Inhibitory mechanisms of two Uncaria tomentosa extracts affecting the Wnt-signaling pathway

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ABSTRACT

Uncaria tomentosa ("uña de gato"; "cat’s claw"), a woody vine native to the Amazon rainforest, is commonly used in South American traditional medicine to treat a broad spectrum of diseases. Although recent studies have reported anti-inflammatory and anti-proliferative properties of different alkaloids extracted from this plant, the underlying molecular mechanisms of these effects have not been elucidated yet. Our study investigates the inhibitory mechanisms of Uncaria tomentosa extracts on the Wnt-signaling pathway, a central regulator of development and tissue homeostasis. A modified cell-based luciferase assay for screening inhibitors of the Wnt-pathway was used for analysis. Three cancer cell lines displaying different levels of aberrant Wnt-signaling activity were transfected with Wnt-signaling responsive Tcf-reporter plasmids and treated with increasing concentrations of two Uncaria tomentosa bark extracts. Wnt-signaling activity was assessed by luciferase activity and by expression of Wnt-responsive target genes. We show that both, an aqueous and an alkaloid-enriched extract specifically inhibit Wnt-signaling activity in HeLa, HCT116 and SW480 cancer cells resulting in reduced expression of the Wnt-target gene: c-Myc. The alkaloid-enriched extract (B/Srt) was found to be more effective than the aqueous extract (B/W37). The strongest effect was observed in SW480 cells, displaying the highest endogenous Wnt-signaling activity. Downregulation of Wnt-signaling by a dominant negative–TCF-4 variant in non-cancer cells rendered the cells insensitive towards treatment with B/Srt. B/Srt was less toxic in non-cancer cells than in cancer cells. Our data suggest that the broad spectrum of pharmacological action of Uncaria tomentosa involves inhibition of the Wnt-signaling pathway, downstream of beta-Catenin activity.

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Introduction

The medicinal vine Uncaria tomentosa (Rubiaceae), native to the Amazon rainforest and commonly known as “uña de gato” or “cat’s claw” has been traditionally used by indigenous tribes to treat a broad spectrum of mental and physical disorders (Keplinger et al. 1999). Today, it is commonly used in tropical American folk medicine to treat viral infections, arthritis, chronic degenerative diseases, gastric ulcers and cancer. Consequently, it has evoked increasing scientific and commercial interest and is widely promoted as an alternative treatment for these ailments (Heitzman et al. 2005).

Despite a growing number of reports describing the clinical and biological effects of U. tomentosa extracts, its pharmacological effectiveness and molecular targets are largely unknown. The most common pharmaceutical forms of U. tomentosa are crude water-soluble or ethanol-soluble extracts derived from its bark or roots for oral consumption as infusions (Keplinger et al. 1999). Active components identified in these extracts include different oxindolic alkaloids, indole alkaloids, glycosides (pentacyclic terpenes with a variety of derivatives such as ursoic acid, quinovic acid glycosides, sterols and prostanoids) and tannins the chemical composition of which can vary depending on their geographical origin and seasonal harvesting (Heitzman et al. 2005). Thus, the diverse pharmacological properties of U. tomentosa reported in the literature might be ascribed to different types and combinations of these compounds.

Most pharmacological in vivo-studies initially investigated how U. tomentosa extracts affect anti-inflammatory, immunomodulatory and DNA-repair mechanisms (Akesson et al. 2003; Keplinger

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Table 1
TOP/FOP-inhibition ratios of cancer cell lines treated with two different extracts of Uncaria tomentosa.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Cell line</th>
<th>0/DMSO</th>
<th>1 µg/ml</th>
<th>10 µg/ml</th>
<th>50 µg/ml</th>
<th>100 µg/ml</th>
<th>200 µg/ml</th>
<th>300 µg/ml</th>
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<tr>
<td>B/W37</td>
<td>HeLa</td>
<td>1.0</td>
<td>-</td>
<td>0.9</td>
<td>1.0</td>
<td>0.8</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>HCT116</td>
<td>1.0</td>
<td>-</td>
<td>1.2</td>
<td>1.1</td>
<td>0.9</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>SW480</td>
<td>1.0</td>
<td>-</td>
<td>0.9</td>
<td>1.4</td>
<td>2.1</td>
<td>1.3</td>
<td>1.1</td>
</tr>
<tr>
<td>B/Srt</td>
<td>HeLa</td>
<td>1.0</td>
<td>1.4</td>
<td>1.2</td>
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<td>1.1</td>
<td>0.8</td>
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<td></td>
<td>HCT116</td>
<td>1.0</td>
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<td>2.2</td>
<td>0.2</td>
<td>0.4</td>
<td>0.0</td>
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</tbody>
</table>

Relative inhibition of Wnt-signaling activity in TOP-luciferase transfected versus FOP-luciferase transfected cells. Ratios, pointing to a specific effect on Wnt-signaling are presented in bold.

Fig. 1. Reduction of Wnt-signaling activity in different cancer cell lines upon treatment with Uncaria tomentosa extracts B/W37 and B/Srt for 24 h. Fold activation of Wnt-signaling was determined by luciferase reporter assays as described in Section “Materials and methods”. Untreated or DMSO-treated cells transfected with FOP-luciferase reporter were set as 1.0. Bars represent the means and standard deviations of six experiments. IC\textsubscript{50(Wnt)} represents the concentration of Uncaria tomentosa extracts, that reduces Wnt-signaling activity to 50% of that in untreated or DMSO-treated cells.
demonstrate cMyc by in reduce Fig. 2. Immunoblot analysis performed on HeLa, HCT116 and SW480 cancer cells treated in the presence or absence of Uncaria tomentosa extracts B/W₃₇ and B/S₉ for 24 h. Expression of the Wnt-signaling target cMyc was clearly down-regulated by both extracts whereas the amount of beta-Catenin in corresponding lysates was unaffected. Twenty micrograms of protein was loaded per lane. Beta-Catenin and cMyc were detected by specific antibodies. Actin expression was determined to demonstrate equivalent loading.

et al. 1999; Sheng et al. 2000, 2001; Spelman et al. 2006). In addition, several studies suggest an anti-tumorigenic activity of both, water-soluble and alkaloid-enriched U. tomentosa extracts: cytotoxic, anti-proliferative and pro-apoptotic effects were reported on different tumor cell lines (Cheng et al. 2007; Garcia Gimenez et al. 2009; Pilarski et al. 2010; Garcia Prado et al. 2007; Gonzales and Valero 2006; Pilarski et al. 2007). Moreover, U. tomentosa preparations with different oxindole alkaloid compositions and hydroalcoholic extracts, respectively were recently shown to reduce tumor growth in in vivo solid tumor animal models (Pilarski et al. 2010; Dreifuss et al. 2010). The mechanism of this antitumor-activity and possible effects on cancer-related signaling pathways in tumor cells however, have not been yet investigated.

The broad spectrum of activities claimed for U. tomentosa extracts suggests that it affects a central regulatory mechanism. One of these major regulators, which are essential for stem cell fate, self-renewal, development and tissue homoeostasis throughout the animal kingdom, is the Wnt-signaling pathway (Logan and Nusse 2004; Nusse 2005). Consequently, alterations on this pathway play a crucial role on human pathophysiology including inflammatory and degenerative diseases, diabetes, and cancer (Janssens et al. 2006; Jin 2008; Logan and Nusse 2004). The canonical Wnt-signaling cascade is activated by binding of secreted Wnt glycoproteins to Frizzled (Fz) receptors via a core set of proteins thereby regulating the ability of beta-Catenin to activate the transcription of T-cell factor (Tcf)-dependent genes. Full activation of Fz receptors requires interaction with LRP (low density lipoprotein receptor-related protein) co-receptors. In the absence of Wnts, most of beta-Catenin is attached to the plasma membrane, where it associates with E-cadherin at adherence junctions. Newly synthesized beta-Catenin is constitutively targeted for degradation by a multi-protein complex. Upon activation of this pathway by specific Wnts, beta-Catenin is released from this complex and transferred to the nucleus where it associates with transcription factors of the TCF/LEF family to activate target genes that regulate cell proliferation, differentiation and genes involved in tumorigenesis (see Wnt homepage for list; http://www.stanford.edu/~rsusse/wntwindow.html). In colorectal epithelium, aberrant activation of Wnt-signaling by mutations is considered to be the major initiating event of cancer growth. In addition, this pathway is commonly affected by mutations in an increasing number of other cancer entities (Behrens and Lustig 2004; Giles et al. 2003; Polakis 2007) as well as in degenerative diseases (Fuerer et al. 2008; Janssens et al. 2006).

Due to its central regulatory role in human disease, the Wnt-signaling pathway is a major target for therapeutic intervention and the development of Wnt-specific inhibitors (Barker and Clevers 2006; Dihlmann and von Knebel 2005; Janssens et al. 2006). This prompted us to investigate whether the mechanism of action of U. tomentosa extracts involves interference with Wnt-signaling activity. Since the preparation and composition of extracts affect its biological activities, which is one of the main problems hindering comparison of different studies, we here used U. tomentosa extracts derived from well characterized and standardized preparation procedures (Pilarski et al. 2007, 2010) which result in biologically active composition of the extracts that are characterized by pentacyclic oxindole alkaloids (Wurm et al. 1998).

Materials and methods

Cell lines

The human colorectal cancer cell line HCT116 was obtained from ECACC (http://www.ecacc.org.uk), the human colorectal cancer cell line SW480, human cervical carcinoma cell line HeLa and human embryonic kidney epithelial cell line 293T were received from the German Cancer Research Centre Tumorbank or CLS Cell Lines Services (Heidelberg, Germany). All cell lines were grown in RPMI 1640 (PAA Laboratories, Germany) supplemented with 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin using standard conditions.

Uncaria tomentosa extracts

Origin of the plant material, as well as preparation and alkaloid composition of the extracts used for this study were described earlier (Pilarski et al. 2007, 2010). Briefly, for preparation of the water-soluble (B/W₃₇) extract, 1 g of bark was extracted in 10 ml of water for 8 h at 37°C. To obtain the alkaloid-rich bark extract (B/S₉), 10 g of bark was extracted with 50 ml of water (6 h, 37°C). Next, the sample was centrifuged at 35,000 rpm and an equal amount of dichloromethane was added to the water supernatant. The organic layer was removed and evaporated under vacuum at 40°C to dryness. For analysis in cell culture experiments, B/W₃₇ was adjusted in cell culture medium RPMI-1640 to prepare a stock solution of 50 mg/ml, B/S₉ was dissolved in DMSO to prepare a stock solution of 50 mg/ml.

Transfection and luciferase reporter assays (TOP/FOP assay)

A modified cell-based assay for screening of Wnt-pathway inhibitors was used to determine the Wnt-signaling activity in cell lines treated with Uncaria tomentosa extracts (Barker and Clevers 2006). Briefly, 2 × 10⁴ cells of each cell line were grown in 6-well plates for 24 h. For reporter assays, 1 × 10⁶ cells of each cell line were transiently transfected in parallel with either 1.5 μg of a Tcf-reporter construct (TOP-luciferase, which responds to aberrant Wnt-signaling by driving high levels of luciferase activity) or 1.5 μg of a mutated reporter construct (FOP-luciferase, which
Fig. 3. Proliferation of HeLa, HCT116 and SW480 cancer cells upon exposure to different concentrations of B/W37 and B/Srt. Uncaria tomentosa extracts for 3, 6, and 24 h. The amount of viable cells as represented by the absorption (A\textsubscript{450} - A\textsubscript{620}) was determined by WST-1 assay as described in Section “Materials and methods”. Error bars represent the standard deviations of three experiments.

is unable to respond to active Wnt-signaling and consequently drives lower levels of luciferase activity). 0.3 µg of a dominant negative pcDNA-dn-hTCF4 was used to biologically downregulate Wnt-signaling, as indicated in the figures. To normalize the transfection efficiency and non-specific toxicity, 0.5 µg of a pRSV-lacZ reporter was included in each sample. All transfections were performed using Fugene HD transfection reagent (ROCHE Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. The TCF-responsive reporter constructs pTOPFLASH and pFOPFLASH were kindly provided by H Clevers; University of Utrecht, The Netherlands. Control plasmid pRSV-lacZ was obtained from DKFZ Heidelberg, Germany. Dominant negative pcDNA-dn-hTCF4 was kindly provided by Bert Vogelstein; The John Hopkins Oncology Centre, Baltimore, MD, USA. Three hours after transfection, cells were harvested and plated out into 96-well plates (1 x 10⁴ cells/well) in 50 µl cell culture medium. Uncaria tomentosa compounds were added to final concentrations as indicated in the figures. Luciferase activity was determined 24 h later by adding 50 µl of OneGlo-Luciferase Assay solution (Promega, Heidelberg, Germany) to each well and incubation for 3 min. Lysates were transferred to white polystyrene plates and luminescence was measured immediately in a 96 well-luminometer (TECAN-Reader, Genios) for 2 s. For normalization, relative beta-galactosidase activity was analyzed from corresponding wells in microtiter plates by using an ELISA reader at 570 nm (Dihlmann et al. 2005). Briefly, 15 µl of each lysate was diluted in 100 µl of phosphate buffer, and 25 µl of a chromogenic substrate (CPRG, ROCHE Diagnostics, Mannheim Germany) was added. Fold activation was determined by normalizing the light units of the luciferase assays versus values derived from absorbance of the beta-galactosidase assay. Mean values of lysates derived from FOP-luciferase-transfected, untreated or DMSO-treated cells were set as 1.0.
WST-1-cell viability assay

Cells were plated in microtiter plates at densities of 2 × 10^3 cells/well and Uncaria tomentosa extracts were added at the desired final concentrations (range from 1 µg/ml to 300 µg/ml). Cell viability was assessed using 10 µl of WST-1 cell proliferation reagent (ROCHE Diagnostics, Mannheim, Germany) after 0, 2, 5, or 23 h of incubation. Cells were grown for 60 min and absorbance was measured using a microplate (ELISA) reader (absorbance at 450 nm versus 650 nm, according to instructions of the manufacturer).

Immunoblotting

Cells were treated as described in Fig. 2. After 24 h, cells were harvested in lysis buffer (150 µl PBS, 100 µM sodium orthovanadate, Proteinase Inhibitor Cocktail (Complete®, EDTA-free, ROCHE Diagnostics, Mannheim, Germany)) and lysed by two freeze–thaw cycles. Protein concentration of lysates was determined by a Protein Assay Kit (BIORAD-Laboratories, München, Germany), and 20 µg of each lysate was applied to SDS-PAGE and blotting using standard procedures as previously described (Dihlmann et al. 2005). Specific proteins were detected by incubation with anti-beta-Catenin (1:1000; Transduction Laboratories (BD Biosciences, Heidelberg, Germany)), anti-c-Myc (1:500; clone 2Q330, Santa Cruz, Heidelberg, Germany) or anti-Actin (1:5000; clone C4; MP Biomedicals, Heidelberg, Germany) antibodies in blocking buffer (5% milk/Tris buffered saline/1% Tween20). After incubation of the blots with rabbit-anti-mouse IgG peroxidase (Dianova, Hamburg, Germany) for 1 h, Western Lightning® Plus–ECL, (Perkin Elmer, Rodgau, Germany) was added as a substrate for visualization by enhanced chemoluminescence.

Results

Inhibition of Wnt-signaling activity by water-soluble (B/W37) and alkaloid-enriched (B/Sr) Uncaria tomentosa bark extracts

We have previously shown that U. tomentosa extracts B/W37 and B/Sr reduce proliferation of various cancer cell lines in different concentrations (Pilarski et al. 2007, 2010). In these studies however, the growth inhibiting activity of B/Sr and B/W37 was very variable. Cancer cell lines of different origins showed great differences in their sensitivity, and no clear correlation between U. tomentosa preparation and cancer cell type was observed. This variation may be due to differently activated cancer signaling pathways. We here applied a cell-based luciferase-reporter assay to determine the ability of U. tomentosa extracts to inhibit beta-Catenin/Tcf-mediated transcription, the outcome of canonical Wnt-signaling. This assay is based on the principle that compounds specifically inhibiting aberrant Wnt-signaling activity will reduce TOP-luciferase activity without (or to a much lower extent) reducing FOP-luciferase activity (Barker and Clevers 2006). Thus, specific Wnt-inhibitors will show TOP/FOP-inhibition ratios to be less than 1.0, whereas unspecific inhibitors will result in TOP/FOP-inhibition ratios greater or equal to 1.0. Fig. 1 shows the effects of a 24-h treatment of HeLa, HCT116 and SW480 cancer cells. In agreement with previous findings (Dihlmann et al. 2001; Huang et al. 2006), endogenous Wnt-signaling in untreated controls was moderately activated in HeLa and HCT116 cells (8–10-fold and 12–20-fold, respectively), whereas it was extensively activated in untreated SW480 colorectal cancer cells (220–250-fold). Upon treatment with B/W37 extract, Wnt-signaling activity was decreased in a dose-dependent manner in all cell lines. SW480 and HCT116 cells were more sensitive than HeLa cells, which is reflected in 50% Wnt-inhibitions (IC50(Wnt)) of 190 µg/ml, 150 µg/ml and 278 µg/ml, respectively (Fig. 1, left panel). Considering the TOP/FOP ratio, however, the effect was only specific in HeLa and HCT116 cells at concentrations > 300 µg/ml (Table 1). In contrast, B/Sr displayed a specific inhibitory effect on Wnt-signaling at much lower concentrations (Fig. 1, right panel). Interestingly, the sensitivity towards B/Srt correlated well with the endogenous Wnt-signaling activity. SW480 cells displaying the highest Wnt-signaling activity were clearly more sensitive than HCT116 and HeLa cells (Fig. 1 and Table 1) with an IC50(Wnt) of 29 µg/ml compared to 60 µg/ml and 38 µg/ml, respectively. This finding further argues for a specific interference of the B/Sr extract with the pathway resulting in down-regulation of beta-Catenin/Tcf-mediated transcription.

Uncaria tomentosa extracts B/W37 and B/Sr down-regulate expression of the Wnt-signaling target c-Myc without affecting beta-Catenin levels

Activation of Tcf-mediated transcription of Wnt-target genes is modulated by beta-Catenin, the level of which is tightly regulated by degradation (Polakis 2007). Over-expression of beta-Catenin, due to mutations that inactivate the beta-Catenin destruction complex or by stabilizing mutations of beta-Catenin itself, results in activation of Wnt-signaling, as seen in the cancer cell lines used in this study (Fig. 1, untreated or DMSO treated cells). To determine, whether the signaling-inhibitory effect of U. tomentosa extracts operates upstream or downstream of beta-Catenin, we investigated beta-Catenin protein levels in HeLa, HCT116 and SW480 cells in response to B/W37 and B/Sr extracts. As shown by immunoblotting (Fig. 2), neither extract affected significantly beta-Catenin levels in all cancer cell lines studied. Furthermore, beta-Catenin degradation products in lysates of U. tomentosa extract treated cells did not increase as compared to the controls (data not shown). Therefore, it is more likely that B/W37 and B/Sr extracts mediate their inhibitory effect by regulating the transcription of Wnt-target genes, through a mechanism independent of the beta-Catenin level, rather than by sequestering beta-Catenin. In contrast, expression of the endogenous Wnt-target cMyc (He et al. 1998) was clearly reduced by both U. tomentosa extracts, confirming the data obtained by the luciferase reporter assays (Fig. 2). In summary, our data provide strong evidence that B/W37 and B/Sr extracts inhibited Wnt-signaling activity downstream of beta-Catenin regulation, thereby reducing the expression of Wnt target genes.

Dose-dependent growth inhibition of HeLa, HCT116 and SW480 cells by B/W37 and B/Sr extracts

To establish, whether the here described Wnt-signaling inhibitory effect exhibited by B/W37 and B/Sr extracts was the cause or the effect of reduced cell growth, the proliferation of treated HeLa, HCT116 and SW480 cells at different time points was determined. As shown in Figs. 3 and 4, proliferation of all cell lines tested was clearly reduced, in a dose-dependent manner, after 24 h of treatment with both extracts. Table 2 shows the concentration of

<table>
<thead>
<tr>
<th>Extract</th>
<th>Cell line</th>
<th>IC50 (µg/ml) 3 h</th>
<th>IC50 (µg/ml) 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/W37</td>
<td>HeLa</td>
<td>280</td>
<td>≥300</td>
</tr>
<tr>
<td></td>
<td>HCT116</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>SW480</td>
<td>165</td>
<td>200</td>
</tr>
<tr>
<td>B/Sr</td>
<td>HeLa</td>
<td>≥200</td>
<td>≥200</td>
</tr>
<tr>
<td></td>
<td>HCT116</td>
<td>≥200</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>SW480</td>
<td>160</td>
<td>95</td>
</tr>
</tbody>
</table>

IC50: concentration of extracts, where cell proliferation is inhibited by 50%.
the extracts required to inhibit cell proliferation by 50% (IC\textsubscript{50}). Similarly to the effect on Wnt-signaling, the strongest inhibitory effect was observed in SW480 cells, resulting in the lowest IC\textsubscript{50}. In addition, the IC\textsubscript{50} for B/Srt after 24 h was lower than that of B/W\textsubscript{37}, indicating a stronger effect on cell proliferation (Table 2). Interestingly, growth inhibition started earlier with B/W\textsubscript{37} than with B/Srt indicating that the ability to enter the cells is important. In contrast, growth inhibition by B/Srt was more intense after 24 h (Fig. 4).

Effect of B/Srt extracts on non-cancer cells

We next investigated the specificity of the alkaloid enriched B/Srt extract in more detail. Since normal, primary cells of the colon are not available and normal epithelial cells of other entities are almost impossible to transfect effectively, we used immortalized human embryonic kidney 293T cells for our analysis. 293T cells are commonly used for studying signaling pathways because they do not harbor mutations in signaling components and are highly transfactory. Due to immortalization by SV40 large T antigen Wnt-signaling is strongly activated in these cells (Fig. 5A, first column). Accordingly, Wnt-signaling activity was effectively inhibited by B/Srt, displaying an IC\textsubscript{50}\textsubscript{(Wnt)} of 43 µg/ml and TOP/FOP ratios, indicating a specific effect at concentrations of not less than 50 µg/ml (Table 1).

To exclude the possibility that the observed down-regulation of Wnt-target gene expression following exposure to U. tomentosa extracts is the result of hitherto unknown growth inhibitory effects we studied time response of Wnt-signaling inhibition by B/Srt. Analysis of time response to 50 µg/ml B/Srt revealed that downregulation of beta-Catenin/Tcf-mediated transcription starts 6 h after treatment, reaching 50% of inhibition at 26 h of treatment (Fig. 5B, continuous line). To investigate whether this effect is indeed dependent on Wnt-signaling activity and not merely a result of growth inhibition, we transfected 293T cells with a well-known inhibiting variant of the Wnt-effector TCF4 lacking the N-terminal beta-Catenin binding region (dn-hTCF4, (Morin et al. 1997)). Dn-hTCF4-transfected 293T cells, displaying a markedly reduced Wnt-signaling activity were insensitive towards 50 µg/ml B/Srt (Fig. 5B, dotted line), indicating that the extract specifically interferes with Wnt-signaling and may thus not affect normal, differentiated cells. Furthermore proliferation was not inhibited by B/Srt in 293T cells, neither in native cells nor when Wnt-signaling was downregulated by dn-hTCF4 (Fig. 5C). In contrast, proliferation was shortly activated within 1 h of B/Srt treatment and returned to control levels during further treatment. Taken together, our results indicate that B/Srt is clearly less cytotoxic to non-cancer cells and to cells without activated Wnt-signaling than to cancer cells with aberrantly activated Wnt-signaling activity.

Discussion

Previous studies on the anti-tumorigenic potential of U. tomentosa extracts focused on inhibition of neoplastic cell growth without analyzing the underlying mechanisms. We here combined investigation of growth inhibitory effects of defined U. tomentosa extracts with analysis of Wnt-signaling, which is a major target for future therapeutic intervention. In general, the anti-proliferative activity of U. tomentosa observed in this study is in good agreement with results obtained with hematopoietic (Bacher et al. 2006; Pilarski et al. 2007; Sheng et al. 1998), breast (Garcia Gimenez et al. 2009; Riva et al. 2001), neurologic (Garcia Prado et al. 2007) and other (De Martino et al. 2006; Pilarski et al. 2010) cancer cell lines, where similar IC\textsubscript{50} values ranging from 25 µg/ml to more than 1000 µg/ml had been determined, depending on the extract preparation and cell line. Differential activity of B/W\textsubscript{37} and B/Srt extracts

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**Fig. 4.** Percentage of viable cells in HeLa, HCT116 and SW480 cancer cells upon exposure to different concentrations of B/W\textsubscript{37} and B/Srt Uncaria tomentosa extracts for 3 h (A) and 24h (B) as derived from the WST1-assay in Fig. 3. Error bars represent the standard deviation of three experiments.
was observed previously, without finding a correlation between IC\textsubscript{50} values and cancer cell types. Particularly, the B/S\textsubscript{nt} extract has been shown to have inconsistent activity in various cancer cell lines which was ascribed to its low solubility in water and/or unknown selectivity for some cell lines (Pilarski et al. 2010). To avoid problems with solubility, we here used B/S\textsubscript{nt} extract dissolved in DMSO, which resulted in good reproducibility of all experiments. Our here presented data strongly suggest that the selectivity of B/W\textsubscript{37} and B/S\textsubscript{nt} extracts on different cancer cell lines may be ascribed to different endogenous Wnt-signaling activities. Cancer cell lines exhibiting high Wnt-signaling activity, such as SW480 cells were clearly more sensitive towards B/W\textsubscript{37} and B/S\textsubscript{nt} than cell lines showing a low endogenous Wnt-signaling activity, such as HCT116 and HeLa cells. Furthermore, inhibition of Wnt-signaling activity by a dominant-negative TCF-4 variant resulted in insensitivity towards B/S\textsubscript{nt} in non-cancerous cells. Finally, in all cell lines tested, the 50\% Wnt-inhibition (IC\textsubscript{50(Wnt)}) was much lower than the 50\% growth inhibition (IC\textsubscript{50}), indicating that the decrease in Wnt-signaling is rather the cause than the result of reduced cell proliferation. The specificity for Wnt-signaling inhibition is therapeutically important, because inappropriate regulation and activation of this pathway is associated with several pathological disorders including cancer, retinopathy, tetra-amelia and arthritis (Janssens et al. 2006). It should be noted, that very low concentrations of both extracts resulted in slight stimulation of both, growth and Wnt-signaling activity (Figs. 1, 3 and 4, Table 2). This response has also been observed in colorectal cancer cells treated with aspirin (Dihlmann et al. 2001), a known inhibitor of colorectal cancer growth (Barker and Clevers 2006; Dihlmann and von Knebel 2005).

In all assays performed in this study, the B/S\textsubscript{nt} preparation was more effective than the B/W\textsubscript{37} preparation. This strongly suggests that the anti-proliferative and Wnt-inhibitory effects of \textit{U. tomentosa} extracts result from oxindole alkaloids, which account for over 50\% of dry mass in B/S\textsubscript{nt} but only 0.43\% in the B/W\textsubscript{37} extract (Pilarski et al. 2010). Moreover, as reported earlier (Pilarski et al. 2010), the alkaloid composition of both extracts is free of tetracyclic oxindole alkaloids and rich in pentacyclic oxindole alkaloids, which are more biologically active (Wurm et al. 1998). So far it is unknown, which of the alkaloids contained in the extracts is mainly responsible for the observed inhibitory effect on Wnt-signaling. Both, the B/W\textsubscript{37} and B/S\textsubscript{nt} preparations contain a high percentage of pteropodine and isomitraphylline (Pilarski et al. 2010). Future testing of single alkaloids will help to identify the constituents responsible for down-regulation of Wnt-signaling. Alternatively, the combination of different alkaloids may be important for the specificity.

In addition to testing the specificity of anti-cancer compounds targeting Wnt-signaling, secondary assays are necessary to ensure their safety and efficiency in vivo. Although comparable studies in humans have not been performed yet, alkaloid-rich \textit{U. tomentosa} extracts and even pure oxindole alkaloids were reported to be non-toxic and safe to use, since LD\textsubscript{50} values determined in mice (Keplinger et al. 1999) point to a lethal poisoning dose for adults of consuming 2 kg of B/S\textsubscript{nt} preparation. Moreover, while this paper was in preparation, we and others reported the capacity

Fig. 5. Effects of B/S\textsubscript{nt} extract on non-cancerous cells. (A) Dose dependent inhibition of Wnt-signaling activity in 293T cells upon treatment with \textit{Uncaria tomentosa} extract B/S\textsubscript{nt} for 24 h. (B) Time course of B/S\textsubscript{nt} activity in 293T cells with active (continuous lines) or inactive (dotted lines) Wnt-signaling. For down-regulation of Wnt-signaling, cells were transfected with a dominant-negative TCF-4 variant (dn-hTCF-4). (C) Time course of cell viability in 293T cells with active (continuous lines) or inactive (dotted lines) Wnt-signaling as determined by WST-1 cell proliferation assay. Data represent the means and standard deviations of six (A and B) or four (C) experiments.
of *U. tomentosa* extracts to reduce solid tumor growth in vivo in different animal models. The B/W37 preparation was shown to significantly inhibit tumor growth in a Lewis lung carcinoma mouse model compared to control groups when administered for 21 days at doses of 5 and 0.5 mg/day (Pilarski et al. 2010). The treatment was well tolerated and non-toxic, as indicated by blood parameters such as leukocyte number, erythrocytes, platelets, and hemoglobin. The B/Sr preparation was likewise well tolerated and non-toxic in the Lewis lung carcinoma mouse model, however, interestingly, it did not reveal any significant anti-tumor activity (Pilarski et al. 2010). A second in vivo study tested the antitumor activity of *U. tomentosa* hydroalcoholic extract in a Walker-256 cancer model in rats (Dreffus et al. 2010). These hydroalcoholic extracts, containing high concentrations of pentacyclic oxindole alkaloids similar to B/Sr, markedly reduced the subcutaneous tumor growth without affecting body weight of the animals. In addition, treatment with these extracts resulted in altered plasmatic levels of urea and hepatic markers, indicating that the antioxidant properties of the *U. tomentosa* extract were responsible for its antitumor effect (Dreffus et al. 2010). The Wnt-signaling activity in Lewis lung carcinoma cells and Walker-256 cells is unknown. However, considering our findings of a specific Wnt-signaling inhibition by B/Sr, it is reasonable to speculate that its ineffectiveness in Lewis lung carcinoma cells may result from low or absent Wnt-signaling activity in these cells. In contrast, the effectiveness of alkaloid-rich *U. tomentosa* extracts on Walker-256 cells might result from higher Wnt-signaling activity. Future analysis will clarify whether different activities of Wnt- and/or other cancer signaling pathways account for the differential effectiveness of various *U. tomentosa* extracts on different tumor entities and which alkaloid-composition is most appropriate.

In conclusion, our data provide strong evidence that some *U. tomentosa* extracts target the Wnt-signaling pathway, explaining in part its broad spectrum of biological activities. In agreement with these findings, we recently demonstrated that *U. tomentosa* extracts affected embryogenesis in a chicken embryo model (Kuras et al. 2009). Bearing in mind that Wnt-signaling plays a central role in embryonic development, it would be interesting to analyze if the observed in vivo changes likewise result from the interaction of *U. tomentosa* extracts with this pathway.

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