Targeting ligand-dependent wnt pathway dysregulation in gastrointestinal cancers through porcupine inhibition

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Abstract

Gastrointestinal cancers are responsible for more cancer deaths than any other system of the body. This review summarises how Wnt pathway dysregulation contributes to the development of the most common gastrointestinal cancers, with a particular focus on the nature and frequency of upstream pathway aberrations. Tumors with upstream aberrations maintain a dependency on the presence of functional Wnt ligand, and are predicted to be tractable to inhibitors of Porcupine, an enzyme that plays a key role in Wnt secretion. We summarise available pre-clinical efficacy data from Porcupine inhibitors in vitro and in vivo, as well as potential toxicities and the data from early phase clinical trials. We appraise the rationale for biomarker-defined targeted approaches, as well as outlining future opportunities for combination with other therapeutics.

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Wnt
gastrointestinal cancer
RNF43
RSPO
clinical trials

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Abbreviations: APC, adenomatous polyposis coli; CAF, cancer associated fibroblast; CMS, consensus molecular subtype; CRC, colorectal cancer; CSC, cancer stem cell; EMT, epithelial-mesenchymal transition; Dkk, Dickkopf; HCC, hepatocellular carcinoma; LGR, leucine-repeat G-coupled receptor; LRP, low-density lipoprotein receptor-related protein; MSI, microsatellite instability; PCP, planar cell polarity; ROR, receptor tyrosine kinase-like orphan receptors; RSPO, Sfrp, Secreted frizzled-related protein; TP53, transformation-related protein 53.

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1. Introduction

Secreted Wnt proteins were originally discovered as key players in development and oncogenesis (Nusse & Varmus, 1982; Nüsslein-Volhard & Wieschaus, 1980; Sharma & Chopra, 1976) and subsequently found to be important in tissue homeostasis, with pivotal roles in stem cell-mediated regeneration of the intestinal epithelium and maintenance of bone integrity (Clevers & Nusse, 2012). Dysregulation of Wnt signaling has been implicated in various cancers, particularly those of the gastrointestinal tract (Jackstadt, Hodder, & Sansom, 2020; Sphyris, Hodder, & Sansom, 2021).

Extracellular Wnts can be divided into canonical or non-canonical according to the cellular pathways they activate (Fig. 1). Canonical Wnts bind Frizzled receptors which, in complex with low-density lipoprotein receptor-related protein (LRP) 5 or 6 and Dishevelled, release β-catenin from a destruction complex of Axin, adenomatous polyposis coli (APC), casein kinase 1α (CK1α) and glycogen synthase kinase 3β (GSK3β). This permits accumulation of β-catenin and its entry into the nucleus to initiate activation of target genes in combination with transcription factor/lymphoid enhancer binding factors (TF/LEF) (Clevers & Nusse, 2012). The pathway is subject to several checks and balances, particularly in the extracellular space (Niehrs, 2012). The transmembrane E3 ligase RNF43 and its close relative, ZNRF3, negatively regulate canonical Wnt signaling through ubiquitylation of Frizzled receptors, which targets them to lysosomes for destruction (Koo et al., 2012). Soluble Dickkopf (Dkk) proteins compete with Wnt binding to LRP5/6 (Kagey & He, 2017) to block canonical signaling. Wnt activation can be fine-tuned by extracellular R-spondins (RSPOs), which bind to leucine-repeat G-coupled receptors (LGR/5/6) on the cell surface, bringing RSPO into contact with RNF43/ZNRF3. This interaction leads to membrane clearance of RNF43/ZNRF3 with a concomitant increase in membrane Frizzled receptors, thus enhancing Wnt signalling.

![Fig. 1. Canonical and Non-canonical Wnt Signaling in Cancer](image)

Both canonical and non-canonical Wnt ligands require Porcupine activity for secretion. Canonical Wnts bind Frizzled receptors in complex with LRP5/6 to activate downstream signaling by phosphorylation and stabilization of β-catenin, which enters the nucleus to activate gene transcript via TF/LEFs. Non-canonical Wnts also act via Frizzled receptors, activating ROR1/2 or recruiting Daam1 to activate the planar cell polarity (PCP) pathways, which can activate gene transcription or promote actin cytoskeleton rearrangements. Both pathways are subject to extracellular regulation, with R-spondins (RSPOs) potentiating canonical signaling, and the ubiquitin-ligase RNF43 (or its close relative ZNRF3) and Dkk acting as negative regulators (depicted in red). sFRPs may act as positive or negative regulators of canonical signalling depending on context. sFRPs and RNF43 can also regulate non-canonical signalling, although Dkk has been shown to activate PCP pathways, in one of several examples of cross-talk between different Wnt pathways. LRP: low-density lipoprotein receptor-related protein, APC: adenomatous polyposis coli, GSK3β: glycogen synthase kinase 3β, CK1α: casein kinase 1α, Daam1: dishevelled associated activator of morphogenesis 1, Dvl: dishevelled; ROR1/2, receptor-tyrosine kinase-like orphan receptor 1/2, TF/LEF: transcription factor/lymphoid enhancer binding factors, sFRP: secreted frizzled-related protein, Dkk: Dikkopf.
(De Lau, Peng, Gros, & Clevers, 2021). Secreted frizzled-related proteins (SFRPs) can sequester Wnts and prevent them binding Frizzled receptors, although they have also been reported to act as agonists, depending on concentration and/or context (Routledge & Scholpp, 2019; Xavier et al., 2014).

Pathways not involving β-catenin are termed non-canonical. One of the most well understood is the planar cell polarity (PCP) pathway, in which non-canonical Wnts such as Wnt5a activate Frizzled and co-receptors such as tyrosine kinase-like orphan receptors (RORs) to activate gene expression via various transcription factors through Jun N-terminal kinase (JNK) activation, or promote rearrangements of the cytoskeleton via dishevelled associated activator of morphogenesis 1 (Daam1) which activates small GTPases such as Cdc42 and RhoA (Mates et al., 2018; Menck, Heinrichs, Baden, & Bleckmann, 2021). Non-canonical Wnts are also subject to direct negative regulation by SFRPs (Satoh, Matsuyama, Takemura, Aizawa, & Shimono, 2008) and RSPOs (Radaszkiewicz et al., 2021; Tsukiyama et al., 2015) but not Dkk1 which exclusively and negatively regulate canonical signaling via LRPs. Indeed, Dkk1 has been suggested to activate non-canonical signaling in a non-direct manner, possibly by releasing Frizzled receptors from LRPs complexes, thereby making them available for non-canonical Wnts (Niehrs, 2006). Dkk1 may also function via other receptors, such as cytoskeleton-associated protein 4, an interaction which may be of relevance in pancreatic and lung cancer (H. Kimura et al., 2016). Cross-talk between Wnt pathways is common (Topol et al., 2003), and it has been proposed that Wnt signaling should be viewed as a complex, integrated network, rather than a linear pathway (Amerongen & Nusse, 2009; Menck et al., 2021).

2. Dysregulation of Wnt Pathways in Gastrointestinal Cancers

Elevated β-catenin, indicative of canonical Wnt pathway activation, has been observed in many gastrointestinal tumors. Such activation may be driven by mutations in upstream pathway components such as RNF43 and RSPO fusions and tumors will remain dependent on Wnt ligand, or mutations in downstream Wnt pathway components such as APC or CTNNB1, the gene encoding β-catenin, where the pathway may be activated independent of Wnt ligand. Deregenerated extracellular Wnt regulators such as Dkk1, SFRPs, and RSPOs play a role in activating the planar cell polarity (PCP) pathway. 

RNF43 (Radaszkiewicz et al., 2021; Tsukiyama et al., 2015) but not in cancers has also been associated with poor prognosis (Chen, Chen, Hlubek et al., 2007). These findings have led to a search for therapeutics targeting Wnt pathways. One of several emerging drug candidates is the membrane-bound protein-serine O-palmitoyltransferase Porcupine, encoded by the PORCN gene. Porcupine is located in the endoplasmic reticulum of stromal or epithelial cells and palmitoylates Wnt ligands, an enzymatic process essential for their secretion and binding to Frizzled receptors (Liu et al., 2013). Since Porcupine targets both canonical and non-canonical Wnts (Madan et al., 2016), inhibitors the potential to hit multiple pro-tumorigenic mechanisms. However, they will only block signaling that remains dependent on secreted Wnts. In the next section, we summarise evidence for the involvement of Wnt pathway aberrations in five leading gastrointestinal cancers, with a focus on ligand-dependent mechanisms.

3. Wnt pathway dysregulation in colorectal cancer (CRC)

CRC is a Wnt-pathway driven disease, with 63-93% of patients having mutations in at least one component of the canonical Wnt pathway (Fig. 2) (Cancer Genome Atlas Network, 2012; Kleeman et al., 2020; Yaeger et al., 2018). The majority of mutations occur in downstream elements of the pathway. Most common are loss of function mutations in APC, which account for 60-80% of Wnt pathway aberrations in colorectal cancers (Kinzler & Vogelstein, 1996; Kleeman et al., 2020; Yaeger et al., 2018), and to a lesser extent, aberrations in other downstream components AXIN1, AXIN2, and CTNNB1. In APC-mutated cancer models, restoration of functional APC protein is effective in reversing tumorigenesis (Dow et al., 2015; Faux et al., 2004; Zilberberg, Lahav, & Rosin-Arbesfeld, 2010), but with current technologies, APC restoration is not a practical therapeutic option. In theory, cancers driven by such downstream activation mechanisms are unlikely to respond to Porcupine inhibition, as they do not depend on Wnt ligand, and are therefore termed ligand-independent. However, there are reports of APC-deficient and CTNNB1-mutant colorectal cell lines that remain dependent on Wnt ligands, and whose proliferation was reduced by Porcupine inhibition (J. Li, Zhang, Wang, & Zhang, 2019; Voloshanenko et al., 2013). Whether the dependency of APC- or CTNNB1-mutant CRC cell lines on Wnt extends beyond in vitro models requires further investigation.

10-20% of CRC patients have pathway aberrations in which a requirement for Wnt ligand is maintained. The majority of these are in RSPO3 or ZNFR3, with RSPO3 mutations found in 9-18% of CRC patients, most of which associate with microsatellite instability and are mutually exclusive from genetic aberrations in APC but strongly associated with BRAF mutations (Giannakis et al., 2014; Yaeger et al., 2018). Many RSPO3 mutations observed in CRC are truncations and hence loss of function, and therefore predicted to activate both canonical and non-canonical Wnt signaling. Certain truncation mutations have been reported to switch the protein from a tumor suppressor into an activator of downstream β-catenin signaling (Spit et al., 2020). Growth of tumorigenic intestinal organoids that lack functional RSPO3 or ZNFR3 remain dependent on local Wnt ligand production (Koo, Van Es, Van Den Born, & Clevers, 2015), and their growth can be blocked by Porcupine inhibitors (Van De Wetering et al., 2015). However, there is debate concerning the most common RSPO3 mutation, G659Vfs*41, a frameshift mutation which is almost exclusively found in microsatellite instability (MSI) patients. Initial reports suggested it was associated with cancer cell growth and/or survival, but it has also been reported to generate fully functional protein, although potentially one that is less stable than wild-type and associated with lower RSPO3 expression (Giannakis et al., 2014; Li et al., 2020; Tu et al., 2019; Yu et al., 2020). Oncogenic effects of this mutation have been reported to be independent of Wnt signaling (L. Fang et al., 2021). It is therefore unclear whether tumors with this particular frameshift retain Wnt ligand dependency in patients or in fact have enhanced Wnt pathway signalling as a result of the mutation.

RSPO2 and RSPO3 fusions are mutually exclusive and are present in 2-10% of CRC, co-occurring with KRAS or BRAF mutations, but only very rarely with APC and mutually exclusive of CTNNB1 and RSPO3 mutations (Giannakis et al., 2014; Seshagiri et al., 2012; Shimamura et al., 2014). The gene encoding RSPO3 fuses with the signal sequence and early section of the PTPRK gene leading to increased expression of RSPO3 and activation of the canonical Wnt pathway. Genetic fusions of RSPO2 have been observed with EIF4E and PIEZO1, and these were associated with increased RSPO2 expression (Hashimoto et al., 2019). Increased stromal RSPO3 expression has also been observed in the absence of gene fusions and this stromal overexpression of RSPO3 is strongly associated with the aggressive mesenchymal consensus molecular subtype 4 (CMS4) CRC phenotype (Kleeman et al., 2020). Targeting RSPO3 overexpression to the murine gut epithelium upregulates Wnt signaling and drives the formation of ectopic niche compartments and intestinal stem cells, leading to adenoma lesions (Hikens et al., 2017). Inhibition of RSPO3 with function-blocking antibodies in PTPRK-RSPO3-fusion tumor xenografts inhibited growth and promoted epithelial differentiation (Storm et al., 2016).
Excessive Wnt signaling can also arise by non-mutational mechanisms. For example, epigenetic silencing of negative Wnt regulators has been observed during CRC cancer progression and was associated with increased nuclear β-catenin (Caldwell et al., 2004; Silva et al., 2014; Suzuki et al., 2004; Zhang et al., 2008). Epigenetic suppression of Wnt feedback loops, including reduced Axin2 expression, has been observed in CRC and low Axin2 expression has been postulated to be a discriminatory biomarker for ligand-dependent disease (Kleeman et al., 2020). However, the expression of some negative Wnt regulators (Notum, Wif1, Dkk3) is very low/absent in normal murine gut tissue (Flanagan et al., 2021) which may suggest epigenetic silencing takes place prior to tumour formation, possibly confounding the premise of further epigenetic silencing in cancer tissue.

Non-canonical pathways are also dysregulated in colorectal cancer, with high expression of Wnt11a or ROR1 associated with poor prognosis (Nishioka et al., 2013; Zhou et al., 2017). Non-canonical Wnt signaling has been linked to proliferation, invasion, survival and metastasis of cancer stems cells (Katoh, 2017; Nishioka et al., 2013), although it should be noted that the role of non-canonical Wnts is context dependent. In certain situations, non-canonical Wnt5a has been shown to be tumor suppressive and promote differentiation, most likely via cross-inhibition of canonical Wnt signaling (Zhou, Kipps, & Zhang, 2017).

4. Wnt pathway dysregulation in pancreatic cancer

RNF43 mutations are the most common Wnt pathway mutations in pancreatic cancer, observed in 5-10% of cases (Fig. 2) (Bailey et al., 2016; Tu et al., 2019; Witkiewicz et al., 2015). Genetic lesions in APC or AXIN1/2 are less common, observed in 1-5% of cases. As expected, RNF43-mutant pancreatic cancer xenografts remained dependent on Wnt ligand. However, a significant proportion of organoids derived from PDAC patients with wild-type RNF43 also remained dependent on Wnt ligands, suggesting opportunities for Porcupine inhibitors and other ligand-dependent therapeutics exist beyond RNF43 mutations in pancreatic cancers (Seino et al., 2018).

At the expression level, upregulation of β-catenin, Wnts and Frizzled receptors was seen in a gene-array analysis of pancreatic ducal adenocarcinomas (Pasca Di Magliano et al., 2007) and elevated nuclear β-catenin is associated with high grade neoplasms and poor prognosis (Al-Aynati, Radulovich, Riddell, & Tsao, 2004; Sano et al., 2016). High epithelial expression of Wnts in pancreatic tumors was also associated with poor prognosis (Seino et al., 2018). Ectopic expression of Wnt1 or β-catenin accelerated tumor growth and decreased survival in a murine pancreatic cancer model (Sano et al., 2016).

Wnt5a is elevated in pancreatic cancer tissue versus surrounding non-cancerous tissues and promotes invasion and proliferation whilst inhibiting apoptosis. Although typically considered a non-canonical Wnt, these effects appear to be via β-catenin signaling (Bo, Gao, Chen, Zhang, & Zhu, 2016; Griesmann et al., 2013; Ripka et al., 2007). The non-canonical pathway receptor ROR1 is overexpressed in circulating tumor cells from pancreatic cancer patients and knockdown of ROR1 reduces invasiveness (Xu, Shen, Xu, Wang, & Ni, 2018). Consistent with non-canonical signaling playing a role in pancreatic cancer, ROR2 expression in tumor epithelium and stroma is associated with poor prognosis in this indication (J. Huang et al., 2015).

5. Wnt pathway dysregulation in gastric cancer

Gastric cancers have one of the highest incidence of Wnt pathway mutations among cancers of the digestive tract (Flanagan, Vincan, & Phesse, 2017). CTNNB1 mutations occur in up to 26% of gastric cancers and are associated with nuclear localization of β-catenin (Clements et al., 2002). Several CTNNB1 single-nucleotide polymorphisms are associated with gastric cancer susceptibility and prognosis (Wang et al., 2018).
Porcupine inhibitors have proven effective in inhibiting proliferation of cell lines and organoids derived from gastric, colorectal, pancreatic and biliary cancers. They also show potent inhibition of tumor xenograft growth in mice (Boulter et al., 2015; Li, Cao, et al., 2018; Madan, Ke, et al., 2016; Mo et al., 2013). Effects are limited to models derived from Wnt-dependent tumors, in particular those with RSP02/3 fusions or RNF43 mutations, consistent with the role of Porcupine in Wnt secretion (Colnot et al., 2004; de La Coste et al., 1998). Another study showed that partial ablation of APC also induced liver tumorigenesis in mice (Buchert, Athineos, Abud, Burke, & Faux, 2010).

In contrast, ZNRF3 mutations were observed in only 3% of HCC (Schulze et al., 2015) and RSP0 fusions are rare, although elevated RSP02 was seen in a subset of HCC patients. Co-amplification of RSP02 and MYC has been observed in HCC, although the functional consequence of this is unclear (Sanchez-Vega et al., 2018). Elevated Wnt3/4/5a and suppression of SFRPs have also been observed, an expression pattern that did not correlate with CTNNB1 mutations but was associated with loss of differentiation and cirrhosis, and activation of PCP pathways (Bengochea et al., 2008). Functional overexpression of RSP02 with combined loss of transformation-related protein 53 (TP53) led to development of liver cancer in mice models (Conboy et al., 2019), implicating a role for extracellular Wnt ligands in liver tumorigenesis.

8. Pre-clinical evidence supporting use of porcupine inhibitors in gastrointestinal cancers

The pivotal role of Wnt signaling in gastrointestinal cancers has led to a search for therapeutics that target this pathway. A major challenge has been finding tractable targets, given the non-enzymatic nature of key intracellular components such as APC, AXIN and β-catenin. The majority of pathway inhibitors explored to date are antibody or biologicals-based approaches such as Frizzled decoy receptors and Frizzled blocking antibodies. Clinical studies of these therapeutics have been limited, hampered by toxicity issues (Table 1). A handful of small molecules have also been developed, such as CWP232291, which degrades β-catenin via an unknown mechanism (Pak et al., 2019), and PRI-724, which has been reported to block β-catenin/CBF interactions (Table 1). However both compounds require intravenous dosing, and reported effects in the clinic to date are minimal, with no direct evidence of target engagement (El-Khouery et al., 2013; Kimura et al., 2017; Ko et al., 2016; Lee et al., 2020). Tankyrase inhibitors, which can antagonise Wnt signaling via Axin stabilisation, have failed to advance into the clinic due to lack of a therapeutically window versus gastrointestinal toxicity, as well as non-Wnt related effects (S.-M. A. Huang et al., 2009; Y. Zhong et al., 2016). Antibodies directed against Dkk-1, such as DKK01 and BHQ880 are also being tested. Although Dkk-1 functions as a naturally occurring negative regulator of Wnt signaling, Dkk-1 expression is often associated with poor prognosis in gastrointestinal cancers, and Dkk-1 blockading antibodies can inhibit tumor growth in pre-clinical animal models (Kagey & He, 2017). Such effects may arise from the complexity of Wnt signaling; for example, Dkk-1 has been linked to promotion of metastasis via activation of the non-canonical PCP-pathway, and also to immune suppression. It is speculated that the role of Dkk1 may be altered in different contexts, including when the canonical Wnt pathway is constitutively activated (Kagey & He, 2017).

More recently, several small molecule drug candidates with attractive pharmacokinetic properties have been developed that target Porcupine (Table 2; Bhamra et al., 2017; B. Chen et al., 2009; J. Jiang et al., 2018; Liu et al., 2013; Madan, Ke, et al., 2016; Profitt et al., 2013). Pre-clinical evidence suggests that Porcupine inhibitors exert antitumor effects via multiple mechanisms, and data from gastrointestinal models is summarised below.

9. Effects of porcupine inhibitors on tumor growth and proliferation

Porcupine inhibitors have proven effective in inhibiting proliferation of cell lines and organoids derived from gastric, colorectal, pancreatic and biliary cancers. They also show potent inhibition of tumor xenograft growth in mice (Boulter et al., 2015; Li, Cao, et al., 2018; Madan, Ke, et al., 2016; Mo et al., 2013). Effects are limited to models derived from Wnt-dependent tumors, in particular those with RSP02/3 fusions or RNF43 mutations, consistent with the role of Porcupine in Wnt secretion (X. Jiang et al., 2013; Koo et al., 2015; Woodcock et al., 2019). Inhibition of tumor proliferation was
accompanied by downregulation of Wnt targets such as Axin2 and c-Myc, and loss of cell cycle and stem cell markers (Madan et al., 2016). In addition, Porcupine inhibitors reduce glucose uptake by cancer cells in vitro and in vivo (Phillips et al., 2021 and Phillips et al, manuscript submitted for publication) and several studies demonstrate elevated caspase 3/7 expression and induction of apoptosis (Bagheri, Tabatabae Far, Mirzaei, & Ghasemi, 2020; Boulter et al., 2015; Mo et al., 2013), all of which may contribute to inhibition of tumor growth. The multi-modal effects of Porcupine inhibitors may explain why established RSPO3-fusion tumours are rapidly cleared following treatment with the porcupine inhibitor LGK974, even though treatment had no effect on the normal growth of intestinal crypts (Han et al., 2017). Effects of Porcupine inhibitors are even though treatment had no effect on the normal growth of intestinal crypts (Han et al., 2017). Effects of Porcupine inhibitors are even though treatment had no effect on the normal growth of intestinal crypts (Han et al., 2017). Effects of Porcupine inhibitors are even though treatment had no effect on the normal growth of intestinal crypts (Han et al., 2017). Effects of Porcupine inhibitors are even though treatment had no effect on the normal growth of intestinal crypts (Han et al., 2017). Effects of Porcupine inhibitors are even though treatment had no effect on the normal growth of intestinal crypts (Han et al., 2017). Effects of Porcupine inhibitors are even though treatment had no effect on the normal growth of intestinal crypts (Han et al., 2017). Effects of Porcupine inhibitors are even though treatment had no effect on the normal growth of intestinal crypts (Han et al., 2017). Effects of Porcupine inhibitors are even though treatment had no effect on the normal growth of intestinal crypts (Han et al., 2017). Effects of Porcupine inhibitors are even though treatment had no effect on the normal growth of intestinal crypts (Han et al., 2017). Effects of Porcupine inhibitors are even though treatment had no effect on the normal growth of intestinal crypts (Han et al., 2017). Effects of Porcupine inhibitors are even though treatment had no effect on the normal growth of intestinal crypts (Han et al., 2017).

Table 1
Wnt Pathway Inhibitors in Cancer Clinical Trials

<table>
<thead>
<tr>
<th>Drug Identifier</th>
<th>Mode of Action</th>
<th>Trial Identifier</th>
<th>Regimen</th>
<th>Cancer Indication</th>
<th>Cancer Indication</th>
<th>Trial Phase</th>
<th>Study Status or Clinical Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMP-54F28 (Ipafriccept)</td>
<td>Fzd8-Fc Decoy receptor</td>
<td>NCT01608867</td>
<td>Monotherapy</td>
<td>Solid tumors</td>
<td>Ovarian cancer; prostate cancer; colorectal cancer</td>
<td>1</td>
<td>Dyseygia and fragility fractures observed. Best response was SD (Jimeno et al., 2017)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT02092363</td>
<td>In combination with paclitaxel and carboplatin</td>
<td>Ovarian cancer; colorectal cancer</td>
<td>1</td>
<td>ORR 76%, comparable with historical data. Development in ovarian stopped due to bone toxicity (Moore et al., 2019)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT02069145</td>
<td>In combination with sunitinib (HCC)</td>
<td>Hepatocellular carcinoma</td>
<td>1</td>
<td>Study completed, no results reported</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT02050178</td>
<td>In combination with Nab-paclitaxel and Gemcitabine</td>
<td>1L pancreatic ductal adenocarcinoma</td>
<td>1</td>
<td>35% PR, 46% SD. Study terminated due to bone toxicity (Dotan et al., 2020)</td>
<td></td>
</tr>
<tr>
<td>OMP1885 (Vantictumab)</td>
<td>Anti-Fzd7 antibody, also neutralizes binding to Wnts to Fzd 1, 2, 5,8</td>
<td>NCT01345201</td>
<td>Monotherapy</td>
<td>Solid tumors</td>
<td>Previously treated Non-small cell lung cancer</td>
<td>1</td>
<td>Study completed, no results reported</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT01957007</td>
<td>In combination with docetaxel</td>
<td>1L pancreatic ductal adenocarcinoma</td>
<td>1</td>
<td>ORR 42%. Study terminated due to bone toxicity (Davis et al., 2020)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT02005315</td>
<td>In combination with Nab-Paclitaxel and Gemcitabine</td>
<td>Locally recurrent/ metastatic breast cancer</td>
<td>1</td>
<td>ORR 31%. Wnt pathway gene signature associated with longer PFS/OS. Bone fractures observed (Diamond et al., 2020)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT01973309</td>
<td>In combination with paclitaxel</td>
<td>Synovial sarcoma</td>
<td>1</td>
<td>Most common adverse events were hematologic disorders. SD in 3/8 patients (Giraudet et al., 2018)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT019476016</td>
<td>monotherapy</td>
<td>Synovial sarcoma</td>
<td>1</td>
<td>Recruiting</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT01469975</td>
<td>monotherapy</td>
<td>Synovial sarcoma</td>
<td>1</td>
<td>Recruiting</td>
<td></td>
</tr>
<tr>
<td>OTSA101</td>
<td>Yttrium90 radiolabelled Anti-Fzd10 antibody</td>
<td>NCT04176016</td>
<td>monotherapy</td>
<td>Synovial sarcoma</td>
<td>RSP03 biomarker-positive metastatic colorectal cancer</td>
<td>1</td>
<td>Best response was SD in 3/7 patients treated with chemo combination (Bendell et al., 2016)</td>
</tr>
<tr>
<td>OMP131R10</td>
<td>Anti-R-spondin3 antibody</td>
<td>NCT02482441</td>
<td>monotherapy</td>
<td>Wnt or without FOLFIRI</td>
<td>1</td>
<td>Recruiting</td>
<td></td>
</tr>
<tr>
<td>Foxy-5</td>
<td>Wnt5a mimetic</td>
<td>NCT02655952</td>
<td>monotherapy</td>
<td>Breast cancer; colorectal cancer; prostate cancer</td>
<td>1</td>
<td>Completed. No efficacy data reported</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT02002091</td>
<td>monotherapy</td>
<td>Breast cancer; colorectal cancer; prostate cancer</td>
<td>1</td>
<td>Completed. No efficacy data reported</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT03883802</td>
<td>As Neoadjuvant with surgery+FOLFOX</td>
<td>Advanced solid cancers</td>
<td>1</td>
<td>No responses reported (El-Khoueiry et al., 2013)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT01302405</td>
<td>Monotherapy</td>
<td>Advanced solid cancers</td>
<td>1</td>
<td>No responses reported (El-Khoueiry et al., 2013)</td>
<td></td>
</tr>
<tr>
<td>PRI-724</td>
<td>Inhibitor of TCF-CBP interaction</td>
<td>NCT01606579</td>
<td>monotherapy or in combination with dasatinib, or cytarabine</td>
<td>Acute and chronic myelogenous leukaemia</td>
<td>1/2</td>
<td>Completed. No results reported</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT01764477</td>
<td>In combination with Gemcitabine</td>
<td>Advanced/metastatic pancreatic adenocarcinoma</td>
<td>1</td>
<td>Rest response was SD in 8/20 patients (Ko et al., 2016)</td>
<td></td>
</tr>
<tr>
<td>CWP232291</td>
<td>Induces degradation of beta catenin. Molecular target not defined</td>
<td>NCT01398462</td>
<td>Monotherapy</td>
<td>Acute Myeloid Leukaemia</td>
<td>1</td>
<td>ORR 3% CI side-effects were most common (Lee et al., 2020)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT03055286</td>
<td>In combination with docetaxel</td>
<td>Acute Myeloid Leukaemia</td>
<td>1/2</td>
<td>Active, not recruiting</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT02426723</td>
<td>Monotherapy or in combination With Lenalidomide and Dexamethasone</td>
<td>Relapsed/refractory multiple myeloma</td>
<td>1</td>
<td>Completed. 4/14 patients with response, 4 had stable disease (Manasanch et al., 2017)</td>
<td></td>
</tr>
<tr>
<td>SM04755</td>
<td>Small molecule Wnt inhibitor</td>
<td>NCT02191761</td>
<td>Monotherapy</td>
<td>Advanced CRC, gastric, HCC and pancreatic cancer</td>
<td>1</td>
<td>Completed. No results reported</td>
<td></td>
</tr>
<tr>
<td>Cimtuzumab</td>
<td>Anti-ROR1 antibody</td>
<td>NCT05155905</td>
<td>In combination with docetaxel</td>
<td>Metastatic castration resistant prostate cancer</td>
<td>2</td>
<td>Not yet recruiting</td>
<td></td>
</tr>
</tbody>
</table>

Source: Clinicaltrials.gov. Data collected on 10th January 2022. Withdrawn trial or trials terminated early are not included. Dkk blocking antibodies such as DKN01 and BHQ880 were not included due to observed effects on other (non Wnt) pathways.
Most studies of Porcupine inhibitors have focused on tumor models with ligand-dependent Wnt pathway activation, under the assumption that downstream pathway activation such as that seen with mutations in APC and β-catenin would be unaffected by extracellular Wnt levels. Indeed, it has been reported that the Porcupine inhibitor LGK974 accelerates tumorigenesis in APC-deficient mice models by selectively blocking wild-type stem-cell proliferation, and thus favoring growth of APC-mutant cells (Huels et al., 2018). However, it has also been reported that Porcupine inhibitors could inhibit growth of a colon cancer cell line with mutations in CTNNB1 (Voloshanenko et al., 2013). Other colon cancer cell lines, including some with APC mutations also appeared dependent on Wnt/Fzd signaling for growth (Vincan et al., 2007). Small molecule Porcupine inhibition in these cell lines resulted in decreased pathway activation, anti-proliferative effects and reduced cell viability. Taken together these data suggest patients with cancers bearing a subset of downstream mutations in the Wnt pathway, traditionally expected to be insensitive to Porcupine inhibitors, may well in fact receive therapeutic benefit from such treatment. However, further research is needed to determine which APC/CTNNB1 mutation subtypes retain Wnt dependence, and the mechanism(s) involved.

10. Effect of porcupine inhibitors on cancer stem cells

Tumor recurrence following chemotherapy occurs through clonal replacement and reactivation of dormant cancer stem cells (CSCs) (Jahanban-Esfahan et al., 2019). Canonical Wnt signaling prevents differentiation of CSC and promotes their retention to fuel tumor expansion by direct effects on CSCs, but also via CSC-stromal/immune interactions (Regan et al., 2017; Vermeulen et al., 2010). These findings go some way to reconcile the paradoxical observation that despite all cells within a tumor harbour Wnt-activating mutation, only a subfraction of cancer cells display CSC properties. Non-canonical Wnt signaling also promotes survival and resistance to therapy to CSCs through PI3K-AKT signaling activation (reviewed in Katoh, 2017). The Porcupine inhibitor LGK974 inhibited the ability of breast cancer stem cells to proliferate and form mammospheres (Zhao et al., 2014), and reduced the cancer stem cell compartment of established cutaneous squamous cell carcinomas in mice (Zimmerli et al., 2018). Mutation of RNF43 or ZNRF3 in mice intestines led to adenoma growth from LG55-expressing stem cells, which was dependent on Wnt3 secreted by Paneth cells, and could be blocked by treatment with a Porcupine inhibitor (Koo et al., 2015). Acute treatment of mice with a high dose of a Porcupine inhibitor (i.e. higher doses than those which have shown anti-tumor effects) induced an initial burst of proliferation of intestinal stem cells as they converted into transit-amplifying cells, with a loss of stem cell self-renewal, supporting the critical function of Wnt signaling to maintain stemness and prevent differentiation in the gut (Kabiri et al., 2018). Consistent with this, Porcupine inhibitors have shown a striking ability to promote epithelial differentiation within the tumor, most notably by upregulation of mucins (X. Jiang et al., 2013; Madan, Ke, et al., 2016).

11. Effect of porcupine inhibitors on invasion and metastasis

Epithelial-mesenchymal transition (EMT) has been linked to invasion and metastasis in intestinal cancers. Reactivation of key developmental pathways, including the Wnt pathway, leads to loss of epithelial architecture and disruption of the basement membrane, which enables cancer cells to invade healthy tissues and migrate into the lymphatic system or bloodstream, leading to metastasis. Canonical and non-canonical PCP Wnt pathways have been implicated in CRC cell migration (Al-Aynati et al., 2004; Katoh, 2017; Y. Zhang et al., 2016; Z. Zhang, Wang, Zhang, & Yang, 2018). The Porcupine inhibitor IWP-2 was shown to reduce migration and invasion of a gastric cancer cell line (Mo et al., 2013), and other Porcupine inhibitors reduced invasion of renal cancer cells in vivo (Li et al., 2020) and prolonged metastasis-free survival in an in vivo model of Ewing sarcoma (Hayashi et al., 2017). Effects of Porcupine inhibitors on migration in vivo may be due to direct effects on cancer cells and/or cell-nonautonomous effects related to interactions with neighboring stromal tissues.

12. Effects of porcupine inhibitors on fibrosis

Another corollary of EMT is the induction of fibrosis. Wnt signaling has long been implicated in fibrosis (Akhmetshina et al., 2012) and stromal signaling plays a key role in cancers, with growing evidence that cancer-associated fibroblasts (CAF) play a role in immunosuppression as well as remodelling (Hilmi, Nicole, Bousquet, & Neuzillet, 2020). Wnt5a functions as a supportive niche factor in gastric cancer, where it is upregulated by the tumor microenvironment (Hayakawa et al., 2013; Maeda et al., 2020). Wnt5a is also associated with the strong desmoplastic reaction observed in pancreatic cancer (Pilarsky et al., 2008). In colorectal cancers, upregulation of endogenous RSP02/3 was
strongly associated with a defined CAF signature and with the CMS4 (mesenchymal) consensus molecular subtype (Kleeman et al., 2020). Although data on fibrosis is lacking from cancer models, Porcupine inhibitors have demonstrated profound anti-fibrotic effects in models of skin, lung and kidney fibrosis (Bunyard et al., 2019; C.-W. Chen et al., 2017; Madan, Patel, et al., 2016).

13. Reversal of immune evasion by porcupine inhibition

Immune evasion plays a key role in tumor development. The presence of Wnt ligands in the microenvironment in gastrointestinal tumors positions them perfectly to impact the local tumor immune response. There is strong association between upregulation of Wnt signaling and downregulation of immune markers in cancers, including those of the gastrointestinal tract (Luke et al., 2019; Spranger, Bao, & Gajewski, 2015). Tumors may be classed as "hot" (i.e. inflamed), or "cold", the latter state characterized by a tumor microenvironment lacking infiltrating lymphocytes and with high levels of suppressive regulatory T-cells (Ochoa de Olza, Navarro Rodrigo, Zimmermann, & Coukos, 2020). Cold tumors do not respond to immunotherapy agents. Of note, several canonical and non-canonical Wnt ligands and Frizzled receptors were upregulated in melanoma patients that failed to respond to anti-PD-1 therapy. The Porcupine inhibitor C59 reversed the ability of melanoma cancer cell-conditioned media to induce generation of dendritic cell-mediated regulatory T-cells in vitro (DeVito et al., 2021). In the immune cold B16F10 (C57BL/6 mice) melanoma tumor model, anti-PD-1 therapy was not effective, but treatment with the Porcupine inhibitor RXC004 was able to reduce tumor size both as a monotherapy and in combination with an anti-PD-1 inhibitor. Flow cytometry showed that the RXC004 treatment reduced the myeloid derived suppressor cells in the tumor microenvironment (Phillips et al., 2019, Phillips et al, manuscript submitted for publication). A combination of the Porcupine inhibitor C59 with anti-CTLA4 antibodies was also shown to have synergistic anti-tumor effects in the same model (Holzthausen et al., 2015). These data suggest that Porcupine inhibitors may be effective in combination with immunotherapy in patients that currently do not respond to such therapies.

Further evidence that Porcupine inhibitors can reverse Wnt pathway-induced immune evasion comes from the immune hot CT26/BALBc colorectal syngeneic model, where RXC004 in combination with anti-PD-1 antibody resulted in a significant increase in the cytotoxic to regulatory T-cell ratio within the tumor. This was not seen in either monotherapy treatment arm (Phillips et al., 2019, Phillips et al, manuscript submitted for publication). The ability to reverse Wnt ligand induced immune evasion in the wider tumor microenvironment may provide an explanation for the observed anti-tumor effects of Porcupine inhibitors even in cases where Wnt pathway is activated by downstream mutations within the tumor cell.

14. Potential toxicities of porcupine inhibitors identified in vivo

Wnt ligands play a vital homeostatic role in stem cell renewal, which means targeting the pathway has the potential for adverse effects, particularly within the intestinal epithelium. Ectopic expression of the negative Wnt regulator Dkk1 in mice caused loss of intestinal architecture (Kuhnert et al., 2004; Pinto, Gregorieff, Begthel, & Clevers, 2003), and high doses of Porcupine inhibitors have adverse effects on stem cell renewal with associated disruption of villi architecture (Kabiri et al., 2018). However, anti-tumor effects of Porcupine inhibitors have been observed at doses that have no effect on the normal intestine (Koo et al., 2015; J. Liu et al., 2013; Phillips et al, manuscript submitted for publication), suggesting there may be a safe therapeutic window for these compounds. The effects of Dkk1 overexpression on intestinal architecture were reversible, consistent with other studies that indicate the intestine has the ability to regenerate after insult (Kuhnert et al., 2004). A possible explanation for this may be redundancy in the stem cell populations, with HOPX-expressing stem cells, typically located higher in the intestinal crypt, able to compensate for loss of LG55 expressing stem cells at the base of crypts, and vice-versa (Takeda et al., 2011), although such plasticity may be a relatively short-lived response to injury. Stem cells located at the intestinal crypt base are exposed to the highest levels of Wnt ligand provided by the niche (Paneth cells and stromal cells), and therefore may be more protected from drug-induced reduction of Wnt secretion (Kosinski et al., 2007). Consistent with this, mice with genetic ablation of PORCN in intestinal epithelial cells retained normal intestinal homeostasis due to compensatory Wnt secretion from neighboring stromal cells (Kabiri et al., 2014). Furthermore, pericytostromal cells have high expression of drug transporters, protecting the stem cell niche from xenobiotics, including Porcupine inhibitors (Chee et al., 2018).

Other in vivo toxicities observed with Porcupine inhibitors include loss of bone volume and density, most likely due to effects on osteoclasts. However, these effects could be mitigated by prophylactic treatment with bisphosphonates (Madan et al., 2018).

15. Porcupine inhibitors in the clinic

To date, five Porcupine inhibitors have entered phase I/II clinical trials (Table 2) in patients with advanced solid tumors. In NCT02521844, a maximum tolerated dose of 30 mg for ETC-159 (previously known as ETC-1922159) was established, limited by high bone marker turnover and compression fractures. Bone effects at lower concentrations were mitigated by prophylactic treatment with denosumab. Dysgeusia (loss or change in taste) was common, even at lower doses. Target engagement was demonstrated by inhibition of Axin2 expression in hair follicles and increased infiltration of T-cells into the tumor microenvironment. There were no objective responses, although 17% (2/12) patients had stable disease (Tan et al., 2018).

The WNT974 (previously LGK974) monotherapy dose-finding study did not find a maximum tolerated dose. Dysgeusia was the most common side effect, although bone effects were also observed in a subset of patients. Target engagement was demonstrated by reduction of Axin2 expression in skin. There were no objective responses; 16% of 98 patients had stable disease (Rodon et al., 2021). In this study, 28 patients were genetically selected for Wnt pathway activation as part of a dose expansion arm, the majority of which had RNF43 mutations. Of these 28 patients, 10 patients had stable disease. This result has led to others concluding that monotherapy Porcupine inhibitor treatment failed even with this enrichment for upstream Wnt pathway aberrations. However, since this dose expansion arm was run in 2015, knowledge of exactly which RNF43 mutations result in loss of function has increased (Spit et al., 2020; Tu et al., 2019; Yu et al., 2020). With our current improved understanding, and excluding patients with co-occurring downstream Wnt pathway mutations, only 8 of these patients would now be considered to have RNF43 loss of function mutations. Of these 8 patients that had a best tumor response between -27 and +20%, suggesting a stringent patient selection strategy is likely needed in the clinic for Porcupine inhibitors (Rodon et al., 2021).

When WNT974 was paired with the anti-PD-1 antibody spartalizumab, dose-limiting toxicities were reported in 2 patients, including one spinal compression fracture. One patient (4%) with triple-negative breast cancer had a partial response, 11 patients (41%) had stable disease, 13 patients (48%) had progressive disease. 75% of patients experienced a treatment-related adverse events, with dysgeusia and osteopenia among the most common (Janku et al., 2020).

Preliminary data from NCT03447470, a dose finding study for RXC004 in solid tumors, has recently been reported. The most common treatment related adverse events were fatigue, nausea, dysgeusia, vomiting and anorexia. No grade 4/5 adverse events or bone fragility events were reported, with patients receiving prophylactic denosumab. Five patients, all with Wnt pathway activated tumors, had stable disease, including one patient with biliary tract cancer, and two CRC.
patients with either an \textit{RNF43} mutation or a \textit{RSPO} fusion (Cook et al., 2021).

These early clinical studies have identified bone loss and dysgeusia as class effects of Porcupine inhibitors. The latter likely arises because Wnt signaling has been shown to be vital for proliferation of taste-progenitors and formation of taste buds (Gaillard et al., 2017; Prochazkova et al., 2017). Prophylactic treatment with bisphosphonates or denosumab has been shown to mitigate increases in bone turnover markers (Cook et al., 2021; Tan et al., 2018). Encouragingly, gastrointestinal side effects appear to be limited and manageable. Furthermore, the limited efficacy observed to date may be attributable to the studies being performed in heavily-pretreated patients with poor prognosis and lack of appropriate patient selection.

16. Conclusions and future perspectives

Porcupine inhibitors have the potential for multiple potentially beneficial effects on tumor biology (Fig. 3). There is substantial preclinical data supporting the use of Porcupine inhibitors in genetically-defined populations of patients with gastrointestinal cancers. Functionally relevant mutations are most common in CRC and pancreatic cancers. Patients with \textit{RNF43} or \textit{ZNRF3} truncations may be amenable to treatment and mutations in \textit{RNF43} are particularly common in MSI high CRC and pancreatic cancers, although it should be noted that the majority of \textit{RNF43} mutations found in MSI disease are the hotspot G659Vfs*41 frameshift, which has been reported to encode functional protein and may be simply a passenger mutation in this context (Tu et al., 2019). Tumors with \textit{RSPO2/3} fusion and \textit{RSPO3} stromal overexpression are also likely to be susceptible to Porcupine inhibition, an intriguing prospect for CMS4 CRC subtype patients, where \textit{RSPO3} overexpression is common. There is also data to suggest Porcupine inhibitors may be effective in patients where Wnt signaling is activated by non-genetic/epigenetic mechanisms, and in certain patients with downstream Wnt pathway aberrations. However further work is needed to understand the processes involved, and to develop biomarkers to robustly identify such patients.

The effects of Porcupine inhibitors in reversing immune evasion provide a strong rationale for their use in combination with checkpoint inhibitors, particularly in patients previously unresponsive to anti-PD-1/ PD-L1 therapy. MSI high CRC patients are currently indicated for anti-PD-1 therapy and exploration of anti-PD-(L)1/Porcupine inhibitor combinations in these patients may also be warranted. Similarly, Porcupine and BRAF inhibitors may be an attractive combination in CRC patients with both \textit{BRAF} and \textit{RNF43} mutations, particularly in light of the recent data from the BEACON study which demonstrated benefits of combining the BRAF inhibitor encorafenib with cetuximab in this setting (Tabernero et al., 2021). Additional combination opportunities are being explored pre-clinically, with synergy observed between ETC-159 and PI3K inhibitors or ETC-159 and PARP inhibition in Wnt-driven cancer models (Kaur et al., 2021; Zhong et al., 2019).

Given the role of Wnt pathway in CSC maintenance, combination of Porcupine inhibitors with chemotherapy in selected patients may provide an interesting avenue of research, based on the rationale that Porcupine inhibitors may overcome chemoresistance.

Initial clinical data is promising in that, despite the homeostatic role of Wnt in the digestive tract, gastrointestinal side-effects at doses that engage the target are limited, indicating the presence of a therapeutic window. Effects on bone turnover can be mitigated with Receptor activator of nuclear factor-κB ligand (RANKL) inhibitors or bisphosphonate treatment, although the additional impact on taste may need to be managed in order to minimize associated weight loss. Since future strategies may include combining Porcupine inhibitors with other therapeutics, the potential for combined toxicities will also need to be managed. However initial data from NCT01351103, where WNT974 was used in combination with anti-PD-1 therapy showed the combination to be well-tolerated (Janku et al., 2020).

\textbf{Porcupine inhibitors block proliferation in genetically defined tumors, promote differentiation and stimulate immune responses}

![Porcupine Inhibitors Have Multiple Effects on Tumor Biology](https://www.biorender.com)

Conclusive clinical efficacy data on Porcpine inhibitors is currently lacking, with studies to date limited to small numbers of late stage, previously-treated patients with relevant somatic mutations. Key questions remain concerning optimal patient selection strategies for Porcpine inhibitors. Different approaches are being explored, including prospectively targeting patients with RSPO2/3 fusions and RN43/ZNRF3 mutations with monotherapy, but also broader populations. WNT974 is under investigation in combination with anti-PD-1 in patients refractory to prior anti-PD-1 therapy, as well as other targeted populations. It is also interesting to note differences in dosing strategies between various Porcpine inhibitors; In NCT01351103, WNT974 was given as a priming dose when used in combination with anti-PD-1. RXC004 is dosed one daily, and ETC-159 demonstrated interpatient variability in pharmacokinetics and was dosed every other day. The impact of distinct dosing regimens and patient selection strategies is yet to be determined. Large scale, randomized, controlled clinical trials with robust biomarker assessments will be needed to elucidate whether Porcpine inhibitors will prove useful in gastrointestinal cancers, and to identify which patients are most likely to benefit from such treatments.

Declaration of Competing Interest

S.W. CE and CP are current employees of Redx Pharma PLC and hold shares or share options in Redx Pharma PLC. D.J.F. and O.J.S have no conflict of interest to declare.

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