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# THE COMPARISON OF THE EFFECTS OF PANOBINOSTAT AND PKF118-310 ON β-CATENIN-DEPENDENT TRANSCRIPTION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA CELL LINES

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**Abstract:** Advanced head and neck squamous cell cancers (HNSCC) have unfavorable prognosis and new therapeutic options are necessary to improve treatment outcomes. The Wnt pathway plays an important role in the pathogenesis and progression of HNSCC. The aim of this study was to assess the effects of a histone deacetylase inhibitor – panobinostat on Wnt-dependent gene expression and cell migration. Cell viability in HNSCC cell lines (BICR6, CAL27, FaDu, H314, SCC-25) was evaluated by MTT assay. The expression of β-catenin-target genes was assessed by qPCR and TCF/LEF-dependent reporter assay. Protein content was evaluated by Western blot. Cell migration was analyzed by the wound healing assay. Panobinostat showed differential modulation of gene expression. It reduced the level of *Axin2* in CAL27 and SCC-25 cells but upregulated its expression in BICR6 and H314 cell lines. Moreover, it diminished the expression of *MMP7* in BICR6, H314 and CAL27 cell lines. In contrast, the inhibitor of β-catenin transcriptional activity – PKF118-310 down-regulated the expression of β-catenin-target genes in HNSCC cell lines. Interestingly, panobinostat had opposite effects on cell migration in CAL27 and FaDu where it inhibited or stimulated migration, respectively. On the other hand, PKF118-310 reduced cell migration. The anti-cancer effects of panobinostat in HNSCC cells are rather not related to the inhibition of Wnt signaling. PKF118-310 attenuates Wnt signaling, but only in a limited number of HNSCC cell lines. Importantly, the inhibition of Wnt pathway reduces the capacity of cells for migration, suggesting that it may potentially reduce cell invasion therapeutically.

**Keywords:** panobinostat, PKF118-310, head and neck cancer, Wnt signaling

Head and neck cancers account for 4-6% of all malignancies, depending on the population analyzed (1). Mean survival rates are above 50%, however, the advanced stages of the disease are still associated with very poor prognosis. Surgery and radiotherapy remain standard treatment options and chemotherapy (chemoradiotherapy) using cisplatin, carboplatin, 5-fluorouracil or paclitaxel may be used in locoregionally advanced cases. However, more effective therapeutics are still required for the treatment of advanced, especially recurrent and metastatic, tumors.

Most head and neck cancers are squamous cell carcinomas (HNSCC). Despite the growing knowledge of the molecular alterations associated with the pathogenesis of HNSCC (2, 3), there are only two targeted therapy options available. The inhibition of EGFR using cetuximab is frequently associated with good response in HNSCC patients however resistance may be developed. Moreover, immunomodulatory anti-PD-1 antibodies have been recently approved by the FDA for the treatment of recurrent/metastatic HNSCC (4). A recent search for compounds that could be effective in the treatment of head and neck cancers identified several hits including panobinostat (5). Panobinostat, next to trichostatin A and vorinostat, belongs to hydroxamic acid non-selective inhibitors of class I, II and IV histone deacetylase (HDAC) inhibitors (HDACi). Several HDACi have already been approved for the chemotherapy of hematologic cancers (6, 7). The treatment of cancer cells with these compounds leads to cell cycle arrest and apoptosis via regulating the acetylation level of histones and non-histone target proteins (8). HDACi affects the transcriptional activity of a relatively broad set of genes which include important regulators of cell growth and apoptosis such as *p21*, *c-MYC*, *CCND1* (encoding cyclin D1) or *BIRC5*/*survivin*. The pharmacological

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effects of panobinostat have not been extensively studied in head and neck cancers so far. It has been shown to reduce the expression of *c-MYC*, *CCND1* and *survivin* and induce cell cycle arrest and apoptosis in HNSCC cell lines (9-11). Moreover, in a recent study panobinostat was found to act as a senolytic drug leading to the death of senescent cells appearing after treatment with cisplatin or paclitaxel (12). The molecular mechanisms of panobinostat action in HNSCC cells are not clear and require elucidation. Since the genes down-regulated by panobinostat in HNSCC may be transcriptionally controlled by the Wnt/β-catenin pathway, it may be hypothesized that panobinostat acts by modulating this pathway. Indeed, HDACi have previously been shown to affect the activity of canonical Wnt signaling in colorectal cancer cells, however, the results are inconclusive and contradictory (13-16). Colorectal cancers are typically related to the aberrant activation of Wnt pathway due to mutations in *APC* or *CTNNB1* (encoding β-catenin). Such genetic alterations are generally not found in HNSCC (17, 18), but there are a few functional studies that corroborate that aberrant Wnt signaling plays a role in the pathogenesis of head and neck tumors (19, 20). Still, the extent of the assumed activation of Wnt signaling in HNSCC is still largely unknown. It is currently hypothesized that the activation of Wnt signaling in head and neck carcinomas is mostly related to the epigenetic silencing of Wnt pathway antagonists. Indeed, the methylation of *SFRPs*, *DKKs*, *WIF1*, *DACH1*, *DACT2*, *DAB2* is detected in a large percentage of HNSCC cases (21-24). On the other hand, the nuclear localization of β-catenin is observed in approximately one-fifth of cases (25, 26), which suggests that the methylation of Wnt antagonists may not be sufficient for the induction of Wnt signaling in HNSCC.

The aim of the present study was to test whether the transcriptional regulation of the genes associated with cell cycle, cell growth, migration and apoptosis by panobinostat may be related to the modulation of the function of canonical Wnt signaling pathway in cancer cell lines derived from different head and neck locations. We compared the effects of panobinostat with the effects exerted by a known inhibitor of β-catenin-dependent transcription – PKF118-310 and a well-described inducer of Wnt signaling – lithium chloride.

## MATERIAL AND METHODS

### Cell culture and viability assay

The BICR6 hypopharyngeal carcinoma cell line, H314 floor of the mouth carcinoma cell line and HCT116 colorectal carcinoma cell line were purchased from ECACC, while CAL27 tongue carcinoma cell line, FaDu hypopharyngeal carcinoma cell line, and SCC-25 tongue carcinoma cell line were purchased from ATCC. With the exception of SCC-25 cell line, the cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Biowest SAS, France) with the addition of 10% FBS (EURx, Poland) and 1% antibiotics (penicillin and streptomycin) solution (Sigma-Aldrich, USA) at 37°C in 95% humidified atmosphere containing 5% $CO_2$. The 1 : 1 mixture of DMEM with 10% FBS/antibiotics and complete Keratinocyte SFM-Medium (Gibco, UK) was used to grow SCC-25 cells.

The effect of PKF118-310 (Sigma, USA) and panobinostat (Cayman, USA) on cell viability was assessed by the MTT assay according to a standard protocol. Cells were seeded in a 96-well plate ($10^4$ cells/well) and, after 24 h of pre-incubation in medium supplemented with 5% FBS and antibiotics, fresh medium containing various concentrations of the tested compounds was added to the wells and the cells were further incubated for 72 h. Then, cells were washed with PBS buffer and incubated for 4 h in the presence of fresh medium containing 0.5 mg/mL MTT salt. Afterward, formazan crystals were dissolved using acidic isopropanol and absorbance was measured at 570 and 690 nm using Infinite M200 plate reader (Tecan, Austria). All the experiments were repeated three times with at least four measurements per assay. This is a standard procedure used in our laboratory for the choice of concentrations of chemicals for cell culture studies (27). The effects on viability accumulate over time, and this reflects well the safe range of subtoxic concentrations.

### Cell treatment and isolation of nucleic acids

Cells ($1\times10^6$) were seeded in 100 mm culture dishes and after 24 h of pre-incubation in medium containing 5% FBS, cells were treated with the tested compounds. Lithium chloride (Sigma, USA) was used as the reference inducer of Wnt signaling at concentrations that show evidence of the induction of Wnt signaling (10 and 20 mM). A small molecule β-catenin inhibitor PKF118-310 was used as the reference Wnt signaling inhibitor at concentrations ranging from 50 nM to 0.5 mM. The concentrations of panobinostat used in the study ranged from 1 nM to 25 nM. Control cells were treated with the vehicle only. Upon termination of incubation, total RNA was isolated using the Universal DNA/RNA/Protein Purification Kit (EURx, Poland) according to manu-

facturer's protocol. All the experiments were repeated twice. Studies were conducted at two time points – a longer incubation was performed in all the studied cell lines, while changes after a shorter incubation time (24 h) were also assessed in two HNSCC cell lines – CAL27 and FaDu, in order to observe time-dependent changes in the observed effects. Based on our previous studies, these time points are optimal for the assessment of β-catenin-dependent changes in gene expression (28).

**Quantitative real-time PCR**

The synthesis of cDNA from RNA samples was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA), according to manufacturer's protocol. Quantitative real-time PCR was performed using the Hot FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne, Estonia) and LightCycler 96 (Roche, Germany). The sequence of the starters which were used for the analysis of the expression of *Axin2*, *BIRC5, CCND1*, *c-MYC*, *MMP7,* and *CTNNB1* has been described previously (28). All qPCR reactions were run in triplicate. The protocol started with a 15 min enzyme activation at 95°C, followed by 40 cycles of 95°C for 15 s; 56°C for 20 s; 72°C for 40 s and the final elongation at 72°C for 5 min, which was followed by melting curve analysis in order to confirm the generation of a single amplification product. As previously described (27), the mean expression of the *TATA-box binding protein* (*TBP*) and *porphobilinogen deaminase* (*PBGD*) housekeeping genes was used for normalization. The relative level of each transcript was calculated using the ΔΔCt method.

**Preparation of cytosolic and nuclear fractions**

The Subcellular Protein Fractionation Kit for Cultured Cells (Pierce/Thermo Fisher Scientific, USA) was used for the preparation of subcellular extracts according to the manufacturer's protocol. Protein concentration was assessed with the BCA Protein Assay Kit (Pierce/Thermo Fisher Scientific, USA) and then the samples were stored at –80°C until further analysis.

**Western blot assay**

The content of β-catenin, phospho-β-catenin (Thr41/Ser45) and Axin2 in cellular extracts was assessed by Western blot as previously described (28). Briefly, cytosolic (β-catenin, phospho-β-catenin, Axin2) or nuclear (β-catenin) extracts were separated on 7.5% SDS-PAGE gels (Bio-Rad, USA) and transferred onto nitrocellulose membrane. After blocking with 10% skimmed milk, the membranes were incubated with primary rabbit polyclonal antibodies (Santa Cruz Biotechnology, USA) directed against β-catenin, phospho-β-catenin or Axin2. The analysis of β-actin or lamin A served as a loading control. After washing, the membranes were probed with alkaline phosphatase-labeled secondary antibodies (anti-rabbit IgG, Santa Cruz Biotechnology, USA) and stained using the BCIP/NBT AP Conjugate Substrate Kit (Bio-Rad, USA). Quantity One software was used to determine the amount of the immunoreactive products and the values were calculated as relative absorbance units (RQ) per mg protein.

**Reporter assay**

FaDu and HCT116 cells were seeded in 96-well plates ($1.5 \times 10^4$/well) and co-transfected with 50 ng reporter (pNL(NlucP/TCF-LEF-RE/Hygro)) and 10 ng control (pGL4.54[luc2/TK] Vector) plasmids using ViaFect transfection agent (Promega, Germany) in a ratio (μg/μL) of either 1 : 6 (HCT116) or 1 : 4 (FaDu), according to manufacturer's recommendations. On the following day, fresh medium containing panobinostat at the concentration of 5 nM (FaDu) or 10 nM (HCT116) was added into wells. Control cells were treated with the vehicle (DMSO). As suggested by the manufacturer, cells were incubated for subsequent 24 h. Afterward, chemiluminescence was detected using Nano-Glo® Dual-Luciferase® Reporter Assay System and GloMax microplate reader (Promega, Germany), according to the manufacturer's protocol. The experiment was repeated twice with three independent replicates per experiment. Relative luminescence (RL) was calculated from the formula:

$$RL = [\text{test compound (NanoLuc / Firefly)} / \text{control (NanoLuc / Firefly)}] \times 100\%$$

**Cell migration assay**

CAL27, FaDu and HCT116 cells were seeded in 24-well plates and left overnight to reach confluence. A wound was performed by scratching the bottom surface of wells with a 10 μL tip, and detached cells were removed by washing wells with PBS buffer. Then, wells were filled with fresh medium containing the indicated concentrations of the tested compounds. Each well was immediately photographed using JuLI FL microscope (NanoEntek, Korea). Plates were incubated for subsequent 24 h, and then, the same area of each well was photographed again. The incubation time was chosen based on the observations of the time, which is

required for the near closure of the wound in control conditions. Moreover, it is not advised to conduct this assay longer than 24 h (29). The JuLI FL software was used to determine the area covered by cells (%) in each well and the difference in cell coverage area between the two time points was calculated. The comparison of the mean difference in cell coverage area between compound-treated cells and vehicle control allowed to determine the relative effect of panobinostat and PKF118-310 on cell migration (compound [area24-area0] / control [area24-area0]).

### Statistical analysis

The Student's t-test was used for the analysis of the significance of differences between the experimental groups and their respective controls, with $p = 0.05$ considered as significant. The analysis was performed using STATISTICA 10 software.

## RESULTS

### Cell viability analysis

The effect of PKF118-310 and panobinostat on cell viability was assessed using the MTT assay (Fig. 1). Panobinostat led to a significant decrease in the viability of all the cell lines at relatively low concentrations, and FaDu and SCC-25 cell lines showed the highest susceptibility to the inhibitory effects of the treatment with this compound. On the other hand, the effects of PKF118-310 were much more diverse. Relatively low concentrations of the compound led to the complete elimination of FaDu cells. On the other hand, BICR6 cell line showed intermediate susceptibility, while H314 cells were most resistant to the cytotoxic effects of PKF118-310.

### The effect of the tested compounds on the expression of β-catenin target genes

Lithium chloride is one of the classical inducers of Wnt signaling. It stimulates the accumulation of β-catenin by inhibiting the activity of GSK-3β. However, it is not fully specific and may also affect other signaling pathways that depend on the function of GSK-3β. In this study, LiCl was tested at two concentrations (10 mM and 20 mM) which have frequently been used for the effective stimulation of Wnt signaling in colorectal and head and neck cancer cell lines (30, 31). All the analyzed head and

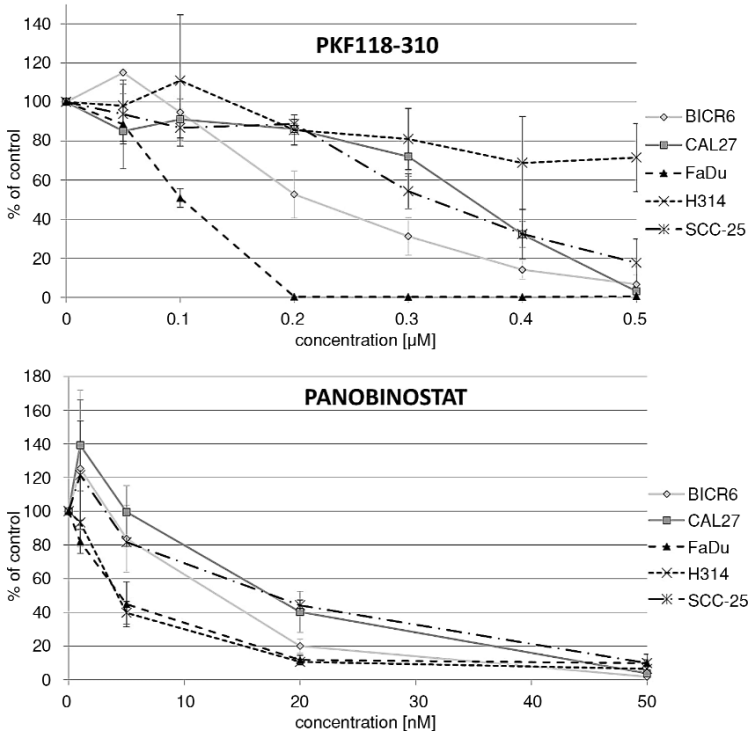


Figure 1. The effect of PKF118-310 and panobinostat on the viability of head and neck carcinoma cell lines assessed with the MTT assay. Mean values ± SEM from three independent experiments are shown.

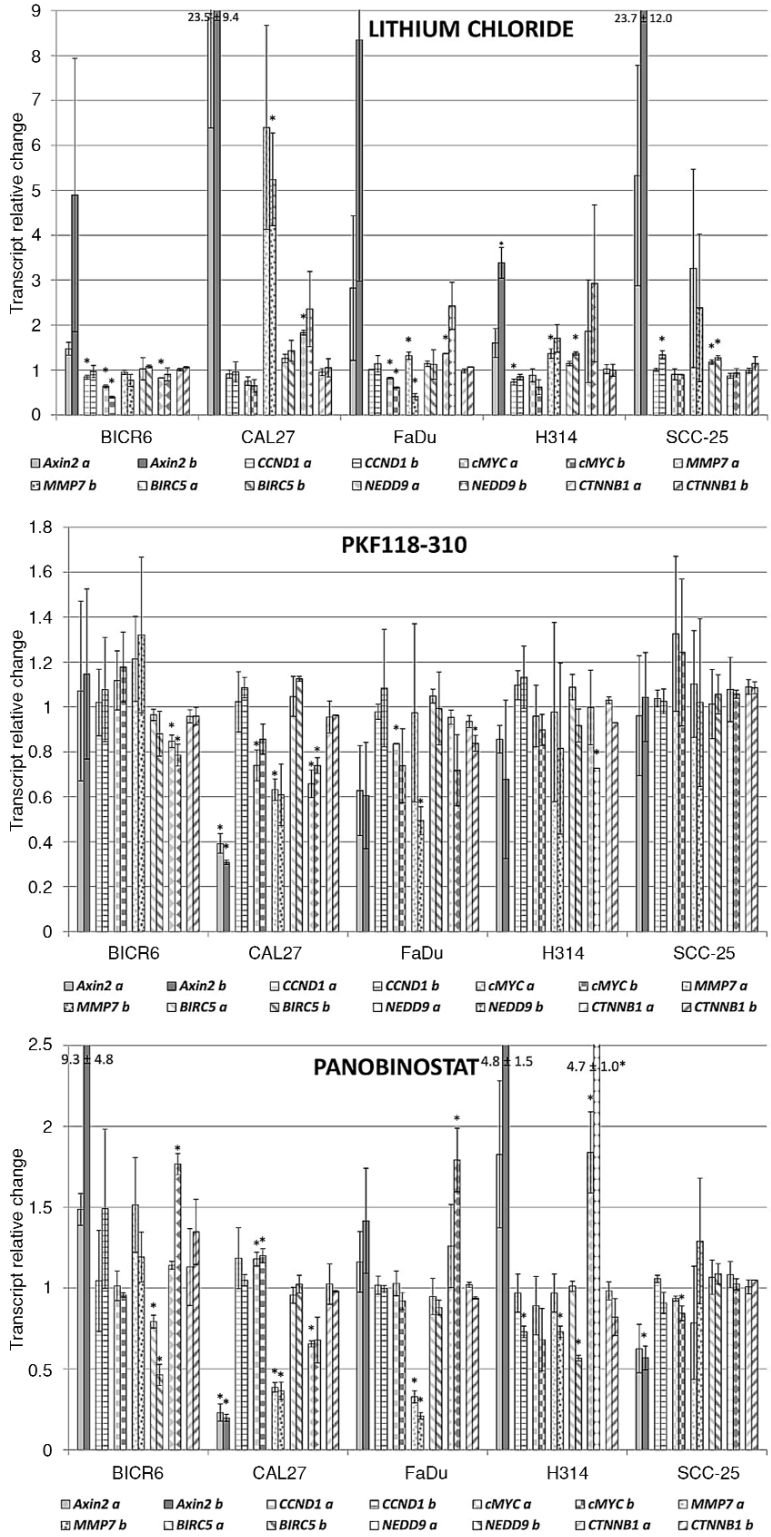


Figure 2. The modulation of the transcript level of β-catenin and its target genes by a 48 h-treatment with lithium chloride or PKF118-310, and a 72 h-treatment with panobinostat. Mean values ± SEM from two independent experiments are shown. The following concentrations of the compounds were used: lithium chloride – a = 10 mM, b = 20 mM; PKF118-310 – CAL27 cell line: a = 0.2 μM, b = 0.3 μM; BICR6 and SCC-25 cell lines: a = 0.1 μM, b = 0.2 μM; FaDu cell line: a = 0.05 μM, b = 0.1 μM; H314 cell line: a = 0.2 μM, b = 0.5 μM; panobinostat – a = 1 nM, b = 5 nM except for CAL27: a = 5 nM, b = 10 nM. Asterisks denote statistically significant changes, p = 0.05.

neck carcinoma cell lines responded to a 48-h incubation of the cells in the presence of LiCl with the induction of the expression of *Axin2* which is considered to be one of the best markers of Wnt pathway activation since its expression is regulated solely by the activity of β-catenin. Moreover, the expression of *MMP7* was induced by LiCl in tongue (CAL27, SCC-25) and floor of the mouth (H314) cancer cell lines. Interestingly, the treatment with LiCl led to a decrease in the level of expression of *c-MYC* (Fig. 2).

PKF118-310 has been developed as an inhibitor of canonical Wnt signaling and it blocks β-catenin-dependent transcription by disrupting the interaction of β-catenin with TCF4. Cells were incubated for 48 h in the presence of PKF118-310 and the concentrations which were used in the experiments have been selected independently for each cell line based on the viability assay. This compound was effective in causing a decrease in *Axin2* expression in CAL27 cells. Such an effect was also observed in the case of FaDu and H314 cell lines however these changes were not statistically significant. Moreover, PKF118-310 diminished the expression of *c-MYC* and *MMP7* in CAL27 and FaDu cell lines. On the other hand, it did not lead to any changes in gene expression in SCC-25 tongue carcinoma cell line. It did not significantly affect the level of expression of *CTNNB1* in any of the cell lines.

Panobinostat led to diverse effects in different cell lines after 72-h incubation. It appeared to inhibit Wnt signaling in CAL27 cells, where it reduced the expression of *Axin2* and *MMP7*. A reduction in *Axin2* expression was also shown in another tongue cancer cell line – SCC-25. On the other hand, the expression of *Axin2* was increased in BICR6 and H314 cell lines. In contrast, the expression of *MMP7* was reduced in FaDu and H314 cells, while the expression of *BIRC5* was diminished in BICR6 and H314 cell lines. Panobinostat also led to a reduction in *CCND1* expression in H314 cells.

**The effect of the tested compounds on the protein level of β-catenin and Axin2**

In order to assess, whether the tested compounds affect the subcellular localization and level of β-catenin, we analyzed the content of active β-catenin and its phosphorylated (inactive) form in cytosolic and nuclear fractions isolated from HNSCC cells (Fig. 3). Lithium chloride increased the level of β-catenin in the nuclear compartment in CAL27, FaDu and SCC-25 cells. Moreover, it reduced the level of phosphorylated β-catenin in FaDu and SCC-25 cells. On the other hand, panobinostat did not affect the levels of β-catenin.

We also analyzed the protein level of Axin2, because its transcript level was most significantly modulated by the studied chemicals. Lithium chloride increased the level of Axin2 by around 20% in CAL27, FaDu and SCC-25 cells. On the other hand, panobinostat increased Axin2 content in BICR6 and H314 cells. Moreover, we verified the effects of PKF118-310 in CAL27, FaDu and SCC-25 cell lines. This compound did not affect the level of β-catenin, however, it reduced Axin2 content in CAL27 and FaDu cells.

**Time-dependent effects of panobinostat and PKF118-310 on gene expression**

Two cell lines, CAL27 and FaDu, were selected for further studies based on the observation of the ability of PKF118-310 and LiCl to inhibit and induce Wnt signaling in these cell lines, respectively. The other three cell lines were inducible by LiCl, however, there was no clear inhibition of the pathway by PKF118-310, what suggests a lower level of activity of Wnt pathway in these cell lines. In order to better characterize the possible effects of PKF118-310 and panobinostat in CAL27 and FaDu cell lines, cells were incubated in the presence of the compounds for 24 h in order to assess whether the observed effects are time-dependent. The results (Fig. 4) were generally similar to those observed after longer incubation times. PKF118-310 diminished the level of expression of *Axin2* and *MMP7* in both cell lines, while the expression of *Axin2* was induced by LiCl. Panobinostat decreased the level of expression of *MMP7* in FaDu and CAL27 cells, and of *BIRC5* in CAL27 cells. The important difference was that panobinostat had an opposite effect on the expression of *Axin2* in CAL27 cells leading to its stimulation and not inhibition as was in the case of the longer incubation time. Additionally, the effects were compared with effects exerted by the compounds in a reference cell line, which shows strong constitutive activation of the canonical Wnt pathway. The HCT116 colorectal cancer cell line bears a heterozygotic mutation in *CTNNB1* gene, which leads to the activation of Wnt signaling by decreasing the degradation of β-catenin since the altered protein is no longer the substrate for phosphorylation by GSK-3β. The treatment of cells with 10 mM LiCl increased the expression of *Axin2* but reduced the expression of *CCND1* and *c-MYC*. PKF118-310 led to a reduction in the level of *Axin2* and *MMP7*. On the other hand, panobinostat increased the level of *Axin2* at the concentration of 10 nM but had no

effect on its expression at a higher concentration of 25 nM. Importantly, it strongly reduced the level of expression of *CCND1*, *c-MYC* and *MMP7* at both concentrations.

In order to further validate the mechanism of action of panobinostat, luciferase-based TCF/LEF reporter assay was performed in FaDu and HCT116 cell lines. Panobinostat increased the level of TCF/LEF-dependent NanoLuc expression by twofold in both cell lines and the changes were statistically significant (Fig. 4D). The relative level of normalized luminescence (RL) was 203% and 232% in FaDu and HCT116 cells, respectively.

**The effect of panobinostat and PKF118-310 on cell migration**

Finally, we wanted to assess whether the modulation of gene expression by panobinostat and PKF118-310 can affect the motility of cells determined in the scratch wound healing assay (Fig. 5). All three tested cell lines showed moderate inhibition of cell migration by PKF118-310. On the other hand, the effects of panobinostat differed among the cell lines. Panobinostat reduced cell migration in CAL27 cells at a concentration of 5 nM. In contrast, panobinostat at a concentration of 5 nM stimulated cell migration in FaDu cells. Also, it showed

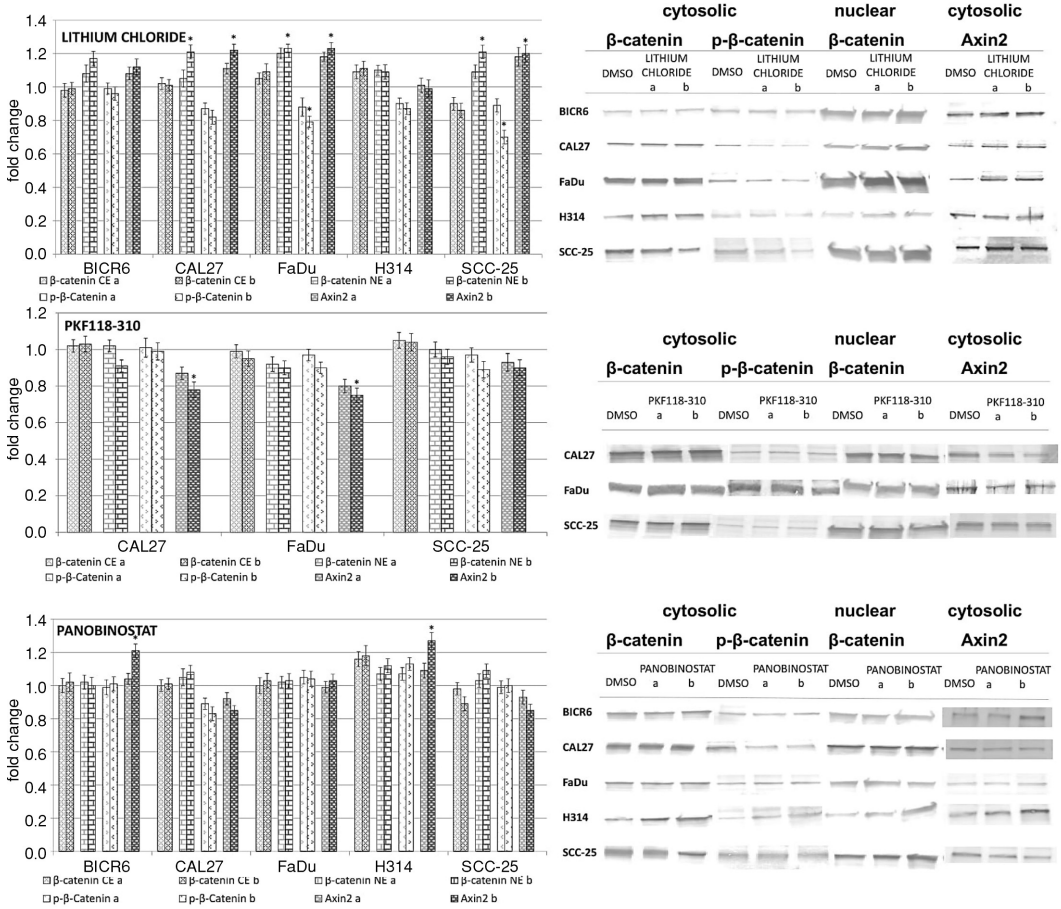


Figure 3. The modulation of the subcellular localization and phosphorylation of β-catenin and Axin2 level by a 48 h-treatment with lithium chloride or PKF118-310, and a 72 h-treatment with panobinostat. Mean values ± SEM from two independent experiments are shown. The following concentrations of the compounds were used: lithium chloride – a = 10 mM, b = 20 mM; PKF118-310 – CAL27 cell line: a = 0.2 μM, b = 0.3 μM; FaDu cell line: a = 0.05 μM, b = 0.1 μM; SCC-25 cell line: a = 0.1 μM, b = 0.2 μM; panobinostat – a = 1 nM, b = 5 nM except for CAL27: a = 5 nM, b = 10 nM. Representative electrophoregrams for the analysis of the cytosolic content of total β-catenin, phospho-β-catenin (Thr41/Ser45) and Axin2 and nuclear level of β-catenin are also presented next to graphs. The results of the analysis of the level of β-actin and lamin A which were used for data normalization are not shown. Asterisks denote statistically significant changes, p = 0.05.

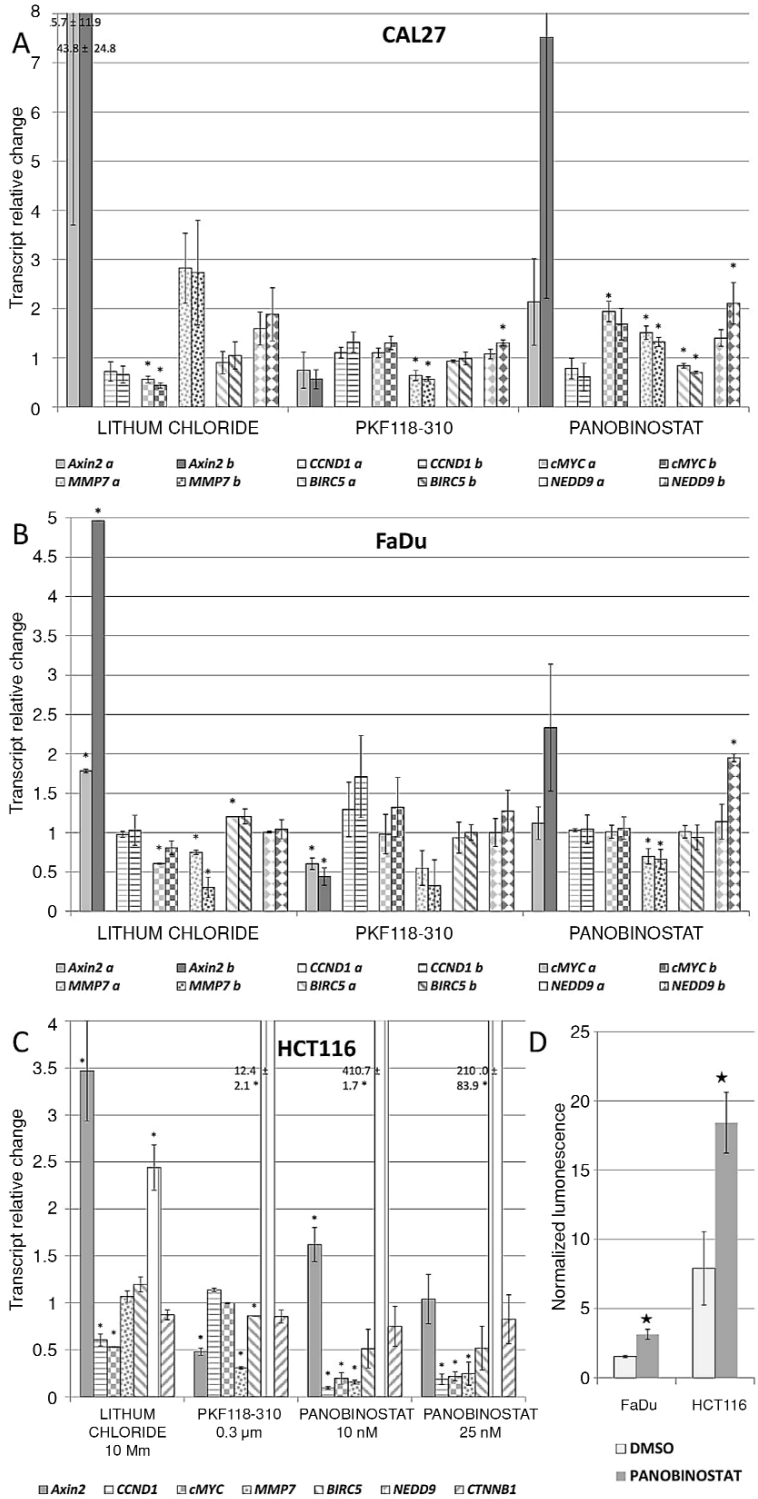


Figure 4. The effect of lithium chloride, PKF118-310 and panobinostat on the transcript level of β-catenin and its target genes in CAL27 (panel A), FaDu (panel B) and HCT116 (panel C) cell lines after 24 h-treatment. Mean values ± SEM from two independent experiments are shown. The following concentrations of the compounds were used: lithium chloride – a = 10 mM, b = 20 mM; CAL27 cell line – PKF118-310: a = 0.2 µM, b = 0.3 µM; panobinostat: a = 5 nM, b = 10 nM; FaDu cell line – PKF118-310 – a = 0.2 µM, b = 0.3 µM; panobinostat – a = 1 nM, b = 5 nM. **Panel D** presents the results of the reporter assay. The effect of panobinostat (5 nM in FaDu cells and 10 nM in HCT116 cells) on the level of luminescence of reporter NanoLuc luciferase normalized to the level of luminescence of control Firefly luciferase is shown as mean values ± SEM from two independent experiments. Asterisks denote statistically significant changes, p = 0.05.

a tendency to diminish cell migration in HCT116 cells at the concentration of 25 nM, although this was not statistically significant.

## DISCUSSION

The canonical Wnt pathway is responsible for the control of cell proliferation, differentiation, migration and cell death by regulating the transcriptional activity of β-catenin through blocking its cytosolic sequestration and degradation (32). In physiological states, β-catenin-mediated transcription is activated by the presence of Wnt ligands, which act on cell membrane Fzd/LRP receptors and block the activity of the cytosolic protein complex consisting of APC, GSK3β, Axin and casein kinase, which is responsible for the phosphorylation of β-catenin resulting in its proteasomal degradation. Upon inhibition of this destruction complex by the action of Wnt ligands, β-catenin undergoes translocation to cell nucleus, where it interacts with the TCF/LEF transcription factors and induces the expression of genes responsible for the regulation of cell cycle (*CCND1*, *c-MYC*), cell migration (*MMP7*) and apoptosis (*BIRC5*) and of other regulators including *Axin2*. The precise regulation of Wnt pathway activity is necessary for the development of oral tissues during embryogenesis, however, the pathway is largely inactive in mature oral keratinocytes (33). Nuclear β-catenin is considered a good marker of Wnt pathway activation and it is detected in both dysplastic keratinocytes and head and neck squamous carcinoma cells (34). Various reports show that approximately one fifth to one quarter of cases may be characterized by the activation of canonical Wnt signaling (25, 26). Most importantly, recent findings stress the key role of Wnt pathway in the occurrence of stem-cell-like properties in small populations of cells in HNSCC, which are responsible for cell-renewal and clinical recurrence (35). These findings indicate that targeting Wnt signaling is a promising therapeutic strategy in HNSCC.

Panobinostat has been recently shown to induce G2/M delay by inhibiting the cell-cycle dependent up-regulation of expression of Polo-like kinase 1 and cyclin B1 in HNSCC cell lines (11). Moreover, high concentrations of panobinostat inhibited *c-MYC* expression and induced the expression of p21 (9). Another study reported that the use of therapeutically attainable concentrations of panobinostat leads to the up-regulation of p21 and p27 and down-regulation of expression of cyclin D1 and survivin and the induction of apoptosis in oral carcinoma cell lines (10). The anti-mitotic and pro-apoptotic effects of panobinostat in HNSCC are rather well-documented however the exact molecular mechanisms of its anti-tumor activity are not clear. Since the pattern of gene expression changes induced by panobinostat in HNSCC cell lines partly matches the set of Wnt/β-catenin target genes, thus we focused on the verification of the hypothesis that the modulation of canonical Wnt signaling may contribute to the effects of panobinostat. The modulation of Wnt signaling by panobinostat and other HDACi has been documented in colorectal cancer and leukemia cell lines although the results are not conclusive. It was shown that HDAC1 is required for Groucho-mediated repression of β-catenin transcriptional activity and trichostatin A relieved this activity (36). Moreover, sodium butyrate, trichostatin A and SAHA induced Wnt signaling by increasing the level of active β-catenin in colorectal cancer cell lines (13). Contradictorily, in another study, SAHA and trichostatin A were shown to downregulate the expression of Wnt target genes (*CCND1*, *c-MYC*, *survivin*) in HCT116 cells, but it was not associated with the changes in the level of acetylated β-catenin (14). On the other hand, the knock-down of *HDAC6* increased β-catenin acetylation and reduced *c-MYC* expression in HCT116 cells. Moreover, panobinostat was shown to decrease the level of *c-MYC* and *survivin* in acute myeloid leukemia cells (37) whereas valproic acid-induced Wnt signaling in acute T lymphoblastic leukemia (38). In this study, we have observed differential responses of head and neck carcinoma cell lines to the modulation of the expression of Wnt target genes by panobinostat, which was used at concentrations which fall within the range of concentrations detected after oral administration of panobinostat (serum peak concentration of 22-65 nM). In the first step, the cells were exposed to panobinostat for 72 h and few changes in gene expression were observed but, importantly, *Axin2* expression was reduced in CAL27 and SCC-25 cells and *MMP7* expression was decreased in CAL27 and FaDu cells. These changes were similar to those exerted by the treatment of cells for 48 h with a known inhibitor of β-catenin – PKF118-310. This compound was previously reported to reduce the reporter gene expression and also decrease the expression of Axin2, cyclin D1 and survivin in HCT116 colorectal cancer, PC-3 and DU145 prostate cancer and U2OS osteosarcoma cells (39-41). In order to better characterize the capacity of panobinostat and PKF118-310 to modulate the expression of Wnt target genes, we treated cells for 24 h with the compounds and

compared the effects with those observed in HCT116 cell line, one of the reference cell lines for the assessment of Wnt signaling. We narrowed the set of the analyzed HNSCC cell lines and selected CAL27 and FaDu because they showed the most pronounced gene expression changes after longer incubation times and were responsive to both the inducing activity of LiCl and the inhibitory action of PKF118-310. In this experiment, lithium chloride evidently induced the expression of *Axin2* which is considered as one of the top Wnt targets, while the level of expression of other genes may be also affected by the activity of other factors. PKF118-310 reduced the level of expression of *Axin2* and *MMP7* in all three cell lines. On the other hand, panobinostat tended either to elevate or not to change the expression of *Axin2* in all three cell lines. Importantly, it showed much stronger effects in

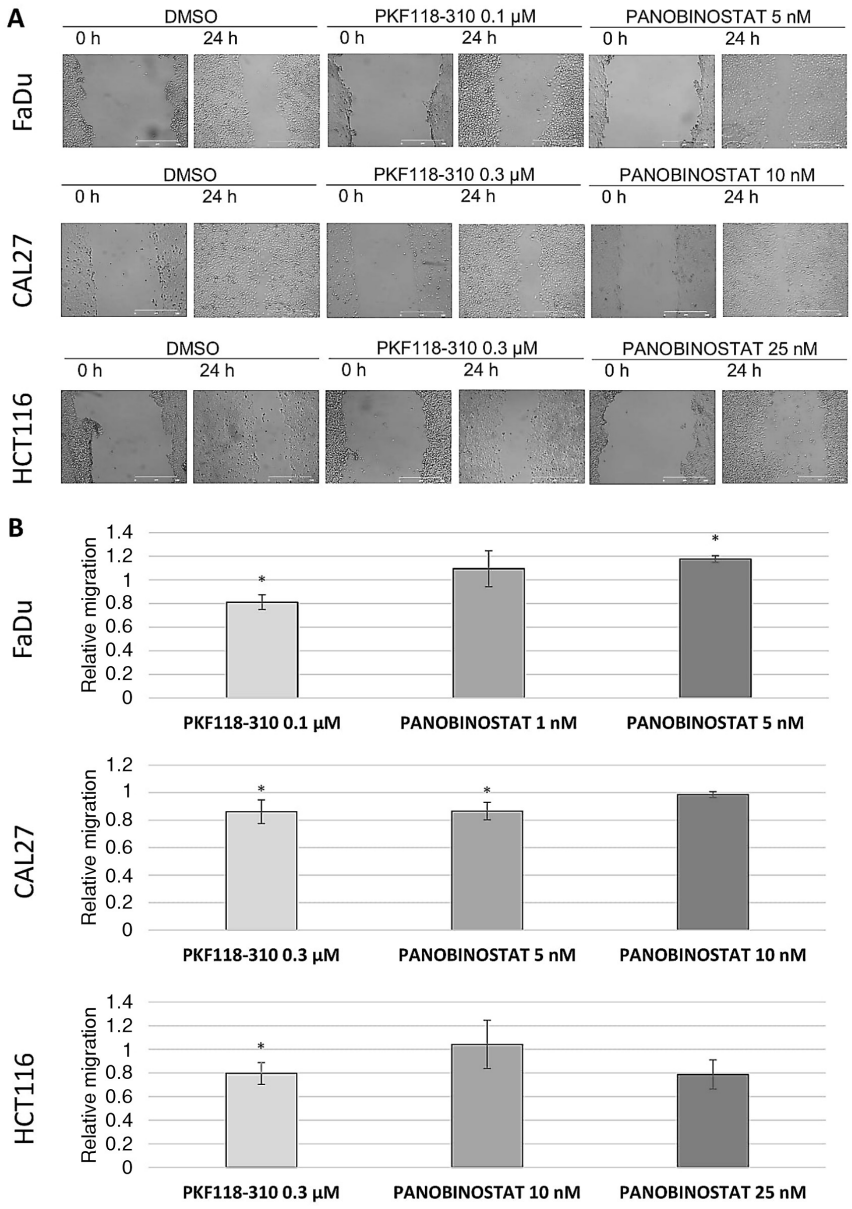


Figure 5. The effect of PKF118-310 and panobinostat on cell migration in CAL27, FaDu and HCT116 cell lines. A – exemplary photographs of wounded cell layers at the beginning and after the termination of a 24-h treatment, B – quantitative results of the scratch assay: mean values ± SEM from three independent experiments are shown. Asterisks denote statistically significant changes, $p \leq 0.05$.

HCT116 cells where it greatly diminished the level of expression of *CCND1*, *c-MYC*, *MMP7* and *BIRC5* (*survivin*) but tended to increase the expression of *Axin2* at the concentration of 10 nM.

Panobinostat affects the acetylation of a broad set of proteins and these pleiotropic effects may contribute to the differences in the pattern of gene modulation between cell lines, taking into account that PKF110-310 showed rather uniform effects. In order to confirm the effects of panobinostat on the activity of Wnt signaling, we performed the Wnt reporter assay in FaDu and HCT116 cells. Unfortunately, we had to leave out CAL27 cells because of the difficulties with obtaining efficient and stable transfection results. Interestingly, panobinostat led to the induction of TCF/LEF-dependent luciferase expression after 24 h incubation. These observations may show that the effects of panobinostat are not only dose-dependent but also time-dependent. Moreover, they significantly depend on the cellular context. Different cytosolic and nuclear HDACs are involved in regulating the function of the components of the canonical Wnt pathway and thus different HDACi with varying specificity towards different HDACs show at least partly different outcomes (42). This may in part explain that the effects of panobinostat are non-linear and may vary in a time-dependent and cell-specific fashion. Although Wnt reporter genes allow the most direct assessment of pathway activation, it has to be taken into consideration that possibly slightly different mechanisms are involved in the regulation of transcription of physiological Wnt targets than in the case of Wnt reporter genes. Moreover, a simpler structure of the promoter in the reporter plasmid eliminates the complexity of the interactome of transcription-regulatory proteins which most likely occurs in native gene promoters. This could explain the differences in gene expression profiles in different experimental conditions. Wnt signaling is affected by multiple factors acting both in the cytoplasm and in the nucleus and specific molecular alterations found in different cell lines may interfere with it. The engagement of different HDACs in the regulation of Wnt signaling requires detailed elucidation in further studies. Thus, it seems that panobinostat and other HDACi cannot be considered as bona fide inhibitors or activators of β-catenin-dependent gene expression because of the complexity of the interactions of HDAC enzymes with other regulatory proteins. The final effect may depend on the actual composition of HDAC protein complexes.

PKF118-310 was shown to diminish cell migration in U2OS osteosarcoma cells (41) and SAHA, a HDACi reduced cell migration and invasion in HNSCC cell lines (43). Thus, in an attempt to better characterize the biological activity of the studied compounds, we tested their influence on cell migration in CAL27, FaDu and HCT116 cells. PKF118-310 moderately reduced cell migration in all three cell lines. This is in line with current knowledge on the influence of Wnt signaling on the regulation of cell migration. The effects of panobinostat were milder suggesting that at the tested concentrations this compound is not likely to significantly affect cell migration.

## CONCLUSION

Overall, panobinostat may not have the ability to directly inhibit canonical Wnt signaling in HNSCC cells, thus its direct anti-cancer effects are rather related to other molecular mechanisms. On the other hand, the attenuation of Wnt signaling can be attained with the use of a specific inhibitor of the transcriptional activity of β-catenin – PKF118-310, however only in a limited number of HNSCC cell lines. Importantly, the inhibition of Wnt pathway reduces the capacity of cells for migration suggesting that this therapeutic strategy may potentially reduce cell invasion potential.

### Compliance with ethical standards

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### Conflict of interest

The authors declare that they have no conflict of interest.

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