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Novel Small Molecule Inhibitors of Cancer Stem Cell Signaling Pathways

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Abstract The main aim of oncologists worldwide is to understand and then intervene in the primary tumor initiation and propagation mechanisms. This is essential to allow targeted elimination of cancer cells without altering normal mitotic cells. Currently, there are two main rival theories describing the process of tumorigenesis. According to the *Stochastic Model*, potentially any cell, once defunct, is capable of initiating carcinogenesis. Alternatively the *Cancer Stem Cell* (CSC) *Model* posits that only a small fraction of undifferentiated tumor cells are capable of triggering carcinogenesis. Like healthy stem cells, CSCs are also characterized by a capacity for self-renewal and the ability to generate differentiated progeny, possibly mediating treatment resistance, thus leading to tumor recurrence and metastasis. Moreover, molecular signaling profiles are similar between CSCs and normal stem cells, including Wnt, Notch and Hedgehog pathways. Therefore, development of novel chemotherapeutic agents and proteins (e.g., enzymes and antibodies) specifically targeting CSCs are attractive pharmaceutical candidates. This article describes small molecule inhibitors of stem cell pathways Wnt, Notch and Hedgehog, and their recent chemotherapy clinical trials.

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Introduction

Tumors are considered as hierarchically organized systems with heterogeneous cell populations, including tumor-initiating, stromal, endothelial, hematopoietic and infiltrating cells with variable self-renewal capabilities, differentiation and tumor propagation potential [1–3]. There are two main rival theories explaining the process of tumorigenesis: the *Cancer Stem Cell (CSC)* and *Stochastic Models* (Fig. 1). The Stochastic Model (or clonal evolution) suggests that many tumor cells have the same potential to give a rise to cancer growth and metastasis [4, 5]. Many years later Canadian researchers found that only a small subset of acute myeloid leukemia (AML) cancer cells were capable of sustaining tumor growth after transplantation into non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID) [6, 7]. This resulted in the development of the CSC model suggesting that some cancer cell populations are reminiscent of somatic stem cells. According to this model cancer develops from small populations of tumor initiating cells through the accumulation of genetic, epigenetic and somatic defects, and altered signaling within the cell's micro-environment [8]. These altered cells are intermixed with the bulk of tumor cells, which may require a different treatment strategy for effective inhibition and suppression of tumor growth and relapse. However, probably both premises play a role in the cancer development and the predominance of a particular mechanism of cancerogenesis

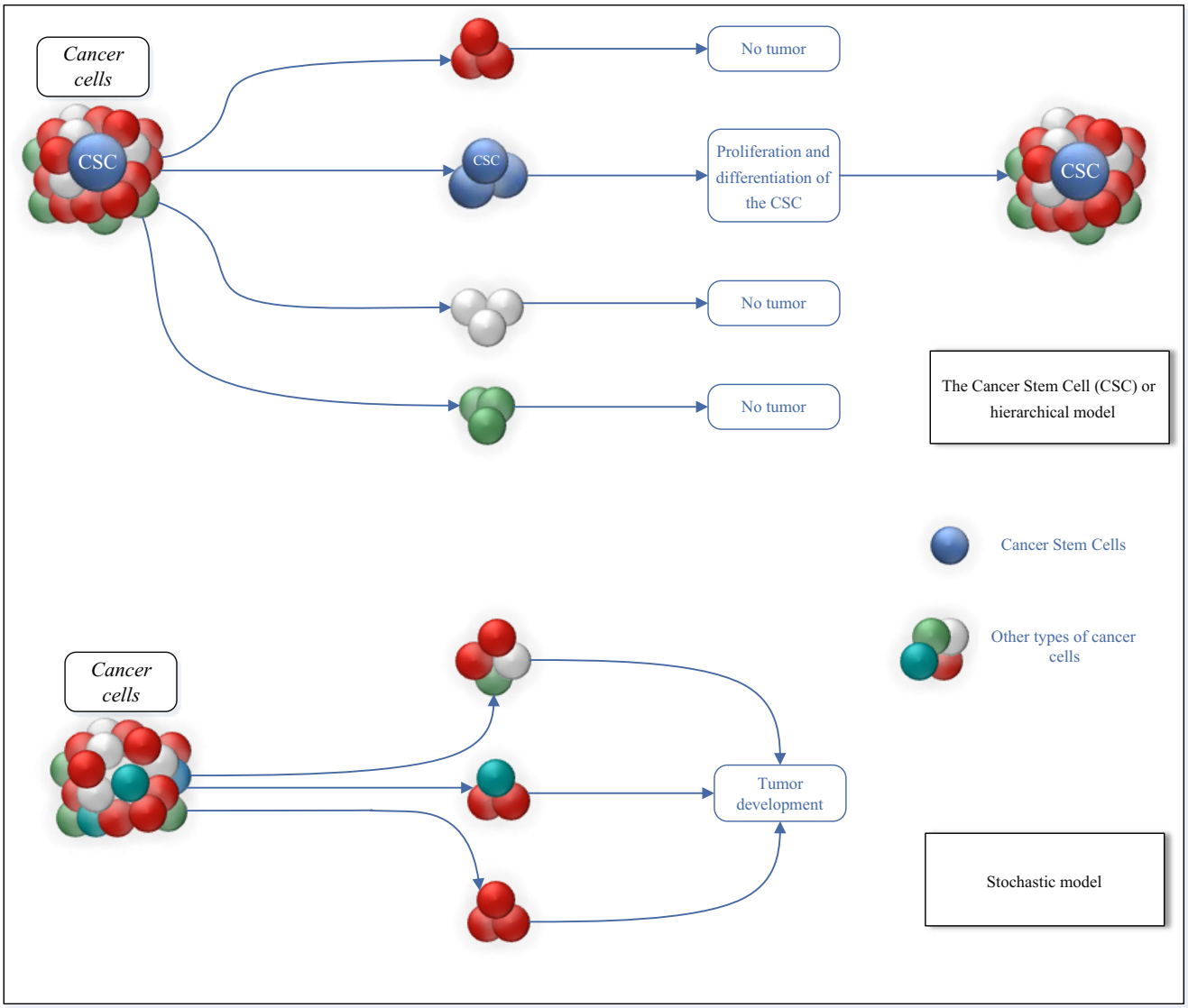

Fig. 1 A schematic comparison between the CSC and Stochastic models. In the Stochastic model all cells within a tumor are biologically equivalent. The chances of cancer development are stochastically dependent on the environmental cues, accumulated mutations and epigenetic abnormalities. Any cell within the tumor bulk has an equal potential to initiate tumorigenesis and differentiate into different subsets of cancer cells. Meanwhile, the Cancer Stem Cell model assumes that cancer cells can be divided into distinct cell populations, where only cancer stem cells are capable to initiate tumorigenesis

depends on micro-environmental cues, cancer types, tumor development stage and other factor types [2, 9, 10].

There are several well characterized molecular signaling cascades in CSCs including Wnt, Notch and Hedgehog pathways [11]. These pathways are broadly involved in self-renewal, proliferation and differentiation mechanisms of CSC. The existence of different populations of heterogeneous cells with different molecular signaling profiles and cell surface phenotypes may be largely responsible for the frequent failure of conventional treatments, contributing to frequent recurrence and relapse. This article will discuss these three CSC signaling pathways and the novel chemotherapeutic candidate drugs that target crucial components of the cascades.

Wnt Pathway-Targeting Chemotherapeutic Drugs

Understanding normal cellular homeostasis is a prerequisite for understanding the molecular mechanisms underlying the origin and development of tumor cells. One of the key signaling cascades involved in cell proliferation and stem cell renewal processes is the Wnt pathway. This signaling cascade can be subdivided into either β-catenin dependent or independent pathways. Both pathways regulate determination of cell fate, proliferation and differentiation mechanisms [12], as well as cell polarity and motility processes [13]. Meanwhile, the dysregulation of these mechanisms has been reported to be the main factor causing development of various cancer types

including colorectal cancer (CRC) [14], acute myeloid leukemia (AML) [15], chronic myeloid leukemia (CML) [16], gastric cancer [17] and many others.

The signaling cascade begins when Wnt ligands bind to the Frizzled (Fz) transmembrane receptor. The complex cooperates with another transmembrane lipoprotein receptor related protein (LRP). Formation of this triple complex inhibits glycogen synthase kinase-3β (GSK-3β) activities, resulting in non-phosphorylated β-catenin cytoplasmic accumulation [18]. Non-phosphorylated β-catenin translocates into the nucleus, where it regulates gene transcription by binding to other transcription cofactors including lymphoid enhancer-binding factors (LEF), T-cell transcription factor (TCF), cAMP response element-binding protein (CREB) [19, 20]. Formation of the machinery triggers expression of downstream targets of the Wnt signaling pathway such as c-MYC, Axin-2 and ASCL2 [21]. Whereas the absence of Wnt ligand results in the formation of the β-catenin destruction complex, which is comprised of GSK-3β, casein kinase 1-alpha (CK1α), axin and adenomatous polyposis coli (APC), causing phosphorylation of β-catenin and degradation in endosomes.

Given the importance of the Wnt pathway in cancer progression, there are a number of chemotherapeutic agents in pre-clinical and clinical trial stages (Table 1). In a series of in vitro cytotoxicity tests and in vivo activity experiments conducted in zebrafish, Chen and colleagues [22] discovered two major types of Wnt pathway antagonists: Wnt ligand production inhibitors (IWPs) and Wnt response inhibitors (IWR). IWP-1 and IWP-2 molecules inhibit the membrane-bound acyltransferase Porcupine, which modifies Wnt ligands through palmitoylation and preventing accumulation of β-catenin molecules in the cytoplasm. This modification seems to be essential for the ligand's signaling activity, such as IWR induced stabilization of Axin molecules, which in turn leads to the suppression of Wnt dependent signaling cascades [22]. Moreover, IWR suppressed Wnt-dependent regeneration processes in resected caudal fin of zebrafish can be reversed by washing out the drug [22]. Recently, Waaler and his colleagues have synthetized JW74 and JW67, two novel Wnt pathway antagonists [23]. Both molecules decreased the level of active β-catenin and increased expression levels of Axin2 molecules. Several molecules like 2,4-diamino-quinazoline and PNU74654 [24, 25] can also act as antagonists of β-catenin and TCF4. A similar effect was achieved by XAV939, which inhibits β-catenin signaling via interactions with the type 1 and 2 tankyrase-binding domain (TBD) of the Axin molecule [26]. However, these drugs have shown low selectivity, therefore further development and optimization is required. Other potential drugs from this series of molecules are ICG-001 analogues, which have shown some promising effects against various cancer stem cell types [27].

Several molecules have been reported to prevent activation of Wnt signaling by specifically targeting the PDZ domain of Dvl. This domain is a protein-protein interaction motif that directly binds to the Fz receptor, thereby activating the signaling cascade [28]. Three potential drugs NSC668036, FJ9 and 3289–8625 have shown to inhibit Dvl and Fz protein-protein interactions.

Table 1 Wnt pathway inhibitors

Molecule	Sponsor	Mechanism of action	Phase/ Clinical Trials. Gov. identifier	Types of cancer stem cells
IWP	N/A	Inhibition of Wnt ligand production	No clinical trials	N/A
IWR	N/A	Stabilization of axin molecules, inhibition of β-catenin accumulation	No clinical trials	N/A
XAV939	National Cancer Institute	Stabilization of axin molecules, inhibition of tankyrase 1 and 2	Phase 1	Neuroblastoma, Breast, Colon cancer SC
2,4-diamino-quinazoline	Parke Davis & Co., Pfizer Inc.	Inhibition of β-catenin/Tcf-4 pathway	Pre-clinical	Not yet investigated
PNU74654	Genentech/ Curis	Inhibition of β-catenin/Tcf-4 pathway	No clinical trials	Colon cancer SC
ICG-001	PrismBiolab	Inhibition of β-catenin/Tcf-4 pathway	Phase 1	Colon cancer SC, Brain tumor SC
NSC668036	US National Cancer Institute	Inhibition of Dvl activity through the PDZ domain	Pre-clinical trials	Breast cancer SC
3289–8625 (BML-286)	Enzo Biochem	Inhibition of Dvl activity through the PDZ domain	No clinical trials	Colon cancer SC
NSAIDs	N/A	Prevention of β-catenin translocation into the nucleus, inhibition of COX2	many	Intestinal, Skin, Basal cell carcinoma CSC

Abbreviations NSAIDs non-steroidal anti-inflammatory drugs, *SC* stem cells, *CSC* cancer stem cell, *COX2* cyclooxygenase 2

Efficacies of these drugs are due to further validation on cancer stem cell lines [28].

Non-steroidal anti-inflammatory drugs (NSAIDs) have also proven to be effective against various cancer types [29] (Table 1). The majority of NSAIDs target cyclooxygenase type 1 and 2 with consequent prevention of nuclear β-catenin accumulation. For instance, sulindac molecules have demonstrated significantly decreasing nuclear localization of β-catenin in patients with familial adenomatous polyposis (FAP) [30].

Chemotherapeutic Agents Targeting Hedgehog Signaling

The Hedgehog (Hh) signaling pathway is an essential signaling cascade mediating cell polarity and migration mechanisms, regulating maintenance of stemness properties and guiding SC differentiation [31]. There are three homologues of the Hh ligand: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh) [32]. The pathway initiates upon binding of Hh to the Patched 1 (Ptch1) receptor. Formation of the complex triggers release of Smoothened (Smo), which drives activation of Gli zinc-finger transcription factor family members (Gli1, Gli2, Gli3). It results in their nuclear translocation and expression of target genes [33].

There are several drugs targeting Hh pathway components that are in preclinical and clinical trial stages (Table 2). The naturally occurring Hh-specific inhibitor, cyclopamine, inhibits Smo activities in Hh-pathway-related tumors such as medulloblastoma, basal cell carcinoma, rhabdomyosarcoma and others [34]. Moreover, in vitro experiments showed a significant decrease of insulin-like growth factor binding protein 6 (IGFBP6) and proliferating cell nuclear antigen (PCNA), simultaneously increasing BCL2-antagonist/killer 1 (Bak1) and BCL2-associated X (Bax) proteins in SW116 cells treated with cyclopamine, which leads to decreased proliferation and initiation of apoptotic mechanisms [35]. Other Smo-inhibiting synthetic molecules, such as GDC-449, IPI-926, Cur-61414 and BMS-833923 are also at pre-clinical and clinical stages. GDC-0449 has recently entered phase II clinical trials on recurrent medulloblastoma, glioma, gastric carcinoma, breast cancer, prostate carcinoma, lung carcinoma and others [36] (Table 2).

Apart from trials with advanced stage patients, the drugs have also been tested on several types of cancer stem cells. The drug has been administered to patients with metastatic cancer, who had previously been treated with FOLFOX

Table 2 Hedgehog pathway chemotherapeutic inhibitors

Molecule	Sponsor	Mechanism of action	Phase/Clinical Trials.gov Identifier	Types of cancer stem cells
Cyclopamine	Generic	Smo antogonist	Phase 1, 2	Glioblastoma, Gastric, Prostate, Leukemic stem cells
GDC-0449	Curis, Genentech, Hoffmann-La Roche	Cyclopamine derivative, Smo antagonist	Phase 2 NCT02371967, NCT02115828, NCT01713218, NCT01604252, NCT02366312, NCT02091141, NCT02073838, NCT01878617, NCT01835626, NCT01774253, NCT01601184	Lung, Pancreatic, Leukemic stem cells
IPI-926	Infinity	Cyclopamine derivative, Smo antagonist	Phase 1, 2 NCT01383538	Primary myelofibrosis SC,
Cur-61414	Curis	Smo antagonist	Phase 1	Basal cell carcinoma, Glioma, Melanoma, Pancreatic CSC
BMS-833923	Bristol-Myers Squibb	Gli inhibition, Smo antagonist	Phase 1, 2 NCT00670189, NCT01218477, NCT01413906, NCT00927875, NCT00909402, NCT01357655, NCT00884546	Basal cell carcinoma SC
LDE225	Novartis	Gli inhibition, Smo antagonist	Phase 1, 2 NCT01350115, NCT00961896, NCT01456676, NCT01033019, NCT00880308, NCT01208831, NCT01125800, NCT01487785	Prostate, Pancreatic, Ovarian, Solid tumors, CML CSC,
PF-04449913	Pfizer	Gli inhibition, Smo antagonist	Phase 1 NCT02038777, NCT02367456, NCT01842646, NCT01841333, NCT01546038	MDS, CML CSC,

Abbreviations MDS myelodysplastic syndrome, *CML* chronic myeloid leukemia, *AML* acute myeloid leukemia

(combination of folinic acid, fluorouracil and oxaliplatin) or FOLFIRI (combination of folinic acid, fluorouracil and irinotecan) [37]. LDE225 (Novartis) and PF-04449913 (Pfizer) have also demonstrated some promising results presented in preliminary data [38]. Phase I trials on patients with advanced solid tumors have shown no dose-limiting toxicities at all given dosages, including up to 800 mg daily [38]. Moreover, decreased expression levels of Gli-1 mRNA in skin and medulloblastomas provided evidence for in vivo Hh pathway inhibition [38]. Phase I trials of PF-04449913 in patients with hematologic malignancies were conducted to determine DLTs and phase II dose recommendations [39]; overall the drug was reported to be tolerated well and even indicated some efficacy against chronic myeloid leukemia patients.

Notch Signaling Pathway Inhibitors

The Notch cascade, may also contribute to dysregulation of homeostasis in cancer stem cells. Mammals possess four Notch receptor homologues (1, 2, 3 and 4) that interact with two different families of ligands: Delta-like ligands (DLLs) and Jagged ligands [11, 40]. Upon formation of the ligand-receptor complexes, conformational changes of the receptor lead to the exposure of cleavage sites to metalloprotease and γ-secretase, releasing the Notch intracellular domain (NICD). The NICD translocates to the nucleus and initiates transcription of target genes including HES (hairy enhancer of split), Myc and p21. Expression levels of Jagged ligands, Notch1 and HES1 were comparable to or slightly higher in cancer stem cells than that in normal intestinal crypt cells [11, 41, 42]. However, another study conducted by Meng and colleagues [42], revealed a positive correlation in HES1, Notch1 and NICD gene expression with the grade of colon cancer progression. Moreover, overexpression of these genes is hypothesized to be involved in chemo-resistance of colon cancer cells.

Notably, γ-secretase inhibition has become a common target for drug development. There are several gamma-secretase inhibitors currently being developed, which inhibit gamma-secretase mediated Notch cleavage in various types of tumors (Table 3). RO4929097 is a gamma-secretase inhibitor in phase I studies in neuroendocrine carcinoma and melanoma patients. The first phase II trial on refractory CRC patients showed a very good tolerability, with minor toxicity levels [43]. The drug showed tumor inhibiting properties in patients with melanomas [43]. The authors suggested that RO4929097 would be more effective in combination with other drugs. Initial experiments of another γ-secretase inhibitor, MK-0752, on T-cell acute lymphoblastic leukemia patients were unsuccessful due to the dose limited toxicity (DLT). Most of the patients had diarrhea and other gastrointestinal symptoms [44]. However, in another phase I trial of MK-0752 in children with refractory malignant CNS tumors, DLT was not observed [45]. Therefore these facts require further investigations and clarification.

Another essential element of the Notch pathway is the DLL4 ligand. It has been demonstrated to play a crucial role in vascularization of tumors [46]. It is believed that the blockade of the vascular endothelial growth factor (VEGF) receptor with monoclonal antibodies has a major therapeutic potential in inhibition of tumor driven angiogenesis [47]. However, severe forms proceed even when these receptors are blocked. Therefore additional angiogenesis-targeted treatments are required. Such therapy had been shown to be feasible by Noguera-Troise and colleagues who demonstrated the importance of DLL4 in the inhibition of tumor angiogenesis and growth in mice [48]. However, the blockade of the DLL4/Notch pathway resulted in enhanced formation of non-functional tumor vessels, thus the DLL4 antibody was concluded to be a negative regulator of tumor vascular growth.

The effect of the anti-DLL4 antibody on xenograft models of CRC patients with oncogenic KRAS mutations has been tested as a combination drug therapy [49]. KRAS mutations in CRC patients are common and are associated with treatment resistance to anti-EGFR therapy. Anti-DLL4 alone, as well as in combination with widely used chemotherapeutic drugs (such as irinotecan), has decreased tumor cell proliferation and angiogenesis in both wild type and KRAS mutant mouse tissue. These results indicate the utility of DLL4 inhibition on treating CRC xenograft tumors. Despite quite a number of experiments and positive effects on cancer patients with this agent there are no reports on dosage and administration regimes for clinical development.

Conclusions and Further Perspectives

The CSC model provides a potential explanation of tumor initiation, progression and metastasis mechanisms in many cancer types. The logic is that failure to eliminate the subset of CSCs within the tumor bulk leads to cancer recurrence, chemotherapy resistance and metastasis. Recent investigations have provided insights into the role and mechanisms of Wnt, Hh and Notch pathways in the development of a number of cancer types. Higher activity of these pathways in CSCs compared to normal somatic cells, as well as the interplay between the molecular pathway components, may contribute to the cellular diversity and complexity of the problem. It is important to emphasize that a number of signaling cascades highly expressed in normal stem cells may also be upregulated in CSCs [50], therefore contributing to CSC driven tumor development and recurrence mechanisms. This fact has hindered the development of effective cancer stem cell-specific therapies. The development of these novel therapeutic approaches may be complicated by significant issues. Despite

Table 3 Notch pathway chemotherapeutic modulators

Molecule	Sponsor	Mechanism of action	Trial	Types of cancer stem cells
MK-0752	Merck	Gamma-secretase inhibitor	Phase 1, 2/ NCT00645333 NCT00572182	Breast, Refractory CNS cancer SC
RO4929097	US National Cancer Institute, Hoffmann-La Roche	Gamma-secretase inhibitor	Phase 1, 2/ NCT01071564, NCT01158274, NCT01238133, NCT01070927, NCT01193868, NCT01193881, NCT01269411, NCT01119599, NCT01122901, NCT01116687, NCT01270438, NCT01198535, NCT01200810, NCT01192763, NCT01232829, NCT01198184, NCT01145456, NCT01218620, NCT01088763, NCT01096355, NCT01131234, NCT01120275, NCT01196416, NCT01216787, NCT01175343, NCT01154452, NCT01251172	Breast, Lung, Glioma, Colon, Prostate, Pancreatic, Solid tumors, Melanoma, Ovarian, Myeloma CSC
LY450139	Eli Lilly and Company	Gamma-secretase inhibitor	Phase 3	Breast, Brain, Myeloid leukemia, Skin CSC,
"Notch Inhibitor"	Eli Lilly and Company	Unspecified Notch inhibitor	Phase 1 NCT01158404	Yet to be investigated
BMS-708163	Bristol-Myers Squibb	Gamma-secretase inhibitor	Phase 2	Breast and other types of CSC
OMP-18R5	OncoMed Pharmaceuticals Inc. in collaboration with Bayer	Targets the Frizzled 7 receptor	Phase 1b NCT02005315, NCT01973309, NCT01957007	Breast, Lung, Pancreatic and others
PF-03084014	Pfizer, Jules Bordet Institute, AIDS Malignancy Clinical Trials Consortium	Gamma-secretase inhibitor	Phase 1, 2 NCT02299635, NCT02338531 NCT02137564, NCT02137564	Breast, Pancreatic, Head and neck sarcoma CSC
OMP-21 M18 (demcizumab)	OncoMed Pharmaceuticals Inc. in collaboration with Celgene Corporation	Targets DLL4	Phase 1, 2	Pancreatic cancer, Non-small cell lung cancer SC and others
OMP-305B83	OncoMed Pharmaceuticals Inc. in collaboration with GlaxoSmithKline	Binds to DLL4 and the VEGF receptor	Phase 1 NCT02298387 Preclinical	Solid tumors. Yet to be studied
OMP-131R10	OncoMed Pharmaceuticals Inc.	Disrupts RSPO-LGR5 pathway	Preclinical	Yet to be studied
MPC-7869	Myriad Genetics & Laboratories; Encore Pharmaceuticals	Inhibition of Notch cleavage by γ-secretase	Phase 3	Prostate, Skin, Intestinal cancer SC
OMP-18R5	OncoMed Pharmaceuticals Inc., Bayer	Targets the Frizzled 7 receptor	Phase 1b	Breast, Lung, Pancreatic and others
OMP-54 F28	OncoMed Pharmaceuticals Inc., Bayer	Binds to Wnt ligands	Phase 1b NCT02092363, NCT02069145 NCT02050178	Ovarian, Pancreatic, Hepatocellular cancers and others
OMP-52 M51	OncoMed Pharmaceuticals Inc., Bayer	Binds to the Notch1 receptor	Phase 1 NCT01778439, NCT01703572	Breast and other solid tumors
OMP-21 M18		Targets DLL4	Phase 1, 2	

Table 3 (continued)

Molecule	Sponsor	Mechanism of action	Trial	Types of cancer stem cells
	OncoMed Pharmaceuticals Inc., Celgene Corporation, Novotech (Australia) Pty Limited		NCT01189968, NCT02259582 NCT01189929, NCT02289898 NCT01952249	Pancreatic, Non-Small cell lung cancer, Ovarian and others
OMP-59R5	OncoMed Pharmaceuticals Inc. collaboration with GlaxoSmithKline	Targets DLL4 and VEGF receptor	Phase 1, 2 NCT01859741	Pancreatic cancer, Small cell lung cancer SC and others

Abbreviations ALL acute lymphocytic leukaemia, *DLL4* Delta-like ligands 4, *VEGF* vascular endothelial growth factor

pioneering works in the field, when scientists used the cell surface phenotype for enrichment and investigations of CSCs, some recent investigations suggest that CSCs undergo dynamic changes at the level of biomarker expression during tumorigenesis [51, 52]. Interestingly, the accumulation of genetic aberrations and instabilities, a common feature of cancer cells, recently has also been extrapolated to cancer progenitor cells [53] suggesting novel mechanisms of cancer transformation and development. For example, chromosomal instabilities and increased expression of c-Myc have been detected in in vivo experiments on fibrosarcoma [54] and non-tumorigenic neural cells [55] leading to the acquaintance of CD133+ phenotype and increased ability to develop cranial malignancies. This makes it difficult to monitor the effects of biologic and therapeutic effects of current drugs on CSCs. Nonetheless, exploring and understanding pathway cross-talk mechanisms may also provide a platform for designing and developing new experimental drugs. It will widen the perspectives of therapeutic options and treatment regimens for effective inhibition of tumor development and relapse.

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