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Regulation of tumorigenic Wnt signaling by cyclooxygenase-2, 5-lipoxygenase and their pharmacological inhibitors: A basis for novel drugs targeting cancer cells?

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ABSTRACT

Canonical Wnt signaling is a highly conserved pathway with a prominent role in embryogenic development, adult tissue homeostasis, cell polarization, stem cell biology, cell differentiation, and proliferation. Furthermore, canonical Wnt signaling is of pivotal importance in the pathogenesis of a number of cancer types and crucially affects tumor initiation, cancer cell proliferation, cancer cell apoptosis, and metastasis.

Reports over the last decade have provided strong evidence for a pathophysiological role of Wnt signaling in non-malignant classical inflammatory and neurodegenerative diseases.

Although, several agents suppressing the Wnt pathway at different levels have been identified, the development of clinically relevant Wnt-inhibiting agents remains challenging due to selectivity and toxicity issues.

Several studies have shown that long-term administration of non-steroidal anti-inflammatory drugs protects against colon cancer and potentially other tumor types by interfering both with the COX and the Wnt pathway.

Our own studies have shown that non-steroidal anti-inflammatory drugs suppress Wnt signaling by targeting the pro-inflammatory enzyme 5-lipoxygenase which is the key enzyme pathophysiologically involved in the synthesis of leukotrienes.

Furthermore, we found a direct link between the 5-lipoxygenase and Wnt signaling pathways, which is essential for the maintenance of leukemic stem cells. Accordingly, genetic and pharmacological inhibition of 5-lipoxygenase led to an impairment of Wnt-dependent acute and chronic myeloid leukemic stem cells.

We believe that 5-lipoxygenase inhibitors might represent a novel type of Wnt inhibitor activating a potentially naturally occurring novel mechanism of suppression of Wnt signaling that is non-toxic, at least in mice, and is potentially well tolerated in patients.

Abbreviations: AML, acute myeloid leukemia; APC, adenomatous polyposis coli; CBP, CREB-binding protein; CK1, casein kinase 1; CLL, chronic lymphatic leukemia; CML, chronic myeloid leukemia; COX, cyclooxygenase; CSC, cancer stem cell; Dvl, disheveled; FLAP, 5-LO-activating protein; Fzd, Frizzled; GSK3β, glycogen synthase kinase 3β; HSC, hematopoietic stem cell; LEF, lymphoid enhancer factor; LSC, leukemic stem cell; 5-LO, 5-lipoxygenase; LRP, low density lipoprotein receptor-related protein; LT, leukotriene; NSAIDs, non-steroidal anti-inflammatory drugs; NF-κB, nuclear factor-κB; PG, prostaglandin; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PPAR, peroxisome proliferator activated receptor; TCF, T-cell factor.

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Contents

1. Introduction

Canonical Wnt signaling is of pivotal importance in the pathogenesis of a number of cancer types and crucially affects tumor initiation, cancer cell proliferation, cancer cell apoptosis, self-renewal of leukemic cells and metastasis. We and others have shown that the Wnt/β-catenin signaling pathway is the key signaling pathway in many cancers, particularly in those involving maintenance of cancer stem cells (CSC) (Muller-Tidow et al., 2004; Wang et al., 2010). CSCs represent a small portion of the total tumor mass in solid as well as hematological cancers (Tirino et al., 2013). They are thought to persist as a distinct population in the remaining tumor tissues after destruction of the bulk of tumor mass by conventional chemotherapy. They might trigger disease relapse and metastasis by generating novel tumor cells (Tirino et al., 2013). For this reason, therapeutics specifically targeting cancer stem cells have the potential for significant improvement of future cancer therapies.

Considerable efforts have been undertaken to identify novel therapeutics that could interfere with oncogenic Wnt signaling (B. Chen et al., 2009). Decades have been spent by both the pharmaceutical industry and academic researchers who have used high-throughput screens to identify small-molecule inhibitors targeted to different Wnt signaling pathway constituents. Regrettably, only one compound, namely PRI-724 (Prism Pharma Co, Ltd/Eisai, Table 1 and Supplementary Table 1), which directly disrupts the transcriptional co-factor function of β-catenin has been developed and entered phase I clinical trials (Safety and Efficacy Study of PRI-724 in Subjects with Advanced Solid Tumors; ClinicalTrials.gov Identifier: NCT01302405). Other Wnt-suppressive drug candidates interfere with Wnt signaling at the level of the Wnt ligand binding or ligand-induced receptor activation. Some of these compounds were/are also included in recent clinical trials (see Section 6). However, the potential side-effects and selectivity of the suppressive effects of this class of agents on the canonical Wnt pathway still remain unclear.

Non-steroidal anti-inflammatory drugs (NSAIDs), such as sulindac, indomethacin, and celecoxib, are able to inhibit Wnt signaling at high concentrations and several mechanisms have been proposed to explain these effects (see Section 7). We recently found that these drugs, at somewhat higher concentrations than those required to inhibit cyclooxygenase (COX) enzymes, also suppress 5-LO product formation suggesting a novel mechanistic linkage between the 5-LO pathway and

Table 1
Selected Wnt signaling inhibitors, their targets and current stage of development.

Agent	Target	Current Stage	References
OMP-18R5	Fzd receptors	Clinical trials (NCT01345201, NCT02005315, NCT01957007, NCT01973309)	(Gurney et al., 2012)
OMP-54F28	Wnt proteins	Clinical trials (NCT01608867, NCT02069145, NCT02092363, NCT02050178)	(Smith et al., 2013; Yeung et al., 2014)
LGK974	Porcupine	Clinical trial (NCT01351103)	(Liu et al., 2013)
IWP-L6	Porcupine	In vivo (zebrafish)	(X. Wang et al., 2013)
Salinomycin	LRP6	In vivo (xenograft model)	(Mao et al., 2014; Wang et al., 2012)
Niclosamide	LRP6	In vivo (xenograft model)	(W. Lu et al., 2011; Osada et al., 2011; Sack et al., 2011)
NSC668036	Dvl	In vivo (*Xenopus* embryos)	(Shan et al., 2005)
3289-8625	Dvl	In vivo (*Xenopus* embryos)	(Grandy et al., 2009)
FJ9	Dvl	In vivo (xenograft model)	(Fujii et al., 2007)
XAV939	Tankyrase 1/2	In vitro (cancer cell lines)	(Huang et al., 2009)
JW55	Tankyrase 1/2	In vivo (mice)	(Waaler et al., 2012)
IWR-1	Tankyrase 1/2, axin	In vivo (zebrafish)	(B. Chen et al., 2009)
Pyrvinium	CK1α	In vivo (xenograft models)	(Thorne et al., 2010; Wiegering et al., 2014)
PKF115-584, CGP049090	β-Catenin/TCF	In vivo (*Xenopus* embryos)	(Lepourcelet et al., 2004)
PNU-74654	β-Catenin/TCF	In vivo (zebrafish)	(Trosset et al., 2006; Buikema et al., 2013; Pradhan & Olsson, 2014)
BC21	β-Catenin/TCF	In vitro (HCT116 cell line)	(Tian et al., 2012)
iCRT3, iCRT5	β-Catenin/TCF	In vitro (colon cancer cell lines, primary human colon cancer specimens)	(Gonsalves et al., 2011)
iCRT14	β-Catenin/TCF	In vivo (xenograft models)	(Gonsalves et al., 2011)
StAx peptides	β-Catenin/TCF	In vitro (colorectal cancer cell lines)	(Grossmann et al., 2012)
Resveratrol	β-Catenin/TCF antioxidant	Clinical trial (NCT00256334, NCT00433576)	(Chen et al., 2012)
ICG-001	CBP	In vivo (xenograft model)	(Emami et al., 2004; Arensman et al., 2014)
PRI-724	CBP	Clinical trials (NCT01606579, NCT01764477, NCT02195440, NCT01302405)	(El-Khoueiry et al., 2013)
SAH-BCL9	β-Catenin/Bcl9	In vivo (xenograft model)	(Takada et al., 2012)
CWP232291	Sam68	Clinical trial (NCT01398462)	JW Pharmaceuticals

oncogenic Wnt signaling (Roos et al., 2014). Furthermore, we and others showed that pharmacological or genetic targeting of 5-LO efficiently interferes with Wnt signaling and maintenance of leukemic stem cells (LSCs) in chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) (Roos et al., 2014).

In the present review, we aim to simplify this recent but rather complex subject in order to appeal to a larger number of readerships and maximize the interest in the topic. We provide a brief introduction to Wnt signaling and its role in tumorigenesis, as well as an overview of available Wnt inhibitors and their molecular mechanisms of Wnt signaling suppression with focus on drugs interfering with eicosanoid biosynthesis. Emphasis is placed on the recently discovered role of 5-LO in regulating the canonical Wnt pathway. Finally, we discuss the possible clinical implications of these findings for new stem-cell specific therapeutics in oncology.

2. The canonical Wnt pathway

The Wnt signaling pathway is a highly conserved pathway with a prominent role in embryogenic development, adult tissue homeostasis, cell polarization, stem cell biology, cell differentiation, and proliferation (Nusse & Varmus, 1992; Cadigan & Nusse, 1997; van de Wetering et al., 2002; Clevers, 2006; Clevers & Nusse, 2012). Traditionally, the Wnt signaling pathway is separated into two routes; the non-canonical and the canonical pathways (see Section 2.1). They differ in their dependency on the protein β-catenin. The non-canonical pathways, such as the planar cell polarity (PCP) and Ca^{2+}-pathway, regulate processes such as cell movement, migration (Kuhl et al., 2001; Mikels & Nusse, 2006; Angers & Moon, 2009), cell orientation (Seifert & Mlodzik, 2007; Angers & Moon, 2009), and function through an β-catenin independent mechanism.

2.1. Overview of the canonical Wnt/β-catenin pathway

In the canonical pathway (Fig. 1), β-catenin is the key effector, which is responsible for the transduction of the signal to the nucleus. At this site, β-catenin triggers the transcription of Wnt-specific genes responsible for the control of cell fate decisions in many cells and tissues (Valenta et al., 2012). In addition to its role in the Wnt signaling pathway, β-catenin is associated with cadherins such as E-cadherin. β-Catenin connects cadherins with the actin cytoskeleton (through α-catenin), forming adherents junctions. It is important for cell stability, cell migration and adhesion (Harris & Peifer, 2005; Wilusz & Majka, 2008).

2.2. Regulation of the Wnt/β-catenin pathway

If not in complex with E-cadherin, β-catenin is targeted for rapid degradation by the proteasome, a protein complex involved in the elimination of mainly dysfunctional cellular proteins (Fig. 1A) (Aberle et al., 1997). This breakdown is mediated by a multiprotein destruction complex which includes the tumor suppressor proteins, adenomatous polyposis coli (APC) and axin, glycogen synthase kinase 3 (GSK3β) and casein kinase 1α (CK1α) (McDonald & Silver, 2009; Clevers & Nusse, 2012), together with the serine/threonine phosphatase A_2 (PPA2) (Luo et al., 2007; Su et al., 2008). APC and axin function as a scaffold, facilitating GSK3β- and CK1α-mediated phosphorylation of critical residues within β-catenin (Kimelman & Xu, 2006; Roberts et al., 2011). In particular, CK1α phosphorylates β-catenin at Ser45, priming the sequential phosphorylation of Thr41, Ser37, and Ser33 by GSK3β (Liu et al., 2002; Xing et al., 2003; Valenta et al., 2012; Stamos & Weis, 2013). Subsequently, β-catenin interacts with the ubiquitin machinery via its phosphorylated Ser33 and Ser37 residues. As a consequence, β-catenin is ubiquitinated and subsequently degraded by the 26S proteasome (Aberle et al., 1997; Hart et al., 1999). The phosphatase PP2A, also associated with axin and/or APC, counteracts the phosphorylation-induced degradation of β-catenin by directly dephosphorylating β-catenin at the critical residues important for proteasomal degradation. Furthermore, PP2A binds APC and is suggested to dephosphorylate the APC protein leading to impaired affinity of APC to β-catenin (Seshacharyulu et al., 2013). Recently, Huang et al. discovered that the two poly-ADP-ribosylating enzymes, tankyrase 1 and tankyrase 2, bind to a highly conserved domain of axin and trigger its degradation through the ubiquitin–proteasome pathway, thereby, releasing β-catenin (Huang et al., 2009).

The Wnt/β-catenin pathway is activated when a secreted Wnt glycoprotein binds to the N-terminal extracellular cysteine-rich domain of a Frizzled (Fzd) receptor (Fig. 1B). Overall, there are currently 19 Wnt ligands and 10 Fzd receptors that have been identified in humans (Silva-Garcia et al., 2014). Upon binding of the Wnt ligand to Fzd, the co-receptor, low density lipoprotein receptor-related protein 5/6 (LRP5/6), interacts with Fzd and initiates a cascade of events that results in disruption of the β-catenin destruction complex. As consequence, β-catenin is stabilized and is facilitated in its nuclear translocation. Activation of the Fzd receptor leads to phosphorylation of disheveled (Dvl) which causes direct binding of Dvl to the receptor complex (Cruciat, 2014). Dvl subsequently multimerizes and induces the formation of the so-called LRP-associated Wnt signalosome (Bilic et al., 2007). This process triggers LRP6 phosphorylation by CK1 (Davidson et al., 2005; Bilic et al., 2007). Phosphorylated LRP6 serves as a docking site for axin (Mao et al., 2001; Tamai et al., 2004; Davidson et al., 2005; Zeng et al., 2005) which recruits the β-catenin destruction complex to signalosomes (Niehrs, 2012). Furthermore, phosphorylation of LRP5/6 leads to suppression of GSK3β activity by direct binding of the enzyme to the co-receptor (Piao et al., 2008). Both events protect β-catenin from GSK3β-mediated phosphorylation, which allows β-catenin to accumulate in the cytoplasm and translocate into the nucleus (Taelman et al., 2010).

Nuclear β-catenin forms a complex with the members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors. This complexed β-catenin acts as a transcriptional co-activator by displacing co-repressors like Groucho and histone deacetylases (HDACs) (Brantjes et al., 2001; Daniels & Weis, 2005; Archbold et al., 2012; Cadigan, 2012) from TCF/LEF. Furthermore, complexed β-catenin recruits other co-activators such as Bcl9/Legless (Lgs), Pygopus (Pyg) (Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002), and CREB-binding protein (CBP)/p300 (Hecht et al., 2000; Takemaru & Moon, 2000) to the TCF/LEF transcription factor. These events trigger DNA binding and transcription of the target genes, including c-myc, cyclin D1 and axin 2 (Lustig et al., 2002; Davidson et al., 2005; Bilic et al., 2007). For further information, the following website offers an extensive list of Wnt target genes: http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes.

3. The role of Wnt signaling in tumor cell growth

Wnt signaling plays a pivotal role in tumor cell growth by regulating a number of genes involved in programmed cell death (Pecina-Slaus, 2010), cell cycle control, and angiogenesis (Clifford et al., 2008).

Gain or loss-of-function mutations of several members of the Wnt/β-catenin signaling pathway that lead to constitutive activation of β-catenin have been shown to have oncogenic effects in solid tumors (Peifer & Polakis, 2000; Giles et al., 2003). Mutations in β-catenin were first uncovered in colorectal cancer (Rubinfeld et al., 1993; Su et al., 1993) and later – with a lower frequency – also in other human tumor entities (Peifer & Polakis, 2000; Giles et al., 2003; Luu et al., 2004). Furthermore, Wnt signaling plays a crucial role in the process of leukemogenesis (see Section 3.4).

3.1. Cell survival

Programmed cell death constitutes a fundamental cellular event involved in the regulation of various physiological processes (Lockshin &

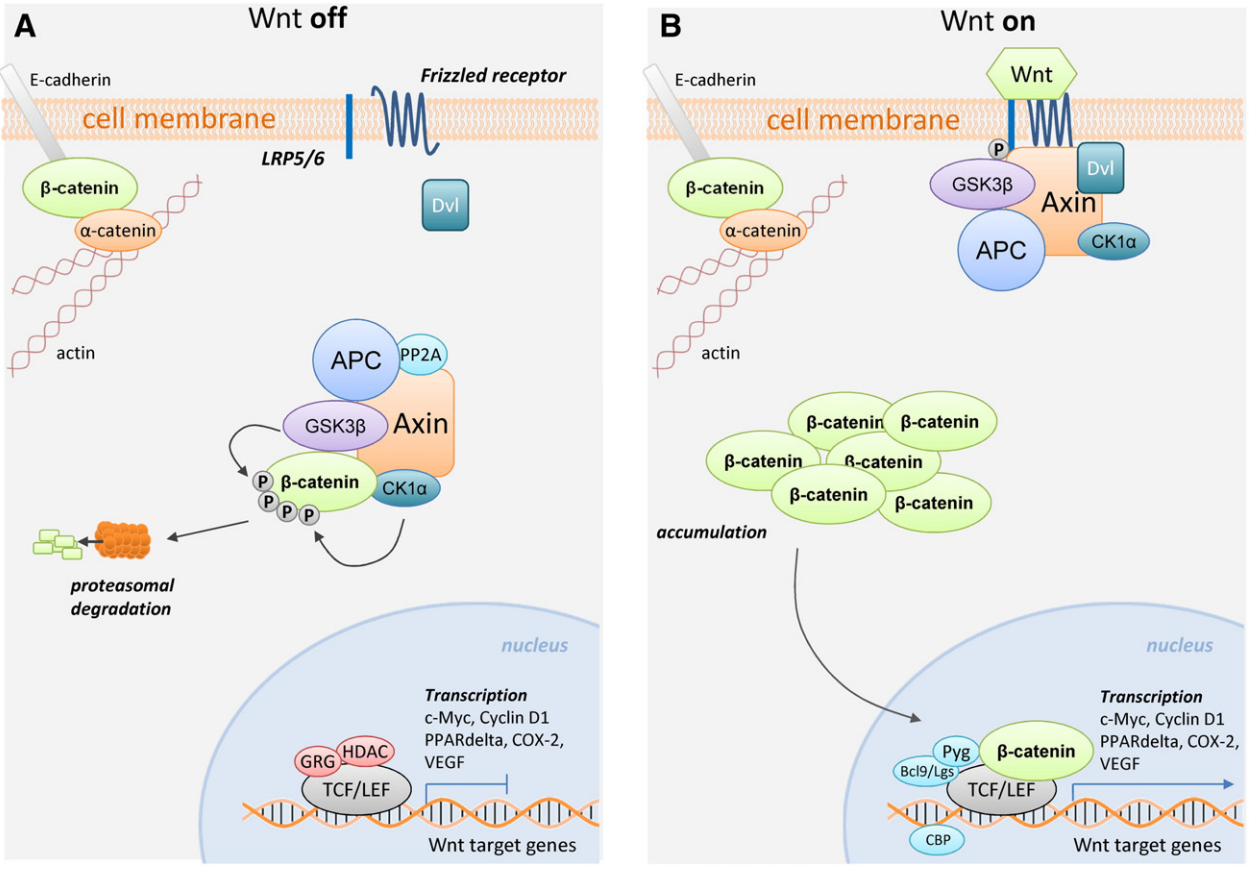

Fig. 1. A simplified overview of the Wnt/β-catenin signaling pathway. In the absence of a Wnt ligand (**A**), β-catenin is either bound to E-cadherins connecting them to the actin cytoskeleton or bound in a multiprotein destruction complex, consisting of axin, APC, GSK3β, CK1α, PP1, and PP2A. In this complex, β-catenin is sequentially phosphorylated (P) at different Thr and Ser residues by CK1α and GSK3β. This phosphorylation creates a binding site for E3 ubiquitin ligase β-TrCP, leading to β-catenin ubiquitination and therefore, proteasomal degradation. The transcription of the Wnt target genes is repressed by binding of co-repressors (i.e. Groucho and HDACs) to the TCF/LEF transcription factor. The binding of a Wnt ligand (**B**) to its receptors, Fzd and LRP5/6, results in the activation of Dvl. Dvl is then recruited to the receptor complex which leads to LRP6 phosphorylation (P) and recruitment of the β-catenin destruction complex to the receptors (currently, there is only evidence for binding of axin, APC, GSK3β, and CK1α to the receptor complex). Thereby, the phosphorylation of β-catenin is inhibited, allowing the free protein to accumulate in the cytosol and translocate to the nucleus where it can act as a co-activator for TCF/LEF-mediated transcription. For this, β-catenin replaces the co-repressors while recruiting various cofactors, including Bcl9/Lgs and Pygopus. APC: adenomatous polyposis coli proteins and; the kinases, GSK3β: glycogen synthase kinase 3β; CBP, CREB-binding protein; CK1α: casein kinase α; Dvl, disheveled; GRG, Groucho; HDAC, histone deacetylase; Fzd, Frizzled; LRP5/6, low density lipoprotein receptor-related protein 5/6; PP2A, phosphatase A_2; Pyg, Pygopus; TCF/LEF, T-cell factor/lymphoid enhancer factor.

Zakeri, 2007). Among the various forms of programmed cell death, apoptosis represents one of the best characterized and is evolutionary highly conserved (Galluzzi et al., 2015). Since tissue homeostasis is the result of a tight balance between cell proliferation and cell death, even small alterations in the rate of apoptosis may significantly impact the overall cell number (Fulda, 2009).

Apoptosis can be viewed as a safeguard mechanism to limit abnormal tissue growth and tumor formation. Consequently, cellular ability to evade apoptosis is one of the hallmarks of cancers (Hanahan & Weinberg, 2011). A number of components of the Wnt signaling pathway such as Dvl, β-catenin, and GSK3β have been shown to modulate apoptosis (Li et al., 2006). Furthermore, β-catenin silencing experiments using siRNA approaches identified several Wnt target genes related to apoptosis, including MYBL2, BAG2, BAG3, PTEN, HIF1A, and DAP3 as well as the anti-apoptotic protein Bcl-XL (Huang et al., 2006). Thus, in the majority of cancer cell types, Wnt signaling is considered to promote cell survival and to inhibit apoptosis, though pro-apoptotic effects due to activated Wnt signaling have also been reported (Benchabane & Ahmed, 2009). In this context, it should be noted that, in cancer, not only is apoptosis dysfunctional, but there is also an increase in the activity of survival pathways that promote proliferation and cell growth.

3.2. Cell cycle regulation

Besides resistance to apoptosis, many cancer cells exhibit dysfunction of the cell cycle machinery with regard to the complex series of protein–protein interactions, protein degradation, and phosphorylation events that regulate the transition to the respective four cell cycle phases (i.e., G1, S, G2, M). The major players at these checkpoints are cyclin-dependent kinases (CDK) which are activated by forming complexes with transiently expressed cyclins.

As described above, a key step in the activation of the Wnt pathway is the formation of a complex between nuclear β-catenin and members of the TCF/LEF family of transcription factors (van de Wetering et al., 1997). The continuous presence of the nuclear TCF/β-catenin complex fosters cancer formation by increasing expression of a number of proto-oncogenes regulating cell growth, inhibition of apoptosis, and genes affecting cell shape and cell migration. Consistent with these findings, disruption of TCF/β-catenin complex formation in colon cancer cells hindered target gene activation and inhibited tumor cell growth in vitro (Tetsu & McCormick, 1999; van de Wetering et al., 2002). Increased stability of β-catenin leads to expression of a number of down-stream target genes, such as the G1 cell cycle control regulators, cellular myelocytomatosis (c-myc), and cyclin D1 (Reya & Clevers,

2005). C-myc is a proto-oncogene overexpressed in a variety of human cancers including leukemia (Dang, 1999) and plays a role in cell cycle progression, apoptosis, and cellular transformation (Hoffman et al., 2002). Cyclin D1 is an oncogenic cyclin, which is frequently overexpressed or amplified in breast, prostate, and colon cancers (Ortega et al., 2002). Nevertheless, some components of the Wnt signaling cascade also function directly to promote formation of the spindle apparatus during mitosis (Niehrs & Acebron, 2012).

3.3. Tumor vascularization

A crucial role for Wnt signaling in tumor vascularization is supported by the presence of Wnt target genes that encode for pro-angiogenic factors. In particular, the gene encoding for vascular endothelial growth factor A (VEGF-A), a potent and well-characterized pro-angiogenic protein, is directly regulated by the TCF/β-catenin complex (Clifford et al., 2008) and is clearly associated with boosted tumor angiogenesis and a poor prognosis (Gratzinger et al., 2007).

3.4. Leukemic stem cell self-renewal

Although the involvement of β-catenin in carcinogenesis was first discovered in solid tumors, β-catenin overexpression is also highly associated with several leukemic subtypes (Chung et al., 2002). Furthermore, the dysregulation of the Wnt//β-catenin signaling pathway was suggested to play an important role in the self-renewal of LSC in at least AML and CML patients (Lane et al., 2011; Mochmann et al., 2011; Siapati et al., 2011; Scheller et al., 2013).

LSCs are typically rare and possess properties that are distinct from most other tumor cells (Lapidot et al., 1994). These cells can undergo self-renewal to maintain an undifferentiated state. They are also multipotent, highly proliferative (Jordan, 2007), and may originate from normal hematopoietic stem cells (HSC) (Krivtsov et al., 2006; Eppert et al., 2011). Although the role of Wnt/β-catenin signaling in the self-renewal of normal HSCs is under debate (Cobas et al., 2004; Jeannet et al., 2008; Koch et al., 2008), several groups have shown that the Wnt/β-catenin signaling pathway is required for the self-renewal of LSCs derived from either HSC or more differentiated granulocyte macrophage progenitors (GMP) (Muller-Tidow et al., 2004; Malhotra & Kincade, 2009; Wang et al., 2010; Luis et al., 2012). Progression, disease relapse, and drug resistance of leukemic cells depend on LSCs that resist treatment. Aberrant activation of the Wnt/β-catenin self-renewal pathway has been identified to drive human blast crisis LSC propagation (Jamieson et al., 2004; Abrahamsson et al., 2009). Suppression of β-catenin reverts the LSC stage to a pre-LSC-like stage and significantly reduces the growth of human leukemic cells (Yeung et al., 2010). The chemotherapeutic agents that are used today effectively eradicate the blast cells, which represent partially differentiated, usually unipotent precursor cells. However, these agents have very little effect on LSCs. Since the canonical pathway is inactive in most normal cells, inhibition of the Wnt/β-catenin pathway holds promise for developing novel, targeted therapies for LSCs.

Finally, Wnt/β-catenin signaling pathways have been shown to be able to control proliferation, survival, and differentiation of hematopoietic cells (Reya et al., 2003). Taken together, the functional versatility of Wnt/β-catenin signaling is remarkable through its influences, on many developmental levels, including organ and cell specification and differentiation as well as maintenance of stem cell activity.

3.5. Tumor prognosis and outcome

In the last couple of years evidence has been accumulating suggesting that an aberrant regulated Wnt signaling pathway is correlated with the clinical outcome of various cancers such as colorectal cancer (CRC) (Ting et al., 2013; Bruun et al., 2014; Aguilera et al., 2015; Nazemalhosseini Mojarad et al., 2015), AML (Fu et al., 2014; Kuhnl et al., 2015), gliomas (Rossi et al., 2011; Wu et al., 2013; Guo et al., 2015), nasopharyngeal cancer (NPC) (Xu et al., 2013; Ren et al., 2015) and breast cancer (Dey et al., 2013). In particular, it could be shown that overexpression of canonical Wnt signaling components is highly associated with the risk of developing metastases in patients with an aggressive form of breast cancer (Dey et al., 2013). Similar results were obtained in a study of potential prognostic factors in glioblastoma patients.

In this case, the investigators demonstrated that β-catenin is a highly predictive marker of malignant behavior and short survival (Rossi et al., 2011). Aberrant expression of β-catenin and other components and target genes of the Wnt pathway, such as sFRP1, Wnt-2, E-cadherin, c-myc and LGR5, have also been correlated with poor prognosis and tumor aggressiveness in NPC, brainstem glioma, AML and thyroid cancer patients (Wu et al., 2013; Xu et al., 2013; Fu et al., 2014; Michelotti et al., 2015; Ren et al., 2015). Yet not only the expression level, but also the membrane/cytosolic or nuclear localization of β-catenin – which is a sign of activated Wnt signaling – can be a negative prognostic factor, as was shown in patients with CRC (Bruun et al., 2014; Nazemalhosseini Mojarad et al., 2015). Additionally, nuclear DKK1, localized at specific chromatin sites of active transcription, is associated with decreased overall survival rate and progression-free survival after chemotherapy administration (Aguilera et al., 2015). Furthermore, single nucleotide polymorphism (SNPs) in APC and β-catenin (CTNNB1) genes, which are known to alter consensus splicing sites sequences, transposable elements and transcription factor binding sites, are correlated with overall survival rates (Ting et al., 2013). Based on the pathophysiological importance of the Wnt signaling pathway in relation to tumor proliferation, suppression of apoptosis, migration, resistance to chemotherapy and maintenance of cancer stem cells (Roarty & Rosen, 2010; Vermeulen et al., 2010; Alison et al., 2012; Debeb et al., 2012), it is not surprising that an aberrantly activated Wnt signaling pathway is, in many cases, associated with poor prognosis of cancer patients. Taken together, all these findings support the concept of Wnt signaling inhibition as a feasible therapeutic strategy.

4. Role of Wnt signaling in inflammatory disorders

4.1. Disease association

Reports over the last decade have provided strong evidence for a pathophysiological role of Wnt signaling in a number of non-malignant classical inflammatory diseases. In particular, several studies have substantiated a crucial role of Wnt-mediated signaling in the activation of fibroblast-like synoviocytes during the pathogenesis of rheumatoid arthritis, triggering destruction of the articular cartilage and bone (Sen, 2005). Furthermore, Wnt signaling pathway and Wnt regulatory proteins, like Dickkopf, may trigger bony fusion in ankylosing spondylitis and thus, may serve as potential biomarkers of this disease (Corr, 2014).

Changes in the expression of several genes of the Wnt signaling pathway were found to be associated with impaired lung function in children with asthma and may contribute to the pathogenesis of the disease (Sharma et al., 2010). Further mechanistic insight was recently provided by Trischer et al. who showed that Wnt signaling via the Wnt10b ligand is an important modulator of T cell activation during asthma pathogenesis (Trischler et al., 2015). Guo et al. demonstrated that intratracheal administration of the Wnt ligand, Wnt3a, or an antibody raised against Dickkopf-1 activating Wnt signaling in alveolar epithelial cells suppressed macrophage and neutrophil infiltration into lungs (Guo et al., 2015). Pathophysiological roles of Wnt signaling have also been identified in chronic obstructive pulmonary disease (COPD) where increased β-catenin activity in fibroblasts was found to be relevant for the diseases-associated remodeling of the extracellular matrix (Baarsma et al., 2011). Interestingly, over-activity of Wnt signaling in primary bronchial epithelial cells sensitized individuals to

cigarette smoke-induced inflammation, potentially triggering COPD (Heijink et al., 2013).

A crucial role of Wnt signaling pathway components is also evident in inflammatory bowel disease (IBD) and the transition to the malignant stage. Several studies found a stage-specific increased or decreased expression of a number of Wnt pathway-related genes, including Wnt ligands, Fzd and Dickkopf-4, in colonic biopsies of subjects with ulcerative colitis or in IBD-associated colorectal neoplasia as well as in cancers (You et al., 2007, 2008; Claessen et al., 2010).

The literature also supports a role of Wnt signaling in a number of other diseases featuring an inflammatory component. Malgor et al. recently provided evidence for an increase in the protein levels of the Wnt5a ligand in advanced arterial arteriosclerotic lesions, when compared with less advanced arterial lesions. Serum levels of Wnt5a were also significantly higher in patients suffering from atherosclerosis compared to healthy controls, suggesting that Wnt signaling enhances the process of atherosclerotic plaque formation at a late stage (Malgor et al., 2014). Polymorphism or reduced expression of the secreted Frizzled-related protein (sFRP)3 has also been observed in patients with osteoarthritis (Loughlin et al., 2004) and Wnt4 ligand induction has been related to kidney damage or polycystic kidney disease (Rodova et al., 2002; Surendran & Simon, 2003; Terada et al., 2003).

Importantly, Wnt signaling appears to play a pathophysiologically relevant role in neurodegenerative diseases, as Wnt-mediated neuroinflammation triggers infiltration of leukocytes and production of pro-inflammatory mediators in the central and peripheral nervous systems, contributing to neuronal cell damage or death as well as to generation and maintenance of chronic pain (Yuan et al., 2012; Ji et al., 2014).

Wnt is an important regulator of neuroinflammation in the brain controlling the dialog between neurons and glia or glial and neural stem/progenitor cells (NPCs) during the inflammatory processes (Marchetti & Pluchino, 2013). The expression of Wnt ligands and Wnt signaling components has only been identified in astrocyte and macrophage/microglia (Halleskog et al., 2011; L'Episcopo et al., 2011) that can harbor Wnt receptors and respond to Wnt in either pro- or anti-inflammatory manner (L'Episcopo et al., 2014a, 2014b). Indeed, deregulation of Wnt/β-catenin signaling pathway, dysregulation of Wnt/Fzd cascade and increase in inflammatory processes have been proposed as common determinants in some neurodegenerative events of schizophrenia (Miyaoka et al., 1999), Parkinson's (PD)/Alzheimer's Disease (AD) (De Ferrari & Inestrosa, 2000; Chong et al., 2007; De Ferrari et al., 2007; Varela-Nallar et al., 2009; Inestrosa & Arenas, 2010; Kim et al., 2011; Purro et al., 2012) and autistic syndrome (Cao et al., 2012).

Wnt1/β-catenin signaling is relevant in maintaining the integrity of dopaminergic neurons by blocking GSK3β-induced phosphorylation and proteasome degradation of β-catenin. Stabilized β-catenin can translocate into the nucleus and associate with transcription factors to regulate the expression of Wnt target genes (Rawal et al., 2009; Inestrosa & Arenas, 2010).

Furthermore, β-catenin levels are markedly reduced in AD patients carrying presenilin-1-inherited mutations (Zhang et al., 1998) and it has been suggested that similar mutations may disturb β-catenin translocation to the nucleus (Nishimura et al., 1999), likely affecting Wnt activity. In this context it is relevant that Aβ has been reported to inhibit Wnt signaling by directly binding to the Frizzled receptors (Magdesian et al., 2008) and to LRP-5 or LRP-6 leading to the inhibition of GSK3β through a cascade of intracellular reactions that involve protein kinases and adaptor proteins (Toledo & Inestrosa, 2010). In addition, genetic studies revealed that LRP6 polymorphisms are causally linked to AD (De Ferrari & Inestrosa, 2000; De Ferrari et al., 2007). Collectively, the literature of the past decade suggests a pathophysiologically relevant modulatory role of components of the Wnt signaling pathway in a number of classical inflammatory or inflammation-associated diseases including rheumatoid arthritis, bronchial asthma, COPD; IBD, atherosclerosis, ankylosing spondylitis and neuroinflammatory diseases.

4.2. Connection with inflammatory signaling pathways

The mechanistic link between Wnt signaling and classical inflammatory pathways, however, is only poorly understood. Yu et al. showed that Wnt5a and Wnt3a ligands are capable of directly triggering pro-inflammatory cytokine production from murine macrophages in some way through the Toll-like receptor (TLR) 4 (C.H. Yu et al., 2014). Conversely, activation of TLR signaling in alveolar epithelial cells by *Mycobacterium bovis*, Bacillus Calmette–Guerin or purified lipopolysaccharide led to suppression of Wnt/β-catenin signaling and decreased transcriptional activity of nuclear β-catenin complexes. Notably, Wnt4 ligands were recently shown to prevent aging-related bone loss and osteoporosis in an animal model by inhibiting pro-inflammatory nuclear factor-κB (NF-κB) signaling (B. Yu et al., 2014). Very recent mechanistic studies support a role of the Wnt pathway in promoting pro-inflammatory IFN-γ signaling and T cell differentiation in the triggering of ulcerative colitis (Sato et al., 2015). Thus, taken together, these recent data suggest an important role of oncogenic Wnt signaling in regulating inflammatory processes.

This mechanistic linkage sheds new light on the previously well-recognized, but poorly understood role of chronic inflammation as a major risk factor in tumorigenesis. The recent findings indicate that chronic inflammation may facilitate carcinogenesis by enhancing infiltration of immune cells and promoting stromal remodeling and this could be maintained by activation of the Wnt signaling pathway (Hanahan & Coussens, 2012; Briso et al., 2013). Conversely, inflammatory processes may trigger activation of Wnt signaling, thereby enhancing tumor cell proliferation. This may explain, at least partially, why colorectal cancer tends to develop on the background of chronic inflammatory bowel disease (Guina et al., 2015). Cutaneous squamous cell carcinomas are also considered to develop on the basis of underlying inflammatory skin diseases, such as lupus vulgaris (Baldursson et al., 1993; Motswaledi & Doman, 2007). Thus, therapeutics that efficiently target Wnt signaling may act to curtail the onset of a series of inflammation-dependent disorders, including classical inflammatory diseases, neurodegenerative diseases as well as malignancies.

5. Components of the Wnt signaling pathway as potential biomarkers of disease

An increasing number of studies document the use of components of the Wnt signaling pathways as possible biomarkers of the stage, severity and progression of several malignant diseases. LEF-1 may function as a potential marker of malignant transformation to aggressive gliomas (Pecina-Slaus et al., 2014). The Wnt5A ligand was found to be a predictive marker of survival in human cervical cancer (Lin et al., 2014), and high serum levels of Dickkopf-1 reliably indicated a poor prognosis in prostate cancer (Rachner et al., 2014). Furthermore, serum DKK1 levels were significantly lower in patients with lung cancer but rapidly normalized after chemotherapy (Xu et al., 2014). The G-protein coupled receptor LGR5 regulating Wnt signaling may also function as a potential prognostic biomarker of colorectal cancer (He et al., 2014) and Fzd receptors may represent novel surface markers for an aggressive type of neuroblastoma cells with stem-cell like features (Cantilena et al., 2011).

Wnt components, such as β-catenin, not only may provide an indication of stage and severity of the disease, but also may have potential as predictive therapeutic markers of chemoresistance and radioresistance in cutaneous squamous cell carcinoma (CSCC) (Y. Zhang et al., 2014). Notably, Wnt components may be useful for monitoring stage and progression of some non-malignant diseases, such as serum levels of Dickkopf-1 to assess inflammatory processes during ankylosing spondylitis (Yucong et al., 2014) or abnormalities of bone metabolisms in chronic kidney disease (Thambiah et al., 2012) and in patients with fluorine bone injury (W. Wang et al., 2013). Furthermore, LRP5 has proven to be a crucial player in murine lung fibrosis and is a marker of disease progression and severity in subjects with idiopathic pulmonary

fibrosis (Lam et al., 2014). It is reasonable to speculate that Wnt pathway biomarkers might well be useful for monitoring the efficacy of Wnt-targeting therapeutics (Serafino et al., 2014), as is true for urinary LTE_4 as a marker of anti-LT therapeutics (Chiu et al., 2014) or the urinary PGE_2 metabolite, PGE-M, to monitor pharmacological COX inhibition (Murphey et al., 2004). Future studies are needed to investigate the effects of the different anti-Wnt therapeutics (see following section) on the diverse Wnt pathway components and to identify possible candidate biomarkers.

6. Inhibitors of Wnt signaling

Considering the enormous impact of canonical Wnt signaling on cancer development and other Wnt-related diseases, like fibrosis, neurological diseases, osteoporosis and regenerative medicine (see Sections 3 and 4), it is not surprising that intensive efforts have been undertaken to understand the fundamental processes and to discover modulators of this pathway (Barker & Clevers, 2006; Verkaar & Zaman, 2011; Niehrs, 2012; Pattabiraman & Weinberg, 2014). Over the last 30 years, a number of druggable components and protein–protein interactions of the Wnt pathway have been identified (targets of selected Wnt pathway inhibitors are illustrated in Fig. 2). However, efficient targeting of Wnt signaling still remains in its infancy (Verkaar & Zaman, 2011; Anastas & Moon, 2013; Kahn, 2014).

In this section, we briefly review the most promising known targets and Wnt pathway inhibitors, the latter consisting of small molecules, peptides, and antibodies (see Fig. 2, Table 1, and Supplementary Table 1). The reader is also referred to several comprehensive reviews on this topic for further thorough reading (Barker & Clevers, 2006; Verkaar & Zaman, 2011; Kahn, 2014; Le et al., 2015).

6.1. Inhibitors acting at the plasma membrane

The Wnt pathway (see Figs. 1 and 2) can be directly targeted at the cell membrane, using antibodies against Fzd receptors and Wnt proteins (Rhee et al., 2002; He et al., 2004, 2005; You et al., 2004; Mikami et al., 2005; DeAlmeida et al., 2007; Gurney et al., 2012).

The monoclonal antibody, OMP-18R5, binds to five distinct Fzd receptors (Fig. 2) through a conserved epitope and prevents binding of Wnt ligands (Gurney et al., 2012; Le et al., 2015). The therapeutic effect of this antibody was assessed in human xenograft models displaying growth inhibition of different tumor types (Gurney et al., 2012). OMP-18R5 is currently in phase I clinical trials as a single agent or for combination therapy (see Table 1).

A very promising candidate is the antibody-based inhibitor OMP-54F28, a truncated Fzd8 receptor fused to the immunoglobulin Fc region (Le et al., 2015). OMP-54F28 inhibits Wnt signaling and tumor growth of a mouse mammary tumor virus (MMTV)-Wnt-1-induced tumors and patient-derived cancer xenograft models (Smith et al., 2013; Yeung et al., 2014). OMP-54F28 is currently in a phase I clinical trial and further trials in combination with chemotherapeutics have been initiated (see Table 1).

Wnt maturation and secretion are specifically dependent on the *O*-acyltransferase, porcupine, which palmitoylates immature Wnt proteins. Inhibition of this enzyme by the selective and orally bioavailable inhibitor, LGK974 (Table 1 and Supplementary Table 1), prevented secretion of Wnt ligands and displayed good efficacy in murine and rat

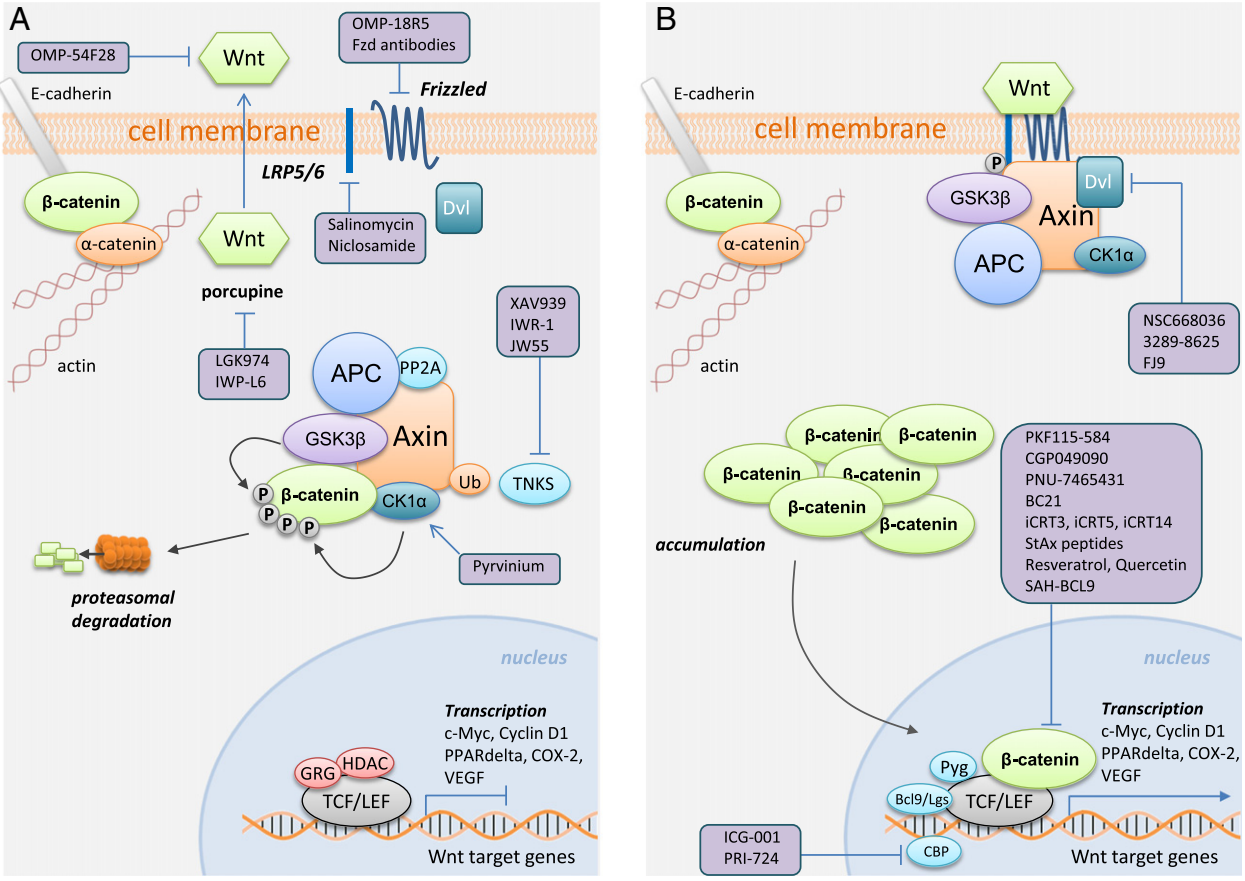

Fig. 2. Mode of action of different inhibitors of Wnt signaling. Agents targeting different pathway components are displayed in purple boxes. For detailed description see Section 6. APC: adenomatous polyposis coli proteins and; GSK3β: glycogen synthase kinase 3β; CBP, CREB binding protein; CK1α: casein kinase α; Dvl, disheveled; GRG, Groucho; HDAC, histone deacetylase; Fzd, Frizzled; LRP5/6, low density lipoprotein receptor-related protein 5/6; PP2A, phosphatase A_2; Pyg, Pygopus; TCF/LEF, T-cell factor/lymphoid enhancer factor; TNKS, tankyrase.

cancer models at well-tolerated doses (Liu et al., 2013). LGK974 is currently in phase I clinical trials for patients with Wnt-dependent malignancies. Inhibitors of Wnt production (IWP) represent a different chemotype of potent porcupine inhibitors (B. Chen et al., 2009; Dodge et al., 2012). IWP-L6 (Table 1 and Supplementary Table 1), which inhibits porcupine at subnanomolar concentrations, prevented posterior axis formation in zebrafish (X. Wang et al., 2013).

Several compounds have been described to act at the level of the Wnt co-receptor, LRP6 (Gupta et al., 2009; M. Chen et al., 2009; D. Lu et al., 2011; W. Lu et al., 2011; Lu et al., 2014). The potassium ionophore salinomycin, a natural product from the fungus *Streptomyces albus*, blocks Wnt-induced phosphorylation of LRP6 and promotes its degradation. Along with reduced β-catenin levels, salinomycin decreased the expression of Wnt target genes. The activity of this agent was further demonstrated in different cancer cell lines and in in vivo studies (Mao et al., 2014; Wang et al., 2012). A similar mode of action was described for the FDA-approved antihelminthic drug, niclosamide. Niclosamide exhibited antiproliferative activity in several cancer cell lines at low micromolar concentrations and inhibited tumor growth and liver metastasis formation in mice (W. Lu et al., 2011; Osada et al., 2011; Sack et al., 2011). However, none of these LRP-targeting agents has entered clinical trials for cancer treatment so far.

A different approach to target the Wnt pathway at the cell membrane is to disrupt the Dvl-Fzd protein–protein interaction (PPI), which is essential for the transduction of the Wnt signal from Fzd to Dvl. In-silico screenings revealed three compounds, NSC668036, 3289–8625, and FJ9, that bind directly to the PDZ domain of Dvl, thereby disabling the Dvl–Fzd interaction (Table 1 and Supplementary Table 1). These compounds inhibited Wnt-dependent signaling in *Xenopus* or mouse xenograft models (Shan et al., 2005; Fujii et al., 2007; Grandy et al., 2009).

6.2. Inhibitors acting at the level of axin

Another promising strategy to inhibit Wnt signaling is the regulation of axin stability. The poly-ADP-ribosyltransferases (PARPs), tankyrases 1/2, were shown to destabilize axin through PARsylation leading to subsequent proteasomal degradation.

Thus, tankyrase inhibitors stabilize axin, consequently enhance degradation of β-catenin, and suppress the growth of colon cancer cell lines (Huang et al., 2009; Lehtio et al., 2013).

Several chemotypes were described as tankyrase inhibitors, including XAV939, IWR-1, and JW55 (B. Chen et al., 2009; Huang et al., 2009; Waaler et al., 2012) (Table 1 and Supplementary Table 1). IWR-1 was active in zebrafish and JW55 reduced the growth of adenomas in mice (B. Chen et al., 2009; Waaler et al., 2012). Despite several attempts to develop promising tankyrase inhibitors, none of them has entered clinical trials so far.

The FDA-approved antihelminthic drug, pyrvinium pamoate (Table 1 and Supplementary Table 1), was described as a Wnt pathway inhibitor in *Xenopus laevis* egg extracts (Thorne et al., 2010). Pyrvinium allosterically activates CK1α, leading to stabilization of axin and degradation of β-catenin, Pyg, and TCF/LEF. It showed a growth suppressive effect on various colon cancer cell lines and delayed tumor growth in vivo (Wiegering et al., 2014).

6.3. Inhibitors of β-catenin interactions

Interrupting the PPI of β-catenin in the nucleus has been a tempting but demanding task (Hahne & Grossmann, 2013; Milroy et al., 2014).

A number of in vitro and in silico high-throughput screens revealed different chemical scaffolds as disrupters of the TCF/β-catenin interaction, among them the natural fungal products, PKF115-584 and CGP049090 (Lepourcelet et al., 2004; Trosset et al., 2006; Z. Chen et al., 2009; Gonsalves et al., 2011; Tian et al., 2012) (Table 1 and Supplementary Table 1). Their activity was demonstrated in colon and prostate cancer cell lines, as well as in *Xenopus* embryos. These compounds were also reported to disturb the β-catenin/APC interaction (Lepourcelet et al., 2004).

An in-silico screen yielded PNU-74654 (Table 1 and Supplementary Table 1) that selectively binds to β-catenin when applied in nanomolar concentrations (Trosset et al., 2006). The biological activity of this compound has been described in zebrafish, during cardiomyocyte differentiation, and in adrenocortical tumors (Durand et al., 2011; Buikema et al., 2013; Pradhan & Olsson, 2014).

Similarly, BC21 (Table 1 and Supplementary Table 1), a copper-containing agent, was identified in a virtual screen, and binds to β-catenin thus disrupting its interaction with TCF4. BC21 decreased the viability and colony formation of HCT-116 cells at low micromolar concentrations (Tian et al., 2012). However, at higher concentrations, BC21 also targets protein phosphatase 2C and the proteasome (Daniel et al., 2004; Rogers et al., 2006).

A high-throughput screen, employing an RNAi-based luciferase reporter gene assay in *Drosophila* cells, identified the oxazole- and thiazole-based compounds, iCRT3, iCRT5, and iCRT14 (Table 1 and Supplementary Table 1) which were shown to disrupt the β-catenin/TCF interaction in vitro and decrease Wnt target gene expression (Gonsalves et al., 2011). iCRT14 demonstrated modest activity in xenograft models (Gonsalves et al., 2011). Moreover, hydrocarbon-stapled peptides (StAx peptides) have been reported to bind directly to β-catenin and specifically disable its interaction with TCF4. These peptides selectively inhibited the Wnt pathway without influencing other developmental pathways, such as Notch, Hedgehog, BMP, and transforming growth factor-β (TGF-β) (Grossmann et al., 2012). Another stapled peptide, SAH-BCL9, selectively targets the β-catenin/Bcl9 interaction. SAH-BCL9 blocked Wnt-dependent gene transcription and decreased proliferation, angiogenesis, and cellular migration in cell culture assays. Moreover, SAH-BCL9 demonstrated promising efficacy in colorectal carcinoma and multiple myeloma mouse xenograft models (Takada et al., 2012).

Some polyphenols, like quercetin and resveratrol (Table 1 and Supplementary Table 1), which are known for their polypharmacology, have also been reported to disrupt the β-catenin/TCF complex, to reduce Wnt target gene expression, and to show activity against different cancer cells (Park et al., 2005; Chen et al., 2012). Resveratrol has already been studied in phase I clinical trials in patients with colon cancer (results are pending).

A different approach to address canonical Wnt signaling at the level of nuclear gene transcription is to prevent β-catenin interaction with the transcriptional coactivator CBP in the nucleus. Emami et al. screened a secondary structure-templated compound library for inhibition of the TOPFLASH reporter. The most promising hit, ICG-001 (Table 1 and Supplementary Table 1), selectively binds to CBP but not to the related p300 and competes with β-catenin for binding to CBP (Emami et al., 2004; Arensman et al., 2014). ICG-001 decreased Wnt target gene expression and selectively inhibited the growth of colon cancer cell lines. In addition, a water-soluble form of ICG-001 reduced tumor volumes in mouse xenografts (Emami et al., 2004).The most promising hit, ICG-001 (Table 1 and Supplementary Table 1), selectively binds to CBP but not to the related p300 and competes with β-catenin for binding to CBP (Emami et al., 2004; Arensman et al., 2014). ICG-001 decreased Wnt target gene expression and selectively inhibited the growth of colon cancer cell lines. In addition, a water-soluble form of ICG-001 reduced tumor volumes in mouse xenografts (Emami et al., 2004).

Further structural development of β-catenin/CBP interaction inhibitors led to PRI-724, which is currently being assessed in phase I/II clinical trials for different types of cancers and liver cirrhosis (El-Khoueiry et al., 2013; Lenz & Kahn, 2014).

Interestingly, the activation of the nuclear receptors for retinoic acid and vitamin D by the respective ligands inhibits Wnt signaling. This influence was attributed to interaction of the nuclear receptors with β-catenin, which sequester β-catenin away from TCF or coactivators like p300 and CBP (Takada et al., 2012).

6.4. Additional targets and agents

Further small molecules and natural products have been described to modulate Wnt signaling (Jaiswal et al., 2002; Kim et al., 2006; Z. Chen et al., 2009; Albring et al., 2013; Zeller et al., 2013). JW Pharmaceuticals is developing a small molecule, CWP232291 (Table 1 and Supplementary Table 1), that targets Sam68 (Src-associated in mitosis), a protein that forms a complex with APC and regulates alternative splicing of TCF1, thereby affecting Wnt signaling (Morishita et al., 2011; Sebio et al., 2014). This agent also modulates NF-κB signaling and alternative splicing of the anti-apoptotic protein Bcl2 (Kahn, 2014). CWP232291 is currently undergoing a phase I clinical trial in patients with acute myeloid leukemia (Pattabiraman & Weinberg, 2014).

In addition, NSAIDs such as celecoxib, aspirin, and sulindac exhibit chemopreventive properties owing to their reduction of the synthesis of prostaglandin E_2 (PGE_2) which has an effect on β-catenin stability (Dihlmann et al., 2001; Grosch et al., 2001; Maier et al., 2005). Potential molecular mechanisms of interference with Wnt signaling by NSAIDs are discussed in more detail in the following section.

Although druggable components of the Wnt pathway exist and potent agents have been developed, only a few drug candidates have reached the clinic so far and no convincing results are available as yet. Apart from off-target effects and poor pharmacokinetic properties, the sluggish progress in the field of Wnt-targeted therapies can be attributed to a variety of issues (Anastas & Moon, 2013; Kahn, 2014). Firstly, the Wnt signaling network is very complex and crosstalk occurs with various pathways. Thus, Wnt signaling components may have redundant functions and/or may be regulated by other pathways. Secondly, the Wnt pathway is essential for embryonic development as well as for tissue homeostasis and repair in adults. Therefore, it is not surprising that the systemic application of Wnt inhibitors is limited by on-target toxicity which might affect hematopoiesis and normal bone and intestine functions. Thus, specificity of action and determination of the appropriate therapeutic window are of major importance to achieve a tolerable balance between anti-tumorigenic effects and possible adverse side effects. Increased understanding of Wnt pathway-related mutations and their effects in patients or the introduction of predictive biomarkers for patient selection will facilitate the choice of an agent that acts upstream or downstream of the destruction complex and may prove crucial to successful targeting of the Wnt pathway. Thirdly, aberrant Wnt signaling does not necessarily correlate with poor patient survival in all types of cancer (Kahn, 2014). Therefore, Wnt-inhibiting drugs must be carefully chosen based on the origin and genesis of the targeted disease. Moreover, Wnt signaling has been linked to cancer chemoresistance and combination of Wnt pathway inhibitors and conventional chemotherapeutics is considered a promising approach to cancer treatment (Anastas & Moon, 2013; Kahn, 2014). Despite these hurdles that still have to be addressed in the future, and the need for improved insight into the clinical applicability of Wnt inhibitors, pharmacological modulation of Wnt signaling remains a promising strategy for the treatment of various diseases, especially cancer. In this regard, already approved drugs, like 5-LO and COX inhibitors, that influence Wnt signaling and thus, provide an acceptable therapeutic window, are likely to be of great value for future research on Wnt-targeted therapies.

7. Non-steroidal anti-inflammatory drugs as Wnt pathway inhibitors

As far back as the early 1980s, evidence was generated showing that long term treatment of cancer patients with NSAIDs causes regression or stagnation of tumor progression (Waddell & Loughry, 1983; Waddell et al., 1983). Large retrospective and prospective studies have confirmed these findings for different NSAIDs, such as aspirin (Thun et al., 1991; Burn et al., 2011; Ng et al., 2015), celecoxib (Steinbach et al., 2000; Phillips et al., 2002), sulindac (Labayle et al., 1991; Giardiello et al., 1993), and exisulind (Arber et al., 2006) in colon cancer patients, mainly in familial adenomatous polyposis (FAP) patients. Furthermore, animal studies using ApcMin mice (a mouse model for human FAP) also indicate that these NSAIDs provide protective effects against cancer (Boolbol et al., 1996; Chiu et al., 1997; Wechter et al., 1997; Mahmoud et al., 1998a, 1998b).

NSAIDs are classified as non-selective or selective inhibitors of COX-1 and/or -2 (Fig. 4B). COX-1, on the one hand, is constitutively expressed in a wide range of tissues and considered to maintain tissue homeostasis, platelet aggregation, and cell signaling in health. The immediate early gene COX-2 is predominantly induced by a number of inflammatory cytokines in inflamed tissues and during cellular stress. COXs are key enzymes in the biosynthesis of a series of (patho)physiologically active prostanoids, such as PGE_2, PGD_2, prostacyclin, and thromboxane A_2. COX-2-derived PGE_2 triggers induction of fever, inflammation-related vasodilatation, and hyperalgesia but also has well-documented mitogenic effects on different cancer cells. However, COX-1-derived PGE_2 maintains mucosal integrity of the gastro-intestinal system by impairing gastric acid secretion and increasing gastric mucus secretion (Simmons et al., 2004).

In numerous cancer types, the mechanisms controlling COX-2 expression are abrogated, leading to overexpression of COX-2 protein and enhanced PG production (Eberhart et al., 1994; Hida et al., 1998; Achiwa et al., 1999; Chan et al., 1999; Mohammed et al., 1999; Molina et al., 1999; Soslow et al., 2000; Khan et al., 2001; Kulkarni et al., 2001). Especially in colon cancer, upregulation of COX-2 has been shown to occur already at the early adenoma stage and is, therefore, one of the earliest mechanisms deregulated during tumor development (Shiff & Rigas, 1999; Dixon, 2003). Thus, enhanced COX-2 expression has been attributed a key role at early stages of cancer development by promoting cell division (Chinery et al., 1999; Fosslien, 2000), inhibiting apoptosis (Sheng et al., 1998; Lin et al., 2001; Nzeako et al., 2002; Tang et al., 2002), altering cell adhesion, and enhancing metastasis (Tsujii et al., 1997; Tomozawa et al., 2000; Chen et al., 2001; Kakiuchi et al., 2002; Li et al., 2002) and neovascularization (Liu et al., 1999; Leahy et al., 2000).

As mentioned previously, several clinical studies have shown that long term administration of NSAIDs protects against colon cancer, especially in FAP patients and ApcMin mice. FAP patients are characterized by mutations in the APC gene, which is one of the main binding partners for β-catenin (see Section 2), and is required for the degradation of β-catenin by the ubiquitin–proteasome pathway (see Fig. 1). Therefore, clinical data suggest that the cancer-protective effect of NSAIDS is both due to their COX-inhibiting potential as well as to interference with the Wnt/β-catenin pathway, independently of COX. Additionally, it has been reported that interdependency also exists between the PGE_2- and Wnt-signaling pathways, which is not only a characteristic of colon cancer but is also conserved in mammalian hematopoietic stem cells and is important for tissue regeneration (Goessling et al., 2009).

7.1. Crosstalk between prostaglandin E_2 and the Wnt/β-catenin signaling pathway

One possibility for NSAIDs to interfere with the Wnt/β-catenin pathway is by inhibiting COX-2 activity. As Fig. 3 illustrates, crosstalk exists between PGE_2 and β-catenin via the binding of PGE_2 to its heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors, EP2 and EP4, at the surface of the cell (Fujino et al., 2002). Sonoshita et al. demonstrated that homozygous deletion of the gene encoding EP2 causes a decrease in the number and size of intestinal polyps in Apc (Delta 716) min mice, whereas homozygous deletion of EP1 or EP3 had no effect on polyp formation in these mice (Sonoshita et al., 2001). Binding of PGE_2 to EP2/4 provokes the release of activated $G\alpha_s$ subunit which binds to axin through its regulator of G-protein signaling (RGS)-domain. The binding of $G\alpha_s$ to axin causes the release

of GSK3β from the β-catenin complex. Furthermore, $G\alpha_s$ activates adenylate cyclase, leading to the formation of cyclic adenosine monophosphate (cAMP) and activation of protein kinase A (PKA) (Fujino et al., 2002). PKA, in turn, phosphorylates β-catenin at Ser675, thereby preventing its degradation by the proteasome pathway (Hino et al., 2005). Additionally, recruited Gβ/γ activates phosphatidylinositol 3-kinase (PI3K), which subsequently stimulates and phosphorylates protein kinase B (PKB/Akt) through protein dependent kinase 1 (PDK1). PKB/Akt causes the phosphorylation and thereby, inactivation of GSK3β, which leads to stabilization and nuclear translocation of β-catenin (Castellone et al., 2005). In the nucleus, β-catenin binds together with TCF/LEF to the promoter regions of several genes which are involved in proliferation, invasion, angiogenesis, survival, or the development of CSC and other tumor cells (Fig. 3). Consequently, inhibition of COX-2 activity by NSAIDs thwarts these effects, which may account for the anti-carcinogenic influence of these substances (Kaur & Sanyal, 2010; Vaish & Sanyal, 2012; S. Zhang et al., 2014).

However, recent studies provided strong evidence suggesting that in addition to COX-2 inhibition, COX-2-independent mechanisms contribute to the anti-tumorigenic effects of these drugs. Apparently, each NSAID influences a more or less separate COX-independent target (Grosch et al., 2001; Grösch et al., 2003; Soh & Weinstein, 2003; Grösch et al., 2005, 2006). NSAIDs also influence the Wnt/β-catenin pathway through a number of COX-independent targets. Some of these targets and related molecular mechanisms leading to interference with the Wnt/β-catenin pathway are described below and illustrated in Fig. 3. The mechanisms of suppression of canonical Wnt signaling by 5-LO inhibitors are described in Section 9.

7.2. Interference of protein kinaseG with the Wnt/β-catenin signaling pathway

In human colon cancer cells, sulindac and its metabolite, sulindac sulfone (exisulind), block the activity of guanosine 3′,5′-monophosphate (cGMP)-specific phosphodiesterases (PDE) 2 and 5, thus, leading to sustained increases in cellular levels of cGMP and activation of cGMP-dependent protein kinase (PKG) (W.J. Thompson et al., 2000).

Active PKG inhibits Wnt/β-catenin signaling, on the one hand, by decreasing β-catenin mRNA expression, and on the other hand, by depriving β-catenin of the TCF/LEF complex, attaching it to active FOXO4

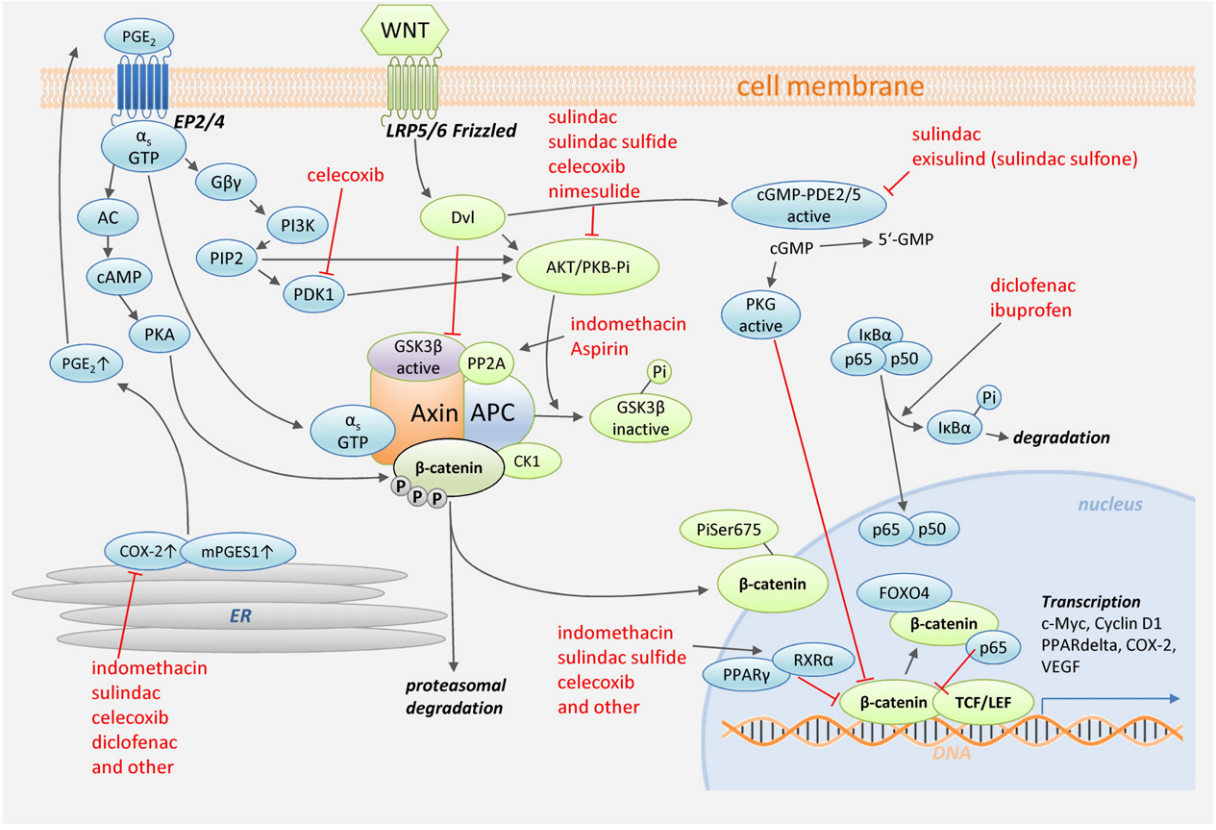

Fig. 3. Modes of action of NSAIDs on the Wnt signaling pathway. In the absence of a Wnt stimulus, the transcriptional coactivator, β-catenin, is degraded by a multiprotein "destruction complex" that includes the tumor suppressors, axin and adenomatous polyposis coli (APC), the Ser/Thr kinases GSK3β and CK1, protein phosphatase 2A (PP2A), and the E3-ubiquitin ligase β-TrCP. When Wnt binds to the surface receptors, Frizzled and LRP5/6 (low-density lipoprotein (LDL)-receptor related proteins 5 and 6), disheveled (Dvl) is activated. This inhibits the β-catenin degradation complex, consisting of adenomatous polyposis coli (APC), axin, and glycogen synthetase kinase 3b (GSK3β) (Moon et al., 2004), by activating PKB (Protein kinase B) which phosphorylates and thereby inactivates GSK3βb (Cohen & Frame, 2001). Other protein kinases, like activated PKG (Protein kinase G), also interfere with the β-catenin pathway. PKG inhibits β-catenin expression on the transcriptional level and blocks its transcriptional transactivation activity, in combination with TCF, by sequestration of β-catenin by FOXO4. Crosstalk between EP2 receptors and the β-catenin pathway: Binding of PGE_2 to EP2 receptors provokes the release of Gβ/γ subunits, which stimulate PKB through PI3K, and $G\alpha_s$ concomitantly binds axin through its regulator of G-protein signaling (RGS)-domain. The binding of $G\alpha_s$ to axin releases GSK3β from the complex and GSK3β is phosphorylated and inactivated by PKB, which leads to stabilization and nuclear translocation of β-catenin. Gαs also activates adenylate cyclase (AC) leading to the formation of cAMP and activation of PKA. PKA stabilizes β-catenin by phosphorylation. In the nucleus, β-catenin binds, together with TCF/LEF, to the promoter region of several genes that are involved in proliferation, invasion, angiogenesis, survival or the development of cancer stem cells (CRC). Transcriptional activity of β-catenin/TCF/LEF complex can be inhibited by several other transcription factors, like NF-κB and PPARγ/RXRα leading to a repression of β-catenin/TCF-dependent genes. A number of NSAIDs (highlighted in red) suppress Wnt signaling by targeting several regulatory proteins at different levels of the Wnt signal transduction cascade. APC, adenomatous polyposis coli protein; cAMP, cyclic adenosine monophosphate; GSK3β; CK1α, casein kinase α; COX, cyclooxygenase; Dvl, disheveled; FOXO4, Forkhead-Box-Protein 4; Fz, Frizzled; GRG, Groucho; GSK3β, glycogen synthase kinase 3β; HDAC, histone deacetylase; LRP5/6, low density lipoprotein receptor-related protein 5/6; mPGES1, microsomal prostaglandin E_2 synthase 1; PDK-1,3-phosphoinositide dependent protein kinase-1; PI3K, phosphatidylinositol 3-kinase; PK A/B/G, protein kinase A/B/G; PP2A, protein phosphatase 2A; PPARγ, Peroxisome proliferator-activated receptor gamma; TCF/Lef, T-cell factor/lymphoid enhancer factor; LEF, RXR, retinoid X receptor.

(Kwon et al., 2010; Li et al., 2013). In ApcMin mice, dietary sulindac eliminated, in particular, intestinal stem cells containing nuclear or phosphorylated β-catenin, indicating that targeting the Wnt/β-catenin pathway is a major mechanism to explain the cancer-preventing effects of this drug (Qiu et al., 2010).

7.3. Interference of protein phosphatase 2A with the β-catenin signaling pathway

Aspirin, diclofenac, sulindac sulfide (the pharmacologically active metabolite of sulindac), and indomethacin diminished Wnt signaling at the level of gene transcription in different human colon cancer cell lines, irrespective of COX-2 expression (Smith et al., 2000; Dihlmann et al., 2003; Gardner et al., 2004). This effect was apparently related to a decrease in nuclear β-catenin localization and enhanced membranous β-catenin staining in the human colon cancer cell line, SW480. In another study, Dihlmann et al. showed an increase in phosphorylated β-catenin protein caused by aspirin that was not only due to enhanced GSK3β activity, but possibly may have been triggered by inhibition of the specific serine/threonine-phosphatase, PP2A (Dihlmann et al., 2003; Bos et al., 2006), which is also a component of the β-catenin multiprotein "destruction complex" (Stamos & Weis, 2013). The phosphorylation status of β-catenin is important for its interaction with various binding partners and also plays a pivotal role in the recruitment of co-activators to the nuclear β-catenin/TCF/LEF complex (Daugherty & Gottardi, 2007). PP2A has been shown to control the binding of CBP/p300 to β-catenin/TCF regulated genes, leading to the maintenance of embryonic stem cell pluripotency in mice (Miyabayashi et al., 2007). These data indicate that the molecular mechanisms of several NSAIDs in modifying Wnt/β-catenin signaling seem to be dependent on the cell type. Thus, further experiments are needed to shed light on the direct molecular targets of these NSAIDs which may relate to their anti-carcinogenic effects and their interference with the Wnt signaling pathway.

7.4. Interference of other transcription factors with Wnt signaling pathway

Some NSAIDs, like diclofenac, aspirin, sulindac (and its metabolites), ibuprofen, and celecoxib, have been shown to exert their anti-carcinogenic effects through manipulation of the NF-κB signaling (Grösch et al., 2006; Gurpinar et al., 2013). Diclofenac and ibuprofen activate the NF-κB pathway, leading to enhanced nuclear expression of p65, which forms a complex with β-catenin, thereby, inhibiting the transcription of β-catenin-dependent genes (Cho et al., 2005; Greenspan et al., 2011). Whether this mechanism also extends to other NSAIDS remains unclear and needs further investigation.

Besides NF-κB, the transcription factor-complex, PPARγ/RXRα (Peroxisome Proliferator-Activated Receptor γ/Retinoid X receptor α), has been shown to be a physical interaction partner of β-catenin, leading to repression of β-catenin-dependent transcription (Lu et al., 2005). Eighteen of nineteen tested NSAIDs inhibit β-catenin-dependent transcription by interference with PPARγ/RXRα (Lu et al., 2005). The molecular mechanism is not clear, but transactivation between PPARγ/RXRα and β-catenin seems to be important, because neither PPARγ nor RXRα alone nor a mutant PPARγ protein, lacking transactivation activity, account for the inhibitory effect of NSAIDs on β-catenin-dependent transcription. For celecoxib, sulindac sulfide, indomethacin and others, a direct interaction with PPARγ or RXRα has been shown. Besides NF-κB, the transcription factor complex, PPARγ/RXRα, has been found to be a physical interaction partner of β-catenin, leading to repression of β-catenin-dependent transcription (Lu et al., 2005).

7.5. Interference of 3-phosphoinositide-dependent protein kinase-1/PDK1/Akt signaling pathway with the Wnt/β-catenin signaling pathway

Fig. 3 indicates that cytosolic β-catenin stabilization/degradation can be regulated by PI3K, PDK1, and protein kinase B (PKB/Akt) in an EP2/4 dependent manner. However, the PI3K signaling pathway is also activated by several other membrane receptors, like integrins, receptor tyrosine kinases (such as EGFR, VEGFR, Her2neu), cytokine receptors (e.g. IL2R), or G-protein coupled receptors, such as the EP2 receptor (for an overview see Hemmings & Restuccia, 2012). All of these receptors have been shown to be involved in cancer development and are often deregulated in different cancer types, leading to constitutive activation of the PI3K→PKB/Akt signaling pathway and cancer progression (Vivanco & Sawyers, 2002; Hennessy et al., 2005). Several NSAIDs have been shown to target PDK1 or PKB/Akt directly, leading to inhibition of this and the downstream signaling pathway and to anti-carcinogenic effects in vitro and in vivo in mice (Arico et al., 2002; Baoping et al., 2004; Kulp et al., 2004; Lee et al., 2005; Fan et al., 2006; Setia et al., 2014). This might also be a major mechanism by which celecoxib interferes with the Wnt/β-catenin pathway, leading to reduced β-catenin expression and transcriptional activity in human cancer cells, irrespective of COX-2 expression and activity (Maier et al., 2005; Sareddy et al., 2013).

In summary, NSAIDs potently inhibit Wnt signaling by different modes of action, which contribute to the anti-carcinogenic effects of these substances in various cancer types. This is of particular relevance for therapy of adenomas and adenocarcinomas in FAP patients and other tumors displaying elevated Wnt signaling due to mutations in pathway-regulatory proteins. Several studies have convincingly indicated a concomitant contribution of β-catenin and PGE_2 signaling pathways to the development of CSC (Ishimoto et al., 2010; Al-Kharusi et al., 2013; Femia et al., 2013) as well as a superior therapeutic efficacy against CRC, particularly for those NSAIDs inhibiting both pathways by COX-dependent as well as COX-independent mechanisms (Qiu et al., 2010).

7.6. Non-steroidal anti-inflammatory drugs affecting Wnt signaling by inhibition of 5-lipoxygenase

As mentioned above, a number of NSAIDs including indomethacin, the COX-2 inhibitor celecoxib, and the active metabolite of sulindac, sulindac sulfide, have been shown to inhibit Wnt signaling (Maier et al., 2005).

Sulindac, indomethacin, or celecoxib are able to inhibit Wnt signaling, but only at high concentrations, exceeding those for the suppression of COX-1/-2. Sulindac sulfide is able to inhibit the 5-LO enzyme directly at clinically relevant concentrations (Steinbrink et al., 2010). Celecoxib also has been reported to suppress the synthesis of 5-LO products by direct interference with the 5-LO enzyme activity (Maier et al., 2008).

Recently, we were able to show that the non-selective COX inhibitor, indomethacin, is capable of suppressing 5-LO product formation in monocytic THP-1 cells at somewhat higher concentrations than those needed for COX inhibition. It is worth noting that for inhibitors mentioned above, including indomethacin and sulindac sulfide, the 5-LO-inhibitory concentrations were similar to those required for suppression of Wnt signaling and the aberrant self-renewal of LSCs (Roos et al., 2014).

This led us to hypothesize that NSAIDs and other 5-LO inhibitors may suppress Wnt signaling by targeting the 5-LO enzyme, as described in Section 9.

To better understand the complex interaction between 5-LO inhibitors, the 5-LO enzyme, and components of the Wnt signal transduction machinery we briefly introduce the 5-LO pathway here.

8. Introduction to the pro-inflammatory enzyme 5-lipoxygenase and its inhibitors

8.1. The 5-lipoxygenase biosynthetic pathway

The enzyme 5-LO (Fig. 4A) is expressed in a tissue- and cell differentiation-specific manner, primarily in leukocytes including

neutrophils, basophils, eosinophils, monocytes/macrophages, dendritic cells, mast cells, and B-lymphocytes (Radmark et al., 2007). The expression level of 5-LO is regulated by promoter methylation and differentiation inducers [dimethyl sulfoxide (DMSO), retinoic acid, 1α, 2,5-dihydroxyvitamin D_3 and TGF-β] upregulate 5-LO in immature myeloid cells (Radmark et al., 2007). Mechanistically, 5-LO catalyzes the insertion of molecular oxygen into polyunsaturated fatty acids, such as arachidonic acid (AA). Upon activation by Ca^{2+} and/or phosphorylation events, 5-LO translocates from the cytosol or the nucleoplasm to the nuclear envelope and (I) catalyzes the insertion of molecular oxygen at C-5 of AA that is provided by the 5-LO-activating protein (FLAP), yielding the intermediate 5(*S*)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5(*S*)-HpETE). Furthermore, it catalyzes (II) the dehydration of 5(*S*)-HpETE to the epoxide leukotriene (LT)A_4 (Radmark et al., 2015). Alternatively, 5(*S*)-HpETE can be reduced by glutathione peroxidases (GPx) to the corresponding alcohol, 5(*S*)-HETE, which can be further oxidized to 5-oxo-ETE (Powell & Rokach, 2015).

Depending on the cell type and enzymes expressed, LTA_4 can be converted by soluble LTA_4 hydrolase to LTB_4 and/or conjugated with glutathione by LTC_4 synthase to yield LTC_4 (Bair et al., 2012).

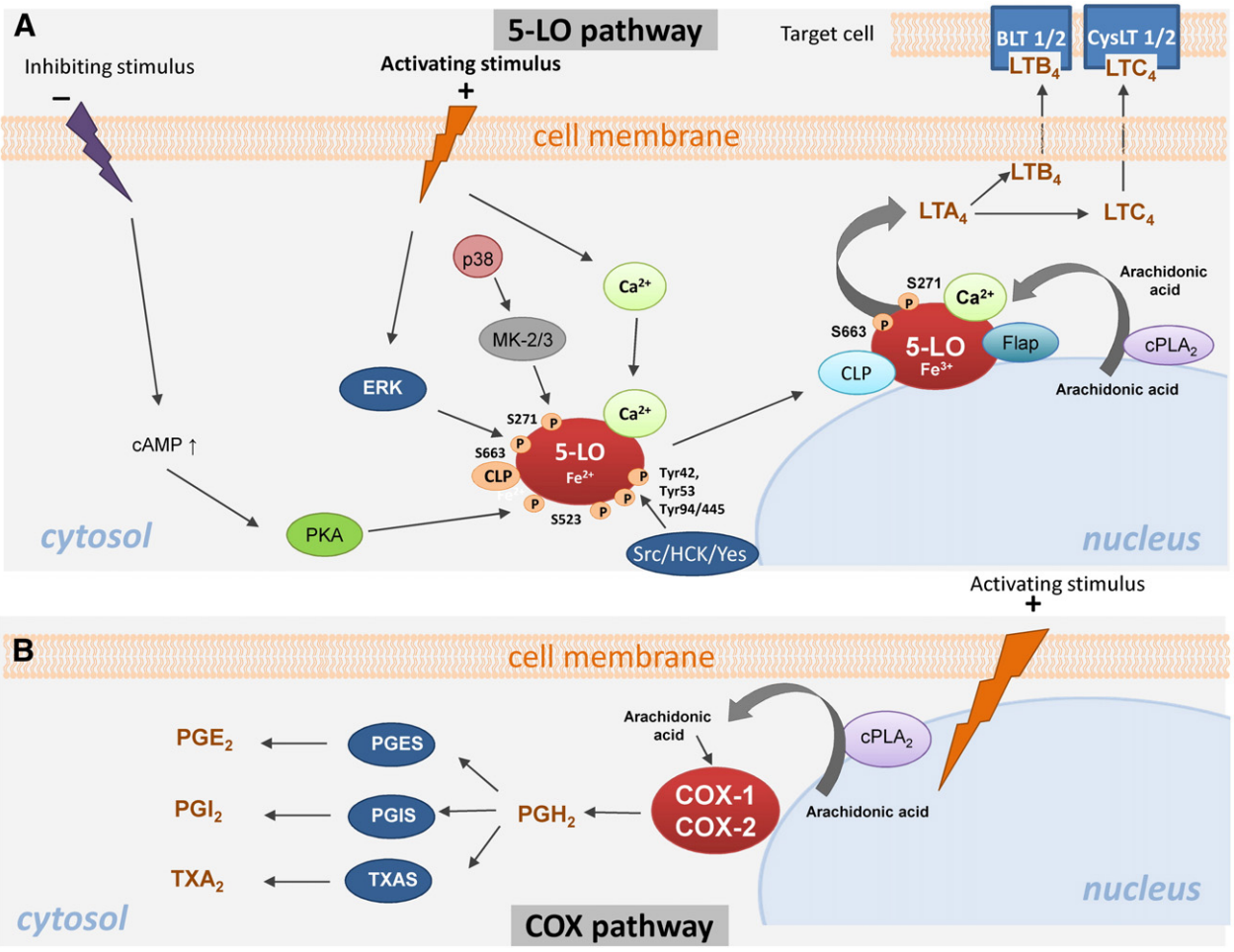

Fig. 4. Regulation of cellular 5-LO and COX activity. (**A**) Depending on the cell type, 5-LO resides as a soluble protein either in the cytosol or in the nucleoplasm of resting cells (O. Werz, 2002). Though nuclear localization sequences are present in the primary sequence of 5-LO, the way in which the distinct subcellular localization is organized is unknown, but systemic factors such as testosterone (Pergola et al., 2008) or cell adherence (Brock, 2005) may affect the subcellular redistribution. In activated cells, however, 5-LO is consistently found at the perinuclear region where 5-LO product synthesis may occur (Werz, 2002; Brock, 2005; Newcomer & Gilbert, 2010; Bair et al., 2012). Upon cell activation by Ca^{2+}-ionophores, soluble agonists like platelet-activating factor (PAF), LTB_4, formyl-methionyl-leucyl-phenylalanine (fMLP) and C5a, as well as phagocytic particles such as zymosan, urate or phosphate crystals, 5-LO translocates to the nuclear membrane (Werz, 2002) where it is anchored to phosphatidylcholine (PC) in a Ca^{2+}-dependent manner via the C2-like domain (Kulkarni et al., 2002). Phosphorylation of 5-LO at the C-terminal catalytic domain can modulate its subcellular localization as well as its activation for product synthesis. The p38 MAPK-regulated MAPK-activated protein kinase (MK)-2/3 (Werz et al., 2000) and ERK1/2 (Werz et al., 2002a, 2002b) phosphorylate 5-LO at Ser-271 and Ser-663, respectively, an action which is strongly promoted by unsaturated fatty acids (i.e. AA) (Werz et al., 2002b). Interestingly, phosphorylation of 5-LO is associated with increased accumulation of 5-LO at the nuclear membrane (Werz, 2002; Werz et al., 2001; Fredman et al., 2014) where it is anchored to phosphatidylcholine (PC) in a Ca^{2+}-dependent manner via the C2-like domain (Kulkarni et al., 2002). Phosphorylation of 5-LO at the C-terminal catalytic domain can modulate its subcellular localization as well as its activation for product synthesis. Protein kinase A was also reported to phosphorylate 5-LO (at Ser523), thus impairing the catalytic activity of 5-LO (Luo et al., 2004). Moreover, moderate stimulation of ERK by androgens reduced 5-LO product formation in neutrophils and monocytes (Pergola et al., 2008; Pergola et al., 2011). Novel phosphorylation sites were recently identified at Tyr42, Tyr53 and either Tyr94 or Tyr445 phosphorylated by the Src kinases Fgr, hematopoietic cell kinase (HCK) and Yes (Markoutsa et al., 2014). In parallel to 5-LO, the Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$) moves to the nuclear membrane and releases AA from phospholipids (Leslie, 2004). The free AA is then transferred by the 5-LO-activating protein (FLAP), an integral nuclear membrane protein, to 5-LO for efficient metabolism to LTA_4 (Evans et al., 2008). FLAP is essential for cellular 5-LO product synthesis, and together with coactosine-like protein (CLP) that interacts with 5-LO as well, FLAP seems to function as a scaffold for 5-LO at the nuclear membrane (Mandal et al., 2008; Bair et al., 2012). In addition, LTC_4S and LTA_4H may participate in this multi-protein assembly to enable subsequent transformation of LTA_4 to LTB_4 and LTC_4, respectively (Newcomer & Gilbert, 2010). (**B**) Cyclooxygenase pathway. Cytosolic phospholipase A_2 ($cPLA_2$) translocates to the nuclear membrane and releases AA from phospholipids. Two isoforms of cyclooxygenase, COX-1 and COX-2, catalyze the conversion of arachidonic acid to prostaglandin H_2 (PGH_2), which is further converted by a number of synthases to various eicosanoids, including PGE_2, PGI_2, and TXA_2. COX-1 is constitutively expressed in many tissues and is important for the maintenance of different physiological processes including gastrointestinal integrity. COX-2 is detected negligibly in most tissues but can be rapidly and strongly induced by cytokines and cellular stress at sites of inflammation. AA, arachidonic acid, CLP, coactosine-like protein; $cPLA_2$, cytosolic phospholipase A_2, FLAP, 5-LO activating protein; COX, cyclooxygenase; HCK, hematopoietic cell kinase; LT, leukotriene; MK-2/3, MAPK-activated protein kinase; PKA, protein kinase A; PGE_2, prostaglandin E_2, PGES, prostaglandin E_2 synthase, PGIS, prostaglandin I_2 synthase, PGI_2 prostaglandin E_2, TXA_2 Thromboxane A_2, TXAS, Thromboxane A_2 synthase.

Release of LTC_4 into the extracellular environment and successive amino acid (γ-glutamyl residue and glycine) cleavage yields LTD_4 and then LTE_4. LTB_4 is a potent chemotactic and chemokinetic mediator, activates granulocytes for degranulation, superoxide and nitrogen oxide production, enhances the phagocytic activity of neutrophils and macrophages, and stimulates secretion of immunoglobulins by lymphocytes (Haeggstrom & Funk, 2011). The cys-LTs, in particular LTD_4, cause smooth muscle contraction, mucus secretion, vasoconstriction, plasma extravasation, recruitment of eosinophils, and fibrocyte proliferation (Peters-Golden & Henderson, 2007; Haeggstrom & Funk, 2011).

Thus, LTs play roles in the immune response and in the pathogenesis of inflammatory diseases, such as arthritis, asthma, allergic rhinitis, and other chronic inflammatory disorders (Peters-Golden & Henderson, 2007; Haeggstrom & Funk, 2011). Furthermore, 5-LO products contribute to atherosclerosis (Poeckel & Funk, 2010) and may regulate proliferation and survival of cancer cells (Greene et al., 2011). Finally, 5-oxo-ETE may play important roles in asthma and allergic diseases, cancer, and cardiovascular disease as well (Powell & Rokach, 2015). The pro-inflammatory and immunomodulatory actions of LTs are mediated by specific G protein-coupled receptors (GPCRs), the BLT1 and BLT2 receptors for LTB_4 and the CysLT1 and CysLT2 receptors for cys-LTs (Back et al., 2011).

8.2. 5-LO inhibitors

In order to intervene pharmacologically with 5-LO product synthesis, inhibition of $cPLA_2$, antagonism of FLAP, and inhibition of 5-LO are all conceivable strategies (see (Pergola & Werz, 2010) for review). Direct 5-LO inhibitors can be categorized into four classes, according to their inhibitory mode of action: (1) redox-active 5-LO inhibitors that reduce the active site iron or scavenge activating hydroperoxides and thereby uncouple the catalytic cycle of the 5-LO enzyme; (2) iron-chelating agents, containing hydroxamic acid or *N*-hydroxyurea groups that form complexes with the active site iron; (3) non-redox-type inhibitors that compete with AA or lipid hydroperoxides for binding to 5-LO devoid of redox properties; and (4) allosteric inhibitors that essentially act at the regulatory C2-like domain, e.g., by antagonizing the stimulatory effect of ATP or by interference with stimulatory effects of phospholipids or diglycerides. In particular, for the latter group, several structurally different compounds with apparently distinct binding sites [e.g. indirubins at the ATP-binding site (Pergola et al., 2014), hyperforin at the PC-binding site (Feisst et al., 2009)] were recently reported with promising molecular mechanisms and favorable pharmacodynamics. Nevertheless, despite intensive efforts to develop 5-LO inhibitors and the resulting plethora of reported compounds, only the iron-ligand chelator, zileuton, has become a marketed drug. In fact, all other 5-LO inhibitors so far investigated failed in preclinical or clinical studies due to side-effects (e.g. methemoglobin formation, liver toxicity), pharmacokinetic issues, or lack of efficacy (Pergola & Werz, 2010). Especially with non-redox-type 5-LO inhibitors, phosphorylation of 5-LO and an elevated oxidative tone in the cell can decrease their potency (O. Werz & Steinhilber, 2005).

9. Modulation by 5-LO and 5-LO inhibitors of Wnt signaling in leukemic stem cells

Over the last couple of years, evidence has been increasing for a key role of deregulated canonical Wnt signaling in the pathogenesis of hematological malignancies (HMs), though the exact underlying mechanisms remain poorly understood (Seke Etet et al., 2013). In AML and CML, the role of Wnt-signal activation for the pathogenesis and the maintenance of the LSCs is well recognized (Zhao et al., 2007; Wang et al., 2010; Heidel et al., 2012). Deregulated activation of Wnt signaling leads to aberrant self-renewal of the LSC in AML and CML and is fundamental to LSC maintenance (Zhao et al., 2000; Passegue et al., 2003; Jamieson et al., 2004). Furthermore, it has been shown that not only Wnt signaling, but also 5-LO is important for the maintenance of LSCs and the pathogenesis of AML and CML (Y. Chen et al., 2009; Roos et al., 2014). Although only a small number of publications have addressed the role of 5-LO in the pathogenesis of HMs, there is increasing evidence for an important role of 5-LO in the development of these diseases.

9.1. Disturbed 5-LO expression in leukemic cells

Differential 5-LO expression has been found in several types of primary leukemic cells from patients with pre-B-cell ALL (Feltenmark et al., 1995; Vincent et al., 2008), CML (Radich et al., 2006; Graham et al., 2007), AML (Valk et al., 2004; Vincent et al., 2008), B-cell chronic lymphocytic leukemia (B-CLL) (Rosenwald et al., 2001; Stratowa et al., 2001; Runarsson et al., 2005; Guriec et al., 2014), and mantle cell lymphoma (MCL) (Boyd et al., 2009; Mahshid et al., 2009). Intriguingly, all these types of HM exhibit a deregulated Wnt/β-catenin signaling (Roman-Gomez et al., 2007; Zhao et al., 2007; Gelebart et al., 2008; Wu et al., 2009; Chung et al., 2010; Trowbridge et al., 2010; Wang et al., 2010; Kuhnl et al., 2011; Perez-Galan et al., 2011; Tandon et al., 2011; Heidel et al., 2012), suggesting an interrelation between 5-LO function and the Wnt/β-catenin signaling pathway.

One of the first studies to indicate involvement of 5-LO in HMs was performed by Tskuada et al. in 1986. In this study, treatment of leukemic cells with the competitive 5-LO inhibitor, AA-861, led to reduced cell proliferation which was accompanied by a reduction in cellular DNA, RNA, and protein synthesis. One of the conclusions of this study was that LTs play an important role in cancer cell viability (Tsukada et al., 1986). Similar results were obtained by Anderson et al., who observed that primary human CML blast cells in culture, as well as leukemic cell lines, showed reduced cell proliferation after treatment with different 5-LO inhibitors (Anderson et al., 1995). Additionally, 5-LO inhibition by AA-861 increased apoptosis in eicosapentaenoic acid (EPA)-treated leukemic HL-60 cells (Gillis et al., 2007).

In a subsequent study, inhibition of 5-LO and FLAP was found to induce apoptosis in MCL cell lines and primary CLL cells, suggesting an important role for 5-LO and/or its products in MCL and other B cell malignancies. This was supported by the fact that 5-LO was up-regulated ~7-fold in MCL cells compared to normal B cells (Boyd et al., 2009). In addition, Runarsson et al. observed that treatment of B-CLL cells with the iron-ligand 5-LO inhibitor BWA4C and FLAP inhibitor MK886 counteracted CD40-dependent activation of these cells by inhibiting CD40-induced DNA synthesis and CD40-induced expression of CD23, CD54, and CD150. This finding was likely LT-dependent, since addition of exogenous LTB_4 almost completely reversed the observed effects (Runarsson et al., 2005).

It was also shown that the highly progressing disease in B-CLL patients is associated with overexpression of 5-LO and other components of the 5-LO pathway, such as $cPLA_2$ and BLT2 (Guriec et al., 2014). The expression levels of BLT1 and LTA_4 hydroxylase were also significantly raised in B-CLL cells compared to healthy B-cells. However, no statistically significant correlation was found with the stage of the disease. Increased $cPLA_2\alpha$ and LTC_4 synthase expression was also found in leukemic granulocytes from AML patients. However, the formation of LTB_4 was also suppressed in the same patient samples (Stenke et al., 1998; Runarsson et al., 2007).

Taken together, it has been shown that in many different types of leukemia there is an altered expression level of 5-LO protein and other protein components involved in LT biosynthesis suggesting a role of the 5-LO pathway in the pathogenesis of leukemia. Additionally, inhibitor studies revealed a function of 5-LO products in proliferation and apoptosis of leukemic cells with particular relevance to B-CLL cells.

9.2. 5-LO and acute myeloid leukemia

Recently, it was shown that 5-LO is strongly upregulated in AML1/ETO-positive AML (DeKelver et al., 2013). The fusion protein AML1/

ETO is the result of the chromosomal t(8;21) translocation (transfer of genetic material between chromosome 8 and chromosome 21), which together with t(15;17) (PML/RARα) is one of the most common translocations associated with AML. These leukemia-associated fusion proteins (LAFP) are also known to induce aberrant activation of canonical Wnt signaling which, as a result, leads to an increased self-renewal capacity of LSCs (Tenen, 2003; Muller-Tidow et al., 2004; Zheng et al., 2004; Zhao et al., 2007; Wang et al., 2010). DeKelver et al. analyzed publically available microarray data from AML M2 patients with and without the t(8;21) translocation (Valk et al., 2004). They found that 5-LO expression was ~2.3-fold higher in t(8;21) patients than in patients without the translocation. Notably, both patient groups had elevated 5-LO levels relative to normal CD34+ control samples (DeKelver et al., 2013). Additionally, it could be shown, by gene expression microarray and chip analysis, that 5-LO is a potential disease-related target gene of AML1/ETO (Lo et al., 2012). AML1/ETO, as well as its C-terminally truncated leukemic isoform, AML1/ETOex9, is able to up-regulate 5-LO via the transcription factor, KLF6 (Krppl-like factor 6), a protein which is also known to be a cell-specific positive regulator of LTC_4 synthase (Zhao et al., 2000). Furthermore, studies with $KLF6^{-/-}$ mice revealed that 5-LO is also critically required for embryonic stem cell differentiation and early hematopoiesis (Matsumoto et al., 2006).

Thus, 5-LO is a direct disease-related and pathophysiologically relevant target gene upregulated by the oncogenic leukemia associated fusion protein, AML1/ETO, which is crucially involved in the induction and maintenance of AML.

9.3. 5-LO and self-renewal capacity of leukemic cells

To analyze the effect of 5-LO on the in vitro self-renewal capacity of the LAFPs AML1/ETOex9, PML/RARα, and MLL-AF9 (translocation t(9;11)), DeKelver et al. performed serial replating assays using wild type and $5\text{-}LO^{-/-}$ murine total bone marrow cells that were transduced with either control vector or LAFPs. 5-LO deficiency led to decreased in-vitro self-renewal capacity with all LAFPs. Remarkably, loss of 5-LO abolished completely the aberrant replating capacity of AML1/ETOex9-positive cells. Nevertheless, AML1/ETOex9-expressing $5\text{-}LO^{-/-}$ cells were still capable of leukemia induction. These results led the authors to hypothesize that in the murine AML1/ETOex9 leukemia induction model, other factors may exist that allow AML1/ETOex9 to overcome these defects. It is also possible that 5-LO expression may be required to a greater extent by AML1/ETO than by AML1/ETOex9 cells in leukemia development, especially since AML1/ETO up-regulates KLF6 more strongly than AML1/ETOex9. Another reason could be that in the in-vitro and in-vivo cell culture experiments, the authors used different cell populations, containing different ratios of HSCs (total bone marrow versus fetal liver cells), suggesting that different subtypes of HSCs respond differently to the introduction of AML1/ETOex9 and 5-LO deficiency (DeKelver et al., 2013).

Taken together, these experiments demonstrated the impact of 5-LO on the self-renewal capacity of LSC. Thus, 5-LO seems to be involved in the maintenance of the undifferentiated state of these leukemic cells which is a characteristic feature of LSCs.

9.4. 5-LO and human leukemic stem cell function

5-LO also seems to be involved in human CML stem cell function, as revealed by microarray studies. In these studies, 5-LO was differentially expressed in human CD34+ CML cells, compared to CD34+ control cells (Radich et al., 2006; Graham et al., 2007). In a recent publication, the expression levels of 5-LO and BLT1 were decreased both in blood samples and CD34+ stem cells from t(9;22)-positive CML patients harboring the BCR/ABL fusion protein, compared to samples from healthy donors (Lucas et al., 2014). On the other hand, it was shown that 5-LO is up-regulated in BCR/ABL-positive $GFP^+Lin^-ckit^+Sca1^+$ LSCs in comparison to non-BCR/ABL expressing $GFP^+Lin^-ckit^+Sca1^+$ cells and that 5-LO is indispensable for the aberrant self-renewal capacity of these LSCs and the development of CML in mice (Y. Chen et al., 2009). This was revealed using a murine BCR/ABL leukemia induction model. Recipients of BCR/ABL transduced bone marrow cells from $5\text{-}LO^{-/-}$ donor mice failed to develop CML, whereas recipients of BCR/ABL transduced bone marrow cells from wild type donor mice developed the disease and died within 4 weeks. In the absence of 5-LO, myeloid leukemia cells gradually disappeared in the CML mice. A functional defect in normal HSCs could not be detected. 5-LO deficiency led only to an impairment of LT-LSCs, preventing these cells from developing CML. This defective function of LSCs in the absence of 5-LO was correlated with a reduction of β-catenin expression levels, suggesting a further regulatory link between 5-LO and Wnt signaling.

The effect of 5-LO deficiency on BCR/ABL-positive LSCs was further investigated in studies using the approved iron-chelating 5-LO inhibitor zileuton. Zileuton led to impairment of BCR/ABL positive LSCs in a similar manner to that observed with 5-LO knock-out. Pharmacological suppression of 5-LO by zileuton in mice with BCR/ABL-induced CML prolonged their survival compared to control mice. An even better therapeutic effect could be achieved with imatinib, a first generation BCR/ABL tyrosine kinase inhibitor, in combination with zileuton. However, BCR/ABL-induced lymphoid leukemia, which originates from more committed lymphoid progenitors than BCR/ABL-induced CML, was not affected by 5-LO deficiency (S. Li et al., 1999), suggesting that 5-LO is specifically required by CML stem cells and not by ALL stem cells (Y. Chen et al., 2009). These results were followed by a clinical phase I study in which zileuton was tested in combination with imatinib for the treatment of CML (Safety of Zileuton (Zyflo) in Combination With Imatinib Mesylate (Gleevec) in CML; http://clinicaltrials.gov trial No. NCT01130688). This study has been completed but the results still remain to be published. Another phase I study for treatment of BCR/ABL-positive CML with zileuton in combination with dasatinib, a second generation BCR/ABL tyrosine kinase inhibitor, is currently ongoing (Evaluating the Safety of Zileuton (Zyflo®) in Combination With Dasatinib (Sprycel®) in Chronic Myelogenous Leukemia; http://clinicaltrials.gov trial No. NCT02047149).

Recently, we demonstrated that 5-LO is also crucial for the maintenance of LSC in a PML/RARα-positive stem cell model of AML (Roos et al., 2014). First, we performed inhibitor studies on Sca1+Lin− murine hematopoietic stem and progenitor cells (HSPC). The HSPC were either transduced with PML/RARα or an empty vector. As 5-LO inhibitors, we used the non-redox type inhibitor CJ-13,610 and zileuton. These inhibitors were both able to abolish the aberrant replating efficiency of PML/RARα-expressing HSPCs in a concentration dependent manner. The same inhibitory effect could be observed on the LT- and ST-stem cell capacity. No cytotoxic effect could be observed after treatment of the non-PML/RARα-positive control cells, which is consistent with the work of Y. Chen et al. (2009). Furthermore, the occurrence of stem cell suppression coincided with inhibition of Wnt signaling. This was shown by immunohistochemical staining of murine spleen colonies induced by PML/RARα against β-catenin. Treatment with CJ-13,610 led to reversal of the PML/RARα-activation of Wnt signaling.

Collectively, these experiments confirm a direct link between the 5-LO and Wnt signaling pathways which is indispensable for the maintenance of LSCs. Accordingly, genetic and pharmacological inhibition of 5-LO led to an impairment of the LSC in AML and CML cells supporting the hypothesis of an regulatory interaction of both pathways. Additionally, pharmacological inhibition of 5-LO significantly increased the therapeutic effect of imatinib, a classical therapeutic agent used in the standard therapy of CML. Thus, a combination of standard therapeutics and 5-LO inhibitors could be beneficial for the clinical outcome of these patients.

9.5. Potential mechanism of 5-LO interaction with Wnt signaling

To confirm and investigate more extensively the involvement of 5-LO in the regulation of the Wnt signaling pathway, we performed co-

immunoprecipitation experiments as well as reporter gene assays and immunofluorescence microscopy in different cancer cell lines. In these studies, we demonstrated that enzymatically inactive 5-LO traps β-catenin at the nuclear envelope (Fig. 5), prevents the translocation of β-catenin to the nucleus, and therefore, suppresses the PML/RARα-induced activation of Wnt target genes. Additionally, we observed that 5-LO directly interacts with β-catenin (Roos et al., 2014), suggesting a connection between Wnt signaling and the 5-LO pathway represented by a 5-LO/β-catenin association. However, an interaction of 5-LO with additional components of the Wnt signaling pathway could not be excluded. Furthermore, the exact molecular mechanism(s) by which inactive 5-LO inhibits the translocation of β-catenin to the nucleus and interacts with β-catenin and possibly other Wnt pathway components still needs detailed experimental investigation.

9.6. 5-LO and Wnt signaling in solid tumors

Increasing evidence in the literature implicates 5-LO as a player in the growth of several solid tumor types, including pancreatic, colorectal, prostate and breast cancers (Kort et al., 1992; Ding et al., 1999a, 1999b; Gupta et al., 2001; Hennig et al., 2002; Jiang et al., 2003; Soumaoro et al., 2006; Ihara et al., 2007; Bishayee & Khuda-Bukhsh, 2013) and the Wnt signaling pathway contributes to the pathogenesis of these cancers (Liu et al., 2000; Milovanovic et al., 2004; Yardy & Brewster, 2005; Yokoyama et al., 2014). However, even though a correlation between 5-LO and Wnt signaling in solid tumors is obvious, at the moment no study has yet been published that directly addresses the relation between 5-LO or the effects of 5-LO inhibitors and the Wnt signaling pathway in these tumors types.

10. Conclusion and future direction

Dysfunctional Wnt signaling is emerging as having pathophysiological significance in a number of diseases, including cancer, classical inflammatory diseases, and neurodegenerative diseases triggered by neuroinflammatory processes.

Numerous high-throughput screens have been employed to obtain small-molecule targeted drugs that would interfere with components of Wnt signaling, mainly directed towards disruption of the binding of β-catenin to its nuclear target, TCF/LEF.

Many compounds with promising activity in cell culture have been identified and characterized, but translation into clinically applicable drugs is still an apparently insurmountable obstacle due to lack of selectivity and a number of pleiotropic off-target effects. This relates particularly to drugs that interfere with the proteasomal degradation of β-catenin, which results in severe side effects in normal tissue as non-nuclear β-catenin contributes to the stabilization of physiologically important intercellular adherens junctions.

The clinical value of silencing Wnt signaling further upstream, i.e., at the level of the Frizzled receptor, remains unclear as a number of tissues depend on a certain basic Wnt activity which may be associated with unknown adverse side effects.

Thus, a complex mixture of problems is encountered in developing Wnt inhibitors using in silico methods or high-throughput screens using compound libraries.

Several inhibitors of eicosanoid signaling, such as NSAIDs, have been shown to interfere in a clinically relevant manner with Wnt signaling, by COX/PGE_2 as well as COX/PGE_2-independent mechanisms and thereby, induce Wnt-dependent anti-tumorigenic effects (see Section 7).

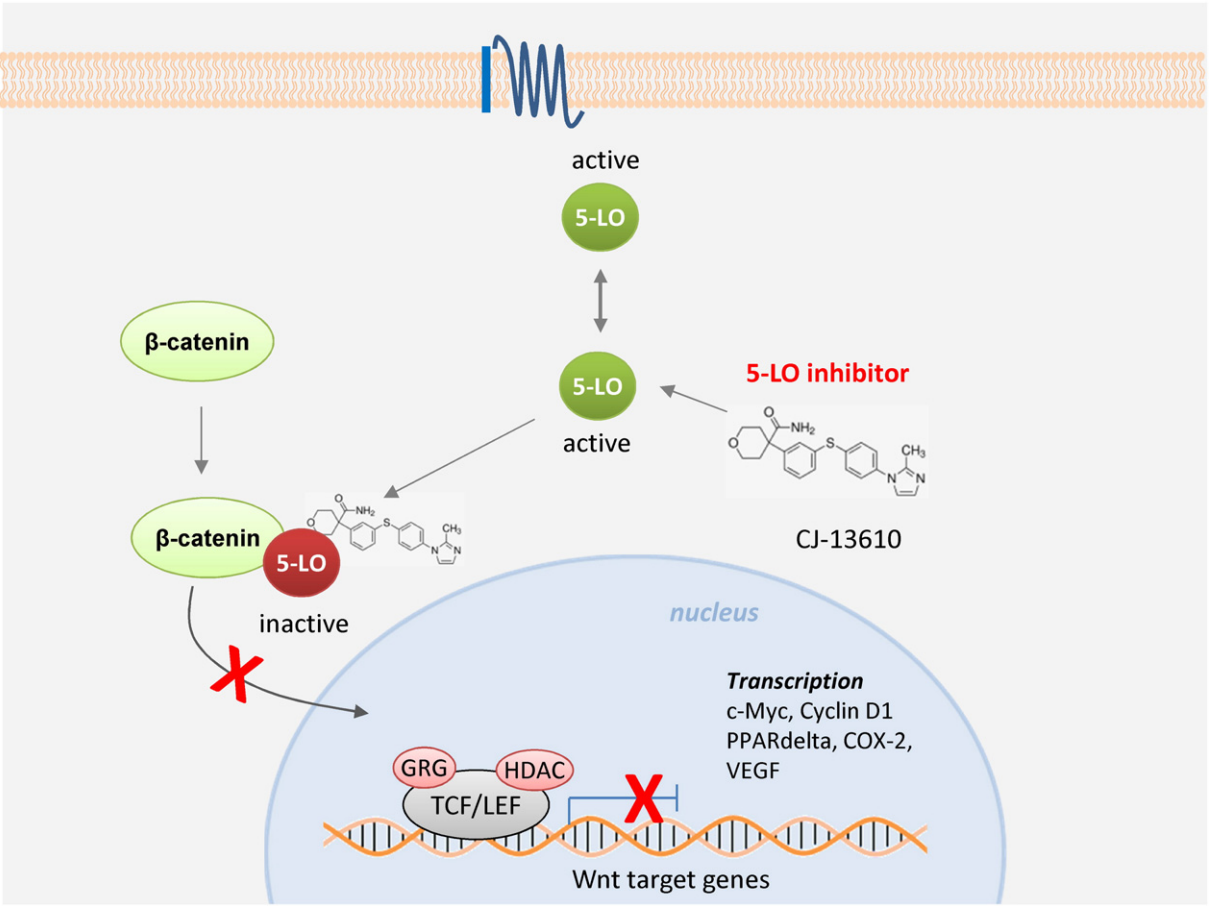

Fig. 5. Proposed mechanism of Wnt signaling suppression by 5-LO. Binding to β-catenin of enzymatically inactive 5-LO, after treatment with the 5-LO inhibitor CJ-13610, inhibits the Wnt signaling pathway by preventing the translocation of β-catenin to the nucleus and subsequent gene transcriptional activity. For further details see Section 9. GRG, Groucho; HDAC, histone deacetylase; TCF/LEF, T-cell factor/lymphoid enhancer factor.

Mechanistically, NSAIDs differ widely from classical Wnt inhibitors in their actions on the Wnt signal transduction cascade and follow novel mechanistic routes of suppression of Wnt signaling. Furthermore, several very recent studies presented here provide valid and comprehensive evidence for an essential role of 5-LO in and interconnection with Wnt signaling in the pathogenesis of leukemia and the maintenance of LSCs in certain sub-types of leukemia.

As a consequence, pharmacological 5-LO inhibition, with concomitant suppression of Wnt signaling, is a potential novel approach to adjuvant stem cell therapy in AML and CML as well as other Wnt-dependent diseases, such as breast cancer, human head and neck squamous cell carcinomas, and some inflammatory diseases (see Section 4) (Liu et al., 2013).

Further insight into the value of anti-5-LO therapy is likely to be gained from two clinical trials — the recently finished study with zileuton and imatinib as combined treatment for CML, as well as the new study with zileuton and dasatinib. Yet to fully understand the role of 5-LO in the Wnt signaling pathway (and possible other leukemia-related pathways), more detailed and comprehensive studies are necessary.

A number of approved NSAIDs, administered at high doses, suppress Wnt signaling in patients but without causing severe concomitant systemic toxicity in the organism. The majority of side effects of these drugs are well documented and derives from suppression of prostaglandin biosynthesis. This includes mainly cardiovascular and thromboembolic events and gastrointestinal ulceration (Bhala et al., 2013). However, there are no reports of adverse effects or toxicity directly arising from the Wnt-inhibitory activity of NSAIDs.

Thus, suppression of Wnt signaling by NSAIDs may follow advantageous mechanistic strategies and NSAIDs as well as 5-LO inhibitors, therefore, should be reassessed as a basis for the design of a new class of Wnt inhibitors.

Following the clarification of the exact molecular mechanism by which these drugs inhibit Wnt signaling, it seems reasonable that chemical structures responsible for Wnt inhibition could be separated from the structural elements causing suppression of COX enzymes. The fact that a number of drugs targeting eicosanoid synthesis concomitantly suppress Wnt signaling independently of COX could further indicate a possible common, but still unidentified key target for the regulation of Wnt activity.

In this respect, 5-LO inhibitors, such as CJ-13610, activate a potentially naturally occurring novel 5-LO-dependent mechanism of suppression of Wnt signaling that is non-toxic, at least in mice, and might be well tolerated in patients.

Future careful studies are needed to clarify the regulatory mechanism linking eicosanoid synthesis pathways to oncogenic Wnt signaling as a potential basis for novel anti-inflammatory drugs that concomitantly suppress Wnt signaling.

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Conflict of interest statement

MJP is a consultant to Leo Pharma a/s and Xellia Pharmaceuticals ApS. The other authors declare that there are no conflicts of interest.

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