Mining the Wnt pathway for cancer therapeutics

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Abstract | Aberrant activation of the Wnt pathway is implicated in driving the formation of various human cancers, particularly those of the digestive tract. Inhibition of aberrant Wnt pathway activity in cancer cell lines efficiently blocks their growth, highlighting the great potential of therapeutics designed to achieve this in cancer patients. Here we provide an overview of the promise and pitfalls of current drug development strategies striving to inhibit the Wnt pathway and present new opportunities for therapeutic intervention.

The Wnt pathway is instrumental in orchestrating proper tissue development in embryos and tissue maintenance in adults. This is achieved by directing a specific set of genes that strictly control temporal and spatial regulation of cell growth, movement and cell survival. Chronic activation of these genes resulting from aberrant activation of the Wnt pathway promotes uncontrolled cell growth and survival, and can consequently drive cancer formation in a range of tissues including colon, skin, liver and ovary. The fact that cultured colon cancer cells stop growing and are forced to differentiate when this aberrant Wnt signalling activity is blocked[1,2], despite the presence of multiple additional mutations in key tumour-suppressor genes and oncogenes in these cells, has fuelled efforts to develop therapeutics capable of recapitulating this in cancer patients. The therapeutic benefits and commercial rewards for developing these drugs are likely to be considerable given the increasing number of human cancers and diseases found to be dependent on Wnt signalling activity. In 2005, colon cancer alone was diagnosed in 148,000 people in the United States, causing approximately 55,000 deaths (according to the American Cancer Society).

The past decade has seen major advances in our understanding of how deregulation of Wnt signalling activity occurs in epithelial tissues, providing a solid platform from which to launch drug development programmes targeting the inhibition of this pathway in cancers. The identification of small-molecule inhibitors of the pathway has been accelerated by the availability of detailed crystal structures for key protein complexes and the generation of synthetic drug-like compound libraries and collections of natural compounds for high-throughput screening (HTS) programmes. Other promising avenues for attacking Wnt-driven cancers include the development of antibodies that block pathway activation at the membrane and targeted expression of suicide genes or selective replication of lytic viruses in these cancers. Furthermore, a largely untapped source of potential therapeutic targets is the set of 300–400 Wnt target genes recently found to be highly expressed in colon cancers as a consequence of aberrant Wnt signalling activity[2,3]. Some of these genes are likely to contribute directly to cancer formation and should warrant further validation as therapeutic targets for selective inhibition. Others encoding surface-expressed proteins might confer no selective advantage to the cancer cells, but will make excellent targets for antibody-based therapies. In this review we summarize the contribution of Wnt signalling to cancer formation and highlight recent progress in developing therapeutics targeting the Wnt pathway as effective cancer treatments.

Also worthy of note, although beyond the scope of this review, are the opportunities for developing drugs eliciting local Wnt pathway activation as a means of stimulating tissue regeneration following injury. Two such opportunities are regeneration of the intestinal epithelium following chemotherapy and stimulation of hair growth.

Wnt signalling and cancer

Secreted signalling proteins of the Wnt family bind to specific Frizzled (Fzd) receptor complexes on the surface of target cells to activate distinct intracellular pathways that are broadly classified as canonical or non-canonical Wnt signalling pathways, where the specific composition of the Wnt/Fzd complex defines which of the two pathways is activated[4]. The canonical pathway, which regulates the ability of the β-catenin protein to drive activation of specific target genes, is better characterized and is generally considered to be more relevant for cancer development (FIG. 1). In brief, in the absence of a Wnt

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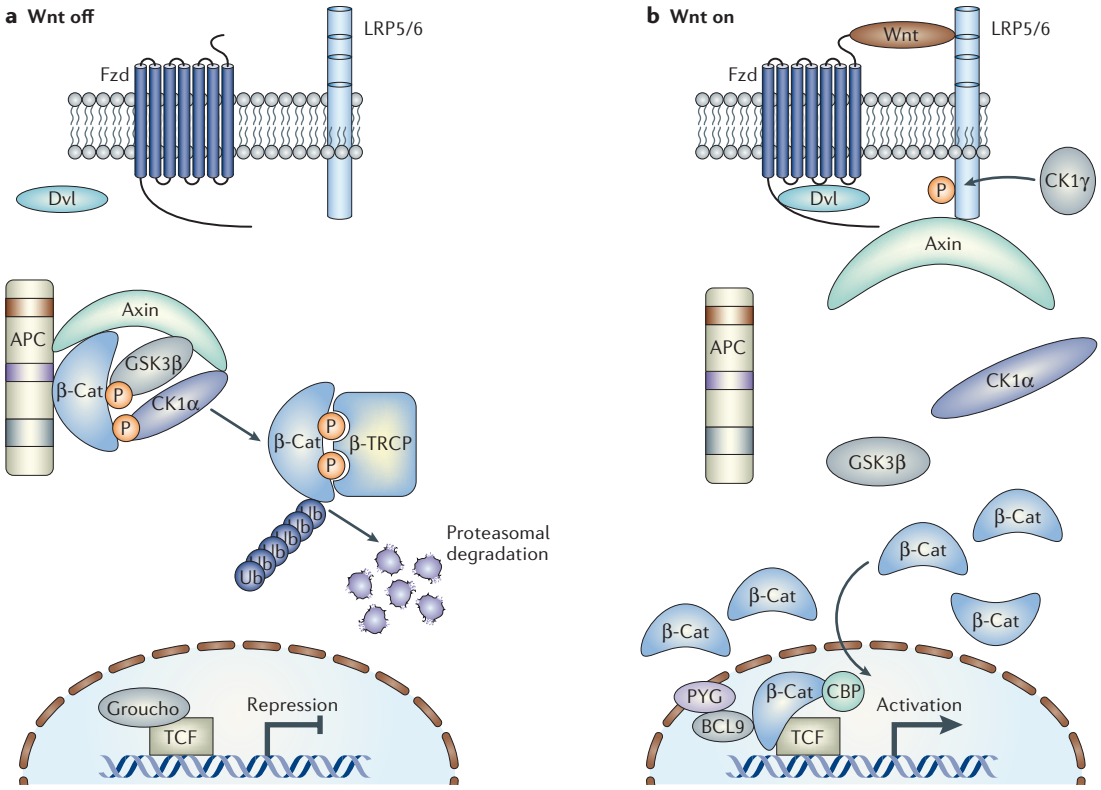


Figure 1 | **An overview of the Wnt signalling pathway. a** | In the absence of a Wnt signal, β-catenin is captured by APC and axin within the destruction complex, facilitating its phosphorylation by the kinases CK1α and GSK3β. CK1α and GSK3β then sequentially phosphorylate a conserved set of serine and threonine residues at the amino terminus of β-catenin[161,162]. This facilitates binding of the β-TRCP, which subsequently mediates the ubiquitinylation and efficient proteasomal degradation of β-catenin[163,164]. The resulting β-catenin 'drought' ensures that nuclear DNA-binding proteins of the Tcf/Lef transcription factor family (TCF1, TCF3, TCF4 and LEF1) actively repress target genes by recruiting transcriptional co-repressors (Groucho/TLE) to their promoters and/or enhancers[165,166]. **b** | Interaction of a Wnt ligand with its specific receptor complex containing a Frizzled family member and LRP5 or LRP6 triggers the formation of Dvl–Fzd complexes and the phosphorylation of LRP by CK1γ, facilitating relocation of axin to the membrane and inactivation of the destruction box[167,168]. This allows β-catenin to accumulate and enter the nucleus, where it interacts with members of the Tcf/Lef family[114,169]. In the nucleus, β-catenin converts the Tcf proteins into potent transcriptional activators by displacing Groucho/TLE proteins and recruiting an array of coactivator proteins including CBP, TBP, BRG1, BCL9/PYG, Legless, Mediator and Hyrax[117–121,123–126,170]. This ensures efficient activation of Tcf target genes such as *c-MYC*, which instruct the cell to actively proliferate and remain in an undifferentiated state[171]. Following dissipation of the Wnt signal, β-catenin is evicted from the nucleus by the APC protein and Tcf proteins revert to actively repressing the target gene program. β-TRCP, β-transducin repeat-containing protein; APC, adenomatous polyposis coli; BCL9, B-cell lymphoma 9; CK1α, casein kinase 1α; CK1γ, casein kinase 1γ; CBP, CREB-binding protein; Fzd, Frizzled; Lef, lymphoid enhancer factor; LRP, low-density lipoprotein receptor-related protein; PYG, Pygopus; Tcf, T-cell factor. For more information we recommend the Wnt Homepage (see Further information).

signal, the transcriptional activator β-catenin is actively degraded in the cell by the actions of a protein complex called the 'destruction box'. Within this complex the Axin and adenomatous polyposis coli (APC) proteins form a scaffold that facilitates β-catenin phosphorylation by casein-kinase 1α (CK1α) and glycogen synthase kinase 3β (GSK3β). Phosphorylated β-catenin is subsequently recognized and ubiquitinylated, resulting in its proteasomal degradation. Levels of free β-catenin consequently remain low, which allows the DNA-binding T-cell factor/lymphoid enhancer factor (Tcf/Lef) proteins to interact with transcriptional co-repressors to block target gene expression in the nucleus (FIG. 1a). Binding of Wnt to Fzd–LRP (low-density lipoprotein receptor-related protein) receptor complexes at the membrane results in the formation of Dishevelled (Dvl)–Fzd complexes and relocation of Axin from the destruction complex to the cell membrane. This allows β-catenin to accumulate and enter the nucleus, where it interacts with members of the Tcf/Lef family and converts the Tcf proteins into potent transcriptional activators by recruiting co-activator proteins ensuring efficient activation of Wnt target genes including *c-MYC* (FIG. 1b).

This pathway was first linked to cancer formation when it was found to be permanently activated in both inherited familial adenomatous polyposis (FAP)[5,6] and spontaneous forms of colon cancer[7,8]. FAP is a hereditary cancer syndrome in which individuals who have inherited one defective APC allele suffer the spontaneous inactivation of the second APC allele at low frequency

in their intestinal epithelial cells[5,6]. The resulting chronic activation of the Wnt pathway in these cells drives their expansion into benign adenomas (also termed polyps), which frequently progress to invasive colon carcinoma following additional genetic mutations later in life. Mouse models of FAP (such as APC^{min}) carrying similar APC defects spontaneously develop multiple intestinal polyps soon after birth, but rarely live long enough for the polyps to progress to malignant cancers[9,10]. Approximately 90% of sporadic colon cancers show aberrant Wnt signalling activity, usually as the result of mutations in APC (80%)[7,11–13], but also less frequently because of mutations in β-catenin[8,14] or Axin 2 (also known as Conductin)[15,16] (FIG. 2). The respective mutations of APC or Axin 2 compromise their function within the β-catenin destruction-box complex, whereas mutation of the conserved phosphorylation sites of β-catenin blocks its targeted degradation by the ubiquitin proteasome pathway.

In addition to these mutations, many colon cancers carry silenced genes encoding members of the secreted Fzd-related protein (SFRP)[17,18] or Wnt inhibitory factor (WIF)[19] families, which act as natural brakes of the Wnt pathway by binding Wnt ligands to block pathway activation at the cell surface. With these brakes removed, Wnt ligands produced by the cancer cells can activate the pathway at the membrane and are thought to amplify the aberrant Wnt signalling activity initiated by mutations in APC, β-catenin or Axin 2. Ultimately, such events lead to accumulation of β-catenin in the nucleus, which drives the chronic activation of the Tcf target gene program (FIG. 3). Aberrant Wnt pathway activation is considered to be the initiating event in colon cancer formation, forcing the transformation of intestinal epithelial cells into immortal adenomas that continue to grow and expand until they acquire additional mutations that facilitate their progression into malignant, invasive and metastatic cancers. Importantly, late-stage colon cancers remain heavily dependent on this chronic Wnt signalling activity to maintain their growth advantage[2]. In the laboratory, suppression of this Wnt signalling activity in malignant colon cancer cell lines by over-expression of dominant-negative Tcf proteins, RNA interference (RNAi)-mediated depletion of β-catenin or re-expression of SFRP proteins efficiently blocks their growth and forces them to differentiate into epithelial cells[1,2,18,20].

A range of other human cancers also show signs of aberrant Wnt signalling activity, although with a lower frequency than colon cancer. In contrast to colon cancers, such aberrant Wnt signalling is very rarely due to loss of APC function. Instead, mutation of β-catenin seems to be the preferred route to chronic Wnt signalling dysfunction in cancers such as liver cancer (hepatocellular and hepatoblastoma), enodometrial ovarian cancer, pilomatricoma skin cancer, prostate cancer, melanoma and Wilms tumour[14]. Axin 1 mutations also account for the aberrant Wnt signalling activity observed in some liver cancers and medulloblastomas[21,22]. Other cancers and diseases show elevated levels of nuclear β-catenin, a hallmark of active Wnt signalling, despite the absence of APC, β-catenin or Axin mutations. Recent reports indicate that this can be achieved through epigenetic silencing of genes encoding natural Wnt pathway inhibitors such as SFRP[23–29] and WIF[30–34], or increased expression of pathway components including Wnt ligands[35–46], Fzd receptors[26,40,42–44,47] and Dvl family members[48–50] (TABLE 1).

Clearly, a strong case is being built for aberrant activation of the Wnt signalling pathway being a major driving force in a broad spectrum of human cancers and diseases, highlighting the urgent need for drugs targeting this pathway. Outlined below are the most promising drug discovery avenues currently being explored in the quest to develop effective Wnt pathway inhibitors.

Existing drugs as Wnt pathway therapeutics

Since the late 1990s, when inappropriate activation of the Wnt pathway was first linked to colon cancer and melanoma, there has been intense interest in the pharmaceutical and biotechnology sectors in developing effective Wnt pathway inhibitors[7,8,51]. Despite this investment, large-scale screening programmes have yet to identify drugs specifically targeting the pathway which are of clinical use. These rational drug design programmes still hold great promise for developing effective cancer therapeutics and will be discussed in detail later in this review. However, a number of drugs that are already on the market or are currently being evaluated for use in treating other diseases, including non-steroidal anti-inflammatory drugs (NSAIDS) and vitamin derivatives, might also directly or indirectly target the Wnt pathway and could be adapted for treatment of cancers 'addicted' to active Wnt signalling.

NSAIDS. Traditional NSAIDS, including aspirin, sulindac and indomethacin, are used worldwide for the treatment of pain, inflammation and fever. More recently, they have attracted considerable interest as potential anticancer drugs. Numerous epidemiological studies have highlighted the benefits of regular use of aspirin and other NSAIDS in reducing the incidence and severity of various human cancers[52,53]. This chemoprotective role is particularly evident for familial/hereditary forms of colon cancer in which uncontrolled Wnt signalling is considered to be a major driving force[54–56]. The efficacy of NSAIDS as anticancer agents can be attributed to a number of effects ranging from inhibition of cancer cell proliferation and induction of apoptosis to curbing cancer cell invasion. Their precise mechanism of action is complex and is likely to be unique for each NSAID class. Suppression of elevated cyclooxygenase (COX) enzyme activity in cancer cells is clearly a key factor in the anticancer activity of many NSAIDS[57] (BOX 1). However, this is certainly not the only route of action, as NSAIDS are also effective against tumour cells lacking COX activity and, conversely, some NSAIDS lacking COX activity demonstrate anticancer activity *in vivo*[58,59].

More recently it has become apparent that a uniting theme of NSAID actions on human cancers might be suppression of aberrant Wnt signalling activity. This was first hinted at when NSAID treatment was found to be effective in reversing polyp growth in patients with FAP and restricting polyp formation in the mouse FAP model

Adenoma
An ordinarily benign neoplasm of epithelial tissue in which the tumour cells form glands or gland-like structures.

Carcinoma
The final, invasive stage of evolution of an epithelial cancer.

APC^{min} mouse
The APC^{min} (multiple intestinal neoplasia) mouse model of human familial adenomatous polyposis carries a germline mutation in the *APC* gene that drives the formation of multiple intestinal tumours.

Epigenetic
Any heritable influence (in the progeny of cells or of individuals) on chromosome or gene function that is not accompanied by a change in DNA sequence.

Apoptosis
Programmed cell death.

APC$^{min\ 60–67}$. More direct support for this was provided in a recent study describing substantial reduction of nuclear β-catenin levels in polyps of FAP patients treated for 6 months with the NSAID sulindac sulphide[68].

Until recently, it was generally assumed that inhibition of COX activity and dampening of the Wnt signal in colorectal cancers were separate effects of NSAIDS that cooperated to effectively block tumour growth. However, it now seems that the activities of the COX and Wnt signalling pathways might be inextricably linked in colon cancers and could be subject to concerted regulation *in vivo* by NSAIDS[69,70]. Elevated COX activity in colon cancer cells drives increases in prostaglandin levels, which might subsequently stimulate Wnt signalling by interfering with the capacity of cells to degrade β-catenin[69,70]. Although the majority of colon cancers already display permanently active Wnt signalling as a consequence of mutations in APC, β-catenin or Axin, the prostaglandin-induced boost of this pathway is likely to further enhance cancer cell growth.

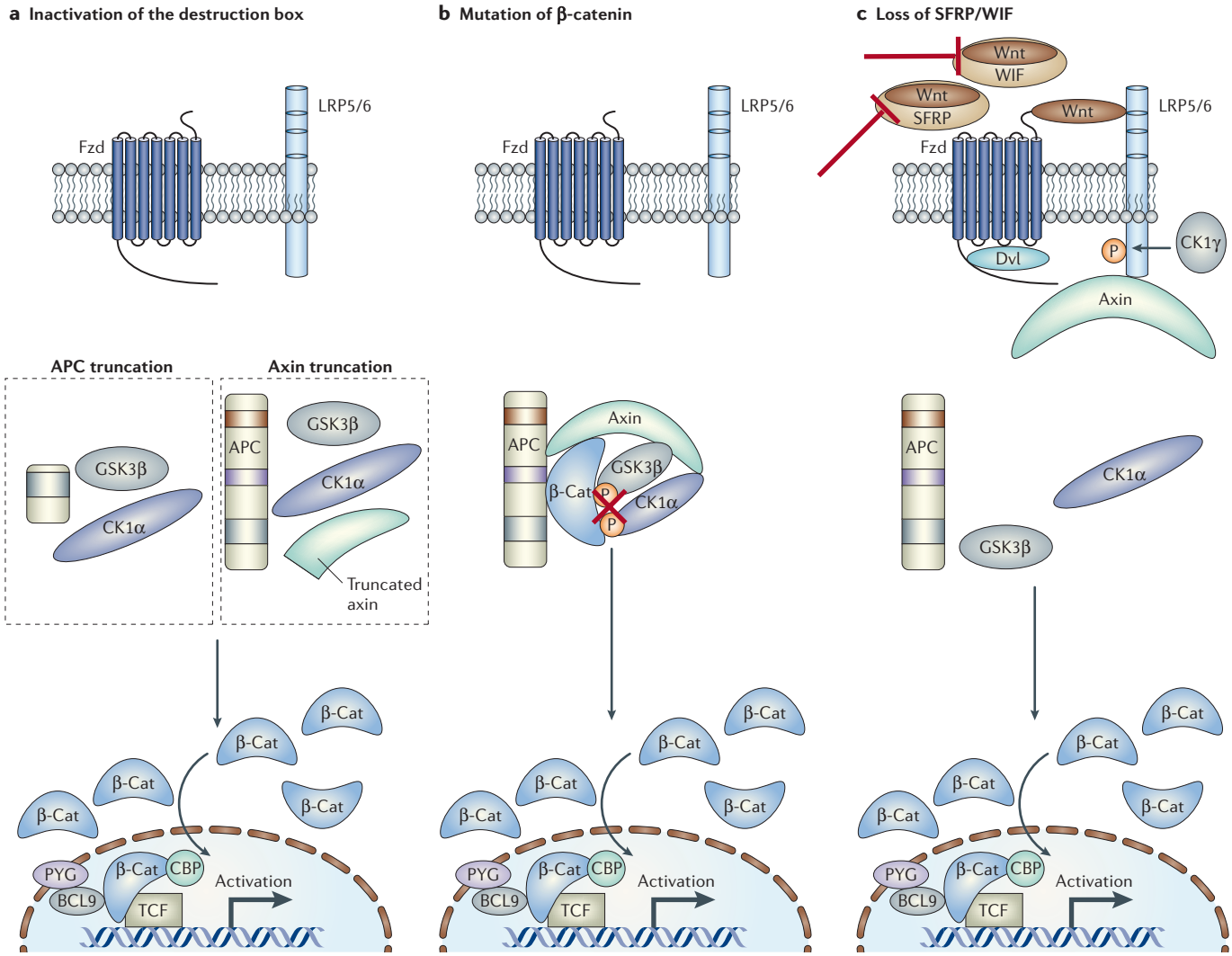


Figure 2 | **Routes to aberrant activation of Wnt signalling in cancer cells. a** | Mutations in *APC* or *axin 1* genes result in the production of truncated scaffold proteins lacking the capacity to bind β-catenin (β-Cat). This prevents the phosphorylation and proteasomal degradation of β-catenin, allowing it to accumulate and form active transcription factor complexes with Tcf/Lef proteins in the nucleus. **b** | Mutation of the conserved serine/threonine phosphorylation sites at the amino terminus of β-catenin blocks its phosphorylation within the destruction complex, thereby preventing binding of β-TRCP. β-catenin consequently evades ubiquitinylation and proteasomal degradation, allowing it to accumulate and form active transcription factor complexes with Tcf/Lef proteins in the nucleus. **c** | Loss of natural Wnt inhibitors such as SFRP or WIF through epigenetic silencing of the corresponding genes allows Wnt proteins produced by the cancer cells to activate the pathway at the membrane. The resulting inactivation of the destruction complex drives accumulation of β-catenin and the formation of active transcription factor complexes with Tcf/Lef proteins in the nucleus. In each case, the uncontrolled formation of Tcf–β-catenin complexes in the nucleus causes chronic activation of the Wnt target gene program, driving cancer formation. β-TRCP, β-transducin repeat-containing protein; APC, adenomatous polyposis coli; Lef, lymphoid enhancer factor; SFRP, secreted Frizzled-related protein; Tcf, T-cell factor; WIF, Wnt inhibitory factor.

It is therefore reasonable to assume that reduction of prostaglandin levels by inhibition of COX activity in the tumour or in the surrounding stroma could account for the observed ability of NSAIDS to dampen the Wnt signalling pathway and curb tumour growth *in vivo*.

However, other NSAIDS lacking the capacity to inhibit COX activity are also reported to target the Wnt pathway in cancer cells, suggesting the existence of additional modes of action. A brief review of the various mechanisms of action proposed for the major NSAID classes is outlined in BOX 2.

New generation NSAIDS. The large-scale prescription of traditional NSAIDS as anticancer agents is currently not feasible as approximately 4% of patients using NSAIDS suffer from severe intestinal bleeding and/or kidney damage. This has prompted the development of safer NSAID derivatives, which retain or increase their anticancer activity while limiting these toxic side effects. These include selective COX2 inhibitors, such as celecoxib (Celebrex; Pfizer) and rofecoxib (Vioxx; Merck), and nitric oxide-releasing NSAIDS (NO-NSAIDS). Celecoxib treatment reduces polyp formation in FAP patients by 28% and is currently the only NSAID approved by the FDA and EMEA for treatment of this condition[65,66]. Treatment of colon cancer cell lines with celecoxib reduces nuclear β-catenin levels, indicating that inhibition of the Wnt signalling pathway accounts for some of this antitumour effect[71]. However, concerns regarding potential cardiovascular side effects of selective COX2 inhibitors might limit their use as anticancer drugs to only the most susceptible groups, such as those with FAP[72,73].

NO-releasing aspirin (NO-ASA) is another success story in the race to develop safe, more effective NSAID-based anticancer drugs. This aspirin derivative is several thousand-fold more effective in inhibiting human colon cancer cell growth than aspirin *in vitro* and is far less toxic[74–77]. NO-ASA also efficiently reduces polyp formation in APC^{min} mice and substantially reduces Wnt signalling in colon cancer cell lines via disruption of Tcf–β-catenin complex formation[78–80]. Importantly, NO-ASA does not cause obvious toxic side effects in the intestine of the APC^{min} mice and has no effect on proliferation of the normal intestinal epithelium when administered at 100 mg per kg per day[78].

Clearly, NSAIDS do show promise for chemoprevention or treatment of some Wnt-driven cancers, but in our view the contribution of Wnt signalling inhibition to this anticancer activity remains to be conclusively demonstrated.

Vitamin A and vitamin D. Vitamin A is converted in the body into a number of different products collectively referred to as retinoids. They are crucial regulators of cell growth and differentiation, and have key functions in embryonic development, reproduction, vision and immune responses. Accumulating evidence from epidemiological studies, clinical trials, rodent cancer models and *in vitro* cellular models also supports the use of retinoids and their synthetic derivatives as

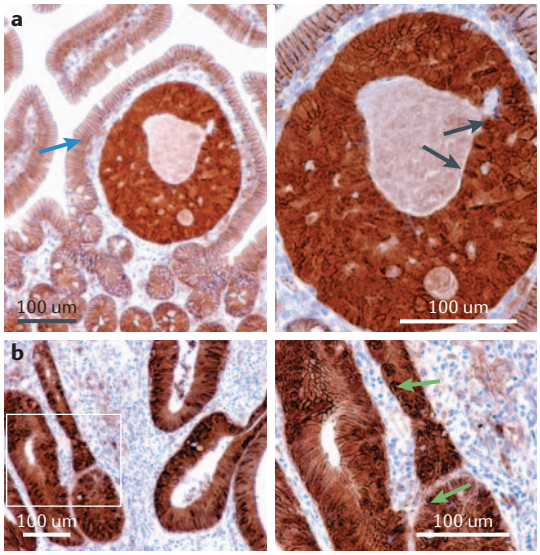


Figure 3 | **Accumulation of β-catenin in adenomas.** **a** | β-catenin is visible only at the membrane of normal intestinal epithelium (blue arrow). In the adenoma, there is a massive accumulation of β-catenin. The presence of β-catenin in the nuclei (black arrows) of the adenoma reflects aberrant activation of the Wnt signalling pathway. **b** | Accumulation of β-catenin in a human colorectal adenoma. Massive accumulation of β-catenin is evident throughout the human adenoma. The presence of β-catenin in the nuclei (green arrows) of the adenoma reflects aberrant activation of the Wnt signalling pathway.

pharmacological agents in cancer therapy and prevention[81]. The anticancer effects of the retinoids might be partly attributed to their capacity to restore cell adhesion by stabilizing components of the adherens junctions and through suppression of oncogenic AP1 and Wnt signalling pathways[82–84].

Retinoids achieve their biological functions by interaction with two families of nuclear receptors, retinoic acid receptor (RAR) and retinoid-X-receptor (RXR)[81]. A number of studies indicate that these retinoid-activated receptors also interact with β-catenin in direct competition with Tcf factors[82–84]. The resulting reduction of Tcf–β-catenin complex formation might contribute to the inhibition of colon cancer growth *in vitro* and in animal models observed following retinoid treatment[85,86]. However, some doubts regarding the therapeutic potential of using retinoids to treat Wnt-addicted cancers were raised by a study that recorded increased intestinal tumour growth in APC^{min} mice treated with retinoic acid[87].

Vitamin D has long been recognized to be important for regulating calcium and phosphorus levels in the body to maintain a healthy skeleton. Epidemiological studies point to stimulation of vitamin D synthesis in the skin by exposure to sunlight as having a protective effect against a range of cancers, including colon, breast and prostate cancers (for a review see REF. 88). The physiologically active form of vitamin D, 1α,25-dihydroxyvitamin D3 (1α,25$[OH]_2$D3), and synthetic derivatives also inhibit the growth of various cancer cells

Table 1 | **Common events linked with aberrant activation of Wnt signalling**

Pathway component	Observed alterations	Disease
Wnt ligands	Increased expression	Colon cancer[98,99]; breast cancer[37,38,40]; melanoma[101]; head & neck cancer[42], non-small-cell lung cancer[36,45]; gastric cancer[38]; mesothelioma[39]; Barrett's esophagus[35]; rheumatoid arthritis[43,44]; schizophrenia[41]
Frizzled receptors	Increased expression	Colon cancer[98,151]; breast cancer[40]; head & neck cancer[42]; gastric cancer[26,27]; synovial sarcomas[152]; rheumatoid arthritis[43,44]
Dishevelled family members	Increased expression	Mesothelioma[50]; non-small-cell lung cancer[49]; cervical cancer[48]
APC	Loss-of-function mutations/reduced expression	Colon cancer[153–155]; Barrett's oesophagus[35]
β-catenin	Gain-of-function mutations	Colon cancer; gastric cancer; hepatocellular cancer, hepatoblastoma; Wilm's tumour; endometrial ovarian cancer; adrenocortical tumours; pilomatricoma[14]
Axin 1	Loss-of-function mutations	Hepatocellular cancer[22,156,157]; hepatoblastomas[21,157]
Axin 2/ Conductin	Loss-of-function mutations	Colon cancer (MSI$^+$)[16,158]; hepatocellular cancer[156]; oligodontia (tooth loss)[15]
SFRP family members	Reduced expression	Colon cancer[17,18]; breast cancer[27,28]; gastric cancer[26]; mesothelioma[24]; non-small-cell lung cancer[23]; Barrett's oesophagus[29]; leukaemia[25]
WIF family members	Reduced expression	Colon cancer[19]; breast cancer[30,34]; prostate cancer[34]; lung cancer[32,34]; bladder cancer[33,34]; mesothelioma[31]
LRP5	Gain-of-function mutations	Increased bone density[159,160]

APC, adenomatous polyposis coli; LRP, low-density lipoprotein receptor-related protein; MSI$^+$, microsatellite instability positive; SFRP, secreted frizzled-related protein; WIF, Wnt inhibitory factor.

in vitro by blocking cell proliferation and inducing differentiation, and demonstrate chemopreventive activity in animal models of colorectal and breast cancer[89–91]. Again, these anticancer activities might be due in part to inhibition of the Wnt signalling pathway, particularly in colorectal cancers. In colon cancers, this cross-regulation of the Wnt signalling pathway is achieved by the interaction of vitamin D derivatives with the vitamin D receptor (VDR) to form a transcription factor complex that efficiently binds β-catenin[92,93]. The ligand-activated VDR also triggers an increase in E-cadherin, which might contribute to the reduction in Wnt signalling by relocating β-catenin to the cell membrane[92]. The net result of these actions is to deplete β-catenin levels in the nucleus, thereby reducing Tcf–β-catenin complex formation and dampening activation of the genetic program maintaining the cancer cells in a permanently proliferating, non-differentiated state. The recent development of non-hypercalcaemic vitamin D derivatives such as EB1089[94] (Leo Pharmaceuticals) should minimize the serious side effects associated with prolonged treatment with vitamin D. However, the observed loss of VDR expression in late-stage colon cancers is likely to be a more serious limitation to the use of vitamin D derivatives in the treatment of Wnt-addicted cancers[95,96]. This could restrict their use to treatment of high-risk groups, such as those with FAP or patients diagnosed with spontaneous colorectal polyps[95,96].

Drugs by design

Although drugs already on the market such as NSAIDS undoubtedly have potential as anticancer agents, their inability to reduce adenoma formation in ~50% of treated FAP patients highlights their limitations in treating Wnt-driven cancers. Their efficacy might well be improved by combining them with traditional chemotherapy treatments. However, the lethal blow to colon cancer growth achieved by blocking the aberrant Wnt signalling pathway in the laboratory suggests that drugs designed to achieve this in patients will have the greatest therapeutic value. Several approaches are currently being explored in pursuit of these selective Wnt pathway inhibitors, the most promising of which are outlined below.

Antibody-based therapeutics. A range of human cancers without mutations in APC, Axin or β-catenin might still utilize an aberrantly activated Wnt signalling pathway by increasing expression of more upstream pathway components such as Wnt ligands, Fzd receptors and Dvl, or by epigenetic inactivation of secreted negative regulators such as SFRPs and WIF (TABLE 1). This presents an opportunity to develop antibodies against the over-expressed Wnt and Fzd proteins as potential cancer therapeutics effecting either inhibition of Wnt signalling or recruitment of immune effectors to the cancer cells[46]. In head and neck cancers, various Wnts and the FZD2 receptor are frequently over-expressed[42]. As a proof of principle, treatment of a head and neck cancer cell line over-expressing WNT1 with a WNT1 monoclonal antibody effectively suppressed Wnt signalling, blocked proliferation and induced apoptosis[42]. WNT1 is also highly expressed in non-small-cell lung cancer (NSCLC) primary tumours and cell lines[36]. Treatment with the same WNT1 antibody again triggered apoptosis and effectively blocked tumour growth in mice[97]. Other cancers including gastric, colon, melanoma, mesothelioma and non-small cell lung carcinoma express high levels of WNT2[38,40,45,98–101]. Treatment of NSCLC, melanoma and mesothelioma cells with a WNT2 monoclonal antibody induces apoptosis *in vitro*, again highlighting the promise of therapeutic antibodies directed against members of the Wnt family[39,45,101]. FZD1 and FZD2 receptors, known to be highly expressed in breast cancers and poorly differentiated colon cancers relative to normal tissue, represent alternative targets for antibody-based therapies[40,98]. The development of such therapeutic antibodies was recently made even more attractive by the discovery that colon cancers also express high levels of various Wnt ligands, which enhance the Wnt signalling pathway already activated through mutation of APC, Axin or β-catenin[18,19]. Indeed, reduction of the Wnt-induced signalling pathway by restoring expression of SFRP or treatment with a WNT1 antibody strongly induces apoptosis in colon cancer cell lines even in the presence of downstream mutations[18,19]. Strikingly,

Box 1 | **Cyclooxygenases in cancer**

Cyclooxygenase 1 (COX1) and COX2 are key enzymes in prostaglandin biosynthesis and have important roles in the protection of the gastrointestinal tract and cardiovascular homeostasis in addition to mediating fever, pain and inflammation. COX2 is considered to be the major villain in many human cancers, with elevated expression evident in 45% of human colon adenomas, 85% of colon carcinomas and in a number of other human cancers, including breast, gastric, lung, oesophageal and hepatocellular cancer[172,173]. COX1 activity is crucial for maintaining a healthy gastrointestinal tract and for proper kidney function. In colorectal cancer, both COX1 and COX2 are considered to cooperate to drive polyp formation[174]. This enhanced COX activity stimulates the production of prostaglandins, which consequently drive tumour growth, angiogenesis and metastasis. Consistent with this, disruption of COX1 or COX2 in APC^{min} mice significantly reduces adenoma incidence, whereas treatment with prostaglandins markedly accelerates adenoma growth[175–177].

aberrant activation of the Wnt pathway as a result of increased Wnt–Fzd complex formation might be the earliest event in colon cancer, accentuating the potential value of antibodies that could block this event[18,102]. Clearly, these preliminary results are encouraging, but the true therapeutic potential of such Wnt/Fzd antibodies will only become evident when their *in vivo* efficacy as antitumour agents is rigorously evaluated in rodent cancer cell models.

Small-molecule inhibitors. It is becoming increasingly clear that a variety of routes are used by human cancers to aberrantly activate the Wnt pathway. A common feature of all these cancers is the constant presence of Tcf–β-catenin complexes in their nuclei, which leads to chronic activation of a genetic program considered to promote cancer formation by stimulating cell growth, blocking apoptosis and altering cell movement. Artificial disruption of Tcf–β-catenin complex formation in colon cancer cells effectively blocks target gene activation and inhibits their growth *in vitro*[1,2]. Drugs designed to mimic this *in vivo* by disrupting Tcf binding to β-catenin are therefore expected to hold great potential for the treatment of a range of Wnt-addicted cancers. Recent successes with developing effective small-molecule inhibitors of protein complexes have fuelled renewed interest in the therapeutic potential of this approach[103,104]. Accordingly, the Tcf–β-catenin protein complex has become a high-priority target for small-molecule inhibitor development in the pharmaceutical and biotechnology sectors.

Crystal structures of Tcf–β-catenin complexes have provided invaluable insight into how Tcf and β-catenin interact to form stable transcription factor complexes in the nucleus[105–107]. The amino terminus of Tcf makes multiple contacts within an extensive domain encompassing armadillo repeat 3–10 of β-catenin to achieve a high-affinity interaction (~8 nM). Disruption of such a stable interaction would seem to be a tall order for a lone small molecule. However, crystal structures and studies comparing the binding capacity of various Tcf or β-catenin single-amino-acid mutants indicate that complex formation *in vivo* relies mainly on a few key amino-acid residues that define interaction 'hot-spots' on the surface of β-catenin and Tcf[105–111] that could be targeted by small molecules. β-catenin is, however, a multifunctional protein that interacts with other proteins such as E-cadherin, APC and Axin. Complex formation with E-cadherin is crucial for cell adhesion, whereas interaction with APC and Axin is essential for regulating β-catenin levels in normal tissues. For small-molecule inhibitors to have real therapeutic value, they must therefore selectively disrupt Tcf–β-catenin complexes while leaving other β-catenin complexes intact to avoid potentially serious side effects resulting from perturbation of cell adhesion and/or inappropriate activation of Wnt signalling in normal tissue. Comparison of crystal structures reveal that β-catenin interacts with Tcf, E-cadherin and APC using substantially overlapping domains, highlighting the potential difficulties in achieving absolute specificity using small molecules[105–107,112,113].

These formidable challenges have not deterred researchers from screening diverse natural and synthetic compound libraries using high-throughput ELISA (enzyme-linked immunosorbent assay) or cell-based assays for identifying effective inhibitors of Tcf–β-catenin complexes (FIG. 4). Well-validated secondary assays designed to evaluate the specificity and *in vivo* efficacy of 'hits' from these primary screens are being used to identify 'lead compounds' with true drug potential (FIG. 5). To the best of our knowledge, no small-molecule inhibitors of Tcf–β-catenin have yet been identified by HTS of large synthetic compound collections. However, three natural compounds (PKF115-584, PKF-222-815 and CPG049090; TABLE 2) were found in HTS of natural compounds and consistently scored as potent inhibitors of Tcf–β-catenin binding in secondary assays. Their capacity to inhibit axis duplication induced by artificial activation of Wnt signalling in *Xenopus* embryos[114] and to selectively block growth of colon cancer cell lines with constitutively active Wnt signalling further supports the notion that these compounds are bona fide Tcf–β-catenin inhibitors with cancer therapeutic potential[115]. Significantly, these three lead compounds share a common core chemical structure, which suggests that they achieve disruption of the Tcf–β-catenin complex by binding to either Tcf or β-catenin in a similar fashion[115]. Computer-simulated docking of these compounds onto the crystal structure of the Tcf–β-catenin complex indicates that they are likely to bind to β-catenin at a Tcf interaction 'hot-spot'[116]. A potential hurdle to future development of these lead compounds as cancer therapeutics is their lack of absolute specificity. The resulting disruption of β-catenin binding to APC might cause deregulation of the Wnt signalling pathway in healthy tissues, thereby potentially promoting cancer formation. Future preclinical studies (for example, using APC^{min} mice) designed to evaluate their *in vivo* efficacy and potential side effects will undoubtedly clarify the therapeutic potential of these three compounds in the near future. Regardless of the final verdict, these compounds will act as a beacon of hope for researchers trawling compound libraries for selective Tcf–β-catenin inhibitors using traditional HTS assays.

The availability of detailed crystal structures for β-catenin bound to its various protein partners has fuelled structure-assisted design approaches towards developing the ultimate small-molecule inhibitors of the Tcf–β-catenin complex. Such rational approaches generally focus on identifying pockets on the surface of β-catenin (or Tcf) in the vicinity of interaction hot-spots, which can theoretically be used to anchor small molecules. Powerful computer-simulation packages (docking programs) are used to predict small molecules that will fit into these pockets. This approach has been successfully used to identify three compounds that were found to reduce Tcf–β-catenin complex formation in biophysical NMR and isothermal titration μ-calorimetry (ITC) assays and the lead compound PNU-74654 is claimed to be active in a cellular Tcf reporter gene assay[116]. Given the very limited biological data in the study, and lack of information regarding the selectivity and *in vivo* efficacy of these compounds, it is dangerous to conclude that they are promising cancer therapeutics. However, it does highlight the considerable potential of virtual or *in silico* screening as an alternative or complementary approach to HTS for identifying small-molecule inhibitors of Tcf–β-catenin complexes amongst large compound libraries.

Box 2 | **NSAIDs as potential Wnt pathway therapeutics**

Aspirin

Aspirin (acetyl salicyclic acid or ASA) is the founding member of the non-steroidal anti-inflammatory drug (NSAID) family and was the first to be identified as possessing anticancer properties in epidemiological studies[52,53]. Like other NSAIDS, aspirin can inhibit cancer cell growth when used at a high concentration and is likely to achieve this by both cyclooxygenase (COX)-dependent and COX-independent means. One of the weapons in its anticancer arsenal seems to be suppression of Wnt signalling, which leads to a reduction in Tcf target gene expression in both APC and β-catenin mutant cell lines[59,178,179]. Aspirin administered at concentrations equivalent to the cardioprotective dose used in humans effectively reduces intestinal tumour growth in APC^{min} mice[180]. This was associated with a reduction in β-catenin levels, which is indicative of a reduction in Wnt signalling in the tumours. *In vitro* studies suggest that aspirin treatment converts β-catenin to a phosphorylated form incapable of activating downstream target genes[181].

Indomethacin

Indomethacin is a reversible COX1/COX2 inhibitor first introduced as an anti-inflammatory and anti-pyretic drug more than 40 years ago[182]. Since then, a substantial body of evidence has accumulated to suggest that indomethacin also has significant anticolorectal cancer activity[183–187]. A link with the Wnt pathway was hinted at by the impressive efficacy with which indomethacin reduces polyp growth (85% compared with controls) in APC^{min} mice[188]. Similarly, indomethacin treatment reduced carcinogen-induced tumour growth and nuclear β-catenin staining in the tumours in a rat colorectal cancer model[183,184]. Limited clinical data also point to indomethacin being effective in causing regression of polyps in familial adenomatous polyposis patients[186,187]. Treatment of human colon cancer cell lines with indomethacin can block cell proliferation and induce apoptosis, although only at concentrations that are unlikely to ever be reached *in vivo*[189]. An observed reduction in β-catenin levels or activity of a synthetic Tcf–β-catenin reporter gene following indomethacin treatment[59,179,181,185] suggested that this effect was mediated via suppression of Wnt signalling. However, its precise mechanism of action remains to be clarified.

Sulindac and derivatives

Sulindac is probably the most intensively studied NSAID in the context of chemoprevention. Treatment of APC^{min} mice with sulindac caused regression of pre-existing polyps in the small intestine, but not in the colon[63,190]. A corresponding reduction in nuclear β-catenin levels in the polyps of the small intestine (but not colon) indicated suppression of Wnt signalling, mirroring results obtained with familial adenomatosus polyposis (FAP) patients[68]. Similarly, sulindac treatment was effective in reducing colorectal tumour formation and nuclear β-catenin in carcinogen-treated rats[184]. Sulindac and its metabolites seem to achieve proteasomal degradation of β-catenin (and therefore block Wnt signalling) in human cancer cell lines in a COX-independent manner that might not require the conserved glycogen synthase kinase 3β (GSK3β) phosphorylation sites present a the amino terminus of β-catenin[191,192].

Sulindac sulphone (also known as Exisulind, Aptosyn and Prevatac) is an oxidative metabolite of sulindac currently being developed as a cancer therapeutic by OSI Pharmaceuticals. Exisulind can selectively induce apoptosis in a range of cancer cells by targeting cyclic GMP phosphodiesterase (cGMP PDE) isoforms 2/5[193–195]. This inhibition of PDE activity drives an increase in cellular cGMP levels, which in turn triggers multiple downstream effects that culminate in apoptosis only in cancer cells. Several studies have demonstrated a dose-dependent reduction in β-catenin levels and Tcf target gene expression in colon carcinoma cells following treatment with Exisulind and higher-affinity analogues such as CP461[191,194,196,197]. Exisulind was originally proposed to reduce β-catenin levels via a novel GSK3β-independent mechanism involving phosphorylation of the β-catenin carboxyl terminus, thereby priming it for proteasomal degradation. This would make Exisulind an ideal pharmacological regulator of β-catenin (and therefore Wnt signalling) in both APC and β-catenin mutant cancers. However, more recent studies indicate that this β-catenin degradation is GSK3β-dependent[196]. Irrespective of its precise mechanism of action, Exisulind and analogues do seem to exert some of their antitumour effects in colon cancer cells by dampening the constitutive Tcf–β-catenin signalling activity present. In agreement with this, Exisulind has shown promise in clinical trials assessing inhibition of polyp formation in FAP patients[198,199]. Despite these promising preclinical and clinical results, Exisulind has not been approved for use in chemoprevention of FAP by the FDA. A recent large-scale trial also showed Exisulind to cause significant regression of sporadic colonic polyps[200], but also revealed significant side effects, such as abdominal pain and liver-related problems, which would be predicted to hamper the long-term use of Exisulind as a chemotherapeutic agent at such high doses.

Isothermal titration μ-calorimetry
Thermodynamic technique for characterizing biomolecular interactions. This measures the heat absorbed or generated when two substances bind, enabling accurate determination of binding affinities and stoichiometries.

Familial adenomatous polyposis
Genetic disorder that is characterized by an increased predisposition to colorectal cancer, associated with germline mutations of the *APC* gene.

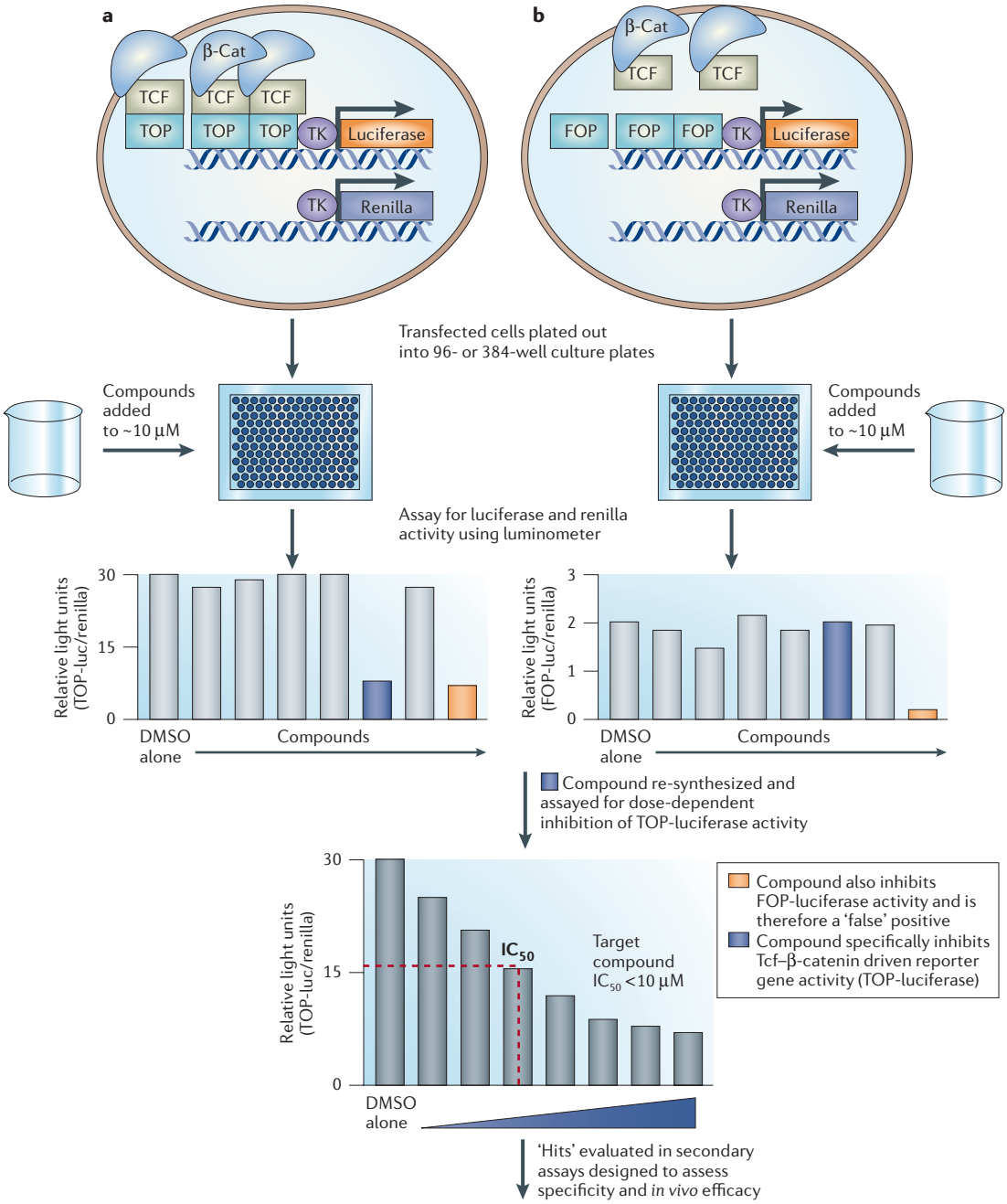


Figure 4 | **A cell-based assay for screening compound libraries for small-molecule inhibitors of the Wnt pathway.** Two reporter cell-lines are generated in parallel. Cell line **a** comprises colon cancer cells transfected with a Tcf reporter gene (TOP-luciferase), which responds to the aberrant Wnt signalling activity by driving high levels of luciferase activity. Cell line **b** comprises colon cancer cells transfected with a control plasmid FOP-luciferase, which is unable to respond to active Wnt signalling and consequently drives much lower levels of luciferase activity. This is included to control for non-specific effects on luciferase activity. Both cell lines are also transfected with thymidine kinase–renilla luciferase (TK–renilla), which drives strong Wnt-independent activity of the *renilla* gene and serves as a measure of cell viability. The reporter cell lines are independently plated out into 96- or 384-well culture plates and compounds dissolved in dimethyl sulphoxide (DMSO) added. After a suitable incubation time (typically 12–24 hours), cells are lysed and luciferase and renilla activities measured using a luminometer. Compounds capable of passing through the cell membrane and specifically inhibiting aberrant Wnt signalling activity will reduce TOP-luciferase activity, without reducing FOP-luciferase activity (blue). Compound-induced changes in cell viability (non-specific toxicity effects) are taken into account by displaying luciferase/renilla activities. 'Hits' are re-synthesized and assayed for dose-dependent inhibition of TOP-luciferase activity in the cell-based assay. Promising 'hits' with IC_{50} typically below 10 μM are subsequently evaluated for specificity and *in vivo* efficacy in a range of secondary assays.
TOP, optimal Tcf-binding site; FOP, Far-from-optimal Tcf-binding site; IC_{50}, concentration required to reduce TOP-luciferase/renilla activity by 50% in colon cancer cells.

Other opportunities for blocking Tcf–β-catenin function in cancer cells include development of small molecules that prevent interaction of β-catenin with essential transcriptional co-activator proteins such as cAMP response element-binding protein (CREB) binding protein (CBP) and B-cell lymphoma 9 (BCL9)/pygopus[117–121]. It is well documented that recruitment of these co-factors into nuclear Tcf–β-catenin complexes is essential for efficient activation of Wnt target genes, and their forced absence is therefore expected to be of therapeutic value in the treatment of Wnt-addicted cancers. A cell-based screen of structurally diverse synthetic compounds identified three closely-related small molecules that partially inhibit Tcf–β-catenin signalling in a colon cancer cell line[122]. The lead compound ICG-001 was found to selectively bind CBP, and prevent its interaction with β-catenin. The resulting displacement of CBP from Tcf–β-catenin complexes in treated colon cancer cell lines probably accounts for the observed efficiency of ICG-001 in reducing Tcf–β-catenin reporter gene activity. Surprisingly, ICG-001 treatment of colon cancer cells resulted in reduced expression of only a limited number of Wnt target genes, including the anti-apoptotic gene *survivin*. Apoptosis was selectively induced in a dose-dependent fashion in colon cancer cell lines but not normal colonic epithelial cells[122]. ICG-001 treatment also inhibited growth of SW480 colon cancer cells *in vitro* and markedly reduced tumour growth *in vivo* in both APC^{min} and SW620 xenograft mouse models of cancer[122].

Other Tcf–β-catenin cofactors, such as TATA-box-binding protein (TBP), Brahma-related gene 1 (BRG1), BCL9, pygopus, Hyrax and Mediator, represent alternative targets for small-molecule-mediated inhibition of Tcf–β-catenin signalling activity in cancer cells[120,123–126]. The crystal structure of a Tcf fragment bound to β-catenin and BCL9 peptides revealed that the β-catenin–BCL9 interface does not overlap with the majority of other β-catenin-interacting proteins, highlighting the potential for selective therapeutic intervention[127]. Indeed, the Genetics Company claim to have successfully generated small-molecule inhibitors of the interaction between BCL9 and β-catenin, which have potent Wnt-inhibitory activity *in vivo* (see Further information). Unfortunately, the lack of accompanying scientific data makes it difficult for us to accurately assess the validity of these claims. Ongoing validation of these small molecules will, we hope, reveal their true therapeutic potential in the near future and pave the way for similar development programmes targeting inhibition of β-catenin–cofactor interactions.

Small-molecule inhibitors of the Tcf–β-catenin complex clearly hold great promise for the treatment of the entire spectrum of Wnt-addicted cancers and human diseases associated with aberrant Wnt signalling. However, small molecules that inhibit more upstream signalling components crucial for relaying the Wnt signal amplified in some cancers and diseases might also have therapeutic value. An example of this is the recent development of small molecules that inhibit the interaction of Dvl with Fzd receptors[128]. Interaction of Dvl with Fzd is crucial for transmitting the Wnt signal initiated by binding of Wnt to Fzd–LRP complexes at the cell surface[129,130]. Dapper, an endogenous inhibitor of the Wnt pathway, binds to a PDZ domain on Dvl and prevents relay of the Wnt signal by inhibiting interaction with Fzd[131]. Using the crystal structure of *Xenopus* Dvl–Dapper complexes as a model, a virtual screen of 250,000 drug-like compounds in the three-dimensional National Cancer Institute database was performed to identify small molecules predicted to bind to the Dvl PDZ domain and inhibit its interaction with Fzd. The most promising candidates were subsequently synthesized and tested for their capacity to interact with Dvl using NMR spectroscopy. One small molecule (NSC668036) bound Dvl, albeit with low affinity (K_d 237 μM), to form a complex with a structure considered likely to prevent interaction of Dvl with Fzd. NSC668036 was shown to partially block embryonic axis duplication in *Xenopus* embryos induced by overexpression of WNT3A but not β-catenin, suggesting that it might indeed reduce Wnt signalling by preventing Dvl–Fzd interaction[128]. It should be noted that the extent of this block in axis duplication and the capacity of the compound to reduce Wnt-induced activation of the Tcf target gene *siamois in vivo* are not very impressive and it is unlikely that this compound will ever be developed as a cancer therapeutic. However, the study does highlight the value of exploring such approaches for developing novel small-molecule inhibitors of the Wnt pathway.

Viral-based therapies targeting Wnt-addicted cancers. The past decade has seen widespread interest in developing viruses engineered to selectively kill human cancer cells as novel cancer therapeutic agents[132]. Such selectivity is typically achieved by restricting infection and/or replication of cell-destroying viruses to the cancer cells (oncolytic viruses), or by selective expression of virally encoded genes that produce toxins or prodrug-converting enzymes in the cancer cells. Although success in clinical trials has been limited to date, the recent approval of the use of an oncolytic virus (H101; Shanghai Sunway Biotech) in combination with chemotherapy for treatment of refractory nasopharyngeal cancer by the Chinese FDA highlights the potential of this approach.

Scientists have adopted this strategy for developing viral-based therapies targeting cancers with constitutive Tcf–β-catenin transcription factor complexes. Such therapies generally involve the use of adenoviruses engineered to express cytotoxic genes under strict control of promoters that contain several Tcf-responsive elements. This approach has been successfully used to develop adenoviruses that selective express the apoptosis-inducing *FADD* (Fas-associated via death domain) gene or cytotoxic genes encoding diphtheria toxin A (DTA) in colon cancer cell lines with hyperactive Tcf–β-catenin signalling[133,134]. The selectivity of the DTA-expressing adenovirus was particularly impressive, with marked cytotoxicity evident in colon cancer cell lines, but not cell lines lacking Tcf–β-catenin signalling[134]. Such impressive cancer-cell selectivity, which is of particular importance

Oncolytic virus
Viruses engineered to selectively replicate in and kill cancer cells.

Prodrug
A pharmacologically inactive compound that is converted to the active form of the drug by endogenous enzymes or metabolism. It is generally designed to overcome problems associated with stability, toxicity, lack of specificity or limited (oral) bioavailability.

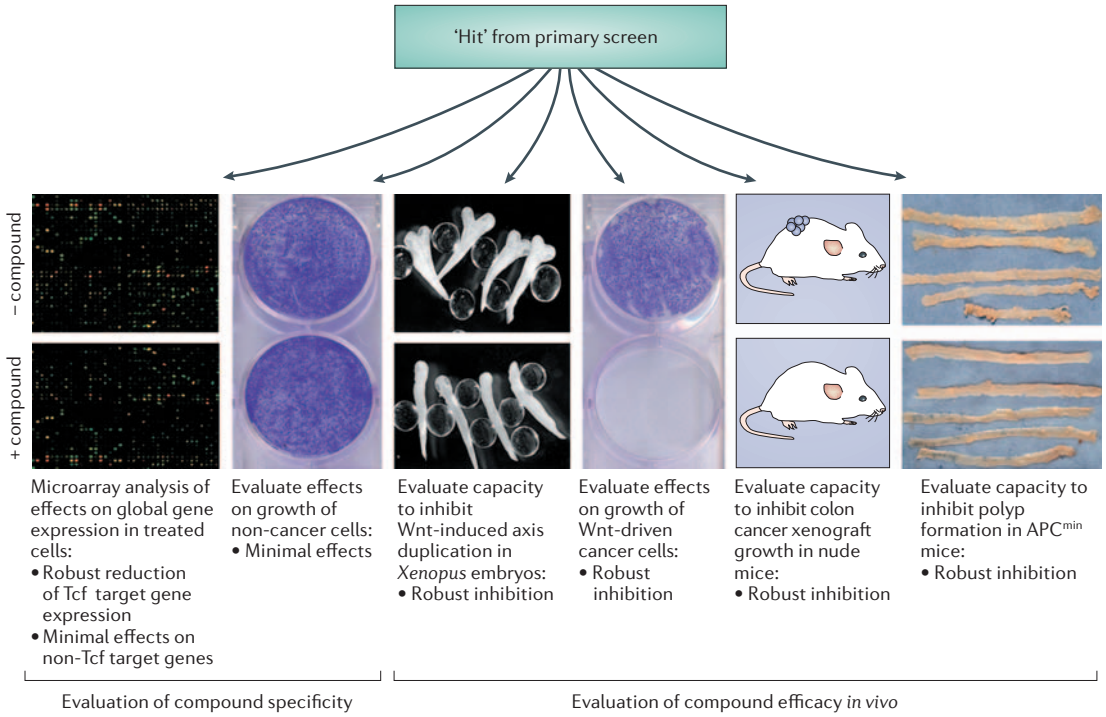


Figure 5 | **Secondary assays for evaluating potential Wnt inhibitors identified in primary high-throughput screens.** Compounds identified as inhibitors of the aberrant Wnt signalling activity in primary high-throughput screens (HTS) are further evaluated for their specificity and *in vivo* efficacy. Selective inhibition of Wnt target gene expression in treated cancer cells and minimal adverse effects on the growth of non-cancer cells in the laboratory are important predictors of compound specificity. Robust inhibition of Wnt-induced axis duplication in *Xenopus* embryos and the capacity to effectively block cancer cell growth in the laboratory and in rodent cancer models are the desired qualities of compounds to be developed as drugs for treating Wnt-driven cancers.

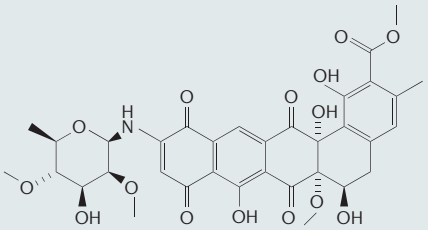
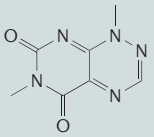
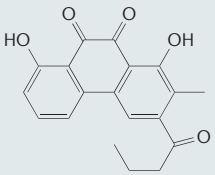
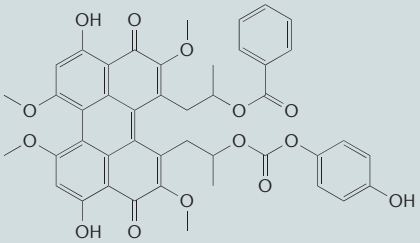
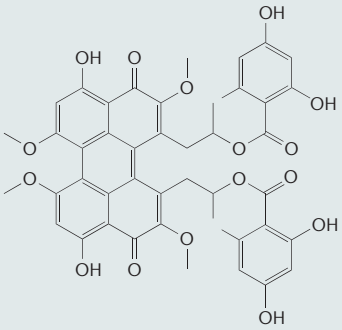
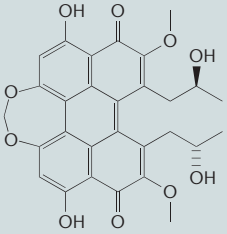
when using a therapeutic regimen based on such potent toxins as DTA or ricin, was achieved through the use of an optimized Tcf-responsive promoter that demonstrated minimal activity in cells lacking Tcf–β-catenin signalling. Using a related approach, researchers have engineered adenoviruses to selectively express enzymes converting prodrugs into potent cytotoxic drugs within cancer cells with active Tcf–β-catenin signalling. One such adenovirus expressing the thymidine kinase (TK) gene under control of a Tcf-responsive promoter selectively killed colon cancer cells treated with the prodrug ganciclovir (converted to an active drug by the actions of TK)[135]. This combination therapy successfully repressed growth of a colon cancer xenograft in immune-deficient mice, with minimal effect on the growth of a liver cancer lacking Tcf–β-catenin activity.

An alternative approach has been to generate oncolytic adenoviruses that selectively replicate in cancer cells with aberrantly high Tcf–β-catenin activity. These adenoviruses infect target cells, replicate and ultimately destroy the host cells by causing them to lyse. The resulting local spread of the virus within the tumour maximizes the therapeutic benefit of the initial inoculation. Selective replication is usually forced on the viruses by replacing the promoters of various essential genes with synthetic promoters containing multiple Tcf-responsive elements. The validity of this approach was confirmed by a study in which adenoviruses engineered to express viral E1B and E2 genes from promoters containing several Tcf-responsive elements were found to replicate efficiently in a colon cancer cell line, but demonstrated a 50–100-fold reduced replication in a lung cancer cell line and normal fibroblasts lacking Tcf–β-catenin signalling activity[136]. Activation of Tcf–β-catenin signalling in the fibroblasts by expression of oncogenic β-catenin permitted efficient replication of the adenovirus. Similar adenoviruses engineered to carry Tcf-responsive elements in the promoters of multiple viral genes show markedly enhanced selectivity (100,000-fold) for tumour cells with constitutive activation of the Wnt signalling pathway[137].

In an effort to increase the therapeutic efficacy of these selectively replicating oncolytic adenoviruses, researchers have further engineered them to express genes that enhance viral spread within the tumours, and to express enzymes that convert prodrugs into highly cytotoxic drugs within the tumours[138]. Recent examples include selectively replicating adenoviruses expressing genes encoding cytosine deaminase, which converts the prodrug 5-fluorocytosine into the highly cytotoxic drug 5-fluorouracil, and *Escherichia coli* nitroreductase which activates the prodrug CB1954 (Cancer Research Campaign/Cobra)[139,140]. These adenoviruses in combination with prodrug treatment efficiently and selectively killed colon cancers with active Tcf–β-catenin signalling, but spared human lung fibroblasts. In the latter study, intravenous injection of the adenovirus followed by treatment

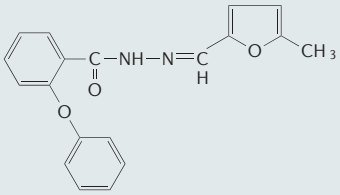
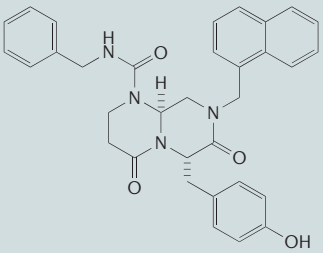
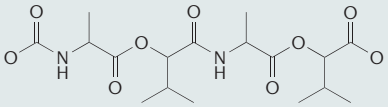
with the CB1954 prodrug successfully suppressed the growth of a colon cancer tumour in a mouse xenograft model[140]. A further marked reduction in tumour growth was achieved by oral administration of the rapamycin inhibitor RAD001 (Everolimus; Novartis) in combination with adenovirus/CB1954 treatment, highlighting the potential of using adenovirus-based combination therapies to achieve maximal therapeutic efficacy[140].

Table 2a | **Small-molecule inhibitors of the Wnt signalling pathway**

Name	Structure	Screening method	IC_{50} (μM)*	Interaction target
ZTM000990		ELISA-based HTS of 7,000 natural compounds[115]	0.64	β-catenin–Tcf
PKF118-310		ELISA-based HTS of 7,000 natural compounds[115]	0.8	β-catenin–Tcf
PKF118-744		ELISA-based HTS of 7,000 natural compounds[115]	2.4	β-catenin–Tcf
PKF115-584		ELISA-based HTS of 7,000 natural compounds[115]	3.2	β-catenin–Tcf
PKF222-815		ELISA-based HTS of 7,000 natural compounds[115]	4.1	β-catenin–Tcf
CGP049090		ELISA-based HTS of 7,000 natural compounds[115]	8.7	β-catenin–Tcf

*Concentration required to reduce Tcf–β-catenin activity by 50% in colon cancer cells. CBP, cAMP-responsive element binding protein (CREB) binding protein; DVL, Dishevelled; ELISA, enzyme-linked immunosorbent assay; HTS, high-throughput screening; ND, not determined; Tcf, T-cell factor.

Table 2b | **Small-molecule inhibitors of the Wnt signalling pathway**

Name	Structure	Screening method	IC_{50} (uM)*	Interaction target
PNU-74654		*In silico* screen of 18,000 synthetic compounds[116]	ND	β-catenin–Tcf
ICG-001		Cell-based HTS of 5,000 synthetic compounds[122]	3.0	CBP–β-catenin
NSC668036		*In silico* screen of 250,000 drug-like compounds[128]	ND	Dishevelled–Frizzled

*Concentration required to reduce Tcf–β-catenin activity by 50% in colon cancer cells. CBP, cAMP-responsive element binding protein (CREB) binding protein; ELISA, enzyme-linked immunosorbent assay; HTS, high-throughput screening; ND, not determined; Tcf, T-cell factor.

Tcf–β-catenin target genes as therapeutic targets. Aberrant Tcf–β-catenin signalling activity is considered to drive cancer formation by altering expression of a limited set of target genes controlling cell proliferation, differentiation, migration and apoptosis. During the past 8 years, the identity of the Tcf–β-catenin target gene program has gradually been revealed, providing valuable clues as to how deregulated Tcf–β-catenin signalling influences cancer initiation and progression (see Wnt Target Gene Overview, Further information). Micro-array analyses of Tcf-dependent gene expression in human colon cancer cells currently suggest that the target gene program comprises 300–400 genes with diverse functions[2,3]. Some of these target genes, such as *c-MYC* and *cyclin D1*, are directly implicated in driving cancer formation. Elevated expression of *c-MYC* in the intestine is thought to disrupt the fine balance between cellular proliferation, differentiation and apoptosis, resulting in unrestricted cell growth and cancer initiation[2,141]. Deletion of *c-MYC* in mouse intestine epithelium efficiently suppresses the increased cell proliferation and altered cell migration that otherwise occurs as a result of aberrant Tcf–β-catenin signalling[127]. This indicates that loss of *c-MYC* removes any selective advantage that aberrant Tcf–β-catenin signalling confers on intestinal epithelial cells and highlights the therapeutic potential of strategies aiming to inhibit *c-MYC* function *in vivo*. Current approaches are focusing on reducing *c-MYC* RNA levels in cancer cells using RNA interference (RNAi) or small membrane-permeable antisense molecules[142]. AVI-4126 (AVI BioPharma) is one example of a *c-MYC*-specific antisense molecule that efficiently suppresses the growth of a variety of cancer cells, including prostate, breast, melanoma and liver[143]. However, the efficacy of such agents in treating Wnt-driven cancers, including colon cancer, remains to be established. Combination therapies with *c-MYC* antisense molecules and chemotherapeutic agents are currently being evaluated for human cancers. Initial studies with colon cancer cell lines highlight the need for caution when designing such treatment regimes because reduction of *c-MYC* levels sensitizes colon cancers to chemotherapeutics such as vinblastine, but reduces the efficacy of others such as 5-fluorouracil and camptothecin[144–146].

Cyclin D1 is an important regulator of the cell cycle that is highly expressed in human cancers as an indirect consequence of Tcf–β-catenin signalling[1,147]. Unlike *c-MYC*, deletion of *cyclin D1* in the mouse small intestine does not prevent the formation of early adenomas resulting from hyperactivation of the Tcf–β-catenin signalling pathway[147]. However, loss of cyclin D1 does efficiently suppress the subsequent growth of these early adenomas, indicating that elevated *cyclin D1* levels probably enhance the growth of established colon tumours *in vivo*. In agreement with this, antisense reduction of cyclin D1 levels in a human colon cancer cell line reduces tumour growth *in vitro* and *in vivo*, highlighting the therapeutic potential of drugs designed to achieve this in cancer patients. The cyclin-dependent kinase (CDK) small-molecule inhibitor *R*-roscovotine (CYC202) is also reported to reduce cyclin D1 protein levels and efficiently suppress the growth of various colon cancer cell lines *in vitro*[148]. Future development of

Antisense
DNA or RNA that is manipulated to be complementary to a target mRNA. Antisense techniques are used to inhibit the expression of genes in a sequence-specific fashion.

specific cyclin D1 inhibitors or inhibitors of its enzyme partners CDK4/CDK6 will hopefully clarify the therapeutic promise of this target.

Other Tcf target genes such as *CD44*, *c-MYB*, peroxisome proliferator-activated receptor-δ (*PPARδ*), *COX2* and matrix metalloproteinase 7 (*MMP7*) are also likely to contribute to cancer formation or progression and might be amenable to therapeutic intervention. However, there are undoubtedly other therapeutic gems waiting to be discovered within the 300–400 genes comprising the Tcf–β-catenin target gene program. Many of these genes are poorly characterized and will require extensive preclinical validation to assess their therapeutic value. Typically this will involve loss-of-function approaches (such as antisense, RNAi and gene knockout) to assess target gene contribution to cancer cell growth *in vitro* or in rodent cancer models such as the APC^{min} mouse. Ideally, loss of function should have minimal effect on non-cancer cell growth to reduce the risk of side effects of future therapeutics. Other target genes might not be directly involved in tumour formation/progression, but will encode membrane proteins that make excellent therapeutic targets for antibody-based therapies. Validated targets for antibody-based therapies should be highly expressed in human cancer tissues relative to matched normal tissue and demonstrate a restricted tissue distribution pattern to minimize the potential for side effects. It is also crucial that expression of these targets is maintained on more advanced cancers and metastases to maximize the therapeutic benefit of the antibody therapeutics. This is certainly not always the case, as highlighted by the Tcf–β-catenin target gene *EPHB3*, which is highly expressed on adenomas and early stage colon cancers, but is dramatically downregulated in more advanced colon cancers and liver metastases[149]. Micro-array analysis recently demonstrated that approximately one-third (121 genes) of the target gene program activated by aberrant Tcf–β-catenin signalling in colon cancer cell lines are more highly expressed in both patient adenoma and adenocarcinoma tissues compared with matched normal tissues[3]. Lists such as this will be an invaluable resource when selecting target genes for further validation as potential therapeutic targets.

Summary and future outlook

The quest for safe, effective drugs that block the effects of constitutive Wnt signalling activity in human cancers has clearly gathered momentum in recent years. The complexity of the Wnt pathway makes it amenable to therapeutic intervention at many levels, ranging from inhibition of receptor–ligand interactions at the cell surface to disruption of Tcf–β-catenin complex formation and inactivation of target genes in the nucleus. Drugs designed to disrupt Tcf–β-catenin transcription factor complexes hold perhaps the greatest promise for treating the entire range of cancers with aberrant Wnt signalling. Clearly, there is still some way to go before these drugs become a reality. However, the natural compounds identified as potent inhibitors of the Tcf–β-catenin interaction in colon cancer cells represent an important landmark in the race to develop specific small-molecule inhibitors of the Wnt pathway[115]. Future challenges will be to identify small molecules with improved selectivity for the Tcf–β-catenin interaction to limit the potential for side effects resulting from disruption of other β-catenin complexes that are essential for maintaining cell adhesion and regulation of the Wnt pathway in non-cancer tissues. Related strategies directed at curbing the transcriptional activity of Tcf–β-catenin complex in cancer cells using small molecules to block the recruitment of essential transcription co-factors are attractive alternatives that deserve more intense exploration. Many of these co-factors, such as CBP, BCL9, TBP, BRG1, Hyrax and Mediator are predicted to bind β-catenin at regions independent of those mediating the interaction with APC and E-cadherin, highlighting the potential for selective inhibition[120,122–126].

HTS of synthetic and natural compound libraries, combined with structure-assisted design of small molecules, should provide new candidates for evaluation as cancer therapeutics in the near future. Encouragingly, the websites of several biotechnology companies (such as Prolexys, Avalon Pharmaceuticals and Curis) boldly herald drug development programmes evaluating novel, proprietary small-molecule inhibitors of the Wnt pathway as potential cancer therapeutics, although such claims should of course be treated with caution in the absence of accompanying scientific data.

The scope for therapeutic intervention in cancers harbouring mutations in the downstream Wnt pathway components APC, Axin or β-catenin broadened considerably recently following the discovery that constant Wnt signalling activity driven by Wnt–Fzd complex formation at the membrane is essential for maintaining the growth of colon cancer cells[18,19]. This should markedly increase the appeal of developing antibody-based therapies that disrupt Wnt–Fzd receptor complexes, or small-molecule inhibitors of essential upstream events such as Fzd–Dsh complex formation, as potential treatments for a wide-range of Wnt-addicted cancers.

Drugs developed to block aberrant Tcf–β-catenin signalling activity undoubtedly have great potential as effective cancer therapeutics, but their prolonged use in patients might carry the danger of side effects in organs such as the skin and intestine, where Tcf–β-catenin signalling activity is crucial for tissue renewal. The challenge will therefore be to define the therapeutic window in which these inhibitors achieve their anticancer effects while minimizing the impact on non-cancer tissues. The recent success in defining the Tcf–β-catenin target gene program inappropriately activated in cancer cells also provides us with an excellent opportunity to develop more selective therapies built around individual target genes. The success of this approach will depend on the identification of target genes whose functions are crucial for cancer cell survival, but dispensable for normal cell growth. Membrane-expressed target genes expressed at high levels on cancer cells are also promising targets for antibody-based therapies.

The development of therapeutics specifically targeting the aberrant Wnt pathway in cancer cells is still largely in its infancy, with no drugs currently in late-stage clinical trials that we are aware of. However, existing drugs such as NSAIDS and vitamin A/D derivatives also show promise in treating Wnt-addicted cancers and have the advantage of already being in clinical use for other disorders. New-generation derivatives of these drugs are currently being evaluated for improvements in safety and efficacy in clinical trials, and will hopefully accelerate the development of effective cancer therapies in the near future.

Given the notorious molecular diversity of cancer, it might be prudent to combine drugs targeting the Wnt pathway with more conventional treatments such as chemotherapy. Combination therapies including Notch pathway inhibitors (γ-secretase inhibitors) are also likely to be very effective treatments for colon cancer[150].

Within the next few years the true potential of drugs targeting the Wnt pathway as cancer therapeutics should be revealed as candidates are (hopefully) pushed out of the laboratory and into clinical trials.

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Competing interests statement
The authors declare no competing financial interests.

DATABASES

The following terms in this article are linked online to:
Entrez Gene:
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
β-catenin | APC | Axin 1 | Axin 2 | BCL9 | BRG1 | CDK4 | CDK6 | CK1α | *c-MYC* | COX2 | cyclin D1 | E-cadherin | *EPHB3* | FADD | FZD1 | FZD2 | GSK3β | Hyrax | *MMP7* | *PPARδ* | RAR | RXR | *survivin* | TK | WNT1 | WNT2 | WNT3A
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CORRIGENDUM

Mining the Wnt pathway for cancer therapeutics

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In Table 2b on page 1009, there is an error in the structure of the compound NSC668036. The correct structure is shown below.