REVIEW ARTICLE

The Canonical Wnt/β-catenin Pathway as a Therapeutic Target in Multiple Myeloma

Ioanna Savvidou[1], Tiffany Khong[1], Karen Alt[2], Andrew Spencer[1,3]

Authors' affiliations:

[1] Myeloma Research Group, ACBD, Monash University & Alfred Hospital, Melbourne, Australia.

[2] ACBD, Monash University & Alfred Hospital, Melbourne, Australia.

[3] Malignant Haematology and Stem Cell Transplantation Service, Alfred Hospital, Melbourne, Australia.

*** Corresponding author:** Andrew Spencer E-mail: aspencer@netspace.net.au

Abstract

It has been more than 30 years since Nusse and Varmus identified a new mouse proto-oncogene int1 (integration 1), conserved across multiple species, and already recognised in Drosophila as Wingless (Wg). Wnts are now known to activate multiple signalling pathways, the best understood and extensively investigated being the canonical/β-catenin dependent pathway. β-catenin signalling has been found dysregulated to varying degrees and via multiple mechanisms in both solid and haematologic cancers, including multiple myeloma (MM). Recently developed inhibitors of the Wnt canonical pathway have proven to be potentially effective against MM, with minimal side effects. There is cautious optimism that some of these inhibitors will be added in our armamentarium against MM in the not so distant future. In this review, we discuss the possible mechanisms of Wnt-canonical pathway dysregulation in the pathogenesis of MM. Furthermore, we summarise the pathway inhibitors that have been validated in the disease in pre-clinical models or clinical trials and their potential challenges.

Introduction

Multiple myeloma (MM) is an incurable neoplastic plasma-cell disorder, accounting for approximately 1% of neoplastic diseases and 13% of haematologic cancers[1]. Despite the improvement in progression-free and overall survival of MM patients after the introduction of high-dose chemotherapy and stem-cell rescue (autologous stem cell transplant [ASCT]) and more effective pharmacotherapies, the available treatment regimens are not curative[2]. Resistance to commonly used therapy, either innate or more often secondary, is the main cause of death in MM[3]. This highlights the significant unmet need for the discovery and development of newer agents, which target pathways important for the pathogenesis and/or progression of MM, that could potentially be used alone or in combination with established anti-MM treatments.

The canonical Wnt signalling pathway modulates the balance between stemness and differentiation in several adult stem cell niches, including haemopoiesis within the bone marrow[4]. Furthermore, it is commonly dysregulated in a range of solid tumours including colon, liver and pancreas carcinoma[5] and haematological malignancies including acute myeloid leukaemia (AML)[5,6], chronic myeloid leukaemia (CML)[7], chronic lymphocytic leukaemia (CLL)[8], and MM[9]. In this review, we discuss the possible role of canonical Wnt pathway activation in the pathogenesis and progression of MM. We also review the current therapeutic inhibitory agents targeting the pathway, both efficacy and the possible limitations for their use.

The Wnt pathway and its role in adult tissues

Wnt signalling is highly conserved in all metazoa[10]. In mammals, the Wnt gene family comprises 19 members, responsible for the production of 19 cysteine-rich secreted glycoproteins, 10 members of the Frizzled family of 7 transmembrane Wnt receptors, as well as several co-receptors such as LRP5/6, Ror1 and Ror2[11]. Traditionally, the Wnt pathway is divided into canonical and non-canonical pathways, more recently referred to as the β-catenin dependent and independent pathways, respectively.

Within the canonical pathway, in the absence of Wnt ligands, cytoplasmic β-catenin, a key player in the pathway, is constantly degraded by the Axin complex (comprised of axin, tumour suppressor adenomatous polyposis coli [APC], casein kinase 1 [CK1], and glycogen synthetase kinase 3 [GSK3]) (figure 1 off-state). The amino terminal of β-catenin, upon phosphorylation by CK1 and GSK3, is subsequently recognised by an E3 ubiquitin ligase subunit, ubiquitinated and degraded by the proteasome. Thus, β-catenin is inhibited from reaching the nucleus where the Wnt target genes remain repressed under the influence of the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins. Conversely, upon the binding of Wnt ligand to the seven-pass transmembrane Frizzled receptor (Fzd) and its co-receptor, low-density lipoprotein receptor related protein 6 (LRP6) or its close relative LRP5, Dishevelled protein (Dvl) phosphorylates LRP6 leading to the recruitment of the Axin complex to the cell membrane (figure 1 on-state). As such, β-catenin remains unphosphorylated, accumulates in the cytoplasm and then translocates into the nucleus, where it binds to the TCL/LEF complex initiating transcription of Wnt target genes[12].

In adult life, Wnt signalling has been shown to contribute to the stem cell-niche interaction, which is responsible for the stem cell maintenance of various tissues, such as the haemopoietic system, colon, skin or mammary glands[13]. Although there is strong evidence for the role of the Wnt pathway in adult stem cells in skin and gut, experimental data supporting its role in haemopoietic stem cells (HSC) is controversial. Recent data suggest that the levels of the Wnt signalling in the haemopoietic organ are significantly lower than those in skin, gut, breast or central nervous system[14]. Thus, mild enhancement (up to 2-3 fold increase) can lead to increased proliferation and repopulating capacity of HSC, whereas greater up-regulation of the pathway results in exhaustion of the HSC pool and inability to accomplish haemopoietic repopulation in recipient mice[14]. Similarly, modest down-regulation of the pathway to approximately 75% of its normal activity, is well tolerated by HSC, whereas complete absence of Wnt signalling seems to be detrimental to HSC function. Besides its role in the regulation of HSC function, Wnt canonical signalling has been implicated in the differentiation process of a number of haemopoietic line-

ages, in a dose dependent manner[4], but with all mature blood cells, with the exception of T lymphocytes, expressing undetectable levels of Wnt canonical pathway activity when utilising a Wnt reporter activity assay.

Recognising the invariable involvement of bone lysis in progressive MM[15] the potential role of the Wnt canonical pathway on bone homeostasis in MM is of significant interest. Indeed, more than a decade ago, several mutations resulting in alterations in bone density (increased or decreased) pointed to the canonical Wnt pathway[16-20] being an important player in the maintenance of bone health. Although the role of canonical Wnt pathway on bone homeostasis is reviewed elsewhere[21], Wnt signaling is thought to enhance the commitment of mesenchymal stem cells to the osteoblastic lineage[22] (responsible for bone formation) while, indirectly repressing osteoclast (responsible for bone resorption) differentiation[23]. However, Wnt canonical pathway can directly influence osteoclasts, in a dosage-dependent manner, favoring the proliferation of osteoclast precursors when under a minimum threshold, while exerting an inhibitory effect at levels above this threshold, whereas pathway attenuation is able to accelerate osteoclastogenensis[24].

Mechanisms of the Wnt pathway dysregulation in multiple myeloma

Several studies have demonstrated aberrant activation of the canonical Wnt signalling pathway in MM with total β-catenin levels in MM cells, as shown by Western Blotting (WB), higher when compared with those in healthy plasma cells, or mature B cell subsets[9,25,26] (naïve, germinal center and memory B cells). Furthermore, the majority of MM samples express the non-phosphorylated and/or nuclear active form of the protein[9,25]. Similarly, immunohistochemical studies (IHC) of MM bone marrow biopsies have revealed nuclear staining for β–catenin in 34% of cases[27]. Interestingly, the same study revealed an absence of nuclear β–catenin in samples derived from patients with Monoclonal Gammopathy of Undetermined Significance (MGUS), a MM premalignant condition, moreover, its up-regulation significantly correlated with more advanced MM disease. Activity of the pathway in MM has been further confirmed by up-regulation of down-stream target genes, such as CD44, c-MYC and Cyclin D1[26]. However, although the Wnt pathway is activated in MM cells, in contrast to the majority of solid tumours, no activating mutations of the major pathway members have been identified. Additionally, the observation that the pathway could be further activated experimentally, by Wnt ligands (e.g. Wnt3a) or GSK3β inhibitors (e.g. LiCl), inducing, in the majority of cases, greater MM cell proliferation[9,25], implies an intact pathway, pointing to possible dysregulation of regulatory components.

Consistent with this, MM cells have been shown to produce their own Wnt ligands (e.g. Wnt3, 4, 7a, 8a, 10a, 11, 14 in HMCL[28], 5a, 10b, 16 in purified primary MM cells[9]) and to overexpress several Fz receptors, establishing an autocrine activation loop for the pathway. Interestingly, data indicate the participation of the non-malignant stromal cells in the activation of the pathway in a paracrine manner, by also producing Wnt ligands, but is not clear if this production is enhanced in MM derived stromal cells as compared non-MM derived stromal cells. Additionally, autocrine and/or paracrine Wnt signalling can be further facilitated by aberrant expression of LGR4 by MM cells, enabling them to hijack R-spondins produced by BM osteoblasts. Under the presence of Wnts, R-spondins binding to LGR4 causes deregulation of Wnt co-receptor turnover, rendering MM cells hypersensitive to Wnt ligands[29].

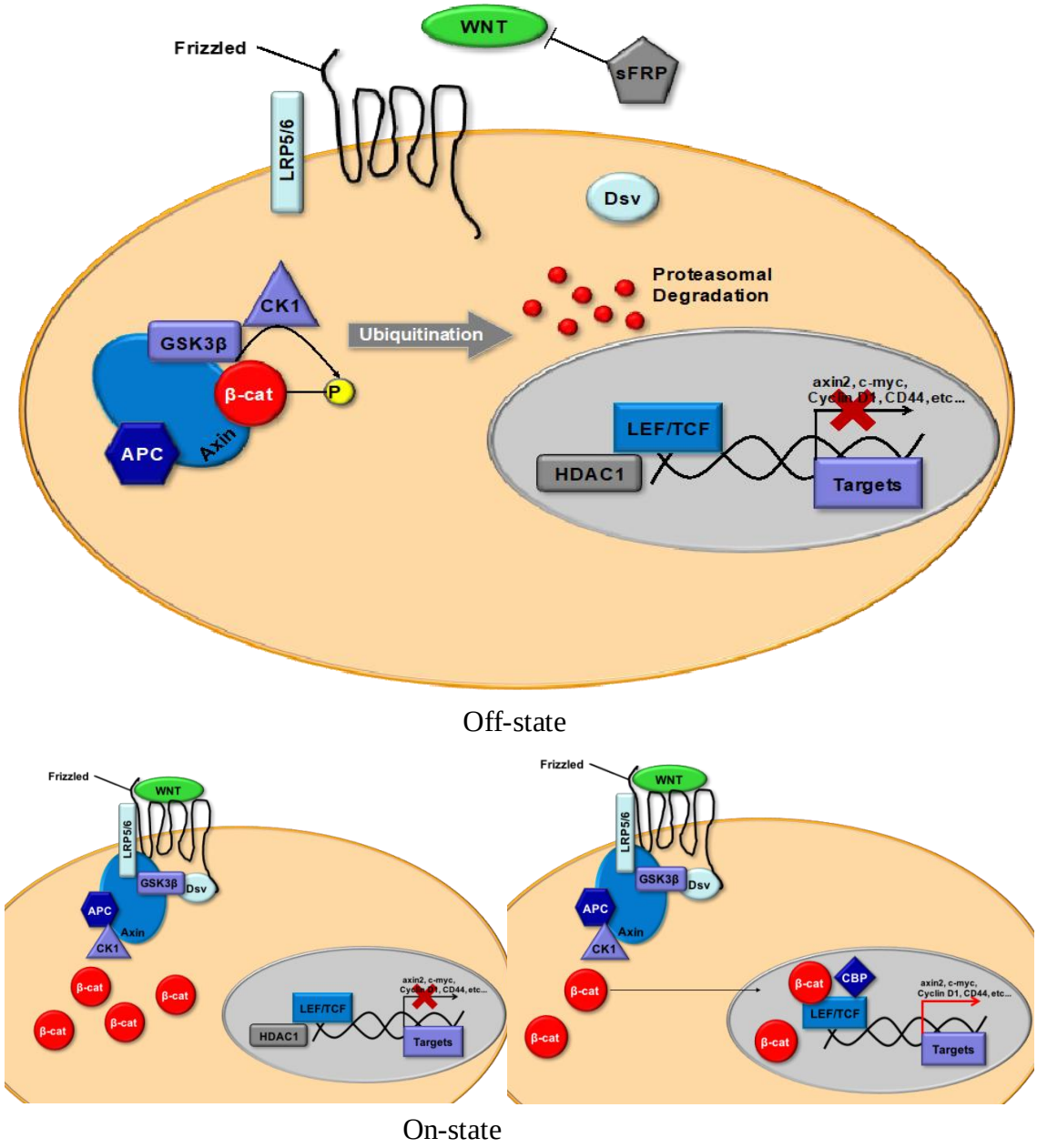

Figure 1: Off-state of Wnt canonical pathway. In the absence of Wnt ligands, cytoplasmic β-catenin is bound to its destruction complex. Thus, it gets phosphorylated and subsequently degraded by the ubiquitin-proteasome degradation system. **On-state of Wnt canonical pathway.** Upon stimulation of the receptor complex and further activation of Dvl, the destruction complex migrates to the vicinity of the receptor, liberating β-catenin. Built-up cytoplasmic β-catenin migrates into the nucleus, where upon binding to the TCF/LEF complex initiates transcription of downstream target-genes.

Although Wnt ligands have been traditionally divided into canonical (Wnt3a, Wnt1, Wnt8) and non-canonical (Wnt5a) pathway activators, depending on their ability to activate the canonical Wnt/β-catenin pathway or not, recent findings argue that the pathways initiated by different Wnts may not depend exclusively on the intrinsic properties of the ligands, whereby, pathway activation could also depend on the context of the receptors expressed on a given target cell[30]. Hence, up-regulation of Wnt ligands

by tumour or tumour stromal cells cannot entirely explain the dysregulation of the Wnt canonical pathway. Furthermore, similar to solid tumours (hepatocellular[31], breast carcinoma[32]) MM cells have been demonstrated to express high levels of LRP6 (and to a lesser extent LRP5) in comparison to lymphoma cell lines[33]. Consistent with this, we have shown that the median expression of the canonical pathway co-receptor LRP6 mRNA is up-regulated in HMCL and primary MM cells when compared to healthy plasma cells, whereas there was no difference for ROR1 and ROR2, the main non-canonical pathway co-receptors. Similarly, LRP6 protein was up-regulated in the majority of HMCL tested, whereas, it was absent in normal plasma cells (unpublished data), implying priming of malignant plasma cells for β-catenin pathway activation.

In addition to positive regulation, the Wnt pathway is also under the control of several antagonists/inhibitors, both secreted and transmembrane. Among these inhibitors, the secreted DKK protein family (DKK1,-2,-3,-4 and DKKL1) is the best characterized[34] and studied largely due to its recognized role in bone metabolism. DKK1 and DKK2 bind to LRP5/6 preventing the Wnt-LRP interaction and disrupting the Wnt-mediated Fz-LRP6 complex formation. In the presence of Kremen receptor (1 and 2 [Krm1/2]) binding of DKK1 can induce rapid endocytosis and removal of LRP6 from the cell surface, thus potentiating the inhibitory effect of DKK[34]. MM cells derived from a subset of newly diagnosed MM patients have been shown to express higher levels of DKK1 as compared to healthy plasma cells or plasma cells derived from patients with MGUS[35]. Moreover, increased serum levels of DKK1 are correlated with the presence of osteolytic bone disease[35,36,37] and have been shown to decrease in patients responding to anti-MM therapy[38,39]. Conversely, and similar to other malignancies[40] (solid[41-45] and haematologic[46-48]), in both HMCL and primary MM samples one or more Wnt pathway inhibitors (including DKKs) have been found to be silenced by promoter methylation[48]. Out of the 42% positive MM samples for methylation, 62% had two or more inhibitors methylated[48], with DKK1 expression in primary MM biopsies being negatively correlated with disease stage (earlty versus late) and the presence of nuclear β-catenin[27]. Thus, available data would indicate that increased DKK1 expression is restricted to a specific, relatively early, stage of MM disease progression[35] similar to that seen in solid tumours[49].

Similar to DKK1, sclerostin, another secreted Wnt inhibitor produced by the osteocytes that inhibits osteoblast-driven bone formation, has recently been found to be elevated in a subset of newly diagnosed MM patients presenting with bone fractures[50]. Its levels are further elevated in relapsed MM patients whereas bortezomib treatment lowers sclerostin levels, independently of its direct anti-MM effect[50]. Interestingly, MM cells have been shown to contribute to the dysregulated expression and secretion of sclerostin not only by osteocytes but also from osteoblasts and mesenchymal stem cells, possibly through DKK1 stimulation[51]. However, clinical data demonstrate only a weak correlation with serum DKK1 levels (r=0.201) [50] implying additional, yet unknown mechanisms for sclerostin dysregulation are in play. Declining renal function (not caused by MM) has also been shown to increase renal sclerostin elimination[52], adding another level of complexity in the regulation of this molecule.

Finally, recent data indicate that β-catenin can be further stabilized in MM, especially in the nuclear compartment, by the process of SUMOylation (small ubiquitin-like modifier). SUMOylation is known to be aberrantly activated in MM, whereas genetic inhibition of the process is able to increase

the destruction of β-catenin by the ubiquitin-proteasome system, implying a competitive effect of SUMOylation to ubiquitin[53]. Thus, while the up-regulation of the Wnt canonical pathway in MM, and its potential role in the proliferation, survival and possibly drug resistance of MM cells, has made it an attractive potential therapeutic target in MM the importance of the pathway for the homeostasis of several organs in adult life, highlights the potential complexities that could ensue from possible off-target effects.

Wnt-canonical pathway inhibitors in MM

Based on the important role of Wnt/β-catenin signalling pathway dysregulation in tumourigenesis, several groups have developed and validated pathway inhibitors (figure 2) with therapeutic intent. These inhibitors are broadly classified into biologics (e.g. monoclonal antibodies, recombinant nucleic acids) and small-molecule compounds[54], the latter further divided into conventional (e.g. NSAIDs, imatinib) and novel agents. For the identification of novel agents high-throughput screens have also been used extensively to identify inhibitors of different pathway constituents[55,56].

There are three screening methods widely used for identification of potent Wnt pathway inhibitors[54,56]. The first method is based on protein-protein interaction (ELISA-based), measuring the ability of compounds to displace TCF4 from β-catenin[57].

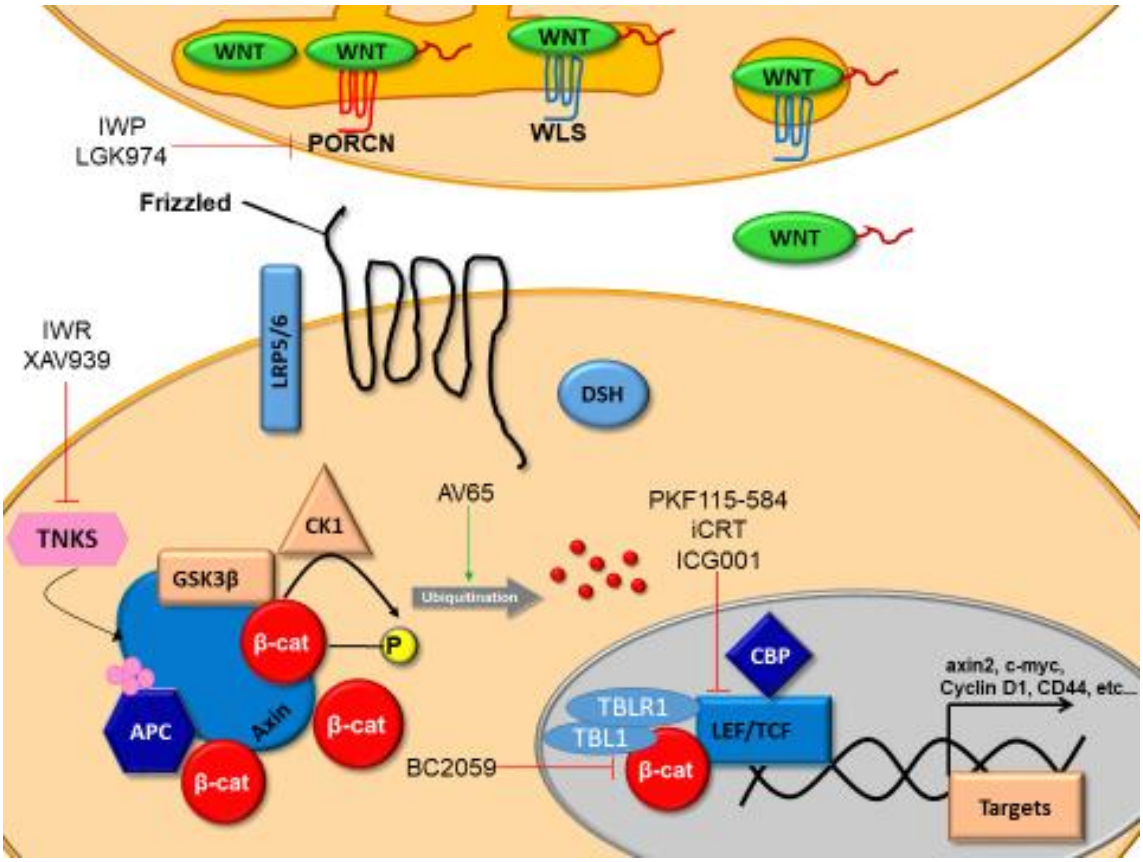

Figure 2: Examples of Wnt pathway inhibitors and their site of interference. Wnts are thought to be post-translationally palmitoleated (red line) by Porcupine (PORCN) in the endoplasmic reticulum, before interacting with Wntless (WLS), that is responsible for their transportation and secretion. Tankyrases (TKNS) promote the ubiquitination of axin leading to its degradation. Inhibition of TKNS leads to stabilization of axin, thus attenuating Wnt signaling.

Among 7000 compounds so screened, 6 were identified as potential, specific, Wnt/β-catenin pathway inhibitors. PFK115-584, one of the compounds identified, has been shown to exert anti-MM effect *in vitro* and *in vivo* but with adverse effects on haemopoiesis and with wasting of some animals[26]. However, neither PFK115-584 nor CGP049090 were associated with evident systemic toxicities when used in 2 alternative murine AML models (C57B1/6 and BALB/c)[58].

The second method utilises cell-based reporter assay screening, where cells are transfected with a TOPFlash reporter containing TCF/LEF binding sites up-stream of luciferase[56]. Compounds that exert an inhibitory effect on the pathway cause down-regulation of TCF/LEF transcriptional activity, expressed as reduction in luciferin. ICG-001[59] and XAV939[60] are two compounds that have been identified by cell-base reporter screening. ICG-001 (also named PRI-724) has been shown to inhibit the binding of CBP (CREB binding protein) coactivator to β-catenin in colon carcinoma cells, inhibiting the transcriptional activity of the latter[59]. Of critical importance to its potential therapeutic utility, ICG-001 does not interfere with p300/catenin dependent signalling and therefore has no impact on normal stem cell populations[61]. However, although ICG-001 has been shown to have anti-MM effects, its pro-apoptotic effect has recently been shown to be Wnt-independent[62]. Nevertheless, PRI-724 is currently being evaluated in six clinical trials (four completed, one study terminated and one withdrawn) for patients with solid tumours, HCV-induced cirrhosis and AML/CML, either as monotherapy or in combination with chemotherapy. Some of the published results describing its use in patients with pancreatic adenocarcinoma concluded that PRI-724 combined with chemotherapy is safe and demonstrates modest clinical activity (NCT01764477)[63]. Nonetheless, there are currently no clinical studies concerning PRI-724 use in MM.

XAV939 is a tankyrase inhibitor which causes axin stabilisation, promoting further phosphorylation and degradation of β-catenin[60]. Although published data have confirmed the up-regulation of Axin protein with a concomitant decrease of total and nuclear β-catenin protein in SW480 and SW620 cells after XAV939 treatment, no cytotoxic effect was seen when XAV939 was used as monotherapy[64]. In accordance, our preliminary validation of XAV939 could not demonstrate any cytotoxic effect against HMCL (unpublished data). Similar to ICG-001, inhibitors of β-catenin regulated transcription (iCRTs), iCRT-3(oxazole) and iCRT-5 (thiazole), were identified by the use of a targeted suppressor screen for their ability to specifically target the transcriptional activity of β-catenin-TCF[65]. iCRT-3 and -5 were able to inhibit MM cell proliferation at doses greater than 15μM *in vitro*. Interestingly, they also inhibited the expression of VEGF and VEGF mediated MM migration[66]. However, no *in vivo* validation of the iCRTs is currently available in MM.

LGK975/WNT974 is a porcupine inhibitor that was derived from cell-based reporter assay screening[67]. Porcupine is a membrane bound O-acyltransferase specifically dedicated to Wnt post-translational acylation, a process necessary for the final maturation and further secretion of Wnt ligands[67]. It has been shown to inhibit Wnt signalling in a coculture assay, where Wnt-secreting cells were cocultured with Wnt reporter cells but with no cytotoxic effect for doses up to 20μM[67]. This inhibitory effect was rescued by the addition of exogenous Wnt3a. Similarly, in our hands, LGK975/WNT974 showed only weak cytotoxicity at doses greater than 1μM and this effect could not be rescued by the addition of exogenous rhWnt3a, implying non-specific cytotoxicity (unpublished data). Although

HMCL and primary MM cells have been shown to secrete Wnt ligands, recent findings argue that porcupine is not necessary for the secretion of all Wnt ligands, and that this dependence is most probably context specific[68]. These authors concluded that the pharmacological inhibition of porcupine may not be an effective strategy to block Wnt release in all human cells[68]. Nonetheless, LGK975/WNT974 is currently being evaluated in a phase I multicentre clinical trial (NCT01351103) for patients with malignancies dependent on Wnt ligands, either alone or in combination with PDR001 (anti-PD-1), an immunotherapeutic agent.

CWP232291 is another Wnt/β-catenin inhibitor that has progressed to early stage clinical trials as monotherapy in patients with relapsed/refractory AML, CML, Myelodysplastic syndrome, Myelofibrosis (NCT01398462) and relapsed/refractory (RR) MM as monotherapy or in combination with lenalidomide and dexamethasone (NCT02426723). Initial reports show single agent safety as well as early evidence of efficacy in subjects with RR MM[69]. However, although the drug was initially introduced as a β-catenin inhibitor, through activation of caspases leading to β-catenin destruction, recent data imply that it exerts a broader effect as a modulator of SRC associated in mitosis of 68 kDa protein (SAM68)[70].

The third method of screening is biomarker-based screening, which validates the effect of different compounds on specific transcriptional activities[71]. With respect to the Wnt pathway, siRNA of β-catenin was used to create an altered transcriptional profile, recognizing nine potential biomarkers as indicators of the response to pathway inhibition[71]. Chemical compounds were then screened for their ability to cause a similar expression pattern. AV-65, derived from this screening approach, was shown to suppress the growth of MM cells and to prolong survival in a mouse model of orthotopic MM[72] by enhancing the ubiquitination and subsequent proteasomal degradation of β-catenin.

BC2059, an AV-65 derivative is an anthracene dioxime compound: 2-((3R,5S)-3,5-dimethylpiperdin-1ylsulfonyl)-7-((3S,5R)-3,5-dimethylpiperidin-1-ylsulfonyl) anthracene-9,19-dione dioxime[73]. It has been shown to interrupt the Wnt/β-catenin pathway by disrupting transducin β-like protein 1 (TBL1) interaction with the coactivator molecule β-catenin[73]. Besides their role as members of the co-repressor silencing mediator for retinoid and thyroid hormone receptor (SMRT)-nuclear receptor co-receptor (N-CoR) complex[74], TBL1 and its related protein (TBLR1) have been shown to play a specific role in the recruitment of β-catenin to the Wnt target-gene promoter[75]. Our group was able to show that BC2059 exerts a potent anti-MM effect *in vitro*, ex-vivo and *in vivo* at clinically feasible doses, whereas a lack of cytotoxic effect on PBMC provided proof of its specificity. Moreover, we demonstrated that BC2059 was able to inhibit the Wnt/canonical pathway by inhibiting its transcriptional activity[25]. Thus, in a highly heterogeneous disease like MM, where multiple changes can lead to upregulation of the same pathway, targeting the final step of this pathway seems a logical approach.

The ability to target the WNT signalling pathway offers enormous promise, however, substantial risks and concerns are ever present with regard to the targeting of such a crucial pathway in stem cell maintenance and tissue homeostasis. In this context, administration of BC2059 in a MM murine model did not have any impact on the gastrointestinal epithelium and haemopoietic system, however we identified histological evidence of disruption of the normal hair cycle, with concomitant alterations of skin structure[25], as would be anticipated from the literature[76]. We further evaluated the effect of BC2059-nanoparticles on bone

homeostasis in the same animal model. MicroCT reconstructions and quantifications of Lumbar vertebrae (L5) after the animals reached study end-point did not reveal any deleterious effect of BC2059 on bone homeostasis when compared to the vehicle treated animals (figure 3), confirming a considerable therapeutic window for the inhibitor. Although it is outside the scope of this review, we cannot overlook the emergence of Wnt pathway enhancers/activators, largely represented by DKKs inhibitors[78]. Based on the growing appreciation for the functions of Wnt in regulating skeletal cell behaviour[79] in combination with the well-proven interaction of MM with the BM microenvironment, there are currently several clinical trials evaluating the role of these inhibitors (BHQ880/990, DKN-01) in MM. Initial results with BHQ880 monotherapy in patients with smoldering MM, showed increased bone anabolic activity with no unexpected adverse events[80]. Similarly, a sclerostin-antibody has recently been confirmed to exert bone anabolic effects in a MM-xenograft model, but without any direct anti-MM activity[51]. In contrast, when used in combination with the proteasome inhibitor carfilzomib, it was able to inhibit tumour burden while increasing bone volume[51].

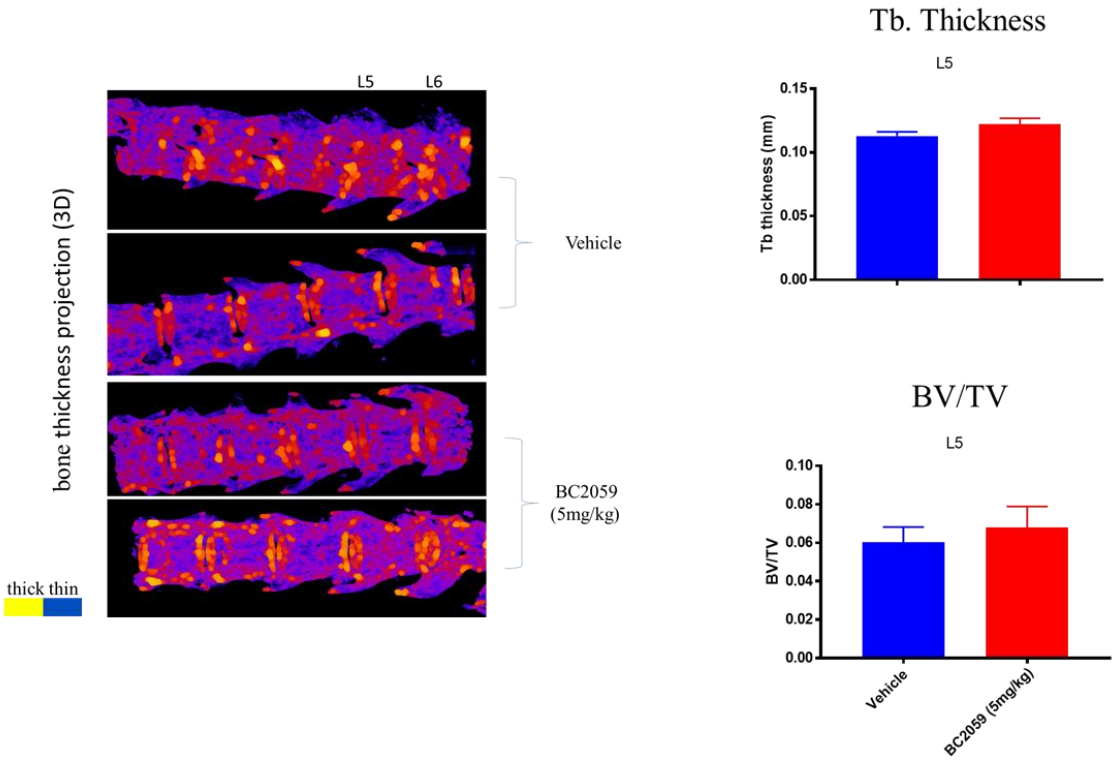

Figure 3: µCT bone studies on the effect of Wnt pathway inhibition on bone homeostasis. Lumbar vertebrae were scanned using micro-computed tomography (µCT) after the mice reached experimental end-point. µCT reconstruction and quantification showed no inferiority of the Wnt inhibitor arm. Tb.:Trabecular, BV/TV: bone volume fraction= bone volume/ total volume. Image acquisition and analysis protocols adhered to the JBMR guidelines[77].

While, the Wnt-canonical pathway is clearly essential for development and tissue homeostasis the high frequency of pathway mutations in several different cancers highlights the significance of Wnt/β-catenin signalling in tumourigenesis[5]. Additionally, an increasing number of tumours, including MM, have been shown to manifest a dysregulated Wnt-canonical pathway through alternate activating mechanisms. Thus, for

the last two decades, high throughput screens have been used to identify inhibitors of different pathway constituents. However, the significant reliance on the same pathway of healthy, highly proliferative tissues mandates that the clinical evaluation of available inhibitors be undertaken with significant caution. These reservations aside, there are several new-generation inhibitors, which have been shown to exert potent anti-MM effects *in vitro* and *in vivo* with minimal adverse events. Moreover, clinical trials are currently evaluating the efficacy of these inhibitors in various tumours and the results are highly anticipated. If proven safe, these inhibitors could represent a powerful tool in our arsenal against cancer including MM.

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