Targeting the Neurokinin-1 Receptor Compromises Canonical Wnt Signaling in Hepatoblastoma

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Abstract

The substance P (SP)/NK-1 receptor (NK1R) complex represents an intriguing anticancer target for a variety of tumors, including hepatoblastoma (HB). Therefore, NK1R antagonists, such as the clinical drug aprepitant, recently have been proposed as potent anticancer agents. However, very little is known regarding the molecular basis of NK1R inhibition in cancer. Using reverse phase protein array, Western blot, Super SFA assays, we identified the AKT and Wnt signaling pathways as the key targets of aprepitant in three human HB cell lines (HepT1, HepG2, and HuH6). Following NK1R blockage, we observed decreased phosphorylation of p70S6K and 4E-BP1/2 and inhibition of the canonical Wnt pathway with subsequent decrease of HB cell growth. This effect was dependent of high baseline Wnt activity either by mutational status of β-catenin or extrinsic Wnt activation. Wnt inhibition seemed to be strengthened by disruption of the FOXM1–β-catenin complex. Furthermore, treatment of HB cells with aprepitant led to reduced expression of (liver) stemness markers (AFP, CD13, SOX2, NANOG, and OCT4) and SFA when grown under cancer stem cell conditions. Taken together, we show for the first time that targeting the SP/NK1R signaling cascade inhibits canonical Wnt signaling in HB cells. These findings reveal important insight into the molecular mechanisms of the SP/NK1R complex as a critical component in a model of pediatric liver cancer and may support the development of novel therapeutic interventions for HB and other Wnt-activated cancers.

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Introduction

Hepatoblastoma (HB) represents the most common malignancy of the liver in childhood (1). Multidrug resistance to established chemotherapeutic regimens is the major problem in children with high-risk hepatoblastoma, ultimately leading to poor prognosis (1, 2). Therefore, novel treatment approaches for this tumor entity are desperately needed.

The AKT axis and the Wnt cascade are two signaling pathways with a pivotal role in regulating cell growth and both have therefore been proposed as promising anticancer targets in HB and colorectal cancer (3–6). When growth factors, e.g., insulin-like growth factor, bind to their receptor tyrosine kinases, mTORC2 and AKT signaling become activated (7). AKT then phosphorylates mTOR, a signal that is further transmitted to 4E-BP1/2 and p70S6K (8). Canonical Wnt ligands bind to LRP5/6 and frizzled co-receptors, inhibiting the β-catenin destruction complex, which results in releasing β-catenin. Free to translocate into the nucleus it acts as a transcriptional regulator (9). High Wnt signaling was previously linked to cancer stem cell (CSC) properties in colorectal cancer (3) and susceptible pancreatic cancer cells (10). Hence, targeting the Wnt pathway supports one of the most promising future anticancer strategies. Unfortunately, efforts to translate AKT or Wnt antagonists into useful clinical drugs have been unsuccessful so far.

The substance P (SP)/NK-1 receptor (NK1R) complex has been identified as an independent anticancer target in humans (11, 12).

NK1R antagonists are known to inhibit tumor growth and induce apoptosis in a great variety of adult and childhood cancer cell lines, including neuroblastoma and rhabdomyosarcoma (13). The clinical drug aprepitant is a small molecule that acts as an inhibitor of NK1R approved by the FDA for the treatment of chemotherapy-induced nausea and vomiting, but at the moment no clinical trial exists to evaluate its efficacy as an anticancer agent.

Currently, very little is known about the role of the SP/NK1R complex in liver tumorigenesis. Our own previous data recently showed that HB cells express NK1R and inhibition of this receptor with small molecules triggers growth inhibition and apoptosis.
(14). Here, we investigate the responsible downstream mechanisms involved after pharmacologic inhibition of NK1R in HB. We found activated upstream AKT and mTOR, but decreased phosphorylation of 4E-BP1/2 and p70S6K, downregulation of FOXM1, and potent inhibition of Wnt activity.

Our findings help the understanding of the effects induced by NK1R signaling at the subcellular level and identify the NK1R inhibitor aprepitant as a potent inhibitor of the Wnt pathway in HB, having important implications for the development of future innovative curative strategies.

Materials and Methods

Cell culture

We used three human HB cell lines (HuH6, HepT1, and HepG2) and one human hepatocellular carcinoma cell line (Huh7). HepG2 was purchased from the ATCC, Huh6 and Huh7 (15) from ICRB (Osaka, Japan), and HepT1 from Dr. Torsten Pietsch, Bonn, Germany (16). All cell lines were grown in RPMI-1640 medium (Invitrogen) supplemented with 10% FCS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37°C in a humidified incubator with 5% CO2. Cells were checked for the known CTNNB1 mutations, but no other authentication was done.

Sphere formation culture

Cells were trypsinized, washed with PBS, and transferred at the density of 75,000 cells/100 mm × 20 mm ultra-low attachment dishes (Corning GmbH) containing sphere formation medium composed of DMEM-F12, 1% penicillin/streptomycin, 10 ng/mL human recombinant BFGF, 20 ng/mL human recombinant EGF, 1% glutamine, and B27 serum-free supplement. After 7 days, the sphere was trypsinized and washed with fresh medium, and subcultured every 3 days. Sphere numbers and sizes were determined under a microscope after 6 to 11 days depending on the cell type.

Drugs

Aprepitant (NK1R antagonist) was purchased from Selleck Chemicals and dissolved in DMSO. SP (NK1R agonist) was purchased from Sigma-Aldrich and dissolved in 0.1 mol/L acetic acid and purified water.

Reverse-phase protein array (RPPA)

Cells were treated for 24 hours with the indicated substrates. Samples were then washed in ice-cold PBS, denatured by 1% SDS (with β-mercaptoethanol) lysis buffer, and stored at −80°C. Further processing of the samples was carried out by the Functional Proteomics RPPA Core Facility at MD Anderson Cancer Center as previously reported (17). Briefly, probes were spotted on nitrocellulose slides and a total of 172 different proteins were analyzed. Densitometry was conducted by normalizing each value for protein loading and data obtained were transformed to linear values for further analysis.

Quantification was conducted by calculating the ratios with the linear protein intensity values of the treated probes and the linear protein intensity values of their respective control probes. Protein ratios that followed the same trend (up- or downregulation) upon aprepitant treatment in all three HB cell lines were displayed separately (at least one value of >1.5-fold induction or repression; Fig. 1B and Supplementary Fig. S1).

Western blot analysis

Western blot analysis was performed as previously described (14). Primary antibodies were used at a 1:1,000 dilution and antibodies against phospho-AKT, total AKT, phospho-p70S6K, total p70S6K, phospho-4EBP1, total 4EBP1, phospho-mTOR, total mTOR, PARP, β-catenin, and β-actin were purchased from Cell Signaling Technologies (CST), and FOXM1 from Santa Cruz Biotechs (SCBT). The secondary antibody, a peroxidase-conjugated goat anti-rabbit IgG antibody from DAKO was added at a 1:2,000 dilution. Antibody detection was performed with an enhanced chemiluminescence reaction (ECL Prime western blotting detection; GE Healthcare).

Western blot band intensity was evaluated by the ImageJ software (NIH) and values of total proteins or ratios of phospho/total proteins were normalized to the respective housekeeping protein after background subtraction.

Immunofluorescence

HepT1, HepG2, and Huh6 were plated onto round cover slips with a diameter of 18 mm (Thermo Scientific) at a density of 75,000 cells/well and treated as indicated. After 24 hours of treatment, cells were fixed in 4% paraformaldehyde for 15 minutes, permeabilized for 15 minutes with 0.15% Triton X-100 in PBS, and blocked for 30 minutes with 1% BSA in PBS. The cells were then incubated overnight at 4°C with mouse primary antibodies against β-catenin (monoclonal, BD Transduction Laboratories) or rabbit antibodies against phospho-AKT (polyclonal, CST), FOXM1 (polyclonal, SCBT), and phospho-mTOR (monoclonal, CST) diluted in blocking solution at 1:50 and 1:25, respectively. After several washing steps, cells were then incubated for 1 hour in the dark with goat anti-rabbit antibody conjugated with Alexa Fluor 488 (Invitrogen) or goat anti-mouse antibody conjugated with Alexa Fluor 594 at a 1:200 dilution. After three washes using PBS, microscopical slides were mounted with Vectashield (Vector Laboratories) containing 4,6-diamidino-2-phenylindole (DAPI). The pictures were acquired using the Olympus Fluoview FV1000 confocal microscope.

RNA isolation and RT-PCR

Total RNA was isolated from spheres or parental cells using TRI-Reagent (Sigma-Aldrich). cDNA was synthesized from 2 μg of total RNA using random primers (Roche Diagnostics) and SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. For each PCR reaction we used 40 ng of cDNA, 500 nmol/L of the primer pair and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories) for a total volume of 20 μL. Thermal cycling consisted of 40 cycles with denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and elongation at 72°C for 30 seconds using the Mastercycler ep gradient S (Eppendorf). Each experimental condition was assayed in duplicate and each experiment was at least repeated twice. Specific primers can be found in the supplementary material (Supplementary Table S1).
TOP/FOP luciferase reporter assay

Luciferase assays were conducted using the Dual-Luciferase Reporter Assay System (Promega). HB cells were plated in 24-well plates at a density of 50,000 cells/well and cultured for 24 hours before starting any treatment. Fugene HD reagent (Promega) and α-Eagle’s Minimum Essential Medium was used to mediate co-transfection with inducible Firefly luciferase expressing SuperTOP or SuperFOP vector (AddGene Plasmids #12456 and #12457) vectors and constitutively Renilla luciferase expressing normalization vector pRL-TK (Promega) at a ratio of 50:1 as a control for transfection efficiency. The cells were simultaneously treated with different concentrations of aprepitant or DMSO. After 24 hours of incubation, total cell lysate was prepared using reporter lysis buffer (Promega) and 5 μL of total protein lysate was used to determine Firefly and Renilla luciferase activities using 25 μL of luciferase substrate LarII solution (Promega) and 25 μL of STOP and GLOW 1x solution (Promega), respectively. The luminescence was measured with an integration time of 12 seconds. T-cell factor-mediated transcriptional activity was determined by the ratio of SuperTOP/SuperFOP luciferase activities, each normalized to the respective luciferase activities of the RL-TK reporter. Each experimental condition was assayed in triplicate.

Statistical analysis

Results are expressed as the mean ± SEM. All statistical comparisons were made with the standard t test using biostatistics software from GraphPad Prism. The criterion for significance was P < 0.05 (*) and P < 0.01 (**) for all comparisons.

Results

NK1R inhibition leads to downregulation of the AKT and Wnt signaling pathways

To examine which signaling pathways are affected by NK1R inhibition, we treated the human HB cell lines HepT1, HepG2, and HuH6 (harboring β-catenin mutations) with 20 and 40 μmol/L aprepitant and screened for changes in protein expression level performing RPPA analysis (17). In this array, samples were analyzed for 172 proteins and phosphoproteins associated with common cancer pathways. We found that most protein levels did not change significantly when compared with
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the untreated control (Fig. 1A, in black; Supplementary Fig. S1), although only approximately 12% were significantly downregulated (green) and approximately 8% were upregulated (red; Fig. 1A). Significantly altered protein ratios after treatment are reported in a separate heatmap with the 40 μmol/L aprepitant treatment (bottom) showing more drastic changes than the treatment with 20 μmol/L aprepitant (top in Fig. 1B). As expected from our previous work, aprepitant treatment induced upregulation of cleaved caspase-7, a major contributor to apoptosis execution (8, 14). However, as shown below, this screening also suggested that several candidate proteins belonging to the AKT/mTOR and Wnt pathways (Fig. 1B) were significantly modified.

In the AKT/mTOR pathway, NK1R blockage with aprepitant induced downregulation of total AKT and the phosphoproteins 4E-BP1/2 (S65), p70S6K (T389), and S6 (S235/236 and S240/244) downstream of the mTORC1 complex (Fig. 1B). Additionally, PRAS40 (T246), a member of mTORC1, and 4E-BP1/2 (T37/T46) were upregulated upon aprepitant treatment (Fig. 1B). Furthermore, calculations of phospho/total protein ratios of the investigated proteins revealed that aprepitant treatment resulted in upregulation of AKT (S473 and T308) as well as downregulation of Rictor (Fig. 1B). The p38 MAPK, which was recently reported to mediate the expression of the Wnt pathway-associated protein and β-catenin destruction complex (S21/S9), an important part of the β-catenin destruction complex (T1135; Supplementary Table S2).

NK1R inhibition comprises FOXM1 expression and subsequently diminishes canonical Wnt signaling

FOXM1 has recently been shown to play an essential role in enhancing nuclear translation of β-catenin as a shuttle protein and subsequent increased activation of the canonical Wnt signaling cascade in glioma cells (18). Our RPPA screening data (Fig. 1B) suggested decreased FOXM1 expression upon aprepitant treatment that we confirmed with Western blot (Fig. 3A) and qPCR analysis (Fig. 3B). In line with this, total β-catenin expression decreased in HepT1 and HuH6 following treatment with aprepitant, thereby supporting our data from RPPA analysis and indicating decreased activation of the Wnt pathway (Fig. 3A).

To further analyze effects of aprepitant treatment on Wnt/β-catenin signaling, we treated cells with aprepitant or SP (70 nmol/L) for 24 hours, stained for β-catenin and analyzed them by confocal microscopy. We observed a depletion of nuclear and cytosolic β-catenin as well as a strong accumulation of membrane-bound β-catenin (green; Fig. 3C, left, white arrow heads and Supplementary Fig. S2A) with aprepitant treatment, indicating an inactivation of canonical Wnt signaling. In contrast, stimulation with SP did not alter Wnt signaling significantly, presumably due to the high baseline Wnt activity in β-catenin-mutated HB cell lines (Supplementary Fig. S2B). In order to investigate the role of FOXM1, we repeated the treatments and stained the HB cells for FOXM1 (red). In contrast to β-catenin, FOXM1 increasingly translocated to the nucleus upon aprepitant treatment (Fig. 3C, white arrows in right), thereby moving in opposite directions to β-catenin (Supplementary Fig. S2A).

For the subsequent experiments, we utilized HuH7 cells, because these cells do not carry a mutation in the canonical Wnt signaling pathway, whereas the other HB cells harbor β-catenin mutations. In accordance with this, we found a significant activation of Wnt signaling in HepT1, HepG2, and HuH6, but not in HuH7 cells as detected by Super TOP/FOP (STF) reporter luciferase assays (Supplementary Fig. S2B). To further investigate the influence of aprepitant on canonical Wnt signaling, we carried out STF assays and, after treatment with increasing doses of aprepitant, found a robust inhibition of Wnt activity in all β-catenin–mutated HB cell lines, whereas Wnt activity in HuH7 remained unaffected (Fig. 3D). These results were validated by qRT-PCR where we found that the Wnt target genes LGR5, CTNNB1, and
AXIN2 were downregulated in a dose-dependent fashion upon aprepitant treatment in HB cell lines to different degrees (Fig. 3E). We reanalyzed our previously described HB xenograft mouse model (14) and detected that LGR5 gene expression was significantly lower and CTNNB1 was lower in some cases in animals treated long-term with aprepitant compared with the respective vehicle-treated animals (Supplementary Fig. S2C). Surprisingly, FOXM1 expression in long-term aprepitant-treated animals was significantly higher than in the control group.

Altogether, these data indicate that antagonism to the SP/NK1R complex by aprepitant in HB cells leads to reduced expression and signaling activity by affecting its shuttle protein FOXM1.

Wnt pathway inhibition by aprepitant disrupts FOXM1–β-catenin interaction

In order to investigate how aprepitant might interfere with the Wnt pathway, we stimulated HEK293T cells with lithium chloride (LiCl), which activates canonical Wnt signaling by inhibiting GSK3β and analyzed Wnt activity by STF assays. LiCl treatment alone significantly increased Wnt activity, although administration of aprepitant decreased Wnt signaling almost to the baseline (Fig. 3F), suggesting that the functional inhibition of Wnt occurs at the level of or downstream to the β-catenin disruption complex. To further analyze this important finding, we transduced HEK293T cells with the lentiviral vector 7XTCf-eGFP//SV40-Puro as reported before, which indicated canonical Wnt signaling by GFP expression (10, 22). Under the same treatment conditions, we found a similar reduction of Wnt activity upon flow cytometric analysis, further strengthening this important point (Supplementary Fig. S2D). Likewise, we were able to show that the β-catenin wild-type cell line HuH7 displayed increased Wnt signaling upon LiCl stimulation by STF (Fig. 3G) and β-catenin IF staining (Fig. 3H). In both cases, aprepitant treatment significantly reduced the effects promoted by LiCl.

As mentioned above, FOXM1 reportedly builds an interaction with β-catenin facilitating the translocation to the nucleus and hence, is critical for enhanced Wnt signaling (18). For further in-depth understanding of how aprepitant might act on this interaction in HB, we treated HuH7 cells with LiCl, aprepitant, and with a combination of both for 24 hours. Cells were then co-stained for FOXM1 and β-catenin (Fig. 3I). We found co-localization of FOXM1 (red) and β-catenin (green) in the cytoplasm of unstimulated HuH7, significantly increased cytosolic β-catenin upon LiCl stimulation (bottom left), and depletion of cytosolic β-catenin with increased free cytosolic FOXM1 when aprepitant was used (right).

Taken together, these findings suggest that treatment with aprepitant inhibits canonical Wnt signaling downstream of GSK3β by disrupting proper FOXM1–β-catenin interaction in HB cells.
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NKIR antagonism inhibits growth of HB cancer stem-like cells

The Wnt pathway is known to be highly activated in CSCs (4, 23). This is intriguing, because therapeutics that specifically target CSCs could potentially alter current treatment strategies especially for highly resistant cancer cells in the case of HB. Therefore, we investigated whether such cells indeed show high activity of Wnt signaling and whether they could be inhibited by aprepitant. We grew HB CSC-like cells as nonadherent sphere cultures that were previously reported to enrich for CSCs (24). First, we confirmed activity of Wnt signaling in sphere cultures compared with parental cell lines by measuring Wnt target gene expression. HepT1 spheres overexpressed LGR5 and AXIN2; HepG2 spheres and parentals expressed similar levels for these markers, and the β-catenin wild-type HuH7 spheres revealed a lower expression of AXIN2 and CTNNB1 (Supplementary Fig. S3A). In order to assess their stemness, cells were analyzed for tumor sphere formation. Aprepitant compromised LiCl-induced Wnt signaling in these cells. A, B, and C, qRT-PCR of HB cells cultured in adherent conditions on Wnt target genes (LGR5, AXIN2, and CTNNB1). Significance levels are as follows: **P < 0.5 and ***P < 0.1 (n=3). D, Relative mRNA levels of β-catenin and FOXM1 in HB cells treated with DMSO (black bar), lithium chloride (LiCl; dark gray bar), or a combination of LiCl and 20 μmol/L aprepitant (light gray bar). E, qRT-PCR of HB cells cultured in adherent conditions on Wnt target genes (LGR5, AXIN2, and CTNNB1). Shown are the relative values to the untreated control sample (black column) as fold changes after normalization to TATA-box binding protein (TBP). Cells were treated for 24 hours with 10 μmol/L (dark gray bars), 20 μmol/L (light gray bars), or 30 μmol/L aprepitant (white bars). Significance levels are as follows: **P < 0.5 and ***P < 0.1 (n=3). F, STF in HEK293T cells: cells were treated with DMSO (black bar), lithium chloride (LiCl; dark gray bar), or a combination of LiCl and 20 μmol/L aprepitant (light gray bar). Aprepitant compromises LiCl-induced Wnt signaling in these cells. n = 3. G, STF assay of HuH7 cells after 24 hours of treatment with DMSO, LiCl (20 mmol/L), or LiCl and aprepitant (Ap). H, the same cells were stained for β-catenin (green) and counterstained with DAPI (blue). I, HuH7 cells were treated with DMSO, aprepitant, LiCl, or a combination of the latter and co-stained for FOXM1 (red) and β-catenin (green). White arrows show increased membrane staining of β-catenin. LiCl increases cytoplasmic β-catenin, whereas aprepitant disrupted FOXM1–β-catenin complexes and accumulated FOXM1 in the cytoplasm. Counterstaining was performed with DAPI (blue).
for their expression of the embryonic stem cell markers SOX2, OCT4, and NANOG (24). We again compared these findings with the parental counterparts and found increased expression of NANOG in all spheres, increased SOX2 in HepT1 and HepG2 spheres, and increased OCT4 in HepT1 and HuH7 spheres (Fig. 4A). In HepT1 spheres SOX2, OCT4, and NANOG were 7-fold and 8-fold overexpressed, respectively, suggesting strong increase of stemness properties of these cells. Interestingly, spheres overexpressed the truncated version of NK1R (tr-TACR1) compared with parental cells, whereas full-length NK1R (fl-TACR1) was grossly unchanged (Fig. 4A).

In order to investigate whether the expression pattern of stem cell markers may change with inhibition of the NK1R receptor, we treated both the CSC-like and their parental cells with aprepitant. First, we found dose-dependent downregulation of the Wnt target genes LGR5, AXIN2, and CTNNB1 upon aprepitant treatment in spheres of all HB cell lines (Fig. 4B). Second, by analyzing pluripotency marker genes and TACR1, we found that in HepT1 and HepG2 parentals and spheres, most these markers were downregulated upon aprepitant treatment, whereas mRNA expressions did not change or increased in HuH7 parentals and spheres after treatment (Fig. 4C and D). Gene expression of the truncated version of NK1R (tr-TACR1) was negatively affected upon aprepitant treatment in HepT1 (par and sph) and HepG2 (sph), whereas its full-length splice variant only changed significantly in HepG2 (par and sph; Fig. 4C and D). However, HuH7 spheres seemed equally unaffected as their parental counterparts; in fact, SOX2 and OCT4 were even

Figure 4. Inhibition of SP/NK1R compromises cancer stemness-associated traits in HB. A, qRT-PCR (relative log2 values standardized to TBP) of pluripotency ESC markers SOX2, OCT4, and NANOG as well as NK1R (tr, truncated; fl, full length; tr-TACR1 and fl-TACR1) in adherent cells (black bars) and spheres of HepT1 (dark gray bars), HepG2 (light gray bars), and HuH7 (white bars). B, HB spheres were treated with increasing concentrations of aprepitant for 24 hours and then evaluated for Wnt target gene expression (LGR5, AXIN2, and CTNNB1). Shown are relative values after normalization to TBP. C, adherent cells were treated for 24 hours with DMSO (black bars) or 20 μmol/L aprepitant. Data are normalized on TBP expression and presented as fold change in gene expression relative to DMSO-treated controls. HepT1 (dark gray bars), HepG2 (light gray bars), and HuH7 (white bars). D, analysis of stemness markers similar to B in spheres. E, spheres were treated with aprepitant for 24 hours and qPCR analysis for liver cancer stemness genes performed. Shown is the mean; error bars, SD. F, similar to Fig. 3B, HB spheres were analyzed for FOXM1 expression on mRNA level after aprepitant treatment. Shown are the relative values standardized to TBP. Significance levels are as follows: *, P < 0.5 and **, P < 0.1 (n ≥ 2). G, sphere-forming assays (SFA) in HB cell lines (simultaneous treatment with aprepitant or SP) reveal inhibited sphere growth. Shown is the relative number of spheres per 500 seeded cells in an ultra low-attachment 96-well plate. H, Western blot analysis of PARP in HB spheres after treatment with aprepitant as compared with untreated controls. Fold changes of protein expression compared with control and standardized to β-actin is shown on top of bands. I, SFA of HB cells. Cells were pretreated with aprepitant (AP) or SP for 24 hours, then viable cells seeded into SFA, and grown without any further treatment for 10 days. Shown is the relative number of spheres per 500 seeded cells. All SFA experiments were performed at least twice in triplicates (n = 3).
upregulated in parental cells (Fig. 4E) as well as SOX2 and NANOG in spheres upon aprepitant treatment (Fig. 4D). In this regard, it seems that the β-catenin–mutated cell lines (HepT1 and HepG2) were more susceptible to aprepitant treatment than the β-catenin wild-type cell line HuH7. Due to the modest changes in HuH7 cells, we included gene expression analysis of previously described liver-specific CSC markers [AFP (25), CD13 (26), CD133 (27), CK19 (28), DLK1 (29), EPCAM (30), and GEP (31)] in our investigation. Here, we found downregulation of AFP and CD13 in all HB spheres upon aprepitant treatment, and decreased expression of CD133, CK19, EPCAM, and GEP in either HepT1 or HepG2 spheres (Fig. 4E). DLK1 was only found to be expressed in HepG2 with no changes (data not shown). Finally, we report that FOXM1 gene expression decreased in a dose-dependent manner in all HB spheres (Fig. 4F) similar to our findings in the respective parental cell lines (Fig. 3E).

Having seen a decreased activity of the Wnt pathway as well as a downregulation of defined (liver) stem cell markers upon inhibition of NK1R by aprepitant, we next investigated whether self-renewal of CSC-like cells could be targeted with aprepitant. In order to do so we performed SFA assays, which assess functional renewal of CSC-like cells, but moreover sustainably induce apoptosis in HB cell lines (14). Taken together, we speculate that the most likely explanation for our observations of late inhibition of sphere formation and simultaneous treatment (Fig. 4G) in all cell lines, including the β-catenin wild-type cell line HuH7. Because aprepitant induces apoptosis in parental HB cells (14), we performed Western blot analysis for PARP in HB spheres after aprepitant treatment. Similarly, we found apoptosis induction in HB spheres as evidenced by increased PARP cleavage upon aprepitant treatment (Fig. 4H). To rule out that inhibition of sphere formation is only executed by apoptosis induction, we pretreated the HB cells and only seeded viable cells for SFAs. Intriguingly, a single pretreatment with aprepitant was sufficient to significantly diminish their sphere formation capacity (Fig. 4I) indicating that aprepitant might not only exert its effect by reducing three-dimensional cell growth or inducing cell death in CSC-like cells, but moreover sustainably influences CSC properties in cancer cells, which support our data obtained by qRT-PCR (Fig. 4C–E).

Taken together, these findings illustrate that in addition to conventional human HB cancers cells, HB cells with stem cell-like properties (CSC-like cells) can be growth inhibited by targeting the NK1R.

**Discussion**

In this study, we investigated the downstream mechanisms following inhibition of NK1R by the small molecule aprepitant on human HB cells. After blockage of NK1R with aprepitant, we identified inhibition of both the AKT and Wnt pathways. To our knowledge, this effect of NK1R blockage has not been described in HB cells.

The AKT pathway is known to be involved in tumorigenesis and has been identified as a potent anticancer target in HB and other cancers (32). We have recently shown that inhibition of HB cell growth can be achieved with rapamycin triggered through inhibition of the AKT pathway by dephosphorylating and deactivating p70S6K (8). In turn, extrinsic activation of the AKT pathway with hepatocyte growth factor/scatter factor (HGF), a ubiquitously expressed molecule that elicits pleiotropic functions on epithelial cells, protected HB cells from the apoptotic effects of chemotherapeutic agents such as cisplatin and camptothecin (33). This effect was mainly dependent on the PI3K/AKT signaling pathway, and inhibition by wortmannin resulted in abrogation of HGF-mediated survival.

In our current study, we found robust inhibition of 4E-BP1/2 and p70S6K, both downstream members of the AKT pathway, indicating strong inhibition of this pathway. This inhibition of both p70S6K and 4E-BP1/2 produced by manipulation at the NK1R is in accordance with Mayordomo and colleagues (20). However, their group treated breast, prostate, and colon cancer cells and used SP antibodies, thereby targeting NK1R’s natural substrate, not the receptor itself. This led to a blockade of the cell cycle progression by inhibition of several cell cycle-related proteins, including mTOR. Likewise, Garcia-Recio and colleagues showed increased early expression of p-AKT in breast cancer cells after treatment with SP (21).

Different from some of these studies, in our study we found p-AKT (S473 and T308) as well as p-mTOR (S2448) to be increased 24 hours after treatment with aprepitant. When we assessed p-AKT by Western blot after 1, 3, and 6 hours of treatment, we observed a decreased expression of p-AKT (data not shown). AKT and mTOR signaling are thought to mediate cell survival in cancer cells through mTORC2 (34) and aprepitant has been shown to induce apoptosis in HB cell lines (14). Taken together, we speculate that the most likely explanation for our observations of late AKT and mTOR activation is a delayed rescue reaction of the tumor cells to escape cell death, although downstream AKT signaling remains suppressed. Another possible explanation is the inhibition of a negative feedback loop. Similar effects have been observed for treatment with everolimus, which inhibits the negative feedback loop on AKT through mTORC1, but not the positive feedback loop of mTORC2 and thus leads to hyperactivation of AKT (35). Together with the data of others, our findings clearly identify the AKT pathway as one major downstream mechanism being inhibited by NK1R antagonism.

In addition to the inhibition of the AKT pathway, we observed a striking reduction of FOXM1, a key interacting protein for the β-catenin and β-catenin–mutated HB cells without prestimulation. Mechanistically, we found that aprepi- tant caused heightened presence of membrane-bound β-catenin, a property seen in epithelial cells with strong cell–cell adhesions (36), as well as a disruption of the FOXM1–β-catenin complex, a known regulatory phenomenon in Wnt signaling (18). Together, this leads to the assumption that NK1R inhibition might reinforce epitelial states of the cell with membrane-bound β-catenin and inhibits canonical Wnt signaling in Wnt-dependent cancer cells with decreased nuclear β-catenin leading to enhanced susceptibility to apoptosis, growth arrest, and decreased stemness.

With respect to the latter aspect, our data show that in vitro aprepi- tant not only diminished the sphere-formation capacity of HB cells when treated simultaneously, but also sustainably by...
Reducing their stemness potential as shown by SEA assays of pretreated HB cells and decreased expression of liver-specific CSC and selected ESC genes. These findings are interesting because increased Wnt signaling has been linked to increased cancer stemness even in β-catenin–mutated cells (3). We here show evidence that NK1R antagonism decreases canonical Wnt signaling by inhibition of FOXM1, including in Wnt-dependent CSC-like cells. This in turn may lead to reduced stemness potential in these tumor cells.

Cross-links between AKT and Wnt signaling have been well described, although not in the setting of NK1R inhibition. Wnt can inhibit GSK3β in order to activate mTOR in the absence of β-catenin during regular cell growth. It has further been described that mTOR is part of the regulatory mechanism used by the TSC1/2 tumor-suppressor complex in order to control protein synthesis. Inoki and colleagues reported that the kinases GSK3β and AMPK cooperate in the activation of TSC2 to inhibit mTOR activity (37). This work clearly showed that the phosphorylation of TSC2 by GSK3β is significantly suppressed by Wnt signaling. These findings suggest that components of the mTOR pathway can potentially be targets for diseases linked to hyperactive Wnt signaling, including cancer (37, 38).

To our knowledge, the data presented here are the first to describe a deactivation of the Wnt pathway by targeting the SP/NK1R complex in HB cells. Therefore, it is difficult to say at this early stage how exactly the SP/NK1R complex fits into the known cross-links between Wnt and AKT signaling. One critical participant in this interaction appears to be FOXM1 (Fig. 5). In our study, we show that upon treatment with aprepitant, the union of FOXM1 and β-catenin is disrupted by pushing β-catenin to the membrane and FOXM1 to the nucleus. Thus, the interaction of FOXM1 with β-catenin becomes greatly impaired and leads to reduced canonical Wnt signaling. mTOR has been shown to facilitate nuclear translocation of FOXK1, another member of the forkhead family by activation at mTOR phosphorylation motifs (39). We speculate here that FOXM1 might be modulated in a similar way as they share a common forkhead domain (40). Unfortunately, at this time our data do not permit to fully answer this intriguing query.

Taken together, our results underline the potential of NK1R antagonists as potent anticancer agents. More importantly, they identify the SP/NK1R complex as a critical inhibitor of the AKT and Wnt signaling pathways and could contribute to the development of future anticancer strategies in HB cells and potentially in other cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: M. Ilmer, E. Alt, R. Kappler, M. Berger
Development of methodology: M. Ilmer, A. Garnier, M. Berger
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Ilmer, A. Garnier, D. von Schweinitz, R. Kappler, M. Berger
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References

# Molecular Cancer Therapeutics

## Targeting the Neurokinin-1 Receptor Compromises Canonical Wnt Signaling in Hepatoblastoma

Matthias Ilmer, Agnès Garnier, Jody Vykoukal, et al.


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