Novel Bispecific Domain Antibody to LRP6 Inhibits Wnt and R-spondin Ligand-Induced Wnt Signaling and Tumor Growth

Heather Jackson1, David Granger2, Gavin Jones2, Louisa Anderson2, Sarah Friel2, Daniel Rycroft2, William Fielies1, James Tunstead1, Michael Steward2, Trevor Wattam2, Adam Walker2, Jeremy Griggs2, Muhammad Al-Hajj1, and Christopher Shelton1

Abstract

Aberrant WNT signaling is associated with the formation and growth of numerous human cancer types. The low-density lipoprotein receptor-related protein 6 (LRP6) is the least redundant component of the WNT receptor complex with two independent WNT ligand-binding sites. Using domain antibody (dAb) technology, a bispecific antibody (GSK3178022) to LRP6 was identified that is capable of blocking stimulation in the presence of a range of WNT and R-spondin (RSPO) ligands in vitro. GSK3178022 was also efficacious in reducing WNT target gene expression in vivo, in both cancer cell line and patient-derived xenograft models, and delays tumor growth in a patient-derived RSPO fusion model of colorectal cancer.

Implications: This article demonstrates the inhibition of a key oncogenic receptor, intractable to mAb inhibition due to multiple independent ligand interaction sites, using an innovative dAb approach. Mol Cancer Res. 14(9): 859–68. ©2016 AACR.

Introduction

The WNT pathway is a high-priority target for development of anticancer therapies. Involvement of the WNT pathway in tumorigenesis was discovered more than two decades ago, with identification of WNT1 as an oncogenic driver in murine mammary carcinogenesis and identification of Adenomatous Polyposis Coli (APC) as a tumor suppressor in colorectal cancer (1, 2). More recently, advances in sequencing methods have revealed that up to one third of solid tumors contain loss-of-function mutations in APC or gain-of-function mutations in β-catenin (CTNNB1; ref. 3). Other genetic lesions have been identified, including R-spondin (RSPO) fusions in colorectal cancer and RNF43 mutations in pancreatic cancer (4, 5). Many tumors show evidence of epigenetic activation of the Wnt pathway through altered expression of negative regulators, such as WIF1 and SFRP1.

WNT ligands are a family of 19 palmitoylated secreted glycoproteins that interact with receptor complexes containing members of two families of cell-surface receptors: 10 related seven-pass transmembrane (7TM) proteins, known as Frizzleds (FZD), and two type I single pass proteins, LRP5 and LRP6 (6, 7). Engagement of WNT ligands with the receptor complex leads to increased cytoplasmic levels of β-catenin through inhibition of a “destruction complex” composed of AXIN2, APC, and GSK3. Cytoplasmic β-catenin translocates to the nucleus and interacts with members of the TCF family of transcription factors to activate expression of target genes that mediate proliferation.

RSPO proteins represent a recently identified second class of ligands capable of stimulating the Wnt pathway (8). RSPO proteins bind to a group of three related 7TMs, LGR4, LGR5, and LGR6, to modulate the activity of two related membrane-bound E3 ligases, RNF43, and ZNRF3. RNF43 and ZNRF3 appear to control the turnover of FZD receptors, and the expression of RNF43 and ZNRF3 is upregulated by WNT stimulation (9). Therefore, RSPO proteins increase WNT signaling by inhibiting the action of a negative feedback loop in the Wnt pathway. A total of 10% of colorectal cancers contain fusions in the RSPO genes that lead to overexpression and enhanced stimulation of the Wnt pathway (4). These fusions are exclusive to β-catenin and APC mutations, providing strong evidence that RSPO alteration can drive tumorigenesis through the Wnt pathway.

Oncogenic Wnt pathway activation can occur through multiple receptor-dependent and -independent mechanisms. Receptor-dependent mechanisms include increased expression of WNT/RSPO ligands, as well as epigenetic repression of other extracellular antagonists, such as DKKs, WIF1, and SFRPs. For example, downregulation of WIF1 or SFRP1 is found in a majority of nonsmall cell lung cancer tumors (10, 11). Receptor-independent mechanisms involve mutations downstream of the receptor complex, typically in components of the destruction complex. In colorectal cancer, with the exception of RSPO fusions, activation of the pathway downstream of the receptor complex is common, typically through mutations in APC, β-catenin, or AXIN2.

LRP5 and LRP6 constitute the least redundant component of the receptor complex and are potential targets for blocking aberrant receptor-dependent activation of the pathway using inhibitory mAb approaches. Biophysical characterization of LRP6 has
revealed two independent WNT-binding sites, and monospecific anti-LRPs mAbs are unable to block both sites simultaneously, indicating that an alternative approach, such as bispecific, is required (12, 13). Using domain antibody (dAb) technology (14, 15), we identified a bispecific inhibitor of LRP6, termed GSK3178022. GSK3178022 is effective at inhibiting both WNT and RSPO stimulation in vitro, abrogates Wnt signaling in vivo, and delays tumor growth in a patient-derived colorectal cancer tumor model with an RSPO fusion.

Materials and Methods

Cell lines
All cell lines were purchased from the ATCC with the exception of EKVX and HEK293/6E, which were obtained from the National Cancer Institute and Biotechnology Research Institute, National Research Centre, respectively. PA-1 and HT-1080 cell lines were grown in Eagle’s Minimal Medium supplemented with 10% FBS, NTERA-2 cell line in DMEM with 10% FBS, and HEK293/6E with FreeStyle 293 Medium (Gibco). All other lines were grown in RPMI1640 with 10% FBS. All lines with the exception of EKVX are banked internally, and authenticity has been confirmed using the Promega Cell ID System.

Reporter assays
HT-1080 or HEK293/6E cells were seeded in tissue culture–treated microassay plates in Eagle’s Minimum Medium supplemented with 10% FBS and incubated overnight at 37°C, 5% CO2. Cells were cotransfected with 75 ng per well TOPFlash or FopFlash plasmid (Millipore) and the appropriate WNT ligand expression plasmid (generated in-house) using Lipofectamine 2000 (Life Technologies) in OptiMEM media (Life Technologies) with 0.5% FBS. Two hours after transfection, cells were treated with anti-LRP6 agents and RSPO ligands (R&D Systems) where appropriate and further incubated for 24 hours. Luciferase activity was measured using Bright-Glo Luciferase Assay System (Promega).

Western blots
HT-1080 cells were seeded in tissue culture–treated dishes in Eagle’s Minimal Medium with 10% FBS and incubated overnight at 37°C, 5% CO2. Cells were treated with anti-LRPs agent for 1 hour and recombinant WNT3a ligand added for an additional 1-hour incubation. Whole-cell lysates were quantitated for total protein content using BCA Protein Assay Kit (Thermo Scientific Pierce). Immunoblots were performed with the following antibodies: anti-phospho-LRP6, anti-LRP6, anti-β-catenin (Cell Signaling Technology), anti-active-β-Catenin (Millipore), and anti-β-actin (Sigma-Aldrich).

Real-time PCR assays
Cell lines were seeded in tissue culture–treated dishes in appropriate growth media with 10% FBS and incubated overnight at 37°C, 5% CO2. Treatment mixes were made in Opti-Mem with 0.5% FBS to contain 200 ng/mL of recombinant WNT3a ligand with or without anti-LRP6 constructs. Frizzled8 CRD (R&D Systems) was used at 10 μg/mL. Growth media were replaced by treatment mixes, and plates were incubated for 24 hours at 37°C, 5% CO2. cDNA was produced and RT-PCR reactions performed using TaqMan Gene Expression Cells-to-CT Kit (Ambion) and validated TaqMan Gene Expression Assays (Life Technologies) for AXIN2 and SP5. Data were analyzed using the ΔΔCt method by normalizing data to β-actin (ACTB) Ct values.

dAb selection and affinity maturation
dAbs were selected from a library using LRP6 ECD (R&D Systems) as described previously (14, 16). Fusion proteins comprising selected dAbs and the Fc-portion of human IgG1 were expressed in HEK293/6E cells, which were maintained in FreeStyle 293 medium (Gibco) at 37°C, 5% CO2, and 125 rpm. dAbs were connected at the N-terminal and C-terminal, using a three amino acid and eight amino acid linker, respectively.

Antibodies were affinity matured by scanning complementarity-determining region (CDR) positions with all codons using site-directed mutagenesis. Plasmid DNA encoding the antibody was used as a template in PCRs with PhiUltra (Agilent). Primers introducing NNB codons were used in reactions and the resultant mutated antibody sequences tested for binding and function in reporter assays.

Surface plasmon resonance
Binding analysis was performed on a BIACORE 4000 instrument (Biacore) at 25°C in HBS-EP buffer (GE Healthcare). dAb molecules fused to human IgG Fc domain were expressed at an unknown concentration and captured from clarified, 0.22-μm filtered cell culture supernatants via IgG by injection at a flow rate of 10 μL/minute for 60 seconds. LRP6 ECD (5 μg/mL) in HBS-EP buffer supplemented with 1% w/v BSA (Sigma) was then injected for 5 minutes at 30 μL/minute, followed by 10 minutes of dissociation. Analysis was performed using the BIACORE 4000 evaluation software (Biacore) using a global analysis 1:1 Langmuir-binding model.

Pharmacokinetics of GSK3178022 in plasma
For pharmacokinetics determination, GSK3178022 was dosed into three groups (n = 3) of male C57Bl6 mice. Each group received an intravenous dose at either 0.25, 1, or 10 mg/kg. Whole blood was taken over a range of time points up to 72 hours postdose for 0.25 mg/kg dose group and up to 168 hours postdose for the 1 and 10 mg/kg dose groups. Whole-blood samples were mixed 1:1 with water containing 5% EDTA to make blood–water and stored at −80°C. Analysis of pharmacokinetic blood-water samples was performed using the Meso Scale Discovery (MSD) platform. LRP6 ECD (in-house) was coated onto 96-well standard plates. Wells were blocked with assay buffer (3% BSA in PBS containing 0.1% Tween 20) and incubated for 1 hour with constant shaking. Quality control samples and blood–water pharmacokinetic samples were added at a range of dilutions. A calibration curve was generated using GSK3178022 diluted in control mouse blood–water (SeraLab). Samples and standards were incubated for 1 hour at room temperature with constant shaking. Bound GSK3178022 was detected with MSD sulfo-tagged mouse anti-human Vx mAb (in-house) or with MSD sulfo-tagged mouse anti-human Fc (in-house). MSD read buffer was added and plates were read on a SECTOR 6000 MSD imager. Final assay results were fitted in WinNonLin by noncompartmental analysis (NCA) according to standard methods. The mean pharmacokinetics profile of GSK3178022 in mice after 0.25, 1, and 10 mg/kg intravenous doses was plotted using GraphPad Prism version 6. Derived pharmacokinetics parameters were obtained from the NCA fit.

In vivo pharmacodynamic and antitumor effect of GSK3178022
NTERA-2 tumors were established by subcutaneous injection of 8 × 106 NTERA-2 cells mixed with Matrigel (BD Biosciences).
tissues. Each mouse was inoculated subcutaneously at the right flank with the selected tumor fragment of 3 × 3 × 3 mm size. The implantation day was denoted as D0. Treatments were started when mean tumor size reached approximately 200 mm³. For antitumor studies, mice were allocated randomly into four experimental groups of 10 mice each according to their tumor sizes. A nonbinding isotype control dual-dAb served as a negative control. Dual-dAbs and ritonectan were administered twice weekly by intraperitoneal injection at 40 mg/kg dose. To measure tumor growth, tumor sizes were determined twice weekly by caliper measurement, and volume was determined, in mm³, using the formula: \( V = \frac{1}{2}ab^2 \), where a and b are the long and short diameters of the tumor, respectively. To determine gene expression, tumors were obtained by necropsy and homogenized prior to isolating RNA using an RNeasy Kit (Qiagen). RNA was converted to cDNA using the High Capacity Reverse Transcription Kit (Life Technologies). RT-PCR reactions were performed using TaqMan Gene Expression Master Mix (Life Technologies) and validated TaqMan Gene Expression Assays (Life Technologies) for AXIN2 and SP5. Data were analyzed using the \( \Delta \Delta C_t \) method by normalizing data to ACTB.

Results

LRP6 contains two independent binding sites for WNT ligand engagement, and biopharmaceutical inhibition of signaling mediated by all WNT ligand classes, therefore, requires inhibition at both sites (12, 13). dAbs are minimal binding regions derived from the variable region of an antibody heavy or light chain (14, 15). To begin construction of a bispecific inhibitor of both WNT ligand-binding sites, monospecific dAbs were first identified that functionally blocked each site of LRP6. To identify dAbs, LRP6-binding dAbs were selected from a phage library using 3 rounds of selection with decreasing antigen concentration to apply selection pressure. Selected dAbs were expressed as bivalent human IgG1 Fc-domain fusion proteins, evaluated for specificity to LRP6 over LRP5 by ELISA and tested for ability to bind to LRP6 ECD using surface plasmon resonance. dAb–Fc fusions were then tested in TOPFlash reporter assays using HEK293/6E cells to identify molecules that functionally blocked different classes of WNT ligands, known to bind each of the two binding sites. This identified a dAb designated k020, which blocked functional activity of WNT3a, and dAbs designated H027, H035, and H037, which blocked functional activity of WNT1 (Fig. 1).

To determine the optimal combination of monospecific dAbs that most effectively inhibited LRP6-mediated signaling by both classes of WNT ligands, "dual-dAb" constructs were generated, comprising dAbs fused to both the N- and C-termini of an IgG1 Fc-domain. All pairings with k020 were generated: N-terminal K020 paired with C-terminal H027, H035, or H037 and vice versa. These bispecific, tetravalent proteins were tested for their ability to inhibit WNT signaling in the human fibrosarcoma cell line, HT-1080, transfected with TOPFlash reporter plasmids and compared...

![Figure 1](https://www.aacrjournals.org/doi/10.1158/1541-7786.MCR-16-0088)
with their monospecific counterparts (Fig. 1). The HT1080 cell line was used because it preferentially expresses LRP6, with no detectable LRP5, by Western blot analysis (data not shown). In this assay, the bivalent, monospecific components of the dual dAbs tested (k020, H035, and H037) were each able to block signaling of one class of WNT ligands but elicited reciprocal potentiation of signaling mediated by the alternative ligand class, as previously reported for monospecific mAbs (12, 13). For example, k020 (10 μg/mL) potentiated WNT signaling mediated by WNT1 (Fig. 1A) but decreased signaling mediated by WNT3A (Fig. 1B). Conversely, and as expected, H035 and H037 each inhibited WNT1-mediated signaling while potentiating signaling by WNT3A. The functional results suggest that the different classes of dAbs interact with different domains of LRP6 that comprise the discrete WNT1 class- and WNT3A class–binding sites. When combined into the dual dAb formats, with the exception of k020-H027, all combinations tested were able to strongly inhibit signaling in the presence of either WNT1 or WNT3A ligands or a combination of both. The reduced activity of the k020-H027 in blocking WNT1 stimulation may reflect a reduced ability of this particular bispecific construct to interact with both epitopes, or unfavorable biophysical characteristics of this construct, although this was not explored further. H027-k020 demonstrated the strongest activity and was selected for further characterization.
Interestingly, each combination demonstrated superior activity over monospecific constructs, potentially due to the avidity effect achieved by the tetravalent, bispecific format. To further improve the properties of H027-k020, the dAb moieties were subjected to affinity maturation by scanning diversified residues within the CDRs by site-directed mutagenesis. Improvements in binding and biological activity of novel variants were initially screened using surface plasmon resonance and single-point TOPFlash assays, which highlighted beneficial CDR mutations, which were then iteratively combined. In addition, it has been demonstrated that residues Leu234-Ser239 of the CH2 domain of human IgG Fc are necessary for FcγR binding (17). The Fc effector functions of H027-k020 were abrogated by mutating the equivalent of Leu-235 and Gly-237 to alanine, abolishing Fc-γ receptor binding and complement deposition. An N-glycosylation site was mutated out of the CDR2 of H027, and a C-terminal threonine residue was incorporated into the molecule to minimize potential immuno- genicity. This yielded the lead molecule GSK3178022. Binding experiments demonstrated that GSK3178022 is highly selective to human LRP6 over murine LRP6, with no significant cross-reactivity to human LRP5 (Supplementary Fig. S1).

To characterize the activity of GSK3178022 against a full range of WNT and RSPO ligands, the HT-1080 cell line and TOPFlash reporter assay were used with a broad panel of WNT ligands. Expression vectors for all 19 WNT ligands were constructed and tested for activity. In this format, 11 WNT ligands were able to stimulate the assay, and the capacity of GSK3178022 to inhibit signaling mediated by each was tested (Fig. 2A). GSK3178022 reduced stimulation by all active WNT ligands except WNT7B, which only weakly stimulated in this assay. It remains unclear whether the lack of inhibition is due to inability of GSK3178022 to block this ligand or whether it reflects a limitation of the assay.

RSPOs increase WNT signaling produced by WNT stimulation. To determine the ability of GSK3178022 to block signaling in the presence of RSPO agonists, all four RSPO family members were proffered in the reporter assay in the presence of representative WNT ligands to each of the binding sites on LRP6: WNT1 and WNT3A (Fig. 2B). All four RSPOs potentiated stimulation by WNT1 and WNT3A. The strongest stimulation was seen when RSPO2 and WNT3A were combined, which approached 50-fold stimulation above baseline. GSK3178022 (5 nmol/L) completely abrogated this signal, confirming that it has broad and potent inhibitory activity against a range of WNT ligands, including when amplified by RSPO ligands.

RSPOs are thought to stimulate WNT signaling by affecting the turnover of EBD receptors (9, 18). WNT ligands lead to phosphorylation of LRP6 and stabilization of β-catenin in the cytosol (19). GSK3178022 may block WNT and RSPO stimulation by affecting turnover of LRP6 or by blocking signaling downstream of the receptor complex. To determine the biochemical mechanism by which GSK3178022 inhibits WNT signaling, a panel of cancer cell lines was used (Fig. 3). A, expression of the Wnt target genes AXIN2 and SP5 in the presence of recombinant WNT3A ligand, with and without GSK3178022 treatment, in a panel of cancer cell lines. B, ability of monospecific dAbs and GSK3178022 to decrease mRNA levels of SP5 in NTERA-2 cells treated with recombinant Wnt3a ligand.
signaling, the levels of total and phosphorylated LRP6 and "active" or unphosphorylated β-catenin levels were measured in HT-1080 cells treated with WNT3A ligand and GSK3178022 (Fig. 2C). WNT3A ligand treatment caused a marked increase in both of these biochemical markers of Wnt pathway activation without changing the levels of either total LRP6 or β-catenin. GSK3178022 blocked phosphorylation of LRP6 as well as the unphosphorylated state of β-Catenin without altering levels of total LRP6. Therefore, GSK3178022 inhibits the receptor complex by blocking the biochemical events resulting from WNT ligand engagement.

To establish the potential effectiveness of GSK3178022 across multiple tumor types and on endogenous target gene expression, inhibitory activity was assessed in a panel of cancer cell lines using RT-PCR against the known Wnt pathway target genes SP5 and AXIN2 (20, 21). Cell lines were stimulated by WNT3A with and without treatment with GSK3178022 and the expression of Wnt pathway target genes SP5 and AXIN2 assessed (Fig. 3A). In all cell lines examined, GSK3178022 inhibited stimulation of SP5 and AXIN2 expression, in most cases returning expression levels to baseline. In five cell lines, expression was reduced below baseline, suggesting inhibition of autocrine WNT signaling in these cell lines.

The human testicular embryonal carcinoma cell line, NTERA-2, was the most responsive to stimulation of the cell lines tested and was therefore used to further characterize the endogenous response of GSK3178022 treatment. Both monospecific dAb–Fc constructs and GSK3178022 were tested. Neither the H027 dAb nor the k020 dAb alone were capable of blocking stimulation of SP5 expression by WNT3A. However, GSK3178022 was highly effective at reducing levels of SP5 below baseline, with an EC50 of around 380 pmol/L (40 ng/mL; Fig. 3B). As a control, FZD8-CRD tested at 10 mg/mL was effective at reducing the stimulation, but not to the levels achieved with GSK3178022. Therefore, GSK3178022 blocks expression of WNT target genes in a range of cancer cell lines with picomolar potency.

To permit detailed analysis of the in vivo activity of GSK3178022, pharmacokinetic studies were performed in mice. GSK3178022 was dosed into three groups of mice, each receiving a single intravenous dose at either 0.25, 1, or 10 mg/kg. Nonlinearity was observed across the dose escalation, with most parameters not scaling as expected with dose, for example, from NCA to NCA

Life obtained in mice was 19.2 hours, with a disposition through interaction with LRP6. The maximum half-life of the 1 mg/kg dose group (Fig. 4) The nonlinearity in pharmacokinetics increased at increasing dose; however, clearance was slowest for a dose-proportional manner. The volume of distribution also increased with dose while the half-lives and MRT increased with dose while the tumor, while others were devoid of signal, indicating reduced levels of SP5 mRNA throughout the tumor. These data indicate xenograft penetration and inhibition of WNT target gene expression by GSK3178022 following intravenous administration. Having determined that acute dosing of GSK3178022 inhibited WNT signaling in xenograft tumors, we tested whether GSK3178022 treatment could affect growth in patient-relevant tumor models. RSPO fusions in colorectal cancer tumors have been found at a frequency of 10% (4). These fusions are exclusive to the more common APC and β-catenin mutations in colorectal cancer, providing strong evidence that the fusions play a role in tumorigenesis. In addition, efficacy has been demonstrated by targeting of RSPOs in patient-derived xenograft (PDx) models selected on the basis of high RSPO expression (22). Because GSK3178022 effectively inhibits both WNT and RSPO stimulation in vitro, PDx models with genetically defined RSPO fusions were identified and used for in vivo testing of the efficacy of GSK3178022 to inhibit WNT signaling and tumor growth.

RSPO fusion colorectal cancer PDx models were identified by examining microarray expression data from a large panel of colorectal cancer models. Models with clearly elevated RSPO expression were tested by PCR as described previously (4). Two models, CR1560 and CR3150, were identified with Crown BioScience as having RSPO3 translocations by PCR and sequencing. The β-catenin and APC genes in CR1560 and CR3150 were sequenced and found to be wild type. Additional oncogenic mutations were present in these PDx models (Supplementary Table S1). Mutations in KRAS, TP53, and EGFR were present in both models. In addition, a mutation in PI3K (PIK3CA) was present in CR3150. To determine the pharmacodynamics and efficacy effect of GSK3178022 treatment, cohorts of mice were established with subcutaneous CR1560 and CR3150 tumors. Tumors were isolated 24 hours after the final treatment dosing in the efficacy groups (below), and the expression of WNT target genes was examined by RT-PCR in tumor homogenates. Strong (>60%) suppression of the WNT target gene AXIN2 was seen only in the CR1560 tumor model, with no effect on AXIN2 expression in CR3150 (Fig. 6A). Serum levels of GSK3178022 in both models were determined and found to be similar: 12.4 μg/mL ± 1.9 (SE) for CR3150 and 11.0 μg/mL ± 0.1 (SE) for CR1560. Therefore, the lack of gene response in CR3150 suggests that this model is insensitive to LRP6 inhibition by GSK3178022.

To establish the effect of GSK3178022 on tumor growth, groups of tumor-bearing mice were treated with an isotype control dual-dAb, GSK3178022, irinotecan, or a combination of GSK3178022 and irinotecan. In both models, treatment with irinotecan alone led to regression in tumor volume, and this effect was not increased in combination with GSK3178022 (Fig. 6B and C). Single-agent treatment with GSK3178022 led to an initial analysis the half-lives and MRT increased with dose while AUC (0–∞) increased with higher doses as expected but not in a dose-proportional manner. The volume of distribution also increased at increasing dose; however, clearance was slowest for the 1 mg/kg dose group (Fig. 4). The nonlinearity in pharmacokinetic parameters obtained suggests possible target-mediated disposition through interaction with LRP6. The maximum half-life obtained in mice was 19.2 hours, with a C_{max} of 94 μg/mL at the 10 mg/kg dose.

Pharmacodynamic studies were undertaken to determine the effect of in vivo treatment on endogenous WNT target genes. NTERA-2 tumor-bearing mice were treated with GSK3178022, and gene expression in tumors was determined by RT-PCR and in situ hybridization. Following a single-dose treatment with GSK3178022, NTERA-2 tumors were harvested at 5, 24, and 72 hours posttreatment. RT-PCR was used to assess the expression of SP5 as compared with tumors harvested from untreated mice (Fig. 5A). We observed a significant decrease in SP5 message levels in NTERA-2 tumors at both 5 and 24 hours posttreatment with GSK3178022. At 72 hours, SP5 message is reduced, but not at a statistically significant level. The pharmacodynamic effect of GSK3178022 is consistent with the relatively short half-life of the molecule (<24 hours).

The tumor penetration and spatial modulation of Wnt gene modulation achieved by GSK3178022 was analyzed by in situ hybridization analysis of SP5 expression in tumor sections (Fig. 5B). Sections of tumor from mice treated with an isotype control dual-dAb, which were incubated with the SP5-specific probe, showed a strong signal over most regions of tumor cells indicating significant levels of SP5 mRNA. Areas of connective tissue and blood within the tumor were devoid of signal. In contrast, sections of tumor from mice treated with GSK3178022 incubated with the SP5 probe showed a significant reduction in signal in most areas of the tumor, while others were devoid of signal, indicating reduced levels of SP5 mRNA throughout the tumor. These data indicate xenograft penetrance and inhibition of WNT target gene expression by GSK3178022 following intravenous administration. Having determined that acute dosing of GSK3178022 inhibited WNT signaling in xenograft tumors, we tested whether GSK3178022 treatment could affect growth in patient-relevant tumor models. RSPO fusions in colorectal cancer tumors have been found at a frequency of 10% (4). These fusions are exclusive to the more common APC and β-catenin mutations in colorectal cancer, providing strong evidence that the fusions play a role in tumorigenesis. In addition, efficacy has been demonstrated by targeting of RSPOs in patient-derived xenograft (PDx) models selected on the basis of high RSPO expression (22). Because GSK3178022 effectively inhibits both WNT and RSPO stimulation in vitro, PDx models with genetically defined RSPO fusions were identified and used for in vivo testing of the efficacy of GSK3178022 to inhibit WNT signaling and tumor growth.

RSPO fusion colorectal cancer PDx models were identified by examining microarray expression data from a large panel of colorectal cancer models. Models with clearly elevated RSPO expression were tested by PCR as described previously (4). Two models, CR1560 and CR3150, were identified with Crown BioScience as having RSPO3 translocations by PCR and sequencing. The β-catenin and APC genes in CR1560 and CR3150 were sequenced and found to be wild type. Additional oncogenic mutations were present in these PDx models (Supplementary Table S1). Mutations in KRAS, TP53, and EGFR were present in both models. In addition, a mutation in PI3K (PIK3CA) was present in CR3150. To determine the pharmacodynamics and efficacy effect of GSK3178022 treatment, cohorts of mice were established with subcutaneous CR1560 and CR3150 tumors. Tumors were isolated 24 hours after the final treatment dosing in the efficacy groups (below), and the expression of WNT target genes was examined by RT-PCR in tumor homogenates. Strong (>60%) suppression of the WNT target gene AXIN2 was seen only in the CR1560 tumor model, with no effect on AXIN2 expression in CR3150 (Fig. 6A). Serum levels of GSK3178022 in both models were determined and found to be similar: 12.4 μg/mL ± 1.9 (SE) for CR3150 and 11.0 μg/mL ± 0.1 (SE) for CR1560. Therefore, the lack of gene response in CR3150 suggests that this model is insensitive to LRP6 inhibition by GSK3178022.

To establish the effect of GSK3178022 on tumor growth, groups of tumor-bearing mice were treated with an isotype control dual-dAb, GSK3178022, irinotecan, or a combination of GSK3178022 and irinotecan. In both models, treatment with irinotecan alone led to regression in tumor volume, and this effect was not increased in combination with GSK3178022 (Fig. 6B and C). Single-agent treatment with GSK3178022 led to an initial
delay in tumor growth in CR1560 (Fig. 6C) but had no effect in CR3150 (Fig. 6B). No in vitro effect of GSK3178022 treatment was seen in cell lines derived from either model, under 2D or 3D growth conditions (data not shown).

Discussion

Using daβ technology, we constructed an anti-LRP6 bispecific molecule, GSK3178022, which potently inhibits signaling by a range of WNT and RSPO ligands in cancer cell lines in vitro. In addition, GSK3178022 reduced WNT-mediated gene upregulation in vivo, both in the cancer cell line Ntera2 and in one of two PDX models selected for activated RSPO status. GSK3178022 delayed tumor growth in the xenograft model that responded molecularly but had no effect on the nonresponding model. This molecule demonstrates the ability to construct bispecific molecules, of reduced molecular weight compared with a mAb, that can functionally inhibit independent ligand-binding sites on the same receptor. In addition, with respect to LRP6, GSK3178022 meets the key challenge of simultaneously inhibiting ligand-mediated signaling through either binding site without the adverse reciprocal increase signaling previously described with conventional anti-LRP6 antibodies.

Both small-molecule and biological attempts have previously been made to develop targeted therapeutics against Wnt stimulation. For example, LGK-974 is a small-molecule inhibitor of Porcupine (PORCN), an enzyme required for lipidation of WNT ligands (23). Because lipidation is a general property of Wnt proteins, and required for secretion, LGK-974 is capable of completely blocking WNT ligand stimulation of the pathway. In addition, OMP-18RS is an antibody in phase 1 trials that recognizes a subset of FZD coreceptors and blocks WNT ligand engagement (24). However, targeting the action of WNT ligands alone may be insufficient in tumors that have alterations in other Wnt pathway modulators, such as in the RSPO/LGR pathway (6, 8). GSK3178022 is capable of blocking activation by both WNT and RSPO ligand classes, and therefore may be effective in a broader range of WNT-activated tumors.

Wnt pathway activity is also required in normal adult tissues, such as the intestine, bone, and liver, and can be modulated by either LRP5 or LRP6 in a tissue-specific manner. For example, WNT signaling in bone regulates osteoblast differentiation and is required to maintain bone density (25). Human genetic data indicate that LRP5 is an essential Wnt signaling component in this tissue and both gain- and loss-of-function mutations in LRP5 have been described that lead to osteopetrosis and osteoporosis, respectively. A role for LRP6 in bone in humans has also been described. Individuals with a loss-of-function mutation in LRP6 develop early onset coronary artery disease, features of metabolic syndrome, and osteoporosis (26). Mice with compound mutations in LRP5 and LRP6 globally or specifically in the embryonic mesenchyme show deficits in bone and limb formation in a dose-dependent manner (27, 28). In the intestine, conditional mouse studies have demonstrated a functional redundancy between LRP5 and LRP6 (29). Intestinal morphology was normal in mice with single conditional knockouts of LRP5 or LRP6, but disrupted in mice with double conditional knockout of both. LRP6 has been described as a more potent biochemical mediator of WNT stimulation than LRP5 (30). Consistent with this, we show that GSK3178022 is broadly inhibitory of WNT3A stimulation in cancer cell lines expressing both LRP5 and LRP6, indicating that LRP5 cannot functionally substitute for LRP6 in these cell lines. However, GSK3178022 may affect normal tissue homeostasis where LRP6 is essential and nonredundant with LRP5. In addition, GSK3178022 may fail to block Wnt pathway activation in tumors that rely on LRP5 for Wnt activation.

Because GSK3178022 is an effective inhibitor of WNT and RSPO stimulation through LRP6, tumors with a genetic RSPO fusion may represent a tumor type responsive to GSK3178022 treatment. Two patient-derived RSPO3 fusion positive colorectal...
cancer xenograft models were tested for sensitivity to GSK3178022. Of the two identified models, one model did not respond at the molecular level, indicating that RSPO fusion status alone is insufficient to predict response. Pathway inhibition in mutationally activated colorectal cancer cell lines has also shown variable dependence on the Wnt pathway (31–33). It is also possible that RSPO ligands can signal through parallel pathways, in addition to effects on the Wnt pathway. However, the second identified RSPO3 fusion positive model demonstrated a response to GSK3178022, both at the level of WNT target gene expression and in tumor growth delay, supporting the hypothesis that RSPO fusions activate Wnt signaling through LR6.

We demonstrated GSK3178022 is a potent inhibitor of signaling mediated by a range of WNT ligands in multiple cell lines, including in the presence of RSPO ligands. We sought to extend these studies into in vivo tumor models, to begin to characterize the pharmacokinetic and pharmacodynamic properties of GSK3178022 in mice and to further explore its therapeutic potential.

GSK3178022 has a half-life of less than 20 hours in mice which may be driven, in part, by target-mediated clearance in normal tissues, as GSK3178022 is cross-reactive for mouse LR6. Nonetheless, administration of a single 90 mg/kg dose was sufficient to achieve a significant reduction in expression of a key WNT response gene, SP5, in NTERA-2 tumors, which was detectable 5 hours after administration and was sustained for at least 24 hours. This data and in situ hybridization analysis were consistent with GSK3178022 achieving tumor penetration and inhibitory activity against WNT signaling in vivo. Similarity, AXIN2 expression was significantly decreased in CR1560 tumors subjected to twice weekly GSK3178022 administration. Despite achieving such functional inhibition of WNT signaling, administration of GSK3178022 under the conditions tested failed to provide a therapeutic benefit. The reason for this observation remains to be determined, but may reflect inadequate potency of GSK3178022 against multiple WNT ligands in an in vivo context or insufficient exposure of GSK3178022 following the treatment regimen used. Further investigation will be required to (i) better characterize the relationship between GSK3178022 pharmacokinetics and pharmacodynamics; (ii) define optimal pharmacodynamic marker(s) for GSK3178022-mediated Wnt inhibition, which predict efficacious tumor response; and (iii) determine the magnitude of WNT response gene inhibition, which is required to affect tumor growth arrest or regression in vivo. Conversely, these data may reflect redundancy between LRP5 and LR6 in this context, which would limit the expected benefit of anti-LRP6 therapy.

Using dAb technology, we demonstrate effective simultaneous inhibition of the two independent ligand-binding domains of the LR6 receptor. We demonstrate that LR6 is necessary for WNT stimulation in a broad range of cancer cell lines and is also necessary for RSPO stimulation of the Wnt pathway. In addition, we found that combining two domain antibodies into a bispecific molecule increased potency compared with admixtures of their monospecific counterparts. Therefore, engineered bispecific molecules using domain antibodies are a promising approach to generating potent inhibitors against complex receptors that mediate multiple ligand interactions.

Disclose of Potential Conflicts of Interest

Funding for the work was provided by GlaxoSmithKline (GSK), the employer of H. Jackson, D. Granger, G. Jones, L. Anderson, S. Fried, D. Rycroft, W. Fiesle, J. Tunstead, M. Steward, A. Walker, J. Griggs, M. Al-Hajj, and C. Shelton. All studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed at the Institutional Animal Care and Use Committee either by GSK or by the ethical review process at the institution where the work was performed.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Jackson, D. Granger, G. Jones, L. Anderson, S. Fried, D. Rycroft, W. Fiesle, T. Wattam, and C. Shelton

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Jackson, D. Granger, S. Fried, D. Rycroft, W. Fiesle, J. Tunstead, M. Steward, and C. Shelton

Writing, review, and/or revision of the manuscript: H. Jackson, D. Granger, S. Fried, D. Rycroft, W. Fiesle, J. Tunstead, M. Steward, A. Walker, J. Griggs, M. Al-Hajj, and C. Shelton

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Shelton

Study supervision: D. Rycroft, C. Shelton

Acknowledgments

The authors acknowledge Crown Bioscience for performing in vivo studies.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 16, 2016; revised June 3, 2016; accepted June 22, 2016; published OnlineFirst July 11, 2016.

References

17. Morgan A, Jones ND, Nesbitt AM, Chaplin L, Bodmer MW, Emtage JS. The N-terminal end of the CH2 domain of chimeric human IgG1 anti-HLA-DR is necessary for C1q, FcγRI and FcγRIII binding. Immunology 1995; 86: 319–324.
Novel Bispecific Domain Antibody to LRP6 Inhibits Wnt and R-spondin Ligand-Induced Wnt Signaling and Tumor Growth

Heather Jackson, David Granger, Gavin Jones, et al.


Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-16-0088

Access the most recent supplemental material at: http://mcr.aacrjournals.org/content/suppl/2016/07/09/1541-7786.MCR-16-0088.DC1

This article cites 33 articles, 13 of which you can access for free at: http://mcr.aacrjournals.org/content/14/9/859.full#ref-list-1

This article has been cited by 1 HighWire-hosted articles. Access the articles at: http://mcr.aacrjournals.org/content/14/9/859.full#related-urls

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, use this link http://mcr.aacrjournals.org/content/14/9/859. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.