Effect of *Galium verum* aqueous extract on growth, motility and gene expression in drug-sensitive and -resistant laryngeal carcinoma cell lines

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Abstract. *Galium verum*, also known as Lady's Bedstraw, is a herbaceous perennial plant of the family Rubiaceae, native to Europe and Asia and used in traditional medicine as an anticancer medicine. It is used as a decoction in most traditional recipes, applied externally as well as internally. We produced a *Galium verum* decoction and applied it *in vitro* to chemosensitive (Hep-2 and HLaC79) and chemoresistant, P-glycoprotein-overexpressing (Hep2-Tax, HLaC79-Tax) laryngeal carcinoma cell lines. It could be demonstrated that *Galium* aqueous extract is cytotoxic for all cell lines. A detailed spheroid-based 3D invasion analysis of Hep2 and Hep2-Tax in semisolid collagen gels and on different extracellular matrix coatings was performed, which showed an inhibition of invasion by sublethal concentrations of *Galium* decoction and proved to be even more pronounced in the more aggressively invading chemoresistant Hep2-Tax cell line. Gelatinolytic activity of MMP-2 was downregulated in three of the four cell lines. Angiogenesis (endothelial tube formation) in contrast, was not affected by *Galium* aqueous extract. Gene expression array on HLaC79 and Hep2 cell lines treated with *Galium* decoction vs. untreated controls revealed no unique pathway activation patterns in these cells. Results are discussed with respect to the use of herbal drugs as a preventive and/or a concomitant therapeutic approach in head and neck cancer.

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Introduction

According to an analysis in 2009 of over 3,000 cases of primary head and neck tumours in Germany 2009, the outcome has not improved significantly from 1995 to 2006, despite changed treatment strategies. Especially the 5-year overall survival rate for carcinomas of hypopharyngeal origin is very low with 27.2% (1). Moreover, in advanced laryngeal and hypopharyngeal cancer the functional and cosmetic deformations produced by surgery can be very disabling for the patients. Chemotherapeutic treatment is meanwhile commonly used for advanced head and neck cancer in order to preserve laryngeal and/or pharyngeal structures. Paclitaxel is one of the agents used and generally seems to be powerful, however, it failed to reach a local-regional tumour control in 12% of patients according to a previously published study (2).

Galium verum, also known as Lady's Bedstraw or Yellow Bedstraw, is an herbaceous perennial plant of the family Rubiaceae, native to Europe and Asia. The dried plants were used to stuff mattresses, and the flowers were also used to coagulate milk for cheese production in the past. Studies on *Galium verum* predominantly originate from the Asian continent, where traditional medicine is more frequently embedded in culture and everyday life. In central European countries or the USA traditional herbal cure has been replaced by modern, science-based therapy. However, traditional phyto-medicine gains more and more attention, especially with respect to alternative treatment of cancer. When mainstream medicine reaches a limit of success, patients search for alternatives.

The cut and dried aerial parts of the plant, *'Herba gallii lutei'*, are used for homeopathic purposes. These are still used for exogenous treatment of psoriasis or delayed wound healing or as a tea with diuretic effect for the cure of pyelitis or cystitis (3). Today the use of *Galium verum* is considered to be obsolete, although it is still referred to in popular non-scientific publications and internet platforms as an anticancer medicine. One example for such literature is the compendium of Treben (4), which is neither science based nor accepted in medicine, but very popular and well sold in German speaking countries of Europe.

Even in non-scientific literature the role of *Galium verum* in treatment of cancer is controversially discussed. While Willfort (5) recommends *Galium* tea for treatment of tongue cancer by mouth rinsing and drinking, Neuthaler (6) disclaims this to be sheer mischief. Treben (4) describes a few cases of tongue and larynx carcinoma patients with remission of tumour and/or sickness caused by *Galium* tea. However, she damped enthusiasm herself by stating, that God's assistance is also necessary for healing of severe diseases.

On scientific level, a variety of bioactive substances have been identified in *Galium verum* plants such as Iridoinglycosides (7-9), flavanoids (8,10,11), anthraquinones (12) and chlorogenic acid (13). *Galium* species are known to have antioxidant [*Galium verum* (14)], antimicrobial/antifungal [*Galium* tricornutum (15)], antifeedant [*Galium* aparine (16)] and insecticidal [*Galium* melantherum (17)] properties.

According to Jonathan Hartwells' survey (18) *Galium verum* was traditionally used in Europe and Northern America for treatment of cancerous ulcers or breast cancer. Amirghofran *et al* (19) showed a cytotoxic effect of *Galium* mite methanolic extracts on K561 and Jurkat cells. Zhao *et al* isolated Diosmetin from *Galium verum* plants and showed protective effects on the thymus of U14-bearing mice (20).

According to popular scientific advice (4) and a number of recommendations circulating in diverse internet platforms, we have tested the influence of a *Galium verum* 'tea' (decoction) on growth and behaviour of head and neck cancer cell lines, either paclitaxel-sensitive or resistant.

Materials and methods

Cell lines and cell culture. The head and neck squamous carcinoma cell line HLaC79 was established from a lymph node metastase of a laryngeal squamous cell carcinoma (21). The cell line was grown with RPMI-1640 medium (Seromed, Munich, Germany), supplemented with 10% fetal calf serum (FCS). HLaC79 cells were treated with 10 nM paclitaxel. A resistant clone was isolated by selective trypsination of single clones. The permanent HLaC79 clonal cell line HLaC79-Tax was cultured in RPMI-1640 medium, supplemented with 10% FCS and 10 nM paclitaxel. Hep2 cells were grown in MEM medium, supplemented with 10% FCS. A paclitaxel-resistant clone was isolated according to the procedure in HLaC79-Tax.

Human umbilical vein endothelial cells (HUVEC) were isolated as described elsewhere (22). They were grown in ECGM (Provitro, Berlin, Germany) medium.

Galium verum decoction. Dried and cut *Galium verum* L. leaves (*Herba gallii lutei*) were kindly provided by Dr Ivo Pischel, PhytoLab GmbH & Co. KG. (Vestenbergsgreuth, Germany). Tea was prepared as follows: 100 ml boiling water were poured over 15 g of dried and powdered *Galium* leaves. After cooling down, the supernatant was cleared by centrifugation and sterile filtration. Aliquots were frozen at -80˚C. One batch of frozen *Galium* extract was used for all experiments.

TLC analysis/sample identification. Samples were analyzed using thin layer chromatography, a physical method to separate complex chemical substance mixtures on a stationary phase with a mixture of solvents. The method relies on dynamic differential migration of substances in the mixture as a result of adsorption characteristics, molecular weight, and solubility (23). As samples for TLC *Galium* decoction, a methanolic *Galium verum* extract (24) and standards were used. They were applied to Silica gel 60 F264 plates (Merckmillipore.com). As solvents ethyl acetate - formic acid - acetic acid - water (100:11:11:27) for flavones or toluene - ethyl acetate - formic acid (50:40:10) for polyphenolcarboxylic acids were used. Compounds were determined and documented by exposure to UV light at 365 nm with a Camag TLC documentation system (Camag.com). The following standards were used: apigenol, luteolin, apigenol-7-O-glucoside, luteolin7-O-glucoside, quercetol and rutoside for flavones; chlorogenic acid, caffeic acid, rosmarinic acid, ferulic acid, and p-coumaric acid for polyphenolcarboxylic acids.

Cell viability and proliferation assay. Cells were seeded at 5000 cells/well in 96-well plates. They were treated with increasing concentrations of *Galium verum* aqueous extract (50 and 100 μl/ml) for 48 h. Controls were kept in medium supplemented with 100 μl/ml water. Cell proliferation was measured after 48 h by replacing the culture medium with medium containing 1 mg/ml MTT. After 4 h of incubation, MTT-staining solution was replaced by isopropanol and cells were incubated at 37˚C for 45 min. The colour conversion of MTT to a blue formazan dye was measured with an ELISA reader at a wavelength of 570 nm. The amount of formazan dye is in direct proportion to the number of metabolically active cells in the culture. Relative toxicity was calculated as % surviving cells by setting control cells treated with vehicle as 100% surviving cells.

Western blot analysis. For western blot analysis, cells were harvested by scraping and dissolved in RIPA (PBS, containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS), supplemented with 10 μg/ml phenylmethanesulfonyl fluoride (PMSF). Protein content was determined according to the method of Lowry (25). Equal amounts of total protein lysates were loaded on 10% SDS-polyacrylamide gels and run at a constant current of 20 mA. Gels were blotted onto nitrocellulose membranes according to the semidry method of Kyhse-Andersen (26). Blots were blocked for 1 h with TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 8.0), containing 5% non-fat dry milk. For detection of p-glycoprotein the monoclonal anti-pGp antibody Clone F4 (Sigma-Adrich, Steinheim, Germany) was used. Primary antibodies were incubated overnight at 4˚C, after washing 3 times with TBST, cells were incubated with corresponding secondary antibodies, coupled to horseradish peroxidase for 1 h. After washing once again, detection of bound antibody conjugates was performed with the enhanced chemiluminescence system (ECL, Amersham Biosciences, Freiburg, Germany), according to the manufacturer's protocol.

In vitro invasion and motility assays. 3D invasion in collagen I/Matrigel® gels. Tumour spheroids were generated by seeding 5000 cells/well of Hep2 and Hep2-Tax cells on ultra-low-attachment (ULA) 96-well round-bottomed plates (Corning, Amsterdam, The Netherlands) (27). HLaC79 and HLaC79-Tax cells did not form stable spheroids. After 3-4 days medium

was replaced by either Matrigel (BD Biosciences, Heidelberg, Germany) or Vitrogen-100 collagen (Cohesion Technologies, Palo Alto, CA, USA) gels, prepared with or without *Galium* extract (33.3 µl/ml). Matrigel was diluted 1:2 with cell culture medium. Vitrogen-100 gels were supplemented with 10% 10X MEM medium (Life Technologies, Carlsbad, CA, USA) and 5% Sodium Bicarbonate (Life Technologies, 7.5% solution for cell culture). Both gels were further supplemented with 25 ng/ml EGF (Sigma-Aldrich, Taufkirchen, Germany). After solidifying gels at 37˚C complete medium with or without *Galium* decoction (33.3 µl/ml) was added on top. Photographs were taken after 1, 24 and 48 h after embedding with a Leica DMI 4000 inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Migration on different substrates. For this tumour spheroid-based migration assay, spheroids of Hep2 and paclitaxel-resistant Hep2-Tax spheroids were placed on different extracellular matrix substrates (28). The surface of flat-bottomed 96-well plates were coated with 0.1% gelatin, 5 µg/ml fibronectin, 50 µg/ml laminin, 50 µg/ml collagen I (all from Sigma-Aldrich, Taufkirchen, Germany) or 125 µg/ml Matrigel (Becton Dickinson, Heidelberg, Germany) for 2 h at room temperature. Wells were washed twice with PBS and subsequently blocked with 1% bovine serum albumin in PBS for 1 h. For 2 days on ULA plates pre-cultivated spheroids (see above) of Hep2 and Hep2-Tax cell lines were transferred to the coated wells with a multichannel pipette. Spheroids were incubated with or without *Galium* decoction (33.3 µl/ml). Migration was recorded by photographing spheroids after 1 and 24 h with a Leica DMI 4000 inverted fluorescence microscope (Leica Microsystems). Quantification of migrated cells was carried out with ImageJ software (National Institutes of Health, NIH, USA).

MMP-2/MMP-9 gelatin zymography. Cell lines were treated with 33.3 µl/ml *Galium verum* extract for 48 h. After 48 h, cells were seeded after incubation with *Galium* extract at equal cell numbers in multiwall plates. Complete medium (MEM or RPMI) was replaced by Optimem (Invitrogen, Karlsruhe, Germany), which is a complete, serum-free medium. Conditioned medium was collected after 18 h. Conditioned (20-60 µl) medium was directly subjected to electrophoresis in 10% SDS-polyacrylamide gels under non-reducing conditions (29), containing 1 mg/ml gelatin (Sigma-Aldrich, Traunstein, Germany). After electrophoresis, gels were renaturated two times for 30 min in 2.5% Triton X-100 and developed overnight in developing solution (50 mM Tris.HCl, pH 6.8, 0.2 M NaCl, 10 mM $CaCl_2$, 0.02% Brij 35) at 37˚C. Subsequently they were stained with Coomassie Brilliant Blue, destained and dried.

Tube formation assay. Using tube formation assays, the ability of endothelial cells to form three-dimensional capillary-like structures was analyzed. Angiogenesis-slides (15-well, Ibidi GmbH, Munich, Germany) were coated with growth factor reduced basement membrane extract (BME; Trevigen, MD, USA). After polymerization of BME, the gels were overlaid with growth medium containing $1x10^4$ HUVECs and the 33.3 µl/ml *Galium* extract. Cells were incubated for 6-8 h and images were taken. Evaluation of pictures was performed by Wimasis GmbH (Munich, Germany). For quantification, four parameters were analyzed: tube length, number of branching points, covered area and number of loops.

cDNA expression array. RNA extraction and RNA quality control. RNA of *Galium*-treated and untreated cell lines Hep2 and HLaC79 (48 h, 33.3 µl/ml) was isolated with the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. RNA quality was assessed with the RNA 6000 Nano kit using the Bioanalyzer 2100 instrument (Agilent, Böblingen, Germany). RNA integrity numbers (RINs) of our samples ranged between 9.4 and 9.9.

Microarray hybridization. For microarray hybridization, 100 ng total RNA were amplified and labelled using the IVT Express kit and hybridized to GeneChip PrimeView Human Gene Expression arrays (both from Affymetrix, Santa Clara, CA, USA). Microarray washing and staining was performed with a Fluidics Station 450; fluorescence intensities were scanned with a GeneChip Scanner 3000 7G (both from Affymetrix). Image processing (microarray grid definition, feature intensity readout) and CEL file generation was achieved with the GeneChip Operating Software (Affymetrix).

Microarray evaluation. Raw microarray data were background corrected, normalized and summarized to probe-set expression values using the Robust Microarray Average (RMA) algorithm (30,31). For each cell line differentially expressed probe-sets resulting from treatment were detected in each cell line by estimating the false discovery rate (FDR) with an empirical Bayesian methodology employing a log-normal normal data modeling (32). Significant (FDR<0.05) probe-sets were subjected to gene set enrichment analysis using DAVID (33). These analyses were performed in the R environment (http://www.r-project.org) using Bioconductor (http://www.bioconductor.org) packages 'affy' and 'EBarrays'.

Real-time PCR. To verify results of arrays, quantitative real-time Taqman^R PCR (AppliedBiosystems.com) was performed. Different cell lines (Hep2, Hep2-Tax, HLaC79, HLaC79-Tax, Fadu) were treated for 48 h with *Galium verum* decoction (33.3 µl/ml) or vehicle. Subsequently cells were rinsed with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4•2 H_2O, 2 mM KH_2PO_4, pH 7.4) and scraped off for RNA isolation, which was performed with the RNeasy kit (Qiagen) according to the manufacturer's instructions. The High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Darmstadt, Germany) was used for cDNA reverse transcription. Real-time PCR was performed in triplicates on a Real-time PCR cycler (Applied Biosystems) using Taqman Gene expression assays for MDR-1, TXNIP, MDM2, JUN, MMP-10 and RND3. Relative quantification was calculated according to the $2^{-\Delta\Delta CT}$ method (34). Expression values were normalized to the expression of GAPDH as an endogenous control which proved to be expressed most stably throughout the cell lines.

Statistical analysis. All statistical analyses and graphs were performed with Graph Pad Prism 4 (Graphpad software, La Jolla, CA, USA)

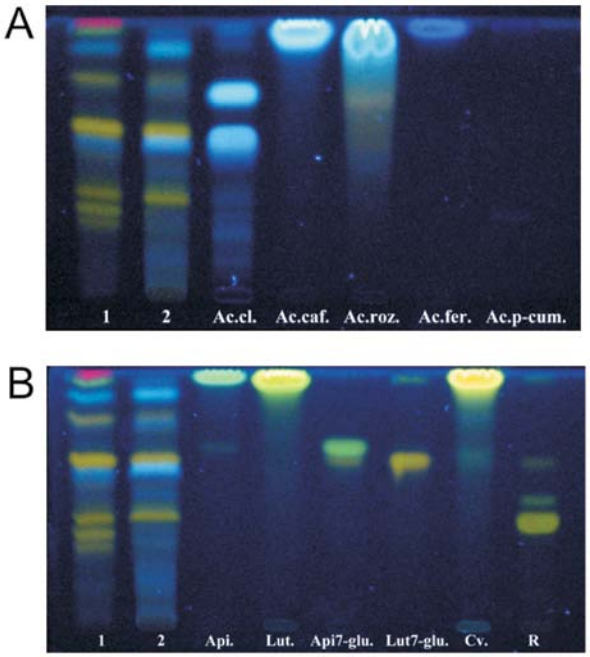

Figure 1. (A) TLC chromatogram for polyphenolic acids of *Galium verum* samples. *Galium verum* - alcoholic extract (1), *Galium verum* - aqueous extract (2), Standards: chlorogenic acid (Ac.cl.), caffeic acid (Ac.caf.), rosmarinic acid (Ac.roz.), ferulic acid (Ac.fer.), p-coumaric acid (Ac.p-cum.). (B) TLC chromatogram for flavons of *Galium verum* samples. *Galium verum* - alcoholic extract (1), *Galium verum* - aqueous extract (2), Standards: apigenol (Api.), luteolin (Lut.), apigenol-7-O-glucoside (Api7-glu), luteolin7-O-glucoside (Lut7-glu), quercetol (Cv), rutoside (R).

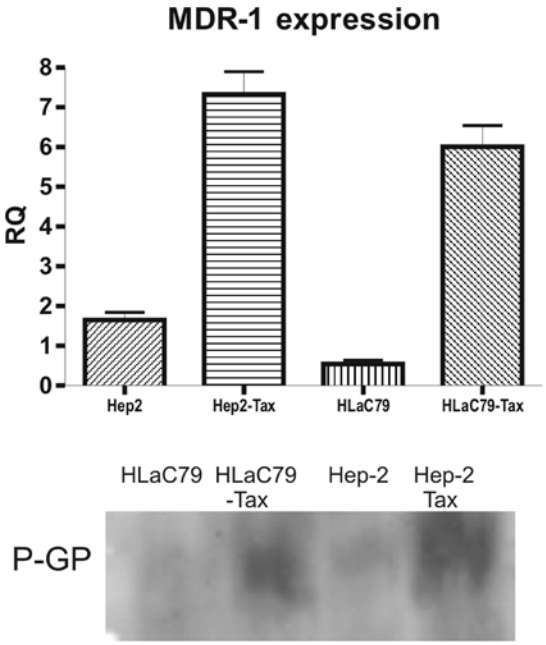

Figure 2. Expression of P-glycoprotein mRNA, measured by Taqman qRT-PCR. RQ=$2^{-\Delta\Delta CT}$. Values are ± SE and normalized to the expression of GAPDH. Results are confirmed by western blot analysis of total cell lysates of HLaC79, HLaC79-Tax, Hep2 and Hep2-Tax cell lines.

Results

Galium verum decoction: sample identification by thin layer chromatography (TLC). Qualitative phytochemical analysis for the *Galium verum* decoction in comparison to a known *Galium verum* methanolic extract (24) was performed by thin layer chromatography, in order to determine the biologically active compounds (polyphenolcharboxylic acids and flavons).

Chromatographic images with polyphenolcarboxylic acid standards clearly demonstrate the presence of chlorogenic acid in both extracts, more pronounced in the aqueous extract (Fig. 1). Luteolin-7-O-glucoside and rutoside (Fig. 2) are present in pronounced amounts in the flavonoid fraction of the aqueous *Galium* extract.

Multidrug resistance. Expression of P-Gp in Hep2, Hep-2-Tax, HLaC79 and HLaC79-Tax was tested by western blot analysis of whole cell lysates and by Taqman qRT-PCR. Both methods confirmed overexpression of P-Gp in HLaC79-Tax and Hep2-Tax cell lines. Lowest amount of the mdr-1 transcript was detected in HLaC79 cells (Fig. 2). Adequate protein bands in western blots were clearly visible in the paclitaxel-resistant clones Hep-2-Tax and HLaC79-Tax. Weak signals came up in HLaC79 and Hep2 protein lysates (Fig. 2).

Cytotoxicity. The four cell lines were treated with increasing concentrations of *Galium* aqueous extract. Cell viability and cytotoxicity of the used drugs were measured with the MTT assay. Mean percentage inhibition was calculated from at least three independent experiments. *Galium* extract significantly suppressed the growth of all cell lines (Kruskal-Wallis-test, $p<0.05$). The multidrug resistant clones of HLaC79 and Hep2, however, tolerated high *Galium* concentrations better than the parental drug-sensitive cell lines (Fig. 3).

Motility. 3D invasion in semisolid gels. Investigation of invasion and motility experiments was carried out using spheroid-based experiments. First these experiments better reflect the solid tumour-microenvironment interaction. Second the widely used Boyden Chamber assay proved to be not reproducible in the actual system.

Spheroids of all for cell lines were grown in ultra-low-attachment-plate (ULA-plate) wells. While Hep2 and Hep2-Tax cells formed round-shaped compact spheroids, HLaC79 cells produced only loose aggregates, resolving on pipetting. In contrast to all other cell lines HLaC79-Tax cells did not even form loose aggregates. Instead, many tiny spheroids appeared within the ULA wells, with cells only loosely attached to each other (Fig. 4). For this reason the examinations were limited to Hep2 and Hep2-Tax cells for detailed motility and invasion experiments. *Galium* decoction was used at a concentration of 33.3 μl/ml which proved to be not lethal to the cell lines.

When embedding Hep2/Hep2-Tax spheroids into solidified Matrigel, with or without EGF, the cells tended not to invade gels. In collagen gels, however, cells did invade. Hep2-Tax cell migration was stronger than the chemosensitive original Hep2 cells (Fig. 5). This mode of invasion was inhibited by *Galium* extract within collagen gels.

Cell migration on extracellular Matrix (ECM) proteins. For quantification of invasion on different ECM substrates Hep2 and Hep2-Tax spheroids were transferred manually to wells, coated with gelatine, fibronectin, laminin, collagen I or matrigel. Images of the cells were taken after attachment to

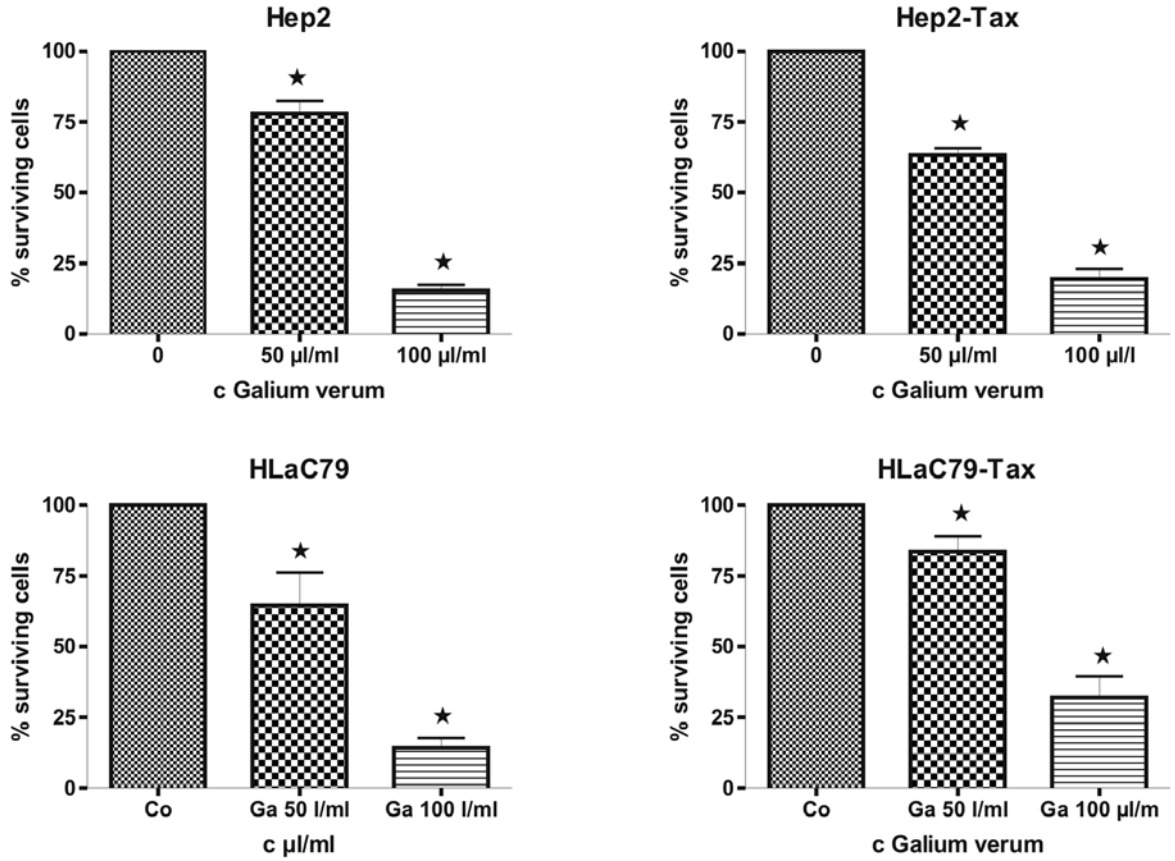

Figure 3. Cytotoxicity of *Galium* decoction on chemosensitive (Hep2, HLaC79) and chemoresistant (HLaC79-Tax, Hep2-Tax) cell lines, measured with the MTT assay. (*p<0.05; Kruskal-Wallis test).

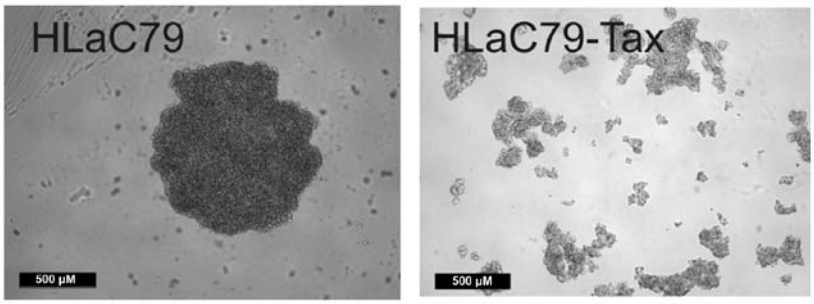

Figure 4. Spheroids produced from HLaC79 and HLaC79-Tax cell lines by growing 5,000 cells/well in ULA-plates.

ECM (t=0) and after 24 h (t=24). For quantification of cells migrating out of spheroids the areas of spheroids at t=0 were substracted from areas measured after 24 h, using ImageJ software (Fig. 6).

For evaluation of cell motility the area at t=0 was set at 100%. The percentage of the migrated area was calculated as the following formula:

$$\% \text{ migrated area} = \frac{100 \times \Delta \text{Area}}{\text{Area}^{t=0}}$$

$$\text{whereas } \Delta \text{Area} = \text{Area}^{t=24} - \text{Area}^{t=0}$$

Migration of Hep2 and Hep2-Tax cells on gelatin, fibronectin, laminin, collagen I and Matrigel substrate with or without *Galium* was measured. Representative examples for Hep2 and Hep2-Tax cells migrating on collagen I and Matrigel with or without *Galium* are shown in Fig. 7. Invasion patterns on gelatin, fibronectin and laminin showed a similar optical appearance, although percentage invasion differed notably between the substrates (Fig. 8). Highest motility was observed on Matrigel substrate in chemosensitive as well as in chemoresistant cells.

Comparing percentage of the migrated areas of Hep2 and Hep2-Tax cell lines revealed higher motility in chemoresistant Hep2-Tax cells, albeit statistically significant only for laminin and collagen I (unpaired t-test, $p<0.05$, Fig. 8) with fibronectin as an exception, where both cell lines migrated approximately equal. Additionally invaded areas between vehicle- and *Galium*-treated spheroids were compared. Results are shown in Fig. 9.

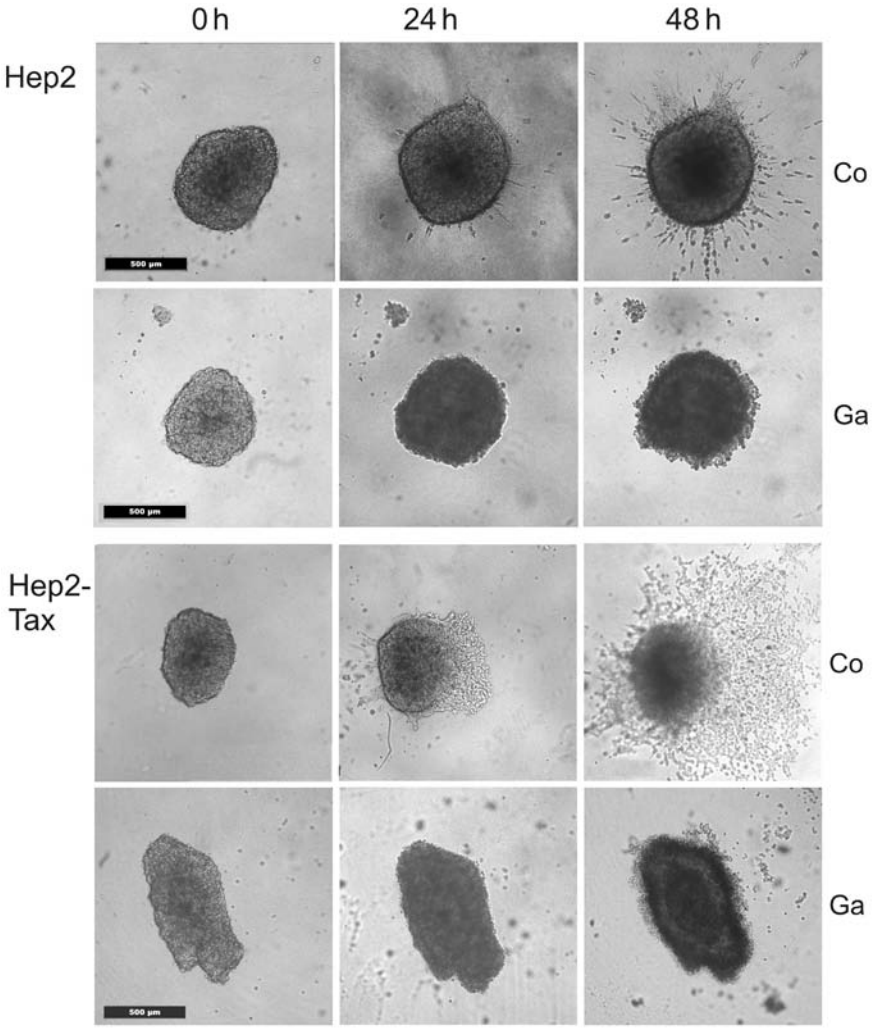

Figure 5. Three-dimensional migration of spheroid cells into Collagen gels with (Ga) or without (Co) *Galium* aqueous extract. Images were taken at 0, 24 and 48 h after embedding.

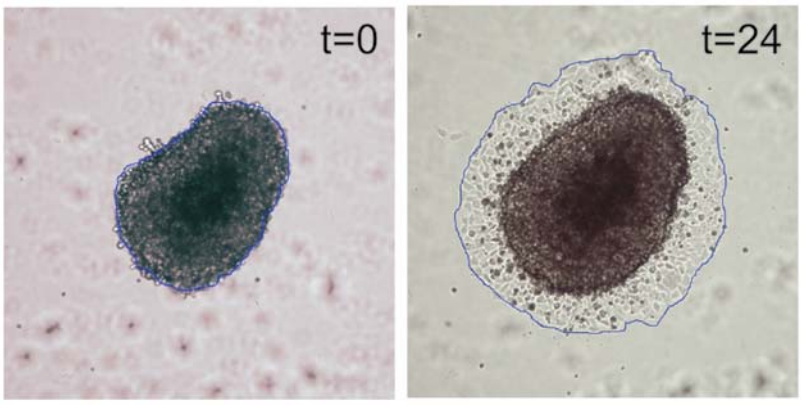

Figure 6. Measurement of invaded areas with ImageJ. For evaluation of cell motility the area at t=0 was set at 100%. The percentage of the migrated area was calculated as described in the Results.

In both cell lines ECM substrate invasion was inhibited significantly by *Galium verum* decoction at sublethal doses of 33.3 μl/ml (unpaired t-test, $p<0.05$). Comparison of the percentage reduction of migrated area caused by *Galium* in the two cell lines revealed a stronger inhibition of invasion in the aggressively invading chemoresistant Hep2-Tax cells (Table I).

Gelatinolytic activity. Gelatinolytic activity of *Galium*-treated cell lines was examined using gelatin zymography. Culture supernatants of Hep2, Hep2-Tax, HLaC79 and HLaC79-Tax cells, treated with sublethal doses of *Galium* or vehicle were separated and developed on gelatin zymographic gels. Results are shown in Fig. 10. Gelatinolytic activity of the active

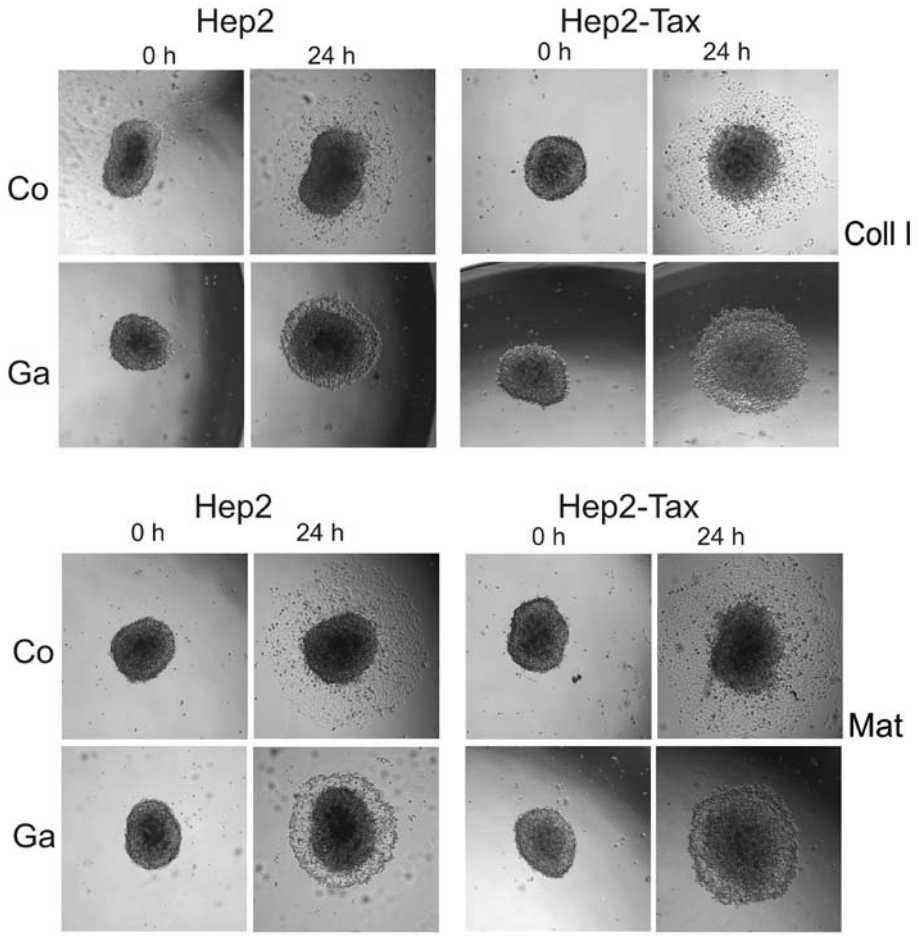

Figure 7. Invasion patterns of Hep2 and Hep2-Tax cells on Collagen I (CollI) or Matrigel® (Mat) coated surfaces at t=0 (0 h after transfer to substrate) and t=24 (24 h later) with (Ga) or without (Co) *Galium verum* aqueous extract.

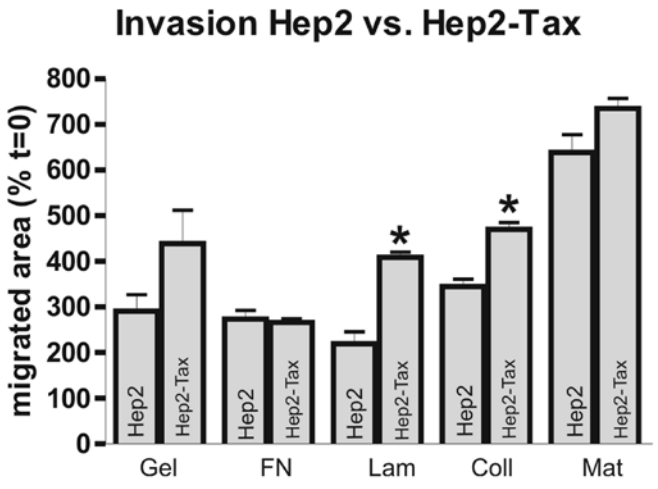

Figure 8. Percentage of invasion of Hep2 or Hep2-Tax cells on different substrates (Gel, Gelatin; FN, Fibronectin; Lam, Laminin; Coll, Collagen I; Mat, Matrigel). Statistically significant values are marked with an asterisk (unpaired t-test, $p<0.05$).

Table I. Percentage reduction of invasion caused by *Galium verum* aqueous extract in Hep2 and Hep2-Tax cell lines.[a]

	Inhibition of invasion Co vs. Ga-treated (%)	
	Hep2	Hep2-Tax
Gelatin	60.75±13.55	102.39±39.43
Fibronectin	77.25±26.83	109.00±15.03
Laminin	95.00±28.84	197.30±21.17
Collagen I	159.70±23.83	273.80±16.36
Matrigel®	368.00±45.52	503.00±36.32

[a]Data are based on the percentage invasion difference between the means of untreated and *Galium*-treated spheroids, calculated by GraphPad 4.

66 kDa MMP-2 was diminished in the cell lines HLaC79, Hep2 and HLaC79-Tax by *Galium* decoction treatment. In the paclitaxel-resistant clone Hep2-Tax no effect of *Galium* treatment on gelatinolytic activity was detectable.

Angiogenesis. Effects on angiogenesis were tested with the tube formation assay, quantifying total tube length, total branching points, total loops and % covered area. There was no statistical significant or visible influence of *Galium* extracts at 33.6 or 66.6 μl/ml on tube formation of HUVECs growing on Matrigel (Fig. 11, here shown for 66.6 μl/ml).

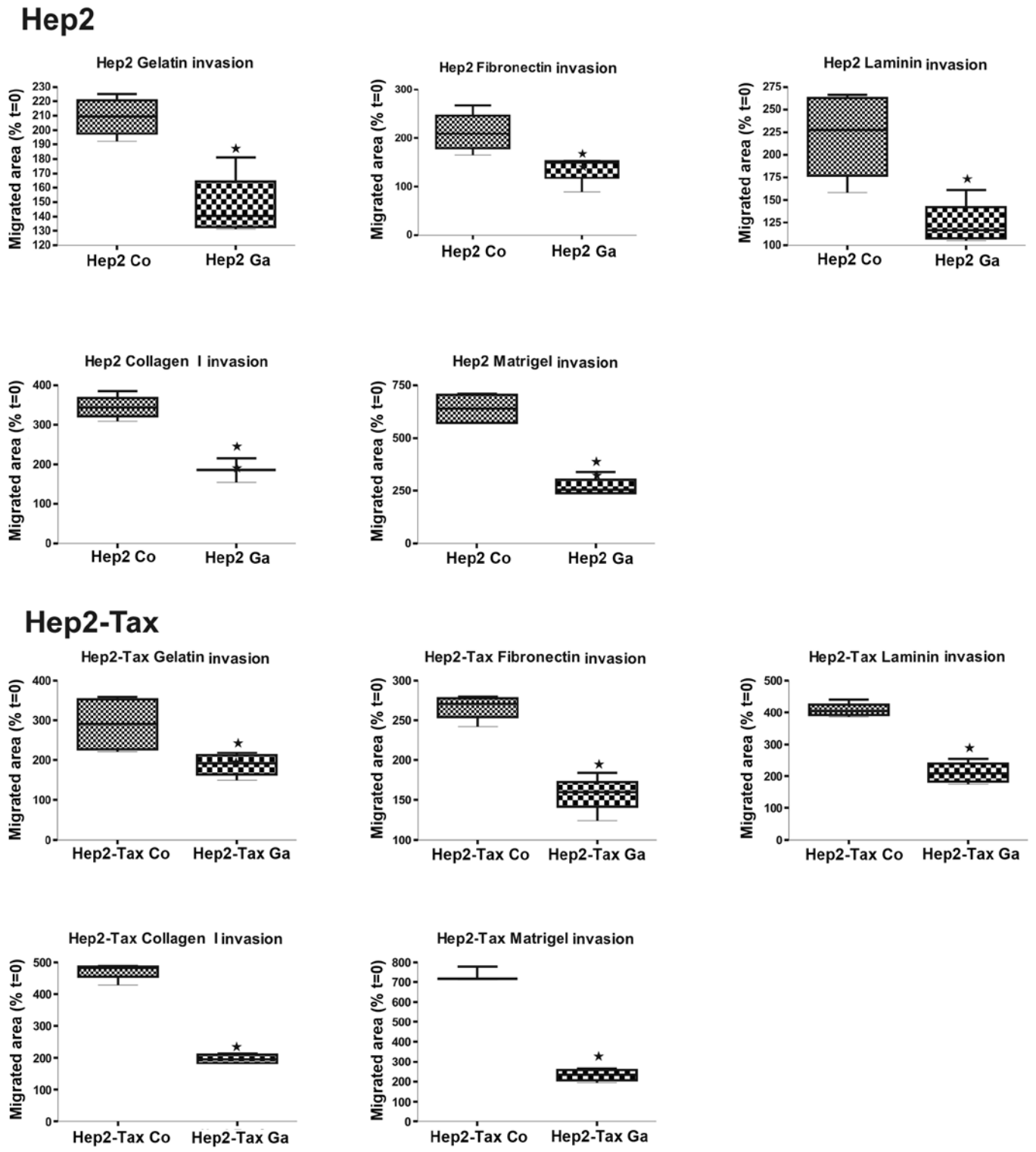

Figure 9. Inhibition of substrate invasion by *Galium* aqueous extract. Hep2 and Hep2-Tax cell lines with (Ga) or without *Galium* (Co) extract on Gelatin-, Fibronectin-, Laminin-, Collagen I- and Matrigel®-coated surfaces were tested. Statistically significant values are marked with an asterisk (unpaired t-test, $p<0.05$).

cDNA array. Using the PrimeView Human Gene Expression array (Affymetrix), the expression profiles of two cell lines (HLaC79 and Hep2) following a *Galium* treatment (33.3 µl/ml, 48 h) in comparison to untreated cells were created. Of 48,736 analyzed genes, 367 were significantly differentially expressed in Hep2 cells and 279 in HLaC79 cells following *Galium* treatment. There were only 11 genes affected significantly in both cell lines (Table III) in this experiment, indicating that the effect of this cocktail of active substances is dependent on cell type and/or cell status (cell cycle, passage number, gene expression profile, differentiation and/or hormonal stage).

The most differentially expressed genes for both cell lines are listed in Table II. For both cell lines, significantly up/downregulated genes are listed in Table III. AKR1C1 and C3

Table II. The most significant up/downregulated genes in Hep2 and HLaC79 cell lines caused by treatment with 33.3 μl/ml *Galium verum* decoction for 48 h.[a]

Gene symbol	Gene name	Function	logFC
HLaC79: *Galium* responding genes			
PHB2	Prohibitin 2	Negative regulation of gene expression	3.10
JUN	Jun proto-oncogene	Apoptosis, protein targeting	-3.09
TMED3	Transmembrane emp24 protein Transport domain containing 3	Protein regulation	3.03
INHBE	Inhibin, βE	Hormone	-3.01
TFRC	Transferrin receptor (p90, CD71)	Membrane organisation, vesicle transport	2.95
HSPA8/SNORD14C/ SNORD14D	Heat shock 70 kDa protein 8/small nucleolar RNA, C/D box 14C/14D	Protein folding, vesicle transport	2.91
ARL6IP4	ADP-ribosylation-like factor 6 interacting protein 4	mRNA splicing regulation (?)	2.68
FNDC3B	Fibronectin type III domain containing 3B	Fat cell differentiation	-2.61
SNRPB	Small nuclear ribonucleoprotein polypeptides B and B1	mRNA splicing	2.49
PTPRM	Protein tyrosine phosphatase, receptor type, M	Cell migration	-2.48
LOC100652805/ 100653302/PGK1	Uncharacterized LOC100652805/100653302/ phosphoglycerate kinase 1	Energy metabolism	2.45
ARHGAP29	Rho GTPase activating protein 29	Small GTPase mediated signal transduction	-2.42
RBBP4	Retinoblastoma binding protein 4	Chromatin remodeling, transcriptional repression	2.41
LMO2	LIM domain only 2 (rhombotin-like 1)	Negative regulation of cell differentiation	-2.40
SNAP91	Synaptosomal-associated protein, 91 kDa homolog (mouse)	Cellular protein complex assembly, vesicle transport	-2.35
MMP10	Matrix metallopeptidase 10 (stromelysin 2)	Invasion, motility	-2.35
SHMT2	Serine hydroxymethyltransferase 2 (mitochondrial)	Mitochondrial thymidylate biosynthesis pathway	2.31
DCLRE1C	DNA cross-link repair 1C	Recombination, DNA repair	2.30
PTPRM	Protein tyrosine phosphatase, receptor type, M	Cell migration	-2.29
ACTG1	Actin, γ1	Cytoskeleton organization, cell motion	2.30
ZNF511	Zinc finger protein 511	Transkriptional regulation (?)	2.26
XYLT1	Xylosyltransferase I	Biosynthesis of glycosaminoglycan	-2.24
NRCAM	Neuronal cell adhesion molecule	Extracellular structure organization, cell motion/adhesion	-2.17
RUFY3	RUN and FYVE domain containing 3	Negative regulation of cell differentiation	-2.16
SATB1	SATB homeobox 1	Negative regulation of gene expression	-2.12
NF1	Neurofibromin 1	Cytoskeleton organization, tumor suppressor	-2.12
TRAPPC6B	Trafficking protein particle complex 6B	Vesicle mediated transport	-2.09
CDS1	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 1	Phosphatidylinositol biosynthesis	-2.09
CPSF6	Cleavage and polyadenylation specific factor 6, 68 kDa	Pre-mRNA 3'-processing	2.11
EDARADD/ENO1	EDAR-associated death domain/enolase 1, (α)	Ectoderm-mesoderm interactions/glycolysis	2.07

Table II. Continued

Gene symbol	Gene name	Function	logFC
Hep2: *Galium* responding genes			
AKR1C2	Aldo-keto reductase family 1, member C2	Xenobiotics metabolism	4.46
HMOX1	Heme oxygenase (decycling) 1	Regulation apoptosis, ARE pathway	4.25
TXNIP	Thioredoxin interacting protein	Regulation apoptosis, drug response, tumor suppressor	4.13
IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	Interferon signalling	-3.91
MDM2	Mdm2, p53 E3 ubiquitin protein ligase homolog (mouse)	Cell cycle	-3.79
AKR1C1	Aldo-keto reductase family 1, member C1	Xenobiotics metabolism	3.63
ASZ1	Ankyrin repeat, SAM and basic/l eucine zipper domain containing 1	Drug response	-3.59
CCPG1	Cell cycle progression 1	Cell cycle	-3.51
AKR1C3	Aldo-keto reductase family 1, member C3	Xenobiotics metabolism	3.28
ENY2	Enhancer of yellow 2 homolog *(Drosophila)*	Chromosome organisation	3.22
CDC42EP3	CDC42 effector protein (Rho GTPase binding) 3	Transcription activation	-3.09
BST1	Bone marrow stromal cell antigen 1		-2.98
HEXIM1	Hexamethylene bis-acetamide inducible 1	Estrogen receptor signalling	-2.93
RBM15	RNA binding motif protein 15	Chromatin organisation (?)	-2.85
LOC100505861/ TPRG1	Uncharacterized LOC100505861/ tumor protein p63 regulated 1		-2.81
NEDD9	Neural precursor cell expressed, developmentally downregulated 9	Cell cycle	-2.73
DHRS2	Dehydrogenase/reductase (SDR family) member 2	Regulation of apoptosis	-2.70
SRP19	Signal recognition particle 19 kDa	Drug response	-2.70
MED18	Mediator complex subunit 18	Transcription activation	-2.68
IGSF10	Immunoglobulin superfamily, member 10		-2.67
HSPA5	Heat shock 70 kDa protein 5	Regulation of apoptosis	-2.66
PDP1	Pyruvate dehyrogenase phosphatase catalytic subunit 1	Protein amino acid dephosphorylation	-2.61
MAT2A	Methionine adenosyltransferase II, α	Drug response	-2.55
PHLDA1	Pleckstrin homology-like domain, family A, member 1	Regulation of apoptosis	-2.55
RND3	Rho family GTPase 3	Cell cycle	-2.54
HIST1H2BC/ BE/BF/BG/BI	Histone cluster 1, H2bc, H2be, H2bf, H2bg, H2bi	Cellular macromolecular complex assembly	-2.54
LARP6	La ribonucleoprotein domain family, member 6		-2.54
DYNLL2	Dynein, light chain, LC8-type 2	Tumor suppressor	-2.50
USP47	Ubiquitin specific peptidase 47		-2.48
MKI67	Antigen identified by monoclonal antibody Ki-67	Cell cycle	-2.46

[a]Gene expression values are indicated as log fold change (logFC), whereas negative values the mean downregulation.

Table III. Significantly up/downregulated genes in both Hep2 and HLaC79 cell lines caused by treatment with 33.3 μl/ml *Galium verum* decoction for 48 h.

Gene symbol	Gene name	Hep2	HLaC79
AKR1C3	Aldo-keto reductase family 1, member C3 (3-α hydroxysteroid dehydrogenase, type II)	+	-
ID1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	-	-
PIR	Pirin (iron-binding nuclear protein)	+	+
HES1	Hairy and enhancer of split 1, *(Drosophila)*	+	-
FNDC3B	Fibronectin type III domain containing 3B	-	-
AKR1C1	Aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-α (3-α)-hydroxysteroid dehydrogenase)	+	-
ENSA	Endosulfine α	-	-
ZNF789	Zinc finger protein 789	+	+
ARHGAP26	Rho GTPase activating protein 26	-	-
CCPG1 /// DYX1C1-CCPG1	Cell cycle progression 1 /// DYX1C1-CCPG1 readthrough (non-protein coding)	-	-
CARD16 /// CAS31	Caspase recruitment domain family, member 16 /// caspase 1, apoptosis-related cysteine peptidase	-	-

+, upregulation; -, downregulation.

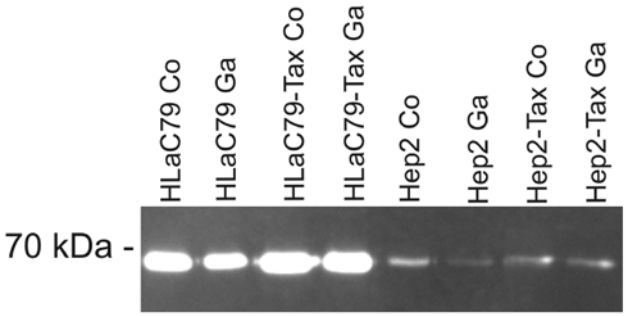

Figure 10. Analysis of gelatinolytic activity using gelatin-zymography. Visible clear bands conform to the 66 kDa activated form of MMP-2.

genes, both coding for proteins needed in detoxification as well as HES1 are oppositionally regulated in Hep2 and HLaC79 cell lines (AKR1C1 HLaC79 logFC= -1.51; Hep2 logFC=3.03; AKR1C3 HLaC79 logFC= -1.43; Hep2 logFC=3.28; HES1 HLaC79 logFC= -2.04; Hep2 logFC=1.75).

qRT-PCR. To test array results on specificity for *Galium* treatment a few interesting candidate genes were picked out and qRT-PCR with *Galium*-treated and untreated cells of the cell lines Hep2, Hep2-Tax, HLaC79, HLaC79-Tax and Fadu was performed. Expression induction/repression of TXNIP, MDM2 and RND3 differentially induced/repressed by *Galium* extract in Hep2 cells and JUN and MMP-10 in HLaC79 cells was tested. Results are summarized in Fig. 12.

Matrix metalloproteinase 10 (MMP-10) and c-Jun (JUN) were downregulated significantly in HLaC79 cells after *Galium* treatment. HLaC79 cells expressed vast amounts of c-jun at the stage of RNA isolation for the cDNA array. In the other cell lines, however, qRT-PCR revealed no clear tendency towards up or downregulation of JUN by *Galium* decoction. It is remarkable, however, that basic expression levels in all other cell lines are much lower than in HLaC79.

MMP-10 was significantly downregulated in HLaC79 cells after *Galium* treatment. There was no amplification of MMP-10 in Hep2 and Hep2-Tax cells. Expression was diminished in HLaC79 cell line. In Fadu and HLaC79-Tax cells, however, MMP-10 expression increased after *Galium* incubation (in Fadu significantly, in HLaC79-Tax cells not significantly, unpaired t-test).

Of the genes differentially affected in expression by *Galium* in Hep2 cells the tumour suppressor gene Thioredoxin-interacting protein (TXNIP) was chosen. TXNIP was significantly upregulated by *Galium* aqueous extract in Fadu, Hep2 and Hep2-Tax cells. In HLaC79 and HLaC79-Tax cell lines, expressing low amounts (HLaC79) or no TXNIP (HLaC79-Tax) induction could not be achieved by *Galium* aqueous extract.

Mouse double minute 2 homolog (MDM2) was significantly downregulated in Hep2 cells 48 h after *Galium* decoction treatment. Negative gene regulation by *Galium* aqueous extract was confirmed to be significant in Fadu cells, in HLaC79 cells without significance. The chemoresistant cell lines showed upregulation of MDM2 (significant in HLaC79-Tax, not significant in Hep2-Tax).

The regulation of RND3/RhoE, which belongs to the Rho family of small GTP-binding proteins was also examined. RND3 was downregulated significantly in Hep2, Fadu and HLaC79 cells following *Galium verum* treatment. Similar

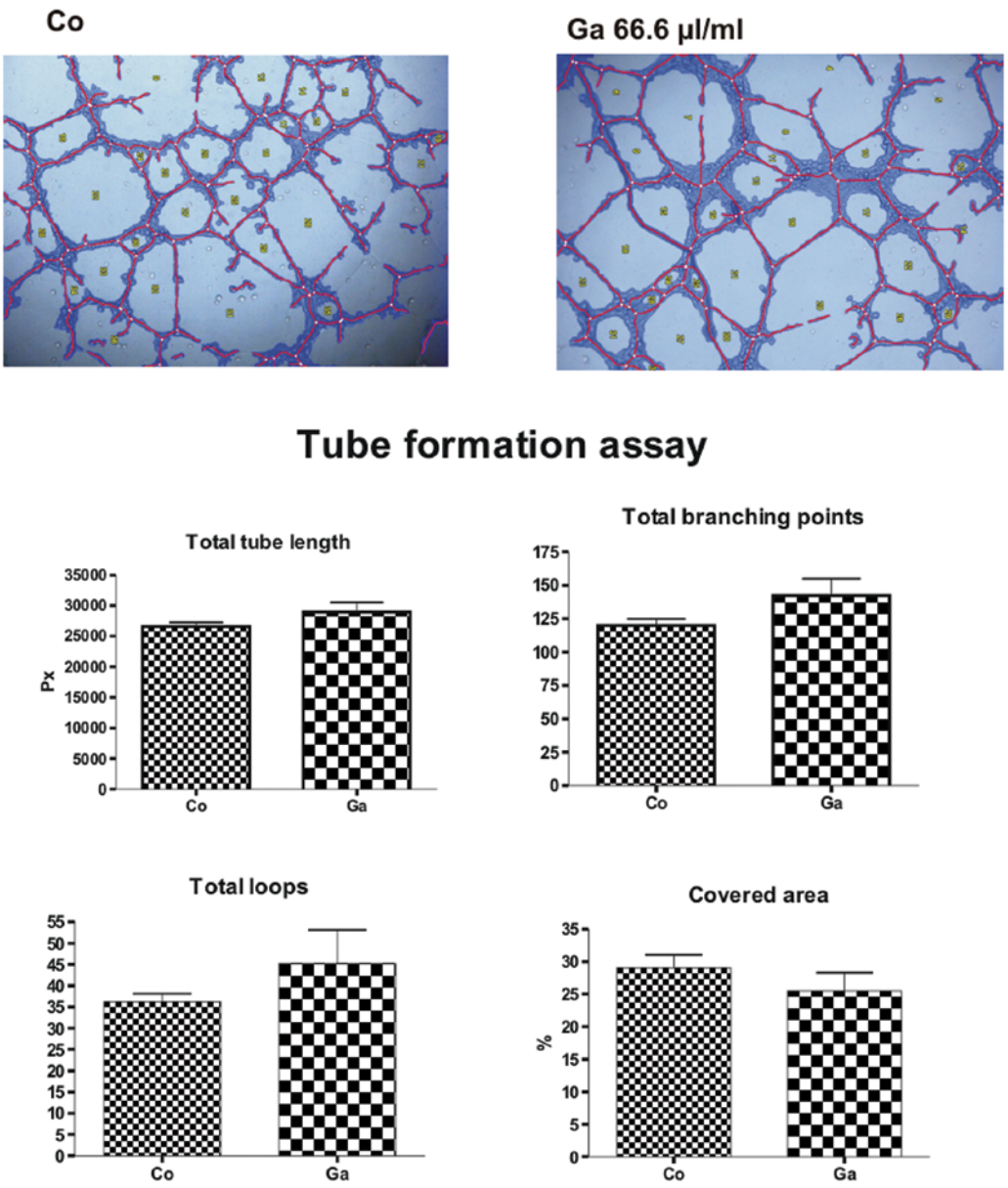

Figure 11. Measurement of angiogenesis with the tube formation assay. HUVEC tube formation was tested with or without *Galium* aqueous extract (66.6 µl/ml). Total tube length (measured in pixels, px), number of branching points, number of total loops and percentage covered area (%) were measured.

to MDM2, an inverse reaction in the chemoresistant clones (significantly upregulated in Hep2-Tax, not significant in HLaC79-Tax) was observed.

Discussion

In advanced laryngeal and hypopharyngeal cancer the chemotherapeutic agent paclitaxel is commonly used for chemotherapy in order to preserve laryngeal and/or pharyngeal structures. Chemotherapeutic failure, frequently observed in head and neck cancer, may be related either to inherited resistance against the drug or/and the acquired resistance during the therapy. One mechanism of drug resistance in cancer cells is the overexpression of multi-drug resistance proteins, such as P-glycoprotein (coded by the multi-drug resistance gene 1, MDR-1, P-Gp), here clearly shown to be upregulated in paclitaxel-resistant clones of Hep2 and HLaC79 cell lines. *Galium verum* is a traditional medical plant commonly used for the exogenous cure of psoriasis, delayed wound healing or as a tea with diuretic effect for the cure of pyelitis or cystitis (3).

Some popular compendia for herbal medicine recommend *Galium verum* for therapy of mouth/neck cancer (4,5). According to Jonathan Hartwells detailed survey (18) *Galium verum* was traditionally used in Europe and Northern America for treatment of cancerous ulcers or breast cancer. In the present study the effect of a simple decoction of *Herba gallii lutei* on chemo-sensitive and -resistant cell lines of laryngeal carcinoma was tested and significant growth inhibition in chemo-sensitive and -resistant cell lines, albeit somewhat extenuated in the paclitaxel-resitant clones was demonstrated. This is in agreement with Amirghofran *et al* (19), showing a cytotoxic effect of *Galium* mite methanolic extracts on K561 and Jurkat cells. Using sublethal doses of *Galium verum* decoction the influence on endothelial tube formation (angiogenesis)

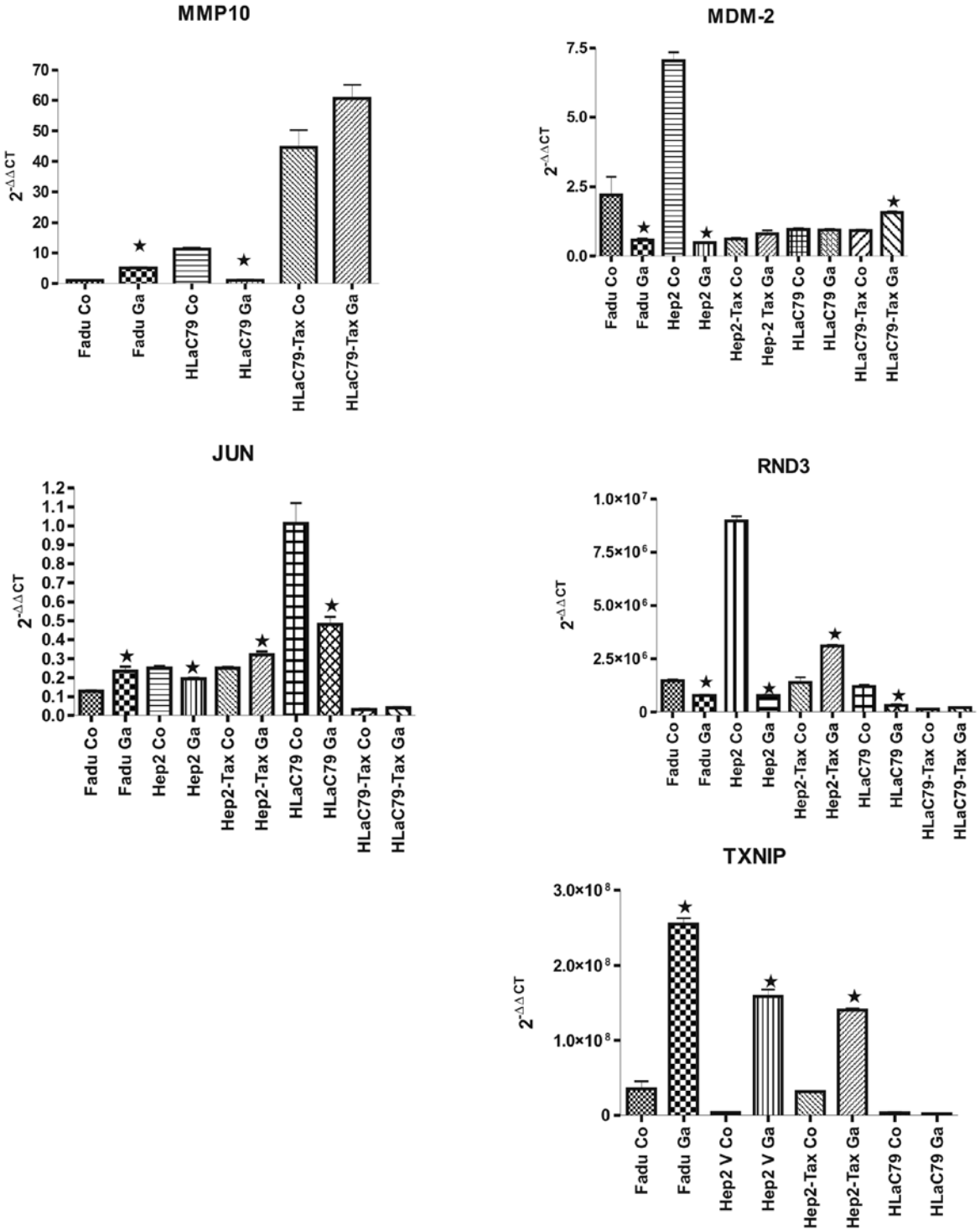

Figure 12. Gene expression of selected genes induced/repressed by *Galium verum* aqueous extract in Hep2 or HLaC79 cells, respectively. Significant differences are marked by an asterisk (unpaired t-test).

was examined, this study revealed no detectable effect. Even higher doses of *Galium verum* decoction did not suppress the development of a visible cordal network.

For the first time, a detailed motility/invasion analysis with tumour spheroids, invading diverse ECM substrates with or without low doses of *Galium* decoction was performed. Invasion was inhibited on all five substrates (gelatin, fibronectin, laminin, collagen I, and Matrigel) by *Galium verum* aqueous extract in chemosensitive as well as in chemoresistant cell lines. Comparing the percentage of reduction in cell migration, a more pronounced inhibition of invasion in the highly motile chemoresistant Hep2-Tax clone was observed. Thus, anti-invasive properties of *Galium verum* are remarkable.

As active compounds, in the *Galium verum* extract used, TLC identified among others chlorogenic acid, Luteolin-7-O-glucoside as well as rutoside. Comparing TLC patterns of methanolic and aqueous *Galium* extract, an enrichment of chlorogenic acid in the aqueous decoction was observed, while

many other substances, such as apigenin, diminished without alcoholic solvent. Yagasaki *et al* (35) showed an obvious inhibition of invasion in rat teratoma cell lines caused by chlorogenic acid at doses, not growth inhibiting. This is in agreement with the present results. Jin *et al* (36) identified chlorogenic acid to be a strong suppressor of MMP-9 activity. In the current study, MMP-2 gelatinolytic activity was suppressed in HLaC79, HLaC79-Tax and Hep2 cells. This effect may be caused by chlorogenic acid, but also by other ingredients.

Luteolin7-O-glucoside has been shown to induce apoptosis in colon cancer cell lines and to effectively inhibit experimental colon carcinogenesis *in vivo* (37). The combination of several migration inhibitory substances may explain the strong inhibitory effect on migration of *Galium verum* aqueous extract.

In the actual study, TLC analysis was performed for identification of the used aqueous extract of *Galium verum*. Only the main flavonoids and phenolic acids were tested. Former detailed chromatographic analyses of *Galium* extracts (throughout non-aqueous extracts) identified many powerful ingredients such as iridoidglycosides (asperuloside, monotropeine, scandoside and daphylloside), flavonoids/flavonoid-glycosides (quercetin-3-glucoside, quercetin-7-glucoside, diosmetin and palustrosid (reviewed in ref. 3), as well as anthraquinones (12) in *Galium* extracts. A detailed HPLC analysis would probably identify more ingredients, qualifying as candidates for the effects observed in our cell lines. The intention, however, was to evaluate the worth of an aqueous extract as a whole, historically used as an anticancer remedy.

Analysis of gene expression revealed no consistent patterns of changes in Hep2 and HLaC79 cells. Of 48,736 analysed genes, 367 were significantly differentially expressed in Hep2 cells and 279 in HLaC79 cells following *Galium* treatment. There were only 11 genes affected significantly in both cell lines, three of them regulated in opposite directions (AKR1C3, AKR1C1 and HES1), although initial expression values did not differ greatly. Enzymes of the aldo/keto reductase superfamily catalyse the conversion of aldehydes and ketones to their corresponding alcohols with specific substrates for each family member. AKRC1 and AKRC3 are involved in the metabolism of progesterone or prostaglandines, respectively. It seems that regulation of these genes is also dependent on hormonal stage of the cell line. Generally it seems that gene activation/suppression caused by *Galium verum* extract is cell-type dependent, rather than the activation of a fixed cascade of genes caused by the ingredients of the extract. A clearer image might appear if expression arrays are performed earlier after induction to capture immediate early gene response. A further approach might be the testing of single ingredients of the extract, such as chlorogenic acid, to focus on common pathways activated by the substance. Nevertheless, some of the significantly *Galium*-regulated genes were picked out and tested in a variety of cell lines, treated with *Galium* aqueous extract vs. untreated controls.

Thioredoxin-interacting protein (TXNIP) showed a tendency to be upregulated in most cell lines upon *Galium* treatment, whereas HLaC79-Tax and HLaC79 cells expressing no or only low amounts of TXNIP. Induction could not be achieved in these cell lines by *Galium* treatment. TXNIP which is frequently repressed in human cancers can also inhibit metastasis (reviewed in ref. 38).

C-jun, coded by the JUN gene is an oncogenic transcription factor (reviewed in ref. 39). In breast cancer c-jun had been associated with proliferation and angiogenesis (40). In the study JUN was significantly downregulated in HLaC79 cells expressing vast amounts of c-jun at the stage of RNA isolation for the cDNA array. In the other cell lines there was no clear tendency towards up or downregulation of JUN by *Galium* decoction. Fadu and Hep2-Tax cells even reacted by increasing JUN expression. Therefore, it seems that the strong inhibitory effect of *Galium* on JUN expression is cell type specific, or responsible for 'normalization' of the punctual strongly upregulated JUN transcription.

Furthermore, the influence of *Galium* aqueous extract on the expression of MMP-10 was tested, which was significantly downregulated in HLaC79 cells due to *Galium* treatment. In Hep2 and Hep2-Tax cells, there was no amplification of MMP-10 transcript detectable either with or without *Galium* treatment. Expression was diminished in HLaC79 cells, in Fadu and HLaC79-Tax cells, however, MMP-10 expression increased after *Galium* incubation (in HLaC79-Tax cells not significantly), and therefore seems not to be a specific candidate for *Galium* caused gene regulation.

Mouse double minute 2 homolog (MDM2) is an E3 ubiquitin ligase, known as oncogene, overexpressed in many cancer types (reviewed in ref. 41). MDM2 was significantly downregulated in Hep2 cells, 48 h after *Galium* decoction treatment. Negative gene regulation by *Galium* aqueous extract was confirmed in Fadu cells as statistically significant, in HLaC79 cells without significance (unpaired t-test with $p<0.05$). The chemoresistant cell lines showed upregulation of MDM2 (significant in HLaC79-Tax, not significant in Hep2-Tax). Qin *et al* (41) reviewed the role of natural products such as flavonoids and isoflavonoids in inhibiting MDM2 expression. Reduction of MDM2 expression was reached with genistein, apigenin and oroxylin A. These flavonoids/isoflavonoids were not reported yet to be ingredients of *Galium* extracts, but other flavonoids or other secondary plant products may also contribute to MDM2 downregulation. The lack of downregulation in the paclitaxel-resistant clones might be caused by P-Gp overexpression and the resulting detoxification. At least it seems likely, that the composition of the chemical cocktail active inside the cell differs between chemo-sensitive and -resistant cell lines.

RND3/RhoE belongs to the Rho family of small GTP-binding proteins. Most Rho family members cycle between a GTP-bound active and a GDP-bound inactive form. RND3/RhoE is an atypical member of the Rho family, regulated rather by expression levels than by GTP/GDP cycling (reviewed in ref. 42). The role of RND3/RhoE in cancer and metastasis is still unclear; controversial results concerning the expression have been published. While RND3 has been shown to be downregulated in hepatocellular carcinoma (43) or prostate carcinoma (44) it has been reported to be upregulated in non-small cell lung cancer (45) or colon carcinoma (46). *In vitro* RND3 expression has been associated with melanoma cell migration and invasive outgrowth (47). In the present study, RND3 was downregulated significantly in Hep2, Fadu and

HLaC79 cells following *Galium verum* treatment. Similar to MDM2, we observed an inverse reaction in the chemoresistant clones (significant upregulated in Hep2-Tax, not significant in HLaC79-Tax). It would, therefore, be interesting to test spheroid-based invasion on different ECM substrates of RND3 knocked out chemo-sensitive and -resistant cells.

Summarizing the results *Galium verum* aqueous extract revealed a growth inhibiting effect on chemo-sensitive and -resistant laryngeal carcinoma cell lines. In non-toxic concentrations *Galium* extract inhibited significantly, invasion of Hep2 and Hep2-Tax cells within collagen gels and on all ECM substrates tested, and was most pronounced in the aggressively invading chemoresistant Hep2-Tax cell line. Angiogenesis, however, was not affected by *Galium* decoction. Gene expression profiling did not reveal unique gene induction/repression patterns in HLaC79 and Hep2 cell lines, suggesting, that hormonal, metabolism and/or differentiation stage of HLaC79 and Hep2 cell lines are too divergent for focussing common pathway inductions. The obvious anti-metastatic effect of *Galium* decoction may justify a concomitant therapeutic use for oral or head and neck cancer.

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