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Development of WNT inhibitors as novel anti-cancer drugs

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RÉSUMÉ

La voie de signalisation WNT joue un rôle essentiel dans le développement embryogénique, où elle contrôle la prolifération, la différenciation et la migration des cellules. En physiologie adulte, son activité se limite aux niches de cellules souches responsables de la régénération. Dans certains cas, cependant, la voie WNT est faussement activée, conduisant à une prolifération cellulaire non contrôlée, également connue sous le nom de cancer. L'activation aberrante de la signalisation WNT se produit également dans le cancer du sein triple négatif (TNBC), un sous-type de cancer du sein très agressif dépourvu de thérapies ciblées. Ce travail propose donc de cibler le TNBC par inhibition de la voie WNT suractivée.

Le candidat-médicament idéal doit prouver qu'il a des effets anticancéreux contre le TNBC, tout en n'étant pas nocif pour la physiologie cellulaire normale. Notre hypothèse de travail est que le ciblage des niveaux de la membrane plasmique, en particulier les principaux récepteurs de la voie, les Frizzleds (FZD), pourrait obtenir de tels résultats. En ciblant le sous-ensemble spécifique de FZD surexprimé dans le tissu tumoral, mais pas dans le tissu sain, il devrait être possible d'éviter l'inhibition des branches WNT nécessaires au fonctionnement physiologique normal. De plus, étant classés comme GPCR, les FZD ont le potentiel d'être hautement médicamenteux, ce qui en fait des candidats cibles idéaux.

Il existe de nombreuses façons d'aborder le développement de nouvelles molécules médicamenteuses : le repositionnement d'entités médicamenteuses approuvées ou le criblage de nouvelles molécules sont deux méthodes prometteuses. Dans ce travail, une bibliothèque de petites molécules a été criblée à l'aide d'une pipeline de test à haut débit spécifique au TNBC pour cibler les GPCR FZD dans le cancer, développé en interne. Cela a conduit à la découverte d'une nouvelle classe de molécules de pyrazole substituées par un diphényle avec des effets inhibiteurs du WNT et des propriétés anticancéreuses *in vitro* et *in vivo*. Des recherches détaillées sur la ou les cibles exactes des molécules nouvellement découvertes ont prouvé qu'elles se lient bien au FZD. Les molécules phares ont des profils de toxicité améliorés par rapport aux inhibiteurs du WNT actuellement connus, ce qui en fait une nouvelle génération très prometteuse d'inhibiteurs du WNT à petites molécules. Pris ensemble, ce travail montre

une approche robuste pour l'identification et le développement préclinique d'inhibiteurs de WNT avec des propriétés anticancéreuses pour le TNBC avec le potentiel d'être étendu à d'autres indications de cancer.

SUMMARY

The WNT signalling pathway plays an essential role in embryogenic development, where it controls cell proliferation, differentiation and migration. In adult physiology, its activity is limited to stem cell niches responsible for regeneration. In some instances, however, the WNT pathway is falsely activated, leading to uncontrolled cell proliferation, also known as cancer. Aberrant activation of WNT signalling also occurs in triple-negative breast cancer (TNBC), a very aggressive breast cancer subtype lacking targeted therapies. This work, therefore, proposes to target TNBC by inhibition of the over-activated WNT pathway.

The ideal drug candidate must prove to have anti-cancer effects against TNBC, while not being deleterious for normal cell physiology. It is our working hypothesis that targeting the plasma membrane levels, especially the main pathway receptors, the Frizzleds (FZDs), could achieve such results. By targeting the specific subset of FZDs overexpressed in the tumour tissue, but not in healthy tissue, it should be possible to avoid inhibition of the WNT branches necessary for normal physiological functions. Additionally, being classed as GPCRs, FZDs have the potential of being highly druggable, making them ideal target candidates.

There are many ways to approach the development of new drug molecules: repositioning of approved drug entities or the screening of new molecules are both promising methods. In this work, a library of small molecules was screened using a TNBC-specific high-throughput assay pipeline for targeting FZD GPCRs in cancer, developed in-house. This led to the discovery of a new class of diphenyl-substituted pyrazole molecules with WNT-inhibitory effects and anti-cancer properties *in vitro* and *in vivo*. Detailed investigations into the exact target(s) of the newly discovered molecules proved that they are indeed FZD binding. The lead compounds have improved toxicity profiles compared to currently known WNT inhibitors, making them a very promising new generation of small molecule WNT inhibitors. Taken together, this work shows a robust approach to the identification and pre-clinical development of WNT inhibitors with anti-cancer properties for TNBC with the potential to be expanded to other cancer indications.

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1. INTRODUCTION

1.1 An introduction to WNT signalling

Multicellular organisms are made up of billions of cells (Bianconi et al., 2013). Each cell knows and retains its place and function, which allows the body to develop and work properly (Hunter, 2000). This amazing process is possible thanks to the various signalling pathways contributing to sophisticated cell-to-cell communication. One such pathway is the evolutionary conserved WNT signalling pathway.

The WNT pathway, as we know it today, carries its name for historical reasons. It was initially discovered in *Drosophila melanogaster*, where X-ray irradiation experiments resulted in, among others, mutant flies without wings which were named *wingless* (*wg*) (Sharma, 1973). These experiments led to the description of the key components of the WNT signalling pathway. Nearly a decade later, investigations of a gene frequently targeted and activated by the mouse mammary tumour virus (MMTV) lead to the discovery of the proto-oncogene called *integration1* (*int-1*) (Nusse and Varmus, 1982). When researchers attempted to isolate the drosophila homolog of *int-1*, they realised that it was identical to the segment polarity gene *wg* (Nusslein-Volhard and Wieschaus, 1980; Rijsewijk et al., 1987). A mnemonic of *wg* and *int*, WNT, was therefore created to allow for a uniform nomenclature for the rapidly growing field of WNT research (Nusse and Varmus, 2012; Nusse et al., 1991).

Since the discovery of WNT signalling, it has become apparent that there are multiple signalling branches (Nusse and Varmus, 2012). All branches share the main pathway components: the main ligands, the WNT proteins (19 homologues in mammals), the main receptors called Frizzled (FZD, 10 homologues in mammals) and the phosphoprotein Dishevelled (3 homologues in mammals), which coordinates intracellular signalling (Foord et al., 2005; Gao and Chen, 2010; Koval and Katanaev, 2011; Willert and Nusse, 2012) . Other components are quite distinct. Classically, the signalling branches are divided into the best described (i) canonical WNT signalling with β-catenin as the central signal transducer, and (ii) the non-canonical pathways, which are β-catenin independent (Nusse and Varmus, 2012).

1.1.1 Canonical WNT signalling

The canonical branch of WNT signalling is particular, in that its "off state" is highly dependent on negative regulation (Hoppler and Moon, 2014). Firstly, the so-called destruction complex in the cytoplasm keeps β-catenin levels low (Kimelman and Xu, 2006). Secondly, in the nucleus, the transducin-like enhancer of split TLE/Groucho in complex with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) represses transcription of the WNT target genes (Chen and Courey, 2000; Roose et al., 1998). As a consequence, mutations of pathway components playing a role in the negative regulation of the pathway often lead to a constitutively activated state (Hoppler and Moon, 2014).

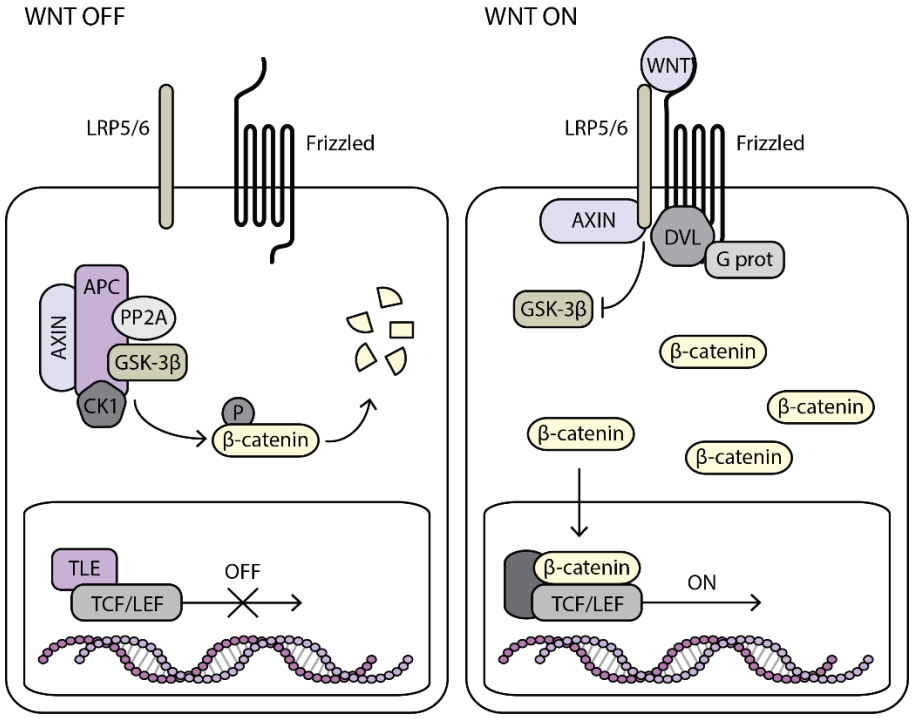

Figure 1 – Schematic representation of the WNT/β-catenin cascade. In the off-state, β-catenin is phosphorylated by the destruction complex and targeted for proteasomal degradation. In the nucleus TLE/Groucho represses transcription by binding to TCF/LEF. The ligand WNT binding to the receptor FZD activates the signalling cascade, leading to the inhibition of the destruction complex. β-catenin accumulates in the cytoplasm and translocated to the nucleus, where it activated transcription of WNT target genes.

In the absence of the main pathway ligand, WNT, β-catenin is phosphorylated by the destruction complex, composed of the threonine/serine kinases glycogen synthase kinase 3β (GSK-3β) and casein

kinase 1 (CK1), the scaffolding protein AXIN, adenomatous polyposis coli (APC) and protein phosphatase 2A (PP2A). β-catenin is subsequently ubiquitinated and targeted to the proteasome for degradation (Fig. 1) (Amit et al., 2002; Hart et al., 1999; Kitagawa et al., 1999; Munemitsu et al., 1995; Rubinfeld et al., 1996).

The event of a WNT protein binding its main receptor Frizzled (FZD) and the co-receptor low-density lipoprotein receptor-related protein 5/6 (LRP5/6) represent the switching of the signalling to its active state. The G proteins, together with Dishevelled (DVL), recruit AXIN to the plasma membrane, which subsequently deactivates the destruction complex (Cliffe et al., 2003; Egger-Adam and Katanaev, 2010). β-catenin is no longer phosphorylated and accumulates in the cytosol. High concentrations of β-catenin allow for its translocation to the nucleus where it displaces TLE and forms a complex together with the transcription factors (TCF/LEF) (Brunner et al., 1997; Molenaar et al., 1996). This complex, together with cofactors such as Bcl-9, Pygopus and CREB/p300 initiates the transcription of over 130 target genes, mainly involved in development, metabolism, proliferation, and differentiation (Nusse, 2020; Willert and Jones, 2006) In vertebrates WNT/β-catenin signalling controls stem cell division, cell fate determination, and the organisation of the primary body axis during embryogenesis (Petersen and Reddien, 2009).

1.1.2 Non-canonical WNT signalling

The term non-canonical signalling encompasses all WNT branches which do not involve transcriptional activation by β-catenin. An attempt to classify them has resulted in the description of several pathways: WNT/PCP, WNT/Ca^{2+}, WNT-RAP1, WNT-ROR2, WNT-PKA, WNT-GSK-3-MT, WNT-aPKC, WNT-RYK and WNT-mTOR (Schulte, 2010; Semenov et al., 2007). However, this classification is not fixed, as there is significant crosstalk between the subbranches and much research is still to be done. The most investigated non-canonical branches are the WNT/PCP (from planar cell polarity) and the WNT/ Ca^{2+} branches.

On a cellular level, the WNT/PCP pathway directs, as its name suggests, planar cell polarity as well as cytoskeletal remodelling and directional cell migration (Humphries and Mlodzik, 2018). The PCP signalling branch involves the functionally conserved WNT/PCP core proteins (Vangl, Prickle, Celsr, Inversin/Diversin) together with WNT, FZD and DVL. Upon WNT binding to FZD, DVL activates downstream signalling through RAC/c-Jun-N-terminal kinase (JNK) leading to activation of downstream transcription factors (Habas et al., 2003; Rosso et al., 2005). Further DVL together with DAAM (Dishevelled-Associated Activator of Morphogenesis) also signals through RAS homologue-gene family member/Rho-associated coiled-coil containing protein kinase (RhoA/ROCK) leading to actin cytoskeletal remodelling (Habas et al., 2001; Marlow et al., 2002; Strutt et al., 1997). In vertebrates, WNT/PCP signalling is responsible for gastrulation, neural tube and eyelid closure and sensory hair orientation in the inner ear (Ciruna et al., 2006; Guo et al., 2018; Rida and Chen, 2009).

The WNT/Ca^{2+} pathway leads to an increase of calcium levels within the cells. This is achieved by multiple WNT/FZD routes (Schulte, 2010). The first involves activation of the cGMP-specific phosphodiesterase 6 (PDE6). This leads to a decrease in cGMP levels, thereby inactivating protein kinase G (PKG), a cGMP dependent kinase, resulting in an intracellular increase in Ca^{2+} levels (Ahumada et al., 2002; Ma and Wang, 2006; Wang et al., 2004). Secondly, WNT/FZD signalling also activates phospholipase C (PLC) via the G proteins. The activation of PLC generates diacylglycerol (DAG) and inositol trisphosphate (IP_3), which in turn leads to a rise of Ca^{2+} levels from the endoplasmic reticulum (Cook et al., 1996; Heldin et al., 2016; Slusarski et al., 1997). The rise in intracellular Ca^{2+} in the cytosol has many effects. On the one hand, it leads to the activation of downstream kinases such as protein kinase C (PKC) and calmodulin-dependent protein kinase II (CaMKII). On the other hand, it activates transcription factors such as nuclear factor of activated T-cells (NFAT) (Saneyoshi et al., 2002; Sheldahl et al., 2003). On the cellular level, the calcium influx affects cytoskeleton remodelling, cell adhesion, cell migration and cell survival (Kühl et al., 2000, 2001). In vertebrates, the WNT/Ca^{2+} signalling is involved in ventral cell fate determination during embryogenesis.

1.1.3 A closer look at key WNT signalling components

1.1.3.1 WNT proteins

The WNT proteins are the main extracellular ligands of the WNT signalling pathway. Evolutionarily conserved, there are a total of 19 WNTs identified in mammals (Cadigan and Nusse, 1997). They are 350 to 400 amino acids long, including 22 conserved cysteines which form disulphide bonds important for protein stability (Janda et al., 2012; MacDonald et al., 2014). With the generation of the first crystal structure of Xenopus WNT8 bound to the CRD domain of mouse FZD8, a palm-like structure was presented (see Fig. 4) (Janda 2012). The N-terminal domain forms a thumb-like protrusion, whereas the C-terminal domain resembles an index-finger. The tips of these finger-like structures, which are exactly where the WNT is in contact with the CRD, contain conserved regions, probably contributing to the general WNT-FZD binding mechanism. The thumb also harbours a typical palmitoleic acid group. This lipid modification, added by the O-acyltransferase Porcupine, is necessary for WNT secretion (Bänziger et al., 2006; Bartscherer et al., 2006; Gao and Hannoush, 2014; Rios-Esteves et al., 2014; Tanaka et al., 2000; Willert et al., 2003). Further, it has been suggested that the lipid moiety is implicated in FZD receptor recognition, dimerisation and signalling (Nile et al., 2017; Willert et al., 2003).

1.1.3.2 Frizzleds

FZDs are considered the primary receptors for the WNT signalling cascade. The 10 human FZD homologues are part of the class F (Frizzled) of GPCRs (Foord et al., 2005; Schiöth and Fredriksson, 2005). Their name stems from the mutant of the *frizzled* gene of *D. melanogaster*, which gave a rather frizzled look to the fly (Schulte, 2010). FZDs are some of the most conserved seven-transmembrane receptors of the animal kingdom (Schiöth and Fredriksson, 2005). They all have in common the extracellular N-terminal cysteine-rich domain (CRD) and a domain spanning the plasma membrane seven times. The CRD contains five conserved disulphide bridges which contribute to its rigid structure (Dann et al., 2001). The seven transmembrane helices are connected by three extra- and three intracellular loops. The three intracellular loops bind downstream interacting partner DVL, together with a PDZ binding domain on the C-terminal tail of FZD (Cong, 2004; Gammons et al., 2016a; Umbhauer,

2000). In comparison to the intracellular loops, the function of the extracellular loops is not known. However, it has been suggested, that they might play a role in the recognition of the ligands (Chen et al., 2004). FZDs are considered atypical GPCRs as they lack some domains that are typically important for G-protein coupling in other GPCRs (Schulte, 2010). However, there is concrete evidence that FZDs do involve the heteromeric G proteins for signalling and that these are involved both in canonical and non-canonical signalling (Egger-Adam, 2008, 2008; Katanaev et al., 2005; Koval and Katanaev, 2011; Koval et al., 2011; Liu et al., 2001; Schulte and Wright, 2018; Slusarski et al., 1997).

1.1.3.3 β-catenin

β-catenin is a scaffolding protein with multitasking abilities. On the one hand, it is the central player of the WNT/ β-catenin signalling branch where it regulates gene transcription in the nucleus (Cong, 2004). On the other hand, it has a structural function as part of the adherens junctions at the plasma membrane together with α-catenin and E-cadherin (Huber and Weis, 2001; Pokutta and Weis, 2000; Valenta et al., 2012). Its third function, albeit less studied, lies within the centrosome, where it promotes mitotic progression (Mbom et al., 2013). The protein is composed of flexible N- and C-terminal domains and twelve central armadillo repeats, creating a super-helix (Huber et al., 1997; Xing et al., 2008). The central region is relatively rigid and contains a long positively charged groove, creating a binding hub for the vast array of binding partners. One of the main questions concerning β-catenin is, how a protein can be involved in three seemingly different processes within a cell, and how the function is regulated. Research has shown that the primary binding partners, E-cadherin, APC and TCF/LEF bind overlapping regions within the central armadillo region, restricting it to one function at a time (Eklof Spink et al., 2001; Graham et al., 2000; Huber and Weis, 2001; Poy et al., 2001). β-catenin may also undergo conformational changes and is post-translationally modified to control its spatial position, its affinities and its stability (Choi et al., 2006; Solanas et al., 2004, 2016; Valenta et al., 2012). This type of regulation is demonstrated by WNT signalling, where in the absence of WNT, β-catenin is phosphorylated in an orchestrated fashion. CK1 primes β-catenin at residue Ser45. This is followed by GSK-3β first phosphorylating Thr41, and then Ser33 and Ser37, to create an amino-terminal recognition

site for the E3-ubiquitin ligase β-TrCP, which in turn adds ubiquitin and targets it for proteasomal degradation (Aberle et al., 1997; Bustos et al., 2006; Liu et al., 2002; Stamos and Weis, 2013; Xing et al., 2003).

1.1.3.4 Dishevelled

The Dishevelled protein is a key component of both canonical and non-canonical WNT signalling. It is a direct binding partner of FZD, where it helps to relay the signal to the downstream components (Boutros and Mlodzik, 1999). In humans, there are three homologues (DVL1-3) with high sequence similarity and partial functional redundancy (Gentzel and Schambony, 2017). With around 750 amino acids in length DVL is composed of three main domains: the N-terminal DIX domain (coined from Dishevelled and AXIN), a central PDZ domain, flanked by a basic and proline-rich region, and the Dishevelled, Eg10 and Pleckstrin (DEP) domain (Gao and Chen, 2010). The DIX domain is needed for DVL head-to-tail polymerisation and recruitment of AXIN to the membrane upon WNT binding to FZD (Schwarz-Romond et al., 2007). The PDZ domain is involved in FZD binding and signal transmission (Pan et al., 2004; Wong et al., 2003). The DEP domain has been suggested to help DVL recruitment to the plasma membrane (Pan et al., 2004; Simons et al., 2009). Further, DEP has also been described to initiate dimerisation of DVL, leading to DVL and AXIN polymerisation through the DIX domains and subsequent inhibition of the destruction complex (Gammons et al., 2016b; Paclíková et al., 2017). DVL not only binds the proteins mentioned above but can also interact with a plethora of others (Gao and Chen, 2010). Ultimately the function of DVL is highly context-specific, and it has been shown that extensive posttranslational modifications, help mediate binding specificity and signalling outcomes (Hanáková et al., 2019).

1.1.3.5 GSK3-β

For the proper functioning of the WNT cascade, there are also several kinases involved. GSK-3β is a Ser/Thr protein kinase and an essential part of the β-catenin destruction complex (Wu and Pan, 2010).

Together with CK1α, GSK-3β phosphorylates β-catenin, leading to its proteasomal degradation and WNT pathway inhibition (Aberle et al., 1997). In humans, there are two GSK-3 paralogs (GSK-3α and GSK-3β). They have been reported to have redundant functions within WNT signalling (Beurel et al., 2015; Wu and Pan, 2010). However, because GSK-3β but not GSK-3α is able to replace the drosophila homologue *shaggy* in transgenic flies, it is has been assumed that GSK-3β is the main WNT isoform (Frame and Cohen, 2001; Ruel et al., 1993). GSK-3β is constitutively active in the absence of an external signal and is inhibited upon receiving a signal from the membrane. Furthermore, it often requires pre-phosphorylation of its substrates by other kinases. For example, β-catenin must be primed by CK1α before phosphorylation by GSK-3β (Beurel et al., 2015; Dajani et al., 2001). GSK-3β has over 500 predicted and around 100 reported substrates, some of which are part of the WNT pathway (LRP5/6, AXIN, APC, and TCF) (Behrens et al., 1998; Hart et al., 1999; Lee et al., 2001; Linding et al., 2007; Zeng et al., 2005). However, many substrates are part of other signalling cascades, such as insulin signalling (where GSK-3β phosphorylates and inhibits glycogen synthetase) and the Hedgehog pathway (Price and Kalderon, 2002; Ross et al., 1999). Being such a promiscuous kinase, it is hard to imagine how it could achieve substrate specificity and not create havoc within the cell. GSK-3β regulation is probably due to a combination of different mechanisms including posttranslational modifications, its subcellular localisation, availability of substrates combined with their pre-phosphorylation state and GSK-3β participation in protein complexes (Beurel et al., 2015).

1.1.4 WNT signalling – additional contributing factors

The pathways described above are a simplified representation of WNT signalling. In humans, there are a total of 19 WNT ligands and 10 FZD receptors (Schulte, 2010; Willert and Nusse, 2012). Many attempts have been made to map the exact WNT-FZD pairs, but it seems that one WNT can bind and activate multiple FZD and that a single FZD can be activated by multiple WNTs (Dijksterhuis et al., 2014, 2015; Schulte, 2015; Voloshanenko et al., 2017). Additionally, the preference of a certain WNT to bind to a certain FZD homologue is modulated by the presence of one of the single-transmembrane FZD co-receptors (LRP5/6, ROR1/2; RYK, PTK7) (Berger et al., 2017; Lu et al., 2004; Mikels and

Nusse, 2006; Oishi et al., 2003; Pinson et al., 2000; Wehrli et al., 2000; Yoshikawa et al., 2003). These co-receptors might also direct the signalling towards the specific branches of WNT signalling. Interestingly, both the WNT/PCP and the WNT/Ca^{2+} signalling result in inhibition of canonical β-catenin signalling, most probably because of the common use of some of the pathway components (Ishitani et al., 2003; Topol et al., 2003; Wang et al., 2013).

In addition to the pathway components, there are multiple WNT inhibitors, that interfere with and control WNT signalling. Secreted Frizzled-related proteins (sFRPs) are homologous to the N-terminal CRD-domains of FZDs (Kawano, 2003; Üren et al., 2000). Therefore, they can bind and sequester WNTs, as well as form dimers with the CRD preventing WNT-FZD interactions. Another WNT pathway modulator, Dickkopf (DKK1-4) contains two CRD domains, enabling DKK to simultaneously bind LRP5/6 and another membrane receptor, Kremen, leading to their combined endocytosis (Bafico et al., 1999; Glinka et al., 1998; Krupnik et al., 1999; Mao et al., 2001, 2002; Semënov et al., 2001). The decreased presence of LRP5/6 induced by internalisation results in inhibition of FZD/β-catenin signalling. Protein WNT-inhibitory factor 1 (WIF1) does not share any sequence homology with FZDs but contains domains similar to LRP5/6 (Hsieh et al., 1999; Kawano, 2003; Patthy, 2000). It forms non-covalent contacts with WNTs and thus preventing them from interaction with FZD and LRP5/6. Further, there is another regulatory system involving the E3 ubiquitin ligases, ZNRF3 or RNF43, which ubiquitinate FZD and target them for degradation. This ubiquitination is stopped by the protein family R-spondin (RSPO1-4) which form a complex with the leucine-rich repeat-containing G protein-coupled receptors (LGR1-4) and E3 ubiquitin ligase, resulting in higher FZD concentrations at the membrane (Chen et al., 2013; Hao et al., 2016). Combined, these facts result in a very complex and, until today, incomplete understanding of WNT signalling integration within the cell.

1.1.5 A fine balance between physiology and pathology

During embryogenesis, WNT signalling is responsible for regulating stem cell division, cell fate determination, cell migration and the organisation of cells into organs (Steinhart and Angers, 2018). It

steers dorsoventral and anteroposterior body axis formation in many species, including vertebrates (Genikhovich and Technau, 2017; Hikasa and Sokol, 2013; Huelsken et al., 2000; Niehrs). In adult tissues WNT signalling retires most of its activity with exception in stem cell niches needed for physiological homeostasis and tissue regeneration. There is a plethora of research showing its involvement in the function of intestine, the skin, the bone, the hematopoietic system, the neuronal system and the mammary gland (Flanagan et al., 2018; Houschyar et al., 2019; Krausova and Korinek, 2014; Lim and Nusse, 2013; Malhotra and Kincade, 2009; Salinas, 2012; Yu et al., 2016).

As much as WNT signalling is necessary for a healthy physiology, under or over activation will tip the balance into pathological states. WNT signalling has been linked to Alzheimer disease and other neurodegenerative diseases, metabolic diseases such as diabetes, cardiovascular health issues, as well as diseases of the liver, kidney, lung, skin and bone (Libro et al., 2016; Ng et al., 2019). Cancer is one of the dire consequences of WNT over-activation, with over 50% of all solid tumours being WNT dependent (Prosperi et al., 2011). The aberrant activation can be the cause of pathway component mutations, as seen in Familial adenomatous polyposis (FAP) patients (APC), in colon cancer (APC, β-catenin, RNF43, R-spo3), leukaemia (FZD5, BCL9) or pancreatic ductal carcinoma (RNF43) (Giannakis et al., 2014; Jiang et al., 2013; Morin et al., 2016; Seshagiri et al., 2012; Wang et al., 2014). Other types of cancer, such as triple-negative breast cancer (TNBC), have overactive WNT signalling because of epigenetically driven up- or downregulation of pathway components (Geyer et al., 2011).

In the intestine, WNT together with BMP, Notch and Hedgehog signalling cascades coordinates renewal of the stem cell niche in the intestinal crypts called crypts of Lieberkühn (Clarke, 2006; van Es et al., 2005). It also promotes the terminal differentiation of the intestinal secretory cells, the Paneth cells, by WNT-induced expression target genes needed for maturation (van Es et al., 2005). Mutations in some WNT pathway components, such as the negative regulator APC, lead to uncontrolled and malignant proliferation in the intestine, as is seen in the patients suffering from FAP (Rowan et al., 2000; Schneikert and Behrens, 2007). In the hematopoietic system, both maintenance of stem cells, as well as lineage commitment of progenitor cells is WNT/β-catenin dependent (Malhotra and Kincade, 2009; Nemeth et al., 2009). In the case of aberrant signalling, WNT can contribute to tumour growth by contributing to tumour immune modulation and immune evasion (Galluzzi et al., 2019; Goldsberry et

al., 2019). In the bone, WNT signalling is a positive regulator of the osteoblast lineage, regulating their differentiation and function (Regard et al., 2012). Loss-of-function mutations of LRP5 induce low bone density and osteoporosis, as seen in patients with the bone disorder osteoporosis-pseudoglioma syndrome (Gong et al., 2001; Kato et al., 2002). On the contrary, persons with dominantly inherited gain-of-function mutations in LRP5 have a high bone mass density (Boyden and Mitnick, 2002; Little et al., 2002; Van Wesenbeeck et al., 2003).

The female mammary tissues are very dynamic, changing during embryogenesis but also post-nataly during puberty, the menstrual cycle, pregnancy and lactaction. The mammary stem cells (MaSC) are responsible for the development and differentiation of the glands during these phases (Visvader, 2009). They are enriched within the basal cell population, giving rise to basal cells or luminal cells upon differentiation. Research has shown that cell fate is to a large extent controlled by WNT signalling (Boras-Granic and Wysolmerski, 2008; Yu et al., 2016). It is only a logical consequence that an imbalance within the WNT signalling pathways of the breast will lead to uncontrolled cell proliferation.

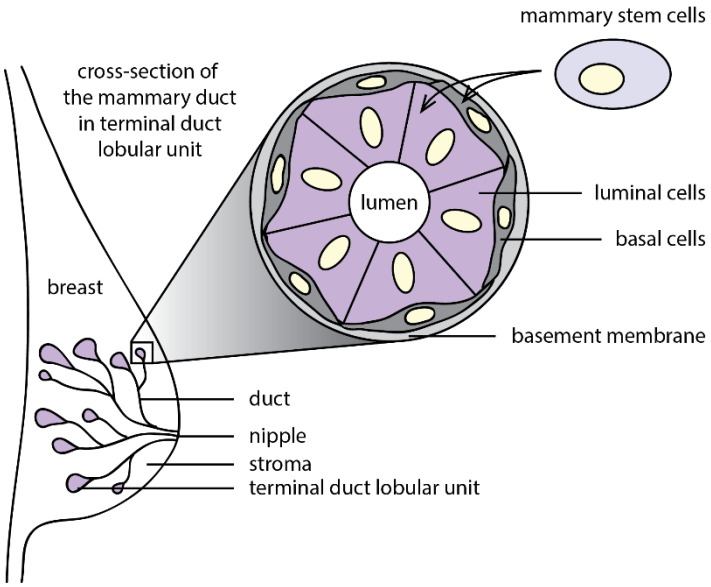

Figure 2 - Cross section of the mammary gland. The terminal duct lobular unit is composed of two main breast cell types, the luminal cells, lining the lumen of the duct and the and the basal cells, lining the basement membrane. Both cell types originate from mammary stem cells. Figure redrawn and adapted from (Wong, 2012).

1.2 Triple-Negative Breast Cancer

Breast cancer is a broad description of a variety of oncological diseases affecting the breast. It can occur in both women and men, though the latter make up less than 1% (Yalaza et al., 2016). In Switzerland, there are yearly about 6000 women diagnosed with breast cancer and around 1300 breast cancer-related deaths per year (BFS, 2018). Worldwide, breast cancer amounted to nearly 2.1 million new cases and 625'000 breast cancer-related deaths in 2018. It is the most prevalent cancer in females, accounting for 24.2% of all cancers and 15% of cancer mortalities (Bray et al., 2018).

1.2.1 Classification of Breast Cancer

There are multiple ways of classifying breast cancers, for example, according to their histology, tumour grade or tumour stage. These classifications were created to help clinicians understand the disease origin and to determine future treatment plans for the patients (Hwang et al., 2019). A distinction is made between the luminal and the basal phenotype of the cancer cells, meaning that the cancer cell's gene expression profile resembles either the luminal cell or the basal cells lining the breast ducts. This phenotype may be determined by their different cytokeratin expressions (CK 8/18 for luminal cells and CK 5/6 and 17 for basal cells) (Perou 2000). In general, basal tumours are considered more aggressive and are found in higher grade tumours. However, the phenotype must not be mistaken for the origin of the cancer cells as for example cancers with a basal phenotype can be of luminal origin (Sims et al., 2007). A more recent classification divides breast cancer into four distinct molecular subtypes defined by their expression patterns of the oestrogen receptor (ER), the progesterone receptor (PR), the human epidermal growth factor receptor 2 (HER2) and the proliferation marker Ki67 (Fig. 3) (Goldhirsch et al., 2011). The four groups are luminal A (ER + and/or PR+, Ki67 low and HER2-), luminal B (ER + and/or PR+, Ki67 high and/or HER2+), HER2-positive (ER-, PR- and HER2+) and triple-negative (ER-, PR-, HER2-).

Most breast cancers are of the luminal A and B subtype (up to 80%) and are treated with endocrine therapy, aromatase inhibitors and selective oestrogen receptor modulators (SERMs) (Fabian, 2007; Noone et al., 2017; Swaby et al., 2007). The HER2+ subtype makes up 10-15% of all breast cancers and

is treated with anti-HER2 therapy, for example, the monoclonal antibody Trastuzumab (Herceptin) (Maximiano et al., 2016). Lastly, TNBC occurs in 15-20% of breast cancer patients (Foulkes et al., 2010). This subtype is mostly of the basal phenotype. As it lacks the ER and HER2, targeted therapy is ineffective, and patients rely solely on traditional treatments such as surgery, radio and chemotherapy (Anders and Carey, 2020). These facts, combined with TNBC being a highly prolific and very aggressive subtype of cancer, leads to a disproportionally high mortality rate (Fallahpour et al., 2017).

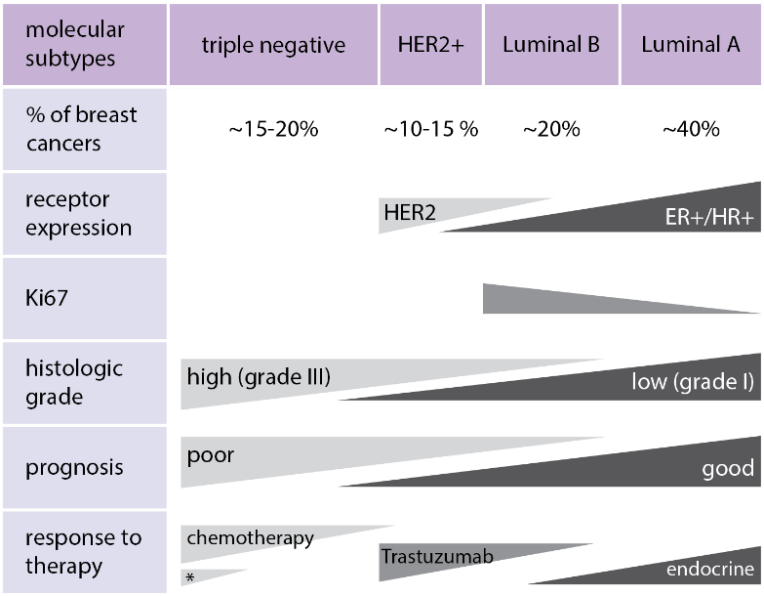

Figure 3 – Molecular subtypes of breast cancer. Based on the expression pattern of the oestrogen receptor, the progesterone receptor, the human epidermal growth factor receptor 2, and the proliferation marker Ki67, breast cancer is divided into four subtypes (top row). The subtypes vary in their histologic grade, their prognosis and their therapy options. The * represents the small group of TNBC patients who may benefit from PD-L1 immunotherapy. Figure redrawn and adapted from (Wong, 2012).

Additionally, unlike hormone-positive breast cancer, where the disease predominantly spreads to the bones, TNBC leads to metastasis occurring mainly in the lungs and brain (Dent et al., 2009; Luck et al., 2008; Maki and Grossman, 2000; Tseng et al., 2013). The median time to distant metastasis is 20.4 months, and median overall survival from the onset of metastasis for TN patients is on average only 9.3 months (Gerratana et al., 2015). The urgency to find a new targeted drug is evident.

1.2.2 Current targeted therapy investigations for TNBC

The race to find a new target for treating TNBC is on. Currently, there are many drugs in development and being tried on TNBC in clinical trials, especially targeted therapies aiming at pathways which are implicated in TNBC. For example, PARP inhibitors for TNBC patients carrying BRCA1 mutation (>70% of BRCA1 mutation breast cancers are triple-negative), inhibitors for the receptor tyrosine kinases VEGFR-, EGFR and FGFR, either as antibodies or receptor tyrosine kinase inhibitors (RTKis), Androgen receptor inhibitors, PI3K/Akt inhibitors, CDK 4/6 inhibitors, Trop-2 inhibitors and the PD1/PDL1 immunotherapies are currently subjects of clinical trials for the treatment of TNBC (Planes-Laine et al., 2019). Cell cycle regulating targets such as Aurora kinases are also being investigated for their potential as anti-TNBC drugs (reviewed in Hwang et al., 2019; McCann et al., 2019). In addition to these targets, some researchers are more interested in targeting pathways regulating cancer stem cells and epithelial-to mesenchymal transition. The notch, hedgehog, TGFβ and WNT pathway, additionally being essential players in embryonic development and normal stem cell physiology, are all targets of drug research programs for TNBC and cancer in general (Shao et al., 2017).

1.2.3 WNT signalling in TNBC

1.2.3.1 WNT pathway components and their implication in TNBC

WNT signalling, while being an integral part of breast physiology, is highly implicated in TNBC (Pohl et al., 2017). It contributes to all stages of cancer, including tumorigenesis, progression, invasion and metastasis. Both the canonical and non-canonical pathways are involved: the canonical pathway is primarily responsible for proliferation and cancer stem cells maintenance, the non-canonical pathway plays a role in invasion and metastasis of the breast cancer. In breast cancer, it is rather an overexpression of oncogenic WNT pathway components or decreased expression of tumour-supressive pathway components that is the cause of WNT deregulation, opposed to the frequent occurrence of pathway mutations as seen in colon cancer (Geyer et al., 2011).

In TNBC cells, knockdown of β-catenin lead to impaired migration, a reduction in cells with stemness-like features and slower growth *in vivo* (Xu et al., 2015). High β-catenin expression has also been linked to increased chemo-resistance (Shen et al., 2019; Xu et al., 2015). Active β-catenin dependent WNT signalling is more likely to be found in patients with TNBC/basal-like breast cancer and is associated with a poor clinical outcome. Analysed tumour tissues showed a lack of membranous β-catenin and higher levels of cytoplasmic and nuclear β-catenin (a biomarker for active WNT signalling). Furthermore, cytoplasmic and nuclear β-catenin correlated with a shorter metastasis-free period and a lower median survival time in breast cancer patients (Geyer et al., 2011; Khramtsov et al., 2010). Patients with WNT-associated TNBC have a higher risk of developing metastasis in the brain and lung than non-WNT-types (Dey et al., 2013).

The expression of 72 WNT target genes was higher in TNBC patient samples compared to those of the luminal A/B or HER2+ type, showing a clear WNT dependency of TNBC (Maubant et al., 2015). A study investigating the expression of WNT pathway components in breast cancer cell lines found that several of the WNT proteins (particularly WNT3A, WNT4, WNT6, WNT8B, WNT9A and WNT10B) were overexpressed and that WNT5a/b and WNT16 were downregulated in TNBC cells (Benhaj et al., 2006). In TNBC patient samples, metastasis of the lymph node, high Ki67 expression and poor recurrence-free survival correlated with negative WNT5a expression (Zhong et al., 2016). Contrary to this, another study showed that the WNT5A-NFκB pathway is upregulated in TNBC cells, contributing to the expression of matrix metalloproteinase-7 (MMP7), migration and invasiveness (Han et al., 2018). Additionally, WNT5A promoted growth and cell migration of TNBC cells by inducing the DVL2/Daam/RhoA pathway (Zhu et al., 2012). Using oligonucleotide microarray analysis, WNT4 and WNT16 were found to be the main upregulated genes in TNBC patient samples linked to recurrence (Tsai et al., 2015). WNT10B has been shown to induce β-catenin signalling and can be predictor of the survival of TNBC patients (Ayachi et al., 2019). Upregulation of canonical WNT signalling was confirmed using a WNT10B-driven TNBC mouse model. Additionally, the transcription and upregulation of HMGA2 and EZH2, proteins that are part of the nuclear transcription factor complex of β-catenin, was shown (Wend et al., 2013). Treating cells with WNT pathway inhibitors or

downregulation of HMGA2 or EZH2 decreased both cell proliferation *in vitro* and visceral metastasis (meaning of the soft internal organs of the body) in a chemo-resistant patient-derived xenograft (PDX) model of TNBC.

The principal receptors of the WNT pathway, the FZDs, are also frequently overexpressed in TNBC. The overexpression of FZD6, typically associated with the non-canonical signalling, is caused by gene amplification (Corda et al., 2017). It correlates with a shorter distant metastasis-free survival time, concomitant with its role in cell migration and invasiveness. Overexpression of FZD7 was observed in TNBC patient tissues and knockdown of FZD7 in TNBC cell lines resulted in inhibition of proliferation *in vitro* and *in vivo* (Yang et al., 2011). Further, ΔNp63, an isoform of transcription factor p63, lacking the N-terminal transactivation domain, induces FZD7 overexpression in mammary stem cells and breast cancer-initiating cells. This leads to an activated WNT pathway, enhancing tumour formation (Chakrabarti et al., 2014). FZD8, on the other hand, has mostly been implicated in chemo-resistance in TNBC. After chemotherapeutic treatment of TNBC lines, the residual cells were predominantly tumour initiating cells (Yin et al., 2013, 2015). These residual cells exhibited increased expression of FZD8 and the transcription factor c-myc, suggesting a role in mediating chemotherapeutic resistance (Yin 2013, Yin 2015). A further study showed that FZD8 is a direct target of miR-100. miR-100 was found to be downregulated in the breast cancer tissues and cell lines, which in turn led to FZD8-induced invasiveness, migration and local metastasis formation (Jiang et al., 2016).

Co-receptors LRP5/6 also play an essential role in TNBC biology. LRP6 expression is upregulated in TNBC (Cho et al., 2017; Lindvall et al., 2009; Liu et al., 2010; Ma et al., 2017a; Yang et al., 2011). Both knockdown of LRP6 in TNBC cell lines using siRNA, and inhibition using Mesd, an LRP6 inhibitor, resulted in reduced growth, migration and invasion (Liu et al., 2010; Ma et al., 2017a). Further, it reduced the expression of S100A4, a WNT target gene known to be involved in TNBC invasion and metastases. Treatment of TNBC cells with methyl-β-cyclodextrins (MβCD), a cholesterol-removing agent to disrupt lipid rafts, reduced proliferation and simultaneously reduced the expression of WNT

pathway components LRP6, β-catenin and target gene c-myc, whereas supplementation of cholesterol upregulated the expression of LRP6 (Badana et al., 2018).

Receptor-tyrosine-kinase-like orphan receptor 1 (ROR1), a non-canonical WNT receptor, is also overexpressed in breast cancer. Higher expression was seen in primary breast cancer tissues which were either ER- or triple-negative (Chien et al., 2016; Zhang et al., 2012). The high expression also correlated with shorter overall survival and disease-free survival times. Silencing ROR1 in TNBC cells resulted in stumped growth *in vitro* and *in vivo* and a reduction of CREB induced gene-expression levels (Cui et al., 2013). Further, the incidence of spontaneous distant metastases was reduced in mice injected with a ROR1 knockout TNBC cell line compared to the wild type line. Pharmacological inhibition of ROR1 using strictinin, a phyto-compound, reduced both growth and migration of TNBC cancer cells via inhibition of PIK3/Akt/GSK-3β signalling axis (Fultang et al., 2019). The high involvement of ROR1 in cancer has led to the development of ROR1 chimeric antigen receptor (CAR) T-cells for therapeutic use (Specht et al., 2018, 2019). They are currently under investigation for therapeutic use against TNBC (NCT02706392). Another homolog of ROR, ROR2, is also overexpressed in 80% of breast cancers. ROR2 positive TNBC patients have a poorer prognosis than other breast cancer patients (Henry et al., 2015).

Negative pathway regulators such as SFRPs and WIF1 are epigenetically silenced in breast cancer leading to overactive WNT/β-catenin signalling (Klarmann et al., 2008). Using breast cancer cell lines, including the TNBC type, this phenomenon was mimicked by transcriptional knockdown of sFRP1, leading to an increase in WNT signalling (Matsuda et al., 2009; Suzuki et al., 2008). The opposite was achieved with overexpression of sFRP1,2 and 5, which significantly decreased cell growth of the cancer cells. This effect was also seen *in vivo*, where overexpression of sFRP1 suppressed tumour growth and metastasis in xenograft mice (Matsuda et al., 2009).

Taken together, the research demonstrates the high implication of active WNT signalling in TNBC. However, much of this research only represents limited "snapshots" of the whole system by picking out individual components. Recent bioinformatical network analysis of over 100 WNT pathway

components using the TCGA and the GTEx gene expression databases has demonstrated, that previous reports of overexpression are not entirely reflected in this study: whereas WIF1 and SRFP1 under-expression was confirmed, FZD7 and LRP6 were downregulated in TNBC when compared to healthy tissues and it was rather FZD9 and FZD2 which showed overexpression (Koval and Katanaev, 2018). The authors concluded that it is an imbalance of the whole WNT signalling and not the up- or downregulation of single components which occurs in breast cancers, especially in TNBC. By correlating the numerous pathway components, the various breast cancer subtypes were given different WNT signatures which could potentially allow for patient stratification and a better design of therapeutics.

1.2.3.2 The role of WNT signalling in therapy resistance and immunomodulation

Approximately 75% of breast cancer-related deaths are caused by the development of metastases (Dillekås et al., 2019). One of the reasons for the cancer recurrence and the formation of metastases, is the development of therapy resistance of cancer cells. Upon chemotherapy, the tumour initially shrinks, which is caused by the cell death of chemo-sensitive cells. However, a subpopulation of cells, including cancer stem cells, becomes chemo-resistant (Zheng, 2017). This chemo-resistance is achieved by the increased expression of efflux pumps involved in cell detoxification and DNA repair mechanisms. It is this pool of cells which then promotes the new tumour growth after the initial shrinkage.

The activation of the WNT pathway induces not only gene expression necessary for the tumour growth, but also the expression of target genes involved in chemo-resistance. For example, multidrug-resistance protein 1 (MDR1 also known as P-glycoprotein or ABCB1), ATP-binding cassette super-family G member 2 (ABCG2) and multidrug resistance-associated protein 2 (MRP2) are all reported as WNT target genes (Chau et al., 2013; Chikazawa et al., 2010; Flahaut et al., 2009; Yamada et al., 2000; Zhang et al., 2016). In TNBC, the WNT inhibitor clofazimine decreases MDR1 expression, and in combination with doxorubicin, its suppression of tumour growth was additive (Ahmed et al., 2019). Further, Pygo2, a transcriptional coactivator of the WNT pathway, was the most upregulated gene in doxorubicin-resistant breast cancer cells, including a TNBC cell line. Pygo2 activated MDR1 via canonical WNT

signalling (Zhang et al., 2016). Inhibition of Pygo2 restored sensitivity towards chemotherapeutic agents and reduced the cancer stem cell population. When comparing patient samples before and after neoadjuvant therapy, a significant increase of both Pygo2 and MDR1 was measured for patients who had relapsed.

Besides chemoresistance, WNT signalling also contributes to radiotherapy resistance. After ionising radiation (IR) therapy, the secretion of WNTs, among other growth factors, is induced in both cancer cells and the stromal cell containing microenvironment, leading to protection against radiation damage (Zhao et al., 2018). Furthermore, in fibroblasts derived from breast cancer patients, the transcriptional response of various WNT pathway components was changed upon IR: the receptor FZD7 was upregulated after only 2 hours, however the Dkk receptor Kremen was downregulated after 24h (Kristin Rødningen et al., 2005). In TNBC cells, IR led to increased WNT3a, LRP6 and β-catenin expression and canonical WNT signalling activation (Yin et al., 2016). Depletion of β-catenin by shRNA and treatment of cells with the WNT inhibitor niclosamide, sensitised the cells to IR by inhibition of β-catenin induced radio-resistance. In mouse xenograft models, niclosamide combined with IR significantly potentiated the tumour growth suppression compared to IR therapy alone.

WNT signalling also contributes towards the dysregulation of the protective anticancer immune responses, resulting in tumour immune modulation and immune evasion. According to research, every step of the very complex tumour-immune cycle, the mechanism to rid our bodies of tumour cells, is blocked by WNT/β-catenin signalling (reviewed by Wang et al., 2018). This affects both the tumour itself and the tumour microenvironment: tumour antigen release, antigen presentation by dendritic cells, T-cell priming and activation, T-cell infiltration and tumour cell elimination are all affected upon aberrant WNT signalling. Although cancer immunotherapy has had some success, there are still challenges to overcome including low response rates and tumour recurrence. It has been postulated that inhibition of WNT signalling could be the way to avoid resistance to immunotherapy or reduce tumour immune evasion (Galluzzi et al., 2019; Martin-Orozco et al., 2019; Wang et al., 2018). For example, high programmed death-ligand 1 (PD-L1) expression, a protein involved in immune evasion was shown

to be regulated by WNT signalling and treatment of TNBC cells with a WNT inhibitor (XAV939) downregulates PD-L1 expression (Castagnoli et al., 2019).

1.2.4 Targeting the WNT pathway in cancer and TNBC

Taken together, the involvement of the WNT pathway in cancer in general and in TNBC in particular, ranging from the tumorigenesis, to tumour immune regulation and its essential role in radio- and chemotherapy resistance makes it an excellent target for new drugs, and numerous attempts have already been made to silence this pathway in cancer (Kahn, 2014; Shaw et al., 2019a). The most successful drugs have reached clinical trials; however, many of them have failed due to their side effects upon administration. The main manifestations are toxicities appearing in areas where stem cell maintenance is controlled by WNT signalling within the adult human body, notably the intestine, the hematopoietic system and the skeletal tissues.

The most successful compounds all have a common feature: they target the areas of the pathway where diverging of signalling occurs, namely at the membrane where all signalling starts, and in the nucleus, where the integrated signal cooperates with various transcription factors, resulting in different outcomes (further discussed in chapter 2.3). At these two levels, the silencing of specific sub-signalling events can be achieved, while leaving physiological signalling mainly intact. As receptors, the FZD proteins are a particularly attractive target. They provide the possibility of targeting a specific subset of FZDs which is cancer-specific. Additionally, opposed to targeting downstream effectors, FZD inhibition allows for both canonical and non-canonical pathway modulation. Further, they offer the advantage of not playing a part in other signalling pathways in contrast to many other downstream components of the WNT pathway. Lastly, FZDs are part of the very druggable GPCR family of receptors suggesting many possibilities to modulate their signalling.

1.2.5 FZDs as pharmacological targets

If one wants to understand the pharmacological landscape and targeting-potential of FZDs, it is necessary to take a closer look at their structures and endogenous ligand binding mechanisms. FZDs are principally composed of two domains, the extracellular N-terminal cysteine-rich domain (CRD) with a cleavable signal sequence for membrane insertion and a seven-transmembrane domain (7TMD) which is connected to the CRD by a linker region (Schulte, 2010). Sequence homology analysis has allowed for their sub-classification into four groups FZD1,2,7 (75% homology), FZD5,8 (70%), FZD4,9,10 (50%) and FDZ3,6 (50%) (Fredriksson et al., 2003). The purification of the full-length receptor is extraordinary difficult, however, purification of the CRD seems to be rather straight forward and therefore this domain was the first to be crystallized. The first crystal structure of the CRD domain was that of mouse FZD8 in 2001 (Dann et al., 2001). Since then, various CRD domains, including the mFZD8 domain in complex with human WNT3A, have been crystallised, giving insight into the 3D structure and function of CRD (DeBruine et al., 2017; Hirai et al., 2019; Janda et al., 2012, 2017; Nile et al., 2017, 2018; Raman et al., 2019; Shen et al., 2015). These publications have shown that the CRD is mostly composed of four α-helices and two small β-sheets. Cysteine-disulphide bridges link the α-helices, making it a tightly packed, rigid structure (Dann et al., 2001). The structure of the 7TMDs was elucidated only recently. In 2018, Yang et al. published the crystal structure of human FZD4 in its ligand-free state (Yang et al., 2018). The structure showed that the conformation corresponds to the typical 7TMD fold with seven α-helices spanning the membrane and an eighth α-helix formed by the C-terminal tail, parallel to the lipid bilayer. However, opposed to the relatively open TMD structures presented by some GPCRs, the three extracellular loops pack tightly together, with ECL2 acting as a plug, blocking most of the opening of the 7TMD pocket, making the binding of ligands to the transmembrane pocket a difficult task. This is also the case for the intracellular part of the molecule, where the three ICLs block the cavity formed by the α-helices. For other GPCRs, this cavity is an allosteric binding site. However, the structure of FZD4 suggests it has a different mechanism of allosteric binding. Furthermore, the structure analysis showed that the pocket of FZD4 is highly hydrophilic and that this feature is very likely to be conserved among all FZD receptors. The authors suggested that this could be another reason for low ligand binding affinity of this region.

So far, two crystal structures have been published of the WNT ligand in complex with the CRD domain of FZD (Hirai et al., 2019; Janda et al., 2012). These showed that WNT forms a hand like structure with a protruding thumb and index finger. It is with these two regions and a palmitoleic acid moiety at the end of the "thumb" that WNT interacts with the CRD. The palmitoleic acid moiety binds the CRD in a specific lipid binding groove. The WNT binding regions of the CRD domain are relatively conserved across the FZD family and the suspected reason why there is such high WNT-FZD promiscuity. This, however, leads to the still unanswered question of how the FZD receptors can differentiate between the various WNTs. It has been hypothesised that because of the high sequence homology of the CRDs, that they act more like a docking station to capture WNTs and that additional binding to the ECLs provides more specificity as they vary significantly from one FZD to the other. This hypothesis receives particular value if one considers the research showing that the CRD domain might actually not be essential for signalling, and may be replaced by other WNT binding domains (Chen et al., 2004; Povelones and Nusse, 2005). Additionally, homodimerisation of CRD domains and heterodimerisation with co-receptors such as LRP5/6 and Ryk are also involved in WNT binding, adding an extra layer of possible interaction and specification (Green et al., 2014; Pinson et al., 2000; Wehrli et al., 2000). In the cytoplasm the ICLs and the variable C-terminal tail are thought to bind and direct the signal to different binding partners such as DVL and heterotrimeric G proteins (Schulte and Wright, 2018; Umbhauer, 2000; Wong et al., 2003).

According to the structural information available, there are multiple binding sites to target FZDs. However, the CRD is by far the most targeted region, both for small molecules and biologicals (Fig. 4). Vantictumab, an antibody already tried in clinical trials, targets five out of ten FZD CRDs. It binds to a discontinuous epitope that spans the lipid-binding groove (Gurney et al., 2012). The lipid-binding groove and the region around it are also targeted by multi-FZD binding antibodies (named F2.I Fab, F7.B Fab and F6 Fab), the FZD subtype-specific designed repeat protein binders (DRPBs), and B12, a four-helix bundle domain protein derived from *Bacillus halodurans* (Dang et al., 2019; Janda et al., 2017; Raman et al., 2019).

Additionally, a natural ligand, the clostridium difficile toxin B (TcdB) also binds the CRD domain, blocking the lipid-binding groove and the WNT-binding site (Chen et al., 2018). A different mode of action is employed for the peptide dFz7-21, which binds CRD-dimerisation interface, inducing a conformational change and the shape of the lipid-binding groove, therefore inhibiting WNT binding (Nile et al., 2018). All these molecules target the "thumb"-site of the WNT binding. However, Lee and colleagues also performed an *in silico* screen and follow up experiments to find small molecules which bind to the "index finger"-binding site (3235-0367; 1094-0205; 2124-0331; NSC36784; NSC654259), successfully inhibiting WNT binding and downstream activation (Lee et al., 2015).

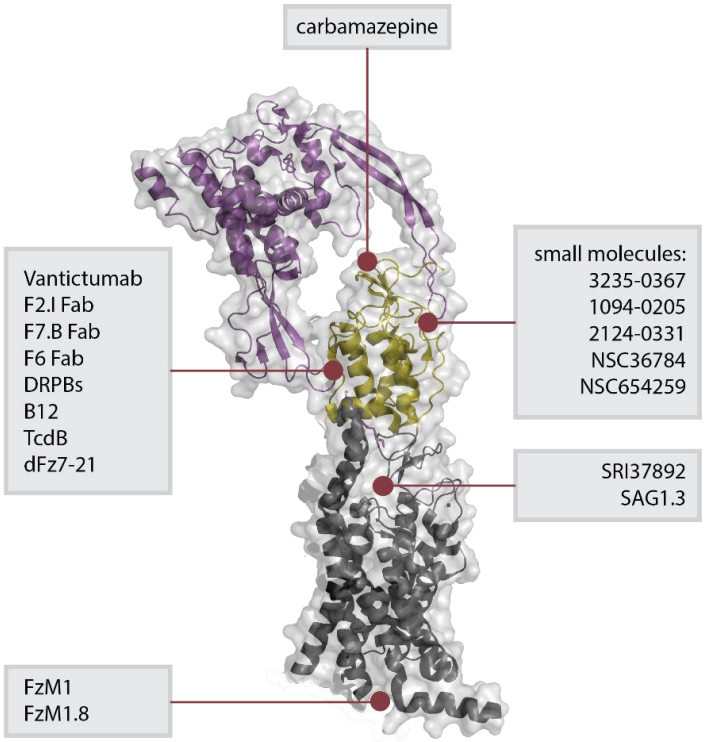

Figure 4 – Molecules targeting FZDs. A model of FZD (based on SMO structure PDB 5L7D) in complex with WNT (purple, based on PDB 4F0A). So far there are 5 main regions of FZD which are targeted by various molecules. The CRD-domain (yellow) has three binding regions, the TMD (grey) is targeted either on the extracellular or on the intracellular region.

Very recently the first crystal structure of a small molecule, carbamazepine, in complex with the FZD8 CRD domain was published (Zhao et al., 2020). This antiepileptic drug, which inhibits WNT/β-catenin

signalling, binds yet another region of the CRD, a pocket between the “finger-” and “thumb”-binding domains.

So far, the extracellular loops and the transmembrane domains have been largely avoided in drug discovery, researchers assuming the region was undruggable due to the tightly-packed ECLs and its hydrophobic nature (Yang et al., 2018). It was not until recently that this notion was proven wrong: first with the small molecule SRI37892 which was predicted *in silico* to target the TMDs of FZD7 and secondly, the FZD6 TMD in was shown in complex with the agonist SAG1.3, a small molecule originally discovered to bind the receptor Smoothened, a further member of the F-class of GPCRs (Kozielewicz et al., 2020; Zhang et al., 2017).

Lastly, two molecules bind the intracellular domain of FZDs, FzM1and FzM1.8 (Generoso et al., 2015; Riccio et al., 2018). These are small molecules that bind ICL3 of FZD4 and disrupt FZD-DVL interaction. Interestingly, they vary in only one moiety (replacement of a thiophene by a carboxylic moiety), but induce the opposite effects: FzM1 is a negative allosteric modulator, and FZD1.8 is an allosteric agonist.

Together these data show that there is no scarcity in FZD binding sites and various options to target these molecules. The challenge remains to produce modulators that specifically target the individual FZDs, which is difficult due to their high homology. Additionally, as to this day, we do not completely understand how WNT signalling diverges at the level of the plasma membrane and which parts of the FZD-molecules are involved in the signalling specificity (CRD, linker domain, extracellular loops, etc.), it is hard to predict which FZD binding sites present the best target.

1.3 Aim of this thesis

The aim of this thesis is to discover and develop small molecule inhibitors to target WNT signalling at the plasma membrane level of the pathway, to allow for selective inhibition of TNBC specific active branches of signalling.

2. ARTICLE SUMMARIES

2.1 Article I: A Second WNT for Old Drugs: Drug Repositioning against WNT-Dependent Cancers (*Cancers, 2016*)

The urgent need to find new specific and efficient therapies for various cancer indications is offset by the long and expensive process of drug development, often lasting more than 10-15 years between the initial discovery and the market-ready drug. To circumvent this challenge, drug repositioning has become a viable alternative to the traditional drug discovery route. The repositioned drugs offer the advantage of already being approved for human use. This means that their safety profiles are known, and large-scale production processes are established, cutting short the time needed for development. Furthermore, as some cancer indications like TNBC are unmet medical needs, the repositioned drugs can be fast-tracked by the Food and Drug Administration (FDA), shortening the approval process even further.

This review article investigates members of the various classes of FDA approved drugs, such as non-steroidal anti-inflammatory drugs, antiparasitics antimicrobials and others with reported anti-WNT properties and their potential uses as anti-cancer drugs. We report sixteen drugs in total, of which some are already being tested in clinical trials for various cancer indications. If known, their involvement in the WNT pathway and possible mechanisms of action against cancer are discussed.

Repositioning of approved drugs is a very time and cost-effective strategy of developing new anti-cancer drugs, however it comes with its on challenges. The use of the drugs might be limited due to intellectual property issues, or the needed drug dose might exceed what is considered safe. Especially when considering WNT-inhibitors, the side effects must be closely monitored, due to the WNT-dependency of regenerative processes within the adult body. With these facts in mind, the option of designing derivatives of the pre-existing drugs to avoid intellectual property issues and to improve efficacy and any potential toxicity is also evoked. Taken together, drug repositioning, although facing its own challenges, is a cost and time effective alternative to the traditional drug discovery routes, and, in the future, more WNT-inhibitors are expected to be discovered this way.

Contributions to the article: design of the review, literature research and writing of sections 1, 2 and 4, contribution to the summary table and final corrections of the article.

2.2 Article II: A high-throughput assay pipeline for specific targeting of Frizzled GPCRs in cancer (*Methods in Cell Biology, 2019*)

Targeting of the WNT pathway is possible at multiple levels: starting with the inhibition of WNT ligand secretion, over the inhibition of pathway components such as β-catenin, down to the inhibition of the most downstream pathway elements such as transcription factors in the nucleus. Even though these approaches might seem viable at a first glance, they do not necessarily confer sufficient specificity when blocking of individual WNT signaling branches is desired. Indeed, these approaches often result in blocking WNT signaling as a whole, simultaneously causing the shutdown of signaling needed for physiological functions of stem cell niches. Therefore, targeting the pathway at the upstream levels, especially the FZD GPCRs, might represent a better alternative. Inhibition of the signalling cascades induced by the various FZDs, specifically overexpressed in the cancer cells, could reduce the inhibition of physiological WNT signalling, therefore avoiding undesirable side effects.

To discover such WNT inhibitors for TNBC, a TNBC-specific high-throughput assay pipeline for targeting FZD GPCRs was developed. The pipeline includes a reporter assay using the TNBC cell line, BT-20, stably transfected with the TOPFlash reporter gene monitoring the activity of the WNT/β-catenin signalling. This primary assay allows the selection of TNBC-specific WNT inhibitors from the initial stages of the selection process. Secondary assays then narrow down the selection of hit molecules to those acting at the membrane level of the pathway and help to determine the anti-cancer properties of the selected molecules. The pipeline described is a powerful and low-cost method to screen for cancer-specific WNT modulators and can serve as a model for the development and adaption of pipelines for other cancer indications.

Contributions to the article: writing of chapter 1 and final corrections of the whole article.

2.3 Article III: Targeting the WNT pathway in cancer: prospects and perils (*Swiss Medical Weekly, 2019*)

Since the discovery that the WNT signalling pathway contributes to cancer development, it has gained attention of drug development projects, in both academia and the pharmaceutical industry. The focus of this review is the involvement of WNT signalling in cancer, especially the correlation between pathway activation in patients and disease outcome. It is generally observed that overexpression of oncogenic WNT pathway components is related to higher grades of the disease, meaning to more aggressive and faster proliferating tumours. Going hand in hand with disease progression is the emerging role of WNT signalling in cancer stemness and therapy resistance.

Compared to other oncogenic pathways, the successful development of an FDA approved drug is still outstanding. Failure to reach advanced stages of clinical trials and approval for market release is due to the side effects induced by nonselective WNT inhibition: bone toxicities, intestinal tract damage and hematopoietic deficiencies, as a result of inhibition of stem cell physiology are common side effects of the drugs. This review also takes a closer look at WNT modulators which have reached clinical trials and reveals that the most successful one are those targeting the divergent levels of the pathway, at the membrane receptors or in the nucleus.

Contributions to the article: Design of the review, literature research and writing of sections 1 and 2, and final corrections of the whole review.

2.4 Article IV: First-in-class small molecules as selective antagonists of FZD in triple-negative breast cancer (*manuscript*)

This article describes a drug discovery project, starting from the discovery phase, leading through to pre-clinical development. It focuses on discovering WNT signalling inhibitors with anti-cancer properties for the development of drugs against TNBC. Using a drug discovery pipeline established in-house, we discovered a a novel class of small-molecules with a pyrazole core and four distinct moieties. Testing of the molecules on a disease representative panel of 6 TNBC cell lines showed anti-tumour

properties *in vitro*. Following the selection of the most stable compounds using microsomal stability assays and pharmacokinetics, the *in vivo* effect was tested. Intramammary tumours treated with the lead compounds FSA and F2-99 grew significantly slower than vehicle-treated tumours. Additionally, the cytoplasmic β-catenin levels were reduced in compound treated tumours, compared to the vehicle-treated tumours, indicating reduction in WNT pathway signalling. Additionally, F2-99 induced only mild bone toxicity compared to other WNT inhibitors, such as the porcupine inhibitors, providing them with a clear advantage over their competitors. Target elucidation using biochemical and cellular assays showed that within the WNT pathway, the CRDs of receptors FZD7,8 and 9 bind FSA/F2-99.

Contributions to the article: Design of the research article, figures 1 and 2, supplementary figures 1b, c, 2, 3, 4, 5, 6a, 9, 13b, and 17 a, c, and supplementary tables 1 and 2 with corresponding methods, text passages and figure legends.

3. DISCUSSION

The aim of the research work presented in this thesis was to develop new drug candidates targeting the WNT pathway in triple-negative breast cancer. More specifically, we aimed at discovering molecules which modulate the WNT pathway at the level of the plasma membrane, to gain more specificity and avoid pan- WNT inhibition and therefore minimise adverse effects. Screening of 1000 molecules led us to the discovery of a novel class of small molecules targeting a subset of FZD receptors, with WNT inhibitory actions and anti-TNBC properties *in vitro* and *in vivo*. Furthermore, the molecules demonstrate an improved *in vivo* toxicity profile compared to previously reported WNT inhibitors, making them attractive candidates for future clinical testing.

Our strategy for the screening and selection of the molecules followed a specific pipeline established in-house (Shaw et al., 2019, chapter 2.2). The primary assay, the so-called TOPFlash assay, is routinely used in research to test for WNT/β-catenin activation and TCF/LEF transcriptional activation of target genes (Veeman et al., 2003). As the TOPFlash assay *per se* is neither tissue- nor cancer-specific, we used the TNBC-cell line BT-20, stably transfected with the TOPFlash reporter plasmid to select TNBC-specific anti-WNT molecules right from initial stages of the selection process. Through the improvement of the Z'-factor, we created a sensitive TNBC-specific screen for WNT modulators. The Z'-factor is a statistical parameter which takes into account the assay's signal dynamic range and the data variation associated with the signal (Zhang et al., 1999). This allows to determine the stability of an assay for high-throughput screening. Additionally, we evaluated the actual expression profile of the WNT and FZD components of the cells.

However, TNBC is a very heterogeneous tumour type, and using one cell line only, of course, bears the risk of specialising the screen towards the "BT-20 subtype" of TNBC. Bioinformatical analysis of the cancer genome atlas (TCGA) and genotype-tissue expression (GTex) databases, which compared the expression of WNT pathway components genes in TNBC cell lines with those of TNBC patient tissues, let us conclude that the cell line HCC 1395 resembles patient expression patterns most (A. Koval, personal communication). This lead to the acquisition and inclusion of this cell line at a later time point of the project. However, BT-20 was second to HCC1395, and still very representative of TNBC patient

tissues as opposed to the cell lines HCC38 and MDA-MB 468, meaning that its employment as the primary screen is still valid. The cell line HCC 1395 could potentially be established as a more representative screening cell line; however, whether it would be suitable due to its low response levels and thus potentially poor Z'-factor remains an open question.

Using the Z' factor to select for the best cell line also meant that the established screen would be sensitive to even weak pathway inhibitors. The screening of FSA-like molecules resulted in various WNT-inhibitory profiles, concerning both IC_{50} and efficacy. In our quest to find the most potent WNT inhibitors, we selected molecules with the highest efficacies and lowest IC_{50}s for successive screening and development. Retrospectively, considering that we now know that FSA and F2-99 bind FZDs, future experiments should look into those few molecules with lower IC_{50}'s regardless of their efficacies (for example F2-52: IC_{50} ~1.55 uM, Efficacy 72.2%). Indeed, these molecules might bind more specifically to an even smaller subset or to individual FZDs, therefore only partially block β-catenin signalling.

The TOPFLash assay results are based on a reporter activity readout measured 18-24h after pathway stimulation with either WNT3a or the GSK-3 inhibitor CHIR99021. As WNT signalling activation induces significant changes in gene transcription, including expression of its own pathway components like FZDs, AXIN2, and TCF/LEF, both potentiating and inhibiting the signalling, its results are only indicative, especially regarding the top-level versus bottom level inhibition by compounds FSA/F2-99 (Hovanes et al., 2001; Jho et al., 2002; Roose et al., 1999; Willert et al., 2002). Therefore, the secondary experiments, testing for β-catenin stabilisation and DVL-phosphorylation and the inhibition thereof, were necessary and validated our findings that FSA/F2-99 inhibited the pathway at the most upstream, likely the membrane protein level.

We further demonstrated that FSA/F2-99 were able to inhibit TNBC cell growth *in vitro,* and replicated these results *in vivo,* both for TNBC cell lines and patient-derived xenografts. Immunohistochemical analysis of the treated tumour tissues clearly indicated that the WNT pathway was deactivated, demonstrated by β-catenin relocation to the plasma membrane. However, the inhibition *in vivo,* although significant, did not result in complete cell growth arrest, rather a delayed growth. As the tumour

concentrations of F2-99 reached levels of nearly 150x the IC_{50} of growth suppression *in vitro,* we decided to rule out insufficient drug concentration as the cause. Since however, TNBC is also dependent on other signalling pathways, such as Notch, Hedgehog and TGF-β signalling, it is possible for the cancer to adapt and eventually compensate for the lack of WNT signalling (Fodale et al., 2011; Giuli et al., 2019; Habib and O'Shaughnessy, 2016; Xu et al., 2018). Cancer cell adaptation is a known phenomenon, and combination therapy is routinely used in the clinical setting to overcome such issues (Mokhtari et al., 2017).

Additionally, as reported in chapter 2.3, WNT signalling is highly involved in cancer stem cell survival and chemo- and radiotherapy resistance. Anthracyclines like doxorubicin, taxanes such as paclitaxel and docetaxel are standard chemotherapeutic treatments of TNBC. However, they are also substrates of the drug pumps and WNT target genes MDR1 and ABCG2 (Nedeljković and Damjanović, 2019). Combining WNT inhibitors with conventional drugs can significantly enhance treatment outcome by increasing the tumour growth suppression and by preventing resistance to cytotoxic chemotherapy. This approach was recently proven successful when mice treated with doxorubicin and the repositioned drug and WNT inhibitor clofazimine. The treatment reduced the expression of ABC transporter MDR1 and resulted in additive anti-cancer effects without additive toxicity profiles (Ahmed et al., 2019). FSA/F2-99 could prove equally as successful in combination therapy, and future experiments *in vitro* and *in vivo* should focus on this aspect.

The WNT pathway is also involved in cancer immune modulation and immune suppression. It has been proposed that high programmed death-ligand 1 (PD-L1) expression, a protein involved in immune evasion is regulated by WNT signalling (Castagnoli et al., 2019; Martin-Orozco et al., 2019). Further, treatment of TNBC cells with selective WNT inhibitors downregulates PD-L1 expression. In the clinical setting PD1/PD-L1 immunotherapy has been approved for many cancers including TNBC: the use of a monoclonal antibody drug targeting PD-L1 in combination with nab-Paclitaxel has received accelerated approval for the treatment of adults with PD-L1-positive, unresectable, locally advanced or metastatic TNBC (Cyprian et al., 2019). Many more trials are currently underway in for PD-L1 positive breast cancer, including the TNBC subtype (NCT02926196; NCT03125902; NCT03197935; NCT0349871; (Di Nicola et al., 2018; Disis and Stanton, 2015; Planes-Laine et al., 2019). With this in mind, it would

be interesting to test if FSA/F2-99 could reduce PD-L1 levels in TNBC or even achieve synergistic effects with PD1/PD-L1 immunotherapy in follow up experiments.

To determine the target of FSA/F2-99 within the WNT-pathway, we investigated the binding of FSA/F2-99 to the CRD domains of the ten FZDs, using pull-down experiments, Biacore SPR analysis and waterLOGSY NMR. The results clearly indicate the interaction of FSA/F2-99 with FZDs, especially FZD7, and 9. FZD8-CRD also showed binding potential, though the functional consequence of this interaction is unclear, due to its mainly uncanonical pathway involvement. Encouraging as they are, these results were done using the soluble CRD domains only, as the mature, full-length receptors are challenging to produce and even harder to purify, necessitating the use of detergents, such as DDM, which actively interfere with ligand binding and are incompatible with some assays (Stetsenko and Guskov, 2017). The question to be asked is therefore if, in a cellular context, FSA/F2-99 also bind the full-length receptors? If yes, would they bind to the same extent and with the same specificity as the CRD domains only? FSA/F2-99 are relatively large molecules, and additional interaction with the linker between the CRD and 7TMDs of FZD and/or with the lipophilic pocket of the 7TM region, as seen for other small molecules might be feasible (Kozielewicz et al., 2020). This binding mechanism could potentially enhance the affinity and confer additional binding specificity, as sequences of the extracellular loops are less homologous than the CRD domains (Phesse et al., 2016).

The binding assays indicate that FZD7, 8 and 9 are the most likely targets of FSA/F2-99. These FZDs signal through both canonical and non-canonical WNT branches (Dijksterhuis et al., 2014). Both the TOPFlash assay and the β-catenin stabilisation assay were used to evaluate canonical WNT signalling inhibition of the FSA/F2-99. As non-canonical signalling regulates cell migration, indications were given by the *in vitro* migration assay, where FSA/F2-99 to some extent inhibited the migration of all but one cell lines tested. However, making a causative connection between the *in vitro* migration assay and uncanonical pathway inhibition is purely speculation, and more specific experiments, such as Ca^{2+} immobilisation assays, NFAT and AP1 dual-reporter luciferase assays (for PKC and PCP signalling respectively), would need to be envisaged to confirm the inhibition of the non-canonical pathway (Ma et al., 2017b; Martineau et al., 2017).

On the same line of research, the future of the project will inevitably focus on the mode of action of FSA/F2-99 on FZD receptors and the specific binding site of the molecules. Does FSA /F2-99 bind the lipophilic PAM site used by WNT-ligands or does it bind an allosteric site to induce a conformational change in CRD dimerisation as recently described by Nile et al., for a FZD7 binding peptide (Janda et al., 2012; Nile et al., 2018)? Competition binding assays or an ELISA using WNT3a (and others) and FSA/F2-99 to bind to purified FZD-CRD domains could give some insight into whether they are orthosteric or allosteric inhibitors (Nile and Hannoush, 2009). If FSA/F2-99 can compete with WNTs, site-directed mutagenesis of residues potentially involved in binding, as predicted by the *in-silico* model, will provide more information. Ultimately the co-crystal structures of a FZD (-CRD) in complex with the molecule, an ambitious feat for sure, would give the most precise answers.

Up until today, the downfall and reason why, even after decades of research, there are no approved WNT signalling inhibitors, are the severe adverse effects induced. As discussed in chapter 2.3, various approaches ranging from small molecule inhibition to the use of engineered biologics have induced a diverse set of toxicities. The most common are bone toxicity, meaning severe osteoporosis, bone fragility and eventually bone fractures, haematological toxicities such as haemophilia and neutropenia and severe gastrointestinal toxicities. Therefore, we treated our mouse models for an extended period with F2-99 to investigate these side effects. Simple observance of the mice indicated that there were no apparent toxicities induced, manifested as the absence of diarrhoea, weight loss, or general malaise. This was reflected upon immunohistochemical analysis of the intestinal wall: the intestinal villi were no different in length or crypt depth or density, meaning that the compounds did not affect physiological WNT signalling of the intestinal epithelium. This is somewhat surprising, as FZD7, the suspected main target of FSA/F2-99, is the main FZD involved in intestinal epithelial Lgr5(+) stem cells located in the intestinal crypts. Furthermore, treatment with FZD7-specific peptides impairs stem cell growth *in vitro* and knockout of FZD7 in adult mouse intestinal epithelium lead to cell death in cultured organoids and *in vivo* (Flanagan et al., 2015; Nile et al., 2018). However, the same body of research also showed that FZD7 knockout *in vivo*, leads to a rapid repopulation of the intestinal epithelium with cells within 30 days and that this phenomenon is FZD7 specific (Flanagan et al., 2015, 2017). They also reported that

FZD7 knockout did not result in loss of the villi, partly due to compensation and upregulation of FZD1 and FZD2 during regeneration. With this knowledge in hand, we can hypothesise that our compounds are indeed FZD7 specific within the gastrointestinal tract, with other FZDs taking over its role once inhibited, leaving the epithelium intact. Additionally, F2-99 might avoid effects on FZD7 due to its favourable tissue distribution, with much lower levels accumulating in the intestine.

We next proceeded to measure the effect of F2-99 treatment on haematological parameters (red blood cell count, platelet count, haemoglobin and haematocrit levels). Except for a slightly elevated platelet count, none of the parameters was significantly different between control-treated mice and F2-99 treated mice, indicating that in general, bone marrow function is not impacted. The measurements did not, however, include white blood cell counts, as the models used were immunodeficient. These parameters should be measured using immunocompetent mice to complete the picture.

Bone toxicity is a major side effect of WNT inhibitors. The porcupine inhibitors WNT974 and ETC-159 severely impact multiple bone properties, reducing the bone mineral density, the cortical area thickness, the trabecular thickness and density, trabecular volume and the cross-sectional thickness (Funck-Brentano et al., 2018; Madan et al., 2018). The effect of F2-99 on these parameters was measured by micro-CT. The F2-99 treated mice showed no difference in five out of six parameters measured. Only the trabecula volume was decreased two-fold. However, the effect is still mild in comparison to the porcupine inhibitors, where the trabecular volume was decreased 4-8 fold after only 3-4 weeks of treatment. The observed bone toxicity is to be expected if one considers that both FZD8 and FZD9 are two of the main WNT-receptors responsible for bone formation and remodelling. Both $FZD8^{-/-}$ and $FZD9^{-/-}$ mice have osteopenia, a weakened bone structure. FZD9 regulates non-canonical signalling in osteoblasts and encourages bone formation, whereas FZD8 signalling through β-catenin reduces osteoclast differentiation, reducing bone resorption (Albers et al., 2011, 2013). The inhibition of both receptors will, therefore, evidently lead to weakened bones, as observed in our *in vivo* experiments. The fact that the effects are not as strong as seen for the porcupine inhibitors might be due to compensation of FZD8/9 by other FZDs involved in bone homeostasis. Indeed, FZD4 was recently discovered to have overlapping functions with FZD8 in osteoblasts (Kushwaha et al., 2020). Our biochemical binding assays and the TOPFlash assay testing the effect of FSA/F2-99 on cells expressing

the individual FZDs suggest that FZD4 binds FSA/F2-99 with the lowest affinity out of all tested FZDs. It is therefore quite possible that in the bone, FZD4 can compensate for FZD8 and 9 inhibition. In a clinical setting, the loss in bone volume could potentially be countered by co-administration of zoledronic acid or vitamin D and calcium supplements, as has been the case for other WNT inhibitors being tested in clinical trials. Taken together, our biochemical data and the mild FZD-specific toxicity observed strongly indicate that FSA/F2-99 indeed only target a small and specific subset of FZD receptors.

Though FSA/F2-99 demonstrates on-target specificity, as discussed in chapter 2.1, even the best of small molecules will have off-target binding affinities and actions, and FSA/F2-99 will be no exception. This is best observed when treating FZD-less cells with our molecules: cell growth and colony formation were still inhibited, even more so than control HEK239 cells. However, searching for additional targets would be a time-costly and challenging endeavour. Furthermore, the interest of doing so is questionable, as so far, we have no reasons to believe they would induce additional severe side effects.

To conclude, we have demonstrated that a novel class small molecules based on a diphenyl-substituted pyrazole core, targets a small and specific subclass of FZDs. This confers them potent WNT inhibitory and anti-cancer properties, with mild adverse effects. Future plans will involve elucidation of the mode of action of FSA/F2-99. This knowledge, together with medicinal chemistry, will help improve the molecules' chemical and pharmacological properties such as solubility and specificity. Another focus will be the combination of FSA/F2-99 with standard chemotherapies to improve the tumour growth suppression *in vivo* and to decrease chemo-resistance. TNBC is not the only WNT-dependent disease, and expansion to other indications, for example, the FZD7 dependent gastric cancer or hepatocellular carcinoma, will allow to increase the scope and impact of the discovered class of small molecules (Cheng et al., 2019; Merle et al., 2004; Wei et al., 2011). These selective FZD small molecules not only have the potential to evolve into potent anti-cancer drugs, but they will also contribute to the WNT-field in general, serving as a powerful tool to increase understanding of WNT-signalling and enhance research.

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Article I

cancers

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Review

A Second WNT for Old Drugs: Drug Repositioning against WNT-Dependent Cancers

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Abstract: Aberrant WNT signaling underlies cancerous transformation and growth in many tissues, such as the colon, breast, liver, and others. Downregulation of the WNT pathway is a desired mode of development of targeted therapies against these cancers. Despite the urgent need, no WNT signaling-directed drugs currently exist, and only very few candidates have reached early phase clinical trials. Among different strategies to develop WNT-targeting anti-cancer therapies, repositioning of existing drugs previously approved for other diseases is a promising approach. Nonsteroidal anti-inflammatory drugs like aspirin, the anti-leprotic clofazimine, and the anti-trypanosomal suramin are among examples of drugs having recently revealed WNT-targeting activities. In total, 16 human-use drug compounds have been found to be working through the WNT pathway and show promise for their prospective repositioning against various cancers. Advances, hurdles, and prospects of developing these molecules as potential drugs against WNT-dependent cancers, as well as approaches for discovering new ones for repositioning, are the foci of the current review.

Keywords: approved drugs; WNT pathway; repositioning

1. Introduction

WNT signaling is one of the essential pathways involved in animal embryonic development, during which it has numerous roles including the regulation of cell proliferation and differentiation [1]. In the healthy adult tissues however, it is largely inactive, with some exceptions such as the renewal of the gastro-intestinal tract [2], as well as haematopoiesis [3] and regeneration after injury [4]. It is to no surprise then that aberrant activation of this pathway can lead to diseases of neoplastic nature such as cancer [1,5].

The signaling is activated by a family of lipoglycoproteins called WNTs, of which 19 can be found in humans and whose production, secretion and diffusion through tissues is tightly controlled [6]. Upon binding to the FZD family of GPCRs [7,8] (ten homologues in humans), various branches of the WNT pathway can be activated, depending on the ligand-receptor combination and cellular context. They are namely the PCP branch mostly involved in cytoskeleton rearrangement, cell polarity and migration; the WNT/Ca^{2+} branch which is known to promote proliferation and antagonize the canonical pathway; and finally the so-called canonical branch [9]. It is the latter, which is mostly associated with disease and cancer and therefore is the focus of many studies touching upon the WNT pathway [1,10]. Upon WNT binding to the FZD-receptor and one of the two single transmembrane co-receptors LRP5/6, the FZD-coupled G-proteins together with Dishevelled (DVL), a multi-domain scaffolding protein, transduce the signal (Figure 1) [11,12]. As a consequence AXIN, part of the

β-catenin destruction complex, is recruited to the membrane [13,14]. The destruction complex is responsible for the phosphorylation of β-catenin and its subsequent degradation. In addition to AXIN, which acts as a scaffold, it also contains adenomatous polyposis coli (APC) and the Ser/Thr kinases casein kinase (CK1) and GSK3β, which in the absence of signaling phosphorylate β-catenin. The phosphorylation of β-catenin results in its ubiquitination and proteosomal degradation [15]. However, upon WNT signaling AXIN is no longer able to form the destruction complex and cytosolic β-catenin accumulates. This leads to its translocation to the nucleus where it exerts its downstream effects by mediating LEF/TCF dependent transcription of WNT-target genes. These include proto-oncogenes such as c-Myc and cyclin D1 [16,17].

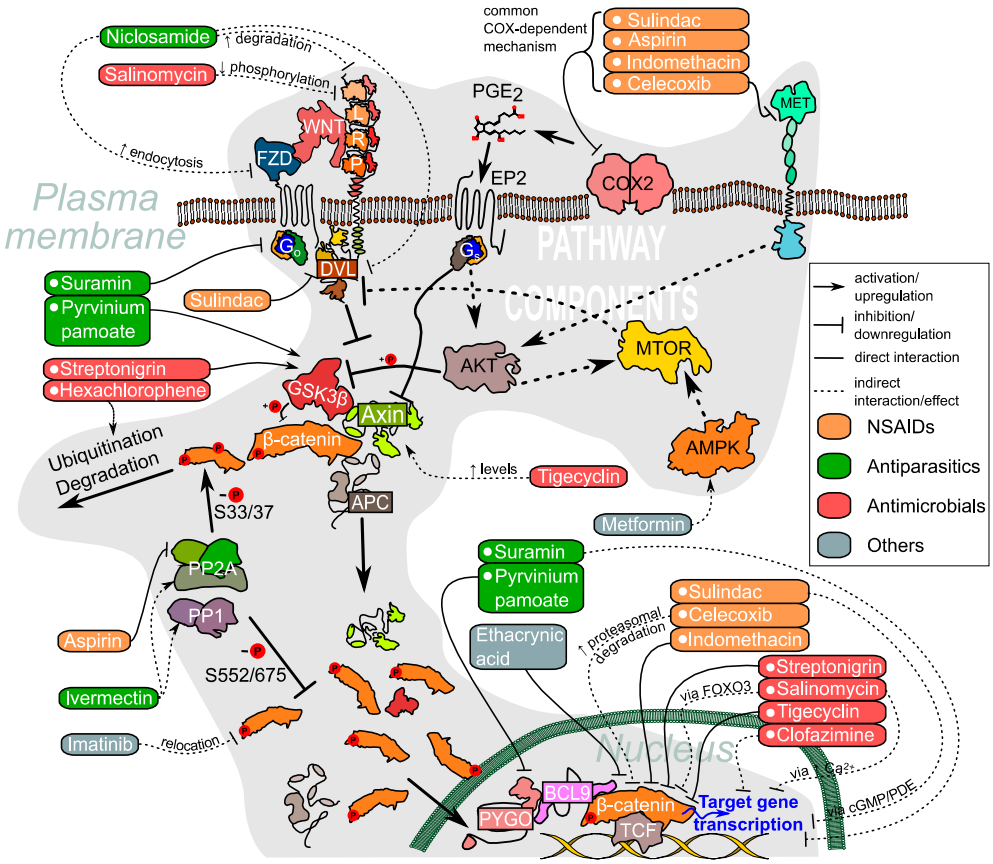

Figure 1. Targets of approved drugs in the context of WNT and related pathways. For detailed descriptions of the activities refer to Table 1 and the corresponding sections in the main text.

To date nearly half of known human tumors show a dysregulation of the WNT signaling pathway [18]. Loss-of-function mutations of APC, which induce adenoma, one of the first steps in the cancerous development, are of the first and probably the best known examples of WNT-dependency in cancer [19]. Since establishing the link between the WNT pathway and tumorigenesis, a broad variety of solid tumors and leukaemias have been shown to either almost entirely or by few subtypes depend on deregulation of the WNT-pathway [18]. Even though the overactivation of the pathway is in some cases due to mutations, in many it is rather the up- or downregulation of pathway components which is the cause. Examples therefor are the upregulation of the WNT receptor FZD_7 found in certain breast cancers and hepatocellular carcinoma [20,21] or the downregulation of the WNT inhibitory factor 1 (WIF1) found in prostate, lung, breast and bladder cancers [22]. More recently, the WNT-pathway has also been shown to be a player in an emerging field of cancer stem cells (CSC), being involved in their maintenance and survival in certain cancers [23,24], resparking the interest of researchers from various fields in this pathway. In several instances CSCs, thought to be tumor initiating cells, have been

demonstrated to be a cause for the occurring drug resistance and metastasis after initial therapy [25]. It is therefore not surprising that in recent years there has been an urgency to discover new drugs targeting this pathway. So far however, no drug targeting the WNT pathway has been approved, and only few have made it into early clinical trials, such as the anti-FZD_7 antibody vantictumab (NCT01345201) and the PORCN inhibitor LGK974 (NCT01351103) [26].

The traditional drug discovery process has become a costly and time-consuming practice [27,28]. On average, de novo discovery and development of a drug costs about 1.8 billion dollars and it takes around 10–15 years for the drug to reach the market [27]. On estimate, only one in ten drugs entering phase I clinical trials is finally approved by the FDA, and this decreases to one in fifteen for drugs with an oncology indication [28]. Drug repositioning, meaning using known drugs for new purposes, has therefore become an attractive drug development strategy, as it has an attractive risk-versus-reward trade-off compared to other business strategies [29]. Indeed, the advantages of repositioning a drug are multiple: not only has the drug already been used in humans, been tested in various stages of the drug development pipeline and therefore offers knowledge on is safety, pharmacology and toxicology, but also in some cases, later stages of the process such as the manufacturing and formulation can be reused for the new drug product [29]. Especially in oncology, where there is an ever-increasing demand for new therapies, drug repositioning could offer a faster and economically more interesting way of fighting this class of disease [30]. The best-known example of successful drug repositioning against cancer is thalidomide. It was initially used to treat morning sickness but was discontinued after being discovered to cause malformations in new-borns. It was later rediscovered to have anti-cancer properties and is currently FDA-approved for multiple myeloma in combination treatment with dexamethasone [30].

Many WNT-dependent cancers, such as triple-negative breast cancer (TNBC), are unmet medical needs. This makes future drugs against these cancers eligible for receiving the fast track designation granted by the FDA. This designation allows the approval process to be accelerated [31]. We propose that the shortened drug discovery process together with fast track designation makes drug repositioning a promising strategy to win the battle against WNT-dependent cancers, bringing help to patients sooner than later.

This review focuses on approved drugs, which have later been found to modulate the WNT pathway. We highlight their mechanism of action and the range of WNT-dependent cancers these drugs may target in vitro and in vivo. We also correlate these data with the pharmacodynamic and pharmacokinetic parameters established for these drugs, and examine the potential for their repositioning against the WNT-dependent cancers. Finally, we discuss the challenges drug repositioning holds and future possibilities of finding new anti-WNT drugs for cancer treatment.

2. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs are a class of drugs marketed for their analgesic, anti-inflammatory and anti-pyretic effects. These effects are achieved by the inhibition of the cyclooxygenase (COX) enzymes, COX1 and COX2, involved in the prostaglandin production. They can be classified as non-selective, as is the case for most of the NSAIDs, or selective COX2 inhibitors, as is the case for celecoxib [32].

Apart from their traditional usage, aspirin and other NSAIDs have shown great promise in lowering the incidence of adenomatous polyposis of the colon and reducing the risk of colon cancer. This conclusion is based on several epidemiological studies of the general population and randomized trials [33–36]. Studied to a lesser extent, the prolonged intake of NSAID has also been linked to a reduction of incidence of various other solid tumors [37,38] such as those of the breast [39,40], lung [41], oesophagus [42], prostate [43], bladder [44], and pancreas [45].

The existence of crosstalk between COX2 and WNT signaling has been demonstrated. Indeed, prostaglandin E2 (PGE_2) induces β-catenin stabilization, leading to its nuclear translocation, and is able to induce β-catenin/TCF/LEF-reporter activity in colon cancer cells [46,47]. Castellone et al. showed that stimulation of the GPCR EP2 by its ligand PGE_2 induces activation of Gαs and its

association with AXIN, leading to the release of GSK3β from the destruction complex. In parallel, the Gβγ component of the initial heterotrimeric Gs protein activates PI3K/AKT, which in turn inhibits GSK3β by phosphorylation. PGE_2 therefore has a dual activating effect on the WNT-pathway, and NSAIDs decrease this effect by inhibiting the COX enzymes [46]. The effectiveness of NSAIDs on cancer is in some cases partly due to the COX-inhibitory effect, which leads to lower levels of PGE_2 production and decreases the β-catenin stabilization [48,49]. In the sections below, we summarize the experimental evidence demonstrating that NSAIDs also target the WNT/β-catenin pathway in COX2-independent manners.

2.1. Sulindac

Multiple studies have shown that sulindac is able to increase β-catenin degradation and decrease its nuclear translocation in breast, lung and colon cancer cells in vitro, leading to reduced expression of the β-catenin/TCF target genes [50–52]. This was also observed in X-RARalpha-expressing cells, reducing their leukemic phenotype and stemness [53]. A metabolite, sulindac sulfide, has demonstrated WNT/β-catenin signaling blockage and inhibition of proliferation of prostate cancer cells [54].

From a mechanistic point of view, sulindac has been shown to directly affect the WNT-pathway independently of the COX expression. Sulindac is also one of the few WNT-active approved drugs for which the exact molecular targets within the pathway have been described. Sulindac is able to specifically bind to the DVL-PDZ domain, which was proposed to directly inhibit DVL's interaction with FZDs. Surprisingly, this interaction is characterized by low micromolar K_d levels [55], while the IC_{50} of the pathway inhibition by sulindac was found to be almost two orders of magnitude higher [50,52]. This discrepancy might be accounted for by the fact that the most prevalent cell-permeable metabolite of this drug (sulindac sulfide) has a reduced affinity to DVL [55]. Additionally, it has been demonstrated that sulindac is a cyclic guanosine 3′,5′-monophosphate phosphodiesterase (cGMP PDE) inhibitor, which leads to elevated levels of cGMP and activated cGMP-dependent protein kinase (PKG). This in turn results in transcriptional suppression of β-catenin and inhibition of the WNT/β-catenin pathway [56]—potentially more powerfully than the inhibition of DVL-FZD interactions.

Further, sulindac has been shown to effectively reduce tumor growth of colon cancer and intestinal cancer cells in vivo and prevent colon cancer metastasis in mouse models [48,57,58]. The daily doses of sulindac tested in vivo were 20–50 mg/kg, however doses above 20 mg/kg have shown significant toxicity. It should be noted that in order to achieve efficient in vivo inhibition of COX2 by sulindac 10- to 20-fold lower doses are used, indicating a very narrow therapeutic window available to achieve maximal anti-WNT effect. Even at borderline to over toxicity dose of sulindac at 20 mg/kg, plasma levels of the drug were just under 20–40 μM [59], which is somewhat lower than the IC_{50} of COX-independent WNT-inhibition in vitro (50–70 μM) [50,52]. However, this might be compensated by the tissue accumulation of sulindac, exceeding plasma levels by 3–5 fold [59]. Sulindac treatment in mice results in reduced transcript and nuclear β-catenin levels [58,60,61]. In humans, familial adenomatous polyposis (FAP) patients treated with a tolerable dose of 300 mg of sulindac per day for 6 months presented lower adenoma nuclear β-catenin levels than adenomas in non-treated patients [50]. However, at this dose sulindac plasma and tissue levels will not exceed 1–10 μM and therefore WNT-pathway modulation in these patients is likely to be a result of COX2 inhibition.

2.2. Aspirin

The chemopreventive effects of aspirin, the only irreversible COX inhibitor, were first reported in a study in 1988 [62] and since, there have been many clinical reports to support such effects [34,36,63]. Diehlmann et al. were the first to demonstrate that aspirin can inhibit β-catenin/TCF transcriptional activity in a luciferase-based reporter assay in colorectal cells lacking COX expression [64]. The drug did not affect the total amount of β-catenin, but the levels of β-catenin phosphorylation (both phospho-S33/S37/T41-catenin and phospho-T41/S45-catenin) were increased, however independently of GSK3β [65]. It was thus hypothesized that aspirin affected β-catenin phosphorylation through

inhibition of a phosphatase, which was later discovered to be protein phosphatase A2 (PP2A), being inhibited by aspirin directly [66,67]. Although not yet clearly demonstrated, it is highly likely that aspirin also affects WNT signaling indirectly through other aspirin affected pathways, for example the NF-κB signaling [68]. In vivo models confirm the influence of aspirin on the WNT pathway. In APC^{min} mice, the murine model of FAP, aspirin treatment decreased tumor formation and lowered β-catenin levels [69]. Noteworthily, the amount of data accumulated on aspirin and its effect on various tumors is tremendous and cannot be reviewed here fully. There are multiple studies confirming its effects on WNT signaling and tumor growth in various cancer types both in vivo and in vitro. These findings are excellently reviewed elsewhere [70,71].

In general, lower millimolar levels (~5 mM) of aspirin are needed for the COX-independent in vitro inhibition of the WNT/β-catenin pathway in human cancer cells [64]. To reach similar concentrations in mouse tumor tissue, high doses (ca. 100 mg/kg) of aspirin must be administered [72]. The chemopreventive effect of aspirin in humans, which has already been shown for doses as low as 75 mg/day [73] is most likely due to the COX inhibitory effects. In order to reach the COX-independent WNT inhibitory effects of aspirin, high doses (>10 g/day) need to be administered [74], which however could lead to toxicity and side effects upon treatment, especially in the long-term [75]. Aspirin's merit as a combination therapy is currently being scrutinized in various trials and retrospective studies, which have already demonstrated its utility in treatment and prevention of notorious WNT-dependent cancers such as breast, colon, prostate and gastric cancers [71]. The first tangible outcome in aspirin repositioning has already been achieved. The US Preventive Services Task Force (USPSTF) recommends a daily low-dose use for individuals with high risk of cardiovascular diseases (CVD) between 50 and 69 years as a mean of both CVD and colorectal cancer chemoprevention.

2.3. Indomethacin

Like aspirin, indomethacin has been shown to inhibit proliferation of colorectal cancer cells independently of COX2 expression; however, the mechanisms of action of the two NSAIDS on the WNT pathway were suggested to be different [76]. Indomethacin concentrations of 100–400 μM significantly decrease the TopFlash transcriptional readout in these cells [64], and higher concentrations intensify this inhibition and are accompanied by a decrease in the total β-catenin protein levels [76,77]. The exact reason or details for these effects remain unclear; however, they might partly result from unusual transcriptional regulation of β-catenin, as mRNA levels of β-catenin were significantly lower in the cells treated with indomethacin. The drug has also shown a differential effect on WNT target genes: while cyclin D1 was expectedly downregulated, c-Myc was upregulated. The latter might be the result of a swift onset of apoptosis due to indomethacin treatment [76]. Another input of indomethacin in WNT inhibition is achieved through disruption of the β-catenin-TCF4 complex formation with DNA in colorectal cancer in vitro models [78].

In the rat model of colon cancer, indomethacin (2 mg/kg) was able to reduce tumor formation [79], eliminating nuclear β-catenin staining while leaving cytoplasmic levels unchanged in these tumors [61]. However, it is likely that the anti-WNT effects seen in these studies are mediated through COX inhibition, since these doses result in plasma levels of ca. 10–30 μM of indomethacin [80], far below of what is required for the strong and direct inhibition of the pathway. Applications of higher doses of unmodified indomethacin are unlikely since they are expected to produce acute toxicity (LD_{50} of the drug is around 14 mg/kg for rats).

2.4. Celecoxib

The COX2-independent effect of celecoxib was demonstrated by the induction of apoptosis in celecoxib-treated HTC-116 cells, a colorectal cell line lacking the expression of COX2. The effect was proposed to be mediated by inhibition of the WNT pathway, since the drug inhibited the TopFlash reporter and cyclin D1 expression [81]. A second study further showed that in colon cancer cells, celecoxib acted downstream of the β-catenin destruction complex, decreasing TCF1 and TCF4 levels

by proteasomal degradation [82]. Complementary to these results, a study demonstrated that in colon cancer cells celecoxib inhibited the c-Met/AKT pathway, resulting in decreased phosphorylation and thus increased activity of GSK3β, leading to an increase in β-catenin phosphorylation [83]. The inhibition of proliferation and the downregulation of the WNT/β-catenin signaling by celecoxib was also demonstrated in glioblastoma and prostate cancer cells [54,84]. In glioblastoma cells, GSK3β phosphorylation was shown to be reduced, leading to β-catenin phosphorylation similarly to the effects observed in the colon cancer cells [84]. Analogous observations were made for hepatoma cells [85] and osteosarcoma cells [86].

Celecoxib also affects CSCs. In colorectal CSCs, celecoxib reduces the chemotherapy-resistant CD133-positive pool, while decreasing WNT activity and expression of stemness markers [87]. In myelogenous leukaemia cells resistant to imatinib, celecoxib sensitized the cells by inhibiting the ABC transporters responsible for drug resistance via WNT and RAS signaling pathways. The study demonstrated downregulation of the WNT activity and pathway components such as GSK3β, β-catenin, LEF1 and TCF4 at protein and mRNA levels [88].

In vivo celecoxib has been shown to prevent the formation of β-catenin accumulated crypts, typical premalignant lesions of colon cancer showing excessive accumulation of β-catenin [89]. Further, celecoxib has been shown to suppress lung cancer cell metastasis in mice through the PGE_2-GSK3β-β-catenin axis [90]. Like sulindac and indomethacin, celecoxib is able to reduce the amount of β-catenin-positive cells in colon cancer in rats [61]. Finally, celecoxib has been shown to suppress WNT-dependent mammary carcinoma, meningioma and Lewis lung carcinoma in mouse models [91,92]. The doses used in these studies correspond to plasma levels of 3–5 μM, which is somewhat below the average 20 μM required for inhibition of tumor growth. However, the drug can accumulate in tissues to concentrations 2–4 folds higher than in plasma and therefore reach the effective dose [93]. FAP patients treated with celecoxib (400 mg/day) showed a 28% reduction in polyps after 6 months [94], and in 1999 the FDA approved this drug for the indication of FAP but later withdrew the approval due to lack of proof of clinical benefit.

3. Antiparasitics

3.1. Niclosamide

Niclosamide is an anthelmintic drug approved by the FDA in 1982 for treating intestinal parasite infections, especially cestodes [95]. In addition to its anthelmintic activity, several studies have described anticancer properties of niclosamide. Its anti-proliferative activity has been demonstrated in a wide array of cancer cell lines representative of WNT-dependent cancers: non-small lung carcinoma [96], multiple myeloma [97], hepatoma [98], adrenocortical carcinoma [99], ovarian cancer [100] and glioblastoma [101]. It also suppresses the growth of $CD34^+/CD38^-$ CSCs of acute myeloid leukemia (AML) and $CD44^+/CD24^-$ CSCs of basal-like breast cancer [102,103].

Niclosamide inhibits the canonical WNT pathway with an IC_{50} of 0.2–0.4 μM, similar to that which mediates inhibition of cancer cells growth (0.33–0.75 μM) [104], suggesting that WNT inhibition is involved in niclosamide's anticancer effects. Several WNT components are involved in the inhibitory action of niclosamide, which vary depending on the cancer subtypes. In osteosarcoma and colorectal cell lines, it inhibits WNT3a-stimulated β-catenin stabilization and LEF/TCF reporter activity through promotion of FZD_1 endocytosis and downregulation of DVL2 [105,106]. However, niclosamide's inhibitory effect for breast and prostate cancer cells seems to involve other components of the WNT pathway. Instead of DVL2 downregulation it induces LRP6 degradation associated with inhibition of cell proliferation, invasion and migration of cancer cells [104].

In addition to inhibiting the canonical WNT pathway, niclosamide may mediate its anticancer activities through several other signaling pathways such as NOTCH [107], MTOR [108], NF-κB [97] and STAT3 [96]. This pleiotropy highlights the need of identifying the relevant targets of niclosamide in different tumors.

The anti-cancer effects of niclosamide have also been tested in vivo. When delivered orally at 200 mg/kg, it induces inhibition of tumor growth and impairs metastases formation in colorectal cancer [106]. WNT pathway inhibition by niclosamide in colorectal and basal-like breast cancer models in mice has been demonstrated by immunohistochemical analysis, where lower levels of cytosolic and nuclear β-catenin were observed for the drug-treated mice [103,106]. When delivered directly into the systemic circulation through intra-peritoneal (IP) injection, it resulted in a significant inhibition of breast tumor growth [109], without manifesting any signs of toxicity or mutagenicity [99,101,103]. However, no information is available on its pharmacokinetics after IP or IV injections. In contrast, the poor oral bioavailability of the drug limits its maximal plasma concentrations achievable by that route of administration to 0.1–0.2 μM [106], one order of magnitude below the effective range; other studies have also shown that the plasma concentrations can vary widely due to variable absorption rates by the gastrointestinal tract [110]. These effects limit the anticancer applications of orally delivered niclosamide. Since the safety profile is only available for oral delivery [110], it is only feasible to use it against gastrointestinal tumors so far. One such study has already been launched: the evaluation of efficacy as a treatment of metastatic colorectal cancers patients in a phase 2 clinical trial, using the same approved dose and oral route of administration (NCT02519582) is ongoing.

3.2. Suramin

First introduced in 1912, suramin was used for the treatment of African sleeping sickness and river blindness in humans [111]. Despite such a long history and appearance of new agents for the same conditions, suramin is still indispensable in the clinical practice as it remains the only treatment for certain subtypes of the diseases [112].

Suramin has demonstrated a dose-dependent anti-proliferative effect in many human cancer cell lines [113–115]. Molecular targets of suramin are numerous. Most relevant for its anticancer effects are the inhibition of binding of many growth factors, e.g., FGF and VEGF, to their cognate receptors [116–118], and the folate metabolism [119]. Recently, we have added to this list inhibition by suramin of at least two targets within the WNT pathway, resulting in its complete blockade [120]. While identification of the downstream target is currently ongoing, we investigated the upstream target, since inhibition of the upstream components of the pathway is a promising approach for increasing drug efficiency against WNT-dependent cancers [26,121,122]. We discovered that suramin acted as a competitive inhibitor of GTP uptake by the heterometric G proteins, in turn regulating internalization of the WNT/FZD complexes, which normally serves to amplify the signaling in the WNT pathway [120,123,124]. We have further shown that inhibition of TNBC growth in vitro and in vivo is more efficient at the concentrations ensuring such inhibition (ca. 200 μM) as compared to lower doses.

Since suramin was already proposed as an anti-neoplastic agent in the late-80s, this drug has an extensive record of clinical trials. Although trials with the focus on the WNT pathway targeting are yet to be done, some have already been performed against cancers which strongly rely on the WNT pathway, such as recurrent breast cancer [125,126], metastatic colorectal cancer [127,128], and lung cancer [129]. Surprisingly, in almost all of these trials suramin failed to show any significant improvements, maximally resulting in only moderate positive response reported in two studies [125,129]. In both, suramin was used at small doses (weekly IV perfusions, ca. 100–150 mg per patient) resulting in plasma levels of 30–50 μM; in these cases treatment was not associated with any significant toxicity. In other studies, the doses (weekly IV, 500–700 mg per patient) used produced plasma levels corresponding to that necessary for WNT inhibition (200–250 μM); unfortunately this resulted in significant side effects, primarily of the neurological character with no significant clinical outcomes for tumors [126–128]. One of the possible explanations for this might be in the unfavourable pharmacokinetics of suramin. It was found to have poor tissue penetration and retention. Suramin concentrations in most tissues were 2–3 times, and in the tumor (pheochromocytoma in this case) almost six times lower than in plasma [130]. It should be also noted that suramin demonstrated

similar negative results against other types of cancers: prostate [131–134], ovarian [130], urinary bladder cancer [135]. Based on this negative data, the FDA has so far refused approval of suramin for therapeutic applications in oncology [134].

Tackling these limitations of suramin can be achieved by several ways. Since systemic administration of suramin results in multiple toxicities and the gastrointestinal tract has shown poor absorption [111], the repositioning of suramin might be achieved by using new routes of administration to avoid systemic treatment. This has led to a phase 1 clinical trial, testing the efficacy of suramin delivered intravesically for urinary bladder cancer patients [135]. Future directions of suramin applications might be through usage of novel targeted delivery systems to create high local concentrations at the tumor site [136] or synthesis of new structural analogues in order to improve potency and overcome the side effects [137–139].

3.3. Pyrvinium Pamoate

Pyrvinium pamoate is an anthelminthic drug approved by the FDA [140]. The anticancer activity of pyrivinium is exhibited through inhibition of colon cancer cell motility and proliferation in vitro and suppression of tumor growth in vivo [141].

Inhibition of the WNT pathway by pyrvinium has also been demonstrated in vitro [142,143] and in vivo [141]. Like for most of the repositioned drugs, WNT inhibition by pyrvinium occurs through multiple components of the pathway. Pyrivinium has been demonstrated to act through activation of an isoform of casein kinase 1α (CK1α), part of the WNT pathway destruction complex. In the same work, the authors identified inhibition of pygopus (PYGO), preventing transcriptional activity of β-catenin, as a second impact of pyrvinium on the WNT pathway. These activities are independent of each other and show comparable IC_{50}'s [143]. However, another study has failed to recapitulate the effects on CK1α by pyrivinium, and instead suggested that the drug acts through the PI3K/AKT pathway in the manner similar to that described above for celecoxib, decreasing GSK3β phosphorylation at Ser9 and thus enhancing its activity [142]. Several other mechanisms, such as the energy metabolism and STAT3 pathway [144,145], glucose deprivation and hypoxia [146], as well as autophagy [147], have been implicated in the anticancer action of pyrvinium.

In vitro studies have identified pyrvinium to be effective against the WNT pathway and cancer cell proliferation within the high-nanomolar range (50–200 nM). When delivered by its standard oral route, pyrvinium's bioavailability is virtually zero [148] and therefore cannot be employed for in vivo anticancer studies. Therefore, it was delivered by daily intraperitoneal injections of 1 mg/kg, which were reported to create acceptable peak plasma levels of 150 nM [149]. Using this dose, efficient suppression of the WNT-dependent colon cancer in vivo was achieved [141]. Unfortunately, this dose is borderline with severe toxicity, since any increase resulted in severe toxic effects [149]. Therefore, phase I safety trials should be launched first in order to verify this novel delivery route in patients; no data has been reported so far for any attempts to run such a trial.

3.4. Ivermectin

First introduced in 1981 as an anti-parasitic for veterinary applications [150], ivermectin was approved in 1987 for the treatment of onchocerciasis and more recently for lymphatic filariasis in humans [151,152]. It has also been reported to activate chloride channels of nematodes, causing parasite paralysis and death [153].

Ivermectin inhibits proliferation of human colon cancer and lung cancer cells both in vitro and in vivo [154]. The anti-proliferative action, affecting both the bulk tumor cells and CSCs, was linked in this study to inhibition of WNT signaling. The mechanism of this inhibition is rather unusual: ivermectin inhibits C-terminal phosphorylation of β-catenin, overactivating by an unknown mechanism protein phosphatases PP2A and PP1. As a result, the activity of β-catenin as a co-factor in transcription of the WNT target genes is reduced [154].

Ivermectin also has a cytotoxic action due to activation of mammalian chloride channels, similarly to its effects in nematodes [155]. Importantly, the anti-WNT IC_{50} of ivermectin is 5–10 times (~1–2 μM vs. 10 μM) lower than that of its toxic effect against chloride channels. Unfortunately, oral bioavailability of the drug, as for other antiparasitic drugs discussed in this section, is very low. Upon normal oral dosing its plasma levels do not exceed 60 nM. Intraperitoneal delivery at 10 mg/kg in the form of a cyclodextrin conjugate, likely achieving high plasma concentrations, was well tolerated and suppressed growth of colorectal cancer in mouse xenograft studies [154]. Toxicity studies in vivo have also demonstrated a wide therapeutic index for ivermectin [151,156]. The scarcity of data regarding the pharmacokinetics and the safety profile of ivermectin delivered to humans by means other than oral delivery make it compulsory for ivermectin to be tested in safety studies before any further clinical interventions.

4. Antimicrobials

4.1. Salinomycin

The anticancer properties of salinomycin, an antibiotic potassium ionophore used to treat poultry, were first discovered in a high-throughput screen on breast cancer stem cells [157]. This study demonstrated the ability of salinomycin to reduce the proportion of breast CSCs in vitro and the expression of genes associated with CSC and poor prognosis. Gupta et al. also showed inhibition of mammary tumor growth in mice treated with salinomycin and the promotion of cell differentiation to an epithelial phenotype after treatment [157]. Since then, salinomycin has been shown to inhibit cell growth in the following WNT-dependent cancer cells in vitro: pancreatic [158], endometrial CSCs [159], chronic lymphocytic leukaemia cells [160], breast and prostate cancer cells [161], osteosarcoma CSCs [162], hepatocellular carcinoma cells [163], nasopharyngeal carcinoma cells [164]. It has also showed promising inhibition of growth of gastric tumors, osteosarcoma as well as hepatocellular and nasopharyngeal carcinoma in mice [162–165].

As the WNT pathway is one of the essential pathways responsible for the survival of CSCs, it has been proposed as one of the targets of salinomycin [160]. Indeed the drug has been shown to downregulate the expression of WNT-target genes such as LEF1, cyclin D1 and fibronectin in vitro [159–161] by inhibiting the WNT-induced phosphorylation of the co-receptor LRP6 and inducing its degradation in WNT-overexpressing cells [160,161]. Further it has been shown that salinomycin is able to activate the transcription factor FOXO3, which then disturbs interactions between β-catenin and TCF, inhibiting the transcription of WNT target genes [166]. One additional suggested mechanism of action of salinomycin is the suppression of the canonical WNT-pathway via an increase of intracellular calcium levels, as it has been shown that non-canonical WNT ligands are able to inhibit canonical WNT-signaling by increasing calcium influx [163]. It should be noted that non-WNT related mechanisms of action of salinomycin on cancer cells are multiple, and excellently reviewed elsewhere [167].

The in vitro IC_{50} of salinomycin varies, depending on the source, cell type used and treatment period, between 0.3 and 10 μM. Up-to-date, there is no comprehensive pharmacokinetic study of salinomycin in animals or humans. Similar to the anti-parasitic drugs, salinomycin is normally delivered orally, however this route is unacceptable for anticancer applications due to low bioavailability and therefore resulting low blood and organ levels. It is also shown that after intravenous injection in mice, salinomycin is rapidly metabolized [168] and therefore frequent injections/infusions are likely necessary, though nothing is known regarding the anticancer activities and pharmacokinetics of its metabolites. Prolonged daily injections of 10 mg/kg of salinomycin in mice grafted with nasopharyngeal carcinoma showed no overt toxicity and resulted in a decrease in the tumor burden, and also in reduced levels of LRP6 and β-catenin [164]. Another group has also reported no toxicity and marked tumor reduction concomitant with decreased GSK3β phosphorylation in an osteosarcoma xenograft model in response to 5 mg/kg salinomycin daily [162]. Treatment with 4 mg/kg salinomycin

reduced tumor burden in an in vivo model of hepatoma. This also corresponded to a significant shutdown of GSK3β phosphorylation with a concomitant β-catenin decrease [163].

Unfortunately, there is currently little knowledge of the toxicity and pharmacology of salinomycin in humans, as it has never been approved for human use. However, in an uncontrolled clinical pilot study employing salinomycin to treat several patients with various metastatic cancers, metastases regression was observed; in another case of squamous cell carcinoma of the vulva, monotherapy resulted in prolonged progression-free disease. Salinomycin was given at 200–250 μg/kg, which corresponds to the initial concentration in blood plasma of ca. 15–20 μM, agreeing with the mouse dose of 1–2 mg/kg. Acute side effects in all cases were minor and included tachycardia and mild tremors with no observed long-term toxicity [169]. Since then however, there have been no further reports of trials involving salinomycin.

4.2. Clofazimine

Our group recently linked the anti-cancer properties of the anti-leprosy drug clofazimine to the inhibition of the WNT pathway. In this study, the library of FDA-approved drugs was screened in silico to identify potential antagonists of the WNT-FZD interaction. Out of the selected higher-scored potential candidates, clofazimine was one of four compounds, which demonstrated significant specific inhibition of the WNT-pathway in vitro when using the TopFlash reporter assay. Despite bioinformatics evidence, the drug was not able to inhibit the WNT-FZD interaction. Instead, it targets the WNT pathway downstream of β-catenin and can inhibit proliferation of TNBC cells [170]. Other potential mechanisms of anticancer effects of clofazimine might be an indirect stimulation of phospholipase A2, resulting in the lysophospholipid-induced apoptotic death [171], or interference of the drug with the respiratory chain [172].

Clofazimine has shown an anti-WNT effect with the IC_{50} in the low-μM range (~3 μM), which is somewhat higher than the usual plasma levels of this drug for anti-leprosy treatment (0.5–1 μM) [173]. However the drug is extremely lipophilic and therefore has a propensity to accumulate in tissues resulting in concentrations of 100–500 μM, which in this case is favourable for the antitumor therapy [170]. While investigation of anti-WNT effects in vivo is now ongoing, these data help to explain previous results of cancer inhibition shown in squamous hepatocellular carcinoma cell cultures [171], in mammary cancer in vivo [174], and in lung cancer in vitro and in vivo [172].

A phase II study has claimed benefits of clofazimine for the indications of unresectable and metastatic hepatocellular carcinoma, where 50% of the patients showed a response or disease stabilization [175]. However, this could not be concluded for the advanced unresectable primary hepatocellular carcinoma, when treated with clofazimine in combination with doxorubicin [176]. Altogether, these studies and the fact that clofazimine is generally considered a well-tolerated and safe drug (its common side-effects include skin discoloration and rashes, palpitations and enteropathies [175,177]) are encouraging for the future repositioning of clofazimine as an anticancer drug directed against highly WNT-dependent tumors such as TNBC. A future challenge will be managing and discovering the effect of clofazimine, when used at high doses for long-term oncology therapy.

4.3. Other Antimicrobials

Salinomycin and clofazimine are not the only antimicrobial drugs in the spotlight for repositioning against WNT-dependent cancers. In this section we review three other compounds approved for human use, which do not benefit from extensive records in scientific literature but have shown promise for targeting the WNT pathway.

Tigecyclin, a tetracycline derivative, inhibits human cervical cancer cell growth in vitro and in vitro, especially when combined with the well-known chemotherapeutic paclitaxel. It decreases both cytoplasmic and nuclear levels of β-catenin and decreases transcription of the WNT-target genes, while increasing the levels of AXIN1 [178].

The antitumor antibiotic streptonigrin was in anticancer trials until 1977 but was discontinued as the toxic effects outweighed therapeutic benefits. The drug's original mechanism of action was mostly due to the induction of DNA damage [179,180]. It has recently been demonstrated that the anti-neoplastic effect might also be achieved through the inhibitory effects of streptonigrin on the β-catenin/TCF complex formation with DNA. However, it seems that this drug has additional targets since suppression of GSK3β phosphorylation and decrease in β-catenin were also observed [181].

Hexachlorophene, a disinfectant previously used as a bacteriostatic skin cleanser, has demonstrated WNT/β-catenin pathway inhibition by promoting degradation of β-catenin through the ubiquitin ligase SIAH1 in colon cancer cells and EBV-infected B-lymphoma, as well as inhibition of cell proliferation in colon cancer cells [182,183].

5. Additional Selected Compounds

5.1. Metformin

Metformin was originally developed as an antidiabetic drug, stimulating the adenosine monophosphate activated protein kinase (AMPK). It was approved by the FDA in 1995. The anticancer effects of metformin have been demonstrated by population-based retrospective studies that reported a decrease in the cancer incidence and a better cancer prognostic outcome in diabetic patients diagnosed with cancer treated with metformin, in comparison to diabetics diagnosed with cancer while not treated with metformin [184,185].

A recent study revealed that anti-proliferative actions of metformin are also associated with the indirect inhibition of the WNT pathway. Surprisingly, its effects are mediated through its original target—AMPK, which then employs the MTOR signaling pathway to promote the ubiquitination and proteasomal degradation of DVL3, one of the principal WNT transducers [186]. This is very encouraging as it means that the drug can be used at its normal dose to exert its anti-WNT effects, and indeed the doses of metformin reported in the study corresponded to those found for AMPK activation in human tissues [187]. However, AMPK is a multi-faceted target, acting not only through the MTOR pathway, but also involved in regulation of the mRNA translation machinery [188]. In addition, metformin's activities may involve perturbations of tumor metabolism and may be mediated by immunomodulatory mechanisms, sustaining the anticancer immune response [189]. Overall, the anti-proliferative action of metformin in cancer cells has been shown in vitro against lung, pancreatic and gastric cancers [190–192] and both in cell lines in and in preclinical models of hepatocellular carcinoma and in ovarian CSCs [193,194].

As the discovery of the anticancer effect of metformin in 2013 was based on clinical data from more than 5000 breast cancer patients (1013 out of them were taking metformin), the results are essentially equivalent to those of a large-scale Phase III clinical trial. This, in combination with no need to significantly escalate the dose or change the delivery route of the drug, expectedly sparked immediate attention of clinicians to metformin. There are 55 clinical trials that have been launched since then, testing the anticancer activity of metformin against a large diversity of cancers in various phases with different endpoints. Any definitive results from these trials should be expected in a few years from now and for details one may consider this excellent review [188].

5.2. Imatinib

Imatinib, known under the trade names of Gleevec/Glivec, is a tyrosine kinase inhibitor targeting BCR/ABL, which is the primary target in chronic myeloid leukemia, and some receptor tyrosine kinases (PDGFR, c-KIT) important in gastrointestinal stromal tumors [195]. Its tyrosine inhibitor function has shown to also affect the WNT/β-catenin signaling in anaplastic thyroid carcinoma cells in a c-ABL dependent manner. Imatinib-treated cells have reduced transcription of the WNT target genes such as cyclin D1. Imatinib also reduced β-catenin levels, inducing its relocation from nucleus

to the plasma membrane, decreasing cell invasiveness [196]. In colon cancer cells, imatinib has shown similar effects indicating that it can be efficient against different WNT-dependent cancers [197].

5.3. Ethacrynic Acid

The WNT-inhibitory effects of the loop diuretic ethacrynic acid (EA) were first discovered in a library screen containing 960 FDA approved drugs. EA inhibited the TopFlash reporter in a dose-dependent manner and was further demonstrated by co-immunoprecipitation studies to target LEF1 and destabilize formation of its complex with β-catenin [198]. In patient-derived chronic lymphocytic leukaemia cells, EA reduced expression of the WNT-target genes such as fibronectin, cyclin D1 and LEF1 [198]. Another study has additionally shown that treatment of myeloma cells with EA results in decreased levels of β-catenin, which points toward existence of several inputs of this drug in WNT signaling inhibition. In vivo EA alone has shown excellent promise and was able to inhibit myeloma growth and prolong survival in mice more efficiently than lenalidomide, the drug currently used in patients with multiple myeloma [199,200].

In humans the maximum dose of EA when administered by intravenous injection is 100 mg/day, which results in plasma levels of around 30 μM [201]. This corresponds to the WNT inhibitory doses used in the in vitro studies [198,199]. In mice, the oral dose of 450 μg/day should result in plasma levels close to those in humans mentioned above, meaning that inhibition of the tumor growth may also be feasible in humans, however no reports of such a study currently exist.

5.4. Riluzole

Several studies reported that in a significant number of melanoma cases the WNT ligand responsible for the invasiveness and metastasis is non-canonical WNT5A which is known to suppress canonical signaling and function through other branches of the WNT signaling [202]. This makes these subtypes of melanoma the only known case of cancer which does not benefit from the elevated levels of canonical WNT signaling. On the contrary, elevated β-catenin levels in corresponding models of the disease have been associated with reduced cell proliferation and improved patient survival, which are the result of induction of cell differentiation [203]. This prompted the screening aimed at finding WNT pathway enhancers, in which the FDA-approved riluzole, a therapeutic for amyotrophic lateral sclerosis, was identified. Further testing of riluzole on melanoma cells in vitro showed that it is indeed able to enhance the ability of WNT3a to inhibit cell proliferation and promote pigmentation. In vivo riluzole was able to decrease metastases formation in mouse models. The authors further identified the glutamate receptor GRM1, a known indirect target of riluzole, to be a regulator of WNT/β-catenin signaling, linking inhibition of GRM1 by the drug to enhancement of the WNT pathway [204]. Patients with GRM1-positive metastatic melanoma were enrolled in a "Phase 0" clinical trial, preliminarily assessing the effects of treatment with riluzole. The study has shown positive dynamics both in regard of pathological responses and biomarkers (pERK and pAKT), favouring further studies in this direction [205]. It should be noted that repositioning of positive WNT modulators is not only attractive against melanoma, but can be extended into the fields of regenerative and anti-ageing medicine where the WNT pathway is in charge of tissue renewal and may be employed to achieve better outcomes [26,206].

6. Challenges and Future Directions for Repositioning WNT Inhibitors in Cancers

As we may conclude, search of the WNT pathway inhibitors among the existing drugs is an idea which excites many minds in the broadly defined field of translational research. Many of them are attracted by the fast-tracking of the results into the clinic, as well as by the usual sheer availability of the mass-produced drug compounds and information on their various aspects such as solubility, metabolic stability and toxicity. This work has already resulted in a considerable amount of promising results reviewed here (Table 1 and Figure 1). However, use of the approved drugs is a double-edged sword, and here we would like to discuss some of the emerging challenges and problems of this approach.

Table 1. List of drugs known to modulate the WNT pathway in cancer.

Drug Category	Drug Name	Mode(-s) of Action of WNT Inhibition	Outcome in Vitro	Outcome in Animal Models	Clinical Results
NSAID	Sulindac	• PGE_2/COX-dependent • Direct binding to DVL3 and likely inhibition of interaction with FZDs • Transcriptional suppression as a consequence of direct cGMP PDE inhibition	Inhibition of proliferation in breast, lung and colon cancer cell lines	Reduction in tumor growth and metastasis in colon (xenograft and chemically-induced and intestine) while decreasing β-catenin levels	Reduction in β-catenin nuclear staining of adenomas in familial adenomatous polyposis (FAP) patients treated for 6 months
	Aspirin	• PGE_2/COX-dependent • Inactivation of PP2A and phosphorylation of β-catenin • Cross-talk with other aspirin-affected pathways (e.g., NF-κB)	Proliferation inhibition in virtually any WNT-dependent cancer	Decreased tumor formation in FAP murine model with concomitant decrease in tumor β-catenin levels	Retrospective studies, especially for colon cancer prevention Multiple trials for combination therapy and chemoprevention Recommended for CRC prevention in people between 50–69 years old
	Indomethacin	• PGE_2/COX-dependent • β-catenin degradation through transcription inhibition • Disruption of β-catenin/TCF4 complex	Inhibition of growth in colorectal cancer cell lines	Reduced tumor burden in chemically induced colon cancer; reduced β-catenin nuclear staining	No data available yet
	Celecoxib	• PGE_2/COX-dependent • Promotion of TCF1 and TCF4 proteasomal degradation • c-Met/AKT pathway cross-talk promoting GSK3β phosphorylation	Impaired proliferation in colorectal cancer, hepatoma, osteosarcoma, glioblastoma and prostate cells lines; Reduction of $CD133^+$ colon cancer stem cells; sensitization of imatinib-resistant leukaemia cells	Inhibition of β-catenin-positive premalignant lesions in the mice colon and in rat colon cancer model Prevention of lung cancer metastasis in mice Suppression of mammary carcinoma and Lewis lung tumor	Reduction of polyps in FAP patients after 6 months of treatment FDA approval for the prevention of cancer in FAP patients retracted due to lacking proof of clinical benefit
Antiparasitics	Niclosamide	• Promotion of FZD_1 endocytosis • DVL2 downregulation • LRP6 degradation	WNT pathway inhibition is associated with reduction of cell numbers in osteosarcoma, colorectal, breast and lung cancer cell lines; also effective against hepatoma, glioblastoma, andrenocortical and ovarian cancers	Lowers β-catenin levels in mice models of colorectal and basal-like breast cancers	No data available yet
	Suramin	• Inhibition of target gene expression via unidentified downstream target • Inhibition of WNT endocytosis through direct inhibition of heterotrimeric G proteins	Tested and found effective against virtually all WNT-dependent in vitro cancer models	Extensive record of in vivo studies involving WNT-dependent cancers	Enrolled in multiple trials; mildly effective or ineffective in a combination therapy against breast cancer; reported multiple toxicities when used in doses comparable to WNT-inhibitory ones
	Pyrvinium pamoate	• Direct CK1α activation • Pygopus inhibition • Direct activation of GSK3β	Efficient against colon cancer	Inhibits tumor growth in colon cancer model	No data available yet
	Ivermectin	• Deactivation of β-catenin by reduced C-terminal phosphorylation through overactivation of PP2A and PP1 phosphatases	Anti-proliferative for colon (including stem cells) and lung cancers	Reduction of tumor growth in the xenograft models of the colon cancer with reduced WNT markers levels in the tumors	No data available yet

Table 1. *Cont.*

Drug Category	Drug Name	Mode(-s) of Action of WNT Inhibition	Outcome in Vitro	Outcome in Animal Models	Clinical Results
Antimicrobials	Salinomycin	• Inhibits LRP6 phosphorylation and induces its degradation • Activation of FOXO3, leading to interrupted β-catenin/TCF interactions • Likely inactivation of canonical WNT pathway by increasing Ca^{2+} levels	Reduction of cancer stem cells in osteosarcoma and breast and endometrial cancers. Anti-proliferative for many WNT-dependent cancer cell lines, e.g., hepatocellular carcinoma, CLL, pancreatic, nasopharyngeal, breast and prostate cancers.	Inhibition of growth of gastric tumors, osteosarcoma, hepatocellular carcinoma and nasopharyngeal carcinoma with signatures of WNT signaling deficiency (reduction of LRP-6 and β-catenin; decreased GSK3β phosphorylation)	Clinical uncontrolled pilot study on several cases with metastatic cancers with positive dynamics such as metastasis regression observed. Minor acute toxicity reported (tachycardia and mild tremors)
	Clofazimine	• Exact mechanism is unknown; is likely involved in inhibition of transcription complex	Growth inhibition of squamous hepatocellular carcinoma and lung cancer	Growth inhibition of lung and mammary cancer growth	Several combination and monotherapy studies on hepatocellular carcinoma with mild positive results.
	Tigecyclin	• Decrease in β-catenin protein • Increase in AXIN1	Cervical cancer cell growth inhibition	Cervical cancer xenografts growth inhibition	No data available yet
	Streptonigrin	• Direct inhibition of β-catenin/TCF binding to DNA • Suppression of GSK3β phosphorylation	Growth inhibition of β-catenin-dependent colorectal and gastric cancer cell lines	No data available yet	No data available yet
	Hexachlorophene	• SIAH1 mediated degradation of β-catenin	Inhibition of colon cancer and B lymphoma cells growth	No data available yet	No data available yet
Others	Metformin	• AMPK-induced proteasomal degradation of DVL3 through MTOR crosstalk	Anti-proliferative in lung, pancreatic, gastric cancer, hepatoma and ovarian cancers	Inhibit tumor growth in hepatocellular carcinoma and ovarian xenografts	Retrospective study of more than 5000 breast cancer patients showing clear survival benefits 55 trial launched, no conclusive data yet
	Imatinib	• Reduction of β-catenin and WNT-pathway target genes • Relocation of β-catenin to plasma membrane	Anti-proliferative in thyroid carcinoma cells and colon cancer	No in vivo WNT effects were reported yet	Approved for use for multiple cancers
	Ethacrynic acid	• Inhibition of LEF1/β-catenin complex formation • β-catenin reduction	Anti-proliferative in CLL and myeloma cells	Reduced tumor growth for myeloma in mice	No data available yet
	Riluzole	• Inhibition of the pathway through target receptor GRM1	Induces melanoma cells differentiation and reduces proliferation	Inhibits metastases	A pilot study assessed safety and efficacy of the compound through biomarkers (pERK and pAKT).

Challenges of the first type are not unique for the WNT signaling but instead concern any attempts to reposition an existing drug for a new purpose. These obstacles of general nature are as follows (also see reviews [29,207]):

- Frequently, for a novel application the drug is required at a higher dose, for an extended treatment period or with a different formulation as compared to the conventional indication in order to demonstrate a significant effect. This may result in unexpected side effects, jeopardizing the idea of the "fast tracking" of the compound due to necessity of a full-scale preclinical and clinical investigation.
- Intellectual property difficulties due to multitude of patents.
- Drug-drug incompatibility: acceptable levels of adverse effects for one application might make the compound useless or uncompetitive for another purpose, as well as incompatible with other treatments for the purpose.
- Different legal statuses of the drug in various countries, e.g., dependence on the region where the disease is widespread or on the socioeconomical status of the population.
- Multiple and controversial mechanisms of novel action, resulting from superposition of the original drug mechanism with the novel one(s).

In addition to that, there are certain challenges, which are specific for drug repositioning for targeting the WNT pathway in cancer:

- The WNT pathway is complex. Many components of the signaling are shared with other pathways, generating cross-talks of varying intensities. Therefore, it is sometimes difficult to clearly distinguish direct influence of the drug on the WNT pathway from its effects on the intersecting pathways.
- Identification of the molecular target is a complicated process, and it is frequently omitted by researchers. Out of the 16 drugs we reviewed here, only EA, suramin, sulindac, pyrvinium pamoate and indomethacin were shown to directly affect identified components of the WNT pathway. Additionally, metformin is known to affect WNT signaling via a cross-talk from its original target AMPK. Delaying the unequivocal identification of the novel molecular target makes it problematic to optimize the drug and evaluate of the scope of its anticancer applications.

It should be also noted that the WNT pathway is not exclusively employed during development or overactivated in cancer. In adults many healthy tissues rely on it for renewal and homeostasis maintenance, most notably the intestine, haematopoietic system, hair, bones and skin. Therefore one might expect adverse reactions in all these organ systems, which has indeed been observed for many WNT-targeting compounds upon attempts to push them into the clinics. The intestine seems to be the most vulnerable in this regard, causing the failure of many anti-WNT agents. As examples, XAV939 and LGK974 result in severe intestinal toxicity in mice, while OMP18RP induces abdominal pain, constipation and diarrhea in patients [208,209].

An interesting and promising direction is the modification of approved drugs for novel diseases. In general, this approach dictates the necessity of full-scale de novo trials, however it still might be considered a future path in drug development. Although the data accumulated for the parent drug cannot be used directly, they will still serve as a strong guide and facilitator in the drug development process. Moreover, frequently there are libraries of the drug derivatives used during the development of the original compound, already available for testing. Efficacy of novel derivatives may allow to overcome many problems we described above for parent molecules, such as multiplicity of mechanisms, dose elevations, and not to forget the hurdles involving the intellectual property.

Of the drugs reviewed here, only some were subjected to medicinal chemistry optimization. Derivatives of niclosamide were synthesized with better metabolic stability without compromising WNT inhibition [210]. In another study, >40 ethacrynic acid derivatives were reported, the best ones

with enhanced WNT inhibitory action were further found to inhibit growth of chronic lymphocytic leukaemia cells [211]. A salinomycin-based drug VS-507 is part of the research portfolio of Verastat, a company whose main focus is the development of anti-CSC therapies [169]. Additional noteworthy attempts to improve salinomycin aimed at reducing its toxicity [212] and improving its potency [213]. There are also reports on derivatives of NSAIDs lacking COX inhibition and showing inhibitory effects on cancer cell lines and tumors in rodent models [57,214,215]. Other derivatization attempts were aimed at overcoming the side effects of NSAIDs, producing nitric oxide releasing aspirin (NO-ASA) and phospho-sulindac, with improved potency and lower gastro-intestinal adverse reactions in mice [216,217].

7. Concluding Remarks

WNT signaling is one of the developmental pathways [216], whose reactivation in many adult tissues underlies oncogenic transformation. Although no drugs against this pathway are yet on the market nor even in advanced clinical trials, the demand for such drugs is urgent. First medications targeting the hedgehog signaling pathway—another embryogenic pathway responsible for various types of adult cancers, previously also evading drug discovery efforts, have recently been approved [26,217]. This success should inspire researchers developing the anti-WNT agents to continue their quest. Clearly, all possible drug discovery approaches (antibodies, de novo screening of synthetic small molecules, rational drug design and in silico screening, natural products, etc.) are welcome in this task [218]. Repositioning of existing drugs for the new indication of treating WNT-dependent cancers is one of such avenues. Examples discussed in this review illustrate the achievements and remaining hurdles on this path, and reflect our cautious optimism that continuation of it may eventually ensure appearance of first-in-class medicines to treat devastating diseases hijacking the WNT pathway for their progression.

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Article II

A high-throughput assay pipeline for specific targeting of frizzled GPCRs in cancer

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Abstract

Frizzleds (FZDs) are a family of GPCRs controlling key events in all branches of the developmental Wnt signaling pathway. In this capacity these receptors are mostly active prenatally and have only a limited set of functions in the human adult. Numerous cancer types and subtypes were shown to be dependent on aberrant Wnt signaling and FZDs in particular. Taken together with their GPCR properties, this makes them an attractive drug target for the development of highly specific and efficient targeted therapies against cancer. Despite that, there are few chemical or other agents described targeting FZDs, and an even smaller number bears any clinical relevance. This sparsity dictates the necessity for broader efforts in order to advance in the Wnt pathway-targeting drug discovery. The current work describes the concepts and methodology of an inexpensive high-throughput screening followed by the pipeline of secondary assays in order to identify anti-FZD agents, which will efficiently deactivate Wnt signaling in cancer cells. Specifically, we describe a process and criteria for the selection, generation and statistical validation of a stable cancer cell line based on the well-described luciferase readout (TopFlash) which is then converted into a disease-representative high-throughput-ready

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screening system. We also provide information on the follow-up test sequence and the post-screening criteria to select FZD-targeting compounds among the hits and to validate them as such. We expect that use of this pipeline will boost the research on clinically valuable Wnt-targeting anti-cancer compounds.

1 INTRODUCTION

Frizzleds (FZDs) are a subfamily of GPCRs (G protein-coupled receptors) that are part of the highly conserved Wnt pathway. They each are made up of an extracellular cysteine rich domain (CRD) responsible for ligand binding, a linker region and a seven transmembrane domain with a conserved carboxyl tail known to be involved in relaying the downstream signal. The 10 homologues of the protein (denoted as $FZD_{1–10}$) found in humans share up to 75% sequence identity and can be divided into five subgroups based on phylogenic analysis: $FZD_{1,2,7}$, $FZD_{3,6}$, $FZD_{5,8}$, $FZD_{9,10}$, and FZD_4 (Schulte, 2010). They are key to the regulation of the various branches of Wnt signaling, namely, the canonical β-catenin Wnt pathway inducing target gene transcription, the PCP (planar cell polarity) pathway involved in cytoskeleton rearrangement, and the Ca^{2+} pathway which is part of the regulation of intracellular Ca^{2+} levels. While the Wnt pathway is involved in regulating various aspects of embryonic development it is mainly silent in adults with the exception of the regulation of stem cell homeostasis. Unbalanced and abnormal activation of Wnt signaling can lead to various pathological conditions such as degenerative diseases and cancer (Nusse, 2005). The canonical pathway is the most studied branch in relation to the oncological occurrences (Giles, van Es, & Clevers, 2003). There is a consensus that in the off-state of the pathway the multiprotein Axin/APC/GSK3β/CK1 destruction complex phosphorylates cytosolic β-catenin, leading to its ubiquitination by β-transducin repeat-containing E3 ubiquitin protein ligase (β-TrCP) and subsequent proteasomal degradation (Kimelman & Xu, 2006). However, upon binding of the lipoglycoprotein Wnt to the FZD receptor and the co-receptor LRP5/6, the signaling cascade is switched on. G-proteins and dishevelled relay the signal leading to Axin's recruitment to the membrane and therefore impairing the formation of the multiprotein destruction complex and phosphorylation of β-catenin (Cliffe, Hamada, & Bienz, 2003; Egger-Adam & Katanaev, 2010; Gao & Chen, 2010). The latter therefore accumulates to the concentrations allowing its translocation to the nucleus where, together with the transcription factor TCF/LEF, it drives transcription of the more than 130 Wnt-dependent target genes involved in cell proliferation, migration and cell fate specification (Nusse, 2018; Willert & Jones, 2006).

Aberrant signaling of the Wnt pathway has been extensively linked to tumor growth and has been shown in almost 50% of human tumors (Prosperi, Luu, & Goss, 2011). Whereas in some tumors the Wnt pathway components such as β-catenin carry mutations and therefore activate the pathway constitutively, other

cancers, for example, those of the breast present an up- or downregulation of expression levels of the pathway components (Fearnhead, Britton, & Bodmer, 2001; Koval & Katanaev, 2018; Yang et al., 2011).

Our recent research has focused on triple-negative breast cancer (TNBC), a particularly aggressive subtype of breast cancer. Representing 15–20% of all BC, it is responsible for an unproportionally high amount of BC related deaths (Liedtke et al., 2008; Oakman, Viale, & Di Leo, 2010). This is due to the lack of expression of the estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (HER2), meaning that current targeted therapies such as tamoxifen and trastuzumab cannot be applied (Penault-Llorca & Viale, 2012). It is therefore imperative to find a new targeted therapy to provide the patients with an alternative to surgery, general chemotherapy and radiotherapy. Wnt signaling is a solid part of breast development and breast stem cell function in adults, but aberrant signaling is linked to all stages of TNBC, including tumor formation, progression and metastases (Yu, Verheyen, & Zeng, 2016). Research has shown that FZD_7 and the co-receptor LRP6 are overexpressed in TNBC and that FZD_7 downregulation in vitro impaired proliferation and invasion of TNBC cells due to reduced levels of nuclear β-catenin. In vivo FZD_7-targeting shRNA stopped tumor formation in mice carrying breast tumor xenografts (Yang et al., 2011).

Our own bioinformatical insights in the relevant TCGA and GTEX datasets have shown that Wnt signaling is radically dysbalanced and that it is not over- or underexpression of individual pathway components that are the cause of aberrant signaling, but rather a lack of checks and balances causing a collapse of the signaling network as a whole. Consequently, these changes lead to overexpression of Wnt target genes in TNBC compared to healthy tissue and Wnt-dependent proliferation of the cancer cells (Koval & Katanaev, 2018).

Though it might be tempting to target the downstream and nuclear proteins of the Wnt pathway, we hypothesize that targeting the upper floors of the pathway, above of the β-catenin destruction complex, especially the FZD GPCRs, is the key to successful and specific inhibition of the Wnt pathway needed for cancer proliferation. Indeed, differences in the expression of FZD combinations at the cell membrane allow, knowing the cellular context, to differentiate between cancerous and non-cancerous tissues (Carron et al., 2003; Driehuis & Clevers, 2017). Therefore targeting downstream, where the signaling converges bear higher risk for inducing adverse effects, damaging tissues where Wnt signaling is needed for tissue regeneration and neuronal functions (Budnik & Salinas, 2011; Whyte, Smith, & Helms, 2012). Furthermore, even though the FZD/β-catenin/TCF branch is often the main focus in the field of oncology, the components of the PCP branch and activation of the Ca^{2+} branch also actively contribute to cancer proliferation, migration and metastasis (Jessen, 2009; Wang, 2009). Targeting specific FZDs or combinations thereof would allow to inhibit not only the canonical branch of the signaling but will also affect the two others, resulting in a specific shut down at the

origin of the cancer-promoting signals and theoretically leaving the physiological signaling intact.

Reinforcing our strategy of targeting the upper vs the lower pathway components, FZDs have the advantage of being part of the GPCR superfamily (Schulte, 2010). GPCRs represent the largest family of membrane receptors and are known to be excellent drug targets. Indeed, GPCRs make up to 12% of all proteins currently targeted by approved drugs, making it the largest drug target group ahead of voltage-gated ion channels (10%), kinases (9%) and ligand-gated ion channels (6%). More impressively, it is estimated that around 700 approved drugs, making up to 35% of the total drug market, target GPCRs (Sriram & Insel, 2018).

So far only few have attempted to develop FZD modulating agents such as small molecule inhibitors (SRI37892), antibody-based inhibitors (Vantictumab), interfering peptides (Foxy-5) and to date, no FZD inhibitor has been approved for commercial use (Canesin et al., 2017; Gurney et al., 2012; Zhang, Lu, Ananthan, Suto, & Li, 2017). There is therefore an urgent need to develop drugs targeting FZDs to combat Wnt-dependent cancers. The pharmaceutical industry, however, has met the potential high druggability of FZDs with caution: due to the 10 receptors and 19 ligands involved and their inter-promiscuity, in addition to receptor homo- and heterodimerization and various FZD co-receptor combinations, there is a lack of complete understanding of Wnt signaling (Dijksterhuis et al., 2015; Dijksterhuis, Petersen, & Schulte, 2014; MacDonald & He, 2012; Nile, Mukund, Stanger, Wang, & Hannoush, 2017). In addition to this, different cancers vary in their FZD subset expression meaning that drug screening assays need to be specifically tailored to each cancer type and subtype and there is not a "one-fit-all" solution (Ueno, Hirata, Hinoda, & Dahiya, 2013). Further, basic technical difficulties still need to be overcome. As an example, many cell lines strongly express multiple FZD paralogs, several of which will respond in parallel to a provided Wnt ligand. This makes identification of the specific FZD-targeting agents—much needed for the anti-Wnt pathway drug discovery, as described above—more difficult (Fig. 1).

Confronted with these difficulties, we have developed and established a drug screening and development pipeline, which is at the same time high-throughput, robust and relatively low-cost. This pipeline is based on a well-known luciferase transcription-based readout assay (TopFlash assay) tailored to specific cancer cell lines, a set of secondary assays determining the level of action of the hits within the pathway, and finally the GTP-binding assay developed in our lab to unequivocally prove that the compounds act on FZD proteins. This pipeline has already permitted us to develop promising new drug candidates against TNBC (pending patent applications; manuscripts in preparation). Along these lines, focusing on a TNBC cell line for the primary assay proved to be the key to pick up TNBC-specific anti-Wnt hit molecules from the very beginning—an approach that could be replicated for other cancer types. Below, we detail the methodology for the setting up and validation of the cancer subtype-specific primary assay, as well as for the secondary assays. We hope this will help other scientists to customize the pipeline for their specific needs and will contribute to the progress in the development of Wnt-modulating agents of high clinical potential.

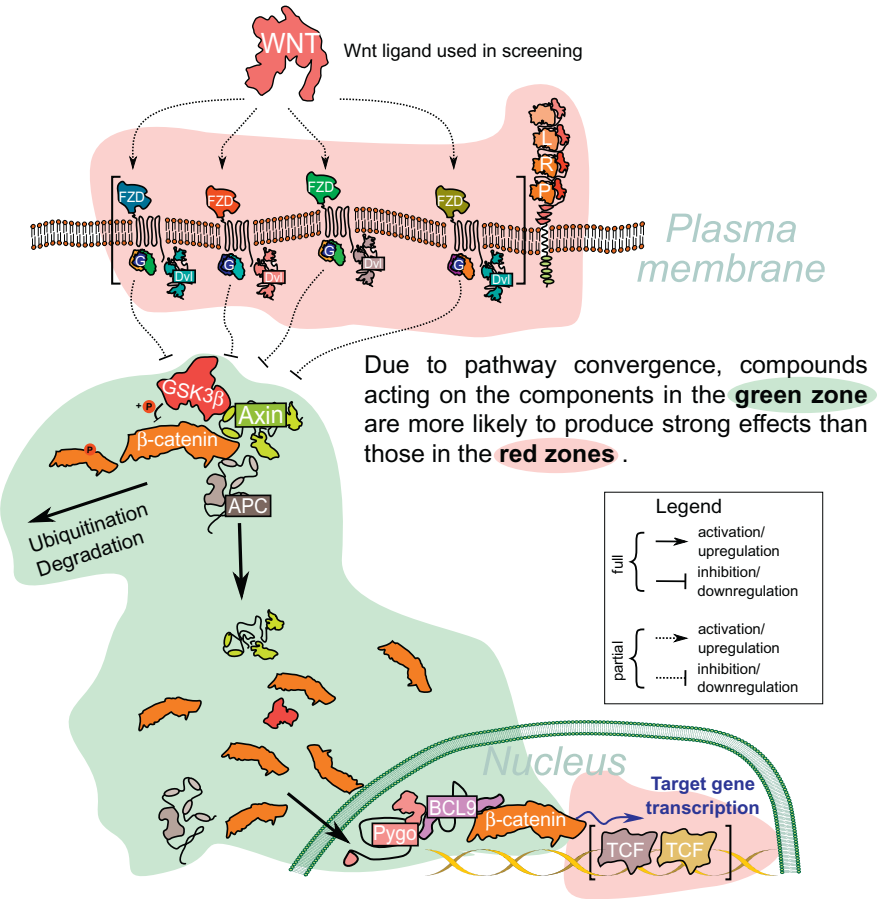

FIG. 1

Pathway branching occurs at the level of Wnt and FZD proteins and, to a lesser degree, at the levels of the TCF/LEF receptors. This is due to the expression of multiple paralogs of the proteins, which frequently transfer the signaling in parallel. Thus inhibitors acting specifically on one or several (but not all) paralogs are unlikely to produce strong inhibition and thus are likely to either be omitted from the hit list or receive a low priority as compared to the hits acting at the unique components responsible for transfer of 100% of signal. Ways to overcome this limitation are discussed in the text.

2 MATERIALS

2.1. Lysis + Luciferase buffer: (25 mM Gly-Gly, 4 mM EGTA, 0.3% Triton X-100, 5 mM $MgSO_4$, 100 μM Luciferin, 1.5 mM ATP, 4 mM DTT)

2.2. Purified Wnt ligand of interest or Wnt-conditioned medium (Koval & Katanaev, 2011; Nusse, 2018; Willert et al., 2003)

2.3. White tissue culture treated plates (e.g., Greiner cat. #655083)

2.4. Transfection reagent and transfection-grade plasmids

3 METHODS

3.1 SELECTION OF THE CELL LINE FOR HIGH-THROUGHPUT SCREENING

In order to target the FZD proteins specific for the particular cancer type or subtype using the TopFlash assay, one needs to use a cell line model. For most of the cancers, more than one model line exists and it is important to use this redundancy for the selection of the cell line which will provide the best quality for the screening and hit compound determination. We can therefore highlight two main criteria for such a cell line:

- mean response and Z' factor in the TopFlash assay;
- representativeness of the Wnt signaling pathway organization and expression landscape.

We thus propose the following pipeline for the selection of the cell line for screening (this protocol assumes use of 96- or 384-well plates, depending on technical feasibility and equipment of the lab). Since an observed response in the TopFlash assay as well as the stability and reproducibility of the signal are difficult to predict, the best way is to test them empirically in order to obtain the most sensitive line for screening. As we have already mentioned, the sensitivity (i.e., ability to identify even weak hits) is a cornerstone of the line which is used to identify FZD-targeting molecules, since the hit compounds targeting FZDs are likely to produce weak inhibitory responses (Fig. 1).

3.1.1. For each candidate cell line, optimize the transfection protocol (seeding density, time, reagent:DNA ratio and respective concentrations) which should ensure at least 70–80% of transfection efficiency to provide an adequate comparison to the future stable line for screening.

3.1.2. Seed and perform the transient transfection with the TopFlash vector (Addgene #12456); each of the cell lines should have at least 6–12 replicates for non-stimulated cells and the same number for each Wnt ligand you intend to use in the screening.

Optional: the specificity of the signal may be verified by introducing additional points with addition of some known Wnt inhibitor (such as the inexpensive, stable and readily available Suramin, Koval, Ahmed, & Katanaev, 2016); additionally, downstream stimulation of the pathway using an GSK3β inhibitor (such as LiCl or CHIR99021) may be used as a reference since it frequently provides maximal levels of the signal.

3.1.3. The day after transfection, the ligands should be supplied. At this stage, Wnt ligands should be used at the concentration, which produces the highest response. Typically, this would be 1–2 μg/mL in case of purified Wnt proteins or will require use of non-diluted Wnt conditioned medium.

3.1.4. After 18–24 h the firefly luciferase measurement should be performed using the lysis + luciferase buffer. The medium should be completely removed from the wells and replaced with the lysis + luciferase buffer (50 µL for 96-well plates and 30 µL for 384-well plates).

Optional: if the cells used are difficult to lyse, prior addition of a lysis solution containing 1% Triton X-100 (Dyer, Ferrer, Klinedinst, & Rodriguez, 2000) may be considered.

3.1.5. Using the values obtained in this assay, the Z' factor (Zhang, Chung, & Oldenburg, 1999) for each Wnt ligand and each cell line used in the experiment should be calculated as follows:

$$Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}, \quad (1)$$

where σ_{c+} and σ_{c-} are standard deviations of positive and negative controls, μ_{c+} and μ_{c-} their corresponding means.

3.1.6. Typically, a single cell line—Wnt ligand combination demonstrating the best Z' should be selected for further development of the stable cell line for screenings. Given the costs of HTS runs and difficulties of combining hit data from runs from two separate cell lines it is typically impractical to develop several screening systems, however, it might be always considered as an option. Additionally, note that Z' is not only defined by an actual mean response levels, but also by the standard deviation of the signal: therefore, a better Z' may be achieved with the less responsive lines (Fig. 2A).

3.1.7. If several lines show a similar Z' factor, an additional selection criteria might be their fit to the patient gene expression data. Such analysis is easily available using RNA-seq data available for both cell lines and for patients and any software (such as R) which can perform Spearman correlation analysis (Fig. 2B).

3.2 GENERATION OF THE SCREENING-READY STABLE CELL LINE (FIG. 3)

The process of selection for such a line is reminiscent but nevertheless significantly different from the typical protocol for other types of the stable cell lines, since it employs clonal variability in order to generate the line with the best statistical characteristics.

3.2.1 For the selected cell line, the antibiotic resistance-bearing plasmid should be chosen. It can be a dedicated plasmid, such as pPur (Clontech, cat. #631601) or an empty vector with an antibiotic cassette, such as pcDNA3.1. For the selected antibiotic, the dose-dependent survival curve should be made and the lowest concentration producing 100% mortality identified.

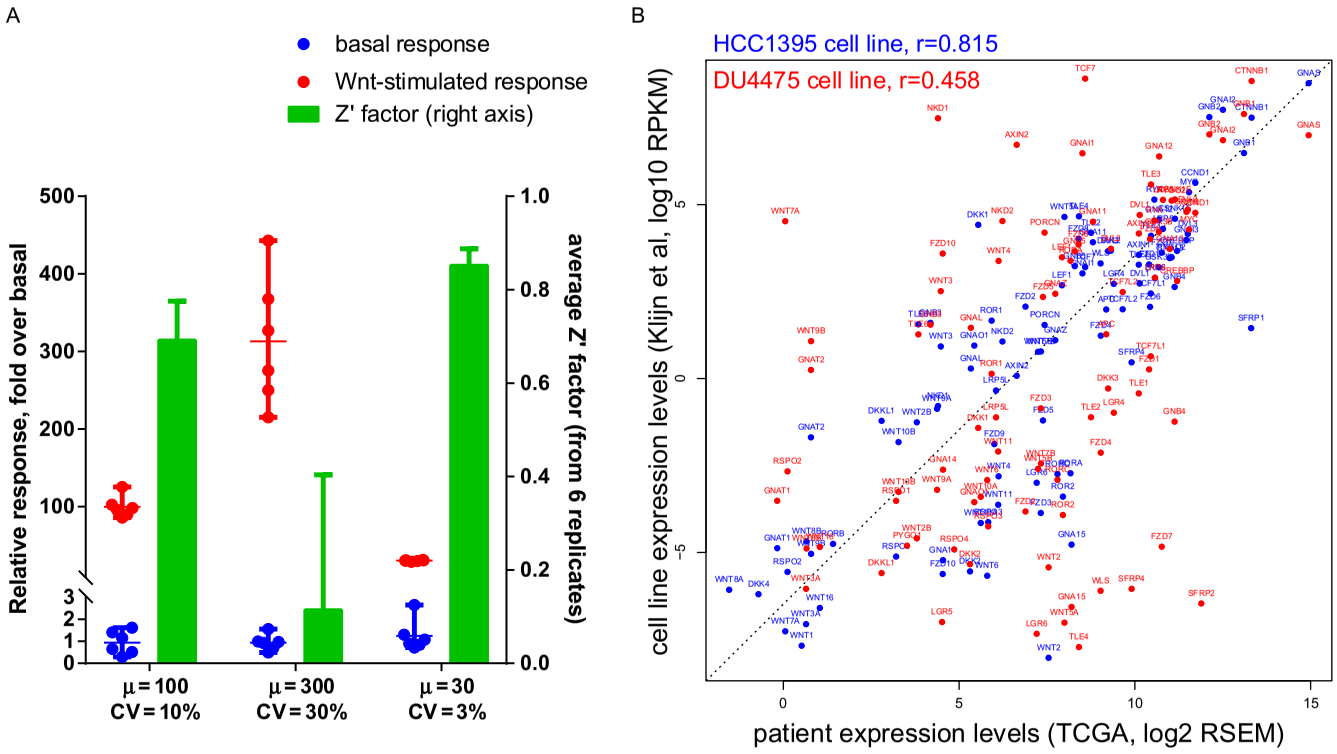

FIG. 2

(A) Dependency of Z' factor on the mean response and variability. Three examples of cell lines with different normalized mean Wnt-mediated responses (μ) and CVs (coefficient of variation or relative standard deviation, σ/μ) were modeled and are shown. In all cases, the variability of the basal response remained constant (CV = 50%). The mean Z' identified from six-point validation of such models is presented as green bars. The figure demonstrates that the input of the assay variance into the Z' factor and thus the suitability of the assay for HTS is much more significant than that of the mean difference between negative and positive control. Moreover, for assay lines with high variance, the Z' factor must be calculated from more repeats in order to have a precise estimate. (B) Correlation between gene expression levels of the Wnt pathway (set of 101 genes, see Koval and Katanaev (2018)) in the primary tumors from patients (data are obtained from TCGA, X axis) and the TNBC cell lines HCC1395 and DU4475 (using data provided by Klijn et al. (2015), Y axis). Higher value of the Spearman correlation coefficient (top left corner) demonstrates that HCC1395 is a significantly more representative model of the Wnt signaling organization in patients' tumors.

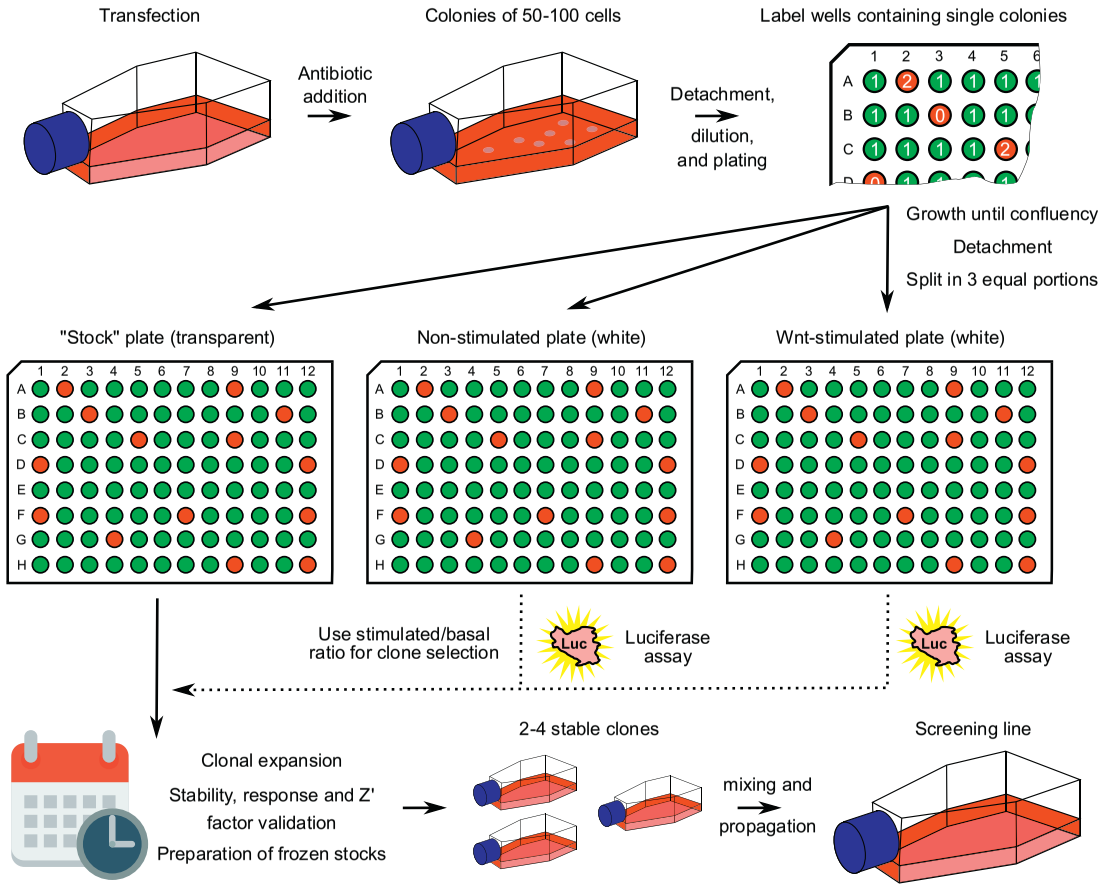

FIG. 3

Scheme of the TopFlash-based screening pipeline development as described in Section 3.2.

3.2.2 Seed the selected cell line in one well of a six-well plate or in a T-25 flask and perform a co-transfection as in Section 3.1 using a 20:1 mixture of the TopFlash plasmid with the resistance-bearing plasmid of choice.

3.2.3 2–3 days after transfection, the antibiotic should be added at the lowest lethal concentration (as identified in Step 1). The medium should be changed with fresh media containing a new portion of antibiotic every 2–3 days.

3.2.4 The selection rate and outcome are highly individual for each cell line. Typically, the selection of the stably transformed lines is successful when most of the cells are dead and few colonies of the stable transformants are observed. At this stage, transparent 96- or 384-well plates should be prepared with a total number of wells corresponding to the number of such colonies (e.g., if there are 100 colonies, one 96-well plate should be used). When the colonies are 50–100 cells large, the T-25 flask should be trypsinized and the cells resuspended in a total of 1 mL of the medium. From this, 20 μL are taken and diluted in a suitable distribution volume of the medium without the selection antibiotic (e.g., 10 mL for one 96-well plate, if 100 μL of the medium should be distributed per well). The suspension is then distributed in the plates and will produce on average one cell per well.

3.2.5 In about 1–2 weeks, depending on the growth rate, the cells will form visible colonies again. At this stage, the wells containing two and more colonies should be labeled to avoid their use in the future. When the colonies are 400–500 cells large, they should be detached by trypsin, resuspended in the wells and put in the fresh medium for attachment to eventually form an even monolayer.

3.2.6 When most of the clones have reached 100% confluency in their wells, the medium should be removed, the clones washed with 100 μL/well PBS and then trypsinized using 10 μL/well Trypsin–EDTA solution. Each well is supplemented with an additional 80 μL/well of the culture medium, the cells are thoroughly detached and resuspended. The volume is then split into three equal parts (30 μL each): one part remains in the original plate for propagation, and the two other parts are transferred to the corresponding wells of two new white tissue culture plates for the use in the TopFlash assay as described in Section 3.1. After the transfer, the total volume of the medium in each well is brought to 100 μL by adding 70 μL of fresh medium.

3.2.7 After attachment, the cells in one of the "copied" white plates are used for the basal signal detection (without Wnt stimulation), while the other white plate should be stimulated by the Wnt ligand at the highest concentration. Next day, the firefly luciferase signal should be measured in both plates. Using the ratio of the stimulated: basal signals in the corresponding wells, clones with the highest relative response should be selected (optimally 10–20). Only clones derived from the single cell (see Step 5) should be considered.

3.2.8 Selected clones should first be scaled-up step-wise until they fill at least one well of a six-well plate. At this stage, each clone should be thoroughly validated: the cells are counted and seeded at the same density in the white tissue culture plates (typically, 10,000–15,000/well for a

96-well plate or 2000–3000/well for a 384-well plate) to provide 5–10 replicates for both the basal and the Wnt-stimulated value. After luciferase activity measurements, the mean relative response and the Z' factors should be determined for each clone. At this stage, some clones will highly likely lose their responsiveness entirely or show an unacceptable Z' (<0.5) and should be discarded. For others, frozen stocks should be established.

3.2.9 The measurement as described in Step 8 should be repeated weekly until at least passage 30. The trend of the relative TopFlash response and the Z' factor should be built, and the clones demonstrating a sustainable trend for a decrease in these characteristics should be discarded. Overall, only the clones showing the stable or at worst slightly decreasing response and Z' until passage 60 should be selected.

3.2.10 Since the monoclonal lines might have idiosyncrasies and be poorly representative of the parental line, 2–4 clones with the best response and Z' factor should be mixed to obtain the final cell line for screening. For this, the frozen stocks of the early passages established at Step 8 should be used. They should be mixed at equal ratios and expanded for at least five passages before freezing.

Optional: if desired, the final cell line might be validated for loss or decrease of response upon knockout or knockdown of the FZD proteins of interest. However, this process might be lengthy and troubled by the lethality caused by such manipulations.

3.3 PRIMARY SCREENING OF THE LIBRARIES

Due to the extreme diversity of the available equipment and the scale of screenings in laboratories, we will, in this section, provide generic guidelines in order to better "tailor" your screen and the treatment of the results for identification of the compounds targeting FZD GPCRs.

3.3.1. The Z' factor of the assay should be determined and optimized using the exact technical conditions of the screening. The factors mostly influencing the Z' value in these cases are the pipetting volumes and the cell density—therefore, test plates should be used in order to test ranges of these variables and their influences on the ultimate Z' values. Finally, the assay should also be analyzed for repeatability using the Bland–Altman method (Bland & Altman, 1986) (Fig. 4A) and the repeatability coefficient RC:

$$RC = 1.96\sigma_{\text{diff}} = 1.96\sqrt{\frac{\sum|r_1 - r_2|^2}{n}},$$

where σ_{diff} is a standard deviation of the difference between the repeats (r_1 and r_2).

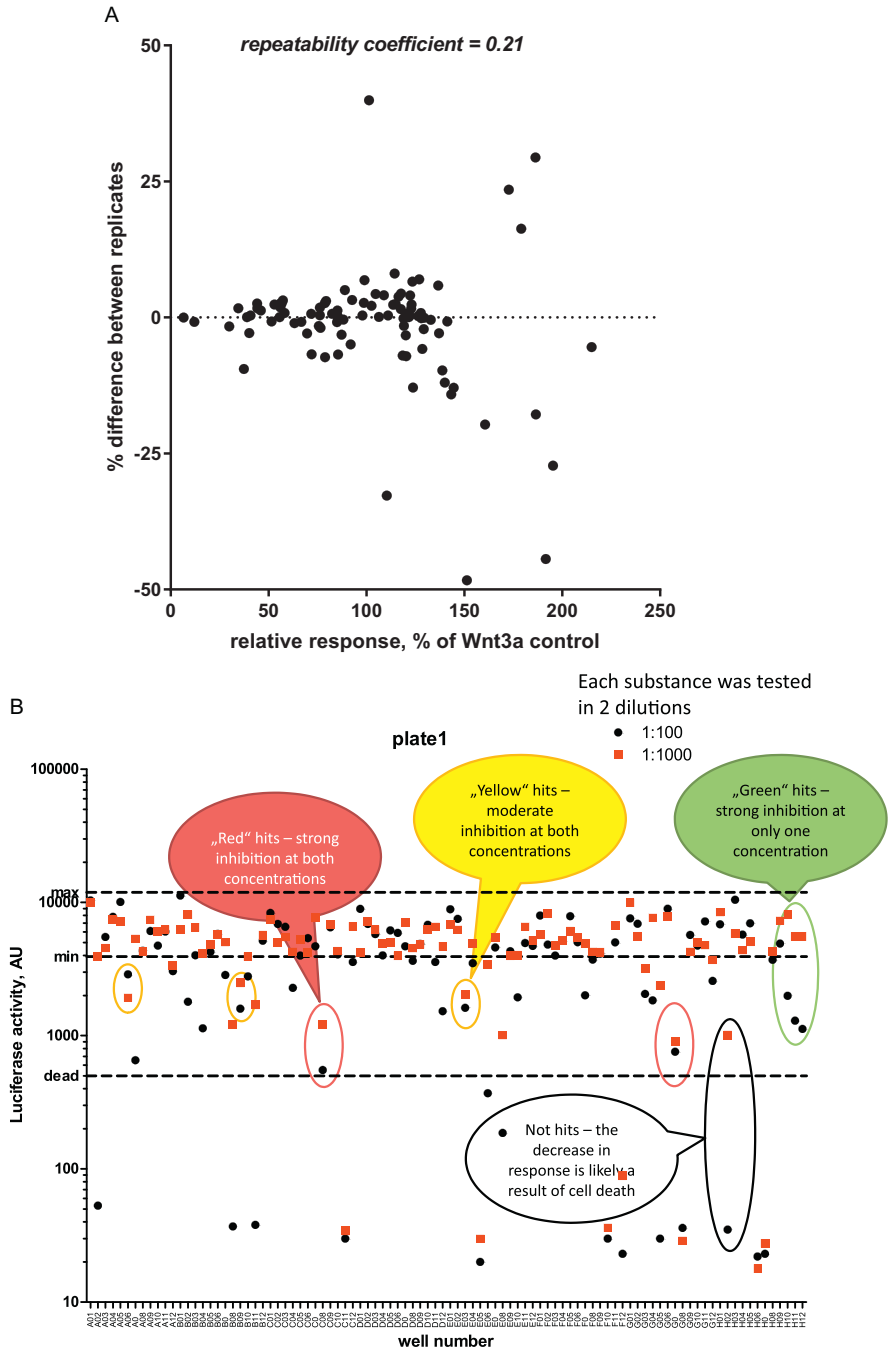

FIG. 4

See legend on opposite page.

3.3.2. Typically, large screens are performed in single repeats to decrease the running costs. However, for small screens a better approach would be the use of the compounds at different dilutions (usually, 10 and 1 μM, see Fig. 4B), which allows to immediately prioritize the primary hits by their prospective potency. Additionally, for the small screenings, the simultaneous transient transfection of constitutively produced *Renilla* luciferase for the dual-luciferase assay can be considered to eliminate the toxic compounds at this first stage.

3.3.3. The FZD proteins are the first to interact with the ligands and transduce the signal downstream. Therefore, to avoid omission of those hits whose kinetics of interaction with FZD may be relatively slow, the compounds must be pre-incubated with the cells for $\geq$1 h before addition of the Wnt ligand.

3.3.4. As illustrated in Fig. 1, the compounds targeting FZD receptors, especially the compounds with maximal isoform specificity (and thus maximal value for subsequent drug development), are not expected to reduce the signal very efficiently due to the presence of multiple FZD isoforms on the cell surface. Indirectly, this issue has already been considered at stage 3.2 (stable cell line selection), since the assay with high Z' factor will reliably permit identification of partial antagonists in addition to full-scale inhibitors. The following factors will further enable identification of FZD-targeting compounds:

- Usage of sub-saturating concentrations of a Wnt ligand will minimize the contribution from less efficient/less expressed FZDs into the overall TopFlash signal. It also reduces the cost of the screening.
- Usage of a chemical analysis software (such as JChem and Instant JChem from ChemAxon, http://www.chemaxon.com/) will permit, based on the structure similarity, to include into further consideration even the less efficient hit compounds. In this case, a sustained/generalized inhibitory effect is expected from groups of chemically similar compounds.

FIG. 4

(A) Bland–Altman plot of the assay repeatability (Bland & Altman, 1986). In order to build the plot, one should plot the actual or normalized values of one repeat on the *X* axis against the difference between the repeats on the *Y* axis. The shape of the plot shows that, as it is very typical for luciferase-based assays, the variability of the assay is increasing with the signal strength. Using these data, repeatability coefficient can be calculated as described in the text. (B) The graph illustrates an alternative approach, which can be used in small-scale screenings: each compound can be tested in two different dilutions allowing quick hit prioritization. Additionally, the graph demonstrates how one can eliminate toxic compounds in case the screening cell line has measurable basal levels of the TopFlash activity (which is typical for Wnt-dependent cancer cell lines).

3.4 FOLLOW-UP SECONDARY SCREENINGS AND ASSAYS TO SELECT FZD-TARGETING COMPOUNDS

In order to select FZD-targeting compounds from the primary hits (which typically will constitute between 0.5% and 3% of the compounds screened) we developed the following assay pipeline:

3.4.1. The screening will inevitably yield some pseudo-hits, which reduce the signal in the TopFlash assay through unspecific cell toxicity or other Wnt-unrelated mechanisms. Therefore, such false positives must be eliminated in the follow-up screen using a cell toxicity assay. In principle, one may choose any suitable assay (such as MTT or its more advanced derivatives MTS, XTT or WST; or resazurin-based protocols), but we strongly recommend to use the dual-luciferase reporter assay. In this simple and sensitive assay, non-specific inhibition is quantitatively measured by the effect of compound on the *Renilla* luciferase (constitutively expressed under the Wnt-independent CMV promoter), while simultaneous TopFlash readout will be a confirmatory secondary screen in order to eliminate stochastic false positives.

Note: some cell toxicity assays are compatible with TopFlash protocol and can be used simultaneously. In case of using an assay not allowing the concomitant TopFlash readout, there might be a necessity to perform a secondary TopFlash screen to eliminate false positives.

3.4.2. Among confirmed hit compounds, the ones targeting downstream elements of the Wnt pathway need to be eliminated. The simplest way to do this is to induce β-catenin accumulation through the direct pharmacological inhibition of GSK3β. Application of such inhibitors (LiCl or CHIR99021 as examples) will likely produce signal levels and Z' factors comparable or exceeding those of the Wnt protein-induced stimulation used in the primary screen and thus will not require an additional assay optimization step. In this scenario, the compounds capable of inhibiting Wnt-induced, but not GSK3β inhibitor-induced, pathway activation, are to be transferred to the next steps of the pipeline.

Optional: it is recommended to re-test the compounds in the Wnt-stimulated assay in parallel to application of GSK3β inhibitors to verify the reproducibility from the prior screening. Additionally, one may add the *Renilla* readout to ensure again that no toxicity is observed.

3.4.3. At the previous steps, only luciferase-based readouts were used. Therefore, it is important to next have an additional verification using a non-luciferase assay. We typically employ the β-catenin stabilization assay (Hannoush, 2008) observing either total or active β-catenin using the corresponding antibody (BD, cat. #610153 and Millipore cat. #05-665). Alternatively, β-catenin translocation to the nucleus can also be probed (Koval et al., 2014).

3.4.4. Finally, the ability of the hit compounds to disrupt FZD GPCRs-mediated signaling should be confirmed in the GTP-binding assay (Fig. 5). GTP

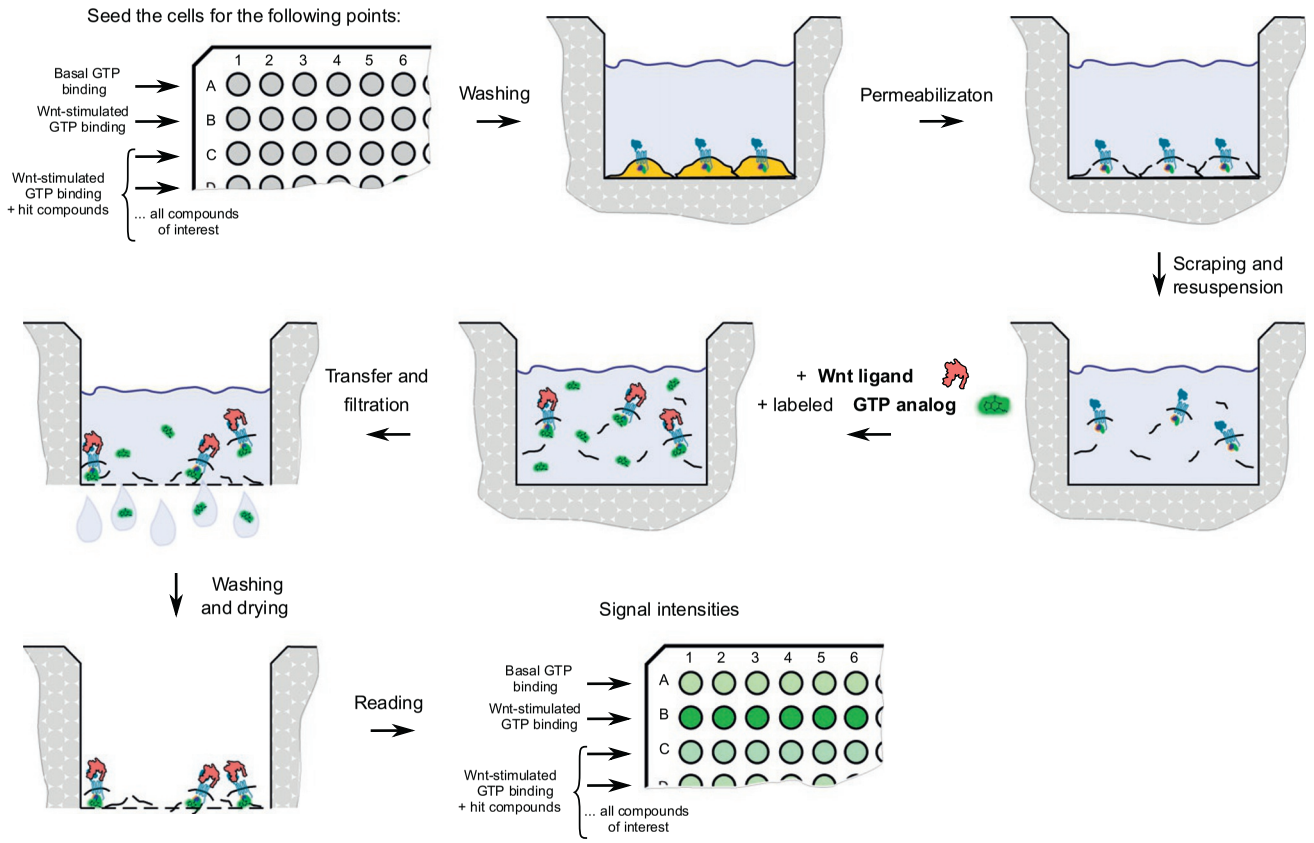

FIG. 5

Workflow and principle of the GTP-binding assay using TRF (time-resolved fluorescence) non-hydrolysable GTP analog (such as GTP-Eu).

analogs, such as Eu chelate-labeled GTP (PerkinElmer cat. #AD0167) or radioactively labeled GTPγS (e.g., PerkinElmer cat. #NEG030X) should be used for this purpose (Koval, Kopein, Purvanov, & Katanaev, 2010). If a reliable signal levels are observed, this assay can be performed without a separate membrane purification step. The protocol for the adherent cell assay in 96-well plates is as follows:

3.4.4.1. Seed the cells at 80–90% confluence in transparent tissue culture 96-well plates. For compound testing, the assay should include the following points (see Fig. 5): non-stimulated binding (in the absence of a Wnt ligand), Wnt-stimulated binding, inhibition of the Wnt binding by the compound. Each assay point should have four to six technical replicates. In some cases, it might be necessary to transfect the cells with Gα subunits in order to observe a reliable signal from endogenous FZDs (Koval & Katanaev, 2011) using your optimized transfection protocol.

3.4.4.2. The next day, the cell medium should be removed, the cells rinsed with 100 μL/well 1 × PBS and permeabilized for 10 min with 0.05% Saponin in 100 μL 1 × PBS + 5 mM $MgCl_2$.

3.4.4.3. The permeabilizing solution should be carefully removed avoiding detachment of the cell monolayer and replaced by 50 μL of fresh DMEM supplemented with 10% FCS, 5 mM $MgCl_2$, 0.05% Saponin, with or without compound of interest. The cell monolayer should be detached by tip scratching or specialized scrapers and resuspended by pipetting up and down.

3.4.4.4. A Wnt ligand or vehicle buffer should be added directly from stock to the final concentration of 1–10 μg/mL. After 5 min incubation, the GTP analog should be supplemented to the final concentration of 10 nM, and the reaction incubated for a further 30 min at RT on the orbital shaker at 200 rpm.

Notes: it might be necessary to optimize the assay conditions (Saponin concentrations (0.01–0.1%), time (between 5 min and 1 h), Wnt ligand and GTP analog concentration). Specificity of the signal might be verified using assay points with sFRP (R&D, cat. # 1169-FR) or soluble CRD domain (such as FZD5-CRD from Adipogene (cat. #AG-40B-0133)) as competitors.

3.4.4.5. The reaction mixture should be resuspended, transferred and directly filtered through GHP filter plates (e.g., Pall cat #Z722251 for TRF (time-resolved fluorescence) detection; Millipore cat. #MANPN2210 for radioactive assays). In the case of radioactive analogs, the scintillation liquid must be added for measurement according to the isotope (^{35}S) and equipment requirements (the counter must be compatible with the plates). The TRF

labels can be measured directly after filtration, though it is recommended to dry the plate at RT for 1–2 h prior to measurement since it amplifies the signal. Background signal from the wells should be measured prior to the filtration and subtracted from the signal values.

3.4.4.6. The data are typically presented as a ratio or percentage of signal (Wnt ligand with or without compound) to basal values.

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Targeting the Wnt signalling pathway in cancer: prospects and perils

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Summary

The Wnt pathway, involved in cancer development and progression, has for a long time been said to be undruggable, owing to its complexity and involvement in stem cell biology. This mindset has shifted in the last few years as new research and insights into the pathway mechanisms specific to tumour cells become apparent, leading to the development of multiple compounds targeting the pathway. In this review, we introduce the Wnt pathway and its connections to cancer biology and therapy resistance. We further dive into the details of drugs that have entered clinical trials, examining their successes and side effects. We show that these drugs all have one thing in common: in order to be successful, the drugs must target tumour specific activated sub-branches of the pathway, either at the receptor level or at the nuclear transcription level.

Keywords: *Wnt signalling, cancer, drug discovery, stem cells, proliferation, chemoresistance, radioresistance, clinical studies*

The Wnt pathway – a double-edged sword

One of the most fascinating features of multicellular organisms is the precise and tightly controlled communication among cells, necessary for the development, coordination and functioning of the individual organs and the body as a whole. To communicate, the cells employ chemical signals, which, when received by the recipient cells, trigger defined intracellular signalling pathways in order to relay the information and provide the adequate response to the external stimuli. This enables the body to coordinate patterning and organ development during embryogenesis, to keep the organism in homeostasis and to respond to external stresses and inputs, and to regenerate after injury. On the cellular level, a signalling cascade is initiated by secreted ligands (e.g., hormones, cytokines, neurotransmitters, growth factors) produced by one cell, which then bind to a receptor on another cell. The receptors in most cases are located on the cell surface, and the signal is then relayed through intracellular components of the pathway, called transducers and second messengers, resulting in the corresponding cellular effect, for example target gene transcription or changes in an enzymatic activity [1].

The Wnt pathway is one of the most important signalling cascades in the early events of embryonic development, where it controls cell proliferation and differentiation [2, 3]. To date, the Wnt signalling is still not fully understood. This is mainly because it is composed of a complicated network of a total of ten GPCR homologue Frizzled (FZD) receptors [4], three transmembrane tyrosine kinases Ryk, ROR and PTK7, muscle skeletal tyrosine kinase (MuSK) [5], the co-receptors LRP5/6 [6], and 19 glycolipoprotein Wnt ligands [7]. There is a high degree of promiscuity in the ligand-receptor interactions, although certain Wnts have higher affinities to certain FZD receptors and co-receptors [4, 8]. To further complicate matters, there are additionally secreted antagonists such as Secreted FZD-related proteins (Sfrp1, 2, 4 and 5), Wnt inhibitory factor (Wif) and Dickkopf 1 (Dkk1) reducing signalling activity, and the agonists R-spondin 1 to 4 potentiating the Wnt signalling through their receptors Lgr4, 5 and 6 [9, 10].

Wnt signalling is generally divided into three distinct branches: the canonical β-catenin/TCF pathway, the planar cell polarity (PCP) pathway and the Ca^{2+} pathway. Whereas some ligands are attributed to one distinct branch, others are competent to initiate signalling in several branches, depending on the receptor-ligand combination. It has also been demonstrated that under certain circumstances the β-catenin branch and the PCP branch antagonise each other [11].

The by far most studied branch is the canonical β-catenin/TCF pathway. It is characterised by accumulation of cytoplasmic protein β-catenin upon pathway initiation. It further translocates to the nucleus to bind the TCF family of transcription factors, leading to specific gene expression. The Wnt-dependent transcriptional programme in the nucleus – in a manner reminiscent of the complexity at the cell surface – is again controlled in a diversified manner. It has been shown that, depending on which co-activators β-catenin recruits, it will either upregulate genes responsible for self-renewal and proliferation (through, e.g., β-catenin binding to CBP) or will lead to upregulation of genes in-

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volved in differentiation (binding to p300) [12]. In the absence of Wnt ligands, a specific complex containing Axin, APC, CK1 and GSK3β phosphorylates β-catenin, targeting it for degradation. In the adult tissues, Wnt signalling is mainly silent with the exception of stem cells, where the pathway regulates replenishment and regenerative processes, for example in the intestinal crypt [13], haematopoietic stem cells [14] and bone [15]. This vital role of the Wnt pathway in maintaining tissues healthy, as one edge of the sword, stands in a sharp opposition to the other edge, which is the key role of the pathway in disease. If not held in check, aberrant Wnt signalling can lead to uncontrolled cell proliferation and cancer [2, 16].

Unlike the canonical signalling, β-catenin is not part of the PCP and Ca^{2+} pathways. The PCP pathway, involving small GTPases and JUN-N-terminal kinase, controls cell polarity, cytoskeletal remodelling, directional cell migration and c-Jun-dependent transcription. The Ca^{2+} signalling branch leads to activation of phospholipase C (PKC) followed by opening of intracellular stores of Ca^{2+}, in turn leading to activation of downstream effectors such as NFAT and CREB transcription factors, also controlling cell migration and cell survival [17]. As these two branches involve cytoskeletal changes and cell migration, it is not surprising that they have been associated with cell invasion and metastasis in cancer [18–20].

The Wnt signalling-dependent cancers can be divided into those that harbour mutations in components of the pathway, and those cancers that have a dysregulation of Wnt signalling due to epigenetically driven up- or downregulation in expression levels of the pathway components. The most famous example of pathway mutations is that of the Wnt pathway suppressor APC. It was first associated with patients with familial adenomatous polyposis (FAP) and occurs in >80% of colorectal carcinomas [21, 22].

Immunohistological analyses of tissues from colorectal patients show that β-catenin relocalisation is a typical sign of the canonical pathway activation. Indeed, the loss of membrane β-catenin (where it plays Wnt-independent functions) is significantly associated with poor prognosis when using overall survival as the endpoint, as shown in a study of 720 colorectal patient samples [23]. Additional reports have demonstrated that loss of membranous β-catenin is especially prominent in the invasive front in colorectal cancer and that membranous localisation in general and in the invasive front in particular are both prognostic markers for longer disease-free survival [24], whereas high nuclear accumulation in colorectal cancer has been associated with worse disease-free and overall survival and higher probability of developing lymph node metastasis [25, 26].

In additional to mutational activation of the Wnt signalling, the pathway can be aberrantly activated by overexpression of the pathway components, such as Wnts or their FZD receptors [27]. Analysis of tumour tissues of 201 patients with colorectal cancer showed high Wnt1 and low expression of the non-canonical Wnt5a correlating with cytoplasmic and nuclear β-catenin; all three characteristics are indicative of shortened disease-free survival. High Wnt1 and nuclear β-catenin also correlated with lower overall survival [25]. In non-small-cell lung cancer, cytoplasmic Wnt1 is also significantly upregulated and correlates with β-catenin, c-myc and cyclin D1 overexpression. Although there was no link between high Wnt1/β-catenin expression and the disease stage, high expression correlated significantly with a lower 5-year survival rate [28].

FZD expression has also been analysed in various studies (reviewed by [29]). As expected, tumour tissues show an upregulation of FZD receptor expression compared with healthy tissues. The expression is even stronger towards later stages of cancer development. For example, in gastric cancer, high FZD7 expression significantly correlates with tumour invasion, metastasis and late stage cancer. In an analysis of 5-year survival, patients with high FZD7 expression had a 30.3% survival rate (median survival 23.5 months) versus 65.4% in patients with low or no FZD7 expression (median survival 77 months) [30].

Analysis of individual Wnt pathway markers, such as select ligands and receptors, is a useful tool for clinicians to predict prognosis and for researchers to determine the molecular mechanisms behind the tumour. However, such analysis often fails to uncover the whole picture. A broader look at the cancer transcriptome of the whole pathway and its numerous target genes is more suitable in this regard, as we have recently done for breast cancer. Exhaustive analysis of the TCGA and GTex databases has revealed that it is not single gene upregulation that is responsible for the aberrant signalling across the patients, but rather epigenetic dysregulation of the whole Wnt system. Such generalised dysregulation is behind the consistent pathway overactivation leading to uncontrolled cancer cell proliferation in breast cancer patients [27]. Network correlation analysis further permitted us to highlight signalling nodes within the Wnt pathway, which emerge as new promising drug targets and biomarkers in clinical studies and personalised medicine treatments [27].

Stemness and therapy resistance in Wnt dependent cancers

Apart from its involvement in tumourigenesis and cell proliferation, the Wnt pathway contributes to chemoresistance and cancer stem cell (CSC) propagation, the two factors ultimately responsible for tumour recurrence after therapy, metastasis and poor patient survival [31]. CSCs are a subpopulation of cancer cells; similarly to normal stem cells, they can self-renew or differentiate [32]. Being activated in CSCs, the Wnt pathway upregulates transcription of genes necessary for proliferation (such as c-myc) [33], cell cycling (such as cyclin-D) [34], anti-apoptosis (e.g., survivin) [31, 35], metabolic switching to aerobic glycolysis (PDK1, MCT-1) [36, 37], and invasion and metastasis (SLUG, MMP) [38, 39]. The role of active Wnt signalling in chemo- and radio-resistance is linked to the survival of CSCs: being relatively dormant, they can better withstand the therapy to repopulate the shrunken tumour, which results in tumour recurrence. There is also a separate mechanism of involvement of the Wnt pathway in cancer chemoresistence, mediated by the ill-famed multidrug resistance protein 1 (MDR1, also known as ABCB1 or P-glycoprotein) [40]. It was first demonstrated in early colorectal cancer that MDR1 is a target gene of the Wnt/β-catenin/TCF4 pathway, thus activation of the pathway led to increased levels of MDR1, increased drug efflux and drug resistance [41]. Similarly, increased MDR1 expression was found to be mediated by FZD1 in neuroblastoma, and a

significant correlation in expression levels of FZD1 and MDR1 was found in patients relapsed after chemotherapy [42]. Other drug pumps involved in chemoresistance, ABCG2 (BCRP) and MRP2, were also shown to be induced by the Wnt pathway [43–45]. Finally, another contribution of the Wnt signalling to drug resistance is mediated by the DNA repair gene O6-methylguanin-DNA-methyltransferase (MGMT) in CSCs [46–48]. MGMT specifically repairs alkylated DNA and therefore upregulation of the protein leads to inefficiency of DNA alkylating agents and PARP inhibitors [49].

Wnt signalling plays several roles in tumour radioresistance. Firstly, radiotherapy induces upregulation of a panel of growth factors including Wnts, both in the tumour and in the surrounding stroma leading to enrichment of the CSC population [50]. Secondly, the Wnt pathway can directly protect against irradiation-induced DNA damage driving expression of DNA ligase 4 (LIG4) in colorectal cancer cells [51]. Further, histone modifier high-mobility group box 1 protein (HMGB1) involved in chromatin remodelling and DNA repair can be induced by Wnt/TCF4 signalling; blocking HMGB1 in oesophageal squamous cell carcinoma cells was found to suppress the Wnt1-dependent radioresistance [50].

Targeting the Wnt pathway is therefore beneficial at multiple levels: inhibition of tumour growth and survival with minimal effects on somatic cells, inhibition of CSC maintenance (and thus, of tumour relapse), and prevention of the development of tumour resistance to chemo- and radiotherapy. Inhibitors of the Wnt pathway are therefore in high therapeutic demand, and platforms dedicated to the search and development of such inhibitors are needed [52]. Although no Wnt-targeting drugs have yet reached the market, some are in preclinical and early clinical stages of development.

Wnt inhibitors in clinical development

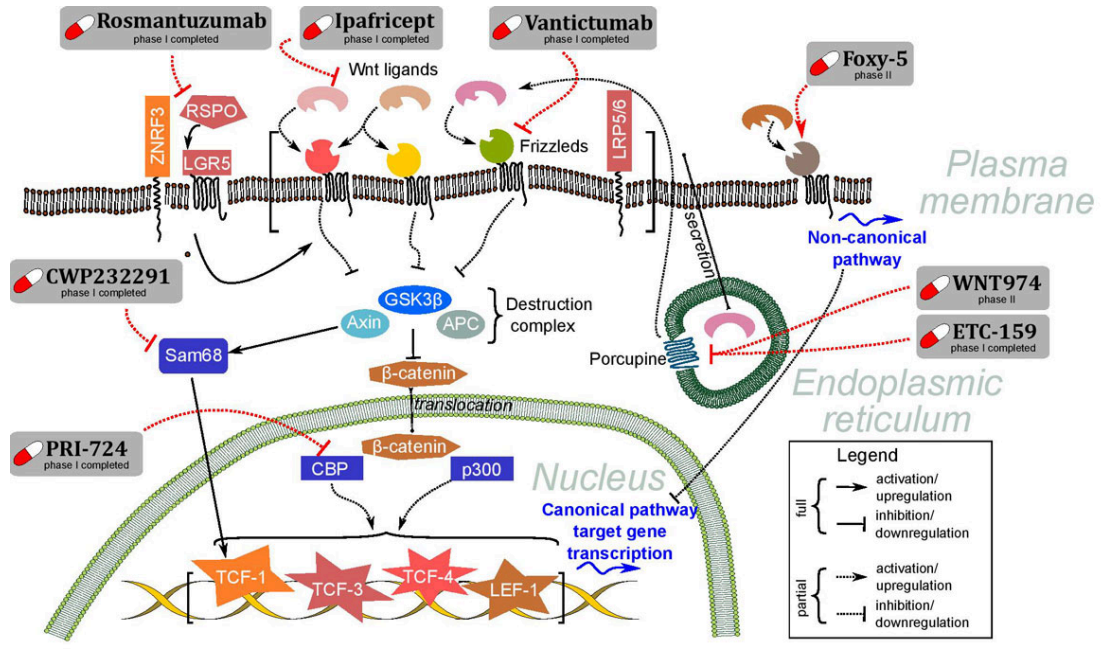
The pathological and physiological roles of Wnt signalling, as well as the complexity of this pathway with its numerous sub-branches utilised in different cell types, underlie the practical difficulties in finding therapeutically relevant Wnt-targeting agents. The total number of Wnt pathway inhibitors active *in vitro* is around fifty [53, 54], many of them having reached different stages of preclinical development, but so far only a few have attained early phase clinical trials.

Figure 1 shows the anti-Wnt agents that have reached clinical trials. It can be appreciated that these agents target the pathway at the levels where the pathway diverges into several sub-pathways (perhaps with the exception of the Porcupine-targeting drugs, but see below). Such diversification into sub-pathways can be found at the level of the plasma membrane, as well as in the nucleus; in contrast, the events in the cytoplasm are poorly diversified and are rather common for all the Wnt signalling subtypes [55, 56]. For a drug candidate being selected for clinical studies, we consider it critical for it to act on Wnt signalling subtypes active specifically in pathological tissues, instead of affecting all Wnt signalling subtypes. Indeed, pan-Wnt inhibitors fail to show acceptable safety profiles at preclinical levels and cannot advance further, as exemplified by attempts to develop tankyrase inhibitors [57, 58] or Dickkopf-1 as a biologic to block the Wnt pathway [59].

Vantictumab, ipafricept and rosmantuzumab

The only agent directly targeting FZDs having entered clinical development is a humanised antibody vantictumab (OMP-18R5). Initially developed against the Wnt-binding CRD-domain of FZD7, it was found to act on FZD1, 2, 5, 7 and 8 – five FZDs out of the ten encoded by the human genome [60]. The preclinical activity profile against a panel of tumour cells [60] prompted it's entry into phase I clin-

Figure 1: Drug compounds of past or current clinical trials target the Wnt pathway at the ligand/receptor level (rosmantuzumab, ipafricept, vantictumab and Foxy-5) or at the transcriptional level (CWP232291 and PRI-724). Porcupine inhibitors (WNT974 and ETC-159) target Wnt secretion.



ical trials (NCT01345201, NCT01973309, NCT02005315 and NCT01957007). All the trials have been completed by now and reports are available for the first three. The first phase Ia study measured the dose escalation effects following the intravenous administration of doses ranging between 0.5mg/kg weekly and 2.5mg/kg once in 3 weeks. The main finding was bone toxicity manifested as a bone fracture on day 110 in one patient. Other adverse events were fatigue, vomiting, abdominal pain, constipation, diarrhoea and nausea of grades 1 and 2, with grade 3 diarrhoea and vomiting reported in one patient. To tackle bone toxicity, the study monitored β-C-terminal telopeptide (β-CTX), a marker for bone degradation, and was able to manage its levels by administering zolendronic acid [61]. The other two studies, phase Ib on pancreatic and breast cancer using vantictumab in combination with paclitaxel (90 g/m^2) or nab-paclitaxel (125 g/m^2), adopted the same strategy for tackling the bone fragility and reported similar adverse effects of grade 2 observed in phase Ia and few additional grade 3 events (neutropenia, leukopenia, pelvic pain, fatigue and nausea). Both studies used increased vantictumab dosages (between 3.5 and 14 mg/kg) and reported further bone fragility events, which required improvements in the zolendronic acid administration regimen and resulted in a temporary halt of the trials in 2014 [62–64].

Similar results were obtained for ipafricept (OMP-54F28) – another anti-Wnt biologic from OncoMed, representing the FZD8 CRD-domain fused to an IgG1 constant fragment. By mechanism, this compound can be expected to possess a certain selectivity towards a subset of Wnt proteins, although its specificity among the 19 Wnts encoded by the human genome is unclear because of the lack of comprehensive data on mutual affinities of Wnt and FZD proteins. Four clinical trials were launched for ipafricept (NCT01608867, NCT02092363, NCT02069145 and NCT02050178) with two of them reporting the results. As with vantictumab, the trials used the scheme with zolendronic acid to counteract the bone-related adverse effects, apparently with more success since only one fracture was recorded at 20 mg/kg; the on-target dose was estimated to be at >10mg/kg. Non-bone-related adverse events with the compound included grade 1 and 2 dysgeusia, decreased appetite, fatigue, muscle spasms, alopecia and vomiting and grade 3 events such as anaemia hypophosphataemia, neutropenia and weight loss [65, 66].

Finally, Oncomed had one more anti-Wnt compound in its portfolio – the R-spondin 3-targeting antibody rosmantuzumab (OMP-131R10). R-spondins are soluble ligands that enhance Wnt signal transduction, especially the canonical branch, through different mechanisms [10, 67]. The phase Ia/b clinical trial (NCT02482441) of the agent demonstrated a set of adverse effects similar to the other two agents of the company: doses from 2.5 to 15 mg/kg every 2 weeks resulted in nausea, decreased appetite, diarrhoea, vomiting and weight decrease of unspecified grades. Additionally, the treatment resulted in changes in bone turnover markers – which is somewhat unexpected since R-spondin 3 (unlike related R-spondin 1 and 2) is not known to be involved in bone formation and maintenance [68, 69]. This might hint towards insufficient specificity of rosmantuzumab, which is difficult to assess since no preclinical report was published for the agent.

Overall, one may conclude that vantictumab, ipafricept and rosmantuzumab, biologics interfering with Wnt signalling at the level of the ligands, receptors and extracellular enhancers, which have been designed to achieve selectivity in targeting different Wnt signalling subtypes, have ultimately revealed poorer specificity than intended. The similar adverse effects of the three drug candidates in safety clinical trials suggest a too-general wiping out of the Wnt pathway instead of the selective inhibition of the pathway subtype active in the tumour. These adverse effects were likely behind the strategic decisions made regarding the drugs: in 2017, Bayer opted out of licensing vantictumab or ipafricept from Oncomed for “strategic reasons”; rosmantuzumab was stated to have “failed to provide compelling evidence of clinical benefit” [70]. These decisions resulted in discontinuation of the clinical development of the three candidates. Since the molecules did not advance beyond the safety trials, conclusions on compound efficacy in human subjects could not be drawn.

Porcupine inhibitors WNT974 (LKG974) and ETC-159 (ETC-1922159)

Another clinically relevant attempt to inhibit Wnt signalling at the upstream levels is currently spearheaded by two competing inhibitors of Porcupine, the acyltransferase responsible for posttranslational modification of all Wnt proteins. By conception, molecules of this type were supposed to be pan-Wnt inhibitors, preventing both autocrine and paracrine signalling since the acylation is considered an absolute prerequisite for the Wnt protein secretion and activity [71]. However, as described below, both molecules show quite acceptable preclinical and clinical safety profiles, which might be explained by novel insights into the signalling by non-acylated Wnts, meaning that inhibition of acylation might affect the pathway only partially [72]. Both competitors from Novartis (WNT974) and Singapore State D3 consortium (ETC-159) have successfully passed preclinical investigations, with significant reduction of the tumour burden and no toxicity – either overt or at the level of tissue morphology following the analysis of several Wnt-dependent tissues. In phase I clinical trials (NCT01351103 for WNT974 and NCT02521844 for ETC-159), both were tested at similar doses – 5 to 30 mg/day for WNT974 and 1 to 30 mg for ETC-159. The more representative study of WNT974, enrolling 94 patients by 2017, showed that it induces grade 1 and 2 dysgeusia, decreased appetite, nausea, fatigue, diarrhoea, vomiting, hypercalcaemia, alopecia, asthenia and hypomagnesaemia. Additionally, in a small number (3–4%) of patients, grade 3 and 4 adverse events included asthenia, fatigue, decreased appetite and enteritis [73, 74]. Analysis of tumour specimens for various markers has shown a profound Wnt inhibitory effect; moreover, in some patients the compound was used in combination with spartalizumab (an anti-PD-1 antibody), giving a positive outlook on potential combination of the anti-Wnt and immune-therapy. Surprisingly, authors do not report any events or even any attempts to follow bone-related effects, which is in contrast to the ETC-159 trial. The latter enrolled 16 patients and the study reported vomiting, anorexia, fatigue, dysgeusia and constipation as the adverse events of unspecified grade identified in >20% of patients. Beta-CTX levels were analysed and found to be expectedly elevated in two patients with con-

comitant loss in bone density, which was counteracted by vitamin D and calcium supplements [75].

Although that WNT974 was shown to affect bone structure in animals [76, 77], the effect does not seem to manifest clinically. Perhaps this is a reason why WNT974 is currently the most advanced anti-Wnt agent and the only one to have moved to a phase II trial (NCT02649530), and therefore might become the first anti-Wnt agent with a comprehensive assessment of clinical pharmacodynamics.

Wnt5a-mimetic Foxy-5

An interesting approach to Wnt inhibition is employed by the WntResearch start-up company from Sweden, which has identified a Wnt5a-mimicking peptide called Foxy-5 as an efficient anti-metastatic agent [78]. Specific to the compound is its intervention into the non-canonical Wnt pathway, suppressing migration and adhesion of breast cancer cells. Therefore, this approach does not target tumour bulk, but is rather oriented to metastasis prevention and is used in combination with surgery, irradiation and other drugs. The compound has passed a phase I clinical trial [79, 80]. According to the sparse information provided, Foxy-5 was reported to be "non-toxic" at any dose, and showing good pharmacokinetics and stabilisation of the levels of circulating tumour cell in patients with metastatic breast, colon, or prostate cancer [81]. Currently the company has reported recruitment of the first patient for the phase II study. This study will compare patients undergoing colon cancer surgery followed by a 6 month regimen with FOLFOX with patients receiving a treatment of Foxy-5 before and after surgery until starting the FOLFOX regimen (NCT03883802).

Downstream pathway component inhibitors PRI-724 and CWP232291

These two drug candidates make use of the "downstream target window" to achieve the necessary specificity (fig. 1). Both compounds affect the pathway at the transcriptional level, but through entirely different mechanisms: the small molecule PRI-724 affects interaction of β-catenin with transcription co-activator CBP, whereas the peptidomimetic CWP232291 (sometimes called CWP-291) binds to Sam68, an RNA-binding protein that regulates alternative splicing of the TCF-1 transcription factor in a complex with CBP. This selectivity of the compounds towards the Wnt pathway components employed by cancer cells allowed both PRI-724 (as its early analogue ICG-001) [82–84] and CWP232291 [85] to succeed in the preclinical setting and enter phase I clinical trials.

PRI-724 was tested in three phase I trials: in patients with advanced solid tumours (NCT01302405), acute and chronic myeloid leukaemia (NCT01606579), and pancreatic cancer (NCT01764477). In the phase I trials including 18 patients, the compound showed a promising safety profile with only dose-limiting grade 3 hyperbilirubinaemia registered in one patient (out of 7 presenting grade 3 events) at the highest dose of the compound (1280 mg/m^2/day). Grade 2 adverse events included diarrhoea, bilirubin elevation, hypophosphataemia, nausea, fatigue, anorexia, thrombocytopenia and alkaline phosphatase elevation. The compound also showed a decrease in survivin expression in circulating tumour cells as an efficacy readout in colon cancer subjects [86]. At the same dosage, no grade 3 adverse events were recorded for refractory leukaemia patients, with only four cases of grade 1 nausea, vomiting and diarrhoea attributed to the drug. Analysis of patient samples demonstrated a 44% median blast decrease [87]. However, in the third study, when combined with gemcitabine against pancreatic adenocarcinoma, the compound induced seven grade 3 and 4 adverse events in 20 patients, inducing abdominal pain, neutropenia, anaemia, fatigue and alkaline phosphatase rise. Stable disease was observed in 40% of patients. Despite this somewhat worse performance, none of the adverse events met the dose-limiting definition, thus the combination was considered overall safe with "modest clinical activity" [88]. Interestingly, since CBP/β-catenin interaction was found to be important during the onset of liver fibrosis, PRI-724 is in clinical trials against this disease as well, reporting similar adverse reactions against the background of a clinical benefit [89]. This later indication is being continued, as currently a phase I/II study is announced for PRI-724 in fibrosis, whereas for its anti-cancer application no follow-up is in sight.

Peptidomimetic CWP232291 was used in a single phase I trial in patients with relapsed and refractory acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS) comprising 56 patients. Grade 3 and 4 events made up 9% of the all recorded adverse events and included fever, nausea and anaphylactic reaction, with the first two being dose limiting. Grade 1 and 2 adverse events included nausea, infusion-related reaction, vomiting, diarrhoea and anorexia. There were also some indications of efficacy, since remission was observed in one patient and reduction in β-catenin and survivin as markers consistently observed in other subjects.

Conclusions and perspectives

The agents described above result from different approaches to inhibiting the Wnt pathway in cancer and are clearly unified by one motif: in order to target the pathway, one needs to identify disease-specific vulnerabilities in it to avoid systemic toxicity. Such specific vulnerabilities in the Wnt pathway are best to be found among its most divergent levels of the pathway – the one at the plasma membrane and the one inside the nucleus [55]. It should be noted that in the current review we have focused on the *de novo* and dedicated Wnt-targeting compounds; however, a wealth of Wnt inhibitors have been found among already approved drugs, prompting attempts to reposition them against Wnt-dependent cancers, as reviewed by us and elsewhere [90, 91]. Our own recent preclinical study shows that clofazimine, known as an anti-leprosy agent with a well-established safety profile, can efficiently inhibit Wnt signalling at the doses comparable to those used against leprosy and is safe to administer in combination with chemotherapy [92, 93]. Other well-known small molecule compounds, such as niclosamide, sulindac, pimozide show promise in various preclinical studies and will hopefully soon appear in clinical studies. Other Wnt inhibitors, including some natural products, may turn out to be promising agents against select Wnt-dependent cancers [94, 95]. Future developments will show whether the new wave of effort to target the "undruggable" Wnt pathway will bear fruit [96]. The main message of our review is that, in order to be suc-

cessful, such efforts should aim not at the Wnt pathway as a whole, but at a particular variant of it, selectively active in a disease state.

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Article IV

Manuscript in preparation

First-in-class small molecules as selective antagonists of FZD in triple-negative breast cancer

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Summary

Although nearly 50% of all cancers are dependent on WNT/FZD signaling, no inhibitors of this pathway have so far advanced beyond early-phase clinical trials. By functional high-throughput screening in triple-negative breast cancer cells (TNBC, one of the cancer types critically dependent on WNT signaling), we discovered a class of small molecules based on a diphenyl-substituted pyrazole core. Lead compounds FSA/99 of the series exert pronounced anti-cancer effects against a panel of TNBC lines. *In vivo*, these lipophilic compounds strongly accumulate in the tumor producing a profound and on-target suppression of the tumor growth in a panel of TNBC models, including cancer stem cells- and patient-derived xenografts. FSA/99 emerge as small molecule modulators of FZD – the principal receptors of the WNT pathway. FSA/99 reveal differential selectivity towards the ten FZD receptors, being more potent towards cancer-relevant FZD (especially FZD7, 8 and 9). This feature is likely behind the strong pharmacodynamics of the drug candidates against the tumor, while sparing physiological WNT signaling in the bone and intestine – the standard on-target effect sites of other, less selective WNT inhibitors. These features spearhead FSA/99 for development towards the first targeted therapy selective to cancer-relevant WNT/FZD signaling.

Main

Even though only 15-20% of all breast cancers (BC) are of the triple-negative subtype, they are responsible for an estimated 50% of all BC deaths[1,2]. Despite this importance, there is to-date no approved targeted drug to treat TNBC. Like other developmental pathways, in addition to being highly active during organism development[3,4], WNT signaling is implicated strongly in the initiation, growth and progression of TNBC and other cancers and is thus a very attractive drug target[5–9]. Canonical WNT signaling is induced by the interaction of one of 19 WNT family lipoglycoproteins with a Frizzled (FZD) G protein-coupled receptor and an LRP5 or 6 co-receptor. The scaffolding protein Dishevelled (DVL, isoforms 1-3 are present in humans) is a key mediator of the signal down to the Axin-based destruction complex (additionally containing APC, casein kinase and GSK-3β) responsible for sequential phosphorylation of cytoplasmic β-catenin, the event marking β-catenin for ubiquitinylation and proteasomal degradation. Activation of the pathway (physiological – through arrival of WNT ligands extracellularly, pathological – through deactivating mutations in components of the destruction complex, or artificial – through chemical inhibitors of GSK-3β) leads to stabilization of cytoplasmic β-catenin, its translocation to the nucleus, and activation of target gene transcription through interactions with TCF/LEF transcription factors[3,4,10].

In the adult, WNT signaling physiologically controls maintenance and renewal in different tissues, e.g. gastrointestinal epithelium, bones, blood and immune systems[3], making the adverse effects induced in these tissues upon blunt blockade of the WNT pathway an important issue. Thus, identifying WNT pathway inhibitors with strong anti-cancer properties but low toxicity has become a major challenge in this domain of drug discovery[11,12]. The increasing knowledge in the field has led research, including our own, to focus on developing drugs targeting the upper pathway levels, especially the FZD receptors[13–17]. Our approach is to harness the complexity of these upstream effectors of the pathway, involving 10 FZD orthologs, in order to find compounds to selectively inhibit WNT signaling in the tumor and leave it functioning in the healthy tissues.

We have established a TNBC-specific high-throughput screening assay using the BT-20 cell line stably transfected with the TOPFlash transcriptional reporter to measure the WNT pathway activation.

As opposed to similar screens performed in pan FZD-expressing healthy cells[18], our assay permits identification of hits acting on the few cancer-relevant FZDs. Using this powerful tool followed by a set of secondary assays, our drug discovery pipeline has successfully yielded an effective and selective WNT pathway inhibitor with *in vivo* anti-cancer properties without adverse effects.

From a compound library to an upstream WNT signaling inhibitor with anti-cancer properties

Following the screening of a library of 1000 GPCR-biased diverse small molecules using the TOPFlash reporter assay in TNBC BT-20 cells[7,19], we focused our efforts on a compound called FSA which inhibited the WNT3a-induced signaling in a concentration dependent manner with an IC_{50} of around 12 μM (Fig. 1a). At the plateau, the pathway inhibition was around 90%. The WNT-independent CMV-Renilla luciferase reporter was not inhibited (Fig. 1a), indicating that the hit compound did not produce acute cell toxicity or affect general cell transcription.

To confirm the WNT-specific inhibition of FSA and to define the pharmacophore, we screened further 151 compounds presenting similar structures. This resulted in the determination of a general scaffold composed of a pyrazole ring with aromatic moieties at positions 1 and 3, as well as a flexible linker at position 4, which allows the attachment of a further aromatic group (see Supplementary Table 1, summarized in Supplementary Fig. 1a). We further found that a synonymous "turned" structure set attaching the flexible-linker arm at position 1, and the two aromatic groups at positions 3 and 5, could also preserve the anti-WNT activity (see Supplementary Fig. 1a with F2-99 as an example). Overall, of the 152 compounds, 47 had the potency to inhibit the WNT3a-induced reporter activity comparable to FSA, 60 showed decreased potency, 24 completely lost the inhibitory activity, 16 were toxic, and 5 increased the WNT3a-induced reporter activity (Supplementary Fig. 1b). Of the active analogs with the efficacy above 80%, none had the potency improved over 1.5-folds over the parental FSA (Supplementary Fig. 1c). The reason why significantly lower IC_{50}s could not be achieved while maintaining high efficacy is probably due to the nature of the molecular target of the compounds, as discussed below.

To determine whether the pathway inhibition by FSA occurs at the plasma membrane level or more downstream, we induced WNT signaling with CHIR99021, a specific GSK-3β inhibitor[20]. Unlike the pathway activation induced by WNT3a, CHIR99021-induced signaling could not be inhibited by FSA (Fig. 1a, b). Activation of the pathway by a less specific GSK-3β inhibitor LiCl is similarly insensitive to FSA in BT-20 cells (not shown). These data provide a preliminary indication that the molecular target of FSA is a WNT pathway component acting above the GSK-3β-containing destruction complex. In agreement with this, WNT3a-induced stabilization of active β-catenin was blocked by FSA, while that induced by GSK-3β inhibition was insensitive to FSA (Fig. 1c, d).

We thus continued to dissect the action of FSA through methods addressing activation of the upstream components more directly. One of the most immediate events post WNT-FZD interaction happening at the plasma membrane is the rapid phosphorylation of DVL isoforms[21]. In the TNBC cell line HCC 1395, we observed that the phosphorylation-induced shift of DVL2 obtained upon stimulation by WNT3a is blocked FSA (Fig. 1e). The same effect could be investigated in L-cells, which provide a more artificial (due to their non-cancerous nature) but more robust readout system, due to non-existent basal levels of WNT signaling. In this system, we could show that WNT3a-induced accumulation of β-catenin, as well as phosphorylation of DVL2 and DVL3 are inhibited by FSA (Supplementary Fig. 2a, b); CHIR99021-induced accumulation of β-catenin was, in contrast, again insensitive to FSA. Thus, we conclude that FSA molecules inhibit the WNT pathway acting on a target close to plasma membrane.

In many published examples, inhibition of WNT signaling in TNBC translates into inhibition of cancer progression[12–15,17,22]. We thus proceeded by testing the ability of FSA to inhibit proliferation of a panel of TNBC cell lines representative of various subtypes of TNBC. Using the MTT assay for measuring cell viability, we showed concentration dependent growth inhibition of all cancer cell lines tested with the IC_{50} varying between 10 μM and 27 μM for FSA, close to the IC_{50}s of the WNT signaling inhibition for both BT-20 and HCC 1395 indicating that the anti-proliferative effect is likely a consequence of WNT inhibition (Fig. 2a, b). Encouraged by this result, we analyzed the anti-proliferative activity of a set of WNT inhibitors from our FSA-like compound library, focusing on

compounds with the potency in the TOPFlash assay above 80% (Supplementary Fig. 1c), and further prioritizing for structurally diverse compounds. This analysis let us choose seven representatives of the FSA scaffold with potent cancer growth inhibitory properties (Supplementary Fig. 3). With the outlook of further *in vivo* application, we also determined the hepatic metabolic stability using microsomes derived from human female livers. Addition of the compounds to the microsomes in combination with the coenzyme NADPH to test for Phase I metabolism and additionally UDPGA to test for subsequent Phase II metabolism resulted in a broad range of metabolic profiles (Supplementary Fig. 4). Four compounds showing the highest microsomal assay stability were passed on to preliminary pharmacokinetic assessment, which revealed that 24 h post-injection, the compound F2-99 was still present in the mouse blood circulation with the AUC value of 285.8 μM*h (Supplementary Fig. 5). The entire set of data described above led us to the selection of F2-99 and the parental FSA itself and as the most promising leads for further detailed investigation.

We have thus assessed activity of these two compounds against our panel of TNBC cell lines in detail. When speaking collectively, we will refer to the compounds FSA and F2-99 as "FSA/99" later on. In addition to the inhibition of proliferation achieved by FSA/99 (Fig. 2a, b), we challenged another hallmark of cancer, the formation of metastasis, with in two additional *in vitro* assays. The clonogenic assay, which monitors the ability of single cells to form viable colonies, showed that both compounds (15 μM) were able to strongly (50% to 90%) inhibit the colony formation in all tested cell lines (Fig. 2c, d). In addition to the decrease in colony numbers FSA/99 loosened the surviving colonies as if the compound-treated cells failed to form proper cell-cell contacts (Supplementary Fig. 6a). The second assay challenged the migratory ability of cells in the presence of 30 μM of the pyrazole molecules. Cancer cell migration is a characteristic regulated by the β-catenin-independent non-canonical WNT signaling rather than the canonical WNT/β-catenin signaling[23-24-25]. We found that FSA/99 were able to slow the TNBC cell movement, down to 50% of the vehicle treated cell migration (Fig. 2e, f). This might indicate that the noncanonical WNT signaling is also affected by the compounds (discussed below). Finally, we have analyzed the effects of FSA/99 on the MCF10A cell line representing immortalized non-cancerous breast epithelium (Supplementary Fig. 6b). As expected, both compounds

had minimal effects on the growth of these cells, being slightly toxic only at the overwhelming doses, with IC_{50}s well above 100 μM.

Lead compounds from the FSA/99 family inhibit tumor growth *in vivo* with minimal adverse reactions.

We have further assessed the FSA/99 compounds as anti-TNBC drug candidates using orthotopic mouse xenograft models: one 'somatic' TNBC model based on HCC 1395 cells, one TNBC cancer stem cells-based model using the IOWA-1T cell line[26], and one patient-derived xenograft (PDX) model. FSA and F2-99 were resynthesized on a large scale (several grams), along with certain specific derivatives (see below). Upon application of FSA/99 to TNBC xenograft-harboring tumors, we observed a dramatic decrease in the tumor burden and a delay in the tumor onset in IOWA-1T (Fig. 3a-b and Supplementary Fig. 7a, b) and HCC 1395 (Supplementary Fig. 7c) TNBC cell line xenografts, as well as in the BRC016 PDX model of invasive ductal triple-negative adenocarcinoma (Fig. 3d and Supplementary Fig. 8a, b). During these trials, we carefully monitored mouse body weight (Fig. 3c and Supplementary Figs. 7d and 8c) as well as other principal behavioral and physiological characteristics of the animals, such as the urine and faeces condition, respiration rate, appearance of orifices, posture and conscience status, detecting no differences between the vehicle-injected and compound-injected groups (data not shown).

We thus moved to investigate the compound actions in animals further. We could confirm that the decrease in tumor burden is associated with concomitant dramatic decrease in cytonuclear β-catenin (Fig. 3e) – a standard readout of canonical WNT pathway activation in tumor tissues[27]. Analysis of F2-99 accumulation in tissues showed a steady accumulation over a 30-day time period with the maximum levels reaching 2 mM in the tumor tissue (Supplementary Fig. 9a), over 150 times the IC_{50} of WNT inhibition and growth inhibition *in vitro*. FSA was less prone to accumulate in the tumors reaching only 2.2+/-1.6μM. Interestingly, the compound preferentially accumulates in a set of tissues including the tumor, neglecting some other tissues including the intestine. This feature may contribute to mollification of adverse effects (see below). The preferential accumulation in fatty tissues is likely

the outcome of the lipophilic properties and high plasma solubility of FSA/99 (Supplementary Fig. 9a-c).

Competitor WNT pathway inhibitors have been reported to produce strong side effects in animals and in phase I clinical trials[12]. Although we do not see any signs of toxicity (see above), we further scrutinized the state of the systems most frequently affected by the blunt targeting of WNT signaling – the bones, hematopoiesis and the intestinal epithelium – in the compound-treated mice. Even after >50-days of daily dosage of F2-99, we didn't detect worsening of the blood parameters (Supplementary Fig. 10a-d). In the bone, the following microCT-measured parameters remained unchanged: cortical area thickness, trabecular thickness, cross-sectional thickness, bone marrow density, and trabecular density (Supplementary Figure 10e, f). One parameter, the trabecular volume, was found decreased about 2-fold. However, even this effect is mild as compared to the effect of porcupine inhibitors observed after only 2 weeks of treatment, resulting in ca. 4-8-fold loss of trabecular volume and drastic effects on other bone health readouts[28,29]. Additionally, we have performed in-depth analysis of 3 intestinal compartments in control and compound-treated mice. It is known that pharmacological WNT inhibition frequently results in severe intestinal damage since it affects the stem cells residing in the crypt[30,31]. However, in our case we observed neither any overt damage to the villi or crypt microstructure (Supplementary Fig. 11a) in any of the compartments analyzed, nor any differences in villus length, crypt depth or density (Supplementary Fig.11b).

The capacity of FSA/99 to suppress tumor growth without serious adverse effects contrasts the observations made with several other WNT pathway inhibitors. The next sections will show that this is likely due to the selectivity of FSA/99 towards the cancer-relevant subset of WNT signaling.

Compounds of the FSA/99 family directly and selectively interact with the CRD domains of FZD receptors.

We thus conducted a set of functional and biochemical experiments addressing the capacity of FSA/99 to directly interact with FZD receptors. First, we made use of HEK293 cells (normally expressing

most of the FZDs) genetically knocked out for all ten FZD genes[32]. Upon retransfection of the FZD-encoding plasmids, it served as a readout of activity of individual FZD proteins, in case it induced a response in the TOPFlash assay. We have identified that all the FZD proteins, with exception of FZD3, 6 and 8 are capable of inducing a WNT3a-induced response. However, the response magnitudes vary somewhat among them (Supplementary Fig. 12a). Fig. 4a and Supplementary Figures 12b, c show the results of the activity profiling for FSA/99 on this cell line against various FZD proteins, revealing the stronger effect, and by inference, affinity for FZD7. For F2-99, the selectivity between the best-inhibited receptor (FZD7) and the worst one (FZD4) reached 4.4 folds.

We have obtained further independent evidence, that the compounds of the FSA/99-like scaffold directly interact with CRD domain of the FZD receptors. We performed an *in silico* screen of the compound library available from MolPort to find the best binders to the 3D structure of the FZD7 CRD. The 25 molecules with the highest scores were further analyzed using the TOPFlash assay in BT-20 cells. We identified 4 compounds that showed specific inhibition, though only 2 of them (#2 and #19, see Supplementary Fig. 13a and Supplementary Table 3) showed inhibition with an IC_{50} at least near 100μM. Subsequently we used the pharmacophores of these 2 compounds, such as the one for #19 shown on Supplementary Figure 13b - note its overall similarity to FSA/99 scaffold - to search for the similar compounds in a more detailed manner. After screening a further 48 high-scoring compounds from the second batch (Supplementary Table 3), we identified 10 active compounds. Moreover, 3 among them, bore a remarkable similarity to the compounds of the FSA/99 scaffold and showed strong and specific inhibition of WNT3A-induced TOPFlash signal.

These results provide a further indication that FSA/99 compounds target FZD proteins directly. In order to confirm the direct and unassisted binding of the compounds to FZD, we synthesized an FSA analog called Sol41, whereby a diaminoethylene linker was added to the FSA scaffold (see Supplementary Fig. 13a). The free primary amino group of the linker serves two functions: it enhances aqueous solubility and permits matrix immobilization for pull-down experiments. However, this modification also led to a 5-fold reduction in potency (Supplementary Fig. 12c, d and 13b). In addition, we synthesized another FSA derivative, Sol42 (see Supplementary Fig. 13a), which contains

a double bond in its linker region which reduces its flexibility and almost entirely removes the inhibitory activity of the compound, (Supplementary Fig. 13b), thus proving that flexibility of the linker region seems to be a prerequisite for efficient interaction. Importantly, Sol41 has a somewhat altered but overall similar selectivity for FZDs as compared to parental compound FSA or F2-99 (see Supplementary Fig. 12b, c).

We created an affinity matrix linking the primary amino group of the 6-carbon spacer arm of Sol41 to NHS-activated sepharose. CRD domains of all 10 human FZD receptors were purified after recombinant production in the baculovirus expression system and applied to the Sol41-sepharose in presence of BSA as a blocking agent; post-incubation, the bound protein was eluted with excess Sol41. As shown on Fig. 4b, three CRD domains – of FZD7, FZD8 and FZD9 – revealed specific interactions. The CRD domains of FZD1 and 10 could not be eluted by Sol41, but still appeared to specifically interact with Sol41-sepharose (Supplementary Fig. 13c). The CRD of FZD7 also showed a starkly reduced interaction with beads loaded with Sol42 – the inactive analog of Sol41 (Supplementary Fig. 13d).

We next aimed at confirming the selective interactions of the FSA/99 compounds with the FZD CRDs by NMR. However, given the poor aqueous solubility of FSA and F2-99 (Supplementary Fig. 9b), only Sol41 demonstrated specific peaks in 1H NMR corresponding to solubility of around 100 μM. We therefore proceeded with waterLOGSY using Sol41 and the CRD domain of FZD7 (CRD7) at 700MHz. As shown on Fig. 4c, CRD7 demonstrated clear and specific binding to Sol41, judged by the phase inversion of the compound-specific peaks (see Supplementary Fig. 15a for peak assignment) in presence of CRD7. The specificity of this interaction was further confirmed by i) presence of the non-inverted peaks of various impurities and DMSO as seen in Fig. 4c and ii) by the same experiment where CRD7 was replaced by BSA – we observed no peak inversion and the spectrum remained essentially identical to that of a free compound (Supplementary Fig. 15b).

As a yet another means to confirm the physical interaction of FSA/99 with the target, we applied the Biacore SPR technology after immobilizing the CRD domains of FZD7 and 9 on the CM5 chip. Unlike in waterLOGSY NMR experiments, the buffer used for Biacore contains a detergent (0.1%

Triton X-100), permitting to reach concentrations up to 80 μM for F2-99 and 250 μM for FSA. As shown in Supplementary Fig. 15c and d, both compounds are capable of interacting with the proteins, though F2-99 shows a very weak interaction with CRD9. Surprisingly, the K_d values identified from these experiments are an order of magnitude higher than the IC_{50}s of the compounds measured from the functional assay (Fig. 4a, Supplementary Fig. 12b, c). We assume that this apparent K_d reduction in the SPR measurements is artificially induced by incorporation of the compounds in detergent micelles, as is well-described for other lipophilic compounds like the popular dynamin inhibitor Dynasore[33].

Cumulatively, our data allow us to conclude that FSA/99 bind directly to the CRD domains of select FZD receptors and identify two of them as the primary targets, FZD7 and 9, as well as to identify FZD4 as the least potent binding partner of these compounds. FZD8 also emerges as a highly potent target. To functionally validate the importance of FZD7 (previously implicated in TNBC and some other cancers[34]) and FZD9 (previously not implicated in TNBC but in hepatic cancer[36], in gastric cancer[37] and astrocytomas[35]) in TNBC cell lines, we designed two independent sgRNA for each of these receptors, cloned them in a pLentiCRISPRv2 lentiviral vector, and transduced the viruses into a panel of TNBC cells; we similarly targeted FZD4 as a negative control. As results, we observe that: 1) FZD7 and even more so FZD9, but not FZD4, are indeed crucial for survival of most TNBC cell lines (Supplementary Fig. 16a); 2) FZD7 and 9 but not FZD4 are important for the high growth rate of TNBC cells (Fig. 4d and Supplementary Figure 16b).

Subsequently, we moved to analysis of the WNT pathway activity in the knockout lines. These results are shown on Fig. 4e and again pinpoint FZD7 and FZD9 as major transducers of the WNT3a-mediated signaling in the cancer cells: their knockout resulted in a significant (often dramatic) loss the response levels.

Finally, we treated the FZD knockout cells with F2-99 to evaluate their response to the treatment on the background of loss of its principal target (Supplementary Fig. 14d). As expected, several FZD7- and FZD9-knockout lines show significant reduction in their sensitivity to the compound. However,

the effect of the compound is not complete and is still resulting in reduction of the cell numbers, likely due to ability of the compound to inhibit different FZDs and switching the surviving cells to them.

Despite that these results clearly indicate that compounds of FSA/99 family are acting through Frizzled proteins in cancerous cells, they clearly also have off-target effects. We have analyzed the potential scope of these off-targets by pulldown experiments using fractionated cell lysed from HCC 1395 cells and Sol41 immobilized on sepharose. LC-MS identification of the bound proteins was performed to assess possible WNT-related and non-WNT-related targets. Although no WNT-related targets were identified, the results indicated some possible binding partners outside the WNT-pathway (Supplementary Table 2) which could be further investigated. Analysis of the membrane fraction was not successful due to instrumentation limitations and therefore our main target the FZD receptors could not be detected in this set of experiments. Additionally, the analysis of 18 pathway reporters showed that the lead compound F2-99 had certain effects on the pathway other than WNT, however most of them are of much lower potency and efficacy (Supplementary Figure 17a) indicating minimal off target effects on other main signaling pathways. Additionally, the pyrazole compounds were tested on a FZD-free cell line to investigate whether the anti-cancer effects are still present. Surprisingly, these cells were even more sensitive to the compound treatment even as compared to parental HEK293 line (Supplementary Figure 15b). Additionally, the colony formation of the cells was also still strongly inhibited (Supplementary Figure 15c). These data indicate that FSA and F2-99 must have additional targets in addition to the FZDs.

To conclude, we have discovered a novel class of small molecules based on a diphenyl-substituted pyrazole core, targeting a small and specific subclass of FZDs. With potent WNT inhibitory and anti-cancer properties, inducing only mild bone toxicity *in vivo*, this novel class of molecules promises to be more advantageous than previously published WNT-signaling inhibitors, making them prime candidates for clinical testing.

Materials and Methods

General

If not stated otherwise, cells were grown in DMEM 10% FBS, 1% PenStrep (Gibco). Cells were incubated at 37°C, 5% CO2, >80% RH. The triple negative cell lines (ATCC) used are: BT-20, HCC 1395, MDA-MB 231, MDA-MB 468, HCC 1806 and HCC 38. For β-catenin stabilization assays and Western Blot analysis, mouse L-cells were also used. FZD-free HEK 239 cells were kindly provided by Benoit Vanhollebeke.

Compound selection

In a first round of screening, 34 randomly chosen compounds resembling FSA, but presenting a large intravariance were tested for their ability to inhibit the WNT pathway, using the TOPFlash reporter assay. Compounds were first tested at concentrations of 5 and 50 μM to show concentration dependency. The IC_{50} values and efficacy of the most promising compounds were then determined for aiding with the selection of the second round of compounds to be screened.

For the second screen the compounds were selected by overall chemical (Tanimoto) and substructural (via generalized structure search) similarity in the ChemDiv collection (ca. 1.5mio compounds). The overall set of similars (ca. 1000 compounds) was clustered using the JChem software and 1-2 representatives of each substructural cluster were selected for analysis, resulting in the comprehensive list of 117 compounds.

TOPFlash assay

For the screens, BT-20 cells, stably transfected with the TOPFlash reporter plasmid were seeded at 15K cells per well in white tissue-culture-treated 96-well plates (Greiner) and incubated overnight. If needed, the cells were additionally transfected with the pRL-CMV plasmid using the X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer's protocol and again incubated overnight. For the additional reporter plasmids, BT-20 WT cells were cotransfected with the reporter

plasmid and the pRL-CMV plasmid according to the same protocol. TOPFlash assays reported for other cell lines, such as HCC1395 and HEK293 FZD-/- were performed at the same cell density upon transfection of the mixture of TopFlash and CMV-Renilla at 4:1 ratio. The cells were pretreated for 1h with DMSO or compound before addition of WNT3a (250ng/ml final concentration) and incubation for 18-24h. The medium was then removed and 12 µl 10% sucrose solution was added to the cells to prevent drying. The plates were then read using the Victor3 Multilabel Counter (PerkinElmer) after injection of the luciferase firefly buffer (50 µl; 25 mM glycylglycine, 15 mM KxPO4, 4 mM EGTA, 2 mM ATP, 1 mM DTT, 15 mM MgSO4, 0.1 mM CoA, 75 µM luciferin, pH 8.0) together with the lysis buffer (15 µl; 25 mM glycylglycine pH 7.8, 1% Triton X-100, 15 mM MgSO4, 4 mM EGTA, 1 mM DTT) followed by the renilla firefly buffer (50 µl; 1.1 M NaCl, 2.2 mM Na_2EDTA, 0.22 M KxPO4, 0.44 mg/mL BSA, 1.3 mM NaN_3, 1.43 µM coelenterazine, pH 5.0). Data was analyzed using the Prism 6 Software (GraphPad).

Proliferation assay (MTT)

TNBC, HEK293 FZD-/- or MCF10A cell lines and were seeded at previously determined concentrations in 96-well plates and incubated for 24h. The next day, the medium was replaced with medium containing either compound or the respective amount of DMSO for the controls. The proliferation was measured after 72h, by addition of a solution of 1mg/ml thiazolyl blue (Roth) in PBS, further incubation for 2h-4h at 37°C, and lysis of the cells by addition of 50 µl DMSO. The absorbance was read at 570 nm using the Victor3 Multilabel Counter (PerkinElmer).

Migration assay

Cellular migration was measured by using the so-called scratch-wound assay. TNBC cell lines were seeded to confluence in clear flat-bottom 96-well plates and incubated over-night. The following day a straight wound was inflicted on the monolayer using a 10 µl pipette tip. The cells were then washed carefully with PBS and treated with media containing the compounds or DMSO. Each well was imaged individually and the cells incubated for 6-18 h. Following this, the wells were again imaged and the migration of the cell front was measured using ImageJ.

Colony forming assay

TNBC cell lines were seeded at previously determined concentrations in 6-well plates and incubated for 24h. The cells were then treated with the compounds or DMSO only and the colony formation followed daily by visual inspection. Once the colonies were big enough (70-100 cells), the cells were fixed with a solution of 4% PFA in PBS pH 7.4. The colonies were then stained using a solution of 1% Crystal violet and images taken of the individual wells to count the number of colonies. The colony counting and analysis was done using ImageJ.

β-catenin stabilization assay and immunoblotting

Cells were seeded to 70-80% confluency in 12-well plates and incubated overnight. The medium was then changed for medium containing compound or DMSO and the cells pre-incubated for 1h. WNT3a for WNT pathway stimulation was added directly to a final concentration of 250ng/ml and incubated to allow for β-catenin stabilization (L-cells, 6h; HCC 1395 and BT-20, 18h), DVL phosphorylation (1.5-2 h) or LRP6 phosphorylation (1.5 h). After washing the cells with ice cold PBS, the cells were lysed by addition of 70 µl RIPA buffer (50 mM Tris pH 7.4, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1mM EDTA, 1 mM DTT, protease inhibitors (Roche)) containing phosphatase inhibitors if needed (4 mM NaF, 4 mM Imidazole, 2.3 mM Na_2MoO_4, 4 mM Na_3VO_4., 8 mM $C_4H_4Na_2O_6$ * $2H_2O$, 2 mM $Na_4P_2O_7$, 2 mM β-Glycerophosphate) and shaking 10 min on ice. The cell lysate was collected and centrifuged for 15 min at 16000g at 4°C to remove cell debris. The samples were equilibrated using the Bradford method and further separated and analyzed by SDS-PAGE and Western blot respectively. The following antibodies were used: anti β-catenin, 1:1000, BD Bioscience #610153; Anti active β-catenin, 1:1000, Merck Millipore #05-665; anti DVL2, 1:1000, Cell Signaling #3223S; anti DVL3, 1:1000, Cell Signaling #3218S anti a-tubulin, 1:2000, Sigma #T6199.

Microsomal assay

Pooled Female Human Liver Microsomes (Sigma) were incubated in 96-well plates together with the compounds (10 µM) in the reaction buffer (10 µl final volume; 100mM phosphate buffer pH 7.4, 2mM

NADPH for CYP metabolism; additionally, 5 mM UDPGA, 50 µg per mg of microsomal protein Alamethicin, 1 mM $MgCl_2$ for UGT metabolism). The reaction was stopped at regular time points by the addition of 30 µl ice cold CH_3CN containing an internal standard. The samples were centrifuged at 16000g, 10min, 4°C and the supernatant was diluted with H_2O to a final concentration of 25% CH_3CN. After repetition of the centrifugation, the samples were quantified by LC-MS on the Q Exactive instrument (ThermoFisher) in positive mode with electrospray ionization. Briefly, 10µl of sample were injected into the reverse-phase HPLC column (Zobrax Eclipse Plus C18, Agilent Technologies) and separated using a gradient from 25 to 95% CH_3CN with 0.1% formic acid. The peaks corresponding to the compounds ([M+H]+) were identified and quantified using the Xcalibur software and with the help of a standard curve (0.3 µM to 100 µM, 6 concentrations) built from known compound dilutions in control blood plasma and extracted in the same batch with the samples.

In vivo pharmacokinetic evaluation

Four NOD/SCID-γ (NSG) mice bearing intramammary tumours were injected IP with FSA, F2-27, F2-95, F2-99 (10 µM theoretical concentration in mouse, in 21% DMSO, 44% H2O, 35% PEG). Blood samples were taken at 2h, 4h, 8h and 24h and the samples measures as described above.

Pharmacodynamic study

The experiments were approved by the Swiss Federal Veterinary Office and were carried out in accordance with the local animal welfare act. Ten NOD/SCID-γ (NSG) mice were each injected intramammary with 50 µl of Matrigel suspension 5×10^5 IOWA-1T cells stably transduced with the pLenti-GFP construct or wild-type HCC1395 cells. For experiments with BRC0016 PDX xenograft, ca. 500cm^3 freshly extracted tumors from 2 donor animals were chopped into small pieces using scissors and digested using Accumax for at 1 hour on a rotary shaker. After sedimentation of the large undissolved chunks, the supernatant was centrifuged at 300g for 5 min, and the resulting cell pellet was resuspended in the matrigel at the ratio of 50µl per recipient mouse. As soon as the tumour volume reached ~100 mm^3, the mice were separated into two equal groups. The mice were injected (IP) daily with either FSA, F2-99 (50mg/kg) or vehicle. The tumour volume was measured at

indicated intervals and *in vivo* imaging was done using the IVIS Lumina II system (Xenogen).The intensity (expressed as integral area brightness) was quantified using the ImageJ software.

Target pulldown assay

NHS-activated sepharose (GE Healthcare) was activated according to the manufacturer's instructions and loaded with either Sol41, or control buffer ($NaHCO_3$, DMSO) subsequently blocked with Tris. HCC 1395 cells were grown to sufficient amounts in culture flasks (4x T175) and harvested using Accutase (Sigma). Cells were washed, supplemented with TBS containing protease inhibitors and lysed on ice using the dounce homogenizer (20 strokes). Nuclear fractions were collected by centrifugation at 1000g for 5min, 4°C, followed by washing with TBS and resolubilisation in TBS, 1% CHAPS. The remaining cytoplasmic and membrane fractions were separated by ultracentrifugation (100'000g, 1h, 4°C). The membrane fraction was resolubilised in TBS, 1% CHAPS. After resolubilisation, membrane and nuclear fractions were again centrifuged at full speed to remove remaining debris. The beads containing Sol41 or control buffer were incubated with the resulting cell fractions, at 4°C spinning, overnight. The next day, the beads were washed using TBS (1%CHAPS for nuclear and membrane fractions) and the bound proteins eluted using Sol 41 or Sol42 or control buffer and supplemented with laemmli buffer. Samples were immediately concentrated and purified by 1D gel electrophoresis. Proteins were then in-gel digested and peptides were analysed by nanoLC-MSMS using an easynLC1000 (Thermo) coupled with a Qexactive Plus mass spectrometer (Thermo). Database search was performed with Mascot (Matrix Science) using the Human Proteome Reference database (SwissProt). Data were analysed and validated with Scaffold (Proteome Software) with 1% of protein FDR and at least 2 unique peptide per protein with a 0.1% of peptide FDR.

Cellular uptake assay

HCC 1395 cells were detached and incubated with media containing 1µM, 25 µM or 100 µM F2-99 at 37°C, 5% CO2, >80% RH for 2 h. After incubation the media was removed and the cells washed in ice cold PBS and transferred to a new Eppendorf tube to avoid contamination by any compound

adsorbed on the tube walls. The media and cells were processed for LC-MS analysis as described above.

Compound solubility

FSA and F2-99 were prepared at concentrations ranging from 1μM to 100 μM in either PBS or mouse plasma. The samples were incubated for 1h at 37°C and then centrifuged at max. speed for 10 minutes at 37°C to remove any undissolved compound. The PBS and plasma were then analysed by LC-MS as described previously.

Baculovirus protein expression and purification

pFastBac Bac-2-Bac system vector was modified by subcloning mellitin secretion peptide leader and sequence encoding fusion of C-terminal 3xHA tag joined to GFP through TEV protease cleavage site (3xHA-TEV-GFP). The resulting vector was called pMel-3xHA-TEV-GFP. CRD domains of human FZD1,5,6,7,9 or 10 and mouse FZD2,3,4 and 8 were subcloned in pMel in frame with mellitin secretion peptide and 3xHA-TEV-GFP tag. The bacmids were prepared using DH10Bac cells according to the manufacturer's protocol for original pFastBac vector and transfected in the Sf9 cells. The resulting viruses were expanded and used at ca. 5-10 MOI to infect cultured HighFive cells in the conical shaker flasks at the density of 1-2x106 cells/ml. After 3 days post-infection the medium was harvested by centrifugation at 10000g for 5 min and total protein was precipitated by addition of 300g/L ammonium sulfate followed by 1h incubation at 4oC. The protein precipitate was separated by centrifugation for 30 min at 25000g and redissolved in 1/10 of original volume in 20mM Tris-HCl pH8.0 buffer. The target protein was then bound to the glutathion beads saturated (ca. 3mg/ml of drained beads) with anti-GFP nanobody fused to GST and washed with at least 100 volumes of 20mM Tris-HCl pH8.0, 150mM NaCl buffer. Further, CRD-3xHA constructs were cleaved directly off the glutathion beads in the same buffer using 10μg/ml of purified TEV protease (PMID 28470608) overnight at 4oC. TEV protease was subsequently removed by incubation with 10μl of Ni-NTA beads

Biacore binding analyis

The analysis was performed using Series S Sensor Chip CM5 and manufacturer's PBS-P+ buffer without DMSO. FSA and F2-99 were directly dissolved in the buffer to the final concentrations of 80 and 250µM correspondingly. CRD7 and CRD9 domains, both at 100µg/ml, were immobilized for 35min at 10µl/min yielding in total 4000-5000RU response. Binding analysis was carried out with indicated concentrations of the compound at 30µl/min with 100-120s contact time.

WaterLOGSY interaction analysis

The analysis was performed in Bruker Avance 700 MHZ Nuclear Magnetic Resonance Spectrometer. Prior to analysis, CRD7 was additionally purified in 1xPBS on Superdex 12 column to avoid any carbon-containing buffer. Buffer in all cases was 1xPBS supplemented with 10% D2O and 5% DMSO-d6. Sol41 was first dissolved in appropriate amount of DMSO-d6 and then dissolved in 1xPBS+10% D2O to the final concentration of 100µM, followed by centrifugation to remove any remaining particles. CRD domain or BSA were added to a final concentration of 1µM. All measurements were carried out at room temperature, waterLOGSY pulse program used was ephogsygpno.2. For comparison with 1D HNMR the spectra were recorded with 10000 scans, for comparison with BSA with1024 scans.

Tissue immunohistochemistry, immunofluorescence and micro-CT

After removal from the animals, tumors were photographed, sliced into fragments of ~300-400mm3 and rinsed in the excess of ice-cold 1xPBS. Subsequently, they were fixed in fresh 10% PFA solution in 1xPBS for 3-5 days at 4oC. Intestinal rolls were prepared after extensive wash in the PBS and overnight fixation in 4% PFA. Femurs were cut at joints, muscles were crudely removed and the bones were fixed in fresh 10% PFA solution in 1xPBS for 3-5 days at 4oC and moved to 1xPBS until measurement. The tissues for IHC and IF analysis were embedded in paraffin blocks and cut into

slices 7-10μm thick. The slices were mounted on glass slides and stored at 4oC. For the staining, the slides were first deparaffinized in three changes each of xylene, sequence of water mixtures of EtOH with decreasing concentrations (95%, 70% and 50%) and finally water. Subsequently, antigen retrieval was performed in 20mM Tris-EDTA, pH 9.0 with 0.1% Tween-20 by heating up the slides to 95oC for 20min and gradual cooling to the room temperature. Further, the slides were blocked in 1xPBS/0.1% Triton X-100 with 3% of normal horse serum and stained with primary antibodies against β-catenin (BD Biosciences) (Sigma) at 1:200 dilution. The slides were mounted using the VectaSchield mounting medium and visualized using AiryScan LSM-880 confocal microscope (Zeiss). μCT analysis was carried out at Quantum GX microCT imaging system in high-resolution mode.

Figure legends.

Figure 1. (a) Concentration-dependent inhibition of WNT3a-induced reporter levels by compound FSA in BT-20 cells. (b) Concentration-dependent inhibition of CHIR99021-induced reporter levels by compound FSA in BT-20 cells. (c) Quantification of protein levels and Western blots (d) of active β-catenin (ABC) levels in BT-20 cells treated with WNT3a or CHIR99021 in presence of FSA (30 μM) or vehicle indicate inhibition of signaling above CHIR99021 activation. Data represent mean ± SD. ns, not significant; ***$p < 0.001$. (e) Immunoblotting of DVL2 of HCC 1395 cells treated with WNT3a show inhibition of phospho-shifted DVL2 (top arrow) in FSA (30 μM) treated cells.

Figure 2. (a) IC_{50} values (red and blue) and the corresponding efficacy values (black and grey) at 100 μM of compounds FSA or F2-99 obtained from MTT assays in a panel of TNBC cell lines. (b) Representative curves of the proliferation assay in cell lines BT-20 and HCC 1395 treated either with compounds FSA or F2-99. (c) Compounds FSA and F2-99 (15 μM) both inhibit colony formation in BT-20 and HCC 1395 cells, as well as other cell lines (d) as seen in the quantification. (e) The migration of BT-20 cells and HCC 1395 cells is inhibited in the presence of 30 μM F2-99. (f) Inhibition of migration in the presence of FSA or F2-99 (30 μM) was also quantified for the other cell lines of the TNBC panel. Data represent mean ± SD. ns, not significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 3. Manual (by electronic calipers) (a) and fluorescence-based (b) measurements of the tumor volume indicate progressive efficiency of FSA and F2-99 against IOWA-1T xenografts correlating with their pharmacokinetic profiles. No notable body weight loss or other overt toxicity signs were noted during the trial (c). Panel (d) shows representative anti-β-catenin immunostainings of paraffin sections prepared from the extracted tumors, which indicate decrease in levels of cytoplasmic β-catenin and its translocation to the plasma membrane. (e) Similar results were obtained during the treatment of patient-derived TNBC xenograft model MX140 provided by TRACE facility from collection of KU Leuven

Figure 4. (a) Concentration-dependent inhibition of WNT3a-induced response by F2-99 in FZD -/- HEK293 cells transfected with individual FZDs. (b) Sol41-linked matrix is capable of specific interaction with certain CRD domains – those of FZD7, FZD8 and FZD9 (green). (c) Sol41 interacts with the CRD domain of FZD7 while DMSO impurity peaks show no interaction as evidenced by waterLOGSY analysis. Absolute height of the waterLOGSY peaks of Sol41 in absence of CRD7 is much smaller than in its presence, indicating that free compound might also be to a degree aggregated or self-interacting – a state, which is disrupted by binding to CRD7. (d) Growth rate of FZD7, 9 and 4 (as control) KO cell lines represented as a heat map. (on this and other panels each FZD was targeted by 2 independent CRISPR constructs, labeled #1 and #2, correspondingly). Only FZD7 and 9 were capable of producing significant systemic slowdown of growth in multiple lines. (e) Analysis of the WNT pathway activity in the knockout lines. FZD7 and FZD9 emerge as major transducers of the WNT3a-mediated signaling in the TNBC cells since their knockouts results in a significant and in many cases dramatic loss of response in the response levels.

Supplementary Figure 1. (a) Compounds representing "removal" of one or several of FSA principal parts – aromatic groups I-IV or linker region between aromatic system I-III and distant aromatic ring IV. These changes in most cases abolish anti-WNT activity. The aromatic groups and flexible-linker arm can be attached to a pyrazole ring in positions 1 and 3 and 5 as well as 4, 5 and 1 correspondingly generating synonymous "turned" structure set (F2-99 compound as an example). (b) Pie chart summarizing the results of the screening of 151 compounds similar to the hit compound FSA using the TOPFlash assay. (c) IC_{50} vs maximal efficacy chart allowed the selection of the best compounds for further testing. FSA remained one of the best compounds in terms of IC_{50} and efficacy. Compounds selected for proliferation assays are marked in blue, and those that were subsequently selected for the microsomal assay are marked in red. Grey dots represent compounds which showed inhibition of Renilla luciferase reporter levels and were excluded due to their potential general toxicity.

Supplementary Figure 2. (a) Quantification of protein levels and representative figures of total β-catenin levels in L-cells treated with WNT3a or CHIR99021 in presence of FSA (30 μM) or vehicle indicate inhibition of signaling above CHIR99021 activation. Data represent mean ± SD. ns, not

significant; ***p < 0.001. (b) Immunoblotting of DVL2 and DVL3 of L-cells treated with WNT3a show inhibition of phospho-shifted DVL2 (top arrow) and phospho-shifted DVL3 (top arrow) in FSA (30 μM) treated cells.

Supplementary Figure 3. Compounds selected from the TOPFlash assay were subsequently tested for the inhibition of proliferation of the BT-20 cell line (MTT Screen 1) allowing for a first elimination for compounds with low efficacy and/or IC_{50} above 30 μM. A secondary screen using more TNBC cell lines allowed for the selection of the best compounds for microsomal stability testing (Screen 2, compounds selected in grey). "n.a." is stated when the compounds had no effect on the cell proliferation. F1-14 was eliminated due to an average low efficacy. F1-32 did not proceed further due to availability issues. Only the compounds tested *in vivo* were tested in HCC 1395 cells, due to later acquisition of the cell line. Data represent mean ± SEM.

Supplementary Figure 4. The metabolic stability of seven selected compounds using human female microsomes. Both the phase I metabolism (CYP,red) and the phase II metabolism (UGT, blue) was tested. The percentage of remaining compound was compared after 60 minutes of incubation with human female microsomes. Compounds FSA, F2-27, F2-95 and F2-99 were most stable and selected for *in vivo* experiments (marked in grey).

Supplementary Figure 5. Mice were injected subcutaneously with 50 mg/kg of the selected compounds (FSA, F2-27. F2-95 and F2-99. The compounds plasma levels were measured at 2h, 4h, 8h, and 24h. The determined area under the curve (AUC) represents the actual exposure to the compound.

Supplementary Figure 6. (a) The colonies (BT-20 and HCC 1395) that formed after treatment with 15 μM FSA and vehicle were of different morphology. Whereas vehicle treated colonies were mostly tightly packed, compound treated colonies were made up of cells which are more dispersed. (b) Survival of the MCF10A cells treated with FSA or F2-99.

Supplementary Figure 7. (a) Photographs of the tumors extracted from the animals at the endpoint of the trial and (b) quantification of their size. (c) Growth of HCC 1395 cells intramammary xenograft is significantly reduced upon treatment with F2-99 without (d) any effect of body weight of the animals.

Supplementary Figure 8. (a) Endpoint MX140 tumor xenograft pictures and (b) quantification of their size. (c) No significant body weight variations were found during the entire 50-day trial on animals bearing MX140 xenograft.

Supplementary Figure 9. (a) Compound tissue concentrations after 30 days treatment of NOD/SCID-γ (NSG) mice with 50 mg/kg F2-99. Tumour levels reached over 2mM, nearly two orders of magnitude higher than the levels found in the lung, intestine and heart.(b) FSA and F2-99 are poorly soluble in PBS, but highly soluble in mouse plasma, whereas in PBS the solubility of FSA and F2-99 is below 1 μM, in plasma the solubility is above 60 μM. (c) Compound cellular uptake was measured using HCC 1395 cells. After 2 h the concentration in the cells was higher that the in the initial media. The uptake plateaus around 260 μM.

Supplementary Figure 10. (a-d) Levels of the principal blood components – red blood cells (RBC, a), platelets (PLT, b), haemoglobin (HGB, c) and haematocrit (HCT, d) - were compared in vehicle- and F2-99-treated animals. For unclear reason, only platelets show increased counts. (e-f) Potential adverse effect of the treatment with F2-99 were assesed by microCT scan of the femur of the animals after 50-day trial. Quantifications of various bone properties shown on 6 graphs on the panel (e) (BV/TV – ratio of bone volume to trabecular volume; BMD – bone marrow density, TMD – trabecular density), and (f) – representative 3D reconstruction of trabecular zone and cross-section. The compound significantly affected only one of these parameters – trabecular volume (expressed as BV/TV) while other remained intact, indicating mild effect of the compound on bone density.

Supplementary Figure 11. (a) Representative images of the intestinal epithelium microstructure and (b) quantification of the villus length, crypt depth and density per mm of intestinal length in ileum show no differences in the vehicle- or F2-99-treated animals.

Supplementary Figure 12. (a) All the FZD except FZD 3, 6 and 8 demonstrate ability to transduce canonical signaling upon their transfection in the FZD -/- HEK293 cells and activation with WNT3a. Concentration-dependent inhibition of WNT3a-induced response by FSA (b) and Sol41 (d) in FZD -/- HEK293 cells transfected with individual Frizzleds. (c) IC_{50} analysis obtained from the graphs on panels (a) and (b) as well as from Figure 4a indicates that FZD7 and 9 can be confirmed as the strongest interaction partners of FSA/99 compounds.

Supplementary Figure 13. (a) Structures of the compounds Sol41 and Sol42, their primary aminogroup linker and double bond are highlighted in green and red, respectively. (b) Anti-WNT activity profile of Sol41 and Sol42 as compared to parental FSA as well as related F2-99. (c) Sol41-linked affinity matrix is capable binding of higher amounts of several other CRD domains of FZDs, which are not eluted specifically by Sol41, but can be identified when analyzing total CRD protein contents bound to the beads. (d) CRD7 only weakly interacts with beads loaded with Sol42 – an inactive analog of Sol41 created by removing necessary flexibility in its structure through introduction of the double bond.

Supplementary Figure 14. (a) Structure of compound #19 identified in batch 1 of the in silico screening of the compounds binding to CRD domain of FZD7 (b) Proposed pharmacophore based on compound #19 with 3 aromatic and hydrophobic groups and 3 H-bond donors (c) 3 specific inhibitors of Wnt pathway identified among 48 best interactors identified in batch 2 of the screening show remarkable similarity to FSA/99 compounds.

Supplementary Figure 15. (a) 1H NMR peak assignment of Sol41 between CD_3OD_3 and H_2O of Sol41. (b) Sol41 interacts with the CRD of FZD7 but shows no interaction with BSA (see the table on the right for peak assignment, also for Fig. 4c) (c-d) Binding profiles and K_d estimations of the FSA (c) and F2-99 (d) and CRD of FZD7 and 9 immobilized on the Biacore CM-5 chip.

Supplementary Figure 16. (a) Viability of the cells directly after viral transduction followed by puromycin selection. These results show that FZD7 and especially 9 are indeed playing crucial role in survival of majority of TNBC cell lines, with death levels of some of the lines exceeding 90% of

control value. (b) Representative set of growth curves for MDA-MB 231 line. (c) viability of the selected FZD-knockout cell lines to the treatment with F2-99. (d) HCC1806 and HCC1395 show statistically significant resistance to the F2-99 action upon removal of target FZD7 and FZD9 proteins.

Supplementary Figure 17. (a) IC_{50} and plateau (or maximum) efficacy observed for treatment of BT-20 cells transfected with a panel of luciferase reporters for different pathways. (b,c) Concentration-dependent survival (including WT HEK293 as a reference) and colony forming assay using FZD -/- HEK293s cells. The cells were incubated with either vehicle or FSA or F2-99.

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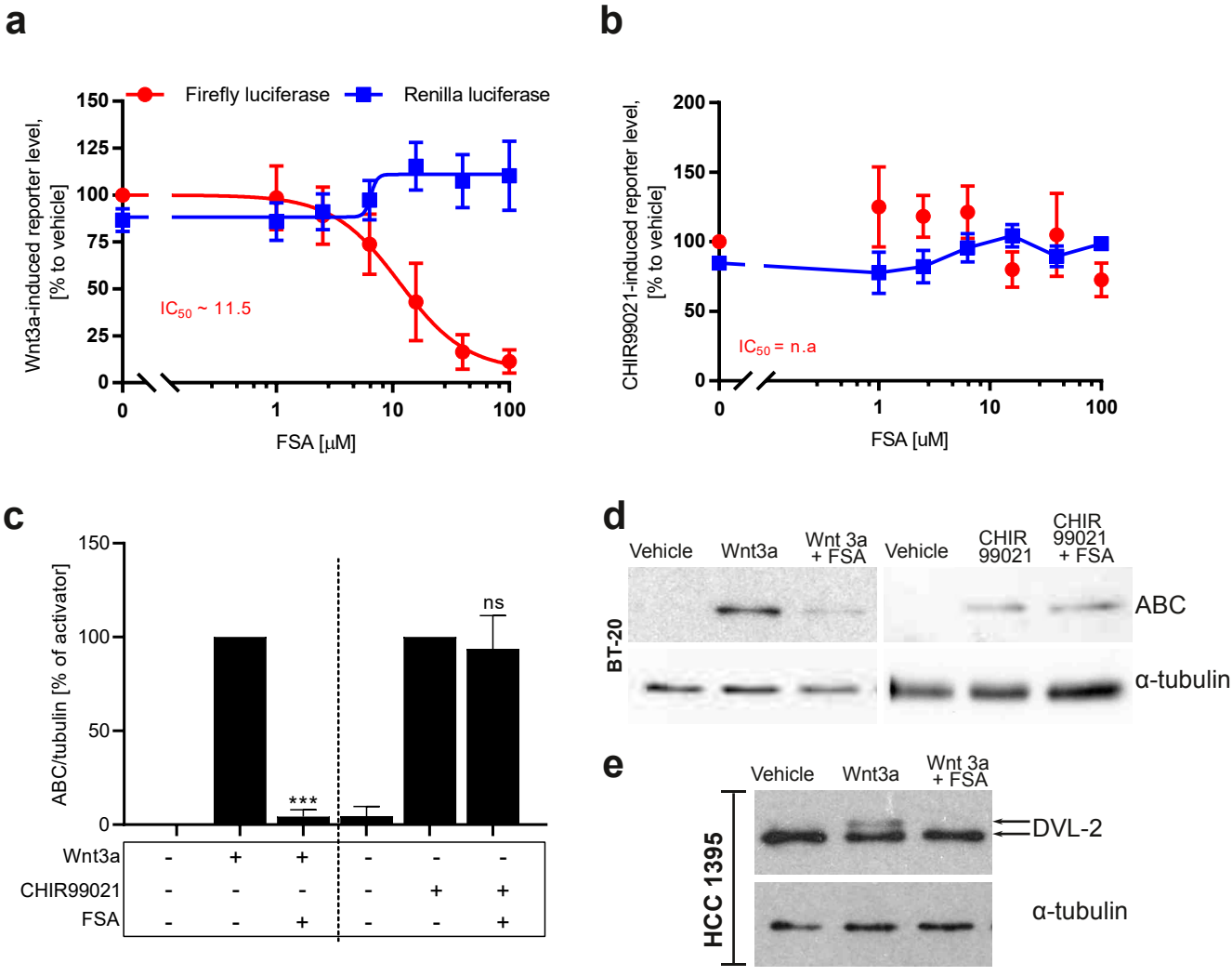
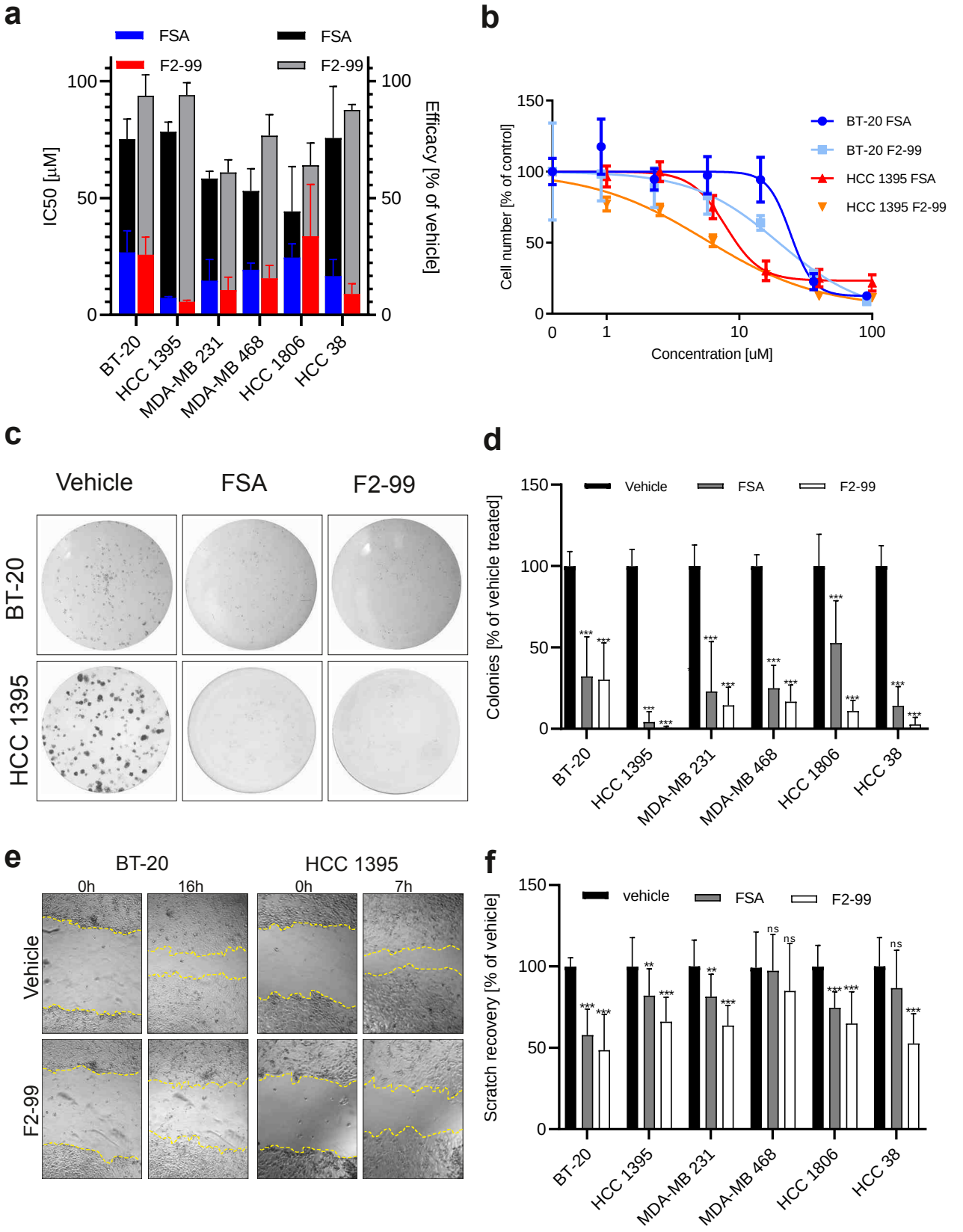


Koval et al, Figure 1



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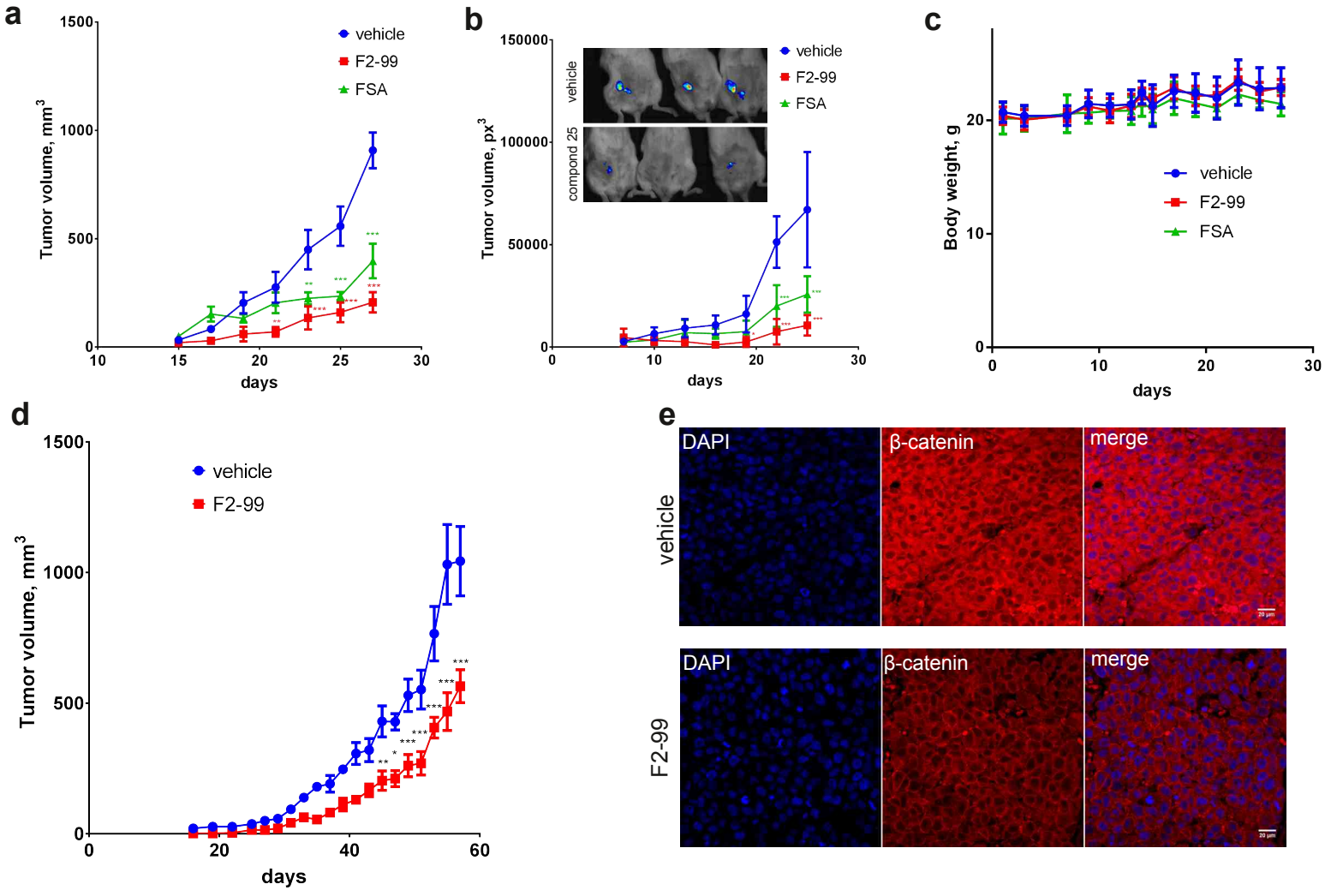

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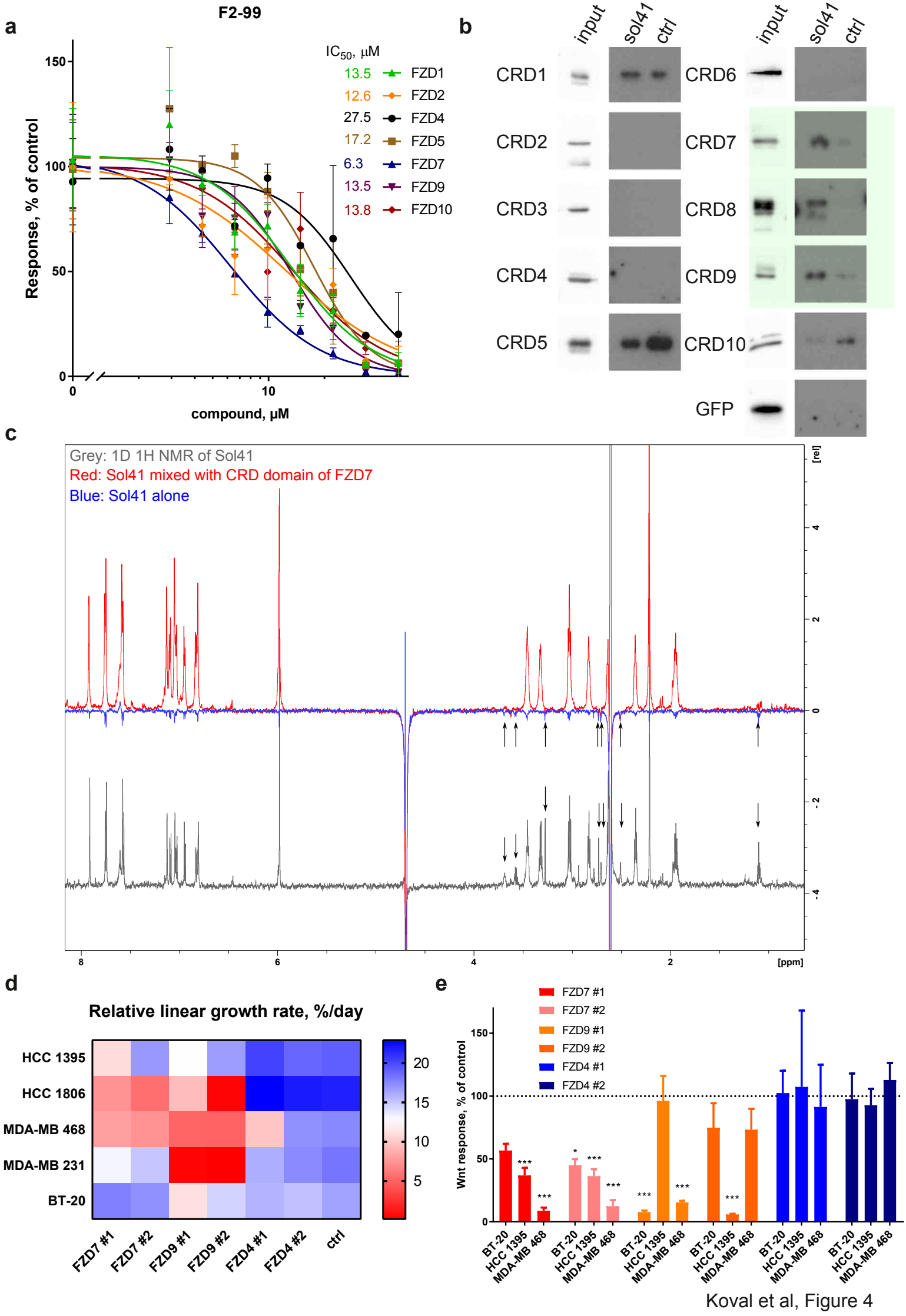

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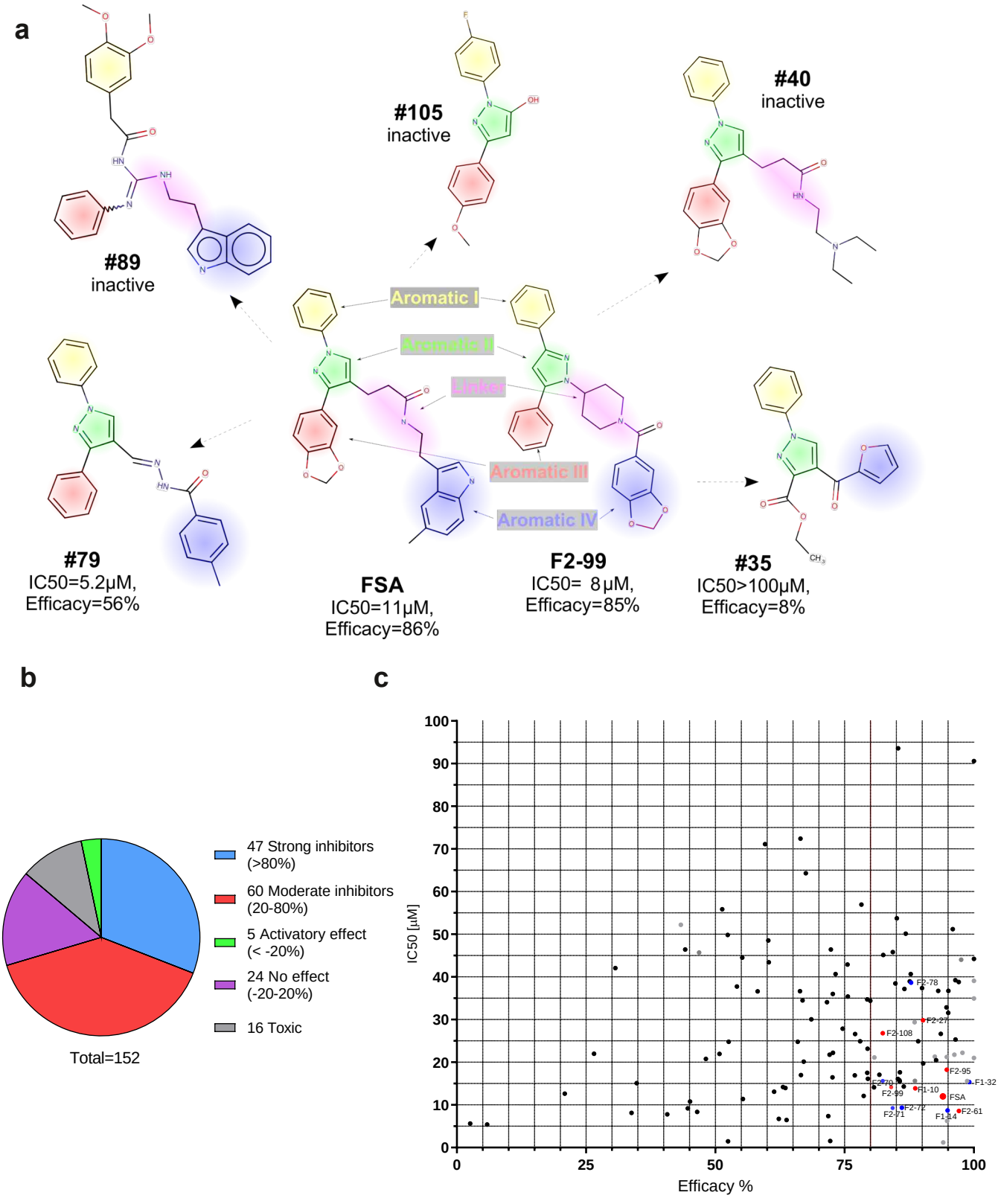

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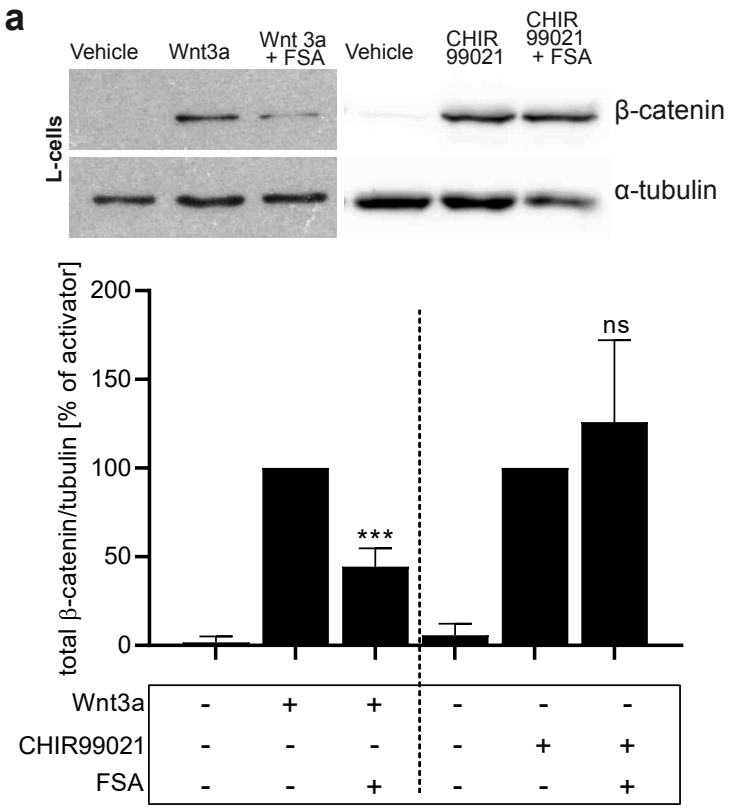
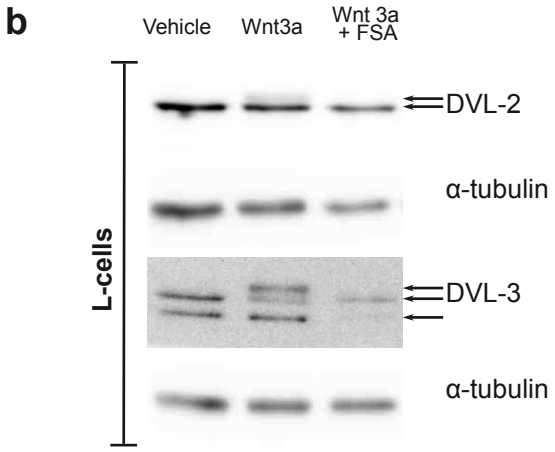
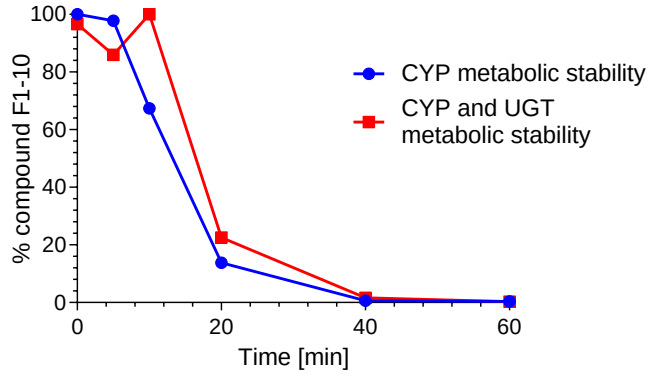


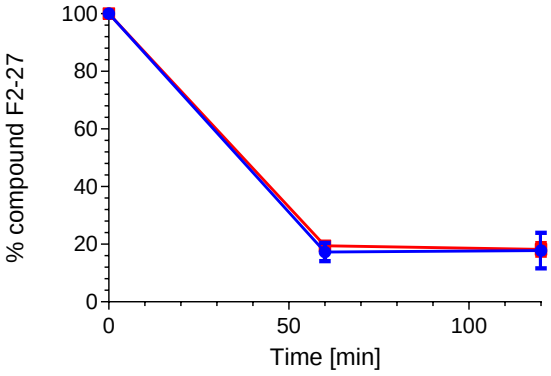


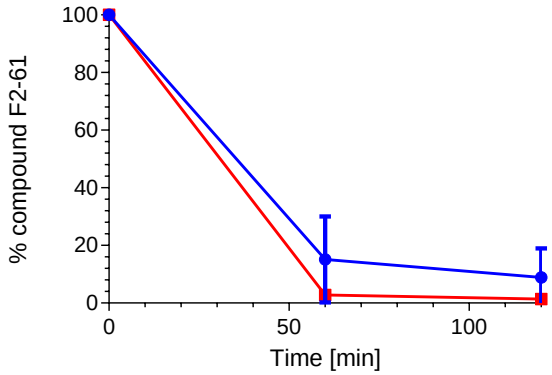
Koval et al, Supplementary Figure 2

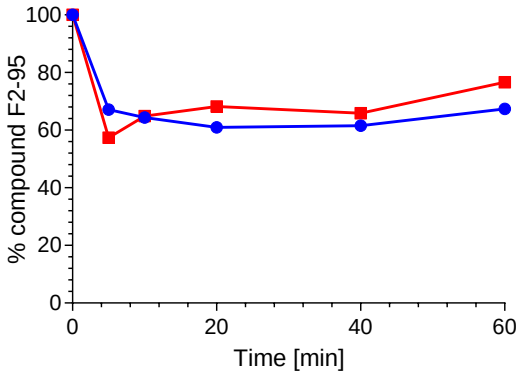
	MTT Screen 1		MTT Screen □						Averages o□ Screens 1&2		Additional MTT	
	B□□20		MDA□□B 231		MDA□□B 468		HCC 1806				HCC 1395	
Com□ound	□C□□ □□□□ □	□□icacy □□]	□C□□ □□□□ □	□□icacy □□]	□C□□ □□□□ □	□□icacy □□]	□C□□ □□□□ □	□□icacy □□]	□C□□ □□□□ □	□□icacy □□]	□C□□ □□□□ □	□□icacy □□]
F1□10	11.1 ± 2.2	68.7 ± 8.6	6.9 ± 0.1	60.4 ± 5.2	8.1 ± 3.0	79.5 ± 12.1	17.3 ± 9.1	64.6 ± 20.2	10,9 ± 2,3	68,3 ± 4,1		
F1□14	10.5 ± 0.9	81.9 ± 4.1	5.5 ± 0.5	54.9 ± 2.4	11.4 ± 2.4	59.6 ± 8.1	19.1 ± 3.5	63.9 ± 8.4	11.6 ± 2,8	65,1 ± 5,9		
F1□□2	22.8 ± 2.1	100.0 ± 7.8	11.2 ± 2.4	100.0 ± 0.0	24.9 ± 3.8	100 ± 12.2	26.8 ± 5.1	100.0 ± 16.3	□1.□ ± 3,5	10□,□ ± □,0		
F□□□7	30.6 ± 7.1	100.0 ± 0.0	19.9 ± 8.4	80.1 ± 15.75	25.7 ± 4.7	99.4 ± 10.6	□38.2	88.6 ± 1.8	28,6 ± □,8	92 ± □,8	10.0 ± 2.2	96.8 ± 7.8
F□□□6	□38.5	27.0 ± 12										
F□□□1	19.3 ± 5.0	87.1 ± 12.8	24.8 ± 11.5	82.3 ± 20.4	20.8 ± 2.7	85.6 ± 6.4	□34.8	47.5 ± 0.8	□□,9 ± 3,5	75,6 ± 9,4		
F□□□0	□90.2	□81.0										
F□□□1	n.a	n.a.										
F□□□2	□38.8	34.5 ± 11.0										
F□□□8	□43.7	100.0 ± 0.0										
F□□□5	23.1 ± 10.6	100.0 ± 21.4	17.5 ± 6.9	100.0 ±18.0	19.1 ± 2.9	100 ±8.5	□36.3	97.5 ± 0.5	□□,0 ± 4,2	99,4 ± 0,6	9.4 ± 0.9	98.6 ± 3.9
F□□□9	25.8 ± 3.8	93.8 ± 4.5	10.7 ± 3.2	61.0 ± 2.7	15.7 ± 3.2	76.7 ± 5.2	33.7 ± 12.8	64.0 ± 5.5	□1.□ ± 5,1	□3,9 ± 7,5	5.7 ± 0.3	94.0 ± 3.1
F2□10□	24.22 ± 9.7	100.0 ± 19.4	26.3 ± 17.3	100.0 ± 29.6	27.7 ± 6.2	100.0 ± 14.4	□36.0	90.0 ± 11.6	28 ,□ ± □,6	97,□ ± 2,5		
FSA	26.8 ± 4.6	75.3 ± 4.3	14.7 ± 5.2	58.3 ± 1.8	19.3 ± 1.7	53 ± 5.5	24.5 ± 3.0	44.3 ± 9.6	□1.□ ± 2,7	□7,7 ± 6,5	7.3 ± 0.3	78.3 ± 2.4

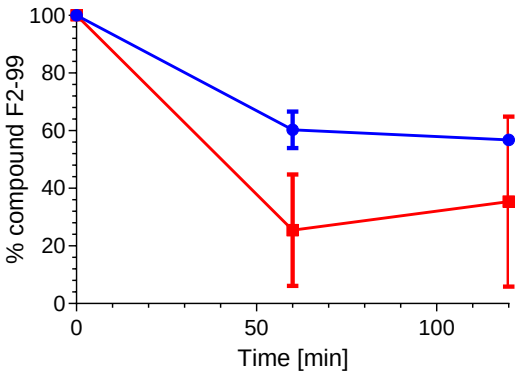
Koval et al, Supplementary Figure 3

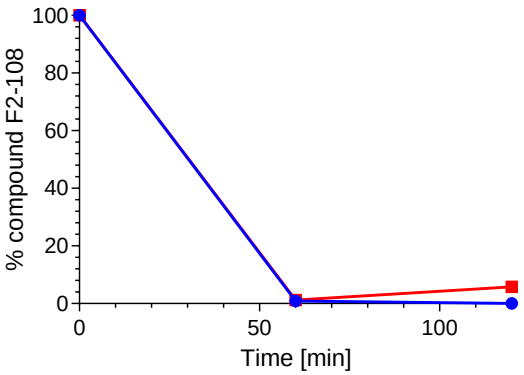


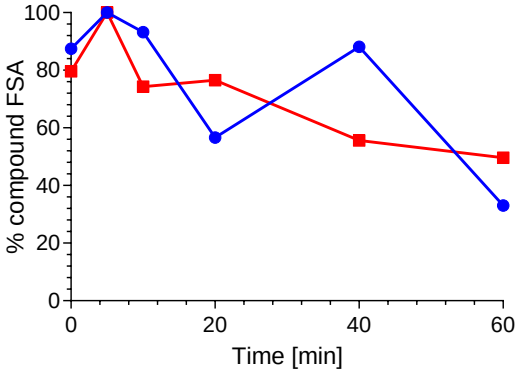




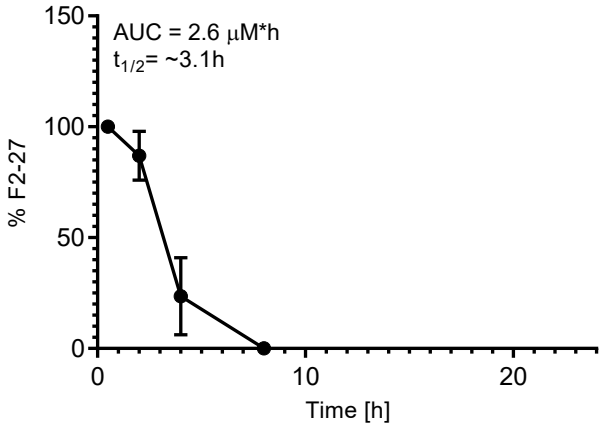


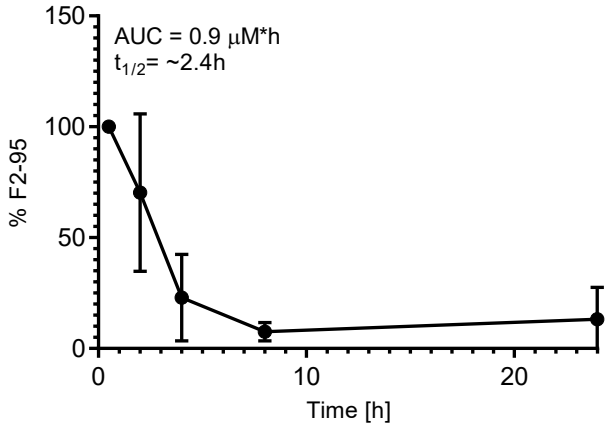


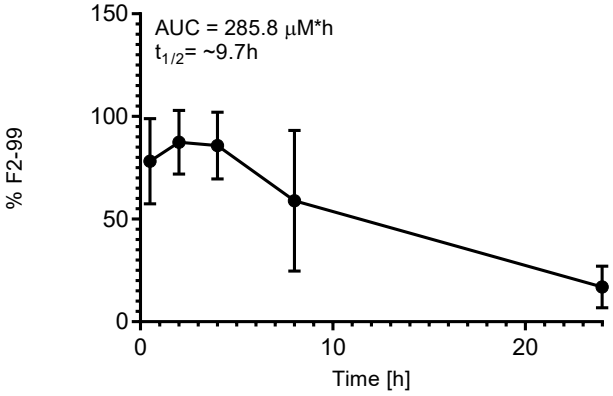


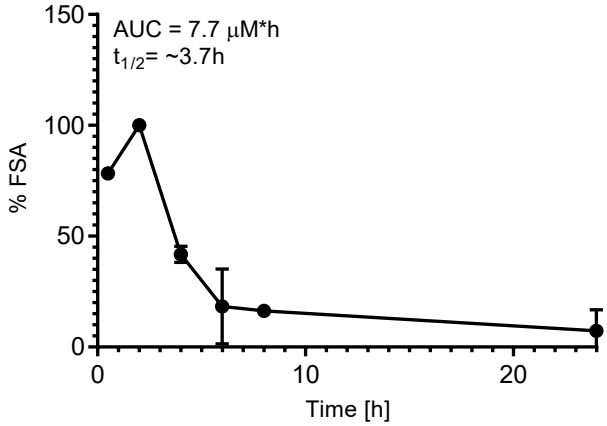


□icrosomal assay	□ com□ound at □□ min	
Com□ound	C□□ stability	C□□ and □□□ stability
FSA	33	49
F1-10	0	0
F2-27	17	19
F2-61	15	3
F2-95	67	76
F2-99	60	25
F2-108	1	1









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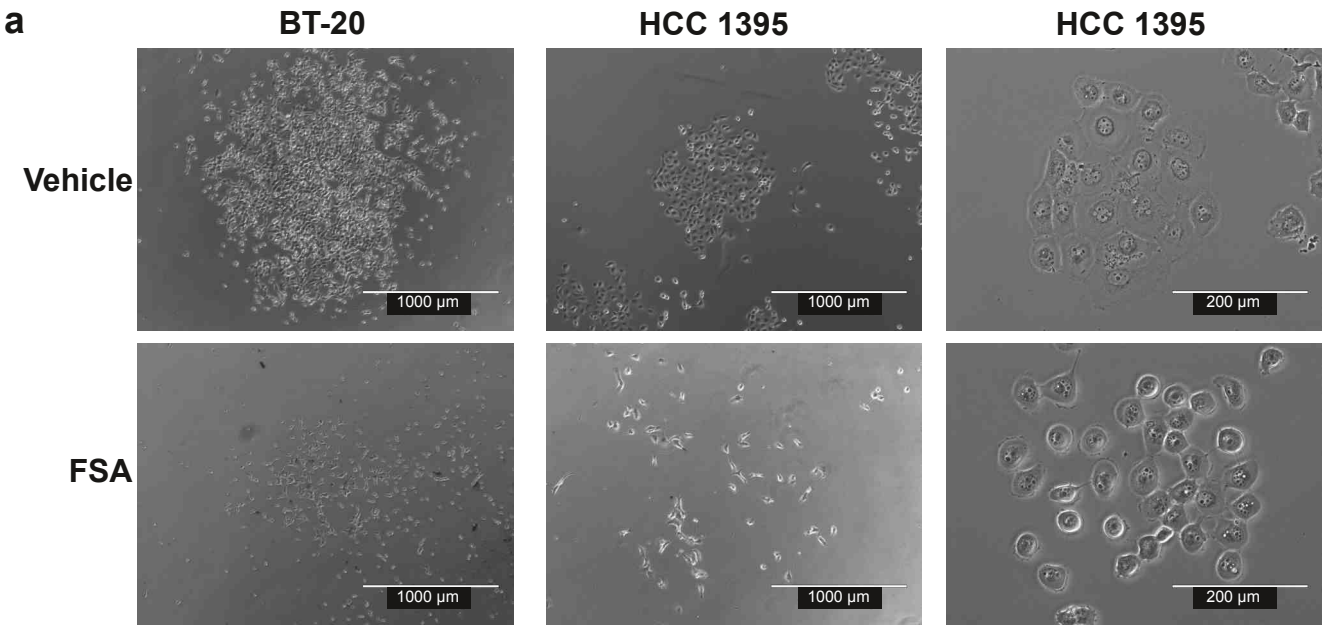

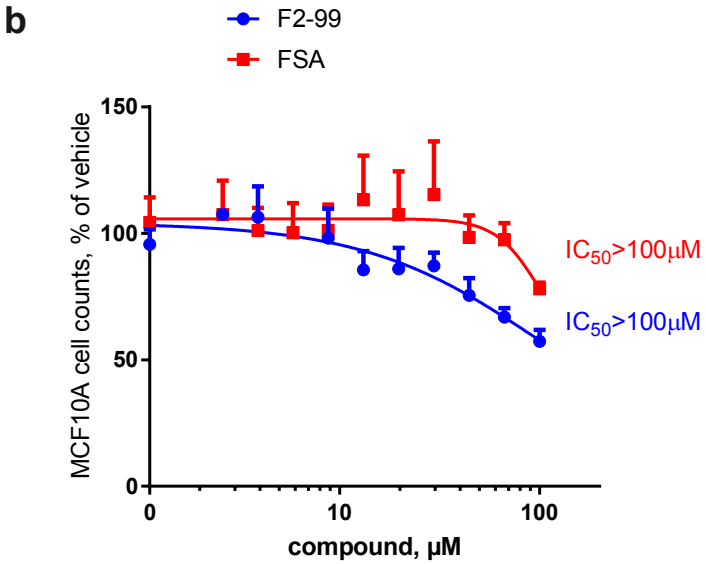

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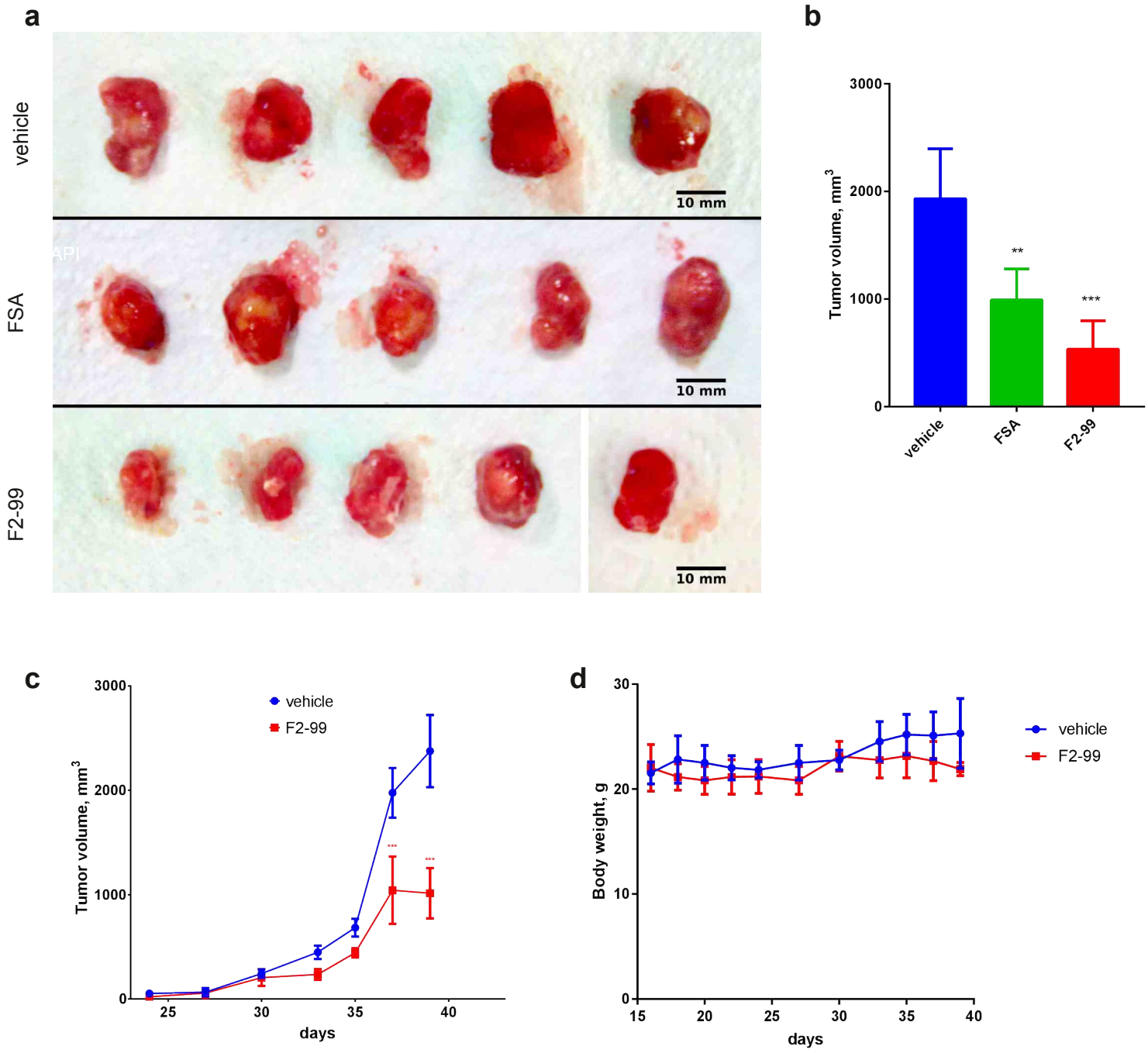

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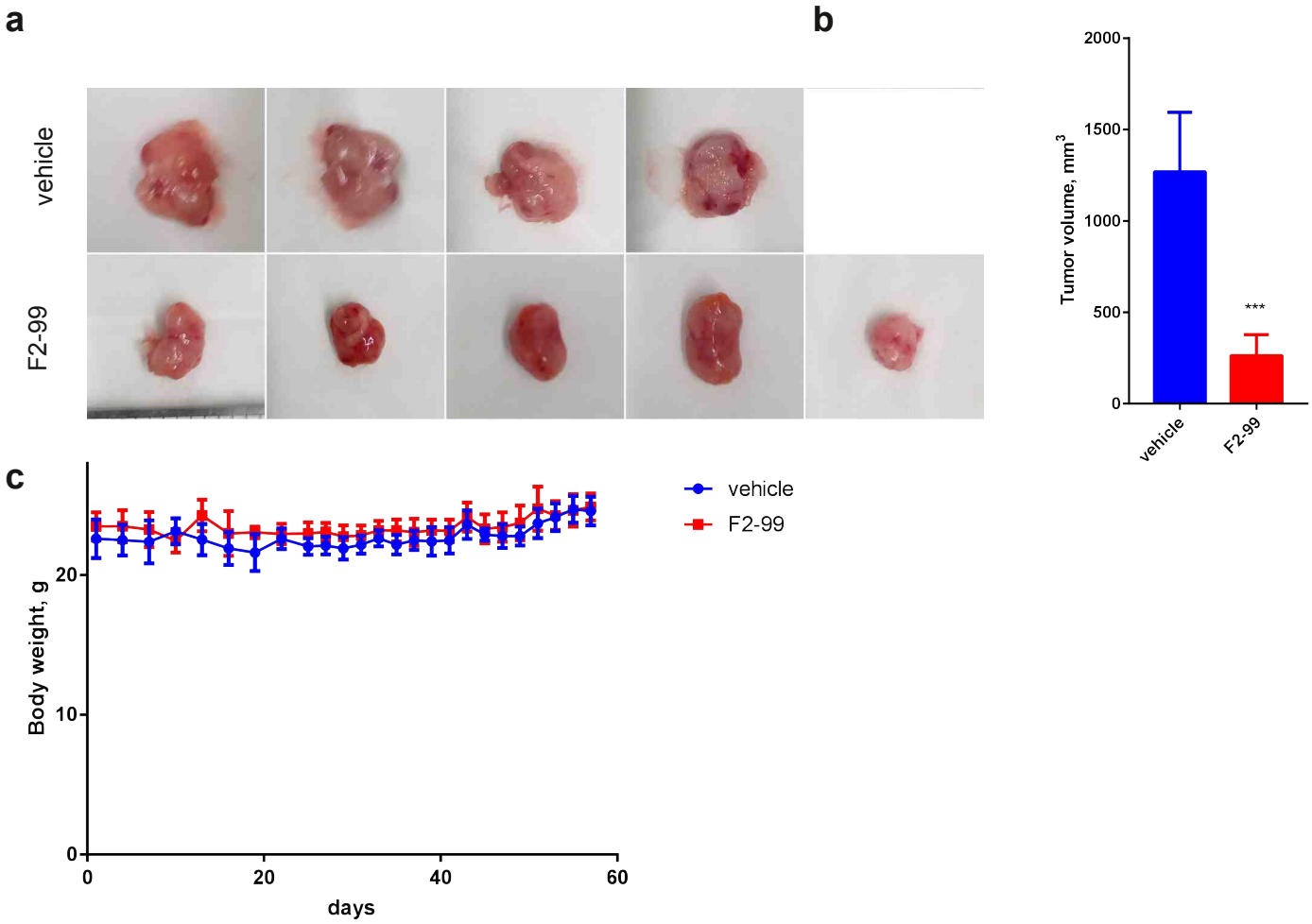

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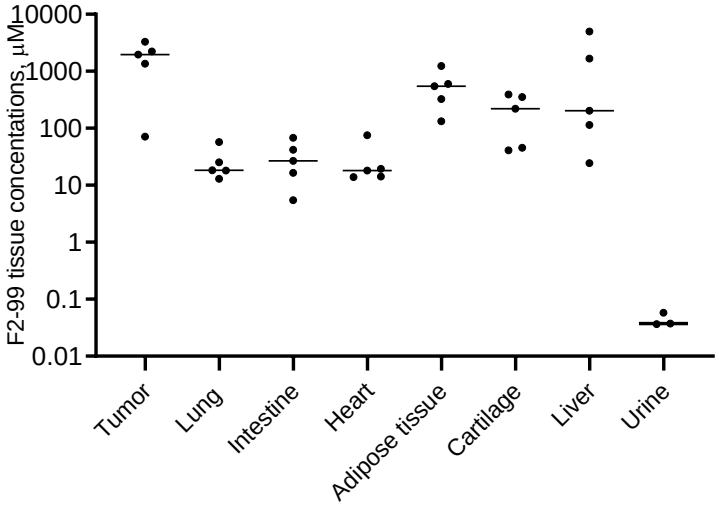
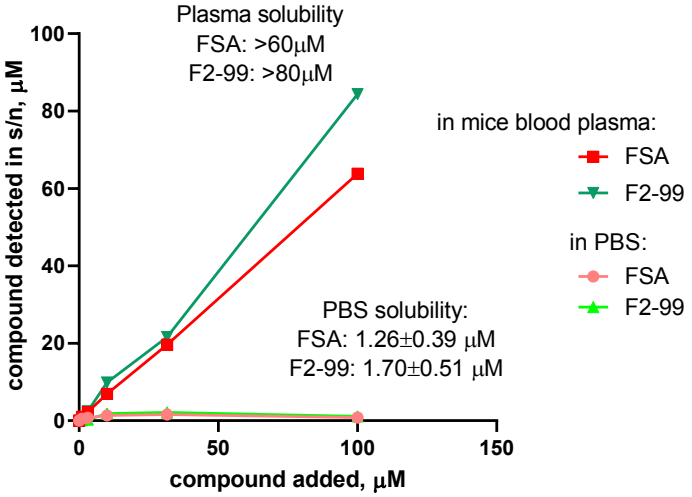


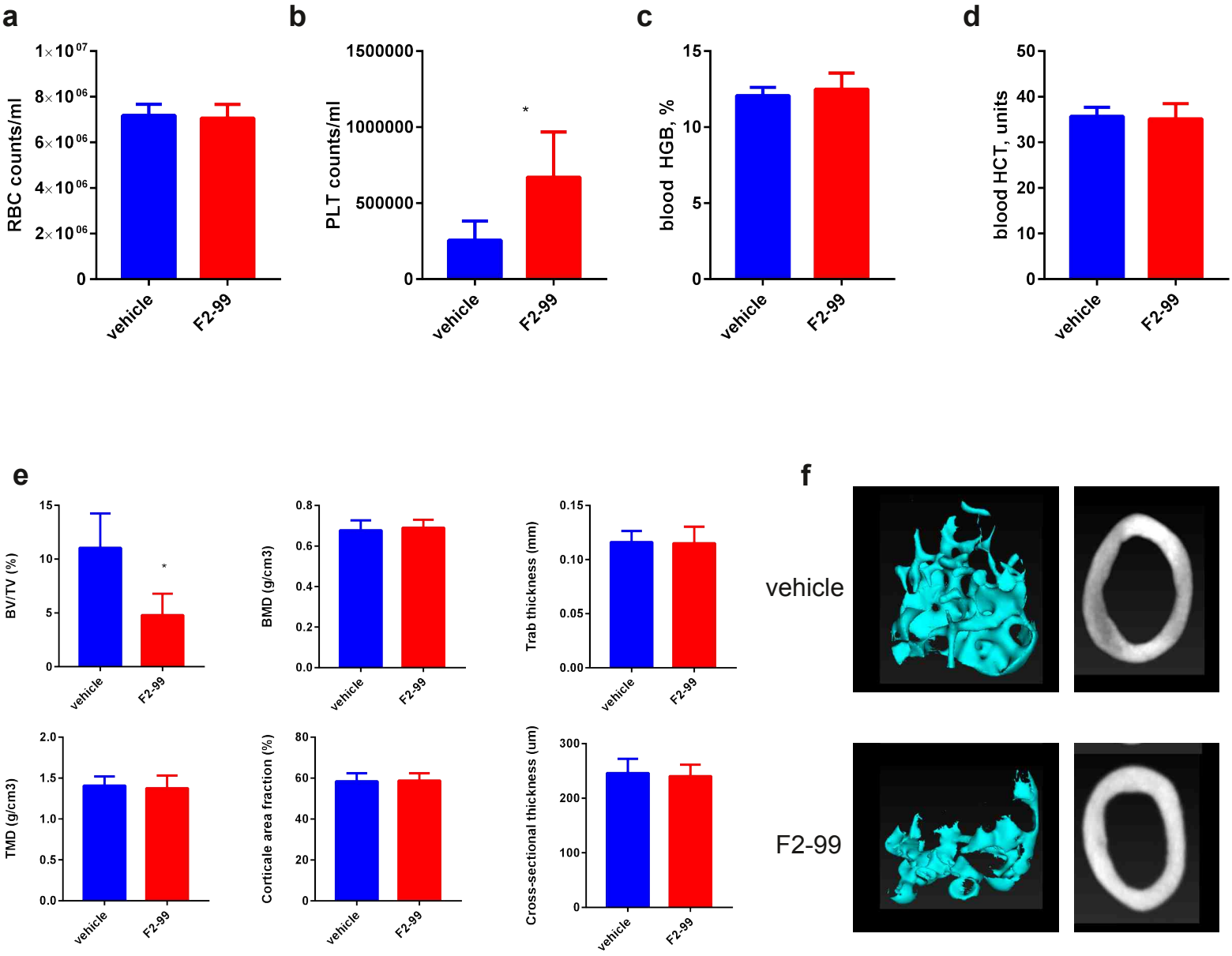
b



c

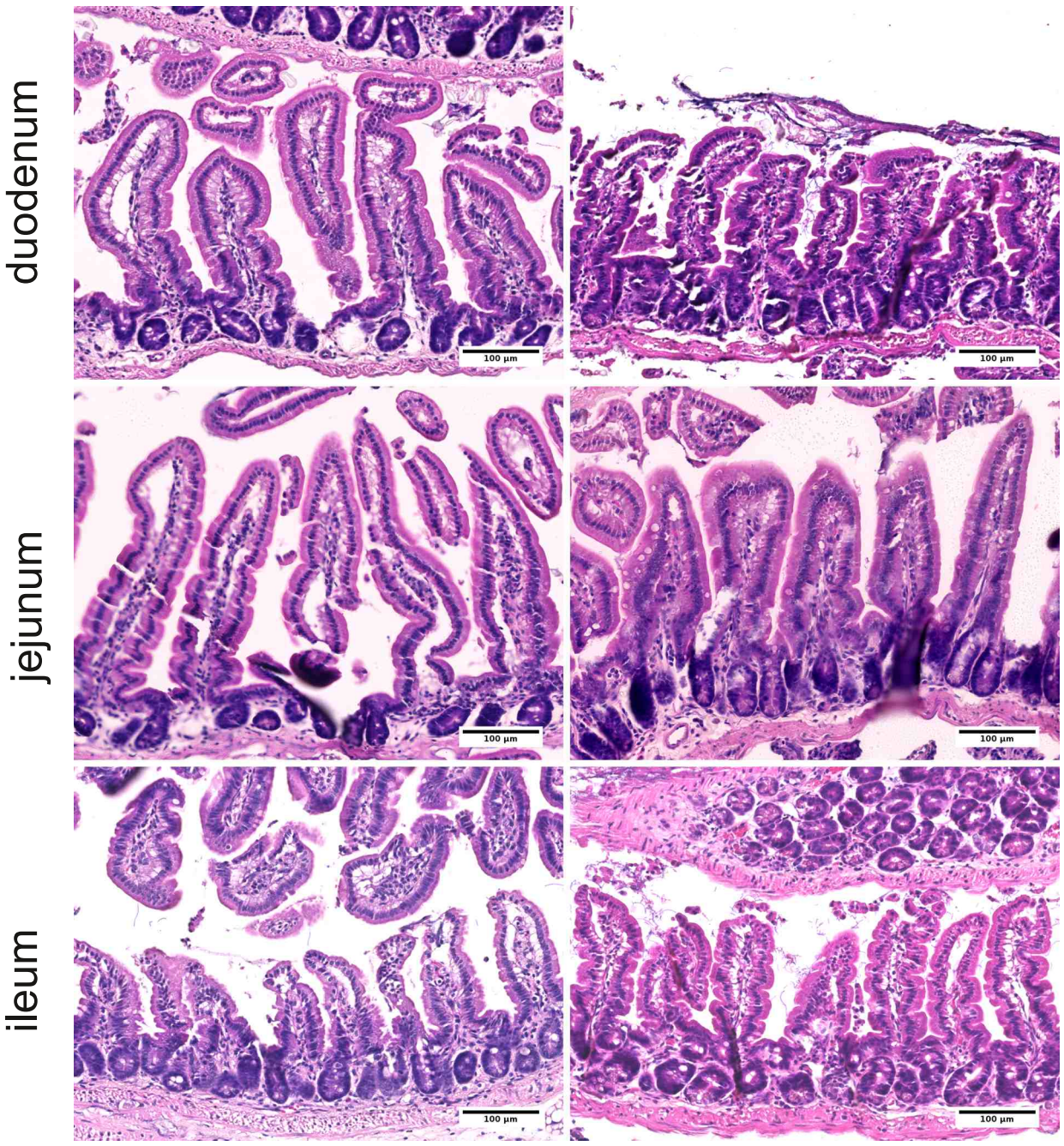
Cellular uptake	F2-99 [µM]		
F2-99 added [µM]	**Medium t=0h**	**Medium t=2h**	**Cells t=2h**
1	1.09	0.90	11.50
25	24.72	15.78	272.67
100	97.82	56.44	255.36

Koval et al, Supplementary Figure 9

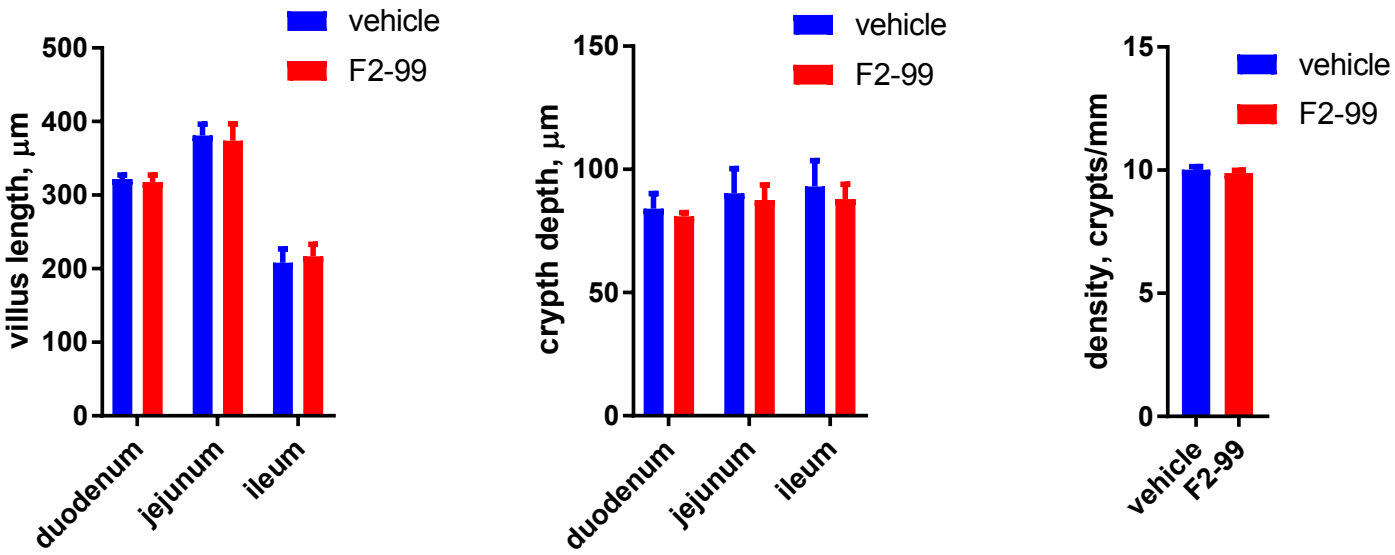


Koval et al, Supplementary Figure 10

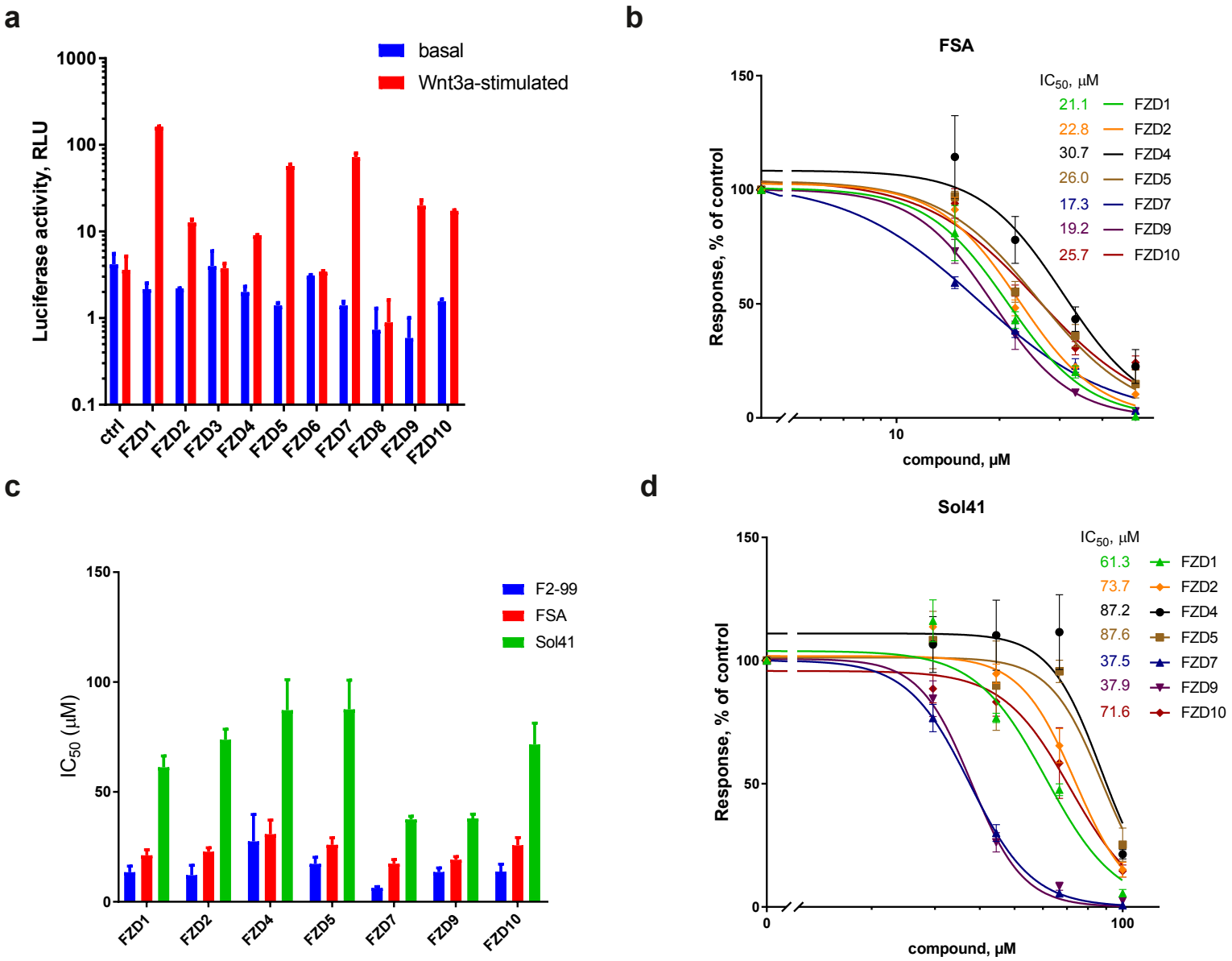
a



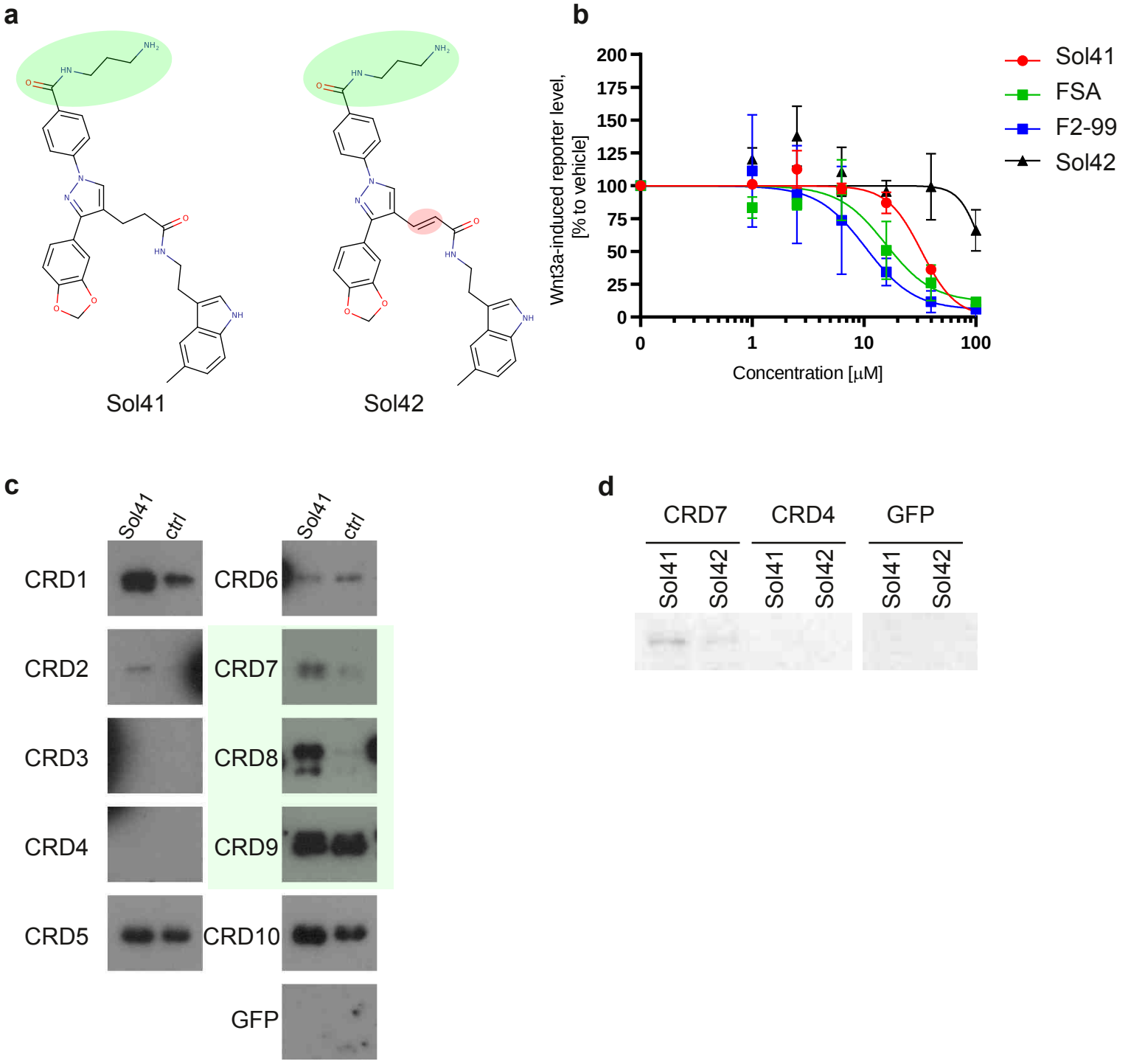
b



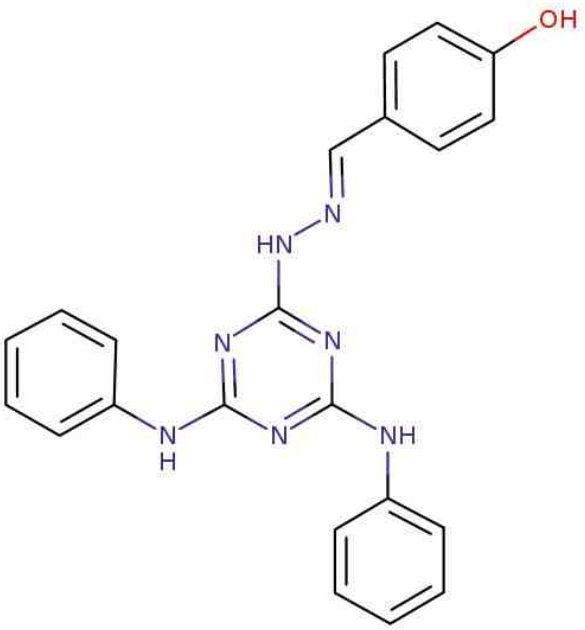
Koval et al, Supplementary Figure 11



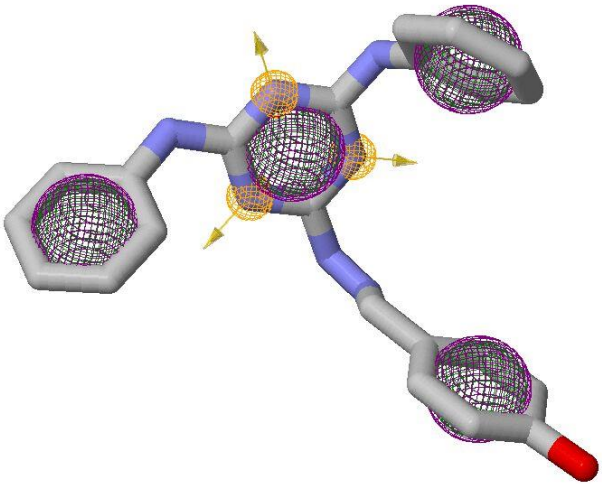
Koval et al, Supplementary Figure 12


a
Sol41
Sol42
b
Wnt3a-induced reporter level, [% to vehicle]
200
175
150
125
100
75
50
25
0
0
1
10
100
Concentration [μM]
Sol41
FSA
F2-99
Sol42
c
Sol41
ctrl
CRD1
CRD2
CRD3
CRD4
CRD5
CRD6
CRD7
CRD8
CRD9
CRD10
GFP
d
CRD7
CRD4
GFP
Sol41
Sol42

a Compound #19, in silico batch 1

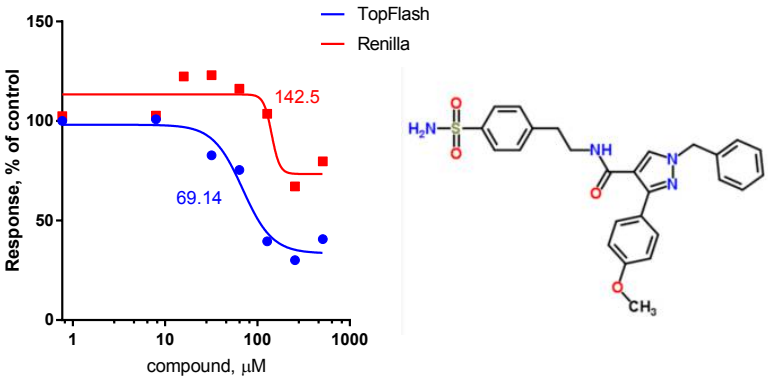


b Pharmacophore used for batch 2 screening

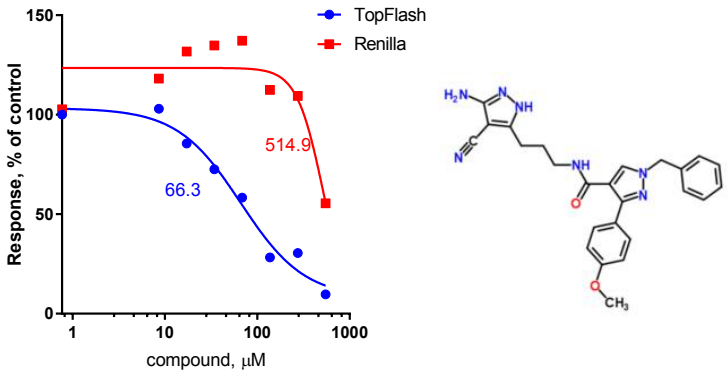


c

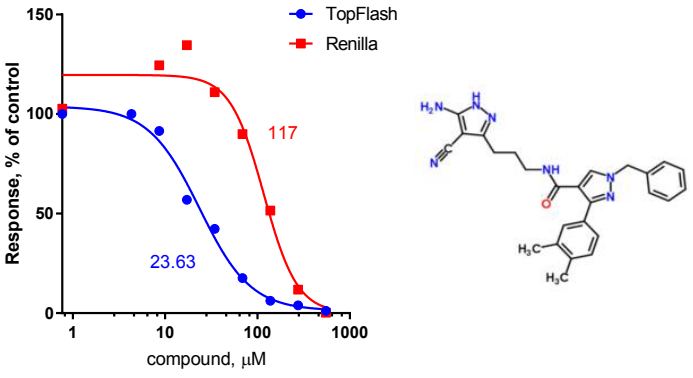
Z88302469



Z223116716

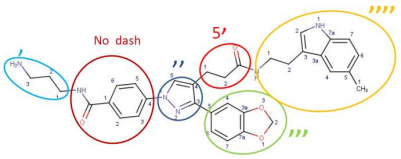


Z51988626



Koval et al, Supplementary Figure 14

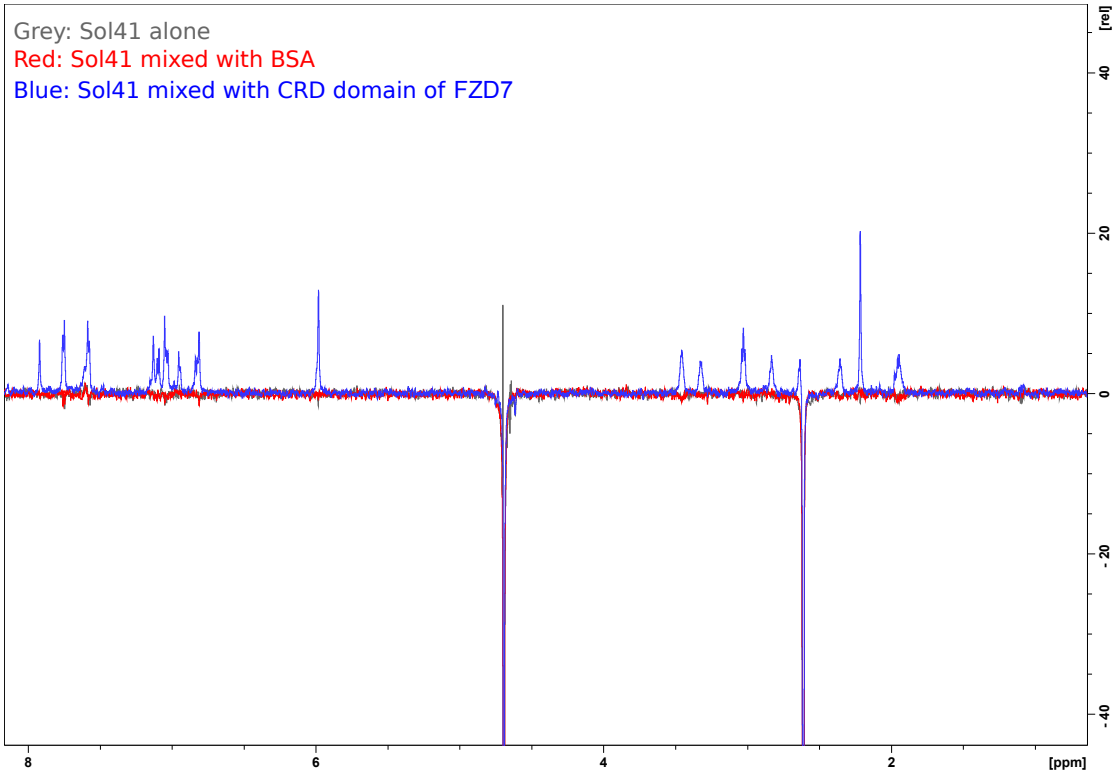
a



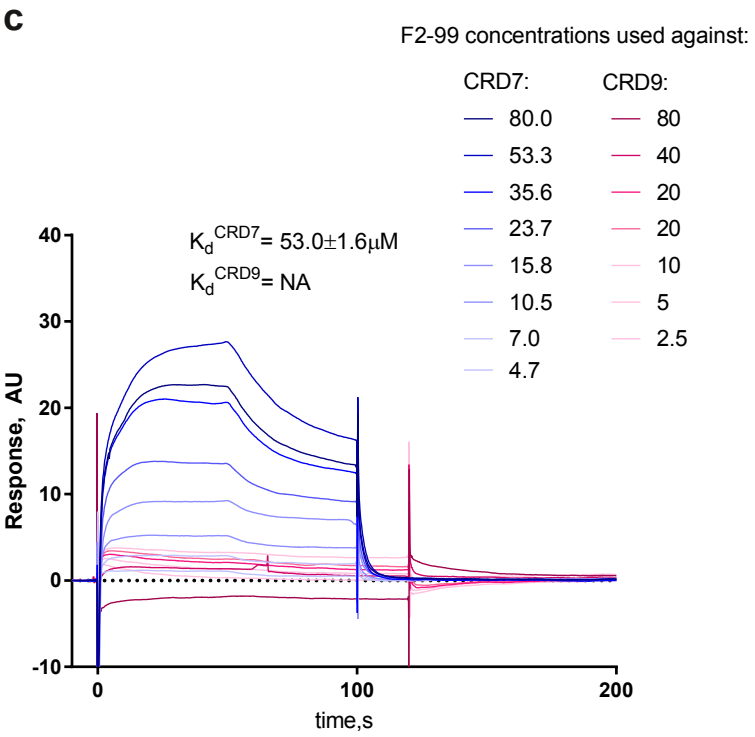
N-(3-aminopropyl)-4-(3-(benzo[d][1,3]dioxol-5-yl)-4-(3-((2-(5-methyl-1H-indol-3-yl)ethyl)amino)-3-oxopropyl)-1H-pyrazol-1-yl)benzamide

Peak shape, group	δ – CD_3OD,	δ – H_2O
s, 1H, HC(5''), pyrazol	8.14	7.92
m, 2H, HC(2, 6), phenyl	8.01-7.93	7.74-7.76
m, 2H, HC(3, 5), phenyl	7.91-7.81	7.56-7.58
s, 1H, HC(2''''), indol	7.30	7.12
m, 3H, HC(4'''', 6'''', 7''''), indol	7.27-7.15	7.02-7.10, 5 peaks
m, 3H, HC(4''', 6''', 7'''), dioxol	6.98-6.87	6.80-6.83, 3 peaks
s, 2H, $H_2C(2''')$, dioxol	6.01	5.98
t, J=6.8 Hz, 2H, $H_2C(3')$, diaminopropan	3.53	3.41, became quadruplet
t, J=7.5 Hz, 2H, $H_2C(2^{3'})$, oxopropyl	3.46	3.45
m, 4H, $H_2C(1')$, $H_2C(1'''')$, diaminopropan, indol	3.06-2.95	3.02+2.92, separated well
t, J=7.2 Hz, 2H, $H_2C(2'''')$, indol	2.88	2.62, overlapping
t, J=7.5 Hz, 2H, $H_2C(1^{3'})$, oxopropyl	2.50	2.35
s, 3H, $H_3C(1'''')$, indol	2.39	2.21
p, J=6.8 Hz, 2H, $H_2C(1')$, diaminopropan	2.00	1.94

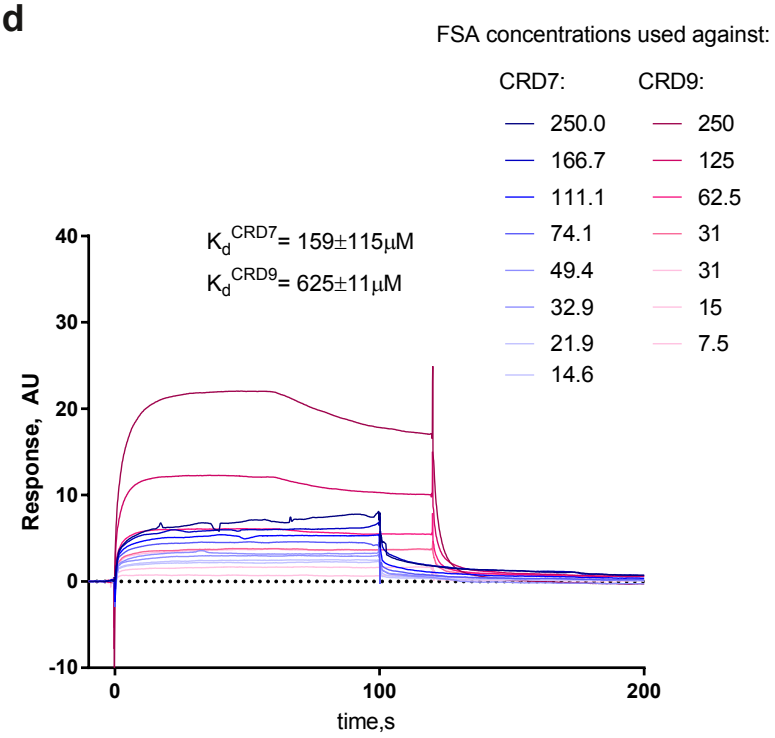
b



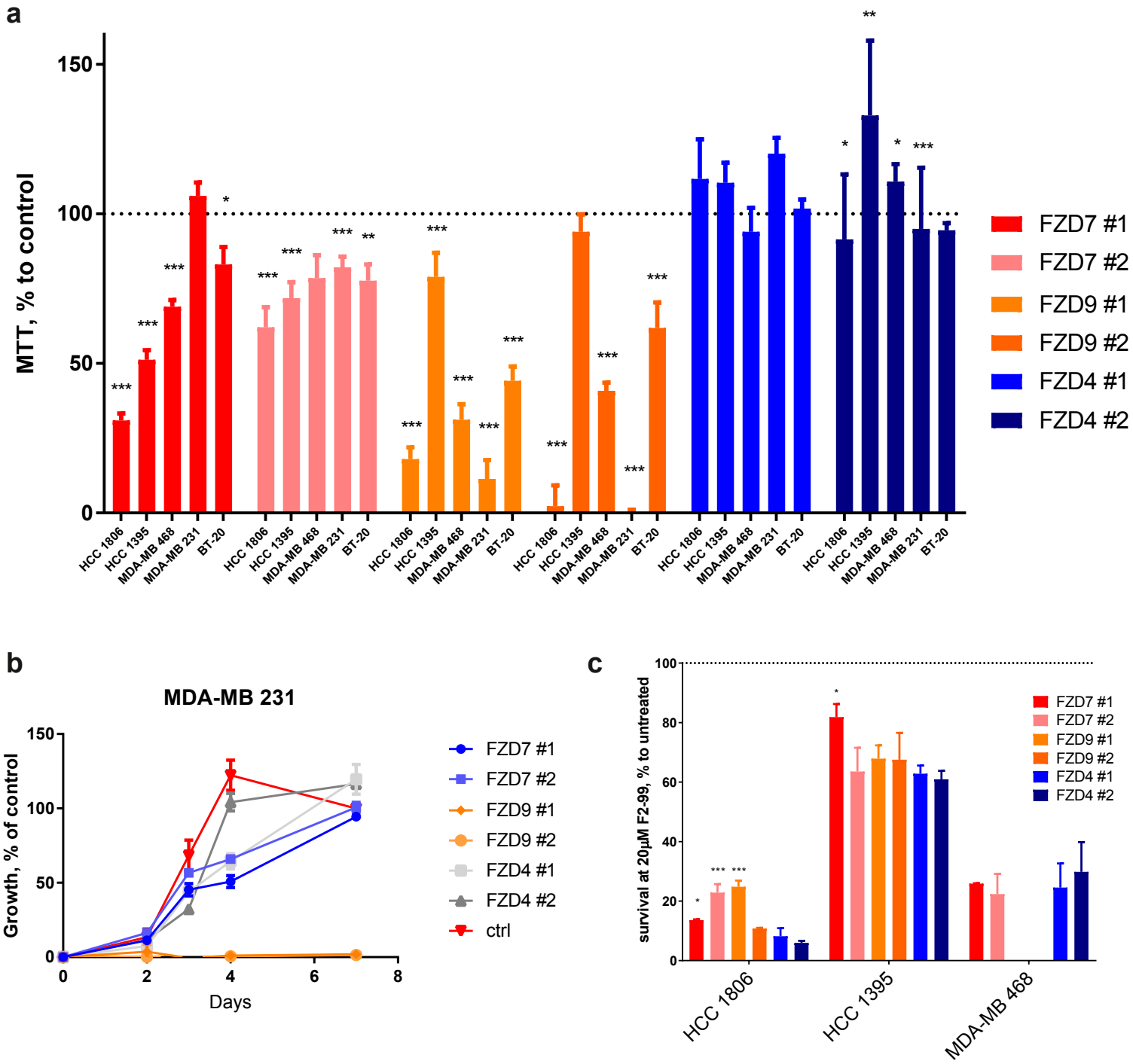
c



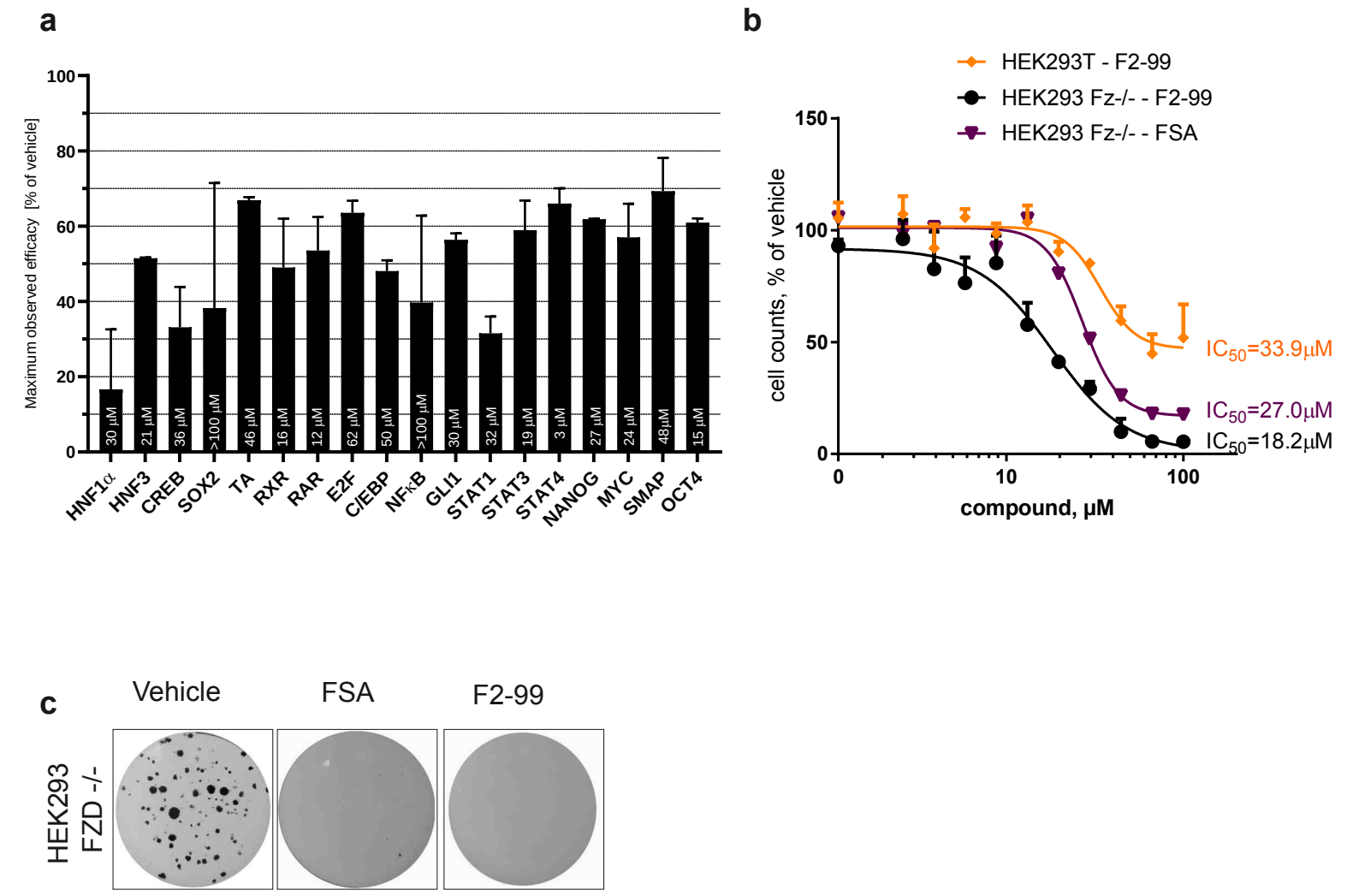
d



Koval et al, Supplementary Figure 15



Koval et al, Supplementary Figure 16



Koval et al, Supplementary Figure 17

Supplementary Table 1

TNBC-specific TOPFlash screening results

= strong inhibitor (>80%)
= moderate inhibitor (20-80%)
= activatory effect (< -20%)
= no effect (-20-20%)
= toxic effect

Library	Compound	Chemdiv N°	Average IC_{50} [µM]	Average Efficacy [%]	Comment
	FSA	F368-0488	11.5	88.63	primary hit compound
1	1	0646-0015	1.20	94.08	toxic
1	2	1683-6432	42.89	75.55	
1	3	8006-2985	n.a	n.a	
1	4	8007-7775	21.77	96.23	toxic
1	5	D296-0002	n.a	n.a	
1	6	F036-0214	34.95	100.00	toxic > 30 uM
1	7	F107-0708	46.41	44.16	
1	8	F107-1752	n.a	n.a	
1	9	F368-0031	13.07	61.36	
1	10	F368-0051	13.88	88.66	
1	11	F368-0052	15.51	85.67	
1	12	F368-0241	90.57	100.00	
1	13	F368-0286	370.64	88.50	
1	14	F368-0371	8.68	94.91	
1	15	F368-0396	46.43	72.33	
1	16	F368-0407	20.99	100.00	toxic
1	17	F368-0417	12.12	78.70	
1	18	F368-0435	16.93	76.98	
1	19	F368-0519	14.12	80.68	
1	20	G117-0261	8.35	46.48	
1	21	G771-0644	n.a	n.a.	
1	22	L153-0292	38.94	87.60	
1	23	M976-0050	n.a	n.a	
1	24	P091-0591	n.a	n.a	
1	25	V002-8085	n.a	n.a	
1	26	V003-8737	51.20	95.91	
1	27	V009-4576	7.36	71.83	
1	28	V029-2426	45.83	84.33	
1	29	Y020-3372	n.a.	n.a.	toxic
1	30	Y020-7424	39.06	100.00	toxic
1	31	Y030-6837	n.a.	n.a.	
1	32	Y040-3267	15.36	99.12	
1	33	Y041-1314	n.a.	n.a.	
1	34	Y041-3378	39.21	96.42	
2	1	2038-0155	17.65	85.70	
2	2	2367-1172	14.17	63.08	
2	3	2556-0793	n.a.	n.a.	
2	4	2556-2159	16.09	85.32	
2	5	3273-6018	n.a.	n.a.	
2	6	3277-0069	21.77	72.10	
2	7	3844-1160	64.30	67.49	
2	8	3935-0561	45.13	82.47	
2	9	3935-0593	52.21	43.33	
2	10	3935-1126	6.24	94.84	toxic
2	11	3935-1502	35.99	72.73	
2	12	3957-0132	21.29	92.44	toxic > 30 uM

2	13	3957-0161	23.16	79.43	
2	14	3957-0601	24.79	52.57	
2	15	3957-1482	29.36	88.55	toxic > 15 uM
2	16	4340-1291	44.53	55.20	
2	17	4341-0248	37.16	86.57	
2	18	4428-0124	50.13	86.82	
2	19	4428-0131	25.31	96.45	
2	20	4463-2645	72.40	66.46	
2	21	4476-1646	38.45	84.81	
2	22	4476-1855	56.95	78.24	
2	23	4476-4087	34.73	79.39	
2	24	4593-7423	40.66	87.80	
2	25	4593-7429	n.a.	n.a.	
2	26	4678-1520	36.71	95.06	
2	27	4678-1587	29.82	90.17	
2	28	4678-1599	93.57	85.36	
2	29	5169-1147	n.a.	n.a.	
2	30	5237-1505	n.a.	n.a.	
2	31	5237-1514	71.10	59.60	
2	32	5493-0072	43.44	60.35	
2	33	5629-1882	36.72	93.10	
2	34	5629-2206	105.75	42.94	
2	35	5756-2264	35.38	75.63	
2	36	6049-2356	55.85	51.34	
2	37	7210-2209	42.05	30.65	
2	38	7999-0674	17.05	81.73	
2	39	8003-7045	24.78	65.93	
2	40	8003-7096	6.47	63.76	
2	41	8007-4473	22.22	72.76	toxic
2	42	8008-5490	10.79	45.10	
2	43	8008-9817	37.76	54.16	
2	44	8010-3686	2.32	-30.50	
2	45	8012-9806	n.a.	n.a.	
2	46	8012-9813	30.07	68.57	
2	47	8013-0582	n.a	n.a.	
2	48	8018-2734	21.10	80.74	toxic
2	49	8519-0008	34.49	66.87	
2	50	8519-0011	6.71	62.27	
2	51	8519-0013	n.a.	n.a.	
2	52	8519-0016	1.55	72.21	
2	53	8519-0022	8.12	33.80	
2	54	8645-0033	34.42	79.98	
2	55	C151-0473	n.a.	n.a.	
2	56	C711-0443	48.56	60.23	
2	57	C797-0529	45.69	46.87	
2	58	C797-0532	6.23	-69.50	
2	59	C797-1033	44.02	97.56	toxic
2	60	D419-2336	22.00	26.54	
2	61	D434-0180	8.59	97.12	
2	62	D481-2402	36.66	66.38	
2	63	E136-1056	11.39	55.34	
2	64	E225-0649	17.00	66.57	
2	65	E512-0207	40.68	73.24	
2	66	F018-0414	13.95	63.55	
2	67	F368-0034	37.38	89.98	
2	68	F368-0043	31.58	95.05	
2	69	F368-0086	34.06	71.59	
2	70	F368-0269	15.62	82.39	
2	71	F368-0277	9.27	84.28	

2	72	F368-0350	9.35	86.07	
2	73	F368-0363	12.63	20.86	
2	74	F368-0395	26.61	77.03	
2	75	F368-0413	38.81	97.09	
2	76	F368-0446	21.94	50.81	
2	77	F368-0459	17.53	79.37	
2	78	F368-0473	38.66	87.84	
2	79	F368-0486	15.08	34.80	
2	80	F368-0490	n.a	100.00	toxic
2	81	F368-0510	27.87	74.63	
2	82	F368-0516	20.15	67.09	
2	83	F368-0520	15.79	85.60	
2	84	F368-0831	9.18	44.66	
2	85	F368-0893	20.76	48.18	
2	86	F368-0924	44.23	100.00	
2	87	F386-0075	n.a	n.a.	
2	88	J006-0932	n.a.	n.a.	
2	89	J006-0944	24.89	77.99	
2	90	K242-0272	19.71	90.19	
2	91	K242-0324	n.a	n.a.	
2	92	L153-0486	15.57	98.69	
2	93	L240-0005	22.23	97.81	
2	94	M475-0735	15.63	88.60	toxic >30 uM
2	95	P076-0599	18.25	94.77	
2	96	P471-0552	5.46	-208.75	
2	97	V001-5676	21.23	94.77	toxic >15 uM
2	98	V006-0629	32.87	94.69	
2	99	V006-4971	14.14	84.01	
2	100	V008-3606	14.30	86.46	
2	101	V008-4709	36.61	58.19	
2	102	V009-6783	20.48	92.70	
2	103	V011-3263	16.10	79.51	
2	104	V011-4610	49.83	52.38	
2	105	V012-0582	53.74	85.10	
2	106	V012-3790	16.47	72.66	
2	107	V019-7986	26.66	93.61	
2	108	V022-3827	26.79	82.39	
2	109	V026-9420	7.43	-90.95	
2	110	V029-9068	n.a.	n.a.	toxic
2	111	Y040-9798	7.80	40.69	
2	112	Y041-0846	24.90	89.22	
2	113	Y070-0646	5.62	2.59	
2	114	Y070-0803	5.41	5.85	
2	115	Y070-1154	1.45	52.44	
2	116	Y070-1393	2.61	-146.60	
2	117	Y070-2040	n.a.	n.a.	

Supplementary Table 2

Analysis:
The following proteins were eliminated:
- more abundant in Ctl sample compared to SOL 41
-more abundant in SOL42 sample compared to SOL41
-SOL41/Sol42 <2
- the membrane samples and cytolic fractions were then compared,
and proteins eliminated if found in CTL or SOL42 of the other sample in comparable amounts.

Proteins found in both membrane and cytosolic SOL 41 fractions.

Membrane fraction Pulldown		% of total spectra		
	Protein	CTL	SOL 42	SOL41
1	BIP_HUMAN	0	0	0.21%
2	C1TC_HUMAN	0.03%	0	0.15%
3	RSSA_HUMAN	0.03%	0.03%	0.13%
4	SYFB_HUMAN	0.03%	0.04%	0.12%
5	ATPB_HUMAN	0	0	0.06%
5	FUBP2_HUMAN	0	0	0.06%
6	PKP1_HUMAN	0	0	0.05%
7	RS21_HUMAN	0	0	0.04%
	SYEP_HUMAN	0	0	0.04%
	DSG4_HUMAN	0	0	0.04%
	HNRPK_HUMAN	0	0	0.04%
	ICAM1_HUMAN	0	0	0.04%
	KPRP_HUMAN	0	0	0.04%
	RBM14_HUMAN	0	0	0.04%
	TXND5_HUMAN	0	0	0.04%
8	SAHH_HUMAN (+2)	0	0	0.02%
	IF4A1_HUMAN (+1)	0	0	0.02%
	CAZA2_HUMAN	0	0	0.02%
	GSDMA_HUMAN	0	0	0.02%
	UCHL1_HUMAN	0	0	0.02%
	H2AJ_HUMAN (+15)	0	0	0.02%
	MTDC_HUMAN	0	0	0.02%
	H2B1B_HUMAN (+17)	0	0	0.02%
	PLD3_HUMAN	0	0	0.02%
	PA2G4_HUMAN	0	0	0.02%
	K2C6C_HUMAN	0	0	0.02%
	EF1B_HUMAN	0	0	0.02%
	SND1_HUMAN	0	0	0.02%
	LRC15_HUMAN	0	0	0.02%
	PGK1_HUMAN	0	0	0.02%
	RPN1_HUMAN	0	0	0.02%
	TMX1_HUMAN	0	0	0.02%

	FXR1_HUMAN	0	0	0.02%
	RS9_HUMAN	0	0	0.02%
	ACLY_HUMAN	0	0	0.02%
	APMAP_HUMAN	0	0	0.02%
	G6PI_HUMAN	0	0	0.02%
	PKP3_HUMAN	0	0	0.02%
	ILVBL_HUMAN	0	0	0.02%
	CELF1_HUMAN	0	0	0.02%

Cytoplasmic fraction pulldown				
		Ctl	SOL42	SOL 41
1	TBA1A_HUMAN	0	0	0.47%
2	ACTN4_HUMAN	0.03%	0.19%	0.29%
3	FAS_HUMAN	0	0.12%	0.27%
4	PYR1_HUMAN	0	0	0.18%
5	FUBP2_HUMAN	0	0.08%	0.17%
6	NU153_HUMAN	0	0.06%	0.12%
7	MOES_HUMAN	0	0.02%	0.11%
8	AHNK2_HUMAN	0.02%	0	0.11%
9	TCPZ_HUMAN	0.02%	0.04%	0.10%
10	HNRPQ_HUMAN (+1)	0	0.02%	0.10%
	PDLI7_HUMAN	0	0.02%	0.10%
11	TRFE_HUMAN	0	0	0.10%
12	CAP1_HUMAN	0.02%	0	0.09%
13	IPO5_HUMAN	0	0.04%	0.08%
13	ACTN1_HUMAN	0	0.04%	0.08%
14	IF4A1_HUMAN (+1)	0	0.02%	0.07%
15	TCPG_HUMAN	0.03%	0.02%	0.06%
16	STRAP_HUMAN	0	0.03%	0.06%
	MAGA4_HUMAN	0	0.03%	0.06%
17	IGKC_HUMAN	0	0	0.06%
18	FUS_HUMAN	0	0.02%	0.06%
	TCPD_HUMAN	0	0	0.06%
19	HPT_HUMAN	0	0	0.06%
20	TCPQ_HUMAN	0	0.02%	0.05%
	CLCA_HUMAN	0	0.02%	0.05%
	SPRE_HUMAN	0	0.02%	0.05%

	SMG9_HUMAN	0	0.02%	0.05%
21	CLCB_HUMAN	0	0	0.05%
	VAT1_HUMAN	0	0	0.05%
	AHSA1_HUMAN	0	0	0.05%
	CPNS1_HUMAN	0	0	0.05%
	HNRDL_HUMAN	0	0	0.05%
22	PRDX6_HUMAN	0.02%	0	0.04%
23	MTAP_HUMAN	0	0.02%	0.04%
24	SEC13_HUMAN	0	0	0.04%
	UBP5_HUMAN	0	0	0.04%
	XRCC6_HUMAN	0	0	0.04%
	COPD_HUMAN	0	0	0.04%
	CAN2_HUMAN	0	0	0.04%
	CAZA1_HUMAN	0	0	0.04%
25	NUP37_HUMAN	0	0	0.03%
	DCTP1_HUMAN	0	0	0.03%
	TIAR_HUMAN (+1)	0	0	0.03%
	P20D2_HUMAN	0	0	0.03%
	UBA1_HUMAN	0	0	0.03%
	PUF60_HUMAN	0	0	0.03%
	ELOC_HUMAN	0	0	0.03%
	RANG_HUMAN	0	0	0.03%
	VINC_HUMAN	0	0	0.03%
	S10AA_HUMAN	0	0	0.03%
	EIF3I_HUMAN	0	0	0.03%
	SYRC_HUMAN	0	0	0.03%
	TPM3_HUMAN (+3)	0	0	0.03%
	CYBP_HUMAN	0	0	0.03%
	EIF3E_HUMAN	0	0	0.03%
	COPZ1_HUMAN	0	0	0.03%
	BAG2_HUMAN	0	0	0.03%
	MYL6_HUMAN	0	0	0.03%
	XPO1_HUMAN	0	0	0.03%
	PYRG1_HUMAN	0	0	0.03%
	SC23B_HUMAN	0	0	0.03%
	BRAT1_HUMAN	0	0	0.03%
	AIMP1_HUMAN	0	0	0.03%
	RADI_HUMAN	0	0	0.03%
	PDXK_HUMAN	0	0	0.03%
	XPOT_HUMAN	0	0	0.03%
	RAN_HUMAN	0	0	0.03%
	I2BPL_HUMAN	0	0	0.03%
	CO3_HUMAN	0	0	0.03%
	CUL3_HUMAN	0	0	0.03%
	WDR5_HUMAN	0	0	0.03%
	A1AT_HUMAN	0	0	0.03%
	RAB5A_HUMAN	0	0	0.03%
	RS21_HUMAN	0	0	0.02%
	SYEP_HUMAN	0	0	0.02%
	CAZA2_HUMAN	0	0	0.02%

	GSDMA_HUMAN	0	0	0.02%
	ICAM1_HUMAN	0	0	0.02%
	UCHL1_HUMAN	0	0	0.02%
	CALM1_HUMAN (+2)	0	0	0.02%
	SSBP_HUMAN	0	0	0.02%
	SUMO2_HUMAN (+2)	0	0	0.02%
	DAZP1_HUMAN	0	0	0.02%
	RAB10_HUMAN	0	0	0.02%
	RBM4_HUMAN	0	0	0.02%
	PSB5_HUMAN	0	0	0.02%
	HNRPL_HUMAN	0	0	0.02%
	IF6_HUMAN	0	0	0.02%
	PDE6D_HUMAN	0	0	0.02%
	MAT2B_HUMAN	0	0	0.02%
	PSA6_HUMAN	0	0	0.02%
	RBMS1_HUMAN (+1)	0	0	0.02%
	PSA7_HUMAN	0	0	0.02%
	AP2B1_HUMAN	0	0	0.02%
	CARM1_HUMAN	0	0	0.02%
	NUDC_HUMAN	0	0	0.02%
	1A68_HUMAN (+2)	0	0	0.02%
	HPRT_HUMAN	0	0	0.02%
	TWF1_HUMAN (+7)	0	0	0.02%
	NIT1_HUMAN (+1)	0	0	0.02%
	ACTZ_HUMAN	0	0	0.02%
	RBBP7_HUMAN (+1)	0	0	0.02%
	TBB2B_HUMAN	0	0	0.02%
	YTHD2_HUMAN	0	0	0.02%
	SRP09_HUMAN	0	0	0.02%
	DNM1L_HUMAN	0	0	0.02%
	SIR2_HUMAN	0	0	0.02%
	AIP_HUMAN	0	0	0.02%
	PAAF1_HUMAN	0	0	0.02%
	TBCB_HUMAN	0	0	0.02%
26	RINI_HUMAN	0	0	0.02%
	RAB14_HUMAN	0	0	0.02%
	PCH2_HUMAN	0	0	0.02%
	FHL2_HUMAN	0	0	0.02%
	UBQL2_HUMAN	0	0	0.02%
	DDX6_HUMAN	0	0	0.02%
	UMPS_HUMAN	0	0	0.02%
	PP1A_HUMAN (+1)	0	0	0.02%
	TTC1_HUMAN	0	0	0.02%
	PANK4_HUMAN	0	0	0.02%
	MTREX_HUMAN	0	0	0.02%
	IGHG3_HUMAN	0	0	0.02%
	PSMD7_HUMAN	0	0	0.02%
	DHYS_HUMAN	0	0	0.02%
	CERU_HUMAN	0	0	0.02%
	6PGL_HUMAN	0	0	0.02%

	PPP6_HUMAN	0	0	0.02%
	RPAB1_HUMAN	0	0	0.02%
	PML_HUMAN	0	0	0.02%
	MCMBP_HUMAN	0	0	0.02%
	SC16A_HUMAN	0	0	0.02%
	AKP8L_HUMAN	0	0	0.02%
	EIF3F_HUMAN	0	0	0.02%
	SYIC_HUMAN	0	0	0.02%
	CSN8_HUMAN	0	0	0.02%
	PSD11_HUMAN	0	0	0.02%
	LA_HUMAN	0	0	0.02%
	CSTF1_HUMAN	0	0	0.02%
	RAE1L_HUMAN	0	0	0.02%
	KC1AL_HUMAN (+1)	0	0	0.02%
	TNPO3_HUMAN	0	0	0.02%
	AKAP8_HUMAN	0	0	0.02%
	CSN1_HUMAN	0	0	0.02%
	ARF4_HUMAN	0	0	0.02%
	PLP2_HUMAN	0	0	0.02%
	PRS6B_HUMAN	0	0	0.02%
	ARPC4_HUMAN	0	0	0.02%
	CNN2_HUMAN	0	0	0.02%

Supplementary Table 3

Batch #1					IC50, μM (for selected compounds)	
Compound #	Catalog #	Supplier	Anti-Wnt activity, 100μM	Renilla levels, 100μM	TopFlash (Wnt)	Renilla (toxic)
1	STK083626	Vitas-M Laboratory, Ltd.	654.6±95.4	188.2±44.8	>100	
2	STK066538	Vitas-M Laboratory, Ltd.	69.3±8.5	165.3±41.1	~79	>100
3	STK577789	Vitas-M Laboratory, Ltd.	145.5±23.6	105.5±20.6	>100	
4	STK576506	Vitas-M Laboratory, Ltd.	35.5±1	99.3±23	>100	
5	40130142	ChemBridge Corporation	30.7±24.7	3.2±2.7		
6	D112-0009	ChemDiv, Inc.	4.1±2.8	0.4±0		
7	Z25180309	ENAMINE Ltd.	109.3±6.3	121.3±33.9		
8	Z295196994	ENAMINE Ltd.	133.1±4.2	130.4±29.6		
9	Z25938887	ENAMINE Ltd.	66.5±8.6	154.7±44.8	>100	
10	F0886-0076	Life Chemicals Inc.	108.9±24.4	126.2±31.3		
11	OSSL_064812	PBMR Labs Ukraine	86.4±2.4	134.9±2.5		
12	PHAR156284	Pharmeks, Ltd.	100.7±4.1	117.3±11.1		
13	PHAR120697	Pharmeks, Ltd.	111.8±20.4	148.7±2		
14	STK604882	Vitas-M Laboratory, Ltd.	175.1±51.6	162.1±18.2		
15	STK585356	Vitas-M Laboratory, Ltd.	219.2±1.8	17±4.3		
16	STK639279	Vitas-M Laboratory, Ltd.	131.2±35.7	156.5±9		
17	STK929967	Vitas-M Laboratory, Ltd.	112.9±3.2	152.8±0.2		
18	STK603960	Vitas-M Laboratory, Ltd.	180.6±4.8	154.3±11.4	>100	
19	STK100086	Vitas-M Laboratory, Ltd.	34.7±11.4	9.6±6.3	~103	~191
20	STL284607	Vitas-M Laboratory, Ltd.	118.3±31.7	130±5.1		
21	STK599194	Vitas-M Laboratory, Ltd.	104.3±3.1	142.6±15.4		
22	STK106214	Vitas-M Laboratory, Ltd.	103.8±2.9	149.7±3.1		
23	STK281263	Vitas-M Laboratory, Ltd.	109.4±34.8	175.1±1.3		
24	STK591642	Vitas-M Laboratory, Ltd.	90.8±20.3	170.8±40		
25	STK053014	Vitas-M Laboratory, Ltd.	181.1±139	159±25.6	>100	

'= inhibitor (0%-80%)'
'=activatory effect (< -20%)'
'=no effect (80-120%)'
= toxic effect

Batch #2				IC50, μM (for selected compounds)		
			Anti-Wnt activity, 100μM	TopFlash (Wnt)	Renilla (toxic)	Max. Inhibition,
1	4116-0024	ChemDiv, Inc.	95.37±5.91			
2	C797-0529	ChemDiv, Inc.	102.02±14.24			
3	K280-0029	ChemDiv, Inc.	85.44±13.85			
4	K280-0321	ChemDiv, Inc.	96.84±22.27			
5	K280-0486	ChemDiv, Inc.	75.76±16.2	8.5±2.22	NA	66.1±10.32
6	Z88302469	ENAMINE Ltd.	17.06±7.22	69.14±32.64	142.5±31.2	33.4±12.48
7	Z223116716	ENAMINE Ltd.	9.52±10.71	66.3±12.61	~519	33.4±12.48
8	Z51988626	ENAMINE Ltd.	4.65±5.15	23.6±13.54	117.0±12.2	7.9±6.4
9	Z26881913	ENAMINE Ltd.	90.44±8.75			
10	Z1157837350	ENAMINE Ltd.	91.65±10.2			
11	Z1162446065	ENAMINE Ltd.	82.34±18.73			
12	Z230128842	ENAMINE Ltd.	94.61±13.52			
13	F0586-0158	Life Chemicals Inc.	90.26±13.42			
14	F0586-0330	Life Chemicals Inc.	95.59±13.73			
15	F3407-1824	Life Chemicals Inc.	114.77±18.96			
16	F3407-1826	Life Chemicals Inc.	90.69±11.96			
17	F3407-1839	Life Chemicals Inc.	98.21±12.47			
18	F3407-1879	Life Chemicals Inc.	91.45±12.15			
19	F3407-1895	Life Chemicals Inc.	95.65±10.86			
20	F3382-5196	Life Chemicals Inc.	109.3±33.66			
21	7114550968	Otava, Ltd.	1.33±0.13			
22	7210460013	Otava, Ltd.	40.83±11.54	6.4±4.64	NA	28.6±7.5
23	PHAR085061	Pharmeks, Ltd.	19.38±9.33	5.3±2.45		29.1±6.2
24	PHAR109426	Pharmeks, Ltd.	5.79±4.05	NA	~100	NA
25	PHAR158313	Pharmeks, Ltd.	29.52±9.32	>100	NA	NA
26	OSSK_497707	Princeton Bio	1.02±0.15	~135	>300	4.5±3.6
27	G-670/3481103	Specs	15.77±21.47	NA	>100	NA
28	N-919/1471211	Specs	119.9±63.7			
29	G-690/1102801	Specs	15.28±0.56			
30	STK705262	Vitas-M Laboratory, Ltd.	90.99±21.9			
31	STK799472	Vitas-M Laboratory, Ltd.	114.3±32.48			
32	STK800440	Vitas-M Laboratory, Ltd.	96.14±36.54			
33	STK817542	Vitas-M Laboratory, Ltd.	83.66±31.92			
34	STK706056	Vitas-M Laboratory, Ltd.	92.53±18.11			
35	STK817855	Vitas-M Laboratory, Ltd.	107.7±43.28			
36	STK817964	Vitas-M Laboratory, Ltd.	101.33±12.95			
37	STK078971	Vitas-M Laboratory, Ltd.	115.35±13.24			
38	STK565523	Vitas-M Laboratory, Ltd.	42.05±11.61	4.43±4.53	NA	66.9±10.6
39	STK553091	Vitas-M Laboratory, Ltd.	87.69±21.71			
40	STK904704	Vitas-M Laboratory, Ltd.	108.7±23.95			
41	STL031962	Vitas-M Laboratory, Ltd.	112.1±37.54			
42	STK369805	Vitas-M Laboratory, Ltd.	98.01±31.33			
43	STK673468	Vitas-M Laboratory, Ltd.	96.87±34.14			
44	STK677929	Vitas-M Laboratory, Ltd.	109.4±33.89			
45	STK063065	Vitas-M Laboratory, Ltd.	41±5.06	10.69±4.6	NA	35.3±17.45
46	STK011318	Vitas-M Laboratory, Ltd.	123.6±13.92			
47	STK063196	Vitas-M Laboratory, Ltd.	108.2±42.1			
48	STK374556	Vitas-M Laboratory, Ltd.	83.75±6.92			