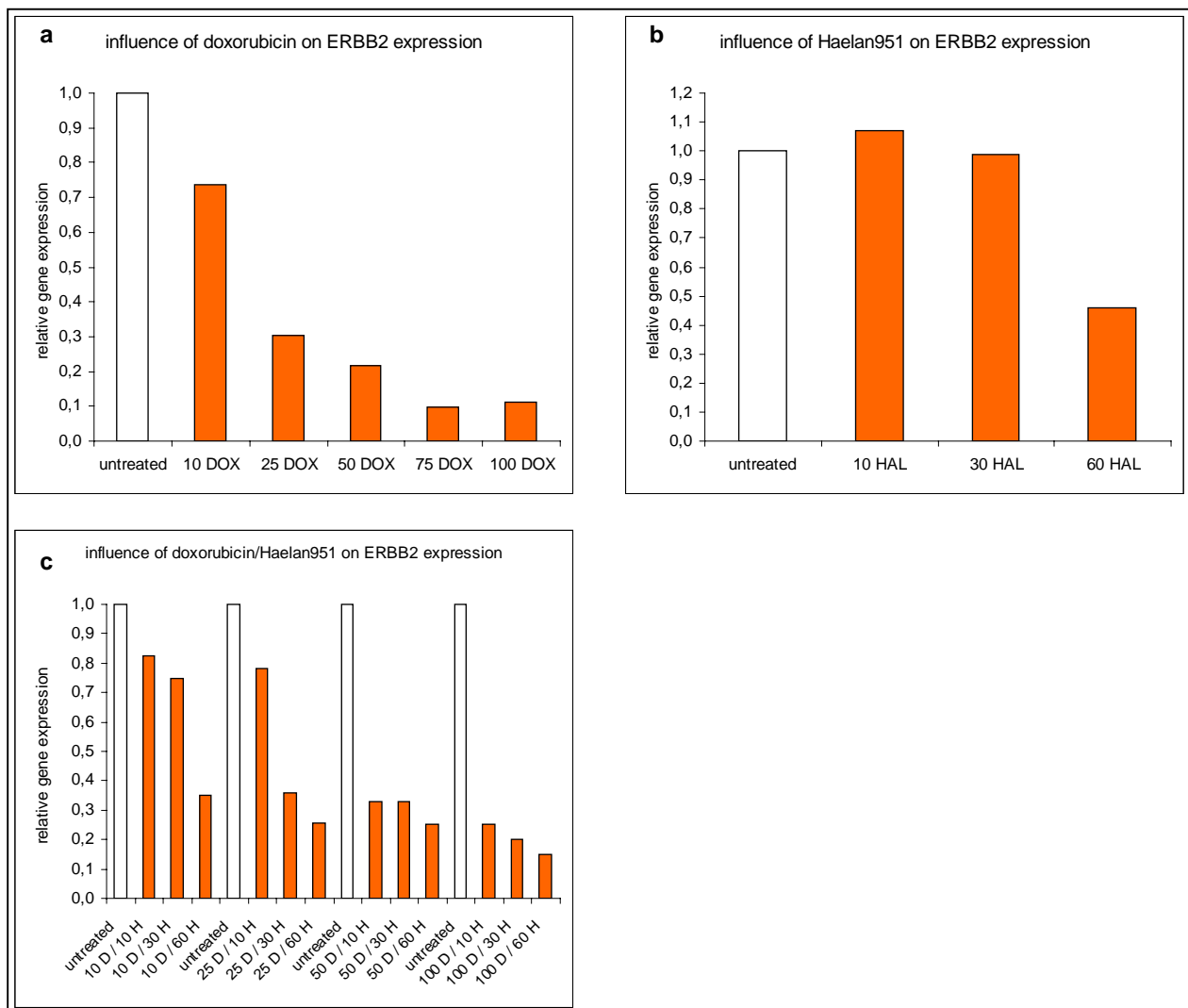


Figure 7: Effect of the test-substances on the expression of ERBB2. The underlying values of these graphs are listed in table A5 in the appendix. D = doxorubicin; H = Haelan 951.

4. Discussion

Present chemotherapy usually requires the administration of combinations of several drugs. Although combination treatment enhances the anti-cancer effect of the therapy, increased side-effects by additive toxicity is a major obstacle of combination treatment. Therefore, therapy regimes have to be determined in clinical trials which are optimized regarding better tumor-response and less side-effects.

The present study should have evaluated a possible combinatorial use of doxorubicin and Haelan 951 for anti-cancer treatment. As a model system, the breast cancer cell line BT₄₇₄ was cultured in the presence of doxorubicin and combinations of Haelan 951 and doxorubicin.

After BT₄₇₄ cells had been treated in monotherapy with doxorubicin or Haelan 951, the survival-rate of the tumor cells declined proportionally with increasing concentrations of either substances. Both substances showed a cytotoxic effect.

In combinations of doxorubicin and Haelan 951, lower concentrations of doxorubicin could be used to achieve an equivalent reduction in the cell-survival-rate. Concordantly, a published study of the Karmanos Cancer Institute described the significantly enhanced inhibition of cell growth and induction of apoptosis after breast cancer cells MBA-MB₂₃₁ were treated with a combination of doxorubicin and the soy-ingredient genistein (Li, Y. et al. 2005).

The most prominent effect of the combination treatment was the influence on the expression levels of the apoptosis markers Bcl2 and Bax. At comparatively low doses of doxorubicin, the addition of Haelan 951 enhanced the downregulation of Bcl2-expression. Combination treatment allowed the reduction of doxorubicin to achieve a comparable effect of higher doxorubicin concentrations on the antiapoptotic gene Bcl2. Moreover, the use of doxorubicin/Haelan 951 combinations raised the expression-levels of the proapoptotic gene Bax two- to three-fold over those of doxorubicin monotherapy. Thus, the combination treatment enhanced the apoptotic condition of BT₄₇₄ cells by downregulation of a gene which inhibits apoptosis (Bcl2) and by upregulation of a gene which promotes apoptosis (Bax). The resulting increased ratio of Bax/bcl2 is considered a crucial determinant for an elevated apoptotic status of the cell. Indeed, workers of the Karmanos Cancer Institute have also demonstrated that the soy isoflavone genistein sensitizes lymphoma cells to chemotherapeutic treatment by increasing the Bax/Bcl2 ratio (Mohammad RM, et al., 2003).

The investigated substances showed interesting effects on topoisomerase IIa. The BT₄₇₄ cell line expresses topoisomerase IIa pretty well and therefore possesses sufficient "drug-target" for doxorubicin, which acts by inhibition of topoisomerase IIa. Increasing addition of Haelan 951 to doxorubicin caused a downregulation of topoisomerase IIa. This effect was most prominent at low doxorubicin concentration. where added Haelan 951 showed the strongest enhancer effect in downregulation of topoisomerase IIa expression. A clinical study with 33 breast-tumor patients described significant downregulation of topoisomerase IIa after response to doxorubicin therapy. In contrast, non-responders showed a raise of topoisomerase IIa expression after completion of therapy (Arpino et. al., 2005). Another study reported significant reduction of cells expressing topoisomerase IIa after successful neoadjuvant anthacycline-based chemotherapy (Tinari et al., 2006). The soy isoflavone genistein is known to inhibit topoisomerase IIa (Markovits J, et al. 1989).

An important factor for the emergence of resistance to doxorubicin based chemotherapy is the MDR1-gene. MDR1 acts as a efflux pump and confers multi-drug resistance by enhancing the transport of toxic compounds out of the cell (Borst P, et al., 2002). In the BT₄₇₄ cells, the expression of MDR1 raised along with increasing doxorubicin exposure. Conversely, except at the highest tested concentration, Haelan 951 did not induce MDR1 expression. Most importantly, if

Haelan 951 was added to doxorubicin, the induction of MDR1 expression could almost be impeded. Even at the highest used doxorubicin concentration, which induced a 17-fold overexpression of MDR1, addition of Haelan 951 led to a obvious reduction of MDR1-expression. In clinical practice, the occurrence of MDR1-related multi-drug resistance is a major obstacle of chemotherapy. Different approaches have therefore been evaluated in pharmacological research to restrict MDR1-induction of doxorubicin. For example, doxorubicin encapsulated in acrylate-nanospheres induced lesser MDR1 in treated cell lines (Laurand A, et al., 2004).

In breast cancer, overexpression of ERBB2 contributes to malignant transformation and correlates with unfavorable prognosis. The BT474 cells used in the present study have the ERBB2 gene amplified which leads to overexpression. Doxorubicin treatment caused a dose-dependent decrease of ERBB2-expression. The addition of Haelan 951 to doxorubicin did neither enhance nor abrogate the effect of ERBB2-downregulation. Therefore, a major influence of Haelan 951 on ERBB2-expression in BT474 is not suggested. In a study of the Japanese National Institute of Health and Nutrition, treatment of ERBB2 overexpressing breast cancer cells with a combination of genistein and doxorubicin resulted in enhanced cytotoxicity and inactivation of ERBB2. It is not clear from that study if the described effect could have been attributed to genistein or doxorubicin.

A strategy to circumvent severe adverse effects of chemotherapy is so called “metronomic” treatment. The drugs are given thereby in lower dosage but more frequently in shorter intervals (Gille J, et al., 2005). The results of our study suggest that the dosage of doxorubicin may be reduced without diminishing its action if administered with Haelan 951 in combination.

Since chemtherapeutic drugs primarily affect proliferating cells and tissues, the novel built blood vessels in a tumor tissue may present a target for this low-dose treatment strategies. Further studies may demonstrate if Haelan 951 could even enhance the action of drugs which inhibit the formation of newly blood vessel in tumors.

A major side-effect of doxorubicin is cardiac toxicity, caused by doxorubicin-catalysed formation of reactive superoxide radicals. Consequently, tissues with low antioxidant defenses like the heart are damaged. Soy isoflavones have been shown to possess antioxidant capacity and to protect doxorubicin-induced heart failure in rats (Ma SF, et al., 2004). Therefore, the addition of Haelan 951 to doxorubicin therapy may provide another advantage to patients by reducing toxic side-effects of the chemotherapy.

5. Summary

- Increasing concentrations of doxorubicin as well as Haelan 951 in the culture medium correlated clearly with a declining survival rate of BT₄₇₄ cells. The addition of Haelan 951 to doxorubicin resulted in a dose dependent reduction in tumor cell viability, which suggests a synergistic action of doxorubicin and Haelan 951.
- Doxorubicin dose-dependently suppressed the gene expression of antiapoptotic Bcl2. If doxorubicin was used in combination with Haelan 951, the same extent of Bcl2 suppression could be achieved at lower doxorubicin concentrations compared to doxorubicin alone.
- Doxorubicin alone had only marginal effects on the expression of the proapoptotic Bax gene. However, the addition of Haelan 951 to doxorubicin caused an two- to threefold increase in Bax-expression. The resulting increase of the ratio between Bax and Bcl2 reflects an enhanced apoptotic status of the tumor cells.
- The cytostatic effect of doxorubicin is known to be based on inhibition of cellular topoisomerase II, which can therefore be considered as the drug-target molecule for doxorubicin. Only higher concentrations of doxorubicin caused a decrease in gene expression of topoisomerase II α . However, medium amounts of Haelan 951 led to a prominent downregulation of topoisomerase II α , and also in combination with doxorubicin. A clinical study in breast cancer patients has recently demonstrated that after successful doxorubicin treatment, topoisomerase II significantly decreases in responders but increases in non-responders.
- High levels of MDR1 in tumor cells confers multidrug-resistance to doxorubicin and many other chemotherapeutic drugs by increasing the drug efflux. A dose-dependent increase of MDR1-expression in doxorubicin-treated BT₄₇₄ cells was observed. On the other hand, only very high Haelan 951-concentrations caused induction of MDR1-expression. Interestingly, addition of Haelan 951 to doxorubicin generally abrogated the dose-dependent MDR1-upregulation caused by doxorubicin treatment. Therefore, this implies the speculation that if chemotherapy is combined with Haelan 951, the occurrence of MDR1-related multidrug-resistance could be reduced.
- No difference on the impact on ERBB2-expression was observed between doxorubicin monotherapy and the combination of Haelan 951 and doxorubicin.

6. Citations

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Appendix

Biometrical data of the experiments. In tables A1 – A6 the measured values and calculations of all gene expression experiments are listed. All measurements were done in duplicate.

The following abbreviations are used:

D = DOX = Doxorubicin.

H = HAL= Haelan = Haelan 951.

CEQ = Cell equivalents compared to the standard curve

Table A1.

Influence of the test-substances on the expression of GAPDH

test-subst.	concentration	GAPDH expr. [CEQ]	mean	relative expr.
	untreated	2419465		
	untreated	2341591	2380528	1
DOX	10 D	1609782		
DOX	10 D	2306311	1958047	0,82
DOX	25 D	1343807		
DOX	25 D	1320282	1332045	0,56
DOX	50 D	357153		
DOX	50 D	312711	334932	0,14
DOX	75 D	68962		
DOX	75 D	40450	54706	0,023
DOX	100 D	117030		
DOX	100 D	101768	109399	0,046
	untreated	3478773		
	untreated	2198702	2838738	1
HAL	10 H	1956930		
HAL	10 H	1340972	1648951	0,58
HAL	30 H	423164		
HAL	30 H	688627	555896	0,2
HAL	60 H	98702		
HAL	60 H	173352	136027	0,048
	untreated	1886056		
	untreated	2362392	2124224	1
DOX / HAL	10 D / 10 H	1857215		
DOX / HAL	10 D / 10 H	2126700	1991958	0,94
DOX / HAL	10 D / 30 H	1101941		
DOX / HAL	10 D / 30 H	728061	915001	0,43
DOX / HAL	10 D / 60 H	300595		
DOX / HAL	10 D / 60 H	121121	300595	0,14
	untreated	1741333		
	untreated	1744027	1742680	1
DOX / HAL	25 D / 10 H	1076048		
DOX / HAL	25 D / 10 H	1189362	1132705	0,65
DOX / HAL	25 D / 30 H	592238		
DOX / HAL	25 D / 30 H	962071	777155	0,45
DOX / HAL	25 D / 60 H	311829		
DOX / HAL	25 D / 60 H	377296	344563	0,198
	untreated	1886056		
	untreated	2362392	2124224	1
DOX / HAL	50 D / 10 H	1095559		
DOX / HAL	50 D / 10 H	599724	847642	0,4
DOX / HAL	50 D / 30 H	471473		
DOX / HAL	50 D / 30 H	855452	663463	0,31
DOX / HAL	50 D / 60 H	325028		
DOX / HAL	50 D / 60 H	354744	339886	0,16
	untreated	3478773		
	untreated	2198702	2838738	1
DOX / HAL	100 D / 10 H	86941		
DOX / HAL	100 D / 10 H	78298	82620	0,029
DOX / HAL	100 D / 30 H	127788		
DOX / HAL	100 D / 30 H	154028	140908	0,05
DOX / HAL	100 D / 60 H	114189		
DOX / HAL	100 D / 60 H	94945	104567	0,037

Table A2:

Influence of the test-substances on the expression of Bcl2

Test-subst.	Conc.	Bcl2 expr. [CEQ]	GAPDH expr. [CEQ]	Normalized ex.	Mean	Relative expr.
	untreated	1165090	2419465	0,48		
	untreated	2477248	2341591	1,05	0,765	1
DOX	10 D	347142	1609782	0,22		
DOX	10 D	1118200	2306311	0,48	0,35	0,4575
DOX	25 D	240792	1343807	0,18		
DOX	25 D	216401	1320282	0,16	0,17	0,2222
DOX	50 D	29423	357153	0,082		
DOX	50 D	29951	312711	0,096	0,089	0,1163
DOX	75 D	3266	68962	0,047		
DOX	75 D	2382	40450	0,059	0,053	0,0693
DOX	100 D	3559	117030	0,03		
DOX	100 D	4036	101768	0,039	0,035	0,051
	untreated	3251848	3478773	0,935		
	untreated	1304573	2198702	0,6	0,77	1
HAL	10 H	2197770	1956930	1,12		
HAL	10 H	1681325	1340972	0,79	0,96	1,2468
HAL	30 H	139345	423164	0,33		
HAL	30 H	266881	688627	0,38	0,36	0,4675
HAL	60 H	1818	98702	0,018		
HAL	60 H	3672	173352	0,021	0,02	0,026
	untreated	1510046	1886056	0,801		
	untreated	2163873	2362392	0,916	0,86	1
DOX / HAL	10 D / 10 H	835380	1857215	0,45		
DOX / HAL	10 D / 10 H	1566915	2126700	0,737	0,59	0,686
DOX / HAL	10 D / 30 H	459033	1101941	0,417		
DOX / HAL	10 D / 30 H	266352	728061	0,366	0,392	0,456
DOX / HAL	10 D / 60 H	7660	300595	0,025		
DOX / HAL	10 D / 60 H	17620	121121	0,145	0,085	0,098
	untreated	1174729	1741333	0,67		
	untreated	1232481	1744027	0,71	0,69	1
DOX / HAL	25 D / 10 H	527873	1076048	0,49		
DOX / HAL	25 D / 10 H	389655	1189362	0,33	0,41	0,5942
DOX / HAL	25 D / 30 H	62787	592238	0,11		
DOX / HAL	25 D / 30 H	72640	962071	0,076	0,093	0,1348
DOX / HAL	25 D / 60 H	7778	311829	0,025		
DOX / HAL	25 D / 60 H	4025	377296	0,011	0,018	0,0261
	untreated	1510046	1886056	0,801		
	untreated	2163873	2362392	0,916	0,86	1
DOX / HAL	50 D / 10 H	120475	1095559	0,11		
DOX / HAL	50 D / 10 H	75065	599724	0,125	0,118	0,137
DOX / HAL	50 D / 30 H	38039	471473	0,08		
DOX / HAL	50 D / 30 H	129769	855452	0,152	0,116	0,135
DOX / HAL	50 D / 60 H	6741	325028	0,021		
DOX / HAL	50 D / 60 H	8248	354744	0,023	0,022	0,026
	untreated	3251848	3251848	1,01		
	untreated	1304573	2198702	0,6	0,81	1
DOX / HAL	100 D / 10 H	1944	86941	0,022		
DOX / HAL	100 D / 10 H	1889	78298	0,024	0,023	0,0284
DOX / HAL	100 D / 30 H	1924	127788	0,015		
DOX / HAL	100 D / 30 H	4475	154028	0,029	0,022	0,0272
DOX / HAL	100 D / 60 H	435	114189	0,0038		
DOX / HAL	100 D / 60 H	407	94945	0,0043	0,0041	0,0051

Table A3

Influence of the test-substances on the expression of Bax

Test-subst.	Conc.	Bax expr. [CEQ]	GAPDH expr. [CEQ]	Normalized ex.	Mean	Relative expr.
	untreated	787339	1741333	0,450		
	untreated	735527	1744027	0,420	0,440	1,00
DOX	10 D	918983	1609782	0,570		
DOX	10 D	801779	2306311	0,350	0,460	1,05
DOX	25 D	619631	1343807	0,480		
DOX	25 D	621720	1320282	0,470	0,480	1,09
DOX	50 D	256556	357153	0,720		
DOX	50 D	300302	312711	0,960	0,840	1,91
DOX	75 D	30714	68962	0,450		
DOX	75 D	16043	40450	0,400	0,430	0,98
DOX	100 D	38608	117030	0,330		
DOX	100 D	28054	101768	0,280	0,310	0,71
	untreated	1097775	3478773	0,320		
	untreated	660935	2198702	0,300	0,310	1,00
HAL	10 H	1073795	1956930	0,550		
HAL	10 H	1385750	1340972	1,030	0,790	2,55
HAL	30 H	414061	423164	0,980		
HAL	30 H	577238	688627	0,840	0,910	2,94
HAL	60 H	116367	98702	1,180		
HAL	60 H	222273	173352	1,280	1,230	3,97
	untreated	789591	1886056	0,420		
	untreated	905012	2362392	0,380	0,400	1,00
DOX / HAL	10 D / 10 H	792519	1857215	0,430		
DOX / HAL	10 D / 10 H	1437841	2126700	0,670	0,550	1,38
DOX / HAL	10 D / 30 H	671331	1101941	0,610		
DOX / HAL	10 D / 30 H	685876	728061	0,940	0,780	1,95
DOX / HAL	10 D / 60 H	182105	300595	0,610		
DOX / HAL	10 D / 60 H	134290	121121	1,110	0,860	2,15
	untreated	787339	1741333	0,450		
	untreated	735527	1744027	0,420	0,440	1,00
DOX / HAL	25 D / 10 H	805868	1076048	0,750		
DOX / HAL	25 D / 10 H	893073	1189362	0,750	0,750	1,70
DOX / HAL	25 D / 30 H	360529	592238	0,610		
DOX / HAL	25 D / 30 H	552019	962071	0,570	0,590	1,34
DOX / HAL	25 D / 60 H	223827	311829	0,720		
DOX / HAL	25 D / 60 H	250576	377296	0,660	0,690	1,57
	untreated	789591	1886056	0,420		
	untreated	905012	2362392	0,380	0,400	1,00
DOX / HAL	50 D / 10 H	479154	1095559	0,440		
DOX / HAL	50 D / 10 H	359653	599724	0,600	0,520	1,30
DOX / HAL	50 D / 30 H	458248	471473	0,970		
DOX / HAL	50 D / 30 H	676632	855452	0,790	0,880	2,20
DOX / HAL	50 D / 60 H	233522	325028	0,720		
DOX / HAL	50 D / 60 H	312726	354744	0,880	0,800	2,00
	untreated	1097775	3478773	0,320		
	untreated	660935	2198702	0,300	0,310	1,00
DOX / HAL	100 D / 10 H	80871	86941	0,930		
DOX / HAL	100 D / 10 H	64311	78298	0,820	0,880	2,84
DOX / HAL	100 D / 30 H	133253	127788	1,040		
DOX / HAL	100 D / 30 H	107687	154026	0,700	0,870	2,81
DOX / HAL	100 D / 60 H	42128	114189	0,370		
DOX / HAL	100 D / 60 H	31168	94945	0,330	0,350	1,13

Table A4

Influence of the test-substances on the expression of Topo II

Test-subst.	Conc.	Topoll expr. [CEQ]	GAPDH expr. [CEQ]	Normalized ex.	Mean	Relative expr.
	untreated	3027953	2419465	1,25		
	untreated	5227736	2341591	2,23	1,74	1
DOX	10 D	5347266	1609782	3,32		
DOX	10 D	7525212	2306311	3,26	3,29	1,8908
DOX	25 D	3409487	1343807	2,54		
DOX	25 D	3038724	1320282	2,3	2,40	1,3793
DOX	50 D	665573	357153	1,86		
DOX	50 D	566539	312711	1,81	1,84	1,0575
DOX	75 D	72833	68962	1,06		
DOX	75 D	51756	40450	1,28	1,17	0,5747
DOX	100 D	41943	117030	0,36		
DOX	100 D	50830	101768	0,5	0,43	0,3909
	untreated	7701371	3478773	2,21		
	untreated	2815125	2198702	1,28	1,74	1
HAL	10 H	2777695	1956930	1,42		
HAL	10 H	2405482	1340972	1,79	1,6	0,9195
HAL	30 H	209050	423164	0,49		
HAL	30 H	223421	688627	0,32	0,4	0,2299
HAL	60 H	52379	98702	0,53		
HAL	60 H	79123	173352	0,46	0,49	0,2816
	untreated	4003162	1886056	2,12		
	untreated	5527138	2362392	2,34	2,23	1
DOX / HAL	10 D / 10 H	5113180	1857215	2,75		
DOX / HAL	10 D / 10 H	6598643	2126700	3,1	2,925	1,312
DOX / HAL	10 D / 30 H	1878997	1101941	1,71		
DOX / HAL	10 D / 30 H	970639	728061	1,33	1,52	0,6816
DOX / HAL	10 D / 60 H	146512	300595	0,49		
DOX / HAL	10 D / 60 H	85112	121121	0,7	0,6	0,2691
	untreated	2029157	1741333	1,17		
	untreated	1814542	1744027	1,04	1,1	1
DOX / HAL	25 D / 10 H	2783028	1076048	2,59		
DOX / HAL	25 D / 10 H	3008326	1189362	2,53	2,56	2,3273
DOX / HAL	25 D / 30 H	519162	592238	0,88		
DOX / HAL	25 D / 30 H	643798	962071	0,67	0,77	0,3008
DOX / HAL	25 D / 60 H	142916	311829	0,46		
DOX / HAL	25 D / 60 H	141506	377296	0,38	0,42	0,3818
	untreated	4003162	1886056	2,12		
	untreated	5527138	2362392	2,34	2,23	1
DOX / HAL	50 D / 10 H	3900452	1095559	3,56		
DOX / HAL	50 D / 10 H	1399053	599724	2,33	2,95	1,266
DOX / HAL	50 D / 30 H	994004	471473	2,11		
DOX / HAL	50 D / 30 H	2265840	855452	2,65	2,38	1,0673
DOX / HAL	50 D / 60 H	380319	325028	1,17		
DOX / HAL	50 D / 60 H	445953	354744	1,26	1,22	0,5471
	untreated	7701371	3478773	2,21		
	untreated	2815125	2198702	1,28	1,74	1
DOX / HAL	100 D / 10 H	170016	86941	1,95		
DOX / HAL	100 D / 10 H	123779	78298	1,58	1,76	1,0115
DOX / HAL	100 D / 30 H	160761	127788	1,26		
DOX / HAL	100 D / 30 H	182947	154028	1,19	1,22	0,7011
DOX / HAL	100 D / 60 H	58233	114189	0,5		
DOX / HAL	100 D / 60 H	47087	94945	0,49	0,49	0,2816

Table A6

Influence of the test-substances on the expression of ERBB2

Conc.	ERBB2 expr. [CEQ]	GAPDH expr. [CEQ]	Normalized ex.	Mean	Relative expr.
untreated	1423204	2419465	0,590		
untreated	3395344	2341591	1,450	1,020	1,000
10 DOX	1130123	1609782	0,700		
10 DOX	1842817	2306311	0,800	0,750	0,735
25 DOX	410095	1343807	0,310		
25 DOX	399310	1320282	0,300	0,310	0,304
50 DOX	89451	357153	0,250		
50 DOX	56725	312711	0,180	0,220	0,216
75 DOX	8807	68962	0,130		
75 DOX	2958	40450	0,073	0,100	0,098
100 DOX	14074	117030	0,120		
100 DOX	8693	101768	0,090	0,110	0,113
untreated	3225931	3478773	0,930		
untreated	1862592	2198702	0,850	0,890	1,000
10 HAL	1680694	1956930	0,860		
10 HAL	1342587	1340972	1,000	0,930	1,069
30 HAL	339642	423164	0,800		
30 HAL	630089	688627	0,910	0,860	0,989
60 HAL	43025	98702	0,440		
60 HAL	60349	173352	0,350	0,400	0,460
untreated	1541349	1886056	0,820		
untreated	2355722	2362392	0,990	0,910	1,000
10 D / 10 H	1139262	1857215	0,610		
10 D / 10 H	1905007	2126700	0,890	0,750	0,824
10 D / 30 H	817095	1101941	0,740		
10 D / 30 H	450938	728061	0,620	0,680	0,747
10 D / 60 H	95428	300595	0,320		
10 D / 60 H	36962	121121	0,310	0,320	0,352
untreated	1617590	1741333	0,930		
untreated	1764263	1744027	1,010	0,970	1,000
25 D / 10 H	870473	1076048	0,810		
25 D / 10 H	842077	1189362	0,710	0,760	0,784
25 D / 30 H	220274	592238	0,370		
25 D / 30 H	302327	962071	0,330	0,350	0,361
25 D / 60 H	101828	311829	0,330		
25 D / 60 H	62026	377296	0,160	0,250	0,258
untreated	1541349	1886056	0,820		
untreated	2355722	2362392	0,990	0,910	1,000
50 D / 10 H	294159	1095559	0,270		
50 D / 10 H	194326	599724	0,320	0,300	0,330
50 D / 30 H	99752	471473	0,210		
50 D / 30 H	323235	855452	0,380	0,300	0,330
50 D / 60 H	68102	325028	0,210		
50 D / 60 H	91384	354744	0,250	0,230	0,253
untreated	3225931	3478773	0,930		
untreated	1862592	2198702	0,850	0,890	1,000
100 D / 10 H	17013	86941	0,200		
100 D / 10 H	18443	78298	0,230	0,220	0,253
100 D / 30 H	23518	127788	0,180		
100 D / 30 H	27309	154028	0,180	0,180	0,202
100 D / 60 H	13058	114189	0,110		
100 D / 60 H	13780	94945	0,140	0,130	0,149